

Fluid Transport Across Leaky Epithelia: Central Role of the Tight Junction and Supporting Role of Aquaporins

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Fischbarg J. Fluid Transport Across Leaky Epithelia: Central Role of the Tight Junction and Supporting Role of Aquaporins. *Physiol Rev* 90: 1271–1290, 2010; doi:10.1152/physrev.00025.2009.—The mechanism of epithelial fluid transport remains unsolved, which is partly due to inherent experimental difficulties. However, a preparation with which our laboratory works, the corneal endothelium, is a simple leaky secretory epithelium in which we have made some experimental and theoretical headway. As we have reported, transendothelial fluid movements can be generated by electrical currents as long as there is tight junction integrity. The direction of the fluid movement can be reversed by current reversal or by changing junctional electrical charges by polylysine. Residual endothelial fluid transport persists even when no anions (hence no salt) are being transported by the tissue and is only eliminated when all local recirculating electrical currents are. Aquaporin (AQP) 1 is the only AQP present in these cells, and its deletion in AQP1 null mice significantly affects cell osmotic permeability (by ~40%) but fluid transport much less (~20%), which militates against the presence of sizable water movements across the cell. In contrast, AQP1 null mice cells have reduced regulatory volume decrease (only 60% of control), which suggests a possible involvement of AQP1 in either the function or the expression of volume-sensitive membrane channels/transporters. A mathematical model of corneal endothelium we have developed correctly predicts experimental results only when paracellular electro-osmosis is assumed rather than transcellular local osmosis. Our evidence therefore suggests that the fluid is transported across this layer via the paracellular route by a mechanism that we attribute to electro-osmotic coupling at the junctions. From our findings we have developed a novel paradigm for this preparation that includes 1) paracellular fluid flow; 2) a crucial role for the junctions; 3) hypotonicity of the primary secretion; and 4) an AQP role in regulation rather than as a significant water pathway. These elements are remarkably similar to those proposed by the laboratory of Adrian Hill for fluid transport across other leaky epithelia.

I. INTRODUCTION

The mechanism of epithelial fluid transport constitutes arguably the last major problem of epithelial function still unsolved. In recent times, much evidence for the paracellular route for fluid flow across leaky epithelia has been dismissed in favor of explanations based on trans-

cellular flow across aquaporins. In contrast, we discuss here the clear-cut evidence for paracellular flow in the corneal endothelium. In this light, we discuss and put in perspective past evidence and interpretations. From our conclusions, the matter is ripe for a pendular swing towards paracellular flow in leaky epithelia, with transcellular flows playing only a compensatory role.

II. FLUID TRANSPORT ACROSS LEAKY EPITHELIA: THE REVIEW CONDENSED

A. Ebb and Flow, ~1970–1998

The enigma covered here is: how does a leaky epithelial layer transfer fluid between two identical solutions? In short, how does this “gradientless flow” take place?

To be noted, gradientless flow is different from trans-epithelial osmosis à la Dutrochet. In this last one, in the presence of an osmotic gradient across an epithelial layer, water obligingly traverses the layer. This is well exemplified by the kidney collecting duct, a tight epithelium for which we accept nowadays that the water goes across both cell plasma membranes in series (29), traversing their aquaporins. There is also the special case of the anuran skin epithelia, whose intercellular junctions are tight, and which water also appears to traverse through cell membrane aquaporins (56, 76).

Having thus hopefully reassured general physiologists and those interested in the ever popular aquaporins, we return to the fact that practically all other epithelia specialized to transport fluid do so in the absence of any external osmotic gradient across their layers; that is, fluid is transported between compartments of similar osmolarity.

1. *The enigma*

According to the father of our field, the late Hans Ussing (personal communication, 1993), the mechanism of fluid transport constitutes the last fundamental question left unanswered in epithelial physiology. In truth, “unanswered” may not be quite correct by now; “unsettled” might be better, as the answer may be already given in one of the explanations offered. The enigma has in fact lasted a very long time, so one hopes the matter can be settled soon.

2. *Solute-solvent coupling*

The notion that transepithelial movement of water depends on the movement of electrolytes arises from a finding by Peter Curran and Arthur K. Solomon (9) that transintestinal water flow (“solvent” flow) depended on the transport of NaCl (“solute” flux) by that layer. That gave birth to the question of how the flow of solute (or “salt”) is linked to the movement of solvent (or “fluid”), or in the short jargon of the field, how solute-solvent coupling arises.

3. *Water channels and osmotic permeability*

The progression of the ideas on fluid transport is linked to those in a parallel field, that of water channels.

After early advances in their characterization (10) and isolation (4), they were molecularly identified by Peter Agre and co-workers in the early 1990s (85, 86), who termed them aquaporins (AQPs). It was subsequently determined that AQPs were present in many fluid transporting epithelia (77) and were also present in water-permeable kidney segments while absent in relatively water-impermeable ones (77). By then, the measurements of osmotic permeabilities of epithelial cell membranes had been refined using video microscopy techniques. The laboratories of Kenneth Spring (working on gallbladders) (84) and of the Welling brothers (working on kidney proximal tubule) (115) found rather high osmotic permeability (or “filtration” permeability, P_f) values (Persson and Spring: 550 and 1,200 $\mu\text{m/s}$ for the apical and basolateral membranes, respectively; Welling: $\sim 300 \mu\text{m/s}$).¹ Both laboratories suggested that, given such high P_f values, a few milliosmoles of osmotic pressure difference across the cell boundaries would suffice to drive the transported fluids through the cells.

4. *The consensus that wasn't*

All the above appeared plausible. In addition, AQPs were increasingly thought of and researched upon. Therefore, these findings led to a coalescence of opinions between video microscopy users and some scientists with interests in aquaporins and in general physiology: water flow might be transcellular, after all, and obey some form of local osmosis. Some textbook writers picked up on this (119), and to this day, they carry this explanation (88), which has been termed “normal science” (94).

Actually, however, such consensus was not general. In fact, there had been all along experimental evidence for the diverging view that fluid transport across leaky epithelia took place via paracellular, transjunctional water flow. That contrary evidence came from the laboratories of Adrian Hill using gallbladder (37, 40), John Pappenheimer and his fellow James Madara using intestine (66), and Guillermo Whittembury and Gerhard Malnic using kidney proximal tubule (118).

B. Fateful 1998

The contrary view of paracellular flow had remained a minority opinion. Still and all, these “rebels” stood their ground, led by an utterly unconvinced Adrian Hill. Considering the divergent views, Kenneth Spring and colleagues decided to take the bull by the horns and use confocal microscopy to look for evidence for or against transjunctional water flow in epithelia. Their reasoning

¹To read about permeability units, Davson's textbook is highly recommended (11).

was appealing: paracellular, transjunctional fluid flow in an absorbing epithelium would lead to significant dilution of a paracellular fluorescent marker trapped in the intercellular spaces, which in turn would be detectable by the optical sectioning methods they mastered; all very elegant, for sure.

And so we come to the paper Spring and colleagues published in May of 1998 (49) reporting that they had found no transjunctional water flow in cultured Madin-Darby canine kidney (MDCK) cell layers. Understandably, their statement had a very large impact. And yet, only some months afterwards, this notion had to be revised as it became clear that the preparation they had chosen presumably transported little if any water. By Spring's own admission in October of the same 1998 (98), "... the fluid transport rate of MDCK cells is only about 1% of that of the renal proximal tubule . . ." More details on MDCK layers can be found below. To spell out the obvious, little or no fluid transport means no transjunctional (or transcellular) water flow either, so in perspective, the findings of Spring and colleagues ("absence of junctional flow") bring no surprise and have no bearing on the issue of the route of fluid flow in general. With the benefit of hindsight, they ought to have determined how much fluid were their preparations transporting while the video observations went on. Studies such as those would still be of value if done in a more representative leaky fluid-transporting layer.

C. From 1998 to the Present

After the demise of the 1998 paper above, doubts about local osmosis continued to be fueled. Adrian Hill had been joined in his criticism of it by Thomas Zeuthen and Ernest Wright. In particular, Zeuthen and co-workers (122, 123) had developed an alternative model for transcellular water transfer based on molecular cotransport through transporters.

Predictably, Hill's views were newly sought out. In a thorough review written with his wife and colleague Bruria Shachar-Hill, they (95) restated the evidence from theirs and collaborating laboratories for junctional flow for *Necturus* and rabbit gallbladder, *Necturus* intestine, *Rhodnius* Malpighian tubule, and rat and rabbit salivary gland. In addition, they (95) gave a convincing account of the evidence consistent with junctional water flow for renal proximal tubule, exocrine gland (salivary, lacrimal), and small intestine. Here we will simply call attention to those arguments and will concentrate on other arguments plus additional evidence of our own.

1. The AQP knockouts

By the end of the 1990s, Alan Verkman's laboratory had been investigating the physiological effects of knock-

ing out AQPs in mice (110). The results of that research were inconclusive with regard to local osmosis; the deletion of AQPs resulted in drastic decreases of cell membrane osmotic permeability, but only in rather mild decreases in rates of fluid transport, and this last to boot only in tissues that transported fluid at high rates. Verkman and colleagues generally discuss those results in a guarded manner, underlining the role of aquaporins as routes for cell water permeability without making pronouncements on the mechanism of transtissue fluid transport. Yet, paraphrasing the comments by Hill and colleagues in another cogent review (39), the effects seen in the AQP knockouts are sometimes difficult to explain, and not commensurate with the deletion of what would be hypothetically a major route for transcellular transtissue water transfer.

In spite of these strong arguments, some colleagues still insist that 1) there may be other substitute AQPs overexpressed and stepping up to compensate for the deletion, and/or 2) the tissue may still function via transcellular fluid flow, somehow, for instance, just increasing the local osmotic gradients.

Argument 1 does not hold: AQP deletion invariably decreases drastically the plasma membrane osmotic permeability, typically by 40–80%. If there is any "compensation" at all by overexpressed unspecified AQPs or other transporters or channels, it must be miniscule.

Argument 2 fares even worse in that it leads to an absurdity. For a secretory epithelium, hypothetically increased osmotic gradients imply also an increased concentration of salt next to its luminal membrane. This in turn means that the salt will diffuse from there towards the bulk at a hefty rate. However, as my colleague Pavel Iserovich has pointed out, the diffusional salt flux thus generated would be impossibly high. As we have shown (23) and we repeat below, for the corneal endothelium such hypothetical flux is 95-fold larger than the experimentally determined electrolyte fluxes! In other words, hypothesizing transcellular osmosis via increased gradients in AQP knockouts apparently leads to violating the laws of physics.

2. Alternative models: transcellular local osmosis and paracellular osmosis

The reader might ask "given this controversy, has anybody given in recent years experimental or theoretical arguments in favor of more classical models of fluid transport?" There are some cases, presented in what follows.

In a 2002 article by Thiagarajah and Verkman (103), they conclude that AQP1 may be involved in fluid transport across mouse corneal endothelium. They base that conclusion on the experiments shown in their Figure 5, in which, to explore corneal thinning by fluid transport, they first make the cornea swell by 20 μm (resting thickness

123 μm in the wild-type mouse). The problem is that they make the cornea swell with a hypotonic solution. Not surprisingly, the subsequent rate of thinning is 1.9 $\mu\text{m}/\text{min} = 114 \mu\text{m}/\text{h}$. They attribute that thinning to endothelial fluid transport, and since the thinning is slower in the AQP1 null mice, they argue that AQP1 is involved in fluid transport.

However, a more likely explanation is that the thinning they observed is due mostly to transcellular, trans-AQP1 osmosis, and that is why it is reduced in the AQP1 null mice. Following corneal exposure to an external hypotonic solution, from $\Pi_0 \times V_0 = \Pi_f \times V_f$, at maximal swelling, the stroma would be hypotonic to the aqueous ($\Pi_f = 258$ vs. $\Pi_0 = 300$ mosM). Given the value of the endothelial osmotic permeability they report ($P_f = 33 \mu\text{m}/\text{s}$), the subsequent rate J_v of osmotic thinning would be $J_v = P_f \times V_w \times \Delta C = 1.5 \mu\text{m}/\text{min} = 90 \mu\text{m}/\text{h}$. This rate is close to the average rate of thinning of 1.9 $\mu\text{m}/\text{min} = 114 \mu\text{m}/\text{h}$ value they report also in their Figure 5. In further support of our alternative interpretation, typical rates of fluid transport are ~ 2.5 times smaller, $\sim 0.72 \mu\text{m}/\text{min} = 43 \mu\text{m}/\text{h}$ (90). In general terms, corneal swelling has this far been induced either by exposure to low ($\sim 4^\circ\text{C}$) temperature (16), or by exposure of the denuded cornea to an isotonic solution (90). Swelling the cornea instead by an osmotic gradient obviously compromises the subsequent conclusions of Thiagarajah and Verkman on the route of fluid transport.

There is also a theoretical paper with a defense of pristine transcellular local osmosis in 2005 by Richard Mathias (67). Basically, he argues that any cellular mechanism to transport fluid will inexorably generate local osmotic imbalances in the neighborhood of the cell, and water will therefore still be going through cellular AQPs to adjust those imbalances. This appears correct; however, the proposals from both Hill's (38) and our laboratory (23) include such imbalances and possible corrections of them through AQPs. What seems more to the point is that the relative amount of water involved in those corrections may be modest. For example, in work done in our laboratory on corneal endothelium, AQP knockouts transported only $\sim 19\%$ fluid less than the controls (53). To repeat the obvious, most of the transtissue water flow appears to be going not through cellular AQPs but through the junctions.

In other important developments, Ussing and Eskesen studied fluid transport in frog skin glands, which drive fluid from the serosal compartment towards the outside. Their paper (106) advanced evidence for electro-osmosis as the driving force for fluid transport, and for Na^+ recirculation as an important element of fluid secretion. Given the detail and sophistication of that study, it constituted a strong call of attention for electro-osmosis. Therefore, in the early 1990s, we began to explore whether corneal endothelium fluid transport was due to

electro-osmosis. The results of our initial attempt (50) were inconclusive. On one hand, there was evidence for electro-osmosis: transendothelial currents in either direction drove fluid (towards the cathode); perhaps that ought to have received closer attention. The problem was that the "short-circuit" current required to arrest fluid transport was nearly four times larger than that calculated for isotonic coupling.

Unfortunately, those results were probably affected by an edge damage artifact. This became apparent only much later, in 2002, when we launched a new attempt, this time using a different mounting procedure. Again, we found electro-osmosis, and this time the coupling calculated made the secretion roughly isotonic (92).

Of course, in the late 1990s that was not known, and explanations for solute-solvent coupling were actively being sought by several laboratories, including Ussing and colleagues. In a 1996 paper by Ussing, Lind, and Larsen (107), a model was presented for isotonic fluid secretion by frog skin glands. They presented novel evidence for paracellular flow, arising from the flow of $^{134}\text{Cs}^+$ from serosa to mucosa: any Cs^+ flowing through the cell would be trapped, so the $^{134}\text{Cs}^+$ flow observed could only be paracellular. The authors attributed it to paracellular solvent drag. The fluid secretion would arise from active Na^+ transport from the cell into the intercellular space, followed by reabsorption of as much as 80% of the Na^+ via apical Na^+ channels, that is, Na^+ recirculation. For solute-solvent coupling and paracellular water flow from serosa to mucosa, the authors resort to a double-membrane model with a complex set of permeability and reflection coefficient requirements that results in fluid being driven by osmosis into the paracellular space across the (serosal side) basement membrane, and driven by pressure through the junctions.

Their experimental system may be too cumbersome to allow for simple answers. One question is if the electrical driving force across the glands is really zero, as they assume. As the frog skin includes several types of cells, the authors need to mask the standard frog skin potential difference (tens of millivolts) using amiloride outside. However, the authors mention residual potential differences of 0.7–1 mV, which in the presence of a paracellular conductance through the glands large enough to account for the solvent drag observed could translate into paracellular Na^+ flow. Another question is whether sizable osmosis can be generated by a NaCl gradient across a basement membrane, and what would be the values of the salt gradients and reflection coefficients necessary for that to happen. Although they assume such osmosis can take place, that is dubious, so additional experimental evidence would be desirable.

This group (Nedergaard, Larsen, and Ussing) also published a paper in 1999 (74) on toad small intestine. Once more, they show clear evidence for transport of

$^{134}\text{Cs}^+$ by convection (from mucosa to serosa) all along the paracellular pathway (cf. their Fig. 6). Their discussion contains a very lucid review of the evidence for convective water flows along the paracellular pathways of other leaky epithelia, citing the cases of gallbladder (37, 117) and kidney proximal tubule (28, 118) (solvent drag is also discussed in sect. II C3). To return to the driving force for paracellular water flow, presumably Nedergaard et al. (74) had in mind a double-membrane type of model, generating water flow by osmosis across the junctions and pressure-driven extrusion through the basal membrane. As that might generate hypertonic fluid, they mention that “isotonicity of the transportate can be maintained by producing a hypertonic fluid emerging from the lateral space combined with reuptake of salt via the cells.”

Hans Ussing unfortunately died in 2000, without seeing the matter resolved. Erik Hviid Larsen has continued to study such models, as exemplified by a recent (2006) review by Larsen and Mobjerg (55). There they present a hypothetical absorptive layer that generates water flow all along the paracellular pathway, driven by osmosis across the junctions, and accompanied by recirculation to adjust the osmolarity. This model constitutes probably the best modern attempt to preserve Curran’s double-membrane hypothesis. To be noted, as the main route of water movement would be paracellular, fluid transport would be little affected in the AQP null mice, in agreement with current literature. Still, their assumption that there can be osmosis through the junctions driven by a salt gradient is crucial and would benefit from experimental corroboration. Findings so far point away from it. In a recent review, Shachar-Hill and Hill (95) give arguments as to why transjunctional osmosis in leaky epithelia must be small. In agreement with that, from work done in our laboratory (F.P.J. Diecke, L. Ma, K. Kuang, P. Iserovich, and J. Fischbarg, unpublished data), we conclude that in cultured bovine corneal endothelial layers the junctional reflection coefficient for NaCl is rather low, perhaps ~ 0.24 .

Still, it seems of interest that three laboratories working in this area have converged on the paracellular pathway as the route of main relevance for water flow across leaky epithelia. Some of their published views are compared in Table 1.

The anuran skin epithelia is a special case. These tight fluid-transporting epithelia are found in the classical preparations of frog and toad skins. The fact that frog

skins transport fluid (from mucosa to serosa) between two identical solutions was recognized as early as 1892 by Weymouth Reid (87) in work that is both exemplary and still amazingly relevant. A good feel for the differences between tight and leaky epithelia can be gotten from Tables 9.2 and 9.3 in the highly recommendable book by Randall House (46).

Some insight can be gotten from calculations linking layer specific resistance and junctional width. We use specific conductance (κ) values (31) of 0.024 and 0.013 S/cm for mammalian (κ_m) and frog (κ_f) Ringer solution, respectively. We consider an idealized epithelium with cells $\sim 20 \mu\text{m}$ wide, such that the sum of the cellular perimeters (pr) is $1,200 \text{ cm/cm}^2$, and the length of the junctions is $j_l = 1 \mu\text{m}$. We assume the width of the junctions is 40 \AA for the leaky epithelium and 3 \AA for the tight one. Tissue specific resistance values are $R = j_l/(\kappa \text{ pr } j_w) = 8.7 \Omega\text{-cm}^2$ for the leaky one and $220 \Omega\text{-cm}^2$ for the tight one, which are of the orders of magnitude expected. In addition, values of the junctional hydraulic conductivity can also be calculated as $L_{pj} = (j_w^3 \times \text{pr})/(12\eta \times j_l)$. Results are $93 \times 10^{-7} \text{ cm}\cdot\text{s}^{-1}\cdot\text{atm}^{-1} = 131 \mu\text{m/s}$ for the leaky epithelium, and $0.027 \times 10^{-7} \text{ cm}\cdot\text{s}^{-1}\cdot\text{atm}^{-1} = 0.04 \mu\text{m/s}$ for the tight one. The L_{pj} for the leaky tissue is close to the value for rat jejunum in House’s (46) Table 9.3, while the L_{pj} value for the tight tissue is much too small to conceive of any significant water flow through its junctions. In this context, the explanation that fluid transport across frog skin proceeds by a Curran-type double membrane local osmotic mechanism (79), with the intercellular spaces as the osmotic coupling compartment (56), appears appropriate. It is still unclear whether there are other tight epithelia that also transport fluid in the absence of an external gradient, and for which local osmosis would therefore emerge as a possible explanation. One case in point is the retinal pigment epithelium, for which recent evidence (in bovine layers) (1) places its specific resistance at the intermediate value of $\sim 140 \Omega\text{-cm}^2$.

3. A standard criticism of paracellular flow

As mentioned above, when dealing with epithelial fluid transport, textbooks have resorted to explain it by local transcellular osmosis. Still, as mentioned above, there is a group of studies that point to the presence of paracellular solvent drag in leaky fluid-transporting epithelia, which is evidence for the paracellular route in-

TABLE 1. Comparison of published views of water flow across leaky epithelium

Laboratory	Water Route	Primary Secretion	Recirculation	Driving Force
Hill	Paracellular	Hypotonic	Implicit?	Junctional mechano-osmosis
Larsen	Paracellular	Hypertonic	Yes	Junction/basement membrane osmosis
Fischbarg	Paracellular	Hypotonic	Yes	Junctional electro-osmosis

stead. It seems strange that all the evidence for paracellular solvent drag can be dismissed in toto by attributing it to unstirred layer artifacts (88). The main argument against such findings is that translateral water movements might concentrate or dilute the substrates in the intercellular spaces, which would be followed by artifactual substrate diffusional flows across the junctions generating pseudosolvent drag. On the other hand, effects of unstirred layers have been found rather modest (82, 83), and the paracellular probe experiments have a very strong theoretical basis (95). In balance, if the role of unstirred layers would be that clear and detrimental, one would expect to see papers showing precisely how unstirred layers would vitiate the conclusions from the use of paracellular probes to tag junctional fluid flow in quite a number of studies (28, 37, 74, 95, 107, 117, 118), or from our findings of paracellular electro-osmosis (92). No such papers apparently exist.

4. Recent evidence supporting our proposal of paracellular, transjunctional, electro-osmotic fluid flow

Since 2002 we have offered experimental and theoretical support for this proposal, as detailed below. In short, we have shown the following for the corneal endothelium, a fluid transporting secretory epithelium.

1) Transendothelial electrical currents produce fluid movements with direction and magnitude linearly linked to the currents (92). These fluid movements require junctional integrity, which suggests they take place across the paracellular, transjunctional route.

2) Short-circuiting the preparation abolishes fluid transport (50), as expected in the electro-osmosis framework given that short-circuiting nullifies the passive paracellular net Na^+ flux while sparing the active transcellular HCO_3^- net flux from stroma to aqueous (120).

3) The direction of fluid movement is reversed by reversing the junctional electrical charges using polylysine (92).

4) Some 25% residual fluid transport persists even when no anions (hence no salt) are being transported by the tissue; hence, such residual fluid transport cannot be explained by salt transport-driven local osmosis. The residual fluid transport is only eliminated when Na^+ recirculation is abolished by the epithelial Na^+ channel (ENaC) blocker benzamil (14), which is consistent with fluid transport arising from recirculating electrical currents and electro-osmosis.

5) A mathematical model of corneal endothelium (22) correctly predicts the experimental results only when based on paracellular electro-osmosis, and not when transcellular local osmosis is assumed instead.

6) AQP1 is the only AQP present in these cells, and its deletion in AQP1 null mice decreases cell osmotic

permeability by 40%, but fluid transport by only 19% (53), which militates against the presence of sizable water movements across the cell.

It is of course theoretically conceivable that the tissue might be operating by local transcellular osmosis and that the electro-osmosis findings would be correct but not relevant. However, the mild effect of the deletion of AQP1 (53) plus the finding of residual fluid transport in the absence of salt transport (14) are very difficult to reconcile with transcellular local osmosis. In a similar vein, short-circuiting makes transcellular transport work most efficiently, while at the same time it abolishes fluid transport, indicating that the latter is related to events in the passive, paracellular limb. Considering the evidence as a whole, the electro-osmosis mechanism proposed is consistently supported, while transcellular local osmosis is not.

D. The Balance at End 2008

Our model based on electro-osmosis (23) has been recently discussed by Hill (34). He identifies three definite merits: 1) it departs from osmotic permeability, 2) it gives a central role to the tight junctions, and 3) junctional electro-osmosis is consistent with and might explain prior findings of paracellular convection. He also points out some experimental criteria that ideally ought to be satisfied by the model and the preparation, such as maintenance of quasi-isotonicity of the secretion with changes in bath osmolarity.

There is some evidence that the primary endothelial secretion might be hypotonic (92). If that is the case, to maintain isotonicity the electro-osmotic secretion would presumably be accompanied by a separate step of osmotic equilibration. These two events might be temporally separate, perhaps in a cyclic sequence.

There is some evidence for that behavior. We note that the driving force for electro-osmosis is the translayer electrical potential difference. In a recent paper (70) we have communicated that the corneal transendothelial electrical potential difference is not constant in time, but instead, it oscillates at frequencies characteristic of a few electrogenic transporters/channels. In more recent work (M. P. Gomez, N. Montalbetti, C. F. Kusnier, and J. Fischbarg, unpublished data), we observe that the oscillations occur in bursts separated by rest periods. These findings are therefore consistent with cyclic electro-osmotic secretion.

These and prior findings raise a tantalizing possibility: could junctional electro-osmosis be the missing link, perhaps the junctional "mechano-osmosis" postulated by Hill's laboratory (95) to account for the driving force for paracellular fluid flow? It was visionary of them to call attention to a coupling mechanism nonosmotic and resid-

ing in the junctions. We may have taken this central matter a step further. These questions remain obvious targets for further investigation. But nothing this far has disproved that electro-osmosis is the missing link.

The preceding remarks apply to leaky epithelia. It appears, however, that fluid-transporting tight epithelia belong instead in a separate track and that for that group transcellular local osmosis is the leading explanation. The case in point is the recent work of Erik Hviid Larsen and colleagues on toad skin (56, 76).

III. FLUID TRANSPORT ACROSS EPITHELIA: THE REVIEW IN DETAIL

A. A Historical Perspective

The idea of transcellular fluid transport started somewhat off-key. An early proposal for it was pinocytosis; alas, in 1960 Adrian Hogben demolished it, calling it “the last refuge of the intellectually bankrupt” (45). In truth, there is no evidence for substantial fluid movements by it (88, 89).

In looking for more logical explanations, one has to consider the two different pathways water can travel across an epithelium: transcellular and paracellular. That there are in some epithelia paracellular pathways with high conductance for water became increasingly clear as the electrical resistance of epithelial layers was studied in the early 1970s. By about that time, epithelia were categorized as tight, intermediate, and leaky, mainly through the work of Frömter and Diamond (27). Subsequently, Whittembury and Reuss (119) pointed out that several epithelia that transport fluid isotonicly were electrically leaky, viz. kidney proximal tubule, gallbladder, intestine, and corneal endothelium. There are, however, other fluid-transporting epithelia such as retinal pigment epithelium, choroid plexus, and ciliary epithelium for which the geometry has so far precluded definitive measurements of its electrical resistance. Hence, it seems prudent to restrict our current arguments to proven leaky epithelia.

In this connection, there have been debates between proponents of the transcellular and paracellular routes for fluid transport. A review by Alan Weinstein and Erich Windhager (114) discusses these issues for kidney proximal tubule, as well as a paper by Subrata Tripathi and Emile Boulpaep (105). This last paper calls attention to the fact that, depending on the relative areas of the lateral versus the basal membranes, the transcellular route can be predominantly transbasal or translateral. The first modern models for epithelial fluid transport were those of Peter Curran and co-workers (47, 79) and Diamond and Bossert (13); in both, fluid was driven by local osmotic gradients at both the apical and basolateral cell membranes and could traverse cell membranes and intercel-

lular junctions. These models set high standards for the field and included transcellular osmosis in a feasible geometrical frame. They began to appear in textbooks as explanations for this phenomenon.

Still, objections began to appear. Adrian Hill (35) pointed out that fluid transported through cells as theorized by Diamond would be hypertonic, while epithelia transported isotonicly. Hill’s objections brought the matter to a standstill. Still, if the flow could not be transcellular, it had to be paracellular, and somehow no consensus for that could be developed either. In several papers, Hill’s and other laboratories showed evidence suggesting solvent drag of solute caused by paracellular, transjunctional water flow. However, there were counter-arguments that a similar drag of solute would take place if fluid would travel via lateral membranes and the paracellular space.

As a result of this impasse, the local osmosis model survived in textbooks, which to this day almost invariably explain fluid transport across leaky epithelia by some version of local transcellular osmosis. The recent avalanche of evidence for the presence of water channels in fluid-transporting epithelia has of course helped this thinking. And yet, things may not be as simple, as we look at the mechanism more closely.

B. Water Channels

For water to traverse cell membranes, it has to be helped to cross the lipid bilayer. So the idea of a plasma membrane water channel emerged early on, championed by Arthur K. Solomon and colleagues (96). As the first water channel protein (AQP1) was characterized (3, 4) and molecularly identified (12, 85, 86), attention turned to its presence in epithelia. As summarized in an earlier section, findings reinforced views from review writers that fluid transport presumably traversed epithelial cell membranes (89, 98).

C. Problems for Transcellular Water Transport Surface and Resurface

If fluid transport traverses epithelial cells via AQPs, one would expect *prima facie* that absence of aquaporins would affect that transport markedly. Yet, that expectation has not been fulfilled.

In the last decade, Alan Verkman’s laboratory in collaboration with several others have extended such studies greatly through the experimental use of AQP knockout mice (110, 112). These results raised similar questions as to whether AQPs are the main route of fluid transport through epithelia. A thorough analysis of the results with AQP knockout mice appears in a review by Hill’s group (“What are aquaporins for?” Ref. 39). A paragraph from it

reads as follows (SPH stands for “simple permeability hypothesis,” in which the role of AQPs is simply to increase the osmotic permeability of membranes above that of the bilayer).

“Observations on such knockouts and mutants have shown a wide range of effects on fluid transfer rates. SPH would predict that the removal of a major pathway of water flux across membranes would have drastic effects on biological function at the cellular, tissue and whole animal level. However, these have been remarkably few, and those effects that have been observed are hard to interpret by the SPH . . .”

We discuss below the evidence for corneal endothelium (53), in which deletion of AQP1 decreased osmotic permeability by ~40% but affected fluid transport by 19%. In a more dramatic example for this same trend (80), in work done on cerebrospinal fluid production by choroid plexus of mice, AQP1 deletion reduced osmotic permeability by ~80% but reduced cerebrospinal fluid production by only ~25%.

A recent review of ours (Fig. 1 in Ref. 23) summarizes the main objections against the transcellular local osmosis explanation for fluid transport across leaky epithelia. 1) Fluid transport is affected little by the absence of water channels. 2) There is no clear evidence for the presence of local concentration gradients. 3) Ussing’s objection (106): he showed that given transcellular flow of water and solute, the friction of water with the cytoplasm fixed elements will mean that the fluid transported will always be hypertonic. From this, transcellular flow could not account for the isotonic fluid transport seen across leaky epithelia.

D. Discriminating Between Paracellular and Transcellular Routes

Given these possible pathways for water across an epithelial layer (Fig. 2 in Ref. 23), there are ways to distinguish between them. Since 1978, there has been a steady succession of papers describing evidence for paracellular water flow. For a review, see Reference 119. More recently, evidence for paracellular flow has been reviewed by Shachar-Hill and Hill (95). They describe studies using paracellular probes in gallbladder, salivary gland, intestine, and Malpighian tubule; the fraction of fluid traversing the paracellular path, including the junction, is ~1 in all these cases. They propose that fluid flow is generated at the junctions by a nonosmotic mechanism. To that list we add the corneal endothelium, for which our own evidence discussed below (92) suggests that fluid transport also traverses the paracellular pathway, including crucially the junctions.

Evidence against paracellular flow appeared in a report finding no water flow across the junctions of cultured

MDCK epithelial layers (49) as determined using a complex optical-computational technique. However, MDCK cell layers are derived (69) from tight epithelia (distal tubule/collecting duct) and fittingly are known to have very poor fluid-transporting ability (104), so that observation (49) does not appear to have relevance for epithelia transporting fluid in normal amounts. In balance, the preponderance of evidence this far suggests that the flow is paracellular in the leaky layers mentioned above.

1. Solute-solvent coupling in the tight junction: the electro-osmosis paradigm

Electro-osmosis has been mentioned before in the literature of fluid-transporting epithelia, not in avalanche proportions, but certainly enough so that interested workers could notice.

Apparently the first such mention of electro-osmosis was that of Adrian Hill in 1975 (36) as a possible mechanism for the solute-solvent coupling underlying fluid transport across epithelia modeled as a double membrane system. That treatment was for coupling at the membrane level rather than the intercellular junctions, but it seems noteworthy that the inherent physics was already there.

Interestingly, electro-osmosis can explain the observations of Kenneth Spring and Charles Paganelli (99) in *Necturus* kidney proximal tubule, although they actually interpreted the observed current-induced fluid movements as due to concentration changes. In another case, also for kidney proximal tubule, electro-osmosis along the paracellular spaces (not the junctions!) was considered and fittingly found insufficient to account for the observed rate of fluid transport (68).

Electro-osmosis was cited in earnest as an explanation of the evidence obtained in experimental papers done with several preparations. That list starts with some relatively isolated observations done in rabbit ileum (72), frog skin glands (75, 106), and rat jejunal epithelium (33). To be noted, for the laboratories of Richard Naftalin and Hans Ussing, the location of the coupling was already the junction. And so we come to the corneal endothelium, for which the evidence is weightier. True, it starts with a speculative brilliant suggestion of electro-osmosis given with scant evidence (62), but it is built up by work we have recently done using several different approaches (23, 70, 92).

Theory, experimental work, and industrial applications of electro-osmosis are well developed. By comparison, the observations above are comparatively few so far, and it would be desirable if more laboratories took an interest in the subject. Eventual application to biology may be complex; for instance, the classical Helmholtz-Smoluchowski treatment developed for ideal capillaries and physicochemical membranes cannot be applied directly, as explained below and elsewhere (26, 91). For a

number of these reasons, electro-osmosis is still viewed with caution. For instance, in recent reviews (88, 89), electro-osmosis is dismissed on the weight of two papers: Wedner and Diamond, 1969 (113) and Van Os et al., 1976 (109). However, from our own recent evidence, the impact from these two papers needs to be reconsidered.

According to Wedner and Diamond, the absorbate generated by transepithelial voltages across mammalian gallbladder was very hypertonic (coupling ratio J_v/J_s was 0.5–1 l/osmol, short of the ideal isotonic coupling of 3.3 l/osmol). In our case, years ago we obtained similar results (50) working with rabbit corneal endothelia mounted in tightly clamped chambers used to determine fluid transport with the Bourguet-Jard technique. We observed electro-osmotic fluid movements (in both directions), with the currents required to generate them being four times larger than required for ideal isotonic coupling. That far, we were in qualitative agreement with the results of Wedner and Diamond. However, more recently, we have reexamined the question using the same rabbit corneal endothelial preparations but a different mounting procedure, that of Dikstein and Maurice (16), which minimizes edge damage. Dramatically, in this case, the current and fluid movements were coupled isotonically (92). The overall coupling ratio reported there (92) was $r = 2.37 (\mu\text{m}/\text{h}) (\text{cm}^2/\mu\text{A}) = 0.066 \text{ cm}^3/\text{Coul}$, from which $r \times F/2 = 3.2 \text{ l/osmol}$ ($F = \text{Faraday's constant}$). Hence, in that system, electro-osmosis can account for isotonic fluid transport.

As for the paper of Van Os et al. (109), it raises the possibility that apparent electro-osmotic fluid movements could be due to current-induced changes in salt concentrations in unstirred layers. However, such concentration changes require a build-up time, whereas electro-osmotic movements are bound to be instantaneous, as discussed by McLaughlin and Mathias (68). In one instance, indeed the rapid development of electro-osmotic fluid flow was reported (72). In this connection, we had described earlier (50) that fluid movements across corneal endothelium responded instantly to changes in current. More recently, we have documented that the fluid movement response to a current pulse takes place in 1 s or less (Fig. 7 in Ref. 92), which is an interval too short for salt buildup (Figs. 8 and 9 in Ref. 92).

In balance, in this area, unstirred layer artifacts can be ruled out by carefully designed experiments. To sort out these matters, again it would be of use to have more and better determinations done of the precise time course of the rate of fluid movement after electrical current step changes. As this may not be exactly easy, results may take a while to appear. In the meantime, we propose that junctional electro-osmosis be considered without prejudice as a possible explanation for fluid transport across leaky epithelia.

2. Recent evidence from our laboratory: paracellular electro-osmotic fluid flow with solute-solvent junctional coupling, the electro-osmosis paradigm

The experimental part of the work has been published (92) and covered in a recent review (21). We have also generated a novel theoretical treatment of electro-osmotic coupling in the tight junctions (26, 91). Here we will highlight some of the main points involved.

A) A BETTER TECHNIQUE? To begin with, the corneal endothelium is a simple layer, as exemplified in Figure 1. This allows one to calculate its geometry with certainty (cf. Refs. 19, 91) and to use such parameters in modeling electrical characteristics and pathways for water transfer (23, 91). In addition, we have detailed (Fig. 1 in Ref. 92) the Dikstein-Maurice dissection procedure and chamber we utilize to work with rabbit corneal endothelium in vitro; the chamber is shown here as well (Fig. 2). As remarked above, with this technique the calculated coupling was found to be near-isotonic, whereas with another technique for fluid transport measurements (25), the coupling was hypertonic (~ 4 times more electrical current was needed to generate similar fluid flows, cf. Ref. 50). To

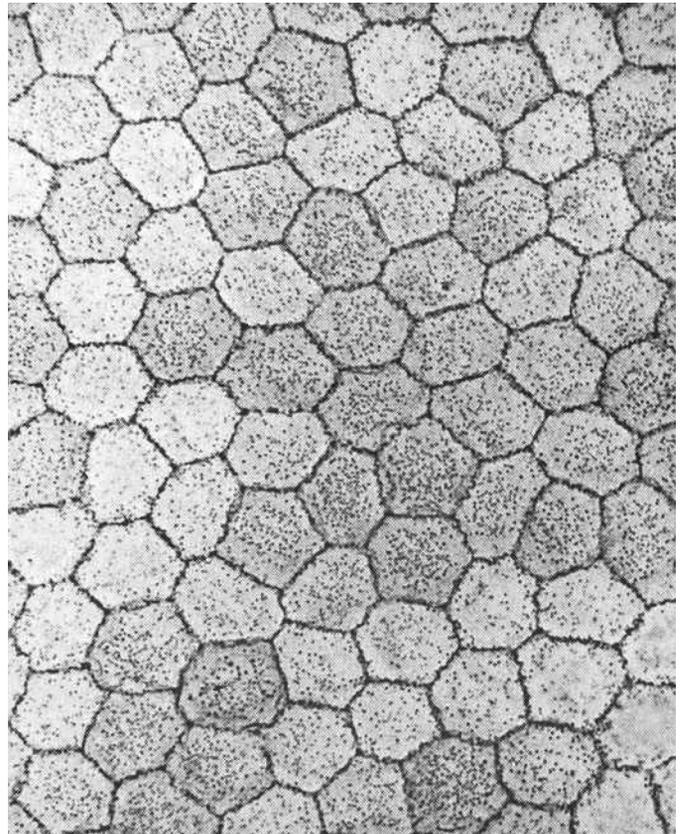


FIG. 1. Schematic drawing of the corneal endothelial cell layer viewed from the apical side. The hexagonal pattern is very characteristic of this epithelium (16). For its histology, see a textbook (44) plus studies made in the laboratory of Ives Pouliquen (41, 42) and the calculations we made for its geometrical parameters (62, 91, 92).

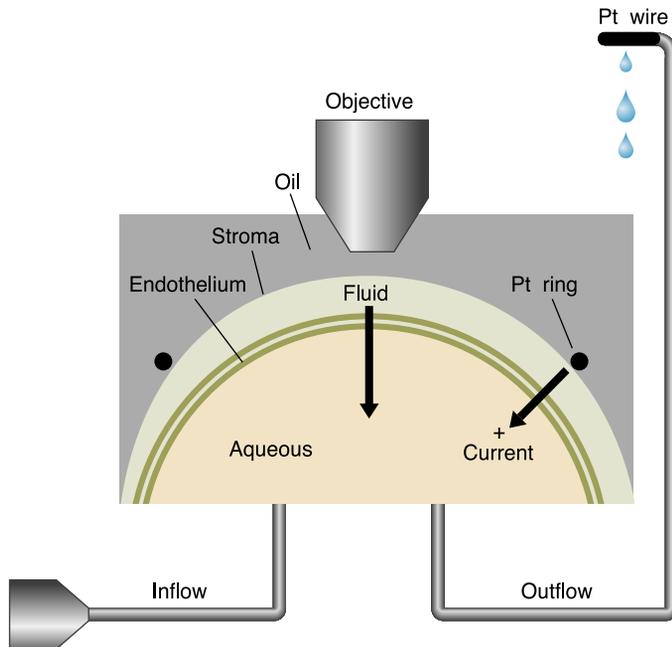


FIG. 2. Experimental arrangement utilized in Sanchez et al. (92). The rabbit cornea is denuded from its epithelium, mounted in a Dikstein-Maurice chamber (16), maintained at 37°C, and perfused with a Krebs-like experimental solution including HCO_3^- and HEPES. A hydrostatic pressure head of 20 cmH_2O at the outflow maintains normal corneal curvature. The external side of the stroma is covered with oil, so water movements take place only across the endothelium and are detected by variations in stromal thickness as determined with a microscope. Electrical currents are applied between the Pt electrodes depicted, one at the outflow opening and the other a ring in contact with the stroma.

be noted, in the Dikstein-Maurice procedure, the clamping force is less, and the corneal stroma remains in its normal, unswollen condition. These factors may account for the improved coupling detected. Moreover, in a normal-thickness (nonswollen) cornea in the steady state, endothelial fluid transport is virtual. In other words, the tendency of the pump to drive fluid from stroma to aqueous is equal and opposite to the tendency of fluid to leak back into the stroma driven by the imbibition pressure of the stromal mucopolysaccharides. For experimental convenience, when so desired, vectorial (that is, nonvirtual) fluid and electrolyte transport rates are determined in preparations in which the corneal stroma is priorly swollen, which eliminates the stromal imbibition pressure.

B) PARACELLULAR ELECTRO-OSMOSIS: EXPERIMENTAL RESULTS.

Figure 3 shows the main results (for more details, cf. Fig. 2 in Ref. 92). As Figure 3A shows, without an intact endothelial cell layer, no electro-osmotic coupling occurs. As Figure 3B shows, in corneal endothelial preparations in steady-state, corneal thickness remains constant. Subsequently, sending external electrical currents across the preparation disturbs the steady state and results in net fluid movements across the endothelium. The direction of the fluid movements corresponds to those of the currents, and the rates of fluid movements correspond to the cur-

rent intensities (Figs. 2 and 3 in Ref. 92). From other experiments (Fig. 4 in Ref. 92), integrity of the intercellular leaky tight junctions is required for electro-osmotic fluid flow.

C) OSMOLARITY OF THE SECRETION. In our current understanding of electro-osmotic coupling, electrical mobile charges in the junction will impart momentum to surrounding water molecules and will thus convect fluid across the junction. It seems reasonable to assume that the fluid convected will assume the composition it had inside the junction. Due to the effects of the macromolecules inside the junction, in our estimate (91), the osmolarity of that fluid would be ~ 0.7 of that in free solution. Of course, upon emergence, this hypotonic primary secretion would undergo osmotic equilibration across the cell membrane (largely via AQP).

This estimate is consistent with a hypothesis from Hill's laboratory for junctional flow generating hypotonic fluid (95). Using their simple estimate for partition of ions and water in the junction based on steric hindrance, one has for the partition factor S

$$S = 1 - \frac{r_s}{r_{ch}} \quad (1)$$

where r_s is the solute radius and r_{ch} is the junctional half-width. Using r values (in Å) of 1.4 for water, 3.56 for

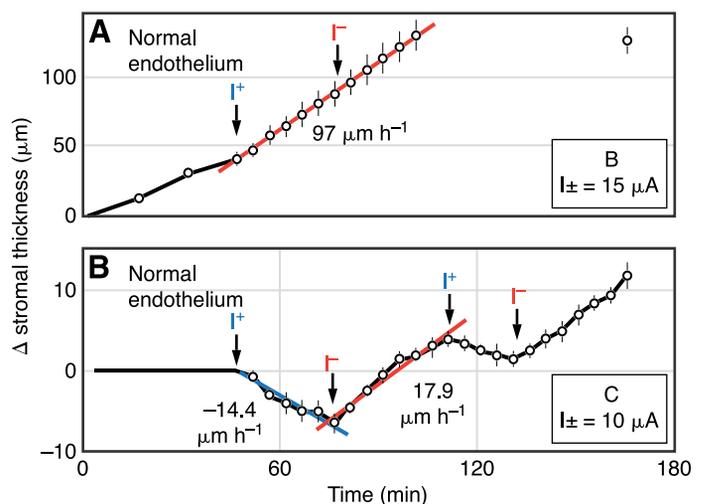


FIG. 3. A: corneal stroma with damaged endothelial layer. B: normal rabbit corneal endothelial preparation. Externally applied electrical currents (I^+ , I^-) generate transendothelial fluid movements evidenced by stromal thickness changes (ordinate). Experimental curves represent the averages of 4 or more experiments. During the control period ($0 < t < 45$ min), the thickness of the stroma remains constant, as the corneal endothelial (virtual) rate of fluid transport (stroma to aqueous) is exactly balanced by the leak (aqueous to stroma) induced by the stromal imbibition pressure. When a current I^+ is applied, fluid moves from stroma to aqueous, and the stromal thickness decreases. The opposite happens when I^- is subsequently applied. Numbers by the fitted lines denote the values of the slopes, which have dimensions of rate of fluid transport (rate of volume change per unit area). The semispherical area of the endothelium is 1.43 cm^2 . [Data from Sanchez et al. (92).]

hydrated Na^+ , and 20 for the junctional half width of the corneal endothelium, the fractional osmolarity would be $S_{\text{Na}}/S_{\text{water}} = 0.88$, or 12% hypotonic. Electrical charges in the junction would make the estimate more hypotonic.

D) SELECTIVITY OF THE JUNCTION. For fluid to move in the same direction of the current by junction-coupled paracellular electro-osmosis (Figs. 6 and 8; cf. also Fig. 10 in Ref. 92), the junction has to have fixed negative charges. There is indeed evidence (61) for some cationic selectivity for the endothelial junctions. To clarify this idea, Figure 9 presents a cross-section of an idealized capillary that is the site of simple electro-osmotic coupling à la Smoluchowski. To be noted, in a resting fluid, positive charges tend to crowd into the fluid boundary layer near the wall, as they are attracted there by the fixed negative charges at the wall. Each mobile ion in the water phase is surrounded by its hydration sphere. As an electric field is imposed (from anode to cathode), positive charges will move to the right, and negative ones to the left. In the bulk of the capillary, these opposite movements roughly cancel each other. However, the events at the wall are highly asymmetric: while the positive charges and their hydration shells move the fluid boundary layer (to the right), the negative charges and the wall are fixed. The momentum this creates in the mobile charges is communicated to the rest of the fluid, which obligingly moves almost as a plug. As the usual adhesion of the boundary layer of fluid to the wall is broken, this turns out to be a highly efficient way to mobilize the fluid column.

This poses the question of whether the fixed negative charges can be experimentally manipulated. We have examined this with the experiments exemplified in Figure 6 of Reference 92. As can be seen there, the same current I^+ is applied to a standard preparation and to another one treated with the polycation polylysine. The direction of fluid flow is the usual one in the untreated preparation (bottom), but reverses in the polylysine-treated one (top). This observation is consistent with paracellular, junction-coupled electro-osmotic flow, with the polylysine agent causing a reversal of the junctional charge and hence a reversal of the direction of current-induced fluid flow.

E) PARACELLULAR ELECTRO-OSMOSIS: THEORY. We have begun to examine the physicochemical mechanisms that might be at play in the paracellular pathway. Within it, we have concentrated our attention on the leaky tight junctions (TJ); they are the site of a very high voltage drop and therefore the likeliest place for paracellular electro-osmotic coupling.

Recent developments on tight junction physiology (108) have provided the necessary background. To our knowledge, ours (91) is the first treatment that analyzes electro-osmosis in the leaky TJs in detail. We assume that TJs possess a significant amount of protruding macromolecules (Fig. 3 in Ref. 91), some of them electrically charged. This results in a partition coefficient that is

smaller for electrolytes in the TJ compared with free solution. This in turn could be a basis for hypotonic secretion, as convection will carry fluid at the TJ local concentration.

Compared with the intercellular spaces in series, we estimate that the junctions constitute some 80% of the total tissue-specific resistance. This has experimental support in that cultured bovine corneal endothelial layers decrease their resistance (initially $28.5 \pm 0.6 \Omega \cdot \text{cm}^2$) by 80% when treated with 10 mM EGTA (51). Calculations based on endothelial geometrical parameters and solution-specific conductivities plus reasonable assumptions agree with these results. We calculate the resistance of the intercellular spaces R_s and the junction R_j as

$$R_s = \frac{sl}{cfs \times per \times sw} = 5.6 \Omega \cdot \text{cm}^2$$

$$R_j = \frac{jl}{cj \times per \times jw} = 20.1 \Omega \cdot \text{cm}^2$$
(2)

where sl is the space length (12 μm), cfs is the specific conductivity in free solution (0.02 S/cm), per is the total cell perimeter (1,020 cm/cm²), sw is the space width (300 Å), jl is the junction length (1 μm), cj is the specific conductivity inside the junctions (0.012 S/cm, estimated to be ~62% of that in free solution), and jw is the junctional width (40 Å).

The voltage drop across the junctions can therefore be calculated to be $R_j/(R_j + R_s)$ of the total potential difference (say 0.74 mV), or 0.58 mV. We assume this takes place across the estimated combined thickness of the strand regions of the junction, 100 nm (91). The resulting electrical field is very large, 5.8 kV/m, which is a determinant in the generation of electro-osmotic coupling.

The model embodies systems of electrohydrodynamic equations for the sterically restricted or strand regions of the TJ. We begin with a modified Brinkman equation (7) (an expression used originally to describe water flow across polymeric materials)

$$\frac{d^2 v_2}{dx^2} + E_2 \rho_{\text{mov}}(x) - K_{\text{fr}} v_2(x) = 0$$
(3)

with boundary conditions as follows

$$\delta_2 = \frac{h_2}{2}; v_2(-\delta_2) = v_2(\delta_2) = 0 \quad (v = \text{at the walls})$$

where $v_2(x)$ is velocity of water in the TJ; h_2 is TJ width; δ_2 is TJ half-width; x and z are coordinates perpendicular and parallel to the direction of water flow, respectively; E_2 is electrical field along the z direction in the strand region of the TJ; $\rho_{\text{mov}}(x)$ is space charge density of the movable charges in the TJ; and k_{fr} is the friction coefficient between water and fixed structures in the TJ.

The second term in *Equation 3* represents the force exerted by the movable electrical charge on the water; this term is absent the original Brinkman equation. If the third term is also absent, then *Equation 3* becomes the classical Stokes equation.

In addition, we introduce the corresponding version of the Poisson-Boltzmann equation

$$\frac{d^2\varphi_2}{dx^2} = \frac{1}{\varepsilon\varepsilon_0} \left\{ Fcn \left[\exp(-F\varphi_2(x)/R_gT) - \exp(-F\varphi_2(x)/R_gT) \right] + \rho_{\text{fix}} \right\} \quad (4)$$

with boundary conditions (Gauss condition)

$$\frac{d\varphi_2}{dx}(-\delta_2) = -\frac{\sigma_2}{\varepsilon\varepsilon_0}; \quad \frac{d\varphi_2}{dx}(\delta_2) = \frac{\sigma_2}{\varepsilon\varepsilon_0}$$

where φ_2 is electrostatic potential in the strand region of the TJ, F and R_g are Faraday's and the general gas constants, respectively; ε is the dielectric constant of water (35°C); ε_0 is the permittivity of free space; c is the ionic concentration in free solution; n is the partition coefficient for an ion in the TJ versus free solution ($n_{\text{Na}} \sim n_{\text{Cl}} \sim n$); T is body temperature; ρ_{fix} is space charge density of macromolecules in the strand region of the TJ; and σ_2 is the surface charge density in the TJ membrane.

After linearizing the Poisson-Boltzmann equation, we obtain analytical solutions for a system of these two equations. We are able to derive expressions for the water flow Q_2 across the TJ and the electrical conductivity in the TJ. For the water flow we have

$$Q_2 = \frac{Fc_{\text{fix}}E_2}{\eta}(L_B)^2f - \frac{\varepsilon\varepsilon_0E_2\zeta_2}{\eta}p \quad (5)$$

$$L_B = \sqrt{\frac{\eta}{k_{\text{fr}}}}; \quad f = \left(1 - \frac{L_B}{\delta_2} \tan h \frac{\delta_2}{L_B} \right);$$

$$\alpha = \frac{L_{D2}}{\delta_2} - \frac{1}{\sqrt{ncos h(F\varphi_D/R_gT)}}$$

$$\gamma_1 = \left[1 - \frac{L_B}{L_{D2}} \cot h \left(\frac{\delta_2}{L_{D2}} \right) \tan h \left(\frac{\delta_2}{L_B} \right) \right];$$

$$\gamma_2 = \left[1 - \left(\frac{L_{D2}}{\delta_2} \right)^2 \right]; \quad p = \alpha \frac{\gamma_1}{\gamma_2}$$

where c_{fix} is the concentration of fixed space negatively charged centers in the strand regions of the TJ, η is water viscosity, L_B is Brinkman length, ζ_2 is zeta potential in the TJ, and L_{D2} is Debye length in the TJ.

In *Equation 5*, the first term represents the influence of the TJ volume charge on the generation of volume flow by the electrical field in the TJ. The second term is similar to the Smoluchowski equation with one important difference: the parameter p takes into account the friction between water flow and the macromolecules into the volume of the TJ.

For the electrical conductivity κ_2 of the strand regions of the TJ we have

$$\kappa_2 = F^2nbc \left[U_{\text{Na}}e^{-(F\varphi_D/R_gT)} \left(1 - \alpha \frac{F\zeta_2}{R_gT} \right) + U_{\text{Cl}}e^{(F\varphi_D/R_gT)} \left(1 + \alpha \frac{F\zeta_2}{R_gT} \right) \right] b = \left[\frac{1 - v_p}{1 + v_p} \right]^2 \quad (6)$$

where φ_D is phase potential, a logarithmic function of c_{fix} (*Eq. 10b* in Ref. 91); U_{Na} and U_{Cl} are ion mobilities; and V_p is fractional volume occupied by the strands.

The factor b represents the fraction by which the ionic mobility in the strand region of the TJ is less than that in free solution. We estimate $V_p \sim 0.2$; hence, $b \sim 0.45$.

From these equations we can go on to compute the total resistance, electrical current (I_2), and electro-osmotic coupling ratio (Q_2/I_2) of the TJ. We illustrate the model by employing geometrical parameters and experimental data from the corneal endothelium. We find a constellation of numerical parameters for which theoretical values of transendothelial specific electrical resistance and water flow agree with experimental ones. In view of the difficulties of the analysis, we deem this agreement quite significant (91).

E. Paradoxically, Aquaporins Are Very Efficient Water Channels, But Most Likely Not the Main Route for Fluid Transport

1. Recent evidence from our laboratory: fluid transport across AQP1 null mice corneal endothelium

A paper with our findings has been recently published (53). As in the prior paragraphs, we comment on some relevant highlights.

As previously reported (Figs. 2 and 3 in Ref. 53), the rates of fluid transport by both wild-type and AQP1 null layers fall at or near a range previously described for normal endothelial preparations ($4.0 \pm 0.5 \mu\text{l}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$, Ref. 73). The rates from both groups show great overlap (Fig. 3 in Ref. 53). The averages were 4.3 ± 0.6 for the wild type and 3.5 ± 0.6 for the AQP1 null. The average rate for the wild type was therefore slightly (19%) larger. With standard statistical handling, that difference was not significant; if one perseveres in the analysis, Lorentz fits to the distributions of the rates yield peaks, with the one for AQP1 null cells falling at $1 \mu\text{l}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$ less than that for wild-type cells. It is conceivable that with more experiments a slight deficit in the AQP1 null cells could be made more apparent. Still, the fact that after nine experiments that is not the case indicates that any such difference is bound to be small and the overlap will dominate.

There is a question of whether other AQPs could be present in the tissue and be compensating for the AQP1 absence. The answer is twofold.

We have looked in corneal endothelium for the presence of mRNA encoding all AQP1 found in the eye (1–5). We found only AQP1 (116); a prior study had reached similar conclusions (30).

The deletion of AQP1 has a very noticeable effect on the endothelial osmotic permeability; the P_f value for the wild-type cells is $74 \pm 4 \mu\text{m/s}$, and that of the AQP1 null cells is $44 \pm 4 \mu\text{m/s}$ (53). From this, AQP1 accounts for ~40% of the cell P_f in the wild type, and the decrease in P_f in the AQP1 null cells has not been compensated (Fig. 4; see also Figs. 5 and 6 in Ref. 53).

From the above, the dichotomy observed in other AQP knockout systems (39) is present here as well: while P_f goes down by 40%, fluid transport decreases by perhaps 19%. Once more, this militates against an explanation of fluid transport involving transcellular local osmosis. A more extensive discussion of transcellular local osmosis is offered in section III F.

2. A role for AQP1 in endothelial fluid transport?

As shown in our paper referenced above (Figs. 5 and 6 in Ref. 53), unexpectedly, volume regulatory decrease is hindered in the AQP1 null mice (by some 40%). In this connection, as we have argued (53), there is evidence that in the endolymph-producing dark cells in the inner ear epithelium, PDZ domain-containing proteins that bind the COOH termini of EphB2 and B-ephrins can also recognize the cytoplasmic tails of anion exchangers and aquaporins (8). It is therefore conceivable that AQPs and volume-sensitive transporters might colocalize in such a way that lack of AQPs might adversely affect the function or the expression of such transporters in plasma membrane domains. In this connection, AQPs have been suggested for roles in regulation of cell processes other than as strict water channels. For instance, AQPs have been proposed

to be involved in determining cell volume set point (100), cell homeostasis (81), or osmosensing (39), so it may be fitting that AQP1 is linked somehow to volume regulation.

As mentioned above, according to estimates (91, 95), the fluid moving through the junctions may emerge hypotonic. That would lead to secondary osmotic equilibration through AQPs. This brings to mind the fact that AQPs are apparently present in every fluid-transporting epithelium. If that equilibration involves some of the mechanisms of volume regulation, that might explain why AQPs are present in leaky epithelia, and why deletion of AQPs has more pronounced effects in those epithelia that generate relatively larger amounts of fluid (39).

In summary, aside from the regulatory roles noted, AQPs do not seem to be otherwise connected to substantial transcellular water flows in leaky epithelia. Some more arguments are given in what follows to support such view.

F. Problems With Apical Osmosis

Given the results with the AQP1 null mice, some advocates of transcellular local osmosis have argued that even with a large decrease in P_f , fluid transport across leaky epithelia could perhaps still proceed if the local osmotic gradient would increase and drive the same amount of fluid as previously. This “gradient increase” proposal has however a glaring flaw. Transcellular local osmosis, say by a secretory epithelium, requires two gradients, one between intercellular spaces and the cell and another one between the cell and the apical compartment. For the case of an absorptive epithelium, the two gradients will be at the apical (mucosal) membrane, and again at the intercellular spaces. The simplest for this argument is to concentrate on the limitations inherent to the hypothetical gradients at the apical membrane, that is, the membrane facing an external, aqueous compartment.

The corneal endothelium has an important advantage: it is a monolayer of remarkably flat cells, with an absence of extensive apical infoldings or villi. This allows one to model the events at the apical cell membrane using a hypothetical flat cell membrane and one-dimensional convection-diffusion equations, as we have done in Figure 11 of Reference 23.

One tenet of local osmosis is that a local osmotic gradient will exist at this apical membrane. If the gradient forms outside the cell, the concentration profile will look as depicted in Figure 11 of Reference 23, rising immediately outside the membrane as electrolyte transport systems lead to accumulation of salt in there, and dissipating along the unstirred layer (of width $\Delta x = 50 \mu\text{m}$) clinging to the cell. With the use of the value for the osmotic permeability for the wild-type mice endothelial cell membrane ($P_f = 74 \mu\text{m/s}$), the concentration gradient ΔC (of,

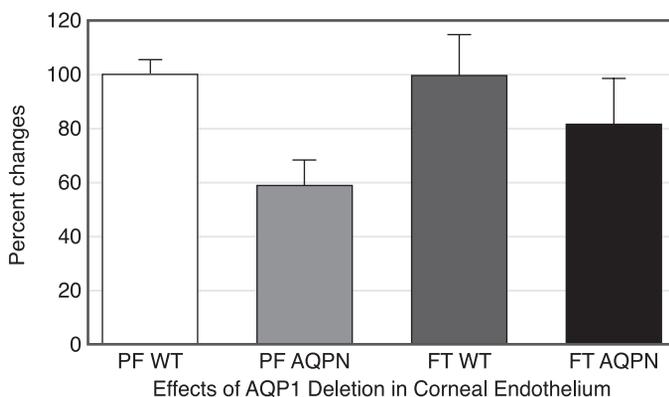


FIG. 4. Comparison of averages for cell membrane osmotic permeability (PF) and rate of fluid transport (FT) in wild-type mice (WT) and AQP1 null mice cells (AQP1N). PF values were determined by light scattering in cells plated on glass coverslips subject to a 10% hypotonic challenge. For determinations of FT, experiments were done with cell layers grown on permeable supports. [Data from Kuang et al. (53).]

say, NaCl) that would have to exist across the apical membrane to drive the experimental rate of fluid transport observed J_v ($4.3 \mu\text{l}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$) is

$$\Delta C = \frac{J_v}{2 \times P_f \times V_w} = 4.5 \text{ mM} \quad (7)$$

However, if the concentration gradient would form only outside of the cell, such gradient would result in a diffusional flux of solute J_{sd} of either ion across the thickness Δx of the unstirred layer (from the membrane boundary towards the bulk of the solution) of magnitude

$$J_{sd} = \frac{D \cdot \Delta C}{2\Delta x} = 48.4 \mu\text{eq} \cdot \text{h}^{-1} \cdot \text{cm}^{-2} \quad (8)$$

The largest flux across this preparation is that of bicarbonate, and that is at most 0.5 (43) to 0.7 $\mu\text{eq}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$ (15). Hence, the solute flux calculated above by assuming an increased gradient would be ~ 70 times larger than the largest experimental flux detected, which appears to violate the First Law of Thermodynamics.

Since the corneal endothelial P_f of the knockout mice ($44 \mu\text{m/s}$) is even some 40% less than what we just used for the “increased gradient” calculation, for the knockout mice the hypothetical local gradient necessary would be correspondingly larger and the calculated flux across the unstirred layer would be 95 times larger than the experimental value. In fact, to put this in perspective, the only apical steady-state gradient that is compatible with the flux and unstirred layer values given is a tiny one of ~ 0.07 mM.

These limitations of the presumed apical local osmosis into or out of a cell through the unstirred layer of an open compartment have appeared before in the literature. Thus, in a review from the Hill group (95), there is a comment that such (local osmosis) process would violate the convection-diffusion equation. In other words, the experimentally determined solute flux should equal the algebraic sum of the convective and diffusional fluxes in the unstirred layer. As the numerical example above shows, the diffusional term would be however almost two orders of magnitude larger than the experimental solute flux, which is a physical impossibility. Our own laboratory examined the issue of apical osmosis already in 1985 (24). We wrote then: “Much as in other models, we confirm that only rather unrealistically high values of the cell membrane permeability lead to isotonic transport. We have also found, however, that isotonic transport can occur at much lower values of the cell membrane permeability if the concentration within the cell differs slightly from that in the ambient medium.”

1. A role for the cell: secretion or absorption?

In other words, apical transcellular local osmosis might operate if the cell is made somewhat anisotonic (say hypotonic for an absorptive epithelium). There are,

however, problems with that possibility as well. Larry Liebovitch and Sheldon Weinbaum (59) examined an absorptive model based on that assumption (lateral spaces definitely hypotonic, cell less so but still hypotonic). One resulting complication was that cells exported fluid simultaneously in two opposite directions, since both the apical and basal compartments were hypertonic to them. Teleologically, it seems too wasteful for a system to have evolved in such manner. The other complication ensues from the value one would have to assume for the cell hypotonicity (perhaps 1% or 1.5 mM). As we have recently reported (54), that amount of anisotonicity would be enough to trigger cell volume regulation, which in turn would diminish or nullify the original gradient.

In view of all this, local osmosis with the cell as a coupling compartment would perhaps require an oscillatory mechanism. In fact, we have once considered a cyclic process for transcellular osmosis (20). However, upon closer analysis, that hypothetical process also presents problems. As there is only AQP1 present in both apical and basolateral membranes of the endothelium (30, 116), for that cyclic mechanism to hold the permeability of the same AQP1 would have to be modulated independently in each membrane. All this seems at the present on the bizarre side and rather unlikely.

On the other hand, oscillations connected with the control of the osmolarity of fluid transported have been hypothesized (95). In this connection, we called attention to our evidence that the primary secretion by corneal endothelium could be hypotonic (21, 92), which carries implicitly that the regulatory mechanisms of the cell would react to such apical hypotonicity. Still, these regulatory oscillations would control paracellular rather than transcellular fluid flows, which does not help the cause of transcellular local osmosis. In summary so far, an unstirred layer cannot be an osmotic coupling compartment for local osmotic fluid transport. The cell itself does not seem to be an ideal coupling compartment either, except if one makes additional assumptions. The standard assumptions in the literature are that the coupling compartments are extracellular, in all likelihood the intercellular spaces. That works somewhat better, but of course leaves still unanswered the question of the transfer mechanism at the apical membrane.

To place all this in context, there are tight epithelia that transport fluid in the absence of an external gradient, and for which the best explanation is that they do that by local osmosis. The limitations noted here for apical local osmosis somehow have apparently not been detailed before. Perhaps these arguments will help lead colleagues working in this field to address the matter both theoretically and experimentally, as no answer appears complete for transcellular local osmosis.

While those questions are central, they are not the only ones that remain for leaky or tight epithelia. It seems

worth pointing out that directions and perhaps even modes of fluid transport might change in a given tissue. One example is the lung alveolar epithelium, which during the fetal period secretes fluid into the alveoli in a process seemingly driven by Cl^- extrusion, while after birth the direction of fluid movement reverses to an absorptive one apparently driven by Na^+ absorption (5). In a similar vein, the intestinal wall is capable of performing both secretion and absorption of fluid (18). Lastly, the corneal epithelium has been found to transport fluid in those two directions. The mature epithelium in situ is tight ($R > 1 \text{ K}\Omega\cdot\text{cm}^2$) and transports very little fluid in the absorptive direction (48). However, when SV40-transformed corneal epithelial cells are grown on permeable supports, the junctions do not develop complete tightness, and the R remains an intermediate one ($R \sim 180 \text{ }\Omega\cdot\text{cm}^2$). Those leakier layers transport an appreciable amount of fluid ($5 \mu\text{l}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$) in the secretory direction (121). This poses the question of whether the elements could be present in a given tissue so that a modification such as a decrease in the specific resistance of the junctions could change the mode and direction of the fluid transport.

In view of this last example, it seems conceivable that a tight tissue would transport fluid by local osmosis, and a leaky one by paracellular flow, perhaps electro-osmosis. Beyond that, the questions of whether absorptive, secretory, leaky, and tight fluid-transporting epithelia have unified mechanisms in common, and precisely which ones are these, cannot be answered in general at present. As will not escape the reader, geometric factors such as the predominance of anion over cation transport and their directions have appeared in the literature; we wonder whether other factors such as junctional resistance might not also have some role. Hopefully future research may be influenced by these considerations.

2. Recent evidence from our laboratory: a corneal endothelial model points to paracellular electro-osmotic flow

There are several mathematical models that describe epithelial transport and electrophysiology (57, 58, 78). To model the corneal endothelium, for convenience we have adopted the methods originated by Leon Moore and colleagues (57), as applied by Alan Verkman and colleagues (32, 111). Their algorithm was suitably modified by us for computational reasons and on the basis of what is known about the presence and distribution of transport proteins in the corneal endothelial membranes. The structure of the program and our results with it are reported in a recent paper (22). Another technical development in that paper that may prove useful was that we started from 5 known parameter values and were able to build a table of 11 parameters using conservation equations (see Table 2 in Ref. 22). As we reported (22), the model is able to

reproduce experimental findings, including the characteristic low electrical potential difference across this preparation and its dependence on bicarbonate concentration (Fig. 10 in Ref. 22).

The model is to us very illuminating. It paints the endothelial cells as devoted not to the net transport of salt but instead to the generation of an intense “open circuit” local current (Figs. 6 and 8; cf. also Fig. 10 in Ref. 92 and Fig. 14 in Ref. 23). In fact, it was the insight emerging from preliminary versions of the developing model that led us to revisit the issue of paracellular electro-osmotic water flows in 2000–2001, leading to the results published shortly afterwards (92).

A sample of the model capabilities is given by how well it accounts for the effect of ambient $[\text{Na}^+]$ on trans-endothelial potential difference (Fig. 13 in Ref. 22). Intuitively, the saturation curve in that figure may have to do with the binding constants for Na^+ at sodium-bicarbonate cotransporter binding sites.

The model also predicts rates of fluid transport by the cell layer. It does so in two different modes: 1) local osmotic mode, in which water movement is given by $J_v = 2J_{s,\text{calc}}/C_{\text{iso}}$, where $J_{s,\text{calc}}$ is the total net monovalent salt transport rate (from stroma to aqueous) calculated by the program; and 2) paracellular electro-osmotic mode, in which $J_v = I_{\text{oc}} \times r$, where I_{oc} is the calculated local open-circuit current density circulating around the cells (Fig. 8) and r is a coupling ratio experimentally determined between current and fluid movements for rabbit corneal endothelium (92).

The predictions of the program are compared with the experimental results obtained when the preparations are bathed in bicarbonate-free medium. Under those conditions, transport of bicarbonate ceases, and for all practical purposes so does transport of salt. On the other hand, about half of the I_{oc} remains, in all likelihood carried mostly by the lateral Na^+ pumps and apical ENaCs and Cl^- channels. As we have reported (Fig. 10, bottom, in Ref. 22), the rate of fluid transport predicted by the model agrees with the experimental ones when paracellular electro-osmosis is assumed, and disagrees when local osmosis is assumed.

3. Recent evidence from our laboratory: fluid transport without solute transport

For many years, the thinking in the area of fluid transport has been influenced by the seminal observation by Peter Curran and Arthur K. Solomon (9) that intestinal fluid transport depended on the integrity of solute transport across that layer. However, upon closer examination, things now appear to be more complex than that. An inkling of the difficulties was brought about by an unexpected discovery: years ago, Michael Doughty and David Maurice (17) reported that the corneal endothelium could

transport fluid in the absence of bicarbonate. That finding was confirmed and attributed to residual endogenous bicarbonate by us (52) or to exogenous bicarbonate by Joseph Bonanno (6). Yet, that was not apparently the case; more recent evidence from our laboratory (14) now gives a clearer picture of the events. Important residual endothelial fluid transport (~50%) does occur in the absence of bicarbonate, as Doughty and Maurice found, and even more, it occurs under conditions when all transport of anions is absent or inhibited (14). Inhibiting Cl^- channels decreases fluid transport another ~25%, and only when Na^+ channels are inhibited is fluid transport finally eliminated (14). These findings are summarized here in Figures 5–7. To us, they mean that fluid transport is truly independent of net vectorial transport of salt and could only occur by a nonlocal osmotic mechanism, such as the paracellular junction-coupled electro-osmosis shown schematically in Figure 8.

G. The Emerging Model for Paracellular Electro-osmotic Fluid Transport in Corneal Endothelium

The model we propose for corneal endothelial fluid transport is summarized in Figure 8. The details of how the endothelial electrogenic transporters and channels contribute to generate an electrical potential difference and a resulting electrical current recirculating around the cells are covered in publications of our laboratory (Fig. 1 in Ref. 22 and Fig. 8 in Ref. 70).

Since the junction is “leaky tight,” with a specific resistance for the layer of ~20 $\Omega\cdot\text{cm}^2$ (60, 63, 91), the local current is relatively intense; for the cross-sectional area of the junctions, we calculate a current density of ~60 mA/ cm^2 . This paracellular current is carried mostly by Na^+

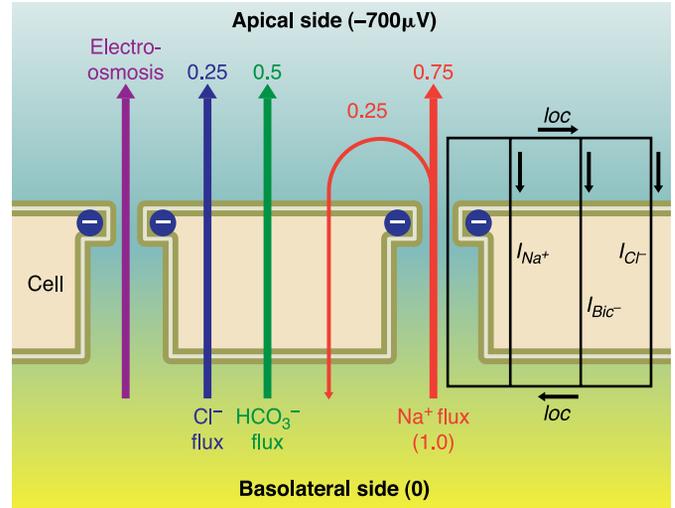


FIG. 6. To the right, a scheme depicting the open-circuit electrical current (I_{oc}) circulating across the junctions and reentering the cell via the apical membrane. I_{Na^+} , I_{Bic^-} , and I_{Cl^-} are the current components carried by Na^+ , HCO_3^- , and Cl^- , respectively (for the identity and location of endothelial transporters and channels, cf. Ref. 22). To the left are the electro-osmotic transjunctional fluid flow and the major ionic fluxes traversing the cell and the paracellular pathway. Numbers by the fluxes denote approximate fractional size of each flux, taking $I_{oc} = 1.0$. By coincidence, these numbers are also close to the chemical magnitude of the fluxes (in $\mu\text{mol}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$).

across the junctions (Fig. 10 in Ref. 92). As the junctions are cation selective (61), the current generates fluid movement by electro-osmotic coupling. For an intuitive assessment, Figure 9 shows how such coupling would take place in an idealized junction; the electric field causes the boundary layer of ions plus their hydration shells next to the membrane to move, and the rest of the junctional water follows in pluglike fashion. In actual junctions, the geometry and therefore the details of the electro-osmotic coupling would be more complex, as described in our recent paper on the subject (91), although the basic phys-

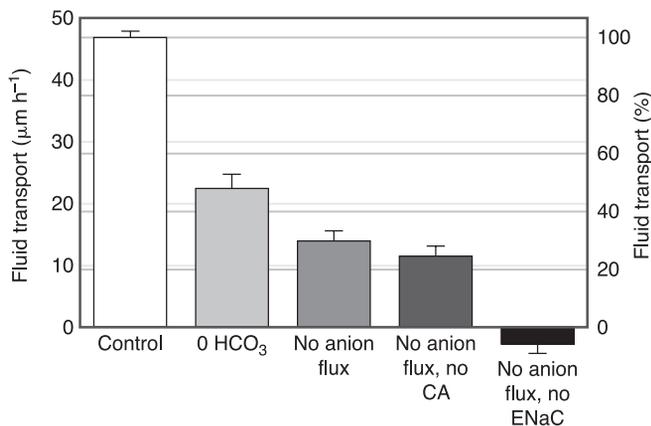


FIG. 5. Transendothelial rate of fluid transport was determined in rabbit corneal preparations with the Dikstein-Maurice procedure (16). No anion flux means that HCO_3^- is absent and Cl^- channels are inhibited. CA, carbonic anhydrase; ENaC, epithelial Na^+ channels. [Data from Diecke et al. (14).]

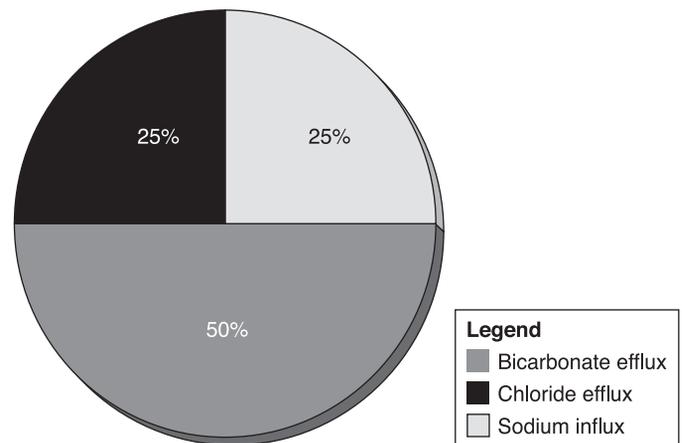


FIG. 7. Proportions of the total open-circuit electrical current carried across the apical membrane of rabbit corneal endothelium by each ion depicted. [Data from Diecke et al. (14).]

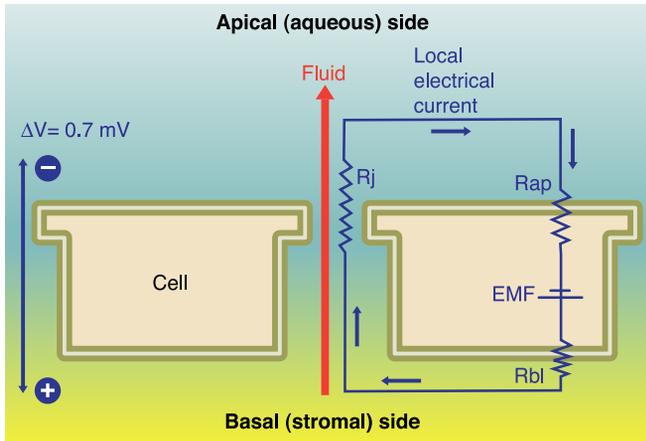


FIG. 8. Model of the electro-osmotic coupling between fluid flow and local open-circuit electrical current density evolved from several recent publications from our laboratory (14, 22, 91, 92). R_j , R_{ap} , and R_{bl} are specific resistances of the junction, apical membrane, and basolateral membrane, respectively. EMF is the equivalent electromotive force generated by cellular transporters. Only a small part of it is detected externally as the transendothelial electrical potential difference (ΔV) depicted.

ical chemistry still rests on the presence of fixed electrical charges as championed by Torsten Teorell (101, 102). As we have previously discussed (91), “. . . efficient electro-osmotic coupling would occur due to unique environmental characteristics to be found only in the strand regions of leaky tight junctions. The picture that appears here is that of a milieu relatively depopulated of ions, and subject to a very intense transverse electric field”

H. Final Considerations

Some teleological considerations appear in order. Perhaps the existence and the location of electrogenic transporters and channels are telling us something very fundamental about the function of these layers. There does not seem to be an explanation of why epithelia in general, and specifically leaky epithelia, would have evolved to have an electrical potential difference across the layer. In principle, salts could simply be transported neutrally. In a similar vein, apical Na^+ channels that allow Na^+ to leak back into the cell would not make sense if the task of an epithelial cell would be to transport salt from the serosal (basal) to the luminal (apical) side. However, both of these apparent incongruencies suddenly make sense if the raison d'être of these epithelia is to perform tasks such as electro-osmosis. The electrical potential might not be an evolutionary leftover but a central feature. The Na^+ channel would not be apical by accident but to help build up the local current meant for electro-osmosis. As mentioned above, aside from the corneal endothelium (62, 92), there is evidence for electro-osmosis in small intestine (33, 72), kidney proximal tubule (99),

and frog skin glands (75). Hence, it would be desirable if the presence of electro-osmosis would be explored in other fluid-transporting epithelia.

Electro-osmotic coupling would result in somewhat (perhaps 30%) hypotonic emerging fluid (23, 91). This entails that the fluid left behind at the intercellular spaces might be correspondingly hypertonic. Such osmolarity difference in turn might be sensed by the cell and trigger mechanisms that would affect sites for regulation at basolateral and apical sites for HCO_3^- and Na^+ transports, and perhaps also at the junction so as to modify the characteristics of the coupling. It is conceivable that such regulation might take place with some degree of periodicity. There may be a role for AQP1 in this regulation, which would explain the mild effects seen on fluid transport in this and other preparations in experiments done with AQP1 null cells. This would explain what has been noted by Verkman and colleagues, namely, that effects of AQP deletion are more pronounced in epithelia that generate higher rates of fluid transport. Thus AQP deletion reduced near-isosmolar fluid transport in kidney proximal tubule (93) and salivary gland (65), where fluid transport is rapid, but not in lung (2, 64), lacrimal gland (71), sweat gland, (97), or corneal endothelium (53) where fluid transport is relatively slow.

As for the other theories proposed for fluid transport, much evidence for paracellular flow for several leaky epithelia was generated by the laboratory of Adrian Hill starting in the late 1970s (as recently reviewed in Ref. 95). They theorized that solute-solvent coupling was due to junctional mechano-osmosis (95).

Importantly, our evidence for electro-osmotic coupling in corneal endothelial fluid transport generated after 2002 (21, 22, 92) is consistent with the mechanism proposed by Hill and colleagues. The junctional coupling we propose is electro-osmotic instead (92). We deem that our

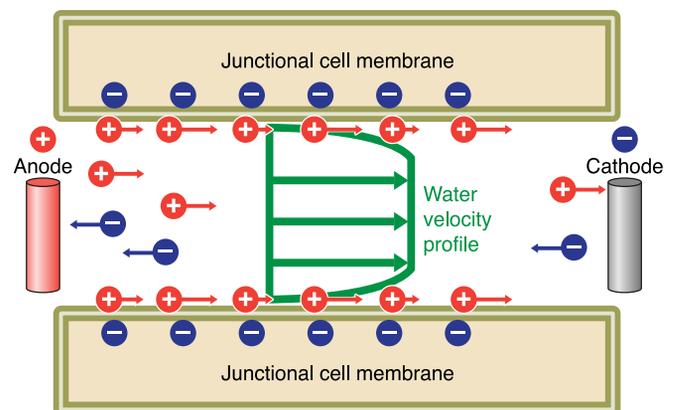


FIG. 9. Idealized scheme of electro-osmotic coupling in a simplified intercellular leaky tight junction. For simplicity, ionic hydration shells, molecular protrusions into the junctional space, and positive fixed charges are not depicted. Negative charges in the membranes are fixed. For a detailed treatment, cf. Ref. 91.

proposal is a qualitative jump that may bring the field very near a solution to these long-standing questions. Interestingly, we have also arrived at a paradigm of paracellular flow, which seems a remarkable convergence for two laboratories using different methodologies, and working independently.

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This review is in honor of the recently deceased Dr. Friedrich P. J. Diecke, a former Chairman of the Department of Pharmacology and Physiology, University of Medicine and Dentistry of New Jersey, New Jersey Medical School. Dr. Diecke and the author had a very fruitful collaboration dating back to 1998. He was to have been a coauthor of this review, but disease prevented that. Among his many virtues, Dr. Diecke counted with a privileged mind, a unique sense of humor, a classical physiological training, and an encyclopedic knowledge of general and comparative physiology and the biological transport literature. Several ideas discussed here carry his brilliant imprint. An outstanding scientist and a gentleman, he will be missed.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author.

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