Reflection Coefficient and Permeability of Urea and Ethylene Gycol in the Human Red Cell Membrane

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ABSTRACT The reflection coefficient ($\sigma$) and permeability ($P$) of urea and ethylene glycol were determined by fitting the equations of Kedem and Katchalsky (1958) to the change in light scattering produced by adding a permeable solute to a red cell suspension. The measurements incorporated three important modifications: (a) the injection artifact was eliminated by using echinocyte cells; (b) the use of an additional adjustable parameter ($K_m$), the effective dissociation constant at the inner side of the membrane; (c) the light scattering is not directly proportional to cell volume (as is usually assumed) because refractive index and scattering properties of the cell depend on the intracellular permeable solute concentration. This necessitates calibrating for known changes in refractive index (by the addition of dextran) and cell volume (by varying the NaCl concentration). The best fit was for $\sigma = 0.95$, $P_o = 8.3 \times 10^{-4}$ cm/s, and $K_m = 100$ mM for urea and $\sigma = 1.0$, $P_o = 3.9 \times 10^{-4}$ cm/s, and $K_m = 30$ mM for ethylene glycol. The effects of the inhibitors copper, phloretin, p-chloromercuribenzenesulfonate, and 5,5'-dithiobis (2-nitro) bensoic acid on the urea, ethylene glycol, and water permeability were determined. The results suggest that there are three separate, independent transport systems: one for water, one for urea and related compounds, and one for ethylene glycol and glycerol.

INTRODUCTION

Historically, three sets of data have been used to support the existence in red cell membranes of aqueous channels that allow the passage of water and small electrolytes: (a) an hydraulic (osmotic) permeability of water that is significantly greater than the diffusive permeability of water; (b) a relatively high permeability with a sharp size cutoff for the small nonelectrolytes (e.g., urea, ethylene glycol), such as would be expected for a molecular sieve; (c) a reflection coefficient for the small nonelectrolytes that is significantly <1. The first set of data indicates that the water molecules are interacting with each other in their passage across the membrane, as would be expected for an aqueous channel. The second set of data is no longer a strong argument for
pores because it has been shown that there are special facilitated transport systems in the red cell for these solutes (Mayrand and Levitt, 1983). The third set of data suggests a pore mechanism because one can derive the general result that if a solute (s) and water (w) are using separate pathways, then the reflection coefficient ($\alpha_s$) should be described by the relation (Kedem and Katchalsky, 1958):

$$\alpha_s = 1 - \left( \frac{\bar{V}_s}{\bar{V}_w} \right) \left( \frac{P_s}{P_t} \right),$$

where $P_t$ is the hydraulic permeability (filtration coefficient), $P_s$ is the diffusive permeability (both in units of centimeters per second), and $\bar{V}_s$ and $\bar{V}_w$ are the molar volumes of solute and water. Substituting in Eq. 1 the known values of $P_t$ (Mlekoday et al., 1983) and $P_s$ (Mayrand and Levitt, 1983) at the solute concentration used experimentally (~400 mosmol for the “zero-time method”), $\alpha$ should be ~0.95 for urea and 0.98 for ethylene glycol. The maximum reported experimental values (Owen and Eyring, 1975) for urea (0.79) and ethylene glycol (0.86) are significantly less than this prediction. If correct, this difference would imply that a major component of the urea and ethylene glycol transport was using a pathway that interacted with the water transport, i.e., an aqueous pore.

However, since the experimental measurement of $\alpha$ is difficult and rather indirect, it is possible that the experimental values are in error (i.e., 10–20% too small). The primary purpose of these experiments is to measure $\alpha$ for urea and ethylene glycol as accurately as possible. The $\alpha$ was determined by fitting the equations of Kedem and Katchalsky (using the previously determined $P_t$) to the time course of cell volume change during an osmotic transient. Three essential modifications of previous attempts to measure $\alpha$ were made: first, as described in the first paper of this series (Mlekoday et al., 1983), echinocytic cells were used to minimize the injection artifact; second, the assumption (used in all previous studies) that the light scattering is proportional to the cell volume is incorrect when permeable solutes are used because it is necessary to correct for the change in refractive index of the cells that results from the change in the intracellular permeant concentration; and third, in the fitting of the kinetic equations it is necessary to correct for the fact that the permeability is not a constant during the experiment, but instead decreases as the intracellular concentration rises and the facilitated transport system becomes saturated. When these three modifications were made, we found that the experimental results were consistent with the $\alpha$ predicted by Eq. 1 for both urea and ethylene glycol.

METHODS

Procedure Used to Determine Membrane Parameters from Light-Scattering Data

The cells were washed, suspended, and treated with lecithin, and the light scattering was measured with the Aminco-Morrow apparatus (American Instrument Co., Silver Spring, MD), as described previously (Mlekoday et al., 1983). The standard “saline” solution consisted of 8 mM KCl, 10 mM Tris (pH 7.28) plus NaCl to produce the desired osmolarity. For the measurement of solute permeability, the cells were usually
suspended in a 220-mosmol saline solution and then challenged with an identical saline solution to which the test solute was added. The refractive index of the dextran, urea, and ethylene glycol solutions was measured directly with a refractometer (American Optical Corp., Buffalo, NY). The values were in excellent agreement with the tables of Wolf et al. (1971). All the results shown in this paper are for blood drawn from one donor (H.L.M.). However, experiments on blood from two other donors had essentially identical light-scattering curves. The blood from one of these donors (R.R.M.) was also used for the tracer experiment measurements of permeability (Mayrand and Levitt, 1983).

The change in light scattering as a function of time for a typical experiment is shown in Fig. 1A. A 0.5% suspension of cells in 220 mosmol buffer is mixed in the stopped-flow apparatus with an equal volume of a solution that has the same

![Figure 1. Light scattering (photocell signal) of lecithin-treated red cells as a function of time. A. Cells in 220 mosmol saline are mixed at t = 0 with an equal volume of 220 mosmol saline plus 400 mosmol urea. Ordinate: 20 mV/div; abscissa: 0.2 s/div. The curved tracing shows the first sweep of the oscilloscope, and the horizontal line indicates the photocell signal after the cells have reached their equilibrium value. The arrow indicates the theoretically predicted deflection from the equilibrium value that results from the change in refractive index of the cells caused by the addition of urea. B. Same as A except the urea concentration of challenging solution is 1,000 mosmol. Ordinate: 50 mV/div; abscissa: 0.2 s/div. C. Control where cells in 220 mosmol saline are mixed with the identical 220 mosmol saline (no permeable solute). Since there is no volume change for this case, this photocell signal will be referred to as an "injection artifact." Ordinate: 10 mV/div; abscissa: 0.1 s/div.](image-url)
electrolyte concentration plus 400 mosmol urea (final urea concentration after mixing of 200 mosmol). Initially, cells shrink because of the osmotic pressure of the urea and then swell as the urea enters the cell. At long times, the cell should return to its original volume because the urea will equilibrate across the cell membrane. However, as can be seen in Fig. 1A, the light scattering does not seem to return to its initial value. Since there is a fairly large injection artifact during the first 20 ms of the curve, the difference between the initial and final light scattering could be simply a result of this artifact. That this is not the case is shown by the experiment in Fig. 1B, which used a higher urea concentration (500 mosmol after mixing) with a correspondingly larger change in cell volume, but with the same injection artifact as in Fig. 1A. The magnitude of the initial injection artifact is shown in Fig. 1C for cells challenged with an identical solution so that there is no volume change (note the change of gain in these photographs). It can be seen that the maximum possible value of this artifact is only ~10–20% of the difference between the initial and final light-scattering value in Fig. 1B. There is no significant injection artifact in the absence of cells.

After puzzling over this result, we realized that it is exactly what would be expected, theoretically. The light-scattering power of the cell depends (in a complicated way) on the difference between the refractive index of the cell and the suspension medium. Thus, for example, the light scattering varies with cell volume because the hemoglobin concentration, and therefore the refractive index of the cell, is a function of cell volume. In the experiment shown in Fig. 1, the cells are initially in a solution in which urea is present only in the suspension medium. At the end of the experiment, urea has equilibrated and is present both inside and outside the cell. The rise in urea concentration in the cell raises the refractive index of the cell relative to the medium and changes its light-scattering properties. Thus, the change in the light scattering during the osmotic transient of Fig. 1 results from two effects: (a) the change in cell volume, and (b) the change in cell urea. Clearly, it is not correct to assume, as has been done in all previous analyses, that the light scattering is directly proportional to the volume. This effect is only important when dealing with permeant solutes and, for example, can be neglected when measuring the hydraulic permeability (Mlekoday et al., 1983). As the permeability of the solute increases, a larger solute concentration is required to get a significant volume change and the importance of this effect increases. For urea, the change in light scattering caused by the change in refractive index from the cell urea is about equal to the change in light scattering caused by the volume change (see Figs. 1A and B). Clearly, an effect of this magnitude cannot be neglected in fitting theoretical curves to the time course of the light-scattering data.

To determine the reflection coefficient, it is necessary to extract the cell volume change from the light-scattering data. Ideally, it should be possible to separate these two effects using light-scattering theory. However, this is not practical because of the critical dependence of the light-scattering behavior on the properties of the detection equipment. Our photocell measures transmitted light. If it were assumed that none of the light scattered by the cells arrived at the photocell, then we would predict from the theory of Latimer (1975) that the light transmission should decrease as the cell volume increases. Actually, just the opposite occurs in our experiments. This is because our detection apparatus allows a fairly wide angle (~10°) of scattered light to reach the photocell. As was shown by Latimer (1975), under these conditions, light transmission should increase as the cell volume increases, as we observe experimentally. Given the poorly characterized properties of our light-scattering equipment, it did not seem profitable to try to develop a detailed theoretical analysis of the light scattering, and instead we developed the following empirical approach.
It is assumed that the change in cell volume ($\Delta V$) and the change in the difference in the refractive index between the cell and medium at constant volume ($\Delta R$) produce linear and independent changes in the photocell signal ($\Delta S$):

$$\Delta S = D_V \Delta V + D_R \Delta R.$$  \hspace{1cm} (2)

The calibration constants ($D_V$ and $D_R$) were directly measured by independently varying the refractive index (by adding dextran to the suspension medium) and cell volume (by varying the impermeant concentration). The above assumption (Eq. 2) was directly tested and verified (see below) for the relatively small changes ($<10\%$) that occurred in our experiments. These two constants are theoretically related because part of the change in light scattering that is associated with the cell volume change results from the change in the cell hemoglobin concentration (which alters the cell refractive index).

The following procedure was used for determining the permeability ($P_n$) and reflection coefficient ($\sigma$) from the time course of the photocell signal ($S$) during the osmotic transient. The flux per cell of volume ($f_v$) and solute ($f_s$) across the red cell membrane is described by the equations of Kedem and Katchalsky (Levitt, 1975):

$$f_v = \frac{dV_c}{dt} = \frac{dV_w}{dt} = A_c \frac{dV_0}{dt} = A_c \frac{dV_0}{dt} = \frac{A_c V_0 P_f}{1 - \sigma} (C_i - C_i^m - \sigma(C_s - C_s^m));$$  \hspace{1cm} (3)

$$f_s = \frac{d}{dt}(V_w C_s) = A_c P_s (C_s^m - C_s) + \tilde{C}_s (1 - \sigma) f_v,$$  \hspace{1cm} (4)

where $A_c$ is the area per cell, $P_f$ is the osmotic permeability (filtration coefficient) determined in a separate experiment as described previously (Mlekoday et al., 1983), $P_s$ is the solute permeability (both in units of centimeters per second), $C_i$ and $C_s$ are the concentrations of the impermeant (i) and permeant (s) in the cell, $C_i^m$ and $C_s^m$ are the impermeant and permeant concentrations in the suspension medium (which are constant), and $\tilde{C}_s$ is the arithmetic mean permeant concentration across the cell membrane. It is assumed that the hydrostatic pressure difference across the cell is negligible.

The analysis is complicated because three different volumes must be considered: the volume of the cell ($V_c$), the water volume of the cell ($V_w$), and the apparent “osmotic water” volume of the cell ($V_{os}$). $V_{os}$ is the effective volume that the impermeant electrolytes are dissolved in (Mlekoday et al., 1983). The impermeant concentration of the cell is defined by:

$$C_i = C_i^0 V_{os} / V_{os},$$  \hspace{1cm} (5)

where $C_i^0$ is the impermeant concentration that the cell was initially equilibrated in (220 mosmol) and $V_{os}$ is the initial osmotic volume of the cell. It is also assumed that:

$$V_c = V_{os} + b V^{iso}_c,$$  \hspace{1cm} (6)

where $b$ is the fraction of the isotonic (290 mosmol) cell volume that is non-osmotic. The water volume of the cell ($V_w$) is defined by:

$$V_w = V_{os} + f V^{iso}_c,$$  \hspace{1cm} (7)

where $f$ is the solid fraction of the isotonic cell volume. The method used to determine the values of $f$ (0.393) and $b$ (0.463) was described previously (Mlekoday et al., 1983). In the following equations, it is assumed that the permeable solute (e.g., urea)
distributes in the total cell water (Macey, 1979). Since the light-scattering properties of the cell depend on the amount of solute inside the scattering surface, it is necessary to use the permeant concentration per cell volume (not water volume) when calculating the change in refractive index of the cell.

Eqs. 3 and 4 are rewritten in terms of the dimensionless variables:

\[ Y_1 = V_w/V^0_w; \]
\[ \tau = tV_wP_Ac^0/V^0_w; \]
\[ Y_2 = c_cV_w/C^m_wV^0_w = Y_1c_c/C^m_m, \]

where the superscript 0 indicates the initial condition of the cells. In the osmotic transient experiment, \( Y_1 \) equals 1 at \( t = 0 \), decreases, and then returns to 1 at \( t = \infty \). \( Y_2 \) is zero at \( t = 0 \) and goes to 1 at \( t = \infty \).

Using Eqs. 5–7, it can be shown that:

\[ V^0_w/V^0_r = (1 - b)c^0/c^0 + b - \tau; \]
\[ V_1/V^0_r = Y_1V^0_w/V^0_w + \tau; \]
\[ V_{os}/V^0_{os} = (b - \tau)(Y_1 - 1)/[(1 - b)c^0/c^0] + Y_1. \]

Substituting Eqs. 8 and 5 into Eqs. 3 and 4:

\[ \frac{dY_1}{d\tau} = V^0_{os}/V_{os} - C^m/C^0 + \sigma(C^m/C^0)(Y_2/Y_1 - 1); \]
\[ \frac{dY_2}{d\tau} = \frac{P_r}{V_wP_A(c^0)}[(1 - Y_2/Y_1) + 0.5(1 + Y_2/Y_1)(1 - \sigma) \frac{dY_1}{d\tau}], \]

with Eq. 11 substituted for \( V^0_{os}/V_{os} \) in Eq. 12. Eqs. 12 and 13 are then integrated numerically by a Runge-Kutta procedure.

There is one additional modification that is required in order to determine \( \sigma \) and \( P_r \). The usual assumption that \( P_r \) is a constant is incorrect for urea and ethylene glycol, which both use facilitated transport systems that are saturable (Mayrand and Levitt, 1983). During the osmotic transient light-scattering experiment, the concentration of the permeant rises in the cell and the system may become saturated, decreasing the effective \( P_r \). From the equation of Regen and Tarpley (1974) for a general facilitated transport system, it can be shown that the effective cell permeability for the case of varying cell concentration \( (c_c) \) and a constant external concentration \( (c^e) \) can be described by (see Appendix):

\[ P_r = P_0/(1 + c_c/K_m), \]

where \( P_0 \) is the permeability in the limit where the cell concentration is zero and the external concentration is \( C^m \), and \( K_m \) is a measure of the apparent “affinity” of the internal site for the permeant. In general, both \( P_0 \) and \( K_m \) will be functions of the external permeant concentration \( (c^e) \). Eq. 14 is then substituted for \( P_r \) in Eq. 13.

There are then three adjustable parameters \( (P_0, K_m, \sigma) \) that must be determined in order to characterize the red cell membrane. For each choice of these parameters, Eqs. 12 (with Eq. 11) and 13 can be integrated to obtain the cell volume and permeant concentration as function of time given the known parameters \( V^0_{os} \) (104 \( \mu m^3 \)) and \( A_e \) (137 \( \mu m^2 \)) (Jay, 1975), \( P_r \) (determined [Mlekoday et al., 1983] in a separate experiment for each cell suspension), \( f \) (0.393), \( b \) (0.463), \( c^e_1, c^m, c^e_m \). Then, substituting the change in permeant refractive index \( (\Delta R \text{ determined from the permeant cell concentration } (c_s) \text{ and index of refraction) and cell volume } (\Delta V) \) into Eq. 2, the theoretical photocell signal can be predicted. Thus, the procedure used to determine \( P_0, K_m, \) and
was to vary and adjust all three parameters to obtain the best fit between the theoretical and experimental photocell signals. The criterion for the best fit was to minimize the fractional least-squares deviation ($E$):

$$E^2 = \frac{\sum (S_i^{\text{obs}} - S_i^{\text{pred}})^2}{\sum S_i^{\text{obs}}^2}.$$  \hspace{1cm} (15)

**RESULTS**

**Calibration**

Fig. 2 shows the results of a typical experiment for determining the dependence of the photocell signal on the difference in the refractive index between the cell and the medium. The slope of this line is the constant $D_R$ in Eq. 2. The refractive index of the medium was changed by adding various amounts of dextran (73,000 mol wt) to the suspension medium. This does not significantly change the cell volume because the change in osmolarity is negligible. In the absence of cells, dextran does not significantly change the light scattering. The cell suspension was injected into both ports of the stopped-flow mixing chamber and the photocell signal was recorded. The standard deviation of each measurement (determined by repeatedly injecting the same solution into...
the mixing chamber after rinsing with water) is ~0.1 cm. A 0.25% cell suspension was used so that the light scattering was equivalent to the stopped-flow experiments when a 0.5% cell suspension is mixed with an equal volume of a cell-free solution. Since the dextran produced a rouleau formation with accompanying changes in light scattering, it was necessary to make the measurements within a few minutes after the dextran addition in order to minimize this effect.

In the urea osmotic transient experiments, at $t = 0$ the urea concentration is 200 mM in the medium and 0 in the cells, whereas at the end of the experiment (when the cells have returned to their original volume), the urea concentration is 200 mM inside and outside the cells. Thus, there is a change in the solute concentration of the cells relative to the medium of 200 mM urea. This corresponds to a refractive index change of $16 \times 10^{-4}$, which (from Fig. 2) should produce a 1.8-cm change in the photocell signal between $t = 0$ and $t = \infty$. This 1.8-cm deflection is marked by the arrow at $t = 0$ in Fig. 1. It can be seen that the experimental light-scattering curve is in good agreement with this prediction.

The calibration constant $D_v$ was similarly determined by recording the change in photocell signal for cells in a suspension of varying NaCl concentration. In the first paper of this series (Mlekoday et al., 1983), this calibration constant was measured by extrapolating the photocell signal back to $t = 0$ for an experiment in which $P_t$ was measured. When these two procedures were compared for the same cells, the two values for $D_v$ agreed within 2%.

The basic assumption of Eq. 2 is that the photocell signal ($\Delta S$) is linearly dependent on $\Delta V$ and $\Delta R$ and the two contributions are independent. As shown in Fig. 2, the dependence of $\Delta S$ on $\Delta R$ is reasonably linear over the experimental range ($0-16 \times 10^{-4}$). The $\Delta S$ was also linear for volume changes of at least 10% (not shown). The independence of the two factors was determined by measuring the photocell signal when both the volume and refractive index of the medium were varied. The change in the photocell signal from the control (cells in 225 mosmol saline) was ~3.2 cm for cells whose volume had been changed by equilibrating them in 265 mosmol saline. The change from control for cells in 1.16% dextran was +1.9 cm. The change from control for cells in 265 mosmol saline plus 1.16% dextran was ~1.2 cm, which is not significantly different from the expected value if the two effects were completely independent (~1.3 cm). These changes in the refractive index and cell volumes are the maximum that could occur in our experiments.

**Determination of $P_0$, $K_m$, and $\sigma$ for Urea and Ethylene Glycol**

Fig. 3 shows the closeness of the fit between the theoretical curves and experimental data for a typical experiment with urea. The experimental data points (*) were determined directly from the photograph of the photocell signal (see Fig. 1). The first experimental data point is at 0.2 s, a time at which the injection artifact is negligible (see Fig. 1C). The experimental and theoretical point at $t = 0$ was determined by adding the theoretical photocell signal due to the change in refractive index of the cell to the equilibrium ($t$...
= ∞) signal when the cells have returned to their initial (t = 0) volume. The curves represent the best fit that could be obtained by arbitrarily varying $P_0$ and $K_m$ for three different values of $\sigma$. The best fit was found with $\sigma = 0.95$ with a corresponding $P_0$ of $8.3 \times 10^{-4}$ cm/s and $K_m = 100$ mM. The value of $E$ (Eq. 15) for this fit was 0.0075, which means that each experimental point deviates from the theoretical values by ~0.75% of the average total deflection. In six other urea experiments, the results were nearly identical to those shown in Fig. 3. The best fit had an $E$ of ≤0.01 and had a $\sigma$ in the range of 0.9–1.0.

![Figure 3. Determination of $\sigma$, $K_m$, and $P_0$ for urea by finding the best theoretical fit to the experimental photocell signal (*) when cells in 220 mosmol saline are mixed at $t = 0$ with an equal volume of 220 mosmol saline plus 400 mosmol urea. For each of the indicated values of $\sigma$, the theoretical curve was obtained by arbitrarily varying $P_0$ and $K_m$ to obtain the best least-squares fit to the experimental data. The best fit (---) was for $\sigma = 0.95$ with a corresponding $P_0 = 8.3 \times 10^{-4}$ cm/s and $K_m = 100$ mM. The value of $E$ (Eq. 15) for this curve is 0.0075. The curve for $\sigma = 0.85$ is for $P_0 = 4.3 \times 10^{-4}$ cm/s and $K_m = 230$ mM ($E = 0.0125$). The curve for $\sigma = 0.75$ is for $P_0 = 3.5 \times 10^{-4}$ cm/s and $K_m = 2,000$ mM ($E = 0.021$)](image)

Fig. 4 shows a similar set of data for ethylene glycol. The best fit is for $\sigma = 1.0$ ($E = 0.0067$; $P_0 = 3.9 \times 10^{-4}$ cm/s, $K_m = 30$ mM).

**Inhibition of Water, Urea, and Ethylene Glycol Permeability**

Macey and Farmer (1970) have shown previously that phloretin inhibits urea permeability without significantly affecting water permeability. We confirmed this result using the fitting procedure described above for a urea osmotic
transient in the presence of $10^{-4}$ M phloretin. Because of the decreased urea permeability, a urea concentration of only 47 mM was needed to produce a cell volume change similar to that produced by 200 mM urea in the absence of phloretin. Since this urea concentration is less than half the $K_m$ of urea ($\sim 100$ mM; Fig. 3), it was assumed that urea transport in the presence of phloretin was not saturable ($K_m = \infty$), so that there were only two adjustable parameters ($\sigma$ and $P$). The best fit ($E = 0.017$) was obtained with $\sigma = 1$ and $P = 1.7 \times 10^{-5}$ cm/s ($\sim 5\%$ of the uninhibited value). Phloretin did not significantly alter the hydraulic permeability of water ($P_f$) or the permeability of ethylene glycol.

![Figure 4](https://example.com/ethanol.png)

**Figure 4.** Theoretical fit to the experimental photocell signal (*) when cells in 220 mosmol saline are mixed at $t = 0$ with an equal volume of 220 mosmol saline plus 206 mosmol ethylene glycol. The best fit (---) was for $\sigma = 1.0$ with a corresponding $P_o = 3.9 \times 10^{-2}$ cm/s and $K_m = 30$ mM ($E = 0.0067$). The curve for $\sigma = 0.9$ is for $P_o = 2.2 \times 10^{-4}$ cm/s and $K_m = 65$ mM ($E = 0.0153$). The curve for $\sigma = 0.8$ is for $P_o = 1.4$ cm/s and $K_m = 170$ mM ($E = 0.031$).

Since copper is a known inhibitor of glycerol permeability and glycerol and ethylene glycol probably use the same transport system (Mayrand and Levitt, 1983), it seemed likely that copper would also inhibit ethylene glycol permeability. Fig. 5 shows the fit of the theoretical curves to the experimental data for an ethylene glycol osmotic transient in the presence of $10^{-4}$ M CuCl$_2$. It was again assumed that the transport was not saturable so that there were only two adjustable parameters. The best fit ($E = 0.0083$) was obtained with a $\sigma = 1.0$ and a $P = 2.9 \times 10^{-5}$ cm/s ($10-20\%$ of the uninhibited value; Fig. 4).

A number of sulfhydryl-reactive reagents (Sha'afi, 1977) were screened for
their effects on water, urea, and ethylene glycol permeability. Results will be described for two of the reagents, \( p \)-chloromercuriphenylsulfonate (PCMBS) and 5,5'-dithiobis(2-nitro) benzoic acid (DTNB), which have markedly different effects on the three transport systems. PCMBS has been shown to inhibit both water and urea permeability (Macey and Farmer, 1970). The time and concentration dependence of this inhibition are shown in Fig. 6. The hydraulic water permeability was determined as described previously (Mlekoday et al., 1983) and the urea permeability was determined by the osmotic transient procedure described above. It can be seen that the two transport mechanisms have markedly different time and concentration dependences, which suggests that there are two different sites of action of PCMBS. PCMBS did not significantly affect the ethylene glycol permeability.

Fig. 7 shows the effect of DTNB on the hydraulic water permeability and the permeability of ethylene glycol. There is a small (30%) inhibition of the water permeability (in agreement with the result of Naccache and Sha'afi, 1974) and an 80% inhibition of ethylene glycol permeability. Since the inhibition was complete by the first time point (10 min), we do not know

![](image-url)
Figure 6. Fractional inhibition of osmotic water permeability (■) and the urea permeability (×) by PCMBS as a function of time. The concentrations of PCMBS used for each curve are indicated in the figure. The curves were fitted to the points by eye.

Figure 7. Fractional inhibition of osmotic water permeability (■) and ethylene glycol permeability (×) by DTNB. The concentrations of DTNB used for each curve are indicated in the figure.
whether the two systems were inhibited with the same time course. DTNB had no significant effect on the urea permeability. The small inhibition of water permeability was not an artifactual result of a change in red cell volume because there was no significant volume change at 10 min.

DISCUSSION

These measurements of the reflection coefficients differ from previous measurements in three important aspects: (a) the cells are made echinocytic to eliminate most of the injection artifact; (b) allowance is made for the contribution to the light-scattering signal of the change in cell refractive index caused by the change in cell permeant concentration; and (c) the theoretical permeability equations that are used to fit the data allowed for saturation of the diffusive transport. It is these differences that presumably explain why the \( \sigma \) for urea and ethylene glycol obtained here is significantly larger than has been previously reported.

This measurement has the major disadvantage that there are three parameters \( (\sigma, P_0, \text{ and } K_m) \) that must be varied to find the best fit to the experimental data. Thus, although the best fit for urea was for \( \sigma = 0.95 \), the fit for \( \sigma = 0.75 \) is probably also acceptable, especially considering the complexity of the fitting procedures and number of calibration constants that must be known. For this reason, these experiments do not provide an accurate measurement of \( \sigma \) for the red cell. However, the important implication of these experiments is that the results are completely consistent with the value of \( \sigma \) (0.95 for urea and 0.98 for ethylene glycol) that is predicted from Eq. 1 for the case in which the solute and water are using separate pathways.

These measurements of \( \sigma \), \( P_0 \), and \( K_m \) were remarkably reproducible, varying by only a small percentage over a period of 2 yr for blood from the same person. The validity of the measurements is difficult to evaluate. One check on these measurements is to compare them with tracer measurements of the \( P_0^{eq} \) and \( K_m^{eq} \) for \([^{14}C]\)urea \((1.16 \times 10^{-3} \text{ cm/s}; 281 \text{ mM})\) and ethylene glycol \((4.8 \times 10^{-4} \text{ cm/s}; 175 \text{ mM})\) (Mayrand and Levitt, 1983). The superscript “eq” indicates that these measurements are of the equilibrium exchange type in which there are equal concentrations of the test solute on both sides of the membrane.

For a general asymmetric facilitated transport system, \( K_{m}^{eq} \) and \( P_{0}^{eq} \) are not equal to those determined in this paper, where there is a net influx. As is shown in the Appendix, these four experimental parameters \( (P_0, K_m, P_0^{eq}, K_{m}^{eq}) \) can be used to estimate the four constants that are required to describe the general asymmetric system (Regen and Tarpley, 1974). Inserting the experimental values into Eq. 5A of the Appendix indicates that the dissociation constant for urea is 117 mM for transport out of the cell and 508 mM for transport into the cell. This asymmetry for urea transport is the opposite of that for thiourea determined by a more direct and accurate tracer approach (Mayrand and Levitt, 1983). Because the parameters of the transport system for urea and thiourea differ so markedly (urea has a low affinity and a high transport rate, whereas thiourea has a high affinity and a low transport rate), their different asymmetry is not surprising. Similarly, the analysis indicates
that the dissociation constant of ethylene glycol is ~42 mM at the internal site and 221 mM at the external site. Considering the complexity of the osmotic transient analysis and its relative insensitivity to the choice of the three parameters, these values for the dissociation constants of the transport systems must be regarded only as qualitative estimates. However, the important implication is that the values of $P_0$ and $K_m$ that correspond to $\sigma = 0.95$ for urea and $\sigma = 0.98$ for ethylene glycol are at least consistent with the tracer flux data.

These measurements of the reflection coefficient suggest that urea and ethylene glycol cross the membrane by a pathway that is not coupled to the major pathway for water movement. In addition, the results with the inhibitors Cu++, phloretin, PCMBS, and DTMB suggest that there are three separate and independent transport systems: one for ethylene glycol and glycerol, one for urea and related compounds (Mayrand and Levitt, 1983), and a third for water. Of the three sets of arguments for an aqueous pore system in the red cell membrane discussed in the Introduction, there is good experimental evidence only for the first (a hydraulic permeability greater than diffusive permeability of water). Thus, although water may be crossing the membrane through aqueous pores, there is probably no significant transport of small nonelectrolytes via this pathway.

APPENDIX

Derivation of Transport Parameters from Equilibrium Exchange and Net Flux Experiments

Equilibrium Exchange

For this case, the concentration of the test solute ($C$) is the same on both sides of the membrane and the flux of a tracer ($C^*$) is measured. The tracer flux ($J^*$) is described by (using the notation and Eq. 9 of Regen and Tarpley, 1974):

$$J^* = FC^*/(1 + C/Bg).$$

(1A)

Comparing this expression with that for the equilibrium exchange experiments (Eq. 2 of Mayrand and Levitt, 1983):

$$F = P_0^\infty B_g = K_m^\infty.$$

(2A)

Net Influx

In these experiments the external concentration ($C_0$) is fixed, whereas the internal concentration ($C_i$) rises during the experiment. The net influx ($J$) is described by (Regen and Tarpley, 1974; Eq. 1):

$$J = F(C_0 - C_i)/(1 + C_0/K_0 + C_i/K_i + C_0C_i/R_B)$$

$$= [F/(1 + C_0/K_0)]$$

$$\cdot(C_0 - C_i)/[1 + C_i(1/K_i + C_0/R_B)/(1 + C_0/K_0)]$$

$$1/R = 1/K_0 + 1/K_i - 1/B.$$
Comparing this expression with that for the net flux experiments (Eq. 14):

\[ P_0 = \frac{F}{(1 + C_0/K_0)} \]

\[ \frac{1}{K_m} = \frac{(1/K_i + C_0/R_B)/(1 + C_0/K_0)}{1/K_m} \]

Finally, by combining Eqs. 2A–4A, one can solve for the Michaelis constant for entry (\(K_0\)) and exit (\(K_i\)) from the cell in terms of the experimental constants \(P_0\), \(P_0^m\), \(K_m\), \(K_m^m\):

\[ K_0 = C_0/(P_0^m/P_0 - 1) \]

\[ \frac{1}{K_i} = \left[ \left(1 + C_0/K_0\right)/K_m + \left(1/K_m^m - 1/K_0\right) \right] \]

\[ \cdot \left(C_0/K_m^m\right)/\left(1 + C_0/K_m^m\right) \]

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