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Isolation of Radio-Iodinated Apical and Basal-Lateral Plasma Membranes of Toad Bladder Epithelium

Hector J. Rodriguez* and Isidore S. Edelman**

Cardiovascular Research Institute and the Departments of Medicine and of Biochemistry and Biophysics of the University of California School of Medicine, San Francisco, California 94143

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Summary. The apical and basal-lateral plasma membranes of toad bladder epithelium were radio-iodinated with the glucose-glucose oxidase-lactoperoxidase system. The covalently bound radio-iodine was used as a marker during subcellular fractionation and membrane isolation. Homogenization conditions that ensured rupture of more than 80% of the cells without substantial nuclear damage were defined by Nomarski optics. The nuclei were separated by differential centrifugation and the apical and basal-lateral components were resolved by differential and sucrose density gradient centrifugation. The apical components yielded two radioactive bands that were identified as glycocalyx and plasma membrane labeled with ^{125}I. The basal-lateral components yielded a heterodisperse pattern made up of at least 3 radioactive bands, but the bulk of the activity of ouabain-sensitive ATPase comigrated with only one of these bands. The mitochondia, identified by assays for cytochrome oxidase and NADH cytochrome *c* reductase activities, were separated from the radio-iodine labeled components by centrifugation in sucrose density gradients under isokinetic conditions. The labeled glycocalyx and the slowly migrating components of basal-lateral labeling were separated from the radio-iodinated membranes by centrifugation at 100,000 × *g* × 1 hr after removal of the mitochondria by the isokinetic method. The labeled membranes were then subjected to ultracentrifugation in sucrose density gradients under isopycnic conditions; the basal-lateral membranes containing ouabain-sensitive ATP-ase were well resolved from the apical membranes by this method. These results provide a relatively rapid method of attaining partial purification of the apical and basal-lateral plasma membranes of toad bladder epithelium.

Two types of markers have been used extensively in membrane isolation: morphological and enzymatic. The use of morphological markers (phase contrast or electron microscopy) to evaluate purification of plasma membranes is complicated by vesiculation during disruption of the cells [6]. The presence of special-

* *Present address:* Renal Division, Department of Internal Medicine, Washington University Medical School, St. Louis, Mo. 63110.

** *Present address and to whom reprint requests should be made:* Department of Biochemistry, College of Physicians and Surgeons, Columbia University, 630 W. 168th St., New York, N.Y. 10032.

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ized elements (e.g., microvilli junctional complexes) facilitates the identification of plasma membrane fragments [10, 20]. These elements, however, are useful in only limited cases. The validity of enzymatic markers relies on the results of histochemical analysis, which assigns a given enzymatic activity to a particular organelle or recognizable membrane element [6, 7]. The limitations inherent in histochemical analysis prompted attempts to develop alternative approaches, including the use of surface antigens, membrane receptors (lectins, hormones, cholera toxin), or covalently attached radioactive labels [3, 5, 24, 31]. We elected to explore the utility of covalent, radioactive membrane markers in analysis of the biochemical properties of epithelial plasma membranes of the urinary bladder of the toad [26].

Isolation and separation of the plasma membranes of toad bladder epithelium may yield new information on molecular mechanisms underlying transepithelial transfer of sodium chloride and its hormonal regulation. In the previous paper, we reported a method for high-yield labeling of the apical and basal-lateral plasma membranes of toad bladder epithelium via iodination of exposed residues on membrane proteins [26]. This paper presents the results of experiments on the isolation and partial purification of the apical and basal-lateral plasma membranes of toad bladder epithelial cells, using covalently attached radio-iodine and specific enzymes as markers.

Materials and Methods

Tissue Preparation and Iodination

All experiments were on urinary bladders of female *Bufo marinus* of Dominican origin. The toads were housed, maintained, and processed as described previously [26], including ventricular perfusion and mounting of the hemibladders as sacs. Paired hemibladders were labeled with ^{125}I-Na on the apical and ^{131}I-Na on the basal-lateral side, as described previously [26]. For the double-label experiments, one hemibladder of the pair was tied to a plastic cannula, apical side out, and the other was mounted basal-lateral side out and the radio-iodine was added to the outer solution. The labeling solution contained: 0.5 U/ml lactoperoxidase; 0.8 μg/ml glucose oxidase; 5 to 10 mM glucose; and 50 to 100 μCi/ml of ^{125}I-Na or ^{131}I-Na (molarities of 2×10^{-8} to 4×10^{-8} M) in a final volume of 50 to 60 ml of frog Ringer's solution [26]. Iodination was at room temperature for 30 min, and the reaction was stopped by transferring the hemibladders to frog Ringer's solution containing 0.1 mM NaI. After 3 to 4 washes with Ca^{++}-free, frog-Ringer's solution, the epithelial cells were scraped with a microscope glass slide and washed extensively with ice-cold Ca^{++}-free frog Ringer's solution by resuspension and sedimentation [26].

The labeled epithelial cells were homogenized in a medium containing: 5.7% sucrose (w/v), 1 mM EDTA, 1 mM $NaHCO_3$, 0.1 mM NaI, 5 mM Tris-HCl, pH 8.1, and 10 μg/ml gentamycin. Cells from one or two hemibladders were suspended in 2.2 ml of the "homogenizing medium" and given a single 5-sec burst with a polytron (Brinkman Instruments, Westbury, N.Y.) at various settings.

Fractionation Protocol

The scheme adopted for membrane isolation (Fig. 1) was as follows: All steps were carried out at approximately 4 °C. The homogenate was centrifuged at 700 × *g* for 15 min. The pellet (*P*1) was resuspended in one ml of homogenizing solution and recentrifuged at 700 × *g* for 15 min. This pellet (*P*2) was resuspended in a small volume (1 to 1.5 ml) of 2.2 M sucrose with 3 mM $CaCl_2$ and centrifuged at 400,000 × *g* for 15 min. The post-nuclear supernatants were applied to a 31-ml continuous (13 to 37%) sucrose gradient and spun until the rotor attained a speed of 100,000 × *g* (~6 min with the brake on) in an SW-25 rotor in the Model L Spinco ultracentrifuge (Beckman Instruments Co.). The gradients were collected in 0.7–1.5 ml fractions from the bottom with 60% sucrose, in an Isco Density Gradient Fractionator Model 183 (Instrumentation Specialties Co.) equipped with an Isco Absorbance Monitor, Model UA-5, and aliquots were assayed for radioactivity or enzymatic activity. The refractive index of the fractions was determined with an Abbe 3-L Refractometer (Bausch & Lomb Instruments, Rochester, N.Y.). The first 6 fractions (~9 ml) were pooled, diluted with homogenizing solution, and centrifuged at 100,000 × *g* for 1 hr in the SW-25 Rotor in the Model L. The resultant pellet was resuspended in 2.2 ml of the homogenizing solution and dispersed with the Polytron (setting 6 × 5 sec). This suspension was loaded onto a 31-ml continuous-discontinuous (13% → 30%:35%:55%) sucrose gradient and centrifuged at 100,000 × *g* for 16 hr in the SW-25 Rotor in the Model L. One and a half ml fractions were collected from the bottom, as described above.

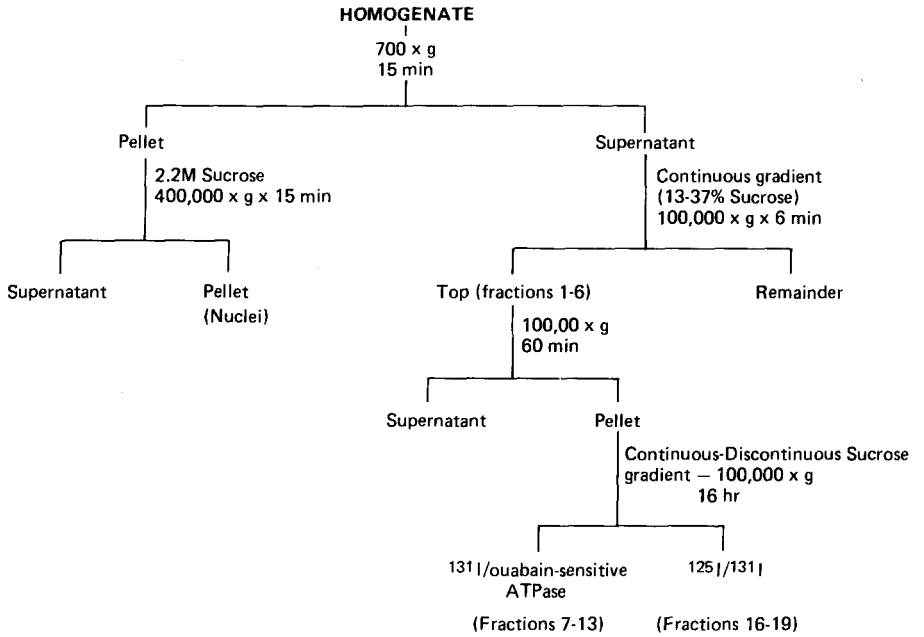

Fig. 1. Flow diagram of the method for isolation of the apical and basal-lateral plasma membranes of toad bladder epithelium. Eighty-five per cent of the radioactivity of the homogenate was recovered in the 700 × *g* supernatant (post-nuclear supernatant). No radioiodine was associated with the isolated nuclei

Sucrose Gradients

The continuous sucrose gradients, in 31-ml cellulose nitrate tubes, were prepared with a Beckman Density Gradient Former (Beckman Instruments, Inc.) at room temperature and cooled to ~4 °C for 1 hr prior to use. The continuous-discontinuous sucrose gradients, in 31-ml cellulose nitrate tubes, consisted of: 6 ml of 55% sucrose, 11 ml of 35% sucrose and 14 ml of a continuous gradient, 13–30% sucrose (prepared with the Gradient Former).

Analytical Methods

$(Na^+ + K^+)$-adenosine triphosphatase (NaK-ATPase) was assayed in the following medium (in mM): 100 NaCl, 10 KCl, 3 $MgCl_2$, 3 ATP (Tris or disodium salt), 5 NaN_3 and 50 Tris-HCl, pH 7.5, in a total volume of 1 ml. Incubation was for 15 min at 25 °C; the reaction was stopped by addition of 10% trichloro-acetic acid (TCA) (final concentration), the protein was separated by centrifugation at 10,000 × *g* for 10 min, and the released inorganic phosphate was measured in the supernatant by the method of Fiske and Subbarow [9]. The Mg-dependent ATPase (ouabain-insensitive) was estimated by adding 5 mM ouabain to the medium. The difference between the total and ouabain-insensitive activities was assumed to represent the NaK-ATPase [29].

Cytochrome Oxidase

This enzyme was assayed by a modification of the method of Wharton and Tzagoloff [32]. Cytochrome *c*, 100 mg, was dissolved in 3 ml of 75 mM potassium phosphate buffer, pH 7.2, with 25 mM sodium ascorbate. After 10 min at room temperature, the reduced cytochrome *c* was separated from ascorbate by gel filtration in Sephadex G-50. The fractions containing cytochrome *c* were pooled and the ratio of optical density at 550/565 nM was determined; reduction was considered adequate for assay purposes when the ratio was greater than 13. The assay was conducted at room temperature in 1-cm cuvettes. The mixture contained 75 mM potassium phosphate buffer, pH 7.2, 0.7 to 1 mg of reduced cytochrome *c*, 0.2 mg/ml lubrol, and appropriate enzyme concentrations. One mM potassium ferricyanide was added to the blank cuvette (all reagents except the enzyme preparation) and the reaction was monitored by determining the decrease in OD at 550 nM. The reaction rates were linear for 90 to 120 sec. Enzyme activity was expressed as the first-order rate constant for oxidation of the reduced cytochrome *c* relative to the oxidation by ferricyanide [25].

NADH-Cytochrome c Reductase

This enzyme was measured spectrophotometrically, at room temperature, by measuring the reduction of cytochrome *c* [18]. The assay mixture contained: (all in mM) 0.1 reduced nicotinamide adenosine dinucleotide (NADH), 0.1 cytochrome *c*, 0.3 KCN and 50 phosphate buffer, pH 7.5, with or without 10^{-6} M rotenone. The reaction was started by addition of the enzyme, and the increase in OD at 550 nM was recorded. Activities were calculated as described for cytochrome oxidase [25].

Protein and DNA

Protein contents were determined by the method of Lowry *et al.* [17], with bovine serum albumin (BSA) as the standard, and DNA by the method of Burton [4], with calf thymus DNA as the standard.

Morphology

Nomarski Optics. Epithelial scrapings were placed in a drop of homogenizing medium on glass slides, covered with cover slips, and examined unstained in a Zeiss Photomicroscope fitted with a 40×/0.65 objective for Nomarksi interference microscopy [22, 33]. The homogenates were examined by the same method at the same time.

Electron Microscopy. All steps were carried out at ~4 °C. Fractions from sucrose gradients were pooled and centrifuged at 100,000 × *g* for one hr. The pellets were resuspended in a small volume of homogenizing solution (0.2 to 0.4 ml), an equal volume of 1.5% glutaraldehyde in 100 mM sodium cacodylate, pH 7.5, was added and fixation allowed to proceed for 30 min. The fixed particulates were collected by centrifugation at 100,000 × *g* for 1 hr. The pellets were washed three times with 0.4 ml, 200 mM sodium cacodylate, pH 7.5, post-fixed in 2% osmium tetroxide in 100 mM sodium cacodylate, pH 7.5 and processed for electron microscopy as previously described [31].

Materials

Carrier-free ^{125}I-Na and ^{131}I-Na were purchased from New England Nuclear (Boston, Mass.); glucose oxidase and lactoperoxidase from Calbiochem (La Jolla, Ca.); crystalline sucrose, density gradient grade (ribonuclease-free), from Schwarz-Mann, (Orangeburg, N.Y.); adenosine triphosphate (ATP), ouabain, NADH, rotenone, cytochrome *c* (type III from horse heart), and lubrol-WX from Sigma Chemical Co. (St. Louis, Mo.). All of the conventional reagents were analytical or spectroquality grade.

Results

Optimal Conditions for Homogenization

Since little information is available on subcellular fractionation of toad bladder epithelial cells, conditions for homogenization were examined. Epithelial scrapings consist largely of sheets of intact cells with a few isolated intact cells and nuclei (Fig. 2, upper panel). Cell scrapings from two hemibladders were washed twice in 10 ml of chilled, Ca-free, frog Ringer's solution and homogenized in various media by a variety of methods. Cellular and nuclear rupture were monitored by Nomarski interference microscopy. The epithelial cells were resistant to rupture (i.e., <50% broken cells) in isotonic sucrose (250 mM), hypotonic bicarbonate (1, 5, 20 mM), or hypotonic Tris-HCL (5, 10, 50 nM) in glass-glass or Teflon-glass homogenizers used either manually or with a motor-driven pestle. In all

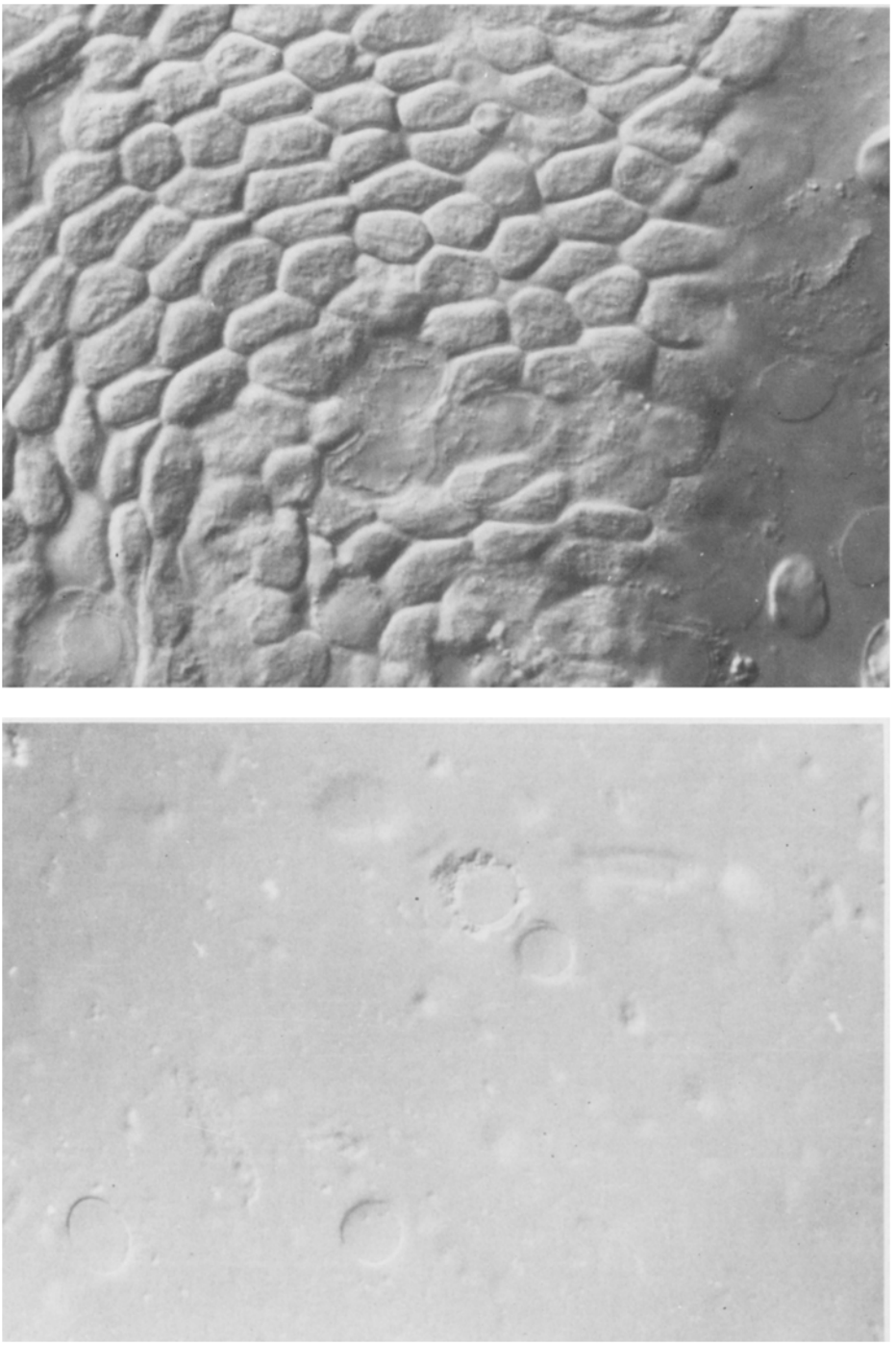

Fig. 2. *Upper panel:* Micrograph of scraped epithelial cells examined by Nomarski interference microscopy. The specimen consists of large sheets of intact epithelial cells and a few isolated nuclei (800×). *Lower panel:* Micrograph of toad bladder epithelial cells examined by Nomarski interference microscopy after homogenization. The scraped cells were homogenized by a single 5-sec burst with the polytron at setting 6. Intact, slightly swollen nuclei are seen; virtually all cells were ruptured (800×)

of these media, sonication with a single burst, at setting 6 of the Branson Sonifier fitted with the microtip, ruptured all of the cells; however, all nuclei were also broken. In the Polytron, a single 5-sec burst at setting 10 ruptured ~50% of the cells suspended in 1 mM $NaHCO_3$ or 5 mM Tris-HCL, pH 8.1, and 100% of the cells suspended in 175 mM sucrose while many nuclei remained intact (Fig. 2, lower panel). On the basis of these results, the following homogenizing medium was chosen (in mM): 175 sucrose, 1 $NaHCO_3$, 1.5 EDTA, 5 Tris-HCl, pH 8.1, and 10 μg/ml gentamycin. The cells were homogenized with a single 5-sec burst at various settings of the Polytron. These homogenates were centrifuged at 700 × *g* for 15 min to sediment unbroken cells, nuclei, and cell debris, and DNA was assayed in both fractions. Virtually all cells were ruptured at Polytron settings of 6–10 as assessed by Nomarksi optics (Table 1). At setting 4, however, ~34% of the cells were intact. At setting 6, 86% of the DNA was recovered in the pellet, whereas at higher settings significantly greater nuclear rupture was encountered as indicated by the lower recoveries of DNA. Thus, a single 5-sec burst at setting 6 was chosen for all subsequent procedures.

Sucrose Gradient Fractionation

To define the distribution pattern of the apical components labeled covalently with ^{125}I in density gradient, the post-nuclear supernatants were analyzed in 31 ml continuous-discontinuous sucrose gradients. NaK-ATPase and cytochrome oxidase activities were used as plasma membrane (basal-lateral) and mitochondrial markers, respectively. The bound ^{125}I was distributed in two discrete peaks; the fast-moving fraction was close to the bottom of the gradient (Fig. 3, top panel). Almost all of the NaK-ATPase activity was confined to a sharp peak in the mid-range that coincided with a minor ^{125}I component and did not overlap with either of the major ^{125}I peaks. The fast-moving ^{125}I peak coincided with a very small NaK-ATPase component.

Table 1. Analysis of homogenization of toad bladder epithelium

Polytron setting	DNA in homogenate (mg/ml)	DNA in 700 × *g* pellet (%)
10	0.339 ± 0.47	47.8 ± 3.7
8	0.359 ± 0.014	68.0 ± 2.9
6	0.397 ± 0.036	85.5 ± 2.2
4	0.384 ± 0.037	91.1 ± 3.8

Cells from two hemibladders were washed twice in Ca-free frog Ringer's solution by centrifugation at 700 × *g* for 10 min. The cells were resuspended in 2.2 ml of 175 mM sucrose, 1.5 mM EDTA, 1 mM $NaHCO_3$ and 5 mM Tris-HCl, pH 8.1, and homogenized by a single 5-sec burst at various settings of the polytron. Cell rupture was monitored by Nomarski interference microscopy. The homogenate was centrifuged at 700 × *g* for 15 min. DNA was determined in the homogenate and in the supernatant and pellet after centrifugation. Virtually all cells were ruptured at settings 6–10 (Nomarski optics); at setting 4 there was a large proportion (30 to 40%) of intact cells. The nuclei were intact and slightly swollen. The values represent the means ± SE of 4 experiments.

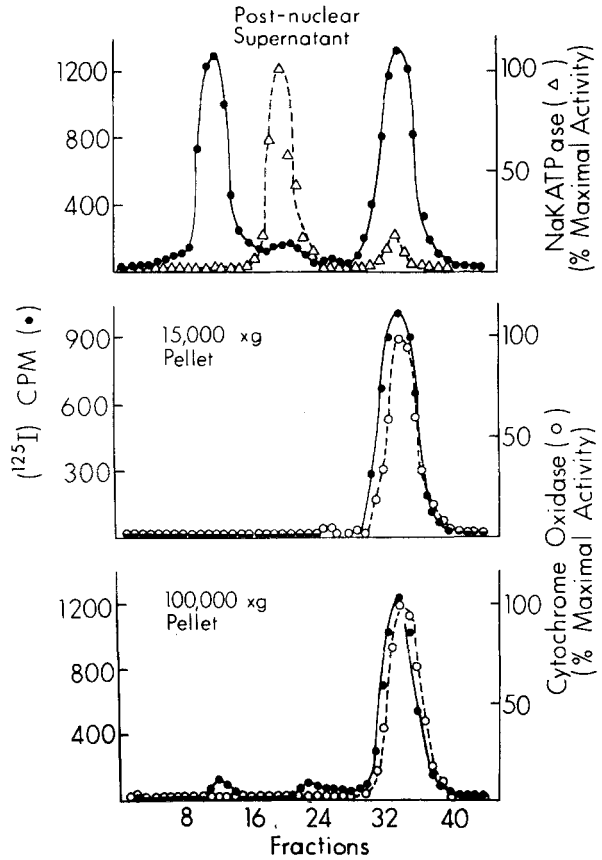

Fig. 3. Fractionation of post-nuclear supernatants of epithelium labeled on the apical side. The post-nuclear supernatants (700 × *g* for 15 min) of cells labeled with ^{125}I on the apical side were centrifuged at 15,000 × *g* for 30 min or 100,000 × *g* for 1 hr. The pellets were resuspended and loaded onto 31-ml continuous-discontinuous sucrose density gradients (from bottom to top: 55%/35%/30% → 13%). After centrifugation at 100,000 × *g* for 16 hr, the gradients were collected and ^{125}I radioactivity (●), NaK-ATPase activity (△), and cytochrome oxidase activity (○) were determined. Fraction volume = 2 ml

In the previous study [26] evidence was obtained that apical labeling included a TCA-soluble macromolecule with the properties expected of the glycocalyx, including recovery of this component is nonmembranous was assessed by two methods: sedimentation and electron microscopy. The post-nuclear supernatants were centrifuged at either 15,000 × *g* or 100,000 × *g* for 1 hr; the pellets were resuspended in the homogenizing medium and analyzed in the continuous-discontinuous gradients. The slow component was not recovered in either of these pellets, although the fast component was recovered quantitatively (Fig. 3, middle and lower panels). Moreover, no membranous elements were found in the slow peak on either negative staining or on direct staining and examination by electron microscopy. These results suggest that the slow component contains ^{125}I-labeled glycocalyx. The fast component contained about 50% of the ^{125}I-labeled

material, presumably apical plasma membrane, and substantial cytochrome oxidase activity indicating comigration of mitochondria (Fig. 3, middle and lower panels).

The basal-lateral surface material labeled with ^{125}I-Na was also analyzed in continuous-discontinuous sucrose gradients. Since epithelial scrapings contain basement membrane, labeling from the serosal side was expected to yield a heterodisperse pattern in the gradients [26]. Sedimentation analysis of post-nuclear supernatants of cells labeled on the basal-lateral aspect with ^{125}I-Na in a continuous-discontinuous sucrose gradient yielded three major bands of radioactivity (Fig. 4). The middle peak coincided with the major NaK-AtPase band (Fig. 4, upper panel). The fast-moving band (peak in fraction 34) sedimented at about the same rate as the fast-moving apically labeled component (*cf.* upper panels of Fig. 3 and 4). When the post-nuclear supernatants were centrifuged at 15,000 × *g* at *g* for 1 hr and the resulting pellets were analyzed in the continuous-discontinuous gradients, almost all the ^{125}I content of the slow band and about 50% of the middle band were lost, whereas the fast band was almost completely recovered in these pellets (Fig. 4, middle and lower panels). The distribution of the activity of cytochrome oxidase was similar to the one obtained in gradients of labeled apical membranes (*cf.* Figs. 3 and 4).

Separation of Mitochondria from Labeled Plasma Membranes

The experiments shown in Figs. 3 and 4 demonstrated that after prolonged centrifugation (16 hr), the activity of cytochrome oxidase, and presumably the mitochondria, were contaminating the radioactive bands corresponding to fast-moving components of both apical and basal-lateral membranes. To separate mitochondria, prior to fractionation of the plasma membranes, an isokinetic method was developed. The post-nuclear supernatants of epithelial cells, simultaneously labled (in separate, paired hemibladders) with ^{125}I-Na (apical) and ^{131}I-Na (basal-lateral) were applied to continuous (13 to 37%) sucrose gradients and centrifuged to a speed of 100,000 × *g* (~6 min) in swinging bucket rotors. Almost all (~85%) of the cytochrome oxidase activity was recovered in fractions 7–24 and ~90% of the ^{125}I and ^{131}I labeled components were recovered in fractions 1–6 (Fig. 5, upper panel). Approximately 90% of total NaK-ATPase activity was also recovered in fractions 1–6 (Fig. 5, lower panel). Separation of the mitochondria from labeled plasma membranes was also assessed by measuring the activity of NADH-cytochrome *c* reductase. The rotenone-sensitive reductase is one of the respiratory chain-linked enzymes and is located in the inner mitochondrial compartment [15, 30]; the rotenone-insensitive reductase is concentrated in the outer membrane and resembles stereochemically the NADH-reductase enzyme of microsomes [23, 30]. In the isokinetic gradient (~6 min), the activity of the rotenone-sensitive reductase coincided with the distribution of cytochrome oxidase, whereas the activity of the rotenone-insensitive enzyme coincided with the distribution of ^{125}I radioactivity (Fig. 6). These results confirm the utility of the isokinetic gradients in separating plasma membranes from the mitochondria and indicate that the slow components contain either outer mitochondrial or microsomal membranes or both, but not inner mitochondrial constituents.

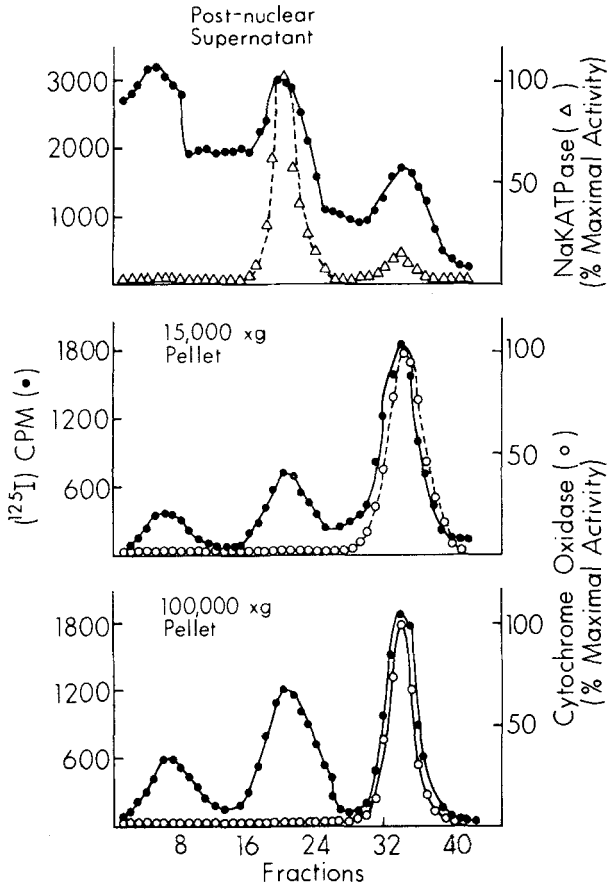

Fig. 4. Fractionation of post-nuclear supernatants of epithelium labeled on the basal-lateral side. The post-nuclear supernatants (700 × *g* for 15 min) of cells labeled with ^{125}I on the basal-lateral side were divided in three portions: one was applied to a 31-ml continuous-discontinuous gradient (55%/35%/30% → 13%), centrifuged at 100,000 × *g* for 16 hr, collected, and the fractions were analyzed for ^{125}I radioactivity (●), NaK-ATPase activity (△), and cytochrome oxidase activity (○). The other two portions were centrifuged at 15,000 × *g* for 30 min and 100,000 × *g* for 1 hr; the pellets were resuspended and analyzed in similar gradients under identical conditions. Fraction volume = 2 ml. All experimental details are given in the text

Isolation of Plasma Membranes in Isopycnic Sucrose Gradients

Based on the information described above, a protocol was designed for the isolation of labeled plasma membranes separated from nuclear and mitochondrial elements. In addition, subfractionation of the differentially labeled apical and basal-lateral components in isopycnic sucrose gradients was evaluated. The complete protocol is summarized in Fig. 1. the nuclei and mitochondria were separated from the plasma membrane fraction by succesive centrifugation at 700 × *g* for 15 min and in a continuous sucrose gradient to speed (100,000 × *g* for ~6

min. The supernatant of the isokinetic gradient centrifugation was cleared of labeled glycocalyx by sedimentation at 100,000 × *g* for 1 hr. These pellets were then analyzed in isopycnic sucrose gradients (continuous-discontinuous, 100,000 × *g* × 16 hr) (Fig. 7). The epithelial cells were simultaneously covalently labeled with ^{125}I from the apical and ^{131}I from the basal-lateral sides. the distribution patterns indicate that minor ^{125}I and ^{131}I-labeled components, with no associated NaK-ATPase, are collected in the first 6 fractions at the top of the gradient (residual slow-moving peaks). The bulk of the NaK-ATPase and about 1/3 of the ^{131}I-labeled components were isolated in the mid-range (fractions 7–13). A minor ^{125}I component was also recovered in these fractions. The remainders of the NaK-ATPase (10 to 15%) migrated with the fast peak that contained almost all of the bound ^{125}I and about 60% of the bound ^{131}I (fractions 16–19). These results suggest that the basal-lateral membranes containing NaK-ATPase are well-resolved from the apical membranes by this system. The residual NaK-ATPase that comigrates with the bulk of the apical membranes may represent contamination

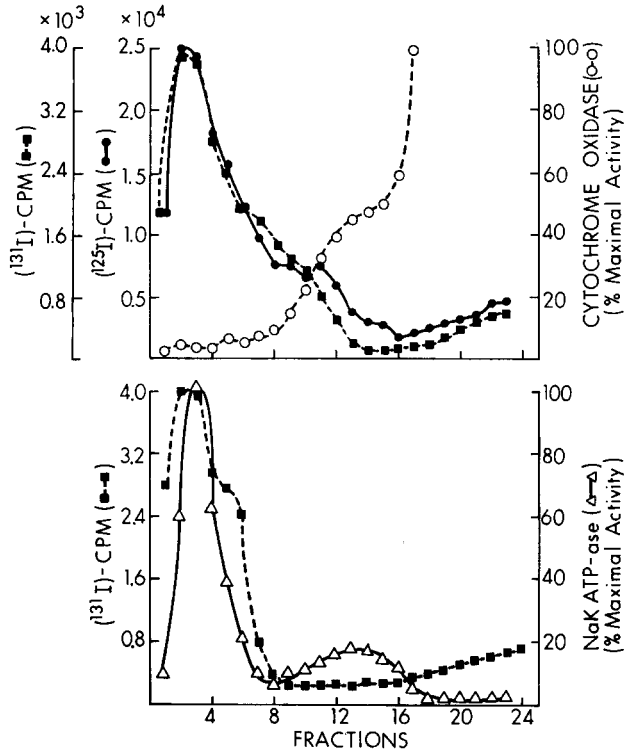

Fig. 5. Distribution of apical and basal-lateral radio-iodine labeled components and enzyme activities in sucrose density gradients. Paired hemibladders were labeled on the apical and basal-lateral side with ^{125}I and ^{131}I, respectively. The epithelial scrapings were pooled, homogenized, and 2.5 ml of post-nuclear supernatants were loaded onto a 31-ml continuous (12% to 37%) sucrose density gradient. The gradients were centrifuged until a speed of 100,000 × *g* (~6 min) was attained. The fractions (2 ml each) were assayed for ^{131}I (■), ^{125}I (●), cytochrome oxidase (○), and NaK-ATPase (△)

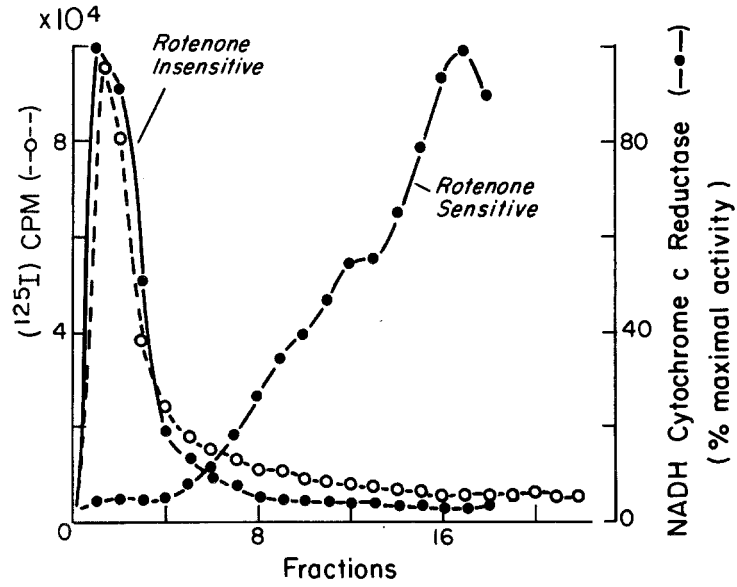

Fig. 6. Distribution of apical ^{125}I-labeled components and activity of NADH-cytochrome *c* reductase in continuous sucrose density gradients. Experimental conditions as described in the legend to Fig. 5. The fractions obtained under isokinetic conditions (to attain a speed of ~100,000 × *g*, ~6 min) were analyzed for ^{125}I (○) and the activity of NADH-cytochrome *c* reductase (●) (in the presence and absence of 10^{-6} M rotenone)

with heavier fragments of basal-lateral membranes or a low abundance of NaK-ATPase in the apical membrane.

Electron Microscopy of Subcellular Fractions

The final gradient used in membrane isolation (after separation of the mitochrondria) (*cf.* Fig. 7) was examined with the electron microscope. Fractions from three different regions of the gradient were pooled as follows: region *I* (fractions 1 through 5), region *II* (fractions 6 through 13) and region *III* (fractions 14 through 21). The pooled fractions were centrifuged at 100,000 × *g* for one hr and processed for electron microscopy. Region *I* did not yield a visible pellet, and negative staining of this region prior to centrifugation failed to disclose the presence of membranous elements. These results confirm the inference that the labeled glycocalyx bands in this position. Regions *II* and *III* produced readily visible pellets, and when examined with the electron microscope vesicular structures resembling plasma membranes were recognized (Figs. 8 & 9). No other subcellular organelles (mitochondria, lysosomes, Golgi) were seen. These vesicular elements cannot be unequivocally identified as plasma membrane fragments, in the absence of distinctive morphologic characteristics, identifiable by electron microscopy [10, 20]. Moreover, the coincidence of these vesicular elements with the membrane markers used in this study does not exclude the presence of unlabeled vesicular elements of nuclear, mitochondrial, or lysosomal origin which would

have a similar microscopic appearance. Nevertheless, the recovery of the bound radio-iodine in regions *II* and *III* indicates that some of the membranous elements are derived from plasma membranes.

Discussion

Although the structural organization and electrophysiological properties of toad bladder epithelium kave been extensively studied by several groups of investigators, few detailed studies on subcellular fractionation are available [11]. In the first study on membrane isolation, neither optimal conditions for homogenization nor contamination with other subcellular organelles were defined [11]. Histochemical analysis of the toad bladder provided identification of mitochondrial and glycolytic enzymes [14]. The conventional membrane marker 5′-nucleotidase was very prominent in serosa, smooth muscle, and capillaries, but the epithelial cells exhibited very low activity which appeared to reside inside the cells and was not limited to the plasma membrane [14]. Attempts to localize the membrane-associated ATPase have yielded conflicting results. Keller [14] concluded that membrane-bound ATPase was confined to the basal-lateral boundary, but other stud-

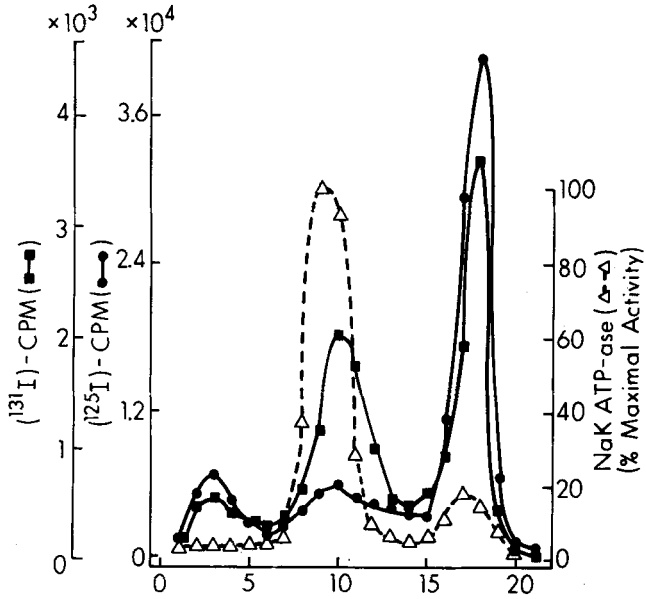

Fig. 7. Isolation of apical ^{125}I-labeled and basal-lateral ^{131}I-labeled components and activity of oubain-sensitive ATPase in sucrose density gradients. The post-nuclear supernatants of cells labeled on the apical side with ^{125}I and on the basal-lateral side with ^{131}I were fractionated in a 31-ml continuous (13 to 37%) sucrose density gradient at 100,000 × *g* for ~6 min. The first 6 fractions (approximately 9 ml) were pooled, diluted, and centrifuged at 100,000 × *g* for 1 hr. The resulting pellet was resuspended and fractionated in a continuous-discontinuous (55%/35%/30% → 13%) sucrose density gradient at 100,000 × *g* for 16 hr. The fractions (2 ml each) were analyzed for ^{131}I (■), ^{125}I (●) and NaK-ATPase activity (△)

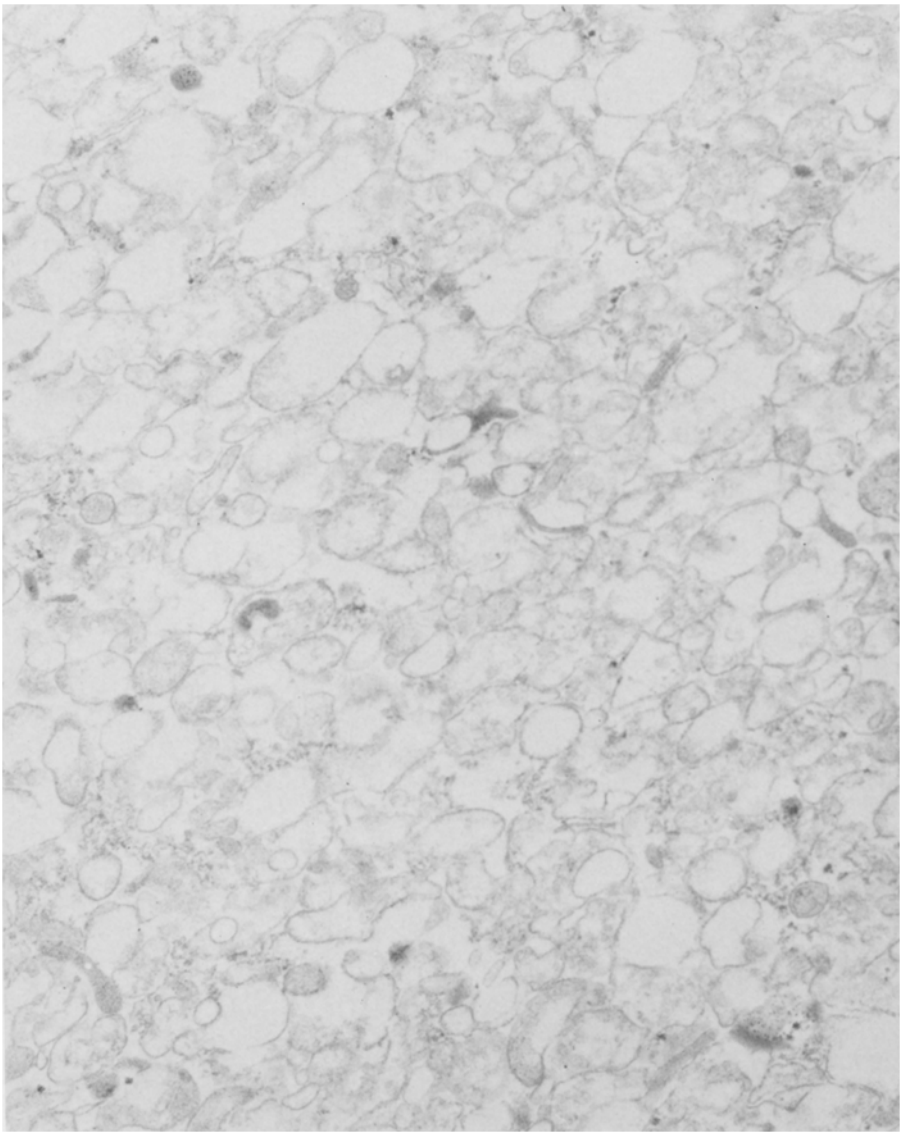

Fig. 8. Electron micrographs of membrane fractions (Region *II*) isolated in sucrose density gradients. Epithelial scrapings labeled with ^{125}I from the apical and ^{131}I from the basal-lateral sides were processed as shown in Fig. 1. Fractions 6 through 13 (Region *II*) of the density gradients shown in Fig. 7 were pooled and centrifuged at 100,000 × *g* for 1 hr. The pellets were fixed in glutaraldehyde, post-fixed in osmium tetroxide, embedded in Epon 812, and cut into 1-μm sections. The sections were examined in a Phillips EM 300 electron microscope. Magnification 39,900×

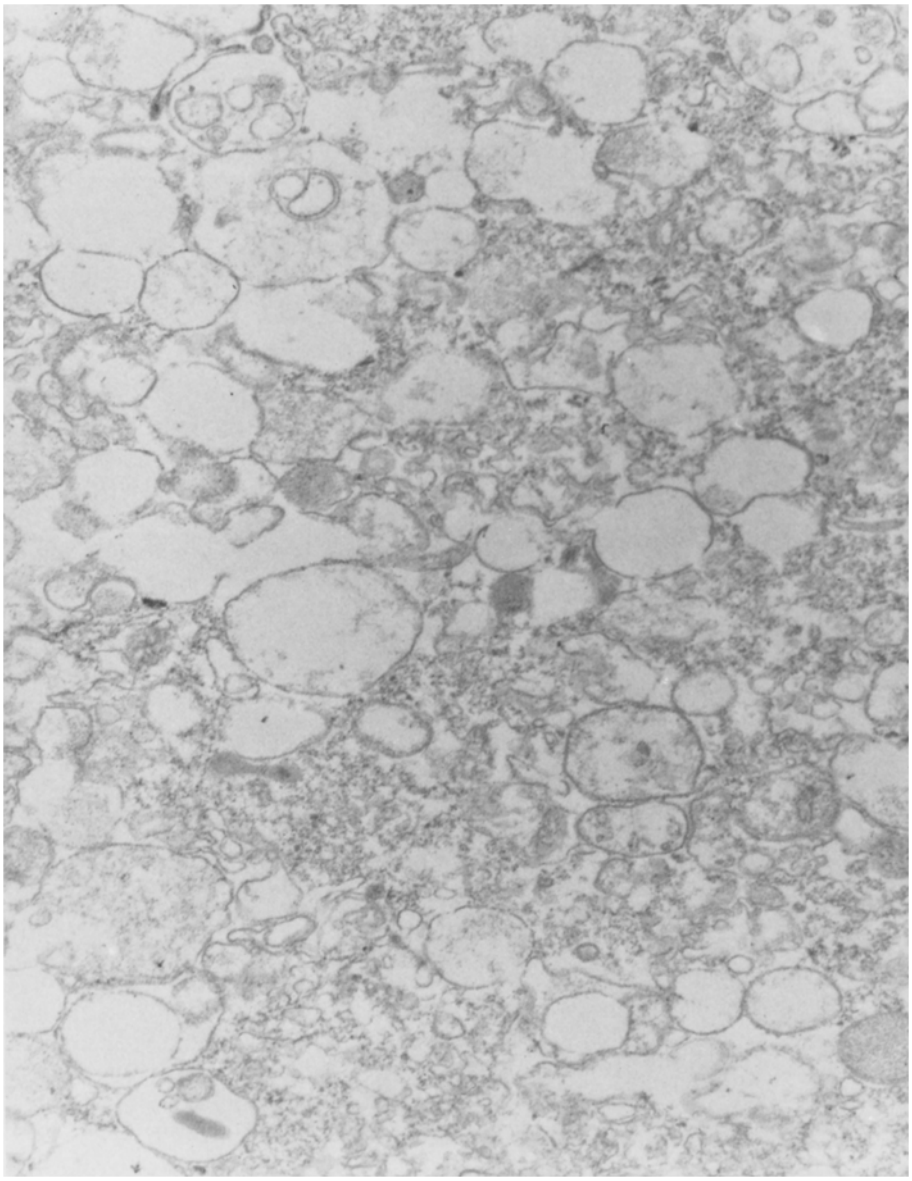

Fig. 9. Electron micrographs of membrane fractions (Region *III*) isolated in sucrose density gradients. The labeling and processing of the epithelial scrapings was as described in the legend of Fig. 8. Fractions 14 through 21 (Region *III*) of the density gradients shown in Fig. 7 were pooled and processed for electron microscopy as described in the legend of Fig. 8. Magnification 39,900×

ies identified ATP-hydrolyzing activities on the luminal membrane as well [1, 21]. This limited and in some respects contradictory information on the histochemistry of the toad bladder complicates the task of membrane isolation in this epithelium, based on enzymatic markers during subcellular fractionation. Moreover, we have obtained evidence that the epithelial cells of the toad bladder possess very low relative activities of enzymes commonly used as markers of subcellular organelles [25], e.g., glucose-6-phosphatase (an endoplasmic reticulum marker) and alkaline phosphatase and 5′-nucleotidase (conventional plasma membrane markers) [6, 7]. In contrast, the activities of mitochondrial enzymes (cytochrome oxidase, NADH-cytochrome *c* reductase) and a glycolytic enzyme (hexokinase) were readily detected [25]. These results are consistent with data obtained by histochemical analysis [14]. Of special interest is the observed difference between the activities of alkaline phosphatase and hexokinase. These enzymes are considered to be markers for segments of the mammalian nephron: alkaline phosphatase for the proximal segment and hexokinase for the distal segment [2, 27, 28]. The fact that the toad bladder appears to have a high relative content of hexokinase and essentially no alkaline phosphatase may be a biochemical expression of the physiological and embryological similarities between the toad bladder and the distal mammalian nephron [16].

The conventional approach of differential centrifugation to membrane isolation was not successful in our first attempts with toad bladder epithelium. Mitochondrial contamination was a major problem which led to membrane aggregation and further hampered purification attempts. Accordingly, we resorted to the use of sucrose density gradients. Fractionation of the radio-iodine labeled apical components on these gradients yielded a clear-cut separation of the glycocalyx and luminal plasma membrane. Separation of mitochondria from apical and basal-lateral label was readily accomplished in sucrose-density gradients by an isokinetic method.

The heterodisperse pattern obtained with radio-iodine labeling of basal-lateral components dictated the use of enzymatic markers to identify radioactive band(s) associated with the plasma membrane. Evidence from electrophysiological studies is consistent with the presence of a ouabain-sensitive, active transport mechanism for Na extrusion at the basal-lateral border of toad bladder epithelial cells [8, 13, 16]. In addition, histochemical studies suggest the presence of ATP-hydrolyzing activities at the basal-lateral border of the epithelium [1, 14]. Thus, a ouabain-sensitive ATPase may be a marker for basal-lateral membranes. The occurrence of a $Na^+ + K^+$-activated ouabain-sensitive ATPase in the basal-lateral plasma membrane is a common property of other epithelial membranes with transport characteristics similar to those of the toad bladder [12, 19].

The fractionation scheme shown in Fig. 1 enabled isolation of two components. The intermediate band labeled with ^{131}I derived from the basal-lateral surface contained almost all of the NaK-ATPase activity. The fast-moving band contained most of the apical ^{125}I-label bound to membranes and about half of the ^{131}I bound to basal-lateral components but was almost devoid of NaK-ATPase activity. This method provides a significant separation of plasma membranes from nuclei and mitochondria and substantial partial purification of the apical and basal-lateral plasma membrane surfaces. The extent of contamination of

these fractions by membranes derived from endoplasmic reticulum, Golgi or lysosomes is unknown, however.

The described method allows rapid isolation and separation of the plasma membranes of the epithelial cells of the toad bladder without the need for elaborate and costly equipment. One difficulty with toad bladder epithelium, that should be emphasized, relates to the relatively low quantities of starting material available under ordinary experimental conditions, which precludes analysis of multiple enzymatic activities in the same preparation. This method, however, may prove to be useful in ascertaining the localization of molecular components, such as hormone receptors or structural constituents of ion specific pathways, that can be identified by various approaches.

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