CORRELATION BETWEEN PINOCYTOSIS AND
HYDROOSMOSIS INDUCED BY
NEUROHYPOPHYSEAL HORMONES AND MEDIATED
BY ADENOSINE 3', 5'-CYCLIC MONOPHOSPHATE

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ABSTRACT
The isolated urinary bladder of the toad responds to neurohypophyseal hormone with a
net increase of water transport from the mucosal to the serosal solution in the presence of
an osmotic gradient. This response is mediated intracellularly by cyclic 3', 5'-adenosine
monophosphate (AMP). The present study demonstrates that hydroosmotically active
substances such as oxytocin, dibutyryl cyclic 3', 5'-AMP, and theophylline, but not hy-
droosmotically inactive substances, induce the uptake of horseradish peroxidase from
the mucosal solution. Peroxidase taken up by the mucosal cells is demonstrable in small tu-
bules and vesicles, and eventually accumulates in lysosomes. The uptake of peroxidase from
the serosal solution into similar bodies in the mucosal cells is not hormone-dependent. It
is also shown that peroxidase does not penetrate the tight junction from either the mucosal
or serosal solution. These results extend previous findings which implicated the apical
membrane of the mucosal epithelium as the site affected by neurohypophyseal hormones. A
mechanism based on secretory phenomena is proposed as a framework for future investiga-
tions of apical membrane permeability changes and pinocytosis.

INTRODUCTION
Neurohypophyseal hormones modify the permea-
bility of the toad urinary bladder to water and
certain other small molecules (27, 32). There is
strong evidence indicating that this action is
mediated by adenosine 3', 5'-cyclic monophos-
phate (cAMP)1 (2, 26, 45). Further, agents such
as theophylline also produce a hydroosmotic re-
sponse (45). Theophylline raises the intracellular
levels of cAMP by inhibiting 3', 5'-phosphodiester-
ase, an enzyme which catalyzes the inactivation of
cAMP (9, 25).

Leaf and Hays (33), on the basis of physiological

1Abbreviations used in this paper: 5'-AMP, 5'-adeno-
sine monophosphate; cAMP, adenosine 3', 5'-cyclic
monophosphate; dibut. cAMP, N6-2'-0-dibutyryl
adenosine 3', 5'-cyclic monophosphate; MVB, multi-
vesicular bodies.
experiments, proposed that the hormone-sensitive permeability barrier is located at the mucosal surface of the epithelial cell, a suggestion which has led to a number of electron microscopic studies of this problem (10, 18, 46, 49). Recent electrophysiological studies by Civan and Frazier (13) provide more direct evidence that the action of neurohypophyseal hormones ultimately involves the apical permeability barrier of the mucosal epithelium.

The present electron microscopic study demonstrates a correlation between physiological changes and morphological changes specifically at the apical mucosal membrane. The neurohypophyseal hormone, oxytocin, is shown to stimulate pinocytosis of an exogenous macromolecule (horseradish peroxidase) by the toad bladder. Furthermore, N2O2-Dibutylryl adenosine 3',5'-cyclic monophosphate and theophylline mimic this oxytocin-induced effect, whereas several hydroosmotically inactive agents, such as the polypeptide (5-valine)-oxytocin, the protein insulin, and the acyclic nucleotide 5'-AMP, failed to do so. The experiments with peroxidase provide further evidence for the tightness of junctions between epithelial cells at the toad bladder lumen. Preliminary reports of the findings have been published (38, 39).

MATERIALS AND METHODS

The following compounds were used in this study: oxytocin, prepared by the solid-phase method of peptide synthesis (40, 41) as described by Manning (36); (5-valine)-oxytocin (56) prepared by the stepwise procedure of peptide synthesis (6). Both neurohypophyseal peptides were checked for their activities in the fowl vasodepressor assay (42) just before use. Insulin was purchased from Eli Lilly and Co., Indianapolis, Calif. (Iletin); N2O2-Dibutylryl adenosine 3',5'-cyclic monophosphate (dibut. cAMP) from Schwarz Bio Research Inc., Orangeburg, N.Y.; 5'-adenosine monophosphate (5'AMP) from Nutritional Biochemicals Corporation, Cleveland, Ohio; theophylline from Mallinckrodt, New York; horseradish peroxidase type II from Sigma Chemical Co., St. Louis, Mo.; 3,3'-diaminobenzidine tetrahydrochloride from K & K Laboratories Inc., Plainview, N.Y.; and cysteine 5'-phosphate from P-L Biochemicals, Milwaukee, Wis.

Preparation of Toad Bladders

Toads, Bufo marinus, supplied by National Reagents, Inc., Bridgeport, Conn., were kept on wet peat moss. The urinary hemibladders were excised from doubly pithed toads and placed in Ringer's solution of the following composition: 110 mm NaCl, 3 mm KCl, 1 mm CaCl₂, 5.5 mm dextrose, 1 mm MgCl₂, 3 mm NaHCO₃, 10 mm Tri(hydroxymethyl)amino methane hydrochloride (Trizma); the pH was adjusted to 8.4 with NaOH. The final tonicity was found to be 242 milliosmols/liter. Bladders were mounted as sacs as described by Bentley (4). In brief, each hemibladder was tied to the end of a hollow glass rod with surgical silk, the mucosal surface facing inward. The mounted hemibladder was filled with 7 ml of Ringer's fluid and placed in a bath of 25 ml Ringer's fluid per bladder for a 2 hr preincubation; preincubation is known to increase the sensitivity of toad bladders to neurohypophyseal hormone (54) and cAMP (19).

Incubation Experiments

Paired hemibladders were used, one of which served as the experimental preparation and the other as the control. The paired hemibladders were exposed to peroxidase (0.2%) with and without an osmotic gradient across the bladder. The following conditions were used:

(a) The mucosal solution used for preincubation was discarded, and both the experimental and control hemibladders were refilled with 7 ml of 5-strength Ringer's fluid containing peroxidase. The experimental hemibladder was then transferred to a fresh bath of full-strength Ringer's fluid containing \(10^{-7} M\) oxytocin, \(10^{-7} M\) (5-valine)-oxytocin, \(10^{-7} M\) insulin, \(1\) to \(5 \times 10^{-3} M\) dibut. cAMP or \(10^{-4} M\) theophylline; the control hemibladders were transferred to a serosal bath of full-strength Ringer's fluid with no additions.

(b) The mucosal solution used for preincubation was replaced by \(\frac{1}{2}\)-strength Ringer's fluid containing peroxidase. The experimental hemibladder was placed in a serosal bath of full-strength Ringer's fluid containing \(10^{-7} M\) (5-valine)-oxytocin, \(1\) to \(5 \times 10^{-3} M\) dibut. cAMP or \(10^{-4} M\) theophylline; the paired hemibladder was placed in a serosal bath of full-strength Ringer's fluid containing \(10^{-7} M\) oxytocin. In addition, an experiment was carried out in which a set of hemibladders containing peroxidase dissolved in \(\frac{1}{2}\)-strength Ringer's fluid was placed in full-strength Ringer's fluid containing \(5 \times 10^{-3} M\) dibut. cAMP, whereas the paired set of hemibladders was placed in full-strength Ringer's fluid containing \(5 \times 10^{-3} M\) 5'-AMP.

(c) After preincubation, the mucosal solution was replaced with full-strength Ringer's fluid containing peroxidase, and then placed in a bath of either full-strength Ringer's fluid containing \(10^{-7} M\) oxytocin (experimental hemibladder) or Ringer's fluid per se (paired control hemibladders).

(d) Preincubated bladders were emptied and re-
filled in one set of experiments with full-strength Ringer's fluid, and in another set with \( \frac{1}{2} \)-strength Ringer's fluid. These bladders were placed into a full-strength Ringer's serosal bath containing peroxidase and \( 10^{-7} \) M oxytocin (experimental hemibladders) or no hormone (paired control hemibladders).

Although the incubations were generally carried out for 1 hr, several incubation experiments were terminated after 10 and 20 min. All bladders were weighed at 30-60 min to determine the rate at which water escaped from the mucosal solution. Bladders from at least three toads were studied with a given compound, except in the case of insulin where bladders of two toads were tested.

**Cytochemical Preparation**

**Peroxidase Activity:** After the incubation period the bladders were cut into 2-mm-wide strips. Several of these strips were fixed in Karnovsky's phosphate- or cacodylate-buffered formaldehyde-glutaraldehyde solution (30). After 2-4 hr of fixation at room temperature and an overnight buffer rinse, the strips were cut into 2-mm\(^2\) pieces. Frozen sections (24), which frequently provide a more uniform reaction product than unfrozen tissue, were difficult to prepare from toad bladder. Therefore, the 2-mm\(^2\) bladder pieces were frozen on the head of a freezing microtome (37), and then incubated for 60 min at room temperature in the medium of Graham and Karnovsky (24). This medium without \( \text{H}_2\text{O}_2 \) or without dianinobenzidine served as a control. As a control for intrinsic peroxidase activity, bladders not exposed to peroxidase were incubated in an identical manner. To exclude the possibility that peroxidase activity in the experimental bladders was exclusively extracellular, bladders were incubated in oxytocin- or dibut. cAMP-containing serosal fluid (but with no peroxidase added to the serosal or mucosal solution) and subsequently fixed, as described above, in Karnovsky's fixative and rinsed overnight. These strips were then soaked in 0.2% peroxidase in Tris buffer for 30 min, washed in three rinses of buffer, and refixed in Karnovsky's solution for 15 min (31). They were then frozen and incubated in the medium of Graham and Karnovsky as outlined above.

**Acid Phosphatase Activity:** To study acid phosphatase activity, several of the 2-mm-wide bladder strips were fixed in 2% glutaraldehyde in 0.1 M cacodylate (53), rinsed in buffer overnight, and cut into 2-mm\(^2\) pieces which were frozen on the microtome head. These pieces were then incubated at 37°C in Gomori medium with cytosine monophosphate as substrate (44); substrate-free medium served as a control. For light microscopy, reaction product was visualized with ammonium sulfide. Electron microscopic preparations were not visualized; they were postfixed in osmium tetroxide and embedded as described below.

**Electron Microscopic Preparation**

After cytochemical incubation the bladder pieces were rinsed in 7.5% sucrose and placed in 1% \( \text{OsO}_4 \) in phosphate or Veronal acetate buffer (47) for up to 2 hr on ice. Dehydration in a graded series of ethanol on ice was followed by propylene oxide and all figures, with the exception of the phase-contrast micrographs 1 and 5, are electron micrographs of mucosal cells of the bladder. All figures show bladders incubated (60 min) for the demonstration of peroxidase, except Fig. 9, which shows acid phosphatase activity (60 min incubation). The bar represents 0.5 \( \mu \) for electron micrographs and 3.0 \( \mu \) for phase-contrast micrographs.

**FIGURE 1** Phase-contrast micrograph of a thick section of a bladder treated with oxytocin in the absence of an osmotic gradient. Peroxidase was present in the serosal solution. The bladder lumen (L) is at the left side of the figure. Reaction product is seen in the submucosa (S) and outlines the basement membrane (B) and intercellular spaces (I) of the mucosal epithelium. Granular bodies (G) within the epithelial cells also contain reaction product. N indicates nuclei. \( \times 1850 \).

**FIGURE 2** Portion of the mucosa of a bladder treated with oxytocin in the absence of an osmotic gradient. Peroxidase was present in the serosal solution. In the granular (G) and mitochondria-rich (M) cell vesicles (V) containing peroxidase reaction product are seen adjacent to the basement membrane (B) and intercellular spaces (I). The lumen (L) and a tight junction (T) show no reaction product. The thin section was not stained. \( \times 29,600 \). Insert (upper left): Portion of a bladder incubated in the presence of an osmotic gradient but not treated with oxytocin. Peroxidase from the serosal solution is demonstrated in the intercellular space (I) but is excluded from the tight junction (T). The thin section was stained with uranyl and lead. \( \times 81,900 \).
embedding in Epon 812 (35). Before dehydration some pieces were stained with uranyl acetate en bloc (23). Silver to gold sections were cut on a Porter-Blum ultramicrotome with a diamond knife. Thick sections of each block were viewed by phase-contrast microscopy to orient the block for cross-sectioning through all the tissue layers. Untainted sections and sections stained with lead citrate (92) or with uranyl acetate (57) followed by lead citrate were viewed with a RCA EMU-3F electron microscope. To eliminate possible nonobjectivity of the microscopist, “blind” experiments were performed in which unidented sections from the different experimental conditions were studied for pinocytic activity. Serial sections of oxytocin-treated bladders (Incubation Experiments [a]) were studied for the intracellular localization of peroxidase activity.

**RESULTS**

**Peroxidase in the Serosal Solution**

Peroxidase placed in the serosal bath is demonstrable in the submucosa and the intercellular spaces of the mucosal epithelium (Figs. 1 and 2). The serosal epithelium is incomplete and the submucosa is readily accessible to peroxidase. Tubules and vesicles containing peroxidase reaction product are seen in the granular cells, the mitochondria-rich cells and the basal cells of the mucosal epithelium (Fig. 2). In addition, many multivesicular and some dense bodies in these three cell types contain peroxidase reaction product (Fig. 3). The marker enzyme is never demonstrable in the tight junction at the luminal border nor on the luminal surface of the cells (Fig. 2). There is no detectable difference between control bladders and those exposed to oxytocin, nor between bladders incubated with or without an osmotic gradient. The appearance of the organelles of the different cell types and the cytoplasmic density do not vary under these diverse conditions; however, the variability in the enlargement of intercellular spaces in hormonally stimulated bladders reported by DiBona and Civan (16) is likewise seen in our preparations.

**Peroxidase in the Mucosal Solution**

Pinocytosis induced by the various biologically active compounds (agonists) was correlated with the hydroosmotic response in the identical hemi-bladder. Typical relative hydroosmotic responses to the compounds are cited in Table I.

**NO OXYTOCIN:** When the bladder was exposed to peroxidase in the mucosal solution, peroxidase reaction product is demonstrable on the luminal surface of the epithelial cell including the evaginations and troughs of the microvilli (Fig. 4). The tight junctional region is free of peroxidase reaction product.

In some sections reaction product is seen in tubules and vesicles within a few cells and in the intercellular spaces. In all these instances we observed, by phase-contrast microscopy that adjacent to the cells which contain vesicles with peroxidase activity was a damaged cell filled with peroxidase reaction product. Furthermore, reaction product outlines the basement membrane regions of these cells and their intercellular spaces up to, but not including, the tight junctions (Fig. 5). This pattern of uptake around damaged cells was a consistent finding in all the preparations containing peroxidase in the mucosal solution discussed below, and is similar to that observed when peroxidase was present in the serosal solution (see above).

The regular finding of damaged cells is not unexpected if one considers the manipulation involved in the preparation of the bladder and normal cellular turnover; basal cells have been suggested as the source for cell renewal at the luminal surface (17). In fact, these limited regions of free passage for molecules may be the structural basis for the small amount of water loss ("basal rate" of water loss) in the absence of hormone (34).

**OXYTOCIN:** When oxytocin was present in the serosal solution, peroxidase reaction product is seen

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**Table I**

| Paired hemi-bladders | Agents tested | Weight loss (mg/min) |
|----------------------|--------------|---------------------|
| A₁                   | Oxytocin (10⁻⁴ M) | 21.0                |
| B₁                   | No hormone added | 0.4                |
| A₂                   | Oxytocin (10⁻² M) | 32.0                |
| B₂                   | (5-Valine)-oxytocin (10⁻⁴ M) | 0.3                |
| A₃                   | Dibut. cAMP (5 × 10⁻⁴ M) | 12.4               |
| B₃                   | 5'-AMP (5 × 10⁻⁴ M) | 0.5                |
| A₄                   | Theophylline (10⁻⁴ M) | 3.0                |
| B₄                   | No methylxanthine added | 0.1                |

* Total weight loss of bladders was determined after 60 min. The mucosal solution (3/4-strength Ringer's) contained 0.2% horseradish peroxidase; the serosal solution (full-strength Ringer's) contained the agents listed in the first column.
in many membrane-delimited bodies within the mitochondria-rich and granular cell types of the epithelium: tubules, vesicles, multivesicular bodies (MVB), and dense bodies (Fig. 7 and 8). Peroxidase is not seen in the tight junction (Fig. 7). The pattern of uptake and the organelles of the epithelial cells were identical irrespective of the fact that some hormone-stimulated bladders were exposed to an osmotic gradient, and therefore experienced a net water flux, while others, not exposed to an osmotic gradient, had not experienced a net water transfer.

To determine whether the tubules and vesicles containing peroxidase reaction product were still connected to the luminal membrane or were truly intracellular, two techniques were used: (a) study of serial sections of bladders stimulated by hormone in the presence of peroxidase, and (b) study of sections of bladders stimulated by hormone, fixed, and subsequently exposed to peroxidase en bloc. The serial sections showed that some of the vesicular images are actually cross-sections of tubules, and that some bodies which appear entirely intracellular in one thin section show continuities with the plasma membrane in another. However, some of the peroxidase-containing tubules and vesicles do not show such continuities, and thus apparently have separated from the surface.

Peroxidase-soaked, fixed bladders likewise showed that many of the vesicles and tubules in question were intracellular; reaction product was present on the luminal surface of the mucosal epithelium, but most vesicles and tubules were not reached by peroxidase. A few of the vesicles at the lateral and basal cell surfaces of the mucosal epithelium contain peroxidase reaction product. In contrast, in the serosal mesothelium many pinocytic vesicles are accessible to peroxidase under the conditions of the soak experiment, and presumably are still attached to the cell surface; these vesicles are seen in hormonally stimulated and unstimulated bladders. It should be noted that the trough between the microvilli on the cell surface seen in Fig. 4 could in cross-section be interpreted as a vesicle or vacuole; but the presence of peroxidase reaction product in such a vesicular image of bladders exposed to peroxidase en bloc would militate against this interpretation.

(5-valine)-oxytocin: (5-valine) - oxytocin, an analog of oxytocin which fails to elicit a hydroosmotic response at a concentration of 10^{-7} m (see reference 19 and Table I) was used to determine the specificity of the pinocytic effect induced by the nonapeptide oxytocin. The bladders exposed to (5-valine)-oxytocin appear identical to control hemibladders placed in Ringer's fluid alone. Peroxidase reaction product is not seen in membrane-delimited bodies in the epithelial cells.

DIBUTYRYL 3',5'-CYCLIC AMP; 5'-AMP; AND THEOPHYLLINE: Exogenous dibut. CAMP (21), a potent stimulant of the hydroosmotic response in the intact bladder (20), also promotes uptake of peroxidase from the mucosal solution (Figs. 10 and 11). The degree of pinocytosis in bladders exposed to dibut. CAMP is essentially indistinguishable from that in bladders treated with oxytocin; peroxidase is seen in tubules, vesicles, MVBs, and dense bodies (Figs. 10 and 11). We used an equal molar concentration of the hydroosmotically inactive 5'-AMP (Table I and see reference 45), as a control for a possible non-specific effect of the active cyclic nucleotide. Bladders placed in a serosal bath containing 5'-AMP do not contain intracellular peroxidase reaction product. Theophylline, which inhibits the breakdown of CAMP, elicits a hydroosmotic response (see Table I). In theophylline-treated bladders we find a few cells in the mucosal epithelium with vesicles and multivesicular bodies containing peroxidase reaction product (Fig. 6).

INSULIN: As a further control for possible non-specific pinocytic stimulation insulin, which is hydroosmotically inactive (31), was tested. The effects of insulin on pinocytosis in other tissues are unclear (3, 55). While insulin may be among the compounds which induce pinocytosis in fat cells, the specificity, frequency, and physiological importance of this effect have yet to be adequately evaluated. In our study, bladders exposed to insulin appear identical to those which did not experience oxytocin treatment.

**Acid Phosphatase**

Since the externally applied peroxidase was found in dense bodies and MVBs as well as in the tubules and vesicles, it was important to determine whether these structures were lysosomes as shown in other tissues (15, 23, 28, 29, 37, 43). Cytochemically, a membrane-delimited body can be tentatively identified as a lysosome if it has demonstrable acid phosphatase activity (43). Acid phosphatase activity is found in the dense bodies of the toad bladder epithelial cells (Fig. 9). Occasionally,
reaction product is seen in vesicles and saccules of the Golgi apparatus and in MVBs. Presumably MVBs containing peroxidase will subsequently be transformed into lysosomes by fusion with acid hydrolase-containing bodies (23).

**DISCUSSION**

Using horseradish peroxidase as an exogenous, macromolecular marker for the origin of intracellular tubules and vesicles, we have demonstrated a neurohypophysyal hormone-dependent and cAMP-mediated change in the apical cell membrane of the toad bladder epithelium. Pinocytosis of the marker molecule from the mucosal solution was paralleled by hormone-induced water permeability in the intact bladder. Oxytocin, dibut. cAMP, and theophylline, all of which when added to the serosal bath elicit a hydroosmotic response along an osmotic gradient in the intact bladder, stimulate the pinocytic uptake of peroxidase into tubules and vesicles. Choi (12), studying bladder primarily from dehydrated toads in which levels of endogenous neurohypophysyal hormone are high (5), likewise found evidence of pinocytic activity. It is noteworthy that bladders treated with theophylline, the weakest agonist of the hydroosmotically active agents tested, show the least amount of peroxidase uptake. The absence of pinocytosis in bladders not exposed to hormone or exposed to agents which are essentially hydroosmotically inactive, such as 10^{-7} M (5-valine)-oxytocin, 5'-AMP, or insulin, supports the contention that the pinocytic effect is a specific response to neurohypophysyal hormones. The oxytocin and cAMP-stimulated pinocytosis observed in the toad bladder may be comparable to the endocytosis of colloid by thyroid follicular cells which is stimulated by thyrotropin in vivo (58) and cAMP in vitro (48) and the increased pinocytosis after epinephrine administration in isolated fat cells (14). As in the thyroid and many other cell types (15), most of the peroxidase pinocytosed by the bladder mucosal cells eventually accumulates in lysosomes, which strongly indicates that the peroxidase in the mucosal cells is truly intracellular.

Further support for this contention comes from serial sections in which peroxidase-containing tubules and vesicles are seen which have no contact with the cell surface. Moreover, bladders soaked in peroxidase after fixation incorporate peroxidase reaction product only into a few of their vesicles and tubules, and in none of their multivesicular and dense bodies, substantiating the absence of continuity of these structures with the cell surface.

**FIGURE 3** Portion of the mucosa of a bladder exposed to an osmotic gradient in the absence of oxytocin. Peroxidase was added to the serosal solution. Intercellular spaces (I) of cells are outlined by reaction product which is also present in a coated vesicle (V), a vacuole-like structure (Va), and a multivesicular body (M). A mitochondrion is seen at Mi, a granule at G, and the basement membrane at B. The thin section was stained with uranyl and lead. X 27,000.

**FIGURE 4** Part of the mucosa of a bladder exposed to an osmotic gradient in the absence of oxytocin. Peroxidase was present in the mucosal solution. Portions of two granular cells including the tight junction between them (T) and a basal cell (BC) are shown. Reaction product outlines the luminal surface (L) including an invagination (I) of the surface of one of the cells. No reaction product is seen in the tight junction (T). The empty vesicles (V) present in the basal cell correspond to the vesicles filled with reaction product in Figs. 2 and 3. Mi, mitochondria and G, granules, are characteristic of granular cells. The thin section was stained with uranyl and lead. X 29,800.

**FIGURE 5** Phase-contrast micrograph of a thick section of a bladder treated with oxytocin in the absence of an osmotic gradient. Peroxidase was present in the mucosal solution. D indicates a damaged cell filled with peroxidase reaction product. Reaction product is seen at the luminal surface of the bladder (L). In the region near the damaged cell, product is also seen at the mucosal basement membrane (B). Little reaction product is evident at the basement membrane (M) of a bladder region distant from the point of damage. Nuclei are indicated by N. The submucosa is seen at S. X 1550.

**FIGURE 6** Portion of a granular cell of bladder exposed to theophylline in the presence of an osmotic gradient. Peroxidase was included in the mucosal fluid. Reaction product is seen in some vesicles (V) and a multivesicular body (M). Mi indicates a mitochondrion and L the luminal surface of the bladder. The thin section was not stained. X 20,400.
Figure 7 Portion of the mucosa of a bladder exposed to oxytocin in the absence of an osmotic gradient. Peroxidase was present in the mucosal solution. Reaction product is present within a granular cell, a tubule (Tu), and some vesicles (V). No reaction product is seen in the tight junction (T) between adjacent cells. The Golgi apparatus is indicated by G, granules in the granular cell by Gr, and the bladder lumen by L. The thin section was stained with uranyl and lead. × 40,000.

Figure 8 Portion of a mitochondria-rich cell from a bladder exposed to oxytocin in the presence of an osmotic gradient. Peroxidase was included in the mucosal solution. Reaction product is seen in tubules (Tu), vesicles (V), a multivesicular body (M), and two dense bodies (D). L indicates the bladder lumen and Mi the mitochondria. The thin section was stained with lead. × 34,000.

Figure 9 Part of a mitochondria-rich cell of a bladder exposed to oxytocin in the presence of an osmotic gradient. No peroxidase was added. The tissue was incubated for acid phosphatase activity. Acid phosphatase reaction product is seen in dense bodies (D). Mi indicates mitochondria and M a multivesicular body. The thin section was stained with uranyl and lead. × 32,000.
Figures 10 and 11 Both pictures show parts of two mucosal cells from bladder exposed to 1 mm dibut. cAMP in the presence of an osmotic gradient. Peroxidase was included in the mucosal solution. Reaction product is seen in multivesicular bodies (M). In Fig. 11 reaction product is seen in a dense body (D), in a tubule (Tu), and a vacuole-like structure (V). At the lumen (L) reaction product is present on the apical cell membrane. In both figures reaction product is absent from the tight junctions (T), from the desmosome (De) (Fig. 10), and the spaces between adjacent cells (S) (Fig. 11); the electron density in the region of the tight junction (T) in Fig. 10 results from the use of uranyl as an en bloc stain. Granules are indicated by G and mitochondria by M. The thin sections were stained in uranyl and lead; Fig. 10, 3 min and 12 min, respectively. × 47,000; Fig. 11, 0.5 min and 10 min, respectively. × 39,400.
The latter experiments also identify the large "vacuoles" previously seen in hormone-treated bladders (46, 49) as enlargements of the intercellular spaces.

Peroxidase added to the serosal bath, instead of the mucosal fluid, also results in the presence of reaction product in pinocytic vesicles and lysosomes. In this situation, pinocytosis of peroxidase occurs whether or not oxytocin was added to the ambient fluid. This result leads us to conclude that the basal and lateral sides of the epithelial cells are freely accessible, not only to water (18, 49) but even to a protein. Whether the peroxidase is pinocytosed from the serosal or mucosal solution, it is destined for intracellular digestion, and apparently is not transported in large quantity across to the opposite cell surface.

Our results with peroxidase demonstrate that the junctions between the epithelial cells are indeed tight to a molecule with a radius of 25-30 Å (31). These findings are in agreement with morphological studies by Peachey and Rasmussen (49) identifying the tight junction as a zonula occludens (22) and electrophysiological investigations by Loewenstein et al. (34), which indicate that small molecules cannot penetrate this region.

Thus it appears that neurohypophyseal hormones change the apical membrane structure, as reflected by pinocytosis. A decrease in DC resistance at this membrane has been described by Civan and Frazier (13). In this context, it is of interest that in the most intensively studied pinocytic system, the ameba Chaos chaos L., a comparable change in cell membrane electrical properties parallels the induction of pinocytosis (7). It is therefore suggested that the correlation of hormone-induced changes in pinocytosis, hydroosmosis, and electrical resistance in the toad bladder involves membrane alterations which have fundamental features in common.

Furthermore, in gland cells alterations in cAMP levels have been reported to influence secretion rates (50 and references therein). There is, moreover, evidence suggesting that a concomitant of increased secretion (exocytosis) is the increased formation of pinocytic-like vesicles from the surface membrane. This has been proposed from morphological studies of the parotid gland (1) and is supported by recent work by one of us (28, 29, Abrahams and Holtzman, unpublished) on peroxidase uptake by nerve endings, and by the rat adrenal medulla. Abrahams and Holtzman find that the adrenalin cells of the medulla become depleted of secretion granules several hours after injection of insulin into a rat; as this occurs the cells show enhanced uptake of peroxidase into tubules, vesicles (coated and noncoated), lysosomes, and related structures. It is possible that membrane alterations in the toad bladder likewise involve an increased rate of fusion of secretion granules with the surface membrane, followed by retrieval of membrane from the surface via the observed pinocytic process. Hence, pinocytosis in the bladder could be viewed as a secondary effect after the increased secretion of granules stimulated by oxytocin or cAMP. This hypothesis provides an intracellular structural link between hormone action at the basal cell surface and expression of the final effect at the apical surface; the membrane permeability change could result from the addition to the apical surface of material derived from the secretion granules. Although information concerning the properties of the secretion granule membrane and contents is lacking, we have noted alterations consistent with an increase in secretion as a result of hormonal stimulation of the bladder. The frequency and distribution of granules changed after the bladder had been incubated for several hours with oxytocin or cAMP; these observations were made on thick Epon sections of mucosal epithelium by phase-contrast microscopy and the PAS technique (11). Further, electron microscopic studies by us and others (12, and Schechter and Schechter, cited in reference 8) revealed an apparent increase in the amount of surface-coating material and in distinctness of the outermost leaflet of the plasma membrane at the apical surface of bladders stimulated with oxytocin.

Unfortunately, such observations are difficult to reproduce and in our hands at least, have not provided a reliable index of secretory activity. Recently we have initiated an electron microscopic survey of bladders under various conditions to determine the frequency of observable exocytosis of secretion granules. Thus far we have found continuities between secretion granule membranes and the plasma membrane in oxytocin-treated material but not in control preparations. If this finding can be substantiated it will provide good evidence for the coincidence of exocytosis and pinocytosis in the bladder.

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