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Evidence for proteic water pathways in the luminal membrane of kidney proximal tubule

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The osmotic permeability of the apical membrane of proximal tubule cells was studied on rat brush-border membrane vesicles by following their rate of shrinkage with a stopped-flow device coupled to light transmission recording. The mercuric sulfhydryl reagent *para*-chloromercuribenzenesulfonic acid (PCMBS) reduced the water permeability of the membrane, in a time- and dose-dependent manner, to 35% of the control value. Mercuric chloride was a more potent inhibitor and decreased the osmotic water permeability of the brush-border membrane to 15% of the control. This inhibition was reversed by an excess of cysteine, while cysteine per se did not modify the rate of vesicle shrinkage. These results suggest that most of the osmotic water movements across kidney brush-border membranes are through polar pathways which involve the integrity of the membrane proteins.

Introduction

The way in which water crosses the kidney proximal tubule is still unknown: paracellular and transcellular pathways have both been proposed but without strong evidence for either alternative [1]. Recently, experiments which dealt with the coupling of water and solute movements indicated that osmotic water flow through rabbit proximal tubules did not carry a measurable amount of solutes [2,3]. These data disagree with the hypothesis of a predominant paracellular movement of water through large pores and contribute to making the transcellular movement of water a more probable hypothesis. The measurement of the large infoldings of the basolateral membrane [4,5], the determination of its osmotic permeability and a comparison with the whole epithelium permeability [6,7,8] also supported the hypothesis of considerable water movement through proximal cells.

Recent investigations added information on the nature of these possible transcellular water pathways. Whittembury et al. [10] reported that the apparent Arrhenius energy of activation of water osmotic permeability of the basolateral membrane was compatible with the existence of channels in which water-water interaction predominates. In addition, they found that the sulfhydryl reagent, PCMBS, which is known to interact with membrane proteins, including those involved in water movement through red blood cells [11–16], inhibited the osmotic permeability of the basolateral membrane by 75%, a strong indication of the presence of protein water channels in the membrane. However, as pointed out by the same authors [10], the water permeability of the apical cell membrane could be the rate-limiting barrier to transcellular osmosis, and their first experiments, made on rabbit proximal tubule, supported this hypothesis of a reduced water permeability of the

Abbreviations: PCMBS, *p*-chloromercuribenzenesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

luminal membrane when compared with the basolateral [17]. We then questioned the possibility of proteic water channels also being involved in the osmotic permeability of the luminal membrane and tried to determine the proportions of water moving by diffusion through lipids and through polar pathways. These points were investigated by measuring the kinetics of osmotic shrinkage of brush-border membrane vesicles by means of a stopped-flow device and light transmission recording. The water pathways were identified in these experiments by the use of mercuric sulfhydryl reagents which are known to react with the proteins involved in water movement leaving the lipid portion of the membrane as an alternative for water transport.

Materials and Methods

Renal brush-border membranes were prepared by the calcium precipitation method from male Sprague-Dawley rats weighing 280–320 g (8–10 weeks old). Slices of kidney cortex were treated as described by Evers et al. [18] with slight modifications [19] and the enrichment factor of the obtained membrane fraction was close to 12 when compared with homogenate. The whole process was conducted at a temperature of 4°C in the presence of a single buffer containing 50 mM sucrose, 10 mM Hepes-Tris (pH 7.5) and NaN_3 0.01%. This buffer was considered as the control solution. Its osmotic pressure, measured by cryometry or with a Wescor vapor pressure osmometer, was 74.1 ± 4.7 mosmol/kg H_2O ($n = 11$).

The internal volume of the vesicles obtained was determined by uptake experiments using the rapid filtration technique [20]. Aliquots of brush-border membrane vesicles (0.2 ml, 1 mg protein/ml) were incubated at room temperature with 4 μCi D-[^{3}H]glucose (Service des Molécules Marquées, CEN Saclay, France) for 90 min. Preliminary kinetic experiments verified that, by this time, the internal compartment was in equilibrium for glucose with the external solution. The suspension was then diluted in 2 ml ice-cold non-radioactive rinsing solution, filtered through a 0.65 μm SM 113 Sartorius filter and washed three times with 5 ml of the previous solution. The binding component of D-[^{3}H]glucose at the surface of the vesicles and on filters was obtained by the same procedure but with no incubation. The equilibrium volumes of the vesicles were measured in control isosmotic conditions and at different external osmotic concentrations obtained by addition of sucrose to the buffer solution. In some experiments, diffusion of sucrose in the vesicles was found, with [^{14}C]sucrose, to be extremely low when compared with [^{3}H]glucose. The final osmotic pressures of the different solutions tested were 126 ± 4, 170 ± 4, 219 ± 3, 258 ± 5, 293 ± 2 and 422 ± 15 mosmol/kg H_2O ($n = 11$). In hyperosmotic conditions, the same solutions were used for rinsing. All incubations were carried out in triplicate. Intravesicular volumes were calculated from the radioactivity found on dried filters and the specific activity of the external D-[^{3}H]glucose solution. The volumes were expressed per mg proteins retained on the filter, the proteins being measured in each experiment with the Lowry [21] or Bradford [22] technique using bovine serum albumin as a reference.

Kinetic measurements of vesicle shrinkage were performed with a stopped-flow apparatus (Durrum D-110 apparatus equipped with a 75 W xenon lamp) interfaced to a Hewlett-Packard 9825A calculator connected to a H-P 3437A system voltmeter and to a storage oscilloscope (type 549, Tektronic Inc.). The wavelength of the emitted light was 450 nm and the change in transmitted light was monitored in the first 2 s. The time-delay for the mixed solutions to reach optical cell was 3 ms. Three runs were usually stored and averaged in each experimental condition. Temperature was 25°C and the final concentration of vesicles in the optical chamber was 0.25 mg protein/ml. All the experiments were performed with freshly prepared vesicles. Means were expressed with their standard errors; statistical significance at $P < 0.05$ was tested with the non-parametric Wilcoxon procedure.

Results

Equilibrium volume determinations

The mean intravesicular volume measured in the presence of control buffer was 2.37 ± 0.11 nl/μg proteins ($n = 8$). The volume dependence of the vesicles on external osmolality is reported in

Fig. 1. It appeared that, in the investigated range of sucrose concentrations, the equilibrium intravesicular volume was inversely proportional to the external osmolality and that the brush-border membrane vesicles behaved like an osmometer. Extrapolation to infinite external osmolalities suggested the existence of a small apparent non-osmotic volume which was significantly different from zero.

Stopped-flow experiments

Rapid mixing of brush-border membrane vesicles suspended in control buffer with an equal volume (200 μl) of the same control buffer did not induce time-dependent change in the transmitted light. The mixing of vesicles and hyperosmotic solution, on the other hand, was followed by a rapid change in the intensity of the transmitted light: light intensity sharply decreased in the first 100 ms and then progressively reached an equilibrium value within the next 2 s (see Fig. 2).

The ΔI, defined as the difference in light transmission between zero time and equilibrium values, was determined for the different hyperosmotic solutions previously used in volume experiments. The results depicted in Fig. 3a showed that ΔI and external osmolality were linearly related. Taking into account the direct relationship between external osmolality and the reciprocal of the volume reported from isotopic determinations (Fig. 3b), ΔI was plotted as a function of volume. As expected, the difference in transmitted light intensity (ΔI) was a linear function of the reciprocal of the intravesicular volume (Fig. 3c); that is:

$$\Delta I = a \cdot (V_0/V) + b \qquad (1)$$

where V_0 is the intravesicular volume before osmotic shock, and V the equilibrium value.

The time derivative of this experimental relationship was:

$$dI/dt = aV_0 \cdot (1/V^2) \cdot dV/dt \qquad (2)$$

Experimental recordings of light intensity changes as a function of time, which were best fitted by the sum of two exponentials, were used to calculate the initial rate in transmitted light intensity variations, i.e. the initial slope $P(0)$ of the double exponential fit. It was considered that at this initial time, the control volume of the vesicles V_0 was the same in all experiments and that the effective osmotic gradient was the nominal one. Then at zero time, Eqn. 2 became:

$$dI/dt(0) = (a/V_0) \cdot dV/dt(0) \qquad (3)$$

that is, the initial change in light intensity, $dI/dt(0)$, is directly proportional to the change in vesicle volume, $dV/dt(0)$. Because the osmotic water flow dV/dt across a membrane is proportional to the osmotic permeability of the mem-

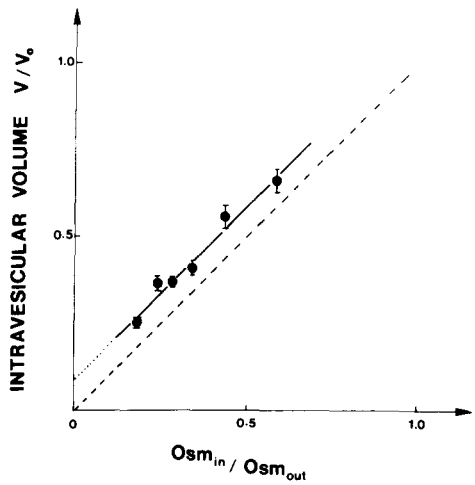

Fig. 1. Volume dependence of vesicles on external osmolality. V_0 is the intravesicular volume before osmotic shock (2.37 nl/μg protein) and V the equilibrium value. Osm_{in} is the intravesicular osmolality before osmotic shock (74 mosmol/kg H_2O) and Osm_{out} the osmolality of the external solution. The dotted line represents the behaviour of an ideal osmometer. Values are the means of eight experiments.

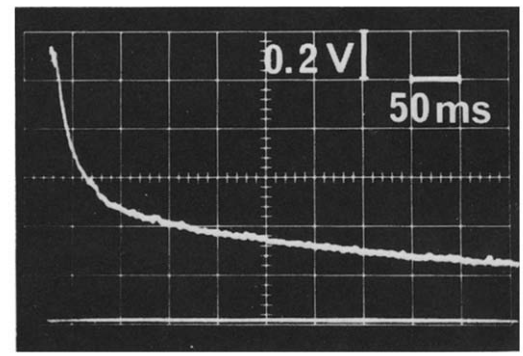

Fig. 2. Representative experiment of change in light transmission intensity in volts as a function of time in ms. Osmotic gradient 145 mosmol/kg H_2O.

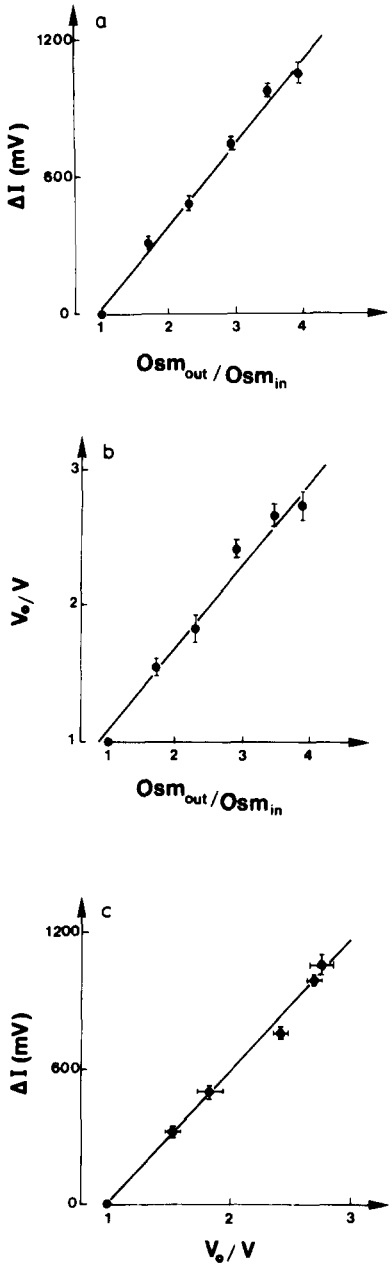

Fig. 3. (a) Dependence of ΔI, the difference in transmitted light intensity, on external osmolality. Osm_{in} is the intravesicular osmolality before osmotic shock (74 mosmol/kg H_2O) and Osm_{out} the osmolality of the external solution, which was increased by addition of sucrose to the control buffer solution. (b) Volume dependence of vesicles on external osmolality. V_0 is the intravesicular volume before osmotic shock (2.37 ± 0.11 nl/μg protein) and V the equilibrium value. (c) Dependence of ΔI on intravesicular volume. The figure was obtained by combining (a) and (b). Values are the means of seven experiments.

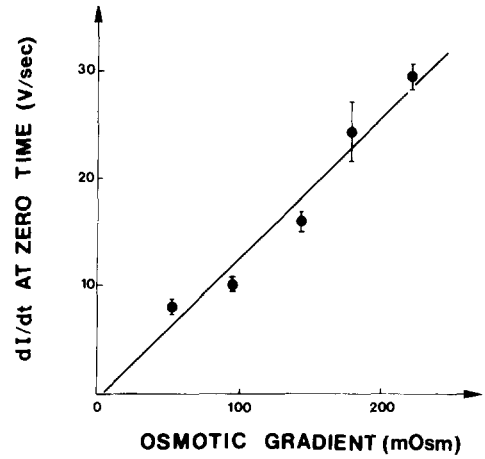

Fig. 4. Dependence of the initial slope $dI/dt(0)$ on the osmotic gradient. Values are the means of seven experiments.

brane L_p, its surface area A and the osmotic gradient (Δosm):

$$dV/dt = L_p ART(\Delta\text{osm}) \quad (4)$$

it is deduced from Eqns. 3 and 4 that $dI/dt(0)$ should be a linear function of external osmolality (assuming that L_p is independent of tonicity):

$$dI/dt(0) = (a/V_0) L_p ART(\Delta\text{osm}) \quad (5)$$

This was experimentally confirmed by plotting the $dI/dt(0)$ calculated from the double exponential fit of the experimental curves, against (Δosm) (Fig. 4).

We concluded that, for a given osmolality, the

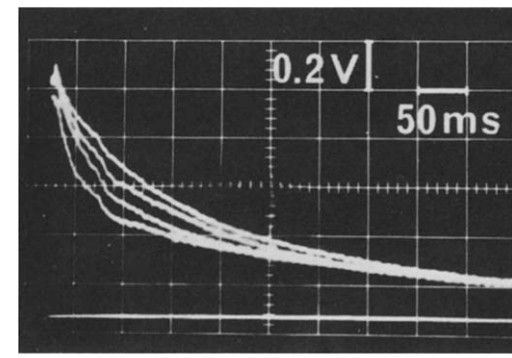

Fig. 5. Representative experiment of the time dependence of water permeability inhibition by PCMBS. One run is done each 5 min. PCMBS concentration was 10 mM. As incubation time increases, the initial rate of cell shrinkage decreases while the equilibrium value is not altered.

change in $dI/dt(0)$ reflects the change in L_p. In the following experiments, the final osmotic gradient was constant (145 mosmol/kgH_2O), and when drug experiments were performed, the tested compound was added in both vesicle suspension and hyperosmotic solution.

Time-dependence of water permeability inhibition by PCMBS

PCMBS (final concentration 10 mM) was added to the brush-border membrane vesicles suspended in buffer solution, the pH of which was readjusted to 7.5, and one run was performed every 5 min. The oscilloscope traces of a representative experiment are shown in Fig. 5. There was a reduction with time in the initial slope of the curve, while the equilibrium values ΔI remained the same, indicating that the extent of the vesicle shrinkage was unchanged, whereas the time needed to reach this value was lengthened by the addition of PCMBS. The mean results from four experiments are shown in Fig. 6. The time course of water permeability inhibition by PCMBS can be characterized by a single-exponential, maximal inhibition being reached at 40 min. The water permeability at this time was reduced to 35% of the control.

Concentration and pH dependence of water permeability inhibition by PCMBS

Vesicle suspensions were preincubated for 45 min with various concentrations of PCMBS. When the pH was readjusted to 7.5, the addition of 2.5 mM PCMBS did not significantly modify the rate of cell shrinkage (Table I) and the first measurable inhibition was found at a concentration of 5 mM. When the pH was fixed at 6.5, the water permeability inhibition was present at lower concentrations. Thus, 2.5 mM PCMBS did reduce the osmotic flow of water out of the vesicles and 5.0 mM PCMBS was 4-times more potent at pH 6.5 than at pH 7.5. However, the maximal inhibition was not significantly increased by low pH.

Control experiments in which pH was changed from 7.5 to 6.5 and the osmotic gradient was also 145 mosmol/kgH_2O showed that pH alone did not alter the rate of vesicle shrinkage ($dI/dt(0) = 16.1 \pm 0.9$ ($n = 8$), pH 7.5, and $dI/dt(0) = 15.8 \pm 0.7$ ($n = 7$), pH 6.5).

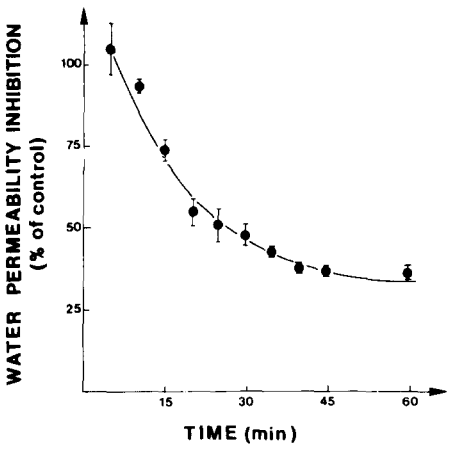

Fig. 6. Time dependence of water permeability inhibition by 10 mM PCMBS. Measurements were made every 5 min and each point is the mean of four experiments.

TABLE I

CONCENTRATION AND pH DEPENDENCE OF WATER PERMEABILITY INHIBITION BY PCMBS

Means are ±S.E. ($n = 3$). Preincubation time with PCMBS was 45 min.

[PCMBS] (mM)	Inhibition (% of control)	
	pH 7.5	pH 6.5
2.5	2.8±2.2	9.4±1.0
5.0	16.4±3.6	61.5±1.8
7.5	65.6±2.9	70.5±0.6
10.0	62.8±1.3	68.8±1.1
15.0	64.8±1.3	68.8±1.2

TABLE II

CONCENTRATION DEPENDENCE OF WATER PERMEABILITY INHIBITION BY $HgCl_2$

Means are ±S.E. ($n = 3$). Preincubation time with $HgCl_2$ was 15 min; pH was 7.5.

[$HgCl_2$] (mM)	Inhibition (% of control)
0.1	5.5±5.8
0.2	61.1±1.8
0.4	83.4±1.8
0.6	84.5±0.7
0.8	84.0±0.3
1.0	84.0±0.3

Water permeability inhibition by $HgCl_2$

The addition of $HgCl_2$ to vesicle suspension considerably reduced the water permeability of the vesicles. The time needed to reach maximal inhibition was less than 3 min. The results of 15 min preincubation of the vesicles with different concentrations of $HgCl_2$ are reported in Table II. It is clear that $HgCl_2$ is a more potent inhibitor of water permeability than PCMBS: the concentration of $HgCl_2$ which gave maximal inhibition was an order of magnitude lower than that of PCMBS and the residual water permeability was 16% of control with $HgCl_2$ in comparison with 35% with PCMBS.

Reversibility by cysteine of the water permeability inhibition, and effect of N-ethylmaleimide

The 15 min addition of 50 mM cysteine to vesicle suspensions preincubated with 10 mM PCMBS for 45 min changed the water permeability inhibition from 68.8% to 10.1% of the control (Table III). The same kind of result was obtained by addition of 20 mM cysteine to 0.4 mM $HgCl_2$, while it was verified that the presence of 50 mM cysteine per se did not significantly alter the rate of vesicle shrinkage (Table III).

N-Ethylmaleimide, a non-mercuric sulfhydryl reagent, added to the vesicles for 60 min at a concentration of 15 mM did not significantly modify the rate of vesicle shrinkage.

TABLE III

REVERSIBILITY BY CYSTEINE OF PCMBS AND $HgCl_2$ WATER PERMEABILITY INHIBITION

Means are ±S.E. ($n = 3$). Cysteine was preincubated for 15 min, 45 min after the addition of PCMBS or 15 min after the addition of $HgCl_2$ to the vesicles suspension.

Compound	Inhibition (% of control)
Cysteine (50 mM)	2.8 ± 4.6
PCMBS (10 mM)	68.8 ± 1.1
PCMBS (10 mM) + cysteine (50 mM)	10.1 ± 0.6
$HgCl_2$ (0.4 mM)	83.4 ± 1.8
$HgCl_2$ (0.4 mM) + cysteine (20 mM)	11.4 ± 5.6

Discussion

The present data indicate that PCMBS and $HgCl_2$ greatly inhibit, in a reversible manner, the water permeability of the luminal membrane of kidney proximal tubule cells. As mercuric sulfhydryl reagents are known to react with proteins, it is concluded that most of the water crosses the luminal membrane proximal cells through pathways which necessitate the integrity of membrane proteins. Assuming that a maximal concentration of $HgCl_2$ effectively plugs all these proteic pathways, one may conclude that water movement through proteic pathways is 8-times greater than that through residual lipidic pathways. This figure is close to that found in red blood cells [11–16].

The aqueous pathway of brush-border membranes showed numerous other characteristics similar to those of erythrocytes. Water movement in both systems was inhibited by sulfhydryl reagent containing a mercuric ligand, whereas the sulfhydryl groups which were accessible to and reactive with *N*-ethylmaleimide were not directly involved in the control of water transfer [11,16]. The time course of water permeability inhibition by PCMBS was, in both membranes, characterized by a single exponential and full effect was reached at similar times, that is 30–75 min [11,15]. In both systems, the effect of PCMBS was activated by low pH [11,15], presumably by increasing the accessibility and reactivity of the membrane sulfhydryl groups, and the concentration needed to block the sulfhydryl groups with $HgCl_2$ was close to 100 μM [14,16]. Finally, the maximal inhibitory effects of the mercuric drug were comparable (80–90% of control permeability) and the inhibition was reversed by cysteine [13]. The only noticeable difference between red blood cells and brush-border membranes was that the PCMBS concentrations needed for maximal inhibition differed: 1.0 mM for the former system and 7.5 mM for the latter. Because PCMBS is virtually insoluble in a lipid environment and is thought to penetrate the membrane through the normal anion permeation channel [13], we suggest that the discrepancy in the concentration dependence of the PCMBS reaction is due either to differences in the structure of water and anion channels in the two membranes or to the low number of anion chan-

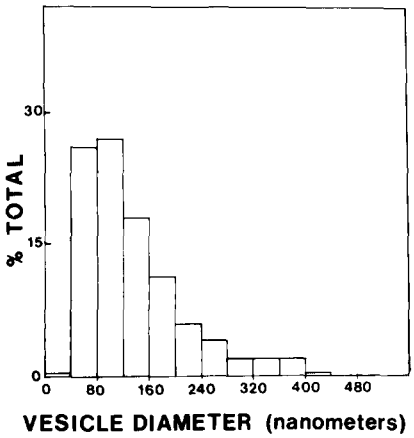

Fig. 7. Distribution of vesicle diameters in a rat kidney brush-border membrane preparation. Measurements were made on freeze-fracture pictures (×45000) of the vesicle suspensions used for water permeability determinations.

nels in brush-border membrane.

Comparison of the PCMBS inhibition of water permeability of luminal and basolateral membranes of proximal cells is also of interest. The latter has recently been investigated by Whittembury et al. [10] on rabbit proximal tubules crimped between micropipettes. These authors also found that the mercuric drug lowered the osmotic permeability of the basolateral membrane in a time- and concentration-dependent manner, with maximal inhibition close to 80% of the control. It suggests that the mechanisms of water permeability through the two membranes in series are basically the same, that is, a predominant water movement across proteic pathways. Furthermore, since the two membranes are of comparable area, their total permeabilities would probably be very similar.

An estimation of the water permeability of the luminal membrane can be obtained from our stopped-flow experiments. Introducing $P_f = L_p \cdot RT/\overline{V}_\omega$ in Eqns. 3 and 4 gives:

$$P_f = (V_0/A)\cdot dI/dt(0)\cdot(1/\overline{V}_\omega \cdot a \cdot \Delta osm) \quad (6)$$

a, the slope of the relation $\Delta I = f(V_0/V)$, is experimentally determined from Fig. 3. $\overline{V}_\omega$ is the partial molar volume of water, V_0 is the volume of the vesicles in the optical cell at zero time and A the surface of water exchange. The critical point was to determine the membrane area corresponding to the intravesicular volume, that is, to ascertain the size of the vesicles. An attempt to measure the mean vesicular diameter was made from freeze-fracture pictures of the vesicles suspensions. The distribution of these diameters, shown in Fig. 7, indicated that the mean vesicle size is 137 ± 36 nm and that 88% of the measured diameters are between 40 and 240 nm. The obtained P_f value calculated from Eqn. 6 and from this mean diameter was 240 μm/s, which is an order of magnitude larger than would be expected from the water permeability of a lipidic membrane [9]. However, the present data do not rule out the possibility that water crosses the brush-border membranes through special lipid arrangements which could be modified by mercurial compounds.

Keeping in mind the possibility that the process of isolation of the membrane vesicles could have changed their properties by alteration of the cytoskeleton or stretching of the membrane, and thus could have impaired a comparison with the whole epithelium, the value of 240 μm/s for luminal membrane permeability appears to be compatible with a large transcellular movement of water across proximal tubule.

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