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Permeability and Reflection Coefficients of Urea and Small Amides in the Human Red Cell

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Abstract. Measurement of the transport parameters that govern the passage of urea and amides across the red cell membrane leads to important questions about transport of water. It had initially been thought that small protein channels, permeable to water and small solutes, traversed the membrane (*see* Solomon, 1987). Recently, however, very strong evidence has been presented that the 28 kDa protein, CHIP28, found in the red cell membrane, is the locus of the water channel (*see* Agre et al., 1993). CHIP28 transports water very rapidly but does not transport small nonelectrolytes such as urea.

The irreversible thermodynamic parameter, σ_i, the reflection coefficient, is a measure of the relationship between the permeability of the solute and that of water. If a solute permeates by dissolution in the membrane, $\sigma_i = 1.0$; if it permeates by passage through an aqueous channel, $\sigma_i < 1.0$. For urea, Goldstein and Solomon (1960) found that $\sigma_{urea} = 0.62 \pm 0.03$ which meant that urea crosses the red cell membrane in a water-filled channel. This result and many subsequent observations that showed that $\sigma_{urea} < 1.0$ are at variance with the observation that CHIP28 is impermeable to urea.

In view of this problem, we have made a new series of measurements of σ_i for urea and other small solutes by a different method, which obviates many of the criticisms Macey and Karan (1993) have made of our earlier method. The new method (Chen et al., 1988), which relies upon fluorescence of the intracellular dye, fluorescein sulfonate, leads to the corrected value, $\sigma_{urea,corr} = 0.64 \pm 0.03$ for ghosts, in good agreement with earlier data for red cells. Thus, the conclusion on irreversible thermodynamic and other grounds that urea and water share a common channel is in disagreement with the view that CHIP28 provides the sole channel for water entrance into the cell.

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Key words: Red cell — Fluorescein sulfonate — Reflection coefficient — Aqueous channel — Urea flux — Amide flux

Introduction

The relationship of the transport of urea, and other small hydrophilic nonelectrolytes, to that of water across the red cell membrane raises very important questions that may be broadly applicable to other cell membranes as well. Following the determination that water crosses the red cell membrane through an aqueous channel (Paganelli & Solomon, 1957; Sidel & Solomon, 1957), Goldstein and Solomon (1960) showed that urea also crossed the red cell membrane in a water-filled channel by determining that the reflection coefficient, $\sigma_{urea} = 0.62 \pm 0.03$. σ_i is the irreversible thermodynamic coefficient that determines whether a solute crosses a membrane by dissolution in the membrane, in which case $\sigma_i = 1.0$ or through an aqueous channel[1] in which case $\sigma_i < 1.0$.

The identity of the protein responsible for the aqueous channel was not established in these early experiments, though it had been suggested that it was the anion exchange protein, band 3 (*see* Solomon, 1989). More recently very strong evidence has been presented indicating that the protein, CHIP28, a small protein of 28 kDa found in the red cell membrane, is the locus of the water channel (Agre et al., 1993). These very small CHIP28 channels transport water very rapidly but are impermeable to small hydrophilic nonelectrolytes, such as urea (Agre et al., 1993). This raises a question as to the identity of the water channels through which urea enters the cell. If the data showing that the corrected

[1] When water can also cross the membrane by other routes, σ_{corr} is used, as discussed in Eq. 3. The condition for passage through an aqueous channel is then, $\sigma_{corr} < 1.0$.

σ_{urea} is <1.0 are accepted, it is then necessary to identify an aqueous channel permeable both to small polar nonelectrolytes and water.

In view of this problem, we have decided to make another measurement of σ_{urea} by a different method. Our published methods of determining σ_{urea} have been criticized by Macey and Karan (1993) and the new method we have devised is responsive to these criticisms. Previously, we measured red cell volume by 90° light scattering. To correct for optical effects that are not related to solute movement, we carried out control experiments in which cells were mixed with isosmotic buffer in the absence of urea, or other small solute, so that cell volume remained constant. We then subtracted these light-scattering changes from those in the presence of solute to obtain the net light scattering arising from solute, or solvent, movement. In the present method, cell volume is determined by the volume dependence of intracellular fluorescein sulfonate (FS) fluorescence measured at 90° by the method of Chen et al. (1988). This technique obviates the need for subtracting results obtained in control runs, although small effects due to changes in refractive index remain. The present results show that the corrected value of σ_{urea} is 0.64 ± 0.03, in good agreement with our previous data. Since this value of σ_i means that urea enters the red cell through an aqueous channel, it is at variance with the conclusion that the only water channel is the CHIP28 aqueous channel which is impermeable to urea.

Toon and Solomon (1990) had also measured σ_i for the series of hydrophilic alcohols and found it to be generally <1.0. However, Macey and Karan (1993) have recently measured σ_i for these solutes by an electron spin resonance method and have found that σ_i is 1.0 for these solutes. In view of this difference, we have used the fluorescein sulfonate method to make new determinations of σ_i for the alcohols. Our present determinations of these values by the fluorescein method also show that $\sigma_i < 1.0$, though σ_i is appreciably larger than the results given by Toon and Solomon (1990).

Materials and Methods

Materials

Fluorescein-5-(and-6) sulfonic acid, trisodium salt (FS), was obtained from Molecular Probes (Eugene, OR). Formamide and propionamide were supplied by Eastman Organic Chemicals (Rochester, NY); acetamide, methylurea, the dihydric alcohols and α-D-glucose by Aldrich Chemical (Milwaukee, WI); urea and all other chemicals (also of reagent grade) by Fisher Scientific (Springfield, NJ). Outdated bank blood was kindly supplied by the Children's Hospital (Boston, MA).

Methods

All fluorescence measurements were performed with a single beam stopped-flow spectrofluorimeter (Applied Photophysics model SF.17MV, Salisbury, England). This instrument has a dead time of the order of 1.5 msec, and was maintained at constant temperature by use of a Constant Temperature Circulator (VWR Model 1145, Preston Industries, Niles, IL). FS fluorescence was excited at 490 nm (slits 1 nm) and emission at 90° monitored through a 540 ± 10 nm bandpass filter (Corion, Holliston, MA).

Outdated blood, after aspiration of plasma and buffy coat, was washed 3 times (1:50 vol) with PBS buffer of the following composition, (in mM): NaCl, 150; Na_2HPO_4, 10; pH 7.4. All procedures were performed at 4°C unless otherwise noted. Washed cells were hemolyzed in 20–40 vols of ice-cold 5 mM Na_2HPO_4, pH 8, (lysis buffer) and centrifuged at 27,000 × *g* for ten min (two- and three-step ghosts had the indicated additional washes in lysis buffer). The pellet of pink (one-step) ghosts was resuspended with an equal volume of 20 mM FS in 20 mM Na_2HPO_4, pH 8.1 and incubated on ice for five min. This was followed by addition of a sufficient amount of 3 M NaCl required to bring the final concentration in the ghost suspension to 150 mM NaCl, and incubation at 37°C for 45 min to reseal the ghosts. After resealing, ghosts were washed an additional three times with PBS, pH 7.4, 4°C (1:80 vols, 12,000 × *g*, ten min). Ghosts were finally resuspended 1:70 v/v in PBS, pH 7.4, and kept on ice at all times. Approximately five min was allowed for the chilled ghosts to equilibrate to the experimental temperature of 24–25°C, after loading the drive syringes.

The Goldstein and Solomon Zero-time Method

The zero-time null method of Goldstein and Solomon (1960) depends upon determination of the permeable solute concentration outside the red cell which exactly balances a cytosolic salt-induced osmotic pressure gradient across the membrane. The equation governing the flow of a single permeant solute and water across a red cell membrane is (Katchalsky & Curran, 1965):

$$J_v = L_P(\Delta P - \sigma\Delta\pi) \tag{1}$$

in which J_v is volume flow in cm sec^{-1} (cm^3 cm^{-2} sec^{-1}), ΔP is the difference in the osmotic pressure of IMPERMEANT solutes across the membrane (mOsm) and $\Delta\pi$ is the difference in osmotic pressure of PERMEANT solutes across the membrane (mOsm). L_P is the hydraulic permeability of the membrane (units of cm^3 $dyne^{-1}$ sec^{-1}). When $J_V = 0$,

$$\sigma = \Delta P/\Delta\pi \tag{2}$$

For σ_i measurements, red cells that contain no permeable solute are exposed to such solutes for the first time when they are mixed with buffer (containing the solute) in the stopped-flow apparatus. Since the only time that cell permeable solute concentration is known exactly is at zero time, when the concentration of these solutes is 0, Goldstein and Solomon determined the time course of the initial rate of red cell volume change as these solutes permeated the cell, and extrapolated the rate to find $(J_v)_{t=0}$. $(J_v)_{t=0}$ is then determined at a number of values of $\Delta\pi$ and displayed graphically so that the value of $\Delta\pi$ for which $(J_v)_{t=0} = 0$ can be determined graphically and σ computed.

Solutions whose optical properties are to be analyzed are propelled into the observation chamber of our single beam stopped-flow spectrofluorimeter by air pressure of 80 psi (pounds/$inch^2$). When two solutions are mixed in the apparatus, the dead time is about 1.5 msec, but when a solution is mixed with a red blood cell suspension, there is a zero-time artifact of 100 msec that often can extend up to 200 msec. We do not know the cause of this artifact. Since our method of analysis to determine the reflection coefficient depends upon extrapolation of the fluorescence data, taken from a cell suspension, to zero time, this 100–200 msec delay raises serious methodological problems. We car-

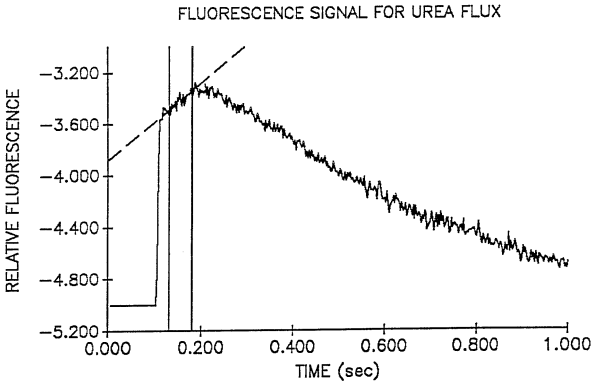

Fig. 1. Relative fluorescence signal for urea flux at 500 mM urea. The two vertical lines are drawn at 0.13 and 0.18 sec and the data are computed from the data between those points as shown in the enlargement in Fig. 2.

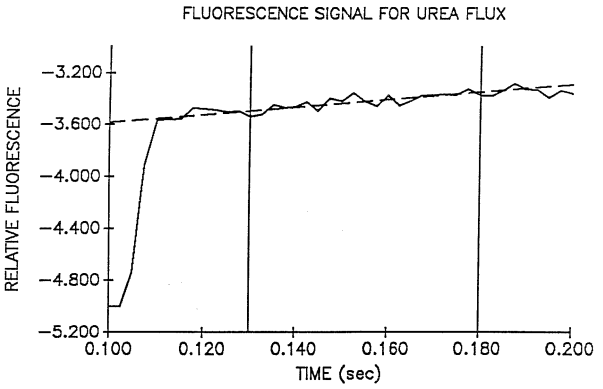

Fig. 2. Enlargement of Fig. 1. The data are obtained from the slope of the broken straight line between 0.13 and 0.18 sec.

ried out a large number of experiments with this procedure, including a great many controls, but finally concluded that the zero time delay was not acceptable. We then replaced the pressure-driven drive by a manual push. With this procedure, the zero time artifact has died down to 22 ± 9 msec and this procedure is the one used for the σ_i experiments reported in this paper. Figure 1 shows an example of the data obtained with a urea concentration gradient of 500 mM. The initial upward displacement is the result of the manual push. The initial 20 msec of subsequent signal contains initial artifacts that are discarded, including any effects due to unstirred layers. The data for analysis are taken between the two vertical lines which are separated by 50 msec. In practice, we start our fits when the initial artifact has died down and extend them only long enough to accrue sufficient representative data for our analysis. In the typical fit to the curve in Fig. 1, illustrated in Fig. 2, fit and experiment virtually cannot be distinguished over the fitting range.

The time course of red cell ghost volume change has been measured by the fluorescein sulfonate (FS) method of Chen et al. (1988). Chen et al. carried out a number of control experiments to validate the FS method. They showed that leakage of FS from rabbit renal brush border vesicles was relatively slow, with a half-time of 45 min at 23°C and >8 hr at 4°C. FS fluorescence is essentially independent of pH above 7.0 and of the presence of neutral solutes such as urea, glucose or sucrose. Chen et al. also showed that the presence of 10 mM FS did not affect the determination of the osmotic water permeability, P_f, by the light-scattering method and further, that P_f determined by the FS method is the same as that obtained by light-scattering. Chen et al. also concluded that vesicle volume determination by FS is independent of the refractive index of the cytosol because final vesicle volume in permeability experiments closely approximated initial vesicle volume. Results of experiments on the refractive index effect on red cell ghosts will be discussed below.

In native red cells, hemoglobin absorption makes it impracticable to measure cell volume by the FS method. We therefore carried out our preliminary experiments with resealed red cell ghosts prepared from bank blood using a modified Steck and Kant (1974) method. Since we can obtain satisfactory data even with some hemoglobin remaining in the ghosts, we used ghosts, prepared after a single wash, called pink or one-step ghosts. FS was sealed into the ghosts by the method used by Ye and Verkman (1989) for sealing a different fluorophore into red cell ghosts. To make sure that the seal is tight in each preparation, we used D-glucose, to which we have found the human red cell to be impermeable in many previous experiments over our limited time scale. As a control, we determine that σ_i is 1.0 for D-glucose in each experiment.

Urea and other amides have a significant effect on the refractive index of red cells, as Levitt and Mlekoday (1983) pointed out. Since the cells comprise less than 1% of the suspension after mixing is complete, the suspending medium may be considered an infinite reservoir. However, the refractive index of the cytosol does change as urea (or another amide), whose concentration in the suspending solution is 0.1–0.6 M, permeates the cell. The resultant change in light-scattering intensity depends upon the geometry of the stopped-flow chamber, and was particularly evident in Levitt and Mlekoday's apparatus. Levitt and Mlekoday observed that their final light-scattering signal was markedly smaller than the original value and they ascribed this difference to the refractive index effect. Thus a simple test of the refractive index effect on cell volume detection methods is afforded by a comparison of apparent initial and final cell volumes. In our previous experiments using the Chasan and Solomon (1985) method, the final cell volume is close to the initial one. For the present experiments, it is necessary to show that Chen et al.'s demonstration that the FS method is independent of cell refractive index also applies to resealed ghosts. In three-step ghost permeability experiments to determine the coefficient, ω, we have found final cell volume to be within 3–7% of initial cell volume, showing that the refractive index change has essentially no effect. However, in one-step ghosts, initial and final cell volume determinations differ by 3–24%, decreasing as the urea concentration increases. To determine whether this change has any effect on the FS determination of relative cell volume, which depends upon the slope of relative fluorescence as a function of π, we measured fluorescence intensity as a function of cell volume in the presence of 100 and 200 mM urea. When red cells or resealed ghosts are suspended in a buffer containing only impermeable solutes, volume is a linear function of π_{iso}/π (π_{iso} is the osmolality of an isosmolal solution). Figure 3 shows that relative fluorescence intensity is linearly dependent upon π_{iso}/π in the presence of equilibrium concentrations of 100 mM and 200 mM urea. In the original data, the 200 mM urea line is displaced vertically from the 100 mM line. When the average displacement of the 200 mM urea line is subtracted from the 200 mM points, all the points are superimposed, as shown in Fig. 3, that is, slope is independent of urea concentration. Thus, the dependence of relative fluorescence intensity on cell volume is independent of equilibrium urea concentration over the 100–200 mM range. Furthermore, as will be discussed in the next section, the null method of determining σ_i minimizes the refractive index effect.

In the present experiments, the reflection coefficient, σ, has been

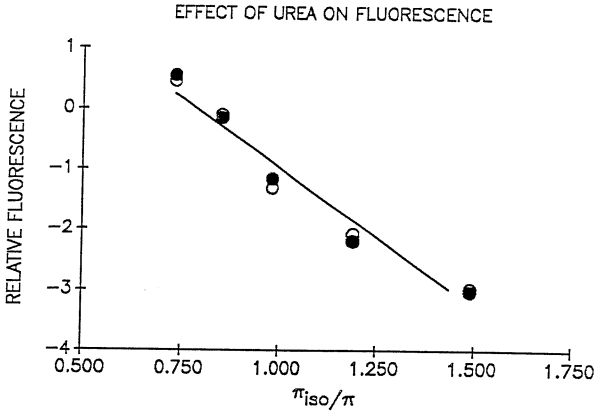

Fig. 3. Effect of urea on relative fluorescence intensity. 100 mM urea, ○; 200 mM urea, ●, points shifted as described in text.

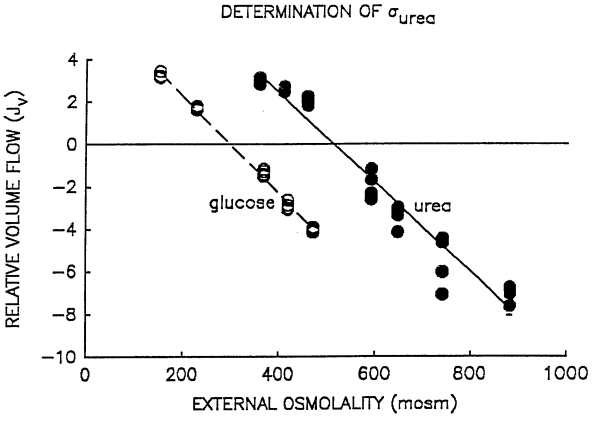

Fig. 4. Method for determination of σ_{urea} in a typical experiment. In this experiment, $\sigma_{glucose} = 1.0 \pm 0.03$ and $\sigma_{urea,corr} = 0.76 \pm 0.08$. Points are measured in triplicate or more.

determined in one-step ghosts by this original zero-time method. For a typical σ experiment, resealed ghosts are exposed in the stopped-flow apparatus to a graded series of 8 buffers containing (after mixing) 150 mOsm impermeant solutes (PBS) and urea (or other solute) concentrations which cover the whole range from swelling to shrinking osmolalities. In preliminary experiments, the averaged data (ten runs) near zero time were fitted empirically with a second order polynomial, from which the initial slope was computed directly (normal operation). In the data used for the tables, single runs were fitted with a linear fit (25–50 msec, manual operation) and the slope was obtained from the graph as in Fig. 2. This typical fitting procedure yields a linear relation between $(J_v)_{t=0}$ and external osmolality, which makes it very easy to determine σ by least squares, as shown in Fig. 4. The null method minimizes errors in the fitting procedure, since it only requires us to discriminate between concentrations that lead to swelling and those that lead to shrinking. The advantage of this consideration applies particularly to the refractive index which affects the amplitude of the signal rather than its direction.

When solutes can also cross the membrane by alternate routes such as dissolution in the membrane, the corrected $\sigma_{i,corr}$ is used (Dainty, 1963) to describe the interaction of solute and water in the aqueous channel.

$$\sigma_{i,corr} = \sigma_i + (\omega_i \overline{V}_{urea}/L_p) \tag{3}$$

in which ω_i is the solute permeability coefficient (units of mol dyne^{-1} sec^{-1}) and $\overline{V}_i$ is the partial molar volume of the solute (cm^3 mol^{-1}). In the native red cell (bank blood), the factor $(\omega_i \overline{V}_i/L_p)$ is relatively small, having a value of 0.099 for urea (Toon & Solomon, 1991). However, in resealed ghosts, as used in our experiments, $(\omega_i \overline{V}_i/L_p)$ is determined for each preparation as discussed in the following section, since small alterations in the resealing procedure may lead to significant variations in $(\omega_i \overline{V}_i/L_p)$. This correction factor is particularly important because the exact corrected condition required for transport through an aqueous channel is $\sigma_{corr} < 1.0$.

Measurement of Red Cell Ghost ω_i and L_p

To compute the correction factor, which is done by the normal method, both the permeability coefficient, ω, for each solute and the water permeability coefficient, L_p, for each preparation must be measured. The experimental procedures for measuring each coefficient have been given before (*see* Toon & Solomon, 1990) and are generally similar, except for the composition of the buffers with which the ghost suspension is mixed in the stopped-flow apparatus. For the L_p determination, the buffer (PBS) to which the ghosts are exposed has an impermeant solute gradient (D-glucose) of 50–200 mOsm after mixing. Data are then averaged for 10 runs and fit by nonlinear least squares to the equation given by Toon and Solomon (1991), with modifications of the cell parameters requisite for ghosts discussed below. The time scale is long enough that the normal method can be used.

For the ω experiments, the buffer (PBS) contains 0.3–0.8 M permeant solute after mixing. The numerical value of ω is determined by the method of Sha'afi et al. (1970) which is based on the value of two parameters determined at the minimum cell volume, V_{min} and (d^2V/dt^2) determined at V_{min}.

The computed numerical values of ω_i and L_p (but not of σ_i) depend upon the volume of the resealed ghost, V_{ghost}. Measurements of the volume of resealed ghosts prepared according to our protocol have been made with the Coulter counter giving values of 76 ± 2 and 88 ± 2 × 10^{-12} cm^3 for one-step and three-step ghosts respectively (three determinations). The volume of ''nonosmotic'' water in the ghosts (V_b) was determined by measuring the dependence of ghost volume on osmotic pressure gradient, as was done by Levin, Levin & Solomon (1980). In the one-step ghosts that Levin et al. characterized, the nonosmotic water was essentially zero, and our measurements in one-step and three-step ghosts also show that V_b is essentially zero ($V_b = 0.023 \pm 0.08 \times 10^{-12}$ cm^3, three determinations).

In a recent article on the permeability properties of short-chain diols, Macey and Karan (1993) criticized our analytical method of determining the initial slope. They tried a number of alternative fitting functions such as an exponential and a third order polynomial and pointed out that the results of the analysis depend upon the analytical function. It is not surprising that different results are obtained for exponential and third order polynomial fitting functions because the curve describing the time course of volume change is neither an exponential nor a third order polynomial. Macey and Karan also report that the fit depends upon the length of the interval chosen for the fit, with which we agree. Our routine procedure is to minimize the length of the fitting interval, subject to obtaining enough data to characterize the curve accurately. The advantage of the null method is that the effect of these differences in fitting interval on σ_{urea} is relatively slight, even for experiments using a pressure drive and a polynomial fit. In such an experiment, σ_{urea} for a 50-msec fitting interval in a trial experiment (normal drive) is 0.49 ± 0.03, as compared to 0.44 ± 0.03 for a 100-msec fitting interval.

Table 1. Reflection coefficient for urea in one-step ghosts

Exp	$\sigma_{glucose}$	σ_{urea}	$\omega\bar{V}/L_p$	$\sigma_{urea,corr}$	L_p [a] $\times 10^{11}$	ω_{urea} [b] $\times 10^{15}$
5-29	0.98 ± 0.03	0.46 ± 0.06	0.25 ± 0.02	0.71 ± 0.10	1.72	96 ± 6
6-1	1.00 ± 0.03	0.41 ± 0.02	0.35 ± 0.03	0.76 ± 0.08	1.41	110 ± 10
6-3	1.00 ± 0.04	0.48 ± 0.03	0.13 ± 0.01	0.61 ± 0.05	2.66	74 ± 3
6-7	1.02 ± 0.04	0.43 ± 0.03	0.18 ± 0.01	0.61 ± 0.04	1.32	53 ± 3
				- - - - - - - -		
			average (error-weighted)	0.64 ± 0.03		

[a] units: $cm^3\ dyne^{-1}\ sec^{-1}$

[b] units: mol $dyne^{-1}\ sec^{-1}$. The error-weighted average for the data in this column is $(69 \pm 2) \times 10^{-15}$ mol $dyne^{-1}\ sec^{-1}$. To convert to units of cm sec^{-1} this figure must be multiplied by RT (2.44×10^{10} dyne cm mol^{-1} at 21°C) which gives $(1.68 \pm 0.05) \times 10^{-3}$ cm sec^{-1}.

Results

The most important question is whether urea and these other solutes enter through an aqueous channel; the tool by which this determination is made is the value of the reflection coefficient, σ_{urea}. Since solutes may be able to cross the membrane by other additional means, such as dissolution in the membrane fabric, the corrected $\sigma_{i,corr}$ ($=\sigma_i + \omega_i\bar{V}/L_p$) is used to refer to passage through both the aqueous channel and the membrane fabric. If the irreversible thermodynamic criterion, $\sigma_{i,corr} < 1.0$, it shows that the solute crosses the membrane, at least in part, through an aqueous channel (*see* discussion by Dainty, 1963).

The first step is to determine the correction term, which is computed separately for each experiment. The water permeability of the membranes, L_p, is dominated by passage through the aqueous channel and is closely similar in one-step ghosts and native red cells. The average L_p for the ghosts, shown in Table 1, is $1.8 \pm 0.6 \times 10^{-11}$ $cm^3\ dyne^{-1}\ sec^{-1}$, which includes one anomalously high value of L_p. In our experience, L_p for fresh cells is $1.8 \pm 0.3 \times 10^{-11}$ $cm^3\ dyne^{-1}\ sec^{-1}$, slightly higher than our usual value for outdated blood, which is in the range of $1.2–1.5 \times 10^{-11}$ $cm^3\ dyne^{-1}\ sec^{-1}$. Thus the water permeability properties of the aqueous channel, measured in one-step ghosts by cytosol fluorescence, are closely similar to those obtained previously both in the native cell and outdated blood.

The solute permeability of the membrane, however, is greater in one-step ghosts than in normal cells. The figure for ω_{urea} which we measured for use in the computations for Table 1 is the average of three determinations at 0.4, 0.5 and 0.6 M urea, so that the average ω_{urea} for Table 1, $69 \pm 2 \times 10^{-15}$ mol $dyne^{-1}\ sec^{-1}$, can be taken as representative of the value at 0.5 M. This is larger than the value in red cells of $30 \pm 8 \times 10^{-15}$ mol $dyne^{-1}\ sec^{-1}$ for a urea concentration of 0.5 M, as given by Toon and Solomon (1991). ω_{urea} includes contributions from passage both through the channels and the membrane fabric. Since σ_{urea} and L_p are little changed in one-step ghosts, we assume that the increase in ω_{urea} primarily reflects the effect of contributions from urea reactions with the membrane. This twofold difference in urea permeability suggests that changes induced in the membrane, or material lost from it, during formation of one-step ghosts, had affected the passage of urea. As will be shown in subsequent Tables 2 and 3, the correction term for urea is larger than that of the other amides and very much larger than that for the diols, which reflects the relatively high solubility of urea in the membrane fabric.

The results of our experiments on σ_{urea} (manual method), given in Table 1, show that the error-weighted average of the corrected figure is $\sigma_{urea,corr} = 0.64 \pm 0.03$, for one-step ghosts, in reasonable agreement with the prior corrected value we had obtained for red cells, 0.73 ± 0.04 (Toon & Solomon, 1991). This agreement is consistent with the view that there is little difference in $\sigma_{urea,corr}$ between the native red cell and one-step ghosts and that there are no large errors in the prior method of σ_{urea} determination that are corrected by the present procedure. As a control we have measured $\sigma_{glucose}$, to which the human red cell is impermeable over our time course (Widdas, 1988). Our finding (column 2) that $\sigma_{glucose}$ is essentially 1.0 confirms the impermeability of the resealed ghosts. The Toon and Solomon (1991) value for uncorrected σ_{urea} is in good agreement with a great many other values previously obtained in this laboratory (*see* Solomon, 1993). A somewhat larger σ_{urea} of 0.79 ± 0.02 had been given by Owen and Eyring (1975). The only higher value in the literature is that of 0.95 given by Levitt and Mlekoday (1983), who consider that a value of 0.75 is also acceptable. Thus the weight of the evidence supports the conclusion that σ_{urea} is appreciably less than 1.0.

We have also determined $\sigma_{i,corr}$ for the short chain hydrophilic amides and methylurea, obtaining the values given in Table 2. These $\sigma_{i,corr}$ values are all less than 1.0 by two standard deviations or more. The statistics of the difference can be evaluated by computing P for the difference of the function $(1 - \sigma_{i,corr})$ from 0. Since $P < 0.02$ (t test) except for propionamide, the most lipophilic of these amides for which $P < 0.2$, there is a significant

Table 2. Reflection coefficient for amides in one-step ghosts

Solute	$\sigma_{glucose}$	σ_i	$\omega\nabla/L_p$	$\sigma_{i,corr}$*
Methylurea	1.05 ± 0.02	0.73 ± 0.02	0.05 ± 0.01	0.79 ± 0.05
Formamide	1.06 ± 0.03	0.35 ± 0.02	0.25 ± 0.01	0.67 ± 0.06
Acetamide	1.03 ± 0.05	0.67 ± 0.04	0.10 ± 0.01	0.77 ± 0.05
Propionamide	1.03 ± 0.05	0.80 ± 0.04	0.10 ± 0.01	0.90 ± 0.05

* Values in this column are computed as the average of separate experiments and may differ slightly from figures computed from the averages given in Table 2.

correlation between the transmembrane flux of the other amides and water, which implies that these solutes also cross the membrane through a water-filled channel. Our finding that this is the case is supported by the observation that the values of $\sigma_{i,corr}$ depend upon the size of the solute, increasing uniformly as the solute becomes larger. Figure 5 shows that $\sigma_{i,corr}$ of the amides and ureas are a linear function of the partial molar volumes of these solutes. The linear correlation is significant with $r = 0.95$, $P < 0.02$, t test. This dependence is similar to the finding of Toon and Solomon (1991) that $(1 - \sigma_{i,corr})$ is linearly dependent upon solute radius. The present values of σ_i for the amides in Table 2 for one-step ghosts have a slightly higher average than those for red cells given by Toon and Solomon (1991) with an average increase of σ_i by 0.13 ± 0.03. Our data also support the observation by Solomon (1993) that 16 short-chain hydrophilic solutes (84 measurements) are characterized by $\sigma_{i,corr} < 1.0$.

We have also determined σ_i for the dihydric alcohols by the new method and obtained the average values given in Table 3. These values are also higher than those previously obtained by Toon and Solomon (1990) with an average increase of σ_i by 0.17 ± 0.03, slightly larger than the average difference observed for the amides. We have also computed the value of P for the probability that $(1 - \sigma_{i,corr})$ is different from 0 and obtained the results in the last column of Table 3. Two of the values are significant with P-values of 0.02 or less and two are not. These findings are consistent with the view that the diols cross the membrane, in part at least, through a water-filled channel. More experiments, in addition to the twelve which comprise Table 3, would be necessary to establish the statistical validity of this conclusion.

Macey and Karan (1993) have determined σ_i for the solutes in Table 3 using a graphical method to evaluate the parameters. The time course of cell volume over a 12- to 20-sec period is compared to the predictions of theory for assumed σ_i values of 0.6, 0.8 and 1.0. Visually the fit appears best for $\sigma_i = 1.0$, except for 2,3 butanediol for which there is no apparent difference between 0.8 and 1.0. Our values, determined experimentally for these four diols all lie between 0.8 and 1.0 and may well be in agreement with the Macey and Karan data.

Discussion

Transport of Water

As we have discussed, a protein of 28 kDa, CHIP28, which Preston et al. (1992) have recently cloned, is tightly bound inside the red cell membrane, where it is believed to serve as the water transport channel (Agre et al., 1993; Zeidel et al., 1994; Shi, Skach & Verkman, 1994), rather than band 3, which had previously been suggested (Solomon et al., 1983). The ability to transport water has caused the class of proteins of structure similar to CHIP28, such as the bovine lens channel protein, MIP26, to be known as aquaporins. CHIP28 is present in the red cell as a tetramer; a single monomer can transport water and such a functional unit has a mass of 30 kDa in the rabbit red cell (van Hoek et al., 1992). Specialized lipids are not required for CHIP28 to function as a water channel. A rare group of individuals has been found who lack normal CHIP28 in their red cell membrane proteins (Colton(a-b-) cells; Preston et al., 1994). Red cell water flux in these individuals was depressed to 20% of normal controls, similar to the 19.1% water flux through the lipids in normals, as estimated by Solomon (1993). Urea flux was unaffected. A similar observation on water was made by Mathai et al. (1995) who have measured the diffusional and osmotic water permeability of red cells that lack CHIP28 because they come from a donor family that has a knockout mutation in the CHIP28 gene. The diffusion permeability of these cells to water is about 35% of that in comparable normal cells. The permeability of these mutated cells to urea has not been determined. The observation that a significant fraction of water transport is not carried by CHIP28 means that there must be an additional mechanism responsible for carrying water across the red cell membrane. The number of copies of CHIP28 is estimated to be in the range of $\approx 2 \times 10^5$ copies/red cell (Agre et al., 1993) to 3.2×10^4 copies/red cell (Mannuzzu, Moronne & Macey, 1993). Most importantly the CHIP28 channel does *not* transport urea (Zeidel et al., 1991, in proteoliposomes; van Hoek and Verkman, 1992, in stripped red cell membranes).

Transport of Urea

In a simple aqueous channel of appropriate dimensions, it is not possible to turn off water flux without affecting urea flux. Although pCMBS (p-chloromercuribenzene sulfonate) inhibits both water and urea fluxes (Macey & Farmer, 1970), it subsequently became clear (Macey,

Table 3. Reflection coefficient for diols in one-step ghosts

Solute	$\sigma_{glucose}$	σ_i	$\omega\nabla/L_p$	$\sigma_{i,corr}$ [a]	P [b]
1,2 propanediol	1.00 ± 0.04	0.92 ± 0.04	0.02 ± 0.01	0.92 ± 0.06	<0.3
1,3 propanediol	1.00 ± 0.05	0.89 ± 0.04	0.01 ± 0.01	0.89 ± 0.07	<0.01
1,4 butanediol	1.00 ± 0.03	0.82 ± 0.04	0.02 ± 0.01	0.86 ± 0.05	<0.1
2,3 butanediol	1.00 ± 0.03	0.68 ± 0.03	0.12 ± 0.01	0.81 ± 0.07	<0.02

[a] Values in this column are computed as the average of separate experiments and may differ slightly from figures computed from the averages given in Table 3.
[b] P values for the probability that the individual values of the function $(1 - \sigma_{i,corr})$ are greater than 0.

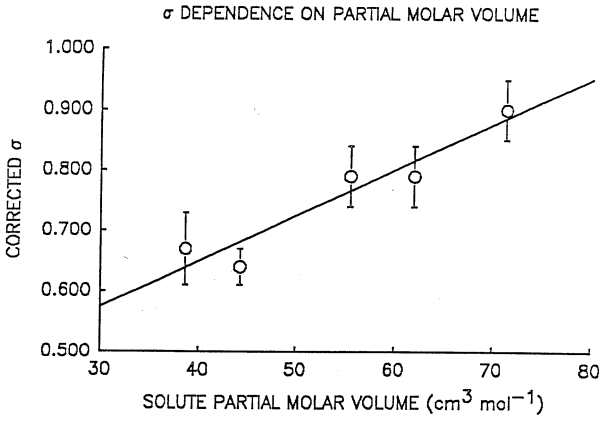

Fig. 5. Dependence of $\sigma_{i,corr}$ on solute partial molar volume for urea and the solutes in Table 2 (in order of increasing molar volume: formamide, urea, acetamide, methylurea and propionamide; average of three experiments or more). The correlation is significant with $r = 0.95$; $P < 0.02$.

1984; Toon & Solomon, 1986) that two separate binding sites are involved, since pCMBS bound much more rapidly to the urea flux inhibition site than to the water flux inhibition site. Further evidence that urea flux did not proceed by simple diffusion had been provided by Wieth et al. (1974), who showed that thiourea flux was inhibited by urea and conversely that urea flux was inhibited by thiourea. Furthermore, both fluxes were shown to be saturable. This evidence is at variance with the concept of a sieve-specific channel that discriminates among hydrophilic nonelectrolytes according to their size and is not saturable.

We have therefore suggested that separate sites are incorporated into a tripartite channel (Toon & Solomon, 1990) with one site governing the passage of hydrophilic nonelectrolytes and the other that of water across the red cell membrane (the ''tripartite mechanism''). The nonelectrolyte constraint consists of two H-bond exchange regions, one at each face of the pore, where the nonelectrolytes, such as urea and its congeners, bind to exchange their water of hydration for H-bonds when they enter and leave the membrane channel. Supporting evidence for two such regions is afforded by Mayrand and Levitt's (1983) finding of separate K_is for thiourea inhibition at the inside and outside face. Mannuzzu et al. (1993) have shown that inhibition of urea flux by mercurials such as pCMBS depends strongly on the lipid solubility of the inhibitor. They assume that the inhibition site is buried in the bilayer or other lipid structure and that inhibition of urea flux depends upon two steps, partition into the lipid and reaction with the inhibition site. They consider the water flux inhibition site to be separate from the urea site.

Results of experiments with red cells from individuals who lack the Kidd antigen (Jk(a-b-) cells) have led to another suggestion about the mechanism of urea transport, since urea transport in these individuals is almost entirely suppressed with no effect on water or anion fluxes. Masouredis et al. (1980), who have estimated that there are about 14,000 copies of the Kidd antigen on red cell ghosts, point out that findings with ghost membranes probably do not provide a faithful representation of the situation on the cell membrane. However, Mannuzzu et al. (1993), who have independently determined the number of sites involved in red cell urea transport to be ⩽32,000/red cell, have supported the suggestion of ''Fröhlich et al. (1991) that the Kidd antigen is also the urea transporter (the ''Kidd mechanism''). Neau et al. (1993) have used experiments with photoaffinity labeling to provide further support for a role of the Kidd antigen in red cell urea transport.

Urea transport proteins have been cloned for the vasopressin-regulated urea transporter in the kidney collecting duct (You et al., 1993). The kidney transporter encodes a 397-amino acid polypeptide, UT2, which is vasopressin sensitive and increases the permeability of oocytes to urea 23-fold. UT2 is not present in red cells, but a related protein, HUT11, has been encoded from a human bone marrow library (Olives et al., 1994) and may play a role in human red cell urea transport. It is a polypeptide of 391 amino acids that exhibits a 63% sequence homology with UT2 and weighs 43,000 Da. The hydrophobicity profiles of both UT2 and HUT11 are very similar and both suggest 10 membrane-traversing segments. In oocyte plasma membranes, HUT11 increased the urea permeability by a factor of 20. Urea

permeability of these oocytes was inhibited by pCMBS and urea analogues, similar to results in human red cells. HUT11 had no stimulative effect on osmotic water permeability in oocytes. Since the initial step in both the proposed urea transport systems (''tripartite and Kidd mechanisms'') is a reaction of urea with a carrier molecule, the existence of these carrier proteins does not argue in favor of either transport mechanism.

In many respects, the mechanism of red cell urea transport proposed by Fröhlich et al. (1991) and Mannuzzu et al., (1993) and their colleagues (''Kidd mechanism'') is similar to ours (''tripartite mechanism''), but there is one essential difference. Both mechanisms include a partitioning (hydration) step in which reactants enter the membrane phase to bind with the carrier, and a reaction step in which there is a carrier/urea reaction which propels the solute on its way across the membrane. The fundamental difference concerns the specificity of the second step which, in our view, is a channel which carries other solutes, particularly water, and in the Kidd antigen view, is a protein which reacts with only a single class of solute, such as urea and its congeners, but not water and other solutes. The urea transport proteins, UT2 and HUT11 are an example of proteins which react specifically with a specific class of solute in the binding step, urea and its congeners. Other solutes will require other proteins.

Coupling Between Urea (or Other Solute) and Water Flux

The view that water transport is mediated by a transport protein that does not transport other solutes such as urea is at variance with evidence of coupled solute/solvent transport. The most direct evidence of coupling between urea flux and water flux can be found by an examination of data in normal human red cells, as was done by Solomon (1993). Water flux is idiosyncratic to individuals, L_p varying between about 1.1 to 1.6 $\times$ 10^{-11} cm^3 dyn^{-1} sec^{-1} in a set of experiments in twelve individuals in whom normal urea flux was also determined. These water flux variations presumably arise from differences in water channel dimensions or numbers. If urea flux is mediated by another protein, independent of the CHIP28 responsible for water flux, the variations in urea flux should be independent of those of water flux. But this is not the case, since Solomon (1993) observed that urea and water fluxes are closely correlated in these data, with a correlation coefficient of 0.9, leading to $P < 0.01$, t test. This correlation provides very strong evidence that human red cell urea and water flux share a common rate-determining element.

Another example of an interaction between urea and water fluxes is provided by anion transport inhibitors of the benzene sulfonate class (Toon & Solomon, 1994) which have been found to stimulate water flux by up to 58% and inhibit urea flux by up to 92%. These interactions are related to binding of the benzene sulfonates to the anion exchange protein since they are inhibited by binding of the anion exchange inhibitor, DIDS (4,4′-diisothiocyanate-2,2′-stilbene disulfonate). The fractional stimulation of water flux is correlated linearly with the inhibition of urea flux ($r = 0.94$, $P < 0.001$, for 4-chlorobenzene sulfonate; similar effects are observed for other benzene sulfonates). These experiments provide convincing evidence of a linkage between the urea and water pathways and also indicate that a common element is shared with the anion transport protein.

A good deal of evidence supports the view that an essential step in hydrophilic nonelectrolyte transport across many cellular membranes is via a mechanism that discriminates among solutes according to size. One example has been given in Fig. 5 which shows that $\sigma_{i,corr}$ for urea and small hydrophilic amides is linearly dependent upon solute molar volume ($r = 0.95$; $P < 0.02$). Previously, in the native red cell, Toon and Solomon (1991) had found that the logarithms of the permeability coefficients of ten ureas and amides were significantly correlated with solute molar volume ($P < 0.01$, t test), in agreement with many previous studies (Solomon, 1993). These dependencies do not identify the rate determining step for solute transport, but they do provide the strongest evidence that there is a rate determining step which discriminates among solutes according to their size.

Problems About Urea Flux Mechanism

It seems likely that the urea binding protein in the red cell membrane, HUT11, is responsible for the first phase of the transport process, which leaves open the nature of the mechanism by which the solute takes its next step in crossing the lipid bilayer. If HUT11 is located within the lipid phase of the membrane, as is probable, it can fulfill Mannuzzu et al.'s (1993) findings about the location and number of urea binding sites. It is only necessary that the number of sites and their individual capacity be sufficient to hydrate enough urea molecules to maintain the transport steady state.

The conflict arises because the CHIP28 water channel is impermeant to urea, as previously pointed out. The experiments discussed in the preceding section all are consistent with the view that urea crosses the red cell membrane in an aqueous channel, as is our present determination that $\sigma_{urea,corr} = 0.64 \pm 0.03$. The evidence that CHIP28 provides the water channel is very strong (Agre et al., 1993) but the observations in red cells that lack CHIP28 indicate that there is an additional mechanism for water transport.

The ratio of osmotic to diffusional permeability (P_f/P_d) of the red cells which lack CHIP28 (Mathai,

1995) is 3.3, which indicates that water flows through a channel. This figure is a substantial fraction of the P_f/P_d ratio of 5.1 at 20°C for the normal red cell (Solomon, 1986), which has been interpreted as an equivalent pore radius of 6.5 ± 0.6 A.

The observation that 20–35% of the red cell water flux of these cells remains after CHIP28 has been knocked out is compatible with the existence of a second mechanism for transporting water. If there were an additional mechanism which was a protein that also transported urea, it could provide a water channel that could account for $\sigma_{urea,corr} < 1.0$. In fact there is one protein (AQP3), a member of the aquaporin family reported by Ishibashi et al. (1994) that is permeable to urea and glycerol. Of course it is not necessary that the 35% of the water flux be carried by an aquaporin, but it is reassuring to find a suggestive protein in the same family.

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