Antigenic Differences in (Na\(^+\),K\(^+\))-ATPase Preparations Isolated from Various Organs and Species*

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Antisera to purified (Na\(^+\),K\(^+\))-ATPase raised in rabbits and in sheep were purified by an absorption procedure employing purified canine kidney (Na\(^+\),K\(^+\))-ATPase. The antibodies were fractionated into two components, one which inhibited catalytic activity, and a second which inhibited ouabain binding. Under certain conditions, the fraction that inhibited ouabain binding also inhibited catalytic activity, and the effectiveness of both was dependent to some extent on the ligands present in the incubation medium. Thus, both antibody fractions appeared to detect conformations of the enzyme that depended upon ligand-induced perturbations. When the antibody raised against catalytic activity was incubated with erythrocyte membrane fragments, an inhibition of the (Na\(^+\),K\(^+\))-ATPase occurred, but only minimal or no effect on potassium influx or on digoxin-induced inhibition of potassium flux in intact erythrocytes was noted. In a similar experiment, however, the antibody against ouabain binding significantly inhibited potassium influx, suggesting specificity in terms of the macromolecular surfaces of the pump which were exposed to the external medium. We concluded that there may be organ and species differences among (Na\(^+\),K\(^+\))-ATPases because of observed differences in the effects of antibodies on various (Na\(^+\),K\(^+\))-ATPase preparations. Antibodies prepared in rabbits and sheep were fractionated by absorption to dog brain enzyme. Both the antibody fraction which bound to the brain enzyme and that which did not bind inhibited the dog kidney (Na\(^+\),K\(^+\))-ATPase, but only the former inhibited dog brain (Na\(^+\),K\(^+\))-ATPase. When the two fractions were recombined, inhibition was restored to the extent of the unfractionated antibody.

Antisera to many enzymes have been produced, and the specificity of the antibodies and the varied nature of their effects have made them useful in the study of enzyme structure and function (1–10). Recently, antisera have been raised to (Na\(^+\),K\(^+\))-ATPase and the results have been reported by several investigators (11–18). Antibodies to the native enzyme appear to inhibit ATP hydrolysis in fragmented membrane preparations (11–18) and sodium efflux from resealed erythrocyte ghosts (13, 15). An antibody has also been produced to a lipid-free heavy chain peptide of the (Na\(^+\),K\(^+\))-ATPase complex that did not affect enzyme turnover but did apparently bind to the portion of the peptide exposed on the inner surface of the vesicular membrane preparation (16).

Antisera to complex macromolecules such as enzymes usually contain "families" of antibodies, each specific for a different portion of the molecule (1, 3, 5). The individual antibodies may have different or even opposing effects upon enzyme activity (1, 3), making the results obtained with antisera complex and often difficult to interpret. This complexity, however, is useful in the case of the (Na\(^+\),K\(^+\))-ATPase, since fractionation of sera may yield antibody components useful as conformation-sensing probes that can help to define specific details of enzyme structure and function. We have reported the purification of two fractions of antibody from (Na\(^+\),K\(^+\))-ATPase-specific serum that exert differential effects upon the ATP hydrolysis and \(^{[3}H\)ouabain-binding properties of the enzyme (17, 18). The present investigation is an extension of these experiments, the data of which suggest that the reactivity of these fractions...
is dependent upon ligand-induced conformations of the enzyme. Effects on energy-linked cation transport in, and digoxin binding to, human erythrocytes are included; these results are consistent with site specificity of the antibody components.

Studies were also carried out on the patterns of cross-reactivity of antisera to dog kidney enzyme with (Na+, K+)-ATPase preparations derived from various organs and species. The data suggest antigenic organ and species differences among (Na+, K+)-ATPases.

**METHODS**

**Production and Fractionation of Antiserum**—The source of enzyme (antigen) used for immunization was highly purified (Na+, K+)-ATPase prepared from canine renal medulla by the method of Lane et al. (19). Rabbits were immunized with this preparation as described previously (17, 18). Sheep were also immunized by intramuscular injections of 1.0 ml of the antigen suspended in Freund’s adjuvant into each haunch on a weekly basis for 6 weeks. Thereafter, the sheep were bled on a bimonthly basis and received booster injections of 1.0 ml into alternate hind legs. Control sera were obtained from unimmunized rabbits and sheep prior to immunization. Globulin was precipitated with ammonium sulfate (20) from rabbit and sheep control sera and antisera and subsequently dialyzed as previously described (17, 18). All globulin fractions from both immune and control sera were adjusted to a final protein concentration of 20 mg/ml by the addition of 150 mM Tris. The globulin fraction obtained from antisera was further fractionated into two antibody fractions as described previously (17, 18). One inhibits catalytic activity and is designated as “anticytolytic antibody.” The second inhibits ouabain binding and is designated as “antidigoxin receptor antibody.”

The globulin fractions of rabbit and sheep antisera raised against dog kidney (Na+, K+)-ATPase were also incubated with an excess of a crude dog brain (Na+, K+)-ATPase preparation (specific activity of 40 to 50) with the following buffer: 150 mM imidazole buffer, pH 7.4; 5 mM MgCl2; and 5 mM P, at 37°C for 30 min. The incubation mixture was allowed to stand at 4°C overnight, and then centrifuged at 14,300 x g at 4°C for 30 min. The supernatant was decanted and the pH lowered to 2.8 by the addition of concentrated HCl. Following 5 min of gentle stirring, the pH was returned to 7.4 by the addition of 2.0 M Tris base. The pellet, containing brain (Na+, K+)-ATPase and bound antibody, was washed by resuspension in a small volume of buffer and appropriate ligands at 37°C for several hours, followed by centrifugation (at 14,300 x g for 30 min) and a final washing by resuspension in a small volume of 0.05 M Tris, pH 7.4, and centrifuged. The washed pellet was resuspended in 140 mM glycine, 140 mM NaCl buffer, pH 2.8, and allowed to stand at room temperature for 1 h. The eluted antibody was then separated from the enzyme by centrifugation at 40,000 x g for 30 min. Both the eluted antibody and the globulin which did not bind to the enzyme preparations (supernatant) were dialyzed against 150 mM Tris buffer, pH 7.4, for 12 h at 4°C with eight changes of buffer (dialysis time of 96 h). Control globulins from rabbit and sheep were also incubated with the dog brain (Na+, K+)-ATPase preparation. No protein, however, was eluted from the pellet upon acid treatment.

**Assays for (Na+, K+)-ATPase Activity, [H]Ouabain Binding, and Protein**—In the studies of the effects of antibody upon (Na+, K+)-ATPase, enzyme activity was routinely determined by the spectrophotometric pyruvate kinase-lactic acid dehydrogenase coupled enzyme assay (21). The assay was also checked by determination of the inorganic phosphate produced (22). The assay medium for the latter contained 5 mM NaATP, 5 mM MgCl2, 100 mM NaCl, 10 mM KCl, and a suitable quantity of enzyme. In the case of the coupled enzyme system, the effects of antibody or control globulin on catalytic activity were examined by adding the fractions while the enzyme was catalyzing ATP hydrolysis or by adding the fractions prior to the onset of hydrolysis, i.e., the fractions were preincubated with enzyme for 30 min at 37°C, added to a 100-ml volume of buffer at the same time, and appropriate ligands at 37°C for 15 min. Where activities were assayed by phosphate determination, antibody or control globulin was preincubated with enzyme for 15 min at 37°C in the assay medium except that ATP was absent.

The effects of antibody upon [H]ouabain binding to the (Na+, K+)-ATPase were studied in the presence of magnesium, sodium, ATP, (Mg2+), (Na+, K+), and ADP in the presence of magnesium and inorganic phosphate (Mg2+P), as previously described (17, 18). Binding was carried out for 30 s to obtain an estimate of the initial binding rate and for 60 min to determine an estimate of the equilibrium level of binding. Protein was determined by the method of Lowry et al. (24).

**Effects of Antibody Components on Intact Erythrocytes**—Human erythrocytes, freshly drawn from a healthy donor, were washed three times in isosmotic choline chloride (pH 7.4); 300 μl of packed cells and 200 μl of antibody or γ-globulin in 150 mM Tris buffer, pH 7.4, were added to the incubation medium (NaCl, 150 mM, Tris buffer, pH 7.4, 10 mM, and glucose, 11.1 mM), and incubated for 1 hour in 10-ml Erlenmeyer flasks in a 37°C shaking water bath. At this time, 30 to 50 μl of [3H]digoxin (Lot 636-126; specific activity, 8.98 Ci/mmol; New England Nuclear Corp., Boston) were added, constituting a final reaction volume of 3 ml, and the incubation continued for an additional 2 hours. Following removal of duplicate 100-μl aliquots for measurement of membrane-bound [3H]digoxin and aliquots for measurement of total [3H]digoxin in the incubation mixture (i.e., in those assays carried out in the presence of drug), 4KCl (0.15 to 0.25 μM) in a volume of 50 to 100 μl was added to each flask. After 1 hour, 100-μl aliquots were removed for potassium influx determinations and for measurements of radioactivity and potassium concentrations in the media (25).

Membrane-bound [3H]digoxin was determined on duplicate 100-μl samples of the suspension by the method of Gardner et al. (25), except that 15 ml of a different liquid scintillation mixture were employed (2.5 g of 2,5-diphenyloxazole, 100 mg of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene, and 333 ml of Triton X-100 made up to 1 liter with toluene). The volume of counted cells was calculated from the hemoglobin concentration of the incubation mixture as measured by the cyanmethemoglobin method (26), assuming that 100 ml of hemolysate contains 34 g of hemoglobin (27).

For potassium influx determinations, duplicate 100-μl samples were washed four times with 300 μl of isosmotic choline chloride in polyethylene tubes in a Microfuge centrifuge (Beckman Instruments, Inc.) at 10,000 x g for 15 s. After the final wash, the cells were counted in an Auto Gamma scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). The specific activity of the potassium in the medium was determined by counting 100-μl aliquots of the incubation mixture (i.e., cells plus medium and any added inorganic phosphate) using the potassium concentration in the supernatant fluid (from all flasks not containing [3H]digoxin) with an Instrumentation Laboratory model 143 flame photometer (Instrumentation Laboratory, Inc., Lexington, Mass.). Since extracellular potassium concentrations in any given experiment varied little from flask to flask, the assumption was made that the potassium concentration in the supernatant fluids of each [3H]digoxin-containing incubation mixture was identical with that in the corresponding incubation mixture not containing [3H]digoxin. Potassium influx was then calculated as previously described (28). 4KCl (CN-1921) was obtained on the day of use from the Cambridge Nuclear Radiopharmaceutical Corp., Princeton, N. J.

**RESULTS**

**Effects of Rabbit and Sheep Antiserum on Dog Kidney (Na+, K+)-ATPase—Immune globulin from antisera of both rabbits and sheep immunized against canine kidney (Na+, K+)-ATPase inhibited the catalytic activity of this highly purified enzyme on which control globulin had no effect (Figs. 1 and 2). When the effects of antibody upon enzyme activity were studied under “enzyme turnover conditions” using the spectrophotometric pyruvate kinase-lactic acid dehydrogenase coupled enzyme system, the inhibition was observed to be time-dependent, requiring 8 to 10 min to reach maximum. The time-dependent nature of the inhibition was most apparent when the concentration of antibody was low (Fig 1, Trace 2). At high concentrations, inhibition was virtually complete within the first min after addition (Fig. 1, Trace 3).

A comparison of the dose responses of purified dog kidney (Na+, K+)-ATPase to rabbit and sheep immune globulin revealed that globulin from both species produced the same maximal degree of inhibition, and, as judged by the amount of globulin protein required to produce 50% inhibition (0.2 to 0.4 μg), the titers of inhibitory activity were equivalent (Fig. 2A). Whole antisera from rabbit and sheep gave similar results (data not shown).
Fig. 1 (left). Time-dependent nature of antibody inhibition of (Na+,K+)-ATPase activity. The tracings represent typical responses of highly purified dog kidney (Na+,K+)-ATPase to control and immune rabbit globulin, assayed by the spectrophotometric pyruvate kinase-lactic acid dehydrogenase coupled enzyme system (21). At time zero, 1.6 μg of enzyme were added to assay media in Cuvettes 1, 2, and 3. At 1 min, 200 μg of control globulin, and 20 μg and 200 μg of immune globulin were added to Cuvettes 1, 2, and 3, respectively. Details of the assay conditions are given under “Methods.”

Fig. 2 (right). Effects of immune globulin from rabbit and sheep on the kidney enzyme at various stages in its purification were examined to determine whether the degree of purity affected the response to antibody (Fig. 2B). The (Na+,K+)-ATPase activities present in the crude “microsomal” preparations were inhibited slightly less than was the purified enzyme. Less inhibition was also noted following glycerol precipitation, despite an 8-fold increase in specific activity and the demonstration of only two polypeptides upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis (19). However, upon the addition of deoxycholate-cholate solution to the enzyme, after glycerol precipitation but prior to fractionation with (NH₄)₂SO₄, the inhibition of the enzyme by antibody reached the maximum that was observed with the fully purified enzyme. Total inhibition, however, of the purified enzyme has not as yet been attained.

The effects of antibody upon catalytic activity were identical for assays using either the spectrophotometric pyruvate kinase-lactic acid dehydrogenase coupled enzyme system or the calorimetric assay for inorganic phosphate production.

Effect of Different Preincubation Conditions on Inhibition of (Na+,K+)-ATPase Activity—As previously shown, those antibodies reactive to (Na+,K+)-ATPase can be separated from the immune globulin fraction (17, 18). Furthermore, these (Na+,K+)-ATPase antibodies can be further separated into two fractions, one of which inhibits catalysis, and a second which inhibits ouabain binding.

The dependency on ligand conditions of the effectiveness of (a) the purified (Na+,K+)-ATPase antibody, (b) its anticitocytic, and (c) its antidigitalis receptor fractions with respect to inhibition of catalysis was studied by preincubation of enzyme with the antibody in the presence of different ligands. The activity of the enzyme thus treated was then assayed by the spectrophotometric pyruvate kinase-lactic acid dehydrogenase coupled enzyme system. While little ligand dependency was found for the inhibition produced by the purified antibody, the two fractions obtained from the pure antibody did show such dependency. The anticitocytic fraction inhibited catalysis strongly under all conditions except in the presence of [Mg²⁺,Na⁺] and [Mg²⁺,K⁺]. It was of interest that the anticitocytic fraction inhibited catalysis when preincubated in the presence of [Mg²⁺,Pᵢ] and [Mg²⁺,Na⁺,ATP], which are the conditions in which this fraction produced no inhibition of [³H]ouabain binding (Fig. 3). The antidigitalis receptor fraction produced little or no inhibition under a number of ligand conditions, including [Mg²⁺,Na⁺,K⁺]. However, the latter fraction did inhibit ATPase activity under several other conditions, including those in which [³H]ouabain binding was also inhibited (i.e. [Mg²⁺,Pᵢ] and [Mg²⁺,Na⁺,ATP]) (Fig. 3).

Enzyme-Antibody Cross-reactivity Studies—A number of (Na+,K+)-ATPase preparations from various organs and species were tested for inhibition by antibodies derived from rabbit and sheep antisera. Details of the preparations used are presented in Table I. These preparations varied widely in specific activity and state of purity, ranging from low activity membrane fractions (cat heart, rat heart, and human red cell) to more highly purified but still heterogeneous preparations (dog heart, brain, and submaxillary gland, rat kidney and brain, beef heart and brain) to still more highly purified enzyme preparations consisting of only two polypeptides plus lipid (dog, sheep, and pig kidney).

In order to facilitate the comparison of effects upon prepara-
tions of widely varying specific activities, the maximal effects produced by rabbit and sheep antibodies upon the activities of these (Na⁺,K⁺)-ATPase preparations are presented in terms of per cent inhibition of control activity (Figs. 4 and 5). Both rabbit and sheep antibodies inhibited canine kidney (Na⁺,K⁺)-ATPase preparations to a greater degree than preparations derived from other species. Rat, sheep, and pig enzyme preparations did not respond uniformly to either antibody. The rabbit antibody inhibited sheep preparations more than pig, and pig preparations more than rat preparations. It was noteworthy that the sheep antibody inhibited the rat and pig enzyme to a comparable level, but had no effect upon the sheep kidney enzyme (Fig. 4A).

The effects of these antibodies upon various brain preparations revealed further complexities (Fig. 4B). For example, sheep antibodies inhibited dog, rat, and beef enzymes to similar degrees. Dog and beef (Na⁺,K⁺)-ATPases were equally inhibited by rabbit antibodies and to the same level as produced by sheep antibodies; however, the former decreased

![Figure 3](image3.png)

**Table I**

<table>
<thead>
<tr>
<th>Species</th>
<th>Tissue</th>
<th>Specific activity (µmol P_i/mg/hr)</th>
<th>Description^a</th>
<th>Method of preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog</td>
<td>Kidney</td>
<td>1000-1400</td>
<td>Two polypeptides</td>
<td>Lane et al. (19)</td>
</tr>
<tr>
<td>Dog</td>
<td>Heart</td>
<td>300-350</td>
<td>Two major polypeptides plus several “contaminant” proteins</td>
<td>Pitts et al. (29)</td>
</tr>
<tr>
<td>Dog</td>
<td>Brain</td>
<td>350-425</td>
<td>Two polypeptides plus minor protein “contaminants”</td>
<td>Modified Pitts et al. (29)</td>
</tr>
<tr>
<td>Dog</td>
<td>Submaxillary gland</td>
<td>100-125</td>
<td>Membrane preparation</td>
<td>Hall et al. (30)</td>
</tr>
<tr>
<td>Beef</td>
<td>Heart</td>
<td>300-350</td>
<td>Two major polypeptide components plus several “contaminant” proteins</td>
<td>Pitts et al. (29)</td>
</tr>
<tr>
<td>Sheep</td>
<td>Brain</td>
<td>250-300</td>
<td>Multiple protein components</td>
<td>Dowd and Schwartz (31)</td>
</tr>
<tr>
<td>Pig</td>
<td>Kidney</td>
<td>700-800</td>
<td>Two polypeptides</td>
<td>Lane et al. (19)</td>
</tr>
<tr>
<td>Cat</td>
<td>Kidney</td>
<td>700-800</td>
<td>Two polypeptides plus trace “contaminants”</td>
<td>Lane et al. (19)</td>
</tr>
<tr>
<td>Rat</td>
<td>Kidney</td>
<td>35-46</td>
<td>Multiple protein components</td>
<td>Matsui and Schwartz (32)</td>
</tr>
<tr>
<td>Rat</td>
<td>Heart</td>
<td>10-15</td>
<td>Membrane preparation</td>
<td>Allen and Schwartz (33)</td>
</tr>
<tr>
<td>Human</td>
<td>Erythrocyte</td>
<td>0.1-0.2</td>
<td>Membrane preparation</td>
<td>Blostein (35)</td>
</tr>
</tbody>
</table>

^a All preparations are particulate in nature and contain substantial quantities of lipid.
the catalytic rate of rat brain enzyme to a greater extent than did the latter.

The effects of antibodies upon (Na+,K+)-ATPases prepared from heart were compared (Fig. 4C). Dog, rat, and beef were inhibited equally by rabbit and sheep antibodies, although to different degrees. Cat heart, however, was affected more by rabbit than by sheep antibodies. Both cat and rat enzymes were inhibited to a greater degree than dog. Dog salivary and human red cell (Na+,K+)-ATPases were inhibited equally and to a high degree by antibodies from both species (Fig. 4D).

Both antibodies produced comparable inhibitory effects on each of the enzymes isolated from dog tissues (Fig. 5). The enzymes from kidney and submaxillary gland were inhibited equally and to a high degree. However, the heart and brain (Na+,K+)-ATPases were both inhibited to a much lesser extent than the enzyme from kidney and salivary gland.

Effects of Antibody Fractions Obtained from Rabbit and Sheep (Na+,K+)-ATPase Antibodies on Brain (Na+,K+)-ATPase—The large differences in the extent to which antibodies to dog kidney (Na+,K+)-ATPase inhibited the enzyme from other canine tissues suggested organ differences in the antigenic composition of (Na+,K+)-ATPase. In an attempt to gain further evidence of such differences, rabbit and sheep antibodies were fractionated by absorption with dog brain enzyme. The purpose of this fractionation was to determine whether immune globulin, specific for kidney, would retain any inhibitory activity against kidney following removal of the inhibitory activity to brain (Na+,K+)-ATPase. Therefore, we examined the maximal effects on catalysis of the fractions of rabbit and sheep antibodies which were obtained by absorption with dog brain (Na+,K+)-ATPase (Table II). Dog brain (Na+,K+)-ATPase was inhibited by antibodies much less than was dog kidney, making a direct comparison of the effects of the fractionations on the two enzymes difficult. Therefore, in Fig. 6, the maximal inhibition produced by the fractions is presented as a per cent of maximal inhibition caused by the unfractionated immune globulins from rabbit and sheep.

Both the rabbit antibodies which did react with the brain enzyme preparation ("brain-reactive") and those that did not react ("brain-nonreactive") were highly inhibitory to dog kidney (Na+,K+)-ATPase (Fig. 6A), reducing the catalytic rate by 88 ± 3% (mean ± S.E.M.) and 79 ± 2%, respectively, of the inhibition produced by the unfractionated antibodies. In the case of inhibition of the brain enzyme activity, the brain-reactive fraction was 97 ± 4% as effective as the unfractionated antibodies; however, the brain-nonreactive fraction was only 21 ± 3% as potent (Fig. 6B). When recombined, the two fractions inhibited both brain and kidney to the full extent of that produced by the unfractionated antibodies. Identical results were obtained with sheep antibody fractions (Fig. 6C and D). The brain-nonreactive fraction retained 73 ± 4% of the effectiveness of the unfractionated sheep antibodies against kidney but only 18 ± 4.1% of that against brain. Conversely, the brain-reactive fraction was 99 ± 3% as effective as the unfractionated antibodies against brain, and 88 ± 3% as inhibitory as kidney. As with rabbit antibody, recombination of the fractions fully restored activity against both brain and kidney. These data indicate that absorption with dog brain (Na+,K+)-ATPase resulted in removal of most inhibitory antibodies to brain, but left considerable inhibitory activity to kidney (Na+,K+)-ATPase.

Effects of Antibodies on Intact Erythrocytes—The antibody raised to the holoenzyme, (Na+,K+)-ATPase, had no effect on either potassium transport or digoxin uptake (Table III). The anticatalytic fraction, which presumably would interact with an "inside" conformation, had only a minimal or no effect on these two parameters, but it was of interest that the antidualalis receptor fraction significantly retarded both potassium influx and digoxin uptake (Table III).

**DISCUSSION**
Antisera from rabbit and sheep raised to highly purified canine kidney (Na+,K+)-ATPase produced nearly complete inhibition of ATP hydrolysis when incubated with this enzyme. These findings are in agreement with those reported by other investigators for antibodies raised against enzyme preparations.
from Escherichia coli (11), rat brain (12), pig kidney (13), dog heart (14), and dog kidney (15).

Enzyme inhibition by antibodies against (Na⁺,K⁺)-ATPase may be produced by (a) binding of a species of antibody directly to the catalytic center; (b) binding to a site near the catalytic center and, while not affecting the center directly, blocking entry of substrate and/or ligands by steric hindrance; (c) binding to site(s) at some distance from the active center on the interior and/or exterior aspects of the complex and "occluding" the enzyme in a conformation that is unresponsive to catalytic stimuli; and (d) binding of antibodies to lipid components of the membrane and inhibiting enzyme function by a perturbation of the membrane (Fig. 7). Considerable lipid is present in the purified enzyme preparation, and the antisera may contain antibodies to lipids. Since the antibody units are bivalent, their interaction with the system may cause aggregation and precipitation of the enzyme complex and, in effect, remove the enzyme from the reaction medium. It is not clear which of these mechanisms, either singly or in combination, is responsible for the inhibition of (Na⁺,K⁺)-ATPase. Monovalent Fab fragments of sheep and rabbit antibodies produced the same level of inhibition as did the intact antibody (data not shown). This finding suggests that inhibition is not the result of the formation of large insoluble aggregates. It is of importance that the purified (Na⁺,K⁺)-ATPase antibody appeared to contain no protein moieties other than immunoglobulin, when examined by immune-electrophoresis employing goat antiserum to whole rabbit serum (Fig. 8).

Table III
Effect of anti-holoantibody (anti-(Na⁺,K⁺)-ATPase antibody), anti-Cat and anti-DR on intact human erythrocytes: potassium transport and digoxin uptake

<table>
<thead>
<tr>
<th>Addition</th>
<th>K+ uptake Control + Digoxin (1.48 × 10⁻⁶M)</th>
<th>Digoxin uptake (1.48 × 10⁻⁶M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mm K⁺/h RBC/hr</td>
<td>pmol/ml RBC</td>
</tr>
<tr>
<td>RGG (0.2 mg)</td>
<td>2.89</td>
<td>1.75</td>
</tr>
<tr>
<td>Anti-NKA (0.2 mg)</td>
<td>3.27</td>
<td>2.42</td>
</tr>
<tr>
<td>Anti-Cat (0.2 mg)</td>
<td>2.18</td>
<td>1.57</td>
</tr>
<tr>
<td>Anti-DR (0.2 mg)</td>
<td>0.70</td>
<td>0.51</td>
</tr>
</tbody>
</table>

*The abbreviations used are: RGG = rabbit γ-globulin (see under "Methods"); anti-NKA = antibody to holoenzyme, canine medulla (Na⁺,K⁺)-ATPase, raised in rabbits; anti-Cat = fraction of antibodies that inhibits catalysis; anti-DR = fraction of antibodies that inhibits glycoside binding.
Antiserum raised against complex protein macromolecules such as enzymes usually contains many different species of antibodies, each being directed against a different region of the molecule (1–3). The conformation, as well as the primary amino acid sequence of these regions, are of great importance in determining the ability of an antibody to “recognize” and bind to its determinant (1–4, 36, 37). The binding of ouabain to (Na⁺,K⁺)-ATPase is believed to result in the enzyme being “fixed” in a conformation different from that which supports ATP hydrolysis (38–40). The ouabain-induced change in conformation might be expected to result in an alteration of the shape of certain areas of the enzyme such that antibodies specific for particular conformations of those areas would be unable to bind to the enzyme-ouabain complex. Consequently, fractionation of the antibody was carried out by absorption of purified antibody with an enzyme-ouabain complex and yielded two fractions. One of these fractions could bind to the complex and, after elution, inhibit catalytic activity. The second could not bind to the ouabain enzyme complex and was found, in separate experiments, to inhibit ouabain binding. It is probable that the latter did not bind to the enzyme-ouabain complex because of a conformational change in the enzyme, but some of this antibody may have been specific for the actual ouabain receptor site and could not bind to the enzyme-ouabain complex simply because the site of interaction was occupied by ouabain.

The two fractions exerted different effects upon catalytic activity and [H]ouabain binding. The failure of one to inhibit [H]ouabain binding supported either by [Mg⁺⁺, Na⁺,ATP] or [Mg⁺⁺, P_i] is probably not the result of that fraction being unable to bind to its determinant(s) on the enzyme under these ligand conditions. This is so because inhibition of ATP hydrolysis was observed upon preincubation of the enzyme with this fraction in the presence of [Mg⁺⁺, Na⁺,ATP] and of [Mg⁺⁺, P_i]. Further evidence in this regard is the increased level of [H]ouabain binding obtained with enzyme exposed to the fraction compared to that with control globulin (17, 18). This stimulation of binding may indicate that these antibodies fixed the enzyme in a conformation more favorable for ouabain binding than that which was present with the ligands [Mg⁺⁺, Na⁺,ATP] alone. It should be noted, however, that this increase in binding was only to the control level reached in the presence of [Mg⁺⁺, P_i]. It is not known whether either of the antibodies binds to the enzyme relative to the “active” site and “ouabain receptor,” or by what mechanism they exert their effects (see Fig. 7). However, the separation of the effects of the fractions upon these two aspects of enzyme function strongly suggests that certain of the molecular determinants necessary for the hydrolysis of ATP are separate from those necessary for ouabain binding.

A number of laboratories have reported effects of antibodies raised to the (Na⁺,K⁺)-ATPase holoenzyme. Askari and Rao (12) observed that antisera to rat brain (Na⁺,K⁺)-ATPase inhibited (Na⁺,K⁺)-stimulated catalysis of ATP and sodium-dependent phosphorylation of the enzyme. The potassium-dependent breakdown of the phosphoenzyme and the potassium-dependent p-nitrophenylophosphatase (p-NPPase) reaction, however, were not affected by their antibody. The authors concluded that the potassium-dependent activities of the (Na⁺,K⁺)-ATPase complex are functions of a component which is antigenically distinct from that involved in phosphorylation and ATP hydrolysis. Jørgensen et al. (13) found that antisera to a pig kidney (Na⁺,K⁺)-ATPase preparation inhibited the ouabain-sensitive sodium efflux from human resealed red cell ghosts, but only when the antisera was present in the resealing medium. Incubation of antisera with resealed ghosts or intact red cells had no effect upon sodium efflux. These investigators concluded that the antibody must bind to some component of the enzyme complex on the interior of the cell membrane in order to inhibit sodium transport. The differential effects of two antibody fractions described here upon catalysis and [H]ouabain support the concept that different partial reactions are controlled by separate portions of the (Na⁺,K⁺)-ATPase complex.

We studied the effect of the holoantibody and its anticytolytic and antidigal digal receptor components on digoxin uptake by and potassium influx into human erythrocytes. The results observed are consistent with the data reported herein on fragmented enzyme preparations. The fact that neither the holoantibody nor its anticytolytic component had any significant effect on digoxin uptake is in accord with the failure of these antibodies to inhibit ouabain binding to purified (Na⁺,K⁺)-ATPase. Since antibodies do not ordinarily enter nonendocytic mammalian cells (41), the failure to inhibit potassium influx is consistent with the hypothesis that the site at which these antibody populations exert their effect is on the inner side of the plasma membrane (42). Inhibition of digoxin uptake by the antibody fraction, which inhibited ouabain binding to isolated (Na⁺,K⁺)-ATPase, is consistent with analogous effects observed with purified enzyme preparations, and strongly suggests that at least some of the antigenic determinants with which this fraction reacts are readily accessible on the outer surface of the plasma membrane. The ability of the same fraction to inhibit potassium influx suggests that the antibody may be inducing structural alterations in (Na⁺,K⁺)-ATPase at a site important for active cation transport.

Calcium-stimulated ATPase of canine cardiac-relaxing system (sarcoplasmic reticulum fragments), magnesium-dependent, ouabain-insensitive ATPase of membranous (Na⁺,K⁺)-ATPase preparations and magnesium-dependent ATPase of mitochondria were not affected by rabbit and sheep antibodies raised against (Na⁺,K⁺)-ATPase. This suggests that there is little antigenic similarity between these classes of ATPases and the (Na⁺,K⁺)-ATPase. Conversely, these antibodies inhibited all (Na⁺,K⁺)-ATPases that were tested (with the important
exception that sheep antibodies did not affect sheep kidney enzyme, indicating that the enzymes in various tissues and from different species share some antigenic determinants with dog kidney (Na⁺,K⁺)-ATPase.

Despite the indication of at least partial homology among (Na⁺,K⁺)-ATPases from various sources, there are strong suggestions of distinct antigenic species and organ differences. (a) All enzymes were not inhibited to the same degree. This variability in the effect of rabbit and sheep antibodies was observed both for enzymes from the same tissue of different species and for enzymes isolated from different tissues of the same species. (b) The exposure of antibody against dog kidney to a dog brain enzyme was successful in removing most of the inhibitory activity toward the brain (Na⁺,K⁺)-ATPase, but left most of the inhibitory activity to kidney. These results suggest that the dog kidney antiserum contained some antibody specific for antigenic determinants of the kidney enzyme which were present in brain but also contained antibodies to determinants that were unique to kidney (Na⁺,K⁺)-ATPase, at least with respect to brain. (c) Antibody raised in sheep against dog kidney (Na⁺,K⁺)-ATPase inhibited the dog enzyme by 85 to 90% but had no effect upon sheep kidney (Na⁺,K⁺)-ATPase, while rabbit antibody against the same canine ATPase was inhibitory to sheep ATPase. This interesting observation is evidence that sheep serum contained no antibodies to determinants of homologous tissue. Inhibition of other (Na⁺,K⁺)-ATPases by sheep antibody must reflect antigenic differences from sheep (Na⁺,K⁺)-ATPase. Such apparent variation in the antigenic profile of (Na⁺,K⁺)-ATPases may be irrelevant in terms of enzyme function. However, the (Na⁺,K⁺)-ATPase of kidney and submaxillary gland, two organs with major ion transport functions, were inhibited equally and to a degree markedly different from that of enzymes from heart and brain.

Varying degrees of inhibition of (Na⁺,K⁺)-ATPase preparations from most tissues other than dog kidney may be explained in several ways. The enzymes from different sources may be structurally different. Partial inhibition also may indicate that the (Na⁺,K⁺)-ATPase activities of heart and brain are the sum of the activities of several subspecies of enzyme; one subspecies being identical to the kidney enzyme and being completely inhibited, while others are distinct subspecies that are unaffected by the antibody to kidney (Na⁺,K⁺)-ATPase. Another explanation is that the preparations differ in purity with respect to the (Na⁺,K⁺)-ATPase. Pure (Na⁺,K⁺)-ATPase preparations are not available from most tissues, and in this study, the enzyme preparations used varied widely as to specific activity, state of purity, and method of isolation. Conversely, it is reassuring that the observed differences in response to antibodies persisted in enzymes that were obtained in a highly purified state, i.e., dog kidney and brain, pig and sheep kidney. The antigenic organ differences in (Na⁺,K⁺)-ATPase may indicate the existence of isoenzymes. It is of interest in this regard that species and organ differences in responses of (Na⁺,K⁺)-ATPase to cardiac glycosides have been reported (43, 44). The well known inactivity of rat heart to cardiac glycosides, for example, has been explained on the basis of an easily dissociable (Na⁺,K⁺)-ATPase-glycoside complex (43, 44).

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