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Quantitative Analysis of Exocytosis and Endocytosis in the Hydroosmotic Response of Toad Bladder

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Summary. This study concerns the timing and magnitude of exocytosis and endocytosis in the granular cells of toad bladder during the hydroosmotic response to antidiuretic hormone. Granule exocytosis at the luminal cell surface is extensive within 5 min of the administration of a physiological dose of hormone. Hydroosmosis becomes detectable during this time period. The amount of membrane added to the luminal surface by exocytosis during 60 min of exposure to hormone can be of the same order of magnitude as the extent of the luminal plasma membrane. Endocytosis, demonstrated by peroxidase uptake from the luminal surface, becomes extensive during the period 15–45 min after hormone administration. Thus, maximal endocytic activity occurs later than the period of most extensive exocytosis and seems to correlate with the onset of the decline in water movement. The amount of membrane retrieved from the luminal surface by endocytosis during 60 min of stimulation is at least three quarters of that added by exocytosis. The bulk membrane movement in ADH stimulated preparations does not require the presence of an osmotic gradient. Colchicine inhibits the hydroosmotic response, the exocytosis of granules, and endocytosis at the luminal surface. These results strengthen our view that the bulk circulation of membrane at the cell surface, via exocytosis and endocytosis, is closely related to the permeability changes occurring at the surface.

Neurohypophyseal hormones (antidiuretic hormones, ADH) increase the permeability of the toad bladder to water, ions and small molecules. The changes in permeability to different substances appear to involve selective and distinct pathways (reviewed in Andreoli & Schafer, 1976). The major permeability barrier to water is the mucosal epithelium, a single layer of cells connected by tight junctions; granular cells comprise 85–90% of the cell population (Peachey & Rasmussen, 1961; DiBona, Civan & Leaf, 1969*a*). Hormone acts on the contraluminal plasma membrane of the mucosal epithelium. One result of this is a subsequent change in the luminal plasma membrane's permeability to water (Hays & Leaf, 1962). An increase in adenosine 3′,5′-cyclic monophosphate (cAMP) precedes the permeability change and appears to mediate the physiological effect of the hormone, with the extent of the response being directly related to the magnitude of the cAMP increment (Handler et al., 1965). The other intracellular events linking the hormone to the permeability change are not known. Freeze-fracture techniques have revealed the aggregation of particles in the luminal plasma membrane during the antidiuretic response (Chevalier, Bourguet & Hugon, 1974; Kachadorian et al., 1977*a*). Our laboratory has reported exocytosis of granules at the luminal surface and endocytic retrieval of luminal plasma membrane (Masur et al., 1971; Masur, Holtzman & Walter, 1972). We proposed that exocytosis is related to the permeability change and endocytosis compensates for the exocytic addition of membrane by retrieving membrane. However, our previous studies on such bulk membrane movement involved long periods of hormone exposure (Masur et al., 1971). The present study examines exocytosis and endocytosis with short term ADH incubations and quantitates the bulk membrane movement. The timing of exocytosis correlates with the onset of the permeability change and endocytosis parallels the waning of water movement. The magnitude of bulk membrane movement suggests that it could alter the plasma membrane extensively. The possible linkage of such movement to the hydroosmotic response is further demonstrated by the fact

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that when colchicine inhibits the ADH-induced hydroosmotic response, it also inhibits the bulk membrane movement. A preliminary account of these findings has been published (Gronowicz, Masur & Holtzman, 1978).

Materials and Methods

The following compounds were used: Horseradish peroxidase (Type II), colchicine and 3, 3′-diaminobenzidine tetrahydrochloride. They were purchased from Sigma Chemical Co., St. Louis, Mo.

Lumicolchicine was prepared according to the method of Wilson and Friedkin (1966) in which colchicine, dissolved in 95% ethanol, is irradiated with ultraviolet light. The decrease in absorbancy which occurs with colchicine's conversion to lumicolchicine was measured at 350 nm with a Beckman spectrophotometer. The concentration of colchicine was determined spectrophotometrically. ADH (pitressin vasopressin injection, USP) was purchased from Parke-Davis and Co., Detroit, Mich.

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 double pithed female toads and placed in Puck's medium (P. Eggena, *personal communication*) of the following composition (in mM): 137 NaCl, 4 $KHCO_3$, 1 $MgCl_2$, 1 $CaCl_2$, and 5.6 dextrose, at pH 7.0. The osmotic activity was 270 mosm. Paired hemibladders were mounted as sacs tied to the end of a hollow glass rod by surgical silk (Bentley, 1958) with the bladder's mucosal surface inward. In most experiments the hemibladders were filled with 7 ml Puck's solution diluted 1:5 with distilled water and suspended in aerated full strength Puck's. Aeration was continued throughout the experiment. Osmotic water movement across isolated hemibladders could then be measured gravimetrically by weighing the hemibladder as water escapes from the mucosal solution into the serosal bath.

Incubation Experiments

One of the paired hemibladders from a toad served as an experimental preparation, and the other hemibladder was a control. ADH was added to the serosal bath of appropriate hemibladders to a final concentration of 50 mU. Hemibladders were weighed at 15-min intervals to determine the rate at which water escaped from the mucosal solution. (Those hemibladders, which were incubated for 5 or 10 min, were weighed after 5 or 10 min.)

Exocytosis: The paired hemibladders were preincubated for 1 hr in full strength Puck's solution. Preincubation increases the sensitivity of the toad bladder to ADH (Schwartz & Walter, 1967). After preincubation the experimental hemibladder was weighed and transferred to a fresh bath with hormone and incubated for 5, 30, or 60 min. The control hemibladder was also transferred to a fresh bath.

Endocytosis: Hemibladders were preincubated for 1 hr, then the mucosal solutions were replaced by fresh 1/5-strength Puck's medium and the hemibladders were weighed and transferred to a serosal bath with ADH. Incubations were ended at 15, 30, 45, and 60 min. At the start of the last 15-min interval before the termination of the incubation, the mucosal solution was removed from the hemibladder and 0.3% horseradish peroxidase (HRP) in 1/5 strength Puck's solution was added. The hemibladders were weighed and returned to their baths. To determine the basal rate of endocytosis, 0.3% HRP in 1/5-strength Puck's was presented to the mucosal surface of an unstimulated hemibladder for 15 or 60 min. Other hemibladders were exposed to ADH in a serosal bath for 60 min with 0.3% HRP present in the mucosal solution (1/5-strength Puck's) for the full period.

Colchicine and Exocytosis: These experiments were initiated because of a report by Taylor et al. (1973) that colchicine inhibits the hydroosmotic response. The paired hemibladders were preincubated for 4 hr with 1/5-strength Puck's inside each hemibladder. The experimental hemibladder was preincubated in a serosal bath containing 1×10^{-5} or 2×10^{-4} M colchicine. The control hemibladder was preincubated in a serosal bath without cochicine. At the end of 4 hr the experimental hemibladder was transferred to a bath containing ADH and 1×10^{-5} or 2×10^{-4} M colchicine. The control hemibladder was placed in a bath with ADH but without colchicine. Incubations were terminated at 10, 30, or 60 min. As additional controls, pairs of hemibladders were treated: one with full-strength Puck's solution and the other with full-strength Puck's with colchicine in the serosal bath, and with 1/5 Puck's as the mucosal solution for 4 hr; these bladders were not exposed to ADH.

Given the limits of accuracy of our measurements and since our interest was in determining the morphological state of bladders in which water movement had been effectively inhibited, we chose not to analyze hemibladders in which colchicine produced less than 30% inhibition of water movement, in our quantitative study of granule loss. Approximately 25% of the colchicine-treated hemibladders showed less than 30% inhibition of water movement. Although these hemibladders were not studied in detail, microscopic examination of these preparations showed similar trends in granule loss as were found in hemibladders with a more appreciable inhibition of the hydroosmotic response.

Colchicine and Endocytosis: For the study of colchicine's effect on endocytosis the same procedure, as described in *Colchicine and Exocytosis,* was used with the following modifications; at the end of the preincubation period, the mucosal solution was removed and 7 ml of 1/5-strength Puck's with 0.3% HRP was added to the inside of both control and experimental hemibladders for the entire length of the incubation period. Incubations were terminated at 15, 30, 45, and 60 min.

Lumicolchicine: Experimental hemibladders were preincubated for 4 hr in 100 ml Puck's with 1 ml lumicolchicine dissolved in 95% ethanol to make 1×10^{-5} or 2×10^{-4} M lumicolchicine in the serosal bath. Control hemibladders were preincubated in a serosal bath with 1 ml of 95% ethanol. Then the hemibladders were transferred to their respective baths containing ADH with or without lumicolchicine. The procedure was modified for the analysis of endocytosis by removing the 1/5-strength Puck's at the end of the preincubation period, and adding 1/5-strength Puck's with 0.3% HRP to the mucosal surface. The incubations were terminated at 15, 30, or 60 min for endocytic analysis and 10 or 60 min for the quantitation of exocytosis.

Cytochemical Preparation: The HRP experiments were terminated by removing the mucosal solution from the hemibladders and adding 7 ml of 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, while immersing the bladder in the same fixative. After 15 min fixation at room temperature the bottom of the bladder's sac was cut into 2 mm-wide strips and placed in fresh fixative for 45 min at 4 °C. In these experiments, as in all others, we analyzed a similar region of the bladder, the bottom of the sac, so as to minimize

the effects of possible variability among bladder regions. After fixation and an overnight buffer rinse at 4 °C, the strips were placed on glass slides and frozen on dry ice for 4 min. Freezing produces a more uniform reaction product (Masur et al., 1971). The bladders were then incubated by the following procedure based on that of Graham and Karnovsky (1966); a preincubation for 30 min at room temperature with a freshly prepared solution of 5 mg of 3, 3′-diaminobenzidine tetrahydrochloride (DAB) in 10 ml of 0.05 M Tris HCl, pH 7.6, followed by a 45 min incubation with a fresh solution of 5 mg of DAB, 10 ml of 0.05 M Tris HCl, pH 7.6, and 1% H_2O_2 at room temperature. Reactions were terminated by rinsing the tissue three times with a cold 7.5% sucrose solution. The preparation was postfixed and processed as described below for morphological preparations.

Morphological Preparations: Bladders were routinely fixed by removing the mucosal solution after the last weighing, replacing it with 7 ml of quarter-strength Karnovsky's fixative (Karnovsky, 1965) and immersing the bladder in the same fixative (1.25% glutaraldehyde and 1% formaldehyde in 0.1 M cacodylate buffer, pH 7.4). After 15 min fixation at room temperature the bottom of the hemibladder's sac (approximately 1 cm) was cut into 2 mm-wide strips and placed in fresh fixative for 45 min.

All hemibladders, including those exposed to HRP, were postfixed with 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.2, for one hour on ice (Palade, 1952). Dehydration in a graded series of ethanol on ice was followed by propylene oxide and embedding in Epon 812 (Luft, 1961). Thick sections of each block were viewed by phase contrast microscopy to orient the tissue properly for thin sectioning through all tissue layers. Silver to gold sections were cut on a Porter-Blum ultramicrotome with a diamond knife. Sections were stained with lead citrate alone (Venable & Coggeshall, 1965) or uranyl acetate (Watson, 1958) followed by lead citrate and examined with a Philips 201 electron microscope.

Carbohydrate Cytochemistry: For the electron microscopic detection of carbohydrates, based on the presence of 1, 2 glycol groups, the silver methenamine procedure of Rambourg (1967) was performed on thin sections of unstimulated toad urinary bladders.

Quantitative Analysis of Exocytosis and Endocytosis: As already mentioned, tissue from the same region of the bladder, the bottom of the sac, was used throughout, and our comparisons are based on analysis of paired hemibladders from the same toad. All bladders studied microscopically were also studied for water transport. To avoid bias in quantitative analysis, after the blocks were sectioned grids were relabeled, so that all microscopy was done blind. To prevent the repetitive sampling of the same cells, only one section per block was analyzed. We chose the section that showed minimal occlusion of mucosal cells by grid bars or section defects and analyzed every cell in that section for granule number or exocytic figures (details are in the figure legends).

For the studies of endocytosis, the membrane delimiting endocytic structures identified by their HRP content was measured with a cartographer's wheel on electron micrographs of each granular cell present along the mucosal surface in a section from each HRP block. Most HRP reaction product from the luminal surface is found in endocytic vesicles and tubules which were shown to be truly intracellular in our previous studies (Masur et al., 1971). We did not include large vacuole-like structures near the luminal plasma membrane since some of these are probably still continuous with the cell surface. We included in our measurements the surface membrane of multivesicular bodies (MVBs) but excluded their interior vesicles, some of which are derived almost certainly from endocytic membrane (Holtzman, 1976). Thus, we may have slightly underestimated the amount of endocytic membrane. We also excluded residual bodies from our measurements since their history is likely to be complex (reviewed in Holtzman, 1976). Extensive pinocytosis occurs at the contraluminal surface of the cells but does not affect the endocytic measurements because HRP is present only in the mucosal solution. HRP is prevented from reaching the contraluminal membrane by tight junctions (Masur et al., 1971; Wade & DiScala, 1971).

In granule enumerations, all granules in each cell were counted. The perimeter of the granules was measured from electron micrographs. The granules are believed to be flattened discs (Wade, DiScala & Karnovsky, 1975); therefore, most of the profiles are those of ellipses and infrequently circles. The circumference of the granules was measured with a cartographer's wheel. We measured all the granule profiles – a total of 278 in thin sections of 10 cells from 5 toads (one section of each cell). From these data we calculated the average perimeter of granule profiles in thin section. The thickness of the membranes of granules and of endoplasmic reticulum (ER) was measured from electron micrographs using an ocular micrometer. Cell dimensions and surfaces were traced on electron micrographs with a cartographer's wheel. From control preparations, such as those treated with lumicolchicine, and from comparing hemibladders treated identically (*see* tables below), it appears that differences of less than 10 to 15% in our quantitative parameters cannot be reliably detected.

Results

Some of the data to be discussed are presented in terms of linear dimensions; however, the measurements made are actually a form of morphometry, and the linear values given are directly proportional to the relative surface areas of the structures in question (Loud, 1962; Weibel & Bolender, 1973; Williams, 1977).

Exocytosis

Many of the granules in the granular cells are located at or near the luminal plasma membrane (Fig. 1). Some granules are found in the supranuclear region, especially in unstimulated hemibladders, and infrequently a few granules are lodged below the nucleus, near the contraluminal membrane. Exocytic ("omega") figures (Fig. 2) represent granules being released at the luminal plasma membrane and are seen within 5 min of the administration of neurohypophyseal hormone. At this time, in 188 cells scanned from three toads, one in seven cells per thin section contained an exocytic profile. 204 cells from paired unstimulated hemibladders had no omega figures. These results imply that exocytosis is extensive in ADH-treated hemibladders soon after hormone exposure. Granule release seems to peak by 10 min but is still found at 30 and 60 min of ADH exposure, and even later (Masur et al., 1972).

The magnitude of granule release was determined by counting the total number of granules present in the mucosal cells with and without ADH exposure

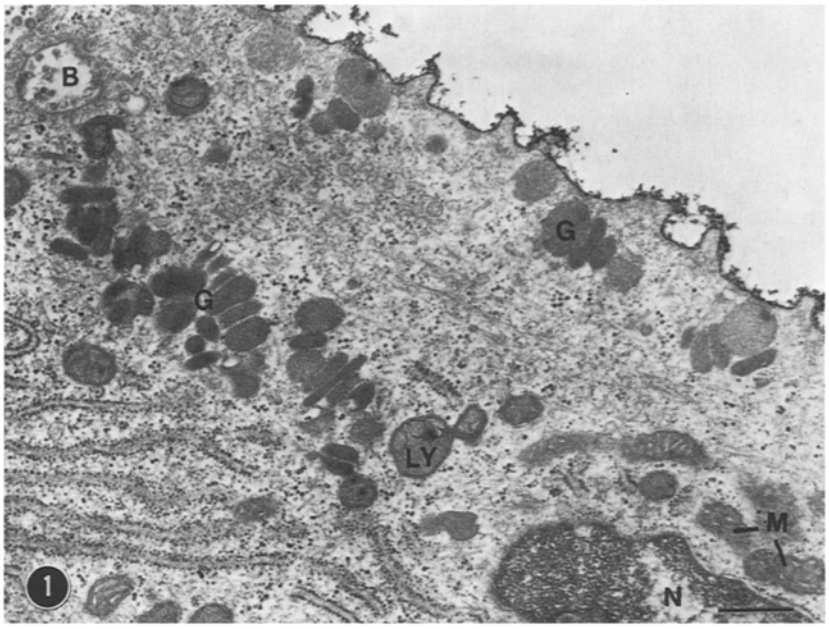


Fig. 1. Numerous granules (*G*) are found in the cytoplasm and at the luminal plasma membrane of a granule cell from an untreated toad bladder. A multivesicular body (*B*), lysosomes (*LY*) and mitochondria (*M*) are also present. *N* indicates the edge of the nucleus. ×20,000. All electron micrographs are from thin sections stained with uranyl acetate and lead citrate, unless otherwise specified in the figure legend. Each bar represents 0.5 μm

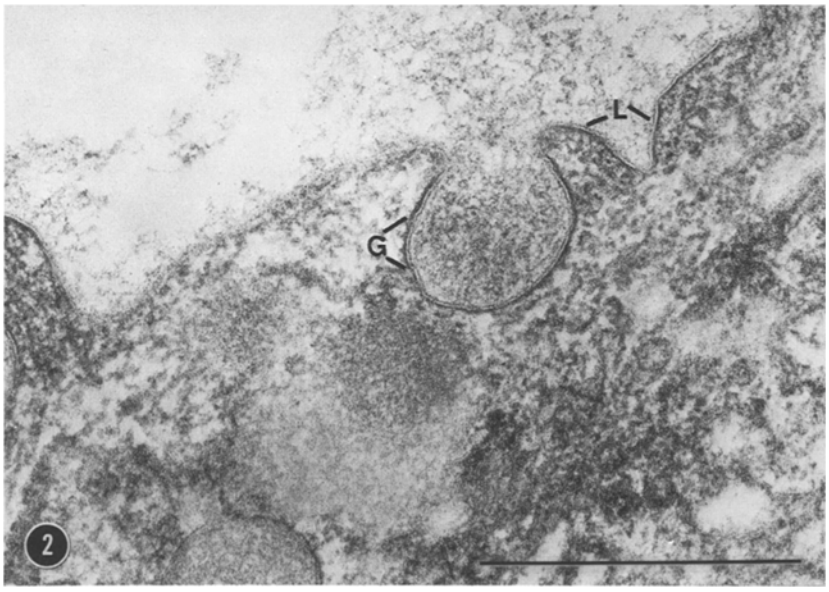


Fig. 2. Exocytic figure in an epithelial cell from a hemibladder exposed to ADH in the presence of an osmotic gradient for 10 min. The limiting membrane of the granule at *G* is continuous with the luminal plasma membrane (*L*). ×85,000

(Figs. 1 and 3). Toads vary in average number of granules per thin section of a cell from 20 to 60 granules. Therefore, it is essential to compare an experimental hemibladder with the control hemibladder from the same toad. (The "no ADH" data demonstrates the similarities between untreated hemibladders from the same toad.) The data in Table 1 indicate the release of 15 granules per thin section of an average cell after 30 min ADH exposure. At 60 min ADH-treated hemibladders have lost an average of 18 granules per thin section of a cell. As is evident from the standard deviations in Table 1, the magnitude of granule loss varied from preparation to preparation (the values for the seven experiments at 60 min were: 17, 30, 41, 46, 52, 58, 78% and the average number of granules lost per cell in thin section ranged from 9 to 43). Note that in all experiments the direction of change was the same and the magnitude of the differences was much greater than seen in hemibladders not exposed to ADH. The percent difference between control and experimental was found to be significant by Student's *t* test ($P < 0.001$). The difference in mean granule number was found to be significant with a $P < 0.01$.

The average perimeter of a granule profile in thin section is 0.89 ± 0.33 (SD) μm. Considering that 18

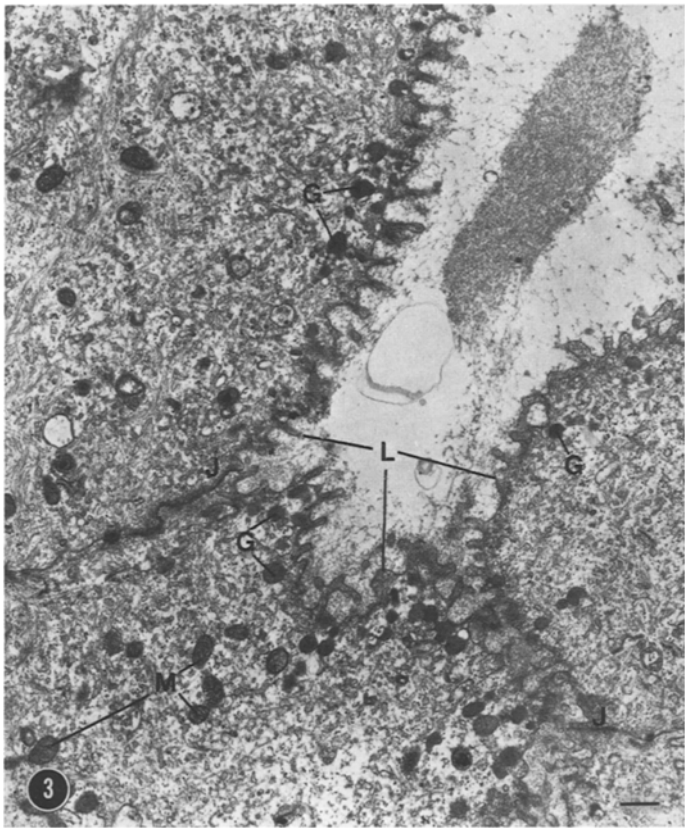


Fig. 3. Portions of several cells exposed to ADH for 60 min in the presence of an osmotic gradient. Most of the granules (*G*) are near the luminal plasma membrane (*L*). Extensive fibrous material resembling the granule content (*cf.* Masur et al., 1972), is seen coating the luminal plasma membrane and in the extracellular space. Junctional complexes (*J*) connect the cells. Mitochondria are seen at *M*. ×10,000

Table 1. Granule release with ADH

	Number of granules released per thin section of a cell	% loss of granules per thin section of a cell	
No ADH	1.6 ± 2.1	5.2 ± 4.6	(4)
30 min with ADH	15.4 ± 12.2	38.5 ± 20.2	(4)
60 min with ADH	18.0 ± 11.1	46.0 ± 18.3	(7)

Hemibladders exposed to ADH for 30 or 60 min were compared to paired unstimulated control hemibladders. The extent of granule release was determined by counting the number of granules present in an average of 20 cells per hemibladder. The no ADH data refer to paired hemibladders, neither of which received hormone. The difference in granule number between the cells in experimental and control hemibladders is indicated in the first column. The second column expresses these data as:

$$\frac{\text{Control hemibladder-experimental hemibladder} \times 100}{\text{control hemibladder}}$$

Data are presented as mean ± SD. The number of toads analyzed is in parentheses.

granules on the average are released per thin section of a cell after 60 min ADH exposure, approximately 16 μm of granule membrane per thin section of a cell are added to the luminal plasma membrane of an average cell. The average length seen in a thin section of the luminal plasma membrane is 22.4 ± 6.0 (SD) μm as measured from electron micrographs of 379 cells in unstimulated bladders from 21 toads. (As noted above, these linear dimensions give relative estimates of the 2-dimensional areas involved.)

The granule population is not completely depleted even after 2 hr of ADH stimulation. In two toads studied at this time, we found the stimulated hemibladders still show 40–50% of the granule content of the unstimulated ones.

It is also interesting to note that the granule membrane before exocytosis is thinner than the luminal plasma membrane. Granule membrane measures 6–7 nm compared to 8–9 nm for the luminal plasma membrane and 5–6 nm for the ER in the same cells.

The granule contents stain intensely by the silver methenamine procedure (Fig. 4), indicating a high concentration of carbohydrates. The luminal cell surface also shows silver methenamine reaction product consistent with the suggestion that the source of the membrane "fuzz" is the granules (Choi, 1963; Keller, 1963; Masur et al., 1972).

While most of our work concerned bladders incubated with an osmotic gradient across the mucosa, hemibladders treated with ADH without a gradient across the bladder also showed exocytic figures and

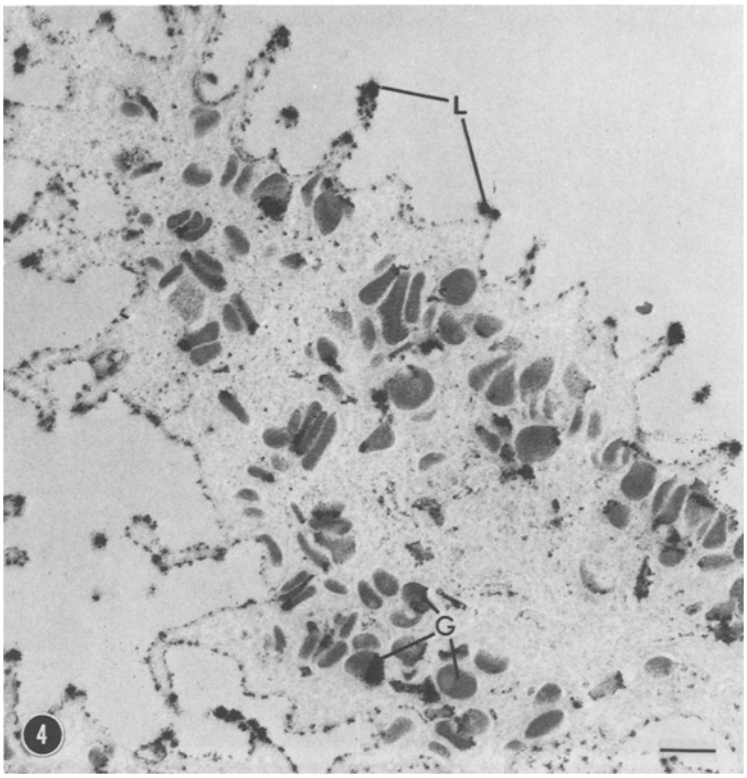


Fig. 4. Portion of an epithelial cell. Carbohydrate-rich material demonstrated by silver methenamine is found in numerous granules (*G*) and at the luminal surface (*L*). The thin section was not stained with uranyl or lead. ×15,000

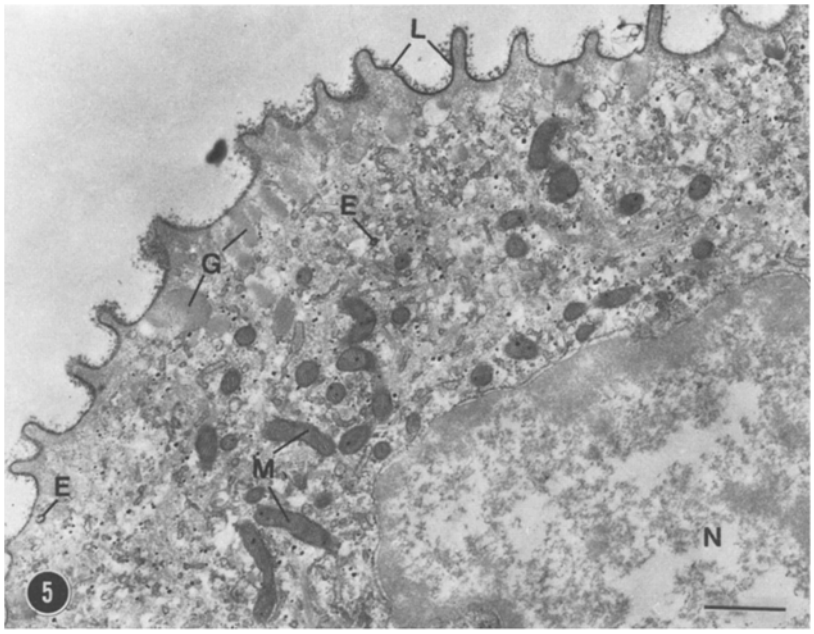
a significant loss of granules compared to their unstimulated control hemibladder. During 30 and 60 min of stimulation with ADH with no osmotic gradient, approximately 16 granules per thin section of a cell are released, which represents a 33.5 ± 17.9 (SD)% difference in granule number between seven ADH-treated hemibladders and their contralateral control hemibladders. (An average of 30 cells from each hemibladder was scanned; $P < 0.05$.) Therefore, the magnitude of granule release appears to be similar to that with a gradient. In previous work (Masur et al., 1971), confirmed in this study, we showed that endocytosis is also stimulated by ADH in the absence of an osmotic gradient.

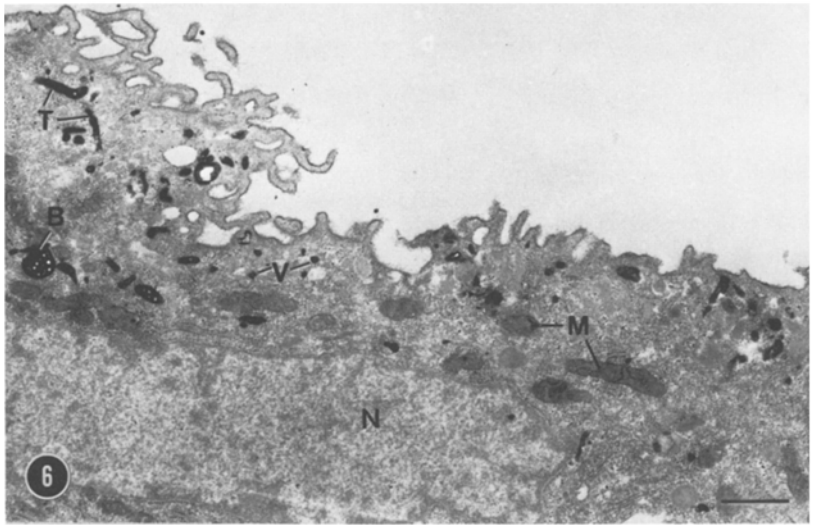
Endocytosis

To evaluate the intensity of endocytosis, we first counted the number of HRP-containing bodies per thin section (Figs. 5 and 6). These included vesicles, tubules with the morphology shown in Fig. 7, and multivesicular bodies. We obtained values for the average number of HRP-containing bodies found per thin section of a cell in preparations exposed to the tracer for the 15-min intervals indicated. In the two experiments for which counts were done systematically (126 cells were scanned), an average of 2 endocytic bodies per thin section of a cell were found to form during the 0–15 min interval with ADH, endocytic bodies during the 15–30 min interval, 10 endocytic bodies during the 30–45 min interval and 5 during the 45–60 min interval. The basal rate of endocytosis for 15 min without ADH was less than one endocytic body per thin section of a cell. Thus endocytosis becomes appreciable during the 15–30 min interval, peaking at about 30 min.

We were primarily concerned with the total amount of membrane retrieved at various timepoints and these data are shown in Table 2. By 60 min, approximately 13 μm of membrane per thin section of a cell were retrieved. As noted in Materials and Methods, this value is an underestimate since we cannot determine reliably how much membrane has been internalized in lysosomes. To do so would require a detailed knowledge of the history of each lysosome. However, since we did include the perimeter of MVBs in our measurements and since at the times studied fewer than 1 in 15–20 of the endocytic structures found is a MVB and fewer than 1 in 30–60 is a residual body, we believe that the underestimation is minor.

The 13 μm of membrane retrieved per thin section of a cell compares with the 16 μm of membrane added





Figs. 5 and **6.** Hemibladders exposed to an osmotic gradient with HRP present in the mucosal solution. The hemibladders were then incubated for the demonstration of HRP. Thin sections were stained with lead citrate only.

Fig. 5. An epithelial cell of a control hemibladder exposed to HRP for 60 min. The luminal plasma membrane (*L*) is coated with the dark HRP reaction product which is also found in a few endocytic vesicles (*E*). The nucleus is seen at *N*. granules at *G* and mitochondria at *M*. ×18,000

Fig. 6. A portion of an epithelial cell exposed to HRP and ADH for 60 min. HRP is seen in numerous tubules (*T*), vesicles (*V*), and a multivesicular body (*B*). The nucleus is seen at *N*, mitochondria at *M*. ×21,000

per thin section of a cell. In terms of area this means than an amount of membrane at least 3/4 of that added to the surface by exocytosis has been retrieved by endocytosis.

Volume Change

Granular cells have been reported to swell during the administration of ADH, due to the transcellular passage of water (Peachey & Rasmussen, 1961; Carasso, Favard & Valérien, 1962; DiBona, Civan & Leaf, 1969*b*). Such volume changes could affect our estimates of the extent of exocytosis or endocytosis but they would have to be quite extensive to affect notably our findings, since our quantitative data concern the entire profile of the cell cut perpendicular to the luminal surface. For example, to produce an apparent halving of granule frequency, the cells' linear dimensions would have to double. Table 3 indicates that, with the fixation conditions we used (fixative on both sides of the bladder, and no hormone present during fixation), there is no discernible change in the lateral dimensions of the cells. There may be a change in the depth of the cells (measured from luminal to contraluminal plasma membrane). This is consistent with the appearance of the mucosal cells with ADH observed in the scanning electron microscope (Davis et al., 1974; Spinelli, Grosso & de Sousa, 1975; Mills & Malick, 1978). However, this change in depth

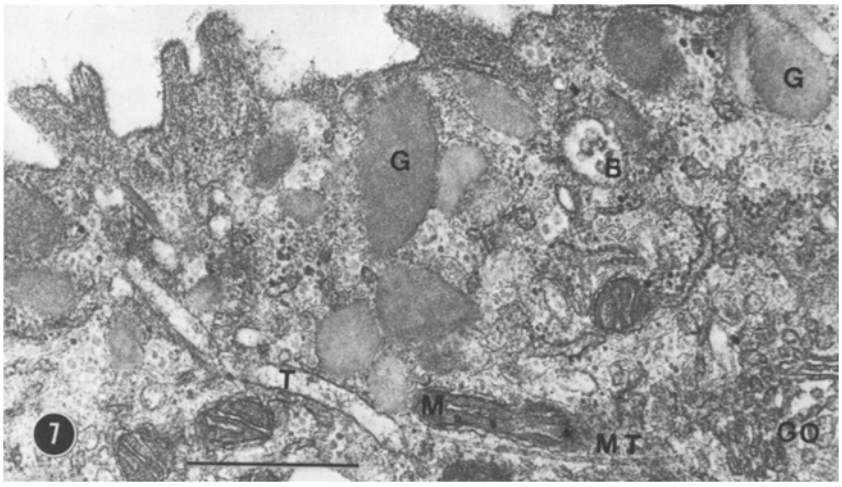


Fig. 7. An endocytic tubule (*T*) near the luminal plasma membrane in a hemibladder exposed to ADH without HRP for 60 min. Such tubules are one of the main sites of location of HRP in the tracer experiments. Microtubules (*MT*), numerous granules (*G*), mitochondria (*M*), multivesicular body (*B*), and a portion of the Golgi apparatus (*GO*) are seen in the cytoplasm of the granular cell. ×46,000

Table 2. Endocytosis with ADH

	Membrane endocytized (μm/thin section of a cell)
No ADH, 60 min with HRP	1.2 ± 1.2 (5)
15 min with ADH and HRP	0.9 ± 2.5 (4)
30 min with ADH and HRP	3.6 ± 2.5 (4)
60 min with ADH and HRP	12.9 ± 7.9 (5)

Endocytic figures from at least 10 cells contiguous along the mucosal surface were quantitated per bladder. The perimeters of HRP-containing endocytic structures were measured with a cartographer's wheel. The data are expressed in terms of μm of membrane internalized per thin section of a cell. The data are presented as mean ± SD. The number of toads analyzed is in parentheses.

Table 3. Relative cell dimensions

	ADH/no ADH	Experimental/control
Lateral dimensions	1.0 ± 0.2 (7)	1.0 ± 0.2 (22)
Depth	1.1 ± 0.3 (7)	1.1 ± 0.5 (22)

The lateral dimensions of the cell were measured with a cartographer's wheel from tight junction to tight junction. The depth of the mucosal cell was the mean of three measurements taken from luminal to contraluminal plasma membrane. At least 10 consecutive cells along the mucosal surface per bladder were measured. The "experimental *vs.* control" data include measurements from colchicine-ADH-treated hemibladders *vs.* their no colchicine-ADH paired control hemibladders; lumicolchicine-ADH-treated hemibladders *vs.* their no lumicolchicine-ADH-paired control hemibladders and ADH *vs.* no ADH-paired control hemibladders. The data are presented as mean ± SD. The number of toads analyzed is in parentheses.

Table 4. Ratio of plasma membrane length to tight junction-to-tight junction length

	30 min	60 min
No ADH	1.3 ± 0.2 (4)	1.4 ± 0.1 (3)
ADH	1.7 ± 0.1 (4)	1.5 ± 0.2 (3)

For each hemibladder the actual luminal plasma membrane length (the contour of the membrane *including* microvilli) of at least 8 consecutive granular cells was measured with a cartographer's wheel. The distance from tight junction to tight junction was measured with a cartographer's wheel by following the cell surface but *excluding* the microvilli and other irregularities. The measurements are expressed as the ratio: mean plasma membrane length/mean tight junction to tight junction distance. (*Note:* as Table 3 indicates, the tight junction to-tight-junction distance is not altered by ADH). Data are presented as mean ± SD. The number of toads analyzed is in parentheses.

Table 5. Rates of Water Loss with ADH

	Weight loss (mg/min)
5 min	8.5 ± 0.3 (4)
15 min	32.8 ± 17.0 (20)
15–30 min	48.9 ± 13.9 (15)
30–45 min	33.9 ± 5.2 (9)
45–60 min	31.0 ± 13.2 (5)
No ADH, 60 min	3.5 ± 3.1 (8)
ADH, 60 min	35.5 ± 7.4 (5)

ADH-treated hemibladders with a mucosal/serosal gradient of 225 mosmol across them were weighed every 15 min. Osmotic water movement across the hemibladder into the serosal bath, was measured as weight loss from the hemibladder. The values are expressed as weight loss per min during the intervals noted. The last two values are the average rates for 60 min. The data are presented as mean ± SD. The number of toads analyzed is in parentheses.

Table 6. Colchicine and exocytosis

	Number of granules per thin section of a cell with colchicine	Number of granules per thin section of a cell, no colchicine	Water loss with cochicine (mg/min)	Water loss, no colchicine (mg/min)
10 min ADH	47.8 ± 4.6	29.8 ± 4.9	19.9 ± 11.0	39.0 ± 11.9 (3)
30 min ADH	42.1 ± 11.1	31.9 ± 7.2	25.3 ± 3.1	44.5 ± 7.2 (5)
60 min ADH	32.5 ± 9.7	20.4 ± 8.8	27.8 ± 1.5	40.8 ± 1.4 (3)
All time points with ADH	41.0 ± 10.4	28.8 ± 8.2	24.5 ± 9.3	42.0 ± 9.9 (11)
No ADH	31.3 ± 10.3	34.0 ± 12.5	0.2 ± 0.01	0.3 ± 0.3 (3)
All time points with lumicolchicine + ADH	19.4 ± 4.4	18.3 ± 3.8	46.8 ± 6.3	47.2 ± 9.8 (4)

The mean number of granules per thin section of an average cell in colchicine-ADH treated hemibladders (column 1) is compared to the number in control hemibladders with ADH but no colchicine (column 2). On the average, 30 cells from the experimental and a similar number from the control hemibladder were analyzed for granule content. Rates of water loss for the same bladders studied in the first two columns are shown in the remaining two columns. Column 3 presents the average rate of water loss (mg/min) in bladders treated with colchicine and ADH. Column 4 presents the average rate of water loss for no colchicine controls, treated with ADH. All data are presented as mean ± SD. The number of toads analyzed is in parentheses.

Table 7. Colchicine and endocytosis

	Amount of membrane internalized with colchicine (μm)	Amount of membrane internalized, no colchicine (μm)	Water loss with colchicine (mg/min)	Water loss, no colchicine (mg/min)
30 minutes ADH	1.7 ± 0.6	4.1 ± 2.8	18.2 ± 10.8	34.6 ± 10.3 (3)
60 minutes ADH	3.8 ± 2.4	8.6 ± 5.3	24.1 ± 12.9	42.1 ± 19.3 (4)
All time points + ADH	2.9 ± 2.0	7.1 ± 4.8	20.1 ± 13.6	38.9 ± 15.4 (7)
All time points with lumicolchicine + ADH	9.1 ± 9.9	10.7 ± 12.8	41.4 ± 11.7	48.9 ± 16.1 (3)

The perimeters of HRP containing endocytic structures were measured with a cartographer's wheel. A minimum of 10 cells were analyzed for membrane uptake in each colchicine-ADH treated hemibladder and the corresponding ADH treated control hemibladder not exposed to colchicine. The data are expressed in terms of the average length of membrane internalized per thin section of a cell. Rates of water loss for the same bladders studied in the first two columns are shown in the remaining two columns. Column 3 presents the average rate of water loss (mg/min) in bladders treated with colchicine and ADH. Column 4 demonstrates the average rate of water loss for no colchicine controls, treated with ADH. All data are presented as mean ± SD. The number of toads analyzed is in parentheses.

would not affect our results. (*Note:* we are not asserting that no volume changes occur, but only that they are not preserved with our procedures.)

Cell Surface Changes

If our data on exocytosis and endocytosis are correct, there should be a change in the amount of membrane at the luminal cell surface that should be especially marked at the 15–30 min interval when much exocytosis has occurred but endocytosis has not yet retrieved much of the membrane. In cells with massive secretory activity, such as mast cells, plasma membrane folds have been correlated with extensive secretion (Burwen & Satir, 1977*b*). In toad bladder, changes in surface topology at 30 min ADH exposure would be expected and such changes are seen in scanning electron micrographs (Davis et al., 1974; Spinelli et al., 1975; Mills & Malick, 1978). Table 4, which presents ratios of the dimensions of experimental and control hemibladders, demonstrates that our prediction is borne out. At 30 min the luminal plasma membrane of ADH-treated hemibladders is expanded in comparison with the controls or with ADH-treated hemibladders at 60 min when more extensive membrane retrieval has occurred. The values for the experimental hemibladders at 30 min were found by Student's t test to be significantly different from controls ($0.001 < P < 0.01$). Those at 60 min were not significantly different ($0.70 < P < 0.80$).

Water Flow

To correlate the morphological observations with the physiology of the hydroosmotic response, we studied

the time course of weight loss, with 50 mU neurophyseal hormone (Table 5). The hydroosmotic response induced by ADH increases water movement at least 10 times that of the basal rate. The hydroosmotic response is detected as early as 5 min after ADH administration. As reported by others, we find the peak of water movement is during the 15–30 min interval and flow declines in the 30–60 min interval (Eggena, Schwartz & Walter, 1968; Kachadorian, Casey & DiScala, 1977).

Effect of Colchicine

At 1×10^{-5} or 2×10^{-4} M, colchicine inhibits water transport and does not interfere with nucleoside or sodium transport in toad urinary bladder (Taylor et al., 1973; 1978). We found that the abundance of granules after exposure to ADH for 10, 30, or 60 min is greater with colchicine than in control hemibladders exposed to hormone for the same length of time without colchicine (Table 6). The difference was statistically significant ($P < 0.001$; Student's t test). Both concentrations of colchicine produced similar effects. Endocytosis is also inhibited significantly in the colchicine-treated hemibladders compared to the control hemibladders (Table 7) ($0.02 < P < 0.05$). Lumicolchicine, the structural isomer of colchicine that lacks colchicine's effect on microtubules (Wilson & Friedkin, 1966; Wilson et al., 1974), does not affect the rate of water loss or the levels of exocytosis and endocytosis engendered by ADH (Tables 6 and 7).

Effects of High Potassium

Our interest in K^+-propionate media was spurred by a preliminary report (Scott et al., 1978) indicating that such media might have interesting effects on the toad bladder; high K^+ is known, for example, to depolarize neurons and engender extensive release of neurotransmitters. Using the K^+-propionate buffer and incubation regimen recommended by Scott and Gennaro (W.N. Scott and J.F. Gennaro, Jr., *personal communication;* the K^+-propionate buffer consists of 10 mM glucose, 89 mM potassium propionate, 17.5 mM $KHCO_3$, 0.8 mM K_2HPO_4, 0.8 mM $MgSO_4$ and 1.5 mM calcium propionate, pH 7.4, and the Ringer's consists of 10 mM glucose, 85 mM NaCl, 17.5 mM $NaHCO_3$, 0.8 mM K_2HPO_4, 4.0 mM KCl, 0.8 mM $MgSO_4$ and 1.5 mM calcium chloride, pH 7.4), we failed to detect granule loss or water loss after as much as 2 hr incubation in the buffer (Table 8); granules were released only when ADH was present.

Table 8. Potassium propionate

	Experiment 1 (number of granules per thin section of a cell)	Experiment 2 (number of granules per thin section of a cell)
A) K/K 60′, R/R 30′	45.7	47.5
R/R 60′, R/R 30′	47.7	43.1
B) K/K 60′, one-fifth K/K+ADH 30′ R/R 60′	38.3	43.5
one-fifth R/R+ADH 30′	36.5	43.6
C) K/K 120′, R/R 30′	44.0	40.4
R/R 120′, R/R 30′	36.7	35.1

In *A*, *B* and *C* one hemibladder was first incubated in high K^+-propionate buffer for the indicated time, while the contralateral hemibladder was incubated in normal Ringer's. The same solutions were present on both mucosal and serosal surfaces. In *A* and *C* the hemibladders were transferred to normal Ringer's for an additional 30 min before fixation, as suggested by Scott and Gennaro (*personal communication*). In *B* the high K^+-propionate hemibladder was transferred to a serosal bath of full strength high K^+-propionate buffer containing 10^{-7} M ADH, while the mucosal solution was changed to 1/5-strength K^+-propionate buffer to provide an osmotic gradient for the additional 30-min period. The contralateral hemibladder was treated identically except that normal Ringer's was used (as expected, we detected a normal hydroosmotic response in both hemibladders upon exposure to ADH). K/K means full strength high potassium Ringer's was present at both surfaces of the hemibladder; R/R means full strength normal Ringer's was presented; one-fifth K or R refers to fifth-strength K^+-propionate or Ringer's presented to the mucosal surface. Granule counts were made on at least 20 cells per hemibladder.

As reported by Scott et al. (1978), exposure of bladders to K^+-propionate buffer does not appear to affect water flow or to markedly diminish the ADH response. When we compared paired hemibladders preincubated in K^+-propionate buffer for 60 min with those preincubated in Ringer's and both then being subjected to a 1:5 Ringer's gradient, we found no significant difference in the rate of water movement (3 pairs of hemibladders; K^+-propionate: $+2.1 \pm 2.8$ (SD) mg/min; Ringer's: $+2.8 \pm 4.7$ mg/min). Preincubation with K^+-propionate for 60 min and transfer to a 1:5 K^+-propionate or Ringer's gradient with 50 mU ADH for 30 min produced a water loss of 27.4 ± 13.2 mg/min in 5 hemibladders compared to 33.9 ± 30.2 mg/min in their contralateral hemibladders which were preincubated in Ringer's followed by a 1:5 Ringer's gradient with ADH. We also studied 2 hemibladders preincubated in Ringer's for 60 min and then subjected to a 1:5 K^+-propionate gradient and observed a net water flux of 0.6 ± 0.3 mg/min compared to a net flux of 0.4 ± 0.2 mg/min in their contralateral hemibladders preincubated in Ringer's and incubated in a 1:5 Ringer's gradient.

All the data from K^+-propionate and control hemibladders are well within the range observed in other experiments. However, K^+-propionate buffer does seem to engender cell swelling and other signs of abnormality due presumably to the extreme nonphysiological conditions (*see also* Hess, Taylor & Maffly, 1975, for their reports of complex and unexplained phenomena seen with propionate). In some preparations the cells' cytoplasm becomes extremely dilute and the nuclei appear washed out and abnormal.

We have also tried to degranulate cells by treating them with 55 mM KCl Ringer's containing 43 mM NaCl. Once again the buffer did not engender detectable loss of granules, although in some cases when the bladder was then transferred back to normal Ringer's we observed a few exocytic figures.

Discussion

These results strengthen our view that bulk membrane movement is closely linked to the change in permeability to water induced by neurohypophyseal hormone at the luminal surface of the bladder: (i) exocytosis at this surface detected both by direct observation of exocytic figures and by quantitation of granule loss, occurs within the first few minutes after hormone addition during the period of onset of the hydroosmotic response; (ii) the amount of membrane added to the surface by exocytosis is substantial; (iii) endocytosis reaches its maximum later than exocytosis, during the period when the transepithelial flow of water begins to decline; (iv) endocytosis retrieves an amount of membrane that is of the same order of magnitude as that added by exocytosis; (v) colchicine, an inhibitor of the hydroosmotic response, also inhibits exocytosis and endocytosis.

Obviously it is not possible from the present information to specify the relationship between bulk membrane movement and the permeability change. Several plausible possibilities as to how they could be linked exist and may be related to findings in other systems (reviewed in Holtzman et al., 1979). For example, the highly impermeable cell surface of epithelial cells in the mammalian urinary bladder is maintained by exocytically inserted specialized membrane (Hicks, 1966; Porter, Kenyon & Badenhausen, 1967; Hicks, Ketterer & Warren, 1974). In fertilized eggs (Epel et al., 1974; Epel, 1975) and perhaps in oxyntic cells (Carlisle, Chew & Hersey, 1978) permeability and transport changes in the cell surface are accompanied by extensive exocytosis.

For toad bladder a number of proposals have been made about the molecular events involved in ADH-induced permeability changes. For example, it has been argued that the route of water movement is through pores and that hormone alters the number or state of such pores (Hays & Leaf, 1962; Finkelstein, 1976). Changes in the conformation or association of proteins by cyclic nucleotide modulated phosphorylation and alterations in membrane fluidity might be involved in the response to hormone (Schwartz et al., 1974; Pietras & Wright, 1975; Andreoli & Schafer, 1976; Pietras, 1976). It has also been suggested that the particle aggregates seen in freeze-fracture studies of the luminal plasma membrane of ADH-treated bladders are the site of enhanced water permeability (Chevalier et al., 1974; Bourguet, Chevalier & Hugon, 1976; Kachadorian et al., 1977*a, b*).

Our results on membrane addition to the luminal surface could relate to these proposals in various ways. It is possible that the granule membrane is itself more permeable to water than the preexisting cell surface or that its addition could change the fluidity or other properties of the plasma membrane pertinent to the hydroosmotic response. Our measurements are not precise enough to establish a direct correlation between the number of granules released in a given toad and the magnitude of the hydroosmotic response: one would have to study granule release in all regions of the bladder, or determine the local permeability changes in the regions studied morphologically. Moreover, it may be the dynamics of the situation – the duration of residence of granule material at the surface – that are decisive. However, we find it interesting that the granule membrane is thinner than the plasma membrane. Membrane thickness reflects membrane composition, particularly lipid content. There are examples in which thin membranes have a higher permeability than thicker ones [e.g., the ER tends to be "leaky" in comparison with usual plasma membranes, and the highly impermeable plasma membrane of the epithelial cells in the mammalian bladder is quite thick (Hicks et al., 1974)]. Little is known about the granule membrane except that it has been reported that granules within mucosal cells reveal few particles in the freeze-fracture preparation (Wade et al., 1975). In fact, the origin of the particle aggregates in the luminal surface seen with ADH administration is still not clear, and while the evidence tying them to the permeability change is intriguing, their role has yet to be elucidated; their relationship, if any, to the granules is still speculative. Intracellular vesicles and tubules delimited by membranes with aggregated particles are found within resting cells and within ADH-treated ones and could be destined for insertion in the cell surface (Humbert et al., 1977) but Wade (1978) reports that such intracellular structures are quite rare in the granular cells of unstimulated bladders. In other cell types the

process of exocytosis has been shown to alter particle distributions in cell surfaces, although this does not produce aggregates of the sort seen in the bladder (Satir & Satir, 1974; Pinto da Silva & Nogueira, 1977; Lawson et al., 1977; Weiss, Goodenough & Goodenough, 1977; Burwen & Satir, 1977*a*).

It is also possible that the carbohydrate-containing granule content, as its spreads across the luminal surface upon exocytosis (e.g., Masur et al., 1972), interacts with the underlying plasma membrane to effect changes. For example, binding of lectins and other ligands to membrane receptors can produce particle aggregation (Gingell, 1976) and conceivably the granule content plays such a role in the hormone-induced particle aggregation in the bladder. Interestingly, ADH-treated bladders demonstrate changes in the distribution of lectin-binding sites (Pietras, 1976). Another possibility is that enzymes released from the granules upon exocytosis modify the extracellular surface of the plasma membrane. However, we find few, if any, of the granules to be acid phosphatase positive, suggesting that they are not the source of acid hydrolases reportedly released from stimulated bladders (Pietras, Naujokaitis & Szego, 1976).

Some amphibia having granular cells in their bladders show no ADH response (Wade, 1980). It would be important to determine in these species whether granules fuse with the surface at a steady rate in the absence of stimulation and thus contribute to maintaining the steady-state permeability of the bladder. In at least some of these species the unstimulated bladders show a high permeability to water (Brodsky & Schlib, 1965; Mullen et al., 1976).

In many cell types endocytosis returns the cell surface to its original area after membrane addition through exocytosis (reviewed in Holtzman, 1976). In the bladder we cannot be sure that the membrane internalized by endocytosis is the same membrane added by exocytosis of the granules. However, it seems plausible that in toad bladder the endocytic process might be removing membrane made permeable through exocytosis or other mechanisms and thus tends to reduce surface permeability. The fact that even at 60 min, when much membrane has been retrieved, water continues to flow across the mucosal cells could be associated with the continuation of both exocytosis and endocytosis which are detectable even after 2 hr exposure to ADH. Or it may mean that the circulating membrane *per se* is not the crucial component; the contents of the granules seem not to be endocytized extensively. It has been suggested that intracellular structures, having aggregated intramembrane particles similar to those seen at the surface, can arise by endocytosis (Humbert et al., 1977). Perhaps these particle aggregates are sites of altered permeability for water movement that have been withdrawn from the cell surface (*see* Pinto da Silva (1973) for evidence that clustered particles are local sites of altered water permeability in red blood cells, but *see also* Kachadorian, Casey & DiScala (1978) for their report that particle aggregate frequency at the cell surface has not declined by 60 min, when much endocytosis has already occurred). Aggregation of particles at local membrane regions slated for endocytosis has been reported in several cell types (Karnovsky et al., 1972; Gingell, 1976; Heuser, 1976).

Once internalized, endocytized membrane could be degraded in lysosomes or eventually recycled to the cell surface (Wade, 1980), as proposed in other systems (Steinman, Brodie & Cohn, 1976; Silverstein, Steinman & Cohn, 1977; Tulkens, Schneider & Trouet, 1977; Farquhar, 1978; Minsky & Chlapowski, 1978; reviewed in Holtzman et al., 1978).

The number of microtubules in bladder mucosal cells increases with ADH administration and decreases with colchicine (Reaven, Maffly & Taylor, 1978). Given these observations and the literature on various cell types (Soifer, 1975), our finding of colchicine inhibition of bulk membrane movement and the reports of colchicine inhibition of hormone-induced alterations in the distribution of intramembrane particles (Kachadorian, 1976) and of lectin binding sites (Pietras, 1976) might reasonably be thought to reflect effects on microtubules.

We are well aware of the possibility that the connection between the permeability change and bulk membrane movement might be indirect or that bulk membrane movement may be involved in changes in permeability to components other than water. It was suggested to us (H. Wyssbrod, *personal communication*) that once the granules are added to the surface some additional alterations might be necessary to affect the granule material and evoke a change in permeability. Or, perhaps exocytosis adds unaltered membrane to the surface in "anticipation" of subsequent endocytic removal of plasma membrane altered as part of the hydroosmotic response by one or another of the mechanisms that have been proposed. Alternatively, the permeability change and bulk membrane movement might be coupled without necessarily being causally related. For example, the granule content might be needed to protect the cell surface. Or, the bulk membrane movement might serve to accommodate changes in cell and bladder geometry attendant upon the removal of water from the bladder's lumen. Changes in cell surface are seen in the mammalian urinary bladder with the storage and release of bladder contents (Hicks et al., 1974; Minsky & Chlapowski, 1978). We did find that exocytosis occurs

whether or not an osmotic gradient is present across the toad urinary bladder. Thus, it does not depend simply upon the osmotically driven net transepithelial movement of water. In this connection it is worth mentioning that preliminary work with hypertonic treatment of hemibladders reveals exocytic figures and a decrease in granule numbers (Masur, Gronowicz & Holtzman, 1979). Hypertonicity has previously been shown to induce a hydroosmotic response (Bentley, 1964; Ripoche, Bourguet & Parisi, 1973), and this was confirmed in our experiments. And finally, although the luminal plasma membrane appears to be the primary barrier to water movement, it might well be that once a threshold for water permeability has been reached at this barrier, other factors in the cytoplasm or at the cell surface could be rate limiting.

The variability in our data suggests that there are experimental or physiological factors we have get to identify. Nonetheless, the point which does seem clear from our findings is that ADH engenders large scale relocations of membrane in the granular cell which must be taken into account in understanding the cell's physiological responses.

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