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# OSMOTIC PERMEABILITIES ACROSS CORNEAL ENDOTHELIUM AND ANTIDIURETIC HORMONE-STIMULATED TOAD URINARY BLADDER STRUCTURES

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**Osmotic permeabilities of several epithelial structures have been determined with novel optical procedures based on specular microscopy. The osmotic permeabilities of several tissue layers were determined by continuously monitoring the position of the apical tissue borders while an osmotic flow was imposed across those layers. The values found were (in $\mu$m/s; mean ± SE): corneal epithelium, 137 ± 30 ($n$ = 5); antidiuretic hormone stimulated toad bladder, 429 ± 64 ($n$ = 6); and corneal endothelium, 711 ± 34 ($n$ = 7). In addition, the osmotically-induced transient change in thickness of the corneal endothelial cells was determined with the help of a computer, and the apparent osmotic permeability measured for the apical membrane was 1420 ± 160 $\mu$m/s ($n$ = 5). It is concluded that the osmotic permeability across the endothelial layer is sizably larger than had been previously detected and that osmotic flows across such layer largely traverse the cellular membranes. With osmotic permeability values (per unit of cell membrane area) as large as presently reported, isotonic fluid transport by epithelia can be explained simply on the basis of local osmotic gradients.**

Measurements of the osmotic permeabilities of epithelial tissues and their cell membranes are important in order to understand basic mechanisms of water permeation and to determine how water and salt movements across fluid-transporting epithelia are coupled. As attention on these measurements has become progressively more focused, some of the techniques utilized earlier for that purpose have come under critical questioning [1,2] due to their relatively low time resolution and due to the uncertainties created by unstirred layers in contact with the tissue structures. In order to confront such problems, we have developed an optical method with which the position of a tissue border can be followed continuously, which allows very great time resolution. We have also minimized the effect of unstirred layers on our measurements. The results we present here are revealing in that the permeability values obtained are larger than had been reported so far.

The present procedures were developed by modifying an earlier technique in which metallurgical or specular microscopy [3] had been successfully applied to the study of corneal thickness [4,5]. The present version allows the resolution of thicknesses of a few $\mu$m; preliminary accounts of this work have been presented [6,7], and a detailed description of the present technique will be published elsewhere (Koester and Fischbarg, in preparation). A schematic diagram is shown in Fig. 1. An adjustable (source) slit was illuminated either with white light from a 75 W xenon arc lamp or with a helium-neon laser (5 mW, polarized, Melles Griot), according to spatial resolution desired and

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Abbreviation: ADH, antidiuretic hormone.

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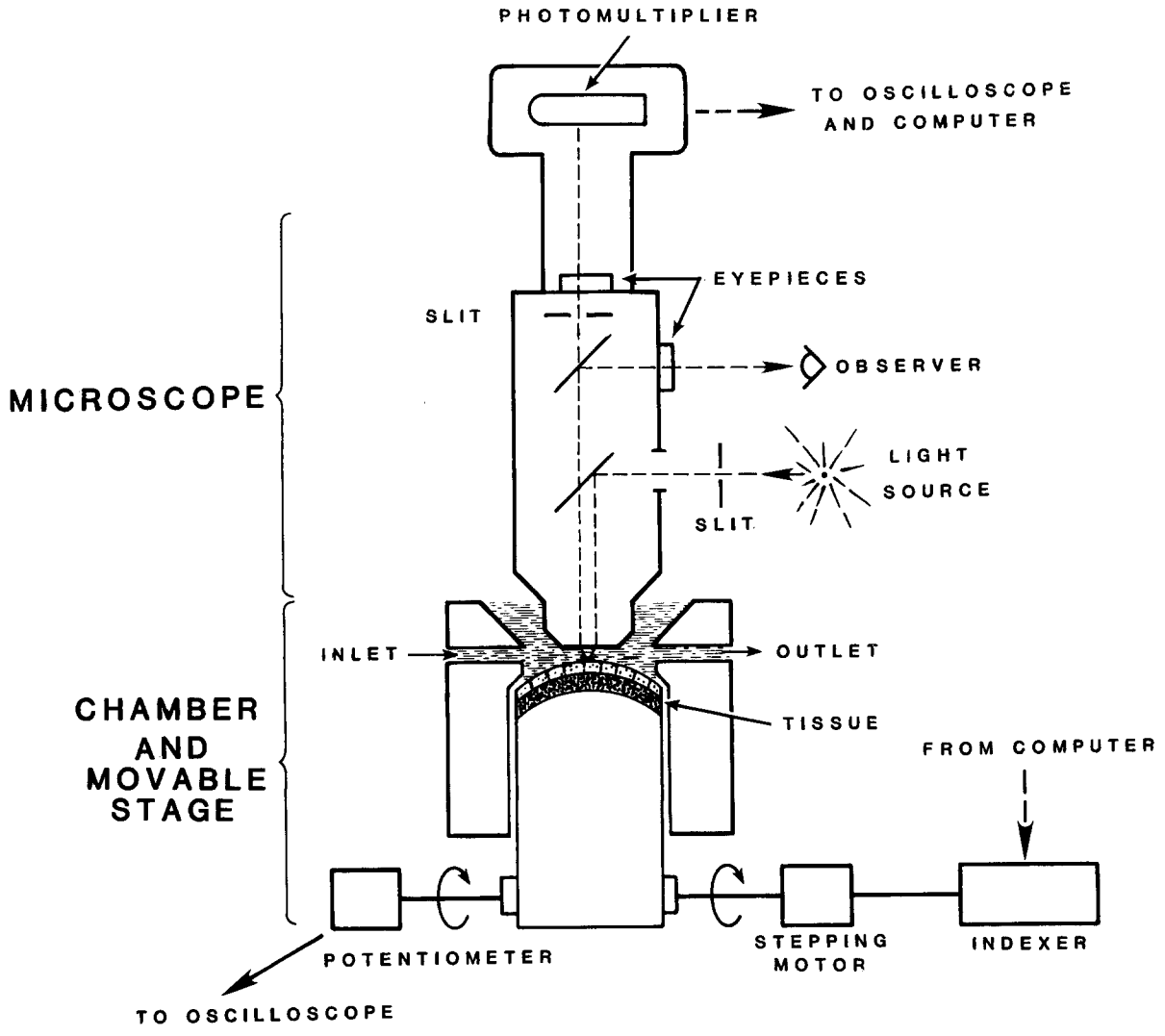


Fig. 1. Schematic diagram of the experimental setup. The photomultiplier assembly can be detached so that the upper slit can be adjusted to coincide with the lower slit as seen through the eyepiece in the microscopic field.

experimental convenience. The slit was projected through the vertical illumination attachment of a Zeiss Universal microscope onto the focal plane of a 40× Leitz water immersion lens (working distance: 1.4 mm). When an optical interface (such as cell water) perpendicular to the optical axis coincided with the focal plane, the slit was reflected by the interface with an intensity proportional to the difference in index of refraction between the two media (cf. also Refs. 4 and 5). The reflection subsequently traversed a second adjustable (image) slit and 7× eyepiece, and was led by a light pipe to a photomultiplier (Hamamatsu R938). The light intensity ($I$) detected with the photomultiplier was displayed on a Tektronix 5103N storage oscilloscope and could be fed to the analog to digital (A/D) converter (AD11-K) of a PDP 11/34A minicomputer (Digital Equipment Corporation). The chamber with the preparation was mounted on the microscope stage, and the micrometric advance of the microscope was driven by an indexer-stepping motor (Superior Electric). The vertical displacement ($z$) of the stage was 0.1 $\mu$m per motor step. In addition, a 25-turn potentiome-

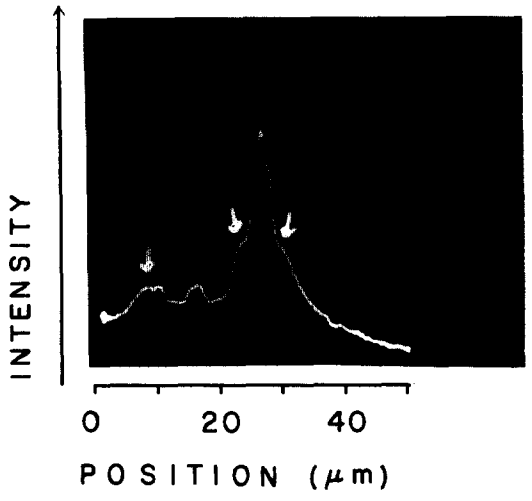


Fig. 2. Plot of $I$ vs. $z$ for the corneal endothelium obtained prior to osmotic challenge. Illumination came from a laser, and the slit was 7 μm wide. From right to left the arrows mark a shoulder at the cell-aqueous interface, another shoulder at the cell-Descemet's membrane interface, and a peak arising from a stromal structure; between the last two is the peak corresponding to the boundary between Descemet's membrane and the stroma. The peak inbetween the two shoulders appears to correspond to an intracellular structure (perhaps the nucleus).

ter was also connected to the micrometer and gave rise to a signal proportional to $z$ which was also displayed on the oscilloscope. Since the A/D converter was activated by the pulses from the indexer, $I$ could be sampled at each 0.1 μm of stage displacement. As far as the technical setup, the use of an adjustable second slit that can be viewed through the eyepiece and a laser for illumination are the main novel features presently introduced.

The measurements performed were of two kinds. The first one consisted of 'normal' or 'space' scans. For this purpose, with the source slit relatively open and illuminated with white light, an area of the preparation about 50 μm wide was examined visually, and the depths at which the cell boundaries and other tissue interfaces were located were noted. Subsequently, in order to increase spatial resolution, the slit was made very narrow (approx. 5 μm wide) and was usually illuminated with the laser. Space scans were then performed by displacing the stage vertically. The intensity of the reflected light thus determined as a function of vertical position ($I$ vs. $z$) was hence correlated with the vertical position of the anatomical accidents of the tissue. Figs. 2 and 3 show examples of $I$ vs. $z$ profiles found for the rabbit corneal endothelium and the urinary bladder epithelium of a toad (*Bufo marinus*). These $I$ vs. $z$ patterns were very characteristic for each given experiment and tissue area, and remained remarkably constant for undisturbed preparations.

The second procedure employed made use of what we will term 'time scans'. The basis for it was as follows: if a given epithelial layer constitutes the movable boundary of a finite compartment, movement of water across such layer would change the volume of the compartment and result in a time-dependent displacement of that boundary. The present method allowed one to monitor continuously the vertical position of the boundary. The procedure utilized was similar for all three epithelial layers examined and is shown schematically in Fig. 3. At first, an $I$ vs. $z$ curve was determined for the purpose of calibration. As can be noted (Figs. 2. 3), the slope of the $I$ vs. $z$ curve is reasonably linear for small tissue displacements away from the objective. Therefore, the light intensity coming from an off-focus position in the vicinity of the cell membrane can be utilized to locate the position of the cell membrane proper with a time resolution practically unlimited. The requisite for this is that osmotic perturbations should not change the $I$ vs. $z$ profile. To help attain such goal, the slit was made about 30 μm wide so as to average light form a larger area. The fact that such goal was achieved can be gathered from the examples shown in Figs. 3A and 3D, when for space scans repeated some 3 min after the osmotic perturbation there was little or no change in the $I$ vs. $z$ curve except for the expected shift due to the displacement of the tissue. After a calibration scan was obtained, the apical cell boundary was placed in focus and $I$ was monitored as a function of time (hence 'time scans'); the stage vertical position now remained undisturbed. At a given time, the bathing solution was quickly replaced by another one of different osmolarity, and the time-transient change in $I$ which ensued was recorded on the oscilloscope screen. Such change in $I$ ($I$ vs. $t$) was therefore determined by the rate of vertical displacement of

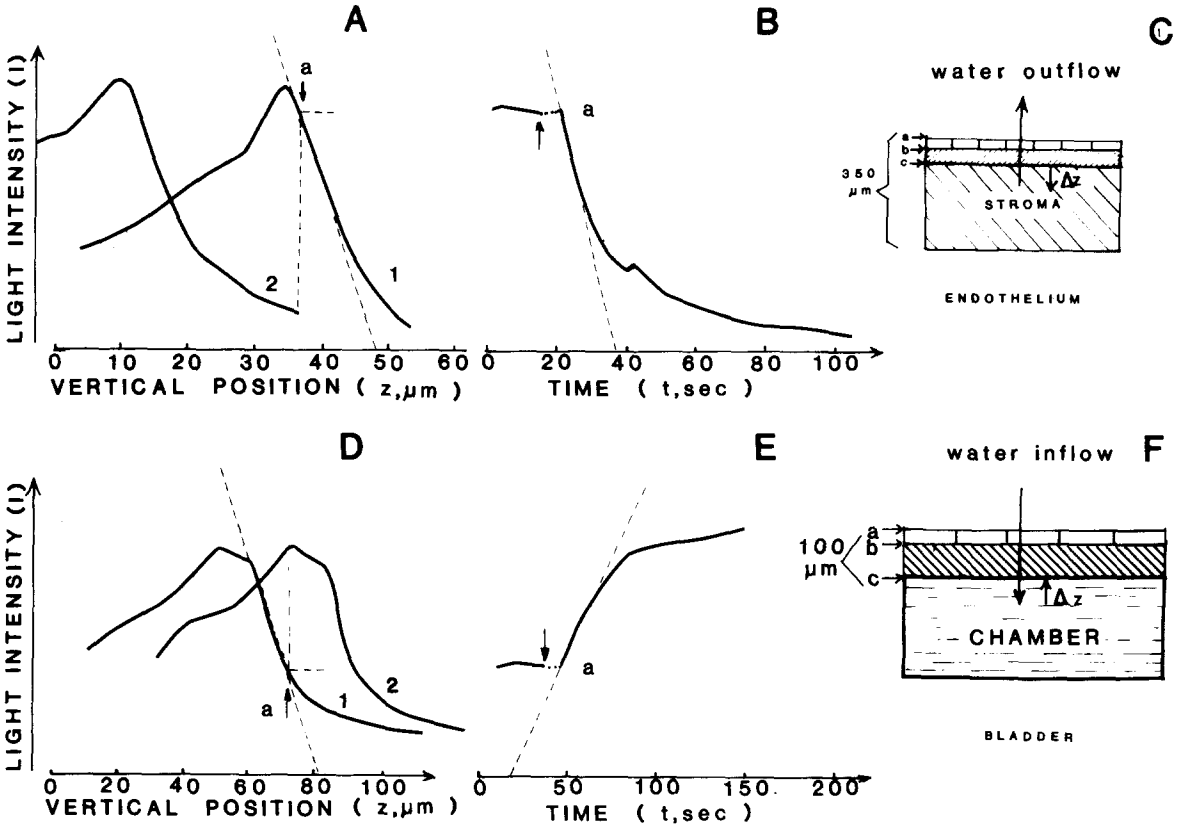


Fig 3. The procedure with which trans-tissue $P_{os}$ measurements based on 'time scans' were done is detailed here for corneal endothelium (A, B, C) and toad urinary bladder (D, E, F). The curves were traced from photographs of the oscilloscope screen, and represent plots of light intensity vs. vertical position (A, D) and light intensity vs. time (B, E) experimentally obtained with white light illumination. For the endothelium, curve 1 in A (space scan, $I$ vs $z$) was obtained before the osmotic transition; $a$ corresponds to the interface between the apical surface of the cells and the bathing medium. In B (time scan, $I$ vs. $t$) the tissue position ($z$) was left fixed with the cell apical boundary in focus ($a$); the light intensity remained constant until the position of the tissue changed in response to the osmotic challenge imposed at the arrow. Some time after hypertonic challenge, curve 2 in A was recorded; stromal thinning had brought about a shift in the curve within the vertical range scanned. Dotted lines illustrate the slopes involved. The scheme in C shows the tissue layers and the direction of the fluid movement; thus a-b: cell, b-c: Descemet's membrane. As water flows upwards, the stroma thins down displacing the apical interface away from the microscope lens (downwards in the graph). For the toad urinary bladder the procedure is similar except for the direction of tissue movement. The scheme in F shows the tissue layers; a-b: cells; b-c; connective tissue. The tissue was mounted stretched over a closed chamber, which resulted in tissue displacement towards the objective lens (upwards in the graph) as water came into the chamber by hypotonic challenge. Curve 1 in D is the control one; $a$ here corresponds to the interface between the cell and the luminal solution. Curve 2 in D was obtained after the osmotic transition. Other details are as above.

the interface (the parameter of interest, $z$ vs. $t$) and by the slope of $I$ vs. $z$ in the prior calibration scans. In consequence, from the initial segment of the $I$ vs. $t$ curve and from the slope of the $I$ vs. $z$ curve, the initial slope of $z$ vs. $t$ could be found. Such a procedure (cf. also Refs. 6 and 7) has not apparently been used before for this purpose.

For the experiments done with corneal limiting layers, corneas were mounted either in their normal position (epithelium upwards) or everted (endothelium upwards). In the last case, the corneas were held in position by a hemispherical plunger which pushed them gently from below. An osmotic gradient was established by replacing the solution in contact with the layer with another one which contained 100 mosM of sucrose added to the

standard components, and the trans-tissue apparent membrane osmotic permeability $P_{os}$ was computed from the $z$ vs. $t$ ($t \to 0$) slopes and the osmotic gradient utilized. The values were (deviations are S.E.): for the epithelium, $137 \pm 30$ $\mu$m/s ($n = 5$), and for the endothelium, $711 \pm 34$ $\mu$m/s ($n = 7$). Given the short times involved, the possible effect of dissipation of the sucrose gradient across the endothelium was neglected (cf. Ref. 13). In addition, the permeability of toad urinary bladder epithelium stimulated with ADH (Pitocin, 30 mU/ml into Frog Ringer's solution) was also determined. Since it is known that the pertinent cell membrane properties remain intact after glutaraldehyde treatment [8,9], the stimulated tissue was fixed for 30 min in 3% glutaraldehyde added to the Frog Ringer's solution in order to prevent contractions of the smooth muscle in the submucosa which otherwise disturbed the optical image. The bladder thus treated separated a closed compartment at its bottom (serosal side) from the overlying solution (luminal side); the cross-sectional area of the chamber was 3.8 cm$^3$. Upon substitution on the luminal side of a Frog Ringer's solution diluted to 1/10 of normal with distilled water, from the subsequent tissue displacement the $P_{os}$ came to be $429 \pm 64$ $\mu$m/s ($n = 6$). From injections of known volumes into the serosal compartment, it was determined that spherical deformation of the tissue required a 10% correction, which is included in the value given. For 9 tissues similarly mounted, their wet and dry weights were, respectively, $48 \pm 10$ mg and $9.6 \pm 3.5$ mg.

The permeability of the apical cell membrane of the corneal endothelium was subsequently studied using space scans. For this prupose, the cornea was mounted in the everted position. In order to increase the intensity of the reflections from the cell boundaries, the preparation was stained for about 30 min with 0.14% ruthenium red dissolved in the normal saline. Such treatment did not apparently affect the pertinent membrane properties, since experiments performed without such staining yielded quite similar results. For the measurements, at a given time a computer program was started that performed continuous scans 51 $\mu$m deep in alternate directions while collecting the $I$ vs. $z$ data. The time resolution of the method was determined by the 0.33-s interval required for each scan. While the scans were being performed automatically, the bathing medium on the apical side was made 2/3 hypotonic by rapidly washing with a solution in which the NaCl had been reduced to 58.4 mM. The set of $I$ vs. $z$ curves was later analyzed and the separation between the 'shoulders' that corresponded to the horizontal cell boundaries (cf. Fig. 2) was plotted as a function of time. As can be seen in the example shown in Fig. 4, the increase in cell thickness after osmotic challenge was very rapid, and the transition was complete in some 2–3s. The cell boundaries separated to an average maximal thickness 1.19 times the original one. In experiments in which the time-course was followed for a more extended period, the cell thickness was seen to return spontaneously over a period of some 2–3 min down to a value close to the original one, presumably due to volume regulation by the cell.

The separation between the two interfaces (cell-aqueous and cell-Descemet's membrane) reflects the accumulation of fluid in the structures comprised between them, that is, cells plus intercellular spaces. If the cell-aqueous interface would remain flat as the cells swell, and if no volume flow would take place initially across either the intercellular junction or the basal membrane (Descemet's), the rate of change of the total volume

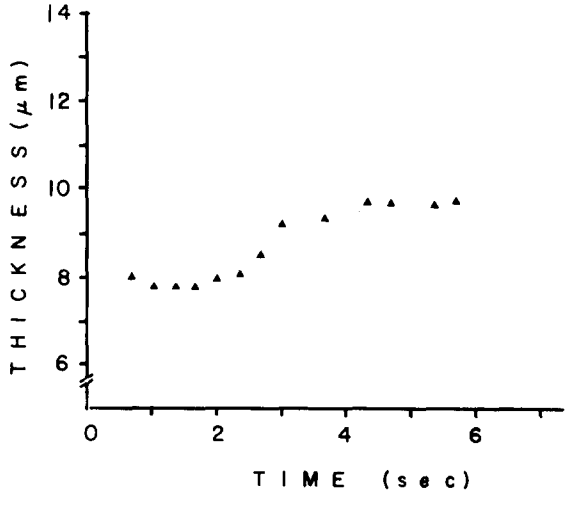


Fig. 4. Plot of the separation of the cellular interfaces vs. time for the corneal endothelium. The data were obtained from consecutive $I$ vs. $z$ scans. A hypotonic solution was perfused immediately after the computer began to perform the scans.

limited by the two interfaces would correspond simply to the rate of fluid flow across the apical membrane. Such apparent apical cell membrane osmotic permeability ($P_{os}$) was therefore determined graphically from the initial rate of separation between the interfaces, and its value was $1420 \pm 160$ $\mu$m/s ($n = 5$).

Given the complexities involved, the value above may differ from the real one. For instance, if the cells would change their shape when swelling to assume 'cobblestone-like' form, the actual Pos may be only one half of the value measured. However, the incidence of such factor may not be that significant. The basolateral area of these cells is about 3.4-times that of the apical membrane. As a consequence, as will be discussed below, if one assumes that all of the osmotic flow traverses the two cell membranes in series, and that the $P_{os}$ value is the same for the apical and basolateral membranes, half of the value measured ($1420/2 = 710$ $\mu$m/s) could not account for the transendothelial overall $P_{os}$ of 741 $\mu$m/s separately measured. In fact, the apparent $P_{os}$ above may be actually underestimated rather than overestimated. This might be due to factors such as: (a) lack of enough temporal resolution to detect the precise initial rate of change; (b) presence of unstirred layers; or (c) volume driven out of the intercellular spaces by hydrostatic pressure buildup in them as the cells are still swelling. Regarding the second factor, the time required to wash out the bulk solution was 30 ms, as determined from an $I$ transition obtained by replacement of a colored solution. From this and the present geometry it was calculated that the unstirred layer near the tissue which NaCl traverses mainly by diffusion was about 34 $\mu$m thick. This would imply a delay of some 0.8 s, and indeed a slight deformation is seen at the 'foot' of the experimental curves (cf. Fig. 4). Although this effect does not extend into the segment of the curves from which $P_{os}$ is presently calculated, it is conceivable that a method with even higher time resolution might allow a more complete description of the swelling curve and allow the determination of the slope at zero time by proper extrapolation. As for the intercellular spaces, they have been seen to swell to a considerable degree [10] under different conditions; whether the same may happen under the present ones remains to be determined. In balance, the uncertainties involved in the experimental determination and the lack of enough information to describe the cellular events in detail dictate some caution in the evaluation of the apparent $P_{os}$ value given above. On the other hand, the first phase of the transient cellular events after osmotic challenge reaches completion very rapidly, in a matter of 2–3 s; we take this to suggest that, even the value presently determined for the cell membrane $P_{os}$ may ben open to scrutiny, its order of magnitude is probably correct.

To focus now on the permeability values across whole tissue layers, the $P_{os}$ value presently found for the stimulated and fixed toad urinary bladder is about 2-fold higher than values usually measured at steady state across preparations which have been stimulated but not fixed. However, the present value is consistent with the observation that such $P_{os}$ shows a sizeable decrease immediately after an osmotic gradient is established across a standard preparation [11]. As for the corneal epithelium, the $P_{os}$ value presently found it is not significantly different from a value of $88 \pm 30$ $\mu$m/s previously reported [12]. This is consistent with the notion that for relatively lower $P_{os}$ values the technical difficulties inherent to these measurements are correspondingly less important. On the other hand, aside from a theoretical suggestion of its high permeability [13], the $P_{os}$ value presently found for the corneal endothelium is sizably larger than all of the values experimentally determined so far (30–220 $\mu$m/s [12,14]; 150–450 $\mu$m/s [15]). Some of the lower values of transendothelial $P_{os}$ we reported in the past [13] were obtained with a technique similar to that employed for other epithelia by several laboratories, namely, steady-state measurements performed during relatively long periods (10–15 min) after an osmotic gradient of sucrose had been imposed. We feel that the lower values such technique yielded in our case may be attributed only in small part to unstirred layers. In spite of statements to the contrary [2], simple unstirred layers cannot explain relatively large discrepancies in trans-tissue, steady-state $P_{os}$ measurements (cf. Ref. 16). Also in this regard, a discrepancy of two orders of magnitude between theoretically expected and observed $P_{os}$ in fluid-transporting epithelia was noted earlier [17] which

remains basically unexplained. We speculate (cf. also Ref. 7) that such discrepancies could be due to a factor not well considered until now, such as the dissipation of the externally imposed osmotic gradient across leaky junctions. For the present case, dissipation of the sucrose gradient imposed across the endothelium is to be expected (cf. Ref. 13), since its intercellular junctions are about 30 Å wide. This would also explain why the $P_{os}$ determined in the steady-state with dextran 40000 mol.wt. (453 $\mu$m/s [15]) comes much closer to the value presently found, since the junctions would be practically impermeant for a molecule of its size. How valid these considerations are for other leaky epithelia remains to be established; obviously, gradient dissipation, unstirred layer effects and tissue morphological changes have to be contended with whenever steady-state osmotic flows are to be measured. As for previous determinations of transendothelial $P_{os}$ based on the detection of time-transient volume changes [12,14], the time-resolution achieved then was presumably insufficient to detect clearly the high $P_{os}$ involved beyond an indication seen at the lower osmotic gradients [14].

In previous communications from this laboratory (cf. Ref. 14), the fact that the hydraulic (i.e., due to a hydrostatic pressure difference) and the osmotic permeability values measured at that time across the endothelium happened to be relatively similar led to the hypothesis that the paracellular shunt could constitute a route for osmotic water flow across that tissue. The results above dictate however a revision of that view. The overall osmotic permeability presently measured across the tissue is seven times larger than the hydraulic one. A paracellular shunt that could account for such a large permeability would have to be some 7.1 nm wide and would be readily visible with the electron microscope, which is not the case. In addition, the reflection coefficient for sucrose of such a structure would be only 0.03, so that the osmotic flows currently seen cannot be explained on that basis. On the other hand, the present data are quite consistent with the possibility that all the osmotic flow across the layer would simply traverse the cellular route. As an illustration, under some simplifying assumptions the relation between the cell volumes after (f) and before (i) the transition would be:

$$V_f/V_i = \frac{C_m(P_A + x \cdot P_B)}{x \cdot P_B \cdot C_m + P_A C_A}$$

where $C_m$ is the concentration in the medium surrounding the basolateral cell membrane (assumed 300 mosM), $C_A$ is the ambient concentration in contact with the apical membrane ($C_A = 200$ mosM), $P_A$ and $P_B$ are respectively the apical and basolateral cell membrane osmotic permeabilities and $x = A_B/A_A$ is the ratio between the basolateral and apical membrane areas, which from electron micrography is about 3.4.

If the thickness change is a reasonable indication of the volume change, from the above one has: $P_B/P_A = r = 0.32$, so that given the measured $P_A$ value of 1420 $\mu$m/s the $P_B$ value would be some 454 $\mu$m/s. Assuming furthermore that the entire osmotic flow traverses the two cell membranes in series, the relation between the overall endothelial permeability $P_{os}$ and that of the cell membranes would be given by:

$$P_{os} = \frac{r \cdot x}{1 + r \cdot x} \cdot P_A$$

The $P_{os}$ computed form this expression is 740 $\mu$m/s, which agrees with the value of 711 $\mu$m/s experimentally measured. Given the assumptions invoked for this treatment, such numerical coincidence may be fortuitous, but even without it the fact remains that both the apical membrane and the entire tissue layer reveal permeability values which are comparatively quite high, and which are of the right order for the total permeability to be thought of as the result of two cell membranes in series. Hence, although some minor flow across the paracellular shunt cannot be excluded, the present results suggest that the osmotic flow across the endothelial layer traverses the cell membranes.

The comparative data reported here show $P_{os}$ values of the order of several hundreds of $\mu$m/s for tissues specialized for water permeation such as the ADH-stimulated toad urinary bladder and the rabbit corneal endothelium, but not for the rabbit corneal epithelium which is not known to transport water in sizable amounts. The value found here for the apparent endothelial apical cell

membrane $P_{os}$ seems also noteworthy. Since this membrane does not present villae or infoldings, the present one constitutes seemingly the largest cell membrane $P_{os}$ value per unit membrane area reported so far (cf. Ref. 18). Large $P_{os}$ values for tissues and, by implication, for cell membranes, had been postulated before [19,1] with less information, but such ideas could not be confirmed until the recent advent of more sensitive microscopic techniques (Ref. 20, this communication). Hence, the experimental measurements of cell membrane permeability that have been made in the recent past (Refs. 21, 22, and 7, this communication) and which point to a high value for such permeability in two fluid-transporting epithelia may be useful to clarify the issues at stake. As for the theoretical implications of these findings, if epithelia specialized for isotonic fluid transport would in general exhibit osmotic permeabilities per unit of cell membrane area of the order reported here, then no special mechanisms beyond simple osmosis across cell membranes would have to be invoked to explain the coupling of solute and solvent movements across them. Relatively small local gradients in the framework of a suitable model (cf. Ref. 23) would be adequate to explain isotonic transport.

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