Retention of Antidiuretic Hormone–induced Particle Aggregates by Luminal Membranes Separated from Toad Bladder Epithelial Cells

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ABSTRACT   Aggregates of intramembrane particles appear in the luminal membranes of renal collecting duct and amphibian bladder cells after stimulation by antidiuretic hormone (ADH). We undertook this freeze-fracture study to determine whether particle aggregates, once in place, remain in the luminal membrane of the amphibian bladder after the membrane is physically separated from the rest of the cell. We found that the aggregates do remain in high yield in isolated membranes stabilized with a bifunctional imidoester (DTBP) followed by fixation with glutaraldehyde, or unfixed but stabilized with DTBP. These findings support the view that the particles are intrinsic membrane components and that their organization in the form of aggregates does not depend on the presence of the intact cell. In addition, the availability of isolated membranes containing particle aggregates provides a starting point for the isolation of the water-conducting proteins.

Antidiuretic hormone (ADH) is believed to initiate a series of fusion events in the receptor cells of the kidney (1) and amphibian bladder (2–4), in which aggregates of intramembrane particles are transferred from the vesicular cytoplasmic structures to the luminal membrane (3, 4, 6). These particles, probably proteins, appear to be the sites of ADH-induced water flow (5). Here we ask whether the aggregates, once in place, remain in the luminal membrane after the membrane is separated from the rest of the epithelial cell. Our interest in this question is two-fold. First, aggregate-containing luminal membranes would provide a relatively simple starting point for the extraction and eventual isolation of water-conducting proteins. In this approach, one can compare aggregate-containing luminal membranes from hormone-stimulated cells to luminal membranes from unstimulated cells which have virtually no aggregates. Second, we would be able to determine whether or not aggregates retain their structure in the isolated membrane.

We compared membranes from control and vasopressin-stimulated bladders under two conditions: (*a*) from bladders whose luminal membranes were stabilized after hormone treatment by the reversible cross-linking agent dithiobispropionimidate (DTBP) (7), homogenized, and then fixed with glutaraldehyde; and (*b*) from membranes taken from DTBP-stabilized bladders which were homogenized and further purified by sucrose density gradient centrifugation but not fixed with glutaraldehyde. We found, upon examination of the two groups of membranes by freeze-fracture electron microscopy, that the isolated luminal membranes did retain aggregates of intramembrane particles to an extent far greater than membranes from unstimulated bladder cells. Preparation of aggregate-containing membranes without employing the irreversible cross-link-

ing agent glutaraldehyde will facilitate the isolation of the aggregate proteins.

MATERIALS AND METHODS

Preparation of Membranes

Four hemibladders from two female toads (*Bufo marinus;* National Reagents, Bridgeport, Conn.) were mounted on glass bungs and rinsed three times, inside and out, with amphibian Ringer's solution (composition: 120 mM Na$^+$, 4 mM K$^+$, 0.5 mM Ca^{2+}, 116 mM Cl$^-$, 5 mM phosphate, 230 mosmol/kg, pH 7.4). The bladders were filled with 10 ml of isotonic Ringer's; 35 ml of Ringer's bubbled with air bathed the serosal surface. Control bladders were incubated for 15 min, and paired vasopressin-treated bladders were incubated for the same time period with 100 mU/ml of arginine vasopressin (Sigma Chemical Co.. St. Louis, Mo.) in the serosal medium. After incubation, the inside medium was removed and replaced by 10 ml of phosphate Ringer's, pH 10, containing 2 mg/ml DTBP (Pierce Chemical Co., Rockford, Ill.) and incubated for an additional 10 min, with the serosal solutions unchanged. DTBP cross-links the surface proteins of the luminal membrane, greatly delaying the disappearance of aggregates following withdrawal of vasopressin (7). The delay in disappearance is greater than that recently reported for bladders incubated in the cold (8). The bladders were then removed from the bungs and rinsed quickly in 1 mM NaHCO₃, pH 7.5. Bladders were then placed, luminal surface outward, over the ends of glass slides, and the epithelial cells were scraped off with a glass cover slip into 1 mM NaHCO₃ at 4°C. The cell sheets were spun down for 1 min in a table-top centrifuge. The supernate was replaced with fresh 1 mM NaHCO₃, and the pellet of cells was resuspended and again spun down. The supernate was then replaced by 3 ml of bicarbonate solution at 4°C and the suspension was homogenized with a tight-fitting Dounce homogenizer in ice until all the cells were broken. This procedure yields large membrane envelopes, easily recognized by phase-contrast microscopy (9).

At this point, two different procedures were followed. To preparation I (control and vasopressin-treated homogenates), glutaraldehyde was added to give a final concentration of 2%. After 15 min, the homogenate was centrifuged in a table-top centrifuge for 1 min; the supernate was then removed and replaced with 0.1 M cacodylate buffer, pH 7.4. The pellet, containing membranes, nuclei, and other

components, was resuspended and then centrifuged at 650 g for 15 min in an International centrifuge (International Equipment Corp., Damon Corp., IEC Division, Needham, Mass.). This was repeated, with replacement of the supernate by fresh cacodylate buffer and resuspension of the pellet. The pellet was then resuspended in 1 ml of cacodylate buffer and kept at 4°C in preparation for freeze-fracture. Preparation II (DTBP-stabilized, unfixed) received enough NaCl to give a final concentration of 140 mM/kg NaCl. 2 ml of the homogenate was then layered carefully over 5 ml of 55% (wt/vol) sucrose, also containing 140 mM NaCl. The tubes were centrifuged for 30 min in a swinging bucket rotor at 1,300 g in a refrigerated International centrifuge at 4°C. The fluffy material at the homogenate-sucrose interface, containing cell membranes approximately fourfold enriched compared to the crude homogenate (R. Kinne, N. Franki, and R. M. Hays, unpublished observations), was then collected, diluted with cacodylate buffer, and spun down for 10 min at 1,300 g. The supernate was removed and replaced with fresh cocodylate buffer, and the pellet was resuspended and centrifuged at 1,300 g for 5 min.

Freeze-fracture

Membranes from groups I and II were suspended in 1 ml of 25% glycerol in 0.1 M cacodylate buffer for 1 h at room temperature. Membranes were then spun down in a table-top centrifuge, and the supernate was discarded. Approximately 4 μl of the pellet was introduced into the central hole of a copper disc sandwich, which was then carefully dried with filter paper and frozen in liquid Freon 22 cooled by liquid nitrogen. The discs were then stored in liquid nitrogen. Freeze-fracturing was carried out in a Balzers freeze-etch unit BAF 301 (Balzers Corp. Hudson, N. H.). Samples were placed in a double-replica specimen holder and cooled down to −150°C. Fracturing was done at −100°C and 2 × 10⁻⁶ torr. Replicas were coated with platinum-carbon at an angle of 43° and, after overnight processing with bleach, were washed and picked up on 200-mesh Parlodion-coated grids (Ernest F. Fullam, Inc., Schenectady, N. Y.) and examined in a JEOL 100 CX electron microscope. Micrographs were taken at × 16,000 of all isolated membranes seen, and enlarged to a final magnification of 45,000. Prints were then arranged randomly, and the number of aggregates was determined by three different observers who did not know whether a given print was from a control or vasopressin-treated bladder. Membrane and aggregate area was determined with a Kontron AM 03 Manual Optical Image Analysing System (Kontron Messgeräte, Munich, W. Germany). Isolated membranes were prepared for scanning electron microscopy by dehydration in alcohol, critical point-drying, and gold palladium coating, and examined in a JEOL JSM-35 scanning electron microscope.

RESULTS

Isolated Membranes: Morphology and Aggregate Frequency

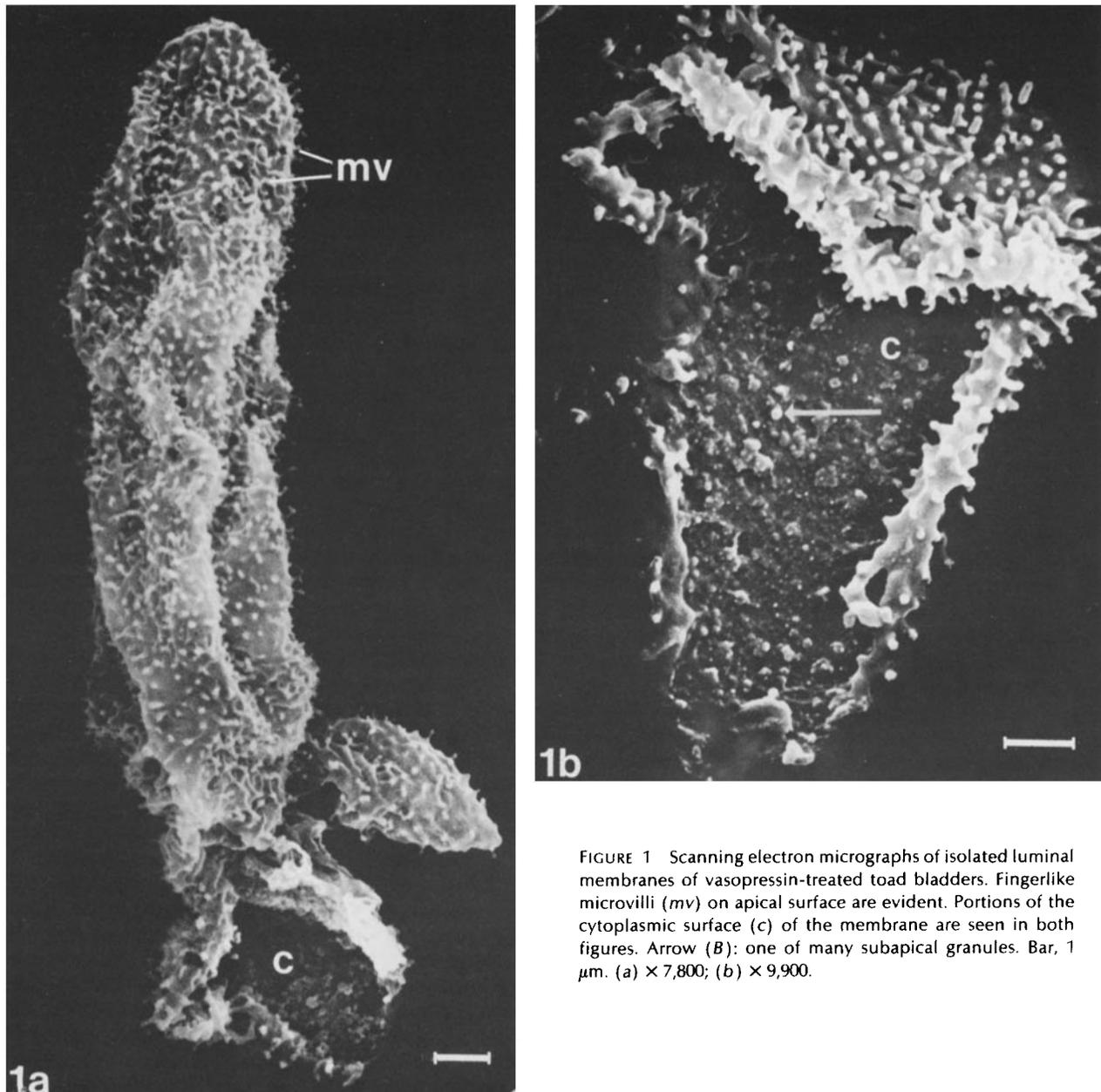Fig. 1 shows two scanning electron micrographs of typical

FIGURE 1 Scanning electron micrographs of isolated luminal membranes of vasopressin-treated toad bladders. Fingerlike microvilli (mv) on apical surface are evident. Portions of the cytoplasmic surface (c) of the membrane are seen in both figures. Arrow (B): one of many subapical granules. Bar, 1 μm. (a) × 7,800; (b) × 9,900.

isolated apical cell membranes, which appear as fusiform cylinders with their edges curled up. Microvilli can be seen on their outer surfaces and serve as markers for the apical cell membrane. In both 1 *A* and *B* of Fig. 1, portions of the

cytoplasmic as well as the external surface are visible; the round sub-apical granules which are characteristic of these cells can be seen particularly well in Fig. 1 *B*.

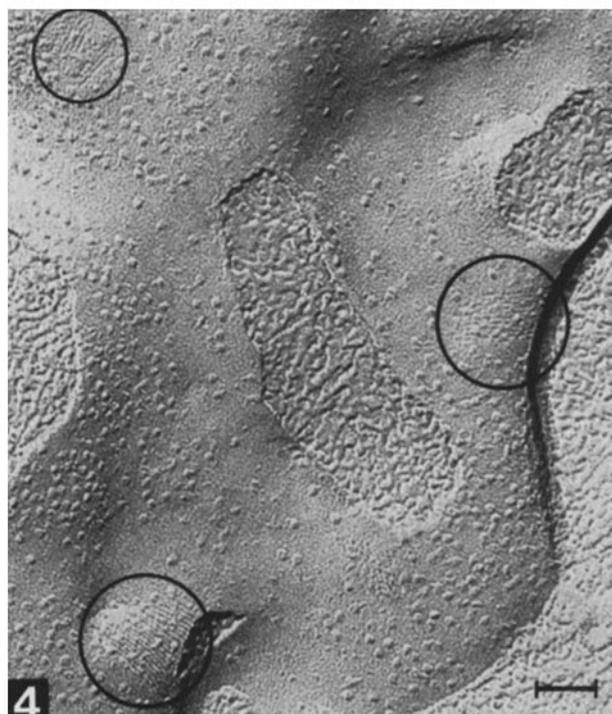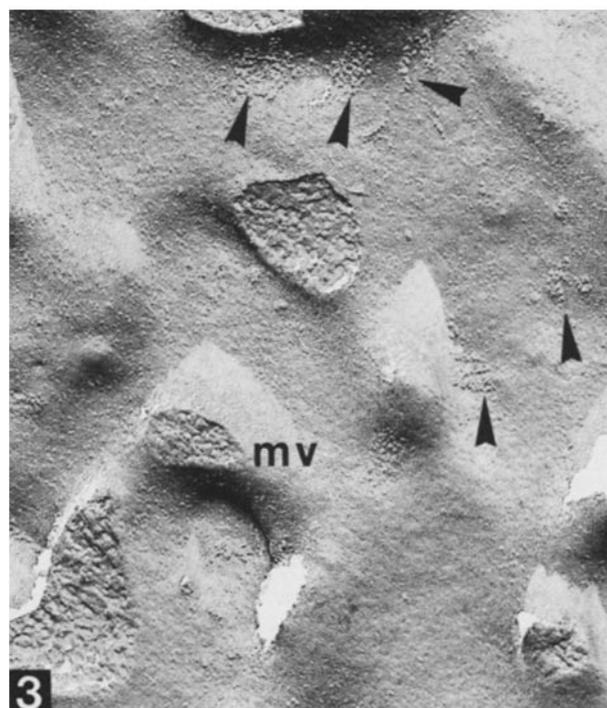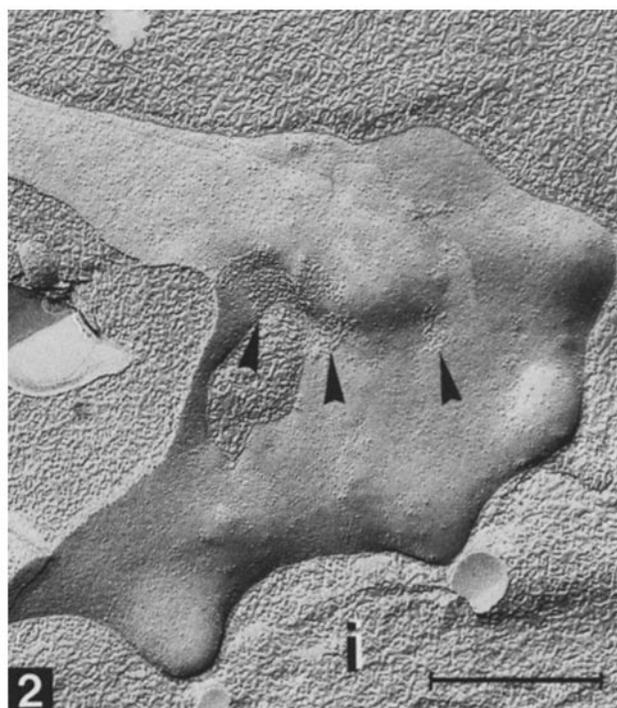Figs. 2–5 are freeze-fracture micrographs of isolated apical

FIGURE 2   Freeze-fracture replica of the P face of an isolated luminal membrane. The intact bladder was stimulated by vasopressin, exposed to DTBP in the luminal medium, homogenized, and then fixed with glutaraldehyde. Several intramembrane particle aggregates are seen (arrowheads). *i*, ice. Bar, 0.5 μm. × 45,000.

FIGURE 3   Isolated luminal membrane; same conditions as Fig. 2. *mv*, microvillus. × 45,000.

FIGURE 4   E face of an isolated luminal membrane; same conditions as preceding two figures. Three typical striae are encircled. Bar, 0.1 μm. × 80,000.

FIGURE 5   Isolated luminal membrane (P face), unfixed, which was stimulated by vasopressin, exposed to DTBP, homogenized, and purified by sucrose density gradient centrifugation. Typical particle aggregates are seen (arrowheads). × 45,000.

membranes from bladders stimulated by vasopressin. Figs. 2 and 3 show membranes stabilized by DTBP, homogenized and fixed with glutaraldehyde (group I). Intramembrane particle aggregates with their longest diameters in the 0.1–0.25 μm range are seen distributed over the P face of the membranes. Linear particle arrays are present in the aggregates. Fig. 4, also from group I, shows typical grooves in the E face of an isolated membrane, which are the complementary images of the aggregate particles in the P face (2, 3). Fig. 5 shows the P face of an apical membrane stimulated by vasopressin and stabilized by DTBP but not fixed by glutaraldehyde (group II). These membranes were further purified by sucrose density-gradient centrifugation. Again, aggregates are present.

An apical membrane from a glutaraldehyde-fixed control bladder (group I) is seen in Fig. 6. In the absence of vasopressin, aggregates are rare, and no aggregates are visible in these membranes. There are occasional small clusters of intramembrane particles which do not resemble aggregates in size or configuration (Fig. 6). Control bladders from group II were similarly devoid of aggregates.

Table I summarizes aggregate frequency in membranes from control and vasopressin-stimulated bladders. In group I (DTBP plus glutaraldehyde), mean aggregate frequency and area in the vasopressin-stimulated membranes were significantly greater than in the controls. The percentage of total membrane area occupied by the aggregates was 0.49%, a value significantly different from that of the controls and approximately half that reported in vasopressin-treated intact bladders (3). Group II membranes from stimulated cells also differed significantly from controls with respect to aggregate frequency, area, and percentage area occupied by aggregates.

DISCUSSION

These studies illustrate that once aggregates are in position in the luminal membrane, they retain their location and configuration even after the membrane is removed from the cell. This is the case for the two experimental conditions described here. Neither DTBP nor glutaraldehyde itself induces aggregate formation, since the control bladders receiving these agents, but no vasopressin, showed virtually no aggregate formation.

Our studies support the view that the particles are intrinsic membrane proteins and not more superficially related to the apical membrane. The evidence for the intrinsic nature of the proteins is far from complete, since there is not yet any proof that the proteins protrude beyond either membrane surface, or that they possess specific external groups which can be recognized (see reference 10). Indeed, it is still an assumption that these elements are proteins at all (11). Nevertheless, it is reasonable to conclude that they remain in the membrane even after homogenization and withdrawal of vasopressin because of their intrinsic nature. It is also possible that the usual cellular mechanisms responsible for the rapid disappearance of aggregates upon withdrawal of vasopressin (7, 12) may not come into play once the luminal membranes are removed from the cell; additional experiments are necessary, however, to establish whether cytoplasmic processes control aggregate disappearance.

The forces that stabilize the aggregates are not known. It is not clear, for example, whether the membrane lipid in which they are located is typical of the luminal membrane as a whole or consists of more specialized patches of membrane derived from the tubular structures from which the aggregates originate.
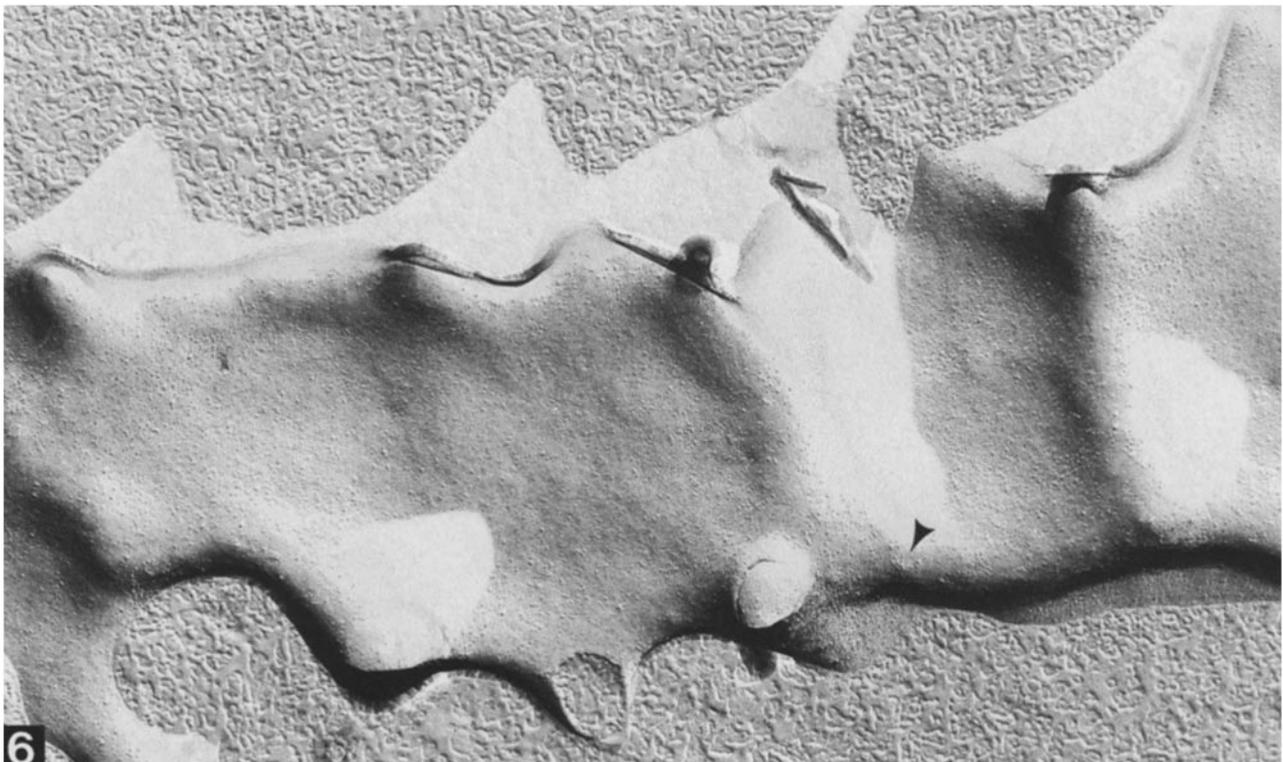
FIGURE 6   Isolated luminal membrane from a control bladder not stimulated by vasopressin. Preparation exposed to DTBP and fixed with glutaraldehyde, as in Figs. 2–4. Scattered intramembrane particles are seen, occasionally in small clusters (arrowhead), but there are no true aggregates. × 45,000.

TABLE I

Aggregate Frequency and Area in Isolated Luminal Membranes

Preparation	Total membranes examined	Total area examined	Mean aggregate frequency	Mean aggregate area	(Mean aggregate area/ Total membrane area) × 100
		μm^2	per 235 μm^2	$\mu m^2/235\ \mu m^5$	%
DTBP + glutaraldehyde					
Vasopressin (3)*	20	189	156 ± 55 (SEM)‡	1.17 ± 0.13‡	0.49 ± 0.05‡
Control (3)	14	190	1.6 ± 1.6	0.04 ± 0.04	0.02 ± 0.02
DTBP alone					
Vasopressin (10)	25	317	35 ± 9‡	0.35 ± 0.11‡	0.15 ± 0.045‡
Control (5)	28	319	2 ± 1	0.01 ± 0.01	0.002 ± 0.002

* Numbers in parentheses indicate the number of experimental runs. Each run contained pooled membranes from four hemibladders.
‡ Significantly different from control by analysis of unpaired values. SEM, standard error of the mean.

Recent studies by Orci et al. (13) of the binding of filipin, a sterol-specific antibiotic, to the luminal membrane of the toad bladder have shown that, while fillipin diffusely labeled the luminal membrane, it did not label areas occupied by the aggregates. While steric factors may have interfered with filipin binding (13), the results suggest that aggregate proteins are situated in patches of membrane that are relatively free of cholesterol. It is possible that these patches aid in maintaining the aggregate structure. In addition, cohesive forces between aggregate particles, or between particles and actinlike protein situated in the membrane, may be important. It would appear that, whatever the cohesive forces, they are closely associated with the isolated membrane.

The fact that glutaraldehyde is not necessary to maintain the particles in the membrane is encouraging for future work in extracting and isolating the water-conducting elements. Glutaraldehyde irreversibly cross-links membrane proteins, making gel chromatography impossible. The use of DTBP, a reversible cross-linking agent, will permit us to break the central S-S bond of the imidoester before extraction of the membrane proteins and their introduction into gel chromatographic systems (14).

REFERENCES

1. Harmanci, M. C., W. A. Kachadorian, H. Valtin, and V. A. DiScala. 1978. Antidiuretic hormone-induced intramembranous alterations in mammalian collecting ducts. *Am. J. Physiol.* 235:F440–F443.
2. Chevalier, J., J. Bourguet, and J. S. Hugon. 1974. Membrane-associated particles. Distribution in frog urinary bladder epithelium at rest and after oxytocin treatment. *Cell Tissue Res.* 152:129–140.
3. Wade, J. B. 1980. Hormonal modulation of epithelial structures. *Curr. Top. Membr. Transp.* 13:123–127.
4. Muller, J., W. A. Kachadorian, and V. A. DiScala. 1980. Evidence that ADH-stimulated intramembrane particle aggregates are transferred from cytoplasmic to luminal membranes in toad bladder epithelial cells. *J. Cell Biol.* 85:83–95.
5. Kachadorian, W. A., S. D. Levine, J. B. Wade, V. A. DiScala, and R. M. Hays. 1977. Relationship of aggregated intramembranous particles to water permeability in vasopressin-treated toad urinary bladder. *J. Clin. Invest.* 59:576–581.
6. Humbert, F., R. Montesano, A. Grosso, R. C. De Sousa, and L. Orci. 1977. Particle aggregates in plasma and intracellular membranes of toad bladder (granular cell). *Experientia (Basel).* 33:1364–1367.
7. Rapoport, J., W. A. Kachadorian, J. Muller, N. Franki, and R. M. Hays. 1981. Stabilization of vasopressin-induced membrane events by bifunctional imidoesters. *J. Cell Biol.* 89:261–266.
8. Kachadorian, W. A., J. Muller, S. Rudich, and V. A. DiScala. 1981. Relation of ADH effects to altered membrane fluidity in toad urinary bladder. *Am. J. Physiol.* 240:F63–F69.
9. Hays, R., and P. Barland. 1966. The isolation of the membrane of the toad bladder epithelial cell. *J. Cell Biol.* 31:209–213.
10. Etemadi, A. H. 1980. Membrane asymmetry. A survey and critical appraisal of the methodology. I. Methods for assessing the asymmetric orientation and distribution of proteins. *Biochim. Biophys. Acta.* 604:347–422.
11. Verkleij, A. J., C. Mombers, J. Leunissen-Bijueit, and P. H. J. Th. Ververgaert. 1979. Lipidic intramembranous particles. *Nature (Lond.).* 279:162–163.
12. Kachadorian, W. A., C. Casey, and V. A. DiScala. 1978. Time-course of ADH-induced intramembranous particle aggregation in toad urinary bladder. *Am. J. Physiol.* 234:F461–F465.
13. Orci, L., R. Montesano, and D. Brown. 1980. Heterogeneity of toad bladder granular cell luminal membranes. Distribution of filipin-sterol complexes in freeze-fracture. *Biochim. Biophys. Acta.* 601:443–452.
14. Peters, K., and F. M. Richards. 1977. Chemical cross-linking: reagents and problems in studies of membrane structure. *Annu. Rev. Biochem.* 46:523–551.