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Characteristics of Antibody Inhibition of Rat Kidney ($Na^+ - K^+$)-ATPase

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Summary. Antibodies which were raised against highly purified membrane-bound ($Na^+ - K^+$)-ATPase from the outer medulla of rat kidneys inhibit the ($Na^+ - K^+$)-ATPase activity up to 95%. The antibody inhibition is reversible. The time course of enzyme inhibition and reactivation is biphasic in semilogarithmic plots.

In the purified membrane-bound ($Na^+ - K^+$)-ATPase negative cooperativity was observed (a) for the ATP dependence of the ($Na^+ - K^+$)-ATPase activity ($n = 0.86$), (b) for the ATP binding to the enzyme ($n = 0.58$), and (c) for the ouabain inhibition of the ($Na^+ - K^+$)-ATPase activity ($n = 0.77$). By measuring the Na^+ dependence of the ($Na^+ - K^+$)-ATPase reaction, a positive homotropic cooperativity ($n = 1.67$) was found.

As reactivation of the antibody-inhibited enzyme proceeds very slowly ($t_{0.5} = 5.2$ hr), it was possible to measure characteristics of the antibody-($Na^+ - K^+$)-ATPase complex: The antibodies exerted similar effects on the ATP dependence of the ($Na^+ - K^+$)-ATPase reaction and on the ATP binding of the enzyme. V_{max} of the ($Na^+ - K^+$)-ATPase reaction and the number of ATP binding sites were reduced while $K_{0.5\,ATP}$ for the ($Na^+ - K^+$)-ATPase activity and for the ATP binding were increased by the antibodies. The Hill coefficients for the ATP binding and for the ATP dependence of the enzyme activity were not significantly altered by the antibodies. The antibodies increased the $K_{0.5}$ value for the Na^+ stimulation of the ($Na^+ - K^+$)-ATPase activity, but they did not alter the homotropic interactions between the Na^+-binding sites. The negative cooperativity which was observed for the ouabain inhibition of the ($Na^+ - K^+$)-ATPase activity was abolished by the antibodies.

The data are tentatively explained by the following model: The antibodies bind to the ($Na^+ - K^+$)-ATPase from the inner membrane side, reduce the ATP binding symmetrically at the ATP binding sites and reduce thereby also the ($Na^+ - K^+$)-ATPase activity of the enzyme. The antibodies may inhibit the ATP binding by a direct interaction or by means of a conformational change at the ATP binding sites. This may possibly also lead to the alteration of the Na^+ dependence of the ($Na^+ - K^+$)-ATPase activity and to the observed alteration of the dose response to the ouabain inhibition.

Antibodies raised against a protein molecule usually consist of a population of different antibodies which bind to different parts of the protein [5]. Only few of them, however, inhibit specific functions of the protein. If, therefore, the antibody effect on a specific protein function is measured, antibodies may be used as specific probes provided the mode and site of antibody action are known.

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Against a series of $(Na^+ - K^+)$-ATPase preparations antibodies have been prepared [1, 12, 15, 17, 19, 24], some of which inhibit the $(Na^+ - K^+)$-ATPase activity. The present study was undertaken to elucidate the mechanism of antibody inhibition of the $(Na^+ - K^+)$-ATPase activity. The results obtained demonstrate that the enzyme activity is inhibited in a reversible manner but that the enzyme antibody complex is very stable. The data suggest that the enzyme activity is inhibited by blocking the ATP binding to the enzyme. It appeared, however, that the mode of antibody action is complex. This is considered to be due to the co-operative nature of the $(Na^+ - K^+)$-ATPase and to general characteristics of antibody action.

Materials and Methods

Preparation of $(Na^+ - K^+)$-ATPase

The outer medulla of rat kidneys [14] was carefully dissected and the membrane-bound $(Na^+ - K^+)$-ATPase was purified according to Jørgensen [11]. Starting from this preparation, the enzyme was solubilized in the presence of ATP with Lubrol (1 mg Lubrol WX/mg protein). The membranes were centrifuged for 1 hr at 180,000 × *g*, and the detergent was removed by placing the supernatant on a Sepharose 6B column (20 mM imidazole-HCl, pH 7.5, 3 mM $MgCl_2$, 2 mM EGTA, 20% glycerol). To remove the adenine nucleotides from the membrane-bound or solubilized $(Na^+ - K^+)$-ATPase, the enzyme was put on a column of Sephadex G 50 which had been equilibrated with a buffer solution containing 50% (vol/vol) glycerol, 20 mM imidazole-HCl, pH 7.5, 3 mM $MgCl_2$ and 2 mM EGTA. The membrane-bound or soluble $(Na^+ - K^+)$-ATPase appeared in the void volume clearly separated from a second peak of nucleotides, which were identified by their absorption ratio 280 to 260 nm and by the Luciferase method [25].

The membrane-bound $(Na^+ - K^+)$-ATPase had a specific activity of 30–36 μmol ATP split per mg protein per min. The Mg^{++}-ATPase as measured in the presence of ouabain and in the absence of NH_4Cl was less than 1%. About 35% of the membrane protein could be solubilized from the membrane-bound $(Na^+ - K^+)$-ATPase. A partial inactivation of the $(Na^+ - K^+)$-ATPase activity during the solubilization could not be avoided so that the specific activity of the solubilized enzyme was 15–20 μmol ATP split per mg per min. The $(Na^+ - K^+)$-ATPase consisted of two different subunits. The amount of extraneous proteins was less than 5% in the purest preparations, as shown in Fig. 1. The molecular weights of the subunits were 120,000 and 60,000 daltons, respectively, as determined in SDS gel electrophoresis [27] using RNA, bovine serum albumin, albumin from chicken eggs, cytochrome *c* and human gammaglobulin as standards. The SDS pattern of the solubilized and membrane-bound $(Na^+ - K^+)$-ATPase were identical.

Immunization

Antibodies were raised in rabbits against a suspension of the purified membrane-bound $(Na^+ - K^+)$-ATPase in a buffer containing 50 mM imidazole-HCl, pH 7.5, 2 mM EDTA, 3 mM ATP (Na_2). Each rabbit received an initial intramuscular (i.m.) injection of the antigen (0.2 mg protein) mixed with an equal volume of complete Freund's adjuvant. After two,

Fig. 1. SDS-polyacrylamide gel electrophoresis of membrane-bound ($Na^+ - K^+$)-ATPase. 20 µg protein has been applied on top of the gel

four, and six weeks further i.m. injections of 0.2 mg antigen protein were given [18]. As no antibodies which inhibit the ($Na^+ - K^+$)-ATPase activity could be detected a week after the last immunization, one month later another immunization scheme [6] was tried in the same animals. The rabbits received four further intraperitoneal injections with increasing amounts of antigen (0.2, 0.4, 0.6, 1.2 mg protein) in intervals of two, four, and six days, respectively. Blood was obtained by cardiac puncture three days after the last immunization.

Purification of Ig G

Gel filtration of the antiserum on Sephadex G 200 (5 × 75 cm, 1 M NaCl, 0.05 M Tris-chloride, pH 7.8) revealed an elution profile consisting of three major peaks. The second peak, which contained the Ig G fraction without contamination of Ig M, was repeatedly dialyzed against water. The protein which was precipitated by this treatment was removed by centrifugation. The remaining protein which contained the Ig G fraction was dialyzed against 50% ammonium sulfate at 4 °C. The protein which was precipitated under these conditions was separated by 20,000 × *g* centrifugation. The precipitate consisted of pure Ig G globulin, which was tested by immunoelectrophoresis [4] against goat anti rabbit serum.

Protein and $(Na^+ - K^+)$-ATPase Assay

Protein was determined after precipitation with 10% trichloroacetic acid (TCA) and dissolution in 1 N NaOH and 10% SDS according to the method of Lowry and coworkers [16]. Bovine serum albumin was used as standard solution. $(Na^+ - K^+)$-ATPase activity was measured by an optical assay, as described by Schoner and coworkers [22]. The reaction mixture contained in 1 ml: 70 µmol imidazole-HCl, pH 7.3, 5 µmol $MgCl_2$, 0.4 µmol NADH, 100 µmol NH_4Cl which was introduced instead of KCl [22], 3 µmol ATP (Na_2), 0.6 µmol phosphoenolpyruvate, 6 units pyruvate kinase, 6 units lactic dehydrogenase, 150 µmol NaCl. Temperature, 37 °C, $\lambda = 360$ nm. In order to compensate for the activity of the Mg^{++}-stimulated ATPase all readings were taken against a control in which NH_4Cl was omitted and 2.4 µmol of ouabain were present in the test. The reaction was started by adding the enzyme to the prewarmed reaction mixture and the initial linear rate of $(Na^+ - K^+)$-ATPase activity was measured within the next 2 min. To measure the ATP dependence of the $(Na^+ - K^+)$-ATPase reaction, the enzyme was freed from ATP by passing through a glycerol column as described above, and the concentration of ATP in the reaction mixture was varied from 0.01 to 10 mM.

Measurement of Antibody Inhibition of the ATP Binding

ATP binding was measured by a rapid mixing centrifugation method [10]. 10 µg enzyme protein, which was either untreated or incubated for 60 min at 0 °C with antibodies before, was incubated at 0 °C with 20 mM imidazole-HCl, pH 7.5, 10 mM EDTA and 0.15 to 11 µM ATP (αP^{32}) in a total volume of 0.25 ml in Eppendorf® tubes. The membranes were spun down at 50,000 × *g* for 20 min. After careful removal of the incubation mixture, the tips of the Eppendorf® tubes were cut off and the sediments solubilized in Soluene-350 Tissue Solubilizer® at 60 °C. After neutralization with concentrated HCl, Rothiscint® as scintillation fluid containing Triton X-100 was added and the radioactivity counted in a Berthold BF 5000 liquid scintillation counter. All results were corrected for unspecific ATP binding by subtracting the value obtained in the presence of 10 mM unlabelled ATP.

Materials

Ethylenediamine-tetraacetic acid (EDTA), ethyleneglycol-bis(β-aminoethylether) NN'-tetraacetic acid (EGTA), Lubrol WX, and human gammaglobulin were purchased from Sigma (London). Nicotinamidadenine-dinucleotide (NADH), lactic dehydrogenase, phosphoenolpyruvate, pyruvate kinase, RNA, and ATP (Na_2) were supplied by Boehringer GmbH (Mannheim). For measurements in the absence of sodium, the ATP sodium salt

was converted to the Tris salt by ion exchange chromatography on a Dowex 50 column. Adenosine 5′ triphosphate-tetra (triethylammonium) salt-[$\alpha^{32}P$] with a specific activity of 17 Ci/mmol was obtained from New England Nuclear (Boston). All other chemicals were purchased from Merck (Darmstadt) or from Serva Biochemicals (Heidelberg).

Results

When the gammaglobulins isolated from the ($Na^+ - K^+$)-ATPase antiserum diffused against solubilized ($Na^+ - K^+$)-ATPase in the Ouchterlony test, no precipitation lines were obtained, regardless of whether the test was performed in the presence or absence of different detergents. The gammaglobulins of the antisera inhibited the ($Na^+ - K^+$)-ATPase activity of both the membrane-bound and the solubilized enzyme. The maximal degree of enzyme inhibition, which was different in the gammaglobuline preparations of different antisera, varied between 80 and 95%. As shown in Fig. 2, maximal inhibition of 20 µg membrane-bound ($Na^+ - K^+$)-ATPase per ml with a specific activity of 32 µmol P_i mg^{-1} min^{-1}, was achieved at a gammaglobulin concentration of 1 to 2 mg/ml. If the concentration of gammaglobulin was increased further, no additional inhibition was observed.

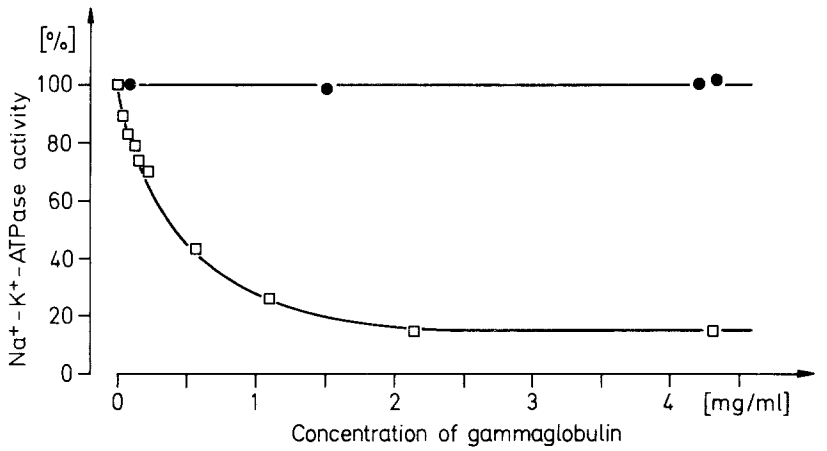

Fig. 2. Dose response curve of membrane-bound ($Na^+ - K^+$)-ATPase to gammaglobulin. 0.05 mg/ml of enzyme which released 32 µmol P_i per mg protein per min was incubated with increasing concentrations of gammaglobulin isolated from ($Na^+ - K^+$)-ATPase antiserum (□——□) or normal rabbit serum (●——●) in an incubation buffer containing 20 mM imidazole-HCl, pH 7.5, 2 mM EGTA, 3 mM $MgCl_2$, 3 mM ATP, 150 mM NaCl, 150 mM KCl, and 1 mg/ml BSA. After 60 min of incubation at 0 °C, the ($Na^+ - K^+$)-ATPase activity was measured

Time Course of Antibody Inhibition and Reactivation

The course of antibody inhibition of the $(Na^+ - K^+)$-ATPase activity was investigated in the membrane-bound enzyme. The antigen antibody reaction was performed at 0 °C in a buffer containing 20 mM imidazole-HCl, pH 7.5, 1 mg/ml BSA, 6 mM phosphate, 3 mM $MgCl_2$, 2 mM EGTA, 3 mM ATP, 150 mM NaCl and 150 mM KCl; the concentration of gammaglobulin was 2.3 mg/ml, that of the protein was 0.02 mg/ml. From Fig. 3*a* it can be seen that the $(Na^+ - K^+)$-ATPase activity was inhibited in two phases. Within one minute the enzyme activity was inhibited to 75% of the final inhibition (Fig. 3*a*). Then in a second slow phase of antibody inhibition, the $(Na^+ - K^+)$-ATPase activity was inhibited further. Within 60 min a steady-state value of enzyme activity was reached. The time required to produce half final inhibition ($t_{0.5}$) was <0.5 min for the rapid phase and 17.1 min for the slow phase of antibody inhibition. In the slow phase of antibody inhibition the rate of $(Na^+ - K^+)$-ATPase activity decreased exponentially with time.

Reversibility of the antigen antibody reaction was tested by examining the reactivation of the antibody-inhibited enzyme when the gammaglobulin concentration was decreased. For these experiments membrane-bound $(Na^+ - K^+)$-ATPase was incubated for 60 min with varying concentrations of gammaglobulin and spun down at $50{,}000 \times g$. The supernatants were 90% removed and replaced either by pure buffer or by buffer containing gammaglobulin at the original concentration of the respective sample. Reactivation of the inhibited enzyme was observed when the gammaglobulin concentration was reduced. More than ten hours, however, were required before equilibrium was achieved. To measure the time course of reactivation, the initial gammaglobulin concentration of 0.8 mg protein per ml was reduced to 0.08 mg/ml at time 0. The initial relative inhibition (i_o) of the $(Na^+ - K^+)$-ATPase activity was 0.71, the final inhibition after 12 hr (i_f) was 0.37. The rate of enzyme reactivation in such a situation where the concentration of the free inhibitor is reduced to a value different from zero can be expressed by the equation [26]:

$$\frac{i_t - i_f}{i_o - i_f} = e \exp \frac{-k_{-1} \cdot t}{1 - i_f} \tag{1}$$

where i_t is the relative inhibition of enzyme activity at a given time and k_{-1} the rate constant of enzyme reactivation. From Fig. 3*b*, in which $(i_t - i_f / i_o - i_f)$ is plotted on a logarithmic scale against time, it can be

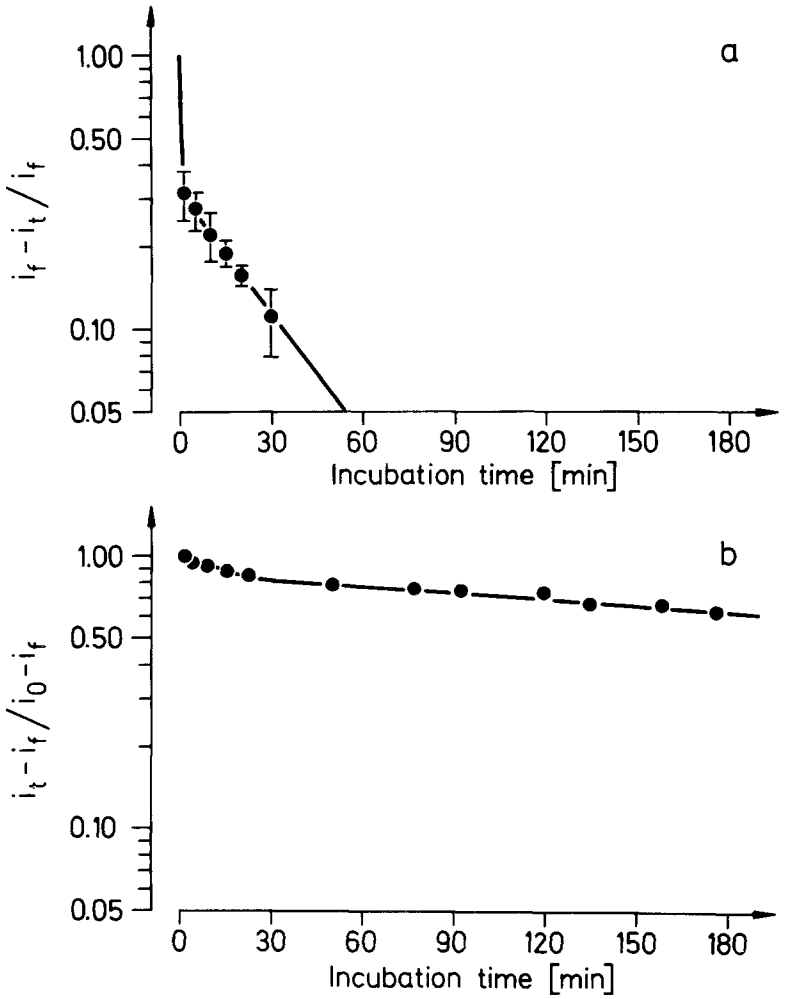

Fig. 3. Time courses of the antibody inhibition of membrane-bound ($Na^+ - K^+$)-ATPase (*a*) and of the reactivation of the antibody inhibited enzyme (*b*), demonstrated in semilogarithmic plots. (*a*): The antibodies have been added to the enzyme at the time 0. At equilibrium the relative inhibition i_f is reached; i_t represents the relative inhibition at the indicated time intervals. The ordinate $i_f - i_t/i_f$ indicates the fraction of the ($Na^+ - K^+$)-ATPase activity which is inhibited. (*b*): At the time 0 with the relative inhibition i_o the gammaglobulin concentration is reduced by dilution; i_f represents the final inhibition which is reached at the equilibrium of the enzyme reactivation and i_t is the relative inhibition at the time intervals indicated on the abscissa. The ordinate $i_t - i_f/i_o - i_f$ indicates the fraction of the ($Na^+ - K^+$)-ATPase activity which remains inhibited

seen that the enzyme reactivation also appears to be biphasic. For the more rapid phase a reactivation constant of 8.8×10^{-5} sec^{-1} ($\tau = 3.2$ hr), and for the very slow reactivation phase a reactivation constant of 1.9×10^{-5} sec^{-1} ($\tau = 14.6$ hr) was estimated.

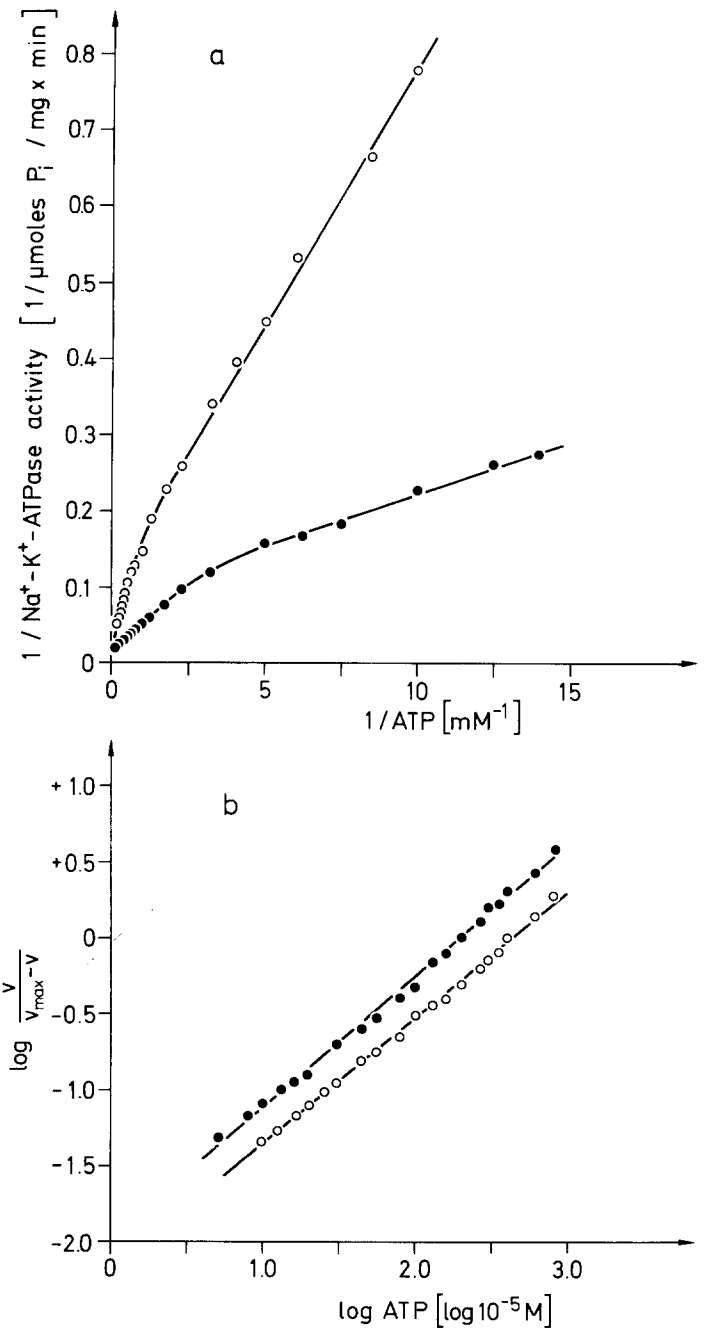

Fig. 4. Effect of ATP concentration on the rate of ATP hydrolysis (v) of untreated (●——●) and partially antibody-inhibited (○——○) membrane-bound ($Na^+ - K^+$)-ATPase. Preincubation with the antibodies was performed in the presence of 3 mM $MgCl_2$ and 6 mM P_i and led to a 51% decrease in the ($Na^+ - K^+$)-ATPase activity. The enzyme activity was measured in the presence of 70 mM imidazole-HCl, pH 7.3, 5 mM $MgCl_2$, 100 mM NH_4Cl, 150 mM NaCl, and the concentration of ATP is indicated. Data are expressed as a Lineweaver-Burk plot (*a*) and as a Hill plot (*b*). The Hill coefficient is 0.86 and 0.82 for the untreated and antibody-inhibited enzyme, respectively

Antibody Action and ATP Dependence of $(Na^+ - K^+)$-ATPase Activity

In the membrane-bound $(Na^+ - K^+)$-ATPase the influence of antibodies on the substrate dependence of the ATP hydrolysis was tested. For this the ATP free enzyme was preincubated with gammaglobulin for 60 min at 0 °C so that the $(Na^+ - K^+)$-ATPase activity was reduced to 49% of the control activity. Then samples were taken, and the initial rate of $(Na^+ - K^+)$-ATPase activity at various concentrations of ATP was measured in the presence of 5 mM $MgCl_2$. Not shown are the identical results obtained if the concentrations of $MgCl_2$ and ATP were varied simultaneously. Figure 4 shows the substrate dependence of untreated and antibody inhibited enzyme. The enzyme does not follow Michaelis Menten kinetics, as can be seen from Lineweaver Burk plots (Fig. 4*a*) where the curves show a downward swing at low concentrations of ATP. In Hill plots (Fig. 4*b*) straight lines were obtained for the untreated and the antibody-inhibited enzyme. About the same Hill coefficient was calculated in both cases (control: $n_{ATP} = 0.86$, antibody-inhibited enzyme: $n_{ATP} = 0.82$). These findings suggest a negative cooperativity which is not affected by the antibodies. For the untreated membrane-bound enzyme a V_{max} value of 59 µmol phosphate liberated per mg per min and a $K_{0.5\,ATP}$ value of 1.9 mM was calculated. In the antibody-inhibited enzyme V_{max} was 29 µmol P_i per mg per min and $K_{0.5\,ATP}$ was 4.3 mM.

Antibody Action and ATP Binding

To find out whether the antibodies inhibit the ATP binding to the $(Na^+ - K^+)$-ATPase, the ATP-free enzyme was first preincubated with gammaglobulin for 60 min so that the $(Na^+ - K^+)$-ATPase activity was reduced to 42% of the control activity. Then the binding of ATP ($\alpha^{32}P$) was measured and compared with that of the untreated enzyme. From the Scatchard plots (Fig. 5*a*) it can be seen that the binding of ATP was reduced by the antibodies. The number of "high affinity" ATP binding sites was reduced to 46% of the control by the antibodies. If the data are plotted according to the Hill equation (Fig. 5*b*), straight lines were obtained, from which Hill coefficients of 0.58 and 0.57 for the untreated and the antibody-inhibited enzyme, respectively, were calculated. Thus, the ATP binding also shows a negative cooperativity which was not influenced by the antibodies. For the untreated and antibody-inhibited enzyme $K_{0.5}$ values for ATP binding of 0.55 and 1.62 µM, respectively, were calculated.

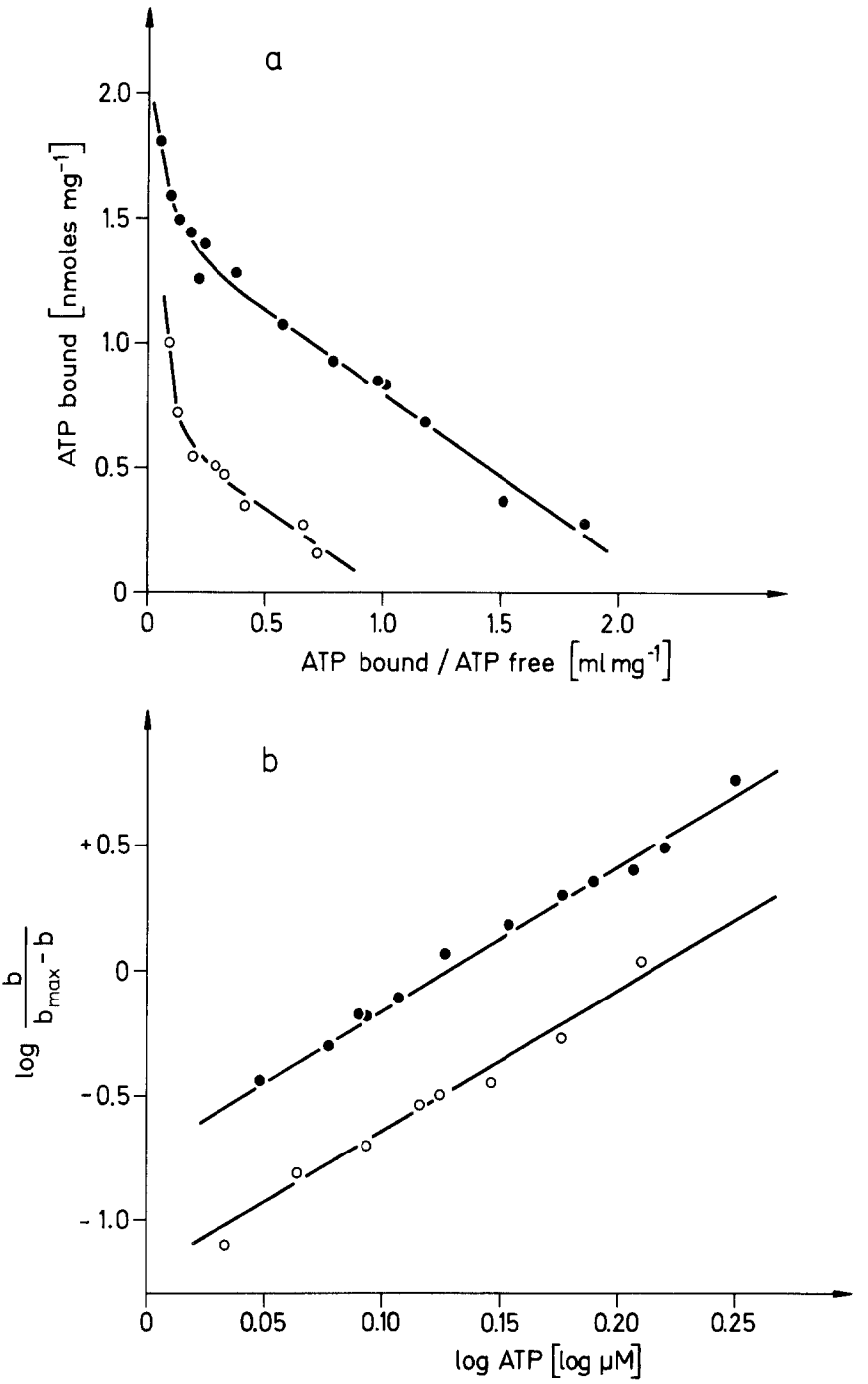

Fig. 5. Binding of ATP (α^{32}P) on the untreated (●——●) and antibody-inhibited (○——○) membrane-bound ($Na^+ - K^+$)-ATPase. The preincubation with the antibodies was performed in the presence of 3 mM $MgCl_2$ and 6 mM P_i. The ATP binding (*b*) was measured in the presence of 20 mM imidazole-HCl, pH 7.5, and 10 mM EDTA. Data are expressed as a Scatchard plot (*a*) and as a Hill plot (*b*). The Hill coefficient is 0.58 and 0.57 for the control and the antibody inhibited enzyme, respectively

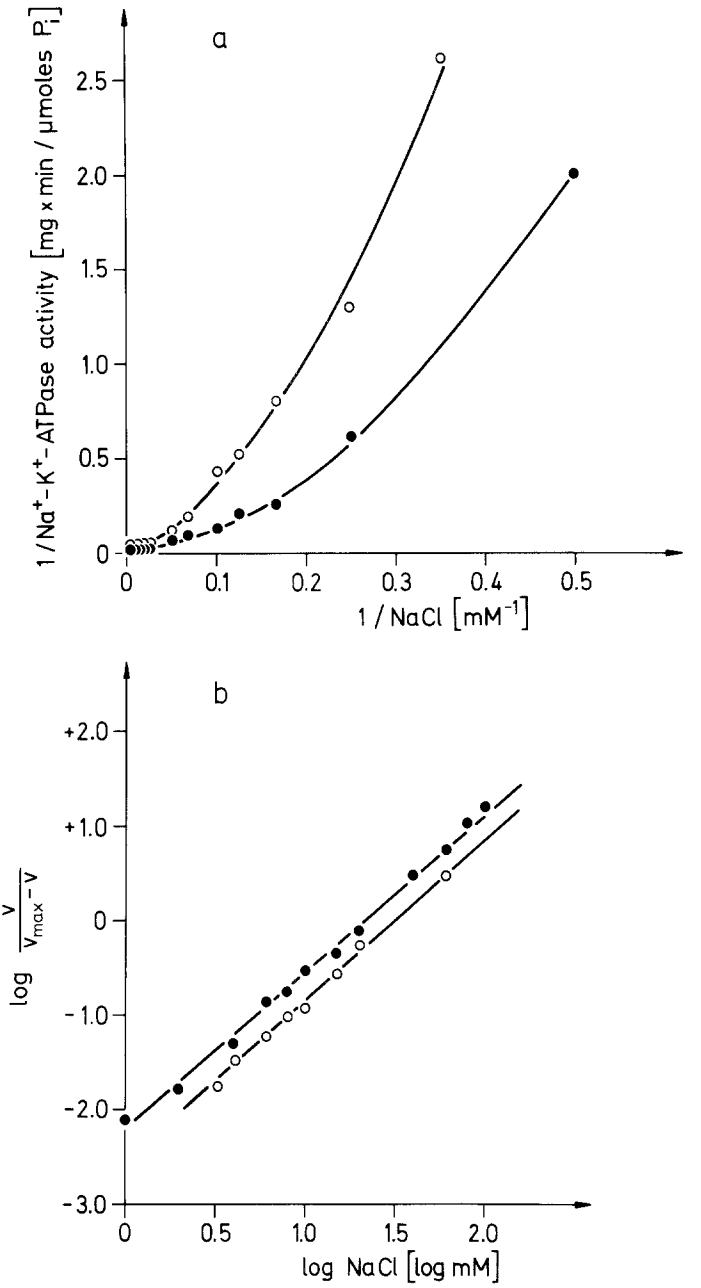

Fig. 6. Effects of NaCl on the ($Na^+ - K^+$)-ATPase activity in the absence (●——●) and presence (○——○) of antibodies. By preincubation with antibodies in the presence of 3 mM $MgCl_2$ and 6 mM P_i, the ($Na^+ - K^+$)-ATPase activity was reduced to 68% of the control. The ($Na^+ - K^+$)-ATPase activity was measured in the presence of 70 mM imidazole-HCl, pH 7.3, 5 mM $MgCl_2$, 100 mM NH_4Cl, 3 mM ATP, and the concentration of NaCl is indicated. The data are presented as in Fig. 4. The Hill coefficient is 1.67 and 1.68 for the untreated and antibody inhibited enzyme, respectively

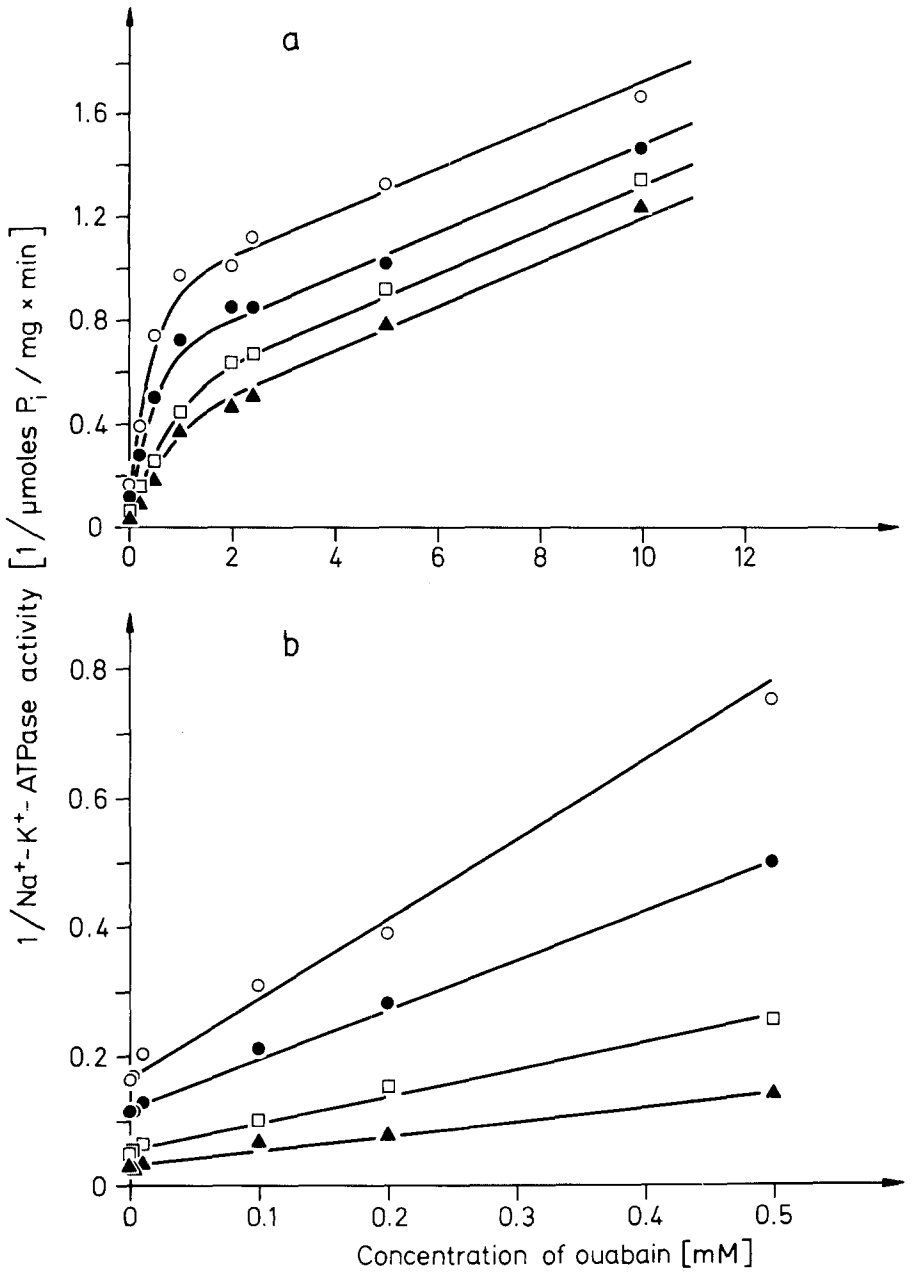

Fig. 7. Dose response of ouabain to the $(Na^+ - K^+)$-ATPase activity. The dose response curves of untreated enzyme (▲——▲) are compared with those of membrane-bound $(Na^+ - K^+)$-ATPase which was preincubated with antibodies in the presence of 3 mM $MgCl_2$ and 6 mM P_i, so that the $(Na^+ - K^+)$-ATPase was reduced to 57% (□——□), 24% (●——●) and 16% (○——○) of the original $(Na^+ - K^+)$-ATPase activity. The data are presented as Dixon plots [(*a*): whole concentration range of ouabain tested; (*b*): low ouabain concentration only] and as a Hill plot (*c*) in which *i* represents the relative inhibition and i_{max} the maximal relative inhibition of the $(Na^+ - K^+)$-ATPase activity

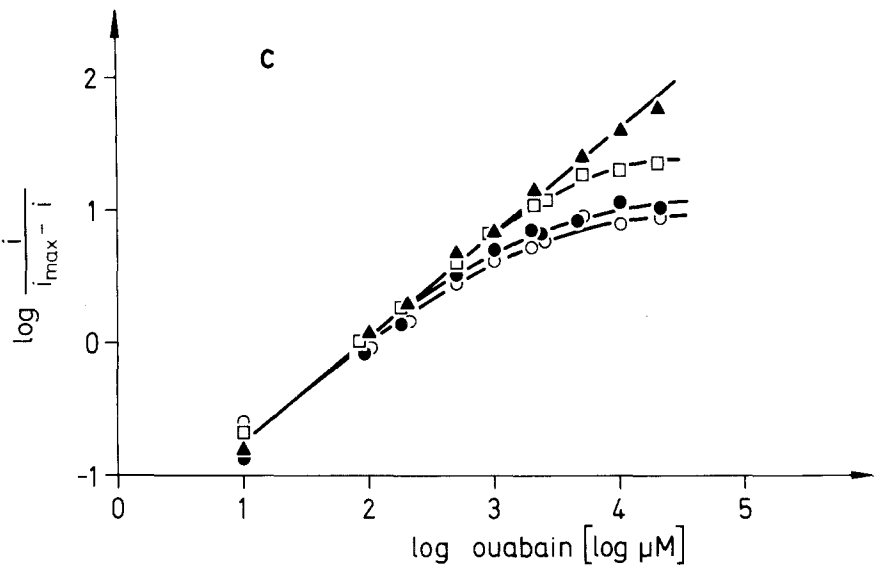

Fig. 7c

Antibody Action and Na^+ Stimulation of the ($Na^+ - K^+$)-ATPase Activity

By measuring the Na^+ dependence of the ($Na^+ - K^+$)-ATPase activity in the presence of 100 mM NH_4Cl, a sigmoidal response was obtained (Fig. 6*a*). A Hill coefficient of $n = 1.67$ and a $K_{0.5\,Na^+}$ of 20.8 were calculated (Fig. 6*b*)[1].

If the enzyme was pretreated with antibodies beforehand (60 min at 0 °C, inactivation 32% of the ($Na^+ - K^+$)-ATPase activity), the $K_{0.5\,Na^+}$ value obtained in the presence of 100 mM NH_4Cl increased to 31.6 mM. The Hill coefficient did not change significantly (antibody inhibited enzyme: $n = 1.68$), indicating that the antibodies did not alter the homotropic positive cooperative effect of Na^+.

Antibody Action and Ouabain Inhibition of ($Na^+ - K^+$)-ATPase Activity

In the untreated and antibody inhibited membrane-bound ($Na^+ - K^+$)-ATPase, the dose response of ($Na^+ - K^+$)-ATPase activity to ouabain was measured. As can be seen from the control values of Fig. 7*a*, a reciprocal plot with different slopes at low and high ouabain concentrations

1 If the measurements were performed in the presence of NH_4Cl concentrations smaller than 100 mM, lower $K_{0.5}$ values and different Hill coefficients were obtained (data not shown).

was obtained. If the data from the untreated enzyme were plotted according to the Hill equation (control of Fig. 7*c*), a straight line was obtained which indicates negative cooperativity for the sites of ouabain inhibition ($n=0.77$). From the Hill plot, a $K_{0.5\,\text{ouabain}}$ value of 89 μM was obtained. Concerning the influence of the antibodies on the ouabain inhibition of the enzyme, it was found that in the range of 0.001 to 0.5 mM ouabain the dose response of the (Na^+-K^+)-ATPase to ouabain was not affected (Fig. 7*a*). At ouabain concentrations higher than 0.5 mM the sensitivity of the (Na^+-K^+)-ATPase to ouabain is decreased by the antibodies (Fig. 7*b*). The straight relationship of the (Na^+-K^+)-ATPase activity to ouabain inhibition in the Hill plot (Fig. 7*c*) was progressively abolished with increasing degree of antibody inhibition.

Discussion

The kinetic data reported in this paper for the untreated membrane-bound (Na^+-K^+)-ATPase from rat kidney demonstrate that the (Na^+-K^+)-ATPase is a cooperative enzyme. They are in agreement with the results reported by other authors [7, 8, 9, 20, 21, 23]. Thus, consistent with other reports [8, 23], we could demonstrate a negative cooperativity for the ATP binding and also for the ATP dependence of the (Na^+-K^+)-ATPase activity. These results indicate that two or more ATP binding sites, which may be also catalytic sites, exist in the functioning enzyme complex. Furthermore, in agreement with previous reports [8, 20, 21], kinetic data on the Na^+ activation of the (Na^+-K^+)-ATPase activity were obtained which could be interpreted as an indication for two or more positive cooperative Na^+-binding sites in the enzyme. Concerning the ouabain binding to the (Na^+-K^+)-ATPase, the data of several authors [7, 9, 23] suggest that more than one ouabain binding site exists in the (Na^+-K^+)-ATPase. The above-described results, which demonstrate a negative cooperativity for the ouabain inhibition of the (Na^+-K^+)-ATPase activity, are consistent with these ouabain binding data. Both sets of experiments together suggest that the different ouabain binding sites of the enzyme must be considered as ouabain inhibition sites.

The inactivation of the (Na^+-K^+)-ATPase activity by the antibodies as well as the reactivation of the antibody-inhibited enzyme proceeds in two phases. Unfortunately, it cannot be decided from our data whether this behavior is due to (a) the action of different antibody populations, (b) to different enzyme populations, or (c) to the inactivation of the different catalytic sites of the (Na^+-K^+)-ATPase. However, the last

possibility seems to be the most probable one, as we also observed biphasic inactivation curves in different membrane-bound and solubilized ($Na^+ - K^+$)-ATPase preparations when (a) the spontaneous inactivation of the ($Na^+ - K^+$)-ATPase activity in dilute solutions was measured (H. Koepsell & F. Hulla, *unpublished*), (b) the ($Na^+ - K^+$)-ATPase activity was inhibited by trypsin (H. Koepsell, *unpublished*), or (c) the enzyme was inhibited with the ATP competitor NbS^6ITP[2] (H. Koepsell & F. Hulla, *unpublished*).

The reactivation of the antibody-inhibited enzyme proceeds very slowly, if the free antibody concentration is reduced. It was therefore possible to study characteristics of the ($Na^+ - K^+$)-ATPase activity of the antibody enzyme complex. The antibody ($Na^+ - K^+$)-ATPase complex shows residual ($Na^+ - K^+$)-ATPase activity. This can be derived from the finding that an increase in $K_{0.5\,ATP}$ was observed if the ATP dependence of the ($Na^+ - K^+$)-ATPase was measured in the antibody-inhibited enzyme under conditions in which no reactivation of the antibody-inhibited enzyme occurred.

It was found that the antibody effect on the ATP dependence of the ($Na^+ - K^+$)-ATPase activity is very similar to the antibody effect on the ATP binding of the enzyme. The antibodies reduce the V_{max} of the enzyme reaction as well as the number of binding sites for ATP. They increase both $K_{0.5\,ATP}$ for the ($Na^+ - K^+$)-ATPase activity and $K_{0.5\,ATP}$ for the ATP binding, but they do not change the negative cooperativity for ATP which was found for the ATP binding as well as for the ATP dependence of the ($Na^+ - K^+$)-ATPase reaction. These data suggest that the ($Na^+ - K^+$)-ATPase activity is inhibited by hindrance of the access of ATP to the ATP binding sites of the enzyme. It cannot, however, be decided from our results whether the antibodies inhibit the ATP binding by direct interaction with the ATP binding sites or by inducing a conformational change of the enzyme in which the ATP binding sites are included. As the degree of negative cooperativity for ATP is not changed by the antibodies, both ATP binding sites seem to be affected in the same way. This could occur if the ATP binding sites are located close to each other so that one antibody molecule could sterically hinder the access of ATP to the different ATP binding sites or if the antibody binding at a different protein region would induce a similar conformational change at both ATP binding sites. As the ATP binding sites are located at the inner surface of the cell membrane, it seems probable that the antibodies which inhibit the

2 6-[(3-carboxy-4-nitrophenyl)thiol]-9-β-D ribofuranosylpurine 5′-triphosphate.

enzyme activity act from this side. In fact, Jørgensen and coworkers [12] have shown that antibodies against a membrane-bound $(Na^+ - K^+)$-ATPase from pig kidney inhibited the ouabain-sensitive outflux of Na^+ from red cell ghosts only if the antibodies were applied from the inner side of the membrane.

It is shown above that the $K_{0.5}$ value for Na^+ activation of the $(Na^+ - K^+)$-ATPase activity was increased by the antibodies and that the positive cooperativity for Na^+ was not changed. Hegyvary and Post [10] have demonstrated that Na^+ stimulates the ATP binding to the $(Na^+ - K^+)$-ATPase in the presence of K^+. If, therefore, the binding of Na^+ to the enzyme has an effect on the ATP binding at the ATP binding sites, one could imagine that, *vice versa*, the antibody action at the ATP binding sites could influence the Na^+ binding. From our experiments it cannot be excluded, however, that the antibodies may act directly at the Na^+ binding sites. By measuring the ouabain inhibition of antibody-treated $(Na^+ - K^+)$-ATPase it was found that the characteristics of ouabain inhibition were altered by the antibodies. With an increasing degree of antibody inhibition, the sensitivity of the $(Na^+ - K^+)$-ATPase to ouabain was progressively reduced at higher ouabain concentrations. This can be explained if one assumes that the binding of antibodies at the inner membrane side leads to a structural alteration at the ouabain binding site(s) at the outer side of the membrane. This structural alteration is not symmetric with respect to the ouabain inhibition sites, so that their negative cooperativity is abolished.

The results obtained are in accordance with the hypothesis that the antibodies inhibit the $(Na^+ - K^+)$-ATPase activity by reducing the ATP binding to the ATP binding sites. The data show that the antibodies can be used to inhibit specific enzyme functions. However, the antibody interaction with the enzyme is complex. This may be due to characteristics of the $(Na^+ - K^+)$-ATPase and/or to the mode of antibody interaction with the enzyme. Further studies with antibodies which are directed against clearly defined peptide segments of the enzyme may lead to unambiguous information about the molecular mechanism of the $Na^+ - K^+$ pump.

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References

1. Askari, A., Rao, S.N. 1972. Na^+, K^+-ATPase complex: Effects of anticomplex antibody on the partial reactions catalysed by the complex. *Biochem. Biophys. Res. Commun.* **49**:1323

2. Cinader, B., Lafferty, K.J. 1963. Antibody as inhibitor of ribonuclease: The role of steric hindrance, aggregate formation, and specifity. *Ann. N.Y. Acad. Sci.* **103**:653
3. Cinader, B. 1965. Antibodies to enzymes – a discussion of the mechanisms of inhibition and activation. *In:* Antibodies to Biologically Active Molecules. B. Cinader, editor. p. 85. Pergamon Press, Oxford
4. Crowle, A.J. 1973. Immunoelectrophoresis techniques. *In:* Immunodiffusion. A. Crowle, editor. p. 323. Academic Press, New York
5. Crumpton, M.J. 1965. The molecular basis of the serological specifity of proteins, with particular reference to sperm whale myoglobulin. *In:* Proceedings of the 2nd Meeting of the Federation of European Biochemical Societies Vienna, 21–24 April. B. Cinader, editor. Vol. I, p. 61. Pergamon Press, New York
6. Finger, H., Günther, O. 1962. Zur Technik der Erzeugung hochtitriger Kaninchenantiseren gegen Affenvirus. *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskr. Hygiene* **185**:155
7. Fricke, U., Klaus, W. 1977. Evidence for two different Na^+-dependent ^{3}H-ouabain binding sites of a $Na^+ - K^+$-ATPase of guinea-pig hearts. *Br. J. Pharmacol.* **61**:423
8. Gache, C., Rossi, B., Ladzunski, M. 1976. (Na^+, K^+)-activated adenosinetriphosphatase of axonal membranes, cooperativity and control. Steady-state analysis. *Eur. J. Biochim.* **65**:293
9. Hansen, O. 1976. Non-uniform populations of g-strophanthin binding sites of ($Na^+ + K^+$)-activated ATPase. Apparent conversion to uniformity by K^+. *Biochim. Biophys. Acta* **433**:383
10. Hegyvary, C., Post, R.L. 1971. Binding of adenosine triphosphate to sodium and potassium ion-stimulated adenosine triphosphatase. *J. Biol. Chem.* **246**:5234
11. Jørgensen, P.L. 1974. Purification and characterization of ($Na^+ + K^+$)-ATPase. III. Purification from the outer medulla of a mammalian kidney after selective removal of membrane components by sodium dodecylsulphate. *Biochim. Biophys. Acta* **356**:36
12. Jørgensen, P.L., Hansen, O., Glynn, J.M., Cavieres, J.D. 1973. Antibodies to pig kidney ($Na^+ + K^+$)-ATPase inhibit the Na^+ pump in human red cells provided they have access to the inner surface of the cell membrane. *Biochim. Biophys. Acta* **291**:795
13. Koepsell, H. 1977. Immunological characterization of Na^+ and K^+ mediated structural states of rat kidney $Na^+ - K^+$-ATPase. *Hoppe-Seylers Z. Physiol. Chem.* **358**:1391
14. Kriz, W., Schnermann, J., Koepsell, H. 1972. The position of short and long loops of Henle in the rat kidney. *Z. Anat. Entwickl.-Gesch.* **138**:301
15. Kyte, J. 1974. The reactions of sodium and potassium ion-activated adenosine triphosphatase with specific antibodies. Implications for the mechanism of active transport. *J. Biol. Chem.* **249**:3652
16. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**:265
17. McCans, J.L., Lane, L.K., Lindenmayer, G.E., Butler, V.P., Jr., Schwartz, A. 1974. Effects of an antibody to a highly purified Na^+, K^+-ATPase from canine renal medulla: Separation of the "holoenzyme antibody" into catalytic and cardiac glycoside receptor-specific components. *Proc. Nat. Acad. Sci. USA* **71**:2449
18. Mitchison, N.A. 1972. Dose, frequency and route of administration of antigen. *In:* North-Holland Research Monographs, Frontiers of Biology. A. Neuberger and E.L. Tatum, editors. Vol. 25, p. 87. North-Holland, Amsterdam
19. Rhee, H.M., Hokin, L.E. 1975. Inhibition of the purified sodium-potassium activated adenosine triphosphatase from the rectal gland of Squalus acanthias by antibody against the glycoprotein subunit. *Biophys. Res. Comm.* **63**:1139
20. Robinson, J.D. 1970. Interactions between monovalent cations and the ($Na^+ + K^+$)-dependent adenosine triphosphatase. *Arch. Biochem. Biophys.* **139**:17

21. Robinson, J.D. 1977. Na^+ sites of the ($Na^+ + K^+$)-dependent ATPase. *Biochim. Biophys. Acta* **482**:427
22. Schoner, W., Ilberg, C. v., Kramer, R., Seubert, W. 1967. On the mechanism of Na^+- and K^+-stimulated hydrolysis of adenosine triphosphate. 1. Purification and properties of a Na^+- and K^+-activated ATPase from ox brain. *Eur. J. Biochem.* **1**:334
23. Schoner, W., Pauls, H., Patzelt-Wenczler, R. 1977. Biochemical characteristics of the sodium pump: Indications for a half-of-the-sites reactivity of ($Na^+ + K^+$)-ATPase. *In:* Myocardial Failure. G. Riecker, A. Weber and J. Goodwin, editors. p. 104. Springer-Verlag, Berlin-Heidelberg-New York
24. Smith, T.W., Wagner, H., Jr. 1975. Effects of ($Na^+ - K^+$)-ATPase-specific antibodies on enzymatic activity and monovalent cation transport. J. Membrane Biol. **25**:341
25. Strehler, B.L., Trotter, J.R. 1954. Determination of ATP and related compounds: Firefly luminescence and other methods. *In:* Methods of Biochemical Analysis. D. Glick, editor. Vol. I, p. 341. Interscience, New York
26. Webb, J.L. 1963. Reversal of inhibition. *In:* Enzyme and Metabolic Inhibitors. J.L. Webb, editor. p. 608. Academic Press, New York
27. Weber, K., Osborn, M. 1969. The reliability of molecular weight determination by dodecyl sulfate polyacrylamid gel electrophoresis. *J. Biol. Chem.* **244**:4406