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Osmotic Water Permeabilities of Human Placental Microvillous and Basal Membranes

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Summary. Literature data suggest that water accumulation by the human fetus is driven by osmotic gradients of small solutes. However, the existence of such gradients has not been supported by prior measurements. Attempts to estimate the size of the gradient necessary to drive net water movement have been seriously hampered by the lack of permeability data for the syncytiotrophoblast membranes. Stopped-flow light scattering techniques were employed to measure the osmotic water permeability (P_f) of microvillous (MVM) and basal membrane (BM) vesicles isolated from human term placenta. At 37°C, the P_f was determined to be $1.9 \pm 0.06 \times 10^{-3}$ cm/sec for MVM and $3.1 \pm 0.20 \times 10^{-3}$ cm/sec for BM (mean ± SD, n = 6). At 23°C, P_f was reduced to $0.7 \pm 0.04 \times 10^{-3}$ cm/sec in MVM and $1.6 \pm 0.05 \times 10^{-3}$ cm/sec in BM. These P_f values are comparable to those observed in membranes where water has been shown to permeate via a lipid diffusive mechanism. Arrhenius plots of P_f over the range 20–40°C were linear, with activation energies of 13.6 ± 0.6 kcal/mol for MVM and 12.9 ± 1.0 kcal/mol for BM. Water permeation was not affected by mercurial sulfhydryl agents and glucose transport inhibitors. These data clearly suggest that water movement across human syncytiotrophoblast membranes occurs by a lipid diffusion pathway. As noted in several other epithelial tissues, the basal membrane has a higher water permeability than the microvillous membrane. It is speculated that water accumulation by the human fetus could be driven by a solute gradient small enough to be within the error of osmolarity measurements.

Key Words placenta · microvillous membrane · basal membrane · permeability · water transport · placental transport

Introduction

To sustain normal growth, the human fetus must acquire water from the maternal compartment at a rate which, on a molar basis, exceeds any other substance transported from the mother to the fetus (Wilbur, Power & Longo, 1978). Investigators have been unable to demonstrate a significant gradient of osmotic pressure across the placenta (Seeds, 1965) and since colloidal (Hinkley & Blechner, 1969) and hydrostatic (Nicolini et al., 1989) pressure differences are likely, if anything, to promote water transfer from fetus to mother, the nature of the driving forces for net fetal water accumulation remains poorly defined. Although human placental water transfer has been modeled (Wilbur et al., 1978), the lack of experimental data in these computer simulations has severely limited their value.

A thorough understanding of placental water transfer requires information concerning the permeability characteristics of the syncytiotrophoblast microvillous and basal membranes. The osmotic water permeability (P_f) of human placental microvillous membranes has been measured previously (Illsley & Verkman, 1986). However, P_f for the basal membrane must be known to estimate water permeability across the syncytiotrophoblast cell. The P_f value obtained previously for the syncytial microvillous membrane was determined using a preparation that is substantially different from that used here, necessitating a repeat measurement. In addition, the results of the previous study were inconclusive with regard to the pathways of water movement since the activation energy suggested movement of water through aqueous channels, while inhibition data and the permeability values were compatible with a lipid-mediated pathway.

The objectives of the present study were to determine the osmotic permeability coefficients for both human syncytiotrophoblast membranes and to unambiguously define the transcellular pathway for water movement in the placenta. For this purpose we used a recently developed preparation (Illsley et al., 1990) which generates a high yield of human syncytiotrophoblast microvillous (MVM) and basal membranes (BM) from the same piece of tissue. To determine P_f, we employed stopped-flow light scattering techniques, used extensively in transport studies in membrane vesicles from a variety of tissues (Rabon, Takeguchi & Sachs, 1980; Worman & Field, 1985; Illsley & Verkman, 1986; van Heeswijk & van Os, 1986; Verkman & Fraser, 1986).

Materials and Methods

Materials

1,6-diphenylhexatriene (DPH) and 9-amino acridine (9-AA) were obtained from Molecular Probes (Eugene, OR). All other chemicals were purchased from Sigma Chemical (St. Louis, MO).

Vesicle preparation

Microvillous (MVM) and basal membrane (BM) vesicles were prepared simultaneously from human term placenta as described previously (Illsley et al., 1990). Briefly, after initial homogenization and centrifugation steps (at 4°C, in 250 mM sucrose, 10 mM HEPES-Tris, pH 7.4) BM were separated from MVM by Mg^{2+} precipitation and further purified on a sucrose step gradient. Samples were frozen in liquid nitrogen and stored at −80°C. This preparation produces membrane fractions with a high enrichment (MVM: 20-fold, BM: ~16-fold), high yield (25%) and minimal contamination by intracellular and nonsyncytial plasma membranes (Illsley et al., 1990).

Fluorescence Measurements

Fluorescence intensity measurements were carried out using a SLM 8000C spectrofluorometer (SLM instruments, Urbana, IL).

Measurements of Vesicular Dimensions

Two fluorescent probes, diphenylhexatriene (DPH) and 9-amino acridine (9-AA), were used to measure the surface-to-volume ratio of the vesicles. DPH is virtually nonfluorescent in aqueous solution but has a high affinity for the hydrophobic core of the membrane where the probe is highly fluoresent (Molitoris, 1987). The increase in fluorescence when membranes are added to a solution of DPH is proportional to the amount of membrane (and therefore surface area). Nonprotonated 9-AA distributes rapidly across vesicle membranes and accumulates in acidic compartments without significant membrane binding (Lee & Forte, 1978). Inside an acidic vesicle the probe is protonated and retained, resulting in probe accumulation and self-quenching. The extent of self-quenching of 9-AA fluorescence is proportional to the volume of the (acidic) vesicle, thus providing a measure of vesicular volume. For both probes, the change in fluorescence following membrane addition (ΔF) was calculated after correction for dilution and nonspecific membrane effects. The following protocol was used in these experiments: 0.4 ml vesicles (5–15 mg protein/ml) were diluted in 10 ml buffer A (100 mM potassium phosphate, pH 5.00 at 23°C). After overnight incubation at +4°C, vesicles were concentrated by centrifugation and resuspended in a small volume (~0.5 ml) of buffer A. In addition, two dilutions (1 : 1 and 1 : 3) were made up for each sample in buffer A. Vesicles from each dilution (25 μl) were added to 2 ml of 5 μM 9-AA in buffer B (100 mM potassium phosphate, pH 8.00 at 23°C) and the change in fluorescence emission (ΔF) at 455 nm (422 nm excitation) was measured. Subsequently, 25 μl of vesicles were incubated in 2 ml of 10 μM DPH for 30 min at room temperature and DPH fluorescence was measured using an excitation wavelength of 328 nm and an emission wavelength of 415 nm. At each dilution, measurements were carried out in triplicate resulting in nine determinations for each sample.

Fraction of Closed Vesicles

In our S/V measurements, open or highly permeable vesicles will contribute to the DPH but not to the 9-AA signal. This could constitute a potential source of error in the S/V-ratio measurements if the fraction of open vesicles differs substantially between MVM and BM. To estimate the fraction of open vesicles, quenching of DPH fluorescence by 2,4,6-trinitrobenzene sulfonic acid (TNBS) was used. TNBS reacts with the amino groups of membrane proteins and since trinitrophenyl-amino groups have an absorption maximum at 415 nm they quench DPH emission (Schroeder et al., 1988). In synaptic plasma membranes very little TNBS permeated over 80 min at 4°C, whereas at 37°C, TNBS permeated quite readily. This is the basis for selectively labeling the outer (4°C, nonpenetrating conditions) and the inner and outer monolayers (37°C, penetrating conditions). Schroeder and co-workers (1988) demonstrated that the TNBS which bound to the outer monolayer was only capable of quenching DPH in that monolayer. Under nonpenetrating conditions, TNBS binds to the inner leaflet only when vesicles are open or highly permeable. Vesicles (25 μl, 5–15 mg/ml) were incubated in 2 ml of 1 μM DPH for 60 min at 23°C. Fluorescence emission measurements (in triplicate) at 415 nm (excitation 328 nm) were acquired over 10 sec at 4-min intervals during this period. Following addition of 1.5 mM TNBS, the fluorescence signal intensity dropped immediately due to partial absorption of the excitation light. Subsequently, the slow fluorescence decrease, due to the quenching of DPH fluorescence by TNBS binding was measured. Measurements were carried out at 4°C and 37°C using control samples to which no TNBS was added.

Membrane Fluidity

To study the influence of increased membrane fluidity on water transport, vesicles were incubated in 50 mM benzyl alcohol for 20 min at 23°C. To evaluate the fluidity effects of this concentration of benzyl alcohol, the steady-state anisotropy of DPH was measured as described previously (Illsley, Lin & Verkman, 1987).

Stopped-Flow Measurements

These were performed on a Hi-Tech SF 51 stopped-flow apparatus (Salisbury, Wiltshire, UK) using a 50-W quartz halogen lamp in series with a single grating monochromator (excitation 500 ± 5 nm) as a light source. Sample temperature was controlled by a circulating water bath and monitored by an indwelling thermistor. After overnight loading (4°C) in 150 mM raffinose, 5 mM HEPES-Tris pH 7.4 (buffer C), vesicles (75 μl; 0.5–1.5 mg protein/ml) were mixed with an equal volume of buffer C to which additional raffinose had been added to produce inwardly directed osmotic gradients. The time course of 90° scattered light intensity at 500 nm was measured and 2,500 data points were acquired per experiment. Single exponential functions were fitted to the time course of scattered light intensity using a nonlinear Newton's method. Experiments were carried out in quadruplicate for each sample and experimental condition. Solution osmolarities were measured using an Osmette A Model 5002 (Precision Instruments, Natick, MA).

P_f Calculations

After imposing an osmotic gradient due to an impermeant solute the change in vesicle volume over time is described by:

$$dV(t)/dt = P_f V_w S(C_i(t) - C_o) \quad (1)$$

where $V(t)$ is the volume of the vesicle, P_f is the osmotic water permeability coefficient, V_w is the molar volume of water, S is the surface area, $C_i(t)$ is the internal concentration of the impermeant and C_o is the constant external concentration of impermeant. Assuming that the intravesicular solute is impermeant over the time course of water movement, the number of intravesicular molecules remains unchanged, thus

$$C_i(t)\ V(t) = C_i(t = o)\ V_o \quad (2)$$

where V_o is the initial volume. As demonstrated subsequently (*see* Results) scattered light intensity (I) is linearly related to the volume of the vesicle:

$$V(t)/V_o = AI + B \quad (3)$$

where A and B are instrumental amplitude and offset. Combining Eqs. (1)–(3) gives:

$$dI/dt = (\{P_f V_w (S/V_o)\}/A)\ (\{C_i(t = o)/(AI(t) + B)\} - C_o) \quad (4)$$

The iso-osmotic volume of the vesicle (V_o) in Eq. (4) was corrected for inactive osmotic space (b) according to van Heeswijk & van Os (1986) using the formula $b = 1 - \{(r - 10)^3/r^3\}$, where r is the radius of the vesicle. Assuming that the membrane vesicles obtained by the current homogenization procedure are approximately spherical, the surface-to-volume ratio is related to the radius of the vesicle by $S/V = 3/r$. Based on the calculated S/V (*see* Results), the osmotic inactive spaces are ~38% and ~31% for MVM and BM. P_f was calculated by comparing single exponential time constants of experimental light-scattering data to curves simulated using Eq. (4) where P_f, A and B were the fitted parameters.

Statistics

Results are given as means ± SD. For statistical analysis Students' t-test for paired samples was used.

Results

Measurement of Vesicular Dimensions

Microvillous membranes prepared by isotonic agitation (Johnson & Smith, 1980; Dicke & Henderson, 1988), a method which shears microvilli from the maternal facing surface of the trophoblast, had a ΔF DPH/ΔF 9-AA of 0.80 ± 0.11 (n = 3). The surface area-to-volume (S/V) ratio for MVM prepared by this method has been calculated by means of electron microscopy to be 3.2×10^5 cm^{-1} (Johnson & Smith, 1980). The fluorescence ratio for membrane vesicles prepared by the current protocol was 1.12 ± 0.15 for MVM (n = 8) and 0.87 ± 0.15 for BM (n = 9). Assuming the estimate made by electron microscopy to be accurate, this value and fluorescence measurements of DPH and 9-AA were used to calculate an S/V ratio of 4.48×10^5 cm^{-1} for MVM and 3.48×10^5 cm^{-1} for BM obtained from the current preparation.

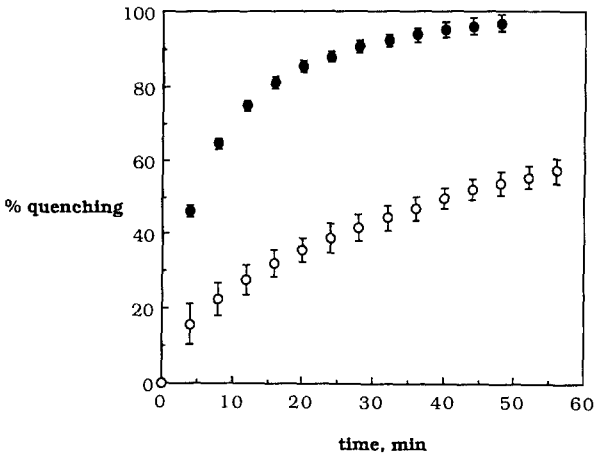

Fig. 1. Time course of DPH fluorescence quenching following addition of 1.5 mM TNBS to BM at 4°C (○) and 37°C (●) (n = 3).

The technique measuring S/V is dependent on a linear relationship between surface area (DPH) and volume (9-AA). This assumption was tested by varying the quantity of vesicles; a twofold increase in protein concentration should produce a twofold increase in ΔF. Indeed, ΔF for both probes was linearly related to the quantity of vesicle with a slope close to 1 (*data not shown*), supporting the validity of the technique.

It is predicted that if all vesicles are sealed, TNBS would quench ~50% of the DPH fluorescence at 4°C. However, if all vesicles are open, TNBS would have access to both bilayer leaflets and therefore would quench virtually all the initial fluorescence. After addition to TNBS to vesicles incubated in DPH, no distinct plateau could be delineated at 4°C, indicating that some TNBS may penetrate under these conditions (Fig. 1). The time course of TNBS binding to the membrane can be described by a saturable function. However, since the TNBS binding to the inner monolayer at 4°C is much slower than the binding to the outer leaflet, the former process will essentially be linear or nonsaturable over the time course studied. Thus, to determine the plateau, curves were fitted to saturable (37°C) and saturable plus nonsaturable (4°C)

Table 1. Time course and degree of raffinose permeation in comparison to the time course of water movement

Type	Temp °C	Δt (sec)	Relative amplitude (t = 20 sec)(%)	τ (sec^{-1})
MVM	23	9 ± 1	98 ± 0.5	0.54 ± 0.03
	37	5 ± 1	89 ± 1	0.19 ± 0.01
BM	23	4 ± 0.5*	80 ± 5*	0.41 ± 0.03*
	37	2 ± 0.5*	53 ± 5*	0.15 ± 0.01*

Means ± SD, $n = 3$ *$P < 0.05$, compared to MVM, paired t-test. Experiments were carried out using a 100 mOsm inwardly directed raffinose gradient. Δt is time from the start of data acquisition at which the intensity of scattered light starts to decrease. Relative amplitude (t = 20 sec) corresponds to $(\Delta I_{(t=20)}/\Delta I_{max}) \times 100$ where ΔI_{max} is the plateau amplitude and $\Delta I_{(t=20)}$ the amplitude at 20 sec of the light-scattering curve.
τ is the exponential time constant for water movement.

functions. In three BM preparations, 100 ± 2% of the DPH signal was quenched by TNBS under penetrating conditions (37°C). Under nonpenetrating conditions (4°C) 51 ± 18% of the initial signal was quenched. In three MVM preparations 100 ± 6% (37°C) and 55 ± 10% (4°C) of the DPH signal was quenched. This suggests, first, that close to 100% of the membrane vesicles in the current preparation are closed and, second, that MVM and BM do not differ in this respect. Consequently, no correction of the S/V values was deemed necessary.

P_f Measured with Light-Scattering Techniques—Methodological Considerations

This section examines the issues which must be addressed in order to measure osmotic permeability coefficients from light-scattering data.

Permeation of the "Impermeant"

Sucrose (mol wt 342) and mannitol (mol wt 180) are usually considered to be impermeant over the time course of water movement in a number of vesicle systems (Illsley & Verkman, 1986; van Heeswijk & van Os, 1986; Verkman & Fraser, 1986). However, in preliminary experiments using sucrose, we observed that under certain conditions the light-scattering intensity curve increased but did not reach a defined plateau. Instead the curve peaked and then started to decrease. This phenomenon, which was particularly evident for BM at 37°C, clearly suggests that sucrose entry into vesicles during the time course of water movement cannot be disregarded. As a consequence, single exponential fits of the data were imprecise. If raffinose (mol wt 594) was used as the impermeant, a constant light-scattering intensity was observed after the initial increase due to vesicle shrinkage, over a time course (Δt) which was a magnitude longer than the time course of water movement (Table 1). Despite this, the light-scattering intensity started to decrease eventually, at an earlier time in BM compared to MVM, suggesting that BM is more permeable to raffinose than MVM. This idea is corroborated by the finding that the light-scattering intensity had decreased to a lower level in BM (53% of maximal value, 37°C) at 20 sec than in MVM (90% of maximal value, 37°C). From these data we conclude that raffinose is impermeant over the time scale of water movement permitting reliable P_f calculations.

Unstirred Layers

In vesicle systems unstirred layer (USL) effects are likely to affect measurements of diffusional permeabilities more than determinations of osmotic permeabilities (Fettiplace & Haydon, 1980). Presence of internal or external USL was tested by increasing the viscosity on the inside or the outside of the vesicle through the addition of 6% dextran (Illsley & Verkman, 1986). In five preparations, increased internal or external viscosity failed to alter the rate of water movement (*data not shown*) suggesting that USL effects on P_f measurements can be neglected.

Linearity between Volume and Scattered Light Intensity

In deriving Eq. (3) it was assumed that scattered light intensity is a linear function of vesicular volume. Figure 2 shows that for MVM at 23°C (n = 3), the amplitude of the scattering signal is linearly related to gradient size in the range of 0–200 mOsm. Similar plots for BM at 23°C, BM at 37°C and MVM at 37°C (n = 3) also demonstrated a linear relationship. In the present study absolute volume was not measured but volume should be linearly related to gradient size provided that the vesicles behave as perfect osmometers. Since this has been shown for a number of vesicle systems (Verkman, Dix & Seifter, 1985; Verkman & Fraser, 1986) including placental MVM (Illsley & Verkman, 1986) within the range of gradients used here, we concluded that the basic assumption in Eq. (3) was valid.

P_f Dependence on Gradient Size

The calculated P_f decreased with increasing osmotic gradient (Fig. 3). Dependence of P_f on gradi-

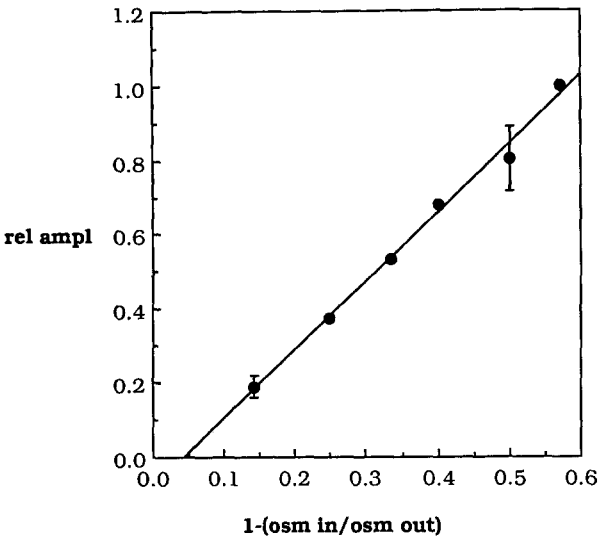

Fig. 2. Relation between gradient size (25–200 mOsm raffinose) and relative amplitude of light-scattering intensity for three MVM preparations at 23°C. The amplitude obtained at 200 mOsm gradient was assigned relative amplitude = 1.0

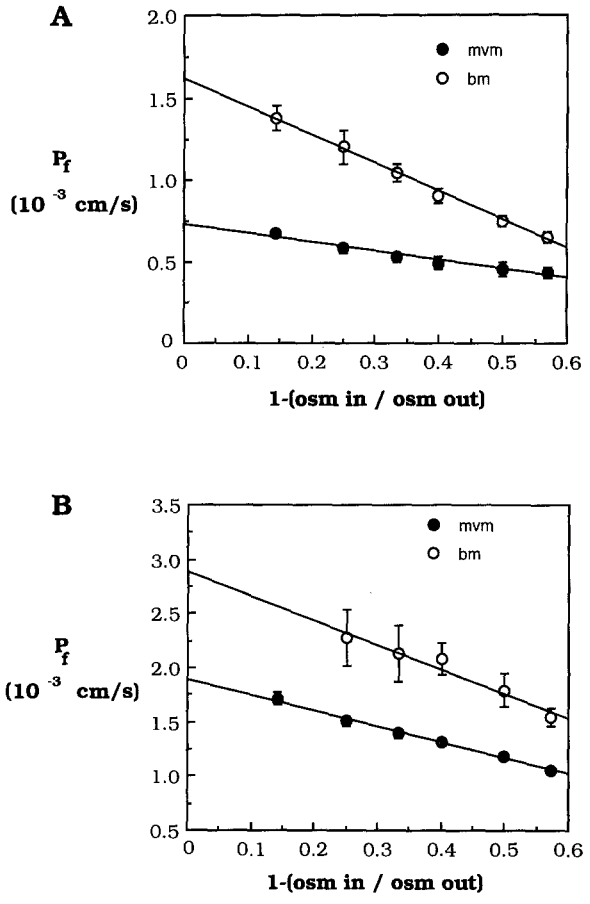

Fig. 3. Relation between gradient size (25–200 mOsm rafinose) and P_f for MVM and BM at 23°C (***A***) and at 37°C (***B***), $n = 3$.

ent size has been described previously for placental MVM (Illsley & Verkman, 1986) and after a thorough examination in that study, a structural restriction mechanism was suggested as a possible cause. In the current investigation, all P_f values were extrapolated to zero gradient using a linear fit (Fig. 3).

Osmotic Water Permeabilities

P_f values, obtained at a 100 mOsm inwardly directed gradient and subsequently extrapolated to zero gradient, are given in Table 2. BM are 128% (23°C) and 63% (37°C) more permeable to water than MVM ($P < 0.001$) A comparison to published P_f values in other vesicle systems is also provided in Table 2. It should be noted that renal membranes have been shown to contain specific carriers for water, explaining the high P_f values in comparison to other membranes in which lipid diffusion is the mechanism which best describes water movement.

Temperature Dependence

The activation energies (E_a) for placental MVM and BM water transport were determined from its temperature dependence data (20–40°C) using a 100 mOsm inwardly directed raffinose gradient. E_a was calculated from the slope of an Ahrrenius plot of ln $1/\tau$ *vs.* $1{,}000/T$. Figure 4 shows a representative plot for one MVM preparation. E_a for MVM was 13.6 ± 0.6 kcal/mol ($n = 3$) and for BM E_a was 12.9 ± 1.0 kcal/mol ($n = 3$).

Inhibition Studies

The effect on water transport of the following agents was investigated: mercuric chloride (250 μM), *p*-chloromercuribenzylsulfonate (2 mM), N-ethyl-malemide (100 μM), fluorodinitrobenzene (100 μM), 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) (100 μM), phloretin (100 μM) and cytochalasin B (100 μM). Solutions of inhibitors were made up fresh at the time of experiment and vesicles were incubated for 30 min prior to experimentation. None of the inhibitors affected water transport at 23°C in three MVM and BM preparations (*data not shown*).

Effect of Benzyl Alcohol

DPH steady-state anisotropy, commonly taken as a measure of membrane fluidity, was measured in

Table 2. Osmotic permeability coefficients in membrane vesicles

Source	Type	Temp °C	P_f ($\times 10^{-3}$ cm/sec)	Reference
Human placenta	MVM	23	0.7 ± 0.04	
		37	1.9 ± 0.06*	
Human placenta	BM	23	1.6 ± 0.05	
		37	3.1 ± 0.20*	
Human placenta	MVM	23	3.0	(1)
Rabbit kidney	MVM/BM	37	11/25	(2), (3)
Rat kidney	MVM/BM	35	35.9/29.1	(4)
Rat small intestine	MVM/BM	35	6/6	(4)
Liposomes		25	0.1–4.9	(5)

Upper panel: Means ± SD. *$P < 0.001$ paired t-test *vs.* MVM. Data from six preparations obtained using a 100 mOsm inwardly directed raffinose gradient and subsequently extrapolated to zero osmotic gradient.
Lower panel: Osmotic water permeabilities in some other studies: (1) Illsley & Verkman, 1986; (2) Verkman et al., 1985; (3) Verkman & Ives, 1986; (4) van Heeswijk & van Os, 1986; (5) Fettiplace & Haydon, 1980.

Table 3. Effect of 50 mM benzyl alcohol on water transport rates and DPH steady-state anisotropies.

		Control	Benzyl alcohol (50 mM)
$\tau(sec^{-1})$	MVM	0.243 ± 0.03	0.203 ± 0.021*
	BM	0.203 ± 0.03	0.193 ± 0.03
r	MVM	0.230 ± 0.005	0.211 ± 0.004*
	BM	0.197 ± 0.007	0.183 ± 0.006*

Means ± SD. $n = 4$. *$P < 0.05$, compared to control, paired t-test. Experiments were performed at 23°C with a 200 mOsm inwardly directed raffinose gradient.
τ is the exponential time constant. r is the steady-state anisotropy of DPH.

control vesicles and found to be lower in BM as compared to MVM (Table 3). Addition of 50 mM benzyl alcohol decreased steady-state anisotropy significantly in both MVM and BM, suggesting an increase in the fluidity. Benzyl alcohol also decreased the exponential time constant obtained for osmotically induced change in light scattering by 16% (increased rate of water transport) in four MVM preparations at 23°C. However, no significant effect was observed in four BM samples (Table 3.)

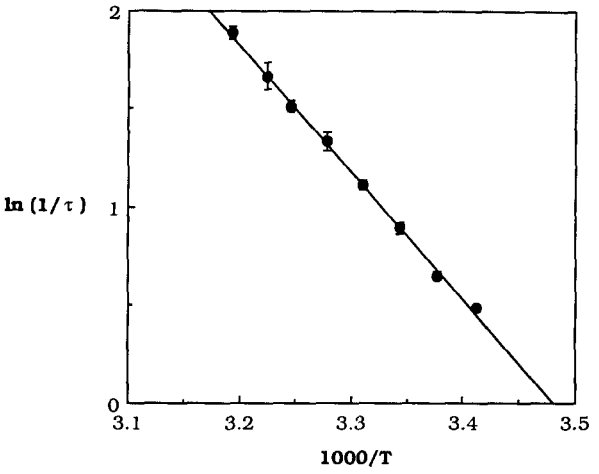

Fig. 4. Temperature dependence of water transport in one representative MVM preparation. Exponential time constants were measured between 23 and 37°C, using a 100 mOsm inwardly directed raffinose gradient. Fitted E_a value was 12.9 kcal/mol. Each measurement was performed in quadruplicate; error bars represent ± SD.

Discussion

This is the first study to report osmotic water permeabilities for both microvillous and basal membranes of the human syncytiotrophoblast. The permeability of placental membranes to water is more than of passing interest since the nature of the driving forces for normal fetal water acquisition is unknown. Furthermore, the pathophysiology behind various disturbances in fetal fluid balance (e.g., oligo- and polyhydramnios) is not fully understood. A more thorough understanding of these conditions can be accomplished only if basic information concerning the water permeability of the placenta is available.

We believe that the preparative procedure employed here produces membrane vesicles that are well suited for the study of water permeability. First, the preparation produces a high yield (~25%) of placental microvillous and basal membranes vesicles (Illsley et al., 1990). The membranes are therefore likely to be representative of the *in vivo* surface rather than consisting of a subpopulation of differing membrane domains. Second, the degree of cross-contamination, contamination by intracellular and nonsyncytial plasma membranes is low (Illsley et al., 1990). Finally, the two syncytiotrophoblast membranes are prepared from the same placenta which will tend to decrease the variability of clinical and genetic origin.

Three technical aspects of the current report deserve brief consideration. First, reliable S/V de-

terminations are critical in order to obtain valid P_f values. The most commonly employed technique, electron microscopy, is time-consuming and relies, for a single S/V value, on the averaging of a wide range of vesicle sizes and shapes. In the present investigation, a novel technique to measure S/V is introduced which involves the use of two fluorescent probes, DPH and 9-AA. In its present form it is dependent on an absolute reference, in this case an S/V value for MVM determined by electron microscopy (Johnson & Smith, 1980), and on a determination of the fraction of open vesicles. As a result of the former, it inherits some of the uncertainties of the electron microscopy technique. The results of the TNBS protocol indicated that a majority of placental membrane vesicles are closed resulting in a S/V for BM that is ~20% lower than for MVM. Although variable, S/V determinations in membrane vesicles originating from other epithelia generally show smaller values for the basal membrane. For example, in membrane vesicles from rat (van Heeswijk & van Os, 1986) and rabbit renal cortex (Meyer & Verkman, 1987) S/V for basolateral membranes were ~50% lower than for brush border membranes. In contrast, other determinations in membranes from rabbit proximal renal tubule (van der Goot, Podevin & Corman, 1989) as well as estimations in rat small intestinal vesicles (van Heeswijk & van Os, 1986) show similar sizes of MVM and BM. It is likely that both the uncertainties in the methods as well as the differences in epithelial structure contribute to this variability. We believe that the DPH/9-AA technique produces an acceptable estimate of the S/V. If further developed and evaluated, this methodology may prove useful as a simple and rapid technique to measure S/V in vesicular systems.

For the light-scattering technique to be valid it is necessary that the "impermeant" does not cross the membrane over the time course of water movement. Sucrose has been generally accepted as an impermeant in this context (Illsley & Verkman, 1986; Verkman & Fraser, 1986; Meyer & Verkman, 1987); however, in preliminary experiments it was found that at 37°C, with sucrose as an impermeant, light-scattering intensity curves started to decrease without reaching a plateau, effects which were more prominent in BM than in MVM. As a consequence, fitting the light-scattering data to exponential functions was difficult and variable. Since it was suspected that permeation of sucrose (mol wt 342) caused these problems, raffinose (mol wt 594) was substituted for sucrose. By comparison with the corresponding sucrose curves, raffinose curves in BM at 37°C had substantially higher amplitudes, higher exponential time constants and a prominent plateau. This suggests that under these conditions sucrose did indeed permeate enough to alter the appearance of the light-scattering curve. Increasing the molecular size of the impermeant to stachyose (mol wt 667) did not change the curves further, indicating that raffinose was sufficiently impermeant to give reliable P_f values. The data in Table 1 lend further support to this conclusion. The effects of sucrose were primarily observed in BM, suggesting a higher permeability to solutes in BM than in MVM. Preliminary results from this laboratory regarding solute permeabilities of human syncytiotrophoblast membranes corroborate this idea (*unpublished observations*).

The third issue which deserves comment is the observation that P_f increased with decreasing size of the osmotic gradient (Fig. 3). This dependence has previously been described for placental MVM (Illsley & Verkman, 1986) and in coated vesicles from bovine brain (Verkman et al., 1989). In contrast, P_f seems to be independent of gradient size in membranes from isolated toad bladder granules (Verkman & Masur, 1988) or in platelets (Meyer & Verkman, 1986). However, in many vesicle systems a possible effect of gradient size on P_f does not appear to have been considered (e.g., Verkman & Fraser, 1986; van der Goot et al., 1989). In the previous report on P_f in placental MVM (Illsley & Verkman, 1986) it was concluded that a structural restriction mechanism impeding vesicle shrinkage was the most likely cause of the decrease in apparent P_f with increasing gradient size. Since the osmotic gradients operating in vivo are likely to be very small, the P_f values reported here are extrapolated to zero gradient. It is possible that P_f is also dependent on gradient size in other vesicle systems in which this relation has not been currently investigated. If so, the reported P_f values may be too low in comparison to the permeability at very small (in vivo) gradients.

P_f values for placental MVM in the present investigation (0.7×10^{-3} cm/sec, 23°C; 1.9×10^{-3} cm/sec, 37°C) are lower than values reported previously (3×10^{-3} cm/sec, 23°C; 4.8×10^{-3} cm/sec, 37°C (Illsley & Verkman, 1986). Although this difference may, in part, reflect the different preparative procedures used in the two studies, the higher molecular weight "impermeant," the correction for inactive osmotic space and the higher S/V ratio used in the current study are all factors that will decrease measured permeabilities. As outlined in Table 2, P_f for human syncytiotrophoblast membranes is similar to values published for liposomes (Fettiplace & Haydon, 1980). However, the osmotic water permeability of placental membranes appears to be lower than P_f in platelets (7×10^{-3}

cm/sec at 37°C; Meyer & Verkman, 1986), rat brain synaptosomes (4.5×10^{-3} cm/sec at 23°C; Verkman & Fraser, 1986) and rat small intestine MVM (van Heeswijk & van Os, 1986).

The finding that placental BM are more permeable to water than MVM is consistent with observations in some other epithelia e.g., rabbit renal membranes (Verkman et al., 1985; Verkman & Ives, 1986; van der Goot et al., 1989). This does not seem, however, to be a generally valid principle since rat small intestine MVM and BM have similar water permeabilities (van Heeswijk & van Os, 1986). It is likely that the higher P_f of placental BM is due to a higher membrane fluidity, compared to MVM. Steady-state DPH-anisotropy was substantially lower in BM than MVM (Table 3) showing that the basal membrane is the more fluid of the two membranes. These data are consistent with findings in other epithelia, e.g., in rat enterocytes (Brasitus & Schachter, 1980), rabbit kidney (Illsley, Lin & Verkman, 1988) and canine kidney (Le Grimellec et al., 1982). As shown by our benzyl alcohol data, an increase in membrane fluidity increases the rate of water transport, corroborating data from other vesicle systems (Brasitus et al., 1986; Worman et al., 1986). Interestingly, the rate of water transport increased significantly only in MVM, a finding that may be related to the lower initial fluidity of this membrane. The lipid composition of artificial lipid membranes has a major influence on its water permeability (Fettiplace & Haydon, 1980). For example, cholesterol addition usually reduces the water permeability of the liposome by several-fold. It is suggested that the higher fluidity and water permeability of the placental BM as compared to MVM is due to differences in lipid composition.

The P_f values for both BM and MVM from human syncytiotrophoblast are substantially lower than values for epithelial membranes shown to contain water channels (e.g., renal proximal tubule membranes; *see* Table 2). This suggests that water channels are not present in placental membranes. The inhibition data lend further support to this conclusion; sulfhydryl reacting agents, which have been shown to inhibit water permeation in membranes containing water channels (Macey & Farmer, 1970; van Heeswijk & van Os, 1986; Meyer & Verkman, 1987) failed to decrease P_f in placental membrane vesicles. Fischbarg and co-workers (1990) have reported the mediation of water transport by facilitative glucose transporters. This was the rationale for investigating possible effects of glucose transport inhibitors on P_f. Neither cytochalasin B nor phloretin, in concentrations that inhibit glucose transport in membrane vesicles, had any effect on P_f in MVM or BM. From these data we conclude that no significant water movement occurs across syncytiotrophoblast membranes via the facilitated glucose carriers.

Additional evidence that water moves across the placental membranes via the lipid regions by means of a solubility-diffusion mechanism rather than through water channels is provided by the temperature dependence data. In membranes containing water channels the activation energy (E_a) for water transport is typically $\leq$ 5 kcal/mol (Macey, 1984; Meyer & Verkman, 1987). In contrast, in membranes lacking water channels, including lipid bilayers (Fettiplace & Haydon, 1980), rat small intestine membranes (van Heeswijk & van Os, 1986) and brain synaptosomes (Verkman & Fraser, 1986), E_a is usually $>$ 8–10 kcal/mol. Thus, the E_a values of ~13 kcal/mol reported here clearly support a lipid-mediated pathway for water permeation in the human placenta. The substantially lower E_a for placental MVM reported previously (5.4 kcal/mol; (Illsley & Verkman, 1986) is at odds with the present findings. Despite this discrepancy, the other findings in the earlier study (low P_f values and lack of inhibition by sulfhydryl reacting agents) are consistent with a lipid-mediated pathway.

The water permeabilities reported here for syncytiotrophoblast membranes will serve as a necessary basis for more accurate modeling of water transfer across the human placenta than previously possible (Wilbur et al., 1978). Syncytiotrophoblast solute permeabilities, necessary for estimation of paracellular cross-sectional area, have not been determined. Consequently, neither the relative contribution of paracellular and transcellular pathways to placental water fluxes, nor the size of the solute gradient necessary to drive net water movement can be calculated with any precision. Nevertheless, assuming hypothetically that the placental barrier consists only of a transcellular route (no paracellular channels), and using published values for placental surface area (Teasdale & Jean-Jacques, 1988), the solute gradient necessary to drive fetal water accumulation of 22 ml/day at term (Hytten, 1979) can be estimated to be less than 0.7 mM. This value will increase somewhat when the effect of paracellular channels is taken into account. This is due to colloidal (Hinkley & Blechner, 1969) and hydrostatic (Nicolini et al., 1989) pressure differences which are likely to promote water transfer from fetus to mother. Although the contribution of the paracellular pathway to transplacental water fluxes is unknown, it is conceivable that water accumulation by the human fetus could be driven by a solute gradient small enough to be within the error of standard osmolarity measurements.

In conclusion, we report values for the osmotic water permeabilities of human placental microvillous and basal membranes which are similar to val-

ues found in membranes that do not contain water channels and substantially lower than in membranes that do. These results, in conjunction with a high activation energy for water transport and failure to inhibit water transport by protein modifying agents, clearly support a lipid-mediated pathway for water permeation across syncytiotrophoblast membranes.

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