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Isolation and Characterization of Specialized Regions of Toad Urinary Bladder Apical Plasma Membrane Involved in the Water Permeability Response to Antidiuretic Hormone

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Summary. Antidiuretic hormone (ADH) increases the apical (external facing) membrane water permeability of granular cells that line the toad urinary bladder. In response to ADH, cytoplasmic vesicles called aggrephores fuse with the apical plasma membrane and insert particle aggregates which are visualized by freeze-fracture electron microscopy. Aggrephores contain particle aggregates within their limiting membranes. It is generally accepted that particle aggregates are or are related to water channels. High rates of transepithelial water flow during ADH stimulation and subsequent hormone removal decrease water permeability and cause the endocytosis of apical membrane and aggrephores which retrieve particle aggregates. We loaded the particle aggregate-rich endocytic vesicles with horseradish peroxidase (HRP) during ADH stimulation and removal. Epithelial cells were isolated and homogenized, and a subcellular fraction was enriched for sequestered HRP obtained. The HRP-enriched membrane fraction was subjected to a density shifting maneuver (Courtoy et al., *J. Cell Biol.* **98**:870, 1984), which yielded a purified membrane fraction containing vesicles with entrapped HRP. The density shifted vesicles were composed of approximately 20 proteins including prominent species of 55, 17 and 7 kD. Proteins of these molecular weights appear on the apical surface of ADH-stimulated bladders, but not the apical surface of control bladders. Therefore, we believe these density shifted vesicles contain proteins involved in the ADH-stimulated water permeability response, possibly components of particle aggregates and/or water channels.

Key Words vasopressin · toad bladder · water flow · antidiuretic hormone · endocytosis · density-shift method

Introduction

In the absence of antidiuretic hormone (ADH), the apical (external-facing) surfaces of the mammalian collecting duct and anuran skin and urinary bladder are remarkably impermeable to water. Addition of ADH to the basolateral (blood-facing) side of these epithelia elicts a large increase in their apical membrane water permeability (P_f), allowing water to flow along an osmotic gradient from a dilute external solution to blood [10]. In all three tissues, the increase in P_f is highly correlated with the appearance of distinctive aggregates of intramembrane particles (particle aggregates) in the apical plasma membrane of ADH-responsive cells [2, 4, 11, 16, 20]. A large body of evidence indicates that particle aggregates represent the site of water channels responsible for the ADH-induced increase in P_f [18, 34].

In the toad bladder, particle aggregates are inserted into the apical membrane of granular cells through fusion of cytoplasmic vesicles called aggrephores. Aggrephores contain particle aggregates within their limiting membranes [15, 27, 32]. In the absence of ADH stimulation, granular cells internalize electron-dense [5, 13, 21, 33] and fluorescent [12, 15] fluid-phase markers from their apical surface at an extremely low rate. Water flow along a large transepithelial osmotic gradient during an interval of ADH stimulation [13, 26] or subsequent hormone removal from the solution bathing the basolateral epithelial surface [25] causes a decrease in apical membrane particle aggregates and P_f and activates endocytosis of fluid-phase markers as apical membrane is internalized. The apical membrane vesicles retrieved during these intervals have been recently shown to contain particle aggregates [5].

To identify proteins involved in the ADH-mediated increase in P_f, the enzyme lactoperoxidase (LPO) was sequestered in particle aggregate-containing vesicles and the proteins were exposed on the inner surfaces of these vesicles labeled with ^{125}I by the intracellular iodination technique of Mellman et al. [22]. The proteins ^{125}I-labeled under internalization conditions were then compared to proteins which were ^{125}I-labeled when the apical surfaces of unstimulated and hormone-treated toad bladders were iodinated with LPO in the absence of internalization [14]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography of these samples revealed that: (i) ^{125}I-labeled

bands of 55 kD, a cluster of proteins ranging from 17 to 14 kD and a 7-kD species appeared only on the apical surface of ADH-stimulated not control bladders, and (ii) identical ^{125}I-labeled proteins were also found to be present on the inner surfaces of vesicles containing particle aggregates.

In this report, we describe the subcellular fractionation of toad bladder epithelial cell homogenates which contain particle aggregate-rich vesicles loaded with horseradish peroxidase (HRP). Using differential and density gradient centrifugation combined with a density shifting technique described by Courtoy et al. [6], we have obtained a highly enriched HRP-containing vesicle fraction. SDS-PAGE analysis of these purified vesicles reveals approximately 20 prominent protein bands that include species of 55, 17, 15 and 7 kD mol wt.

Materials and Methods

HRP Uptake and Homogenization Protocol

HRP was internalized into granular cells as previously described [5, 13]. Briefly, hemibladders mounted as sacs were stimulated by addition of ADH to a final concentration of 50 mU/ml in the solution bathing their basolateral surfaces. After 15 min of incubation at 23°C when the P_f response is maximal, the isotonic solution bathing the bladder's apical surface was replaced with a solution made hypotonic (40 mOsm/kg) by reducing the NaCl content of an isotonic amphibian Ringer's solution (*see* [13] for composition) containing 10 mg/ml of HRP (Type II, Sigma Chemical Co., St. Louis, Mo.). The HRP-containing solution had been dialyzed overnight against a 100-fold excess of the same buffer. Five minutes after addition of the HRP, ADH stimulation was terminated by three exchanges of the basolateral solution. The incubation was continued for an additional 10 min, whereupon the HRP was removed and the bladders were rapidly chilled to 2°C by addition of cold Ringer's solution to both apical and basolateral surfaces. The apical surface was then successively rinsed with five additions of cold isotonic Ringer's solution followed by five additional rinses with distilled water made isotonic (6% wt/vol) with sucrose. This was necessary to remove adherent HRP. The epithelial cells were then scraped from the bladder with a glass slide. The cells were suspended in homogenization buffer (5 mM Tris, 0.1 mM EDTA, 5.7% sucrose at pH 8.0, 180 mOsm/Kg H_2O) and pelleted at 4,500 × *g* for 5 min. This swelled the cells and removed trace amounts of remaining adherent HRP. All further procedures were carried out using solutions cooled by ice. Five hundred microliters of packed cells were resuspended in 2.5 ml of homogenization buffer and ruptured in a Polytron (PCU-2, Brinkmann Instruments, Westbury, N.Y.) at a setting of 5 for a duration of 5 sec. In agreement with Rodriguez and Edelman [30], toad bladder granular cells required both swelling in a hypotonic homogenization solution and disruption in a polytron apparatus for efficient breakage of the cells (*data not shown*).

Membrane Fractionation

The resulting cell homogenate was centrifuged for 15 min at 700 × *g* (*see* fractionation scheme Fig. 1). The pellet containing mostly unbroken cells and nuclei was termed the *residual pellet*. The supernatant was removed and centrifuged at 10,000 × *g* for 10 min to yield a pellet (*intermediate pellet*) and supernatant (*intermediate supernatant*). In some instances, the intermediate supernatant was further fractionated by centrifugation at 100,000 × *g* for 1 hr in a Beckman 65Ti rotor (Beckman Instruments, Palo Alto, Calif.). This centrifugation yielded a *high speed pellet* and a *high speed supernatant*. These pellet fractions were resuspended by gentle agitation, layered on 10 ml 29–62% (wt/vol) linear sucrose gradients buffered by homogenization solution and centrifuged in SW-41Ti rotor (Beckman Instruments, Palo Alto, Calif.) at 22,500 rpm for 4 hr at 4°C. The gradients were fractionated by flotation on 65% sucrose.

Density Shift Protocol

The density shift maneuver was carried out as outlined by Courtoy et al. [6] with the following modifications. Sucrose gradient fractions containing HRP activity were pooled and divided into several equal portions. Routinely, 0.8 ml of HRP-rich sucrose gradient fractions derived from intermediate pellet in approximately 50% buffered sucrose was added to 3.2 ml of the 3,3'-diaminobenzidine (DAB) solution which contained 2.56 mg/ml of DAB in 25% sucrose buffered with 3 mM imidazole at pH 7.0. Twenty microliters of 6% H_2O_2 was then added to this solution and the reaction carried out in the dark at 23°C. for 30 min with intermittent gentle agitation. Samples that contained sequestered HRP and the complete reaction mixture rapidly changed from a pale yellow to a light rusty color. After the incubation, the samples were loaded on a second set of sucrose gradients identical to those from which they were originally harvested and recentrifuged and fractionated as before. In tubes containing the samples reacted with the complete reaction mixture, there was a small brown pellet which was gently resuspended in 80 μl of 62% sucrose solution and processed for SDS-PAGE or electron microscopy.

Protein Quantitation

Protein was measured either by the method of Bradford [3] (Biorad Labs, Richmond, Calif.) or, where the samples contained detergent or high concentrations of sucrose, the BCA protein assay reagent (Pierce Chemical Co., Rockford, Ill.). In both cases, the micro protocols detailed in company literature were used for all assays. Both methods were standardized with bovine γ-globulin in the appropriate sample buffer.

SDS-PAGE and Autoradiography

Protein samples from membrane fractions were dissolved in Laemlli solubilizing buffer [17] and electrophoresed on 12% acrylamide gels with a 30/0.8 monomer/bis ratio in a Mighty Small apparatus (Hoeffer Sci., San Francisco, Calif.). The gels were fixed in 50% methanol, 10% acetic acid and 40% water solution for 30 min and stained using either: (i) 0.25% Coomassie blue R-

250 in fixation solution with destaining in 50% methanol, 1% acetic acid and 49% water solution; (ii) silver stain [23] using the Biorad silver stain kit (Biorad Labs, Richmond, Calif.); or (iii) a combination of these two stains as detailed by Dzandu et al. [7]. Gels were dried down onto filter paper and autoradiographed using Kodak XR-2 film (Kodak, Rochester, N.Y.) as described by Bonner [1].

Electron Microscopy

Samples from membrane fractions were processed for electron microscopy by one of two protocols. The first monitored the composition of membrane fractions by morphology alone, while the second allowed identification of vesicles which contained HRP. Two protocols were necessary because of the poor penetration of DAB into a glutaraldehyde-fixed centrifuged membrane pellet [M.C. Willingham, *unpublished observation*]. In the first protocol, an equal volume of 2.5% glutaraldehyde, 0.1 M sodium cacacodylate at pH 7.4 was added to selected membrane fractions. After a fixation interval of 10 min at 23°C, the samples were centrifuged at 13,000 × *g* for 5 min, yielding a pellet which was stored in phosphate buffered saline (PBS), pH 7.4, at 4°C until further processing. The second protocol began by attaching the components of membrane fractions to polylysine-coated 35-mm plastic tissue culture dishes (Corning Glass, Corning, N.Y.) prior to fixation. The dishes were first incubated for 30 min at 23°C with a 10-mg/ml solution of polylysine hydrobromide (Sigma Chemical Co., St. Louis, Mo., average mole wt 175,000) dissolved in phosphate-buffered saline, pH 7.4. After decanting the polylysine solution, membrane fractions were added to the plate and the components allowed to settle and attach to its surface. This process was observed by phase-contrast microscopy. The membrane fraction solution was then decanted, and the plate was immediately filled with fixation solution (*see above*) and incubated for 10 min at 23°C. The plate was then rinsed with five changes of PBS and stored for further processing in PBS at 4°C.

Glutaraldehyde-fixed pellets were fixed in 1% osmium tetroxide for 30 min, washed in water and dehydrated thorough a series of ethanol solutions followed by incubation in propolyene oxide. The pellet was then embedded in Epon 812, and sections were cut with a diamond knife. These were counterstained with lead citrate and examined under 40 kV with a 400T Phillips electron microscope. Dishes containing fixed membrane fragments were first incubated for 10 min with a solution of DAB (4 mg DAB in 10 ml of PBS) to which was added 3 μl of 30% H_2O_2. After rinsing in PBS, the contents of the dish were treated in an identical manner as the fixed pellets, except the dish contents received no propolyene oxide dehydration.

Radioactive Labeling Techniques

(1) The apical surface of unstimulated toad bladders was ^{125}I-labeled using diazotized ^{125}I-iodosulfanilic acid synthesized from ^{125}I-sulfanilic acid (NEX-121, Dupont/New England Nuclear, Boston, Mass.) which was diazotized following the instructions provided by the manufacturer. Eighty microliters of 50 mM $NaPO_4$, pH = 7.5, was added to neutralize solution containing the diazotized reagent, which was then further diluted to a final volume of 0.6 ml by addition of isotonic Ringer's solution. The apical surface was routinely iodinated as described in reference [8] using 2.5 mCi of diazotized ^{125}I-sulfanilic acid for 15 min at 2°C. The reaction was terminated by removal of the reaction mixture and rinsing of the apical surface with cold isotonic Ringer's solution followed by homogenization buffer. The bladder epithelial cells were then removed by scraping and treated in the homogenation protocol as detailed above. (2) HRP was iodinated using a mixture of LPO, H_2O_2 and $Na^{125}I$, which were added to HRP in a 50 mM Na_2HPO_4, pH 7.5, solution and allowed to incubate for 30 min at 4°C. The labeled protein mixture was separated from large amounts of $Na^{125}I$ by gel filtration using Sephedex G-10 (Pharmacia, Uppsula, Sweden) and from ^{125}I-labeled LPO by chromatography on Sephadex G-100 in the same buffer. The purified ^{125}I-labeled HRP contained approximately 7 × 10^4 cpm/μg of protein. ^{125}I was routinely quantitated using a Beckman Gamma-9000 counter (Beckman Instruments, Irvine, Calif.).

Enzyme Assays

β-hexosaminidase was assayed according to Li et al. [19]. Cytochrome oxidase was determined by the method of Rodriguez and Edelman [30]. HRP activity was quantitated as previously detailed [13].

Miscellaneous Techniques

Sucrose density was determined using an Abbe-56 refractometer (Bausch and Lomb, Rochester, N.Y.).

Results

Subcellular Fractionation of HRP-Loaded Toad Bladder Epithelial Cells

We have previously shown that a wave of granular cell apical membrane endocytosis is induced by water flow from a large transepithelial osmotic gradient during ADH stimulation [13] or subsequent removal of hormone from the solution bathing the basolateral surface of the toad bladder [12]. Recent studies using a novel label-fracture approach have directly demonstrated that within the first 10 min of membrane retrieval, a large proportion of the vesicles filled with fluid phase markers acquired by contact with the solution bathing the apical surface contain particle aggregates within their limiting membranes [5]. However, 60 min after initiation of membrane retrieval, fluid-phase markers are transferred to multivesicular bodies (MVB) [5, 12]. Therefore, we loaded the vesicles with HRP, then after 10 min of endocytosis rapidly (within 2 min) cooled the tissue to 2°C to prevent further intracellular processing before the epithelial cells were harvested and disrupted.

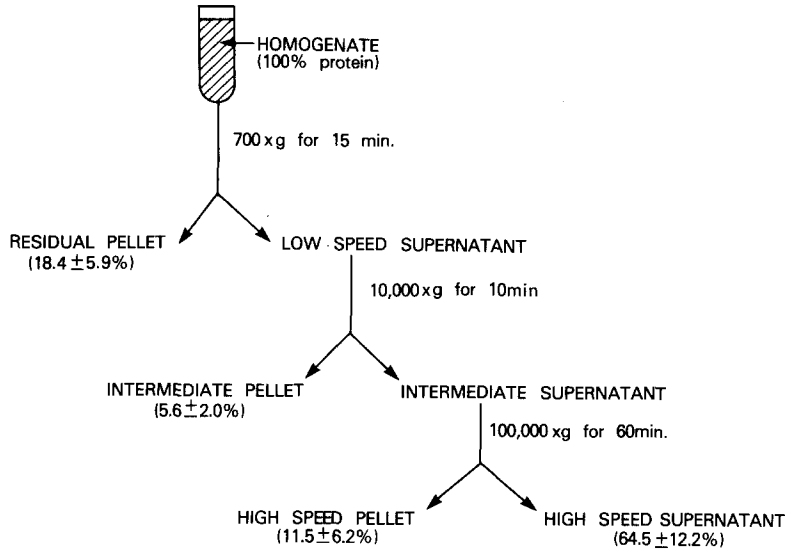

Fig. 1. Fractionation scheme using differential centrifugation of toad bladder epithelial cell homogenate. Isolated epithelial cells were homogenized in a Polytron apparatus for 5 sec on a setting of "5" and subjected to centrifugation as detailed below. Numbers within the parentheses indicate the distribution of homogenate protein ± SD ($n = 4$)

Table 1. Distribution of peroxidase activity in subcellular fractions[a]

Subcellular fraction	Fraction (sp act × 10^{-3})	Enrichment[b]	HRP distribution[c] (% total HRP)
Homogenate			
Control	7.8 + 1.8		
Experimental	25.0 + 1.3	1.0	100
Residual pellet			
Control	28.0 + 4.8		
Experimental	25.0 + 3.1	−0.2	37
Intermediate pellet			
Control	2.4 + 1.1		
Experimental	90.0 + 5.5	5.1	27
High speed pellet			
Control	2.3 + 0.9		
Experimental	37.0 + 3.8	2.0	22
High speed supernatant			
Control	10.0 + 0.3		
Experimental	14.0 + 0.3	0.2	14

[a] *Peroxidase activity* was measured in subcellular fractions prepared from toad bladders that had either undergone ADH stimulation and removal in the absence (control) or presence (experimental) of 10 mg/ml of horseradish peroxidase in the solution bathing the bladder's apical surface (*see* Materials and Methods for details). Peroxidase activity is expressed as OD/min/μg protein × 10^{-3} and represents the values obtained from four separate experiments.

[b] *Enrichment* is defined as the following ratio of peroxidase activities: (experimental-control) fraction/(experimental-control) homogenate. This number represents the increase or decrease in HRP within any given fraction compared to that present in the HRP-loaded experimental homogenate.

[c] *HRP distribution* was calculated by subtracting the peroxidase activity of control from experimental bladders, then factoring the result by the distribution of total proteins given in Fig. 1.

Bladders were either loaded with HRP by ADH stimulation and removal (experimental) or were exposed to the same HRP solution on their apical surface but in the absence of hormone (control). Under the latter conditions, there is essentially no endocytosis of HRP [12]. Epithelial cells were collected, homogenized and initially fractionated by differential centrifugation as shown in Fig. 1. An initial low speed centrifugation step produced a pellet (residual pellet) that contained unbroken cells and nuclei as assayed by phase-contrast microscopy (approximately 18% of total homogenate protein). The su-

pernatant was centrifuged for 100,000 × g · min to yield an intermediate pellet and intermediate supernatant. Further fractionation of the latter by a third centrifugation for 600,000 × g · min produced a high speed pellet and supernatant. Sixty four percent of total homogenate protein remained soluble, while approximately 17% was in the intermediate and high speed pellets. There were no differences between control and experimental bladders in the yield for any of the above fractions. Total protein recovery averaged 84 ± 17% (n = 4) for both control and experimental bladders.

Table 1 shows the distribution of peroxidase activity among the subcellular fractions of control and experimental bladders. As previously shown [12, 13], scraped epithelial cells possess endogenous peroxidase activity. Peroxidase activity in control (HRP exposed but not internalized) bladders does not significantly differ from bladders without HRP exposure. Table 1 indicates that peroxidase activity varies among these various subcellular fractions from control and experimental bladders. Thus, the difference between experimental (HRP loaded) and control (HRP exposed but not internalized) peroxidase values from any given fraction divided by the difference between the peroxidase values of experimental and control whole homogenates should be a measure of the increase or decrease of exogenous HRP within that fraction as compared to that present in whole homogenate. The experimental homogenate shows approximately 2.7 times more peroxidase activity than does control. Both intermediate and high speed pellets are enriched for exogenous HRP. Together, they comprise approximately 49% of the total internalized HRP of the homogenate. Overall recovery of peroxidase activity averaged 92 ± 16% (n = 4).

The variable amounts of endogenous peroxidase distributed throughout these subcellular fractions complicate an estimate of exogenous HRP which has been released as a result of vesicle breakage due to the homogenation and centrifugation steps. Vesicle lysis probably does occur during the fractionation procedure since 14% of the internalized HRP is found in the high speed supernatant (Table 1). However, HRP-loaded vesicles present in the intermediate pellet appear to be stable since the sucrose gradient shown in Fig. 3 (*see below*) contains only a small amount of HRP that does not migrate with the vesicle fraction.

SDS-PAGE analysis of fractions obtained by differential centrifugation are displayed in Fig. 2. Because the pattern of protein bands visualized was dramatically different, depending on whether we stained with Coomassie blue or silver, we employed a double staining procedure [7] to simultaneously

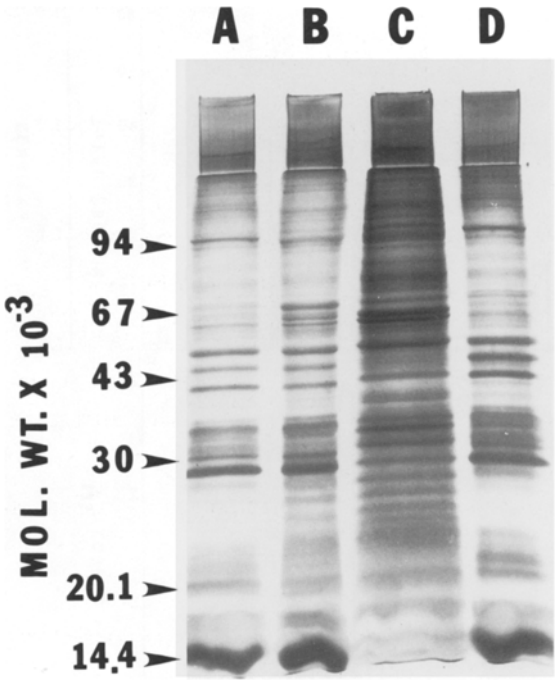

Fig. 2. Comparison of the protein composition of subcellular fractions obtained from homogenized epithelial cells. The subcellular fractions prepared from experimental bladders as described in Fig. 1 were analyzed by SDS gel electrophoresis and stained with a double staining technique [7] using both silver stain and Coomassie blue. Each lane received 10 μg protein: lane *A*, total homogenate; lane *B*, residual pellet; lane *C*, intermediate pellet; and lane *D*, intermediate supernatant. The position of mol wt markers are indicated with arrows

detect both groups of stained bands within a single gel. This demonstrates that, while the residual pellet and intermediate supernatant fractions have similar proteins, the intermediate pellet fraction differs from these fractions in the distribution of a large number of protein bands. Thus, the intermediate pellet is a fraction that contains a selected portion (5%) of total homogenate protein where approximately 27% of the HRP internalized by epithelial cells is sequestered.

Both intermediate and high speed pellets from experimental bladders were resuspended, layered on sucrose gradients and centrifuged for approximately 1 × 10^7 × g · min. The top panel of Fig. 3 shows fractionation of the gradient containing intermediate pellet assayed for both peroxidase and total protein. The majority of protein and peroxidase are found within the lower half of the gradient. Similar gradient separation of intermediate pellet fractions from control bladders showed an identical distribution of protein, but virtually all peroxidase activity was located within the first 10 fractions at the top of

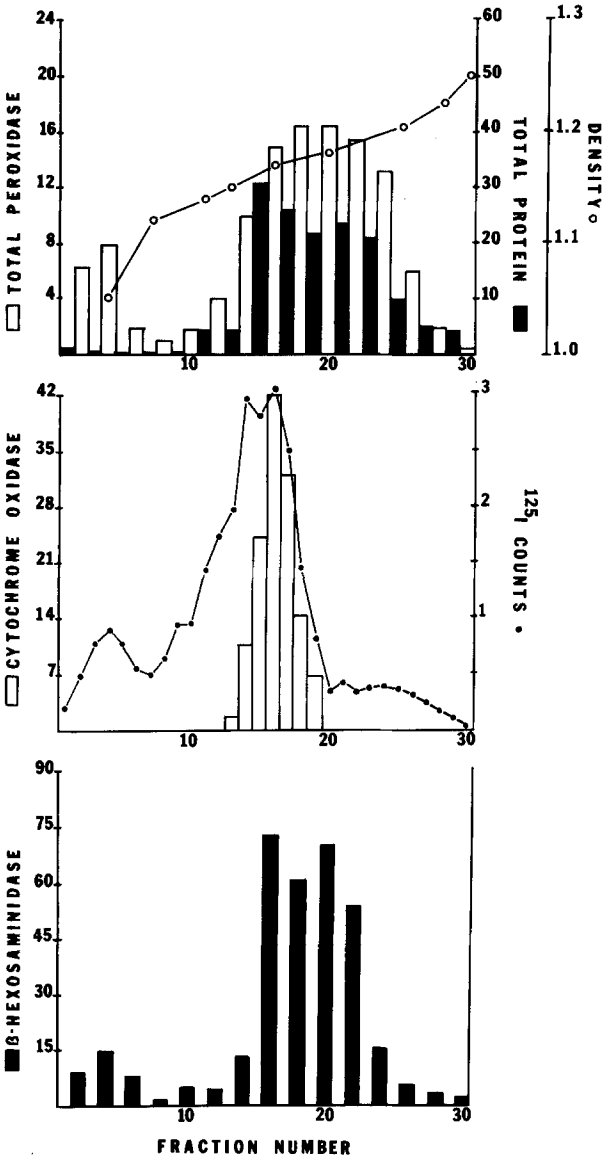

Fig. 3. Fractionation of a sucrose gradient containing HRP-loaded intermediate pellet. 400 μg of the intermediate pellet fraction were layered on a continuous 29 to 62% sucrose gradient and centrifuged for $1 \times 10^7 \times g \cdot$ min. The resulting gradient was then fractionated and subjected to the following assays. *Top panel:* total peroxidase (OD/min/μg protein $\times 10^1$), total protein (μg/fraction) and density (g/ml); *Middle panel:* cytochrome oxidase (OD/min/fraction $\times 10^2$) and ^{125}I-labeled apical membrane protein (cpm $\times 10^{-3}$); and *Bottom panel:* β-hexosaminidase (OD/min/fraction $\times 10^4$)

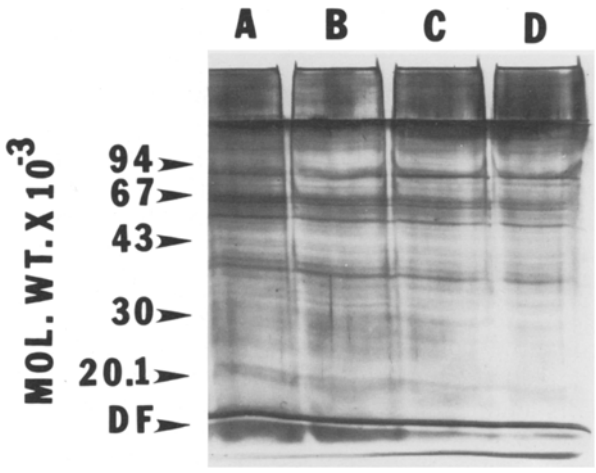

Fig. 4. SDS gel analysis of selected fractions of sucrose gradient containing intermediate pellet. Samples from the sucrose gradient detailed in Fig. 3 were fractionated by SDS gel electrophoresis and stained with a combination of silver and Coomassie blue [7]. Refer to Fig. 3 for fraction numbers: lane *A*, fraction 3; lane *B*, fraction 15; lane *C*, fraction 20; and lane *D*, fraction 22. The position of mol wt markers are indicated with arrows

the gradient. In contrast to the intermediate pellet fractionation, all the peroxidase and protein of the high speed pellet of experimental and control bladders were located in the top 10 fractions of gradient (*data not shown*). The high speed pellet was not characterized further because of: (i) greater enrichment of HRP in intermediate pellet (Table 1) and (ii) the likely presence of unsequestered HRP which would render it unsuitable for the density shifting maneuver (*see* Discussion). The contents of the intermediate pellet fraction was further characterized as shown in the middle and lower panels of Fig. 3 and Table 2. The distribution of mitochondrial (cytochrome oxidase), lysosomal (β-hexosaminidase) and exposed apical membrane proteins from unstimulated bladders (^{125}I-labeling of apical membrane with diazotized iodosulfanilic acid) [8] were assayed in fractions from differential and density gradient centrifugation. The intermediate pellet fraction is enriched for ^{125}I-labeled apical membrane, mitochondria and lysosomes as well as HRP (Table 2). All three markers band rather closely together at a density of 1.15–1.175 and substantially overlap the broad peak of peroxidase activity centered at approximately 1.175 g/ml (Fig. 3). SDS-PAGE analysis of fractions across the gradient show only minor changes in the apparent protein composition (Fig. 4).

Density Shifting of HRP-Loaded Vesicles from the Intermediate Pellet Fraction

Both marker enzyme and electron microscopic analysis (*see below*) showed that, although the specific activity of peroxidase had increased to approximately 132 times (fraction #18, Fig. 3) that of homogenate, gradient fractions #18–24 were still

Table 2. Summary of radioactive and enzyme markers in subcellular fractions of ADH-stimulated toad bladder[a]

Subcellular fraction	^{125}I-counts cpm/μg	E.F.	Cytochrome oxidase[b] OD/min/μg	E.F.	β-hexosaminidase[c] OD/min/μg	E.F.	Peroxidase[d] OD/min/μg	E.F.
Homogenate	42.9	1.0	8.9	1.0	4.6	1.0	3.5	1.0
R. Pellet	5.4	0.13	8.6	4.9	1.06	4.3	1.2	
I. Pellet	96.2	2.24	52.1	5.84	4.78	8.9	2.5	
I. Supernt.	38.5	0.90	ND		2.2	0.47	2.2	0.6

[a] Measurements were performed on subcellular fractions prepared from ADH-stimulated toad bladders that had internalized horseradish peroxidase (n = 2). ^{125}I counts represent proteins labeled on the apical surface of unstimulated bladders with diazotized ^{125}I-iodosulfanilic acid. The following abbreviations are used: E.F.–enrichment factor normalized the specific activity of the homogenate to 1; R. Pellet – residual pellet; I. Pellet – intermediate pellet; I. Supernt. – intermediate supernatant. *See* Materials and Methods and text for details. Cytochrome oxidase activity expressed as OD/min/μg protein $\times 10^{-5}$.
[b] β-hexosaminidase activity expressed as OD/min/μg protein $\times 10^{-5}$.
[c] Peroxidase activity expressed as OD/min/μg protein $\times 10^{-2}$.

substantially contaminated with other intracellular organelles. HRP was used as a fluid phase marker for membrane particle aggregate-rich vesicles so that we could employ a density shifting technique [6] to further purify these organelles. Briefly, this method relies on the fact that oxidized and polymerized diaminobenzidine (DAB) has a density of >1.34 g/ml. Entrapped intravesicular HRP converts reduced membrane permeant DAB to intravesicular oxidized and polymerized DAB, which is trapped and shifts the equilibrium density of that vesicle fraction (*see* Materials and Methods for details). Fraction #'s 18–24 were pooled and diluted with a sucrose solution containing DAB. H_2O_2 was then added to selected samples and the mixture incubated for 30 min. In those samples where the combination of HRP-loaded vesicles, DAB and H_2O_2 was present, there was a change in color of the mixture from a pale yellow to a lightly rusty brown. The samples were then layered on top of sucrose gradients of identical composition as in Fig. 3 and recentrifuged as described above. In the bottom of the tubes from samples that underwent the color change there was routinely a small brown pellet.

After careful removal of sucrose from the centrifuge tubes, SDS-solubilizing buffer was added to the bottom of each tube and its solubilized contents analyzed by SDS-PAGE and stained with Coomassie blue (Fig. 5). Centrifuge tubes with samples from control bladders (lanes *A* and *B*) incubated with DAB in the presence (lane *B*) or absence (lane *A*) of H_2O_2, yielded gels with minimal protein bands. In contrast, when fractions from experimental bladders (lanes *C* and *D*) were incubated with DAB in

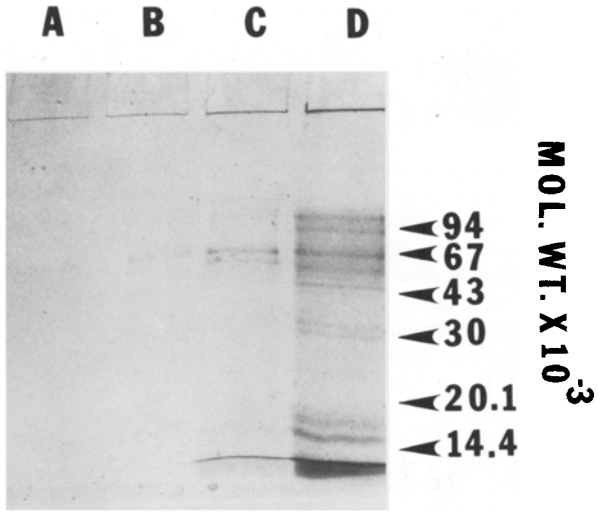

Fig. 5. SDS gel analysis of density-shifted sucrose gradient fractions. Pooled samples of control and experimental intermediate pellet (*see* text) from sucrose gradient fractions #18–24 (Fig. 3) were diluted into a buffer containing 2.56 mg/ml of DAB with or without H_2O_2 and incubated for 30 min. The samples were then layered on a second sucrose gradient identical to the first and centrifuged as before. A visible pellet appeared only in samples containing experimental intermediate pellet and the complete reaction mixture. SDS-solubilizing buffer was added to the bottom of each of the centrifuge tubes, and after incubation at 100°C for 5 min the samples were electrophoresed SDS gels which were stained with Coomassie blue. The two left lanes were obtained from control intermediate pellet fractions incubated without (Lane *A*) and with (lane *B*) H_2O_2. Samples from experimental intermediate pellet fractions incubated without (lane *C*) and with (lane *D*) H_2O_2 are shown on the right. Note the appearance of protein bands in lane *D* when HRP-loaded vesicles are incubated with the complete density shifting reaction mixture. The position of mol wt markers are indicated by arrows

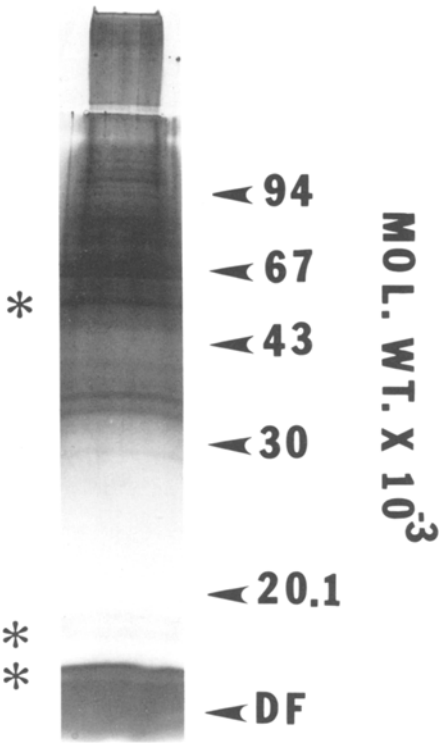

Fig. 6. An SDS gel of density-shifted sucrose gradient fractions from HRP-loaded intermediate pellet. The pellet obtained after the density shift maneuver was performed on fractions containing HRP-loaded vesicles (*see* Fig. 5) was subjected to SDS gel electrophoresis and stained with a combination of silver and Coomassie blue. Molecular weight markers are indicated by the arrows. Asterisks indicate bands at 55, 17 and 15 kD and 7 kD. *DF* is the dye front of the gel where the intense staining is attributable to DAB

the presence (lane *D*) but not the absence (lane *C*) of H_2O_2, there was a dramatic appearance of 15 major protein bands within the gel. Both their appearance and distribution were highly reproducible in completely separate experiments (n = 6). Analysis of SDS gels using the double staining method revealed approximately 20 bands (Fig. 6) in a distribution very different from the original gradient fractions (Fig. 4). Of note are protein bands of 55, 17, 15 and 7 kD (co-migrates with the dye front) which comprise a major portion of the protein present. We have iodinated proteins of identical molecular weights both on the apical surface of ADH-stimulated bladders but not on the apical surface of controls as well as during intracellular iodination of vesicles that contain particle aggregates [5, 14]. Thus, density shifted HRP-loaded vesicles contain proteins previously identified as likely participants in the ADH-induced water permeability response.

As discussed by Courtoy et al. [6], the specificity of the density shifting method can be jeopardized if the preparation is contaminated with soluble or externally exposed HRP activity which may result from vesicle lysis or absorption of HRP to membrane fragments. This may result in the rapid agglutination of the preparation and nonspecific precipitation of its contents. This possibility was investigated by mixing ^{125}I-labeled apical membranes from control bladders harvested from fractions #18–20 with unlabeled fractions #18–22 from experimental bladders as used in Fig.3 . The density shifting maneuver was performed on the combined fractions, and the centrifuge tubes were monitored for the presence of a pellet and distribution of ^{125}I counts (Table 3*A*). A visible pellet was produced only when H_2O_2 and DAB were added to the combined fractions as compared to DAB addition in the absence of H_2O_2. In all cases less than 10% of the total ^{125}I counts were found in the pellet with the bulk of the radioactivity sedimented to the same position as in the original gradient. Therefore, we conclude that these fractions do not contain free HRP that will agglutinate the preparation and produce nonspecific precipitation of membrane fragments.

It is also possible that the sequestered HRP contributes to the proteins visualized on the gels shown in Figs. 5 and 6. To test for this, we iodinated HRP with lactoperoxidase using standard techniques [24]. The ^{125}I-labeled HRP was then sequestered in toad bladder epithelial cells which were homogenized and fractionated as in Fig. 3. Fractions #18–22 from the initial sucrose gradient were pooled and subjected to the density shifting maneuver with and without the addition of H_2O_2 (Table 3*B*). In the peroxide-treated preparation where a visible pellet was observed, it contained 43% of the ^{125}I counts. In contrast, when no H_2O_2 was added, no pellet appeared and 89% of the ^{125}I counts resedimented to an identical position in the second sucrose gradient. Autoradiography of the SDS gel from the density shifted pellet demonstrated that none of the radioactivity entered the running gel. It was all localized in a high molecular weight band at the top of the gel (*data not shown*). These experiments demonstrate that the density shift maneuver was specific and the proteins present in the SDS gel of the resulting pellet are not fragments of HRP.

Electron Microscopy of Membrane Fractions

The intermediate pellet fraction was examined by thin-section electron microscopy for both morphology and the nature of membrane vesicles that sequestered HRP (Fig. 7). It contained a variety of membranous structures, including large sheets of

Table 3. Horseradish peroxidase (HRP) mediated density shift of ^{125}I-labeled membrane fractions

	Visible pellet[a]		Gradient fractions #10–20[b]		
Density shift	^{125}I counts	% total	^{125}I counts	% total	^{125}I recovery (%)
A) ^{125}I-labeled apical membrane					
+	+616	7.6	8,091	99.4	106
−	−171	2.1	7,579	92.5	94.6
B) ^{125}I-labeled HRP					
+	+230	43.1	209	39.2	82.4
−	−10	1.9	473	89.2	91.1

[a] *Visible pellet* – The presence (+) or absence (−) of a visible pellet is indicated, and the number of total ^{125}I counts the bottom of the centrifuge tube contained is shown to its immediate right.
[b] *Gradient fractions #10–20* – Membrane fractions (#10–20) were isolated from an initial sucrose gradient, pooled and subjected to the density shift maneuver. These were layered on a second sucrose gradient of identical composition to the first. After centrifugation, the second gradient was fractionated identically to the first and the ^{125}I counts in fractions #10–20 were determined.

plasma membrane, mitochondria, dense granules and a collection of smaller vesicles. Vesicles which sequestered HRP were detected after attaching the contents of intermediate pellet to polylysine-coated tissue culture dishes where they were fixed and developed using DAB and H_2O_2. Here we observed both round and elongated vesicles, which were intensely stained with DAB reaction product (Fig. 7*a*). Examination of HRP-rich fractions from the initial sucrose gradient showed the dominant organelle was mitochondria together with fragments of plasma membrane and small vesicles (Fig. 7*b*). Vesicles that stained intensely for DAB reaction product were more numerous than in the unfractionated intermediate pellet. Both round and elongated vesicles were observed (Fig. 7*c*). Thin sections of the fixed density shifted pellet showed numerous round and elongated vesicles of various sizes filled with DAB reaction product (Fig. 7*d*) together with occasional membranes that did not contain peroxidase reaction product as indicated by the asterisk.

Discussion

The results reported here confirm the observations of Rodriguez and Edelman [30] with respect to both the homogenization conditions and behavior of toad bladder membrane fractions after disruption. A period of cell swelling followed by homogenization of cells in a polytron apparatus produced efficient disruption of epithelial cells.

The purification strategy detailed above relies on the specificity of apical membrane retrieval during a 10-min interval after removal of hormone from ADH-stimulated bladders. During ADH stimulation in the absence of a transepithelial osmotic gradient, aggrephores fuse with the apical membrane [9, 25], increasing its surface area by as much as 25% [28, 31], but there is no measurable apical endocytosis [13, 21]. In contrast, high rates of osmotic water flow induce retrieval of apical membrane via a process stimulated further by the removal of ADH [12, 13, 21, 25, 27]. Electron microscopic examination of thin sections shows that vesicles initially retrieved from the apical membrane and thus containing HRP are both tubular vesicles whose morphology is identical to aggrephores observed in freeze-fracture electron microscopy [5, 12, 13, 33] and spherical vesicles, a portion of which are in fact tubular vesicles cut in cross-section [12].

Recent work [5] using a novel label fracture approach has allowed simultaneous visualization of internalized markers such as HRP or colloidal gold and structures within the lipid bilayer of the same vesicle. That study examined the nature of the vesicles retrieved from the toad bladder apical membrane during intervals of ADH stimulation and removal identical to those used in the present study. Coleman et al. [5] observed three types of vesicles retrieved during these intervals: (i) long tubular vesicles morphologically identical to aggrephores which contained membrane particle aggregates, (ii) spherical vesicles which also possessed particle aggregates but whose limiting membrane appeared to be derived from apical membrane which was distinct from that of aggrephores, and (iii) a less frequent type of spherical vesicle which had no detectable particle aggregates and also appeared to be derived from the apical membrane. Thus, the epi-

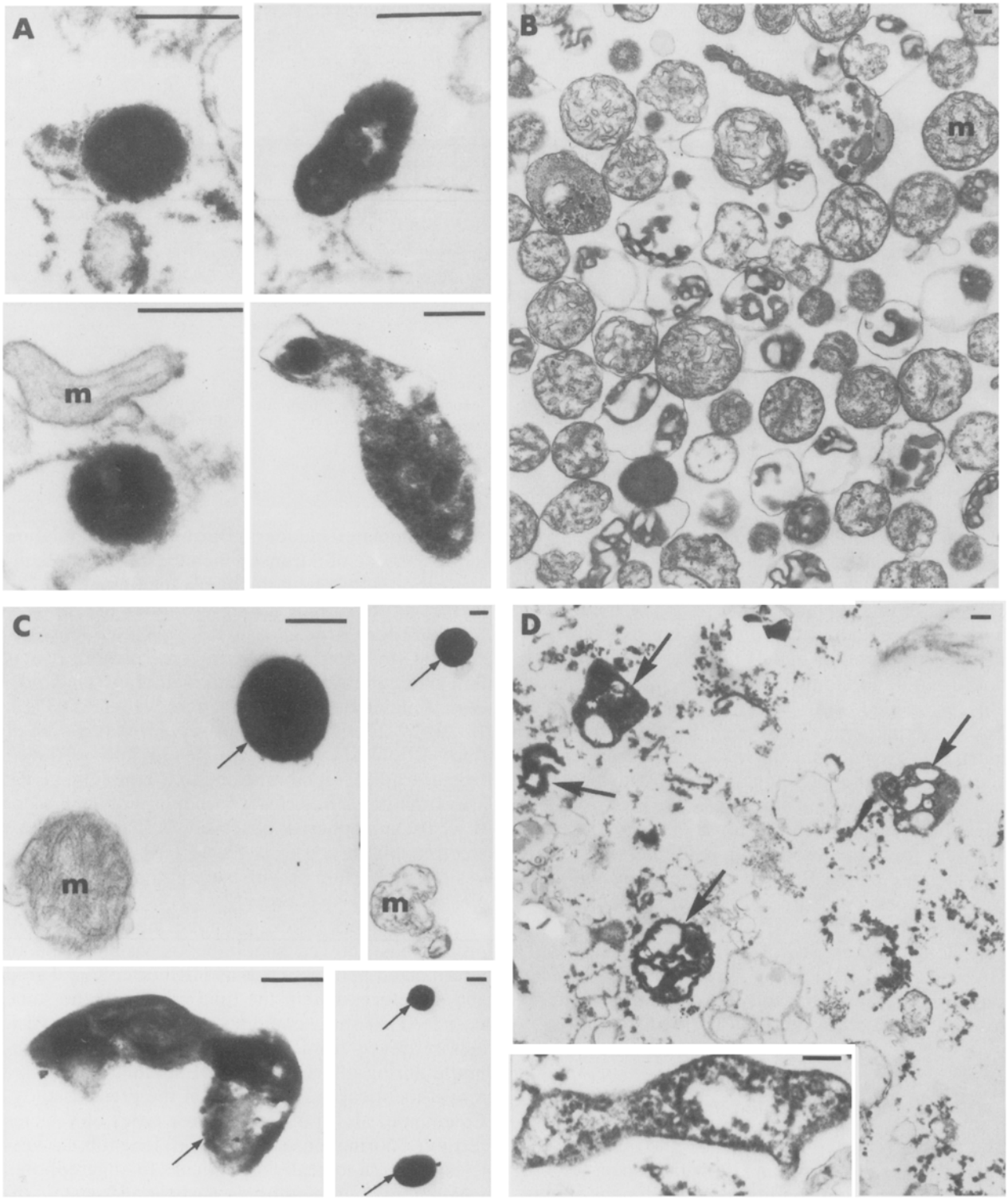

Fig. 7. Morphology exhibited by HRP-containing membrane fractions. Samples from intermediate pellet (*A*), sucrose gradient (*B* and *C*) and the pellet of the density shift maneuver (*D*) were prepared for electron microscopy either as a fixed centrifuged pellet (*B* and *D*) or attached to the surface of a polylysine-coated dish, fixed and HRP localized before embedding and sectioning (*A* and *C*). All bars measure 0.2 μm. Magnifications for each photo in panels *A–D* in clockwise direction are: (*A*) 1, 2, 3–100,000×; 4–57,000×; (*B*) 15,600×; (*C*) 1,3–57,500×; 2, 4–15,600×; (*D*) 15,600*x*. *m* = mitochondrion. The four arrows in *D* indicate vesicles containing sequestered HRP, while an asterisk shows a nonperoxidase-containing membrane contaminant. The inset shows an elongated vesicle that resembles the morphology of aggrephores, which has entrapped HRP. The dark granular material interspersed throughout the field is polymerized DAB

thelial cells harvested from bladders subjected to the protocol used in this study should contain HRP-loaded vesicles, the majority of which contain membrane particle aggregates within their limiting membranes.

Electron micrographs of the intermediate pellet, sucrose gradient and density-shifted fractions clearly show that HRP is sequestered in both elongated and apparently spherical vesicles (Fig. 7). Their specific relationship to those elongated and spherical vesicles visualized by label fracture analysis [5] remains to be determined. Since the vesicles shown in Fig. 7 derive from cells whose contents have been subjected to high shear forces during homogenization, hyperosmotic conditions of density gradient centrifugation and the density shifting protocol, it is not possible to directly relate their morphology to the HRP-rich vesicles present in intact granular cells. The forms that the different types of vesicles visualized by label fracture analysis of intact cells take after these multiple fractionation steps will be the subject of further study.

It is difficult to estimate the final purity of the final vesicle fraction produced by density shifting pooled sucrose gradient fractions. Assays of marker enzymes for known organelles after density shifting is not reliable. As demonstrated by Quintart et al. [29], marker enzymes are variably affected by exposure to the reagents of the density shift maneuver. It is also not possible to determine HRP activity enrichment after the density shift maneuver. Electron microscopy demonstrates that vesicles containing entrapped HRP undergo substantial enrichment by both density gradient and density shifting steps in purificaiton. However, examination of the density shifted pellet also shows that despite this multiple step fractionation scheme there are still some membranes that do not contain sequestered HRP and contaminate this final fraction. Perhaps the best criterion for purification is the dramatic change in the protein composition of the density shifted fraction (Figs. 5 and 6). Although the SDS gel patterns of intermediate pellet (Fig. 2) and sucrose gradient fractions derived from it (Fig. 4) are considerably different than the homogenate (Fig. 2), the SDS gel pattern of the density shifted pellet is radically different. Experiments detailed in Table 3 show the change in protein composition is not due to nonspecific agglutination of the reaction mixture or to fragments derived from HRP. Thus, we conclude that the density shifted pellet from ADH-stimulated toad bladders exposed to HRP represents a highly enriched fraction of vesicles derived from specialized regions of apical membrane.

SDS gel bands of molecular weights 55 kD, a group of bands clustered from 17 to 14 and 7 kD were ^{125}I-labeled in the absence of water flow only on the surface of ADH-stimulated but not control bladders [14]. An identical pattern of ^{125}I-labeled bands was also observed on the inner surface of vesicles internalized under conditions identical to those examined by the label fracture analysis [5] and used in this study. As shown in Figs. 5 and 6, protein bands of 55, 17, 15 and 7 kD are clearly visible among the approximately 20 major species which constitute the density shifted fraction. Since this group of proteins has repeatedly been associated with membrane particle aggregate-containing vesicles and the ADH-induced P_f, we conclude that the density shifted pellet represents a purified vesicle fraction which is enriched for a selected set of proteins involved in the ADH-induced P_f.

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References

1. Bonner, W.M. 1984. Fluorography for the detection of radioactivity in gels. *In:* Methods in Enzymology. W.B. Jakoby, editor. Vol. 104, pp. 460–466. Academic, New York
2. Bourguet, J., Chevalier, J., Hugon, J.S. 1976. Alterations in membrane-associated particle distribution during antidiuretic challenge in frog urinary bladder epithelium. *Biophys. J.* **16**:627–639
3. Bradford, M.M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254
4. Brown, D., Grosso, A., DeSousa, R.C. 1983. Correlation between water flow and intramembrane particle aggregates in toad epidermis. *Am. J. Physiol* **245**:C334–C342
5. Coleman, R.C., Harris, H.W., Jr., Wade, J.B. 1987. Novel application of the label-fracture method to studies of internalized tracers in toad urinary bladder. *J. Cell Biol.* **103**:1222 *(abstr.)*
6. Courtoy, P.J., Quintart, J., Baudhuin, P. 1984. Shift or equilibrium density induced by 3,3'-diaminobenzidine cytochemistry: A new procedure for the analysis and purification of peroxidase-containing organelles. *J. Cell Biol.* **98**:870–876
7. Dzandu, J.K., Deh, M.E., Barratt, D.L., Wise, G.E. 1984. Detection of erythrocyte membrane proteins, sialoproteins and lipids in the same polyacrylamide gel using a double staining technique. *Proc. Natl. Acad. Sci. USA* **81**:1733–1737
8. Ekblad, E.B.M., Strum, J.M., Edelman, I.S. 1976. Differential covalent labeling of apical and basal-lateral membranes of the epithelium of the toad bladder. *J. Membrane Biol.* **26**:301–317
9. Ellis, S.J., Kachadorian, W.A., DiScala, V.A. 1980. Effect of osmotic gradient on ADH-induced intramembranous particle aggregates in toad bladder. *J. Membrane Biol.* **52**:181–184
10. Handler, J.S., Orloff, J. 1973 The mechanism of action of antidiuretic hormone. *In:* The Handbook of Physiology. Renal Physiology. Section 8, Chap. 24, pp. 791–814. American Physiological Society, Washington, D.C.

11. Harmanci, M.C., Stern, P., Kachadorian, W.A., Valtin, H., DiScala, V.A. 1980. Vasopressin and collecting duct intramembranous particle clusters: A dose-response relationship. *Am. J. Physiol.* **239:**F560–F564
12. Harris, H.W., Jr., Wade, J.B., Handler, J.S. 1986. Fluorescent markers to study membrane retrieval in ADH treated toad urinary bladder. *Am. J. Physiol.* **251:**C274–C284
13. Harris, H.W., Jr., Wade, J.B., Handler, J.S. 1986. Transepithelial water flow regulates apical membrane retrieval in ADH-stimulated toad urinary bladder. *J. Clin. Invest.* **78:**703–712
14. Harris, H.W., Jr., Murphy, H.R., Willingham, M.C., Handler, J.S. 1987. Identification and isolation of proteins involved in the toad urinary bladder's water permeability response to ADH. *Kidney Int.* **31:**169 (*abstr.*)
15. Humbert, F.R., Montesano, A., Grosso, A., DeSousa, R.C., Orci, L. 1977. Particle aggregates in plasma and intracellular membranes of toad bladder (granular cell). *Experientia* **33:**1364–1367
16. Kachadorian, W.A., Wade, J.B., DiScala, V.A. 1975. Vasopressin: Induced structural change in toad bladder luminal membrane. *Science* **205:**910–913
17. Laemelli, U. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (London)* **227:**680–685
18. Levine, S.D., Jacoby M., Finkelstein, A. 1984. The water permeability of toad bladder: II. The value of $P_f/P_{d(w)}$ for the antidiuretic hormone induced water permeation pathway. *J. Gen. Physiol.* **83:**543–561
19. Li, Y.-T., Li, S.-T. 1972. α-mannosidase, β-N-acetylhexosamidase and β-galactosidase from jack bean meal. *Methods Enzymol.* **28:**702–713
20. Lorenzen, M., Kubat, B., Reale, E. 1986. Quantification of vasopressin-induced intramembrane particle aggregates in isolated cortical collecting tubules (ICCT's) of rabbit kidney. *Kidney Int.* **29:**419 (*abstr.*)
21. Masur, S.K., Cooper, S., Rubin, M.S. 1984. Effect of an osmotic gradient on antidiuretic hormone-induced endocytosis and hydroosmosis in the toad urinary bladder. *Am. J. Physiol.* **247:**F370–F379
22. Mellman, I.S., Steinman, R.M., Unkeless, J.C., Cohn, Z.A. 1980. Selective iodination and polypeptide composition of pinocytic vesicles. *J. Cell Biol.* **86:**712–722
23. Merril, C.R., Sedman, S.A., Ebert, M.H. 1981. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science* **211:**1437–1439
24. Morrison, M. 1978. The determination of the exposed proteins on membranes by the use of lactoperoxidase. *Methods Enzymol.* **32:**103–109
25. Muller, J., Kachadorian, W.A. 1984. Aggregate-carrying membranes during ADH stimulation and washout in the toad bladder. *Am. J. Physiol.* **247:**C90–C98
26. Muller, J., Kachadorian, W.A. 1985. Regulation of luminal membrane water permeability by water flow in toad urinary bladder. *Biol. Cell.* **55:**219–224
27. Muller, J., Kachadorian, W.A., DiScala, V.A. 1980. Evidence that ADH-stimulated intramembrane particle aggregates are transferred from cytoplasmic to luminal membranes in toad bladder epithelial cells. *J. Cell Biol.* **85:**83–93
28. Palmer, L.G., Lorenzen, M. 1983. Antidiuretic hormone-dependent membrane capacitance and water permeability in the toad urinary bladder. *Am. J. Physiol.* **244:**F195–F204
29. Quintart, J., Courtoy, P.J., Baudhuin, P. 1984. Receptor-mediated endocytosis in rat liver: Purification and enzymic characterization of low density organelles involved in uptake of galactose-exposing proteins. *J. Cell Biol.* **98:**877–884
30. Rodriguez, H.J., Edelman, I.S. 1979. Isolation of radio-iodinated apical and basal-lateral plasma membranes of toad bladder epithelium. *J. Membrane Biol.* **45:**215–232
31. Stetson, D.L., Lewis, S.A., Alles, W., Wade, J.B. 1982. Evaluation by capacitance measurements of antidiuretic hormone induced membrane area changes in toad bladder. *Biochim. Biophys. Acta* **689:**267–274
32. Wade, J.B. 1978. Membrane structural specialization of the toad urinary bladder revealed by the freeze-fracture technique. *J. Membrane Biol.* **Special Issue:** 281–296
33. Wade, J.B. 1985. Membrane structural studies of the action of vasopressin. *Fed. Proc.* **44:**2687–2692
34. Wade, J.B., Stetson, D.L., Lewis, S.A. 1981. ADH action: Evidence for a membrane shuttle hypothesis. *Ann. N.Y. Acad. Sci.* **372:**106–117

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