Characterization of a New Photoaffinity Derivative of Ouabain: Labeling of the Large Polypeptide and of a Proteolipid Component of the Na,K-ATPase†

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ABSTRACT: We have synthesized 2-nitro-5-azidobenzoyl (NAB) derivatives of ouabain as photoaffinity labels of the cardiac glycoside binding site of Na,K-ATPase. [^{3}H]NAB-ouabain was found to bind to the same number of sites on Na,K-ATPase (purified from pig kidney outer medulla) as ouabain (1.9 nmol/mg), with approximately the same affinity (K_d(ouabain)/K_d(NAB-ouabain) $\simeq$ 1.6), and ouabain was fully competitive with NAB-ouabain at these sites. NAB-ouabain binding and inhibition were reversible in the dark, but on exposure to ultraviolet light (310–370 nm) 30–40% of the binding and inhibition became irreversible; this binding was shown to be covalent by stability to trichloroacetic acid, organic solvents, and heat denaturation. Covalent labeling was prevented by photolysis of NAB-ouabain prior to the experiment, or by prior incubation of the enzyme with ouabain. On sodium dodecyl sulfate–polyacrylamide gels of labeled Na,K-ATPase, about half of the covalently bound [^{3}H]NAB-ouabain migrated with the large polypeptide (molecular weight $\simeq$ 95 000), and half migrated with a small polypeptide (molecular weight $\simeq$ 12 000); noncovalently bound NAB-ouabain (60–70% of total label) ran with the tracking dye. A similar labeling pattern was obtained utilizing NaI microsomes prepared from pig kidney outer medulla. The small polypeptide was characterized as an acidic proteolipid by extractability into acid chloroform/methanol; labeling of this component by NAB-ouabain is the first demonstration that it is directly associated with the Na,K-ATPase. The results of our characterization of NAB-ouabain show that it has the required specificity, covalency, and efficiency of labeling for application in structural studies of Na,K-ATPase subunit interactions.

The Na,K-ATPase[1] complex, responsible for the active transport of Na and K across animal cell membranes, has been shown to contain both a large polypeptide of mol wt $\simeq$ 95 000 and a smaller glycoprotein of mol wt $\simeq$ 45 000 (e.g., Hokin et al., 1973). The stoichiometry of ligand binding is such that for each pair of large polypeptide molecules, one ATP or one cardiotonic steroid molecule may be bound (Jorgensen, 1974b), or one of the polypeptide chains may be phosphorylated from ATP (Hokin et al., 1973). Investigation of the structural aspects of the ATP and cardiac glycoside binding sites requires affinity analogues of the ligands that will remain attached at their sites under conditions that denature the enzyme, i.e., detergent solubilization, gel chromatography and electrophoresis, and chemical conditions for peptide mapping. The affinity labeling analogues must exhibit high specificity for their sites, both with regard to their substrate (ATP) or inhibitory (cardiac glycoside) actions and with regard to their covalent attachment. The conditions for covalent labeling must be mild enough to ensure that the ligand–enzyme interaction is unaltered, and the process should be reasonably rapid. Lastly, the efficiency of labeling, that is the fraction of reversibly bound ligand that becomes covalently bound, should be as high as possible.

Although a number of affinity derivatives of cardiotonic steroids have been prepared, none has met the above criteria for use in structural studies of Na,K-ATPase. Haloacetyl cardiac aglycones were found to irreversibly inhibit the enzyme, but to be nonspecific alkylating agents as well (Hokin, 1969), and a photoreactive strophanthidin derivative proved to be similarly nonspecific in its action (Tobin et al., 1975). Photolysis of 3-azidoacetylstrophanthidin with Na,K-ATPase produced an increment of 10% in irreversible inhibition (Tobin et al., 1976); however, labeling studies using radioactive material have not been reported. Ruoho & Kyte (1974) used short wavelength UV light to covalently attach tritiated derivatives of cymarin to the Na,K-ATPase and found that the large subunit but not the glycoprotein was covalently labeled. The extent of labeling, approximately 1% of total binding, was severely limited by photo-cross-linking of the protein in the short wave light, a problem that would complicate subsequent structural studies. Furthermore, the mechanism of attachment is unclear, since a derivative not containing the diazo group was more effective than the intended photoaffinity compound (Ruoho & Kyte, 1974). Hegyvary (1975) achieved a high degree of covalent labeling of partially purified Na,K-ATPase by sodium borohydride reduction of presumed Schiff bases formed between tritiated, oxidized ouabain and the enzyme binding site; again label was found predominantly at the position of the large subunit on NaDodSO$_4$ gels. We have been unsuccessful in attempts to use this method to label enzyme purified from pig kidney outer medulla, although we have tried various periods of reduction and a range of pH (Forbush, Kaplan, & Hoffman, unpublished results). In any case, the method is not applicable to studies involving simultaneous determination of covalent ouabain binding and of phosphorylation, since the acyl phosphate intermediate is unstable

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[1] Abbreviations used: Na,K-ATPase, magnesium dependent, sodium plus potassium stimulated, adenosine triphosphatase (EC 3.6.1.3); ATP, adenosine triphosphate; NAB, 2-nitro-5-azidobenzoyl; NAB-ouabain, 2-nitro-5-azidobenzoyl ouabain; NAB active ester, *N*-hydroxysuccinimide ester of 2-nitro-5-azidobenzoic acid; ED-ouabain, ethylenediamine-ouabain adduct; P_i, inorganic phosphate; MeOH, methanol; Tris, tris(hydroxymethyl)aminomethane; NaDodSO$_4$, sodium dodecyl sulfate; BSA, bovine serum albumin; TLC, thin-layer chromatography; UV, ultraviolet; % T, w/v % of total acrylamide; % C, 100 × bis(acrylamide)/acrylamide.

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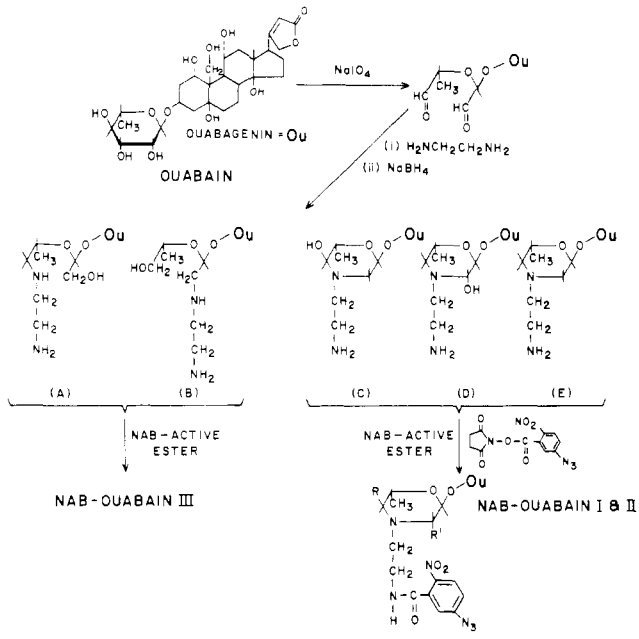

FIGURE 1: Synthetic route to ouabain photoaffinity labels. The reductive amination of oxidized ouabain leads to an array of products (A–E). Following reaction with NAB-active ester, open-chain amines yield NAB-ouabain III, while the morpholino derivatives yield NAB-ouabain I and II, where R and R′ are H or OH.

under the reduction conditions. Finally, two laboratories have reported that nonderivatized cardiac glycosides remain attached to the large subunit when it is solubilized in $NaDodSO_4$ and submitted to gel chromatography (Alexander, 1974) or electrophoresis (Kott et al., 1975). Others, including ourselves, have been unable to confirm this behavior on polyacrylamide gels, and Lane (1976) has proposed that it may be an artifact caused by anomolous behavior of digitoxin and ouabain in some electrophoretic and chromatographic systems.

Two attributes have led to the frequent choice of aryl azides for use in photoaffinity derivatives (Knowles, 1972; Cooperman, 1976). (1) Their absorption spectrum usually extends into the long wave UV (>320 nm) enabling the use of light that is not absorbed by the biological material and that does not in itself affect the sample. (2) The highly reactive photoreleased nitrene can readily insert into nearby C–H bonds without a requirement for a reactive group at the biological site. However, this high reactivity may also be responsible for side reactions, resulting in attachment efficiencies that are usually less than 30%. In this paper we present the synthesis and biological characterization of a nitroazidobenzoyl (NAB) derivative of ouabain that appears to satisfy the above requirements for structural studies of Na,K-ATPase. A brief report of this work has been presented previously (Forbush et al., 1978).

Methods

Chemical Procedures. Cardiac steroid was assayed by the method of Kedde (1947). To a 0.05–0.2-mL sample was added 2 mL of 1.2% 3,5-dinitrobenzoic acid in methanol followed by 0.5 mL of 1.5 N NaOH. After 5 min the optical density at 540 nm was determined. This assay is quite specific for the cardenolides and requires an intact lactone ring. Primary amine was assayed by the method of Inman & Dintzis (1969). To a 0.05–0.2-mL sample was added 1 mL of 0.3% 2,4,6-trinitrobenzenesulfonic acid followed by 1 mL of saturated $Na_2B_4O_7$ and after 5 min the optical density at 420 nm was measured. Secondary amines were detected by the method of Siggia (1963), modified by the omission of extraction into an organic phase. An aliquot (2 mL) of CS_2–pyridine–isopropyl alcohol (7:5:13) was added to 1 mL of 0.04% $CuCl_2$ in H_2O–pyridine (50:50). A small volume (25–100 μL) of an aqueous or ethanolic solution of the sample (ca. 10^{-2} M) was added, the solution was shaken vigorously, and after 10 min the absorption spectrum measured in the range 300–500 nm using an Aminco DW-2 spectrophotometer. Secondary amines are characterized by an absorption peak in the 440–450-nm range, tertiary amines do not react in the assay, and primary amines do not produce the absorption peak in this region. The NAB group was determined from the change in absorption at 320 nm on complete photolysis with 310–350-nm light, using $\Delta\epsilon(\pm \text{ light}) = 7.4 \times 10^3$. TLC was routinely performed on precoated silica gel 60 sheets (E. Merck, Darmstadt, West Germany) using ethyl acetate–methanol–water, 75:25:5 (approximate R_f's: ouabain 0.22; oxidized ouabain 0.35; NAB-ouabain I 0.42; NAB-ouabain II 0.45; NAB-ouabain III 0.09; ED-ouabains < 0.05) or $CHCl_3$–methanol, 4:1 (approximate R_f's: NAB-ouabain I 0.42; NAB-ouabain II 0.59). Products were detected by long wave UV absorption (NAB compounds), I_2 vapor (NAB and ouabain compounds), 2,4,6-trinitrobenzenesulfonic acid/$Na_2B_4O_7$ sprays (primary amines), 3,5-dinitrobenzoic acid/NaOH or 1,3-dinitronaphthalene/NaOH sprays (cardiac steroids), and ^3H counting. All operations involving NAB compounds were performed in dim yellow light. Unless otherwise noted, chemical procedures were carried out at room temperature.

Chemical Synthesis. The synthesis of NAB-ouabain is outlined in Figure 1. Schiff base formation between ethylenediamine and the aldehyde groups of oxidized ouabain (Smith, 1972) was followed by reduction with $NaBH_4$ and passage over anionic and cationic exchange resins to break up borate complexes (Abdel-Akher et al., 1951). After separation of three ouabain amines using ion-exchange chromatography, reaction with *N*-hydroxysuccinimide ester of 2-nitro-5-azidobenzoic acid (NAB active ester, Galardy et al., 1974) yielded three NAB derivatives of ouabain.

Ethylenediamine–Ouabain Adducts (Ed-Ouabain I, II, III). In the following description, the first quantity given in each set of parentheses refers to a large scale preparation with a trace amount of [^3H]ouabain and the second quantity refers to a small scale preparation of much higher specific activity. Ouabain (2.5 g, 3.43 mmol; or 10 mg) and [^3H]ouabain (0.05 mCi; or 2 mCi) was dissolved in hot water (50 mL; or 0.5 mL) and allowed to cool. $NaIO_4$ (2.5 g, 11.7 mmol; or 10 mg in 0.1 mL of H_2O) was added and the oxidation was allowed to proceed for 30 min in the dark; oxidation was quantitative as determined by TLC. The solution was passed over Dowex 1-X8, Cl^- form (250 mL bed volume; or 3 mL), and evaporated (to 20 mL; or to 1 mL). Ethylenediamine (0.6 g, 9.9 mmol in 33 mL, pH 9 with HCl; or 2.5 mg in 0.14 mL) was added, followed in 5 min by $NaBH_4$ (2.5 g, 66.0 mmol; or 10 mg in 0.1 mL of H_2O). After 15 min the mixture was cooled to 0–4 °C and brought to pH 1 by slow addition of 0.2 N HCl (0 °C). After an additional 5 min, the solution was neutralized with 1 M triethylamine in 20% ethanol and passed over Dowex 1-X8, acetate form (250 mL bed volume; or 3 mL). The eluate was combined with a water wash (1 L; or 20 mL) and applied to a bed of Dowex 50-X8, H^+ form (250 mL; or 3 mL). This time the eluate and water wash (1 L; or 30 mL) were discarded; they contained about 15% of the starting material. The mixture of amines was eluted with 1 M triethylamine in 20% ethanol (600 mL; or 25 mL), repeatedly extracted with methylene chloride, and evaporated to an oil. While the recovery of lactone ring was 70% at this stage, only 40% of the starting tritium

remained; the balance of the tritium was found in the traps of the rotary evaporator following the last step. The amine mixture was brought to pH 5 with acetic acid (in 50 mL of H_2O; or 2 mL), applied to a column of CM-Sephadex (2.6 × 100 cm; or 0.4 × 150 cm) and eluted with a linear gradient of 0.1–1.0 M triethylammonium acetate, pH 5.5 (3 L; or 80 mL) over 5 days. Three ethylenediamine–ouabain adducts (ED-ouabain I, II, III), representing 10%, 20%, and 20% of the starting lactone ring and 5%, 10%, and 10% of the tritium, were found. Collected fractions were pooled and dried to a glass by repeated additions of water and evaporation.

The synthetic route outlined in Figure 1 includes a range of products (A–E) which could result from the reductive amination of oxidized ouabain with $NaBH_4$ and ethylenediamine. In the present work the ratio of primary amine to lactone ring was found to be 1:1 in all three ED-ouabain products, so that structures not showing this substituent stoichiometry have not been included. ED-ouabain III was identified as an open ring derivative, either A or B or a mixture of both, by a positive in the secondary amine assay and by lability of the acetal in dilute acid. Hydrolysis of ED-ouabain III in 0.2 N HCl (k = 0.003 s^{-1}, 100 °C) yielded a lactone ring product free of amine and identical (by TLC) with the hydrolysis product of periodate-oxidized ouabain, presumably ouabagenin. ED-ouabain II was negative in the secondary amine assay, negative for aldehyde by reaction with 2,4-dinitrophenylhydrazine (Siggia, 1963, p 124), and was stable to acid (0.2 N HCl, 100 °C, 20 min). Thus ED-ouabain II is presumed to be E, the product expected by analogy to that obtained following the reductive amination of oxidized adenosine 5′-monophosphate (Khym, 1963; Brown & Read, 1965). Ed-ouabain I was negative for secondary amine and gave a positive aldehyde test by reaction with 2,4-dinitrophenylhydrazine in acidic methanol in 3 h (but not in 30 min). This behavior would be expected of carbinolamines (C) and (D) which should be in equilibrium with open chain aldehyde/secondary amines, especially at low pH. On the other hand the stability of ED-ouabain I to acid (0.2 N HCl, 100 °C, 20 min) and resistance to further reduction with $NaBH_4$ are inconsistent with the carbinolamine forms, so the structure of ED-ouabain I remains uncertain. Chromatographically ED-ouabain I and II are very similar, and, unlike ED-ouabain III, the former pair elute one after the other at about 0.6 M triethylammonium acetate whereas the latter is greatly separated, eluting at about 1 M. The similarities and differences are also observed in the TLC behavior and solubility properties of the respective NAB derivatives.

Analysis (theoretical) (C and D) $C_{30}H_{49}N_2O_{10}\cdot H_2O$: C, 58.52; H, 8.35; N, 4.55. (E) $C_{30}H_{49}N_2O_9$: C, 61.95; H, 8.49; N, 4.82. (A and B) $C_{30}H_{51}N_2O_{11}$: C, 58.52; H, 8.35; N, 4.55. Found: (for ED-ouabain I) C, 58.46, H, 7.9, N, 4.3; (for ED-ouabain II) C, 59.88, H, 8.5, N, 4.81; (for ED-ouabain III) C, 55.40, H, 8.47, N, 4.84.

2-Nitro-5-azidobenzoyl Derivatives of ED-ouabains (NAB-Ouabains I, II, III). NAB active ester (gift of Dr. Richard Galardy) reacted readily with the primary amine of the ED-ouabains. Typically, 1–60 μmol of ED-ouabain in 0.5 mL of methanol and 35 mL of triethylamine was added to a 1.5-fold excess of NAB-active ester in 1 mL of dioxane and the reaction was allowed to proceed for 12 h. Water (5 mL) was added and the solution was alternately extracted with ether and benzene five times; then the pH was brought to 2 with HCl and the extractions were repeated. After raising the pH to 10, NAB-ouabain I or II was extracted into methylene chloride (3 mL, 5–10 times), dried on the rotary evaporator, dissolved in 5% Me_2SO at 0.1 mM, and stored at −20 °C. A single spot was detected on TLC; it contained 96% (NAB-ouabain II) or 88% (NAB-ouabain I) of the ^3H applied. The NAB to lactone ring ratio was found to be 1:1 for both compounds and the ultraviolet absorption spectra were consistent with 1:1 summation of the spectra of ouabain and of NAB. NAB-ouabain III was not purified beyond the initial benzene and ether extractions. On TLC (ethyl acetate–methanol–water, 15:5:3) it appeared as a spot containing 50% of the applied ^3H; the rest of the ^3H trailed to the origin. All three NAB-ouabain products were negative in the primary amine assay.

Photolysis. The output of a 1000-W mercury arc lamp (Ushio 1005D, Oriel Corp., Norwalk, Conn.) was filtered (3-mm UG-11, Schott Glass; 13-mm 4.2% $CuSO_4$; 8 cm H_2O) to transmit light with a peak wavelength of 342 nm and a half bandwidth of 60 nm. Photolysis of NAB-ouabain was complete within 10 s when the lamp was focused on a 2.5-cm spot (approximately 0.44 μE/(cm^2·s), determined using the ferrioxalate actinometer of Hatchard & Parker (1956)), or within 60 s when the light was diffusely reflected onto a 5-cm spot. In preliminary experiments the long wave lamp of a hand held mineral light (UVSL-25, Ultraviolet Products, San Gabriel, Calif.) was used; photolysis was complete within 10 min at a 2-cm distance. Samples (4 °C or room temperature) were photolyzed in polystyrene (1 cm i.d.) or glass (1.4 cm i.d.) test tubes without stirring, or on the surface of Nucleopore filters. In no case was there more than 50% attenuation of the 340-nm light by absorption in the sample.

Enzyme Preparation. Purified Na,K-ATPase was prepared by the method of Jorgensen (1974a) with two minor modifications: (1) Outer medulla dissected from coronally bisected fresh pig kidneys was homogenized for 15 s in a Waring blender and filtered through cheesecloth before homogenization in the Teflon pestle homogenizer. (2) The density gradient centrifugation was performed in a Sorvall Ti865 angle rotor (2 h, 65 000 rpm) on a 19-mL 20–36% sucrose gradient. Fractions between 30% and 33% sucrose were pooled and treated as the zonal rotor fractions of Jorgensen. The specific activity of freshly prepared material was 800–1000 μmol of P_i/(mg·h). NaI microsomes were prepared according to Lane et al. (1973) with the microsomal fraction isolated in the above procedure; the specific activity was 80–100 μmol of P_i/(mg · h). Protein was determined by the method of Lowry et al. (1951), without Cl_3CCOOH precipitation, with BSA standards.

Enzyme Assay. The assay medium routinely contained 125 mM NaCl, 25 mM KCl, 4 mM Na_2ATP (Sigma, grade II), 4 mM $MgCl_2$, 1 mg/mL BSA, 60 mM Tris-Cl, 0.75 mM EDTA, pH 7.5 (37 °C), approximately 1 μg of Na,K-ATPase, and optionally 10^{-3} M ouabain in a final volume of 0.6 mL. After 10 min incubation at 37 °C the reaction was stopped by addition of 1 mL of freshly prepared 1.5% ammonium molybdate, 2% silicotungstic acid in 1.4 N H_2SO_4, and 2 mL of isobutyl alcohol/hexane (1:1) and immediately vortexed for 10 s (Martin & Doty, 1949). An 0.8-mL aliquot of the upper phase was diluted with 1.6 mL of 2% H_2SO_4 in 95% ethanol and reduced with 0.1 mL of freshly diluted 1.2% $SnCl_2$ in acidic ethanol. The optical density of reduced phosphomolybdate at 670 nm was linear with P_i in the assay medium to beyond 1 mM (≃ 1 o.d.).

Ouabain and NAB-Ouabain Binding. This was done in the presence of Na, Mg, and ATP or in the presence of Mg and P_i. Unless otherwise specified the first medium contained (final) 120 mM NaCl, 3 mM NaATP, 3 mM $MgCl_2$, 0.2% BSA, and 25 mM Tris, pH 7.5 (37 °C); the second contained 5 mM $MgCl_2$, 3 mM H_3PO_4, 2 mM EDTA, 0.2% BSA, and 30 mM Tris, pH 7.25 (37 °C). The [^3H]ouabain or [^3H]NAB-ouabain concentrations (10^{-8} to 10^{-5} M), enzyme concentration

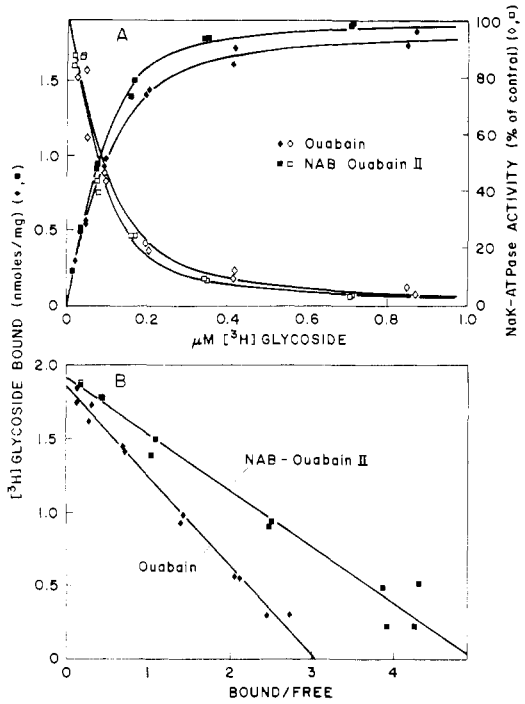

FIGURE 2: Comparison of ouabain and NAB-ouabain II binding to the Na,K-ATPase. (A) Na,K-ATPase (0.0587 mg/mL) was incubated with [^{3}H]ouabain (◇, ◆) or [^{3}H]NAB-ouabain II (□, ■), (0.01–0.88) μM, in 0.12 mM Mg, 0.12 mM P_i, 30 mM Tris, pH 7.25, in a final volume of 0.3 mL. After 1 h at 37 °C the samples were diluted by the addition of 2 mL of 10 mM KCl, 10 mM Tris (0 °C). Two 100-μL aliquots were then removed for assay of Na,K-ATPase activity (open symbols) and the remainder of the sample was filtered on a Nucleopore filter and rinsed with 1 mL of cold 10 mM KCl, 10 mM Tris to determine [^{3}H]glycoside binding (solid symbols). Control Na,K-ATPase activity was 600 μmol of P_i/(mg·h) after the 1-h incubation; it was 870 μmol of P_i/(mg·h) in fresh enzyme. The lines are theoretical curves describing bimolecular binding using the parameters obtained from the straight lines in B. (B) The ^{3}H binding data of A are replotted with the abscissa indicating the ratio of bound to free [^{3}H]glycoside. The straight lines are least-squares fits of the data.

(5–1500 μg/mL), and the incubation time at 37 °C (5–60 min) were varied to suit the requirements of individual experiments and they are reported in text and figure legends. Because NAB-ouabain adsorbs strongly to cellulosic membrane filters (e.g., Millipore), polycarbonate Nucleopore filters were used to separate NAB-ouabain bound to protein from free NAB-ouabain. At least 95% retention of enzyme resulted when either 0.08-μm pore filters were used with the purified Na,K-ATPase, or the 0.2-μm pore size with NaI microsomes. Filtration (at $\Delta P \simeq 150$ mmHg, on a 3025 Millipore sampling manifold or on glass frits) usually required about 15 min. Samples were rinsed (0–4 °C) with 10 mM KCl, 10 mM Tris (pH 7.5), or with binding media containing nonradioactive ouabain, as specified in the text. Addition of BSA to binding media and dilution of samples prior to filtration or photolysis were effective in reducing nonspecific binding and photolabeling of the enzyme by NAB-ouabain to less than about 5% of total binding, as evidenced by results of experiments shown in Figure 2 and Table I, below. In an experiment similar to that of Figure 2, filtration was compared with ultracentrifugation (3 h, 300 000*g*, 0 °C); the results were identical within experimental error. The purity (fraction of ^{3}H as cardiac glycoside) of [^{3}H]ouabain was 89–91% and that of [^{3}H]NAB-ouabain II was 97–98%, as determined by the method of Hansen & Skou (1973). Corrections for impurity were applied in calculation of the binding data in Figures 2 and 3. All experiments with NAB-ouabain were performed in dim yellow light.

NaDodSO$_4$–Polyacrylamide Gel Electrophoresis. Samples (final protein 0.2–2 mg/mL) were solubilized in 2% NaDodSO$_4$, 20% sucrose, 20 μg/mL Pyronin Y, and 40 mg/mL dithiothreitol at room temperature. For removal of protein from Nucleopore filters, the filter was placed in a 4-mL glass test tube with 200 μL of this solution and subjected to six freeze-thaw-vortex cycles, after which the filter was pressed free of excess solution, rinsed with an additional 40 μL, and removed. When [^{3}H]NAB-ouabain photo-bound to Na,K-ATPase was solubilized in this way, less than 2% of the radioactivity remained on the filter.

The buffer system of Fairbanks et al. (1971; 0.2% NaDodSO$_4$) was used with gradient slab gels formed in apparatus modeled after that of Margolis & Kenrick (1968). Most often gradients from 2.5% T, 3.7% C to 13.5% T, 3.7% C or from 4.5% T, 3.7% C to 17% T, 9% C were used, where %T is the percent acrylamide by weight and % C is bis(acrylamide) as a percent of acrylamide. The latter system, with a gradient of cross-linking as well as acrylamide concentrations gave significantly better resolution, but the middle two-thirds of the gel had a slight haze (0.3 o.d. in 2 mm thickness) as a result of the high cross-linking. Gels were stained with 0.05% Coomassie blue R in 10% acetic acid, 30% methanol, and destained in the same solvent. Gel tracks were sliced for counting either before or after staining, as noted below. Generally, 60–80 μL sample buffer/track was applied to 3-mm thick gels intended for slicing and counting, and 20–25 μL sample buffer/track was applied to 1-mm thick gels to be stained. Stained tracks were scanned in a Gilford 20-cm gel scanning attachment for the Beckman DU spectrophotometer (0.5 mm slit, 550 nm). Correction was made for the background haze in the highly cross-linked gel by digitally subtracting a scan at 480 nm from that at 550 nm using an attached Hewlett Packard 9820 desk calculator. The baseline of a blank gel was 0.05 ± 0.05 o.d. after this subtraction.

Radioisotope Counting Procedures. Samples were treated as follows. Aqueous solutions were counted in ACS (Amersham/Searle). Nucleopore filters were dissolved in 1 mL of Protosol and counted in 10 mL of Econofluor (New England Nuclear). Polyacrylamide gel slices were digested in 0.5 mL of 30% H_2O_2 for 3–24 h at 80 °C and counted in 4 mL of ACS in minivials. Internal standards ([^{3}H]ouabain, calibrated with a [^{3}H]toluene standard) were used in all counting solutions. The specific activities of the cardiac steroid samples used throughout this study were 145, 47, 60, and 71 Ci/mol for [^{3}H]ouabain, and [^{3}H]NAB-ouabains I, II, and III, respectively.

Results

In preliminary experiments comparing [^{3}H]NAB-ouabains I, II, and III, the three compounds were found to bind to, inhibit, and photolabel the Na,K-ATPase in qualitatively the same manner. NAB-ouabain II was selected for most intensive study because it was obtained in highest purity and yield.

Binding of NAB-Ouabain and Inhibition of Na,K-ATPase. As shown in Figure 2A, when incubated with the Na,K-ATPase in the presence of MgP_i, [^{3}H]NAB-ouabain II bound to the enzyme and inhibited the subsequently measured activity. The number of binding sites for [^{3}H]NAB-ouabain II is the same as for [^{3}H]ouabain, 1.9 nmol/mg, approximately 30% more than the number of sites reported by Jorgensen (1974b) in enzyme of comparable activity. At [MgP_i] = 0.12 mM, the dissociation constants obtained from the Scatchard plots in Figure 2B are 3.6×10^{-8} and 2.2×10^{-8} M for ouabain and NAB-ouabain II, respectively. For NAB-ouabain I the K_d was 3.5×10^{-8} M (data not shown). These values are

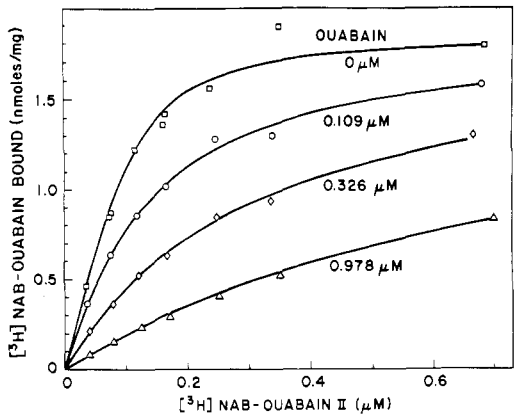

FIGURE 3: Competition of ouabain and [^{3}H]NAB-ouabain II. Binding as in Figure 2, except that [^{3}H]NAB-ouabain II was 0.03–0.7 μM and unlabeled ouabain was added to a final concentration of 0 (□), 0.109 (○), 0.326 (◇), or 0.978 (△) μM. The lines are theoretical curves for the competitive binding of two substrates to a homogeneous population of binding sites using [sites] = 1.9 nmol/mg, K_d(ouabain) = 0.26 μM and K_d([^{3}H]NAB-ouabain II) = 0.22 μM.

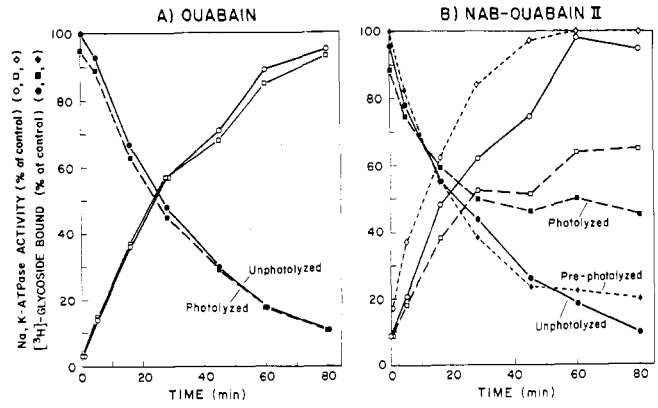

FIGURE 4: Reversibility of ouabain and NAB-ouabain II binding and inhibition. Na,K-ATPase (1.63 mg/mL) was incubated with 4 × 10^{-6} M cardiac steroid for 5 min at 37 °C in Na,MgATP binding medium (final volume 92 μL) and then diluted into (12 mL, 0 °C) 0.4% BSA, 60 mM imidazole. Samples were (□, ■) or were not (◇, ◆, ○, ●) photolyzed for 1 min. After warming rapidly to 37 °C at t = 0, duplicate samples were taken for immediate ATPase assay (0.25 mL; 8 min assay; open symbols) or for filtration on Nucleopore filters and scintillation counting (0.5 mL; 0.5 mL rinse with 10 mM KCl, 10 mM Tris, 0 °C; solid symbols). (A) Ouabain. (B) NAB-ouabain II. (◇, ◆) NAB-ouabain II was photolyzed prior to incubation with the enzyme.

comparable to those obtained by Hansen & Skou (1973) for ouabain binding to brain Na,K-ATPase at low [MgP_i]. In the presence of 120 mM Na, 3 mM MgATP, the dissociation constants for ouabain and NAB-ouabain II were approximately 6 × 10^{-9} and 1 × 10^{-8} M, and, as with MgP_i, inhibition of the enzyme was directly proportional to cardiac glycoside binding (data not shown).

In control samples in experiments such as that shown in Figure 2, addition of 10^{-4} M ouabain to the binding medium containing 0.6 μM [^{3}H]NAB-ouabain decreased [^{3}H]NAB-ouabain II binding to about 2%. A further test of competition is shown in Figure 3 where the effect of low concentrations of ouabain on [^{3}H]NAB-ouabain II binding is examined. The data fit the theoretical curves for competitive binding of two substances to a single site on the enzyme (cf. Best-Belpomme & Dessen, 1973, eq 1 and 2). Since the site and both substances are present in similar concentrations, a simple inverse type of analysis (e.g., Scatchard or Lineweaver–Burk) does not provide a theoretical linearization; instead when the transformation of Best-Belpomme & Dessen (1973, eq 7) is applied, the data satisfy the criteria of linearity (slope = 1.2; correlation coefficient = 0.993) and of intercept at the origin (Y intercept = 0.03).

To demonstrate reversibility of NAB-ouabain action, conditions were chosen that resulted in the most rapid dissociation of cardiac glycoside from the enzyme: binding in the presence of Na and MgATP followed by dilution into (or centrifugation and resuspension into) a buffered medium containing no Na, K, Mg, or ATP (cf. Wallick et al., 1977). As seen in Figure 4 (squares), both ouabain and NAB-ouabain II dissociate with a half-time near 20 min under these conditions and reversal of inhibition is 90% complete within 80 min.

Photoaffinity Labeling with NAB-Ouabain. When enzyme-bound NAB-ouabain II was photolyzed, 30–40% of the compound became irreversibly bound and remained inhibitory after dilution (Figure 4B). This was not due to an effect of light on the Na,K-ATPase because photolysis did not alter reversibility of [^{3}H]ouabain binding and inhibition (Figure 4A). When NAB-ouabain II was photolyzed before incubation with the enzyme its binding and inhibition were fully reversible showing that the prephotolyzed species is not able to covalently bind, but that photolysis must occur with the enzyme and photolabel present at the same time.

Table I presents the results of four experiments demonstrating that irreversible photolabeling is covalent, as judged by stability to treatment with 5% Cl_3CCOOH. Na,K-ATPase (0.8 mg) was incubated for 15 min at 37 °C in MgP_i binding medium (0.4 mL) with 10^{-6} M NAB-ouabain II, ouabain, or prephotolyzed NAB-ouabain II and then diluted into 15 mL of 120 mM NaCl, 0.1–0.4% BSA, 30 mM Tris (pH 7.5) containing in addition ouabain, NAB-ouabain II, or ouabain in A, B, and C, respectively. In this way all three incubation media contained the same final amounts of ouabain and NAB-ouabain II, but prior addition of ouabain prevented the binding of NAB-ouabain II to the enzyme in sample B. Photolysis did not alter the total amount of NAB-ouabain II bound (line 3), as was the case in the experiment of Figure 4B. Denaturation with 5% Cl_3CCOOH for 10 min dissociated the NAB-ouabain II enzyme complex in all unphotolyzed samples; however, when Cl_3CCOOH treatment followed photolysis, 35–41% of the label remained on the precipitated enzyme. In other experiments when the enzyme was washed on Nucleopore filters with either 20% dimethyl sulfoxide for 10 min at 70 °C, or with 50% dimethyl sulfoxide/20% acetic acid for 30 min at room temperature, the results were identical with treatment with 5% Cl_3CCOOH.

Pattern of Photolabeling. Photolyzed samples from one of the experiments summarized in Table I (line 3) were concentrated by centrifugation and the pellets were washed in binding medium (4 °C) to remove free photolyzed NAB-ouabain II and most of the BSA, then solubilized in $NaDodSO_4$ (Na,K-ATPase 1.3 mg/mL, NaI microsomes 2.0 mg/mL) and run on polyacrylamide gels. As shown in Figure 5 (peak a), NAB-ouabain II was bound to the large subunit of the enzyme (molecular weight ≃ 95 000), the ^{3}H in this peak accounting for 15% of the NAB-ouabain II originally bound to the enzyme. Prephotolyzed NAB-ouabain II that was only reversibly bound to the enzyme prior to $NaDodSO_4$ solubilization (sample C) migrated with the tracking dye, Pyronin Y, as did the photolyzed NAB-ouabain II that did not bind covalently in sample A (peak d). This is an important control, since Lane (1976) has shown that under certain gel conditions [^{3}H]ouabain alone may run at the position of the large polypeptide. Note furthermore that the counts bound to the large chain are not removed by staining and destaining in 30% MeOH/10%

TABLE I: Effect of Cl_3CCOOH on [^{3}H]NAB-Ouabain Binding (% of Control A or A′ ± SEM, n = 4).

	purified Na,K-ATPase			NaI microsomes		
binding medium:	A [^{3}H]NAB-ouabain	B ouabain	C photolyzed [^{3}H]-NAB-ouabain	A′ [^{3}H]NAB-ouabain	B′ ouabain	C photolyzed [^{3}H]-NAB-ouabain
diluted into:	ouabain	[^{3}H]NAB-ouabain	ouabain	ouabain	[^{3}H]NAB-ouabain	ouabain
control	100	4 ± 1	97 ± 2	100	8 ± 1	98 ± 2
+ Cl_3CCOOH	2 ± 1	2 ± 1	3 ± 1	4 ± 2	2 ± 1	6 ± 1
+ light	103 ± 1	8 ± 1	98 ± 4	101 ± 2	13 ± 2	102 ± 4
+ light + Cl_3CCOOH	41 ± 3	7 ± 3	5 ± 1	35 ± 1	5 ± 1	7 ± 2

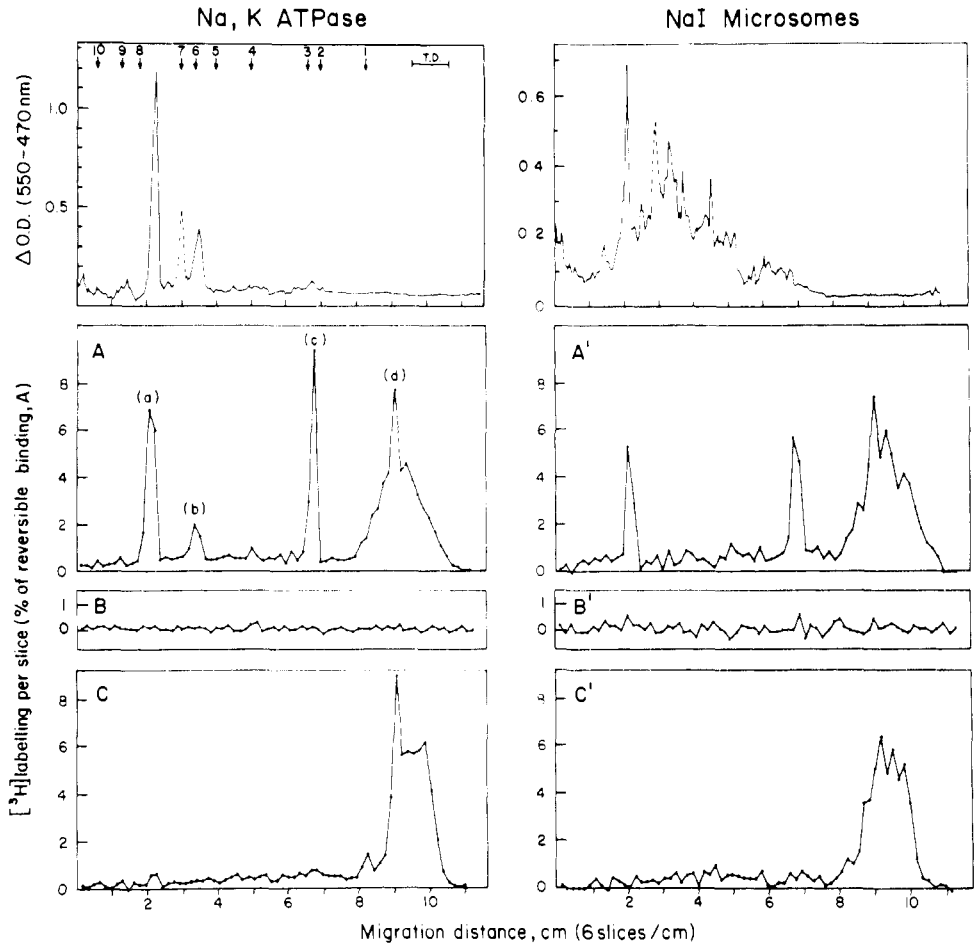

FIGURE 5: $NaDodSO_4$ gel electrophoresis of Na,K-ATPase and NaI microsomes covalently labeled with [^{3}H]NAB-ouabain II. As described in the text, the samples for electrophoresis were prepared from one of the experiments of Table I. Upper: Scans of Coomassie blue stained gels. The absorbance peak due to residual BSA is shown dotted. Molecular weight markers were as follows: (1) insulin, 5700; (2) cytochrome c, 12 300; (3-10) molecular weight standards (BDH Chemicals, Poole, England) 14 300, 29 000, 43 000, 57 000, 72 000, 106 000, 159 000, 212 000. T.D. marks the position of the tracking dye, Pyronin Y. Lower: Labeling per slice is plotted as the percent of total [^{3}H]NAB-ouabain II originally bound to sample A or A′ (Table I, line 1). In panel A, peaks are identified at the positions of (a) large polypeptide, (b) glycoprotein, (c) proteolipid, (d) free photolyzed [^{3}H]NAB-ouabain II.

acetic acid, in contrast to the counts at the bottom of the gel (see Figure 6, below).

Labeling at the position of the glycoprotein (peak b in Figure 5) was about 4% in sample A, although this component is usually considerably smaller ($\simeq$1%; see Tables II and III below). Since only minimal counts appear at this position in the $NaDodSO_4$ gel of NaI microsomes (Figure 5A′), the significance of the low level binding in the purified enzyme is questionable. Labeled fragments resulting from limited acid hydrolysis or from tryptic digestion of the large polypeptide have the same mobility as the glycoprotein (unpublished observations), suggesting the possibility of a small amount of proteolysis in the purified preparation.

When gels of high acrylamide concentration and high percent cross-linking were used as in Figures 5 and 6, a peak (c) of specifically bound NAB-ouabain II was resolved from the free NAB-ouabain II (d) at the bottom of the gel, corresponding to a band weakly stained with Coomassie blue. Radioactivity in the peak was about 13% of the originally bound NAB-ouabain II, and together with labeling at the position of the large polypeptide accounted for most of the Cl_3CCOOH stable counts. When compared with cytochrome c (molecular weight = 12 300) run in neighboring tracks, the polypeptide ran just ahead in low density gels and on the trailing edge in the highly cross-linked, high density gels. We have identified this material as an acidic proteolipid as defined by its solubility

in acidic chloroform/methanol (cf. Folch-Pi & Stoffyn, 1972): Following a slight modification of the procedure of MacLennan (1974), the polypeptide was found to remain with the solids when phospholipids were extracted with 90% MeOH (3 mg of protein in 0.8 mL of 250 mM sucrose, 7.2 mL of MeOH, 0 °C) followed by $CHCl_3$/MeOH 2:1 (3 mL, four times); it was then dissolved in four extractions with $CHCl_3$/MeOH acidified with 10 mM HCl (3 mL each). Like the proteolipid from sarcoplasmic reticulum (MacLennan, 1974), the proteolipid was extractable into neutral $CHCl_3$/MeOH (30% in four extractions) when the 90% MeOH step was omitted.

Figure 6 illustrates the results of the extraction procedure performed on Na,K-ATPase photolabeled with NAB-ouabain II. Coomassie blue stained gel tracks of the labeled enzyme before extraction, of the pellet remaining after extraction, and of the acidic $CHCl_3$/MeOH extract are shown in the photograph at the top, and the corresponding distributions of ^3H in an unstained, sliced, and counted companion gel are presented below. It is seen that [^3H]NAB-ouabain II covalently bound to the proteolipid is extracted into acidic $CHCl_3$/MeOH, whereas label on the large polypeptide remains in the pellet. A partial redistribution of protein and ^3H from peak a to peak b was also observed in the pellet, presumably as a result of acid hydrolysis of the large polypeptide during extraction. During staining and destaining of the gels about two-thirds of the counts at the position of the proteolipid were lost (Figure 6, uppermost radioactivity profile); this is probably due to diffusion of the proteolipid from the gel into the staining solution (30% MeOH/10% acetic acid), although we have no direct evidence on this point.

The Effect of Binding Conditions on the Pattern of Photolabeling. The rate of dissociation of ouabain from the Na,K-ATPase depends on ligands in the binding medium, e.g., MgP_i or Na,MgATP, and also on ligands present in the dissociation medium (cf. Yoda & Yoda, 1974). It was of interest to see if such differences in the ouabain–enzyme interaction would be reflected in differences in the photolabeling pattern with NAB-ouabain II. In the experiments presented in Table II, after binding NAB-ouabain II in the presence of either MgP_i or Na,MgATP, the enzyme was filtered on Nucleopore filters and washed for 10 min with the same medium containing in addition 10^{-4} M ouabain (rinse A, 4 °C). The rinse solution was then changed to rinse B (37 °C) for 1–10 min; control experiments had shown the half-time of ouabain dissociation to be about 20 min under the rinse B conditions of lines 3 and 8, and 90–120 min for the other conditions. Following a 1-min photolysis, and a final 5-min rinse with 10 mM KCl, 10 mM Tris (4 °C), the protein was solubilized in $NaDodSO_4$ and run on gels. Labeling of the three components identified on gels is expressed as percent of total ^3H counts in the solubilized samples (i.e., as a percent of reversible binding to the enzyme. As shown in Table II, there were changes in the labeling pattern under the condition of line 3 compared with the control in line 1, and under the conditions of lines 7, 8, and 9 compared with the control in line 4. These differences presumably reflect conformational changes of the enzyme–NAB-ouabain complex during rinse B, but it would be premature to speculate how they may be related to changes in glycoside dissociation rates and specificities under similar conditions (Yoda & Yoda, 1974).

Comparison of Photolabeling from NAB-Ouabain I, II, and III. The relative effectiveness of NAB-ouabains I, II, and III as photolabels was compared in experiments similar to those just described. As seen in Table III, whereas the three compounds photolabeled the proteolipid to about the same level, NAB-ouabain I was less than half as effective as NAB-ouabain II in photobinding to the large polypeptide. This is surprising

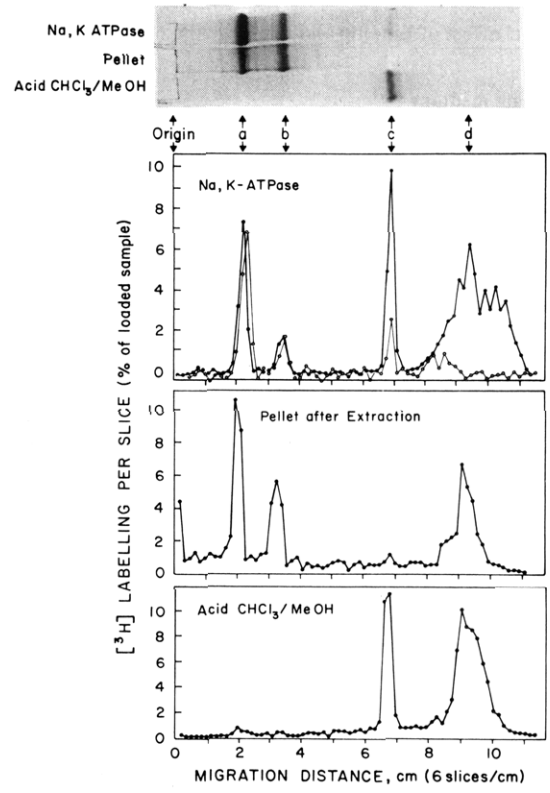

FIGURE 6: $NaDodSO_4$ gel electrophoresis showing the results of acidic $CHCl_3$/MeOH extraction of Na,K-ATPase. Na,K-ATPase (7 mg) was incubated (37 °C, 15 min) in MgP_i binding medium (5 mL) with 10 nmol of [^3H]NAB-ouabain II, diluted to 35 mL in binding medium and pelleted (2 h, 350 000g), resuspended in 2.5 mL of 10 mM Tris, and photolyzed for 1 min. After addition of sucrose (to 250 mM) 3 mg of protein was extracted as described in the text. Fractions from the extraction sequence were dried on a rotary evaporator and dissolved in sample buffer (5 min, 100 °C), and run on polyacrylamide gels as in Figure 5. Tracks of the 90% MeOH and $CHCl_3$/MeOH extractions are not shown; they exhibited no visible staining and contained less than 5% of the counts that ran behind photolyzed NAB-oubain II. Peaks a–d are identified as in Figure 5. Upper: photograph of Coomassie blue stained gel tracks. Lower: Labeling is plotted as radioactivity per slice as a percent of [^{3}]NAB-ouabain applied to each gel track. (●) Slices without staining; (○) slices after staining and destaining.

in view of the proposed similarity in chemical structure of the two compounds (Figure 1); apparently the small difference in the linkage of the diamine to the sugar translates into a large difference in the interaction of the NAB group with the binding site. A direct evaluation of the labeling with NAB-ouabain III is not possible, because of the impurity of that compound.

Discussion

In this paper we have reported the synthesis of a new photoaffinity derivative of ouabain, NAB-ouabain; we have shown that it binds to and inhibits the Na,K-ATPase in the dark (Figure 2), that it is competitive with ouabain (Figure 3), and that on photolysis 30–40% of the tritiated compound becomes covalently bound to the enzyme (Table I and Figure 4). The label is approximately evenly distributed between the large polypeptide, or "catalytic subunit", and a small proteolipid that was not previously known to be directly associated with the Na,K-ATPase (Figure 5).

The photolabeling efficiency of 30–40% reported here is relatively high compared with that of most previous investigations in which both the amount of reversible binding and the extent of covalent labeling by aryl azide derivatives have been determined; the efficiency has usually been found to be between 10% and 30% (cf. Haley, 1975; Katzenellenbogen et al.,

TABLE II: Effect of Binding and Rinse Media on Labeling of Na,K-ATPase Components by [^{3}H]NAB-Ouabain II.

binding medium and rinse A[a]	rinse B[b]	time rinse B (min)	labeling on gels (% of total binding ± SEM)[c] large polypeptide	glycoprotein	proteolipid
1 Na,MgATP	no change	7	18.3 ± 0.9 (3)	1.2 (1)	15.5 ± 0.6 (3)
2 Na,MgATP	KCl	6	14.8 ± 0.8 (3)	1.1 (1)	13.7 ± 0.9 (3)
3 Na,MgATP	buffer	1	11.8 ± 0.1 (3)	0.8 (1)	12.9 ± 1.5 (3)
4 MgP_i	no change	10	15.3 ± 0.8 (5)	1.0 (1)	14.1 ± 1.2 (5)
5 MgP_i	K,MgATP	6	16.2 ± 0.0 (3)	1.1 (1)	15.9 ± 0.2 (3)
6 MgP_i	Na,MgATP	6	15.9 ± 0.7 (3)	0.4 (1)	14.8 ± 1.0 (3)
7 MgP_i	K,ATP	5	17.0 ± 2.0 (2)	0.6 ± 0.2 (2)	9.8 ± 0.2 (2)
8 MgP_i	Na,ATP	5	19.0 ± 0.5 (6)	0.7 ± 0.3 (6)	11.6 ± 0.9 (5)
9 MgP_i	buffer	5	14.9 ± 0.9 (2)	0.3 ± 0.1 (2)	7.2 ± 0.2 (2)

[a] Binding (10 min, 37 °C) was performed as in Methods with 0.45 μg of Na,K-ATPase, 1.5 nmol of [^{3}H]NAB-ouabain in 0.9 mL; 0.25 mL was filtered on each Nucleopore filter. [b] Rinse media contained, in addition to 60 mM imidazole and 1 mM EDTA, buffer with no additions; KCl–25 mM KCl; K,MgATP–5 mM KCl, 2 mM MgATP; Na,MgATP–120 mM NaCl, 2 mM MgATP; K,ATP–5 mM KCl, 2 mM ATP; Na,ATP–120 mMNaCl, 2 mM ATP. In lines 1 and 4, rinse A was continued for the rinse B time period. [c] Total binding was determined as ^{3}H in the $NaDodSO_4$ solubilized sample. This amounted to >90% of the reversible binding before the rinse sequence in all cases, except line 8 where it was about 80% of the original reversible binding. Data are presented as mean ± SEM of n single experiments, except in line 8 where two sets of triplicates were used.

TABLE III: Labeling of Na,K-ATPase Components by [^{3}H]NAB-Ouabains I, II, and III.

[^{3}H]-NAB-ouabain	binding & rinse medium[a]	covalent labeling (% of total binding ± SEM)[b] large polypeptide (n = 2)	glycoprotein (n = 1)	proteolipid (n = 2)
I	Na,MgATP	6.2 ± 0.6	0.9	13.2 ± 2.2
	MgP_i	6.0 ± 0.8	0.9	14.8 ± 4.0
II	Na,MgATP	16.8 ± 3.2	1.7	17.4 ± 1.7
	MgP_i	15.0 ± 1.8	1.5	18.2 ± 0.8
III	Na,MgATP	9.5 ± 0.7	0.6	18.2 ± 0.0
	MgP_i	9.2 ± 0.4	0.7	16.4 ± 1.5

[a] Binding conditions as in Table II. The rinse media (10 min, 4 °C) contained 10^{-4} M ouabain and no NAB-ouabain in addition to constituents of the binding media. [b] Binding is expressed as in Table II.

1977; Cooperman, 1976) and exceptionally 60% (Bridges & Knowles, 1974). In order to increase incorporation some workers have used repetitive cycles of binding-photolysis-wash, each time using fresh photolabel (cf. Cooperman & Brunswick, 1973; Lau et al., 1977). Failure of the aryl azides to give higher yields of attachment to polypeptides has been attributed to other pathways of reaction of the photo-produced nitrene, most likely through reaction with water (Knowles, 1972; Cooperman, 1976).

We have demonstrated the specificity of photolabeling of the Na,K-ATPase by NAB-ouabain by taking advantage of the slow reversibility of cardiac glycoside binding. For example, in the experiments of Table I and Figure 5 the only difference between the labeled sample and the control was the order of addition of ouabain or NAB-ouabain, the total concentrations at photolysis being the same in both samples. This type of experiment is not feasible in the more usual case of a rapidly reversible ligand–enzyme interaction. In other experiments free NAB-ouabain was removed by extensive washing of the enzyme on filters or by centrifugation prior to photolysis, so that 98% of reversibly bound NAB-ouabain was competitive with ouabain. When this material was photolyzed (e.g., Tables II and III), labeling was due to NAB-ouabain which was specifically bound to the ouabain site.

While the absolute determinants of inhibitory potency of cardiotonic steroids are found in the lactone and steroid ring system, the presence of the glycoside moiety slows the dissociation rate by two orders of magnitude compared with the aglycones (Yoda & Yoda, 1977); up to three sugar rings and their substituents may be involved in the interaction (Yoda & Yoda, 1975). Thus the ouabain binding site can be considered to consist of a primary binding site that recognizes the aglycone, and a secondary area that binds the sugar portion of the inhibitor. Since the NAB group in NAB-ouabain is at approximately the same distance from the steroid as is the terminal sugar in a cardiac triglycoside (e.g., digitoxin), NAB-ouabain probably photolabels the secondary area.

Covalent labeling of the Na,K-ATPase by NAB-ouabain II is equally distributed between the large chain and a proteolipid, and the question may be raised as to whether the primary ouabain binding site is on one or the other of these components or is in a cleft between the two. To approach this problem a derivative with the photoaffinity group near the lactone ring would be desirable, although it is not clear how such a compound can be made with good inhibitory potency. Covalent attachment of oxidized ouabain and of derivatives of cymarin may involve reaction of groups closer to the steroid than the NAB group of NAB-ouabain; however, due to considerable nonspecific labeling and lack of resolution of low molecular weight peptides in the polyacrylamide gels, it is not possible to tell from the published data if specific labeling of the proteolipid occurred with these compounds (Ruoho & Kyte, 1974; Hegyvary, 1975). Our own work indicates that, when [^{3}H]ouabain bound to Na,K-ATPase is photolyzed in 254 nm ultraviolet light, an exposure similar to that of Ruoho & Kyte (1973), a small fraction becomes covalently bound to the large chain, with no labeling of the glycoprotein or proteolipid (Forbush & Hoffman, manuscript in preparation). Thus, in agreement with the previous authors, our results are consistent with the large polypeptide as the locus of the primary ouabain binding site.

We have considered the possibility that the labeled material identified here as proteolipid is not a separate entity in native Na,K-ATPase, but is a cleavage product of some component of the enzyme, perhaps the large polypeptide. If proteolysis occurred during purification of the enzyme, it would be expected that the ratio of ^{3}H in the large polypeptide peak to ^{3}H in the proteolipid peak would depend on the method of puri-

fication; however, as seen in Figure 5 (compare A and A′) the labeling ratio for purified Na,K-ATPase is similar to that for NaI microsomes. Also, proteolysis during storage does not appear to be a factor since the labeling ratio was the same in an experiment conducted 45 days after purification (ratio = 1.15; Figure 5A) as in a duplicate experiment performed 3 days after purification (ratio = 1.12, data not shown). Production of a labeled fragment by a photolytic or photocatalytic process is unlikely, since the appearance of the low molecular weight band in Coomassie blue stained gels of purified Na,K-ATPase does not depend on photolysis or on the presence of NAB-ouabain; the stained band comigrates with photolabeled material in two gel systems (Tris/sodium acetate, pH 7.4; Tris-acetate/cacodylate, pH 5.5, unpublished observations) at various acrylamide concentrations, and coextracts in organic solvents. Fragmentation of the large polypeptide on NaDodSO_4 gels is also improbable since, when labeled large polypeptide isolated by small scale NaDodSO_4 gel electrophoresis was rerun on an NaDodSO_4 gel, no labeling was found below the position of the large polypeptide (unpublished data). Finally, loss of a 12 000 molecular weight fragment from the large polypeptide would be expected to leave an 83 000 molecular weight piece which might then be degraded further. We have not observed a peak near 83 000 on NaDodSO_4 gels, although we would be able to detect an amount of Coomassie blue staining $\lesssim$3% of the large polypeptide.

Proteolipids, i.e., proteins that are extracted into chloroform/methanol from water (cf. Folch-Pi & Stoffyn, 1972), have been shown to be components of the proton translocating ATPase of mitochondria (Steckhoven et al., 1972), chloroplasts (Nelson et al., 1977), and bacteria and yeast (Sierra & Tzagoloff, 1973) and of the Ca-ATPase of sarcoplasmic reticulum (MacLennon et al., 1973). Racker & Eytan (1975) have shown that the proteolipid of the Ca-ATPase causes an increased Ca^{2+} permeability when it is reconstituted into phospholipid vesicles and Nelson et al. (1977) have noted that the chloroplast ATPase proteolipid acts as an *N,N′*-dicyclohexylcarbodiimide-sensitive proton channel in reconstituted liposomes. Racker (1976) has suggested that proteolipids may form the functional ion translocating channel both in the above ATPases and in the Na,K-ATPase. Prior to the present investigation, however, there was no direct evidence that proteolipid is a component of the Na,K-ATPase. In NaDodSO_4-polyacrylamide gels of their purified Na,K-ATPase, Hokin et al. (1973) found that material running below bromophenol blue in the Laemmli gel system constituted the only significant Coomassie blue-staining band other than the large polypeptide and the glycoprotein; however, they considered it an impurity and did not pursue it further. Lane et al. (1973) and Jorgensen (1974b) did not notice low molecular weight material in their preparations; however, Racker (1976) has reported a 10 000 molecular weight polypeptide, and Dowd et al. (1976) found phosphorylation by exogenous protein kinase of an 11 700 molecular weight component in a preparation of Na,K-ATPase from beef heart. It should be noted that in gels of low acrylamide concentration the proteolipid runs as a diffuse band near or with the tracking dye; furthermore, the Coomassie blue stain may be removed from the band if the gels are destained overly long. While the proteolipid is usually the third most prominently staining band on NaDodSO_4 gradient gels of our Na,K-ATPase purified according to Jorgensen (1974a), this is only weak evidence that it is an integral part of the enzyme. Rather, this paper establishes that the proteolipid is a component of the Na,K-ATPase because of its close association or identity with the NAB-ouabain binding site. The fact that NaI microsomes exhibit the same labeling pattern as the highly purified enzyme demonstrates that the association of the proteolipid with the Na,K-ATPase is not an artifact of purification.

A photoaffinity labeling analogue of ATP, 8-azido-ATP, has previously been synthesized in this laboratory and shown to be a good substrate for Na,K-ATPase in human red cell ghosts and for Na,K-ATPase purified from duck salt gland (Haley & Hoffman, 1974). It is anticipated that an approach using NAB-ouabain, 8-azido-ATP, and phosphorylation of the enzyme from [^{32}P]ATP or $^{32}P_i$ will provide information as to the structural and functional interactions of the subunits of Na,K-ATPase.

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CORRECTIONS

Fluorescence-Quenching Study of Glucose Binding by Yeast Hexokinase Isoenzymes, by Isaac Feldman* and Douglas C. Kramp, Volume 17, Number 8, April 18, 1978, pages 1541–1547.

On page 1542, in line 2 of the caption to Figure 1, E_0 should equal 142 μg/mL, not 42 μg/mL as printed. On page 1543, in line 2 of the caption to Figure 4, E_0 should equal 111 μg/mL, not 11 μg/mL as printed.

Size and Shape of the Model Lipoprotein Complex Formed between Glucagon and Dimyristoylglycerophosphocholine, by Andrew J. S. Jones, Richard M. Epand,* K. Frank Lin, D. Walton, and W. J. Vail, Volume 17, Number 12, June 13, 1978, pages 2301–2307.

The equation on the last line of page 2305 should read:

$$(\bar{v}_2 + \delta_1 v_1^0)$$

A previous correction to this article (*Biochemistry 17*(*15*), 3176) was in error.