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# Iodination ($^{125}I$) of the Apical Plasma Membrane of Toad Bladder Epithelium: Electron-Microscopic Autoradiography and Physiological Effects

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*Summary.* The apical plasma membrane of toad bladder epithelial cells has been enzymatically iodinated, using lactoperoxidase, $H_2O_2$ (generated by a glucose-glucose oxidase system) and NaI. The site of labeling was demonstrated by electron-microscopic autoradiography; the silver grains ($^{125}I$) were found exclusively overlying the luminal plasma membranes of the epithelium. The iodination reaction reached completion in less than 5 min. The dependence of the degree of iodination on NaI concentrations (range = $6.3 \times 10^{-8}$ to $6.3 \times 10^{-2}$ M) in the mucosal medium was determined. The results suggest that three classes of sites are iodinated within this concentration range. At concentrations of NaI of $6.3 \times 10^{-6}$ M or less, iodination of the apical membrane had no significant effect on either the fine structure of the epithelium or on electrophysiological properties. The baseline short-circuit current (SCC) remained steady and the response to vasopressin was unimpaired. At concentrations of $6.3 \times 10^{-5}$ M NaI and greater, the baseline SCC was depressed and the response to vasopressin was partially inhibited. The results indicate that $^{125}I$ may serve as a covalent marker (specific for tyrosine and histidine residues) of the apical plasma membrane of epithelia.

The two-barrier series model of Koefoed-Johnsen and Ussing (1958) proposes that transepithelial active $Na^+$ transport is determined by rate-limiting steps at the apical and basal-lateral plasma membranes. In this model, the electrochemical gradient provides the driving force for $Na^+$ entry across the apical plasma membrane and $Na^+$ is extruded across the basal-lateral surfaces by a $Na^+$ pump. Reagents that bind covalently to plasma membranes could serve two functions: (1) as markers in the separation of the apical from the basal-lateral surfaces, and (2) as probes for studying the physiological roles of the separate surfaces in the regulation of active $Na^+$ transport.

Iodination of the apical plasma membrane of the toad bladder was investigated for several reasons: A convenient and rapid method of iodinating

membrane using an enzymatically regulated redox system was described by Hubbard and Cohn (1972). The carbon-iodine bond is stable and specifically located ortho to the hydroxyl group of tyrosine, although histidine residues are also iodinated to a lesser extent (Morrison, Bayse & Danner, 1970). $^{125}I$ is suitable for autoradiography at the electron-microscopic level (Kayes, Maunsbach & Ullberg, 1962; Forberg, Odeblad, Soremark & Ullberg, 1964).

Hubbard and Cohn (1971, 1972) exploited the lactoperoxidase (LPO), glucose-glucose oxidase (GO) system to iodinate plasma membranes of erythrocytes, platelets and L cells in tissue culture, and reported the technique did not provoke cell damage. Owing to molecular size, the enzymes LPO and GO are excluded from the interior of cells which ensures that the highly reactive iodine is released on the outside of the plasma membrane.

Our objectives were to: (1) demonstrate by electron-microscopic autoradiography that iodination of toad bladder epithelium was limited to the apical plasma membrane surface when the reagents were added to the mucosal medium; (2) determine the time-course of the iodination reaction; (3) define the concentration-dependence of membrane iodination; (4) examine the effect of iodination on the baseline short-circuit current (SCC); and (5) test the effect of iodination on the electrophysiological response of the toad bladder to vasopressin.

## Materials and Methods

Urinary bladders from the toad, *Bufo marinus* (Tarpon Zoo, Florida) were used in all experiments. The toads were kept at room temperature and were maintained on $H_2O$ without food for 2 days to 3 weeks before use. After the toads had been double-pithed, hemibladders were removed and transferred into frog-Ringer's solution (NaCl 111 mM; KCl 3.4 mM; $KHCO_3$ 2.4 mM; $CaCl_2$ 2.7 mM; osmolarity 224 mOsm/liter; pH 8.0 in air) and then carefully mounted over Lucite rings, 2.54 $cm^2$ in diameter (Bogoroch, 1969). The hemibladder-ring assembly was inserted as a diaphragm in glass chambers, such that the serosal surface was supported by nylon mesh stretched across the orifice.

Paired hemibladders mounted in the glass chambers were incubated for 30 min in aerated frog-Ringer's solution. The bathing medium was then changed to a modified bicarbonate buffer (NaCl 104 mM; $NaHCO_3$ 12 mM; KCl 3 mM; $MgSO_4$ 0.5 mM; $KH_2PO_4$ 0.5 mM; $CaCl_2$ 1 mM, glucose 5 mM; 220 mOsm/liter), and maintained at pH 7.2 by gassing with a mixture of 97% $O_2$-3% $CO_2$ (Handler, Preston & Orloff, 1969). After 1 hr the buffer was replaced with fresh bicarbonate buffer and an additional $1^1/_2$ hr equilibration period was instituted before iodination.

### *Enzymatic Iodination, SCC and PD*

Enzymatic iodination of the apical epithelial plasma membrane was accomplished by adding the following to the luminal side of the experimental hemibladders (final concentrations): 0.2 μg/ml glucose oxidase (fungal type IV, Cal Biochem., San Diego, Calif.), various concentrations (100 or 200 μC/ml) of carrier-free $^{125}I$, Na salt (New England Nuclear, Boston, Mass.), and 3 μg/ml lactoperoxidase (gift from Dr. S. J. Klebanoff)[1]. The total

1 Lactoperoxidase is commercially available from Cal Biochem.

voume of the luminal fluid was 15 ml. The iodination reaction was initiated by the addition of the lactoperoxidase. The glucose-glucose oxidase served as a controlled $H_2O_2$-generating system (Klebanoff, 1967). To compensate for the possible effects of changes in the amount of bathing media in the chambers, control hemibladders were treated at the same time by adding identical amounts of buffer.

The transepithelial SCC and electrical potential difference (PD) were measured across each isolated hemibladder at 5-min intervals, by the method of Ussing and Zerahn (1951).

## *Light and Electron-Microscopic Autoradiography*

The iodination reaction was allowed to proceed for 10, 15 or 30 min before the hemibladders were washed in 3 volumes of fresh pre-gassed bicarbonate buffer. The hemibladder-ring assemblies were then removed from the glass chambers and immersed in 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 1 hr at room temperature. Different areas from the exposed portion of the hemibladder were removed and cut into small pieces for an additional 1-hr fixation. The tissue was transferred to ice-cold, 0.2 M sodium cacodylate buffer, pH 7.4, for 18 hr and then post-fixed at 4 °C in 2% $OsO_4$ in 0.1 M sodium cacodylate. After dehydration in a graded series of aqueous ethanol solutions, the tissue was embedded in Epon 812 (Luft, 1961).

For autoradiography at the light microscope level, 1-μ-thick Epon sections were cut and placed on gelatin-alum coated slides. The slides were dipped into a 1:1 aqueous dilution of melted Ilford L4 emulsion (Ilford Ltd., Ilford, Essex, England), dried and stored in light-tight boxes containing Drierite. After exposure for 4 days to 2 weeks at 4 °C, the sections were developed in Dektol (Eastman Kodak Co., Rochester, N.Y.) cleared in Kodak Rapid Fixer, washed in water and stained with 1% toluidine blue "0" buffered to pH 7.2.

For electron-microscopic autoradiography, sections were cut on a Sorvall MT-2 ultramicrotome at a thickness that gave a light gold interference color, and placed on carbon-coated copper grids. The loop method of Caro and van Tubergen (1962) was used to coat the thin sections with Ilford L4 emulsion. Following exposure for 2 weeks to 6 months at 4 °C, the sections were developed for 4 min in Kodak Microdol-X, cleared in 15% sodium thiosulfate, washed, and stained with 0.5% uranyl acetate in 30% ethanol (Caro & van Tubergen, 1962). These sections were examined in a Philips EM 300 electron-microscope.

## *Time-Course of Enzymatic Iodination*

The time-course of the iodination reaction was defined by varying the period of exposure to the reactants to 5, 15, 30 or 60 min. At the end of each time period, the media were replaced three times with fresh, pre-gassed bicarbonate buffer solutions. After the washing procedure, hemibladder-ring assemblies were removed from the chambers and the central areas (approximately 2 $cm^2$ in diameter) of the bladders were carefully excised. The epithelial cells were scraped from the mucosal surface with the edge of a clean glass microscope slide and the scrapings were placed in frog-Ringer's solution in an Eppendorf tube. The cells were washed twice with frog-Ringer's solution and the proteins were precipitated by adding equal volumes of ice-cold 20% trichloroacetic acid. The precipitates were collected by centrifugation at 5,000 rpm for 10 min and then dissolved in 1 ml of 1 N NaOH. The average protein recovery was 0.5 mg per hemibladder. These Eppendorf tubes were inserted into holders that fit the well of the Packard gamma spectrometer, Model 578, and assayed for total $^{125}I$ content. After radioassay, 0.1 ml aliquots were taken for analysis of protein content by the method of Lowry, Rosebrough, Farr and Randall (1951).

### *Concentration-Dependence of Iodination*

In the concentration-dependence studies, the enzymatic reaction was allowed to proceed for 5 min in the presence of $Na^{125}I$ with varying amounts of carrier NaI: the final iodine concentrations ranged from $6.3 \times 10^{-9}$ M to $6.3 \times 10^{-2}$ M. The epithelial cells were scraped from the exposed area of the hemibladder, and radioactivity and protein in each sample was determined as described above.

### *Electrophysiological Effects of Iodination*

The toxicity of the iodination reaction was evaluated by determining its effect on the baseline SCC and PD, and comparing the response to vasopressin of iodinated *vs.* control hemibladders. The apical surfaces of experimental hemibladders were iodinated for 5 min as described above, with nonradioactive NaI at final concentrations ranging from $6.3 \times 10^{-8}$ to $6.3 \times 10^{-2}$ M. After exposure to the iodinating solution for 5 min, the bathing media were removed and replaced three times with fresh pre-gassed bicarbonate-buffer solutions. To test the effect of enzymes alone, hemibladders were exposed to glucose oxidase and lactoperoxidase in the absence of NaI. An additional 45-min stabilization period was allowed before vasopressin (Pitressin, Parke, Davis & Co., Detroit, Mich.) was added to the serosal side (final concentration = 50 mU/ml) of the control and experimental hemibladders. The SCC and PD responses were then recorded for an additional 45 min.

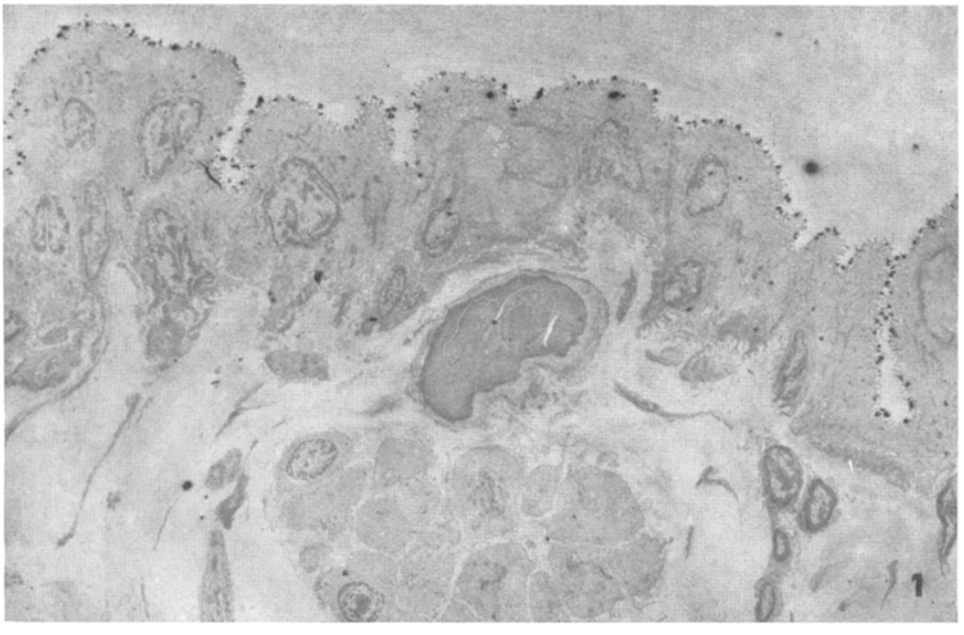



Fig. 1. Electron-microscopic autoradiograph of iodinated toad bladder epithelial cells at very low magnification. The toad bladder was exposed for 30 min to the enzymes and to $Na^{125}I$ at a concentration of $6.3 \times 10^{-8}$ M. The epithelium borders the lumen and $^{125}I$ grains are seen overlying the apical surfaces. Only a few background grains are evident elsewhere in the tissue. Exposed to Ilford L-4 emulsion for 6 months. × 1,600

## Results

### *Autoradiography*

In light microscopic autoradiographs, silver grains indicative of radioiodination were localized along the luminal epithelial cell surfaces. In electron-microscopic autoradiographs, the grains were found specifically overlying the apical membranes and microvilli of the epithelial cells (Figs. 1–4). The apical surfaces of the mitochondria-rich cells, however, consistently showed fewer silver grains than did the granular epithelial cells (Figs. 2 and 3).

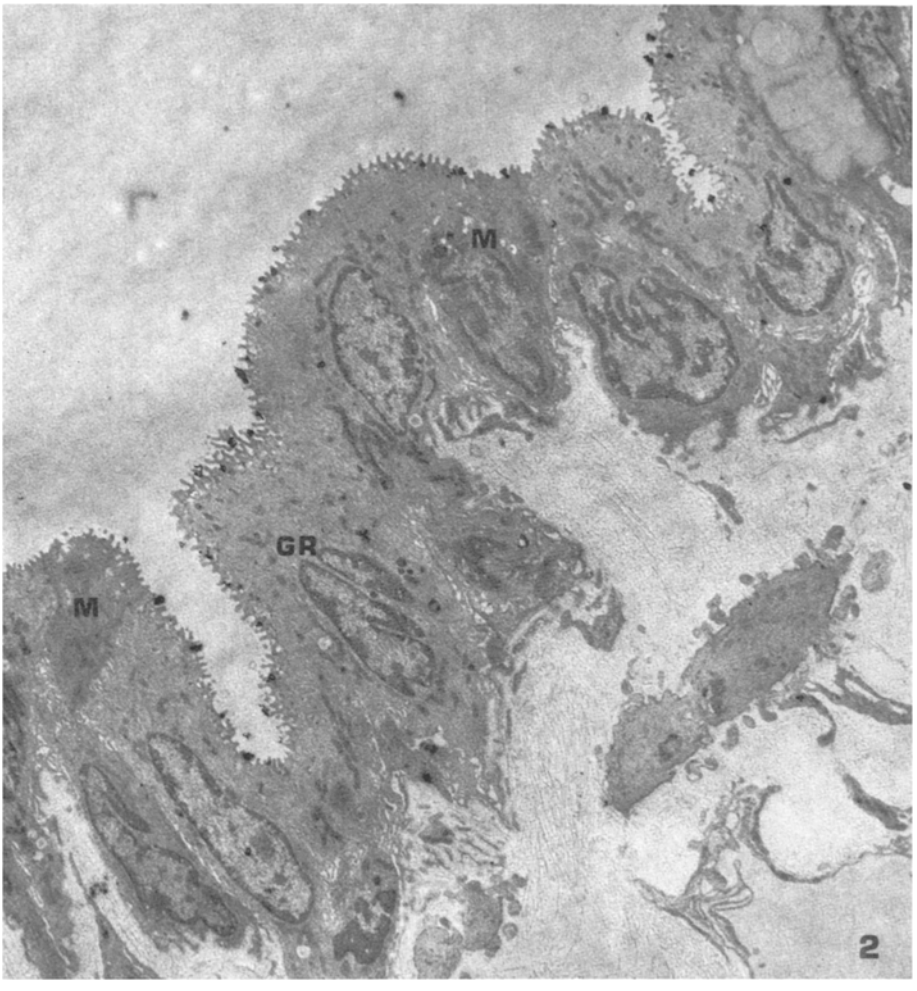



Fig. 2. Low magnification electron-microscopic autoradiograph of enzymatically radioiodinated ($^{125}I$) toad bladder epithelium. The toad bladder was exposed for 30 min to the enzymes and to $Na^{125}I$ at a concentration of $6.3 \times 10^{-8}$ M. The surfaces of the granular cells (GR) consistently showed more silver grains per unit surface area than the mitochondria-rich cells (M). Exposed for 3 weeks. × 3,040

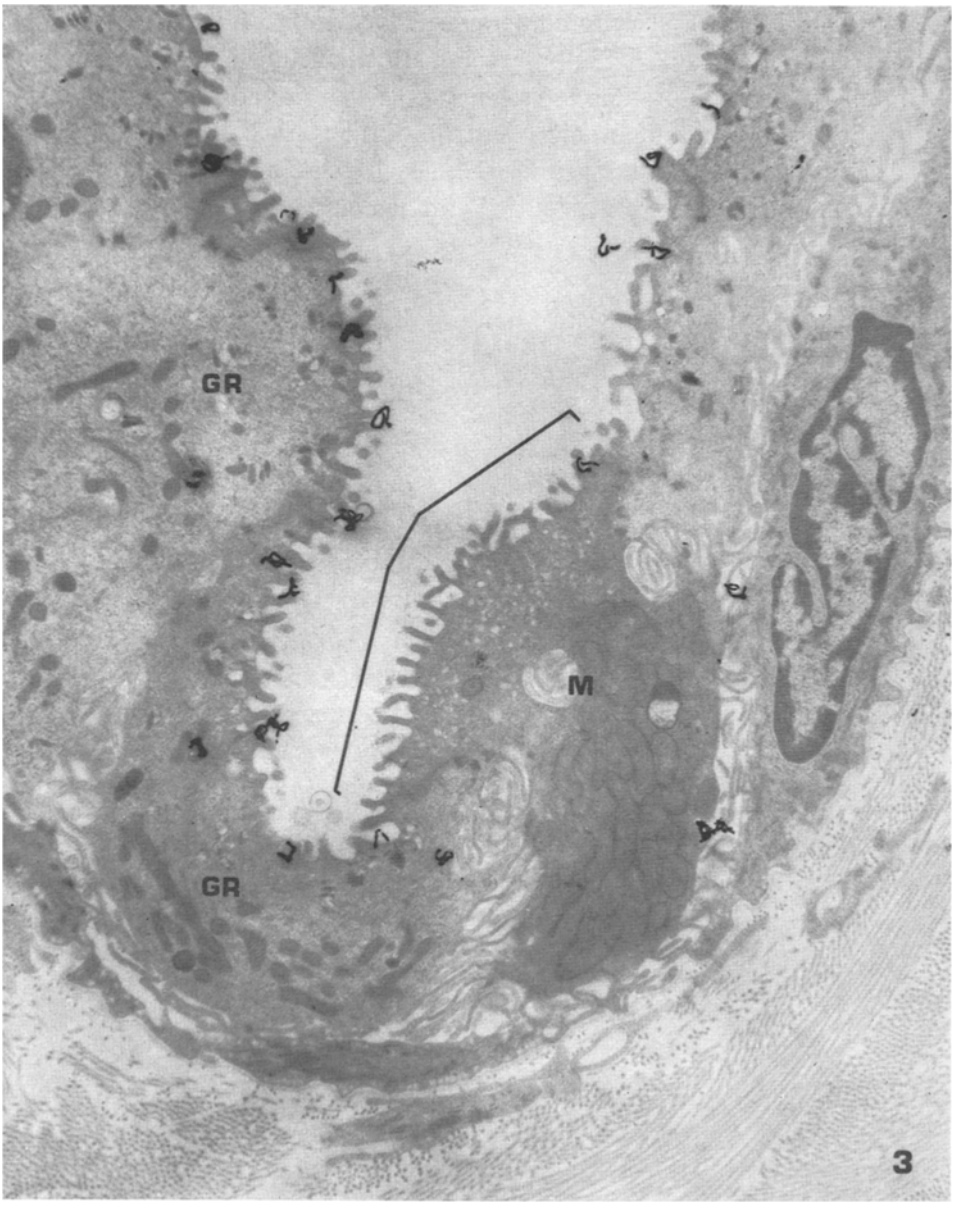



Fig. 3. Electron-microscopic autoradiograph illustrating the difference in the number of $^{125}I$ grains localized over the apical surfaces of granular epithelial cells (GR) as compared with a mitochondria-rich cell (M). The toad bladder was exposed to the enzymes and to $Na^{125}I$ at a concentration of $1.26 \times 10^{-7}$ M. The bracket indicates the extent of the apical border of the mitochondria-rich cell. Exposed for 3 weeks. × 8,500

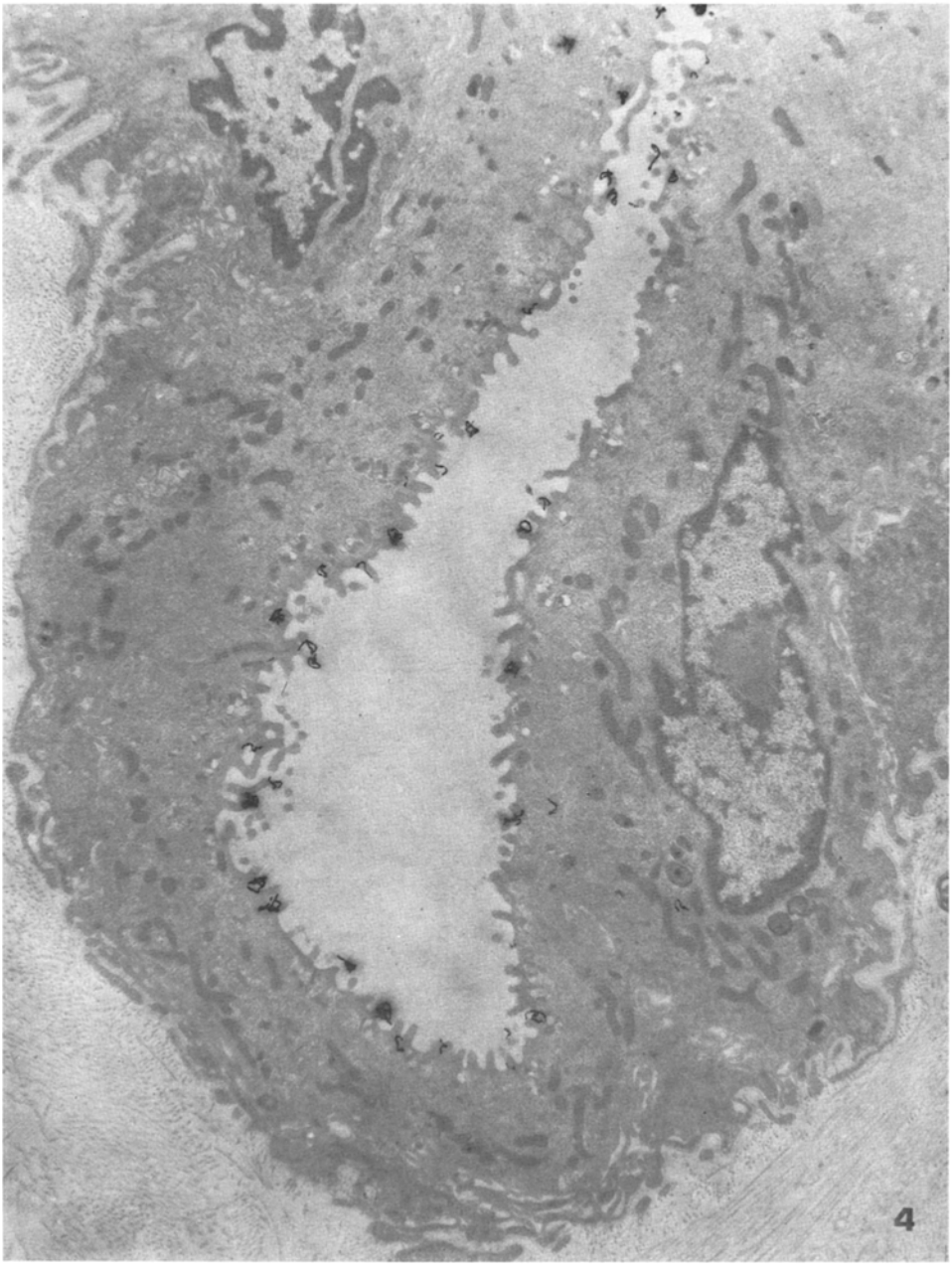



Fig. 4. Electron-microscopic autoradiograph of $^{125}I$ grains overlying the apical plasma membranes and microvilli of toad bladder granular epithelial cells. The toad bladder was exposed to the enzymes and to $Na^{125}I$ at a concentration of $1.26 \times 10^{-7}$ M. All the cells in this section are granular cells. Almost no background grains are evident. Exposed for 2 weeks. × 5,900

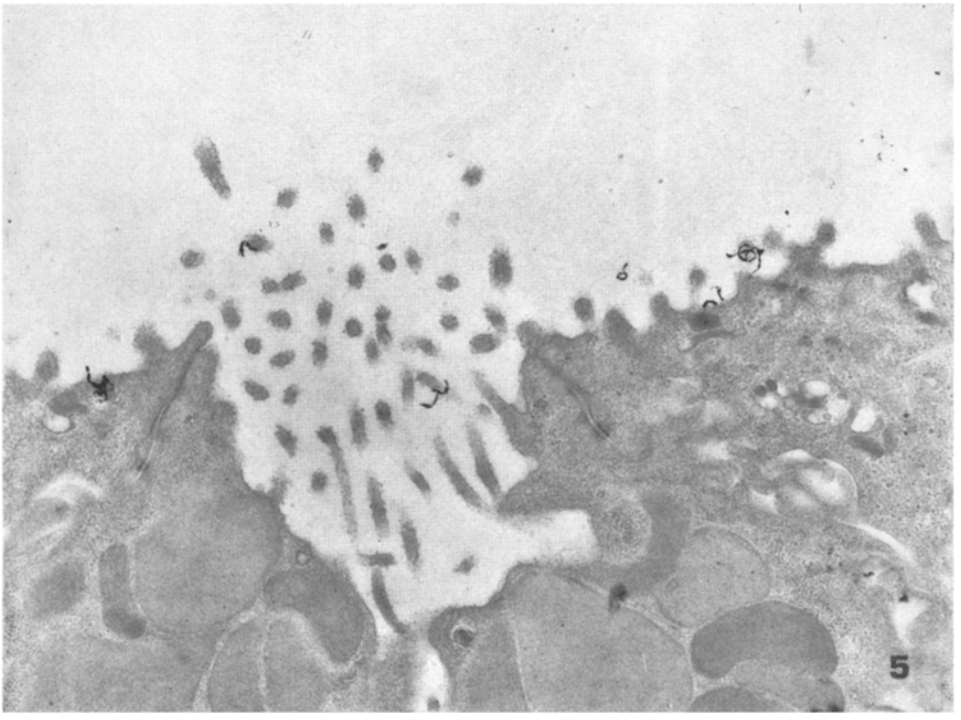


Fig. 5. High magnification electron-microscopic autoradiograph of apical portion of a mucous cell within the toad bladder epithelium. The toad bladder was exposed to the enzymes and to $Na^{125}I$ at a concentration of $1.26 \times 10^{-7}$ M. These cells, which constitute only a small percentage of the cells bordering the lumen, also contained silver grains over their long microvilli. Note the large mucin-containing granules just below the apical surface. Exposed for 2 weeks. × 14,500

This was most obvious at higher magnifications (Fig. 3). The apical surface of mucous cells (which make up only a small percentage of the cell types bordering the lumen of the toad bladder) usually contained a few silver grains (Fig. 5). Other than an occasional background grain, the iodine label was confined exclusively to the apical surface (Fig. 4). The toad bladders shown in Figs. 1–5 were exposed to iodinating solutions for 30 min. Identical radioautographs were obtained in bladders exposed for 10 min. Thus, progressive penetration is not apparent for at least 30 min.

### *Time-Course*

Fig. 6 illustrates the incorporation of $^{125}I$ into membrane protein of toad bladder epithelial cells, as a function of time. It is evident that the reaction has reached completion in less than 5 min. This is in accord with the known rapidity of oxidation of $I^-$ to $I^0$ and the reaction of $I^0$ with tyrosine

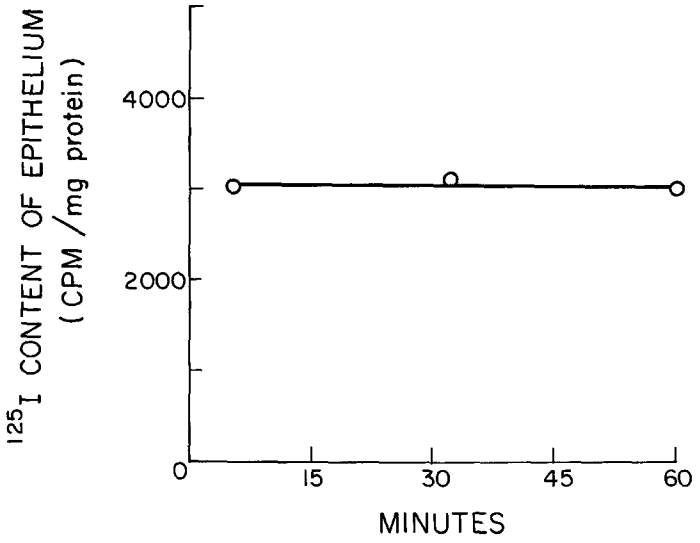



Fig. 6. Time-course of iodination of the toad bladder epithelium. The hemibladders were incubated in bicarbonate-buffer, pH 7.2. The luminal solution contained glucose = 5 mM, glucose oxidase = 0.2 μg/ml, Na$^{125}$I (carrier-free) = $6.3 \times 10^{-8}$ M. At time zero, lactoperoxidase (final concentration = 3 μg/ml) was added to the luminal solutions to initiate the reactions. At the indicated times, hemibladders were washed free (3 ×) of the reagents and assayed for bound $^{125}$I. Each point represents an average of 3 hemibladders

residues (Morrison & Bayse, 1970). The initial $^{125}$I concentration ($6.3 \times 10^{-8}$ M) corresponded to a radiochemical concentration of 100 μC/ml. Thus, less than 1% of the total $^{125}$I pool was covalently bound to the epithelium. No attempt was made to determine why the reaction was "complete" in 5 min when only a small fraction of the $^{125}$I was incorporated.

### *Dependence on NaI Concentration*

The dependence of the extent of iodination of the plasma membrane on NaI concentrations (glucose, glucose oxidase and lactoperoxidase are in excess) is shown in Fig. 7. These studies represent 5 min of exposure to the reagents. It is possible that longer exposure times would alter the concentration-dependence of labeling since "completeness" of the reaction was not determined at concentrations of NaI greater than $6.3 \times 10^{-8}$ M. Plateau regions appear to be present at concentrations of $6.3 \times 10^{-8}$ to $6.3 \times 10^{-7}$ M, and $6.3 \times 10^{-5}$ to $6.3 \times 10^{-4}$ M. The overall pattern suggests a reaction with multiple classes of reactive sites but further experiments would be necessary to establish this point. Even at $6.3 \times 10^{-2}$ M saturation of the iodination sites was not achieved. Thus, to minimize heterogeneity in labeling, low concentrations of Na$^{125}$I should be used (e.g., $\sim 10^{-8}$ M).

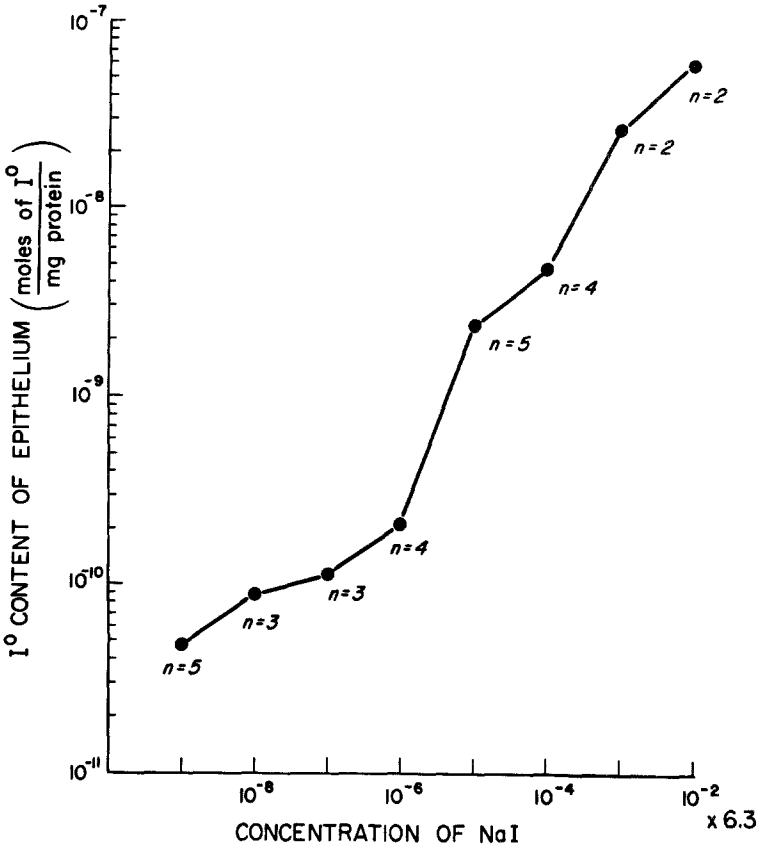



Fig. 7. Dependence of the extent of iodination on the NaI concentration. The conditions for iodinating the apical plasma membrane with $^{125}I$ was as described in text. The contact time with LPO was 5 min. The concentration of $Na^{125}I$ in the mucosal medium is indicated on the ordinate in moles/liter. "N" denotes the number of hemibladders sampled at each concentration

### *Electrophysiological Effects of Iodination*

The format used to determine the effects of iodination on the baseline and vasopressin-stimulated SCC is shown in Fig. 8. Iodination at a NaI concentration of $6.3 \times 10^{-8}$ M had no effect on either the baseline SCC or the increase in SCC evoked by vasopressin. As shown in Fig. 9, iodination at a concentration of $6.3 \times 10^{-2}$ M NaI resulted in a significant depression in the

Fig. 9. Effects of iodination (high concentration of NaI) of the apical plasma membrane on the baseline SCC and the response to vasopressin. The time of additions and the conventions used in this figure are the same as in the legend of Fig. 8

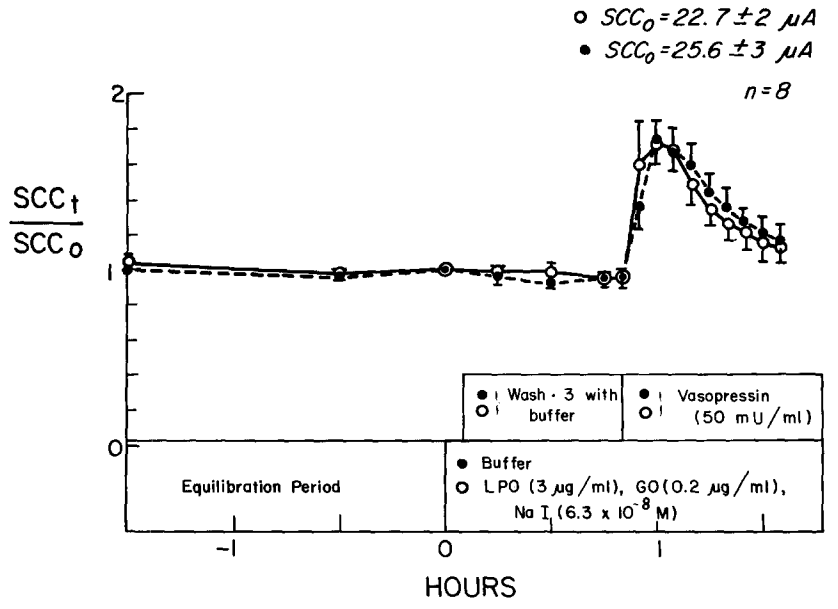
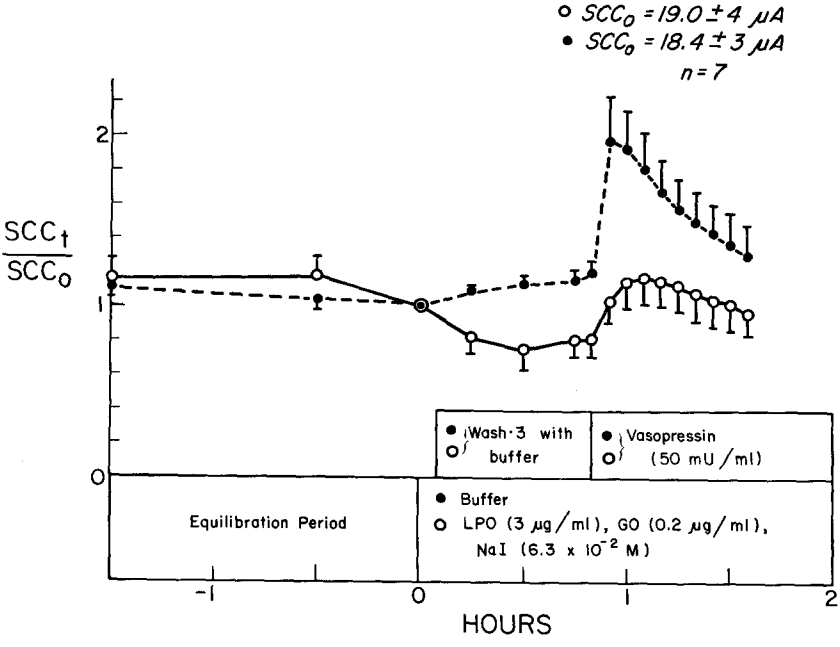



Fig. 8. Effects of iodination (low concentration of NaI) of the apical plasma membrane on the baseline SCC and the response to vasopressin. The enzymes and NaI ($6.3 \times 10^{-8}$ M) were added to the mucosal solutions of the experimental hemibladders at time zero. Vasopressin (50 mU/ml) was added to the serosal solutions of both hemibladders 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 hemibladders. $SCC_0$ is the absolute SCC at time zero in units μAmp/2.54 $cm^2$, given as the mean ± SE



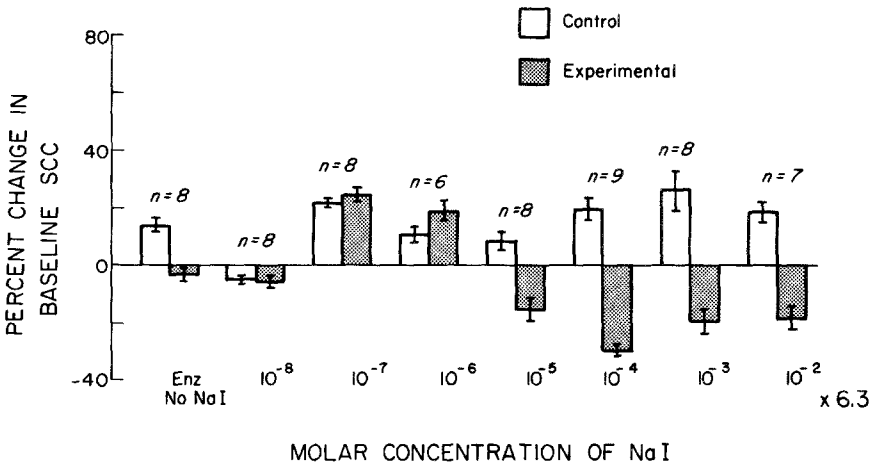



Fig. 10. The effects of iodination of the apical plasma membrane at various concentrations of NaI on the baseline SCC and the response to vasopressin. The protocol used in these studies is illustrated in Figs. 8 and 9. The reference SCC was recorded at time zero and the effect of iodination recorded at 50 min. The per cent change in SCC in the control hemibladders is indicated by the height of the clear bars and in the experimental hemibladders by the shaded bars. The vertical line represents $\pm 1$ SE. The concentration of NaI used in the iodination of the experimental hemibladders is indicated on the abscissa. "N" denotes the number of paired hemibladders in each group. Variations in the height of the clear bars indicate the variations in spontaneous drifts in SCC in the control populations

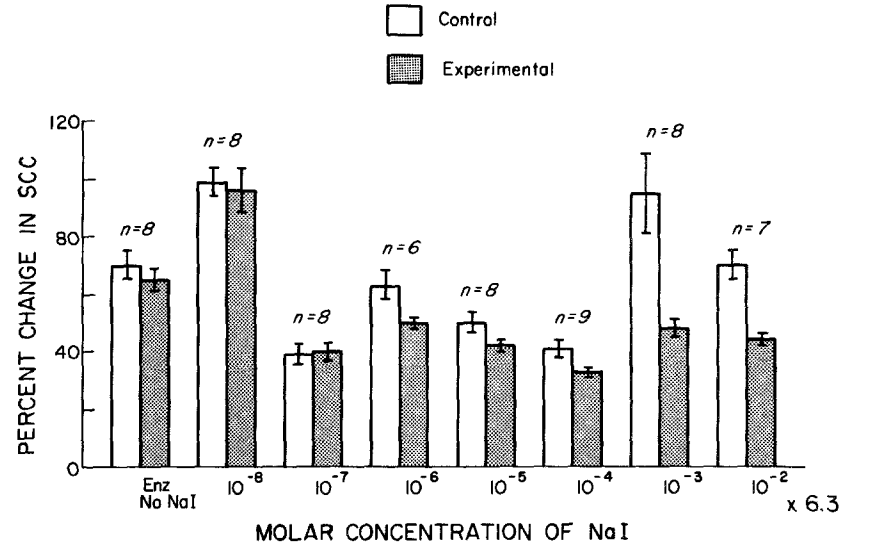



Fig. 11. The effects of iodination of the apical plasma membrane at various concentrations of NaI on the SCC response to vasopressin. The per cent change in SCC was computed from the value recorded at the time of addition of vasopressin to the medium and that at the maximum of the response as shown in Figs. 8 and 9. The conventions used in this figure are described in the legend of Fig. 10

baseline SCC and marked impairment of the response to vasopressin. The concentration-dependence of the "toxic" effects are shown in Figs. 10 and 11. At concentrations of NaI of $6.3 \times 10^{-6}$ M or less, no effect on either the baseline SCC or the increment elicited by vasopressin was apparent. At concentrations of NaI greater than $6.3 \times 10^{-6}$ M, the baseline current was inhibited by 25 to 45% (Fig. 10). Impairment of the response to vasopressin was also evident at concentrations greater than $6.3 \times 10^{-6}$ M but the degree of suppression was marked only at concentrations greater than $6.3 \times 10^{-4}$ M (Fig. 11). In the absence of NaI the addition of lactoperoxidase, glucose and glucose-oxidase had very little effect on either baseline SCC or the response to vasopressin (Figs. 10 and 11). Both the time-course and the dependence on NaI concentration of the effects of iodination on transepithelial PD were similar to those on SCC as shown in Figs. 8–11, except that the inhibitory effects on PD (when they occurred) tended to be somewhat more pronounced. Thus, at concentrations of NaI greater than $6.3 \times 10^{-6}$ M, iodination of the apical membrane tended to increase total transepithelial conductance.

## Discussion

$^{125}I$ emits gamma rays, Auger electrons and internal conversion electrons. The electrons are monoenergetic, low energy particles with short path lengths that fall within the range of sensitivity of Ilford L4 emulsion (Kayes *et al.*, 1962). These low energy electrons are suitable for electron-microscopic autoradiographs and provide resolution similar to that of $^{3}H$, approximately 750 Å, or about 5 times the thickness of the plasma membrane. This isotope, therefore, is suitable for distinguishing between intracellular and plasma membrane labeling.

It was unlikely, a priori, that the enzymes lactoperoxidase (78,000 mol wt) and glucose-oxidase (115,000 mol wt) would cross the plasma membrane. The possibility existed, however, that $H_2O_2$ and $^{125}I$ might enter the cell and iodinate intracellular components. In addition, if the procedure breached the apical permeability barrier intracellular iodination might eventuate. The autoradiographic results, however, clearly demonstrate that the apical plasma membrane surface of toad bladder epithelial cells is selectively labeled by the enzymatic iodination procedure of Hubbard and Cohn (1972). The $^{125}I$ silver grains were localized exclusively over the apical plasma membranes and microvilli of the epithelial cells (Figs. 1–5).

Lactoperoxidase catalyzes iodination of exposed tyrosine (and histidine) residues on proteins (Morrison *et al.*, 1970). In their identification of the iodinated species of the red cell membrane, Hubbard and Cohn (1972) found

that more than 95% was monoiodotyrosine and no label was found associated with histidine or diiodotyrosine. Although we have no information on the iodinated species in the apical membrane of toad bladder epithelial cells, it is probable that proteins of the external coat also known as the "glycocalyx" (Bennet, 1963) or "fuzz" (Ito & Winchester, 1963) were labeled as well as the proteins within the plasma membrane. The outer face of the apical plasma membrane of toad bladder is covered with an abundant glycocalyx which is susceptible to proteolytic enzymes (Miller, Bogoroch & Edelman, *unpublished observations*). These proteins would surely be exposed to the reactive iodine. That the proteins of the plasma membrane proper are also iodinated is suggested by the electrophysiological effects noted above.

The labeling procedure is convenient, reproducible, rapid and gentle: No detectable ultrastructural changes in the membranes or interior of the epithelial cells were detected even at high NaI concentrations. These findings agree with those of Hubbard and Cohn (1972) who reported less than 0.5% lysis of erythrocytes during a 60-min incubation in which ~250,000 atoms of iodine were incorporated per cell. Moreover, iodinated red cells remained intact on storage at 4 °C for from 18 to 48 hr (Phillips & Morrison, 1970; Hubbard & Cohn, 1972).

The stability of the iodination product, presumably mostly monoiodotyrosine, is indicated by its ability to withstand displacement by multiple washes (3 ×) in the chambers, and by fixation, dehydration, and embedding in plastic. The bond is also stable to acid as indicated by the $^{125}I$ content of the TCA precipitates of the epithelium (Fig. 7).

Lactoperoxidase catalyzed iodination of proteins exposed on the luminal surface of the epithelium reached completion in less than 5 min. This is in contrast to the finding of Hubbard and Cohn (1972) where incorporation of $^{125}I$ into erythrocytes was linear up to 30 min.

The concentration-reaction product curve (Fig. 7) suggests that there may be multiple classes of iodination sites in the membranes of toad bladder epithelial cells. To minimize heterogeneity in labeling, low concentrations of NaI may be necessary ($\cong 10^{-7}$ or $10^{-8}$ M). At low concentrations of NaI ($6.3 \times 10^{-6}$ M or less), no effect was recorded on either the baseline SCC or the increase in SCC evoked by vasopressin. At concentrations greater than $6.3 \times 10^{-6}$ M, both the baseline SCC and the response to vasopressin were inhibited. The degree of inhibition, however, was not very different at NaI concentrations between $6.3 \times 10^{-4}$ and $6.3 \times 10^{-2}$ M. The mechanism of inhibition may involve specific transport pathways in that the pattern of the effect differed significantly from that seen with polylysine (Mamelak, Wissig, Bogoroch & Edelman, 1969). Exposure of the luminal surface of the toad

bladder to high molecular weight polylysine resulted in a rapid fall in the baseline SCC but the stimulatory effect of vasopressin on $Na^+$ transport was relatively preserved when expressed in terms of the percentage increase in SCC. Concentrations of the polycation sufficient to inhibit baseline SCC produced epithelial cell lysis in proportion to concentration and duration of exposure to the polymer. These results implied an "all or none" loss of epithelial cells. In contrast, apical iodination had no effect on the ultrastructure of the epithelium. Electron-micrographs of toad bladder iodinated at NaI concentrations of $6.3 \times 10^{-4}$ M revealed no morphological abnormalities. The mechanism of inhibition by iodination of the apical membrane of both the baseline SCC and the response to vasopressin remains to be elucidated.

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