Immunochemical Evidence for a Transmembrane Orientation of Both the (Na^+,K^+)-ATPase Subunits†

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ABSTRACT: Antibodies were raised against the large catalytic subunit (apparent M_r 96 000) and the glycoprotein (apparent M_r 60 000) of the sodium- and potassium-dependent adenosine triphosphatase [(Na^+,K^+)-ATPase] from *Bufo marinus*. The specificity of each antiserum was assessed by two-dimensional immunoelectrophoresis using toad kidney microsomes or the purified holoenzyme as a source of antigen and by indirect immunoprecipitation of detergent-solubilized (Na^+,K^+)-ATPase subunits from radioiodinated or biosynthetically labeled kidney holoenzyme, microsomes, or postnuclear supernatant. The anticatalytic subunit serum reacted exclusively with a 96 000-dalton protein. The antiserum to the glycoprotein was rendered specific to this subunit by absorption with purified catalytic subunit. The two antisera were agglutinating and lytic in the presence of complement when toad erythrocytes were used as targets, indicating that antigenic determinants of both subunits were exposed on the cell surface. The specfiic reactivities with surface-exposed antigenic determinants of both subunits could be absorbed with toad red blood cells. Such absorbed antisera still reacted with detergent-treated or untreated kidney microsomes, revealing the presence of cytoplasmic and/or intramembranous antigenic sites. Our immunochemical data demonstrate that the glycoprotein subunit of (Na^+,K^+)-ATPase spans the lipid bilayer and confirm the transmembrane orientation of the catalytic subunit postulated from functional studies.

The sodium–potassium-activated adenosine triphosphatase (ATP phosphohydrolase, E.C. 3.6.1.3) which mediates the active transport of Na^+ and K^+ ions across the plasma membrane of animal cells has been isolated from a number of species of at least four classes of vertebrates. In fish, the enzyme was purified from the electric organ of *Electrophorus* (Dixon & Hokin, 1974; Jean et al., 1975) and the rectal gland of *Squalus acanthias* (Hokin et al., 1973; Skou & Esmann, 1979); in amphibian, from kidney of *Bufo marinus* (Geering & Rossier, 1979); in birds, from the nasal salt gland of a duck (*Ana platyrhinchos*) (Hopkins et al., 1976); and in various mammalian species, from the kidney (Jørgensen, 1974), brain (Sweadner, 1979), or heart (Pitts & Schwartz, 1975). In all species studied, the (Na^+,K^+)-ATPase complex consists of at least two subunits. A larger polypeptide (apparent molecular weight ~100 000) termed the catalytic subunit is phosphorylated during turnover by intracellular ATP and participates in the binding of cardiac glycosides (Forbush et al., 1978). This subunit is exposed to the cytoplasm and the cell surface and therefore spans the membrane. A smaller glycoprotein (apparent molecular weight ~55 000) is firmly associated with the cell membrane, but its sidedness has not been determined (Kyte, 1974). More recently, a smaller proteolipid (apparent molecular weight ~12 000) has been shown to bind covalently a photoactivatable derivative of ouabain and thus is believed to be part of the (Na^+,K^+)-ATPase complex (Forbush et al., 1978).

Several investigators have prepared antibodies directed against the holoenzyme or its two subunits in order to analyze the orientation of the enzyme complex in the cell membrane, to relate specific protein domains on each polypeptide to known functions of the enzyme, and to understand ligand-induced conformational changes of the enzyme. From the literature, it is evident that the effects produced by such antibodies on measurable functions of (Na^+,K^+)-ATPase are variable and sometimes contradictory [see review by Lauf (1978)]. These includes studies with anti-holoenzyme antibodies which reveal variable or no effect on ouabain and ATP binding (Askari, 1974; Michael et al., 1977; McCans et al., 1975), phosphorylation of the catalytic subunit from [^{32}P]ATP (Askari, 1974; Jean & Albers, 1976), ATP–ADP exchange (Askari, 1974), and ATP hydrolysis (Askari & Rao, 1972; Koeppsel, 1978; Lane et al., 1973; Smith & Wagner, 1975). Anti-catalytic antibodies decreased ATP hydrolysis (Jean & Albers, 1976) and ouabain binding (Rhee & Hokin, 1979). Anti-glycoprotein antibodies partially inhibited ATP hydrolysis (Jean & Albers, 1976, 1977; Rhee & Hokin, 1974), but not ouabain binding (Rhee & Hokin, 1979). Finally, the effects of many such antibodies, usually anti-holoenzyme antibodies, on the transport of monovalent cations have been extensively studied (Glynn et al., 1974; Koeppsel, 1979; Lauf, 1975; Smith & Wagner, 1975).

Unfortunately, most antibody preparations were insufficiently characterized in terms of their immunochemical specificity. The variable and sometimes conflicting results reported in the literature could be explained by a lack of specificity of an antibody population for a given subunit.

In the present study, we have raised polyclonal antibodies in rabbits to the catalytic and glycoprotein subunits of detergent-purified toad kidney (Na^+,K^+)-ATPase. The specificity of each antiserum was tested by independent immunochemical techniques using detergent-solubilized enzyme at different stages of purification from toad kidney. The sidedness of both subunits was analyzed by serological methods using antibodies directed against various antigenic sites on the two (Na^+,K^+)-ATPase subunits.

Experimental Procedures

Animals. Male or female toads (*Bufo marinus*) were obtained from C.P. Chase, Miami, FL, and kept at 25 °C. Animals were killed by double pithing. New Zealand white female rabbits, 3–4 months old, were used for immunization.

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Reagents. Na_2ATP was obtained from Boehringer Mannheim, Federal Republic of Germany; bovine serum albumin, powder V, was from Armour Pharmaceutical Co., Chicago, IL; Enzymobeads and Bio-Gel A 1.5m were from Bio-Rad Laboratories, Richmond, CA; glucose oxidase (1175 units/mg) and lactoperoxidase (140 units/mg) were from Sigma, St. Louis, MO; Nonidet (NP40) was from Shell Chemie, Zurich, Switzerland; DEAE-cellulose D52 was from Whatman Biochemicals Ltd., Maidstone, England; protein A–Sephadex and agarose LM were from Pharmacia Fine Chemicals, Uppsala, Sweden; $Na^{125}I$ (carrier free) and [^{3}H]leucine (130–190 Ci/mmol) were purchased from New England Nuclear, Boston, MA; $Na_2{}^{51}Cr_2O_7^{2-}$ (1 mCi/mL) was from E.I.R., Würenlingen, Switzerland; Millex GS and cellulose ester filters were from Millipore Corp., Bedford, MA.

Preparation of (Na^+,K^+)-ATPase from Toad Kidney. The preparation of microsomes from toad kidney was performed as described before (Geering & Rossier, 1979). Further purification of the (Na^+,K^+)-ATPase was achieved by a modification of previously described methods (Geering & Rossier, 1979; Jørgensen, 1974). Briefly, microsomes were incubated at a final protein concentration of 1.2 mg/mL in a solution containing 3 mM Na_2ATP, 2 mM EDTA, 20 mM Tris-HCl (pH 7.5), and sodium dodecyl sulfate ($NaDodSO_4$), added with constant stirring to final concentrations of 0.70–0.75 mg/mL. After the preparation stood for 30 min at 20 °C, 6 mL was layered on a discontinuous sucrose gradient consisting of 16 mL of 20% (w/v), 9.6 mL of 15% (w/v), and 6.4 mL of 10% (w/v) sucrose in 20 mM Tris and 1 mM EDTA, pH 7.5. The gradients were centrifuged in an SW 27 rotor in a Sorvall OTD-2 ultracentrifuge at $88600g_{av}$ for 210 min at 4 °C. The resulting pellet was resuspended in 30 mM DL-histidine, 5 mM EDTA, 18 mM Tris, and 200 mM sucrose (pH 7.4) and stored at −80 °C. Specific enzyme activities around 1200 μmol of P_i (mg of protein)$^{-1}$ h^{-1} were routinely attained with this procedure.

Purification of the Catalytic Subunit and the Glycoprotein. (*a*) *$NaDodSO_4$–Polyacrylamide Gel Electrophoresis.* Aliquots of 100 μg of sodium dodecyl sulfate treated enzyme were electrophorized on cylindrical 10% polyacrylamide gels (Geering & Rossier, 1979). Gels were run at 1 mA/gel until the tracking dye had entered the resolving gel and then at 2.5 mA/gel. In order to achieve better resolution of the catalytic and glycoprotein subunits, electrophoresis was continued for another 2 h. The gels were then immediately scanned at 280 nm (Zeiss KM2 chromatogram spectrophotometer), thus enabling the localization of the two enzyme subunits (Figure 1, arrows) which were then cut out of the gel.

(*b*) *Gel Filtration.* The subunits were also obtained by gel filtration of purified (Na^+,K^+)-ATPase on Bio-Gel A 1.5m equilibrated with 0.1 M Tris-HCl buffer, pH 7.4, containing 1% $NaDodSO_4$, 1 mM phenylmethanesulfonyl fluoride (PMSF), and merthiolate (diluted 1:10000) (Hopkins et al., 1976).

Preparation of Antisera. Pieces of gels containing 100 μg of $NaDodSO_4$–polyacrylamide gel electrophoresis purified subunits were homogenized with a Polytron homogenizer in Pi/NaCl containing 0.5% Nonidet P40 and 1 volume of complete Freund's adjuvant. The homogenates containing ~100 μg of antigen were injected subcutaneously (multiple sites) in the back of rabbits at 2-week intervals. Two months later, the rabbits were bled from the ear-marginal vein. The rabbits were further bled each month and boosted 1 week before each bleeding. The antisera were heat inactivated (30 min at 56 °C), filtered through a 0.22-μm Millipore filter, and pooled according to their titer measured by hemagglutination (see below).

Absorption. The anti-glycoprotein serum was absorbed 2 hours at 37 °C and overnight at 4 °C with 500 μg of purified catalytic subunit per mL and centrifuged at $100000g_{av}$ for 20 min at 4 °C. For removal of antibodies reacting with cell surface exposed sites, the antisera were absorbed 2 h at 37 °C and 1 h at 4 °C with washed toad red blood cells at a concentration of 5×10^9 cells/mL. Alternatively, this could also be achieved by absorption with toad serum. Toad blood obtained by cardiac puncture was allowed to coagulate. The serum was then lyophilized, and the powder was added to the antisera in order to minimize dilution by the toad serum. The serum-absorbed antisera were then centrifuged at $100000g_{av}$ for 20 min.

Immunological Methods. (*a*) *Hemagglutination* was performed in 96 well microplates (Costar, Cambridge, MA) by using 200000 washed toad erythrocytes per well and serial 2-fold dilutions in Ringer's solution of the sera directed against the anticatalytic or the -glycoprotein subunit.

(*b*) *Solid-Phase Binding Assay.* The wells of poly(vinyl chloride) microplates (Milian Instruments S.A., Geneva, Switzerland) were filled with 100 μL of toad kidney microsomes (2 mg of protein/mL and 10 mM Tris-HCl, pH 7.4) and incubated for 1 h at room temperature, in order to allow attachment of the membranes to the plastic. The wells were subsequently rinsed 3 times with 100 μL of 0.1 M Tris-HCl, pH 7.4, buffer containing 0.5% bovine serum (Tris–albumin buffer). Antiserum (100 μL) diluted 1:1000 with Tris–albumin buffer supplemented with 0.5% nonimmune or preimmune rabbit serum was added to the wells in triplicates, and the plates were incubated overnight at 37 °C at 100% humidity. The plates were then cooled to 4 °C for 1 h and the wells rinsed 3 times with Tris–albumin buffer. Antiserum (100 μL) containing 100000 cpm of ^{125}I-labeled protein A was added to each well, and the plates were further incubated for 6 h. The wells were rinsed 3 times with 100 μL of Tris–albumin buffer. Each well was cut, and the radioactivity was counted in a Packard γ counter for 2 min.

(*c*) *Complement-Mediated Cytotoxicity.* Cytotoxic tests with ^{51}Cr-labeled erythrocytes from various species as targets were performed according to a modification of the technique previously described (Bron & Gallagher, 1974). In brief, 5×10^6 washed erythrocytes in 200 μL of Ringer's buffer containing 5% fetal calf serum (Ringer's) were incubated with 0.5 mL of $Na_2{}^{51}Cr_2O_7^{2-}$ (1 mCi/mL) for 30 min at 30 °C for amphibian cells or 37 °C for mammal cells. The labeled cells were washed once, suspended in 5 mL of Ringer's, kept for 1 h at 30 °C, and finally washed 3 times with Ringer's. Guinea pig serum absorbed with 1 volume of washed erythrocytes was used as a source of complement. The tests were performed in 4-mL plastic tubes containing 20 μL of various dilutions of antiserum, 20 μL of complement diluted 1:4 with Ringer's, and 10^5 cells in 20 μL of Ringer's. After 90 min of incubation at 30 or 37 °C, 2 mL of cold Ringer's was added, the tubes were centrifuged at 100*g* for 10 min, and the ^{51}Cr released in the supernatant was counted in a Packard γ counter for 5 min. The percent of specific lysis was calculated by using the formula

$$\frac{\text{experimental } {}^{51}\text{Cr release} - \text{spontaneous release} \times 100}{\text{maximal release} - \text{spontaneous release}}$$

(*d*) *Two-dimensional immunoelectrophoresis* (Converse & Papermaster, 1975) was performed as modified by Nigg et al. (1980).

(*e*) *Immunoprecipitation.* The *Staphylococcus aureus* protein A mediated immunoprecipitation procedure (Kessler, 1975) as modified by Maccechini et al. (1979) was used to immunoprecipitate (Na^+,K^+)-ATPase and its subunits from purified (Na^+,K^+)-ATPase, toad kidney microsomes, or postnuclear supernatant.

Labeling Procedure. (*a*) [^3H]*Leucine Incorporation into Kidney Slices.* Toads were killed by pithing and subsequently perfused through a heart puncture with oxygenated Ringer solution (Rossier et al., 1979). The kidneys were removed under sterile conditions, rinsed in serum-free culture medium (Handler et al., 1979), and sliced into small cubes (2 mm a side) with a razer blade. The kidney blocks (~800 mg) were incubated for 20 h at 25 °C with continuous shaking in 15 mL of leucine-free incubation medium containing 120 μCi/mL of [^{3}H]leucine under a 95% O_2 and 5% CO_2 atmosphere. The tissue blocks were then washed 3 times with 20 mL of leucine-free incubation medium and incubated for an additional 4 h with 15 mL of leucine-containing incubation medium. The tissue blocks were then frozen in liquid nitrogen. Postnuclear, microsomal, and detergent-treated fractions were prepared as described elsewhere (Geering & Rossier, 1979).

(*b*) *Radioiodination of Purified* (Na^+,K^+)-*ATPase.* Purified holoenzyme was iodinated by a solid-phase procedure using lactoperoxidase and glucooxidase (Karonen et al., 1975). Prior to iodination, 25–50 μg of purified (Na^+,K^+)-ATPase was solubilized in 50 μL of 100 mM sodium phosphate buffer, pH 7.4, containing 1% $NaDodSO_4$ and then centrifuged at 130000g_{av} for 20 min at 20 °C in order to remove nonsolubilized material. Enzymobead suspension (30 μL), 1 mCi of $Na^{125}I$, and 30 μL of D-glucose (10 mg/mL) were sequentially added. After 25 min at room temperature, 100 μL of 25 mM NaN_3 and 50 μL of 100 mM NaI were used to stop the reaction. Radioiodinated proteins were separated from free iodine by chromatography on Sephadex G25 (14-mL column) equilibrated with 0.5% $NaDodSO_4$ in 0.1 M Tris-HCl, pH 7.4. Incorporation of ^{125}I into proteins was on the order of 5%, and the specific activity for each subunit ranged between 1 and 5 μCi/μg.

Morphological Methods. (*a*) *Negative Staining.* A 10-μL sample of purified (Na^+,K^+)-ATPase from kidney microsomes (200 μg of protein/mL) in 0.1 M Tris-HCl, pH 7.4, mixed with 10 μL of 0.1 M Tris buffer containing bacitracin (300 μg/mL) was applied onto glow discharge treated carbon–formvar-coated copper grids. After 1 min, the grids were stained 15–20 s with 2% phosphotungstic acid adjusted at pH 7.2 with KOH, the droplets drained off, and the grids dried (Deguchi et al., 1977).

(*b*) *Thin Sections.* Purified ATPase (300 μg) was diluted to 4 mL with 25 mM imidazole and 1 mM EDTA, pH 7.5, and centrifuged at 40000 rpm for 60 min. The pellets were fixed with 2% glutaraldehyde, postfixed with 1% OsO_4, dehydrated in alcohol, and embedded in Epon.

(*c*) *Freeze Etching.* Unfixed and fixed ATPase pellets were suspended in 20 μL of imidazole buffer, containing 30% (v/v) glycerol, 1 h before freezing. Droplets were placed on support disks, frozen in Freon 22, cooled to liquid nitrogen temperature, and fractured at −100 °C in a BAF 300 Balzer apparatus. Shadowing was performed with platinum–carbon immediately after fracturing or 10 min later. Replication was completed by evaporation of carbon, and the replicas were cleaned with Clorox and distilled water and collected on carbon–formvar–coated grids.

(*d*) *Morphometric Analysis.* Micrographs were observed in a dissecting microscope at a final magnification of 180000× to measure particle diameters to the nearest 0.1 mm and center to center distances between particles. Particle density was estimated by a combination of particle counts with morphometric estimation of membrane surfaces.

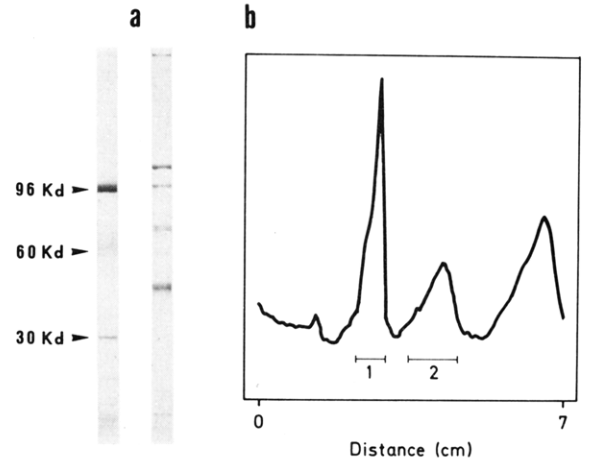

FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoresis pattern of purified toad kidney microsomes (Na^+,K^+)-ATPase. (a) Coomassie blue staining of a 5–13% polyacrylamide gradient slab gel loaded with 50 μg of enzyme. The three main bands (left slot) correspond to apparent molecular weights of 96000, 60000, and 30000. Myosin (M_r 220000), β-galactosidase (M_r 135000), phosphorylase *a* (M_r 96000), bovine serum albumin (M_r 68000), and ovalbumin (M_r 45000) served as protein markers (right slot). (b) Scan at 280 nm of an unstained 10% polyacrylamide cylindric gel containing 100 μg of purified (Na^+,K^+)-ATPase from toad kidney. Bars indicate areas of the catalytic (1) and the glycoprotein (2) subunits which were subsequently cut out of the unstained gel. The peak at the bottom of the gel did not contain proteinaceous material.

Results

Properties of Purified (Na^+,K^+)-*ATPase.* Purified toad kidney (Na^+,K^+)-ATPase exhibited a specific activity around 1200 μmol of P_i (mg of protein)$^{-1}$ h^{-1}. When analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis, the enzyme was resolved into three major bands, with apparent molecular weights of 96000, 60000, and 30000, and smaller molecular weight bands (Figure 1). The 96000-dalton band was identified as the catalytic subunit by its phosphorylation in the presence of [^{32}P]ATP and Na^+ and the 60000-dalton band as the glycoprotein by its staining with periodic acid–Schiff reagent (Geering & Rossier, 1979). The purified enzyme preparation was examined morphologically by transmission electron microscopy, negative staining, and freeze fracturing. The purified (Na^+,K^+)-ATPase appeared as vesicular, flat, or cup-shaped membrane sheets most often with a unit membrane structure and sometimes as a thin electron-opaque line (Figure 2). The diameter of the membrane particles, their center to center distances, and their density were estimated and compared with values published for mammalian (Na^+,K^+)-ATPase (Deguchi et al., 1977). On a negatively stained preparation, the overall frequency of particles was asymmetrical with respect to the two aspects of the membrane. On one face, the density was 2.5-fold higher than that reported for rabbit (Na^+,K^+)-ATPase, although the particle diameter and the center to center distance were only slightly larger (1.2-fold) (Table I, Figure 3). The other face exhibited a smaller overall frequency, which was evident on folding of some membrane, exposing simultaneously both membrane surfaces. Estimation of intramembranous particle density obtained by freeze fracturing purified (Na^+,K^+)-ATPase (Figure 4) was also asymmetrical with respect to the two fracture faces, and the overall frequency was similar to that of the mammalian

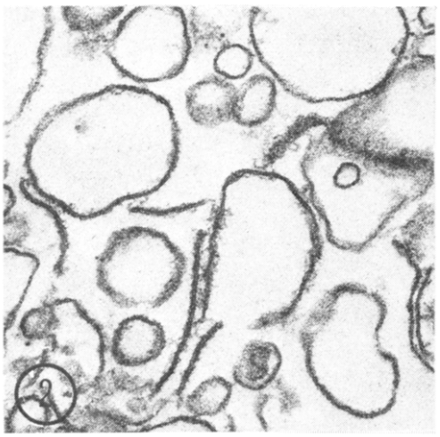

FIGURE 2: Thin section of purified (Na^+,K^+)-ATPase from toad kidney, which appears as vesicular, cup-shaped, or flat membranes. Most membranes are triple layered; some appear as a single electron-opaque line. Magnification, 44 500×.

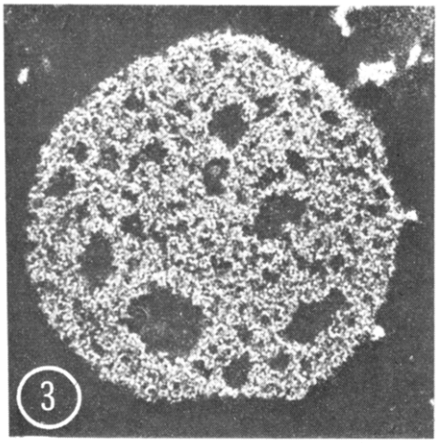

FIGURE 3: Negative staining of purified (Na^+,K^+)-ATPase. On this surface, the particles are uniformly distributed with an overall frequency of ~29 000 particles/μm^2. Magnification, 90 000×.

Table I: Morphometry of Particles in (Na^+,K^+)-ATPase Preparations Purified from Toad Kidney[a]

		freeze fracture	
	negative staining	A face	B face
diameter (nm)	3.2 ± 0.4 (281)	9.9 ± 1.8 (128)	9.8 ± 2.2 (85)
center to center distance (nm)	5.4 ± 0.9 (254)	9.95 ± 2.2 (128)	ND[b]
density (particles/μm^2)	29500 ± 2150 (28)	3852 ± 380 (10)	420 ± 85 (10)

[a] Results represent mean values ± standard error of the means with the number of independent measurements in parentheses. [b] ND = not determined.

enzyme complex (Deguchi et al., 1977). Since no attempt to orientate the membrane was made, we cannot correlate the fracture faces to the recommended nomenclature (E and P faces).

Specificity of the Antisera. The activity of each antiserum, i.e., anti-catalytic subunit and anti-glycoprotein sera, was tested by two-dimensional immunoelectrophoresis using as antigens either purified (Na^+,K^+)-ATPase or detergent-solubilized kidney microsomal proteins resolved on $NaDodSO_4$–polyacrylamide gel electrophoresis. In order to further characterize the specificity of each antiserum, biosynthetically labeled or radioiodinated kidney postnuclear supernatant, microsomes, or purified holoenzyme was solubilized in $NaDodSO_4$, incubated with each antiserum, and precipitated with a suspension of *Staphylococcus aureus*. The immune precipitates were then resolved on $NaDodSO_4$–polyacrylamide gel electrophoresis and analyzed by autoradiography.

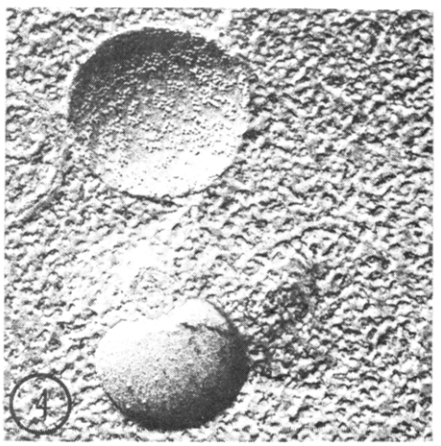

FIGURE 4: Purified (Na^+,K^+)-ATPase membranes fractured with a cryoprotectan (Glycerol) and no fixative. The A face (top) exhibits higher intramembranous particle density than that on the B face (bottom), where the density is much lower. Magnification, 53 500×.

The anti-catalytic subunit serum reacted exclusively with the catalytic subunit. When toad kidney microsomes were resolved on $NaDodSO_4$–polyacrylamide gel electrophoresis and then electrophoresed onto agarose containing the anti-catalytic serum, a rocket corresponding to a 96 000-dalton protein was observed (Figure 5a). The same antiserum precipitated from the postnuclear supernatant of [^{3}H]leucine-labeled toad kidney (Figure 6, lane 1) one major protein, which migrated on $NaDodSO_4$–polyacrylamide gel electrophoresis as a 96 000-dalton band (Figure 6, lane 2). From biosynthetically labeled holoenzyme (Figure 6, lane 3), only the large catalytic subunit precipitated, and the band comigrated with the 96 000-dalton band as seen in lane 2 (Figure 6). In contrast, the same anti-catalytic serum precipitated three major bands with apparent molecular weights of 96 000, 60 000, and 38 000 (Figure 6, lane 7) from radioiodinated purified holoenzyme (Figure 6, lane 6). No material was precipitated with preimmune serum.

The anti-glycoprotein serum reacted mainly with a 60 000-dalton protein when tested against kidney microsomes resolved on $NaDodSO_4$–polyacrylamide gel electrophoresis (data not shown). When purified (Na^+,K^+)-ATPase was used as an antigen, the presence of contaminating antibodies to the catalytic subunit could clearly be detected (Figure 5c). Such antibodies were removed by absorption with purified catalytic subunit as illustrated in Figure 5d. Such an absorbed anti-glycoprotein was systematically used in all subsequent assays. The anti-glycoprotein serum precipitated from biosynthetically labeled (Na^+,K^+)-ATPase a single protein comigrating with the 60 000-dalton glycoprotein (Figure 6, lanes 5 and 9). Preimmune serum did not precipitate any material (Figure 6, lane 10).

The two anti-catalytic and anti-glycoprotein sera were also tested against toad plasma or serum by immunoelectrophoresis (Figure 7). The anti-catalytic subunit or the glycoprotein sera formed a similar precipitation line against toad serum and to a lesser extent against plasma, suggesting the presence of (Na^+,K^+)-ATPase antigenic determinants in serum or plasma. This is supported by the disappearance of the precipitation lines when the two antisera were absorbed with purified holoenzyme or the corresponding subunits. Interestingly, the lines also disappeared when the antisera were adsorbed with washed toad

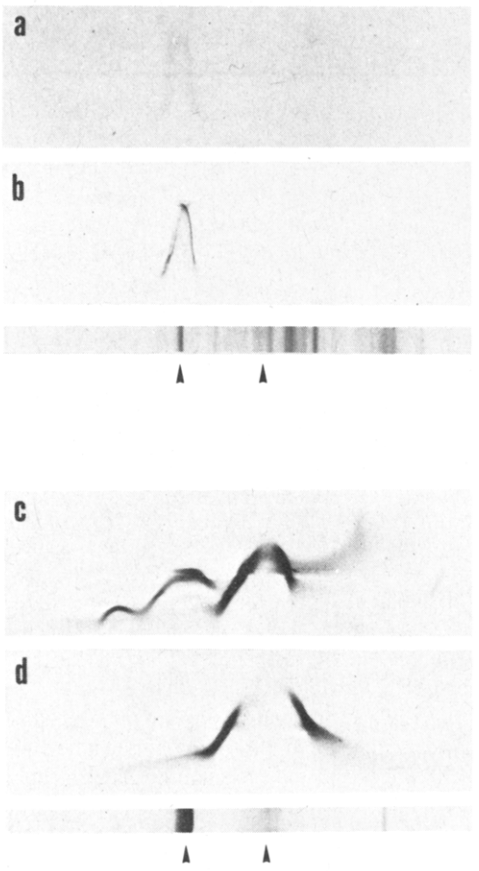

FIGURE 5: Characterization of anti-catalytic subunit and anti-glycoprotein serum by crossed $NaDodSO_4$–polyacrylamide immunoelectrophoresis. $NaDodSO_4$-solubilized kidney microsomes (30–100 μg) (50 μmol of P_i mg^{-1} h^{-1}) (a and b) or 75 μg of purified holoenzyme (c and d), was used as a source of antigen and run in the first dimension of a 5–13% $NaDodSO_4$–polyacrylamide gel (lower panel). The position of the enzyme subunits is indicated by arrows. (a) The anti-catalytic subunit serum was diluted 5-fold in 1% agarose containing 200 mM Tris-HCl, 40 mM acetate, and 4 mM EDTA, pH 7.4. A single rocket is generated from kidney microsome corresponding to the apparent molecular weight of the catalytic subunit. (b) Absorption of the anti-catalytic subunit with toad erythrocytes (5×10^9 cells/mL) or lyophilized toad serum (3–4 volumes/mL) did not change the pattern seen in (a). (c) Two-dimensional electrophoresis with the anti-glycoprotein serum using purified holoenzyme as a source of antigen. A strong precipitation line is formed with the 60 000-dalton protein; reaction with the 96 000-dalton protein, although less intense, can be clearly visualized, indicating the presence of contaminating anti-catalytic subunit antibodies in the antiserum. Due to the high concentration of antigen (75 μg) applied on the first dimensional gel, aggregation of the catalytic subunit occurred which results in an additional small rocket. (d) Same conditions as in (c) with anti-glycoprotein serum absorbed with the catalytic subunit (400 μg/mL of antiserum). The strong precipitation rocket with the 60 000-dalton glycoprotein subunit is still present, but the precipitation lines with the catalytic subunit disappeared, indicating that the contaminating antibody has been efficiently removed. A single rocket was also seen when kidney microsomes were resolved on the first dimensional gel or when the anti-glycoprotien was absorbed with toad red blood cells or toad serum as in (b).

erythrocytes, indicating that the antigenic determinants present in serum are cell surface exposed sites as will be discussed later. Finally, the lines were not observed when the antisera were absorbed with toad serum (20% v/v).

Sidedness of the (Na^+,K^+)-ATPase Subunits in the Lipid Bilayer. (a) Antibodies Directed against Cell-Surface Antigenic Sites. In order to detect antigenic determinants of both the catalytic and glycoprotein subunits exposed at the cell surface, intact toad red blood cells were used as targets, since it has been established that (Na^+,K^+)-ATPase is associated

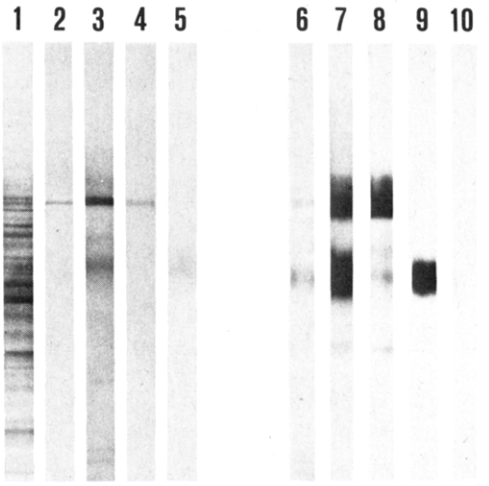

FIGURE 6: Immunoprecipitation of the (Na^+,K^+)-ATPase subunits by the corresponding antisera from biosynthetically labeled kidney microsomes (lanes 1 and 2), holoenzyme (lanes 3–5), or radioiodinated holoenzyme (lanes 6–10). [^{3}H]Leucine was incorporated into kidney slices, and postnuclear supernatant, microsomal, or holoenzyme fractions were prepared as described under Experimental Procedures. $NaDodSO_4$-solubilized (Na^+,K^+)-ATPase was radioiodinated, and 2 μCi of labeled enzyme was incubated with each antiserum. The immunoprecipitates from biosynthetically labeled or radioiodinated material were resolved on a 5–13% $NaDodSO_4$–polyacrylamide gel electrophoresis gradient. The gels were dried and fluorographed or autoradiographed at −80 °C on preactivated Kodak Royal-X-O-Mat films. Intensifying screens were used with radioiodinated material. (Lane 1) [^{3}H]Leucine-labeled postnuclear supernatant. (Lane 2) A single band of apparent M_r 96 000 immunoprecipitated by the anti-catalytic subunit serum; absorption of the antiserum with toad erythrocytes or toad serum (see Figure 5b) did not alter the immunoprecipitation pattern. (Lane 3) Biosynthetically labeled purified holoenzyme is resolved into 96 000-, 60 000-, and 30 000-dalton bands. (Lane 4) Immunoprecipitation of biosynthetically labeled enzyme with nonabsorbed anti-catalytic subunit. (Lane 5) Immunoprecipitation of biosynthetically labeled enzyme with anti-glycoprotein (absorbed with the catalytic subunit). (Lane 6) ^{125}I-labeled (Na^+,K^+)-ATPase is resolved into 96 000-, 60 000-, and 30 000-dalton bands. (Lane 7) Immunoprecipitation with the nonabsorbed anti-catalytic subunit serum generated the 96 000-dalton band and two additional 60 000- and 38 000-dalton bands when compared to lanes 2 or 4. The 60 000-dalton band almost completely disappeared when the antiserum was absorbed with red blood cells or toad serum as shown in lane 8. A single 60 000-dalton band was precipitated by the absorbed and the unabsorbed anti-glycoprotein serum as illustrated in lane 9. (Lane 10) No bands can be seen with preimmune serum using biosynthetically or radioiodinated antigen.

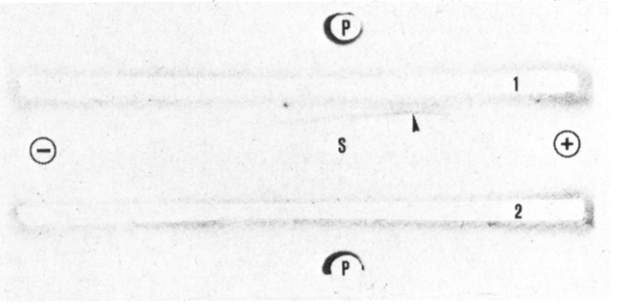

FIGURE 7: Immunoelectrophoresis of anti-catalytic subunit serum tested against toad plasma (wells P) or toad serum (well S). The anti-catalytic subunit serum (trough 1) formed a precipitation line with a β mobility when tested against toad serum (arrow). The line was weaker with toad plasma. The line disappeared upon absorption of the antiserum with purified catalytic subunit, purified holoenzyme (trough 2), toad erythrocytes, or toad serum. The immunoelectrophoretic pattern was identical with that for anti-glycoprotein serum.

with erythrocytes, although at a much lower density than on other cell types (Lauf, 1975). Both the anti-catalytic subunit and the anti-glycoprotein sera were agglutinating at dilution

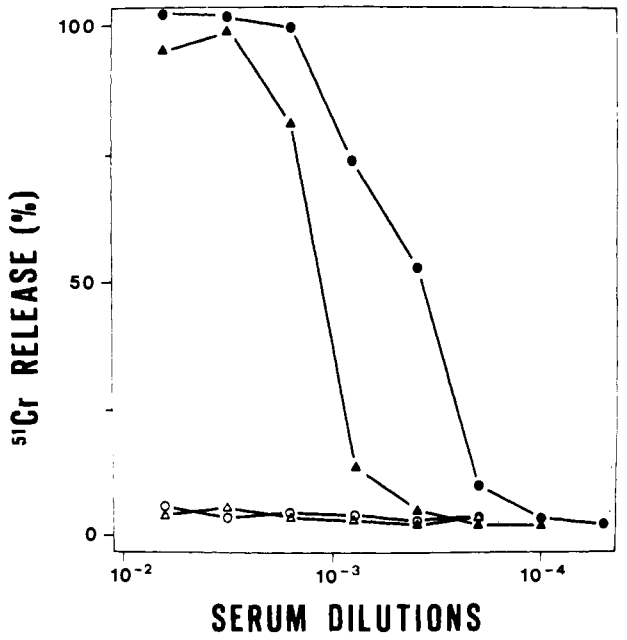

FIGURE 8: Complement-mediated cytotoxicity with ^{51}Cr-labeled toad red blood cells from *Bufo marinus* and anti-catalytic serum (●), anti-catalytic serum absorbed with washed toad red blood cells (○), anti-glycoprotein serum (▲), or toad erythrocyte absorbed anti-glycoprotein serum (△). The anti-catalytic serum was lytic up to 1:640 and the anti-glycoprotein up to 1:320 with *Bufo marinus* red blood cells. Preimmune sera were not lytic.

up to 1:1000 when 5×10^5 erythrocytes were incubated per well. In the presence of complement, 100% of toad erythrocytes were lysed up to 1:400 dilutions with both antisera (Figure 7). Similar experiments were carried out with *Bufo bufo*, *Xenopus laevis*, *Rana ridibunda*, rat, and human red blood cells. Cells from *Bufo bufo* and *Xenopus laevis* were lysed with the anti-catalytic serum at lower dilutions (1:100) whereas the anti-glycoprotein serum was lytic only for *Bufo bufo* erythrocytes. Neither antiserum lysed *Rana ridibunda*, rat, or human red blood cells (Girardet et al., 1981).

(*b*) *Antibodies Directed against Cytoplasmic Exposed Antigenic Sites.* Absorption of the anti-catalytic subunit and the glycoprotein sera with washed toad red blood cells completely abolished the hemagglutinating activity of each antiserum, as well as the complement-mediated cytotoxicity (Figure 8). This indicated that the absorption procedure efficiently removed antibodies reacting with cell surface exposed sites. Interestingly, the two absorbed antisera still reacted with the corresponding detergent-solubilized (Na^+,K^+)-ATPase subunits from purified holoenzyme or kidney microsomes and generated identical rockets by two-dimensional immunoelectrophoresis. In contrast, immunoprecipitation of radioiodinated holoenzyme using absorbed anti-catalytic serum resulted in a reduced precipitability of the 60 000-dalton band but no significant change in the precipitation of the 96 000- and the 38 000-dalton bands (Figure 6, lane 8). When scanned, the residual density over the 60 000-dalton region using absorbed anti-catalytic serum represented 10% of the density observed with the unabsorbed serum (Figure 7, lanes 7 and 8). Since a single 96 000-dalton protein was immunoprecipitated from biosynthetically labeled enzyme by the anti-catalytic serum, but two additional 38 000- and 60 000-dalton bands were recovered from radioiodinated enzyme by using the same antiserum, cleavage of the catalytic subunit must occur during radioiodination, yielding the two smaller peptides. In addition, in view of the significant decrease in the precipitability of the 60 000-dalton fragment when absorbed anti-catalytic subunit was used, we conclude that this fragment carries the cell surface exposed antigenic sites. In order to rule out the possibility that antibodies from antisera absorbed with toad erythrocytes react with new antigenic sites exposed as a result of detergent treatment which is required for immunoprecipitation or two-dimensional electrophoresis, we decided to analyze the interaction of toad erythrocyte-absorbed antisera with native holoenzyme present in kidney microsomes. For this purpose, a solid-phase binding assay was developed in which untreated kidney microsomes noncovalently adsorbed at the surface of plastic wells were allowed to react with toad erythrocyte-absorbed and -nonabsorbed antisera diluted 1:1000. After the reacting antibodies were washed, they were revealed with radioiodinated protein A. As shown in Table II, the unabsorbed antisera were able to react with (Na^+,K^+)-ATPase contained in kidney microsomes. Upon absorption with washed toad red blood cells, 70% of the binding activity of the anticatalytic subunit serum was retained, and more than 60% for the anti-glycoprotein serum (Table II).

Table II: Binding Assay on *Bufo marinus* Kidney Microsomes[a]

serum	unabsorbed	absorbed with *Bufo marinus* erythrocytes
anti-catalytic	20 190 ± 1 340 (4)	16 346 ± 1 546 (4)
anti-glycoprotein	31 995 ± 1 760 (4)	20 456 ± 1 125 (4)

[a] Results are expressed as specific counts per minute (total − nonspecific counts per minute) per well and represent mean values ± standard error of the mean with the number of measurements in parentheses. The nonspecific binding (preimmune sera) was 2450 ± 680 cpm (6).

Discussion

Specific probes for the (Na^+,K^+)-dependent ATPase, such as antibodies, have been widely used to study the relationship between various domains on the enzyme subunits and the expression of defined functions of the enzyme (Jean & Albers, 1977; Rhee & Hokin, 1979), ligand-induced conformational changes (Glynn et al., 1974; Koepsell, 1979; Lauf, 1975; Smith & Wagner, 1975), or the topographical distribution of the enzyme complex on cells in fixed tissues (Kyte, 1976a,b). In most studies, however, no attempts were made to characterize the reacting antibodies in terms of their immunological specificities. The use of reagents insufficiently characterized may explain the variable and sometimes contradicting results reported in the literature. In addition, the distribution of the enzyme subunits by using such probes has not been studied on viable cells or tissue, because antibodies reacting with cell surface exposed sites of (Na^+,K^+)-ATPase were not characterized. In this study, polyclonal antibodies were raised in rabbits against the (Na^+,K^+)-ATPase catalytic and glycoprotein subunits purified from toad kidney (*Bufo marinus*). The specificity of each antiserum was assessed by cross-immunoelectrophoresis and indirect immunoprecipitation using radioiodinated or biosynthetically labeled antigens from kidney fractions or purified holoenzyme. The two monospecific antisera served to analyze the sidedness of each enzyme subunit in the lipid bilayer by using serological methods.

The specificity of the *anti-catalytic subunit serum* for the large catalytic subunit, identified by its phosphorylation in the presence of [^{32}P]ATP, was clearly demonstrated by the single precipitation rocket obtained with detergent-solubilized kidney microsomes (Figure 6a). In addition, a single band, comigrating with the catalytic subunit, was immunoprecipitated from the biosynthetically labeled kidney postnuclear supernatant (Figure 7, lane 2) or the purified holoenzyme (Figure

7, lane 4). In contrast, two additional bands were recovered from radioiodinated holoenzyme (Figure 7, lane 6) which migrated with apparent molecular weights of 60 000 and 38 000. These two fragments were generated by proteolytic cleavage of the catalytic subunit which occurred in the absence of protease inhibitors. Such inhibitors minimized the degradation of the catalytic subunit, but they could not be used because they interfere with enzyme-mediated iodination. The proteolytic generation of the two fragments is consistent with other workers' description of a 60 000-dalton fragment of the catalytic subunit, comigrating with the glycoprotein (Esmann et al., 1979), and the recently reported proteolytic fragmentation of photoaffinity-labeled (Na^+,K^+)-ATPase into two components of 58 000 and 41 000 apparent molecular weights (Farley et al., 1980).

The *anti-glycoprotein serum* reacted mainly with a 60 000-dalton protein identified as a glycoprotein by its staining with the periodic acid–Schiff reagent (Geering & Rossier, 1979). Some cross-reaction occurred, however, with the catalytic subunit (Figure 6c). Since cleavage of the catalytic subunit into a 60 000-dalton fragment can occur upon detergent solubilization of the enzyme in the absence of protease inhibitor, one would expect such a fragment to copurify with the glycoprotein by elution from $NaDodSO_4$–polyacrylamide gel electrophoresis. It therefore would also elicit antibodies to the catalytic subunit as well as to the glycoprotein. The presence of such contaminating antibodies in anti-glycoprotein sera could explain the various effects on ouabain binding reported in the literature (Rhee & Hokin, 1974, 1979). Importantly, absorption of the anti-glycoprotein serum with purified catalytic subunit removed the contaminating antibodies (Figure 6d) without altering the properties of the remaining anti-glycoprotein antibodies.

Evidence for antibodies present in both antisera reacting with externally exposed cell surface antigenic sites of (Na^+,K^+)-ATPase came from the hemagglutination and the complement-mediated cytotoxicity experiments using red blood cells as targets. These antibodies appear to be useful probes to study the topographical distribution of (Na^+,K^+)-ATPase on viable cells, since they can label the plasma membrane of intact cells.[1] The two antisera also agglutinated or lysed in the presence of complement red blood cells from *Bufo marinus* and *Bufo bufo*, but not from *Rana ridibunda*, rabbit, rat, or human. Cells from *Xenopus laevis* were lysed by the anti-catalytic subunit, but not by the anti-glycoprotein serum.

Phylogenically, the surface-exposed antigenic determinants of the enzyme complex appear to be restricted to closely related species. In vertebrates, the functional domains of (Na^+,K^+)-ATPase seem to be highly conserved as reflected by the great similarity in the half-maximum stimulation constants for sodium and potassium and the affinity constant for ATP, whereas great variations exist in the inhibition constant for ouabain (from 50 μM in the toad to 0.5 μM in the rabbit) (Geering & Rossier, 1979), which binds to a cell-surface domain of the catalytic subunit. Therefore, it is possible that cytoplasmic domains of the enzyme are more conserved than are surface-exposed sites. This remains to be documented, and experiments are currently under way to test cross-reactivities of antibodies with cytoplasmic-oriented antigenic sites from various species. The cell surface reacting antibodies were removed by absorption with toad red blood cells. The large amount of erythrocytes, i.e., 5×10^9 cells/mL of antiserum, required to abolish hemagglutination and complement-mediated cytotoxicity of both antisera can be expected from the low enzyme activity measured in toad erythrocyte membranes [specific activity 0.2–0.5 μmol (mg of protein)$^{-1}$ h^{-1}]. Surprisingly, absorption of hemagglutinating and cytolytic antibodies was also achieved by using large amounts of toad serum, suggesting the presence in serum of (Na^+,K^+)-ATPase antigenic determinants. This was confirmed by immunoelectrophoresis of toad serum. The precipitation line formed between toad serum and the anti-subunit antisera disappeared when each antiserum was absorbed with the corresponding subunit. Furthermore, the line also disappeared with toad erythrocyte absorbed antisera, indicating that the antigenic sites present in toad serum were similar to the cell surface exposed sites of (Na^+,K^+)-ATPase. The two antisera reacted to a lesser extent with ultracentrifuged toad plasma. Taken together, these observations suggest that small vesicles which carry at their surface (Na^+,K^+)-ATPase antigenic determinants are present in serum. These vesicles might be generated from platelets during the clotting process (Warren & Vales, 1972).

The antibody population left after absorption with toad erythrocyte or serum was still able to interact with detergent-solubilized (Na^+,K^+)-ATPase (Figures 6 and 7). This could result from exposure of new antigenic sites on the cell surface exposed domains of each subunit consecutive to detergent treatment required for cross-immunoelectrophoresis or indirect immunoprecipitation. This is unlikely, however, since the absorbed anti-catalytic serum did not immunoprecipitate the 60 000-dalton fragment from radioiodinated enzyme but only the intact catalytic subunit and a smaller 38 000-dalton fragment. The significant decrease in the precipitability of the 60 000-dalton fragment with absorbed anti-catalytic subunit indicates that the fragment carries the cell surface exposed antigenic determinants mediating agglutination and cytolysis and not new antigenic sites exposed by $NaDodSO_4$ treatment. To test whether the absorbed antisera against both subunits were still able to recognize sites on the native holoenzyme, we developed a binding assay in which antibodies were allowed to interact with immobilized kidney microsome. The binding capacity of each antiserum was decreased by about one-half following removal of the hemagglutinating and cytolytic antibodies. We interpret these results as an indication for the presence of at least two antibody populations, one reacting with cell surface exposed sites and another with cytoplasmic-oriented domains of both (Na^+,K^+)-ATPase subunits.

In conclusion, we have shown immunochemically that the two (Na^+,K^+)-ATPase subunits span the lipid bilayer. Thus, the glycoprotein becomes a potential constituent of transmembrane channels required for the translocation of the monovalent cations across the lipid bilayer. Such a functional role for the glycoprotein has already been suggested by others (Lo & Titus, 1978; Zaheer et al., 1981). Further evidence for a transmembrane orientation of the glycoprotein subunit has been provided by two recent reports. Montecucco et al. (1981) have shown that the hydrophobic domains of the catalytic and glycoprotein subunits were labeled by using photoreactive phosphatidylcholines and amphiphatic photoreactive derivatives of glycosamine and glycine. Spector and his co-workers (Spector et al., 1981) have demonstrated the phosphorylation of the glycoprotein subunit by membrane-bound kinases in Ehrlich ascites tumor cells.

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