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Diffusive Water Permeability in Isolated Kidney Proximal Tubular Cells: Nature of the Cellular Water Pathways

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Summary. The diffusive water permeability (P_d) of the plasma membrane of proximal kidney tubule cells was measured using a ^{1}H-NMR technique. The values obtained for the exchange time (T_{ex}) across the membrane were independent of the cytocrit and of the Mn^{2+} concentration (in the range 2.5 to 5 mM). At 25°C the calculated P_d value was (per cm^2 of outer surface area without taking into account membrane invaginations) 197 ± 17 μm/sec. This value equals 22.3 ± 1.9 μm/sec when the invaginations are taken into account. Cell exposure to 2.5 mM parachloromercuribenzenesulfonic acid, *p*CMBS, (for 20 to 35 min) reduced P_d to 45% of its control value. Five mM dithiothreitol, DTT, reverted this effect. The activation energy for the diffusive water flux was 5.2 ± 1.0 kcal/mol under control conditions. It increased to 9.1 ± 2.2 kcal/mol in the presence of 2.5 mM *p*CMBS. Using our previous values for the osmotic water permeability (P_{os}) in proximal straight tubular cells the P_{os}/P_d ratio equals 18 ± 1, under control conditions, and 3.2 ± 0.3 in the presence of *p*CMBS. These experimental results indicate the presence of pathways for water, formed by proteins, crossing these membranes, which are closed by *p*CMBS. Assuming laminar flow (within the pore), from P_{os}/P_d of 13 to 18 an unreasonably large pore radius of 12 to 15 Å is calculated which would not hinder cell entry of known extracellular markers. Alternatively, for a single-file pore, 11 to 20 would be the number of water molecules which would be in tandem inside the pore. The water permeability remaining in the presence of *p*CMBS indicates water permeation through the lipid bilayer. There are similarities between these results and those obtained in human red blood cells and in the apical cell membrane of the toad urinary bladder.

Key Words diffusive water permeability · kidney tubules · water channels · epithelia · nuclear magnetic resonance (NMR) · single-file diffusion

Introduction

Mammalian kidney proximal tubules reabsorb large amounts of water at rates of the order of 10 to 50 nl/cm^2 tubular wall · sec (Weinstein & Windhager, 1985). Part of this reabsorptive flux (Carpi-Medina et al., 1984*b*; Whittembury et al., 1985) or all of it (Berry, 1985) must occur through the cells. Water can cross cell membranes by diffusion/solution in the lipid bilayer and/or through specialized regions which may be related to transmembrane proteins (Stein, 1986). The water permeability of unmodified lipid bilayers is high enough to explain water movement across most cell membranes, making unnecessary the presence of specialized regions for water movement (Stein, 1986; Finkelstein, 1987). However, the latter seems to be required in mammalian proximal tubule (Whittembury et al., 1984), red blood cells (*see* Stein, 1986 for extensive references) and amphibian urinary bladder under ADH stimulation (Parisi & Bourguet, 1983; Levine, Jacoby & Finkelstein, 1984; Finkelstein, 1987).

The purpose of the present work is to explore further this problem in the proximal tubule of the mammalian kidney. Several strategies can be used to distinguish between the two ways mentioned above in which water can cross cell membranes (*cf.* Andersen & Procopio, 1980). (I) One involves the comparison of P_{os}, the water osmotic, and P_d, the water diffusive permeability coefficient. In the absence of unstirred layer effects, there is now agreement in that $P_{os}/P_d > 1$ indicates the presence of a pore or channel (Fettiplace & Haydon, 1980; Solomon et al., 1983 and references therein; Solomon, 1986; Finkelstein, 1987). A $P_{os}/P_d > 1$ indicates either the pore radius if laminar flow is conceived to occur within the pore (Longuet-Higgins & Austin, 1966) or the number of water molecules within the pore if the latter is so narrow that water molecules cannot overtake and therefore must go in tandem through it (Hodgkin & Keynes, 1955; Heckmann, 1965; Finkelstein & Rosenberg, 1972; Parisi & Bourguet, 1983; Hille, 1984; Stein, 1986; Finkelstein, 1987).

(II). The second criterion emerges from E_a, the Arrhenius energy of activation of these permeabilities. In free solution E_a is 4.2 to 4.8 kcal/mole for viscous and/or diffusive water movement because the movement of water is then determined almost

entirely by the temperature dependence of the viscosity of the diffusing medium (Vieira, Scha'afi & Solomon, 1970; Stein, 1986, Finkelstein, 1987). Therefore, values for E_a near those obtained in free solution would indicate a low degree of interaction between water molecules and membrane, as would be expected if there were continuous water channels crossing the cell membrane. For diffusion/solution in the lipid bilayer E_a will be much higher than that observed in free solution, because the increase in temperature will increase the diffusion coefficient by an increase in the overall formation of holes into which water moves (Hildebrand, 1971; Stein, 1986). Values for $E_a \geq 10$ kcal/mole have been observed in nontreated lipid bilayers, illustrating that the degree of interaction between lipid and water is very large during movement of water across a membrane containing no pores. Additional tools in the exploration of water movement in membranes are (III) the use of blockers of water movement, and (IV) a study of the interaction between water and solutes crossing the membrane.

Measurements of P_{os} in mammalian proximal tubule cells (Gonzalez, Carpi-Medina & Whittembury, 1982; Welling, Welling & Ochs, 1983; Carpi-Medina et al., 1984*a*; González et al., 1984; Whittembury et al., 1984; Carpi-Medina, 1986) give seemingly high values per cm^2 of basement membrane area, which turn out to be of the same order of magnitude of those of human red cells (RBC) when corrected for membrane invaginations and infoldings (Whittembury et al., 1984). The osmotic permeability of the apical and of the basolateral membrane of the proximal tubular cells is reduced in a dose-dependent manner by paracloromercurybenzenesulfonic acid (*p*CMBS), not only in whole tubules (Carpi-Medina, 1986; Whittembury et al., 1984) but also in apical plasma membrane vesicles (Pratz, Ripoche & Corman, 1986; van Heeswijk & van Os, 1986). E_a for water fluxes across the basolateral plasma membrane (Whittembury et al., 1984) under control condition is similar to that measured for free water movement, and increases with *p*CMBS to values near those obtained in artificial lipid membranes without pores (Cass & Finkelstein, 1967; Cohen, 1975; Fettiplace & Haydon, 1980; Stein, 1986), indicating the presence of water pathways made up by proteins (which can be blocked by *p*CMBS, Whittembury et al., 1984), in addition to water permeation through the bilayer.

However, as mentioned above, other strategies are required to further characterize the water pathways. For this purpose, measurements of P_d were undertaken. P_d measurements can be carried out by using either fast reaction methods to study THO diffusion or proton nuclear magnetic resonance (NMR). With this latter technique used to measure P_d in RBC (Conlon & Outhred, 1972, 1978; Fabry & Eisenstadt, 1975, 1978; Pirkle, Ashley & Goldstein, 1979) and in epithelia (Steward & Garson, 1985) measurements are carried out at equilibrium avoiding problems of external unstirred layers, that have introduced errors in many P_d evaluations (Fettiplace & Haydon, 1980; Barry & Diamond, 1984). Briefly, the method measures the rate constant for diffusive water efflux from the cell interior (where water proton relaxation is very slow) to the extracellular fluid (where proton relaxation has been accelerated several-fold by the presence of a low concentration of Mn^{2+}). In this way the signal from the cells is unmasked. Its decay as a function of time allows to calculate P_d.

Our original aim was to evaluate separately the P_d of basolateral cell membranes working with a suspension of kidney tubules (Vinay, Gougoux & Lemieux, 1984). However, uncertainties as to the magnitude of the luminal tubular cell surface that (in our preparation) was contributing to the water fluxes and as to the degree of penetration of Mn^{2+} in the tubular lumen in experiments which were primarily intended to evaluate the basolateral membrane P_d, led us to prefer working with isolated cells in spite of the fact that with this preparation measurements yield an average P_d of the whole membrane of the isolated cells, which does not distinguish apical from basolateral membrane water movement. Our results with isolated cells agreed with our previous estimates with isolated tubules. However, for the reasons just mentioned, the emphasis of this paper is on the results obtained with isolated cells. In addition, we studied the E_a of P_d and the effect of *p*CMBS on them. These results are compared with our previously measured values of P_{os}. It is concluded that specially narrow water pathways, formed by proteins, where water molecules form single files pierce the proximal tubule cell membrane. The patency of these pathways depends on the state of sulfhydryl groups of the transmembrane proteins. The permeability remaining in the presence of *p*CMBS must reflect the water permeability of the lipid bilayer part of the membrane.

Part of this work has been presented (Carpi-Medina, 1986; Carpi-Medina et al., 1984*a*; 1987*a*,*b*,*c*). Since this work was completed a publication has appeared which essentially agrees with our measurements (Verkman & Wong, 1987).

Materials and Methods

Proximal kidney cells were isolated by a modification of the method of Nagineni et al. (1984) in which hyperosmolar solutions containing 0 nominal Ca^{2+} concentration are used together with gentle mechanical disruption of the tissue. Briefly, New Zealand

rabbits weighing 1.5 to 2 kg were injected with heparin and sacrificed about 10 min later. Both kidneys were perfused by means of a peristaltic pump through their renal arteries with a solution that contained (in mM): $NaHCO_3$ 9.0, HEPES 10.0, K_2SO_4 7.0, K_2HPO_4 44.0, D-glucose 180, mannitol 212, at room temperature, until the solution coming out of the kidney was clear. This required at least 30-min perfusion. Superficial cortical slices of both kidneys were then cut with a hand microtome. They were manually homogenized in the same solution using a Potter-Elvehjiem homogenizer with a leaky pestle. At the end of the homogenization procedure the suspension consisted mainly of long, well-preserved proximal tubular segments, which were collected on top of an 80-μm pore size nylon mesh filter and incubated for 30 min at room temperature in the same solution with gentle stirring. The suspension was again filtered through the 80 μm and then through a 40-μm filter. The preparation, consisting now of isolated proximal cells, was incubated in a 1% Trypan blue isosmotic solution to study its viability. An aliquot was separated to obtain the cytocrit value by centrifugation (at 1000 $\times$ g for 2 min) and a drop was extended on a microscope slide to measure the average cell diameter by examining 100 cells by means of an optical microscope provided with a 65$\times$ objective. If the viability was adequate (>90%)[1] the cells were incubated in artificial bathing solution (ABS) containing (in mM): NaCl 116.4, $NaHPO_4$ 4, KCl 5, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 1.9, D-glucose 8.3, L-alanine 5 and 6% bovine serum albumin.

Before the measurements were carried out an aliquot of cells (or tubules) was incubated in ABS in which part of the NaCl had been isosmotically replaced by the required amount of $MnCl_2$. Cells were never used if they had been in the presence of Mn^{2+} for more than 1 h (*see* Results). A special double concentrical tube consisting of two tubes, one inside the other, was used for the NMR measurements. The cells were placed in the inner tube. The space between the inner and outer tubes contained D_2O for lock. On the top of the tube a 95% O_2/5% CO_2 atmosphere maintained the adequate O_2 supply to the cells and the pH. The same tube was used with the same atmosphere for measurements with ABS alone. In our experiments, 5 mM $MnCl_2$ was added to the ABS at least 1 week prior to the experiments to insure a stable reduction in the free Mn^{2+} concentration, since Mn^{2+} is known to interact with bovine serum albumin (Midwan & Cohn, 1963).

The T_2 relaxation time was measured at 100 MHz in a Varian XL-100 spectrometer equipped with a Nicolet computer and a computer-controlled pulse generator. The Carr-Purcell-Meiboom-Gill sequence was used for the T_2 measurement. Thirty different times and 12 acquisitions for each time were needed for the cell measurements (the total acquisition time was 15 min) while only 12 times were needed for the ABS measurements. The relaxation curves were fitted by a double-exponential nonlinear fitting program in a computer. Standard equations by McConnell (1958) for two sites exchange were used to calculate the exchange times using the predetermined values for both the cytocrit and the plasma relaxation time.

Equations

The original statement of the problem is (Conlon & Outhred, 1972, 1978; Steward & Garson, 1985):

$$dM_i/dt = -(K_i + K_x)M_i + K_yM_o$$
$$dM_o/dt = -(K_o + K_y)M_o + K_xM_i$$

where M is the net magnetization, subscript i stands for intracellular and o for extracellular compartment, K_x and K_y are the exchange constants across the membrane; $K_i = 1/T_i$ and $K_o = 1/T_o$ are the intracellular and extracellular relaxation times, respectively. At equilibrium influx is equal to efflux:

$$K_xN_i = K_yN_o$$

where N_i and N_o are the number of intracellular and extracellular protons. The solution of the above equations expressing the measured magnetization ($M_i + M_o$), normalized to 1 for $t = 0$, is:

$$(M_i + M_o) = (1 - H)\exp(-\phi_A t) + H\exp(-\phi_B t)$$

where the values are:

$$\Phi_A = (\tfrac{1}{2})(K_i + K_o + K_x + K_y) + (\tfrac{1}{2})[(K_i - K_o + K_x - K_y)^2 + 4K_xK_y]^{1/2}$$
$$\Phi_B = (\tfrac{1}{2})(K_i + K_o + K_x + K_y) - (\tfrac{1}{2})[(K_i - K_o + K_x + K_y)^2 + 4K_xK_y]^{1/2}$$

and the intercept H at time 0 is $H = [f_i(K_o - K_i) + \phi_A - K_o]/[\phi_A - \phi_B]$ and $f_i = N_i/(N_i + N_o)$ is the intracellular proton fraction.

In the presence of an appropriate manganese concentration ϕ_A and ϕ_B simplify to $\phi_A = K_o + K_y$ and $\phi_B = K_i + K_x$. P_d can be calculated from K_x as $P_d = K_x V/A$, where V/A is the cell volume-to-area ratio. T_{ex}, the exchange time is the time required for the cell signal to decay to one-half its value. $T_{ex} = 0.693/K_x$.

Results

Relaxation Curves

Figure 1 illustrates a relaxation curve obtained in the artificial bathing solution, ABS, with 5 mM Mn^{2+}, which can be fitted with one exponential, and a curve obtained after cells were added to the same solution. It may be seen that, in the presence of cells a second, slower, distinct exponential is present, which can be clearly separated from the first one at this Mn^{2+} concentration.

Effect of Mn^{2+} Concentration and Cytocrit on the Measured Exchange Times

In order to avoid possible negative effects of Mn^{2+} on the cell characteristics (Araujo, Persechini & Oliveira-Castro, 1986) and on the exchange times (Pirkle et al., 1979) (which appear at high Mn^{2+} concentrations, $[Mn^{2+}]$), we used as low a $[Mn^{2+}]$ as possible. First we tested several $[Mn^{2+}]$ ranging from 1.25 to 25 mM. $[Mn^{2+}]$ of 10 to 25 mM resulted

[1] This degree of exclusion of Trypan blue was shown to correlate with adequate characteristics of oxygen consumption by the cells.

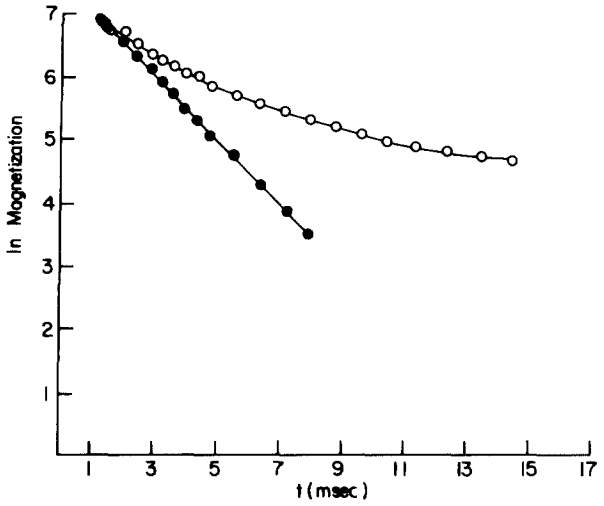

Fig. 1. A computer-fitted semilog plot of the magnetization curves obtained in the presence of ABS only (filled circles) and in the presence of ABS plus cells (open circles). ABS contained 5 mM Mn^{2+}. Temperature 17°C. Circles represent the actual data

Table 1. Exchange time (T_{ex}) and P_d values (expressed per unit outer surface area, no invaginations were taken into account) for isolated proximal tubule cells incubated at various temperatures under control conditions

Temp (°C)	T_{ex} (msec)	P_d (μm/sec)
35.6	14.2 ± 1.3	147 ± 13
23	13.6 ± 1.1	197 ± 17
17	14.4 ± 1.6	145 ± 17
13	20.0 ± 1.5	134 ± 10
7	22.8 ± 2.1	117 ± 10

in prolongation of the exchange times. With a [Mn^{2+}] of 1.25 mM it was difficult to separate the two exponentials, which were, however, clearly discernible with 2.5 and 5 mM Mn^{2+}. With 2.5 and with 5 mM Mn^{2+} exchange times were not statistically different from each other. However, as data analysis was easier with 5 mM Mn^{2+}, this was the Mn^{2+} concentration used in the experiments reported here. Figure 1 illustrates how closely a computer-fitted double-exponential curve follows the original data. Preliminary experiments showed that the intracellular T_2 in the presence of 5 mM Mn^{2+} was stable for 2 to 3 hr. After 3 hr of incubation with Mn^{2+} the intracellular T_2 showed a slight decline. The intracellular T_2 decreased by about 20% after 4-h incubations. As a precaution, in the experiments herein reported only cells that had been in the presence of Mn^{2+} containing solutions for less than 1 hr were used, since it was clear that during incubation for 1 hr no change in the intracellular T_2 was detected. In short, we believe that because of the absence of drift in the relaxation data for times as long as 120 to 150 min and because the same results were obtained with [Mn^{2+}] ranging from 2.5 to 5 mM, any adverse effects of Mn^{2+}, if present, were either minimal or undetectable. Over cytocrit ranges from 15 to 40% results were independent of this parameter. A cytocrit of 30% was typically used, since preliminary experiments had shown that data analysis was more difficult with cytocrits smaller than 15%.

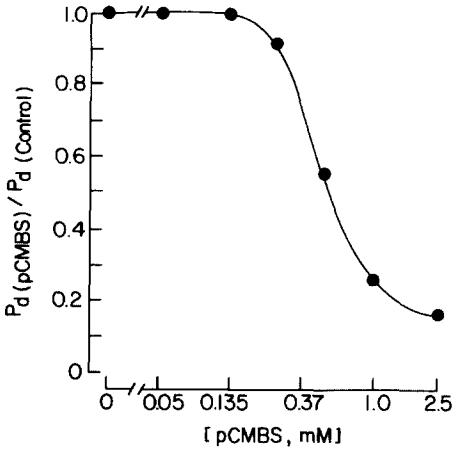

Fig. 2. Dose-response curve for the reduction of P_d by *p*CMBS. Incubation time for each point was 20 to 35 min. Each point was studied in a new cell suspension

Measurements of P_d and Effect of *p*CMBS on P_d

Results of experiments performed under control conditions in the presence of 5 mM Mn^{2+} at different temperatures are shown in Table 1. Results of the P_d measurements are expressed in units of 10^{-4} cm^3/cm^2 of membrane · sec. They are abbreviated as μm/sec. At room temperature the average value is 197 ± 17 μm/sec.[2] To study the effect of *p*CMBS on the diffusive water permeability, different groups of cells of the same preparation were incubated for 20 to 35 min at increasing *p*CMBS concentrations. The experiments were carried out in dextran (3%, mol wt 20,000) solutions instead of ABS (a procedure that does not affect P_d) in order to avoid possible interaction between *p*CMBS and ABS, which might have altered the free amount of *p*CMBS present in the solution. At room temperature the average P_d value in the presence of *p*CMBS is 116 ± 8 μm/sec, i.e. some 59% of the control value. The dose-response curve for *p*CMBS effect is shown in Fig. 2.

[2] To compare these values with P_{os} measurements, usually expressed as 10^{-4} cm^3/cm^2 of membrane · sec osmolar, the molar water volume 0.018 liter/mole was used.

Table 2. Exchange time (T_{ex}) and P_d values (expressed per unit outer surface area, no invaginations were taken into account) for isolated proximal tubule cells incubated at various temperatures in the presence of 2.5 mM *p*CMBS

Temp (°C)	T_{ex} (msec)	P_d (μm/sec)
31	15.2 ± 0.3	124 ± 2
24	16.2 ± 1.2	116 ± 8
14	32.4 ± 6.2	58 ± 11
9	35.6 ± 3.0	53 ± 4

Table 2 shows the effect of the presence of 2.5 mM *p*CMBS as a function of temperature, on the exchange time and P_d values. Notice that at every temperature the P_d values in the presence of *p*CMBS are significantly smaller than the control values ($P < 0.001$). Not shown is the observation that the action of *p*CMBS could be reversed within 10 min by addition of 5 mM dithiothreitol to the preparation. Table 3 shows the E_a values obtained under control conditions and with *p*CMBS. Figure 3 gives the plots used in the calculation of E_a. It can be seen that both in control and *p*CMBS experiments P_d values obtained at temperatures higher than 25°C were lower than those expected from an extrapolation of the values measured at lower temperatures. It is possible that this phenomenon is due to the presence of some cell damage rather than to a real break in the Arrhenius plot. In the calculation of E_a these points were not taken into account.

Table 3. Apparent activation energy values for P_d in isolated proximal tubule cells

Control	5.18 ± 1.00 (n = 4)[a]	kcal/mol
+ 2.5 mM *p*CMBS	9.06 ± 2.22 (n = 3)	kcal/mol

[a] n = number of temperature data used in the calculation.

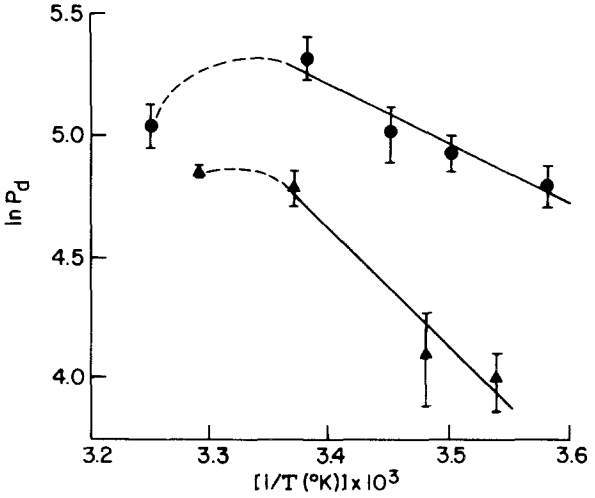

Fig. 3. Arrhenius plots of ln P_d (expressed in μm/sec) as a function of ($1/T$) of control cells (circles) and cells incubated with 2.5 mM *p*CMBS for 20 to 35 min (triangles). The continuous lines have been drawn to fit control and *p*CMBS data to calculate E_a at temperatures lower than 25°C ($1/T = 3.45 \times 10^{-3}$). Data at the highest temperatures are not included in the calculation. A new cell suspension was used for each temperature. Data from Tables 1 and 2

Discussion

The control P_d value obtained at 24°C, when expressed per cell outer surface area (not taking the membrane infoldings into account) was 197 ± 17 μm/sec or 3.58×10^{-4} cm³/cm² sec · osM. This value corrected for the real cell membrane area using the morphometric data of Welling and Welling (1975), yields values for P_d of 22.3 ± 1.9 μm/sec, which can be compared with the values of 36.7 μm/sec obtained in RBC (Moura et al., 1984; *see also* Solomon et al., 1983; Stein, 1986) and of 16 μm/sec for *Necturus* gallbladder epithelial cells (membrane infoldings taken into account, Steward & Garson, 1985).

Effects of Unstirred Layers

Although diffusion delays outside and inside the cell may influence the measurements of P_d (Barry & Diamond, 1984), the NMR technique for the measurement of cell diffusive permeability eliminates the effect of external unstirred layers (USL), because Mn^{2+} has ample time to equilibrate in the extracellular space. The influence of inner cytoplasmic USL will now be evaluated. Using Eq. (7) and Fig. 3 of Hansson-Mild and Lovtrup (1985) in a cell with a measured P_d of 200 μm/sec and a radius of 6 μm, the intracellular compartment can be considered as well stirred if the water diffusion in the cytoplasm is larger than 0.2×10^{-5} cm²/sec, a value which is 7% of the free diffusion coefficient for water. As there are no measured values for water diffusion in proximal cell cytoplasm that we are aware of we use the intracellular diffusion figure measured in hepatocytes of 0.9×10^{-5} cm²/sec at 20 to 22°C (Alpini et al., 1986). This value would indicate that no correction for USL effects is required. However, in the calculations that follow of P_{os}/P_d, as a margin of security we use a possible error of 30%, which would give a highest limit for the P_d values of 30 μm/sec. The possible influence of USL will be smaller in the experiments in which longer T_{ex} were observed.

Comparison of P_d and P_{os}

P_{os} has been measured in isolated proximal straight rabbit tubules (González et al., 1984; Whittembury et al., 1984; Carpi-Medina, 1986) with a high space and time resolving method (Lindemann, 1984*a*; Whittembury et al., 1985) obtaining the following values, expressed per cm^2 of real cell membrane area (cell membrane infoldings being taken into account): apical plasma membrane P_{os}, 297 μm/sec and basolateral plasma membrane P_{os}, 496 μm/sec (this latter value is of the order of that obtained by Welling et al., 1983).[3] Therefore, the average cell P_{os} of 396 μm/sec, yields a ratio of net to tracer water permeability, P_{os}/P_d under control conditions of 18 (=396/22.3), with a lower limit of 13 (if a value for P_d of 30 μm/sec is used in the calculation) clearly $\gg 1.00$. To further advance our knowledge of the geometry of the water pore, we need to consider our results of P_{os}/P_d in the context of solute-solvent interactions. We know that strongly hydrophilic solutes with molecular radii ranging from 4 to 6 Å like mannitol, sucrose and raffinose, do not cross the cell membrane. Therefore the water pore must be about 4 Å in radius or at most about 7 Å if one water molecular is allowed as layer of solute hydration (Solomon, 1986). Now, P_{os}/P_d of 13 to 18 lead to the calculation of a pore radius of 12 to 15 Å, which would let through the solutes mentioned above. Therefore this calculation has to be rejected as unlikely. The situation is analogous to that described in the gramicidin channel (*cf.* Andersen & Procopio, 1980). The alternative interpretation (*see* Introduction) is that the calculation of a pore radius from P_{os}/P_d is inappropriate if the pores in the membrane are too small to allow water molecules to overtake. In such a case, the ratio of the net permeability (measured as P_{os}) to the unidirectional permeability (measured with isotope as P_d) is related to n' the number of water molecules passing in tandem through the pathway (Hodgkin & Keynes, 1955; Heckmann, 1965; Longuet-Higgins & Austin, 1966; Finkelstein & Rosenberg, 1979; Finkelstein & Andersen, 1981; Finkelstein, 1987). This then means that some 11 to 20 water molecules single file through these pathways.[4] The pore radius, r must be bound by the radius a and the diameter $2a$ of the water molecule, $a < r < 2a$ (i.e. between 1.5 and 3.0 Å), so that water molecules do not overtake within the pore. Single-file behavior can also occur in somewhat larger pores if water molecules are prevented from passing each other (Lindemann, 1984*b*).

2.5 mM *p*CMBS reduces P_d to 50% of its control value at room temperature. The *p*CMBS concentration that causes 50% inhibition of the water permeabilities was 0.5 mM for P_d and 0.1 mM for P_{os} (Whittembury et al., 1984). Thus the sulfhydryl reagent, *p*CMBS, reduces both P_{os} and P_d but the change is more marked on the osmotic permeability. The values obtained for both permeabilities in the presence of 2.5 mM *p*CMBS, expressed per cm^2 of real cell membrane area, are for P_d, 10 μm/sec (present work) and P_{os}, 32 μm/sec (Carpi-Medina, 1986) yielding a P_{os}/P_d ratio in the presence of *p*CMBS of 3.2 ± 0.6. Thus *p*CMBS perturbs the single-file water channels. The observation that dithiothreitol reverses the inhibitory action of *p*CMBS on P_{os} and P_d indicates that water pathways stay opened when sulfhydryl groups are kept in a reduced state.

Number of Pores per Cell Membrane Area

With limits for r of 1.5 and 3.0 Å, and the values for P_d and P_{os} given above the number of pores per cm^2 of real membrane area is estimated to range from 10^{11} to 10^{12}. The permeability per pore would be 1.3×10^{-13} cm^3/sec, or 2.3×10^{-15} cm^3/sec Osmolar.

Water-Membrane Interactions

The E_a values obtained for P_d under control and *p*CMBS conditions parallel the results obtained for P_{os} (Whittembury et al., 1984). E_a for P_d under control conditions is 5.2 ± 1.0 kcal/mol for P_d (Table 3) and 3.2 ± 1.4 kcal/mol for P_{os} (Whittembury et al., 1984), not different from the value for free water movement of 4.2 to 4.8 kcal/mol (Stein, 1986). With *p*CMBS E_a for P_d increases to 9.1 ± 2.2 (Table 3) and for P_{os} to 9.2 ± 2.2 kcal/mole (Whittembury et al., 1984), not different from the figures obtained in artificial membranes (Cohen, 1975; Cass & Finkelstein, 1967; Stein, 1986). These values are also very similar to those obtained in RBC (Vieira et al., 1970; Conlon & Outhred, 1978; Pirkle et al., 1979), and indicate a small interaction between water and membrane under control conditions and a larger interaction in the presence of *p*CMBS similar to that encountered in black lipid films (Fettiplace & Haydon, 1980). A water molecule in free solution

[3] The hydraulic conductivity of the tubular basement membrane (Welling & Grantham, 1972) is 300 to 800 times greater than our P_{os} measurement; therefore, no correction of its effect on P_{os} has been used.

[4] The exact relation between P_{os}/P_d and n' depends on the mechanism of water transfer and on the number of vacancies within the channel (Hodgkin & Keynes, 1955; Heckmann, 1965; Kohler & Heckmann, 1980).

makes 4 hydrogen bonds with its nearest neighbors (Hille, 1984). On entering the single-file pore, two of them must break (since inside the pore there will be only one neighbor in front and one behind), resulting in an E_a value > 4.5 kcal/mole. It is possible that the water pore in the kidney proximal tubule cells has similar characteristics to the gramicidin pore interior, that probably has no regions to which the water molecule strongly binds such that water molecules can rotate, thus moving within the pore practically as in free solution (Fornili, Vercauteren & Clementi, 1984), presenting less of a barrier to water diffusion than expected. The residual P_d value of 10 μm/sec observed with *p*CMBS represents water movement mainly through the bilayer part of the membrane. This value could still have a small contribution from specific pathways, since P_{os}/P_d, in the presence of *p*CMBS is 3.2 ± 0.6, significantly larger than 1.00.

Location of the Water Channel

The present experiments point out the presence of water pathways, formed by proteins, sensitive to *p*CMBS, occupied by 11 to 20 molecules of water in a single file, which can be reasonably accommodated in a pore spanning the lipid bilayer (*see* Stein, 1986). Other geometries (alway including a single-file region) are compatible with the value of P_{os}/P_d of 13 to 18 (Levine et al., 1984; Finkelstein, 1987). Direct studies of P_{os}/P_d in apical and basolateral plasma membrane vesicles should help distinguish among some of these possibilities. The present values of P_{os}/P_d make the proximal tubule cell membrane pores similar to those of the RBC and of the ADH-stimulated toad urinary bladder, where a comparable single-file behavior has been proposed (Parisi & Bourguet, 1983; Levine et al., 1984; Moura et al., 1984). The P_{os} figures of RBC and ADH-stimulated toad urinary bladder coincide also with the values reported here when expressed per cm^2 or real cell membrane area, and per cm^2 of basement membrane area, respectively, to make the comparison feasible (Parisi & Bourguet, 1983; Solomon et al., 1983; Levine et al., 1984; Macey, 1984). Clearly many more experiments are needed in order to ascertain how close are these similarities. As an example, Moura et al. (1984) have proposed a two-state model to explain the interaction between *p*CMBS and the proteins that make the water pathways across the RBC membrane, in which the water pathways close in an all-or-none way. Using the inhibition of P_{os} by *p*CMBS as a function of the *p*CMBS concentration in a Hill plot, they have calculated that N, the number of *p*CMBS molecules that interact with one water pathway to inhibit its permeability, equals 1. This suggests a 1 : 1 stoichiometry for the *p*CMBS membrane receptor complex. Using the same assumptions (but realizing the weaknesses of this approach, Klotz, 1985), values of N of 2 to 4 for the inhibition of P_d (Fig. 2) and of P_{os} (Fig. 2 of Whittembury et al., 1984) by *p*CMBS may be calculated indicating that more than one, probably 2 to 4, *p*CMBS molecules are needed to close each water pathway in the proximal tubular cells. Band 3 and band 4.5 proteins (Benga et al., 1983; Solomon et al., 1983; Benz, Tosteson & Schubert, 1984; Solomon, 1986) are being associated with the water channel in the red cell because of the inhibition of water permeability by *p*CMBS, while in the corneal endothelium inhibitors of glucose transport also curtail water transport (Fischbarg, Liebovitch & Koniarek, 1987). It remains an open question, whether this is also the case of the cell membrane of the proximal tubule, in view of the differing values for N mentioned above, of the several transmembrane proteins and of the presence of two cell membranes in series in this nephron segment. Given the dimensions required for the water pathway, water could move through interstices in the proteins, which might even be located in regions closer to the protein-lipid interface, provided the microenvironment of such regions is sufficiently hydrophilic.

Another important difference with red cells and toad urinary bladder emerges from the interaction between water and urea in the water pores in the proximal tubule. The effect of urea as an osmotic agent is clearly smaller than that of mannitol, raffinose and PEG 6000 in the proximal tubule (González et al., 1982), pointing out that the reflection coefficient for urea is <1.00. In the red cell there seem to be conflicting views as to whether urea shares the same pathway with water (Macey, 1984; Solomon, 1986). In the toad urinary bladder and the collecting duct urea does not permeate the water pore (Finkelstein, 1987; Kondo & Imai, 1987). Further experiments along these lines are required in kidney proximal tubule cells.

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