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Immunochemical Studies for the Characterization of Purified (Na^+, K^+)-ATPase and Its Subunits with a Special Reference of Their Effect on Monovalent Cation Transport in Reconstituted (Na^+, K^+)-ATPase Vesicles

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ABSTRACT

A highly purified (Na^+, K^+)-ATPase from the rectal gland of *Squalus acanthias* and from the electric organ of *Electrophorus electricus* has been used to raise antibodies in rabbits. The 97,000 dalton catalytic subunit and glycoprotein derived from the rectal gland of spiny shark were also used as antigens. The two (Na^+, K^+)-ATPase holoenzymes and the two shark subunits were antigenic. In Ouchterlony double diffusion experiments, these antibodies formed precipitation bands with their antigens. Antibodies prepared against the two subunits of shark holoenzyme also formed precipitation bands with their antigens and shark holoenzyme, but not with eel holoenzyme. These observations are in good agreement with inhibitory effect of these antibodies on the catalytic activity of (Na^+, K^+)-ATPase both from the shark and the eel, since there is very little cross-reaction between the shark anticatalytic subunit antibodies and the eel holoenzyme. The maximum antibodies titer of the anticatalytic subunit antibodies is found to be 6 weeks after the initial single exposure to this antigen. Multiple injections of the antigen increased the antibody titer. However, the time required to produce the maximum antibody titer was approximately the same.

These antibodies also inhibit catalytic activity of (Na^+, K^+)-ATPase vesicles reconstituted by a slow dialysis of cholate after solubilization of the enzyme in a presonicated mixture of cholate and phospholipid. In these reconstituted (Na^+, K^+)-ATPase vesicles, effects of these antibodies on the fluxes of Na^+, Rb^+, and K^+ were investigated. Control or preimmune serum had no effect on the influx of $^{22}Na^+$ or the efflux of $^{86}Rb^+$. Immunized sera against the shark (Na^+, K^+)-ATPase holoenzyme, its glycoprotein or catalytic subunit did inhibit the influx of $^{22}Na^+$ and the efflux of $^{86}Rb^+$. It was also demonstrated that these antibodies inhibit the coupled counter-transport of Na^+ and K^+ as studied by means of dual labeling experiments. However, this inhibitory effect of the antibodies on transport of ions in the (Na^+, K^+)-ATPase vesicles is manifested only on the portion of energy and temperature dependent alkali metal fluxes, not on the portion of ATP and ouabain insensitive ion movement. Simultaneous determination of effects of the antibodies on ion fluxes and vesicular catalytic activity indicates that an inhibition of active ion transport in reconstituted (Na^+, K^+)-ATPase vesicles appears to be due to the inhibitory action of the antibodies on the enzymatic activity of (Na^+, K^+)-ATPase molecules incorporated in the vesicles.

These findings that the inhibitory effects of the antibodies specific to (Na^+, K^+)-ATPase or to its subunits on ATP and temperature sensitive monovalent cation transport in parallel with the inhibitory effect of vesicular catalytic activity by these antibodies provide direct evidence that (Na^+, K^+)-ATPase is the molecular machinery of active cation transport in this reconstituted (Na^+, K^+)-ATPase vesicular system.

Key Words: Antibodies to (Na^+, K^+)-ATPase, (Na^+, K^+)-ATPase vesicle

Abbreviations: (Na^+, K^+)-ATPase, sodium and potassium actived adenosine triphosphatase (E.C. 3.6.1.3); Anti (Na^+, K^+)-ATPase antibodies, Antibodies to (Na^+, K^+)-ATPase holoenzyme; Antiglycoprotein antibodies, Antibodies to glycoprotein subunit of (Na^+, K^+)-ATPase; Anticatalytic Subunit antibodies, Antibodies to (Na^+, K^+)-ATPase catalytic subunit.

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INTRODUCTION

It is now generally agreed that (Na^+, K^+)-ATPases from various tissues consist of two polypeptides subunits-a catalytic α-and a glycosylated β-subunit (Kyte, 1971a; Hokin *et al.*, 1973; Jørgensen, 1974b). In most of cellular systems investigated, these two subunits are synthesized coordinately and are assembled into (Na^+, K^+)-ATPase in a specific stoichiometric ratio of the two subunits (Geering *et al.*, 1989). The higher molecular weight peptide chain is phosphorylated during turnover (Jørgensen, 1974a; 1974b; Kyte, 1971b; Lane *et al.*, 1973; Uesugi *et al.*, 1971) and contains binding sites for cardiac glycosides (Ruoho and Kyte, 1974). The smaller peptide is a glycoprotein (Hokin *et al.*, 1973; Jørgensen, 1974b; Kyte, 1972). Circumstantial evidence that the glycoprotein is a subunit of the enzyme has been reported in our previous paper (Rhee and Hokin, 1979). Little is known about the functional role of these subunits in energy dependent coupled transport of sodium and potassium, though this enzyme is intimately involved with the active transport of Na^+ and K^+ in most animal cell membranes (Glynn, 1957; Skou, 1960; Sen and Post, 1964; Whittm and Ager, 1965).

An immunochemical approach is useful for an investigation of the function and structure of the enzymes (Arnon, 1967; Cinader, 1967; Michaeli, 1969a; 1969b; Richards *et al.*, 1975) because of its high specificity. (Na^+, K^+)-ATPases with various degree of purity from different tissues and species have been used to produce antibodies in laboratory animals. Antibodies against rat brain (Na^+, K^+)-ATPase inhibited (Na^+, K^+)-ATPase activity and sodium dependent phosphorylation of the enzyme, but it had little or no effect on potassium dependent p-nitrophenylphosphatase activity and potassium dependent breakdown of phosphorylated intermediate (Askari and Rao, 1971). Antibodies deposited inside of the resealed red blood cell ghosts inhibited Na^+ efflux (Jørgensen *et al.*, 1973). Antibodies against (Na^+, K^+)-ATPase from canine renal medulla had no effect on ouabain inhibitable $^{86}Rb^+$ uptake in canine renal slices and intact human erythrocytes (Smith *et al.*, 1973). Antibodies have been reportedly separated into two distinct globulin components, one of which specifically inhibited ouabain binding to (Na^+, K^+)-ATPase and another inhibited catalytic activity of (Na^+, K^+)-ATPase without affecting ouabain binding to this enzyme (McCans *et al.*, 1974). Antibodies specific for lipid-free larger subunits prepared by sodium dodecyl sulfate (SDS) chromatography from kidney appeared to bind to the inner surface of the kidney microsomes (Kyte, 1974). However, they did not inhibit (Na^+, K^+)-ATPase activity at saturating concentration. Jean *et al.* (1975) reported that catalytic and glycoprotein subunits prepared by SDS-gel column from the purified (Na^+, K^+)-ATPase of the electric organ of *E. electricus* were all antigenic and antibodies against these two subunits inhibited (Na^+, K^+)-ATPase activity to a certain degree in concentration dependent manner.

In this present paper we report the inhibitory effect of antibodies against (Na^+, K^+)-ATPase holoenzyme from electric organ of electric eel and the catalytic subunit of (Na^+, K^+)-ATPase from shark rectal gland on catalytic activity of (Na^+, K^+)-ATPase. We also demonstrate that the antibodies inhibit the coupled translocation of Na^+ and K^+ in the reconstituted (Na^+, K^+)-ATPase vesicular system, of which properties and characterization have been reported elsewhere (Hilden *et al.*, 1974).

MATERIALS AND METHODS

Materials

(Na^+, K^+)-ATPases from the rectal gland of *Squalus acanthias* and from the electric organ of *Electrophorus electricus,* and their glycoproteins and catalytic subunits were used as antigens. These enzymes were prpeared and purified according to previously described methods (Dixon and Hokin, 1974; Hokin *et al.*, 1973; Perrone *et al.*, 1975). Antibodies against these antigens were raised in female white New Zealand rabbits. Immunodiffusion studies were carried out on immunodiffusion discs containing 0.9% agarose in 50mM imidazole buffer. Sephadex G50 and agarose were bought from the Sigma Chemical Co.; ouabain and N-ethylmaleimide from the Aldrich Chemical Co.; egg lecithin from Schwartz-Mann or EM Laboratories, Inc. $^{22}Na^+$ (carrier-free), $^{86}Rb^+$ (2.8 mc/mg) and $^{42}K^+$ (1.6 mc/mg) were purchased from the New England Nuclear Corp.

Production of antibody

Immunization procedures and treatment of antisera were described elsewhere (Rhee and Hokin, 1979). All the antisera were destroyed its complements by heating them at 56° for 30 min and dialyzed against 50 to 100 volumes of 0.15M im-

idazole buffer (pH 7.0) for 3 days at 4° with frequent changes of dialysis medium. This procedure effectively eliminated inorganic phosphate in serum, which interferes with the determination of (Na^+, K^+)-ATPase activity by the conventional Fiske and SubbarRow method (1929). Either antisera or purified globulin as antibodies was used as indicated in each experiment.

(Na^+, K^+)-ATPase and vesicular (Na^+, K^+)-ATPase assay

Protein was determined by the Lowry method (1951) with bovine serum albumin as standard so that known amount of (Na^+, K^+)-ATPase or globulin could be used. (Na^+, K^+)-ATPase assay with antibody was performed as described (Hokin *et al.*, 1973) with a few modifications. An appropriate amount of ammonium sulfate-treated enzyme from shark or electric eel was preincubated with or without various fractions of antibodies at room temperature for 30 min in a final volume of 1ml of 30mM imidazole buffer (pH 7.0) containing 5mM $MgCl_2$, 10mM KCl and 100mM NaCl. The reaction was started by the addition of 5mM ATP after 5 min preincubation at 37°. The reaction was terminated by the addition of 1ml of 5% trichloroacetic acid in $CHCl_3$-CH_3OH (1:1). After centrifugation of the mixture, an aliquot of the aqueous upper phase was assayed for inorganic phosphate by the method of Fiske and SubbarRow (1929). In each assay, tubes for phosphate standard and blank tubes with 10^{-4}M ouabain were included. For the determination of vesicular ATPase activity during transport study at 25°C, an aliquot of incubation mixture in a transport medium (5mM $MgCl_2$, 100mM KCl, 20mM NaCl and 30mM imidazole buffer, pH 7.0) instead of normal assay medium was used so that (Na^+, K^+)-ATPase catalytic activity and cation transport could be correlated simultaneously.

Reconstitution of (Na^+, K^+)-ATPase into vesicles and transport study

Detailed techniques for the preparation of (Na^+, K^+)-ATPase vesicles were described previously (Hilden *et al.*, 1974; Hilden and Hokin, 1975). In brief, three times recrystallized cholate and egg lecithin were sonicated till clear. This mixture was added to (Na^+, K^+)-ATPase to give a final concentration of 20 mg/ml egg lecithin, 10 mg/ml cholate and 1 mg/ml (Na^+, K^+)-ATPase from the rectal gland of the shark in a 30mM imidazole buffer (pH 7.0) containing 5mM $MgCl_2$, 20mM NaCl and 100mM KCl (transport medium). In some cases, 60 mg/ml egg lecithin, 30 mg/ml cholate and 1mg (Na^+, K^+)-ATPase per ml of transport medium buffered with 30mM cholate and 1mg (Na^+, K^+)-ATPase per ml of transport medium buffered with 30mM imidazole was used. This mixture was dialyzed with constant stirring against its 500 to 1000 volumes of transport medium with frequent changes of the dialysis medium. After two to three days dialysis, the inside content of the dialysis bag, hereinafter referred to as (Na^+, K^+)-ATPase vesicles, was used to study cation transport. For ion transport study with this reconstituted (Na^+, K^+)-ATPase vesicle, an aliquot of (Na^+, K^+)-ATPase vesicles preloaded with $^{22}Na^+$, $^{86}Rb^+$, or $^{42}K^+$ was usually used. However, in most of $^{22}Na^+$ transport study, vesicles were used without a preload of $^{22}Na^+$. The transport reaction was started by the addition of $^{22}Na^+$ (0.5-2μc) and 5mM ATP to (Na^+, K^+)-ATPase vesicle in a final 1ml of the transport medium (pH 7.0) at 25°C. Antibodies were incubated with radioactive ion-preloaded vesicles at room temperature for 30 min and active transport was started by the addition of 5mM ATP at 25° in a final volume of 1ml with the transport medium. After 10 to 60 min incubation, depending upon the amount of (Na^+, K^+)-ATPase vesicles and radioactive labelling, an aliquot of the incubation mixture was passed over a Sephadex G 50 column (Fine Pharmacia) and eluted with the trasnport medium at room temperature. The flow rate was 1ml per min and each 1ml fraction was collected by a Gilson fractionator (Middleton, Wis.). The radioactivity which emerged under a void volume peak was counted in Packard tricarb liquid scintillation spectrometer (model 3380) after mixing with 5ml of Bray's solution (Bray, 1960). In double labelled experiments with both $^{22}Na^+$ and $^{42}K^+$, since the half life of $^{42}K^+$ is 12.3 hours in comparison to 1.6 years of $^{22}Na^+$, counting was repeated after the $^{42}K^+$ had decayed (14 half-lives). The radioactivity eluting in the void volume was expressed as a percent of the total radioactivity applied to the column in the absence or in the presence of 5mM ATP. The ratio of percent of $^{22}Na^+$ influxed to the percent of $^{42}K^+$ effluxed was computed in double labelled experiments because this ratio is a sensitive index for the monitoring of the simultaneous coupled change of Na^+ and K^+ concentrations in the vesicles.

RESULTS

Effect of antibodies on the catalytic activity of (Na^+, K^+)-ATPase

Antibodies raised in rabbits against purified (Na^+, K^+)-ATPase prepared from the electric organs of electric eels inhibited the enzymatic activity of (Na^+, K^+)-ATPases derived from both shark rectal glands and the electric organ of electric eels as shown in Table 1. There was little inhibition of shark (Na^+, K^+)-ATPase with antibodies against electric eel (Na^+, K^+)-ATPase.

Table 1. Effect of Antibodies to Electric Organ (Na^+, K^+)-ATPase on the Catalytic Activity of Shark and Eel (Na^+, K^+)-ATPase

Rabbit #	(Na^+, K^+)-ATPase (% inhibition)		
	N[2]	Eel (Na^+, K^+)-ATPase	Shark (Na^+, K^+)-ATPase
6	4	81 ± 1.4[3]	7 ± 1.8
8	4	77 ± 2.0	17 ± 1.8
9	4	88 ± 2.1	39 ± 2.2

[1] Twenty μg of eel or shark (Na^+, K^+)-ATPase was assayed with 0.5ml of individual antisera as described under Experimental Procedure. Initial specific activity of the eel and the shark (Na^+, K^+)-ATPase was 1231 and 949μm Pi/mg/hr, respectively.

[2] Indicate number of replication.

[3] Mean ± S.E.

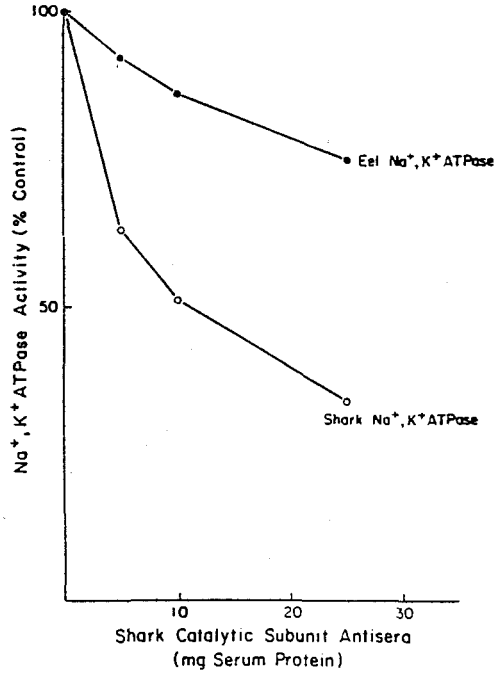

Fig. 1. Inhibition of Na^+, K^+-ATPase by anticatalytic subunit antibody. Twenty μg of shark or eel Na^+, K^+-ATPase were mixed with indicated amount of antishark catalytic subunit antisera at room temperature for 30 min in a final volume of 1ml of an assay medium containing 5mM $MgCl_2$, 10mM KCl, 100mM NaCl and 30mM imidazole buffer (pH 7.0). The reaction was started by the addition of 5mM ATP at 37° for 5 min after preincubation at 37° for 5 min. Inorganic phosphate released by the Na^+, K^+-ATPases was determined as described under Materials and Methods.

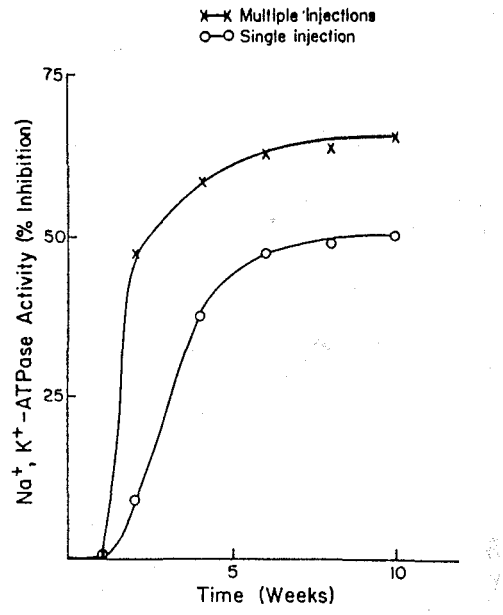

Fig. 2. Effects of the frequency of antigen challenge on antibody titer in the inhibition of Na^+, K^+-ATPase in vitro. Rabbits were immunized singly or several times against the shark catalytic subunit as reported previously (38). Na^+, K^+-ATPase assay in the presence of the anticatalytic antibody was carried out as described in Fig. 1.

ATPase, although there was a considerable variation between animals. For instance, antibodies from rabbit 6 inhibits eel (Na^+, K^+)-ATPase as much as that from rabbit 9, but produces practically no inhibition of shark (Na^+, K^+)-ATPase. There was good cross-reaction between eel and shark (Na^+, K^+)-ATPases with antiserum from rabbit 9. The inhibitory effect of anti-shark catalytic subunit antibodies on the catalytic activity of both eel and shark (Na^+, K^+)-ATPases is illustrated (Fig. 1). Shark catalytic subunit antibody also cross-reacted with the eel (Na^+, K^+)-ATPase, but it was less significant than the corss-reaction with antibodies against shark holoenzyme (Rhee and Hokin, 1979). However, the inhibitory effect of the anti-shark catalytic subunit antibodies on the shark (Na^+, K^+)-ATPase was as great as the inhibition with antibodies against shark holoenzyme.

Fig. 2 illustrates production of antibody titer after an immunization with the shark catalytic subunit. A single immunization of the shark catalytic subunit produced antibody slowly. The titer of the antibody was reached at a steady state level 5 to 6 weeks after an initial exposure to the antigen. However, the maximum inhibition of (Na^+, K^+)-ATPase with the antibodies after a single immunization was significantly below than that seen after weekly multiple exposure to the antigen. The latter procedure produced high antibody titers within two weeks after an initial exposure to the antigen. It was of interest to note that the time required to reach the maximum antibody titer was not really different after the single or multiple exposure to the antigen.

Fig. 3 shows Ouchterlony double diffusion between the shark rectal gland (Na^+, K^+)-ATPase catalytic subunit and its antibody. The shark catalytic subunit, like the purified membrane (Na^+, K^+)-ATPase, was apparently diffusable into 0.9% agarose medium without the aid of solubilizing agents, as reported previously (Rhee and Hokin, 1975). The control serum (well 2) did not form any precipitation band. However, anti-shark catalytic subunit antibody formed clear precipitation lines with

Fig. 3. Ouchterlony double diffusion pattern of the shark catalytic subunit and its antibodies. Well 1 contained 30μl of 0.15M imidazole buffer; 2, 30μl of control serum; 3, 4, 5 and 6 contained 10, 20, 30 and 40μl of the antishark catalytic subunit antisera. And the center well contained 100μg of the purified shark catalytic subunit. Diffusion pattern was photographed after 24 hrs at room temperature as described (38).

Fig. 4. Double diffusion pattern of electric eel holoenzyme and its antibodies. Well 1 contained 30μl of 0.15M imidazole buffer; 2, 30μl of control sera; 3, 4, 5 and 6 contained 10, 20, 30 and 40μl of the anti-eel holoenzyme antiserum. The center well contained 100μg of the purified eel Na^+, K^+-ATPase. Diffusion conditions were the same as Fig. 3.

its antigen within 24 hours at room temperature. By increasing the amount of antiserum from 10μl to 40μl, the diffusion bands were thicker, as the diffusion time became longer. The third well (10μl) of antiserum formed the precipitation line near the side well (number 3) due to an excess of antigen in the center well. However, the precipitation band between the center well and well number 6 was formed in the middle.

A similar double diffusion pattern was observed between the electric organ (Na^+, K^+)-ATPase and its antibody as shown in Fig. 4. Under identical diffusion conditions, the precipitation lines were very sharp, compared to those obtained from the shark catalytic subunit and its antibody (see Fig. 3 and 4). The glycoprotein of the shark rectal gland (Na^+, K^+)-ATPase also formed a precipitation band with its antibodies in similar manner (data are not shown).

Fig. 5 shows the diffusion pattern of the shark rectal gland holoenzyme and its catalytic subunit antibody (between center well and well no. 3). The anti-glycoprotein antibody (well 4) and the anti-holoenzyme antibody (well 6) also interacted with the shark holoenzyme. However, no clear precipitation band was noted between the shark rectal gland (Na^+, K^+)-ATPase holoenzyme (center well) and antibodies to the electric organ (Na^+, K^+)-ATPase (well 5), even though the amount of antiserum used was the same

Fig. 5. Double diffusion pattern of purified shark Na^+, K^+-ATPase holoenzyme and various antibodies. Each well contained 30μl of indicated substance. Well 1 contained 0.15M imidazole buffer; 2, control serum; 3, anti-shark catalytic subunit antibody; 4, anti-shark glycoprotein antibody; 5, anti-eel Na^+, K^+-ATPase holoenzyme antibody and 6, anti-shark holoenzyme antibody. The center well contained 100μg of the purified shark Na^+, K^+-ATPase holoenzyme. Diffusion conditions were the same as in Fig. 3.

Table 2. Effect of Various Antibodies on $^{22}Na^+$ Uptake in Purified (Na^+, K^+)-ATPase Vesicles[1]

Antisera	N[2]	$^{22}Na^+$ Uptake (% inhibition)
Control Antibody	6	0 ± 0[3]
Antishark Holoenzyme Antibody	6	27 ± 4.0
Antieel Holoenzyme Antibody	5	29 ± 5.4
Antishark Glycoprotein Antibody	4	14 ± 4.1
Antishark Catalytic Subunit Antibody	4	17 ± 4.2

[1] (Na^+, K^+)-ATPase vesicles (200μg) was mixed with 0.5ml of various antisera at room temperature for 30 min in a final volume of 1ml of transport medium containing 5mM $MgCl_2$, 20mM NaCl, 100mM KCl and 30mM imidazole buffer (pH 7.0). $^{22}Na^+$ uptake reaction was started by the addition of 5mM ATP and $^{22}Na^+$ (0.5μc or greater) at 25°C for 15 min. An aliquot of the incubation mixture was passed over a Sephadex G 50 column and $^{22}Na^+$ eluted in a void volume was counted as described under Materials and Methods.

[2] Indicates number of replication.

[3] Mean ± S.E.

as in other wells. Likewise, the electric organ (Na^+, K^+)-ATPase also did not form clear precipitation lines with either antibodies to the shark glycoprotein moiety or to the shark catalytic subunit (data are not shown). These observations are consistent with the fact that a minimal inhibition of eel (Na^+, K^+)-ATPase was obtained by the antibodies against the shark catalytic subunit and glycoprotein.

A test has been made to obtain a precipitation line between (Na^+, K^+)-ATPase antibodies and (Na^+, K^+)-ATPase in the membrane stage. A typical Ouchterlony band could not be obtained presumably due to the difficulty of the crude membrane to diffuse into 0.9% agarose medium. However, as reported previously (Rhee and Hokin, 1979) antibodies against shark holoenzyme still inhibit (Na^+, K^+)-ATPase activity in the membrane.

Inhibitory effect of antibodies on monovalent cation fluxes in reconstituted (Na^+, K^+)-ATPase vesicles

Serum from the control animals as well as preimmune serum had no effect on the influx of $^{22}Na^+$ into the (Na^+, K^+)-ATPase vesicles reconstituted with shark (Na^+, K^+)-ATPase by incorporation into the phospholipid and cholate mixture as reported (Hilden *et al.*, 1974). Antisera against shark (Na^+, K^+)-ATPase and its two subunits and anti-eel (Na^+, K^+)-ATPase antibody inhibited the influx of $^{22}Na^+$ as shown in Table 2. However, these antibodies had no effect on $^{22}Na^+$ influx in the absence of 5mM ATP (data are not shown). Previously we suggested that this ATP independent, ouabain and temperature insensitive $^{22}Na^+$ influx in the reconstituted (Na^+, K^+)-ATPase vesicles is chiefly due to the passive diffusion of $^{22}Na^+$ (Hilden *et al.*, 1974). Thus, the inhibition of $^{22}Na^+$ uptake by these antisera was manifested only on the energy linked active transport of $^{22}Na^+$, not on the ATP insensitive passive diffusion of $^{22}Na^+$ in the vesicular system.

Since the previous study from this laboratory indicated that ATP dependent $^{22}Na^+$ influx was linked to $^{42}K^+$ or $^{86}Rb^+$ efflux, vesicles were preloaded with $^{86}Rb^+$ by incubation with $^{86}Rb^+$ for 2 to 4 hours at 4°. To determine effects of these antibodies on the $^{86}Rb^+$ efflux, an aliquot of $^{86}Rb^+$ preloaded vesicles was incubated with or without 5mM ATP in the presence of these antibodies. In control experiments, 0.41% of the total $^{86}Rb^+$ applied to the Sephadex G 50 column was retained inside of the 200mg of (Na^+, K^+)-ATPase vesicles as shown in Table 3. By incubating the same amount of the vesicles under identical conditions with 5mM ATP, 0.15% of $^{86}Rb^+$ was effluxed so that only 0.26% of $^{86}Rb^+$ was retained by the vesicles. An addition of control serum to this system did not change the $^{86}Rb^+$ efflux pattern. However, an addition of antibodies against shark (Na^+, K^+)-ATPase or its subunits inhibited this parameter significantly so that $^{86}Rb^+$ efflux was reduced up to 57% of that with control sera. Glycoprotein antibody inhibited 14% of active transport of Rb^+, which is consistent with its ability to inhibit Na^+ active uptake (Table 2). Catalytic subunit antibody also inhibited Rb^+ efflux by as much as 30%.

In this experiment it is noted that ATP indepen-

Table 3. Inhibition of $^{86}Rb^+$ Efflux by Antibodies Raised Against Shark Holoenzyme or its Subunits[1]

Experiments	$^{86}Rb^+$ Retained in Vesicle (%)		%	
	Without 5mM ATP (a)	With 5mM ATP (b)	Net $^{86}Rb^+$ Efflux (a-b)	Inhibition
Vesicles (200mg)	0.41	0.26	0.15	—
Vesicles + Control Serum	0.37	0.23	0.14	0
Vesicles + Antishark Holoenzyme Antiserum	0.73	0.67	0.06	57
Vesicles + Antishark Glycoprotein Antisera	0.66	0.54	0.12	14
Vesicles + Antishark Catalytic Subunit Antisera	0.59	0.49	0.10	29

[1] (Na^+, K^+)-ATPase vesicles were preloaded with $^{86}Rb^+$ by the incubation of the vesicles in the presence of $^{86}Rb^+$ at 4°C. An aliquot of $^{86}Rb^+$ loaded vesicles was incubated with 0.6ml of various antisera at 25° for 15 min in the absence or in the presence of 5mM ATP. $^{86}Rb^+$ retained in the vesicles was determined as described in Table 2.

[2] Each value represents mean of at least 6-7 determinations.

Table 4. Do Antibodies Bind $^{22}Na^+$ Nonspecifically?[1]

	$^{22}Na^+$ Eluted in a Void Volume on Sephadex G-50 (cpm)		
	Without 5mM ATP (a)	With 5mM ATP (b)	Active $^{22}Na^+$ Transport (b-a)
200μg Vesicles	3247	4520	1373
200μg Vesicles plus 0.6ml control globulin	3329	4964	1635
0.6ml control globulin	234	313	79

[1] Six tenth ml of control globulin, 200μg of (Na^+, K^+)-ATPase vesicles with or without 0.6ml control globulin was incubated in a final 1ml of the transport medium at 25° for 30 min. The Na^+ uptake reaction was started by the addition of 5mM ATP and $^{22}Na^+$ as described in Table 2.

dent $^{86}Rb^+$ retention by the vesicles fluctuated from 0.37% to 0.73%. Even though these vesicles were not in complete equilibrium with $^{86}Rb^+$, effect of the antibodies on the efflux of $^{86}Rb^+$ is quite clear. This still raises the possibility that serum may bind $^{22}Na^+$ or $^{86}Rb^+$ somehow and elute in a void volume so that the parameter may not represent a true ion transport through the vesicles. Therefore, effect of control sera on the $^{22}Na^+$ eluted in a void volume was tested in the presence or in the absence of 5mM ATP under conditions identical to the transport study. Table 4 summarizes the results. The vesicles (200μg) showed an ATP dependent $^{22}Na^+$ influx in addition to ATP independent passive $^{22}Na^+$ diffusion. Addition of 0.6ml control sera did not affect significantly the influx of $^{22}Na^+$ in the presence or absence of 5mM ATP. Without vesicles, 0.6ml control serum showed little radioactivity associated and eluted in a void volume. There was no ATP dependent increment of $^{22}Na^+$ counts without the vesicles.

Effect of antibodies on the coupled transport of $^{22}Na^+$ and $^{42}K^+$

Although the coupled transport of $^{22}Na^+$ influx and $^{42}K^+$ efflux was studied separately (Hilden and Hokin, 1975) in (Na^+, K^+)-ATPase vesicles, the effect of antibodies on the simultaneously coupled $^{22}Na^+$ and $^{42}K^+$ counter transport has not been investigated in the same preparation. Vesicles were loaded with $^{22}Na^+$ and $^{42}K^+$ simultaneously by preincubating them with these ions for 12 hours at 4°C. Aliquots of these $^{22}Na^+$ and $^{42}K^+$ preloaded vesicles were used to determine their contents of $^{22}Na^+$ and $^{42}K^+$ after incubation with or without ATP for indicated time intervals as shown in Fig. 6. The radioactivity of $^{22}Na^+$ and $^{42}K^+$ at zero time without incubation indicates the level of these ions due to their passive diffusion. This level is also shown by the ratio

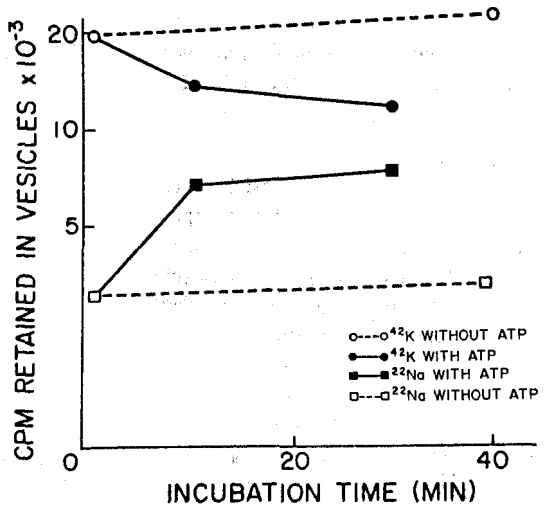

Fig. 6. Coupled transport of $^{22}Na^+$ and $^{42}K^+$ in (Na^+, K^+)-ATPase vesicles. (Na^+, K^+)-ATPase purified from the rectal gland of the hark was used to reconstitute Na^+ and K^+ transport system by incorporating the enzyme into cholate-lipid vesicle as reported (25). $^{42}K^+$ was preloaded for 12 hrs at 4°C in the transport medium and an aliquot (1ml) of the vesicles was used to determine the coupled transport of $^{22}Na^+$ influx and simultaneous efflux at $^{42}K^+$ at 25°C as described in Methods. The radioactivity due to retained $^{22}Na^+$ and $^{42}K^+$ by the vesicles was determined after addition of 5mM ATP. The same procedure was repeated without 5mM ATP for 40 minutes.

of $^{22}Na^+$ retained in vesicles to total radioactivity due to $^{22}Na^+$ applied to the column.

As a result of the incubation of the veshicles at 25°C in the presence of 5mM ATP, $^{22}Na^+$ counts were increased from 3000 cpm to 6800 cpm, while $^{42}K^+$ decreased from 20,000 cpm to 11,000 (Fig. 6). This ATP dependent increase of $^{22}Na^+$ counts which occurs simultaneously with the decrease of $^{42}K^+$ is direct evidence of the energy linked active coupled transport of Na^+ and K^+ in (Na^+, K^+)-ATPase vesicles. Incubation of the vesicles for 20 min produced a greater increase in $^{22}Na^+$ influx with a concomitant decrease of $^{42}K^+$. However, the rate of $^{22}Na^+$ increment was slow, compared to the initial 10 min period, which is reflected in a decrease of the ratio of $^{22}Na^+$ to $^{42}K^+$. $^{22}Na^+$ and $^{42}K^+$ counts did not change in the vesicles when they were incubated without 5mM ATP for 40 min so that the ratio of $^{22}Na^+$ to $^{42}K^+$ remained unchanged (Fig. 7).

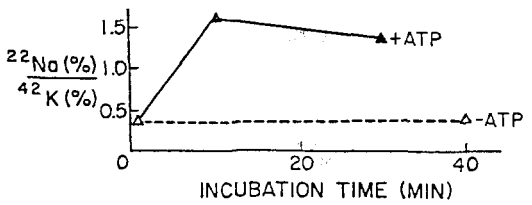

Fig. 7. ATP dependency on coupled transport of $^{22}Na^+$ influx and $^{42}K^+$ efflux in reconstituted (Na^+, K^+)-ATPase vesicles. (Na^+, K^+)-ATPase vesicles were prepared as in Fig. 6 using the shark enzyme. The coupled transport of $^{22}Na^+$ and $^{42}K^+$ was started by the addition of 5mM ATP at 25°C. The percent of $^{22}Na^+$ or $^{42}K^+$ retained by an aliquot (1ml) of vesicles from the total $^{22}Na^+$ or $^{42}K^+$ applied to the column was determined as in Method and the ratio of the percent of $^{22}Na^+$ to $^{42}K^+$ was calculated. The passive fluxes of $^{22}Na^+$ or $^{42}K^+$ were also estimated without the addition of 5mM ATP under identical conditions.

In this actively coupled transport system, effect of antibodies was studies as shown in Table 5. The passive diffusion of $^{22}Na^+$ and $^{42}K^+$ gave the ratio of Na^+ to K^+, 0.41, in the control experiment without 5mM ATP. By the addition of 5mM ATP to this system, there was threefold increase of $^{22}Na^+$ with concomitant reduction of $^{42}K^+$ in 10 min so that the ratio was increased to 2.25. Control globulin did not significantly affect the ratio. However, the same quantity of anti-shark (Na^+, K^+)-ATPase or anti-shark (Na^+, K^+)-ATPase glycoprotein antibodies reduced the ratio significantly.

Since the mechanism by which (Na^+, K^+)-ATPase is inhibited by these antibodies is not known, it was not clear whether the inhibition of active transort of monovalent cations in reconstituted (Na^+, K^+)-ATPase vesicles by these antibodies is related to the catalytic activity of (Na^+, K^+)-ATPase molecules incorporated into the vesicles. Therefore, it was logical to study whether the magnitude of inhibition of ac-

Table 5. Inhibitory Effect of (Na^+, K^+)-ATPase Antibodies on the Coupled Transport of $^{22}Na^+$ and $^{42}K^+$ in Reconstituted (Na^+, K^+)-ATPase Vesicles[1]

Experiments	$^{22}Na^+$ and $^{42}K^+$ Retained in Vesicles (%)			
	5mM ATP	$^{22}Na^+$	$^{42}K^+$	Ratio of $^{22}Na^+/^{42}K^+$
Vesicle	–	0.33	0.80	0.41
Vesicle	+	0.90	0.40	2.25
Vesicle + Control Globulin	+	0.83	0.40	1.83
Vesicle + Antishark Holoenzyme Antibody	+	0.67	0.45	1.49
Vesicle + Antishark glycoprotein Antibody	+	0.64	0.38	1.68

[1] Na^+, K^+-ATPase vesicles were preloaded with $^{22}Na^+$ and $^{42}K^+$ by incubating them with the vesicles for overnight at 4°C. These $^{22}Na^+$ and $^{42}K^+$ preloaded vesicles were mixed with indicated antibodies at room temperature for 30 min in a final volume of 1ml of the transport medium. Transport reaction was started by the addition of 5mM ATP at 25° for 15 min. Percent of $^{22}Na^+$ and $^{42}K^+$ eluted simultaneously in a void volume was calculated as described in Table 2. The ratio of $^{22}Na^+$ to $^{42}K^+$ was also computed because this ratio is a sensitive index of simultaneous countertransport of Na^+ and K^+.

[2] Indicates the ratio of $^{22}Na^+$ and $^{42}K^+$ uptake, therefore, there is no unit.

tive transport of cations by these antibodies was in agreement with the inhibition of vesicular (Na^+, K^+)-ATPase activity in a single experiment. In a test tube, after incubation of the vesicles with or without antibodies under normal transport experiment conditions at 25°C, an aliquot of vesicle mixture was used to determine the catalytic activity of vesicular (Na^+, K^+)-ATPase while the rest of the vesicle mixture was used to measure influx of $^{22}Na^+$ as described previously. The result of vesicular enzymatic activity with various antibodies is summarized in Table 6. As the experiment with normal assay medium at 37°C, control serum did not affect the vesicular (Na^+, K^+)-ATPase activity, although ATPase activity was very low due to high concentration of K^+ (100mM) and low concentration of Na^+ (20mM) at 25°C. Antibodies to eel (Na^+, K^+)-ATPase, shark (Na^+, K^+)-ATPase or its two subunits reduced the vesicular (Na^+, K^+)-ATPase activity up to 34% with anti-shark holoenzyme antibodies. Antibodies to shark glycoprotein had the least effect on catalytic activity of (Na^+, K^+)-ATPase vesicles, which is in good agreement with the effect of anti-shark glycoprotein on $^{22}Na^+$ influx (Table 2). The effect of other antibodies on vesicular (Na^+, K^+)-ATPase activity and their effect on $^{22}Na^+$ influx strongly suggests that the

Table 6. Inhibition of Vesicular (Na^+, K^+)-ATPase Activity in Reconstituted (Na^+, K^+)-ATPase Vesicles by Various Antibodies[1]

Antibodies	N[2]	Na^+, K^+-ATPase Activity (% inhibition)
Control serum	5	0±0[3]
Antishark holoenzyme antiserum	4	34±3.6
Antieel holoenzyme antiserum	3	22±3.0
Antishark glycoprotein antiserum	4	8±2.9
Antishark catalytic subunit antiserum	4	11±3.1

[1] During the transport experiment as described in Table 2, an aliquot of Na^+, K^+-ATPase incubation mixture was utilized to measure the content of inorganic phosphate released by the vesicular Na^+, K^+-ATPase activity as described under Materials and Methods. The control vesicular Na^+, K^+-ATPase activity was 380μm pi released per mg of Na^+, K^+-ATPase incorporated into the vesicles per hour.

[2] Indicate number of replication.

[3] Mean±S.E.

Table 7. Comparison on the Effect of Various Antisera on Catalytic Activity of Na^+, K^+-ATPase in Assay Medium and Trasnport Medium[1]

Antisera	Na^+, K^+-ATPase Catalytic Activity (% inhibition)			
	Assay Medium[2]		Transport Medium[3]	
	Shark	Eel	Shark	Eel
Control Serum	0 (1218)[4]	0 (1529)	0 (267)	0 (424)
Antishark Holoenzyme Antiserum	80	70	48	21
Antieel Holoenzyme Antiseraum	62	88	16	65
Antishark glycoprotein Antiserum	40	5	18	4
Antishark Catalytic Subunit Antiserum	43	10	41	1

[1] Twenty μg of Na^+, K^+-ATPases from the rectal gland of sharks or the electric organ of electric eels was incubated with 0.5ml of various antibodies in a final volume of 1ml of either assay medium or transport medium at room temperature for 30 min. The reaction was started by the addition of 5mM ATP at 37°C. Determination of inorganic phosphate released by the Na^+, K^+-ATPase was carried out as described under Materials and Methods.

[2] Assay medium contains 5mM $MgCl_2$, 10mM KCl, 100mM NaCl and 30mM imidazole buffer, pH 7.0.

[3] Transport medium contains 5mM $MgCl_2$, 100mM KCl, 20mM NaCl and 30mM imidazole buffer, pH 7.0.

[4] Parentheses indicate specific activity in μm pi released per mg protein/hr.

Table 8. Effect of N-ethylmaleimide (NEM) of $^{22}Na^+$ Uptake in Reconstituted (Na^+, K^+)-ATPase Vesicles and Vesicular ATPase Activity[1]

	N[2]	$^{22}Na^+$ Uptake (% inhibition)	Vesicular ATPase Activity (% inhibition)
1mM NEM	3	56.0±1.41	52±5.2

[1] One mM N-ethyl maleimide was added to the Na^+, K^+-ATPase vesicles at the beginning of the transport experiment. The transport reaction was started by the addition of 5mM ATP and $^{22}Na^+$ at 25° for 15 min. $^{22}Na^+$ uptake and vesicular Na^+, K^+-ATPase activity were determined as described in Tables 2 and 6.

[2] N indicates number of replication.

inhibitory mechanism of vesicular (Na^+, K^+)-ATPase activity by these antibodies is also responsible for the inhibition of $^{22}Na^+$ influx by these antibodies in reconstituted (Na^+, K^+)-ATPase vesicles.

In these reconstituted (Na^+, K^+)-ATPase vesicles, the vesicular enzymatic activity was very low in comparison to that of the intact enzyme when the vesicles were assayed in the transport medium at 25°C instead of 37°C in the normal assay medium. Therefore, effect of these antibodies on enzymatic activity of (Na^+, K^+)-ATPase was compared in normal assay medium at 37°C and in transport mediums at 25°C. The results are summarized in Table 7. The inhibitory effect of antibodies at 25°C was not significantly different from that at 37°C (Rhee and Hokin, 1979, and also see Fig. 1) in both eel and shark (Na^+, K^+)-ATPase, although inhibitory effect of anti-shark holoenzyme antisera on eel enzyme was somewhat greater than the usual value. In the transport medium which contained 100mM KCl, the inhibitory effect of the antibody was significantly reduced. Especially, the cross-reactivity was reduced remarkably. For example, anti-shark holoenzyme antibodies inhibit 70% of eel (Na^+, K^+)-ATPase in the assay medium, but there was only 21% inhibition in the transport medium.

In order to correlate further the inhibitory effect of antibodies on transport of cations and (Na^+, K^+)-ATPase activity in the reconstituted (Na^+, K^+)-ATPase vesicular system, we selected another agent, which is known to inhibit (Na^+, K^+)-ATPase activity so that the magnitude of the inhibitory effect of this agent on these two parameters could be correlated. As presented in Table 8, at the concentration of 1mM, N-ethylmaleimide inhibited 56% of $^{22}Na^+$ influx in comparison to the appropriate control experiment, while it reduced 52% of the vesicular (Na^+, K^+)-ATPase activity which was assayed simultaneously in an identical test tube as described previously.

DISCUSSION

In a short communication (Rhee and Hokin, 1979), we reported that antibodies raised against shark rectal gland (Na^+, K^+)-ATPase holoenzyme inhibited the catalytic activity of (Na^+, K^+)-ATPase. In this paper we also reported some immunochemical properties of antibodies against shark (Na^+, K^+)-ATPase glycoprotein subunit. A similar result was reported with antibodies raised against (Na^+, K^+)-ATPase in various degree of purity from rat brain tissue (Askari and Rao, 1971), pig kidney (Jørgensen *et al.*, 1973), canine heart (Wikman-Coffelt *et al.*, 1973) and canine renal medulla (Kyte, 1974; McCans *et al.*, 1974; Smith *et al.*, 1973). In this present paper, the inhibiory effect of antibodies against shark rectal gland catalytic subunit on the catalytic activity of (Na^+, K^+)-ATPase, as well as their effect on the transport of monovalent cations in reconstituted (Na^+, K^+)-ATPase vesicles were discussed. All these four polypeptide molecules—eel (Na^+, K^+)-ATPase, shark (Na^+, K^+)-ATPase, and their catalytic and glycoprotein subunits—were very antigenic. These antibodies inhibited (Na^+, K^+)-ATPase activity from both eel and shark in a concentration dependent manner and precipitated their antigens in immunodiffusion gel. This observation is basically in agreement with Jean *et al.* (1975), though they could not demonstrate the direct precipitation between their antigens and antibodies.

Kyte (1974) reported the properties of antibodies raised against a lipid-free large chain (comparable to our catalytic subunit reported in this present study) of purified (Na^+, K^+)-ATPase from canine renal medulla. These antibodies bound to the antigen based on complement fixation, but these antibodies did not inhibit their own antigen, (Na^+, K^+)-ATPase, at any concentration. This failure to inhibit (Na^+, K^+)-

ATPase by the anti-large chain antibody is difficult to understand because at least one more laboratory (Jean *et al.*, 1975), besides this present study showed a significant inhibition of (Na^+, K^+)-ATPase by anticatalytic subunit antibodies. It may be possible that extensive extraction of inrinsic membrane lipid by the treatment of a chloroform-methanol mixture might change the large chain in such a way that the antigenic property or the three dimensional structure of the antigen is unable to direct the formation of its specific antibody. This possibility is supported by the study that a certain species of antibody inhibits catalytic activity of (Na^+, K^+)-ATPase, while another species of antibody inhibits the binding of cardiac glycoside to this enzyme without interference with catalytic activity (McCans *et al.*, 1974). Immunochemical determinants of a protein are not necessary in the linear structure of amino acids because it has been shown that two antigens of identical primary structure (sperm whale and pork insulin) reacted differently to an identical human antisera (Ball and Loftice, 1987).

In the present study, in spite of the same immunization procedure, a certain rabbit produced a very high titer of antibodies and other animals produced antibodies which were cross-reacted more with shark (Na^+, K^+)-ATPase than with eel (Na^+, K^+)-ATPase (Table 1). therefore, a caution is required in drawing a conclusion on the basis of experiments with a limited number of experimental animals.

In this study, inhibitory effect of various antibodies of shark or eel (Na^+, K^+)-ATPase agreed well with the data on the formation of antigen-antibody precipitation lines studied by double diffusion tests. There was a considerable cross-reaction between antishark (Na^+, K^+)-ATPase antibody and eel (Na^+, K^+)-ATPase, or vice versa, although there was far less cross-reaction between antishark catalytic subunit antibody and eel (Na^+, K^+)-ATPase. The least cross-reaction was noted between anti-shark glycoprotein antibodies and the eel (Na^+, K^+)-ATPase. This degree of cross-reaction also correlated well with the intensity of immunodiffusion precipitation bands between these antigens and antibodies, because it was impossible to see the precipitation line formed between antishark glycoprotein antibodies and the eel holoenzyme under conditions used for eel holoenzyme and its antibody diffusion test.

As reported previously (Hilden *et al.*, 1974), reconstituted (Na^+, K^+)-ATPase vesicles with purified shark (Na^+, K^+)-ATPase demonstrated an ATP dependent, temperature sensitive and ouabain inhibitable influx of $^{22}Na^+$. This active $^{22}Na^+$ influx is clearly coupled with counter translocation of $^{42}K^+$ or $^{86}Rb^+$ in this reconstituted (Na^+, K^+)-ATPase vesicular system (Fig. 5). The energy dependent active coupling of $^{22}Na^+$ and $^{42}K^+$ was significantly inhibited by the addition of various antibodies, although the antibodies against shark glycoprotein had the least effect on ion transport. Preimmune or control serum had no effect on the transport of monovalent cations. The inhibitory effect of these antibodies cannot be explained by the inhibition of passive movements of these cations, because these antibodies did not significantly affect the fluxes of $^{22}Na^+$ or $^{42}K^+$ in the absence of 5mM ATP.

Here, questions are raised whether the inhibition of (Na^+, K^+)-ATPase activity by specific antibodies is relevant to the inhibition of the active transport of alkali metals in the reconstituted (Na^+, K^+)-ATPase vesicles. We developed a system, in which we were able to demonstrate the active transport of ions in relation to enzymatic activity of the (Na^+, K^+)-ATPase containing vesicular system. This system contains (Na^+, K^+)-ATPase and phospholipid solubilized in cholate with minimal contaminants of other impurity (Hilden *et al.*, 1974). In this active transport system, the inhibitory effect of antibodies on (Na^+, K^+)-ATPase molecules incorporated into phospholipid vesicles could be related to the inhibition of the active transport of monovalent cations. It appears to us that this is the case. Although the specific activity of (Na^+, K^+)-ATPase vesicles was very low due to 100mM KCl in transport medium and low incubation temperature (25°C) instead of 37°C, an excellent correlation between the degree of inhibition of $^{22}Na^+$ accumulated and that of the specific activity of vesicular ATPase was observed (Table 2, 6). That is, these antibodies against (Na^+, K^+)-ATPase or its subunits inhibit the active transport of $^{22}Na^+$ in (Na^+, K^+)-ATPase veiscles by inhibiting the (Na^+, K^+)-ATPase molecules in the vesicles regardless of the mode of inhibition of (Na^+, K^+)-ATPase by these antibodies. This conclusion is further supported by the experiment with N-ethylmaleimide (Table 8), which inhibited (Na^+, K^+)-ATPase vesicular catalytic activity and $^{22}Na^+$ influx in parallel fashion.

Although the exact mechanism by which the antibodies inhibit (Na^+, K^+)-ATPase activity is not clear at this time, a number of possible mechanisms has been suggested in other system (Celada and Strom, 1972). It may be reasonable to consider that the binding of antibodies to (Na^+, K^+)-ATPase must precede any manifestation of the antigen and antibody interaction (Cinader, 1967). In case of ribonuclease, antibodies against the ribonuclease bind to

ribonuclease and inhibit it, while some of these antibodies do not inhibit and even compete with the same species of antibodies which inhibits the enzyme for the binding site (Richards *et al.*, 1975). This antibody could bind the enzyme at the catalytic active center or near the active center so that the conformation of the enzyme-antibody complex might no longer be suitable for the approach of substrates. In (Na^+, K^+)-ATPase, the lipids probably are necessary to preserve specific antigenic determinants because a lipid-free large chain produces antibodies which bind to its holoenzyme without inhibition of catalytic activity (Kyte, 1974). In the interaction of (Na^+, K^+)-ATPase and its antibody, the possibility has been ruled out that the mechanism of (Na^+, K^+)-ATPase inhibition by antibodies is due to the precipitation of an antibody-enzyme complex, because Fab fragments obtained by the digestion of antibodies with papain had a similar inhibitory action and specificity as the original antibody (Smith *et al.*, 1973). This finding suggests that the minimum distance between strong immunogenic determinants and the catalytic active center in (Na^+, K^+)-ATPase may be close because, if they are far apart, univalent antibodies (90 Å) and divalent antibodies (250 Å) must show a different effect on (Na^+, K^+)-ATPase as studies in the other system (Almeida *et al.*, 1965).

In many senses, the (Na^+, K^+)-ATPase vesicles are an analogous system to red blood cells, although the design of our experiment is directed to (Na^+, K^+)-ATPase moleculaes oriented backwards (Hilden and Hokin, 1975). In red blood cells (Postand Jolly, 1957) the flux of Na^+ and K^+ dependes upon an energy source and is tightly coupled in a ratio of 3 to 2 (Na^+:K^+), although this could vary from tissue to tissue (Windhager, 1969). In the present study it was possible to estimate directly the ratio of Na^+ influx to K^+ efflux in duel labelled experiment (Fig. 6). The vesicles had 0.59% of Na^+ and 1.34% of total radioactive K^+ after a 12 hr preloading period at 4°C. After 10 min incubation at 25°, the count of $^{22}Na^+$ was raised to 1.45% and that of $^{42}K^+$ was decreased to 0.9%, so that the ratio of Na^+ to K^+ became 0.89. This ratio is still quite low in comparison to 1.5 found in intact red blood cells. Recently, the ratio estimated by the amount of Na^+ and K^+ transported per unit of ATP hydrolysis was improved to 1.4 reaching the value obtained in red blood cells (Hilden and Hokin, 1975). Golden and Tong (1974) also reported such a low efficiency of pumping action with their reconstituted (Na^+, K^+)-ATPase prepared from canine renal medulla. However, the active influx of $^{22}Na^+$ in their vesicles was not coupled with the efflux of K^+, but cotranslocated with Cl^- in the same direction in a ratio of 1:1 (Na:Cl) to maintain the electrochemical neutrality. Therefore, these authors consider the possibility that their purified (Na^+, K^+)-ATPase or reconstituted vesicles may either have a nonfunctional "K^+" or lack the pump altogether.

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