J. Membrane Biol. 26, 301–317 (1976)

Differential Covalent Labeling of Apical and Basal-Lateral Membranes of the Epithelium of the Toad Bladder

E.B. Margareta Ekblad, Judy M. Strum, and Isidore S. Edelman

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

Received 6 August 1975; revised 3 November 1975

Summary. The apical (luminal) plasma membrane of toad bladder epithelial cells has been labeled with (^{125}I) diazo-diiodo sulfanilic acid (^{125}I-DDISA) as demonstrated by electron-microscopic autoradiography. The silver grains (^{125}I) were localized exclusively to the apical surface. At concentrations of DDISA of 10^{-3} M or less, binding to the apical membrane had no significant effect on the fine structure of the epithelium. At concentrations of DDISA of 10^{-6} M or less, the baseline short-circuit current (SCC), and the response to cyclic 3′,5′-adenosine monophosphate (cAMP) plus theophylline were unimpaired. At 10^{-5} M, baseline SCC was unchanged and the response to cyclic AMP plus theophylline was enhanced. At concentrations of 10^{-4} M and greater baseline SCC was depressed and the response to the nucleotide inhibited.

The basal-lateral epithelial plasma membranes were labeled by exposing the serosal side to pyridoxal phosphate and reducing the resultant Schiff base with sodium borotritide (^{3}H-$NaBH_4$). In electron-microscopic autoradiographs, the silver grains (^{3}H) were found over the basal and lateral surfaces of the epithelium. At concentrations of pyridoxal phosphate of 10^{-4} M and ^{3}H-$NaBH_4$ of 10^{-3} M, there were no significant changes in the fine structure of the epithelium. Addition of pyridoxal phosphate (10^{-4} M) and $NaBH_4$ (10^{-3} M) to the serosal side decreased the baseline SCC significantly but not the response to vasopressin.

Covalent attachment of the ^{125}I and the ^{3}H was indicated by resistance to elution in the preparation of the sections for electron-microscopy and the reagent requirements for binding.

In the two-barrier in-series model of Koefoed-Johnsen and Ussing [18] transepithelial active Na^+-transport is determined by rate-limiting steps at the apical and basal-lateral plasma membranes. The driving force for Na^+ entry across the apical plasma membrane is presumed to be provided by the electrochemical gradient. The Na^+ is then extruded across the basal-lateral membrane by a Na^+ pump. Labeling of the

apical and basal-lateral membranes with nonpenetrable covalently bound reagents could serve two functions: 1) as markers in the separation of apical from basal-lateral plasma membranes, and 2) as probes for studying functional aspects of the separate barriers.

DDISA was chosen as a candidate for covalent labeling of the apical plasma membrane for several reasons: This reagent reacts rapidly with proteins under mild conditions [13, 14, 15, 24]. The resultant C-N-bond is stable and located ortho to the hydroxyl group of tyrosine, though to a lesser extent other amino acids also react with diazonium compounds. ^{125}I was used as the radiolabel because of its suitability for electron-microscopic autoradiography [12, 17]. Sulfanilic acid was used as the parent compound in that the negative charge retards entry into cells. Thus, Pardee and Watanabe [21] used the diazonium salt of 7-amino-1,3-naphthylene disulfonic acid to demonstrate that the sulfate binding protein of *Salmonella typhimurium* is located on the outside of the plasma membrane. Berg [3] used diazo-sulfanilic acid (DSA) and Sears *et al.* [25] used DDISA to label erythrocyte membranes. Bender *et al.* [1] found that reaction of DSA with intact human red cells inhibited the membrane-bound acetylcholinesterase as well as the facilitated diffusion of glucose. In these studies no evidence was adduced of intracellular penetration of the reagent.

In an earlier study, Strum and Edelman [26] reported covalent labeling of the apical plasma membrane of toad bladder with ^{125}I activated by the glucose oxidase-lactoperoxidase system. The availability of multiple iodine isotopes and the prospect of group specific labeling in the same membrane (i.e., the apical leaflet), with two different reagents prompted us to explore the potential utility of ^{125}I-DDISA. Recently, Berg and Hirsch [4] described an improved method for synthesizing ^{35}S-labeled DSA that provides an additional potential means of labeling epithelial as well as other cell surfaces.

Tritium was chosen as a label for the basal-lateral plasma membranes to enable simultaneous differential labeling of both surfaces of the epithelium. Tritiation of the basal-lateral membranes was attempted by reducing a Schiff base (formed between free amino groups of proteins and pyridoxal phosphate) with ^{3}H-$NaBH_4$ [8, 10]. Rifkin *et al.* [22] used this method to incorporate ^{3}H into the coat proteins of influenza virus. Since phosphate esters penetrate cells slowly [22], pyridoxal phosphate should form Schiff bases primarily with proteins of the plasma membrane and consequently the ^{3}H label should be restricted to proteins thus situated.

The present study was designed: 1) to evaluate by electron-microscopic autoradiography whether the radioactive compounds had labeled the desired membrane surface, i.e., the apical membrane by ^{125}I-DDISA and basal-lateral plasma membranes by pyridoxal phosphate ^{3}H-$NaBH_4$; 2) to examine the effect of these reagents on transepithelial Na^+ transport; and 3) to test the effect of these compounds on the electrophysiological response of the toad bladder to cyclic-AMP, theophylline and vasopressin.

Materials and Methods

Urinary bladders from the toad *Bufo marinus* (Tarpon Zoo, Florida) were used in all experiments. The toads were kept without food at room temperature and were maintained on tap water for about 2 weeks before use. After double-pithing the toads, the hemibladders were removed and transferred (NaCl, 111 mM; KCl, 3.4 mM; $KHCO_3$, 2.4 mM; $CaCl_2$, 2.7 mM; osmolarity, 224 mOsm; pH 8.0) to frog-Ringer's solution. For the SCC experiments, the paired hemibladders were mounted in Lucite double chambers, with areas of exposure of 2.54 cm^2, and the serosal surface was supported by nylon stretched across the orifice. For autoradiography, the bladders were mounted on Lucite rings, 2.54 cm^2 in diameter [5], and this assembly was inserted as a diaphragm in glass chambers.

Paired hemibladders were incubated for 1 hr in aerated frog-Ringer's solution. The bathing media were then changed and the bladders were incubated for an additional 2–3 hr before exposure to DDISA or pyridoxal phosphate. The media were adjusted to pH 7.5 for the experiments with pyridoxal phosphate and $NaBH_4$. Active Na^+ transport was measured periodically by the SCC technique of Ussing and Zerahn [27].

Preparation of DDISA and ^{125}I-DDISA

Diiodosulfanilic acid (DISA) was prepared by the method of Helmkamp and Sears [13] and the resultant salmon-colored crystals were used to prepare the diazonium salt (DDISA) by the method of Higgins and Harrington [14] and Saunders [24]. For autoradiography, DISA was treated with $Na^{125}I$ and ICl, which gave the radioactive product by exchange and the ^{125}I-DISA was then diazotized [13]. Crystalline, dried DDISA was stored in the dark for up to 2 months before use, during which time the product is stable [24]. ^{125}I-DDISA was used within 24 hr of preparation.

Light and Electron-Microscopic Autoradiography

^{125}I-DDISA was dissolved directly in frog-Ringer's solution and added to the mucosal media at final concentrations of 10^{-3} or 10^{-6} M. The reaction was allowed to proceed in the dark for 10 or 30 min, and the reaction was terminated by removal of the hemibladder-ring assemblies from the reaction mixture and immersion in 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 1 hr at room temperature. Various areas of the exposed portion of the hemibladders were excised, cut into small pieces and fixed in the glutaraldehyde-cacodylate buffer for an additional hour.

Tritiation involved the following steps: 1) the serosal or mucosal side of the hemibladders was exposed to pyridoxal phosphate (final concentration, 10^{-4} M) for 20 min at room temperature. 2) The chambers were then cooled to 4 °C and ^{3}H-$NaBH_4$ (2.8 Ci/mmole) (final concentration 10^{-3} M) was added to the appropriate side for 15 min. 3) The concentration of pyridoxal phosphate was then increased to 10^{-3} M in order to remove residual ^{3}H-$NaBH_4$. The hemibladders were washed three times in frog-Ringer's solution and fixed in the cold, or at room temperature for a total of 2 hr in 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. The epithelium was scraped free of the connective tissue in some of the hemibladders after labeling with ^{3}H-BH_4 but prior to fixation. The scrapings were fixed as described above. All of the tissue samples were transferred to ice-cold 0.2 M cacodylate buffer, pH 7.4, for 18 hr and postfixed at 4 °C in 2% OsO_4 in 0.1 M sodium cacodylate buffer, pH 7.4. After dehydration in a graded series of aqueous ethanol solutions, the tissues were embedded in Epon 812 [19]. Light and electron-microscopic autoradiographs were obtained as described previously [26].

Analysis of Autoradiographs

Electron-microscopic autoradiographs of ^{3}H-labeled scraped epithelium were analyzed by the grain density distribution technique of Budd and Salpeter [6]. The electron-micrographs were enlarged 33,000× and grain densities were tabulated within normalized units of half density distance (HD) from the suspected source (i.e., the basal and lateral plasma membranes). HD is the distance within which 50% of the grains fall, and for this study (using sections 1000 Å thick, or less, a monolayer of Ilford L4 emulsion, and Microdol-X developer) an HD value of 1500 Å has been established [23]. A similar distribution was then obtained in units of HD by measuring the distances from developed grains to points on a superimposed grid, calibrated to 1 intersection point/0.6 μ^2. The number of grains per unit distance was then divided by the number of points in the same unit distance to obtain a true density distribution.

Electrophysiological Effects of DDISA

The functional consequences of the action of DDISA were evaluated by determining its effect on the baseline SCC and the electrical potential difference (p.d.), and the response to cyclic AMP and theophylline. The apical surface of the experimental quarter-bladders was exposed to DDISA (final concentrations $=10^{-6}$ to 5×10^{-3} M) for 1 hr. The experimental and control quarter-bladders were then challenged with cyclic AMP (final concentration $=5\times10^{-3}$ M) plus theophylline (final concentration $=10^{-3}$ M) by additions to the serosal media. The SCC and p.d. were monitored for another hour. All of these experiments were carried out in the dark at 24 °C. The inactivated form of DDISA was prepared by prior exposure to fluorescent light for 2 hr. Inactivation was revealed by a change in color from light yellow to dark brown. The photoproduct is *p*-hydroxy sulfanilic acid (PHSA) [11]. PHSA was used as a control reagent in electrophysiological studies as described above for DDISA.

Electrophysiological Effects of Pyridoxal Phosphate and Sodium Borohydride

The effects of formation and reduction of the Schiff base on the baseline SCC and p.d. as well as on the response to vasopressin were evaluated in experiments analogous

to those with DDISA. The apical or basal-lateral surfaces of the experimental quarter-bladders were reacted with pyridoxal phosphate (final concentration $=10^{-4}$ M) for 30 min at which time $NaBH_4$ (final concentration $=10^{-3}$ M) was added to the appropriate side. After an additional 15 min the experimental and the control quarter-bladders were given vasopressin (final concentration = 20 mU/ml) in the serosal media. The pH in these experiments was 7.5, obtained by titration of the frog-Ringer's solution.

Reagents

$Na^{125}I$ (17 Ci/mg) was obtained from New England Nuclear Co. and ^3H-$NaBH_4$ (6.4 Ci/mmole) from Amersham-Searle Co. Sulfanilic acid was purchased from Matheson Co., pyridoxal PO_4, $NaBH_4$, and 3′,5′-cyclic AMP from Sigma, theophylline from Nutritional Biochem. Corp. and vasopressin (Pitressin®) from Parke-Davis and Co. All of the conventional reagents were analytical grade.

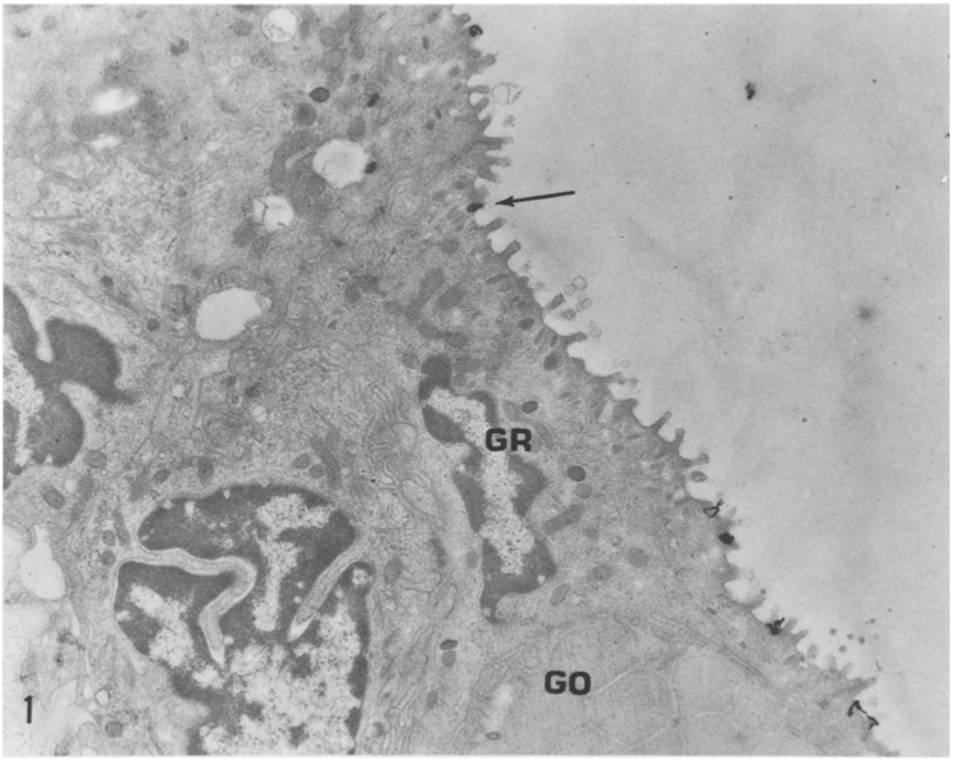

Fig. 1. Low magnification electron-microscope autoradiograph of the mucosal epithelial surface of a toad bladder exposed to ^{125}I-DDISA (10^{-3} M, 60 μCi/ml) for 10 min. Silver grains (arrow) are observed only along the apical membrane surfaces of the cells. Granular cell (*GR*), goblet cell (*GO*). Autoradiographic exposure 8 weeks. Magnification = 8,400×

Results

Autoradiography

In light-microscopic autoradiographs, the silver grains of ^{125}I-DDISA were localized along the luminal epithelial cell surfaces. In electron-microscopic autoradiographs, the grains were over the apical membranes and microvilli of the epithelial cells (Figs. 1 and 2). All three luminal epithelial cell types (granular, mitochondria-rich, and mucous) [7] were labeled but quantitative assessment of the degree of labeling by cell type was not attempted. Except for an occasional background grain (less than 10% of the total), the ^{125}I label was confined exclusively to the apical epithelial surface. The toad bladders shown in Figs. 1 and 2 were exposed to ^{125}I-DDISA for 10 min. Identical autoradiographs

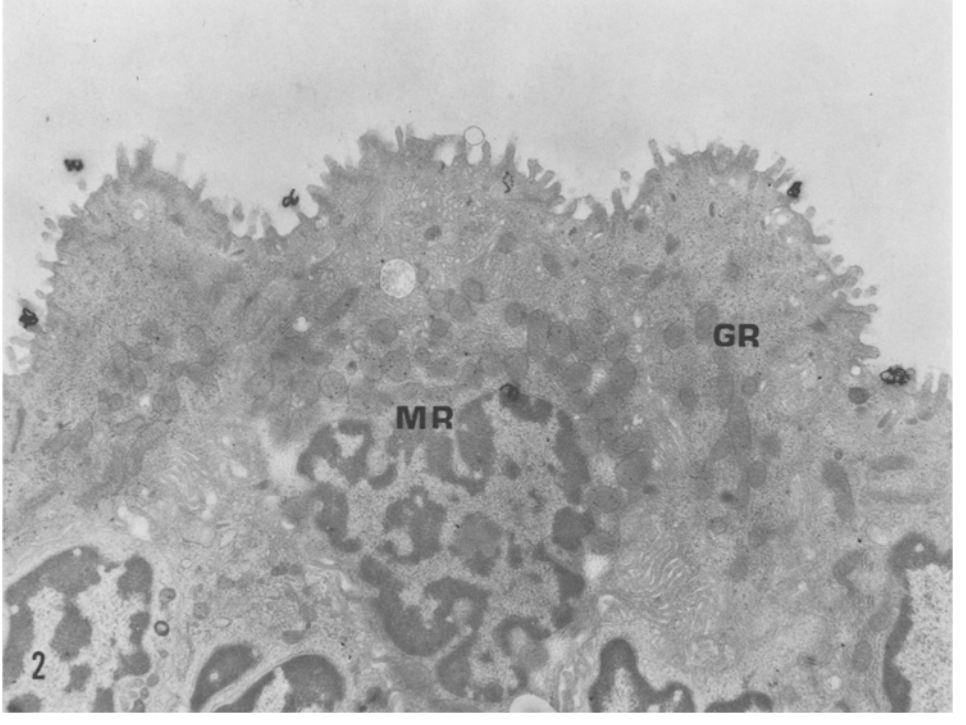

Fig. 2. Electron-microscope autoradiograph of toad bladder epithelium exposed from mucosal side (as in Fig. 1) to ^{125}I-DDISA. Silver grains overlie the apical microvilli of the granular epithelial cells (*GR*) and the mitochondria rich (*MR*) cells. (The apical membrane surfaces of goblet cells were also labeled in these preparations.) Exposed for 9 weeks. Magnification = 9,000 ×

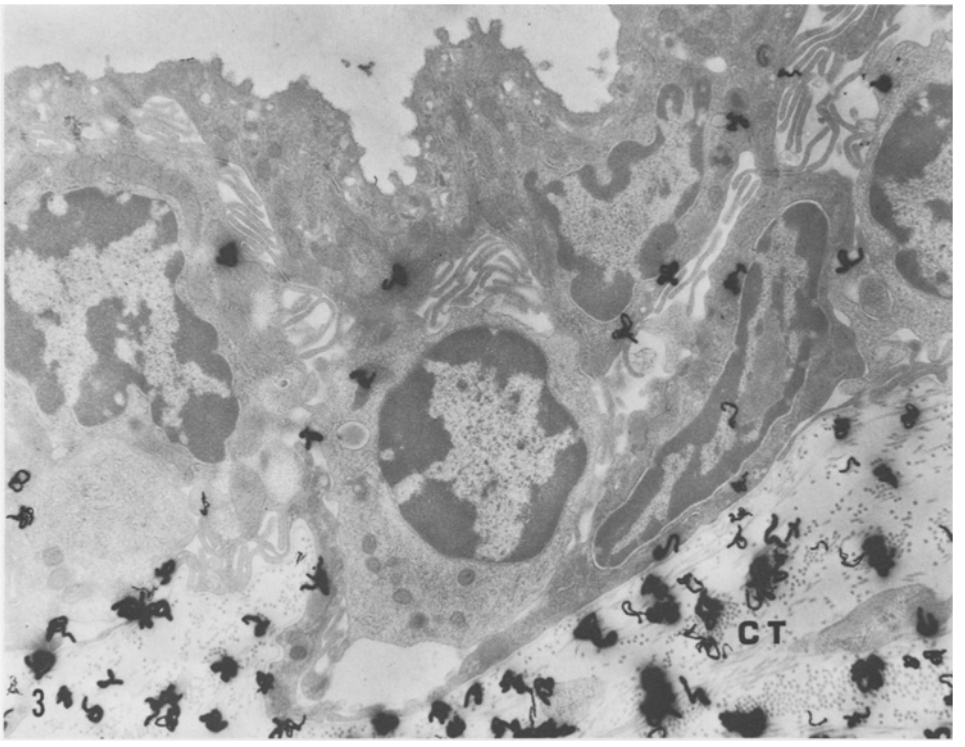

Fig. 3. Electron-microscope autoradiograph of toad bladder epithelium, tritiated by adding pyridoxyl phosphate and ^{3}H-NaBH$_4$ from the serosal surface. Most of the silver grains associated with the epithelium overlie the lateral and basal cell membranes. The connective tissue (*CT*) underlying the epithelium contains many silver grains. Exposed for 8 weeks. Magnification = 8,800 ×

were obtained in bladders exposed for 30 min. Thus, progressive penetration into the cell interior was not apparent for at least 30 min.

Autoradiographs of sections of intact bladders labeled from the serosal side with pyridoxal phosphate-^{3}H-NaBH$_4$ contained silver grains over the connective tissue layer outside the basement membrane as well as over the basal and lateral membranes of the epithelial cells and the intercellular spaces within the epithelium (Fig. 3). The basal surface is opposed to the basement membrane and the lateral membranes bound the intercellular spaces [7]. To delineate the distribution of the label within the epithelium, the epithelial cells were scraped free of the underlying connective tissue and processed for autoradiography. The autoradiographs of isolated epithelial layers revealed many silver grains associated with the basal and lateral cell membranes and almost all of the cells

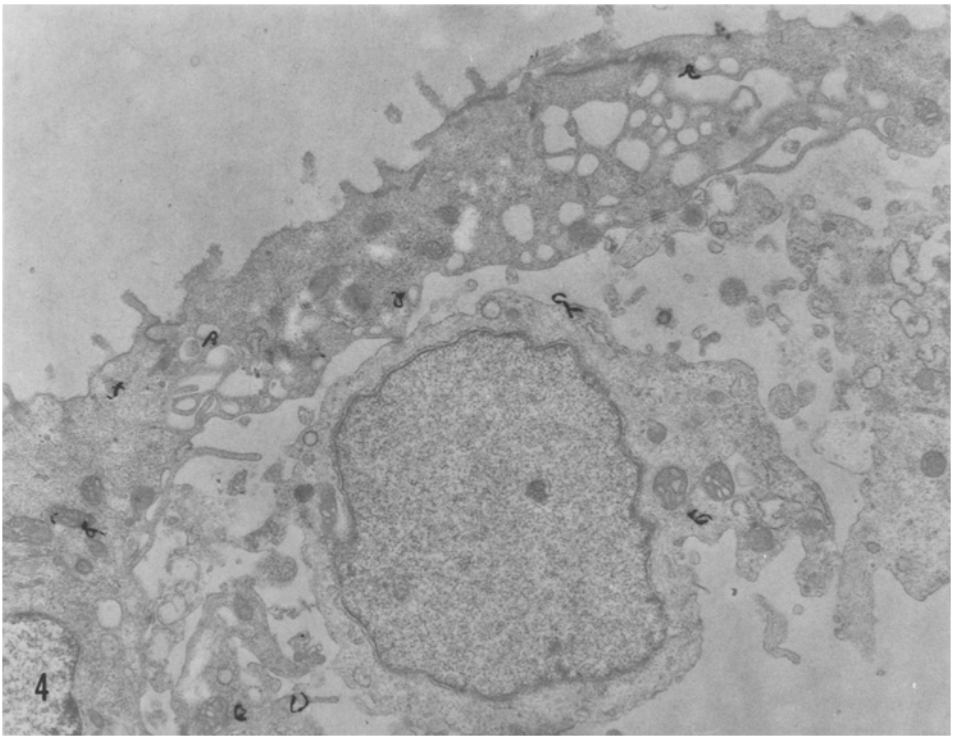

Fig. 4. Electron-microscope autoradiograph of toad bladder epithelium scraped from the underlying connective tissue after labeling from the serosal surface with pyridoxyl phosphate and ^3H-$NaBH_4$. Silver grains are associated with the basal and lateral epithelial cell membranes. Exposed 4 weeks. Magnification = 10,900 ×

contained membrane-associated label (Figs. 4 and 5). The density distribution analysis showed a peak of grain density over the basal and lateral plasma membranes (Fig. 6). The skewed distribution indicates that 15% of the label penetrated the intracellular compartment. In the intact hemibladder, significant numbers of silver grains were seen over the interior of 4.7% of the epithelial cells (of 318 cells counted), and 14.5% of the cells in the isolated epithelial preparation (of 577 cells counted) contained intracellular label. Silver grains were rarely seen within smooth muscle cells, leucocytes, or granular epithelial cells, although the label often penetrated the interior of the erythrocytes. Exposure of the mucosal side of the bladder to pyridoxal phosphate-^3H-$NaBH_4$ resulted in labeling of the apical plasma membrane surfaces of the epithelium but in relatively low abundance. Penetration of the label into the epithelial cells was rare in these preparations.

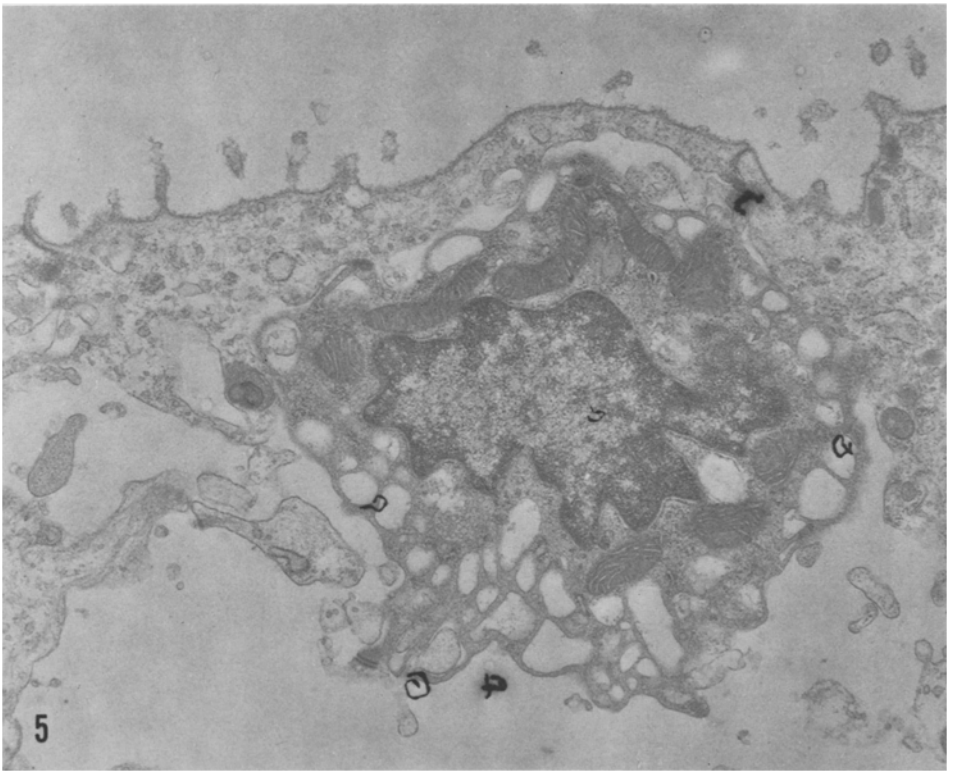

Fig. 5. Electron-microscope autoradiograph of toad bladder epithelium, scraped free of connective tissue, and tritiated by exposure to pyridoxal phosphate and ^3H-$NaBH_4$. Silver grains are most abundant along the basal and lateral plasma membranes of the mitochondria-rich cell. Exposed for 4 weeks. Magnification = 13,400×

Electrophysiological Effects of DDISA

The effects of mucosal addition of DDISA on SCC and p.d. were tested at concentrations of 10^{-6} to 5×10^{-4} M. At 10^{-6} M, DDISA had no effect on either the baseline SCC or the response to cyclic AMP plus theophylline. At 10^{-5} M, this reagent similarly had no effect on the baseline SCC but markedly enhanced the response to the nucleotide (Fig. 7). Irradiation of DDISA was used to obtain the photoproduct PHSA which lacks a group capable of initiating covalent binding to membrane proteins. Thus, PHSA was used in analogous experiments to determine whether augmentation of the response to cyclic AMP plus theophylline elicited by DDISA could be attributed to a covalent binding reaction. As shown in Fig. 8, PHSA (10^{-5} M) had no effect on either

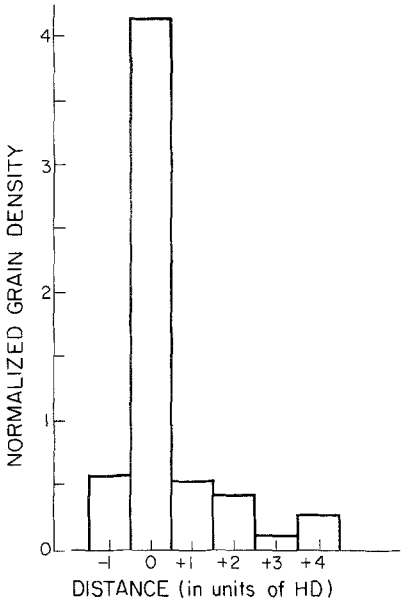

Fig. 6. A histogram of the grain density distribution relative to basal-lateral epithelial cell membranes in scraped epithelial layer from toad bladders labeled with pyridoxal phosphate and ^{3}H-NaBH$_4$. The distance from the plasma membranes is given either as – (outside cell) or + (inside cell). The Half Density Distance (HD) is 1,500 Å and the superimposed grid was calibrated to 1 grid point/0.6 μ^2 at a magnification of 33,000×. The histogram represents 131 grains counted for 231 grid points

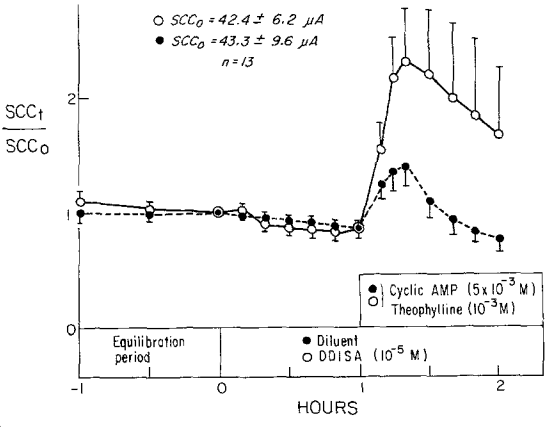

Fig. 7. Effects of DDISA, at low concentration, on the baseline SCC and the response to cyclic-AMP plus theophylline. The DDISA (10^{-5} M) was added to the mucosal solutions of the experimental quarter-bladders at time zero. Cyclic-AMP (5×10^{-3} M) plus theophylline (10^{-3} M) were added to the serosal solutions of control and experimental quarter-bladders as indicated. SCC_t/SCC_0 denotes the ratio of the short-circuit currents recorded at time "t" and time zero. Each point and vertical line represents the mean ± SE. "n" denotes the number of pairs of quarter-bladders. SCC_0 is the absolute SCC at time zero in units μAmp/2.54 cm^2, given as the mean ± SE

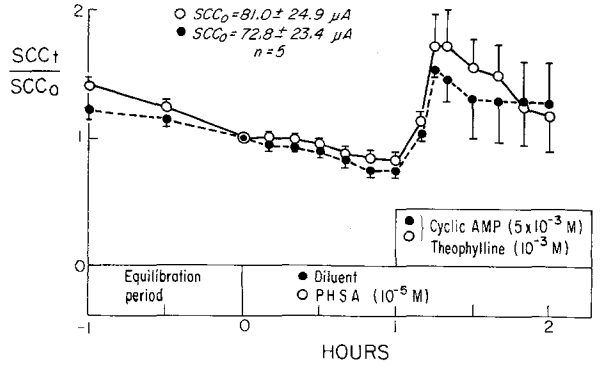

Fig. 8. Effects of PHSA, at low concentration, on the baseline SCC and the response to cyclic-AMP plus theophylline. The time of the additions and the conventions used in this Figure are as in the legend of Fig. 7

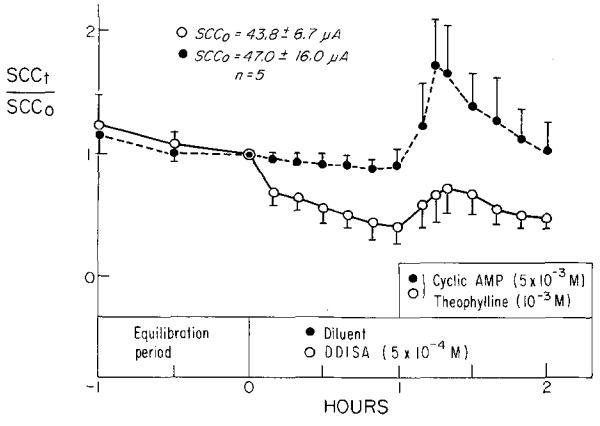

Fig. 9. Effects of DDISA, at high concentration, on the baseline SCC and the response to cyclic-AMP plus theophylline. The time of the additions and the conventions used in this Figure are the same as in the legend of Fig. 7

the baseline SCC or the response to the nucleotide. At high concentrations, DDISA (5×10^{-4} M) inhibited both the baseline SCC and the response to cyclic AMP plus theophylline (Fig. 9). As summarized in Fig. 10, DDISA evoked a complex biphasic response. At low concentrations no effects are seen. At intermediate concentrations only augmentation of the response to cyclic AMP plus theophylline is seen. At high concentrations, both baseline SCC and the response to the nucleotide are significantly inhibited.

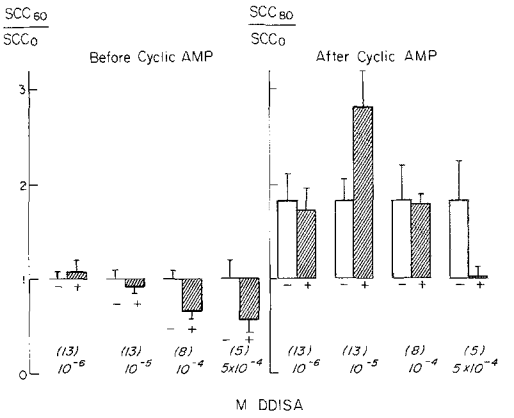

Fig. 10. Effects of DDISA at various concentrations on the baseline SCC and the response to cyclic-AMP plus theophylline. The protocols used in these studies are illustrated in Figs. 7 and 9. The changes in the SCC_{60}/SCC_o and SCC_{80}/SCC_o in the control quarter-bladders are indicated by " − " and in the experimental by " + ". The vertical line represents ± 1 SE. The concentrations used in the experimental quarter-bladders are indicated on the abscissa. The number of paired quarter-bladders in each group is given in brackets

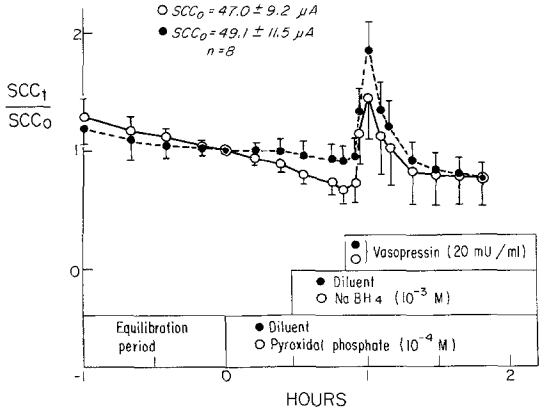

Fig. 11. Effects of pyridoxal phosphate plus $NaBH_4$ on the baseline SCC and the response to vasopressin. Pyridoxal phosphate (10^{-4} M) was added to the serosal solutions of the experimental quarter-bladders at time zero. Thirty minutes later the $NaBH_4$ was added to the same media. Vasopressin (20 mU/ml) was added to the serosal solutions of experimental and control quarter-bladders as indicated. The conventions used in this Figure are the same as in the legend of Fig. 7

Electrophysiological Effects of Pyridoxal Phosphate and $NaBH_4$

The sequential addition of pyridoxal phosphate (10^{-4} M) and $NaBH_4$ (10^{-3} M) to the serosal media appeared to depress the baseline SCC

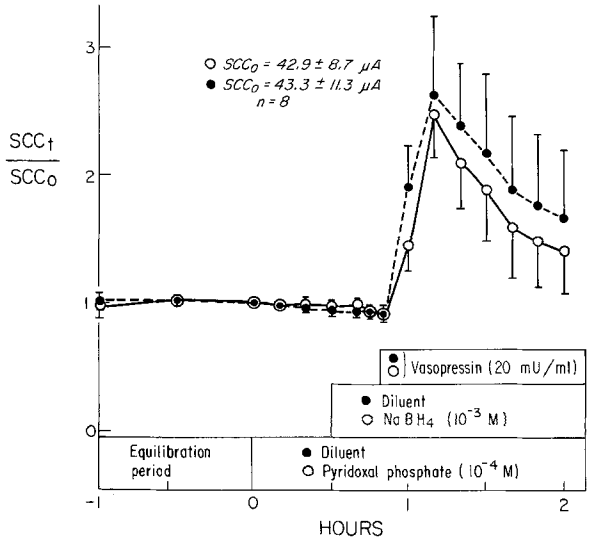

Fig. 12. Effects of pyridoxal phosphate plus $NaBH_4$ on the baseline SCC and the response to vasopressin. The pyridoxal phosphate and $NaBH_4$ were added to the mucosal solutions, otherwise the protocol and conventions used in this Figure are the same as in the legend of Fig. 11

but the differences between the SCC ratios of control and treated quarter-bladders did not achieve statistical significance (Fig. 11). Moreover, the rise in SCC elicited by vasopressin, expressed as the ratio of SCC at the peak to the baseline SCC, was the same in the treated and control populations. These reagents added at the same concentrations and in the same time sequence to the mucosal media, had no discernible effect on baseline SCC or on the vasopressin-induced rise in SCC.

Discussion

The low energy electrons emitted by ^{125}I, fall within the range of sensitivity of Ilford L4 emulsion, and therefore this radionuclide may be used for electron-microscopic autoradiographs [17]. The utility of this radionuclide in defining the site of labeling was demonstrated earlier with ^{125}I labeling of the apical surface of the toad bladder catalyzed by the glucose oxidase-lactoperoxidase system [26].

The autoradiographic results with ^{125}I-DDISA demonstrate that the apical plasma membrane surface of toad bladder epithelial cells is selectively labeled, without any apparent preference for cell type (Figs. 1, 2).

The low rate of penetrance of ^{125}I-DDISA into the epithelial cells is presumably a consequence of the net negative charge contributed by the sulfonate group. The exclusion of grains from the cell interior also indicates that any residual unreacted reagent, ^{125}I-DDISA, that might have been adsorbed to the surface did not cross the membrane barrier during fixation with glutaraldehyde.

The apical plasma membrane of the toad bladder epithelium is covered by an outer coat of "fuzz" or glycocalyx [2, 7, 16][1]. This glycocalyx contains sugars and proteins in abundance as indicated by staining characteristics and susceptibility to proteolytic enzymes.[1] DDISA in all likelihood will react with these surface proteins. In addition, the electrophysiological effects of DDISA, and, in particular, augmentation of the response to cyclic AMP plus theophylline imply a reaction involving proteins intrinsic to the plasma membrane.

Labeling of the apical plasma membrane with DDISA was convenient, fast, reproducible and nontoxic. The electron-microscopic autoradiographs suggest that the reaction with DDISA is complete within 10 min, since increased labeling was not observed by exposing the cells for 30 min. The ultrastructure of the membranes and the interior of the epithelial cells were unaltered even at high concentrations (10^{-3} M) of DDISA. Berg [3] reported lysing of red blood cells with DSA, presumably as a result of increased permeability to Na^+ and K^+. Lysis was not seen in our preparations although an inhibitory effect on SCC was seen at 5×10^{-4} M DDISA (Fig. 9). Further studies are needed to elucidate the nature of this inhibitory effect.

That covalent binding was achieved with DDISA is indicated by the finding that the label withstood displacement by fixation, dehydration and embedding in plastic. A further indication of covalent attachment is the failure of the photoproduct, PHSA, to have any electrophysiological effects.

The available evidence indicates that vasopressin stimulates Na^+ transport by the following sequence of events: 1) activation of an adenyl cyclase enzyme system on or near the basal-lateral side of the epithelial cells; 2) increased intracellular concentration of cyclic AMP; 3) enhanced entry of Na^+ across the apical plasma membrane by the nucleotide or a reaction product; 4) stimulation of extrusion of Na^+ across the basal-lateral boundary by the access of the augmented intracellular Na^+ concentration to a 'transport pool' [9, 20]. Theophylline enhances the

1 Also, F. Miller, R. Bogoroch and I.S. Edelman, *unpublished data.*

Table 1. Effects of DDISA on total transepithelial conductance of the toad bladder

n (pairs)	DDISA	ΔSCC	$\Delta\lambda$	$\frac{\Delta SCC}{\Delta\lambda}$
13	0	23.5	285	0.082
	10^{-5}	54.1	248	0.218
5	0	32.5	415	0.078
	5×10^{-4}	10.0	293	0.034

SCC and conductance (λ) vaues are given as μamps and μmhos/2.54 cm^2. The values used for these computations are taken from the experiments depicted in Figs. 7 and 9. $\Delta SCC = SCC_{80} - SCC_{60}$ and $\Delta\lambda = \lambda_{80} - \lambda_{60}$. λ was computed from the ratio of SCC to the open-circuit pd at each time point.

accumulation of cyclic AMP by inhibiting phosphodiesterase activity [20].

To assess the functional consequences of the binding of DDISA to the apical plasma membrane, cyclic AMP plus theophylline were used as probes. As shown in Fig. 10, DDISA augmented the response to cyclic AMP (plus theophylline) at an intermediate concentration and inhibited the response at a high concentration, implying heterogeneity in the groups titrated or in their functional roles. A further analysis was done on the nature of this heterogeneity by computing total conductance (λ) from the ratio of the SCC to the open-circuit p.d. The results in Table 1 indicate that augmentation of the SCC response to the nucleotide was achieved without a corresponding increase in conductance. These results lend support to the inference that DDISA titrates a variety of membrane groups with distinct differences in their functional roles in the transport process. The nature of these groups and their roles in transepithelial Na^+ transport remain to be elucidated.

The electron-microscopic autoradiographs clearly demonstrate that pyridoxal phosphate-^{3}H-$NaBH_4$ penetrated through the serosal, connective tissue-muscularis layers and the basement membrane of the bladder when the reagents were added to the serosal media. The autoradiographs also clearly establish that tritiation of the basal and lateral membranes of the epithelium was achieved. The histogram in Fig. 6, however, reveals a skewed distribution of grains although the peak is centered over the basal and lateral plasma membranes. The direction of the asymmetry in grain distribution implies significant but small penetration ($\sim$15%) into the intracellular compartment. These grain counts were done on scraped epithelia to facilitate identification of the basal and lateral mem-

branes, which are often obscured by infoldings and interdigitations in the intact preparations. Rifkin *et al.* [22] reported no detectable intracellular labeling with these reagents. We, therefore, considered the possibility that scraping resulted in some cell damage, which facilitated the entry of the reagents into a small number of damaged cells. This possibility was evaluated by estimating the proportion of cells that contained intracellular grains in scraped epithelia and in the epithelium of the intact preparation. The proportion of cells that contained intracellular grains was about 3 times greater in the isolated as compared to the intact preparations (i.e. 14.5% *vs.* 4.7%). Based on these counts and the histogram of the autoradiographic distributions, we conclude that the pyridoxal phosphate-^{3}H-$NaBH_4$ method provides a valid means of differential labeling of the basal and lateral plasma membranes. The resistance of tritium labeling to elution during preparation of the sections for electron-microscopy is an indication that the label was covalently bound.

The availability of techniques for differential covalent labeling of the apical and basal-lateral membranes should facilitate the development of methods for physically separating these surfaces and analyzing their enzymatic as well as other properties (e.g., conductance determinants, role of specific components in the responses to hormones).

It is a pleasure to thank Vivien Ho and Roxie Chaney for their skillful assistance during these studies. This work was performed with the financial assistance of USPHS Grant No. HL-06285 and USPHS Training Grant No. HL-05725 presented to E.B.M.E. by the National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland.

References

1. Bender, W.W., Garan, H., Berg, H.C. 1971. Proteins of the human erythrocyte membrane as modified by pronase. *J. Mol. Biol.* **58**:783
2. Bennett, H.S. 1962. Morphological aspects of extracellular mucopolysaccharides. *J. Histochem. Cytochem.* **11**:14
3. Berg, H.C. 1969. Sulfanilic acid diazonium salt: A label of the outside of the human erythrocyte membrane. *Biochim. Biophys. Acta* **183**:65
4. Berg, H.C., Hirsch, D. 1975. Synthesis of diazotized (^{35}S) sulfanilic acid of high specific activity: A label for the outer surface of cell membranes. *Anal. Biochem.* **66**:629
5. Bogoroch, R. 1969. Studies on the intracellular localization of tritiated steroids. *In*: Autoradiography of Diffusable Substances. L.J. Roth and W.E. Stumpf, editors. p. 99. Academic Press, Inc., New York
6. Budd, G.C., Salpeter, M.M. 1969. The distribution of labeled norepinephrine within sympathetic nerve terminals studied with electron microscope radioautography. *J. Cell Biol.* **41**:21
7. Choi, J.K., 1963. The fine structure of the urinary bladder of the toad. *J. Cell Biol.* **16**:53

8. Churchich, J.E. 1965. Energy transfer in protein pyridoxamine-5-phosphate conjugates. *Biochemistry* **4**:1405
9. Civan, M.M., Frazier, H.S. 1968. The site of the stimulatory action of vasopressin on sodium transport in toad bladder. *J. Gen. Physiol.* **51**:589
10. Cooper, D., Reich, E. 1972. Neurotoxin from venom of the cobra, *Naja Naja Siamensis*. *J. Biol. Chem.* **247**:3008
11. Fieser, L.F., Fieser, M. 1961. Advanced Organic Chemistry. Reinhold, New York, p. 723
12. Forberg, S., Odeblad, E., Soremark, R., Ullberg, S. 1964. Autoradiography with isotopes emitting internal conversion electrons and Auger electrons. *Acta Radiol. Ther. Phys. Biol.* **2**:241
13. Helmkamp, R.W., Sears, D.A. 1970. A label for the red cell membrane: Diazotized diiodosulfanilic acid. *Int. J. Appl. Radiat. Isot.* **21**:683
14. Higgins, H.G., Harrington, K.J. 1959. Reaction of amino acids and proteins with diazonium compounds. II. Spectra and protein derivatives. *Arch. Biochem. Biophys.* **85**:409
15. Howard, A.N., Wild., F. 1957. The reaction of diazonium compounds with amino acids and proteins. *Biochem. J.* **65**:651
16. Ito, S., Winchester, R.J. 1963. The fine structure of the gastric mucosa in the bat. *J. Cell Biol.* **16**:541
17. Kayes, J., Maunsbach, A.B., Ullberg, S. 1962. Electron microscope autoradiography of radioiodine in the thyroid using the extra-nuclear electrons of I^{125}. *J. Ultrastruct. Res.* **7**:339
18. Koefoed-Johnsen, V., Ussing, H.H. 1958. The nature of the frog skin potential. *Acta Physiol. Scand.* **42**:298
19. Luft, J.H. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9**:409
20. Orloff, J., Handler, J. 1967. The role of adenosine 3′,5′-monophosphate in the action of antidiuretic hormone. *Am. J. Med.* **42**:757
21. Pardee, A.B., Watanabe, K.J. 1968. Location of sulfate-binding protein in *Salmonella typhimurium*. *Bacteriology* **96**:1049
22. Rifkin, D.B., Compans, R.W., Reich, E. 1972. A specific labeling procedure for the proteins on the outer surface of membranes. *J. Biol. Chem.* **247**:6432
23. Salpeter, M.M., McHenry, F.A. 1973. Electron microscope autoradiography, analysis of autoradiograms. *In*: Advanced Techniques in Biological Electron Microscopy. J.K. Koehler, editor. p. 113. Springer-Verlag, New York
24. Saunders, K.H. 1949. The Aromatic Diazocompounds and their Technical Application. Edward Arnold, editor, 2nd edition. Longmans, Green & Co., London
25. Sears, D.A., Reed, C.F., Helmkamp, R.W. 1971. A radioactive label for erythrocyte membranes. *Biochim. Biophys. Acta* **233**:716
26. Strum, J.M., Edelman, I.S. 1973. Iodination (^{125}I) of the apical plasma membrane of toad bladder epithelium: Electron-microscopic autoradiography and physiological effects *J. Membrane Biol.* **14**:17
27. Ussing, H.H., Zerahn, K. 1951. Active transport of sodium as the source of electrical current in the short-circuited isolated frog skin. *Acta Physiol. Scand.* **23**:110