Biochimica et Biophysica Acta, 730 (1983) 327–341
Elsevier Biomedical Press

BBA 71615

LACTOPEROXIDASE-CATALYZED IODINATION OF SODIUM AND POTASSIUM ION-ACTIVATED ADENOSINE TRIPHOSPHATASE IN THE MADIN-DARBY CANINE KIDNEY EPITHELIAL CELL LINE AND CANINE RENAL MEMBRANES

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(Received October 11th, 1982)
(Revised manuscript received January 17th, 1983)

Key words: ($Na^+ + K^+$)-ATPase; Iodination; Membrane-protein orientation; Subunit asymmetry; (MDCK cell)

Experiments are described in which the large chain of ($Na^+ + K^+$)-ATPase is labeled by lactoperoxidase-catalyzed iodination either at its extracytoplasmic surface exclusively or at both its extracytoplasmic and its cytoplasmic surfaces simultaneously. The former was accomplished by labeling intact cells of the Madin-Darby canine kidney line, and the latter by labeling open membrane vesicles, also from canine kidney. A comparison of the specific radioactivities for the large chain from the open membranes and the large chain from the Madin-Darby canine kidney cells reveals that the former was labeled approximately 5-fold more extensively. This indicates that the large chain of ($Na^+ + K^+$)-ATPase is situated in the membrane such that more of its mass protrudes into the cytoplasm than into the extracytoplasmic environment.

Introduction

Sodium and potassium ion-activated adenosine triphosphatase (($Na^+ + K^+$)-ATPase) is a membrane-bound enzyme which actively transports sodium ions out of and potassium into the cell with concomitant hydrolysis of ATP. ($Na^+ + K^+$)-ATPase has been purified to homogeneity from several diverse sources [1–5]. The purified complex has been reconstituted into closed membrane vesicles and shown to transport Na^+ and K^+ actively [6], a fact which demonstrates that the purified enzyme contains all of the components necessary for transport function. In all known cases, the enzyme has been shown to be a molecular complex of two polypeptide chains, present in equimolar amounts in at least the canine complex [7]. The smaller chain of the enzyme is a sialoglycoprotein, about 510 residues in length [7], of unknown function. The larger chain, about 1100 residues in length [7], is phosphorylated specifically during the turnover of the enzyme [8] and contains the amino acid side chains which surround the cardiac glycoside binding site [9]. Two other very closely related, mammalian active transport enzymes, proton and potassium ion-activated

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Abbreviations: ($Na^+ + K^+$)-ATPase, sodium and potassium ion-activated adenosine triphosphatase; ($H^+ + K^+$)-ATPase, proton and potassium ion-activated adenosine triphosphatase; Ca^{2+}-ATPase, calcium ion-activated adenosine triphosphatase; MDCK, Madin-Darby canine kidney; Gn·Cl guanidinium chloride; SDS, sodium dodecyl sulfate; phosphate-buffered saline, 0.15 M NaCl, 0.1 mM EDTA, 20 mM sodium phosphate, pH 7.4; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Abs-4B, Sepharose 4B to which antibodies from antisera raised against large chain of ($Na^+ + K^+$)-ATPase have been attached; Gn·Cl pool, peak of radioactivity from guanidinium chloride elution of immunoadsorbent.

adenosine triphosphatase ($(H^+ + K^+)$-ATPase) from gastric mucosa [10], which also has a phosphorylated intermediate [11], and skeletal muscle calcium ion-activated adenosine triphosphatase (Ca^{2+}-ATPase) [12], which possesses a three residue amino acid sequence around the phosphorylation site identical to that in the large chain of $(Na^+ + K^+)$-ATPase [13], both contain only large chains. Therefore, only the large chains of $(Na^+ + K^+)$-ATPase may be necessary for the catalysis of active transport.

The vectorial nature of the cation transport across the cell membrane catalyzed by $(Na^+ + K^+)$-ATPase illustrates the enzyme's functional asymmetry which, presumably, results from a structural asymmetry. In this regard, electron microscopic investigations have shown that anti-large chain antibody binds only to the cytoplasmic surface of membrane vesicles containing $(Na^+ + K^+)$-ATPase [14], and from this it was concluded that all large chain molecules are inserted into the cell membrane oriented in the same direction. In addition, the ability to bind antibody from the cytoplasmic surface and strophanthidin from the extracytoplasmic surface simultaneously demonstrates that the large chain of $(Na^+ + K^+)$-ATPase spans the plasma membrane [15].

So far, structural studies on $(Na^+ + K^+)$-ATPase have focused primarily on the question of whether the active, native enzyme is oligomeric [7,15–17]. Information concerning the disposition of the enzyme relative to the plane of the membrane has not been reported *. The purpose of these experiments was to quantify the extent to which the large chain of $(Na^+ + K^+)$-ATPase was iodinated in intact Madin-Darby canine kidney (MDCK) cells and open membranes also from canine kidney. Since the large chain in the former preparation has been labeled only on its extracytoplasmic surface, and that in the latter, on both its cytoplasmic and extracytoplasmic surfaces, and since both had been labeled under identical circumstances, a comparison of their specific radioactivities provides an indication of the distribution of the enzyme's mass across the plane of the membrane.

* Subsequent to submission of this manuscript, two articles have appeared which address this general question using different methodology (Jørgensen, P.L., Karlish, S.J.D. and Gilter, C. (1982) J. Biol. Chem. 257, 7435–7442; O'Connell, M.A. (1982) Biochemistry 21, 5984–5991).

Experimental procedures

Materials

Chemicals. Sepharose 4B and Sephadex G-150 were purchased from Pharmacia Fine Chemicals; Dowex 1-X2, from Bio-Rad; acrylamide and *N,N'*-methylenebisacrylamide, from Eastman; guanidinium chloride of highest purity, from Heico; sodium dodecyl sulfate (SDS) and type V glucose oxidase, from Sigma; bovine serum albumin, from Pentex; purified grade lactoperoxidase, from Calbiochem; aquasol, [^{14}C]formaldehyde (52 mCi · mmol^{-1}), and Na^{125}I (17 Ci · mg^{-1} in the low pH, high concentration form), from New England Nuclear; and Freund's complete adjuvant, from Difco Incorporated. Amberlite XAD4 was a gift to Dr. Jack Kyte, Department of Chemistry, University of California, San Diego, from Rohm and Haas Corporation.

MDCK cells. Cells and supplies for cell culture were generously provided by Dr. Milton H. Saier, Jr., Department of Biology, University of California, San Diego. Trypsin, Dulbecco's modified Eagle's medium, fetal calf and horse serum, were obtained from the Grand Biochemical Company. Plastic tissue culture ware was from LUX Scientific.

Methods

Maintenance of cells. Cells were maintained as monolayers in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, or 2.5% fetal calf + 7.5% horse serum, as well as $1.9 \cdot 10^2$ IU · ml^{-1} of penicillin, 0.2 mg · ml^{-1} of streptomycin and 25 μg · ml^{-1} of ampicillin, in a 5% CO_2 incubator at 37°C [18]. Petri dishes of 150 mm diameter were routinely used. Cell density in suspensions was determined with a hemacytometer. Cells were plated at a density of $3.6 \cdot 10^5$ cells per plate. Under these conditions, the doubling time is approx. 24 h and time to confluency generally seven days with one change in medium on day four. On day six, when cells were harvested, the density was about $2 \cdot 10^5$ cells · cm^{-2}.

Enzyme assays. $(Na^+ + K^+)$-ATPase activity was assayed according to Kyte [1]. Lactoperoxidase

and glucose oxidase activities were measured according to Hubbard and Cohn [19]. One unit (U) of glucose oxidase or lactoperoxidase activity is the amount of enzyme necessary to produce (glucose oxidase) or consume (lactoperoxidase) 1 μmol H_2O_2 per min under the assay conditions.

Iodination with lactoperoxidase. Twenty-four dishes ($7 \cdot 10^8$ cells) are harvested just prior to confluency (day six) by exposure of the MDCK cells to 0.10 mM EGTA in 150 mM NaCl, 0.1 mM EDTA, 20 mM sodium phosphate, pH 7.4 (phosphate-buffered saline) for 45 min at 37°C. After this incubation cells are squirted off the plate with a Pasteur pipette and $CaCl_2$, in a 0.5 molar excess over the EGTA and EDTA, is immediately added. Cells are concentrated by centrifugation in a clinical centrifuge. This combined EGTA-Ca^{2+} treatment yields cells with maximum ($Na^+ + K^+$)-ATPase activity and causes only a slight decrease in the total amount of protein relative to a scraped preparation *. Reagents and cells are mixed such that each final ml will contain $1.7 \cdot 10^6$ cells, 80 μmol glucose, 20 mU lactoperoxidase, 0.25 μmol KI, and 12.5 μCi ^{125}I. The reaction is then initiated immediately by the addition of 32 mU of glucose oxidase. All reagents are diluted with phosphate-buffered saline. Labeling is allowed to proceed for 30 min at 23°C with gentle swirling on a gyrotory shaker and cell viability, measured by dye exclusion, remains > 95%. The reaction is terminated by centrifugation and by four washes with termination buffer (phosphate-buffered saline containing 10^{-5} M sodium thiosulfate) to remove the labeling enzymes and unbound ^{125}I [22].

Open membranes from canine kidney medulla, which permitted accessibility of the reagents to both the extracytoplasmic surface and the cytoplasmic surface, were prepared according to the procedure of Jørgensen, as modified by Munson [23], and contained ($Na^+ + K^+$)-ATPase and only a few minor contaminating proteins. A specific enzymatic activity of 1500 μmol $P_i \cdot mg^{-1} \cdot h^{-1}$ was obtained. Iodination of the open membranes proceeded simultaneously to that of the MDCK cells but in a separate flask containing 10% of the labeling reagents and 10% of the cells at the same final concentrations. After the 30 min incubation, the dummy cells were removed by low speed centrifugation ($1100 \times g$) and the open membranes collected as a pellet at $100000 \times g$ for 3 h. These were resuspended by homogenization in the presence of 10 mM Tris-HCl (pH 7.7); 2-mercaptoethanol and SDS were added to final concentrations of 1% and 4 mg per mg protein, respectively, and the sample was brought to 100°C for one min.

Cytoplasmic labeling. The cytoplasmic proteins were distinguished from the proteins of the plasma membrane by the following procedure. After labeling, an aliquot of the iodinated, intact MDCK cells were removed and lysed. This homogenate was centrifuged at $1500 \times g$ and the supernate centrifuged at $5500 \times g$. The resultant supernate was then centrifuged at $100000 \times g$ to collect a membrane fraction while the cytoplasmic proteins remain in the supernate. The final pellet, which had been resuspended in phosphate-buffered saline, and supernate were mixed with trichloroacetic acid (final concentration of 6.4%, w/v) to precipitate the proteins in each. After centrifugation, the protein fractions were counted for radioactivity, the protein concentrations were determined, and the specific radioactivities were calculated.

Lipid extraction of iodinated cells. The lipids of iodinated MDCK cells were extracted by the chloroform-methanol procedure of Weinstein et al. [24]. The chloroform layer, which contains the lipids, was washed with water made 5 mM in KI [25] to remove nonlipid material and the majority of the unbound ^{125}I from the extract. Complete removal of unbound ^{125}I from the lipid extract was effected by addition of silver nitrate (and urea) to precipitate the iodide. The proteins of the iodinated cells precipitated during the initial chloroform-methanol extractions and were easily separated from the chloroform layer by centrifugation. Unbound ^{125}I was removed from the protein portion by dialysis against 5 mM KI.

Immunological procedures. Active ($Na^+ + K^+$)-ATPase was denatured in SDS and separated into

* This method of harvesting MDCK cells, which was developed for these experiments and which prevents loss of enzymatic activity, is now used routinely in the laboratories of Drs. Saier [20], Yguerabide [21], and Fortes at the University of California, San Diego. The popularity it has gained is evidence of its suitability for this particular cell line.

large and small chains by gel filtration on Sepharose 6B in buffers containing SDS [26]. Antisera were raised by injection of the antigen suspension, emulsified in Freund's complete adjuvant, into the lymph nodes of several rabbits [27]. The γ-globulin fraction of the antiserum was isolated by ammonium sulfate precipitation [28]. Complement fixation assays were performed by the method of Levine and Van Vunakis [29].

Antibodies were coupled to Sepharose 4B essentially according to the procedure of March et al. [30]. Sepharose 4B was activated with BrCN and immediately mixed with an antibody solution (5 $mg \cdot ml^{-1}$) in phosphate-buffered saline in the proportions of 5 mg γ-globulin per ml packed resin. After mixing for 20 h at 4°C, glycine was added and allowed to mix for 4–5 h at 4°C to mask any unreacted sites on the activated resin. This immunoadsorbent (Abs-4B) was washed with 1 M ammonium sulfate and phosphate-buffered saline until A_{280} equalled background in order to remove noncovalently bound antibodies.

Glycinium chloride or acetic acid at pH 2–3 will not elute the large chain from Abs-4B. 4 M guanidinium chloride in phosphate-buffered saline [31] does, however, successfully elute large chain.

Removal of sodium dodecyl sulfate. Partial removal of SDS was performed by use of XAD4 beads * and for complete removal of bound SDS, the procedure of Weber and Kuter [32] involving Dowex 1-X2 resin was utilized. The total concentration of detergent (bound and unbound) was measured by an adaptation of the methylene blue assay of Mukerjee [33]. To a sample solution in a volume < 0.5 ml and containing between 0–10 μg total SDS, is added 1 ml of methylene blue solution (24 $mg \cdot l^{-1}$), 4 ml of chloroform and the phases are mixed for 1 min. After the phases have separated, the organic layer is carefully transferred to a cuvette. Absorbance at 655 nm is then measured against the organic layer from a sample containing no detergent as the blank.

Preparation of plasma membranes from MDCK cells. Iodinated cells were lysed by swelling the suspension of the MDCK cells with water, followed by several passages through a 27 gauge (1/2 inch) needle. A crude plasma membrane fraction could then by isolated from this homogenate by the sucrose gradient method developed for hepatoma cells in culture [34]. The 1.6–1.8 M sucrose and 1.8–2.2 M sucrose interfaces were collected separately, diluted with 10 mM Tris-HCl (pH 7.7) to decrease the surcrose concentrations, and centrifuged at 234000 × *g* for 1 h. The pellets were resuspended in 10 mM sodium phosphate (pH 7.2), dissolved in 2-mercaptoethanol (1% final concentration) and SDS (final concentration 4 mg per mg protein), and the sample was brought to 100°C for one min.

Protein determinations. Precise protein concentrations were determined by amino acid analysis on a Beckman 118C analyzer by the method of Spackman et al. [35] with the inclusion of norleucine as an internal standard. Protein concentrations were routinely estimated by the method of Lowry et al. [36] as modified by Bailey [37], after an initial precipitation with trichloroacetic acid. A solution of bovine serum albumin, whose protein concentration was evaluated by quantitative amino acid analysis, was used as the standard.

Polyacrylamide gel electrophoresis. Cylindrical gels, 5% in polyacrylamide, were run in buffers containing SDS (SDS gel electrophoresis) according to the method of Shapiro et al. [38] as modified by Weber and Osborn [39]. These gels were scanned on a Zeiss PMQII fitted with a linear transport system. All mobilities reported for components separated by electrophoresis on SDS-polyacrylamide gels (R_F) were relative to the middle of the tracking dye band. Radioactive gels were sliced manually into 1.3 mm slices and then counted directly in gamma vials with a Beckman Biogamma II. Iodinated proteins from particular regions of the gel were subjected to a second electrophoresis by placing the appropriate slices on top of a second SDS-polyacrylamide gel, overlaying with 5 μl of 2-mercaptoethanol and 75 μl of a 1 : 1 mixture (v/v) of glycerol and tracking dye (Bromophenol blue), and running as usual. Iodinated proteins contained in slices from the second 5% SDS-polyacrylamide gels were eluted for 24 h at 37°C with a buffer composed of 0.01% SDS, 10 mM NH_4HCO_3, (pH 7.9) and made 2.5% in 2-mercaptoethanol prior to use [1]. The elution

* This method was developed by J. Kyte, University of California, San Diego.

was repeated and the eluants pooled. The recovery of radioactivity averaged 74%. The pools were dialyzed, lyophilized, and redissolved, the radioactivity was measured, and, finally, aliquots were hydrolyzed for quantitative amino acid analysis. Blank polyacrylamide gels are treated identically to those with protein.

Results

Lactoperoxidase-catalyzed iodination of MDCK cells

Impermeant reagents may label the lipids of the membrane as well as the proteins. It is important to assess the distribution of the radioactive label between protein and lipid in order to be certain that the reaction conditions favor iodination of proteins. A sample ($8.5 \cdot 10^7$ cells) of iodinated MDCK cells was submitted to lipid extraction [24]. After unbound label was removed from the lipid extract and from the proteins, it was determined that the lipid fraction contained 28% (49 000 cpm) and the protein fraction 72% (127 000 cpm) of the covalently-bound label.

Also, essential to any labeling study using an impermeant reagent is a demonstration that the reagent has not breached the permeability barrier of the cell membrane. A comparison of the specific radioactivities of a membrane fraction and the supernate from a centrifugation at $100\,000 \times g$, both from the same lactoperoxidase-iodinated MDCK cells, revealed that the value for the former, which contains the plasma membranes, was 8-fold that of the latter, which contains the cytoplasmic proteins. This value is a lower limit of the true number because the membrane preparation is not pure plasma membrane and small plasma membrane vesicles may contaminate the supernate. The conclusion drawn from this experiment is that lactoperoxidase-catalyzed iodination proceeds with high selectivity for the external surface of the MDCK cells.

Isolation of iodinated large charge chain from the labeled cells

After iodination and washing, the intact MDCK cells were dissolved completely by the addition of SDS to a 3-fold weight excess over total cell protein and 2-mercaptoethanol to a 1% final concentration, and the sample was brought to 100°C for 2 min to prevent proteolytic degradation during denaturation [40]. The necessity of utilizing this drastic procedure to dissolve the entire cell followed from several results. First, the relatively low specific $(Na^+ + K^+)$-ATPase activity found in crude plasma membranes from MDCK cells (2–4 μmol $P_i \cdot mg^{-1} \cdot h^{-1}$) indicates that these cells possess very few copies of the enzyme, and plasma membrane purification would, therefore, be quite costly. Second, the $(Na^+ + K^+)$-ATPase activity was very unstable in all crude and partially purified membrane preparations ($t_{1/2} = 14$ h at 4°C) and this suggests that intracellular proteases are released upon initial rupture of the intact cells. Third, this rapid loss of enzymatic activity could not be prevented by any of the conventional methods for the inhibition of proteolysis [41]. Lysis of the cell with SDS causes rupture of the nuclear membrane followed by release of DNA which forms visible, gelatinous strands which were removed by filtration of the iodinated, detergent-dissolved cell solution through glass wool in a plastic syringe.

The iodinated cell solution was concentrated by lyophilization and applied to a Sepharose 4B column (4.1 cm × 45 cm) equilibrated with 0.2% SDS and 40 mM Tris sulfate (pH 8.0). Gel filtration chromatography in the presence of SDS provided a convenient method for the separation of the proteins according to their length [42] as well as the removal of the lipids from the large chain of $(Na^+ + K^+)$-ATPase [26]. The elution position of large chain was determined with both ^{14}C-labeled large chain and nonradioactive large chain at A_{280}. Fig. 1 shows both a typical calibration and the profile of an iodinated sample. Those fractions containing large chain were pooled, based upon the prior calibration results. In addition to the large chain, this pool contains cytoplasmic and membrane-bound proteins which have coincident elution positions. The pool was concentrated for the next step by lyophilization, redissolved and bovine serum albumin was added to a final concentration of 0.1%.

Unbound SDS was removed from this pool by the use of XAD4 beads *. This procedure removes

* See footnote on p. 330.

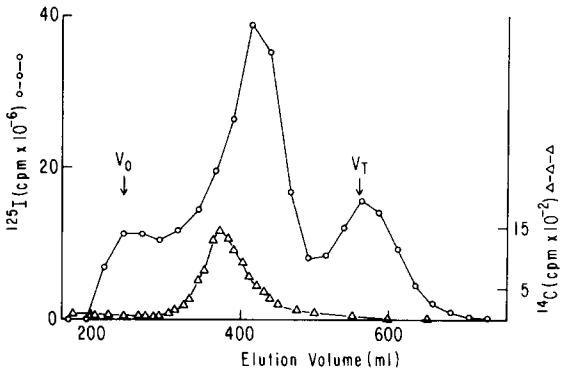

Fig. 1. Calibration of Sepharose 4B column with large chain of $(Na^+ + K^+)$-ATPase and distribution of the radioactivity from an iodinated MDCK cell solution. About $7 \cdot 10^8$ MDCK cells were labeled by lactoperoxidase-catalyzed iodination and washed four times with phosphate-buffered saline containing 10^{-5} M $Na_2S_2O_3$. The iodinated cells were dissolved completely by the addition of SDS to give a 3-fold weight excess of SDS over total cell protein and 2-mercaptoethanol to a 1% final concentration, and the sample was brought to 100°C. Strands of DNA were removed by filtration of the solution through glass wool and the filtrate was concentrated by lyophilization. The iodinated sample, in 2.5 ml, was applied to a Sepharose 4B column (4.1 cm×45 cm) equilibrated with 0.2% SDS and 40 mM Tris sulfate (pH 8.0) [26], and run at 7 ml·h^{-1}. Selected fractions were counted directly for ^{125}I (O———O). The void volume (V_0) and total volume (V_T) has been determined by Dextran blue and 2-mercaptoethanol. A large chain standard, prepared from intact kidney medulla and denatured in SDS [26], was rendered radioactive by reductive alkylation [43] with [^{14}C]formaldehyde and was used to determine the elution position of the iodinated large chain from labeled MDCK cells. Selected fractions were analyzed by liquid scintillation counting for ^{14}C (Δ———Δ).

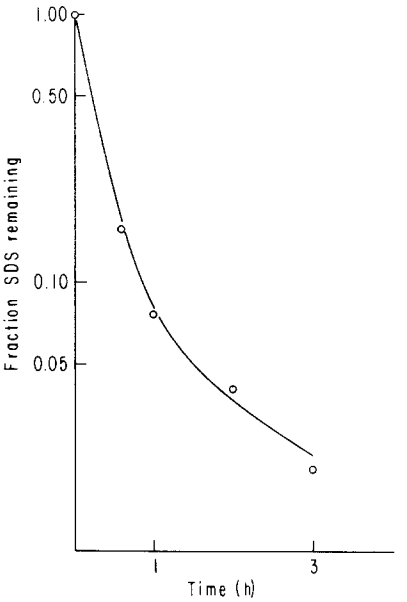

Fig. 2. Removal of SDS by XAD4 beads. Those fractions, from a Sepharose 4B column (Fig. 1), which contain large chain were pooled, concentrated by lyophilization, and redissolved. A 10% aliquot (240 μl) of this iodinated sample, at an SDS concentration of 4.6% (11 mg SDS), was stirred with 100 μl of XAD4 beds which has been well washed with methanol and equilibrated with buffer. At selected times during the 3 h incubation, the stirring was stopped briefly and 1 μl of the solution was removed, diluted, and assayed for SDS [33]. The fraction of the initial SDS which still remained in the solution is plotted as a function of the time of incubation with the beads.

all the unbound SDS and a portion of the SDS bound to the protein, and permits quantitative recovery of the protein with no increase in sample volume. The total concentration of SDS both before and during the incubation with the beads can be followed by a methylene blue assay [33]. Fig. 2 presents a plot of removal of detergent as a function of time of incubation with the beads. The curve is not a simple exponential function, and this observation, and others not shown, suggest that the initial, rapid uptake of SDS from the sample solution represents the removal of unbound SDS by the beads and the slower kinetic components result from the stripping of bound SDS from the protein. Because the methylene blue assay is very fast, the decrease in the SDS concentration during the incubation with the beads can be followed while the reaction progresses.

After XAD4 treatment, iodinated samples were brought to 0.5% bovine serum albumin and drained into a 10 ml plastic syringe containing 5 ml of packed Abs-4B. After 1 h the column was rinsed with phosphate-buffered saline until radioactivity reached a constant level. Elution of large chain was effected with 4 M guanidinium chloride and terminated when the radioactivity in the eluate again declined to the previous, constant level. All of the above manipulations were performed at 4°C. The peak of radioactivity from the guanidinium chloride elution was pooled (Gn · Cl pool) and dialyzed against 6 M urea to remove the guanidinium chloride. After the addition of SDS to the dialysis bag, the pool was dialyzed against an SDS/sodium phosphate buffer to remove the urea and finally lyophilized.

An aliquot of the Gn · Cl pool was subjected to electrophoresis on 5% SDS-polyacrylamide gel and the gel was sliced and counted for ^{125}I. The results from a typical gel (Fig. 3) reveal that there are more peaks of radioactivity than the large chain and that the background level of radioactivity throughout the gel is quite high. Additionally, the Gn · Cl pool contained more than a single peak of protein when its polypeptide composition was examined by the staining of another 5% SDS-polyacrylamide gel with Coomassie brilliant blue (Fig. 4). The stained gel also demonstrates, however, that there is a distinct, significant peak of protein from the Gn · Cl pool which migrates with a R_F identical to that of the large chain.

Since the final isolation of the iodinated large chain from the MDCK cells could not be effected completely by immunoadsorption, an additional purification step was required. Slices from the region of the SDS gel to which the large chain migrates, as determined by prior calibration, were submitted to a second electrophoresis on another

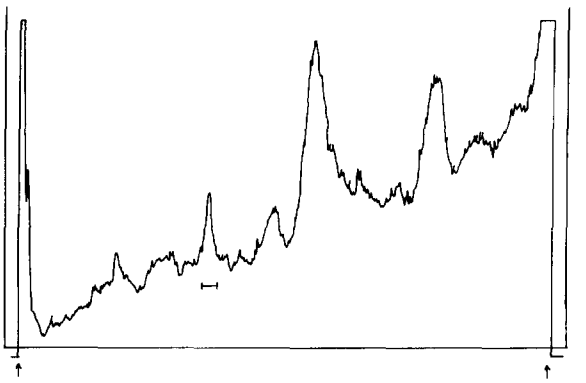

Fig. 4. Scan of an SDS-polyacrylamide gel of the guanidinium chloride (Gn·Cl) pool from iodinated MDCK cells. An aliquot, containing $2 \cdot 10^5$ cpm of ^{125}I, of the same Gn·Cl pool from labeled cells used in Fig. 3 was subjected to SDS gel electrophoresis on a 5% SDS-polyacrylamide gel. After electrophoresis, however, the gel was stained with 0.25% Coomassie brilliant blue. Absorbance at 515 nm is recorded as a function of distance migrated. The direction of electrophoresis is from left to right; arrows mark top and bottom of the gel. The bar marks the R_F of a large chain standard run on a parallel 5% SDS-polyacrylamide gel.

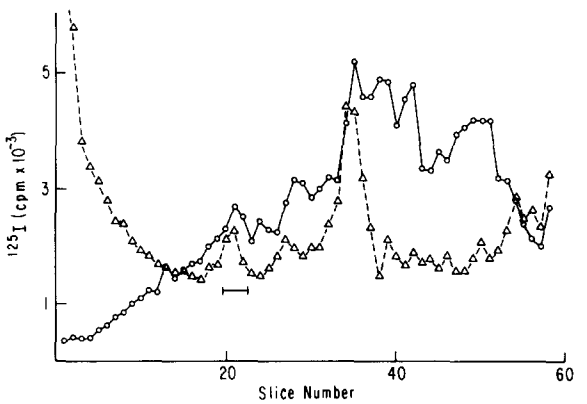

Fig. 3. Superimposition of the radioactivity profiles of SDS-polyacrylamide gels of guanidinium chloride (Gn·Cl) pools from iodinated MDCK cells (○——○) and from iodinated open membranes (△— — —△), respectively. An aliquot, containing $2 \cdot 10^5$ cpm of ^{125}I, from the Gn·Cl pool from labeled MDCK cells and an aliquot, containing $5 \cdot 10^5$ cpm of ^{125}I, from labeled, open membranes dissolved in SDS were each submitted to SDS gel electrophoresis of 5% SDS-polyacrylamide gels prepared by the method of Weber and Osborn [39]. After electrophoresis, the gels were sliced into 1.3 mm disks, and each slice was counted directly for ^{125}I. The direction of electrophoresis is from left to right. The bar marks the R_F of a large chain standard run on parallel 5% SDS-polyacrylamide gel stained with Coomassie brilliant blue, and destained by diffusion.

5% SDS-polyacrylamide gel. The results from the second gel (Fig. 5) show that the high level of background radioactivity has been eliminated, and no radioactivity remains in the original gel slices. Furthermore, a significant portion of the radioactivity applied to the gel (> 50%) migrated with an R_F identical to that of the large chain. This peak of the radioactivity also co-migrated with a large chain standard (Fig. 6).

The nature of the undesired peaks of radioactivity was explored by the following approach. An iodinated sample which has been removed prior to immunoadsorption was reacted with a nonimmune Abs-4B and eluted in the usual manner. Table I compares the results of this experiment with that of one using the immunoadsorbent and shows that with equivalent volumes of each resin there was about a 7-fold increase in the amount of radioactivity in the Gn · Cl pool from the immune Abs-4B over that from the nonimmune Abs-4B. A comparison of the profiles of radioactivity from the Gn · Cl pools from the immune Abs-4B and the nonimmune Abs-4B upon SDS gel electrophoresis revealed that the latter pool does not contain any radioactive peaks on a comparable scale. This

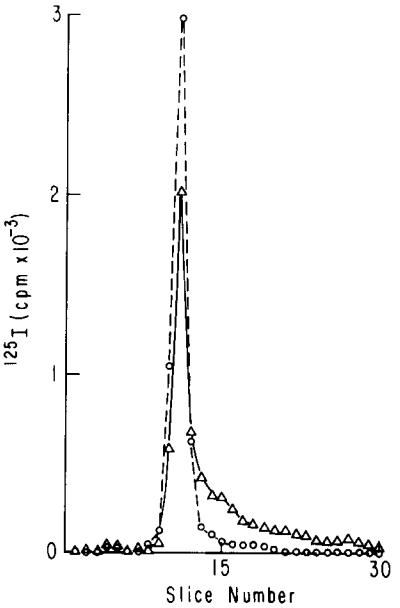

Fig. 5. Superimposition of the radioactivity profiles from SDS-polyacrylamide gels of selected slices from the first SDS gel electrophoresis (Fig. 3) of the guanidium chloride (Gn·Cl) pool from iodinated MDCK cells (△———△) and of iodinated open membranes (○— — —○), respectively. Three SDS-polyacrylamide gel slices from the iodinated MDCK cells (Nos. 20, 21, and 22 in Fig. 3), containing $7.4 \cdot 10^3$ cpm of ^{125}I, were submitted to a second SDS gel electrophoresis. Likewise, three SDS-polyacrylamide gel slices from the iodinated open membranes (Nos. 20, 21, and 22 in Fig. 3), containing $6.2 \cdot 10^3$ cpm of ^{125}I, were subjected to a second SDS gel electrophoresis. After electrophoresis, the gels were sliced into 2.6 mm disks, and each slice was counted directly for ^{125}I. The direction of electrophoresis is from left to right.

experiment demonstrates that the additional peaks of radioactivity from the MDCK cells are not removed by the normal rabbit γ-globulins attached to the resin and suggests that these peaks of radioactivity may represent fragments of the large chain which still possessed intact antigenic determinants.

Purification of large chain from open membranes

Open membranes, purified by the procedure of Jørgensen as modified by Munson [23], which had been iodinated and dissolved in SDS detergent were also subjected to electrophoresis on 5% SDS-polyacrylamide gels. Since the only polypeptide components present in significant quantities in these purified membranes are the two chains of $(Na^+ + K^+)$-ATPase, the large chain was readily

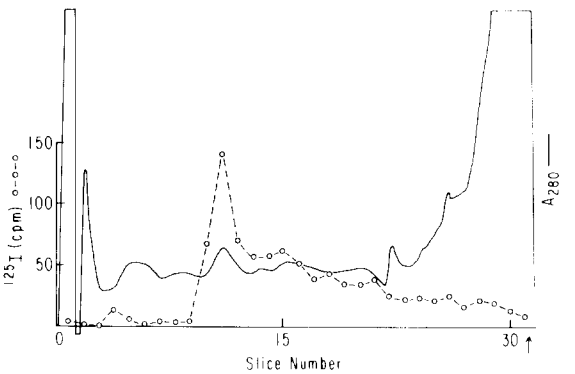

Fig. 6. Scan and superimposed profile of radioactivity from an SDS-polyacrylamide gel of selected gel slices from the first SDS gel electrophoresis of an aliquot of the guanidinium chloride (Gn·Cl) pool from iodinated MDCK cells to which a large chain standard had been added. Large chain standard (30 μg) was added to an aliquot of a Gn·Cl pool from labeled MDCK cells. This mixture was subjected to SDS gel electrophoresis on a 5% SDS-polyacrylamide gel (Fig. 3). Three gel slices, with R_F identical to those selected in Fig. 3, were subjected to a second SDS gel electrophoresis. After electrophoresis, the gel was scanned without staining [9] at 280 nm (A_{280}, ———), was sliced into 2.6 mm disks, and each slice was counted directly for ^{125}I (○— — —○). The direction of the electrophoresis is from left to right; arrow marks the bottom of the gel and zero marks the top.

TABLE I

COMPARISON OF ^{125}I-LABELED MDCK CELL SAMPLE ADSORBED TO AND ELUTED FROM IMMUNE Abs-4B AND NONIMMUNE Abs-4B

MDCK cells were iodinated, dissolved, and lyophilized. The concentrated solution was submitted to gel filtration, radioactivity with the mobility of large chain was pooled (Fig. 1), and the SDS was removed by XAD4 treatment (Fig. 2). Samples from this concentrated pool were adsorbed to Abs-4B made from immune or nonimmune γ-globulin (5 mg per ml packed resin). Gn·Cl, guanidinium chloride.

	Immune Abs-4B	Nonimmune Abs-4B
Radioactivity added to resin (cpm)	$163 \cdot 10^6$ [a]	$560 \cdot 10^4$ [a]
Amount of resin used (ml)	5.0	0.10
Radioactivity (cpm) per μl resin	$32 \cdot 10^3$	$56 \cdot 10^3$
Gn·Cl pool (total cpm)	$7.4 \cdot 10^6$ [a]	$2.1 \cdot 10^4$ [a]
Gn·Cl pool cpm per μl resin	1500	200

[a] All cpm were corrected to the day of labeling.

identified and had the same R_F as nonradioactive large chain. This demonstrates that iodination, as expected [44], does not influence the electrophoretic mobility of the large chain in the presence of SDS. A problem with background radioactivity, similar to that with the cell preparation, was encountered (Fig. 3) and again could be eliminated by submitting the gel slices which contain the large chain to a second electrophoresis (Fig. 5). Fig. 3, which represents the superimposition of the profiles of radioactivity from the first electrophoresis of each preparation, MDCK cells and open membranes, shows that identical regions on the SDS-polyacrylamide gels were selected to be subjected to a second electrophoresis. Fig. 5, in which the profiles of radioactivity from the second electrophoresis of each preparation are superimposed, demonstrates that the iodinated large chains from the MDCK cells and the open membranes migrate identically when rerun. Again, a significant portion (> 70%) of the radioactivity reran in the large chain position in the case of the protein from open membranes.

An initial SDS-polyacrylamide gel of the radioactive open membrane preparation was stained with Coomassie brilliant blue, destained, scanned at A_{550}, sliced and counted for ^{125}I. A comparison of the gel scan from the labeled open membranes with that from the same preparation prior to iodination (Fig. 7) shows that after labeling there is a dramatic loss of the large chain relative to the small chain and a significant increase in the stained material which will not enter the 5% SDS-polyacrylamide gel.

Other plasma membrane proteins from iodinated MDCK cells

In order to distinguish between a low level of iodination of the large chain due to its disposition in the membrane as opposed to a general ineffectiveness in the labeling procedure, it was necessary to determine the specific radioactivities of other plasma membrane proteins from the MDCK cells. Membranes were isolated from iodinated MDCK cells. By the criteria of either Coomassie brilliant blue staining patterns or radioactive profiles of sliced gels, the pellets from the 1.6–1.8 M and 1.8–2.2 M interfaces are identical. Gels of the iodinated samples displayed the same problem of a high background of radioactivity which has been encountered with the iodinated large chain samples. This was again eliminated by a second electrophoresis of the appropriate gel slices. Four regions of a 5% SDS polyacrylamide gel of these membranes were selected to be resubjected to electrophoresis because they possessed Coomassie brilliant blue staining material which coincided with a

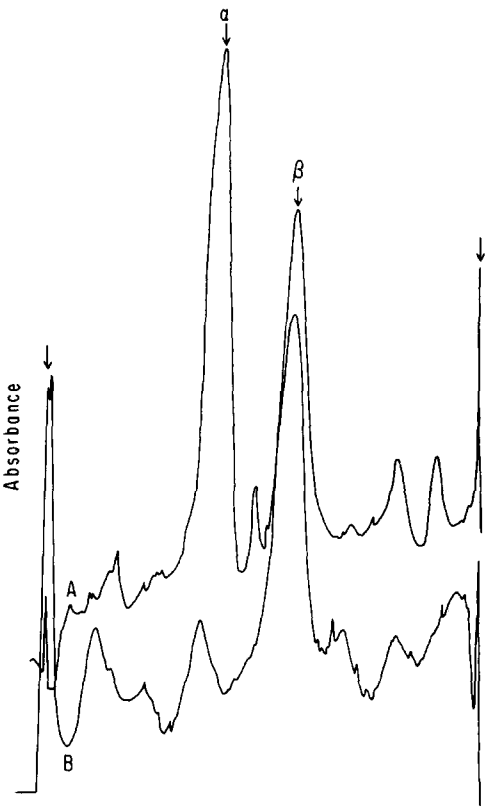

Fig. 7. Scans of SDS-polyacrylamide gels of pre-labeling and post-labeling samples from open membranes. Aliquots from the open membranes prior to labeling and from the open membranes after iodination were both dissolved by the addition of SDS (4 mg per mg protein) and 2-mercaptoethanol to a 1% final concentration, and the sample was brought to 100°C. They were submitted to SDS gel electrophoresis on SDS-polyacrylamide gels prepared by the method of Weber and Osborn [39]. After electrophoresis, the gels were stained with 0.25% Coomassie brilliant blue. Absorbances at 550 nm from the prelabeling open membranes (gel A) and at 530 nm for the post-labeling open membranes (gel B) are recorded as a function of distance migrated. The direction of electrophoresis is from left to right; arrows mark top and bottom of the gels. The positions of the large chain and the small chain of $(Na^+ + K^+)$-ATPase are indicated on the scans as α and β, respectively.

TABLE II

SPECIFIC RADIOACTIVITIES OF IODINATED PROTEINS FROM MDCK CELLS AND FROM OPEN MEMBRANES OF INTACT KIDNEY MEDULLA

Sample	Protein hydrolyzed [a] (μg)	Specific radioactivity [b] (cpm·μg^{-1})	Relative specific radioactivity
Experiment 1 [c]			
Large chain from open membranes	≤ 1	≥ 49590	≥ 6.6
Large chain from MDCK cells	5	7490	1.00
Peak 1 from MDCK cells	≤ 1	≥ 31890	≥ 4.2
Peak 2 from MDCK cells	≤ 1	≥ 33000	≥ 4.4
Peak 3 from MDCK cells	3	37120	4.9
Peak 4 from MDCK cells	4	32510	4.3
Experiment 2			
Large chain from open membranes	23	22560	5.2
Large chain from MDCK cells	10	4350	1.00
Peak 1 from MDCK cells	9	32090	7.4
Peak 2 from MDCK cells	8	36670	8.4
Peak 3 from MDCK cells	36	26930	6.2
Peak 4 from MDCK cells	47	24370	5.6

[a] Samples were hydrolyzed under vacuum in 6 M HCl for 20 h [35].
[b] All cpm were corrected to the day of labeling.
[c] In this experiment MDCK cells were not present during the iodination of the open membranes.

significant peak of radioactivity, and they did not migrate with the mobility of the large chain, the small chain or any combination of the two chains. In addition to permitting an evaluation of the effectiveness of the labeling of MDCK cells, these proteins provided an estimate of the lower limit of the specific radioactivity of fully exposed, extracytoplasmically oriented, plasma membrane proteins for comparison with the specific radioactivity of the large chain isolated from the same cells.

Specific radioactivities

Table II lists the specific radioactivities of the six iodinated protein samples. Values for the other membrane proteins from MDCK cells range from 24000 to 37000 cpm per μg protein and are 4-fold to 8-fold greater than the values for the large chain from the same cells. The ratio of specific radioactivities between the large chain from open membranes and the large chain from MDCK cells is greater than 6.6-fold is the first determiantion and 5.2-fold in the second determination.

Discussion

One of the criteria essential to the acceptance of any structural information obtained from this labeling study is that iodination occurred with acceptable preference for the extracytoplasmic surface residues of $(Na^+ + K^+)$-ATPase in the MDCK cells. Several arguments, in addition to the results described, pertain to this criterion. First, the MDCK cells are quite durable to manipulation and were demonstrated to be intact, prior to the iodination, by dye exclusion. Second, the central component of the labeling reaction, the enzyme lactoperoxidase, which produces the iodinating agent continuously, cannot cross the membrane of an intact cell. Third, any iodinating agent which penetrated the surface of the cell would react with the membrane lipids before reaching the cytoplasmic surface of $(Na^+ + K^+)$-ATPase. Fourth, any iodide transported into the cell and perhaps converted to iodinating agent would have reacted with the cytoplasmic proteins which comprise the vast majority of the cell's proteins and act as a

convenient sink to the label. Nevertheless, a comparison between the specific radioactivities of a cytoplasmic protein fraction and of a crude plasma membrane fraction was made and it demonstrates that no significant labeling of the cytoplasmic proteins in iodinated MDCK cells occurred. Thus, there was no significant amount of contramembrane labeling of plasma proteins during the lactoperoxidase-catalyzed iodination of the MDCK cells.

At the outset, it was hoped that the immunoadsorption step would yield completely purified large chain; however, other radioactive components were present in the elution pool (Fig. 3). This problem of contaminating labeled material isolated during an immunoadsorption step is not exclusive to the purification of the large chain [45–48]. Experimental results were presented (Table I), however, which suggest that much of the contamination is antigenic. An additional problem, that of a high background level of radioactivity in iodinated preparations subjected to electrophoresis on SDS-polyacrylamide gels, has been encountered before in experiments with Ca^{2+}-ATPase from sarcoplasmic reticulum [49] and brush-border membranes of epithelial cells from the intestine [50].

Furthermore, the yield of large chain from the iodinated open membranes, in which the two chains of $(Na^+ + K^+)$-ATPase were nearly the only polypeptides present, was disappointing. Gel scans of pre-labeled and post-labeled preparations (Fig. 7) reveal that after iodination there is a dramatic increase in the amount of stained material not entering the gel and a significant loss of the large chain relative to the small chain. It would appear that the large chain has been preferentially cross-linked into polymeric forms of a size sufficient to be excluded from the 5% polyacrylamide gel. The nature of this crosslinking reaction was not explored. In this context, however, cross-links may have occurred between tyrosine residues on neighboring polypeptides chains as has been demonstrated in the presence of hydrogen peroxide with horseradish peroxidase [51], ovoperoxidase [52], or lactoperoxidase [53].

On the positive side, the results presented in Fig. 7 demonstrate that iodination of the large chain while in the membrane does not change its mobility on an SDS-polyacrylamide gel. The radioactive products isolated from either the MDCK cells or the open membranes from intact kidney medulla both co-migrated with a nonradioactive large chain standard upon SDS-polyacrylamide gel electrophoresis (Figs. 5 and 6). Moreover, the results of the specific radioactivity determinations (Table II) proved to be quite satisfactory and internally consistent. First, the absolute values obtained for four membrane protein samples from the MDCK cells compare favorably with those reported in the literature for membrane proteins from other cells. Levels of iodination for erythrocyte [19] and fibroblast [22] membrane proteins averaged about 10000 cpm per μg protein, while those for membrane proteins hepatoma cells [34] were about 16000 cpm per μg protein. With this in mind, it can be concluded that the protocol established for MDCK cells yields efficiently labeled plasma membranes. Second, a comparison of the values for the large chain from open membranes and the large chain from MDCK cells reveals that the former was labeled at least 5-fold more extensively.

A priori, the large chain from open membranes is expected to have a higher specific radioactivity since amino acid residues at both the extracytoplasmic and cytoplasmic surfaces are accessible to the labeling reagents, whereas with the MDCK cells only the amino acid residues at the extracytoplasmic surface are available for labeling. The relative specific radioactivities, however, indicate that the large chain is situated in the membrane such that more of its mass, at face value four to five times as much, protrudes into the cytoplasm than into the extracytoplasmic environment. Finally, a comparison of the values for four membrane protein samples from MDCK cells and for the large chain from the same cells reveals that the former were also labeled 4-fold to 8-fold more extensively. Since the specific radioactivity of a fully exposed, extracytoplasmically oriented, membrane-bound protein in the labeled cells would certainly be greater than the values obtained, the tabulated ratio in specific radioactivities is the minimum ratio. The magnitude of this ratio, therefore, supports the conclusion drawn from the comparison of the specific radioactivities of the two large chain preparations, that the amount of mass

of the large chain on the extracytoplasmic surface is small.

What factors might complicate this interpretation of the data? Implicit in the proposal is the assumption that the available tyrosine residues of the large chain are distributed at each surface in equal quantities per unit mass. A clustering of accessible tyrosines in a short length of sequence at the cytoplasmic surface would invalidate any conclusions about the distribution of mass. The utilization of additional labeling reagents which react with different amino acids would help to resolve any question of clustering of the enzyme's tyrosine residues. Relevant to this question of the distribution of tyrosine residues in the sequence of the large chain of $(Na^+ + K^+)$-ATPase is information obtained from a closely related membrane-spanning transport protein, Ca^{2+}-ATPase of skeletal muscle. Ca^{2+}-ATPase from sarcoplasmic reticulum and the large chain of $(Na^+ + K^+)$-ATPase share a number of similarities. They both contain an identical, three-residue peptide in their active site [13]. They comigrate when submitted to electrophoresis on SDS-polyacrylamide gels [7]. Since the polarity of the two amino acid compositions is almost identical, this co-migration indicates that these two polypeptides are the same length. Further correspondences are apparent when other comparisons are made; for example, three membrane-affiliated regions and the site of phosphorylation on the large chain of $(Na^+ + K^+)$-ATPase appear in positions on the protein very similar to those on Ca^{2+}-ATPase [54–60]. Therefore, all the structural information known about these two polypeptides strongly suggests that their amino acid sequences are very similar. Although it has not yet been demonstrated conclusively, the results of several experiments suggest that the three major, sequenced regions of Ca^{2+}-ATPase, which together comprise about 50% of the total sequence, are located on the cytoplasmic surface [58]. The mole present of tyrosine in the major, sequenced regions is identical to that in a number of small peptides which have been sequenced but not aligned [58] demonstrating that the distribution of tyrosine residues of Ca^{2+}-ATPase between the extracytoplasmic and cytoplasmic surfaces would be representative of its distribution of mass at these surfaces.

Unfortunately, because $(Na^+ + K^+)$-ATPase rapidly deteriorates in lysed MDCK cells, open membranes from MDCK cells could not be employed for labeling experiments. This raises the possible ambiguity of whether or not specific radioactivity determinations of the large chain from an established cell line and an intact kidney can be compared. Since the MDCK cells do possess specific $(Na^+ + K^+)$-ATPase activity, were derived originally from a canine kidney [61], and do display the extreme characteristics of a transporting epithelium [62–65], it is fair to assume that the enzyme from this established cell line is identical in sequence and structure to that in the intact kidney. As far as the open membranes are concerned, it is extremely unlikely that any additional tyrosine residues have become accessible as a result of a rearrangement of the enzyme in the phospholipid bilayer during the purification of the membranes prior to the labeling. The SDS used to purify these membranes is at a concentration below the critical micelle concentration, the mass ratio of SDS to protein is well below that necessary to denature a protein [66], and the specific activities of these preparations of the enzyme are the highest reported. Electron micrographs of this preparation reveal that it is composed entirely of large sheets and vesicles of membrane, presumably unaltered from the native state at the molecular level [67]. Furthermore, the removal of any loosely bound membrane proteins during purification of the open membranes is inconsequential to the labeling of the large chain. The actual iodinating species is a small, penetrating, oxidized form of iodide and no evidence that a ternary complex exists between lactoperoxidase, iodide, and the protein which is being labeled has ever been presented. That so much lipid is labeled during the reaction, presumably at the olefin positions, demonstrates that steric accessibility is of little consequence to the promiscuous species actually iodinating the protein. Finally, the open membranes were labeled under conditions identical to those used for the MDCK cells even to the point of including MDCK cells at the same concentration. For all these reasons, a comparison of the specific radioactivities from these two samples of large chain should be valid.

To further complicate the situation, the large

chain from the open membranes appears to have been cross-linked by the labeling reaction (Fig. 7) while that from the MDCK cells has been degraded (Fig. 3). Both of these consequences are chemically independent of the *ortho* iodination at tyrosine itself, however, and will not produce, therefore, a change in the specific radioactivity of that unaltered large chain which runs with the proper mobility.

A final possible point of contention concerns the purity of the large chain isolated from MDCK cells. Unfortunately, the most convincing types of evidence for the purity of a protein, such as a single NH_2-terminal analysis and peptide mapping, require far greater amounts of the protein than were obtained (Table II). Thus, the criteria available here are the antigenicity, and the mobility on an SDS-polyacrylamide gel. Polyacrylamide gel electrophoresis in SDS detergent solutions is a powerful technique for the separation of protein mixtures, however, a single stained band or single peak of radioactivity does not guarantee the presence of only one polypeptide.

In order to assess the extent to which the specific radioactivity of the large chain from MDCK cells may have been affected by contaminating material, the fraction of contaminating radioactivity and the fraction of contaminating protein were roughly estimated from the radioactivity profile (Fig. 3) and from the scan of the gel stained with Coomassie brilliant blue (Fig. 4), respectively, by calculating the areas above and below the broad, sloping envelopes in the region of the gel which contains the large chain. The value for the contaminating radioactivity has been corrected for the contribution from the unbound ^{125}I by using the data in Fig. 5. The estimated value for the contaminating protein is necessarily a maximum value because no correction could be made for the contribution to the absorbance area from non-protein substances which bind Comassie brilliant blue. When the contribution of the contaminating radioactivity and protein to the observed specific radioactivity of the large chain from MDCK cells is removed, the value for the large chain increases by a maximum factor of about 1.6 to 7000 cpm per μg protein. The ratio between the specific radioactivities of the two large chain preparations is reduced from a value of 5.2-fold (Table II), in which no contamination is assumed, to the mininum value of 3.2-fold in which the maximum contamination is assumed. The true ratio most likely lies between these two limiting values, Therefore, even when the least favorable interpretation of the data is made, the general conclusion drawn here concerning the distribution of the mass of the large chain across the plane of the membrane is not changed.

Since $(Na^+ + K^+)$-ATPase is only one of many proteins which are associated with the phospholipid bilayer, it is worthwhile to examine how common or how unique its distribution of mass across the plane of the membrane is. Experiments utilizing surface labeling, proteolytic cleavage, electron microscopy, or amino acid sequence techniques have revealed that some membrane-bound proteins possess the majority of their protein mass on the extracytoplasmic side, such as erythrocyte glycophorin [68,69] and aminopeptidase from intestinal brush border [70–72], others on the cytoplasmic side, such as erythrocyte anion carrier [73–75] and Ca^{2+}-ATPase of skeletal muscle [49,58], and for at least one protein, bacteriorhodopsin [76], the majority of the protein mass is embedded within the bilayer. Therefore, for membrane-bound proteins there is no one type of distribution of mass across the plane of the bilayer. It is interesting to note that erythrocyte anion carrier, muscle Ca^{2+}-ATPase, and the large chain of kidney $(Na^+ + K^+)$-ATPase all appear to have the majority of their protein mass on the cytoplasmic side of a membrane. Therefore, this indeed may be a structural feature shared by all transport proteins as was proposed previously [77].

The results of the lactoperoxidase-catalyzed iodination of the large chain of $(Na^+ + K^+)$-ATPase described here suggest that there is 2.2- to 4.2-times more mass of the large chain at the cytoplasmic surface than at the extracytoplasmic surface. If about 25% of the mass of the large chain also is embedded in membrane, as estimated from the binding of [^{3}H]Triton X-100 to $(Na^+ + K^+)$-ATPase [78], then the portion of its total mass present at the extracytoplasmic surface will be between 14% (for 4.2) and 23% (for 2.2) and at the cytoplasmic surface between 61% and 52%. If the large chain from $(Na^+ + K^+)$-ATPase does traverse the membrane several times and does possess only

a small portion of its mass on the extracytoplasmic side, as suggested by the results of previous and present labeling studies, respectively, then it will be most interesting to discover how the small amount of mass contained in the several loops protruding on the extracytoplasmic surface functions as the cardiac glycoside binding site as well as the extracytoplasmic portion of the cation transport site.

Acknowledgments

The author wishes to thank, first and foremost, Dr. Jack Kyte, in whose laboratory this work was conducted, for his advice and support. Lorraine Chuman and Dr. Michael Rindler are thanked for their assistance with the MDCK cells. I also wish to thank Henry Rodriguez, John Winslow, William Craig, and the rest of the laboratory members for helpful discussion and encouragement. The research presented in this publication was supported by Grants PCM-78-24284 from the National Foundation and HL-17879 from the National Institutes of Health to Jack Kyte, University of California, San Diego, as well as Institutional Grants GM-07169 and AM-07233 from the National Institutes of Health; the author was a predoctoral trainee on Training Grant T32 GM-07313 from the National Institutes of Health.

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