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Radio-Iodination of Plasma Membranes of Toad Bladder Epithelium

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Summary. The present report describes high yield enzymatic radio-iodination of the apical and basal-lateral plasma membranes of toad bladder epithelium, by a procedure that does not breach the functional integrity of the epithelium, as assessed by the basal and vasopressin-sensitive short-circuit current (SCC). Restriction of the label to the membrane surface was ascertained by light and electron-microscopic autoradiographs. On the apical surface, the grains were over the glycocalyx and the plasma membrane. Analysis of the labeled glycocalyx by agarose gel filtration, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as well as enzymatic and pH-dependent hydrolysis indicated that the glycocalyx is a trichloro-acetic acid-soluble macromolecular complex of high molecular weight composed of a peptide moiety attached to large prosthetic groups (presumably carbohydrates) by O-glycosidic bonds. Analysis of the labeled apical plasma membrane components by agarose gel filtration and SDS-PAGE disclosed the presence of six major species of apparent molecular weights: 23,000, 28,000, 37,000, 44,000, 68,000, and 95,000. More than half of the membrane-associated radio-iodine was in two bands of molecular weights 37,000 and 44,000.

Concentrations of vasopressin and cyclic AMP sufficient to increase the SCC significantly did not modify the extent of membrane labeling or the distribution of the label among the apical membrane components (presumably proteins) as assessed by SDS-PAGE. Iodination in the presence of amiloride inhibited incorporation but did not change the pattern of the distribution of the label among the components resolved by SDS-PAGE.

Iodination of basal-lateral plasma membranes, at a yield comparable to that obtained with apical labeling, was attained after about 30 min of exposure of the intact bladder to the labeling solutions. Approximately 25% of the basal-lateral labeling was lost when the epithelial cells were harvested after collagenase treatment, implying that some iodination of the basement membrane had taken place. Less than 10% of iodination of the apical or basal-lateral surfaces was accounted for by lipid-labeling. Analysis of the labeled apical

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and basal-lateral species by enzymatic digestion and thin layer chromatography disclosed that virtually all the radioactivity was present as mono-iodotyrosine (MIT).

Transepithelial transport of NaCl and water apparently depends on the unique structural and functional properties of the apical and basal-lateral plasma membranes. In the toad bladder and frog skin, for example, electrophysiological studies suggest that the apical boundary constitutes a saturable diffusion barrier, whereas the basal-lateral border contains an active transport system, the Na^+ pump [8,16,17]. Definition of the chemical determinants of the properties of the two surfaces should be facilitated by isolation and purification of these components. Differential, covalent labeling of the apical and basal-lateral plasma membranes of epithelia should aid in their separation and purification. In addition, covalent labeling of these membranes may provide information on the nature of exposed proteins that function as part of specific ion permeation pathways, hormonal receptors, transport enzymes, or surface antigens.

In a previous study [40], the luminal surface of toad bladder epithelium was radio-iodinated with ^{125}I by the enzymatic method of Phillips and Morrison [28], as modified by Hubbard and Cohn [12]: The label was entirely confined to the plasma membrane, assessed by electron-microscopic autoradiography [40]. The enzymatic radio-iodination method has also been used in analysis of membrane proteins of other epithelia (e.g., small intestinal and gastric mucosal cells) [3, 18, 35].

In addition to radio-iodination, the apical and basal-lateral surfaces of toad bladder epithelium have been differentially covalently labeled with ^{125}I-diazo-diiodosulfanilic acid and pyridoxal phosphate + ^{3}H-$NaBH_4$ [6]. In these, as well as the studies with enzymatic radio-iodination, labeling of the surfaces did not perturb the basal or vasopressin-stimulated short-circuit current (SCC) of the toad bladder [6, 40]. The radioactive yields of the labeled membranes, however, were too low for biochemical characterization of the labeled product and limited the utility of these procedures for isolation of the separated surfaces.

The present study concerns definition of the conditions required for high yield, differential, radio-iodination of the apical and basal-lateral surfaces of toad bladder epithelium. The functional consequences of labeling of these surfaces was assessed by the SCC method, including challenge with vasopressin. The products were characterized by solvent extraction, amino acid analysis, sodium dodecyl sulfate (SDS) agarose gel chromatography and SDS polyacrylamide gel electrophoresis.

Materials and Methods

Tissue Preparation

All of the experiments were performed on urinary bladders of female toads of Colombian and Dominican origin. The animals were maintained at room temperature in

tanks with running tap water and were starved for three to fifteen days before the experiments. The toads were double-pithed, and each animal was perfused, via the ventricle, with 200 to 400 ml of frog-Ringer's solution of the following composition: NaCl, 90 mM; $NaHCO_3$, 25 mM; KCl, 3 mM; $CaCl_2$, 1 mM; KH_2PO_4, 0.5 mM; $MgSO_4$, 0.5 mM and gentamycin 10 μg/ml. The pH was adjusted to 7.8 ± 0.1 by bubbling with a gaseous mixture of 97% O_2/3% CO_2. The osmolarity was 230 ± 10 mosM/kg H_2O. The bladders were tied, as sacs, to the end of plastic cannulae with either the apical (mucosal) or basal-lateral (serosal) side facing the outside. The bladder sacs were filled with 5 ml of Ringer's solution containing 10 mM glucose and incubated at room temperature for 30 to 60 min with vigorous stirring of the outside solution. This step was required to wash away mucous or other substances overlying the bladder surfaces. The bladders were then iodinated and analyzed biochemically or by measurements of the PD and SCC.

Iodination

Iodination was achieved with the glucose, glucose oxidase, lactoperoxidase system, which has the advantage of generating a continuous micromolar supply of H_2O_2 without accumulation of damaging amounts of peroxide [12, 40]. The labeling solution contained the following substances, unless indicated otherwise: 0 to 10 mM glucose, 0 to 0.4 units/ml lactoperoxidase, 0 to 0.5 μg/ml glucose oxidase, 10 to 100 μCi/ml ^{125}I-Na, 4×10^{-9} to 4×10^{-8} M, and frog-Ringer's solution in a final volume of 50 to 60 ml. The outside solution was gassed with 97% O_2, 3% CO_2. In the control incubations, glucose, glucose oxidase or lactoperoxidase were omitted. The reaction was started by addition of one of the reagents, usually glucose oxidase; hemibladders tied to plastic cannulae were quickly immersed into the labeling solutions and the reaction allowed to proceed for 5 to 30 min, with continuous magnetic stirring. When the solutions were not vigorously stirred, the extent of labeling was unsatisfactory. The reaction was terminated by transferring the bladders to Ca-free frog Ringer's solution containing 0.1 mM NaI. The mucosal and serosal bathing solutions were changed 3 to 4 times, the bladders were removed from the cannulae, gently blotted, and stretched on a Lucite block covered with parafilm, mucosal side up. The epithelial cells were scraped with a microscope glass slide and harvested into 10 ml of chilled Ca-free, 0.1 mM NaI, frog Ringer's solution. All subsequent steps were carried out at 4°C. The epithelial scrapings were washed three times in the same solution by centrifugation at 1,000 × *g* for 10 min. The final pellet was resuspended in 5 ml of frog Ringer's solution and sonicated at setting 6, with 2 to 3 five second bursts with a microtip Branson Sonifier. Two ml of the sonicates were assayed for DNA content by the method of Burton [4] and for protein content by the method of Lowry [20]. The yield of radio-iodination was assayed by adding an equal volume of 20% ice-cold trichloro-acetic acid (TCA) to the sonicates. The precipitates were collected by centrifugation at 10,000 × *g* for 10 min and washed 3 times with ice-cold 10% TCA containing 0.1 mM NaI. The final pellet was dissolved in 1 N NaOH and the radioactivity assayed in a Packard gamma spectrometer (Packard Instruments Co., Inc., Downers Grove, Ill.).

Autoradiography

After iodination the hemibladders were washed 3 to 4 times in frog Ringer's solution, removed from the cannulae, carefully mounted over Lucite rings, 2.54 cm in diameter,

and fixed in 1.5% glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.5) for 1 hr at room temperature. The bladders were then washed in ice-cold 200 mM sodium cacodylate buffer (pH 7.5) for 18 to 20 hr and post-fixed in 2% osmium tetraoxide in 100 mM sodium cacodylate (pH 7.5). The tissues were dehydrated in a graded series of aqueous ethanol solutions and embedded in Epon 812, as previously described [40]. Sections were cut for light and electron microscopic autoradiography, coated with Ilford L4 emulsion (Ilford Ltd., Ilford, Essex, England), exposed for 1 to 3 weeks, stained with 1% toluidine blue for light microscopy or with 0.5% uranyl acetate in 30% ethanol, for electron microscopy, and developed as previously described [40].

Electrophysiological Measurements

The effect of iodination on the functional capacity of the tissue was assessed by measuring the transepithelial SCC and PD across isolated hemibladders mounted as sacs [42]. The SCC was measured continuously except for brief interruptions of 5 sec every 10 to 15 min for the measurement of the transepithelial PD. After an equilibration period of 1 hr, the bladders were iodinated and 1 hr thereafter challenged with vasopressin (20 mU/ml final concentration) or the diluent added to the serosal bathing solution.

Preparations for Analysis of Labeled Components

The labeling conditions for these preparations as well as for SDS-agarose gel filtration chromatography and SDS-polyacrylamide gel electrophoresis (PAGE) were as follows: 0.4 U/ml lactoperoxidase, 0.4 μg/ml glucose oxidase, 10 mM glucose, 3.2×10^{-8} M NaI (^{125}I, 80 μCi/ml) for 30 min at 25°C.

The labeled epithelial scrapings were resuspended in the "homogenizing solution" that contained sucrose, 5.7 g/100 ml; EDTA, 1.5 mM; $NaHCO_3$, 1 mM; NaI, 0.1 mM; Tris HCl, 5 mM, (pH 8.1); gentamycin, 10 μg/ml; and lima bean trypsin inhibitor, 0.1 μg/ml (to limit losses due to endogenous protease). The resuspended cells were dialyzed, in cellulose tubing, against 200 volumes of the homogenizing solution at 4°C for 16 hr and then homogenized at setting 6 with a single 5-sec burst in a Polytron (Brinkmann Instruments). Virtually all of the cells were disrupted, as assessed by Nomarski Interference Microscopy [30]. To ensure binding of the ^{125}I to membrane components, the homogenates were centrifuged at 100,000 × *g* for 60 min, in an SB-283 rotor in the B-60 IEC ultracentrifuge. Aliquots of the pellets (particulate fraction) and supernatants (cytosol fraction) were assayed for ^{125}I content as described above. Additional aliquots of the supernatant and pellets were made up to 10% TCA (v/v) and centrifuged at 10,000 × *g* for 30 min in an RC-B Sorvall centrifuge. The resultant pellets were assayed for ^{125}I activity as described above.

Gel Filtration Chromatography

Homogenates (labeled and prepared as described in the "Preparations" section) were processed by washing the pellets once with an equal volume of the homogenizing solution and the supernatants by dialysis in cellulose tubing, against 200 volume of the "homogenizing solution" for 16 hr at 4°C. These fractions were then made up to 1% SDS (w/v), 5 mM mercaptoethanol, and 1.5 mM EDTA and heated for 2 min at 100°C. The solutions

were then loaded onto 5 M Agarose (50 × 1.1-cm column) previously equilibrated with: SDS, 1% (w/v); glycine, 200 mM; Tris HCl, 100 mM, pH 8.8. The samples were eluted with the same solution as the equilibration buffer at room temperature. The columns were calibrated with Blue-Dextran (mol. wt $\geqq 2 \times 10^6$) and myoglobin (mol. wt = 17,000) [7]. The fractions, 0.5 ml each, were analyzed for ^{125}I activity (*see* above).

SDS-Polyacrylamide Gel Electrophoresis

The labeled homogenates (*see* "Preparations" section) were dialyzed against 200 volumes of the homogenizing solution for 16 hr at 4°C. The dialyzed suspensions were centrifuged at 100,000 × *g* for 1 hr in the SB-283 rotor. The pellets (particulate fractions) were resuspended in 0.1 to 0.2 ml of the same solution; the supernatants (cytosol fractions) were concentrated by lyophilization and redissolved in a similar volume. All samples were solubilized in 1% SDS, 5 mM mercaptoethanol, 1.5 mM EDTA, 10% glycerol (v/v), heated to 100°C for 2 min and then applied to 5 mm × 100 mm SDS-Tris-glycine-polyacrylamide gels [26]. The total acrylamide concentration ranged from 7% to 12% and the ratio of acrylamide to N, N-methylene-bis-acrylamide (cross-linking) was varied between 20:1 and 37:1. Electrophoresis was carried out at constant current set to deliver 0.5 W per gel, for 4 to 5 hr. The chamber was cooled to 15°C. The gels were fixed and stained for 1 hr in a solution of 2.5% Coomasie Blue, 50% methanol, 10% acetic acid; destained in 7.5% acetic acid, sliced, and the radioactivity associated with each slice counted in a Packard Gamma Spectrometer (Packard Instruments Co., Inc., Downers Grove, Ill.). The molecular weight markers included phosphorylase *a*, mol wt = 94,000; bovine serum albumin, mol wt = 68,000; ovalbumin, mol wt = 44,000; glycerol phosphate dehydrogenase, mol wt = 40,000; chymotrypsinogen, mol wt = 26,000; and myoglobin, mol wt = 17,000 [7, 43]. The standard proteins were solubilized in SDS-mercaptoethanol-EDTA, heated, and run in parallel with the sample from iodinated cells.

Hydrolysis of Soluble Labeled Components

The apical surface was labeled with ^{125}I and processed as described in the "Preparations" section. The labeled components were hydrolyzed either enzymatically or by treatment with acid or alkali.

For enzymatic hydrolysis, the cytosol fractions of the homogenized labeled cells were treated with proteolytic enzymes (5 μg/ml) in the following buffers: 100 mM potassium phosphate (pH 7.5) for trypsin, chymotrypsin and pronase digestion, and 100 mM potassium phosphate with 5 M cysteine and 1 mM EDTA (pH 6.5) for papain digestion. Toluene, 25 μl, was added to all tubes to suppress bacterial growth [38], and the mixtures were incubated at 37°C for 48 hr. The samples were then lyophilized, and the pellets were resuspended in a small volume of water, centrifuged at 15,000 × *g* for 30 min (to remove excess enzyme), and loaded on an 0.5 M agarose column (33 × 1.1 cm) equilibrated with: 10 mM TES, 10 mM EDTA, 0.02% sodium azide and 300 mM KCl (pH 7.5). Radioactivity in the collected fractions (0.5 ml each) was determined as described above.

For acid hydrolysis, the cytosol fractions were dialyzed in cellulose tubing against 200 volumes of the "homogenizing solution" for 16 hr at 4°C. The dialyzed supernatants were acidified to pH 2.5 with 2 N HCl and heated at 95°C for 10 to 45 min. The tubes were immersed in ice water and then centrifuged at 10,000 × *g* for 10 min in an RCB Sorvall

centrifuge. These supernatants were neutralized with 2 N NaOH and chromatographed under nondenaturing conditions in a 0.5 M Agarose column as described above (section on "Enzymatic Hydrolysis").

For alkaline hydrolysis, the dialyzed 100,000 × *g* supernatants were alkalinized to pH 12 with 2 N NaOH and heated to 95° for 10 to 45 min. The mixture was placed in ice water, neutralized with concentrated HCl, and chromatographed as described above (section on "Enzymatic hydrolysis").

Labeled Amino Acids

The apical and basal-lateral surfaces were labeled with ^{125}I independently, as described in the "Preparations" section. The labeled, scraped cells were dialyzed in cellulose tubing for 24 to 36 hr against 400 volumes of Tris-HCl, 50 mM (pH 7.5), EDTA, 5 mM. The cells were then resuspended in a small volume (approximately 0.5 to 1 ml containing 50 to 100 μg protein) of potassium phosphate, 100 mM (pH 7.5), and sonicated at setting 6 by two 5-sec bursts in a Bronson Sonifier with the microtip. Pronase (final concentration of 5 μg/ml) and toluene (12 to 25 μl) were added and the mixture was incubated at 37°C for 48 to 72 hr. The enzymatic digest was acidified with 1 N HCl, centrifuged at 10,000 × *g* for 10 min (RCB Sorvall centrifuge), and the supernatant extracted 4 times with 3 volumes of acidified butanol. The butanol extract was dried under nitrogen, resuspended in a small volume of acidified butanol, and chromatographed on flexible gel plates (cellulose MN 300 and silica gel G) with a solvent system of *n*-butanol/acetone/1 N NH_4OH (1:4:1) [27]. The plates were dried, cut in 0.5-cm strips, and counted in a Packard gamma spectrometer. Standards or radioactive mono-iodotyrosine ($M^{125}IT$), diiodotyrosine ($D^{125}IT$) and ^{125}I-Na were chromatographed simultaneously in adjacent positions to the enzyme digest sample.

Labeling of Membrane Lipids

The apical and basal-lateral surfaces were independently labeled with ^{125}I as described in the "Preparations" section. The labeled scraped cells were freed of unbound ^{125}I by dialysis and disrupted by sonication as described in *Labeled Amino Acids*. Aliquots of the sonicates were acidified with TCA (10%, final concentration) and centrifuged at 10,000 × *g* for 30 min. The pellets were extracted with organic solvents (*see* below). Aliquots of the sonicates were also extracted with organic solvents, without prior treatment with TCA. The extraction procedure was as follows: Thirty-seven volumes of ethanol/ether (3:1) or acetone/water (10:1) were added, the mixtures maintained at 4°C for 1 hr, centrifuged at 15,000 × *g* at −20°C for 30 min, and the supernatants dried under nitrogen. The precipitates were resuspended in the same solvent and the above extraction repeated [12, 13]. The final pellets were dissolved in 1 N NaOH, and the radioactivity in the pellets and dried supernatants were counted in a Packard gamma spectrometer.

Analyses and Statistics

The protein assays were done by the method of Lowry *et al.* [20] with bovine serum albumin (BSA) as the standard and DNA by the method of Burton [4] with calf thymus

DNA as the standard. All results are expressed as mean ± SEM and evaluated for statistical significance by the unpaired Student "*t*" test [37].

Materials

Toads (*Bufo marinus* of Colombian origin) were supplied by the Tarpon Zoo (Tarpon Springs, Fla.) and of Dominican origin by National Reagents (Bridgeport, Conn.) or by Pond Life Research (Grass Valley, Calif.). Carrier-free ^{125}I-Na was purchased from New England Nuclear Corp. (Boston, Mass.) and used within two to three days. Glucose oxidase, lactoperoxidase, pronase, trypsin, chymotrypsin, papain and sodium deoxycholate (DOC) were obtained from Calbiochem (La Jolla, Calif.). Lactoperoxidase was dissolved in Ca^{++}-free Ringer's solution and kept frozen at −20°C; glucose oxidase was dissolved in Ca^{++}-free Ringer's solution (pH adjusted to 7 with diluted HCl) and stored frozen in small samples at −20°C. Phosphorylase *a*, ovalbumin, BSA, calf thymus DNA, chymotrypsinogen, lima bean trypsin inhibitor II-L, and lubrol WX were purchased from Sigma Chemical Co. (St. Louis, Mo.); SDS, acrylamide, bis-acrylamide, N, N, N′, N′-tetramethylethylenediamine (TEMED), ammonium persulfate and agarose from BioRad Laboratories (Richmond, Calif.); myoglobin (horse heart) from Schwarz/Mann (Orangeburg, N. Y.); glycerol phosphate dehydrogenase from Boehringer Mannheim (Germany); Triton-X-100 from Rohm and Haas (Philadelphia, Pa.); glycerol from Eastman Kodak Co. (Rochester, N.Y.); and Pitressin from Parke Davis (Detroit, Mich.). 3,5-Di(^{125}I) iodo-L-tyrosine and 3-(^{125}I) iodo-L-tyrosine were supplied by Amersham and Searle Corporation (Arlington Heights, Ill.). Amiloride was a gift from Dr. C. A. Stone of Merck, Sharp and Dohme Co. The conventional chemicals were all of reagent grade.

Results

Optimal Conditions for Iodination

To document optimal mixing, pairs of hemibladders were mounted, mucosal surface out, as sacs and labeled enzymatically with ^{125}I-Na (30 μCi/ml, 1.2×10^{-8} M) in 10 mM glucose, 0.25 U/ml lactoperoxidase, 0.25 μg/ml glucose oxidase, at room temperature for 5 min or exposed to the same solutions devoid of the enzymes. The external medium of all hemibladders was bubbled with 97% O_2-3% CO_2, and, in addition, one of each pair was subjected to vigorous magnetic stirring of the external medium. In the absence of augmented stirring, incorporation of ^{125}I into TCA insoluble radioactivity of scraped epithelial cells was (all in cpm/hemibladder) 510 ± 70 (control) and 1,030 ± 90 (with enzymes). In contrast, with vigorous stirring, incorporation was 460 ± 80 (control) and 21,400 ± 2,500 (with enzymes) (n = 5 experiments). In all subsequent labeling experiments, therefore, augmented magnetic mixing was used routinely.

To ensure that adequate concentrations of glucose were provided as substrate for the glucose oxidase reaction, the concentration of glucose in the external medium was varied from 0 to 10 mM, and the effect on ^{125}I labeling was assessed. Hemibladders were mounted, mucosal (apical) side out, and labeled for 12 min in 50 ml of Ringer's solution with 0.35 U/ml lactoperoxidase, 0.4 μg/ml glucose oxidase, and ^{125}I Na (40 μCi/ml, 1.6×10^{-8} M) (n = 4 experiments). Incorporation

into the TCA precipitable fraction of scraped epithelial cells was $0.75 \pm 0.14 \times 10^{-15}$ mol/mg protein and 6.6 ± 0.9 to $7.0 \pm 0.6 \times 10^{-15}$ mol/mg protein in the absence and in the presence of 1-10 mM glucose, respectively. Thus, for routine labeling we used either 5 or 10 mM glucose in the medium.

Enzyme requirements were evaluated by addition of fixed concentrations of one enzyme and varying the concentration of the other. Paired hemibladders were mounted, mucosal side out, as sacs and labeled for 10 min at room temperature. In the presence of glucose (10 mM) and lactoperoxidase (0.4 U/ml), there was a linear increase in the incorporation of ^{125}I as the concentration of glucose oxidase was increased from 0.1 to 0.4 μg/ml (Fig. 1). Higher concentration (> 0.4 μg/ml) of glucose oxidase did not result in further increases in the rate of incorporation. Similar results were obtained with lactoperoxidase as the variable (0.02 to 0.35 U/ml) when the concentration of glucose oxidase was maintained constant (0.4 μg/ml). To provide for maximal rates of iodination, the routine procedure was to use 0.4 to 0.5 U/ml of lactoperoxidase and 0.4 μg/ml of glucose oxidase.

Lactoperoxidase catalyzed iodination of the free amino acid, L-tyrosine, proceeds rapidly and is essentially complete in a few minutes [23]. By contrast, iodination of tyrosine residues in membrane proteins of erythrocytes increases linearly with time [12]. To characterize the effects of time and ^{125}I concentration on iodination, hemibladders were labeled from the apical side for periods varying from 5 to 30 min at three temperatures (4, 15, and 25°C) and at three isotope concentrations (3.8×10^{-9} to 3.2×10^{-8} M). Incorporation of ^{125}I into TCA insoluble radioactivity was linear for 20 min when the reaction was conducted at 25 or 15°C, although iodination was slower at 15°C (Fig. 2). At 4°C virtually no iodination occurred. The time dependence of labeling was confirmed by light-microscopy autoradiography [30]. Iodination of the apical surface also increased in proportion to the increase in concentration of ^{125}I from 10 to 80 μCi/ml (molarity of 3.8×10^{-9} to 3.2×10^{-8} M) (Fig. 2). In the following experiments, we limited the concentration of ^{125}I to <100 μCi/ml (4×10^{-8} M).

Electron Microscopic Autoradiography

Iodination was accomplished with the usual complement of enzymes (*see* legend of Fig. 3) and ^{125}I (80 Ci/ml, 3.2×10^{-8} M) and vigorous stirring of the mucosal medium for 20 min.[1] The silver grains were confined to the apical plasma membrane; intracellular labeling was minimal (Fig. 3). The stability of the bound ^{125}I is indicated by its ability to withstand displacement by the rigorous extraction procedures required for preparation of the autoradiographs [40]. These results indicate that under our experimental conditions (high yield) iodination is limited to the apical surface (membrane and glycocalyx).

Effect of Apical Iodination on Transepithelial Na^+ Transport

The utility of covalent labeling of plasma membranes is extended if the labeling process does not perturb the functional state of the system. In a previous

[1]We are indebted to Dr. J. Strum for preparing and obtaining these autoradiographs.

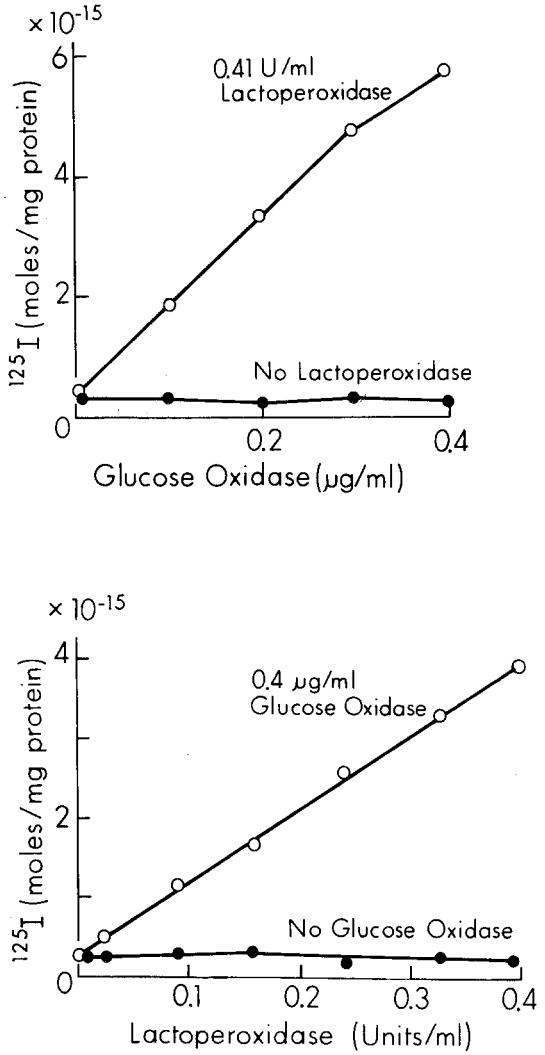

Fig. 1. Effect of various concentrations of glucose oxidase (upper panel) and lactoperoxidase (lower panel) on labeling of toad bladder apical membrane. Paired hemibladders were mounted mucosal side out and labeled for 10 min at room temperature in 50 ml of Ringer's solution containing 10 mM glucose, 30 to 35 μCi/ml ^{125}I-Na (1.2×10^{-8} to 1.4×10^{-8} M). ^{125}I incorporation into TCA-insoluble radioactivity was determined in homogenates of the epithelial scrapings. The values plotted are the means of 3 to 4 experiments

study [40], iodination of the apical plasma membrane at concentrations of NaI $<10^{-5}$ M had no effect on the basal PD or SCC, or the responses to vasopressin. To ensure that high yield iodination did not impair epithelial transport, hemibladders were iodinated apically for 20 min at ^{125}I concentrations of 1.6×10^{-8} M with vigorous stirring. Neither the basal nor vasopressin-stimulated SCC's were changed by apical iodination, over a 4-hr period (Fig. 4). Identical patterns were also obtained in the PD measurements.

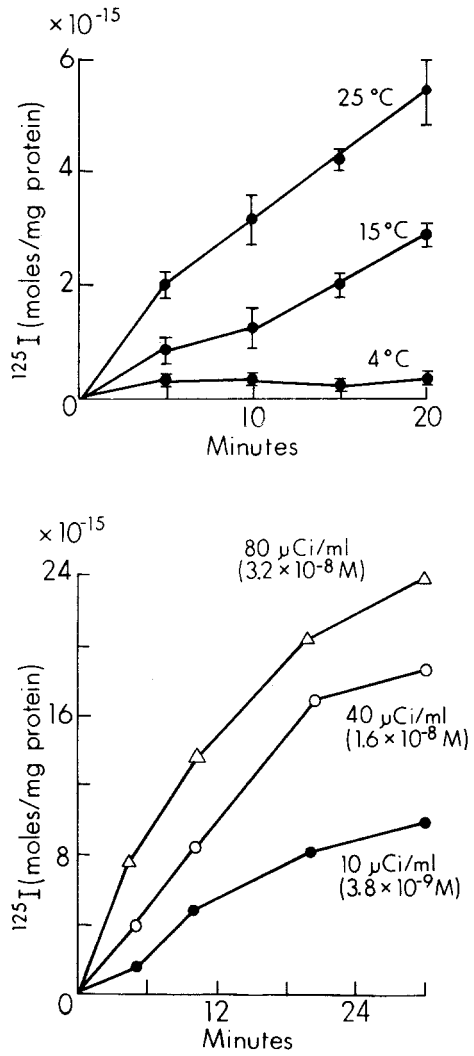

Fig. 2. Effects of time and temperature (upper panel) and ^{125}I concentration (lower panel) on labeling of apical membrane. *Upper panel:* Hemibladders (mucosal side out) were incubated in 50 ml of Ringer's solution with 10 mM glucose at 25, 15, or 4°C for 1 hr. At the end of this period, the labeling enzymes (0.35 U/ml lactoperoxidase, 0.5 μg/ml glucose oxidase) and ^{125}I (10 μCi/ml corresponding to 3.8×10^{-9} M) were added, and the reaction was allowed to proceed for varying times. ^{125}I incorporation into the TCA-insoluble fraction was determined in homogenates of the epithelial scrapings. The values are the means ± SE of 4 experiments at each temperature. *Lower panel:* Hemibladders were labeled at 25°C in 50 ml of Ringer's solution with 10 mM glucose, 0.4 U/ml lactoperoxidase, 0.5 μg/ml glucose oxidase, and various concentrations of ^{125}I-Na. The values plotted are the means of 3 to 7 observations for each experimental point.

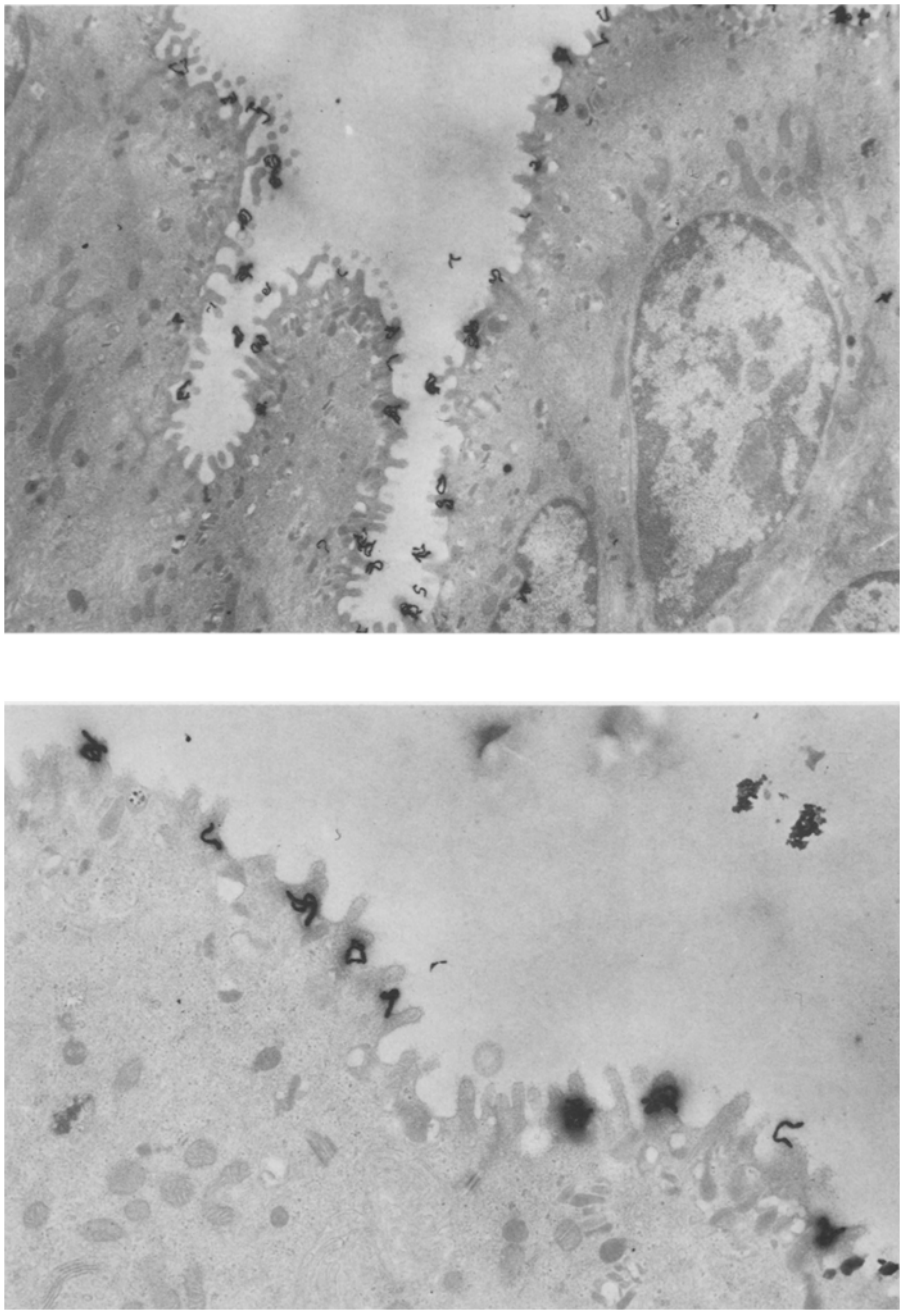

Fig. 3. Electron-microscopic autoradiographs of iodinated apical surfaces of toad bladder epithelium. Hemibladders were labeled for 20 min at room temperature in 50 ml of Ringer's solution containing 0.4 U/ml lactoperoxidase, 0.4 μg/ml glucose oxidase, 5 mM glucose, and 80 μCi/ml (3.2×10^{-8} M) of ^{125}I-Na. Almost no background grains are evident. The silver grains are essentially confined to the luminal surface of mitochondria-rich and granular cells (upper panel) and to the interdigitations of the microvilli in the luminal surface (lower panel). The autoradiographs were exposed to Ilford L-4 emulsion for 3 weeks. Magnification: upper panel, 4,600 ×; lower panel, 8,100 ×

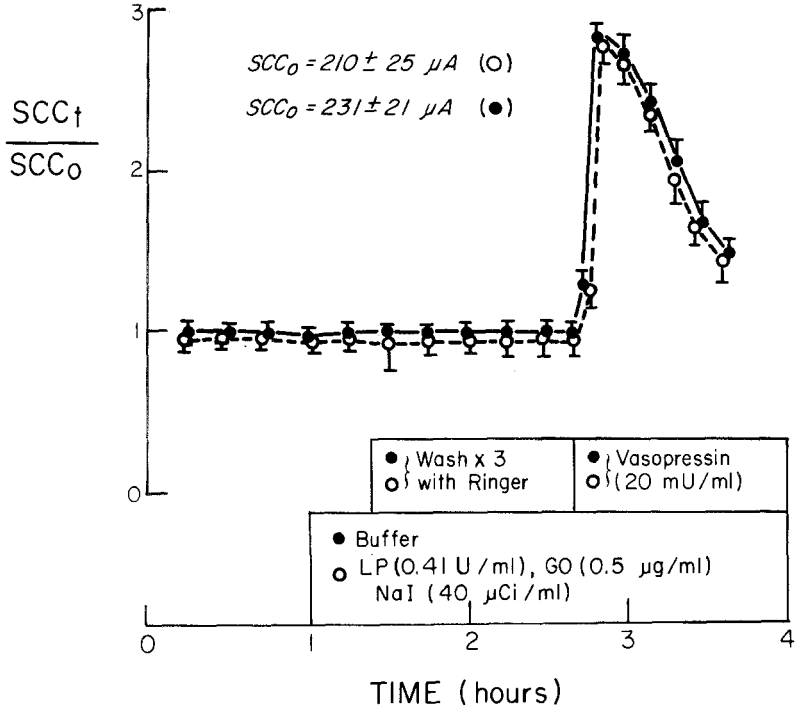

Fig. 4. Effect of iodination of the apical plasma membrane on the baseline SCC and the response to vasopressin. Paired hemibladders were mounted, mucosal side out, equilibrated for 1 hr and iodinated for 20 min (^{125}I concentration: 1.6×10^{-8} M). Vasopressin was added to the basal-lateral solution, SCC_t/SCC_o denotes the ratio of the SCC recorded at time "t" and time "zero". Each point and vertical line represents the mean ± SE of 3 pairs of hemibladders. SCC_o is the absolute SCC at time "zero" in μAMP/hemibladder

Analysis of Labeled Components in the Apical Membrane

In preliminary experiments, the scraped epithelial cells, labeled apically, were dialyzed, homogenized, and the entire homogenates were treated with 10% TCA. Almost 55% of the bound ^{125}I was associated with TCA-soluble components. To obtain more information on the nature of the labeled components, the epithelial cells were labeled either from the mucosal (apical) or the serosal (basal-lateral) sides, dialyzed, homogenized, and divided into particulate and cytosol fractions (Table 1). Basal-lateral labeling yielded products that were predominantly particulate (85%). In contrast, 46% of the apically labeled components were recovered in the cytosol fraction. Almost all of the ^{125}I in the cytosol fractions was TCA soluble; 91% with apical labeling and 100% with basal-lateral labeling, whereas almost all of the ^{125}I bound to the particulate fractions were TCA insoluble. To determine whether TCA-soluble ^{125}I-labeled components recovered in the cytosol fractions were small molecules, the TCA supernatants of these fractions were dialyzed against 200 volumes of distilled H_2O for 16 hr at room temperature. All of the ^{125}I content of the cytosol of the basal-lateral labeled fraction and none of the apical labeled fraction were dialyzable. These results indicate that apical labeling

Table 1. Distribution of ^{125}I radioactivity in 100,000 × *g* pellet and cytosol fractions of apical and basal-lateral membranes

	% ^{125}I radioactivity	
	Cytosol	Particulate
Apical label	46 ± 5	50 ± 3
Basal-lateral label	12 ± 5	85 ± 8
	% TCA precipitable radioactivity	
	Cytosol	Particulate
Apical label	9 ± 2	92 ± 8
Basal-lateral label	—	95 ± 6

Hemibladders were labeled for 30 min at room temperature (0.4 U/ml lactoperoxidase, 0.4 μg/ml glucose oxidase, 10 mM glucose, 80 μCi/ml (3.2×10^{-8} M) ^{125}I. The labeled cells were dialyzed, homogenized with polytron, centrifuged at 100,000 × *g* for 1 hr, and the pellet (particulate) and supernatant (cytosol) fractions were assayed for ^{125}I. Aliquots of the fractions were acidified to 10% TCA, centrifuged at 10,000 × *g* for 30 min, and the pellet and supernatant fractions were counted. The values represent the means ± SE of 8 experiments, each in duplicate.

involves significant binding of ^{125}I to a large molecular weight, nonmembranous TCA-soluble component, possibly the glycocalyx.

Gel Filtration Chromatography

To prepare the ^{125}I-labeled fractions for analysis by gel filtration or PAGE, the conditions required to solubilize the ^{125}I bound to the particulate fractions were defined. Hemibladders were labeled with ^{125}I either from the mucosal or the serosal side. The scraped cells were dialyzed extensively, homogenized, and particulate fractions were obtained by centrifugation at 100,000 × *g* for 1 hr. The resuspended particulates were incubated at 4°C with a 1% solution of the following detergents: SDS, Triton X-100, DOC or Lubrol. After 15 min of incubation, the tubes were centrifuged at 100,000 × *g* for 1 hr, and the radioactivity in the pellet and supernatant fractions was determined. Control tubes, in which detergent was omitted, were run in parallel. SDS solubilized virtually all of the membrane-associated radioactivity; Triton X-100 and DOC were somewhat less effective, whereas Lubrol solubilized less than 20% of the bound ^{125}I (Table 2). Thus, SDS was chosen for chromatographic analysis of the labeled components.

Gel filtration chromatography (Agarose 5M) was performed on the apically labeled components: SDS-solubilized particulate fractions and SDS-treated cytosol fractions. The ^{125}I-labeled components recovered in the cytosol fraction were, for the most part, eluted with the void volume (89% in fractions 20-25), and 9% was recovered in the internal volume (some of which had apparent molecular weights as low as 17,000 (Fig. 5). The particulates treated with SDS, however, gave la-

Table 2. Solubilization of ^{125}I-labeled components of apical and basal-lateral membranes by detergents

	% ^{125}I radioactivity in supernatant	
	Apical label	Basal-lateral label
SDS	94.3 ± 3.6	95.2 ± 2.1
Triton X-100	72.1 ± 2.0	75.2 ± 5.2
DOC	73.7 ± 2.0	82.1 ± 3.2
Lubrol	21.2 ± 1.8	18.5 ± 3.6

Labeling conditions are as described in the legend to Table 1. The epithelial scrapings were dialyzed, homogenized in 5.7% sucrose, 1.5 mM EDTA, 1 mM $NaHCO_3$, 5 mM Tris-HCl, pH 8.1, 0.1 mM NaI, and 10 μg/ml gentamycin with a single burst (5 sec) of the polytron at top speed. After centrifugation at 100,000 × *g* for 1 hr, the particulate fractions were resuspended in homogenizing solution and detergents were added (10 mg/ml). The tubes were kept at 4 °C for 15 min, centrifuged at 100,000 × *g* for 1 hr, and the radioactivity in the pellet and supernatant was assayed. Control tubes (without detergent) were analyzed to monitor recovery (97% ± 0.5%). the values are the means ± SE of 4 experiments.

beled components that chromatographed in the region between Blue Dextran and myoglobin; 82% was eluted coincident with myoglobin (17,000 Daltons), 11% in the intermediate region, and 5% with the void volume. These results indicate that the nondialyzable ^{125}I recovered in the cytosol fraction was bound to high molecular weight macromolecules resistant to further dissociation by SDS. In the particulate fraction, however, SDS generated labeled components that chromatographed in the range of 15,000-75,000 daltons.

SDS-PAGE Analysis

To provide estimates of the molecular weights of the ^{125}I-labeled membrane components, a series of noniodinated standard proteins were treated with SDS-mercaptoethanol-EDTA, heated to 100°C for 2 min, and analyzed by SDS-PAGE in parallel with the SDS-solubilized apical components. The standard curve is shown in Fig. 6. The apically labeled components were prepared for SDS-PAGE as described in the section on gel filtration chromatography. The ^{125}I-labeled components of the particulate fraction were resolved into at least 6 bands with apparent molecular weights of 23,000, 28,000, 37,000, 44,000, 68,000, and 95,000. A small fraction failed to penetrate into the gel (Fig. 7). The minor bands (I, II, V, and VI) accounted for 24% of the ^{125}I that penetrated the gel, and the major bands (III and IV) accounted for 56%. The resolution of these components, however, is not sufficient to conclude that these 6 bands represent 6 unique peptides.

Analysis of the SDS-treated cytosol fraction revealed that only trivial amounts of ^{125}I-labeled material penetrated into the gel (Fig. 7). This finding confirms the inference based on SDS-Agarose-5M gel filtration chromatography that the nondialyzable ^{125}I recovered in the cytosol fraction is bound to a high molecular weight surface component.

Digestion of the ^{125}I-Labeled Soluble Component

Enzymatic iodination of the apical surface labels a TCA-soluble, high molecular weight component. Enzymatic and pH-dependent hydrolysis was used to obtain further information on the nature of these components. Hemibladders were labeled with ^{125}I from the apical side, homogenized, dialyzed extensively, and the cytosol fractions were incubated with trypsin, chymotrypsin, pronase, papain, or enzyme-free buffers for 48 hr at 37°C. The products were chromatographed on Agarose-5M under nondenaturing conditions (Fig. 8). As shown previously (*cf.* Fig. 5), virtually all the soluble ^{125}I was bound to components that eluted with the void volume. After treatment with pronase and papain most of the bound ^{125}I eluted as low mol wt components; 75% migrated at mol wt <17,000 and only 10% was recovered in the void volume. Similar results were obtained with trypsin in that 60% was recovered as the low mol wt species, but chymotrypsin yielded only

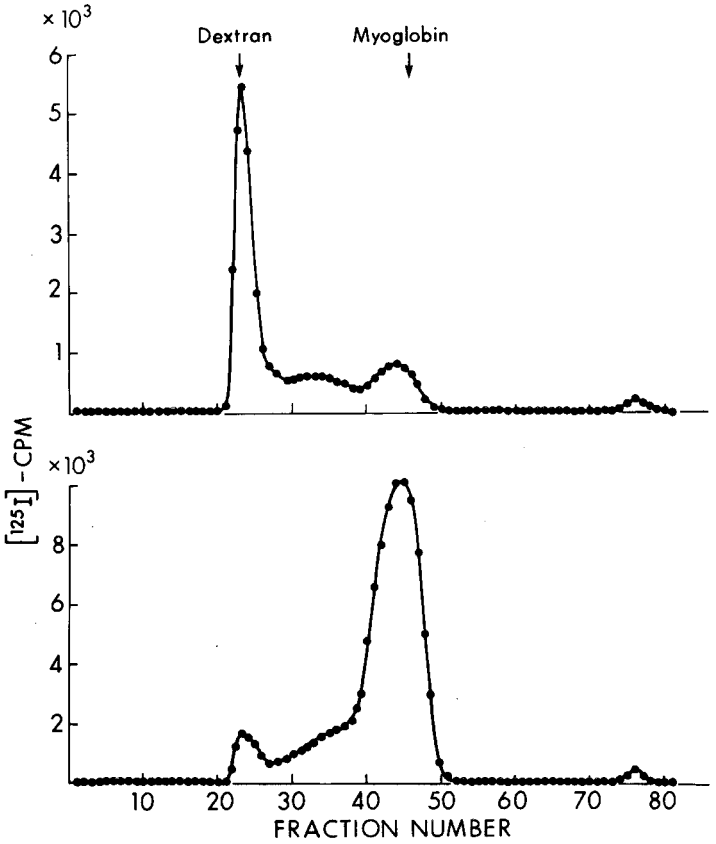

Fig. 5. Gel filtration of iodinated apical membranes. The labeling and homogenization conditions are described in the legend of Table 2. The pellets (100,000 × *g* for 1 hr) and dialyzed cytosol fractions were treated with SDS and chromatographed on Agarose 5M. The column was equilibrated and eluted with Tris-glycine-SDS buffer. The upper panel depicts the results obtained with the cytosol fraction, and the lower panel with the high-speed pellet. The arrows indicate the elution position of Blue Dextran and myoglobin

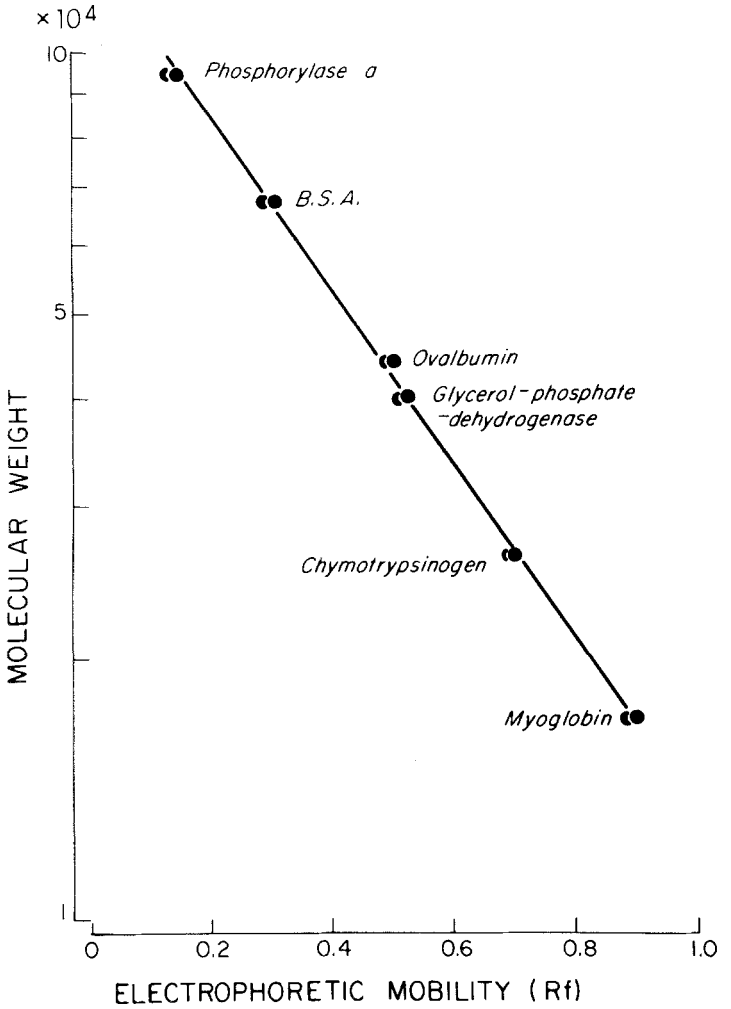

Fig. 6. Electrophoretic mobility as a function of molecular weight of protein standards. The protein standards were denatured by treatment with SDS-mercaptoethanol-EDTA and heating. Electrophoresis was carried out in 0.1% SDS, 12% acrylamide and 3% bis-acrylamide. The SDS front was used to calculate the relative mobilities (R_f). The molecular weights of the protein markers are: phosphorylase *a*, mol wt = 94,000; BSA, mol wt = 68,000; ovalbumin, mol wt = 44,000; glycerol phosphate dehydrogenase, mol wt: 40,000; chymotrypsinogen, mol wt = 26,000; and myoblobin, mol wt = 17,000 [7,43]

25% in the low mol wt form (data not shown) [30]. These results imply that the TCA-soluble component is a conjugated protein made up of a small peptide attached to large prosthetic groups.

To test whether the prosthetic group might be a carbohydrate, the TCA-soluble component was subjected to mild acid and alkaline hydrolysis. As shown in Fig. 9, after 10 min of acid hydrolysis 20% of the radioactivity eluted in a position

corresponding to low mol wt peptides, and this percent remained essentially unchanged after 20 min (21%) and 45 min (24%) of hydrolysis. Since peptide bonds formed by aspartyl residues are susceptible to mild acid hydrolysis [36], the low mol wt radioactivity probably corresponds to peptides cleaved at aspartic acid residues. Mild acid hydrolysis for less than an hour should also remove terminal

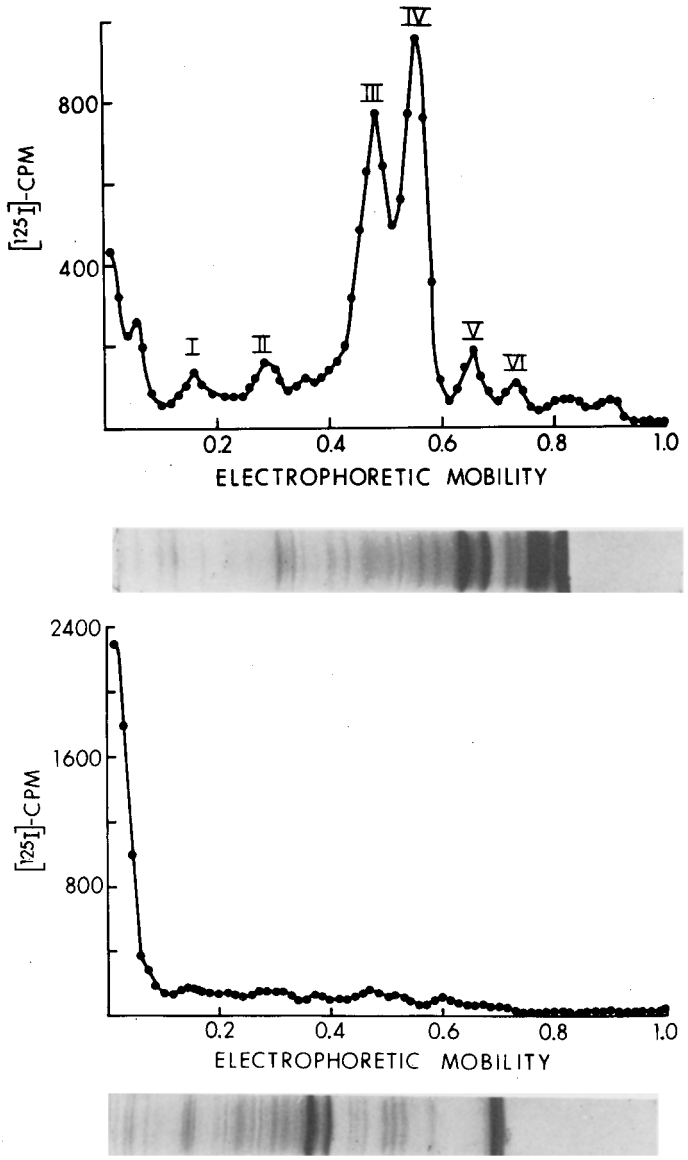

Fig. 7. SDS-polyacrylamide gel electrophoresis pattern of 100,000 × *g* pellet (upper panel) and cytosol fractions (lower panel). Labeling conditions were similar to those described in the legend to Table 1. Gels were 0.1% SDS, 12% acrylamide, 3% bis-acrylamide. The SDS front was used to calculate the electrophoretic mobility

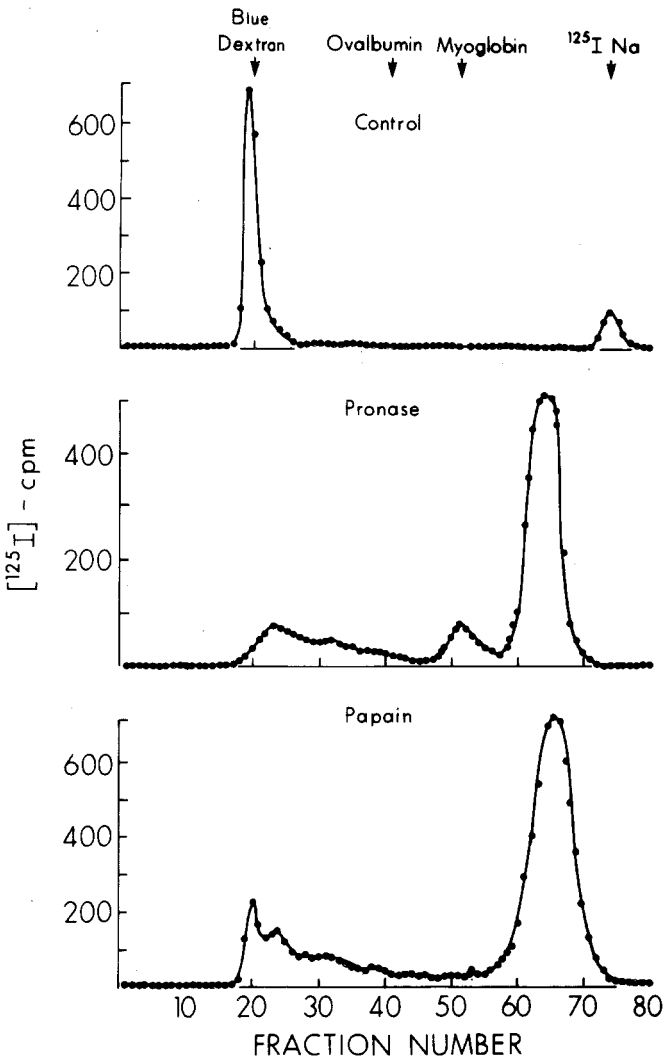

Fig. 8. Enzymatic digestion of soluble components from the apical surface. Epithelial scrapings labeled with ^{125}I from the apical side were homogenized, dialyzed, and centrifuged at 100,000 × *g* for 1 hr. The cytosol fractions were subjected to protease digestion and chromatographed under nondenaturing conditions by gel filtration through Agarose 0.5M. The columns were equilibrated and eluted with TES-EDTA-KCl buffer. Controls were incubated but without proteases, and chromatographed under the same conditions

sialic acid and to a lesser extent galactose residues [39]. The broadening of the radioactivity eluting in the position of the void volume may present loss of terminal sialic acids. It seems unlikely, however, that a substantial number of asparagine-*n*-acetyl glucosamine linkages exist in the molecule, because of the relative resistance to acid hydrolysis [36].

Alkaline hydrolysis resulted in a dramatic change in the chromatographic be-

havior of the iodinated TCA-soluble component; after 10 min, more than 95% of this material was degraded into smaller components, whose molecular weights were less than 40,000 [Fig. 10]. Hydrolysis for only 2-1/2 min produced a similar pattern (data now shown) [30]. Forty-five min at a pH of 12 converted virtually all the material into polydisperse low mol wt components. These results demonstrated that the TCA-soluble cytosol macromolecules contain alkali-labile bonds, most likely glycosidic [39]. This component is probably derived from the glycocalyx since it is labeled from the luminal side.

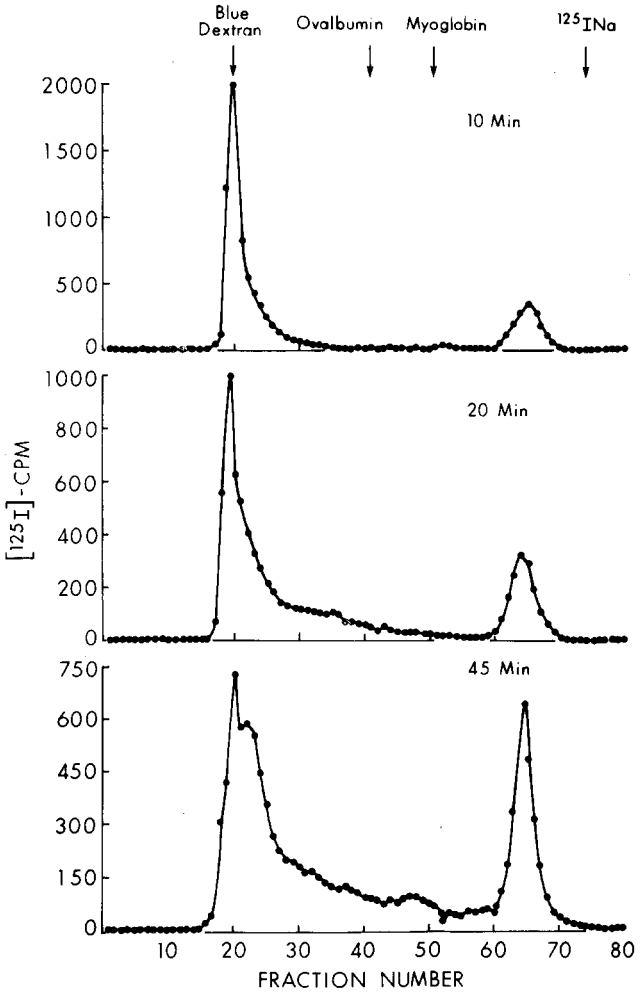

Fig. 9. Effect of acid hydrolysis on soluble components from the apical surface. Epithelial scrapings labeled with ^{125}I from the apical surface were homogenized, dialyzed, and centrifuged at 100,000 × *g* for 1 hr. The cytosol fractions were subjected to mild acid hydrolysis (in 0.2 N HCl) at 95°C for 10, 20, or 45 min, and chromatographed under nondenaturing conditions by gel filtration through Agarose 0.5M. The columns were equilibrated and eluted with TES-EDTA-KCl buffer

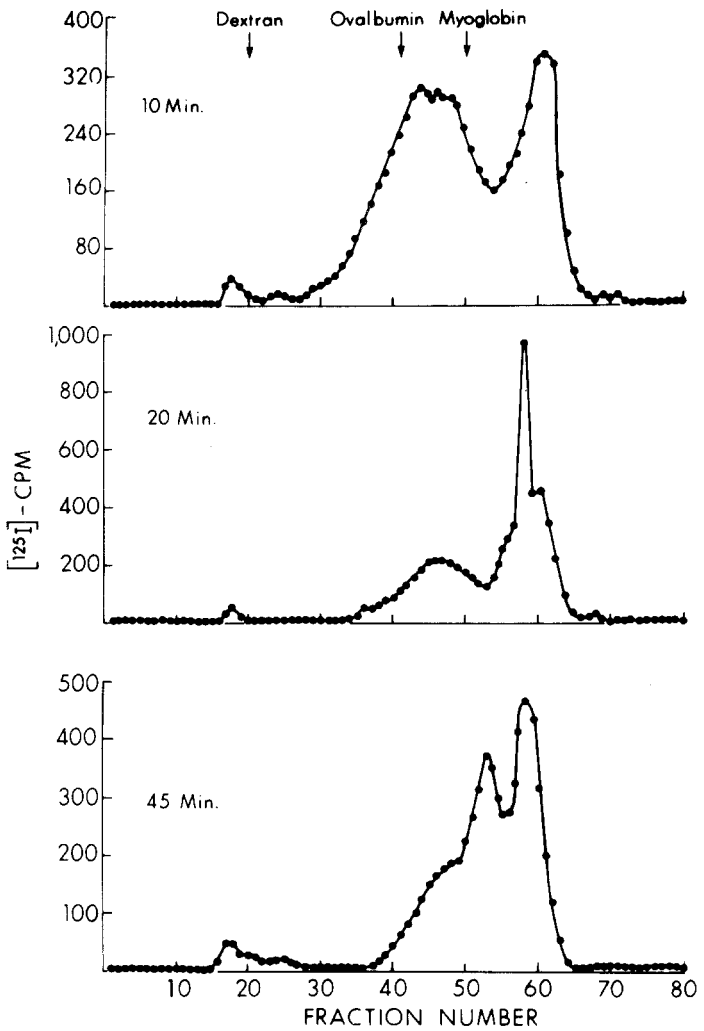

Fig. 10. Effect of mild alkaline hydrolysis on soluble components from the apical surface. Epithelial scrapings labeled with ^{125}I from the apical side were homogenized, dialyzed and centrifuged at 100,000 × *g* for 1 hr. The cytosol fractions were treated with 0.2 N NaOH at 95°C for 10, 20, or 45 min and chromatographed in Agarose 0.5M under nondenaturing conditions

Effects of Agents that Modify Apical Membrane Conductance

Vasopressin and cyclic-AMP increase Na transport in the toad bladder apparently by increasing the permeability of the apical membrane to Na^+ [17, 25]. On the other hand, amiloride inhibits active Na transport in this tissue by reversible interaction with components of the luminal boundary, presumably the Na^+ conductance channel proteins [2, 9].

As shown in Table 3, neither vasopressin nor cyclic AMP, at concentrations

Table 3. Effect of vasopressin, amiloride and cyclic-AMP on iodination of apical membrane

	^{125}I ($\times 10^{-15}$mol/mg protein)		
	Total	Cytosol	Particulate
Control ($n = 16$)	11.9 ± 1.3	5.0 ± 0.5	7.0 ± 0.8
50 mU/ml vasopressin ($n = 11$)	11.2 ± 1.6	4.5 ± 0.6	6.7 ± 1.0
10^{-4} M amiloride ($n = 11$)	7.0 ± 1.3	4.1 ± 0.8	2.8 ± 0.5[a]
10^{-3} M cyclic-AMP ($n = 6$)	12.8 ± 1.8	5.5 ± 0.8	7.4 ± 1.0

Hemibladders were labeled for 30 min at room temperature as described in the legend of Table 1. Vasopressin and cyclic-AMP were added to the serosal bathing solution immediately before labeling. Amiloride was added to the mucosal bathing solution 30 min prior to labeling, and the same concentration of the drug was present throughout the labeling period. The labeled cells were homogenized in 5.7% sucrose with 1.5 mM EDTA, 1 mM $NaHCO_3$, 0.1 mM NaI, 10 μg/ml gentamycin, 0.1 μg/ml lima bean trypsin inhibitor and 5 mM Tris-HCl, pH 8.1, by a 5-sec single burst with the polytron at top speed. A sample of the homogenate was treated with 10% TCA, and the radioactivity associated with the protein pellet was determined. The remainder of the homogenate was dialyzed against the same solution, centrifuged at 100,000 × *g* for 1 hr, and the radioactivity of the pellet (particulate) and supernatant (cytosol) fractions were determined. *n* = number of hemibladders.

[a] Statistically significant difference from control ($P < 0.02$).

that affect Na and water transport, produced any detectable difference either in the incorporation of ^{125}I into TCA-insoluble material or in the distribution of the label between the particulate (100,00 × *g* pellet) and the glycocalyx fractions (100,000 × *g* supernatant). In contrast, amiloride decreased overall iodination by about 40% and almost all of this decrease is a result of inhibition of labeling of the particulate fraction.

An attempt was made to define the sites of amiloride inhibition of ^{125}I labeling of the particulate fraction by SDS-PAGE. The gels were loaded with varying amounts of protein to provide approximately the same total quantity of bound ^{125}I for analysis. The distribution of ^{125}I among the 6 identifiable bands, however, was unchanged by either vasopressin or amiloride (Fig. 11).

Iodination of the Basal-Lateral Membranes

Ekblad *et al.* [6] provided evidence of covalent labeling of the basal-lateral membranes of toad bladder epithelium with pyridoxal phosphate and ^{3}H-$NaBH_4$, as indicated by electron microscopic autoradiography. For the purpose of biochemical analysis, however, the yield of radioactive labeling of these membranes was low. Our attempts to use this method to obtain high yields of radioactive labeling were unsuccessful, owing to very high background labeling obtained with

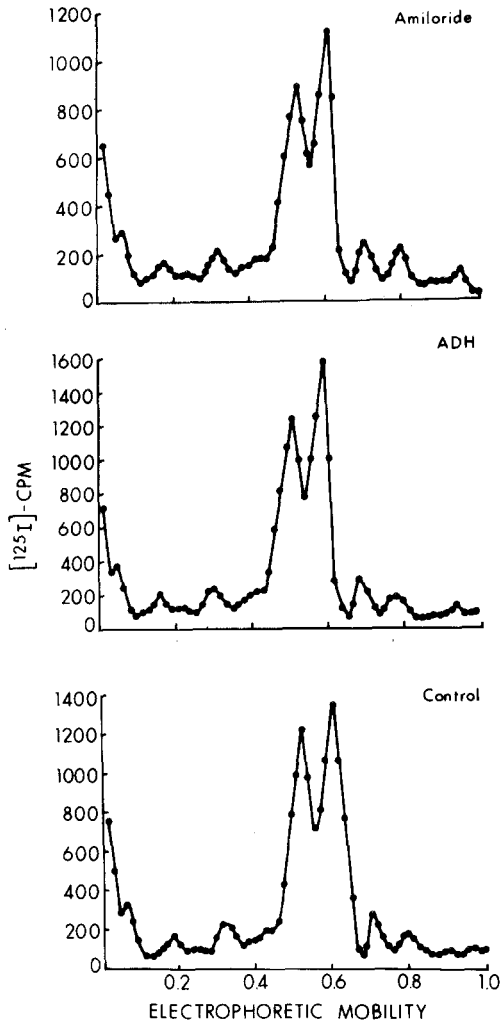

Fig. 11. Effects of vasopressin (ADH) and amiloride on the ^{125}I-labeling pattern of apical membranes. Hemibladders were labeled with ^{125}I from the apical side for 30 min and processed as indicated in the legend to Table 3. The membrane fraction (100,000 × *g* pellet) was dissolved in 1% SDS-buffer and analyzed by SDS-PAGE in gels of the following composition: 0.1% SDS, 12% acrylamide, and 3% bis-acrylamide. A similar pattern was obtained in bladders stimulated with cyclic-AMP

only ^{3}H-NaBH$_4$, presumably due to reduction of unsaturated lipids. Almost all of this "background" labeling was extractable with ethanol-ether, as reported with red cells [5]. Accordingly, we explored the possibility of applying the enzymatic iodination method to covalent labeling of the basal-lateral membranes.

Paired hemibladders were mounted as sacs with the serosal side out and labeled with ^{125}I (50 μCi/ml, 2×10^{-8} M), with vigorous magnetic stirring, for 5 to 30 min at room temperature. The external surfaces of the hemibladders were

washed with 6 to 8 exchanges of the serosal medium (i.e., until ^{125}I content of the wash was <20 cpm/ml). The epithelial cells were harvested by scraping and homogenized by sonication. The residual serosal wall was digested by incubation in 1 N NaOH at room temperature for 3 to 4 days. The epithelial homogenates were made up to 10% TCA, at 4°C, and the precipitable material was collected by centrifugation. The results in Table 4 indicate time-dependent iodination of the submucosal structures. Thirty minutes were required, however, to achieve labeling of the epithelium, at a yield comparable to that obtained with apical labeling (*cf.* Fig. 2 and Table 4). These results imply progressive penetration of the lactoperoxidase through the various serosal layers during the labeling period. By light-microscopy autoradiography, after 5 min of labeling only the outermost layers of the submucosa-serosa were labeled; after 30 min, labeling of the submucosa-serosa was extensive and the silver grains overlay the basal-lateral aspect of the epithelial cells [30].

When the epithelial cells are collected by scraping without collagenase treatment, the basement membrane remains attached to the cells [19, 21]. Pretreatment of the serosal side of the bladder with collagenase, however, results in complete digestion of the basement membrane with preservation of the integrity of the epithelial cells [19, 21]. This treatment was used to evaluate the possible contribution of labeling of the basement membrane to basal-lateral ^{125}I activity.

Paired hemibladders ($n = 3$) were mounted serosal side out and labeled with ^{125}I-Na (100 Ci/ml, 4×10^{-8} M), lactoperoxidase (0.41 U/ml), glucose oxidase (0.5 μg/ml) and glucose (5 mM) for 30 min at room temperature. The serosal media were then exchanged for Ca^{++}-free Ringer's solution, collagenase (0.5 mg/ml) was added to the serosal medium of one of each pair and incubated for 40 min at room temperature. The epithelia were harvested by scraping, sonicated, and made up to 10% TCA at 4°C. The pellets of the control epithelia incorporated 77.7×10^{-15} and the collagenase treated, 56.9×10^{-15} mol/mg protein. Thus, 73% of basal-lateral labeling was collagenase resistant.

Table 4. Iodination of toad bladder epithelium from the basal-lateral (serosal) side

Labeling time (min)	^{125}I ($\times 10^{-15}$ mol/mg protein)	
	Epithelial cells	Serosal and sub-mucosal layers
5	0	7.4
10	0	39.6
30	14.4	540.5

Paired hemibladders were mounted serosal (basal-lateral) side out and labeled in 50 ml of Ringer's solution with 10 mM glucose, 0.4 U/ml lactoperoxidase, 0.5 μg/ml glucose, and 50 μCi/ml (2×10^{-88} M) ^{125}I-Na for different times. The hemibladders were washed until radioactivity in the supernatant was at background level (~20 cpm). The epithelial scrapings were homogenized and precipitated with 10% TCA. The serosal-submucosal layers were digested in 1 N NaOH. The values represent the means of three experiments.

Effect of Basal-Lateral Iodination on Transepithelial Na^+ Transport

The functional consequences of labeling of the basal-lateral membranes with a limited concentration of NaI was tested by the electrophysiological methods used in evaluating apical labeling (*see* above).

Hemibladders (serosal side out) were iodinated with NaI (5×10^{-8} M) for 30 min at room temperature and monitored for PD and SCC for an additional 90 min before challenge with vasopressin (Fig. 12). Neither the basal nor vasopressin-stimulated SCC's were perturbed by basal-lateral labeling, and the same patterns were obtained in the PD measurements.

Comparison of Apical and Basal-Lateral Labeling

a. Extent of the reaction. For the purpose of providing membrane markers in isolation and purification of the apical and basal-lateral plasma membranes, ap-

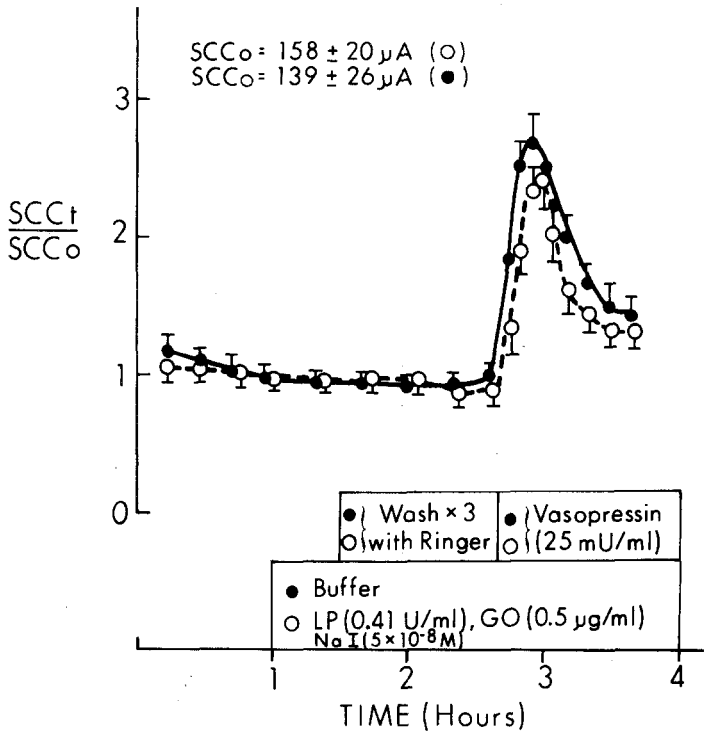

Fig. 12. Effects of iodination of the basal-lateral plasma membrane on the basal and vasopressin-stimulated SCC. Paired hemibladders were mounted serosal side out, equilibrated for 1 hr, and iodinated for 30 min. The bladders were then challenged with vasopressin added to the basal-lateral side. SCC_t/SCC_0 denotes the ratio of the SCC recorded at time "t" and time "zero". Each point and vertical line represents the mean ± SE of 3 pairs of hemibladders: SCC_0 is the absolute SCC at time "zero" in μAmp/hemibladder

proximately equal yields of radio-iodination are desirable. To assess the relative yields, one of each pair of hemibladders was mounted mucosal (apical) side out and the other serosal (basal-lateral) side out and both were labeled for 30 min under identical conditions: (0.41 U/ml lactoperoxidase, 0.5 μg/ml glucose oxidase, 5 mM glucose and 100 μCi/ml (4×10^{-8} M) ^{125}I-Na at room temperature ($n = 5$). The epithelial scrapings were homogenized, and the homogenates were made up to 10% TCA at 4°C. The pellets were analyzed for ^{125}I, protein and DNA. The yields were 135 ± 39 and $128 \pm 34 \times 10^{-15}$ mol/mg protein for apical and basal-lateral labeling, respectively. On a paired basis the ratio of the yields expressed as apical/basal-lateral was 1.08 ± 0.12. When normalized to DNA content, the yields were 643 ± 182 and $651 \pm 166 \times 10^{-15}$ mol/mg DNA for apical and basal-lateral labeling, respectively. The paired ratio (apical/basal-lateral) was 0.99 ± 0.09. These results indicate identical extents of reactions, provided that sufficient time (30 min) is allowed for the reactants and enzymes to reach the basal-lateral surface.

b. Lipid extraction. To provide information on whether the apical and basal-lateral labeling involved similar cell surface components, lipid and amino-acid analyses were completed. Labeling of lipids was estimated by extraction methods. One of each pair of hemibladders was mounted mucosal (apical) side out and the other, serosal (basal-lateral) side out, and both were labeled under identical conditions: (0.4 U/ml, lactoperoxidase, 0.5 μg/ml glucose oxidase, 10 mM glucose, and 90 μCi/ml (3.2×10^{-8} M) ^{125}I-Na, for 30 min at 25°C. The epithelial scrapings were dialyzed, homogenized, and extracted with either ethanol or acetone-water before and after precipitation of the labeled components with 10% TCA. Approximately 10% of the bound ^{125}I was extracted by either solvent system, regardless of either the side of labeling or of prior precipitation with TCA (Table 5). Negligible membrane lipid labeling has also been reported in iodinated erythrocytes and mouse L cells [12,13].

c. Identification of Labeled Amino Acid Residues. If both apical and basal-lateral labeling are largely confined to membrane proteins, the radio-iodine should be located primarily on tyrosine residues since this amino acid is selectively iodinated by lactoperoxidase [22].

Paired hemibladders were labeled, either on the mucosal or serosal sides, and processed under the same conditions as in the lipid extraction experiments. The entire homogenates were extensively digested with pronase. Preliminary experiments showed that, after incubation with pronase for 48 to 72 hr, over 90% of the radioactivity became TCA soluble, indicating that the proteins had been extensively hydrolyzed. The enzymatic digest was then subjected to thin-layer chromatography. Monoiodotyrosine accounted for virtually all of the acid-soluble radioactivity; diiodotyrosine and NaI accounted for less than 10% (Table 6). Similar results have been reported in the plasma membranes of erythrocytes and mouse L cells [12, 13].

Discussion

Under appropriate experimental conditions, high yield enzymatic iodination of the apical and basal-lateral plasma membranes of toad bladder epithelium can be

Table 5. Lipid extraction of ^{125}I-labeled apical and basal-lateral components

	% ^{125}I extracted	
	−TCA	+TCA
3 : 1 Ethanol/ether		
Apical	7.3 ± 2.1	9.6 ± 1.3
Basal-lateral	10.2 ± 0.5	9.6 ± 0.5
10 : 1 Acetone/water		
Apical	10.1 ± 1.8	10.2 ± 2.1
Basal-lateral	9.8 ± 2.3	11.5 ± 1.2

Hemibladders were labeled from either side (apical or basal-lateral) in 50 ml of Ringer's solution at room temperature for 30 min, as described in the legend of Table 1. The epithelial scrapings were dialyzed, sonicated, and the lipids extracted prior to TCA treatment (−TCA) or after precipitation of the protein with 10% TCA (+TCA), in 37 volumes of ethanol-ether or acetone water. The values represent the percent of ^{125}I radioactivity recovered in the organic solvent phase with respect to the total ^{125}I content of the homogenate. Each value is the mean ± SE of 4 to 7 experiments.

accomplished, without evidence of functional impairment of the tissue. The high yields enabled characterization of the apically labeled species and partial purification of the two membrane surfaces [31].

Iodination of toad bladder epithelium should be conducted ideally in Ringer's solution at close to neutral pH to minimize cell damage which may result in labeling of intracellular sites [14]. Although chloride ions may inhibit glucose oxidase and the optimal pH for lactoperoxidase is ~5, iodination of toad bladder epithelium was accomplished under conditions which minimize tissue damage [12,23,32].

Iodination of epithelial membranes poses problems not encountered with cell suspensions. The presence of mucous and the glycocalyx may interfere with labeling by acting as a barrier to diffusion of the enzymes. The increase in the

Table 6. Enzymatic digestion and identification of labeled amino acid residues

	% ^{125}I Recovered		
	M^{125}IT (R_f = 0.4)	D^{125}IT (R_f = 0.20)	^{125}I Na[a] (R_f = 0.85)
Apical	90	5	2
Basal-lateral	92	2	2

Hemibladders were labeled as described in the legend of Table 1. The epithelial scrapings were dialyzed, sonicated, pronase-digested, and chromatographed on thin layers of cellulose silica gel. The R_f values were calculated from samples run adjacent to the enzymatic digest. Carrier amounts of cold MIT and DIT were routinely added to minimize degradation. MIT = monoiodotyrosine; DIT = diiodotyrosine. The values given are the mean of 3 separate experiments.

[a] R_f values for NaI and some other iodides (e.g., iodothyronines) in this system are similar; hence, radioactivity identified as NaI may actually represent an organic iodide.

yields attendant on vigorous stirring of the luminal solution may be a result of the presence of these components on the apical surface.

Lactoperoxidase catalyzed iodination of cell membranes has been used extensively in cell suspensions of erythrocytes, platelets, adipocytes, lymphocytes, fibroblasts, and in myelin sheaths [10,11,12,24,29,41]. Strum and Edelman [40] showed that low-yield iodination of the apical side of the toad bladder had no adverse effects on basal or vasopressin-stimulated SCC and that the silver grains were restricted to the apical surface. Iodination of the basal-lateral surface of rat small intestine and of the brush border of mouse duodenum has also been reported [3,18]. Saccomani *et al.* [35] found that enzymatic iodination of the mucosal surface of the dog stomach produced a single radioactive band (mol wt: 82,000), by SDS-PAGE analysis. A large fraction of the bound ^{125}I failed to penetrate the gels, a finding similar to ours and attributable to labeling of the glycocalyx. As suggested previously [1], the composition and structure of the glycocalyx may be similar in epithelial tissues of different origins. That enzymatic iodination of the glycocalyx accounts for a significant fraction of the yield on the apical side is indicated by the studies on distribution in the fractions (cytosol *vs.* particulate), acid-solubility, release of low molecular weight components by protease or acid and susceptability to alkaline digestion. These results imply peptide attachment to large prosthetic groups, presumably carbohydrates, by O-glycosidic bonds. Proper identification of these species, however, will require the use of special reagents. A molecular species of this structure (e.g., small peptide moiety with high molecular weight prosthetic groups) may explain its acid-soluble character as well as its behavior when analyzed by SDS-PAGE. Recently, it has been reported that treatment of the apical side of the isolated toad bladder with periodate and NaB^3H_4 resulted in selective inhibition of urea transport [34]. If periodate oxidation predominantly affects carbohydrate moieties, the glycocalyx may play a role in some of the transport properties of this epithelium [34].

The acid-insoluble fraction of apical labeling apparently represents iodination of the plasma membrane. Nearly 80% of the membrane-associated radioactivity was distributed among 6 major molecular species whose molecular weights ranged from 23,000 to 100,000. More than half (~60%) of the membrane associated radioactivity was in two bands with molecular weights of 37,000 and 44,000. In red cells most of the radioactivity is attached to a large molecular weight protein (90,000 Daltons, band 3) [12]. Band 3 is clearly involved in some of the transport functions of the cell membrane (e.g., anion transport, sugar transport) [33]. The predominantly labeled species of the apical surface of the toad bladder, however, may not be involved in ion permeation pathways. Amiloride decreased apical membrane iodination by about 40% but did not modify the pattern of iodination assessed by PAGE. Amiloride, at a concentration of 10^{-4} M, markedly inhibits iodination of BSA [30]. Hence, the inhibitory effect of this drug on membrane labeling may be due to inhibition of the labeling enzymes rather than to interaction of the amiloride with proteins in the membrane surface.

Vasopressin and its intermediate, cyclic AMP, had no effect on the extent or pattern of labeling of apical components. Recent studies with freeze-fracture electron microscopy have shown that vasopressin and cyclic-AMP stimulation of isolated toad bladders results in aggregation of intramembranous particles in or-

derly linear arrays in the apical membrane [15]. The lack of effect of vasopressin and cyclic-AMP on the pattern of iodinated membrane proteins may indicate that the species that participate in ion translocation are intrinsic membrane proteins and possess short exposed segments on the apical boundary. As a result, labeling of these sites may be obscured by a large background of heavily labeled extrinsic luminal proteins. In this regard, it is of interest that the particle aggregation observed in freeze-fracture studies occurs in the cytoplasmic surface of the fracture [15].

Exposure of the basal-lateral aspect of the toad bladder to the labeling reagents for 20 to 30 min resulted in isotope incorporation into scraped epithelial cells of comparable magnitude to that observed with luminal iodination. The difficulty with basal-lateral labeling stems from the fact that heavy labeling of the submucosal structures also occurs, including the basement membrane. The heavy labeling also complicates analysis of label localization by light and electron microscopic autoradiography. The observation that, after collagenase digestion, a large proportion of the label was recovered in the epithelial scraping, however, provides evidence that iodination of the plasma membrane was accomplished.

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