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Characteristics of Antibodies to Guinea Pig $(Na^+ + K^+)$-Adenosine Triphosphatase and their Use in Cell-Free Synthesis Studies

Alicia A. McDonough*, Andrew Hiatt, and Isidore S. Edelman
Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, New York 10032

Summary. Antibodies have been produced, in three rabbits, to Na/K-ATPase purified from guinea pig renal outer medulla. Each rabbit produced antibodies to both the α (catalytic) and the β (glycoprotein) subunits of Na/K-ATPase. The titers of the anti-α and anti-β antibodies varied with time and between rabbits. None of the antisera inhibited Na/K-ATPase activity under various preincubation conditions. A method is presented for separating small amounts of anti-α subunit from anti-β subunit antibodies. There was no cross-reactivity of antibodies to one subunit with the other subunit. The α subunit of the Na/K-ATPase was cleaved into a 41,000-dalton peptide (that contains the ATP phosphorylating site) and a 58,000-dalton hydrophobic peptide as described by Castro and Farley (Castro, J., Farley, R.A., 1979, *J. Biol. Chem.* **254**:2221–2228). Anti-α antibodies from all of the rabbits reacted with both proteolytic fragments. The anti-guinea pig Na/K-ATPase antisera (pooled) cross-reacted with the α subunit of Na/K-ATPase from human, cow, dog, rabbit, rat, mouse, turtle, and toad; and with the β subunit from human, rat, and mouse. The loci of cross-reactivity were investigated using partially purified canine kidney Na/K-ATPase cleaved with trypsin as described above. The antisera from rabbits 1 and 2 cross-reacted with the 41,000-dalton peptide from the dog but very little with the 58,000-dalton peptide. No cross-reactivity was observed with antiserum from rabbit 3 to either fragment. Guinea pig kidney RNA was translated in a rabbit reticulocyte lysate system followed by immunoprecipitation with the antisera. The molecular weight of the cell-free synthesized α chain was 96,000 daltons. Its identity was established with purified anti-α antibodies and by immunocompetition with purified Na/K-ATPase and Ca-ATPase. Translation of the β subunit was not detected in this system.

Key words Na/K-ATPase · antibodies · biosynthesis · cross-reactivity · cell-free synthesis

Introduction

The sodium pump is an intrinsic and vital plasma membrane-bound oligomer conisting of a catalytic subunit (α) and a glycoprotein subunit (β) with Na^+ and K^+ activated adenosine triphosphatase activity (Na/K-ATPase[1]). Considerable information is available on the enzymatic, transport, and structural characteristics of this enzyme. Studies have also been reported on the regulation of Na/K-ATPase activity and content by hormones, such as triiodothyronine and steroids, and by ions [2, 5, 7, 8]. To examine the regulation of transcription and translation of Na/K-ATPase and to study the processing and assembly of the subunits, a cell-free synthesis system for the enzyme must be developed. The detection of primary translation products of Na/K-ATPase mRNA(s) is dependent upon the availability of antibodies, specific for the α and β subunits of the enzyme. Moreover, the preparation of well-characterized anti-Na/K-ATPase antibodies may also provide useful probes for the analysis of structure-function relations of the enzyme. Antibodies have been prepared against a number of Na/K-ATPase preparations, some of which inhibit enzyme function [9, 11, 13, 14, 18]. In these studies, functional assays, complement fixation, and immunoprecipitation have been used to assay for antibodies to the holoenzyme. These methods are limited in their ability to detect antibodies against low level contaminants in the antigen preparation, and little information is available on antibodies against separated α and β subunits. The latter is crucial in the use of antibodies to detect translation of the subunits in cell-free systems.

The present report concerns the preparation and

* *Present address:* Department of Physiology and Biophysics, University of Southern California School of Medicine, Los Angeles, California 90033.

[1] *Abbreviations:* Na/K-ATPase = $(Na^+ + K^+)$-dependent adenosine triphosphatase, E.C. 3.6.1.3. Ca-ATPase = $(Ca^{++} + Mg^{++})$-dependent adenosine triphosphatase. DPT = diazophenylthioether. SDS = sodium dodecyl sulfate. PAGE = polyacrylamide gel electrophoresis. DATD = N,N′diallytartardiamide. EDA = ethylene diacrylate. *Staph a* = staphalococcus aureus. Protein A = the surface protein extracted from *Staph* α that binds to the *Fc* fragment of IgG. TPCK-trypsin = L-1-tosylamide-2-phenylethyl chloromethyl ketone-treated trypsin. BSA = bovine serum albumin. Hepes = N-2-hydroxy ethylpiperazine-N-2-ethanesulfonic acid. PPO = 2,5-diphenyloxazole.

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characterization of antibodies to highly purified Na/K-ATPase from guinea pig renal outer medulla. Evidence is presented that these antibodies are specific for the Na/K-ATPase subunits, and information was obtained on binding to defined fragments of the α subunit. The cross-reactivity of the antibodies to Na/K-ATPase from various species is described as well as a method for separating subunit specific antibodies. The antibodies were also used to identify α chains synthesized in cell-free reticulocyte lysates.

Materials and Methods

Purification of Na/K-ATPase

Na/K-ATPase was purified from the outer medulla of frozen guinea pig kidneys (Pel-Freeze, Rodgers, Ark.) in a membrane-bound form by extraction with SDS followed by density gradient centrifugation in a zonal rotor, as described by Jorgensen [10]. The purity of the preparation was analyzed by SDS-PAGE by the Laemmli method [15], and the molecular weights of the subunits were determined by SDS-PAGE according to Weber and Osborn [26]. Enrichment of enzyme activity was assessed by Na/K-ATPase assays as described by Lo et al. [16]. Purified enzyme[2] was stored in 25 mM imidazole, 1 mM EDTA (pH 7.5) at −70 °C.

To assess the binding of the antibodies to Na/K-ATPases of various species, membrane fractions enriched in Na/K-ATPase were prepared from Balb c mouse kidney, and renal outer medulla of Sprague-Dawley rat, New Zealand rabbit, mongrel dog, cow, and man, as well as from the urinary bladder of the toad (*Bufo marinus*) and turtle (*Pseudemys scripta* elegans). The kidney samples were stored at −70 °C prior to use and the membrane fractions were prepared by homogenization and differential centrifugation in sucrose-histidine, by the method of Jorgensen [10]. Plasma membrane preparations enriched in basal-lateral membranes that contain much of the Na/K-ATPase activity were prepared from epithelial scrapings of toad and turtle urinary bladders by sucrose gradient centrifugation according to Palmer and Edelman [19]. The membrane preparations were assayed for Na/K-ATPase activity by the method of Lo et al. [16], analyzed by SDS-PAGE [15], and stored at −70 °C pending use. Sarcoplasmic reticulum enriched in Ca-ATPase was prepared by dissecting skeletal muscle from the extremities of an exsanguinated guinea pig and processing through the R1 step of the method of MacLennan [17]. The Ca-ATPase peptide was identified by SDS-PAGE [17].

Immunization

Antibodies were raised in three rabbits against suspensions of purified membrane-bound guinea pig Na/K-ATPase in 25 mM imidazole, 1 mM EDTA buffer (antigen). The antigen preparations were estimated to contain less than 5% impurities as judged by SDS-PAGE and had Na/K-ATPase activities (V_{max}) of 1,799–2,100 μmol P_i/mg protein/hr. The rabbits were injected at multiple sites subcutaneously along the back with the antigen mixed with an equal volume of Freund's complete adjuvant. Each rabbit received 0.5 mg antigen initially and was reinjected 2, 3, 5, and 9 weeks and then monthly with 0.2 mg antigen.

The production of antibodies against Na/K-ATPase was determined by the method of Renart et al. [22]. Na/K-ATPase subunits were resolved by SDS-PAGE (gels crosslinked with DATD or, in one case, EDA) transferred, and covalently attached by blotting to DPT paper prepared according to B. Seed[3]. The DPT paper is also available commercially from Collaborative Research, Inc. The resolved peptides coupled to DPT paper were incubated with antiserum diluted 1:50 to 1:200, washed, and incubated with ^{125}I-Protein A (0.25 μCi/gel slot) followed by another wash [22]. Immune complexes labeled with Protein A were visualized by autoradiography using Kodak XAR-5 film and DuPont Cronex Lightening-plus XL screens at −70 °C. Protein A was iodinated using BioRad Enzymobeads.

Proteolytic Fragmentation

The α subunit was cleaved into a 41,000-dalton peptide and a 58,000-dalton peptide by the method of Castro and Farley [4] which entails the incubation of purified membrane-bound Na/K-ATPase with a 10-fold excess (wt/wt) of TPCK-trypsin in the presence of 150 mM KCl, 25 mM imidazole and 1 mM EDTA at pH 7.5 for 6 min at 37 °C. The trypsin reaction was stopped with a threefold excess of soy bean trypsin inhibitor. The fragments were resolved by SDS-PAGE, transferred to DPT paper and tested with isolated anti-α antisera as described above [22]. The 58,000-dalton peptide has a similar R_f as the β subunit in the SDS-PAGE system. Any ambiguity in identifying reactivity with antibodies, however, was resolved by testing binding to specific anti-α antibodies that display no cross-reactivity with the β subunit.

Antibody Separation

To separate and recover small amounts of anti-α and anti-β antibodies, the DPT paper with coupled α or β subunits was used as an affinity ligand. Purified Na/K-ATPase was dissolved in SDS gel sample buffer applied all along the top of an SDS slab gel, electrophoresed, and blotted as described by Renart et al. [22]. Strips containing only α subunit and only β subunit were cut from the DPT paper, incubated with anti-serum (diluted 1:20 to 1:40) and washed for 12 hr at 37 °C [22]. The specific antibodies were eluted by incubation of the washed strips in 3 to 5 ml of 200 mM glycine-HCl, 0,25% BSA (pH 3.3) for 3 to 30 min at 0 or 20 °C and neutralized to pH 7.0 by the addition of 2 volumes of 2 M Tris-HCl (pH 9.1) to the elution medium.

Preparation of RNA

English short-hair guinea pigs (~2 kg body weight) were anesthetized by ether inhalation, killed by a sharp blow to the head, exsanguinated through a cardiac incision, and the kidneys were immediately removed by surgical dissection. Total RNA from either whole kidneys or from outer medulla harvested by sharp dissection was prepared according to Chirgwin et al. [6]. Briefly, tissue was homogenized at room temperature using a Brinkman Polytron at full speed in 4 mM guanidinium thiocyanate, 0.1 M 2-mercaptoethanol, 0.5% Sarkosyl, 25 mM sodium citrate, pH 7.0. The homogenate was centrifuged at 10,000 × g for 10 min, the supernatant diluted with 0.75 vols of absolute ethanol, and this solution incubated at −20 °C overnight. The precipitated nucleic acids were dissolved in 7.5 M guanidine HCl, reprecipitated by the addition of 0.5 vols of absolute ethanol and allowed to stand for 3 hr at −20 °C. Re-solution and re-precipitation were carried out two more times to obtain purified RNA. Poly(A)$^+$RNA was isolated by chromatography on oligo-dT cellulose by the method of Chirgwin et al. [6]. The poly(A)$^+$RNA was ethanol precipitated, redissolved in 0.1 mM EDTA, and stored in liquid N_2.

[2] "Purified" membrane bound Na/K-ATPase denotes enzyme preparations with activities (V_{max}) greater than 1,700 μmol P_i/mg protein hr.

[3] Personal communication from Brian Seed, California Institute of Technology, Pasadena, California.

Cell-free Translation

Nuclease-treated rabbit reticulocyte lysate was prepared as described by Pelham and Jackson [21]. The translation mixtures contained 50 μl of lysate, 58 mM K-acetate, 1.0 mM $MgCl_2$, 0.5 mM spermidine, 20 mM Hepes, pH 7.6, 1 mM Na_2ATP, 0.2 mM Na_2GTP, 12 mM creatine phosphate, 50 U/ml creatine phosphokinase, 30 μM unlabeled amino acids excluding methionine, 50 to 100 μCi ^{35}S-methionine (sp act ~1,000 Ci/mM). In some experiments, rabbit reticulocyte lysate purchased from Amersham was used. All RNA samples were first pre-incubated in 10 mM methylmercury hydroxide for 5 min at room temperature prior to translation [20]. Translation was allowed to proceed for 1 to 3 hr, depending on the desired yield, after initiation by the addition of RNA, at 30 °C. After 1 hr of incubation, incorporation of ^{35}S into peptides was generally 10-fold greater in lysates with added RNA than in lysates without this addition. The lysates were then centrifuged at 180,000 *g* for 60 min to pellet polysomes containing nascent chains. Released chains in the supernatant were then immunoprecipitated by the addition of 10 volumes of PBSE/NP-40 (10 mM KH_2/K_2PO_4, pH 7.0, 150 mM NaCl, 5 mM Na_2EDTA, 0.5% NP-40, 5 mg/ml BSA) and 3 μl of undiluted antiserum. After standing at 4 °C overnight, twenty μl of 50% Sepharose-protein A in PBSE/NP-40 was then added to the lysate. After 30 min on ice, the Sepharose-protein A beads were collected by centrifugation for 1 min at room temperature in a Beckman microfuge and were washed four times with 1 ml each of PBSE/NP-40 (without BSA). Thirty μl of SDS solubilizing buffer (0.2% SDS, 1% 2-mercaptoethanol, 1 mM EDTA, 10 mM phosphate buffer at pH 7.0 [26] was then added to the beads. After boiling for 3 min, the solution was loaded onto SDS polyacrylamide gels prepared according to Laemmli [15] and electrophoresed for 4 hr at 125 V. The gels were then fixed in methanol/H_2O/acetic acid (5:4:1) for 6 to 12 hr, and stained in 0.1% Coomassie R-250 in fixer for 1 hr, destained in 7.5% acetic acid. The gels were saturated with PPO [3], and the bands were made visible by placing the dried gels against Kodak X-Omat film for 2 days at −70 °C.

Materials

All of the conventional chemicals were reagent grade, spectroquality or electrophoresis purity reagents. SDS-PAGE reagents were obtained from BioRad. BSA (Fraction V powder), Sarkosyl and soy bean trypsin inhibitor were from Sigma Chemicals. DPT paper was prepared using chemicals from Aldrich. EDA was obtained from Pfaltz and Bauer, Stamford, Conn., TPCK-trypsin from Worthington, protein A and sepharose-protein A from Pharmacia. English short hair guinea pigs (~2 kg body wt) were obtained from Camm. Oligo-dT cellulose and NP-40 were from Bethesda Research Labs, rabbit retuculocyte lysate and ^{35}S-methionine (sp act >1,000 Ci/mmol) from Amersham, guanidine HCl from Schwartz Mann, and guanidine thiocyanate (Fluka purum) from Tridom, Inc.

Results and Discussion

Na/K-ATPase Purification

Guinea pig renal outer medulla yielded microsomal preparations with Na/K-ATPase activities of 20 to 40 μmol P_i/mg protein/hr, and the activities of the enzyme purified by the Jorgensen procedure [10] ranged from 1,700 to 2,100 μmol P_i/mg protein/hr. This 50-fold enrichment in activity corresponds to that reported by others for purified renal Na/K-ATPase [13]. In our purified preparations, there was no detectable Mg-ATPase activity measured as the ouabain-insensitive activity. Analysis of one of the purified enzyme preparations by SDS-PAGE [15] is shown in Fig. 1. The amount of extraneous protein detectable by SDS-PAGE [15] was minimal in the purified preparations. The apparent molecular weights of the subunits, determined by SDS-PAGE using the Weber and Osborn method [26] were 110,000 for the α subunit (catalytic) and 67,000 for the β subunit (glycoprotein). When analyzed by Laemmli [15] discontinuous SDS gels, the subunits had lower apparent molecular weights: 96,000 for α and 55,000 for β. These values are all in the range of those reported for other mammalian preparations of Na/K-ATPase when analyzed by SDS-PAGE [25].

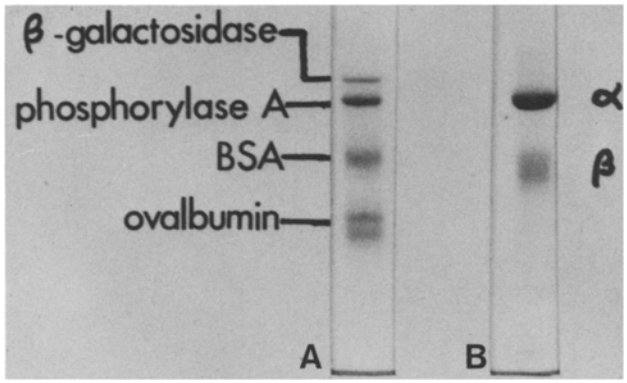

Fig. 1. SDS-PAGE [15] of guinea pig kidney Na/K-ATPase. (*A*) Molecular weight standards: β-galactosidase = 135,000; phosphorylase *a* = 94,000; BSA = 68,000; ovalbumin = 44,000. (*B*) Na/K-ATPase: α = catalytic subunit; β = glycoprotein subunit

Production of Antibodies

Three rabbits (*R*1, *R*2, *R*3) were immunized with the purified membrane-bound Na/K-ATPase. The specificity of the antibodies for the subunits of Na/K-ATPase was tested by resolving the subunits by SDS-PAGE, transferring them to DPT paper, incubating the paper with antisera diluted 1:100, and detecting antibody binding by attachment of ^{125}I-protein A [22]. The results obtained on antibody fractions from each rabbit are shown in Fig. 2*A*. Clearly, each rabbit produced antibodies against the α and β subunits. The titers of antibodies against the two subunits varied over the time course of immunizations and bleedings. The highest titer antibodies were obtained early after the appearance of antibodies (for *R*1 on 8–3 and for *R*3 on 8–24). *R*2 died on 8–24. The titers in *R*1 and *R*3 remained constant for the next few months (Fig. 2*A*).

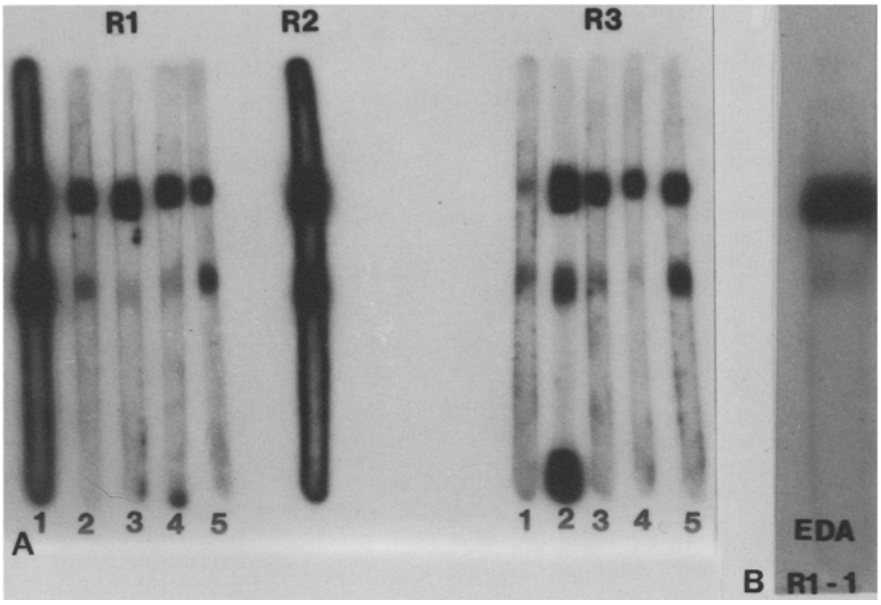

Fig. 2. Testing antisera to Na/K-ATPase. (*A*) Na/K-ATPase subunits were resolved by SDS-PAGE crosslinked with DATD and covalently coupled to diazotized paper. The paper was subsequently incubated with antisera (diluted 1:100) drawn on the dates indicated below, washed, and incubated with ^{125}I-protein A followed by autoradiography. *R1*: (*1*) 8–3, (*2*) 8–24, (*3*) 9–28, (*4*) 10–17, (*5*) 11–29. (*B*): Na/K-ATPase was resolved by SDS-PAGE crosslinked with EDA and treated as described above; incubated with *R1* 8–3 antiserum diluted 1:50

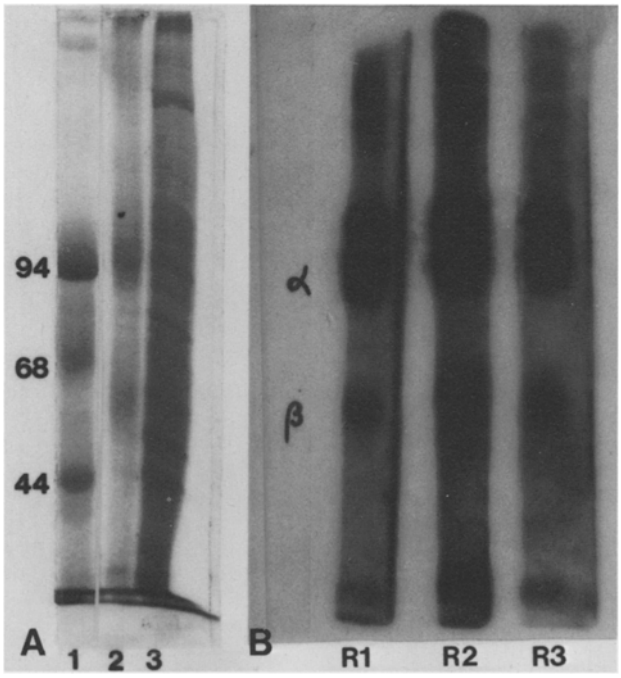

Fig. 3. Specificity of anti-Na/K-ATPase antibodies. (*A*): SDS-PAGE of (*1*) molecular weight markers, (*2*) purified Na/K-ATPase, (*3*) crude microsomes from guinea pig renal outer medulla. (*B*): Autoradiogram of microsomal fraction tested with 1:100 dilutions of *R1* 8–3, *R2* 8–3, and *R3* 9–4 as described in Fig. 2

The gels crosslinked with DATD were incubated with 2% periodic acid for 1 hr to dissolve the crosslinks prior to transfer. In order to test whether this treatment was altering antigenic sites on the Na/K-ATPase subunits, the enzyme was resolved on gels crosslinked with EDA [22] and incubated with 0.25 M ammonium hydroxide prior to blotting. As can be seen in Fig. 2*B*, there was very little Protein A binding in the region of the β subunit subsequent to incubation with antiserum. Whether this was due to a less efficient coupling of β to the DPT paper or to less antibody binding to β was not determined. The β subunit is a highly glycosylated peptide. Conceivably, the periodic acid, through cleaving portions of the oligosaccharides of β, exposes sites that can be coupled to the DPT paper or exposes antigenic sites that are now available for antibody binding. Whether, in addition, there are antigenic sites that are sensitive to periodic acid has not been determined.

The binding of antibodies to the proteins of a crude microsomal preparation of guinea pig outer medulla using the DPT transfer technique was performed to evaluate the specificity of the antibodies (Fig. 3). In the stained gel of the microsomal preparation, the α and β subunits cannot be distinguished from the background of proteins (Fig. 3*A*). However, binding of antibodies is seen in the region of both the α and the β subunits with all three antisera (Fig. 3*B*). In addition, there is antibody binding to material slightly above the α subunit. Evidence suggesting that this is part of the Na/K-ATPase system will be presented in this paper.

The effects of the antisera on enzyme activity were assessed in three different preincubation solutions (1 hr at 0 °C). At dilutions of antisera of 1:5 to 1:10, inhibition of Na/K-ATPase activity was not apparent (Table 1). In previous studies both inhibitory and noninhibitory antibodies have been described. At dilutions of antisera of as high as 1:15, Jorgensen [12], and Koepsell [13] noted greater than 80% inhibition of Na/K-ATPase activity. Jean and Albers [9] and McCans et al. [18] were able to demonstrate a similar

Table 1. The effect of antisera on Na/K-ATPase activity

Preincubation mixture:	I	II	III
	Na/K-ATPase activity of control		
Preimmune sera	100%	100	100
Rabbit 1	116	97	95
Rabbit 2	90	112	103
Rabbit 3	119	108	100

Na/K-ATPase activity is expressed as per cent of the control activity assayed in the presence of combined preimmune sera. Ten μg of purified guinea pig Na/K-ATPase was preincubated for 1 hr at 0 °C prior to Na/K-ATPase assay in the following mixtures: I: 120 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 3 mM Tris-ATP, 15 mM Tris-HCl (pH 7.4 at 37 °C) and 1:5 dilution of sera. II: 25 mM imidazole, 1 mM EDTA (pH 7.5 at 25 °C) and 1:5 dilution of sera. III: 150 mM KCl, 6 mM KH_2PO_4, 20 mM imidazole, 2 mM EGTA (pH 7.5 at 25 °C), 3 mM Tris-ATP and a 1:10 dilution of sera

inhibition using an antibody (IgG) to enzyme ratio of 12 (mg/mg). The conditions used in this study are equivalent to a weight ratio of 200. In contrast, Kyte [14] detected no inhibitory activity in rabbit anti-dog Na/K-ATPase antisera. The determinants of the variations in inhibitory activity of various antisera are obscure at the present time.

Separation and Cross-Reactivities of Anti-α and Anti-β Antibodies

The separated α and β subunits coupled covalently to the DPT paper proved to be useful as affinity ligands for separating and collecting small amounts

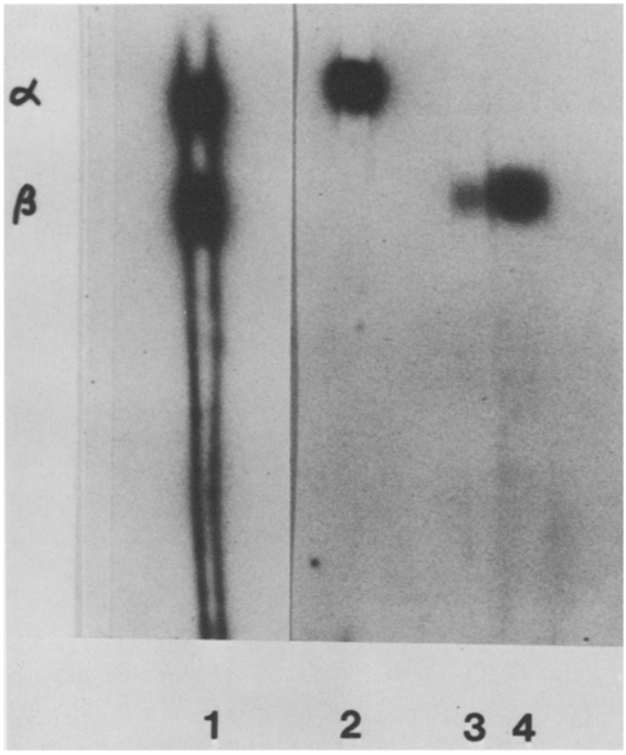

Fig. 4. Separation of anti α- and anti β-antibodies. Strips of DPT paper with only α or only β subunit attached were incubated with *R2* antiserum and washed. The anti-α or anti-β antibodies were eluted from the papers by incubation in 200 mM glycine-HCl, 0.25% BSA (pH 3.3), and neutralized by the addition of 2 volumes of 1 M Tris-HCl (pH 9.1). The separated antibodies were retested for binding to both subunits linked to DPT paper. *1–R2* antiserum before separation; *2*-anti-α antibodies; *3*-anti-β antibodies removed with 3 min incubation at 0 °C. *4*-anti-β antibodies removed with 30 min incubation at 22 °C

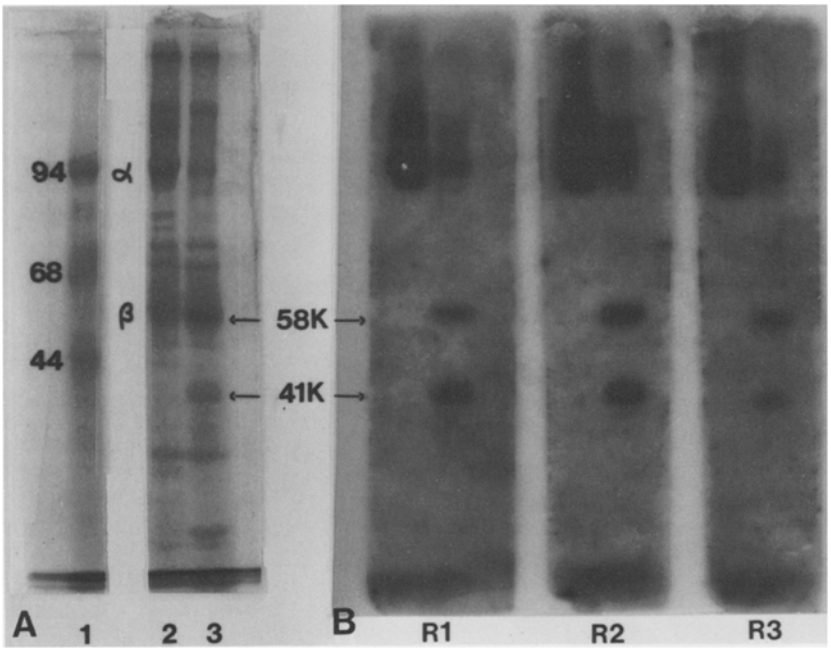

Fig. 5. Antibodies to proteolytic fragments of α. (*A*): SDS-PAGE of: (*1*) molecular weight markers, (*2*) partially purified Na/K-ATPase, (*3*) Na/K-ATPase following incubation with KCL and TPCK-trypsin as described in methods. (*B*): Identical samples to those shown in *A2* and *A3* were transferred to diazotized paper and tested with purified anti-α antibodies *R1* 8–3, *R2* 8–3 and *R3* 9–4. For purification of the anti-α antibodies, twice as much *R3* 9–4 serum was processed (1:20 dilution) and incubated with these samples as the *R1* and *R2* antisera (1:40 dilutions)

of anti-α and anti-β antibodies. After transferring the resolved subunits, strips of DPT paper with α or with β subunit attached were incubated with *R*2 antiserum (diluted 1:40) and washed. The antibodies were eluted from the paper with the acid glycine buffer and re-tested for binding to both subunits linked to DPT paper. Elution of the anti-α antibodies from the strips was essentially complete in 3 min at 0 °C, and elution of the anti-β antibodies was essentially complete in 30 min at 20 °C, indicating a higher affinity of the latter for its antigen. The results in Fig. 4 show that there is a clean separation of the two populations of antibodies. There was no apparent cross-reactivity of the anti-α chain antibodies with the β subunit or the anti-β chain antibodies with the α subunit, suggesting that there is little homology in the two subunits at least with respect to antigenic determinants.

Antibody Characterization

In the presence of KCl, TPCK-trypsin cleaves the α subunit into a 41,000-dalton hydrophilic peptide and a 58,000-hydrophobic peptide [4, 8]. The binding of separated anti-α antibodies from each of the three rabbits to intact α and trypsin-cleaved α subunit was evaluated by the DPT transfer technique (Fig. 5). Twice as much *R*3 antibodies as *R*1 and *R*2 was used in this experiment because of the lower apparent titers of *R*3. All three populations contain antibodies against both the 41,000- and 58,000-dalton fragments.

As discussed earlier, there was antibody binding to material of higher molecular weight than α. This binding was seen again in this experiment with the purified anti-α antibodies. In addition, this binding decreased when the sample was digested with trypsin, suggesting that the material was derived from α subunit. Sweadner [24] has reported that there are two molecular forms of α in the brain. We doubt that the antibody binding that we detected involves two forms of α, for two reasons. Firstly, Sweadner [24] did not find two forms of α in canine kidney, although the antibody binds to a higher molecular weight component in canine kidney (*see* Fig. 7 and Discussion). Secondly, to resolve the two forms of α, Sweadner [24] had to employ 5–6% acrylamide gels while the bands seen here are resolved using 10% acrylamide gels, indicating that this material has a higher molecular weight than the $\alpha+$ observed by Sweadner [24]. Thus, the higher molecular component in our gels may be an $\alpha\beta$ monomer. Experiments are being conducted with purified anti-β antibodies to test this hypothesis.

Since Na/K-ATPase is a ubiquitous and well-conserved enzyme, the cross-reactivity of the anti-guinea pig antibodies against other species was explored. Na/K-ATPase-containing membrane fractions from various animals were compared to an equivalent amount of guinea pig Na/K-ATPase in their ability to bind antibodies (Fig. 6). In this study, peak antisera from *R*1, *R*2, and *R*3 were combined for testing. The anti-α antibodies cross-reacted with all of the α subunits tested, albeit with varying intensities. The anti-β antibodies, however, apparently bound only to the rat, mouse, and human subunits. These results suggest that there is more similarity between species in the structure of the α subunit than in the β subunit, at

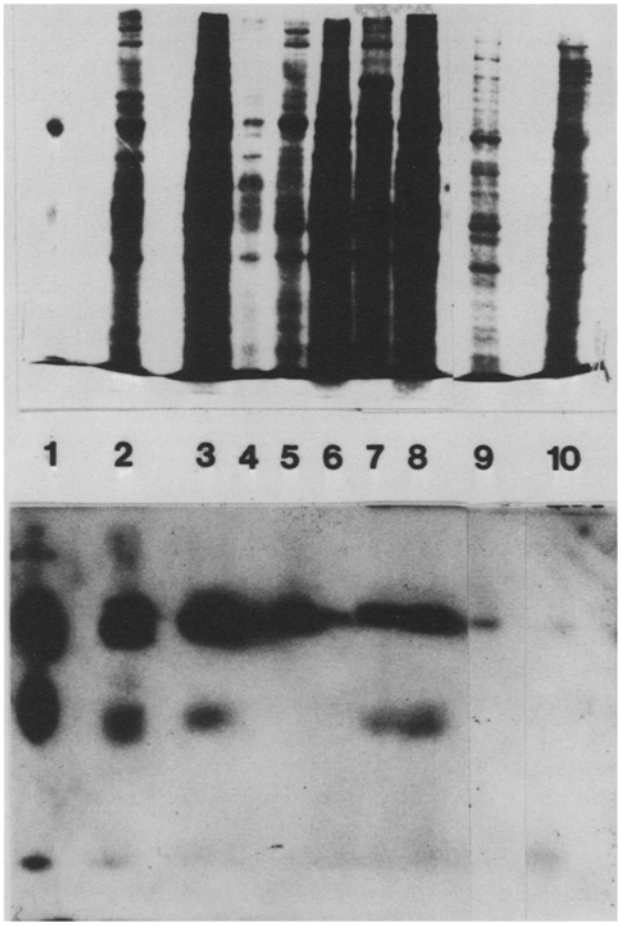

Fig. 6. Cross reactivity of anti-guinea pig Na/K-ATPase antibodies with Na/K-ATPase from various species. Membrane fractions from various animals were prepared as indicated in the methods section and subjected to SDS-PAGE. The same amount of Na/K-ATPase activity was applied to each lane – 1.0 μmol P_i/hr/gel lane. The upper was stained with Coomassie blue. The lower photograph is a duplicate gel that was transferred to diazotized paper, incubated with pooled antisera from *R*1, *R*2, *R*3 diluted 1:100, washed, incubated with ^{125}I-protein A. The resulting autoradiogram consists of lanes that contain: *1* – purified guinea pig Na/K-ATPase, *2* – guinea pig renal outer medulla membranes, *3* – human outer medulla membranes, *4* – beef outer medulla membranes, *5* – dog outer medulla membranes, *6* – rabbit outer medulla membranes, *7* – rat outer medulla membranes, *8* – mouse kidney microsomes, *9* – turtle bladder membranes, *10* – toad bladder membranes

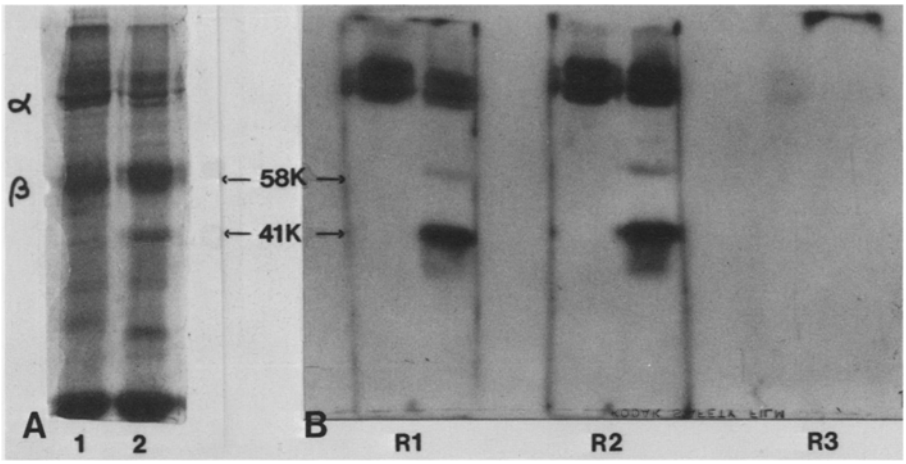

Fig. 7. Antibodies to proteolytic fragments of canine α. (*A*): SDS-PAGE of: (*1*) partially purified canine kidney Na/K-ATPase; (*2*) canine Na/K-ATPase following incubation with KCL and TPCK-trypsin as described in methods. (*B*): Identical samples to those shown in *A* transferred to diazotized paper and tested with unpurified antisera *R1* 8–3, *R2* 8–3 and *R3* 10–3 diluted 1:50

least in the antigenic portions of the subunits. Past studies on cross-reactivities of antibodies with respect to inhibition of Na/K-ATPase activities, ouabain binding, and ion flux have also shown considerable affinity for this system within mammalian Na/K-ATPases but not with highly divergent species [2, 12, 18].

To examine the loci of the cross-reactivity, purified dog kidney Na/K-ATPase was digested with TPCK-trypsin in the presence of K^+, transferrred to DPT paper, and tested with unpurified antisera from each of the three rabbits (Fig. 7). *R*1 and *R*2 antisera reacted negligibly with the 58,000-dalton fragment and strongly with the 41,000-dalton fragment. *R*3 antiserum did not cross-react with the dog Na/K-ATPase at all. None of the antisera cross-reacted with the β subunit. These results suggest that the 41,000-dalton fragment is either more highly conserved than the 58,000-dalton fragment or its antigenicity is less dependent on the degree of denaturation. Since the 41,000-dalton fragment contains the phosphorylation site [4], this region should be highly conserved.

The anti-guinea pig Na/K-ATPase antisera react primarily with the 41,000-dalton fragment of dog Na/K-ATPase. These antibodies could conceivably be used without purification to specifically probe the 41,000-dalton peptide in dog. Exploiting the cross-reactive properties of antibodies may be a preferred alternative to generating fragment specific antibodies.

Again, a component with a higher molecular weight than the subunit reacted with the antisera. The binding to this material was reduced by the trypsin treatment. In addition, *R*3 antiserum did not bind to the high molecular weight component, strongly suggesting that the material is related to the α subunit rather than a contaminant that binds antibodies unrelated to the Na/K-ATPase system.

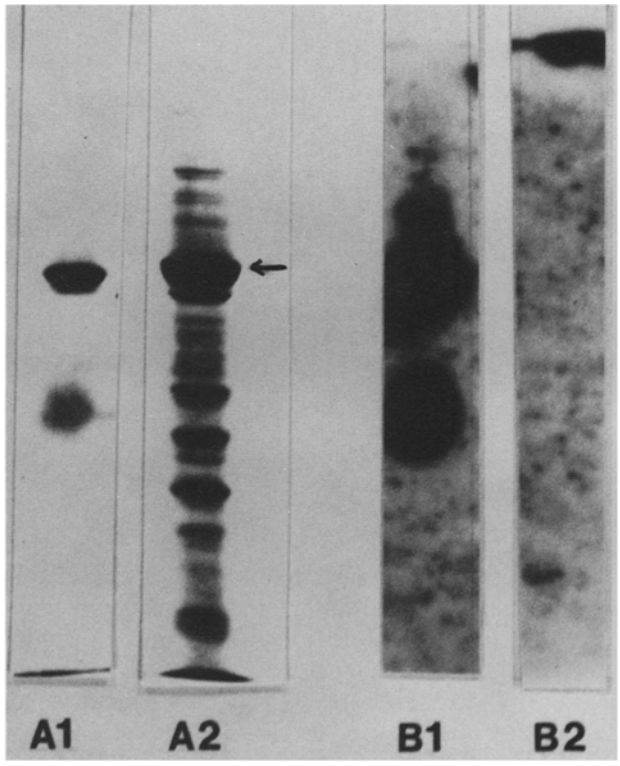

Fig. 8. Cross reactivity of anti-guinea pig Na/K-ATPase antibodies with guinea pig Ca-ATPase. SDS-polyacrylamide gel of: *A1* = purified guinea pig Na/K-ATPase and *A2* = guinea pig Ca-ATPase as the major band at 94,000 daltons. *B1* = duplicate gel (Na/K-ATPase) transferred to diazotized paper, incubated with pooled antisera from *R*1, *R*2, *R*3 diluted 1:100, incubated with ^{125}I-protein A and subjected to autoradiography. *B2* = duplicate gel (Ca-ATPase) treated exactly as *B1*

Cross-reactivity was also studied with Ca-ATPase resident in sarcoplasmic reticulum. Ca-ATPase was chosen for study because of its many similarities to the α-subunit of Na/K-ATPase, including molecular weight, amino acid composition, specific cation-de-

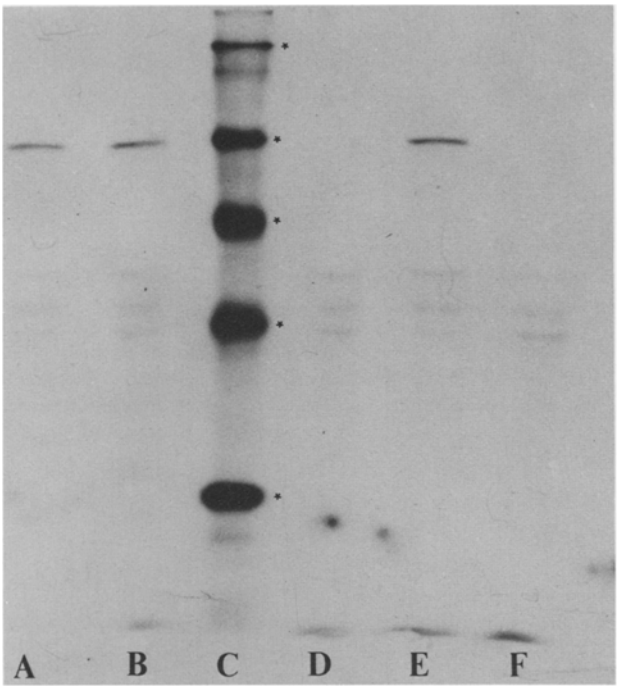

Fig. 9. Immunoprecipitation of cell-free synthesized guinea pig peptides. Cell-free translation was carried out in reticulocyte lysates as described in Materials and Methods. The [^{35}S]-methionine labeled translation products were immunoprecipitated and separated by SDS-PAGE. Peptides in the gel were then made visible by fluorography. Lanes *A*, *B*, and *D* show the immunoprecipitates obtained using *R*1, *R*2, and *R*3 antisera, respectively. *F* is the immunoprecipitate obtained with normal rabbit serum. *E* shows an immunoprecipitation using *R*1 antiserum in the presence of 10 μg of purified guinea pig muscle Ca-ATPase. C contains ^{14}C-labeled molecular weight standards. The molecular weights of the bands marked with an asterisk are, from top to bottom: 200,000, 96,000, 69,000, 46,000 and 30,000

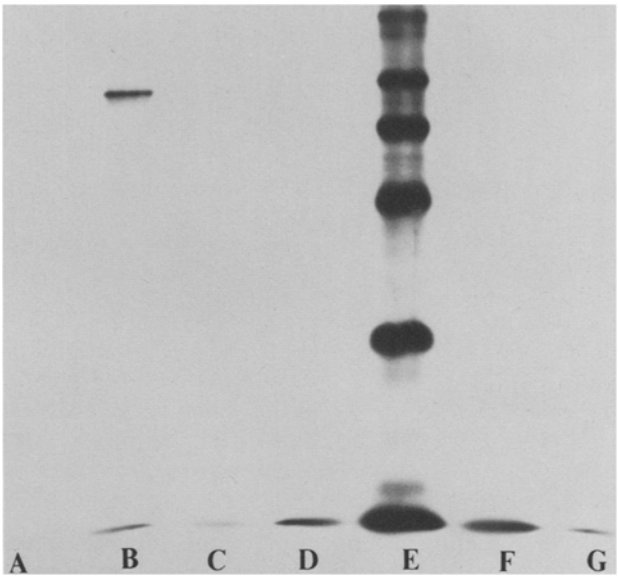

Fig. 10. Immunocompetition of cell-free synthesized 96,000 dalton peptide. Cell-free synthesized peptides in the lysate were immunoprecipitated with either *R*1 antiserum or *R*1 antiserum in the presence of 1 μg purified guinea pig Na/K-ATPase. Lane *A* is the immunoprecipitate using normal rabbit serum; *B*, the immunoprecipitate using *R*1 antiserum; *C*, *D*, *F*, and *G* are immunoprecipitates using *R*1 antiserum in the presence of purified guinea pig Na/K-ATPase. *E* shows the same molecular weight standards as in Fig. 9. Four different highly purified Na/K-ATPase preparations were used in the immunocompetition experiments

pendent formation of a phosphorylated intermediate, Mg dependence, and as an integral membrane protein [11]. Ca-ATPase isolated from guinea pig sarcoplasmic reticulum is the major band in Fig. 8*A*. As can be seen in Fig. 8*B*, there was no cross-reactivity of Ca-ATPase with the anti-Na/K-ATPase antisera, indeed with none of the integral membrane proteins of sarcoplasmic reticulum. Although Ca-ATPase and Na/K-ATPase may have evolved from a common precursor ATPase molecule, they no longer share common antigenic determinants detectable with these antisera.

In vitro Translation of Na/K-ATPase α Subunit mRNA

Total cellular RNA as well as poly(A)$^+$ RNA isolated from either whole guinea pig kidneys or outer medulla was used to direct cell-free translations. The translated peptides were immunoprecipitated with rabbit antisera directed against the Na/K-ATPase holoenzyme. As compared to the pre-immune serum, three antisera (*R*1, *R*2, *R*3) each yielded a specifically immunoprecipitated peptide (by adsorption to Sepharose-protein A, analyzed by SDS-PAGE) (Fig. 9). The molecular weight of the immunoprecipitated band when compared to ^{14}C-labeled molecular weight standards is 96,000. In SDS-PAGE the immunoprecipitated band co-migrates with mature α subunit. Variable quantities of additional bands were occasionally observed, and when present the same bands were invariably seen in tandem control immunoprecipitations with pre-immune serum and therefore are nonspecific precipitates. One of the anti-sera (*R*3) immunoprecipitated approximately 10-fold less of the newly synthesized 96,000-dalton peptide. Although the three antisera contain equivalent titers of antibodies against both subunits of guinea pig Na/K-ATPase, no specifically immunoprecipitated peptides were detected in cell-free translations that would correspond to the expected size of the β subunit peptide (25,000–35,000 daltons).

When purified guinea pig Ca-ATPase was used as an immunocompetitor with *R*1 antiserum, the intensity of the 96,000-dalton band was indistinguishable from control (Fig. 9). As an additional test of the specificity of immunoprecipitation of the Na/K-ATPase peptide, purified guinea pig Na/K-ATPase was added with the antiserum to the lysate after trans-

lation. The highly purified guinea pig Na/K-ATPase completely eliminated the 96,000-dalton immunoprecipitated band under standard conditions of fluorography (1 day exposure, Fig. 10). To verify the ability of guinea pig Na/K-ATPase to compete for the antibodies that bind to the 96,000-dalton product, a variety of purified guinea pig Na/K-ATPases of varying specific activities were used in immunocompetition experiments. The SDS-PAGE profiles of these preparations are all very similar to that in Fig. 1. All of the preparations bound sufficient antibody to eliminate the 96,000-dalton band from the immunoprecipitations (Fig. 10). These results imply that the 96,000-dalton band represents the α subunit.

Antibody Separation

The immunoprecipitated peptide was identified with polyclonal antibodies to both subunits of the Na/K-ATPase. It was necessary therefore to determine if the cell-free translated peptide is a precursor of the α or of the β subunit, in that the 96,000-dalton peptide could conceivably be a precursor of either subunit [23]. To explore this possibility antibodies against each subunit were separated by adsorption to α or β subunits covalently attached to DPT paper. Cell-free translated peptides in the lysate were then adsorbed to the separated antibodies and the immunoprecipitated peptides were analyzed by SDS-PAGE and fluorography. Antibodies against α subunit but not β subunit immunoprecipitated the 96,000-dalton peptide (Fig. 11).

The antibodies produced and characterized as described above are presently being used as specific probes to examine the cell-free synthesis and processing of the subunits and to screen cDNA clones by hybridization-specific cell-free translation. The specificity of the three antisera for various proteolytic fragments of the α and β subunits is also being established in an attempt to define which portions of the enzyme are recognized as antigenic in both the nascent synthesized peptides and the mature enzyme.

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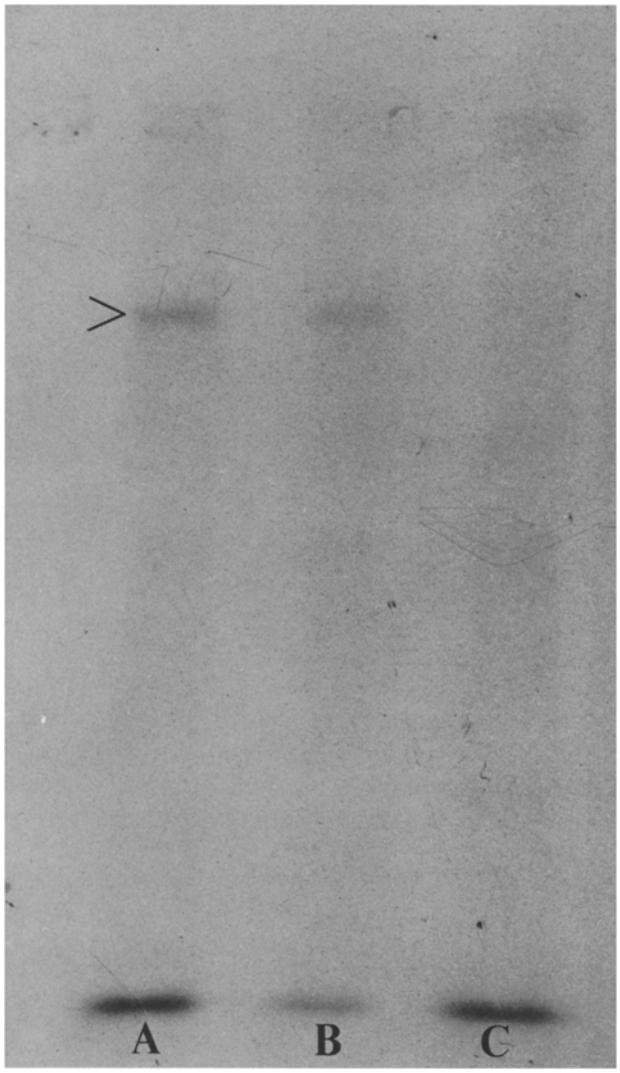

Fig. 11. Immunoprecipitation with purified antibodies. Antibodies against either α or β subunit were separated by adsorption to α or β subunits covalently attached to DPT paper. Cell-free translated peptides in the lysate were then adsorbed to the separated antibodies and the immunoprecipitated peptides were analyzed by SDS-PAGE and fluorography. Lane *A* shows an immunoprecipitation with 0.5 µl of *R*1 antiserum diluted to 9 ml with 0.5 M Tris-HCl (pH 7.0); *B* is an immunoprecipitation with anti-α; *C*, an immunoprecipitation with anti-β. > marks the position of the 96,000-dalton molecular weight standard

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