J. Membrane Biol. 104, 241–251 (1988)

The Journal of
Membrane Biology

Very Low Osmotic Water Permeability and Membrane Fluidity in Isolated Toad Bladder Granules

A.S. Verkman and Sandra K. Masur
Cardiovascular Research Institute, University of California, San Francisco, California 94143 and Department of Physiology and Biophysics, Mount Sinai School of Medicine, New York, New York 10029

Summary. Osmotic water permeability of the apical membrane of toad urinary epithelium is increased greatly by vasopressin (VP) and is associated with exocytic addition of granules and aggrephores at the apical surface. To determine the physiological role of granule exocytosis, we measured the osmotic water permeability and membrane fluidity of isolated granules, surface membranes and microsomes prepared from toad bladder in the presence and absence of VP. P_f was measured by stopped-flow light scattering and membrane fluidity was examined by diphenylhexatriene (DPH) fluorescence anisotropy. In response to a 75 mM inward sucrose gradient, granule size decreased with a single exponential time constant of 2.3 ± 0.1 sec (SEM, seven preparations, 23°C), corresponding to a P_f of 5×10^{-4} cm/sec; the activation energy (E_a) for P_f was 17.6 ± 0.8 kcal/mole. Under the same conditions, the volume of surface membrane vesicles decreased biexponentially with time constants of 0.13 and 1.9 sec; the fast component comprised ~ 70% of the signal. Granule, surface membrane and microsome time constants were unaffected by VP. However, in surface membranes, there was a small decrease (6 ± 2%) in the fraction of surface membranes with fast time constant. DPH anisotropies were 0.253 (granules), 0.224 (surface membranes) and 0.190 (microsomes), and were unaffected by VP. We conclude: (1) granules have among the lowest water permeabilities of biological membranes, (2) granule water permeability is not altered by bladder pretreatment with VP, (3) granule membrane fluidity is remarkably lower than that of surface and microsomal membranes, and (4) rapid water transport occurs in surface membrane vesicles. The unique physical properties of the granule suggests that apical exocytic addition of granule membrane may be responsible for the low water permeability of the unstimulated apical membrane.

Key Words toad bladder · osmotic water transport · light scattering · fluorescence · membrane fluidity

Introduction

Epithelial polarity is characterized by separation of the plasma membrane into apical and basolateral membrane domains by tight junctions. The apical membrane of the bladder epithelium of the well-hydrated toad has low osmotic water permeability, while the basolateral membrane is highly water permeable. Vasopressin binding to basolateral receptors causes activation of adenylate cyclase, cAMP production, protein kinase activation, and ultimately, by a series of poorly understood events, a marked increase in apical membrane water permeability. Granule-rich cells make up 90% of the mucosal surface of the bladder and in their apical membranes reside the hormonally regulated water transport properties (DiBona, Civan & Leaf, 1969). Upon vasopressin stimulation, two types of membranous organelles fuse with the apical surface: aggrephores and granules (Masur, Holtzman & Walter, 1972; Muller, Kachadorian & DiScala, 1980). Granule exocytosis is also stimulated by stretch, mucosal phorbol ester and lectin application (Brown, Montesano & Orci, 1981; Masur, Sapirstein & Rivero, 1985; Masur, Greenberg & Howell, 1987).

The physiological role of the toad bladder granule is not known. It has been suggested that granule exocytosis is important for the hydroosmotic response to vasopressin, for insertion of 'average' apical membrane or for addition of glycocalyx to the apical membrane. A procedure for isolation of purified granules from toad bladder cells has been developed recently using cell homogenization and Percoll® gradient centrifugation methods (Masur et al., 1986). We report here the measurement of water permeability and membrane fluidity of the isolated granules and other bladder cell membrane fractions. We find that granule membranes have a very low osmotic water permeability, lower than most other biological and artificial membranes. Granule water permeability is unaffected by pretreatment of toad bladders with vasopressin. This is consistent with the granule playing a role as precursor to the impermeable apical membrane. In addition, compared with other membranes, the granule has a very low

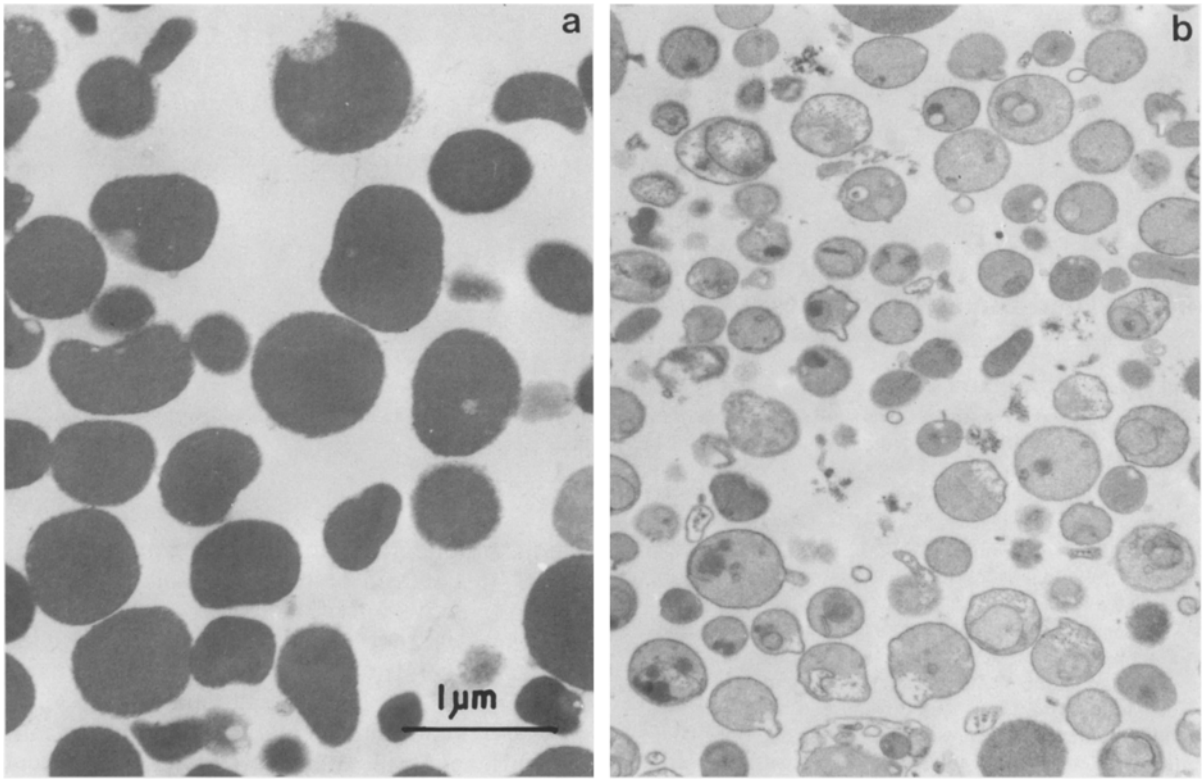

Fig. 1. Electron micrographs of isolated toad bladder granules. (*a*) Granules were fixed using 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, postfixed with 2% reduced OsO_4, dehydrated, and embedded in Epon (Masur et al., 1986). Silvergold sections were examined in a JEOL 100 B electron microscope. Stained with uranyl acetate and lead citrate. (*b*) Electron micrograph of isolated granules suspended in hypotonic media and needle homogenized (*see* Materials and Methods). The samples were fixed by addition of glutaraldehyde (2%) to the hypotonic medium. Note the decrease in granule diameter and electron density as compared to Fig. 1*a*, which is in the same magnification

membrane fluidity, a characteristic which may maintain segregated functional domains in the apical membrane.

Materials and Methods

Dominican toads (*Bufo marinus*) were purchased from National Reagents (Bridgeport, CN) and were maintained on damp peat moss. 1,6-diphenyl-1,3,5-hexatriene (DPH) was obtained from Molecular Probes (Eugene, OR). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Solution osmolarities were measured on a 3W2 Advanced Instruments Osmometer (Needham Heights, MA).

Membrane Preparation

Vesicle membranes were prepared from toad bladder as described previously (Masur et al., 1986). Toads (15–20) were killed by double pithing and bladders were suspended for 15 min in amphibian HEPES-buffered saline solution (110 mM NaCl, 18 mM D-glucose, 10 mM HEPES, 1 mM Na_2HPO_4, pH 7.5, 23°C) in the presence or absence of 50 mU/ml pitressin (Park Davis, vasopressin). Epithelial cells were scraped from the bladder and washed twice in 10 mM NaCl, 132 mM sucrose, 10 mM HEPES, 2 mM EGTA, 1 mg/ml bovine serum albumin, pH 7.5, ~240 mOsm (buffer A) at 4°C. Cells were homogenized, centrifuged differentially and fractions were separated using a series of four Percoll density gradients (Masur et al., 1986). Phenylmethylsulfonyl fluoride (PMSF) was added at each step at a final concentration of 0.2 mM. Membranes were maintained at 4°C until the time water transport and fluidity measurements were performed (<24 hr).

All fractions were examined biochemically and by electron microscopy. Enzyme enrichments were similar to those reported previously (Masur et al., 1986). Morphologically, the initial granule fraction recovered from Percoll was relatively homogeneous (Fig. 1*a*) with mean granule diameter of 0.9 μ. For measurement of osmotic water permeability, granules were placed in hypotonic K-HEPES buffer (65 mM sucrose, 5 mM HEPES, pH 7.4, ~70 mOsm, buffer B) and homogenized by passage through 23 and 26 gauge needles. The resultant granules in Fig. 1*b* are smaller (mean diameter 0.44 μ) and their contents are less electron dense. The surface membrane fraction, made up of apical and basolateral membrane vesicles, consisted of a heterogeneous mixture of electron dense cylindrical bodies (0.1 × 1 μ), smaller membrane vesicles (0.35 μ diameter), and a few larger spherical vacuoles enclosing smaller membranous organelles, including granules. The microsomal fraction consisted predominantly of small spherical vesicles with diameters 0.13–0.36 μ with a few large vacuoles of 0.6 μ diameter. There was no significant altera-

tion in the ultrastructure of surface membranes and microsomes after resuspension in hypotonic K-HEPES as described above. Biochemically, the microsomal fraction consists of surface-like membranes with >fourfold alkaline phosphatase enrichments over crude homogenate.

Gel Electrophoresis

Granule membranes and contents were separated by suspending granules in distilled water with 1 mM dithiothreitol (DTT) after 12 hr in 500 mM NaCl, 10 mM HEPES, 1 mM EDTA, 0°C. Membranes were pelleted at 12,000 × *g* for 3 min. Triton X-114 phase fractionation was performed to identify the hydrophobic membrane proteins, presumably the intrinsic membrane proteins (Bordier, 1981). The granule membrane pellet was resuspended in 1% prewashed Triton X-114 in 110 mM NaCl, 10 mM Tris, 1 mM DTT, pH 7.5 and incubated at 4°C for 30 min. The mixture was then incubated at 37°C for 5 min and the resultant cloudy mixture centrifuged for 3 min at 12,000 × *g*. The heavy, detergent-rich phase was separated from the lighter, detergent-depleted phase. A second temperature-mediated phase partitioning was performed on each phase as described by Bricker and Sherman (1984). Polyacrylamide gel electrophoresis was performed according to the procedure of Maizel (1971) on 7.5% acrylamide mini-gels (Hoefer Scientific) as described in Masur et al. (1986). One μg protein samples of granules, granule contents, granule membranes, Triton extracted proteins and aqueous phase proteins were compared in gels stained with silver (Merril, Goldman & VanKeuren, 1984).

Osmotic Water Transport Measurement

Osmotic water permeability was determined by the stopped-flow light-scattering technique (Verkman, Dix & Seifter, 1985; Meyer & Verkman, 1987). Seventy-five μl of vesicles (40–80 μg protein/ml) in 65 mM sucrose, 5 mM HEPES/Tris, pH 7.4 (buffer B) were mixed with an equal volume of hyperosmotic buffer in a Hi-Tech stopped-flow apparatus (Wiltshire, England) to give specified inwardly directed sucrose gradients. Solutions were degassed prior to experiments to prevent cavitation. The instrument dead time was <2 msec and the electronic response time was <1 msec. Temperature was controlled by a circulating water bath and monitored by an indwelling thermistor. The sample was excited with monochromatic light at 550 nm (4 nm bandpass) from a 100 watt tungsten-halogen bulb powered by a deep cycle 12V marine battery. The time course of 90° scattered light intensity was measured and recorded by a MINC/23 computer (Digital Equipment Corp., Maynard, MA) for analysis. 512 data points were acquired in each experiment with a maximum rate of data acquisition of 25 kHz.

The time course of scattered light intensity was fitted to single (granules) or biexponential (surface membranes and microsomes) functions. For granules, the osmotic water permeability coefficient (P_f in cm/sec) was calculated from the exponential time constant and the average vesicle surface-to-volume ratio (1.4×10^5 cm^{-1}, assuming a 0.44 μ diameter) by comparing exponentials fitted to experimental and calculated time courses (Meyer & Verkman, 1986; Illsley & Verkman, 1986). For surface membranes and microsomes, absolute P_f for the fast component was estimated using a surface-to-volume ratio of 2.5×10^5 cm^{-1} (*see* Discussion).

Fluidity Measurements

Measurements of DPH fluorescence in vesicles were carried out using an SLM 48000 multifrequency phase-modulation fluorimeter (SLM Instrument, Urbana, IL) as described previously (Illsley, Lin & Verkman, 1987, 1988). Vesicles were diluted in N_2 gassed buffer B to concentrations of ~100 μM phospholipid. DPH (0.3 μM) was added to vesicles while vortexing from a 2 mM stock solution in acetone. The suspension was incubated for 1 hr under N_2 at 23°C in the dark prior to fluidity measurements.

Steady-state DPH anisotropy was measured in the T-format using 350 nm excitation (4 nm bandpass) and >408 nm emission (KV408 cut-on filters, Schott Glass Co., Duryea, PA). Less than 1% of detected light was due to vesicle and background scattering. Depolarization caused by emission scattering was absent as shown by the independence of anisotropy on a fivefold dilution of the vesicle/DPH suspension.

DPH lifetime heterogeneity experiments were carried out using 12 modulation frequencies (1–150 MHz) with He-Cd laser excitation (7.5 mW at 325 nm, Liconix Corp., Sunnyvale, CA). A dilute solute of 1,4-bis(4-methyl-5-phenyloxazol-2-yl) benzene (POPOP) in ethanol (lifetime 1.3 nsec) was used as reference solution. The sample was excited with vertically polarized light; emitted light was detected through sequentially placed KV408 and GG420 filters and a polarizer oriented at 54.7 degrees from the vertical. Ground state heterogeneity analysis was performed using software provided by SLM Instruments Inc. Differential depolarization measurements were carried out in the T-format using 15 modulation frequencies. Differential phase and modulation data were fitted to the anisotropy decay predicted for an isotropic hindered rotator using a maximum DPH anisotropy of 0.39 in the absence of depolarizing rotations.

Results

The granule fraction consists of membrane-bound electron dense granules with few (<5%) contaminating lysosomes and mitochondria (Masur et al., 1986). Electron microscopy confirms the homogeneity of the granule fraction and the successful removal of Percoll by centrifugation twice in >100 volumes of isosmotic sucrose (100,000 × *g*, 90 min) Fig. 1*a*). The mean diameter of the isolated granules used for osmotic water transport measurements was 0.44 μ after resuspension in hypotonic buffer and needle homogenization (*see* Materials and Methods) (Fig. 1*b*).

For comparison of granule proteins with those reported for other toad bladder membrane fractions, SDS-PAGE of granule proteins was performed (Fig. 2). Granule membranes and contents were separated by hypotonic shock prior to electrophoresis. The broad band at 68–72 kilodaltons is a granule content marker consistent with the observations of Wade, McCusker and Coleman (1986). The majority of granule peptides appear to be tightly associated with the membrane under these conditions. Further separation into intrinsic and peripheral membrane proteins was performed. The gel

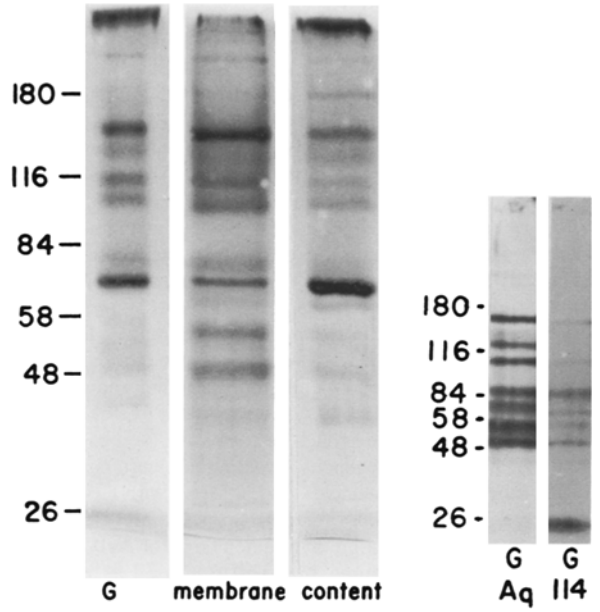

Fig. 2. *Left:* SDS-PAGE of isolated intact granules (*G*) and granule membrane and content separated by osmotic shock. M_r standards are indicated at the left in kDa. *Right:* SDS-PAGE of granule membrane proteins separated on basis of hydrophobicity using temperature-induced phase separation of proteins in Triton X-114 (*see* Materials and Methods). Two classes of membrane proteins are identified as components of the osmotically shocked granule membrane: "*G* 114" are the lipophilic proteins that partition into the detergent phase. "*G aq*" are the hydrophilic proteins found in the aqueous phase after multiple temperature-dependent extractions

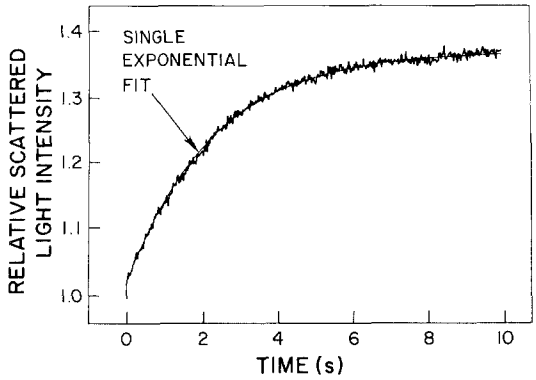

Fig. 3. Osmotic water transport in isolated toad bladder granules. Granules (40 μg protein/ml, 65 mOsm buffer) were subjected to a 75 mOsm inward sucrose gradient at 23°C as described in Materials and Methods. Light scattering was measured at 550 nm. Data were fitted to a single exponential function with time constant 2.3 sec, corresponding to a P_f of 5×10^{-4} cm/sec

shows a 14–160 kDa range of intrinsic proteins, which includes proteins of 17 and 55 kDa, corresponding to two out of the three most prominent proteins reported by Harris et al. (1987) in density shifted endosomes from toad bladder.

Osmotic Water Permeability in Granules

Vesicle osmotic water transport was measured from the time course of vesicle volume decrease after imposing rapidly (<1 msec) an inward sucrose gradient in a stopped-flow apparatus. The transient in vesicle volume was followed optically from the time course of scattered light intensity at 550 nm. These conditions were determined empirically by measurements of the relative sensitivity of scattered and transmitted light intensity at a series of wavelengths (350–650 nm) to changes in granule volume. Similar methodology has been used to measure quantitatively osmotic water transport in red cells (Terwilliger & Solomon, 1981), platelets (Meyer & Verkman, 1986), and membrane vesicles from kidney (Verkman et al., 1985; Meyer & Verkman, 1987), intestine (Worman & Field, 1985), trachea (Worman et al., 1986) and placenta (Illsley & Verkman, 1986).

Figure 3 shows the time course of scattered light intensity following exposure of granules to a 75 mM inwardly directed sucrose gradient at 23°C. There is a single exponential time course of increasing scattered light intensity in granules corresponding to osmotic water efflux and vesicle shrinkage. Following the exponential increase, the signal remains constant for >90 sec, indicating that granules are impermeant to sucrose (*not shown*). In four separate preparations of granules, measured time constants were 2.3 ± 0.2 sec (SD, $n = 4$), 2.5 ± 0.2, 2.3 ± 0.1 and 2.1 ± 0.2 sec (23°C), indicating a good degree of biological reproducibility. In one preparation tested, the time constant for a 75 mM inward osmotic gradient was not dependent on the neutral impermeant used to induce water flow (sucrose 2.3 ± 0.2 sec [SD, $n = 4$], raffinose 2.3 ± 0.1 sec and mannitol 2.1 ± 0.2 sec).

To determine granule P_f quantitatively from the light-scattering data, the relationship between scattered light intensity and vesicle volume was established. Figure 4 (top) shows the dependence of relative scattered light intensity, determined from the amplitude of single exponentials fitted to the light-scattering time course, on vesicle volume, assumed to equal the ratio of intravesicular to solution osmolarities. This 'perfect osmometer' assumption has been confirmed by ^{3}H-glucose space measurements in a wide variety of cells and vesicles. As found for other vesicles systems, the data show a negative linear relationship between scattered light intensity at 550 nm and granule volume. This relationship

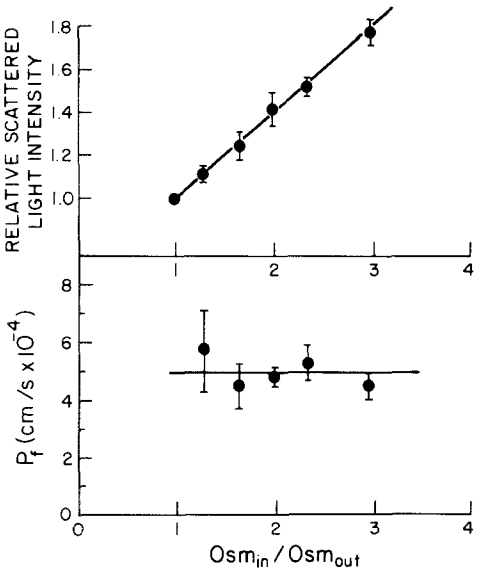

Fig. 4. Dependence of relative scattered light intensity and apparent P_f on osmotic gradient size. *Top:* Abscissa is the ratio of intravesicular/external osmolarities immediately after mixing before osmotic water flow has occurred. Ordinate is the amplitude of a single exponential fitted to the time course of scattered light intensity. A linear regression is shown. *Bottom:* Apparent P_f was calculated for each osmotic gradient as given in Materials and Methods. All data are mean ± SD for quadruplicate determinations

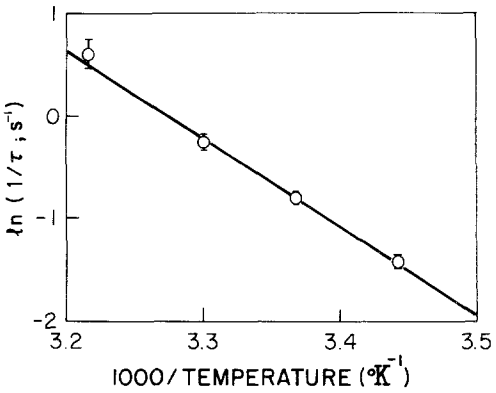

Fig. 5. Temperature dependence of granule osmotic water permeability. Experiments were performed as in Fig. 2 at varying temperatures. Data are given as an Arrhenius plot with fitted activation energy of 17.6 ± 0.8 kcal/mole. Data points are mean ± SD of quadruplicate P_f measurements

was used to calculate P_f from the scattering *vs.* time data (Illsley & Verkman, 1986; Meyer & Verkman, 1986).

At 23°C, P_f was calculated to be 5×10^{-4} cm/sec for a 75 mOsm inward gradient using a granule surface-to-volume ratio of 1.4×10^5 cm^{-1} determined from electron micrographs. To investigate whether this value might be lower than the true membrane P_f because of nonmembrane restrictions to osmotic water flow (e.g. vesicular contents or cytoskeletal constraints), the dependence of P_f on osmotic gradient size was measured (Fig. 4, bottom). A decrease in apparent P_f with increasing osmotic gradient size might occur if significant nonmembrane barriers to osmosis exist (Illsley & Verkman, 1986). The data show no significant effect of osmotic gradient size on P_f.

The very low granule P_f suggests absence of water channels and presence of a specialized water-impermeable membrane (*see* Discussion). Based on osmotic water transport measurements in a wide variety of biological and artificial membranes, it is found that the activation energy (E_a) for P_f is high for lipid-mediated transport (>10 kcal/mole) and low for water passage through pores or channels (2–6 kcal/mole) (Finkelstein, 1987). As shown in Fig. 5, there is a marked effect of temperature on the rate of granule osmotic water transport. The data fit well to a single activation energy of 17.6 ± 0.8 kcal/mole (15–45°C), supporting the absence of facilitated water transport through channels in the toad bladder granules.

Table 1. Osmotic water permeability in toad bladder membranes[a]

	TAU_{fast}	TAU_{slow}	A_{fast}/A_{tot}
Granules			
− vasopressin	—	2.4 ± 0.1	0
+ vasopressin	—	2.3 ± 0.2	0
Surface membranes			
− vasopressin	0.14 ± 0.01	1.8 ± 0.3	0.70 ± 0.02
+ vasopressin	0.14 ± 0.02	2.0 ± 0.1	0.65 ± 0.02
Microsomes			
− vasopressin	0.16 ± 0.01	1.3 ± 0.1	0.75 ± 0.02
+ vasopressin	0.15 ± 0.01	1.4 ± 0.1	0.74 ± 0.01

[a] Experiments were carried out as in Fig. 3. Time constants are given in seconds. A_{fast}/A_{tot} represents the fractional pre-exponential amplitude of the fast exponential process. Data are expressed as mean ± SE for quadruplicate measurements performed on four independent membrane preparations.

Osmotic Water Transport in Surface Membranes and Microsomes

In response to a 75 mM inward sucrose gradient, the time course of scattered light intensity in surface membranes and microsomes fitted closely to a biexponential function (Fig. 6); a single exponential fit was inadequate. Fitted exponential time constants and pre-exponential factors are summarized in Table 1 for four separate membrane preparations. For

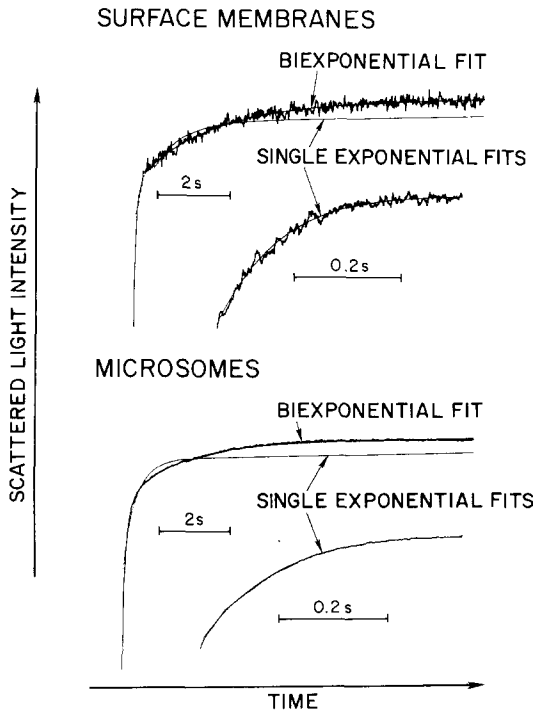

Fig. 6. Osmotic water transport in surface membrane and microsomes. Experiments were carried out as in Fig. 3. Fitted single and double exponentials are shown along with an expanded view of early time points. Because of the high signal-to-noise ratio for microsome data, the experimental and fitted curves are difficult to distinguish. Fitted parameters for the double exponential fits are summarized in Table 1

both membrane types the majority of the signal amplitudes was the faster exponential process with average time constant 0.15 sec, 15-fold faster than the time constant measured for granule osmotic water transport measured under identical conditions. Assuming an average surface-to-volume ratio for these membranes of 2.5×10^5 cm^{-1}, a 0.15 time constant corresponds to a P_f of 6×10^{-3} cm/sec at 23°C.

To examine further the mechanisms of water transport in the surface membrane fraction, the activation energies for the fast and slow processes were determined. In the temperatures range 16 to 38°C, single activation energies of 9.1 ± 0.6 and 19 ± 1 kcal/mole were measured for the fast and slow components, respectively ($r > 0.98$ for linear fits to Arrhenius plots, *data not shown*). The very different activation energies for the fast and slow processes indicate that the different rates of water transport represent differences in membrane composition, and not in vesicle geometry.

Vasopressin Effects

Vasopressin has been shown to increase exocytosis of aggrephores and granules to the apical surface of toad bladder cells (Masur et al., 1972; Muller et al., 1980) as well as increase endocytic internalization of apical membrane (Masur et al., 1971; Gronowicz, Masur & Holtzman, 1980). The net effects of vasopressin action are an increase in apical membrane surface area and expression of active water channels possibly by insertion of channels into specialized regions or microdomains of the apical membrane surface. It is not known whether there is, in addition, chemical modification of the water channels (e.g. phosphorylation or dephosphorylation) causing channel activation or inactivation. To examine whether vasopressin results in a change in granule water permeability or in the relative fraction of fast and slow components of water transport in surface membranes and microsomes, osmotic water transport was measured in vesicles isolated from toad bladder which had been stimulated by saturating vasopressin concentrations.

Table 1 shows no effect of vasopressin stimulation on the exponential time constants for osmotic water transport in each membrane type. There was no effect of vasopressin on the relative signal due to the fast component in microsomes; however, there was a small but significant decrease in the fast component measured in surface membranes with vasopressin (*see* Discussion).

Table 2. Steady-state DPH anisotropy in toad vesicles[a]

	− vasopressin	+ vasopressin
Granules	0.253 ± 0.004	0.255 ± 0.007
Surface membranes	0.224 ± 0.006	0.218 ± 0.012
Microsomes	0.192 ± 0.003	0.195 ± 0.005

[a] Steady-state DPH anisotropy was measured at 23°C as described in Materials and Methods. Data are expressed as mean ± SE for measurements performed on three independent membrane preparations. Errors for individual measurements are under 0.004 (SD).

Membrane Fluidity Measurements

To correlate water permeability with the physical state of the vesicle membranes, membrane fluidity was assessed by steady-state DPH anisotropy. Table 2 summarizes the steady-state anisotropies obtained in four separate sets of membrane preparations. The granules show a remarkably higher DPH anisotropy than surface membranes or microsomes, indicating a lower degree of membrane fluidity (*see* Discussion). There was no significant effect of vasopressin stimulation on DPH anisotropy in each of the membranes.

To examine further the physical state of the

granule membrane, measurements of DPH lifetime and rotational heterogeneity were performed. Because steady-state DPH anisotropy measurements give composite information about DPH lifetime, rotational rate and rotational freedom, differences in anisotropy among membrane types cannot be interpreted rigorously in terms of differences in membrane fluidity (Verkman & Ives, 1986; Illsley et al., 1987). Time-resolved fluorescence measurements using differential polarization allow definition of the anisotropy decay law, providing independent information about DPH lifetimes and rotational characteristics.

The results of the lifetime and differential polarization analyses are given in Table 3. For granules and surface membranes, the data fitted well to a single DPH lifetime in the usual range 8–11 nsec plus scattering components with shorter lifetimes. A chi squared value of <1.2 indicates excellent correspondence between theory and experiment. For microsomes, the best-fit model was that of triple lifetime decay with DPH lifetimes of 8.5 and 15.4 nsec, plus a scattering component. Differential phase and modulation data were fitted to the anisotropy decay law for a single isotropic hindered rotator (Lakowicz & Prendergast, 1978); because of imperfect membrane homogeneity, the use of more detailed anisotropy decay laws containing ground state rotational heterogeneity (Illsley & Verkman, 1988) was unwarranted. Therefore bearing in mind the approximate nature of the anisotropy decay model, the differential polarization data showed that the limiting anisotropies paralleled the steady-state anisotropies, supporting the conclusion that granules have a very low membrane fluidity. Additionally, the rotational correlation time for DPH in granules was longer than that in surface membranes and microsomes, indicating that both the DPH rotational rate and motional freedom are low in granules.

Table 3. Lifetime and differential polarization measurements of DPH

	Granules	Surface membranes	Microsomes
Lifetime analysis			
Double decay:			
τ_1	10.8 nsec	10.3 nsec	9.5 nsec
τ_2	0.8 nsec	1.8 nsec	1.6 nsec
f_1	0.95	0.96	0.94
f_2	0.05	0.04	0.06
Chi-squared	1.1	2.6	3.2
Triple decay:			
τ_1		10.7 nsec	15.4 nsec
τ_2		3.9 nsec	8.5 nsec
τ_3		0.8 nsec	1.4 nsec
f_1		0.91	0.19
f_2		0.07	0.77
f_3		0.02	0.04
Chi-squared		1.2	0.8
Differential polarization analysis			
ϕ	1.36 nsec	0.77 nsec	0.98 nsec
r_∞	0.232	0.209	0.165

Symbols: τ_i: lifetime of ith component; f_i: fractional amplitude of the i^{th} lifetime component; ϕ: rotational correlation time; r_∞: limiting anisotropy.

Discussion

The present studies were performed to examine the water permeability and fluidity properties of granule membranes isolated from toad bladder cells. Stopped-flow light scattering was used to measure the rapid time course of vesicle volume following exposure to an osmotic gradient. From these measurements, the membrane osmotic water permeability coefficient (P_f) was determined quantitatively. Fluorescence anisotropy measurements of the fluidity-sensitive probe DPH were used to assess granule membrane fluidity. Results obtained in granules were compared with those from parallel experiments performed in two other toad bladder cell fractions: surface membranes and microsomes.

The time course of osmotic water transport in granules is described well by a single exponential increase in scattered light intensity. The absence of measurable heterogeneity in the light-scattering data suggests that granule membranes are functionally homogeneous with respect to intrinsic water permeability and surface-to-volume ratio. The relative homogeneity of the granule fraction and the uniformity of granule size seen in the electron micrograph (Fig. 1*b*) support the adequacy of a single exponential fit to the data.

At 23°C the calculated granule osmotic water permeability (P_f) is 5×10^{-4} cm/sec, among the lowest values reported for biological membranes. The activation energy (E_a) was high, 17 kcal/mole. The independence of P_f on osmotic gradient size and the linear Arrhenius plot suggests that the measured P_f value represents true membrane P_f and not the composite resistances of the membrane and a series nonmembrane barrier. Fettiplace and Haydon (1980) reviewed the P_f and E_a values measured for a series of planar and liposomal bilayers. P_f values ranged from 2×10^{-4} cm/sec for cholesterol-containing bilayers to 5×10^{-3} cm/sec for phosphatidylcholine bilayers (values corrected to 25°C). E_a values ranged from 8 to 28 kcal/mole but were usually in the range 11–20 kcal/mole. Thus the

granule P_f and E_a values are similar to those measured in lipid bilayers.

The granule P_f and E_a values are notably different from those in many biological membranes. In membranes thought to contain water channels, P_f values have been reported to be 2×10^{-2} cm/sec for red cells (Terwilliger & Solomon, 1981), $1-4 \times 10^{-2}$ cm/sec for proximal tubule brush border and basolateral membranes (Carpi-Medina, Gonzalez & Whittembury, 1983; Welling, Welling & Ochs, 1983; Meyer & Verkman, 1987), $2-3 \times 10^{-2}$ cm/sec for the vasopressin-stimulated toad bladder (Levine & Kachadorian, 1981) and kidney collecting tubule (Kuwahara, Berry & Verkman, 1988), and 3×10^{-2} cm/sec for vasopressin-induced endosomes isolated from renal medullary collecting tubule (Verkman et al., 1988). Interestingly, the granule P_f of 5×10^{-4} cm/sec is lower than that measured for biological membranes which do not contain water channels including platelets ($3-6 \times 10^{-3}$ cm/sec; Wong & Verkman, 1987), placental brush border membranes (5×10^{-3} cm/sec, Illsley & Verkman, 1986) and small intestine brush border and basolateral membranes (6×10^{-3} cm/sec, van Heeswijk & van Os, 1986) but similar to the unstimulated toad bladder (2.8×10^{-4} cm/sec, Levine & Kachadorian, 1981). Granule E_a (17.6 kcal/mole) is similar to that measured in pure lipid bilayers but distinctly higher than E_a in membranes containing water channels including red cells (5 kcal/mole, Macey, 1984), proximal tubule brush border and basolateral membranes (2–4 kcal/mole, Meyer & Verkman, 1987), intestinal vesicles treated with gramicidin (Worman & Field, 1985) and the vasopressin-stimulated toad bladder (4 kcal/mole). In addition, the estimated granule diffusional water permeability (P_d) is similar to granule P_f,[1] providing further support for the lack of a water channel in granule membranes.

Water transport in surface membranes and microsomes was functionally heterogeneous. The light-scattering data were described well by biexponential functions with time constants of 0.15 and 1.4–2.0 sec; most of the kinetic signal (~70%) consisted of the fast component. Because of the size heterogeneity of surface membranes and microsomes, it is difficult to assign unambiguously time constants to the vesicle morphologies seen on electron micrographs. The large difference in activation energies of the fast and slow components of the light-scattering signal in surface membranes indicates that the functional heterogeneity represents a difference in intrinsic membrane transport properties rather than in vesicle geometry. If the fast time constant is assigned to the small vesicles making up >70% of the surface membrane fraction, then the small vesicles have $P_f \sim 6 \times 10^{-3}$ cm/sec with E_a 9.1 kcal/mole. These values are very different from those measured in granules and are consistent with rapid water transport in surface membranes. If the fast time constant in surface membranes is assigned to larger vesicles, then P_f would become even larger. Additional separation methods are required to define with certainty the water transport properties of the individual toad bladder apical and basolateral membranes.

There was no effect of vasopressin stimulation of the intact toad bladder on the rates of water transport in each of the vesicle fractions. These results indicate that the membrane of the toad bladder granule has not been modified by vasopressin, at least prior to fusion of the granule with the apical cell surface. While there was no effect of vasopressin stimulation on the time constants for surface membrane water transport, there was a small but significant ($P < 0.05$) decrease in the fraction of the signal with the shorter time constant. It is difficult to interpret this result at present because a clear assignment of time constants in the surface membrane fraction has not been made. Possible explanations for the decrease in the amplitude of the rapid surface membrane signal with vasopressin are: (1) with vasopressin, apical vesicle water transport becomes very fast and unresolvable, leading to a decreased amplitude of the fast component; (2) with vasopressin, there is an increased slow component due to an increase in apical surface area, some of which has the low water permeability of the granule membrane; and (3) vasopressin causes a redistribu-

[1] If granule osmotic (P_f) and diffusional (P_d) water permeability coefficients are equal, then the diffusional exchange time (t_{ex}) for intravesicular water would be ~10 msec ($1/t_{ex} = P_d S/V$). P_d could not be measured by nuclear magnetic resonance in granules because of the small quantities of membrane obtainable. We estimated granule P_d using the H_2O/D_2O exchange method described by Lawaczeck (1984) in which the intensity of scattered or transmitted light is an instantaneous function of intravesicular H_2O/D_2O composition. Granules (125 μg/ml) in 75 mOsm D_2O buffer were diluted fivefold with isosmotic H_2O buffer in the stopped-flow apparatus. To maximize signal amplitude, the time course of transmitted light at 400 nm was measured at a data acquisition rate of 0.2 msec/point. There was a rapid decrease in transmitted light intensity with a half-time of 6–10 msec with a signal-to-noise ratio of 3 : 1; there was no consistent change in transmitted light intensity upon mixture of granules in H_2O buffer with isosmotic H_2O. These results are consistent with equal granule P_f and P_d values; however, the signal quality for fast diffusional exchange with limited vesicle quantity did not permit rigorous fitting of the data.

tion of intracellular membranes altering the density profiles and thus the separation on Percoll gradients.

The physical state of the lipid bilayer has been shown to be an important determinant of water permeability (Carruthers & Melchior, 1983; Worman & Field, 1985; Verkman & Ives, 1986; Worman et al., 1986; Illsley et al., 1987). For water transport via lipid diffusion, water permeability increases with increased membrane fluidity; for water transport through channels, effects of fluidity on transport are unpredictable and depend upon the interactions of the water channel with surrounding phospholipid. Above the phase transition temperature of artificial lipid membranes, addition of cholesterol results in a decrease in water permeability (Finkelstein & Cass, 1967; Jain, Touissaint & Cordes, 1973), consistent with the cholesterol-induced decrease in membrane fluidity.

Because of the strong correlation between water permeability and membrane fluidity, we examined the fluidity of each type of toad bladder vesicle by DPH fluorescence anisotropy. DPH has been used widely to assess membrane fluidity or 'microviscosity' because of its excellent membrane partitioning, brightness and sensitivity of rotational characteristics to changes in the physical state of the membrane. Steady-state DPH anisotropies of granules (0.25) were markedly higher than those of surface membranes (0.22) and microsomes (0.19). Compared to values in other membranes, DPH anisotropies in granule membranes are at the upper limit of those reported for epithelial brush border membranes at 23–25°C (0.23–0.25, Worman et al., 1986; Illsley et al., 1987, 1988), and higher than those measured for epithelial basolateral membranes (0.21–0.23, LeGrimellec et al., 1982; Hise, Mantulin & Weinman, 1984; Verkman & Ives, 1986), nonepithelial plasma membranes (e.g., red cells and hepatic plasma membranes, 0.16–0.20) and intracellular organelle membranes. The low granule fluidity arises likely from the high cholesterol and low protein content of the granule membrane (Masur et al., 1987), and possibly from specialized lipid phase structures within the membrane. Analysis of the lipid composition of granules and of the gel/fluid structure of the granule membrane are required to define further the cause of the very low granule membrane fluidity.

To resolve the lifetime and rotational components of steady-state DPH anisotropy in toad bladder membranes, multi-frequency fluorescence lifetime and differential polarization measurements were performed. While DPH lifetimes were similar in each vesicle type, the limiting anisotropy of DPH in granule membranes was higher than that in surface membranes and microsomes. These results confirm the interpretation of steady-state DPH anisotropy data in terms of membrane fluidity.

Our data indicate that the toad bladder granule has a uniquely low water permeability and membrane fluidity which is not altered by pretreatment with vasopressin. These results suggest that the granule does not contribute water channels to the apical membrane during the hydroosmotic response to vasopressin. The homology between toad bladder and mammalian distal nephron is expressed in their hydroosmotic response to vasopressin. However, the low permeability to water and small ions of the resting toad bladder is seen in the luminal membrane of the mammalian urinary bladder as well as in the luminal membrane of the resting mammalian distal nephron. Structural and chemical analysis of the low permeability mammalian bladder luminal membranes are of interest in light of the extremely low osmotic water permeability of toad bladder granules. Discoidal vesicles apparently translocate between the cell surface and the cytoplasm during expansion and contraction of the mammalian bladder (Hicks, Ketterer & Warren, 1974; Minsky & Chalpowski, 1978). A similar role could be postulated for toad bladder granule exocytosis to accommodate bladder volume change or simply as precursor to the impermeable luminal membrane.

Our data do not rule out the possibly that the granule contributes proteins which are chemically modified at the apical surface, or specialized lipids which are important for the increase in apical water permeability caused by vasopressin. The contributed proteins or lipid may be involved in the modulation of the activity of other channels or transport proteins not involved in osmotic water permeability. It is more likely however that the granule contributes 'average' low permeability apical membrane important for the low basal water permeability of the toad bladder. This interpretation is consistent with the temporal dissociation of PMA-induced granule exocytosis and induced osmotic water permeability. Further measurements of water permeability of toad bladder aggrephores and purified surface membranes are required to extend current understanding of the mechanism of the hydroosmotic response.

We thank Stephen Massardo for his expertise in cell fractionation and electron-microscopy procedures. This work was supported by NIH grants DK35124, DK39354, DK25110 and a grant-in-aid from the American Heart Association with funds from the Long Beach California Chapter. ASV is an established investigator of the American Heart Association. SKM is an Irma T. Hirschl Career Scientist.

References

Bordier, C. 1981. Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* **256:**1604–1607

Bricker, T.M., Sherman, L.A. 1984. Triton X-114 phase fractionation of membrane proteins of the cyanobacterium Anactys Nidullans R2. *Arch. Biochem. Biophys.* **235:**204–211

Brown, D., Montesano, R., Orci, L. 1981. Stretch induces granule exocytosis in toad urinary bladder. *Cell. Biol. Int. Rep.* **5:**275–285

Carpi-Medina, P., Gonzalez, E., Whittembury, G. 1983. Cell osmotic water permeability of isolated rabbit proximal convoluted tubules. *Am. J. Physiol.* **244:**F554–F563

Carruthers, A., Melchior, D.L. 1983. Studies of the relationship between bilayer water permeability and bilayer physical state. *Biochemistry* **22:**5797–5807

DiBona, D.R., Civan, M.M., Leaf, A. 1969. The cellular specificity of the effect of vasopressin on toad urinary bladder. *J. Membrane Biol.* **1:**79–91

Fettiplace, R., Haydon, D.A. 1980. Water permeability of lipid membranes. *Physiol. Rev.* **60:**510–550

Finkelstein, A. 1987. Water Movement Through Lipid Bilayers, Pores, and Plasma Membranes. Distinguished Lecture Series of the Society of General Physiologists. Volume 4. John Wiley and Sons, New York

Finkelstein, A., Cass, A. 1967. Effect of cholesterol on the water permeability of thin lipid membranes. *Nature (London)* **216:**717–718

Gronowicz, G., Masur, S.K., Holtzman, E. 1980. Quantitative analysis of exocytosis and endocytosis in the hydroosmotic response of toad bladder. *J. Membrane Biol.* **52:**221–235

Harris, H.W., Jr., Murphy, H.R., Willingham, M.C., Handler, J.S. 1987. Isolation and characterization of specialized regions of toad urinary bladder apical plasma membrane involved in the water permeability response to antidiuretic hormone. *J. Membrane Biol.* **96:**175–186

Hicks, R.M., Ketterer, B., Warren, R.C. 1974. The ultrastructure and chemistry of the luminal plasma membrane of the mammalian urinary bladder: A structure with low permeability to water and ions. *Philos. Trans. R. Soc. London B* **268:**23–38

Heeswijk, M.P.E. van, Os, C.H. van 1986. Osmotic water permeabilities of brush border and basolateral membrane vesicles from rat renal cortex and small intestine. *J. Membrane Biol.* **92:**183–193

Hise, M.K., Mantulin, W.W., Weinman, E.J. 1984. Fluidity and composition of brush border and basolateral membranes from rat kidney. *Am. J. Physiol.* **247:**F434–F439

Illsley, N.P., Lin, H.Y., Verkman, A.S. 1987. Lipid domain structure correlated with membrane protein function in placental microvillus vesicles. *Biochemistry* **26:**446–457

Illsley, N.P., Lin, H.Y., Verkman, A.S. 1988. Lipid phase structure in epithelial cell membranes: Comparison of renal brush border and basolateral membranes. *Biochemistry* **27:**2077–2083

Illsley, N.P., Verkman, A.S. 1986. Serial permeability barriers to water transport in human placental vesicles. *J. Membrane Biol.* **94:**267–278

Illsley, N.P., Verkman, A.S. 1988. Phase modulation analysis of fluorophore rotational heterogeneity in synthetic and biological membranes. *Biophys. J.* **53:**489*a*

Jain, M.K., Touissaint, D.G., Cordes, E.H. 1973. Kinetics of water penetration into unsonicated liposomes. Effects of *n*-alkanols and cholesterol. *J. Membrane Biol.* **14:**1–16

Kuwahara, M., Berry, C.A., Verkman, A.S. 1988. Rapid onset of vasopressin-induced hydroosmosis in perfused collecting tubule measured by a new fluorescence technique. *Biophys. J. (in press)*

Lakowicz, J.R., Prendergast, F.G. 1978. Quantitation of hindered rotations of diphenylhexatriene in lipid bilayers by differential polarized fluorometry. *Science* **200:**1399–1401

Lawaczeck, R. 1984. Water permeability through biological membranes by isotopic effects of fluorescence and light scattering. *Biophys. J.* **45:**491–494

LeGrimellec, C., Giocondi, M.C., Carriere, B., Carriere, S., Cardinal, J. 1982. Membrane fluidity and enzyme activities in brush border and basolateral membranes of the dog kidney. *Am. J. Physiol.* **242:**F246–F253

Levine, S.D., Kachadorian, W.A. 1981. Barriers to water flow in vasopressin-treated toad urinary bladder. *J. Membrane Biol.* **61:**135–139

Macey, R.I. 1984. Transport of water and urea in red blood cells. *Am. J. Physiol.* **246:**C195–C203

Maizel, J.V. 1971. Polyacrylamide gel electrophoresis of viral proteins. *Methods Virol.* **5:**179–246

Masur, S.K., Cooper, S., Massardo, S., Gronowicz, G., Rubin, M.S. 1986. Isolation and characterization of granules of toad bladder. *J. Membrane Biol.* **89:**39–51

Masur, S.K., Greunberg, J., Howell, K.E. 1987. Endosomal compartment of toad bladder epithelium. *Am. J. Physiol.* **252:**C115–C120

Masur, S.K., Holtzman, E., Schwartz, I.L., Walter, R. 1971. Correlation between pinocytosis and hydroosmosis induced by neurohypophyseal hormones and mediated by adenosine-3′,5′-cyclic monophosphate. *J. Cell Biol.* **49:**582–589

Masur, S.K., Holtzman, E., Walter, R. 1972. Hormone stimulated exocytosis in the toad urinary bladder. *J. Cell Biol.* **52:**211–219

Masur, S.K., Sapirstein, V., Rivero, D. 1985. Phorbol myristate acetate induces endocytosis as well as exocytosis and hydroosmosis in toad urinary bladder. *Biochim. Biophys. Acta* **821:**286–296

Merril, C.R., Goldman, D., VanKeuren, M.L. 1984. Gel protein stains: Silver stain. *Meth. Enzymol.* **104:**441–447

Meyer, M.M., Verkman, A.S. 1986. Human platelet osmotic water and non-electrolyte transport. *Am. J. Physiol.* **250:**C549–C557

Meyer, M.M., Verkman, A.S. 1987. Evidence for water channels in renal proximal tubule cell membranes. *J. Membrane Biol.* **96:**107–119

Minsky, B.D., Chalpowski, R.J. 1978. Morphometric analysis of the translocation of lumenal membrane between cytoplasmic and cell surface of transitional epithelial cells during the expansion-contraction cycle of mammalian urinary bladder. *J. Cell Biol.* **77:**685–697

Muller, J.W., 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:**83–95

Terwilliger, T.C., Solomon, A.K. 1981. Osmotic permeability of human red cells. *J. Gen. Physiol.* **77:**549–570

Verkman, A.S., Dix, J.A., Seifter, J.L. 1985. Water and urea transport in renal microvillus membrane vesicles. *Am. J. Physiol.* **248:**F650–F655

Verkman, A.S., Ives, H.E. 1986. Water transport and fluidity in renal basolateral membranes. *Am. J. Physiol.* **250:**F633–F643

Verkman, A.S., Lencer, W., Brown, D., Ausiello, D.A. 1988. Endosomes from kidney collecting tubule contain the vasopressin-sensitive water channel. *Nature (London)* **333:**268–269

Wade, J.B., McCusker, C., Coleman, R.A. 1986. Evaluation of granule exocytosis in toad urinary bladder. *Am. J. Physiol.* **251:**C380–C386

Welling, L.W., Welling, D.J., Ochs, T.J. 1983. Video measurement of basolateral membrane hydraulic conductivity in the proximal tubule. *Am. J. Physiol.* **245:**F123–F129

Wong, K.R., Verkman, A.S. 1987. Nuclear magnetic resonance measurement of diffusional water permeability in human platelets. *Am. J. Physiol.* **252:**C618–C622

Worman, H.J., Brasitus, T.A., Dudeja, P.K., Fozzard, H.A., Field, M. 1986. Relationship between lipid fluidity and water permeability of bovine tracheal epithelial cell apical membranes. *Biochemistry* **25:**1549–1555

Worman, H.J., Field, M. 1985. Osmotic water permeability of small intestinal brush-border membranes. *J. Membrane Biol.* **87:**233–239

Received 7 March 1988; revised 2 May 1988