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Single Water Channels of Aquaporin-1 Do not Obey the Kedem-Katchalsky Equations

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Abstract. The Kedem-Katchalsky (KK) equations are often used to obtain information about the osmotic properties and conductance of channels to water. Using human red cell membranes, in which the osmotic flow is dominated by Aquaporin-1, we show here that compared to NaCl the reflexion coefficient of the channel for methylurea, when corrected for solute volume exchange and for the water permeability of the lipid membrane, is 0.54. The channels are impermeable to these two solutes which would seem to rule out flow interaction and require a reflexion coefficient close to 1.0 for both. Thus, two solutes can give very different osmotic flow rates through a semi-permeable pore, a result at variance with both classical theory and the KK formulation. The use of KK equations to analyze osmotic volume changes, which results in a single hybrid reflexion coefficient for each solute, may explain the discrepancy in the literature between such results and those where the equations have not been employed.

Osmotic reflexion coefficients substantially different from 1.0 cannot be ascribed to the participation of other 'hidden' parallel aqueous channels consistently with known properties of the membrane. Furthermore, we show that this difference cannot be due to second-order effects, such as a solute-specific interaction with water in only part of the channel, because the osmosis is linear with driving force down to zero solute concentration, a finding which also rules out the involvement of unstirred-layer effects. Reflexion coefficients smaller than 1.0 do not necessitate water-solute flow interaction in permeable aqueous channels; rather, the osmotic behaviour of impermeable molecular-sized pores can be explained by differences in the fundamental nature of water flow in regions either accessible or inaccessible to solute, created by a varying cross-section of the channel.

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Key words: AQP1 (aquaporin-1) — Osmosis — Kedem-Katchalsky equations — Water channels — Reflexion coefficient — Red cell.

Introduction

The Kedem-Katchalsky equations (Kedem & Katchalsky, 1958) are considered to represent flows of water and solute in a transmembrane pore and, applied to cell membranes, the phenomena of osmotically induced cell swelling or shrinkage. These equations are based on the assumption that Onsager symmetry applies between osmosis and ultrafiltration in aqueous pores (Staverman, 1951) leading to the result that the reflexion coefficients for osmosis and ultrafiltration are equivalent. There is, however, virtually no experimental evidence for this widely accepted theory and none that demonstrates equivalence in a unit osmotic channel. The equations embody a model of osmosis in which the osmotic flow rate, described by the reflexion coefficient, is a function of the interaction between osmolyte and water in a channel. By adopting a single mechanism of water transfer through very small pores this assumption can be given a semi-rigorous proof (Levitt, 1975) although, if viscous flows are involved, symmetry cannot be demonstrated as a general phenomenon. With the discovery of aquaporins, which dominate the water permeability of most cell membranes by creating single water channels spanning the lipid membrane, the KK equations have been used to analyze osmotically-induced changes in volume of erythrocytes and other cells. The results have shown that, where they are used, they usually give a value of near 1.0 for all solutes investigated. This is interpreted to mean, in terms of the model underlying the KK theory, that water and solutes do not interact within the channels but use separate pathways in traversing the membrane. This would seem to fit the fact, established certainly for aqua-

porin-1 (AQP1), that the channels are impermeable to most small solutes tested (Coury et al., 1998; Van Hoek & Verkman, 1992; Whittembury et al., 1997).

There are extensive data, however, which do not fit this pattern. For many years Solomon and co-workers have measured reflexion coefficients by the 'minimum' method which does not use the KK equations explicitly, although they attest to their validity. These results have shown a spread of σ-values for small solutes significantly lower than 1.0 (Toon & Solomon, 1990; Toon & Solomon, 1991; Toon & Solomon, 1996). Recently we have been conducting similar experiments and have obtained similar results. To investigate the relationship between σ and other parameters more closely, and to test the applicability of KK theory, we measured the membrane parameters for the solute methylurea, which permeates red cell membranes but cannot pass the channel of AQP1, and compared them with those for NaCl, which is impermeable and is considered to give the maximum osmotic flow or $\sigma = 1.0$. Osmotic flow was also measured over a range of osmolarities because the effect of osmolyte concentration on the reflexion coefficient determines the degree of solute-water interaction in any part of the channel and provides another important constraint for alternative explanations to KK theory.

We discuss the various forms of the KK equation that have been used to analyze osmotic flow in membranes, either explicitly or implicitly, and question whether these equations and the theory behind them are capable of describing water flow through aquaporin channels or similar structures.

Materials and Methods

Using human red-cell ghosts (HRCG), the addition of methylurea or NaCl to HRCG suspensions caused cells to shrink and, with methylurea, to re-swell. From the ratio of the initial slopes the reflexion coefficient σ for the membrane was obtained. The shape of the methylurea curves at the minimum volume or turning point allowed determination of the permeability ω of the membrane to methylurea. Finally θ, the fraction of the total P_f of the membrane due to AQP1 was measured by comparison of the initial shrinkage slopes created by NaCl in the presence and absence of 1 mM $HgCl_2$. This is based upon the assumption that mercurials such as $HgCl_2$ and pCMBS are potent inhibitors of AQP1, which is responsible for most of the nonlipidic P_f of the HRCG membrane. From σ, ω and θ the reflexion coefficient σ_c of the AQP1 channel can be calculated (*see below*).

Membrane Preparation and Solutions

Phosphate Buffered Saline (PBS) in mM: NaCl 150, Na_2HPO_4 10, $MgCl_2$ 0.5. pH = 7.4. Lysis Buffer Solution (LB) in mM: Na_2HPO_4 5, $MgCl_2$ 0.5, pH = 8–8.1. Reconstitution Buffer Solutions (RB) in mM: Na_2HPO_4 5, $MgCl_2$ 0.5 and the required osmolyte was added for each experiment. Fluorescein Sulfonate Buffer: (FSB): FS (sodium fluorescein-5 sulfonate: Molecular Probes, Europe) was dissolved at 20 mM in the required RB for each experiment.

Red Blood Cell ghosts were prepared by a modification of the method used by Toon & Solomon (1996). Fresh blood was taken from a single donor for each experimental session. The blood was spun at 4°C and 2,500 × G for 3 min and the plasma removed by suction. The red cells were washed 3 times with 50 volumes of PBS and spun at 4°C and 2,500 × G for 3–5 min to remove the buffy coat. Cells were lysed by adding them to 50 volumes of iced LB and stirring vigorously for 2 min. The suspension was spun for 10 min in iced tubes at 4°C and 35,000 × G. The ghosts were washed 3 times in 50 volumes of iced LB. At this stage they could be kept at 0–4°C overnight without any effect on their properties.

Loading with fluorescein sulfonate: FSB was added to the ghost suspension so that the final FS concentration was not less than 10 mM and this was kept on ice for 5 min to allow equilibration. The ghosts were 're-sealed' in the loading solution for 45 min at 37°C. The sealed ghosts were washed 3 times in the appropriate reconstitution buffer and spun for 10 min at 12,000 × G at 4°C. They were then re-suspended in approximately 80 volumes of the required RB to give a 2% haematocrit and kept on ice. Aliquots were taken for each experimental run.

Stopped-Flow Measurements

All fluorescence measurements were performed using an SF-61 single mixing stopped-flow system (Hi-Tech Scientific, Salisbury UK). Fluorescence was excited at 490 nm with an F/4 Czerny-Turner monochromator and detected using a 530 nm cut-off filter. All measurements were made at a temperature of 25°C maintained by a circulating water bath (RTE111 Neslab, Portsmouth, NH).

The time course of red cell ghost volume change was determined by the concentration-dependent self-quenching of entrapped fluorescein sulfonate (FS) according to the method described by Chen, Pearce & Verkman (1988) and as used by Toon & Solomon (1996) for HRCG. Changes in fluorescence with time were recorded by a computer interface with the Hi-Tech SF-61 using a dedicated software package (Hi-Tech Scientific, Salisbury, UK). The relationship between fluorescence intensity and relative cell volume was determined by mixing aliquots of FS loaded ghosts in RB (150 μl) with equal volumes of 0.05, 0.1, 0.2, 0.5 and 1.0 M NaCl. Mixing was achieved using the stopped-flow apparatus pneumatic drive syringes at a mixing pressure of 2 bar. For each concentration 10 replicate curves were recorded and averaged to produce a mean curve. Final equilibrium fluorescence values from the mean curves were plotted against π_{iso}/π for each NaCl concentration to produce a calibration curve for fluorescence against relative volume assuming ideal osmotic behaviour.

Effect of Osmolyte on the Fluorescence

The effect of methylurea on the fluorescence was tested with FSB (20 mM FS). FSB with or without the osmolyte methylurea at 1.0 M was mixed with RB in a 1:1 ratio and the change in fluorescence measured. No detectable change in the fluorescence was seen after mixing, i.e., release from self-quench was instantaneous. There was no significant difference between the two sets of measurements [238.8 SEM 18 mV, $n = 5$ (− methylurea) *v.* 217.7 SEM 13 mV, $n = 5$ (+ methylurea), ratio 1.09 NS]. This is in accord with the finding that small neutral solutes have no detectable effect on the fluorescence or self-quenching of FS (Chen et al., 1988). The results from the red cell experiments described here confirm this in two ways. (1) In shrinkage experiments the fluorescence signal returns to the initial starting value (baseline) after the cells have re-swollen to their original volume. Before shrinkage there is no methylurea in the ghosts to which the FS is confined; afterwards the two solutes are mixed. If there were an effect of methylurea on FS fluorescence then there would be no return to baseline. (2) The initial

shrinkage rates at $t = 0$ that dominate the final values of the reflexion coefficient (*see* Table) are also determined before methylurea and FS have mixed inside the ghosts; in addition, the shrinkage rates are highly linear with methylurea concentration (*see* Fig. 2*B*) which would not be found if methylurea was affecting the behaviour of FS, i.e., the slope would revert to that obtained with NaCl (*see* Fig. 2*A*) as the driving force (concentration) of methylurea approached zero.

Data Analysis

From the calibration data comprising fluorescent signal *v.* cell volume, taken for each HRCG batch on each day, a third-order polynomial was fitted and used to convert spectrometer output to volumes. After conversion to relative volumes, sections of the curves at short time (0–0.1 sec) and the volume minima (turning points for methylurea exps) were cut out and fitted to polynomials: second-order for short times, which are quasi-linear, and fourth-order for the turning points. Package software (Mathematica) was used to extract the relevant derivatives and co-ordinates.

The derivation of the following expressions and the symbols used are given in the Appendix. The initial rates of volume change $(d\bar{v}/dt)^0$ are used to determine the osmotic permeabilities of the whole RBC membrane for NaCl and methylurea. Assuming that the reflexion coefficient of NaCl is 1.0 these overall permeabilities are derived from Eqn. A1

$$P_{os(\mathrm{NaCL})} = P_f = -(v^0/a)(d\bar{v}/dt)^0/\Delta\pi_i^0 \quad ; \quad \Delta\pi_p^0 = 0 \tag{1}$$

$$P_{os(\mathrm{methylurea})} = \sigma P_f = -(v^0/a)(d\bar{v}/dt)^0/\Delta\pi_p^0 \quad ; \quad \Delta\pi_i^0 = 0 \tag{2}$$

and σ for the membrane was calculated from

$$\sigma = P_{os(\mathrm{methylurea})}/P_{os(\mathrm{NaCl})} \tag{3}$$

From the minimum of the curve ω is given by

$$\omega = (v^0/a)^2 \frac{\bar{v}^{\min}(d^2\bar{v}/dt^2)^{\min}}{P_f(\pi_{ii}^0/\bar{v}^{\min} - \pi_{oi})} \tag{4}$$

The reflexion coefficient of the channel was then calculated from

$$\sigma^c = \sigma + \frac{\omega\bar{V}_s}{P_f^c\bar{V}_w} - (1-\sigma)\frac{P_f^{rm}}{P_f^c} \tag{5}$$

(*see* Appendix) where $P_f^c = \theta P_f$ and $P_f^{rm}/P_f^c = 1/\theta - 1$. The second RHS term corrects σ for the total solute volume transfer and the third for the water flow through the 'rest of the membrane' P_f^{rm}, i.e., the nonmercurial sensitive pathways.

Results

Reflexion Coefficients

In Fig. 1 representative curves of relative volume are shown for methylurea and for NaCl as a function of time after mixing. In Fig. 2*A* the initial slopes $(dv/dt_{(t=o)})$ obtained with NaCl at different osmolarities are plotted against driving concentration and can be compared with those obtained with methylurea, Fig. 2*B*. The slopes are highly linear and each regression line extrapolates to a flow not significantly different from zero. The ratio of the slopes of shrinkage with methylurea *v.* NaCl (Eqn. 3) yields a reflexion coefficient of 0.55. The linearity shows that the experiments are not complicated by unstirred layers adjacent to the membrane surfaces, which are highly nonlinear in their effects. In addition, this linearity, with its extrapolation to zero, has direct implications for the mechanism of osmotic flow in AQP1 as discussed below.

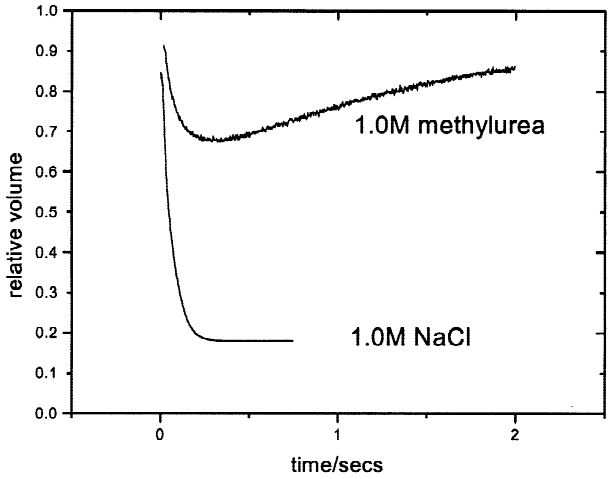

Fig. 1. Shrinkage of the HRCG with hypertonic solutions of NaCl and methylurea corrected to relative volumes using a fluorescent standardization curve after removal of a short mixing perturbation.

$P_f\bar{V}_w$ in these experiments varied from 0.7–1.1 $cm^4/osm \cdot sec$ using a volume/area ratio of 4.67×10^{-5} cm for the isotonic HRBC (Macey & Karan, 1993; Sha'afi et al., 1970). The permeability of methylurea from the minimum has a value of $\omega = 3.50 \pm 1.085 \times 10^{-4}$. The θ-value as determined from the use of mercuric chloride on the initial NaCl shrinkage slope is 0.9 ± 0.1[1]. These values can be compared with $\omega = 3.6 \times 10^{-4}$ (Toon & Solomon, 1996) and $\theta = 0.9$–0.93 (Macey & Karan, 1993; Mathai et al., 1996; Toon & Solomon, 1986; Zeidel et al., 1992). There are no other proteins known in the HRBC that make any significant contribution to the P_f of the membrane. The P_f which is above that of the lipid membrane is almost wholly attributable to AQP1 (CHIP28) and is abolished by mercurials (Van Hoek & Verkman, 1992). In view of the fact that these parameters are determined on different preparations (though all on human red cell membranes) and the basal permeability of ghosts is dependent upon the degree of sealing and internal osmolarity, the similarities are quite

[1]There may be a very small amount of AQP3, which is mercurial-sensitive, present in the HRBC (Roudier et al., 1998). Strictly therefore, the values of σ and θ determined here include it.

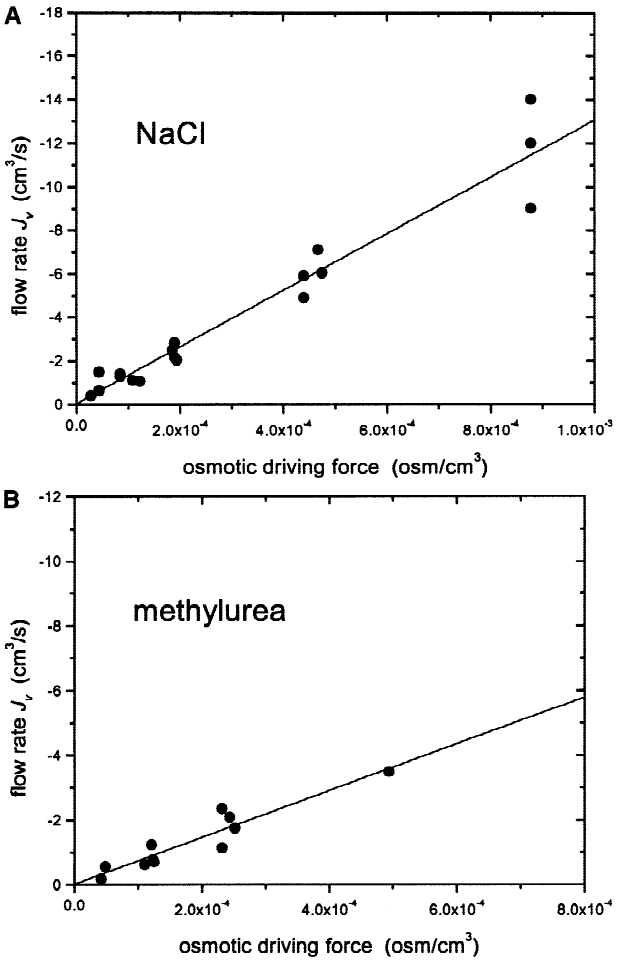

Fig. 2. Initial shrinkage rates $(dv/dt)^0$ for the two solutes. (*A*) NaCl regression: $y_0 = 0.045$, slope = 13010.4, R^2 0.96, $P < 0.0001$. (*B*) Methylurea regression: $y_0 = 0.023$, slope = 7213.9, R^2 0.94, $P < 0.0001$.

acceptable. The important point is that in this study parameters for methylurea and NaCl are compared in aliquots from batches prepared under similar conditions.

The σ_s from these slopes represents the net volume exchange across the membrane and to derive the σ-value for the channel σ_s has to be corrected for the component of volume transfer by solute diffusion. Further to that, it has to be corrected for the volume of water flow across the lipid element of the membrane. The two corrections according to Eq. 5 are shown in the Table together with the final value. It is apparent that the corrections are quite small, and because they are of opposite sign, they tend to cancel each other. The ratio of initial slopes is thus clearly different and is a reasonably good measure of σ_s of the AQP1 channel itself.

Table. The corrections to sigma from Eqn. 5.

σ	$\omega\bar{V}_s/P_f^c\bar{V}_w$	$-(1-\sigma)\,P_f^{rm}/P_f^c$	σ^c
0.558 ± 0.085	0.025 ± 0.007	−0.048 ± 0.0085	0.54 ± 0.087

The following experimentally derived values were used: $\omega = 3.50 \pm 1.085 \times 10^{-4}$ cm/sec; $P_f^c\bar{V}_w\,(=\theta P_f\bar{V}_w) = 0.858 \pm 0.039$ cm^4/osm · sec; $\theta = 0.9 \pm 0.1$ and $P_f^{rm}/P_f^c = (1-\theta)/\theta$.

Discussion

KK Fits

The KK equations for osmotic flows in which pressure-driven flow is absent

$$Jv = \sigma P_f \Delta\pi \tag{6}$$

$$Js = \bar{c}(1-\sigma)Jv + \omega\Delta\pi \tag{7}$$

may be cast in a slightly different form

$$Jv = \sigma_s P_f \Delta\pi \tag{8}$$

$$Js = \bar{c}(1-\sigma_f)Jv + \omega\Delta\pi \tag{9}$$

in which a distinction has been made between the 'osmotic' coefficient σ_s and the 'ultrafiltration' coefficient

σ_f. In KK theory these are set equal, $\sigma_s = \sigma_f$, on general grounds of symmetry. It is quite clear, however, that $\sigma_f = 1.0$ because the channel is impermeable to all small solutes including methylurea. Most of the leading contenders for the water channel in the HRBC have been effectively ruled out, such as band-3 (the anion exchanger) (Zhang et al., 1991) GLUT1 (band-4.5, the glucose transporter) (Zeidel et al., 1992; Zhang et al., 1991) or the HUT11 (the urea transporter) (Sidoux-Walter et al., 1999; Wintour, 1997). The mercurial-sensitive AQP1 is responsible for virtually all the P_f of the membrane and the AQP1 channel is impermeable to a range of small molecules right down to urea which is smaller than methylurea (Coury et al., 1998; Van Hoek & Verkman, 1992; Whittembury et al., 1997). In KK theory this would demand that σ_s is also 1.0 but this is clearly not the case. The value for methylurea given by Eqn.A1, either before or after adjustment for solute movement, is very far from that for NaCl (assuming $\sigma_{NaCl} = 1.0$). If it is clear on physical grounds that there is no equality (symmetry) then the KK equations (6–7) cannot be used to analyze the data *a priori.* However, if there is equality between the coefficients, and σ_f is independently set to 1.0 (using Eqns. 8–9) σ_s should also yield a value of 1.0 but this is not the case.

It is not clear to us why generalized fits of KK equations to volumetric data (albeit inappropriate for mosaics) have led to values for σ_s close to 1.0. As indicated below, fits can sometimes ignore the fact that the membrane is a mosaic. However, in our hands fits of a single pair of KK equations to our data do not give values of $\sigma = 1.0$ for methylurea. We can only offer the suggestion that for impermeable pores there is no relationship between J_s and J_v which imposes $\sigma = 1$ by Eqn. 7 and that this constrains σ in Eqn. 6 towards unity as well. In addition, the values of ω obtained by KK fits are not independent of the fitted values of σ and P_f as they are all recovered from a three-parameter minimization. The use of the 'minimum' method has been criticized on grounds of its accuracy, involving as it does a preliminary curve fit—usually a polynomial (Macey & Karan, 1993). For third- and fourth-order polynomials we have found this to be a robust method of extracting ω. In fact, where estimation of the reflexion coefficient for the channel is concerned, the precise value of ω is of minor importance because the correction for solute permeation is small and largely cancelled out by the correction for lipid background (Table).

A problem with curve-fits based upon the KK equations as used in much published work is that they do not apply to a mosaic membrane made of parallel elements, such as the one we consider here, composed of aqueous channels and a lipid pathway, each of which may have its own reflexion coefficient σ and conductance P_f. Even when each element is considered to obey a separate KK equation (which for the aqueous channel is probably incorrect, as shown above) there is no symmetry for the ensemble (*see* Appendix). If Eqns. 8–9 are applied to a membrane composed of two KK elements the values for the reflexion coefficients in each equation are

$$\sigma_s = \frac{\sigma_1 P_{f1} + \sigma_2 P_{f2}}{P_{f1} + P_{f2}} \tag{10}$$

and

$$\sigma_f = \frac{\sigma_1^2 P_{f1} + \sigma_2^2 P_{f2}}{\sigma_1 P_{f1} + \sigma_2 P_{f2}} \tag{11}$$

where σ_1, σ_2 and P_{f1}, P_{f2} are the values for the separate elements each of which are taken to satisfy Eqns. 6 and 7. For methylurea the values found here of $\sigma = 0.55$, assuming $\sigma_{lipid} = 1$ and the ratio $\theta = 0.9$ or $P_{f(lipid)}/P_{f(channel)} = 0.11$ indicate that the difference between the two σ is of the order of 10% which is not negligible in this case.

In the derivation of the 'minimum' expression for ω symmetry equations were used and the rest of the membrane was not considered (Sha'afi et al., 1970). What is then the status of the 'minimum' method, particularly as it is applied in this paper? At the turning point $J_v = 0$ and the term containing $(1\text{-}\sigma)$ in Eqn. 7 or 9 is zero. In the Appendix an identical minimum expression is derived by specifically choosing to omit the interaction term in $1\text{-}\sigma_f$ and is therefore independent of any KK assumptions.

We consider now three explanations for the fact that $\sigma < 1.0$ in a channel that is impermeable. (i) There may be 'hidden' osmotic channels in parallel. (ii) There is water-solute interaction in only a part of the channel. (iii) The mechanism of water flow (its viscous component) is changing with osmolyte size and thus penetration depth. These explanations will be examined in greater detail and we shall argue that (i) and (ii) can be eliminated as possible explanations for the low value of σ for methylurea.

Hidden Osmotic Channels

These would be additional membrane channels which have a low reflexion coefficient but whose contribution to the overall value of σ has yet to be demonstrated. They may be membrane components that are already well known or ones yet to be discovered. This explanation has been advanced in relation to the reflexion coefficient of urea (Toon & Solomon, 1996). The explanation given by these workers who found $\sigma_{urea} = 0.64$ is that, as AQP1 is impermeable to urea and by KK theory $\sigma_{urea(AQP)} = 1$, there must be additional aqueous channels mediating the osmotic flow. In the case here of methylurea any parallel channels have to posses a

σ-value lower than 0.54, which means that by KK theory they must be permeable to methylurea. This rules out, on known functional grounds, such membrane proteins as band-3 and the urea and glucose transporters. Furthermore, with the same analysis as used here, it is possible to obtain experimental values of $\sigma_s < 1.0$ for a whole range of small solutes after correcting for solute permeability (Toon & Solomon, 1996; unpublished results of ours); this would require 'hidden' channels with a large spectrum of permeabilities to virtually all solutes or a multi-solute channel, a situation which we consider impossible.

There are also constraints on the osmotic permeability of any parallel system. By Eqn. 10 the reflexion coefficient for methylurea would be given by $\sigma_{\text{methylurea}} = \sigma_1\theta_1 + \sigma_2\,\theta_2$ where $\theta_1 = P_{f1}/(P_{f1} + P_{f2})$ and $\theta_1 + \theta_2 = 1$; if the first channel is the AQP1 and $\sigma_{1(\text{AQP1})} = 1.0$ we obtain $1 - \theta_2\,(1 - \sigma_2) = 0.54$ where σ_2 is the reflexion coefficient of any hidden channel(s). For $\sigma_2 = 0$, the lowest value possible, $\theta_2 = 0.46$ or 46% of the non-lipidic P_f. Any hidden channel with a higher coefficient ($\sigma_2 > 0$) would require a progressively larger value for θ_2. For this to be true these channels would have to make a significant contribution to the overall P_f of the membrane although virtually all the non-lipid water permeability of the red cell membrane is attributable to AQP1. Clearly, 'hidden' osmotic channels are not an explanation.

Water-Solute Interaction

If there is penetration of solute into part of the channel (e.g., accessible end sections) although an inaccessible section excludes the solute (e.g., a central section), there may be friction with water or a partial occlusion of the channel resulting in a lower σ than that shown by a reference solute which is totally excluded ($\sigma = 1.0$). A possible explanation for a spread of σ-values in an impermeable channel is that the channel is not of uniform cross-section: there could be solute-specific interaction between the water flux and stationary solute in part of the channel architecture without the channel possessing an overall solute permeability.

To make this point clear we develop a simple frictional argument for the channel. In the steady state the osmotic driving force on the water F_w is balanced by the partial frictional interactions f between water (w), membrane (m) and solute (s), moving with velocity v:

$$F_w = f_{wm}v_w + f_{ws}\,(v_w - v_s). \tag{12}$$

When the pore is impermeable to solute $v_s = 0$, but there is still solute access to parts of the channel, and using the relationship $c_w f_{ws} = c_s f_{sw}$ (Katchalsky & Curran, 1965: chap. 10) the osmotic flow rate is proportional to v_w where

$$v_w = \frac{F_w}{f_{wm} + f_{sw}c_s/c_w} \tag{13}$$

in which c_s/c_w is the mean ratio of solute to water concentration in the accessible parts of the channel. The dependence of the osmotic flow rate is not a linear function of solute concentration but clearly falls off as c_s rises. A value of $\sigma < 1.0$ for methylurea, were it due to interaction between moving water and stationary solute, would be due to the departure from linearity and this would be dependent on the solute. In general, smaller solutes would penetrate the accessible channel regions more than larger ones and at higher concentration per unit channel water. This relationship has been noted for several solutes in the HRBC membrane (Toon & Solomon, 1996).

The linear relationships between the osmosis (J_v) and the osmolyte driving forces shown in Fig. 2 indicate that Eqn. 13 is not the explanation for the lowered reflexion coefficient. The flow intercept, which is not significantly different from zero, indicates the osmotic permeability (and σ) is not solute dependent and has a constant value over the experimental range. The effect of interaction in channel regions is probably present to some extent, indeed it is difficult to see how it could not be, but the size of the terms in Eqn. 13 are such that $f_{wm} \gg f_{sw}c_s/c_w$ and the curves are within an effectively linear domain.

σ_S and The Mechanism of Water Flow

In the KK theory the mechanism of osmotic water flow is not specified but it is assumed to be the same as that for pressure driven flow. In that theory a single coefficient f_{wm} for water-membrane interaction is used to describe both processes (Kedem & Katchalsky, 1965). This coefficient is very different for diffusive and viscous flows, being higher for the former. If both flows are involved in channel osmosis then the situation is more complicated and the use of a single (unspecified) coefficient cannot be supported. Consideration of the osmotic flow in 'leaky' channels has led to the conclusion that the presence of solute gradients within a such a channel can only result, in this case, in diffusive water transfer (Hill, 1982). In channels impermeable to solute the water flow is hydraulic (viscous) and takes place at a greater rate for the same solute gradient imposed across the membrane. It has been argued that in channels of molecular size such as AQP1 there is no difference between diffusive and viscous flow (Longuet-Higgins & Austin, 1966) but calculations (albeit derived from macroscopic theory) do not support this; in fact, calculation

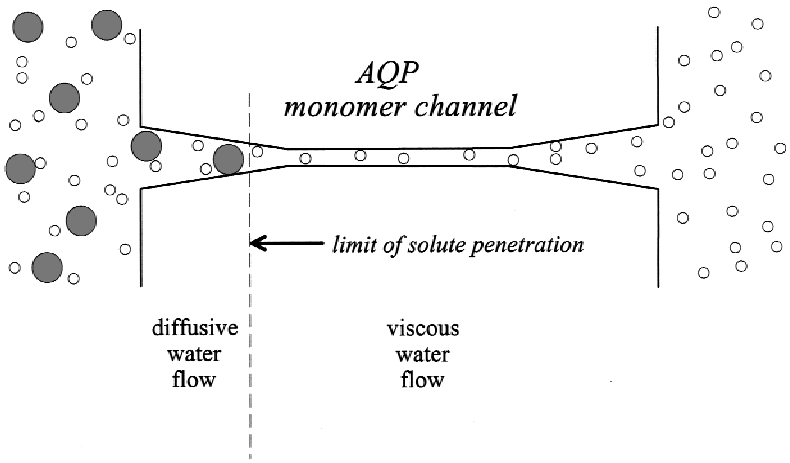

Fig. 3. Solute access in relation to pore cross section. If the presence of solute (filled circles) creates a diffusive flow of water but in solute-free regions the flow is hydraulic (Hill, 1995) the overall rate of osmosis is given by Eqn. 14.

of the P_f of AQP1 and gramicidin channels with an extension of macroscopic theory yields values in remarkably good agreement with experiment (Hill, 1994).

Water channels are undoubtedly variable in cross section and from this it follows that solutes may make incursions into the channel without traversing the whole because they cannot enter the central section—solutes of smaller 'cylindrical' radius will have longer accessible channel lengths than larger ones. If the water flow is indeed diffusive in these accessible sections, as opposed to hydraulic elsewhere in the channel, then the overall rate of osmotic water flow is the result of both diffusive and hydraulic flow in series. The extent of these flows is dependent on the solute cylindrical radius, the smaller the solute the slower the overall resultant flow. Beyond a certain size (that of the effective channel mouth) all solutes will set up hydraulic flow throughout the channel and show the maximum flow rate, i.e., $\sigma = 1.0$. This situation is shown diagrammatically in Fig. 3. If there are two sections in series the overall osmotic permeability P_{os} of the channel is given by

$$\frac{1}{P_{os}} = \frac{1}{\sigma P_f} = \frac{l_1}{p_d} + \frac{l_2}{p_f} \tag{14}$$

and so

$$\sigma = \frac{1}{P_f}\left(\frac{p_d p_f}{l_1 p_f + l_2 p_d}\right) \tag{15}$$

where l_1 and l_2 represent the accessible and inaccessible section lengths. p_d and p_f are the diffusive and hydraulic permeabilities per unit length (these are averages because the channel is not uniform in cross-section). If solute is completely excluded ($l_1 = 0$), l_2 becomes the total pore length, and as the maximum osmotic flow (like pressure-induced flow) is hydraulic, $p_f/l_2 = P_f$ and we arrive at $\sigma = 1.0$. Simple though Eqn. 15 is, it summarizes the 'bimodal' approach to osmotic flow (Hill, 1995). Methylurea would be expected to have reasonably deep access to the AQP1 channel but be unable to cross the pore structure from phase to phase, i.e., $\sigma < 1.0$.

Conclusions

The volume responses of red cell membranes to osmotic challenges with methylurea and NaCl lead to the following conclusions:

1. KK equations cannot be used to analyze time curves of osmotically-induced volume change without a prior knowledge of the system structure, e.g., the mosaic properties.

2. KK theory does not apply to any channel where there is not symmetry, except for the trivial case where $\sigma = 1.0$. Four parameters P_f, ω_s, σ_s and σ_f are required in general to describe the overall system involving osmosis and pressure flow, ultrafiltration and diffusion. For osmolyte-impermeable channels, such as AQP1, $\sigma_f = 1$ but σ_s is substantially less than 1.0.

3. σ_s-values less than 1.0 do not imply solute-water flow interaction in solute-permeable channels. Such values are most probably generated by changes in the mechanism of water flow in parts of the channel.

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Appendix

Equations Used in the Paper

a	area (constant)
v	volume
v^0	partial molar volume of solute or water
σ, σ^c	reflexion coefficient of permeable solute for membrane or channel
σ_s, σ_f	σ for osmosis or ultrafiltration
P_f, P_{os}	hydraulic permeability or osmotic permeability
P_f^c, P_f^{rm}	P_f of channel or 'rest of the membrane'
ω	permeable-solute permeability
$\pi_{oi,ii}$	outer, inner osmolarity of impermeable solute
$\pi_{op,ip}$	outer, inner osmolarity of permeable solute
π_{ii}^0	inner osmolarity of impermeable solute at $t = 0$
θ	fraction of total osmotic permeability due to channel

Cell Volume with Impermeant and Permeant Osmolyte at Constant Area

The volume and solute exchange (j_s) are given by

$$\frac{dv}{dt} = -P_f a[(\pi_{oi} - \pi_{ii}) + \sigma(\pi_{op} - \pi_{ip})] \tag{A1}$$

$$j_s = \omega a(\pi_{op} - \pi_{ip}) = \frac{d(\pi_{ip} v)}{dt} = v\frac{d\pi_{ip}}{dt} + \pi_{ip}\frac{dv}{dt} \tag{A2}$$

Eliminating π_{ip} and converting v to relative volume by $v \rightarrow v/v^0$ leads to

$$\frac{(v^0)^2 v}{P_f a}\frac{d^2 v}{dt^2} + \frac{(v^0)^2}{P_f a}\left(\frac{dv}{dt}\right)^2 + v^0\left(\pi_{oi} + \sigma\pi_{op} + \frac{\omega}{P_f}\right)\frac{dv}{dt} - \omega a\frac{\pi_{ii}^{(0)}}{v} + \omega a\pi_{oi} = 0 \tag{A3}$$

In this derivation no use has been made of the KK equation because interaction terms including $(1 - \sigma)$ have been specifically omitted from Eqn. A2 on the physical grounds of channel impermeability. When dv/dt is zero at the minimum, Eqn. A3 yields

$$\omega = (v^0/a)^2 \frac{\bar{v}^{\min}(d^2\bar{v}/dt^2)^{\min}}{P_f(\pi_{ii}^0/\bar{v}^{\min} - \pi_{oi})} \tag{A4}$$

In this derivation it should be noted that although the absolute volume:area ratio (v^0/a) of the cell features in all expressions for P_f (Eqns. 1 & 2) and ω (Eqn. A4), when cast in relative volume the expressions for σ such as Eqn. A6 are relative parameters and thus independent of this ratio; its exact value does not need to be determined here.

Conversion of Membrane σ to Channel σ^C

The ratio of osmotic volume flow created by a permeant species to that created by an impermeant one is a definiton of the reflexion coefficient i.e.

$$\frac{J_v^\pi}{J_v^P} = \sigma = \frac{(\sigma^c P_f^c + \sigma^{rm} P_f^{rm})\Delta\pi}{(P_f^c + P_f^{rm})\Delta\pi_i}$$

thus

$$\sigma^c = \sigma(1 - P_f^{rm}/P_f^c) - \sigma^{rm}\,(P_f^{rm}/P_f^c) \tag{A5}$$

where $\Delta\pi = \Delta\pi_i$. For the 'rest of the membrane' without the channel in question

$$\frac{J_v^{\pi}}{J_v^P} = \sigma^{rm} = \frac{(P_f^{rm}\overline{V}_w - \omega\overline{V}_s)\Delta\pi}{P_f^{rm}\overline{V}_w\Delta\pi_i} \quad \text{or} \quad \sigma^{rm} = 1 - \frac{\omega\overline{V}_s}{P_f^{rm}\overline{V}_w}$$

and substituting σ^{rm} into Eqn. A5 we have

$$\sigma^c = \sigma + \frac{\omega\overline{V}_s}{P_f^c\overline{V}_w} - (1-\sigma)\frac{P_f^{rm}}{P_f^c} \tag{A6}$$

KK Equations and Mosaic Membranes

For osmotic flow across a mosaic membrane with two elements, each of which obey a KK equation we have by Eqns. 6 and 7

$$J_{vm} = J_{v1} + J_{v2} = (\sigma_1 P_{f1} + \sigma_1 P_{f2})\Delta\pi \tag{A7}$$

where the membrane conductance $P_{fm} = P_{f1} + P_{f2}$. The membrane reflexion coefficient is given by $J_{vm}/\Delta\pi$ so that

$$\sigma_s = \frac{\sigma_1 P_{f1} + \sigma_2 P_{f2}}{P_{f1} + P_{f2}} \tag{A8}$$

Similarly, for solute flow

$$J_{sm} = J_{s1} + J_{s2} = \overline{c}(1-\sigma_1)\,J_{v1} + \omega_1\Delta\pi + \overline{c}(1-\sigma_2)\,J_{v2} + \omega_2\Delta\pi \tag{A9}$$

If this is set equal to $\overline{c}(1 - \sigma_f)J_{vm} + \omega\Delta\pi$ where $\omega = \omega_1 + \omega_2$ then it follows that

$$\sigma_f = \frac{\sigma_1^{\,2}P_{f1} + \sigma_2^{\,2}P_{f2}}{\sigma_1 P_{f1} + \sigma_2 P_{f2}} \tag{A10}$$