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Cardiac Glycoside Interaction with Solubilized Myocardial Sodium- and Potassium-Dependent Adenosine Triphosphatase

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SUMMARY

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Canine myocardial microsomal membranes were exposed under optimum binding conditions to 8 nM[^{3}H]ouabain, a concentration producing only partial inhibition of the ($Na^+ + K^+$)-ATPase activity in this preparation. Microsomal membrane components were then solubilized with the nonionic detergent Lubrol-WX. After centrifugation at 100,000 × *g* for 1 h and gel permeation chromatography on Sepharose 6B, [^{3}H]ouabain was found exclusively in fractions containing ($Na^+ + K^+$)-ATPase activity, and closely paralleled the enzyme activity profile. In Lubrol-solubilized preparations, bound [^{3}H]ouabain penetrated the gel with a component of apparent molecular weight 600,000. Sucrose density gradient centrifugation and liquid isoelectric focusing of Lubrol-solubilized preparations also resulted in close correspondence between the presence of [^{3}H]ouabain and ($Na^+ + K^+$)-ATPase activity. Lubrol-solubilized ($Na^+ + K^+$)-ATPase interacted with ouabain in a manner similar to the membrane-bound enzyme, as judged by identical half-maximal inhibitory concentrations of 60 nM. Thus solubilization of myocardial microsomal membrane components resulted in preservation of ouabain binding and did not disclose any high-affinity receptor separable from ($Na^+ + K^+$)-ATPase by these techniques.

INTRODUCTION

A great deal of interest has been focused in recent years upon ($Na^+ + K^+$)-ATPase as a possible receptor for the therapeutic and toxic effects of digitalis on the heart. Since the studies of Schätzmann (1) and Skou (2) it has been apparent that cardiac glycosides inhibit monovalent cation transport as well as ($Na^+ + K^+$)-activated ATPase activity, and Repke (3) postulated on the basis of his early studies that this inhibition might underlie the effects of digitalis on the intact heart. A number of previous studies have shown Na^+-, Mg^{++}-, and ATP-dependent binding of cardiac glycosides to membrane fractions containing ($Na^+ + K^+$)-ATPase activity (4–10), and ouabain binding has been shown to parallel ($Na^+ + K^+$)-ATPase inhibition (7, 9, 11).

Observations of cardiac glycoside binding to myocardial ($Na^+ + K^+$)-ATPase have thus far been limited to studies of membrane or "microsomal" preparations, leaving open the possibility that cardiac glycosides might

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be specifically bound to membrane components other than (Na^+ + K^+)-ATPase. In view of continuing controversy regarding the role of (Na^+ + K^+)-ATPase in the mediation of cardiac glycoside-induced inotropy (12–15), the present studies were undertaken to test the hypothesis that some molecular species other than (Na^+ + K^+)-ATPase might be present in microsomal preparations, which would specifically bind cardiac glycosides and thus provide an alternative receptor site.

In these studies advantage was taken of recently developed techniques for the solubilization and partial purification of canine myocardial microsomal (Na^+ + K^+)-ATPase (16), allowing delineation of ouabain binding to solubilized components of the microsomal membrane fraction.

METHODS

Microsomal (Na^+ + K^+)-ATPase Preparation

Canine myocardial microsomal preparations containing (Na^+ + K^+)- ATPase were prepared through the second deoxycholate extraction step by minor modification (16) of the method of Schwartz *et al.* (17). This preparation was used in Lubrol-WX solubilization procedures as described subsequently.

[^{3}H]*Ouabain Binding to Microsomal Preparations*

[^{3}H]Ouabain (12 Ci/mmole) was obtained from New England Nuclear Corporation. Thin-layer chromatography on silica gel G using a choloroform–methanol–water (63:30:5) solvent system showed that at least 90% of tritium counts ran with the same R_F as that of crystalline ouabain (obtained as the octahydrate, Sigma).

Prior to detergent solubilization procedures, canine myocardial microsomes were suspended to a final concentration of 2 mg/ml in 5 ml of buffer containing 50 mM NaCl, 1.25 mM $MgCl_2$, 5 mM disodium ATP, 1 mM H_2Na_2 EDTA, and 50 mM Tris-Cl, pH 7.4. A low concentration of [^{3}H]ouabain (8 nM), sufficient to cause only partial inhibition of (Na^+ + K^+)-ATPase activity, was added, and the mixture was warmed to 37° for 15 min. The suspension was then chilled rapidly on ice and centrifuged at 100,000 × *g* for 30 min. After careful removal of the supernatant solution, centrifuge tube walls were rinsed gently with buffer and residual droplets were removed with filter paper or a small cotton swab. The pellet was then resuspended in preparation for solubilization procedures.

Microsomal (Na^+ + K^+)-ATPase Solubilization

In solubilization experiments, 10 mg of microsomal protein were suspended in a volume of 10 ml containing 0.32 M sucrose, 1 mM H_4 EDTA adjusted to pH 6.9 with Tris base, and 0.2% Lubrol-WX (donated by ICI America, Inc., Bridgeport, Conn.). The mixture was homogenized by hand with 10 strokes in a ground glass homogenizer and allowed to stand on ice for 15 min. Five additional strokes were then applied, followed by 15 min longer on ice. The contents were then transferred to centrifuge tubes and spun for 60 min at 100,000 × *g*. The supernatant solution was carefully removed and used as solubilized enzyme. A thin band of whitish material was present at the top of the centrifuge tube in some experiments and was discarded. Eighty to ninety per cent of the (Na^+ + K^+)-ATPase activity present in the microsomal preparation remained in the supernatant phase under these conditions, and stable activity was maintained for at least 1 week when stored at 0°.

Further details of these procedures and discussion of solubilization criteria are presented elsewhere (16).

Gel Permeation Chromatography

Further fractionation of myocardial microsomal membrane components was accomplished by gel permeation chromatography on 6% agarose. Sepharose 6B (Pharmacia Fine Chemicals) was extensively washed with distilled water and 0.15 M NaCl to remove azide, then equilibrated with buffer containing 320 mM sucrose, 40 mM NaCl, 1 mM disodium EDTA, 50 mM Tris-HCl (pH 6.8), and 0.2% Lubrol-WX. A 2.5 × 60 cm gel column was eluted at constant pressure under downward flow at a rate of 8 ml/hr.

Samples were applied in a volume of 4 ml, and constant volumes were collected by drop counting.

Calibration of columns was accomplished by application of 10 mg each of the following proteins: porcine thyroglobulin (Sigma), apoferritin (Calbiochem), rabbit γ-globulin (Pentex), and ovalbumin (Calbiochem). The void volume was determined with blue dextran T-2000 (Pharmacia), and K_{av} was calculated according to Laurent and Killander (18). Tritiated water was used in each run to mark the salt volume and to monitor the constancy of the column elution pattern.

Sucrose Density Gradient Centrifugation

Step gradients were formed with varying concentrations of Schwarz/Mann ultrapure sucrose in 1 mM H_4 EDTA adjusted to pH 6.9 with Tris base. Nitrocellular tubes (1 × 3 in.) were prepared with 5 ml each of 25%, 22.5%, 20%, 17.5%, and 15% (w/v) sucrose in 1 mM H_4 EDTA, pH 6.9, and 5 ml of Lubrol-solubilized enzyme in 5% sucrose. The tubes were centrifuged at 24,000 rpm for 24 hr, using an SW 25.1 rotor in a Beckman/Spinco model L2 preparative ultracentrifuge. Gradients were bled by puncturing the bottom of the gradient tube and collecting 5 ml fractions. Sucrose concentration in individual fractions was estimated from measurement of the refractive index, using a Zeiss Abbé refractometer.

Isoelectric Focusing

Proteins were fractionated by liquid isoelectric focusing with an LKB 8102 electrofocusing column (440 ml, LKB Instruments, Stockholm) at 4° (19). A stabilizing gradient of 0–46% sucrose (Schwarz/Mann, ultrapure) was used; carrier ampholytes, at a concentration of 0.4% in the pH range 4–6, were included. The pH gradient was established prior to the introduction of the sample, which was added in 9 ml of 1 mM H_4 EDTA, 0.2% Lubrol-WX (pH 7.0), and 10% sucrose, using capillary tubing. A power supply was utilized which maintained the power applied constant at 3 W (20), and the time of presence of the sample in the column never exceeded 12 hr.

The contents of the column were eluted by gravity, and the absorbance at 280 nm was monitored continuously with an LKB Uvicord II spectrophotometer equipped with a microflow cell and a 14-cm linear recorder. The pH of the effluent was measured with a Radiometer model 26 pH meter. Blank focusing columns were run in the pH range 4–6 for the same length of time and under the same conditions in order to establish a correct baseline for the distribution and absorbance of ampholytes.

Protein Concentration Determination

Protein concentrations were determined by the method of Lowry *et al.* (21), except where noted, using crystalline bovine serum albumin as standard. When required, standard solutions were made in sucrose–Lubrol-WX–Tris buffers to match samples to be measured. Lubrol-WX at concentrations from 0.01 to 1.0 mg/ml did not interfere with protein measurements under these conditions. Protein elution from the 6% agarose column was monitored by reading the absorbance at 280 nm on a Zeiss model PMQ II/M4Q III spectrophotometer.

Protein concentrations of fractions from isoelectric focusing experiments could not be determined by the Lowry procedure because of interference by ampholyte content, and were therefore measured by fluorometry (22) on a Hitachi Perkin-Elmer fluorescence spectrophotometer, model MPF-2A, equipped with a Hitachi recorder, model QPD 33, again using bovine serum albumin as standard. This procedure also proved useful in measuring protein concentrations of solutions containing Lubrol-WX, which at higher concentrations also interfered with protein determinations by the Lowry technique. The presence of ampholytes or Lubrol-WX did not interfere with protein determination by fluorometry, which had a sensitivity of 5 μg/ml and consistently agreed within 15% with protein concentrations measured by the Lowry procedure.

Enzyme Activity Measurements

$(Na^+ + K^+)$-ATPase activities were measured by the following techniques as noted in individual experiments.

Linked enzyme assay (7). Hydrolysis of ATP to ADP and P_i was coupled through the presence of excesses of phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase to an NADH-NAD^+ transition, which was followed at 340 nm with a Gilford recording spectrophotometer, the cuvette compartment of which was thermostated at 37.0° ± 0.2° with a Haake constant-temperature circulator. Concentrations in each cuvette, in a final volume of 2.0 ml, were: NaCl, 100 mM; KCl, 10 mM; $MgCl_2$, 5 mM; phosphoenolpyruvate, 2.5 mM; NADH, 0.5 mM; Tris-ATP, 2.5 mM; pyruvate kinase–lactate dehydrogenase (Sigma), 20 μl; Tris-HCl, 50 mM, adjusted to pH 7.4. The reaction was initiated by the addition of 10–50 μg of enzyme protein. After a constant slope of A_{340} vs. time plot for at least 10 min had been recorded, ouabain was added to a final concentration of 0.1 mM, and a constant slope was again recorded for at least 10 min. The difference in slope before and after addition of ouabain was calculated and converted to (Na^+ + K^+)-ATPase activity. In ouabain concentration-effect studies, enzyme inhibition was calculated from slopes recorded over a 10-min period beginning 30 min after addition of ouabain to give final concentrations varying from 10 nM to 0.1 mM. This assay method was used for samples which contained 0.2% or more Lubrol-WX because of interference by Lubrol-WX with colorimetric determination of inorganic phosphate.

Inorganic phosphate assay. The reaction was carried out in duplicate in a volume of 1.0 ml containing 100 mM NaCl, 10 mM KCl, 50 mM Tris-Cl (pH 7.4), 1 mM EDTA, and 5mM $MgCl_2$ in the presence or absence of 0.1 mM ouabain. An amount of enzyme protein shown to provide a linear rate of P_i liberation for at least 30 min was first incubated for 5 min at 37°, and the reaction then was initiated by the addition of ATP to a final concentration of 2.5 mM. After 30 min the reaction was stopped by the addition of 1 ml of ice-cold 20% trichloracetic acid. After standing on ice for 10 min, tubes were centrifuged at 3000 × *g* for 20 min.

One-milliliter aliquots of the supernatant fraction were then assayed for P_i content by the method of Fiske and SubbaRow (23). Blanks were run by adding enzyme immediately prior to trichloracetic acid addition. Standard curves were constructed using 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mM KH_2PO_4 in assay buffer solutions.

Other

Water used in all experiments was glass double-distilled. All reagents other than those specifically described were of the highest available commercial grade and were used without further purification.

RESULTS

(Na^+ + K^+)-ATPase activity in the canine myocardial microsomal preparation was highly sensitive to ouabain, as shown in Fig. 1. Solubilization of the enzyme with Lubrol-WX had essentially no effect on ouabain sensitivity, with both microsomal and solubilized preparations showing 50% inhibition at 60 nM ouabain. Further purification by density gradient centrifugation was was also without effect on ouabain sensitivity as plotted in Fig. 1. Lubrol solubilization did have a pronounced effect on the percentage of total enzymatic activity which was ouabain-

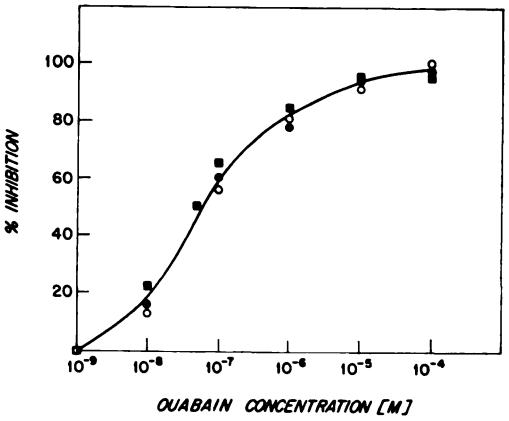

FIG. 1. *Ouabain concentration-effect curves for canine myocardial microsomal (Na^+ + K^+)-ATPase* (○), *Lubrol-WX-solubilized enzyme* (●), *and Lubrol-WX-solubilized enzyme further purified on a sucrose density gradient* (■)

Zero and 100% inhibition refer to values obtained in the absence and in the presence of 0.1 mM ouabain, respectively. Values are means of duplicate determinations, which differed by less than 5%.

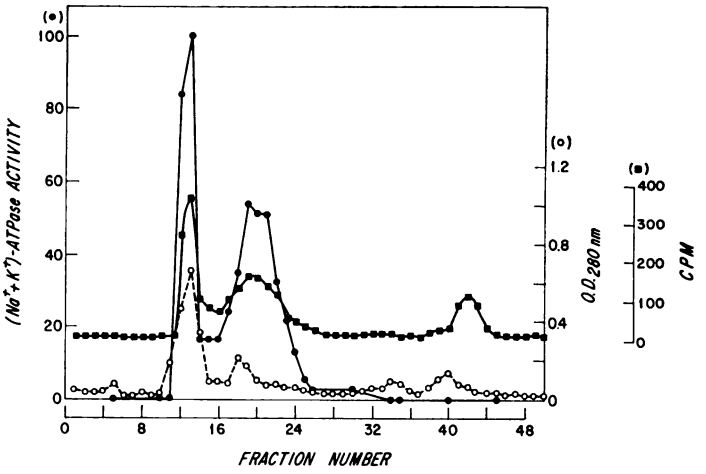

FIG. 2. *[³H]Ouabain distribution after binding of 8 nM [³H]ouabain to canine myocardial microsomal fraction followed by solubilization with 0.2% Lubrol-WX and gel permeation chromatography on Sepharose 6B*

○, absorbance at 280 nm; ●, $(Na^+ + K^+)$-ATPase activity as a percentage of the most active fraction; ■, [³H]ouabain distribution (counts per minute).

inhibitable. This value rose from an average of 41 ± 5% (SD) in five microsomal preparations to 82 ± 4% for Lubrol-solubilized enzyme.

Relation of [³H]Ouabain Binding and Solubilized $(Na^+ + K^+)$-ATPase Activity in Gel Permeation Experiments

When microsomes labeled with 8 nM [³H]ouabain were solubilized in 0.2% Lubrol-WX and partially purified by 6% agarose gel permeation chromatography, [³H]ouabain counts closely followed the elution profile of $(Na^+ + K^+)$-ATPase activity. [³H]Ouabain appeared at the front in conjunction with a peak of nonsolubilized $(Na^+ + K^+)$-ATPase activity, which ran in the void volume (Fig. 2). A second [³H]ouabain peak invariably appeared coincident with $(Na^+ + K^+)$-ATPase activity which penetrated the gel. This peak had an apparent molecular weight of 600,000 as compared with a series of marker proteins, as shown in Fig. 3. However, the amount of Lubrol-WX remaining associated with the solubilized enzyme has not been determined, so that the value of 600,000 may be an overestimation if substantial amounts of detergent remain bound. The amount of [³H]ouabain closely paralleled $(Na^+ + K^+)$-ATPase activity in each fraction, and 85–90% of total ³H counts were found in the two peaks of enzyme activity. The remaining 10–15% of counts were found in the salt volume and presumably represented free [³H]ouabain. No evidence of [³H]ouabain binding to fractions other than those which contained $(Na^+ + K^+)$-ATPase was observed. A late free [³H]ouabain peak coincided with the elution volume of tritiated water.

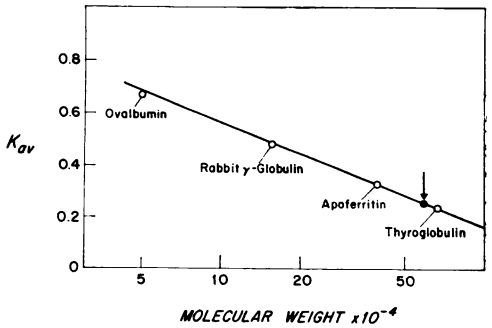

FIG. 3. *Molecular weight estimation of [³H]-ouabain binding component which penetrated Sepharose 6B, (↓, ●) compared with marker proteins*

K_{av} was calculated according to Laurent and Killander (18). $(Na^+ + K^+)$-ATPase activity appeared coincident with this [³H]ouabain peak. See the text for further details.

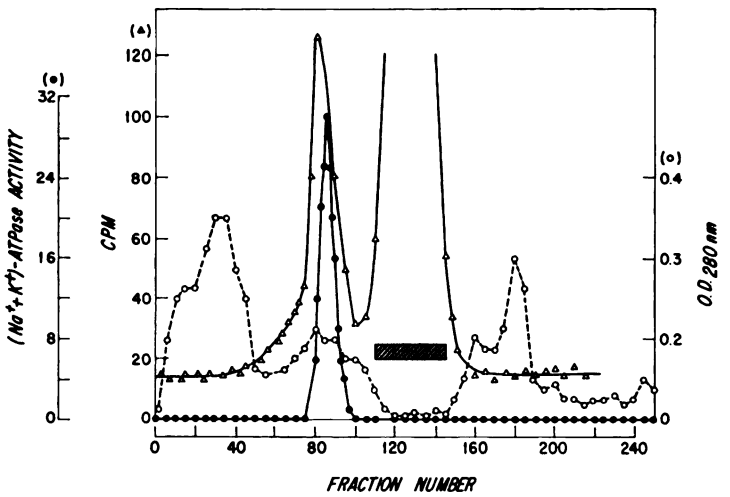

FIG. 4. *[³H]Ouabain distribution in liquid isoelectric focusing of canine myocardial microsomes labeled with 8 nM [³H]ouabain and solubilized with 0.2% Lubrol-WX*

Isoelectric focusing was carried out on a pH 4–6 gradient as described in the text. ●, (Na^+ + K^+)-ATPase specific activity, in micromoles of P_i per milligram of protein per hour; ○, optical density at 280 nm, corrected for ampholyte absorption; △, [³H]ouabain counts. The cross-hatched bar indicates the distribution of fractions with [³H]ouabain counts above 1.5 times background for a column run in identical fashion but in the absence of the microsomal extract.

When these experiments were repeated using microsomes labeled with 1 μM [³H]-ouabain, an identical distribution of ³H counts was observed.

Relation of [³H]Ouabain Binding and (Na^+ + K^+)-ATPase Activity after Isoelectric Focusing

[³H]Ouabain bound to microsomes with subsequent Lubrol-WX solubilization and isoelectric focusing was eluted from the column in close conjunction with (Na^+ + K^+)-ATPase activity (Fig. 4). A second, relatively broad peak of free [³H]ouabain was present at the location where the preparation was applied to the column. There was essentially no protein present at the location of the free [³H]ouabain peak, although binding to a minor component buried within this region cannot be excluded with certainty.

Relation of [³H]Ouabain Binding and (Na^+ + K^+)-ATPase Activity after Density Gradient Centrifugation

(Na^+ + K^+)-ATPase activity, [³H]ouabain binding, and protein concentration after centrifugation of a [³H]ouabain-labeled and subsequently Lubrol-solubilized preparation on a discontinuous sucrose gradient are plotted in Fig. 5. As with preparations partially purified by agarose gel permeation and isoelectric focusing, [³H]ouabain distribution closely paralleled the profile of (Na^+ + K^+)-ATPase activity. No evidence was noted of binding to fractions other than those with (Na^+ + K^+)-ATPase activity. When [³H]ouabain was added to membrane preparations in the absence of ATP, Mg^{++}, and Na^+, 95% of the tritium counts were recovered in the top two gradient fractions.

DISCUSSION

An impressive weight of circumstantial evidence has accumulated in recent years implicating the myocardial (Na^+ + K^+)-ATPase system in the mechanism of at least some of the actions of cardiac glycosides on the intact heart (24–26). Other workers have called attention to apparent dissociations between inotropic (15) and electrophysiological (27) actions of cardioactive steroids and (Na^+ + K^+)-ATPase inhibition, and alternative possible mechanisms of action have been hypothesized (12). It is apparent that cardiac glycosides, particu-

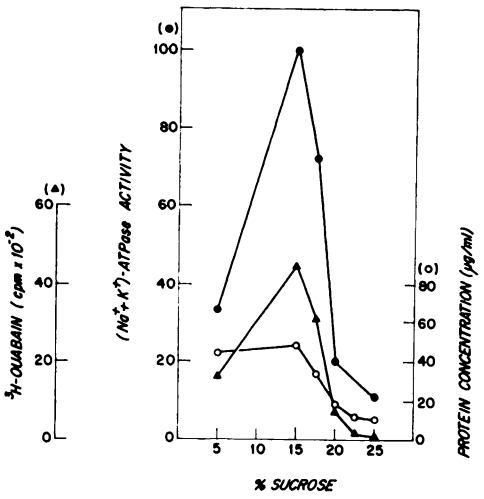

FIG. 5. *Distribution of [³H]ouabain bound to canine myocardial microsomes, followed by solubilization with Lubrol-WX and sucrose density gradient centrifugation*

●, $(Na^+ + K^+)$-ATPase activity as a percentage of the most active fraction; ○, protein concentration estimated by the Lowry procedure; ▲, [³H]ouabain counts.

larly those of relatively low polarity, are found in myocardium at other sites in addition to the $(Na^+ + K^+)$-ATPase complex (28, 29). However, the fact that concentrations of cardiac glycosides of the order of 10–100 nM produce readily apparent inotropic and electrophysiological effects on the myocardium implies a relatively high-affinity receptor or receptors. Because previous studies of cardiac glycoside interactions with myocardial $(Na^+ + K^+)$-ATPase have used microsomal preparations of marked molecular heterogeneity, the present studies were undertaken to test the hypothesis that a receptor might be found which was dissociable from membrane-bound $(Na^+ + K^+)$-ATPase activity after solubilization of myocardial microsomal preparations.

As shown in Fig. 1, solubilization and partial purification of myocardial microsomes exerted no measurable effect on the interaction between ouabain and $(Na^+ + K^+)$-ATPase as judged by the concentration effect curves. The unchanged half-maximal inhibitory concentration of 60 nM documents the high affinity of the $(Na^+ + K^+)$-ATPase complex for ouabain, as previously observed (30, 31). Kline *et al.* (32) have also reported ouabain sensitivity of $(Na^+ + K^+)$-ATPase in Lubrol extracts of beef brain microsomes, which was similar to that of their microsomal starting material. Although the brain enzyme required substantially higher ouabain concentrations (0.7–1.0 μM) to produce 50% inhibition, this difference is probably due, at least in part, to differences in methods used to determine ouabain effects on $(Na^+ + K^+)$-ATPase activity.

Binding of [³H]ouabain to the microsomal membrane fraction used in these studies was carried out under conditions which result in a maximum yield of [³H]ouabain binding to the entire member fraction (30). However, gel permeation chromatography of Lubrol-solubilized myocardial microsomes after exposure to [³H]ouabain under these conditions resulted in a distribution of ³H counts which closely paralleled the distribution of $(Na^+ + K^+)$-ATPase activity. There was no evidence of a high-affinity binding site separable from $(Na^+ + K^+)$-ATPase activity by this technique. A low concentration of [³H]-ouabain (8 nM) was used in these experiments in order to allow simultaneous determination of the distribution of [³H]ouabain and $(Na^+ + K^+)$-ATPase. No lower-affinity binding sites were observed in experiments using 1 μM [³H]ouabain.

Fractionation by isoelectric focusing (Fig. 4) and by sucrose density gradient centrifugation (Fig. 5) of Lubrol-solubilized myocardial microsomes previously exposed to [³H]ouabain also provided no evidence of a high-affinity receptor site separable from $(Na^+ + K^+)$-ATPase. In each instance the distribution of [³H]ouabain counts closely paralleled the distribution of $(Na^+ + K^+)$-ATPase activity. Thus bound [³H]ouabain may be a useful marker in purification procedures.

It must be pointed out that while these experiments provide no evidence for a high-affinity cardiac glycoside binding site other than the $(Na^+ + K^+)$-ATPase complex, they do not rigorously exclude the existence of an alternative receptor site or sites in intact myocardium. Such a site could have been removed or rendered less able to bind [³H]-ouabain at any of the multiple steps leading to the detergent-solubilized preparations, or

could have bound [^{3}H]ouabain only under different liganding conditions. Furthermore, one cannot exclude the possibility that a high-affinity receptor complex distinct from $(Na^+ + K^+)$-ATPase might copurify by each of the fractionation techniques used in these studies. Finally, even if specific cardiac glycoside binding in myocardium were limited to the $(Na^+ + K^+)$-ATPase complex, it would not necessarily follow that the effects of cardiac glycosides are exerted only by inhibition of Na^+ or K^+ transport by this enzyme system.

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