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Research Article

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Ouabain-Specific Antibodies: Immunochemical Properties and Reversal of Na^+, K^+-Activated Adenosine Triphosphatase Inhibition

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ABSTRACT Antibodies with high affinity and specificity for the cardiac glycoside ouabain were raised in rabbits. The antigen used was a conjugate of ouabain linked through its rhamnose moiety to terminal α-amino groups of poly D,L alanyl-human serum albumin. Ouabain-specific antibodies were present as early as 3 wk, and rose steadily in titer over the initial 20–33 wk of immunization. Levels as high as 6.5 mg specific immunoglobulin per ml antiserum were reached in one rabbit at the end of 45 wk. The average intrinsic association constants for ouabain were 1.3×10^9 M^{-1} and 1.6×10^9 M^{-1} in antisera studied in detail, and there was evidence of restricted heterogeneity of binding site affinities. A high degree of specificity was demonstrated. Significant cross-reactivity occurred only with other cardioactive steroid compounds such as acetyl strophanthidin, digoxin, and digitoxin, while endogenous steroids did not cross-react even when present in 1000-fold excess. A rapid and convenient radioimmunoassay procedure for plasma or urine ouabain concentrations was developed using these antibodies. Competition between ouabain-^3H tracer and unlabeled ouabain for specific antibody binding sites allowed the measurement of ouabain concentrations as low as 0.1 ng/ml or less without need for extraction procedures. The high association constants observed in these studies permit antibody reversal of established myocardial effects of ouabain. Both blockade and reversal of ouabain inhibition of canine myocardial microsomal Na^+, K^+-activated ATPase by antibody were documented, suggesting a possible mechanism for reversal of cellular effects.

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INTRODUCTION

The rapidly acting cardioactive steroid glycoside ouabain has been widely used in both clinical and physiological investigations, as well as in the management of patients with congestive heart failure and certain cardiac rhythm disturbances. To the present time, however, the only means of quantitating the minute concentrations of the drug present in plasma or urine of animals or human subjects given usual pharmacologic doses has been the administration of tritiated ouabain (ouabain-^3H) with subsequent measurement of radioactivity by liquid scintillation counting (1–3).

This report describes the immunization of rabbits with a conjugate of ouabain covalently linked to poly D,L alanyl-human serum albumin. Ouabain-specific antisera obtained after this challenge were characterized in terms of affinity, heterogeneity, specificity, and time course of rise in titer. A specific and convenient radioimmunoassay was developed which permits the measurement of picomolar concentrations of ouabain in biological fluids including plasma and urine.

The high association constants observed enhance the sensitivity of the assay and allow antibody reversal of established cellular effects of ouabain (4). In order to explore the mechanism of antibody reversal of cardiac glycoside effects on myocardium (4–8), blockade and reversal of ouabain inhibition of canine myocardial microsomal Na^+, K^+-activated adenosine triphosphatase (Na^+, K^+ ATPase)[1] by ouabain-specific antibody were also studied.

METHODS

Crystalline ouabain and acetyl strophanthidin were donated by Dr. C. T. Chiu, Eli Lilly & Co. (Indianapolis, Ind.).

[1] *Abbreviations used in this paper:* HSA, human serum albumin; Na^+, K^+ ATPase, Na^+, K^+-activated adenosine triphosphatase; PBS, phosphate-buffered saline.

Crystalline digoxin and digitoxin were the gifts of Dr. Stanley T. Bloomfield, Burroughs Wellcome and Co. (U. S. A.), Inc. (Tuckahoe, N. Y.), and Dr. Paul Boyles, Wyeth Laboratories (Philadelphia, Pa.), respectively.

Ouabain-^{3}H (11.7 Ci/mmole) was obtained from New England Nuclear Corp., Boston, Mass.

Cortisol, cholesterol, dehydroepiandrosterone, 17β estradiol, progesterone, and testosterone were kindly supplied by Prof. Lewis Engel.

Activated charcoal (Norit, neutral) coated with dextran of molecular weight 80,000 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) was prepared according to Herbert, Lau, Gottlieb, and Bleicher (9) except that the volume of buffer in which the coated charcoal was suspended was decreased to provide a fourfold greater amount of charcoal per unit volume.

Crystalline human and bovine serum albumins were obtained from Pentex, Inc., Kankakee, Ill.

Pyruvate kinase-lactic dehydrogenase suspension, reduced nicotinamide adenine dinucleotide (NADH), phosphoenol pyruvic acid, and tris-ATP were obtained from Sigma Chemical Co., St. Louis, Mo.

Other reagents used were of the highest available commercial purity and were used without further purification. Glass double distilled water was used throughout all experiments.

Statistical evaluations of all data, including least squares linear regression analyses, were performed by conventional techniques (10) with the aid of an IBM 360-65 time sharing computer.

Synthesis of antigen

A summary of the sequence of reactions used in this synthesis is shown in Fig. 1. A modified carrier protein for the ouabain hapten was synthesized by reaction with N-carboxy D,L alanine anhydride by minor modifications of the method of Schechter, Bauminger, Sela, Nachtigal, and Feldman (15). 3 g of human serum albumin (HSA) were dissolved in 240 ml 0.05M Na_2HPO_4 adjusted to pH 7.0 with H_3PO_4. 5 g of fresh N-carboxy D,L alanine anhydride (Pilot Chemical Co., Watertown, Mass.) were then dissolved in 50 ml anhydrous dioxane. A small amount of insoluble residue was removed by brief centrifugation and the supernatant solution added dropwise to the HSA solution at 0° in an iced beaker. This mixture was allowed to react for 24 hr with magnetic stirring at 4°C, followed by exhaustive dialysis against seven changes of 4 liters of distilled water at 4°C. The resulting slightly cloudy solution was adjusted to pH 7.0 with 1 M NaOH and centrifuged for 15 min at 15,000 *g*. The clear supernatant solution was lyophilized and the resulting colorless poly D,L alanyl-HSA stored at − 20°C.

This product was characterized by amino acid analysis before and after deamination with nitrous acid (16) to determine the number and length of poly D,L alanine side chains added to the HSA carrier. 6 mg of poly D,L alanyl-HSA was dissolved in 0.5 ml distilled water and 1.5 ml of a saturated aqueous solution of $NaNO_2$ added, followed by addition of 0.5 ml glacial acetic acid. The mixture was then allowed to stand overnight at room temperature. After exhaustive dialysis against distilled water, the preparation was lyophilized. Samples of this deaminated product and of poly D,L alanyl-HSA were then hydrolysed with constant boiling HCl in evacuated sealed tubes at 110°C for 24 hr. Amino acid composition was determined by the method of Spackman, Stein, and Moore (17) with a Beckman Model 120B amino acid analyzer (Beckman Instruments, Inc., Fullerton, Calif.). The change in recovery of the multifunctional initiator lysine showed that an average of 50 poly D,L alanyl side chains had been attached per HSA molecule. The increase in alanine content indicated an average poly D,L alanyl side chain length of 9.5 residues.

Ouabain was then coupled to terminal α-amino groups of this carrier by Schiff base formation after periodate oxidation of vicinal hydroxyl groups on the rhamnose moiety of ouabain (14, 18, 19). 1 g poly D,L alanyl-HSA was dissolved in 36 ml distilled H_2O, adjusted to pH 9.5 with 5% K_2CO_3. Ouabain, 591 mg, was separately dissolved in 36 ml H_2O; 0.125 mCi ouabain-^{3}H was included to facilitate quantitation of coupling yield. 36 ml of freshly dissolved 0.1 M aqueous $NaIO_4$ was then added to the ouabain solution dropwise with magnetic stirring at room temperature. After 45 min, 1.1 ml 1 M ethylene glycol was added and allowed to react for 15 min. This reaction mixture was then added dropwise to the poly D,L alanyl-HSA solution and allowed

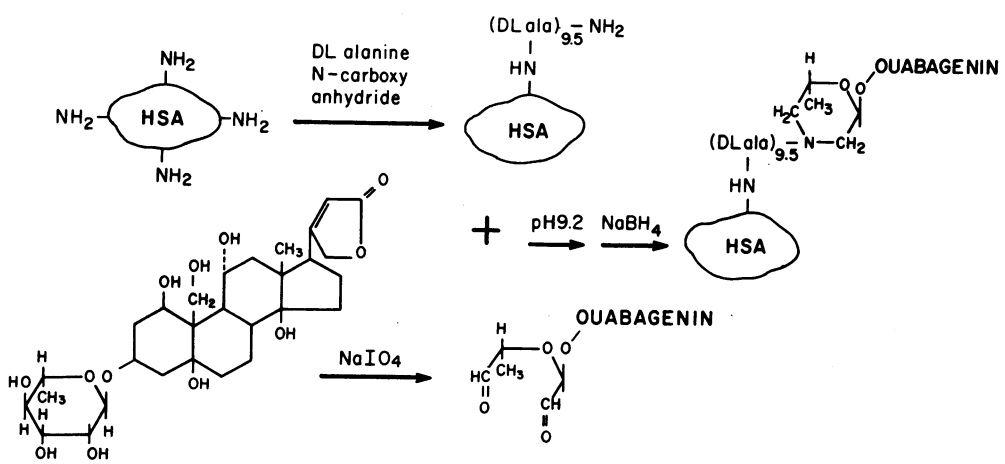

FIGURE 1 Sequence of steps used in synthesis of ouabain-poly D,L alanyl-HSA antigen. The rhamnose C_3' carbon is released as formic acid by periodate (11). Evidence for coupling through the morpholino derivative has been presented previously (12–14).

to react at room temperature for 1 hr, with addition of sufficient 5% K_2CO_3 to maintain the pH between 9.0 and 9.5. 0.54 g $NaBH_4$ freshly dissolved in 36 ml H_2O was then added. After 3 hr, 1 M formic acid was slowly added to lower the pH to 6.5. Evolution of gas had subsided after 1 hr and the pH was raised to 8.5 by addition of 1 M NH_4OH. Preliminary purification of the product was achieved by dialysis against six 6-liter changes of distilled H_2O at 4°C over a period of 48 hr. After lyophilization, 250 mg of the resultant colorless, fluffy product was dissolved in 10 ml 0.05 M NH_4HCO_3 and chromatographed on a 50×2 cm column of Sephadex G-75 (Pharmacia Fine Chemicals, Inc.) previously equilibrated with 0.05 M NH_4HCO_3. 95% of the ouabain (identified and quantitated by the tritium label) emerged in the void volume coupled to poly D,L alanyl-HSA while 5%, representing noncoupled ouabain which had not been removed by dialysis, eluted in the salt volume.

Measurements of tritium radioactivity were made with a Packard Model 3003 liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.), using a Triton X-100-toluene (2:1 v:v) based scintillation medium containing 5.5 g 2,5-diphenyl oxazole and 0.1 mg 1,4 bis-[2-(4-methyl-5-phenoxyazolyl)] benzene per liter. Analysis of ouabain-^{3}H content of weighed samples of the lyophilized ouabain-poly D,L alanyl-HSA product showed that an average of 4–5 moles of ouabain were coupled per mole of carrier.

Immunization procedure

Ouabain-poly D,L alanyl-HSA (2 mg/ml) was dissolved in 0.85% NaCl and an emulsion formed with an equal volume of complete Freund's adjuvant which contained 1 mg/ml killed tubercle bacilli.

Immunization of three New Zealand albino rabbits was initiated by injection of 0.1–0.2 ml of the antigen preparation into each of four toe pads weekly for 4 wk, followed by i.m. injections of 0.4 ml every 2 wk. Animals were bled at 2-wk intervals just before booster injections and serum was separated and stored at −20°C.

Characterization of antisera

Determination of titers. Detection and quantitation of ouabain-binding antibody in sera from rabbits challenged as described above was carried out as follows. Ouabain-^{3}H was diluted with 95% ethanol to a concentration of 4×10^{-8} g/ml. 50 μl of this solution (containing 2 ng ouabain-^{3}H) was then added to 1 ml volumes containing 0.5 ml normal human plasma (citrate anticoagulant) and 0.5 ml phosphate buffered saline (PBS) (0.01 M Na_2HPO_4, 0.15 M NaCl, adjusted to pH 7.4 with H_3PO_4). Heparin anticoagulated plasma and serum gave results identical to those obtained with the citrate anticoagulant. 50 μl of antiserum serially diluted with PBS was then added and the mixture allowed to incubate at room temperature for 30 min. Dextran-coated charcoal (0.3 ml) was then added to selectively adsorb free ouabain-^{3}H. After 5 min the charcoal was firmly pelleted by centrifugation at $8{,}000 \times g$ for 15 min and the supernatant phase containing antibody-bound ouabain-^{3}H was decanted into 15 ml toleuene-detergent base liquid scintillation medium (Instagel, Packard Instrument Co., Inc.) and counted. Quenching correction was made by subsequent addition of a known amount of ouabain-^{3}H or by the use of a ^{226}Ra external standard. The percentage of ouabain-^{3}H tracer bound by antibody was then plotted as a function of the dilution of antiserum. The titer of an individual antiserum was defined as the dilution at which 50% of total tracer counts were bound.

Determination of antibody concentration and affinity. Since antibody titer is a function of both concentration and binding characteristics, further methods were necessary to determine concentration of ouabain-specific antibody as well as to measure the average intrinsic association constant (K_o). Two techniques were used for this purpose. Equilibrium dialysis was carried out as previously described (19), using 0.2–1 μl antiserum in 1 ml buffer volume on one side of the dialysis membrane and amounts of ouabain-^{3}H varying from 10^{-10} to 10^{-8} g on the other. Another method was developed in which dextran-coated charcoal was used to achieve separation of antibody-bound from free ouabain-^{3}H. This method yielded similar data to those obtained by equilibrium dialysis, with considerably greater rapidity and convenience. Constant amounts of antiserum (0.2–1 μl) were added to test tubes containing varying amounts of ouabain-^{3}H (10^{-8}–10^{-10} g) in 1 ml of the 0.5 ml plasma-0.5 ml PBS system described above. After a 1 hr incubation at room temperature, dextran-coated charcoal was added and centrifuged, also as described above. Portions of the supernatant phase were counted to determine the antibody-bound ouabain-^{3}H concentration. Control tubes containing serum obtained from New Zealand albino rabbits before immunization were handled in the same way to allow correction for variation in charcoal binding with varying concentrations of free ouabain-^{3}H. Free ouabain-^{3}H concentration at equilibrium was calculated from the difference between total counts added and bound counts recovered in the supernatant solution.

Antibody concentration was estimated by the use of reciprocal bound vs. reciprocal free plots (20). Average intrinsic association constants and heterogeneity indices were obtained by the use of the modified Sips equation (20–22), $\log [r/(n-r)] = a \log C + a \log K_o$, where r equals moles of hapten bound per mole of antibody at free hapten concentration C; n equals moles of hapten bound at binding site saturation; and a is the heterogeneity index, reflecting distribution of association constant values about the average intrinsic association constant K_o.

Ouabain radioimmunoassay

In order to quantitate picomolar concentrations of unlabeled ouabain in plasma, conditions were selected under which ouabain-^{3}H tracer and unlabeled ouabain would compete for a limited number of antibody binding sites. The procedure is outlined schematically in Fig. 2.

The following protocol was designed to achieve good resolution over a concentration range from 0.1 to 10 ng/ml, using 0.5 ml plasma volumes. If desired, ouabain-^{3}H tracer and antibody quantities can be adjusted to allow assay of smaller plasma portions (down to 0.1 ml or less) or measurement of still lower ouabain concentrations. To a 1 ml volume containing 0.5 ml of unknown or standard plasma was added 0.5 ng of ouabain-^{3}H in 50 μl 95% ethanol. Plasma standards were made up at the time of each assay by the addition to the system of 10 μl of 95% ethanol containing 0, 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, or 5 ng ouabain, prepared by serial dilution of a 1.00 mg/ml solution. An amount of ouabain-specific antibody (12 wk bleeding from rabbit B) in 50 μl PBS was then added which would bind 30–40% of the ouabain-^{3}H label in the absence of any competing ligand, and the mixture allowed to incubate for 30 min at room temperature. Dextran-coated charcoal was added and, after 5 min, centrifuged as described above. The supernatant

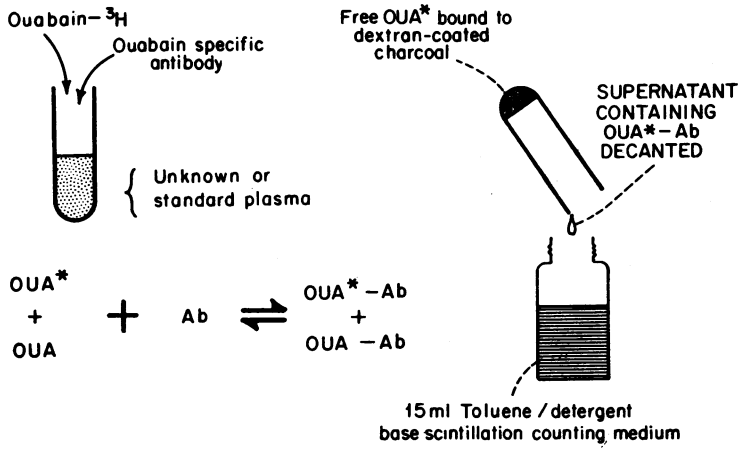

FIGURE 2 Outline of ouabain radioimmunoassay procedure. OUA*, ouabain-^{3}H; Ab, ouabain-specific antibody. Details of procedure as described in text.

phase was then decanted into 15 ml Instagel liquid scintillation medium and counted for 2 min, followed by a 1 min ^{226}Ra external standard count for quenching correction. Blanks were also included in which 50 μl PBS were added without antibody, to allow correction for the small amount of free ouabain-^{3}H tracer not bound by the dextran-coated charcoal. Ouabain concentrations in unknown samples were determined by comparison with a simultaneously run standard curve, as described in the Results section.

Portions of urine between 1 and 500 μl can be assayed in this system by substitution of an appropriate volume of urine for PBS. The size of the portion selected should be such that a portion of the standard curve with optimal resolution is employed. Since urinary ouabain concentrations are approximately two orders of magnitude greater than plasma concentrations at comparable times, it has not been necessary in our pharmocokinetic studies (45) to use urine portions greater than 50 μl.

Hapten inhibition studies

Specificity of the assay method was assessed by addition to the system of varying concentrations of several potentially competing ligands before addition of antibody. Compounds tested included the cardiac glycosides digoxin and digitoxin as well as the cardioactive aglycone acetyl strophanthidin, and the endogenous steroids cholesterol, cortisol, dehydroepiandrosterone, 17β estradiol, progesterone, and testosterone. The radioimmunoassay procedure described above was then completed.

Na^+, K^+ ATPase studies

Partial purification of myocardial microsomal Na^+, K^+ ATPase was carried out as described by Schwartz, Nagano, Nakao, Lindenmayer, and Allen (23). Dogs were sacrificed and hearts were immediately removed and placed in ice. Minced left ventricular myocardium was subjected to two deoxycholate extractions followed by treatment with sodium iodide. The resulting preparations had a specific activity of 10–15 μmoles inorganic phosphate liberated per mg protein per hour, with 90–95% of total ATPase activity inhibitable by 10^{-4} M ouabain. Enzyme protein concentrations were measured by the method of Lowry, Rosebrough, Farr, and Randall (24).

Studies of the effects of ouabain-specific antibodies on ouabain inhibition of Na^+, K^+ ATPase were carried out using a linked enzyme assay which allowed continuous recording of ATPase activity (23, 25). A Gilford Model 240 recording spectrophotometer was used, thermostated at 37° by a Haake constant temperature bath. Assay cuvettes contained 5 mM Mg Cl_2, 100 mM NaCl, 25 mM tris-HCl (pH 7.4), 2.5 mM tris-ATP, 0.6 mM NADH, and 2.5 mM phosphoenolpyruvic acid in a final volume of 2.0 ml. An ammonium sulfate suspension of pyruvate kinase and lactic dehydrogenase (Sigma Chemical Co.) was added in a volume of 20 μl. The resulting 22 mM NH_4 concentration acted as a K^+ analog, allowing activation of the K^+-dependent Na^+, K^+ ATPase and pyruvate kinase enzyme systems.

Ouabain-specific antibody used in blockade and reversal experiments was added in the form of whole antiserum or as a γ-globulin fraction obtained by precipitation with 33% ammonium sulfate and re-solution in PBS. Identical results were obtained with both types of antibody preparations.

About 10 μg enzyme protein was added to the cuvette and the initial Na^+, K^+ ATPase activity determined by the rate of conversion of NADH to NAD^+, recorded at 340 nm. In experiments designed to test the ability of ouabain-specific antibody to block ouabain inhibition of Na^+, K^+ ATPase, specific antibody or nonspecific control rabbit serum was added in a volume of 50 μl after 30 min, followed by ouabain to a final concentration of 5×10^{-7} M after an additional 50 min of stable control enzyme activity observation. Reversal experiments were also initiated by addition of Na^+, K^+ ATPase, followed by addition of ouabain to a final concentration of 5×10^{-7} M after a 30 min period of control enzyme activity observation. After attainment of maximal enzyme inhibition, a two- to five-fold molar excess of ouabain-specific antibody or equivalent amount of nonspecific control rabbit serum was added and observations were continued for an additional 1–2 hr.

RESULTS

Characterization of antisera. The responses of the three rabbits immunized with ouabain-poly D,L alanyl-HSA are shown in Fig. 3. Detectable antibody was present in rabbit A at 3 wk. Titers in all three animals

rose over the initial 12–20 wk. Two then showed a tendency to plateau, while the third continued to rise steadily over a period of 33 wk.

Two antisera were selected for more detailed study; these were from rabbit B at 12 wk and from rabbit C at 45 wk. Results of equilibrium dialysis studies of the rabbit B antiserum are shown in Figs. 4 and 5. Fig. 4 is a plot of reciprocal bound vs. reciprocal free hapten concentration, which extrapolates linearly to the zero intercept on the reciprocal free axis. This allowed an accurate estimate of binding sites occupied at infinite free ouabain concentration, thus defining the concentration of binding sites occupied at full saturation. The calculated binding site concentration in undiluted antiserum was 2.5×10^{-5} M. Assuming a molecular weight of 75,000 per antibody binding site, this corresponds to 1.9 mg of ouabain-specific immunoglobulin per ml of antiserum.

Replotting these data according to the Sips equation, as shown in Fig. 5, the average intrinsic association constant (K_o) was found to be 1.3×10^9 M^{-1}, while the heterogeneity index (a) was 1.0, indicating relative homogeneity of binding site affinities.

This antiserum was also studied by the charcoal binding method described above. The reciprocal bound vs. reciprocal free plot obtained from these experiments also extrapolated linearly ($r = 0.98$) to the zero intercept on the reciprocal free axis, yielding a binding site concentration of 2.4×10^{-5} M, in good agreement with the value of 2.5×10^{-5} M obtained by equilibrium dialysis. Moreover, when these data were plotted according to the Sips equation, a K_o value of 1.4×10^9 M^{-1} was found, also in quite good agreement with the value of 1.3×10^{-9} M derived from equilibrium dialysis data.

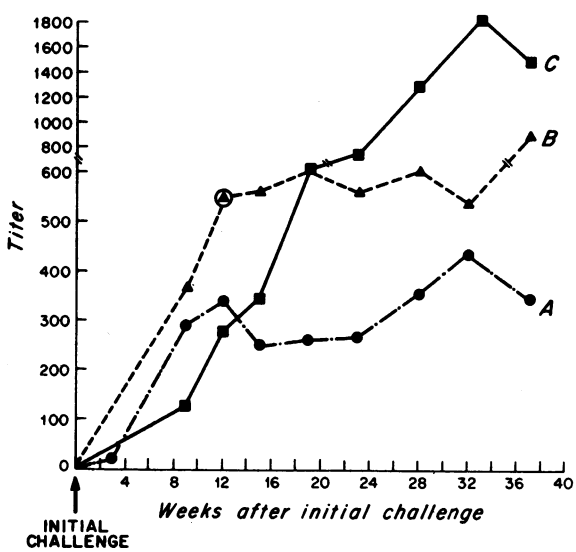

FIGURE 3 Ouabain-specific antibody response as a function of time in rabbits A, B, and C. The encircled triangle represents the 12-wk antiserum from rabbit B which was characterized by equilibrium dialysis experiments and used in the radioimmunoassay procedure.

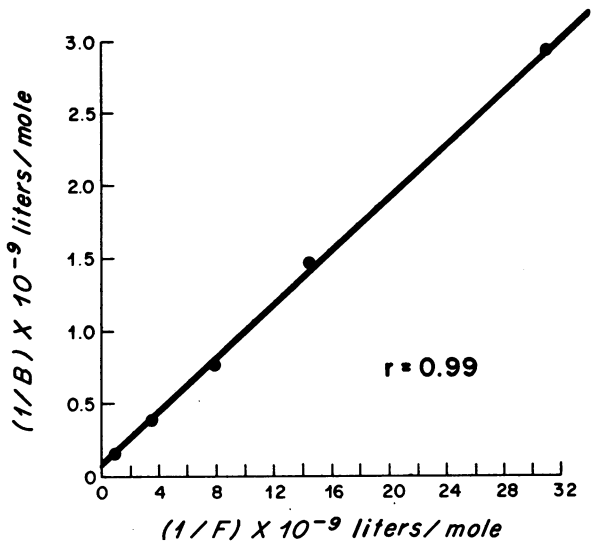

FIGURE 4 Reciprocal antibody bound (1/B) vs. reciprocal free (1/F) ouabain-^{3}H concentrations from equilibrium dialysis experiments. A constant amount of rabbit B antiserum (12 wk) on one side of the membrane was dialyzed against varying amounts of ouabain-^{3}H on the other. Points are the average of duplicate values which agreed within 5%. r, correlation coefficient of line plotted by least squares linear regression.

The second antiserum studied in detail was from a later bleeding of rabbit C, at 45 wk. Data plotted in Figs. 6 and 7 were obtained using the charcoal binding method for separation of antibody-bound from free ouabain-^{3}H. Antibody binding site concentration in undiluted antiserum had risen to 8.6×10^{-5} M, corresponding to a ouabain-specific immunoglobulin concentration of 6.5 mg/ml. K_o was 1.6×10^9 M^{-1}, similar to the value determined for the earlier bleeding from rabbit B. A somewhat greater degree of heterogeneity of binding site affinities was indicated by an a value for the Sips plot of 0.83.

Ouabain radioimmunoassay. Competition between ouabain-^{3}H tracer and unlabeled ouabain in normal human plasma for ouabain-specific antibody binding sites resulted in highly reproduceable semilogarithmic standard curves of the sort shown in Fig. 8. Using the conditions described above, the sensitivity of the assay was such that 0.1 ng of ouabain produced an easily demonstrable displacement of ouabain-^{3}H from antibody binding sites. Still greater sensitivity was obtainable by the use of smaller amounts of tracer and antibody, but at the expense of some decrease

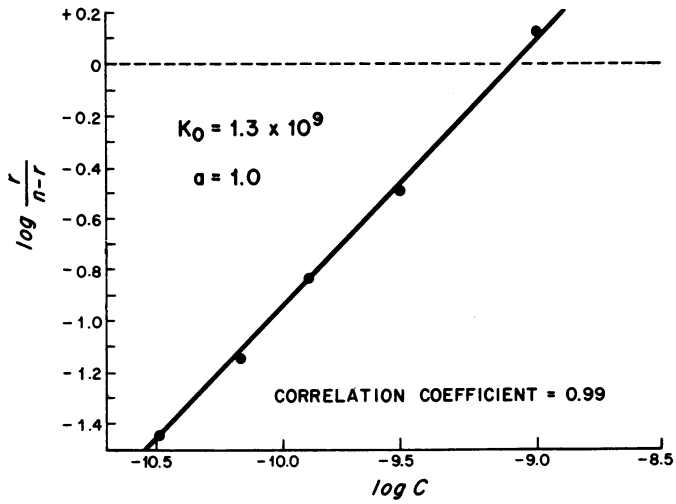

FIGURE 5 Equilibrium dialysis data (rabbit B, 12 wk) plotted according to the modified Sips equation. K_o, average intrinsic association constant (M^{-1}); a, heterogeneity index. See text for details.

in precision. Resolution was satisfactory over the range from 0.1 to 10 ng/ml. The precision of the method is indicated by the range of duplicate determinations shown. Individual concentrations determined for sets of 10 replicate samples had a standard deviation of 5% or less over the range from 0.1 to 10 ng/ml.

Since sigmoid standard curves of the type shown in Fig. 8 were somewhat inconvenient to plot and to adapt to computer usage, the rectilinear standard curve plot shown in Fig. 9 was also used. In practice, raw counts per minute from the liquid scintillation spectrometer were corrected for blank values and quenching by a computer program written for an IBM 360-65 time sharing system, which then plotted reciprocal antibody bound disintegrations per minute against unlabeled ouabain concentration and determined the line of best fit by least squares linear regression analysis. Correlation coefficients for the lines obtained were 0.985 or better for 18 consecutive standard curves. The linearity of this type of plot is a consequence of the restricted heterogeneity of antibody binding site affinities.

Specificity of both antibody binding site and radioimmunoassay system were defined in hapten inhibition experiments. As shown in Fig. 10, of the compounds tested, acetyl strophanthidin competed most effectively with ouabain for antibody binding sites. A twofold greater molar concentration of acetyl strophanthidin was required to displace 50% of the ouabain-^{3}H counts bound in the absence of any competing ligand. Digoxin and digitoxin, which differ from one another only in the presence or absence of a hydroxyl group at the C12 position of the steroid nucleus, behave essentially identically. Both required a 15-fold excess compared with ouabain to produce 50% displacement of ouabain-^{3}H.

In marked contrast to the significant cross-reactivity shown by these cardioactive compounds, endogenous steroids including cholesterol, cortisol, dehydroepiandrosterone, 17β estradiol, progesterone, and testosterone did not cross react to a measurable extent even when present in concentrations more than 1,000-fold in excess of ouabain concentrations which produced evident displacement (see Fig. 10). Further evidence for the specificity of the method was the complete absence of false positive plasma or urine ouabain concentrations in samples from more than 30 human subjects not receiving cardiac glycosides.

Na^+, K^+ ATPase studies. Ouabain-specific antibody was able to completely block the inhibition of myocardial Na^+, K^+ ATPase. As shown in Fig. 11, addition of ouabain-specific antibody or nonspecific control rabbit serum had no effect on Na^+, K^+ ATPase activity as reflected by the unchanged slope of the plot of NADH oxidation vs. time. When 5×10^{-7} M ouabain was subsequently added, marked enzyme inhibition was observed in the sample containing control rabbit serum, with only 15% of initial enzyme activity remaining when full ouabain effect was manifest. In contrast, the presence of ouabain-specific antibody completely blocked ouabain inhibition of the enzyme. This complete blockade of ouabain inhibition by specific antibody was observed in each of four similar experiments,

while the degree of residual activity in the presence of nonspecific control serum varied from 13 to 18%. The latter range was also observed in the presence of 5×10^{-7} M ouabain with no added nonspecific serum or γ-globulin.

The effect of ouabain-specific antibody on established ouabain inhibition of Na^+, K^+ ATPase is shown in Fig. 12. Addition of 5×10^{-7} M ouabain resulted in inhibition of both enzyme samples to 20% of initial activity. Nonspecific rabbit serum had no further effect, whereas addition of ouabain-specific antibody resulted in a gradual return to 91% of the original enzyme activity. In three identical experiments, the range of residual activity after addition of 5×10^{-7} M ouabain was 16–21%; samples to which ouabain-specific antibody was added recovered 85–91% of initial activity. At least part of the small decrement in final Na^+, K^+ ATPase activity compared with initial values may be attributed to a minor loss of activity approximating 10% during 3-hr incubations at 37°C in which no intervention was carried out.

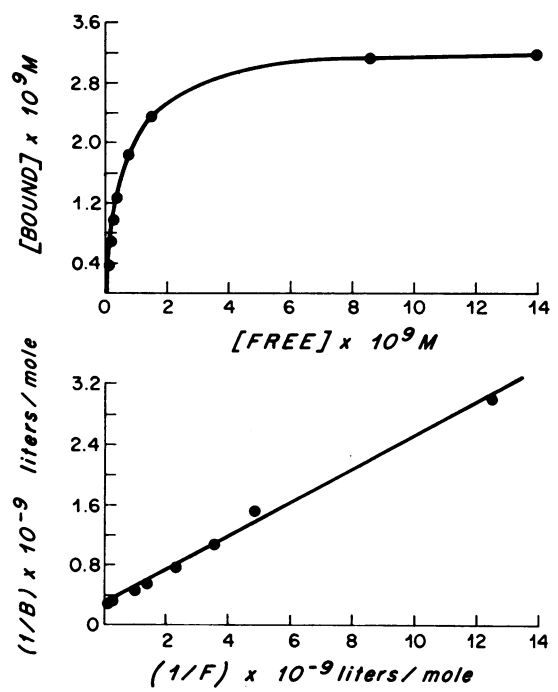

FIGURE 6 Ouabain-^{3}H binding by antiserum from rabbit C. This antiserum, from a bleeding 45 wk after initial antigenic challenge, was studied by charcoal separation of antibody-bound and free ouabain-^{3}H. Above, molar concentrations of antibody-bound and free ouabain-^{3}H. Plotted points are means of duplicate determinations which agreed within 4% or better. Below, data as above plotted as reciprocal bound vs. reciprocal free concentrations. Correlation coefficient = 0.99.

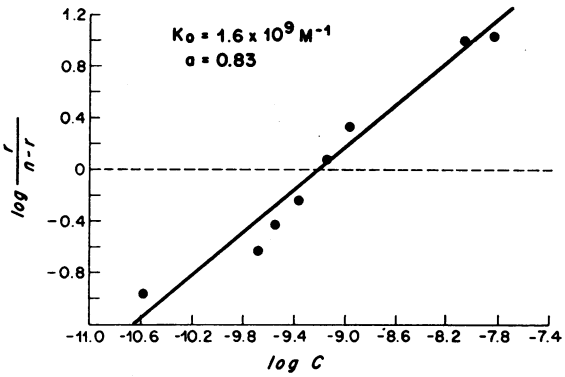

FIGURE 7 Data as in Fig. 6, plotted according to the modified Sips equation. Correlation coefficient = 0.98.

DISCUSSION

Recent work has shown that steroids or steroid glycosides covalently coupled to carrier proteins can elicit specific antibodies in animals appropriately challenged with such antigens (18, 19, 26–29). The studies reported here add further support to the concept that such antibodies may be of quite high affinity and specificity for steroid haptenic determinants, and that they may be used in radioimmunoassay systems for the quantitation of sub-nanogram amounts of the hapten (27, 30, 31, 29).

The ouabain-specific antisera described in this report are similar to digoxin-specific antisera previously studied (19) in that other cardioactive steroids show significant cross-reactivity, while endogenous steroids cross-react minimally or not at all. This may be related to the

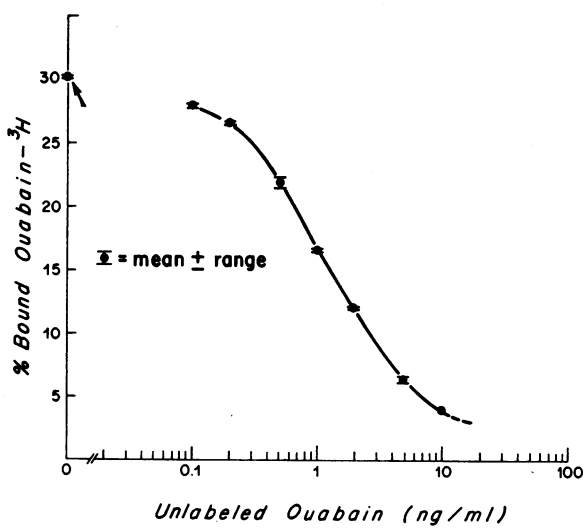

FIGURE 8 Standard curve for ouabain radioimmunoassay. This semilogarithmic plot shows adequate resolution over the range from 0.1 to 10 ng/ml. Details of assay as described in text. The arrow on the vertical axis denotes binding of ouabain-^{3}H in the absence of unlabeled ouabain.

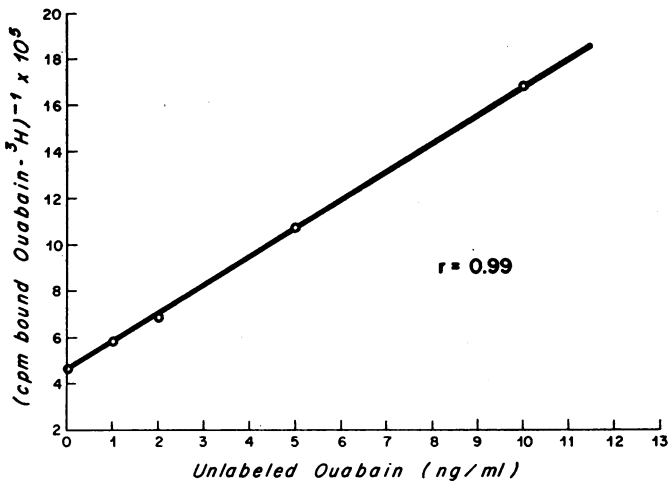

FIGURE 9 Reciprocal plot of standard curve for ouabain radioimmunoassay. Data shown in Fig. 8 have been plotted as reciprocal antibody bound ouabain-^{3}H counts against a linear scale of unlabeled ouabain concentration. r, correlation coefficient for line obtained by least squares linear regression analysis. Individual points are means of duplicate determination with agreement as shown in Fig. 8.

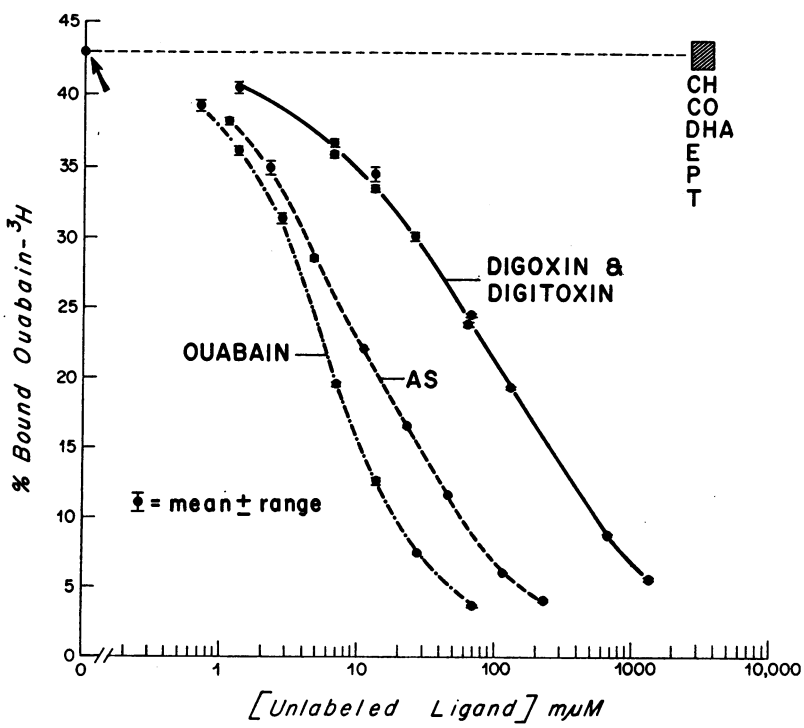

FIGURE 10 Displacement of ouabain-^{3}H from ouabain-specific antibody by various steroid and steroid glycoside compounds. The arrow on the vertical axis denotes binding in the absence of any potentially competing ligand. All values for endogenous steroids fell within the crosshatched area. AS, acetyl strophanthidin; CH, cholesterol; CO, cortisol; DHA, dehydroepiandrosterone; E, 17β estradiol; P, progesterone; T, testosterone.

fact that although acetyl strophanthidin, digoxin, and digitoxin differ from ouabain in both glycoside content and functional groups in the aglycone moiety, all share the C13 methyl and C14 hydroxyl groups, the cis fusion of C and D rings, and the α, β-unsaturated lactone ring (32). These characteristics are, of course, lacking in endogenous steroids from animal species.

The antigen used in these studies differs in a potentially important respect from antigens previously used for similar purposes (18, 19, 27, 33). Because our earlier work had shown that the most important antigenic determinants of digoxin coupled to protein carriers were those most distal to the protein backbone (19), the ouabain hapten was attached to serum albumin through an extended polypeptide side arm consisting of an average of 9.5 D,L alanine residues. Since no experiments were undertaken with direct ouabain-HSA antigens, however, it is not possible to state whether the extended D,L alanyl side chain played a decisive role in determining the specificity of the antibody population elicited.

It is of interest that K_o values of these ouabain-specific antisera were about an order of magnitude lower than those of certain late digoxin-specific antisera previously studied (19). Since hydrophobic interactions between antibody combining sites and steroid haptens might be expected to be an important factor in determining affinity, it is perhaps not surprising that the more polar ouabain hapten interacts somewhat less strongly with its binding site.

The ouabain radioimmunoassay procedure reported here is analogous to methods previously described for digoxin (30, 34, 35) and digitoxin (31). Given an antibody population of sufficiently high affinity and specificity, the limiting factor in sensitivity of the system is

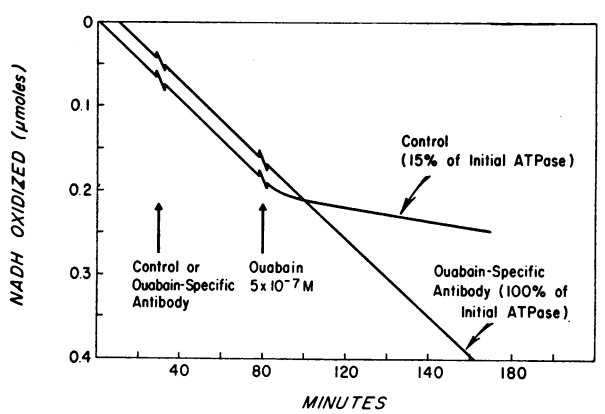

FIGURE 11 Blockade of ouabain inhibition of canine myocardial microsomal Na^+, K^+ ATPase by ouabain-specific antibody. Rate of ATP cleavage was followed continuously by a linked enzyme assay (23, 25); each mole of NADH oxidized represents the hydrolysis of 1 mole of ATP. Assay conditions as described in text.

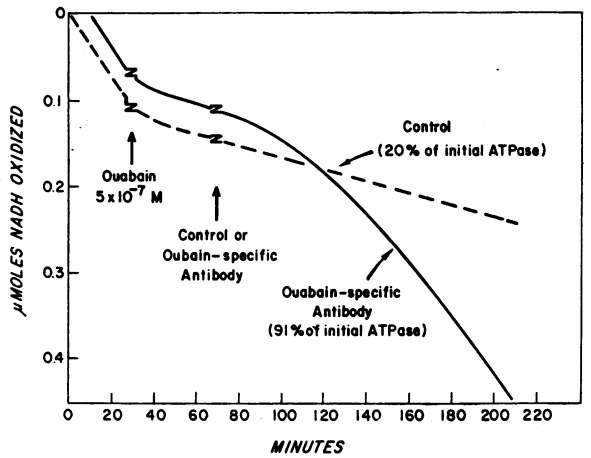

FIGURE 12 Reversal of established ouabain inhibition of Na^+, K^+ ATPase by ouabain-specific antibody. See text for details.

the specific activity of the tracer compound. The availability of ouabain-^{3}H of high specific activity (11–12 Ci/mmole) therefore allows picomolar concentrations of unlabeled ouabain to be quantitated without difficulty.

Methods which require an initial extraction step with water-immiscible organic solvents have been successfully applied to the measurement of serum or plasma digoxin and digitoxin concentrations (27, 36–40). In the case of the more water-soluble compound ouabain, however, efficient extraction from plasma might be quite difficult to achieve. Lack of need for any extraction procedure in the ouabain radioimmunoassay system described here may therefore have special significance.

Compared with the numerous useful studies of the pharmacokinetics of digoxin and digitoxin in both experimental animals and man (41–43), relatively few studies dealing with ouabain have been published (1–3, 44). The radioimmunoassay described here has therefore been used to define the pharmacokinetics of ouabain in dogs and in human subjects (45).

The high affinity of these ouabain-specific antibodies has also been useful in other studies of general biomedical interest. Watson and Butler have shown that digoxin-specific antibodies can prevent the effect of digoxin on cellular potassium transport (46) and Schmidt and Butler have demonstrated both prevention (47) and reversal (5) of potentially lethal digoxin-induced rhythm disturbances in intact animals. The studies of Mandel, Butler, and Bigger (6), Skelton, Butler, Schmidt, and Sonnenblick (8), and Curd, Smith, Jaton, and Haber (7) have further documented the ability of digoxin-specific antibodies to reverse established cellular effects of digoxin. It has been found that the ouabain-specific antibodies described here will reverse established ouabain and acetyl strophanthidin-induced rhythm disturbances

and inotropy in isolated cardiac muscle (4). In addition, preliminary studies by Doctors J. V. Temte and L. D. Davis suggest that toxic alterations in the transmembrane action potential induced by ouabain can be reversed. Of note in this regard are the studies of Ciofalo and Ashe (33), who observed that rabbits immunized with a ouabain-bovine serum albumin conjugate appeared to be protected against otherwise lethal doses of ouabain. As is the case for digoxin-specific antibodies, the high affinity ouabain-specific antibodies described here can not only block potential cardiac glycoside effects, but can reverse typical myocardial effects which have already become established (4).

Several recent studies have focused on Na^+, K^+ ATPase as a possible receptor for cardiac glycoside action (48–53), although the calcium activated ATPase thought to reside in sarcoplasmic reticulum has also been implicated as a possible mediator of inotropic effects (54). In addition to blockade of inhibition of Na^+, K^+ ATPase by 5×10^{-7} M ouabain, ouabain-specific antibodies produced near-complete reversal of established ouabain inhibition of the enzyme (Fig. 12). We have observed comparable reversal of digoxin inhibition of the enzyme by digoxin-specific antibody. Thus, the capacity of high-affinity cardiac glycoside specific antibodies to reverse established Na^+, K^+ ATPase inhibition offers a possible explanation for the reversal of cardiac glycoside effects in a number of experimental systems including erythrocyte ion transport (7, 46), isolated myocardial preparations (4, 6–8), and digitalis-toxic cardiac rhythm disturbances in intact dogs (5, 7).

These experiments also support the previously advanced contention (55, 56) that the interaction between ouabain and Na^+, K^+ ATPase is reversible.

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