Water and solute permeabilities of medullary thick ascending limb apical and basolateral membranes

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Rivers, Rickey, Anne Blanchard, Dominique Eladari, Francois Leivel, Michel Paillard, Rene-Alexandre Podevin, and Mark L. Zeidel. Water and solute permeabilities of medullary thick ascending limb apical and basolateral membranes. Am. J. Physiol. 274 (Renal Physiol. 43): F453–F462, 1998.—The medullary thick ascending limb (MTAL) reabsorbs solute without water and concentrates NH4 in the interstitium without a favorable pH gradient, activities which require low water and NH3 permeabilities. The contributions of different apical and basolateral membrane structures to these low permeabilities are unclear. We isolated highly purified apical and basolateral MTAL plasma membranes and measured, by stopped-flow fluorometry, their permeabilities to water, urea, glycerol, protons, and NH3. Osmotic water permeability at 20°C averaged 9.4 ± 0.8 × 10^-4 cm/s for apical and 11.9 ± 0.5 × 10^-4 cm/s for basolateral membranes. NH3 permeabilities at 20°C averaged 0.0023 ± 0.00035 and 0.0035 ± 0.00080 cm/s for apical and basolateral membranes, respectively. These values are consistent with those obtained in isotonic tubules and account for known aspects of MTAL function in vivo. Because the apical and basolateral membrane unit permeabilities are similar, the ability of the apical membrane to function as the site of barrier function arises from its very small surface area when compared with the highly redundant basolateral membrane.

The medullary thick ascending limb of Henle (MTAL) reabsorbs a substantial proportion of the filtered load of NaCl without its accompanying water, diluting the urine (2, 12, 32). This epithelium also reabsorbs NH4 while apparently excluding NH3, so that a lumen to interstitium gradient of NH4 can be formed despite a luminal pH that is acidic with respect to that of the plasma (5, 8, 9, 25). Finally, this epithelium exhibits a low permeability to urea (11). The low transepithelial permeabilities of this segment permit it to dilute the urine, create the solute gradients (with the countercurrent multiplier) that drive the concentrating mechanism, and generate the high levels of interstitial NH4 that are needed to excrete the body’s daily acid load.

It is hypothesized that the barrier function of this epithelium is localized primarily in the apical membrane (30). However, nearly all of the relevant data have come from studies of isolated perfused tubules and cell suspensions (2, 8, 9, 25). These studies indicate that the apical membrane is the site of barrier function, but they do not reveal whether the lower apical permeabilities result from differences in surface area or differences in permeability per unit surface area. This problem can be generalized to other barrier epithelial cells, where it is also unknown whether the permeability properties of the membrane domain itself or differences in surface area dominate in determining the rate of flux across the apical or basolateral domain of the plasma membrane. What is needed are studies of the permeability properties of apical and basolateral membranes in isolation.

Until recently, it has been difficult to examine MTAL apical and basolateral membrane permeabilities independently, because they have not been available in highly purified fractions. However, Attmane-Elakeb et al. (1) have developed a method relying on calcium binding and differential centrifugation to isolate both apical and basolateral membrane fractions. The resulting final fractions exhibit 10-fold or greater purification of apical membrane or basolateral membrane-specific marker enzymes. Moreover, the activities and protein labeling of Na+/H+ exchanger (NHE) isoforms (NHE-1 and NHE-3) are localized appropriately in these fractions, with NHE-3 markedly enriched only in the apical membrane fraction and NHE-1 markedly enriched only in the basolateral membrane fraction (1).

The present studies used these highly purified apical and basolateral membrane fractions from the rat MTAL to define the passive permeability properties of these membranes. Stopped-flow measurements of permeabilities to water, urea, glycerol, protons, and NH3 were performed, and the results are compared with those obtained in prior studies using perfused tubules and cell suspensions, as well as to permeabilities in other barrier epithelia.

METHODS

Preparation of apical and basolateral membrane vesicles. Suspensions of MTAL tubules were prepared from the inner stripes of the rat outer medulla exactly as described previously (1); the procedure was performed in the same laboratory by the same workers as previously described. The inner stripe of the outer medulla of male Sprague-Dawley rats was excised under the dissecting microscope and subjected to collagenase digestion. Because MTAL segments are far more resistant to this treatment than other segments from the inner stripe of the outer medulla, this step results in a highly purified suspension of MTAL (see Ref. 1). Thus nearly all (95–98%) of the tubules in these suspensions were labeled with antibody directed against the Tamm-Horsfall protein. Suspensions were homogenized in a buffer containing 250 mM sucrose and 20 mM tris(hydroxymethyl)aminomethane.
(Tris)-N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), pH 7.4, containing 1 mM dithiothreitol and 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride. To permit permeability studies, 10 mM 5,6-carboxyfluorescein (CF; Molecular Probes, Juncion City, OR) was added to the homogenization buffer, and the pH was adjusted to 7.0 with solid Tris. Fractionation of the homogenate and purification of apical and basolateral membrane fractions were performed exactly as described (1) at 0–4°C. The preparation was then express mailed from Paris to Pittsburgh on ice in homogenization buffer containing 10 mM CF. Because of variability in international express mail service, the preparations arrived 1–5 days after they were made. Permeability values did not vary in any way with different travel times. Upon arrival in Pittsburgh, the suspensions were washed and resuspended in 250 mM sucrose and 20 mM HEPES buffered with NaOH, pH 7.40.

To determine further the purity of the apical and basolateral membrane preparations, bumetanide-sensitive 22Na+ uptakes were measured at room temperature using a rapid filtration technique as described previously (1). Apical and basolateral membrane vesicles were equilibrated in buffer containing 300 mM sucrose and 100 mM Tris-HEPES, pH 7.0. They were then diluted 1:11 into either the same medium or media containing 22NaCl, 200 mM sucrose, 50 mM KCl, 100 mM Tris-HEPES, pH 7.0, 1 mM amiloride, and 0 or 1 mM bumetanide. When present, bumetanide was added to both the preincubation and incubation solutions. The flux measurements were terminated by rapid filtration of the vesicles through Millipore 0.45-µm HAWP filters, and the filters were washed with ice-cold 250 mM potassium gluconate and 20 mM Tris-HEPES, pH 7.4. In all experiments, vesicle uptake was corrected for nonspecific isotope binding to the filter. Uptake was determined by detecting radioactivity in a scintillation counter; filters were suspended in 3 ml of scintillant (Filter Count; Packard).

Permeability measurements. Permeability measurements were performed as described (14, 18) using a stopped-flow spectrofluorometer (SF.17MV; Applied Photophysics, Leatherhead, UK) with a measured dead time of 0.7 ms to monitor the fluorescence quenching of CF entrapped within the vesicles. Extravesicular fluorescence was quenched completely using anti-CF antibody. The excitation wavelength was 490 nm. The emitted light was filtered with a 515-nm cutoff filter. Fluorescence data from the stopped-flow device from 5 to 10 individual determinations were averaged and fit to a single exponential curve using software supplied by Applied Photophysics. The software utilizes a nonlinear regression (Marquardt) algorithm calculated from the time course using the “Curfit” routine.

Before stopped-flow permeability measurements, each preparation was examined on a standard spectrofluorometer (SLM-Aminco 500C; SLM-Aminco, Rochester, NY). The concentration of entrapped CF was defined by measuring the increase in CF fluorescence that occurred when the vesicles were lysed with Triton X-100. The change in relative fluorescence was compared with a CF quench curve and confirmed, for each preparation, that the concentration of CF inside the vesicles was 10 mM. In addition, the osmotic behavior of each preparation was examined by sequentially raising extravesicular osmolality with small aliquots of a 2 M sucrose solution. As osmolality rose by each increment, the vesicles shrank, as indicated by increasing CF quenching (reduced fluorescence).

Over the osmolalities used in these studies, the relationship between relative fluorescence and the relative volume (predicted from the external osmolality) was linear. From the CF quench curve, the concentration of CF inside the vesicles could be calculated and appeared to rise in a manner entirely consistent with the predicted shrinking of the vesicles if they acted as perfect osmometers (see Fig. 1 and Refs. 14, 18, 19).

Water and small nonelectrolyte permeabilities. Osmotic water permeability (Pw) was determined by abruptly doubling the osmolality of the extravesicular solution (homogenization solution) with additional sucrose and monitoring the time-dependent quenching of entrapped CF. Upon mixing, intravesicular CF concentration increased as water efflux occurred, leading to concentration-dependent CF quenching. Urea permeability was measured by preloading the vesicles with 600 mM urea and abruptly diluting the vesicles in a buffer of the same osmolality as that inside but containing 300 mM urea and 300 mM additional sucrose as a balancing osmoticant. After mixing, the urea effluxed down its concentration gradient. As intravesicular urea concentration fell, an osmotic gradient was created, leading to efflux of water, vesicle shrinkage, and self-quenching of entrapped CF.

Pw was calculated from the time course of relative fluorescence by comparing single-exponential time constants fitted to simulated curves in which Pw was varied. Simulated curves were calculated using a commercially available software package (MathCad) from the osmotic permeability equation

\[ \frac{dV_{rel}}{dt} = (P_w)(SAV)(MVW) \cdot \left[ \frac{C_{in}}{V(t)} \right] - C_{osm} \]  

where Vrel is the relative volume of the vesicles at time t, SAV is the vesicle surface area-to-volume ratio, MVW is the molar volume of water (18 cm³/mol), and Cin and Con are the initial concentrations of total solute inside and outside the vesicle, respectively (3, 6, 14, 20). Because the volume within the vesicle was small compared with the volume outside, it was
assumed that $C_{\text{ext}}$ remained constant throughout the experiment. Parameters from the exponential fit (amplitude and end point) were used to relate relative fluorescence to relative volume using boundary assumptions that relative fluorescence and volume are 1.0 at time 0 and that relative volume reaches a known value (if at time 0 the osmolality outside is double that, inside, the relative volume reaches 0.5) at the end of the experiment.

Calculations of small nonelectrolyte (urea or glycerol) permeabilities were performed using the following equation

$$\frac{dV_{\text{rel}}}{dt} = P_{\text{solute}}(SA/V_0)(1/900)(1,200/V_{\text{rel}} - 1,500)$$

which has been derived and validated previously (3, 6, 14, 20). $P_{\text{solute}}$ is the permeability coefficient (cm/s) for solute, $V_0$ is the relative volume of the vesicles at time 0, and SA is the surface area of the vesicles. By use of parameters from the single-exponential curve fit to the data, $P_{\text{solute}}$ was solved using MathCad.

Proton permeability. Proton permeability ($P_{\text{H}^+}$) was determined by monitoring pH-sensitive CF quenching on the stopped-flow device (14, 18). Because CF at high concentrations (10 mM) is a poor pH indicator, vesicles were incubated at 4°C for 24 h in 200 µl of homogenization buffer lacking CF to reduce intravesicular CF concentration to 0.1–100 µM. After addition of sufficient extravesicular antibody to quench all extravesicular CF fluorescence, the relationship between relative fluorescence and pH was defined by equilibrating the vesicles with successively lower pH values on the fluorescence spectrophotometer. Over the range of pH values used, pH and relative fluorescence were linearly related (see Fig. 1). Buffer capacity was determined by measuring the change in intravesicular pH effect by addition of 10 mM sodium acetate as described (14, 16). $P_{\text{H}^+}$ was determined by abruptly reducing the extravesicular pH from 7.4 to 6.5 and monitoring the fall in intravesicular pH to the same level. As noted above, the solution lacked bicarbonate. Fluorescence data from the stopped-flow device were averaged and fit to single exponential curves, and fitting parameters were used to solve the equation for $P_{\text{H}^+}$. In some cases, there was a slow linear slope to the data after the initial rapid drop in fluorescence. Under these circumstances, the curves were fitted with single exponentials truncated after 85% of the excursion was completed or with single exponentials with a linear component added on. These approaches led to close fitting of the initial pH drop data and did not lead to wide variations in rates. The general equation for $P_{\text{H}^+}$ is

$$J_{\text{H}^+} = (P_{\text{H}^+})(SA)(\Delta C) = (\Delta pH/\text{l})/(\text{BCV})$$

where $J_{\text{H}^+}$ is the flux of protons, $\Delta C$ is the initial difference in concentration of protons between the inside and outside of the vesicle, $\Delta pH$ is the excursion in pH when time equals 1/10 t, t is the time constant of the single exponential curve describing the initial change in fluorescence as a function of time, and BCV is the buffer capacity of an individual vesicle.

NH$_3$ permeability ($P_{\text{NH}_3}$) was determined by monitoring pH-sensitive fluorescence of CF on the stopped-flow device after rapid mixing of vesicles at pH 6.8 with ammonium chloride at a final concentration of 10 mM (14, 18). The small amount of NH$_3$ present in the ammonium chloride permeates the membrane and titrates intravesicular protons, forming NH$_4^+$ and thereby raising intravesicular pH. The final intravesicular pH was determined from the linear correlation between relative fluorescence and pH. With the use of the rate of change of intravesicular pH, the final intravesicular pH, and the buffer capacity, $P_{\text{NH}_3}$ was calculated as described (14, 18).

Determining vesicle size. Vesicle diameters were determined by quasi-elastic light scattering using a Nicomp model 270 submicron particle analyzer.

Statistics. Each flux measurement was performed 5–10 times, and the curves were averaged to form a single curve. These averaged curves were then fitted, and permeabilities were calculated. An “n” consists of a single preparation. Groups were compared using Student’s t-test, and P values < 0.05 were considered statistically significant.

RESULTS

Purity of the vesicle preparations. Because of the presence of CF in the preparations and the need to maximize yield for permeability studies, the preparations used for permeability studies were not evaluated for purity using enzyme markers. However, enzyme markers are monitored routinely and were performed on other preparations contemporaneous with those sent to Pittsburgh. In these studies, apical membrane vesicles were enriched 10.5 ± 0.5-fold for the luminal marker γ-glutamyltransferase and were de-enriched 0.65 ± 0.03-fold for the basolateral marker Na-K-ATPase. By contrast, basolateral membrane vesicles were enriched 10.2 ± 1.0-fold for Na-K-ATPase and were marginally enriched 1.7 ± 0.2-fold for γ-glutamyltransferase (n = 4 preparations). These values are entirely comparable to those published previously (1).

To define further the Na$^+$ permeability properties of these preparations, the effect of extravesicular KCl and bumetanide on Na$^+$ uptake was examined. For apical membrane vesicles, $^{22}\text{Na}^+$ uptake averaged (in pmol·mg$^{-1}$·s$^{-1}$) 15.0 ± 0.9 in the absence of KCl (control), 29.0 ± 1.2 in the presence of KCl, and 11.5 ± 0.9 in the presence of both KCl and bumetanide. By contrast, for basolateral membrane vesicles, uptake averaged 20.3 ± 1.6 for control, 22.1 ± 1.2 with KCl, and 15.5 ± 2.4 with KCl and bumetanide. For all values, n = 6, and uptake determinations were performed on two individual preparations of apical or basolateral membranes. KCl stimulated and bumetanide then inhibited $^{22}\text{Na}^+$ uptake significantly only in the apical membrane preparation.

Size of vesicle populations. To determine permeabilities from fluorescence quenching data, it is essential to define the diameters of the vesicles. Figure 2 shows representative size histograms for apical and basolateral membrane vesicles. As is standard for such measurements, the abscissa scale is logarithmic. Diameters of apical membrane vesicles averaged 250 ± 38 nm, whereas those of basolateral membrane vesicles averaged 306 ± 27 (SD) nm (n = 4). The P value comparing vesicle sizes (P < 0.055) was slightly too high to denote a statistically significant difference in sizes among the two populations. These values are entirely consistent with those obtained by measuring the profiles of the vesicles on electron micrographs (1). In addition, the range of diameters is relatively tight and resembles that obtained in lysosomes, gastric apical membranes, and liposomes in our laboratory (18, 31, 33).

$P_{\text{i}}$ of MTAL apical and basolateral vesicles. Figure 3 shows water flux measurements in apical and basolateral membrane vesicles at 20 and 37°C. Apical mem-
brane $P_f$ averaged (in cm/s) $9.37 \pm 0.77 \times 10^{-4}$ (n = 5) at 20°C, and two values obtained at 37°C were 33.7 and $33.2 \times 10^{-4}$ cm/s. $P_f$ values for basolateral vesicles at these temperatures were $11.9 \pm 0.5 \times 10^{-4}$ cm/s (n = 5) at 20°C, and two values at 37°C were 42.2 and $43.3 \times 10^{-4}$ cm/s. At 20°C, apical membrane $P_f$ was significantly lower than basolateral membrane $P_f$ (P < 0.0003). $P_f$ measurements were performed at multiple temperatures, ranging from 10 to 37°C. These values were used to determine the activation energy for water flow (Fig. 4), which averaged $14.1 \pm 0.4$ for apical and $14.3 \pm 0.6$ kcal/mol for basolateral membrane vesicles.

Small nonelectrolyte permeabilities of MTAL apical and basolateral vesicles. Figure 5 shows urea and glycerol permeability measurements performed at 20°C in apical and basolateral vesicles. Urea permeability averaged $5.33 \pm 1.1 \times 10^{-7}$ cm/s (n = 4) in apical and $6.59 \pm 1.2 \times 10^{-7}$ cm/s (n = 3) in basolateral vesicles. In addition, glycerol permeability was measured and averaged $2.73 \pm 0.87 \times 10^{-7}$ cm/s (n = 3) in apical and $2.15 \pm 0.09 \times 10^{-7}$ cm/s in basolateral vesicles. Both urea and glycerol permeabilities did not differ significantly when apical and basolateral membrane permeabilities were compared.

Proton and NH$_3$ permeabilities of apical and basolateral vesicles. Figure 6 shows the rate of change in intravesicular pH over time when extravesicular pH was abruptly lowered from 7.4 to 6.5 for apical and basolateral membrane vesicles. Figure 6, A–B and C–D, shows fluxes in the absence and presence of 10 µM gramicidin, respectively. Gramicidin creates a pore in the membrane that conducts protons rapidly. It is apparent that $J_{H^+}$ were far more rapid in the presence of gramicidin than in its absence, indicating that movement of protons through the membrane was the rate-limiting process in the studies performed in Fig. 6, A and B (absence of gramicidin). At 20°C, $P_{H^+}$ averaged $0.0080 \pm 0.0045$ (n = 3) cm/s for apical vesicles and $0.0007 \pm 0.0003$ cm/s (n = 3) for basolateral vesicles. Proton permeabilities were not significantly different between apical and basolateral membrane vesicles.

To simplify the conditions for measurement of $J_{H^+}$, we did not employ potassium and valinomycin to

Fig. 2. Quasi-elastic light-scattering profiles of apical (A) and basolateral (B) membrane vesicles from rat medullary thick ascending limb. Ordinate shows percentage of vesicles while abscissa shows vesicle diameter. Both preparations behave as single populations, permitting permeability measurements.
dissipate any voltage gradients that might form during 
J_{H^+} measurements. In multiple prior studies in vesicles 
using identical protocols, we have found little difference 
in J_{H^+} between studies performed in the presence and 
absence of valinomycin (3, 14, 19). The marked increase 
in J_{H^+} observed in the presence of gramicidin provides 
strong evidence that voltage gradients set up by proton 
influx in the absence of gramicidin did not significantly 
impede proton entry into the vesicles. Nevertheless, it 
is possible that we have modestly underestimated P_{H^+} 
in these studies.

Figure 7 shows the rate of change in intravesicular 
pH over time when vesicles equilibrated at pH 6.85 
were abruptly exposed to 10 mM NH_4Cl/NH_3. The 
extended time course of these experiments (not shown) 
showed an initial rapid rise in intravesicular pH as 
NH_3 entered the vesicles and titrated free protons, 
followed by a more gradual fall in intravesicular pH as 
the resulting pH gradient was dissipated. The gradual 
fall in intravesicular pH gave a time course roughly 
similar to that obtained in the J_{H^+} measurements. In 
these studies, Cl^- and K^+ were absent from all solu-
tions. Under these conditions, the initial rapid rise in 
intravesicular pH permits estimation of P_{NH_3}, without 
interference from fluxes of NH_4^+ via Na-K-2Cl cotrans-
porters or Na-K-ATPase. At 20°C, P_{NH_3} averaged

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**Fig. 4.** Activation energy determinations for apical (A) and basolateral (B) membrane vesicles. Water fluxes were measured as shown in Fig. 2 at several different temperatures. Ordinate, natural log of the rate constant from the fitted single exponential curve; abscissa, reciprocal of temperature in °K. Data are the means ± SD for 2 determinations.

**Fig. 5.** Time courses of urea (A) and glycerol (B) fluxes in apical and basolateral vesicles. Vesicles preequilibrated 
with permeant solute were abruptly diluted in buffer containing less of the 
permeant solute with osmolality equal inside and outside the vesicles. As the solute effluxed down its concentration 
gradient, water efflux occurred, leading to further self-quenching of entrapped carboxyfluorescein.
0.0023 ± 0.00035 cm/s (n = 3) for apical membrane vesicles and 0.0029 ± 0.00066 cm/s (n = 3) for basolateral membrane vesicles (not significant). Corresponding values at 37°C were 0.015 ± 0.003 cm/s (n = 3) and 0.010 ± 0.008 cm/s (n = 3) for apical and basolateral membrane vesicles, respectively (not significant). To ensure that exchange of H⁺ for NH₄⁺ via the NHE did not account for the rise in pH upon abrupt exposure of the vesicles to external NH₄⁺/NH₃, experiments were repeated in the presence of 1 mM amiloride. For both apical and basolateral membrane vesicles, the rise in pH as a function of time was identical in the absence and presence of amiloride (not shown).

**DISCUSSION**

Several epithelia, such as those lining the mammalian and amphibian bladders, the stomach, and portions of the nephron, are capable of generating or maintaining large chemical gradients for substances that are normally highly permeant across biological membranes (3, 6, 8, 9, 17, 20, 30, 32). These substances include water, small nonelectrolytes such as urea and...
glycerol, NH₃, and protons. The function of the MTAL depends critically on its ability to generate and maintain osmotic gradients as well as gradients for urea and NH₃. Thus this segment dilutes the urine and transports solute without water into the interstitium, permitting the generation of osmotic gradients for concentrating the urine (2, 12, 32). If the epithelium did not exhibit a strikingly low permeability to water, its ability to dilute the urine and participate in urine concentration would be abolished.

In addition, the ability of the MTAL to reabsorb NH₄⁺ without the need to create a favorable pH gradient provides the driving force which, along with the counter-current multiplier, permits the accumulation of NH₄⁺ in the medullary interstitium (5, 8, 9, 25). This accumulated NH₄⁺ is trapped in acidic urine in the collecting duct as NH₃ crosses this segment, and NH₃ is formed in the acidic lumen.

Prior studies have documented low permeabilities of the MTAL to water, small nonelectrolytes, and NH₃, but they have provided little information on the relative contributions of the apical and basolateral membrane domains to barrier function. On the basis of studies in the collecting duct and amphibian bladder, which revealed low apical and high basolateral membrane permeabilities to water (6, 22), it has been assumed that the thick ascending limb barrier to water flow occurs in the apical membrane (8, 30). However, the constitutive presence of water channels in the basolateral membrane of the collecting duct (and perhaps in the amphibian bladder) limits the applicability of these studies to the MTAL (4). In the case of NH₃, measurements of intracellular pH have suggested that less NH₃ occurs across the apical membrane than across the basolateral membrane (5, 8, 9).

To examine the permeability properties of these membranes, they were isolated, and their permeabilities were measured by stopped-flow fluorometry. Prior and present studies of these preparations reveal that they are highly enriched for markers specific for apical or basolateral domains. Moreover, as described previously, they exhibit NHE isoforms that are appropriate to their membrane localization (1). Finally, as shown in the present study, the apical membrane and not the basolateral membrane preparation exhibits KCl-dependent, bumetanide-sensitive Na⁺ uptake, demonstrating activity of the Na-K-2Cl cotransporter only in the appropriate (apical) membrane fraction.

To facilitate comparisons, Table 1 compiles permeabilities of barrier epithelia, both in intact cells and isolated membranes. Values for intact cells are corrected for actual membrane surface areas, as measured by morphometry (13) or capacitance measurements (17). It is apparent that both the apical and basolateral membrane MTAL preparations exhibit strikingly low permeabilities, which are comparable to those of other barrier epithelia. We have recently published permeability measurements using a membrane fraction from the kidney, renal lysosomes, which exhibit far higher permeabilities to water and urea (19), values comparable to those obtained in the brush borders of the proximal tubule and the small intestine. These results demonstrate that the MTAL membranes, like those of other barrier epithelia, are specialized and exhibit lower permeabilities than other membranes (24).

Permeability measurements indicate that basolateral P₇ is slightly (28%) but significantly higher than apical P₇. However, there is 10- to 14-fold more surface area of basolateral membrane than of apical membrane (13). Therefore, the differences in surface area of the two membrane domains contributes far more than differences in intrinsic permeability properties in determining the rate of water flux across the epithelium.

Transepithelial water flux was measured in rabbit MTAL by Burg and Green (2). Because of the large difference in apical and basolateral membrane surface areas and the results of our studies, which reveal similar permeabilities of the two membrane domains, the apical membrane is assumed to act as the barrier to water flow, and the osmotic gradient applied by these investigators is assumed to apply across the apical membrane. Correction of their calculated P₇ at 37°C for the membrane surface area measured by morphometry (13) gives a value of 11.6 ± 3.6 × 10⁻⁶ cm/s. Comparing this value with our measured value requires that we consider the effects of unstirred layers in the perfused tubule preparation. The relationship between measured permeabilities in the perfused tubule and the actual apical membrane permeability is

\[ 1/P_{\text{TUB}} = 1/P_{\text{AM}} + 1/P_{\text{UL}} \]  

where P₇ is the permeability coefficient for water flow measured in the tubule, P₇ is the actual value of apical membrane permeability, and P₇ is the P₇ across the unstirred layers. Substituting the value for P₇ available from the perfused tubule data and our measured value for P₇, we obtain a value for P₇ in

Table 1. Comparative permeability values among barrier epithelia

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<td>P₇ (×10⁻⁶)</td>
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Units are cm/s. P₇, water permeability; P₃, urea permeability; P₃, NH₃ permeability; P₄, proton permeability; MTAL AM, medullary thin ascending limb (MTAL) apical membranes; MTAL and CCD, intact MTAL or cortical collecting duct, respectively, corrected for apical membrane surface area per mm tubule length; toad bladder AM, toad bladder apical membranes; Mamm Bladder, rabbit urinary bladder; Mamm Bladder AM, apical membranes isolated from rabbit bladder; Gastric AM, porcine stomach apical membranes. Data are from Refs. 2, 6, 7, 11, 18, 20, and 22.
perfused tubules of $17.9 \times 10^{-4}$ cm/s. Using the diffusion coefficient of water through water at 37°C ($D_W = 3.13 \times 10^{-5}$ cm²/s; see Ref. 17) and the calculated $P_f$ of the unstirred layer in the perfused tubule preparation, $P_{UL}$, it is possible to estimate the thickness of the unstirred layer

\[ \text{Thickness} = \frac{D_W}{P_{UL}} \]  

(5)

The value obtained is 175 µm. This value compares favorably with that obtained by Schafer and Andreoli (20a) in cortical collecting ducts perfused at rates 10 times faster than the rates used in the perfused MTAL studies (110 µm). Therefore, our measured value for apical membrane $P_f$ is reasonable and predicts the ability of this epithelium to serve as a barrier to the flux of water.

The proton/hydroxyl permeability values obtained in the present study were low when compared with those obtained in unilamellar artificial liposomes of widely varying composition (14), lysosomes (19), and gastric apical membranes (15). It is notable that the permeability measurements in all of these studies were performed under nearly identical conditions, with similar pH gradients, similar methods for determining vesicle size and for determining buffer capacity, and identical instrumentation. The similarities in experimental design enhance our ability to compare these results. In the liposome measurements, reducing the fluidity of the liposome preparations markedly reduced permeabilities to water, small nonelectrolytes, and NH₃ but had little effect on proton permeabilities, suggesting that proton permeation across the lipid bilayer occurs via a mechanism distinct from that for other substances (14). We have observed far greater differences in biological preparations, with values in lysosomes 100-fold higher than those observed in this study and values in gastric apical membranes 10-fold higher than those observed in this study. From these results, it appears likely that membrane proteins play a critical role in governing the passive permeability to protons.

Studies of ammonium/ammonia fluxes in MTAL cells provided evidence that the apical membranes of MTAL cells exhibited strikingly low permeabilities to NH₃ and relatively high permeabilities to NH₄⁺, because exposure of these cells to NH₄⁺/NH₃ led to a sharp fall in intracellular pH rather than a rise (8, 9, 25). In all cells studied before the MTAL, exposure to NH₄⁺/NH₃ led to prompt alkalinization of cell pH, because the entry of NH₃ was so much more rapid than that of NH₄⁺ (8). In preliminary studies, Watts and Good (26) have measured the NH₃ permeabilities of apical and basolateral membranes of isolated perfused rat MTAL and obtained values (corrected for morphometric values of apical and basolateral membrane surface areas as well as cell volume per unit tubule length) of 0.005 cm/s for the apical membrane and 0.001 cm/s for basolateral membranes. The apical membrane value is two- to threefold lower than ours, and the basolateral membrane value is 10-fold lower. The reason for these differences is not entirely clear. In the case of the basolateral membrane, it is possible that the time constant for diffusion of NH₃ into the basolateral infoldings is somewhat rate limiting under perfused tubule conditions (D. Good, personal communication).

Several aspects of these measurements deserve comment. First, it is possible to calculate from the permeability values available whether addition of NH₄⁺/NH₃ to the apical surface will lead to acidification or alkalinization of intracellular pH. If 10 mM NH₄⁺/NH₃ is added to the apical surface at pH 7.4, the flux of NH₄⁺, $J_{NH₄⁺}$, will be

\[ J_{NH₄⁺} = P_{NH₄⁺} \times [NH₄⁺] \times SA \]  

(6)

where $P_{NH₄⁺}$ is the NH₄⁺ permeability of the apical membrane, $[NH₄⁺]$ is the concentration gradient for NH₄⁺ at time 0 (external NH₄⁺ concentration is 9.86 mM and internal concentration is assumed to be 0), and SA is calculated per millimeter tubule length (0.088 × 10⁻² cm²/mm; see Ref. 13). $P_{NH₄⁺}$ can be calculated from the maximal rate of apical membrane NH₄⁺ entry measured by Kikeri et al. (9) and is 0.00038 cm/s. From these values, we obtain an anticipated $J_{NH₄⁺}$ of $3.3 \times 10^{-9}$ mmol·s⁻¹·mm⁻¹ tubule length.

Similarly, using $P_{NH₃}$ of 0.015 cm/s and $[NH₃]$ of 0.097 mM at pH 7.4 and 10 mM NH₄⁺/NH₃, flux of NH₃ ($J_{NH₃}$) is anticipated to be $1.28 \times 10^{-9}$ mmol·s⁻¹·mm⁻¹, a value 2.6 times lower than that of the $J_{NH₄⁺}$. Indeed, because the $J_{NH₄⁺}$ was estimated from the rate of acidification, which overcame NH₃-induced alkalinization (9), it can be anticipated that the actual value of NH₃ entry was 4.6 × $10^{-9}$ mmol·s⁻¹·mm⁻¹ tubule length, a value 3.6-fold higher than the $J_{NH₄⁺}$. These calculations indicate that the permeability values that we have obtained can account for the observed behavior of the intact MTAL when it is exposed to NH₄⁺/NH₃. Because of a lack of direct data on $J_{NH₃}$ across the basolateral membranes of intact cells, similar calculations cannot be performed for the basolateral membrane. However, it appears that the rate of NH₄⁺ entry via the basolateral membrane is slower than that across the apical membrane, whereas that for NH₃ is higher (9, 25).

An additional issue concerns the site of the transepithelial barrier to $J_{NH₃}$. One way to estimate the barrier properties of the individual membrane domains would be to calculate the flux anticipated if either the apical or basolateral surface were exposed abruptly to NH₃/NH₄⁺. The anticipated $J_{NH₃}$ for apical or basolateral membranes is

\[ J_{NH₃} = P_{NH₃} \times (\Delta NH₃) \times SA \]  

(7)

Using the conditions outlined above and the permeabilities obtained in the present study, the anticipated $J_{NH₃}$ for the apical membrane is $1.37 \times 10^{-9}$ mmol·s⁻¹·mm⁻¹, whereas that for the basolateral membrane is $10.2 \times 10^{-9}$ mmol·s⁻¹·mm⁻¹. As was the case for water permeation, the huge difference in surface area of the two membrane domains accounts for the lower anticipated flux across the apical membrane.
Comparison of NH$_3$/NH$_4^+$ transport in the MTAL with that of the cortical collecting duct reveals that the MTAL concentrates NH$_3^+$ in the interstitium without generating a pH gradient, whereas the cortical collecting duct entraps NH$_3$ in the lumen by means of luminal acidification (5). Comparison of apical membrane water and NH$_3$ permeabilities of these segments (Table 1) indicates lower values of $P_{\text{NH}_3}$ across the cortical collecting duct apical membrane (28). Therefore, the reason that the MTAL can concentrate NH$_3^+$ in the interstitium without a pH gradient is its very rapid transport of NH$_3^+$, which overwhelms the backflux of NH$_3$ through the apical membrane bilayer. By contrast, in the cortical collecting duct, transport rates of NH$_3^+$ are low (7), so that flux of NH$_3$ across the epithelium, although comparable to that of the MTAL, dominates, leading to pH-dependent entrapment of NH$_3^+$ in the lumen for excretion into the urine.

It has been generally believed that the apical rather than the basolateral membranes of barrier epithelia represent the major barrier to permeation, and this barrier function has been attributed to differing lipid structure of apical as opposed to basolateral membrane domains (15, 30, 32). In support of this concept, Strange et al. (23) measured the osmotic water permeabilities obtained can account for the behavior of this segment in vivo and in isolated perfused tubule studies. The results indicate that the barrier properties of the apical membrane result primarily from its low surface area and not from any specialized lipid composition of this domain. The ability of the MTAL to create gradients for NH$_3^+/\text{NH}_3$ without favorable pH gradients appears to arise less from low NH$_3$ permeability and more from high rates of active transport of NH$_3^+$ compared with NH$_3$.

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