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Interrelation of ethylene glycol, urea and water transport in the red cell

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The reflection coefficient, σ_j, which measures the coupling between the jth solute and water transport across a semipermeable membrane, varies between 0 and 1.0. Values of σ_j significantly less than 1.0 provide irreversible thermodynamic proof that there is coupling between the transport of solute and solvent and thus that they share a common pathway. We have developed an improved method for measuring σ and have used it to determine that $\sigma_{\text{ethylene glycol}} = 0.71 \pm 0.03$ and $\sigma_{\text{urea}} = 0.65 \pm 0.03$, in agreement with many, but not all, previous determinations. Since both of these values are significantly lower than 1.0, they show that there is a common ethylene glycol/water pathway and a common urea/water pathway. Addition of first one and then two methyl groups to urea increases σ to 0.89 ± 0.04 for methylurea and 0.98 ± 0.4 for 1,3-dimethylurea, consistent with passage through an aqueous pore with a sharp cutoff in the 6–7 Å region.

Introduction

The reflection coefficient, σ_j, provides an irreversible thermodynamic measure of coupling between the transport of the jth solute and water across a semipermeable membrane. When $\sigma_j = 1.0$ (except for a small correction factor) there is no coupling, which shows that water and the jth solute cross the membrane by entirely independent paths. When σ_j is significantly less than 1.0, there is coupling between the transport of solute and solvent which proves that they share a common pathway. Goldstein and Solomon's [1] determination that $\sigma_{\text{eth gly}} = 0.63 \pm 0.03$ in the human red cell showed that ethylene glycol permeated the membrane through an aqueous channel. This conclusion was strengthened by the subsequent determination of Owen and Eyring [2] that $\sigma_{\text{eth gly}} = 0.86 \pm 0.03$, larger than the original value but still significantly less than 1.0. More recently, Levitt and Mlekoday [3] reported that $\sigma_{\text{eth gly}} = 1.0$, using a method in which two permeability coefficients and a binding constant were determined from a curve fitting program applied to the data from a single experiment, and concluded, using this and other arguments, that ethylene glycol was transported across the human red cell membrane through a path that was independent of the water channel.

We have now found techniques to improve the Chasan and Solomon [4] method of determining σ and report that $\sigma_{\text{eth gly}} = 0.71 \pm 0.03$ in good agreement with the original measurement of Goldstein and Solomon [1]. We have also determined that $\sigma_{\text{urea}} = 0.65 \pm 0.03$ in excellent agreement with Chasan and Solomon's value of 0.70 ± 0.02. These experiments show that there is significant coupling between ethylene glycol, urea and water transport across the red cell membrane and thus that each nonelectrolyte shares a common pathway with water.

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Materials and Methods

Materials

All chemicals were of reagent grade. Methylurea was obtained from Aldrich Chemical Co. (Milwaukee, WI), ethylene glycol from J.T. Baker (Phillipsburg, NJ), 1,3-dimethylurea from Eastman Kodak (Rochester, NY), Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and Dextran T70 from Sigma (St. Louis, MO). All other chemicals were supplied by Fisher Scientific (Fairlawn, NJ). Outdated bank blood was kindly supplied by the Children's Hospital (Boston, MA).

Methods

Outdated whole blood, after aspiration of plasma and buffy coat, was washed three times with a buffer of the following composition, in mM: NaCl, 142; KCl, 4.4; Hepes-NaOH, 20; $CaCl_2$, 1.2; $MgCl_2$, 0.5; pH 7.4, 300 ± 5 mosM. The calibration buffer consisted of 0.15 M NaCl and 0.02 M Hepes-NaOH (pH 7.4) ($\pm$urea or dextran). Osmolalities of all solutions were determined with a Fiske Model OS osmometer (Uxbridge, MA). Refractive indexes were measured with an Abbé-3L refractometer (Bausch and Lomb Optical Co, Rochester, NY).

The time-course of red cell volume changes was measured by 90° scattered white light using the stopped-flow apparatus described by Terwilliger and Solomon [5]. The analog data were digitized and averaged by a Hewlett Packard Model 217 computer which was also used for the data analysis.

Cells at 2% hematocrit were mixed with an equal volume of buffer made hyperosmolal by the addition of either NaCl or permeant solute. For each mixing solution, 25 consecutive runs were averaged to reduce random fluctuations. The average cell volume/time records were corrected for scattered light intensity changes not associated with volume changes by subtraction of control runs in which the blood suspensions were mixed with isosmolal buffer solutions as described by Levin et al. [6]. All experiments were carried out at 20–23° C.

Calibration

Our data is obtained as the amplified analog voltage from a photodiode, which is digitized by the computer and plotted as light intensity in 'light-scattering units' (lsu). We treat these lsu as linearly proportional to red cell volume which, though not absolutely necessary, makes for a great simplification in interpreting the data. Terwilliger and Solomon [5] showed that lsu were related to cell volume by a curvilinear function but the curvature was so slight around the isosmotic volume that they approximated it by a linear equation (their equation 17). Levin, Levin and Solomon [6] confirmed this finding and also used a linear relation which they considered to introduce an error of 1–3%. We have made similar measurements, as shown in Fig. 1A (control). The linear approximation is justified since the data fit reasonably well over a relative hematocrit range of 1.0 to 0.8, though there are significant departures as the cells shrink further.

In the experiments to determine σ, urea and ethylene glycol have replaced NaCl in the shrinking buffer, producing changes in the refractive index. The refractive index of an 0.25 M NaCl solution is $n = 1.3358$, as compared to a computed $n = 1.3400$ when 0.5 M urea is added to the solution. When Sha'afi et al. [7] used the stop-flow method to measure urea permeability, they showed that the addition of 397 mosM of urea (on both sides of the membrane) displaced the calibration curve upwards but had no effect on the slope. Terwilliger and Solomon [5] measured the effect of refractive index changes produced by sucrose (in the suspending medium alone) on lsu and their equation 17 can be used to determine the predicted effect of refractive index changes on lsu. This shows that addition of 0.5 M urea should produce an increase in lsu equivalent to an apparent 9.5% increase in cell volume. The first question we addressed was whether such a difference affected the calibration curve. The upper line in Fig. 1A shows the calibration curve obtained when cells are shrunk by NaCl in the presence of 0.5 M urea (at equilibrium across the red cell membrane). The data have been fit by least squares to a line ($r = 0.99$) with a slope of 11.0 ± 1.4 lsu · (0.1 unit of $Hct/Hct_o)^{-1}$, the same as the slope of 10.9 ± 0.4 lsu · (0.1 unit of $Hct/Hct_o)^{-1}$ in the absence of urea ($r = 0.98$). Addition of 0.5 M urea displaces the curve upward by an amount equivalent

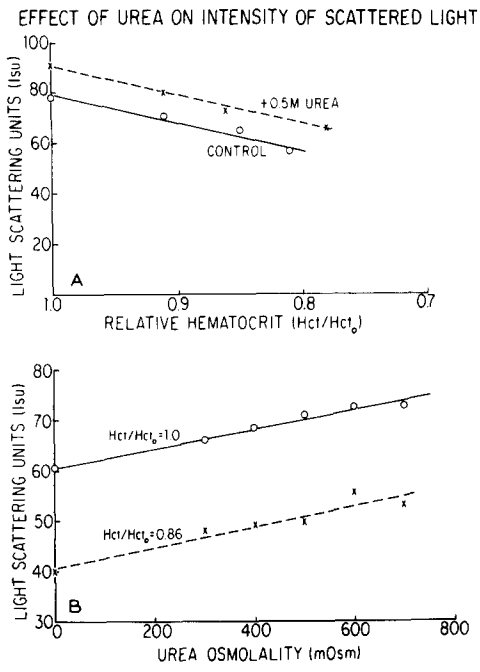

Fig. 1. Effect of urea on the intensity of scattered light. (A) Calibration experiment (one of two) in which red cells, having reached equilibrium volume in buffer (± 0.5 M urea) at 2% hematocrit, were mixed with equal volumes of the identical buffer in the stop-flow apparatus. The average signal for 25 runs was recorded over a 200 ms period and the average value in light-scattering units (lsu) was estimated by eye. Buffers were varied to cover the cell volume ratio (Hct/Hct_0) from 1.0 to 0.8. Hematocrits were measured by centrifugation in quadruplicate. The curve in the absence of urea has a slope of 1.1 ± 0.1 lsu·(0.1 unit of $Hct/Hct_0)^{-1}$ which does not differ from that in the presence of urea: 1.1 ± 0.4 lsu·(0.1 unit of $Hct/Hct_0)^{-1}$. (B) The experiments were carried out as above at two cell volumes, $Hct/Hct_0 = 1.0$ and $Hct/Hct_0 = 0.86$, and the urea concentration was varied over the range, 0 to 700 mosM. The slope of the line for the isosmotic cells, 0.019 ± 0.001 lsu·mosM^{-1} does not differ from that of the shrunken cells, 0.020 ± 0.03 lsu·mosM^{-1}.

to an apparent 10.7% of cell volume, in good agreement with the prediction of the Terwilliger and Solomon equation. Since the slope of the two lines is the same, the addition of urea has no effect on the calibration as used in the initial slope calculation.

In the experiments to determine σ_{urea} the cells were suspended in the normal medium containing 0.14 M NaCl together with urea in concentrations between 0.3 M and 0.6 M. It was possible that the varying urea/NaCl ratio could affect the calibration in a urea dependent fashion. To examine this possibility we compared the lsu of cells with $Hct/Hct_0 = 1.0$ with the lsu of cells with $Hct/Hct_0 = 0.86$ as a function of urea concentration (at equilibrium across the red cell membrane) over the range from 0.0 M to 0.7 M urea. Fig. 1B shows that the slopes of the two lines are the same, 0.019 ± 0.002 lsu · mosM^{-1} for $Hct/Hct_0 = 1$ and 0.020 ± 0.03 lsu · mosM^{-1} for $Hct/Hct_0 = 0.86$. Thus, when urea is at equilibrium across the cell membrane, the calibration is independent of the urea concentration over the ranges we have studied.

In our calibration experiments urea was present on both sides of the red cell membrane whereas in the σ_{urea} experiments, urea was present in the suspending medium and entered the red cell in a time-dependent way. Levitt and Mlekoday [3] pointed out that the contribution of cellular urea could not be neglected * and studied the effect by using high molecular weight Dextran solutions which altered the extracellular refractive index with a negligible contribution to osmolality. We have used this technique in the experiments in Fig. 2A which compare the effect of Dextran-induced changes in the refractive index of the suspending solution with changes produced by urea on both sides of the membrane. The experiment was carried out under two conditions: isosmotic volume

* Levitt and Mlekoday [3] treated their red cells with phosphatidylcholine to make them spherical in order to minimize the noise in the light-scattering signal caused when the forward motion of the suspension is suddenly arrested. After these cells had been exposed to urea concentration gradients and then allowed to reach their equilibrium volume, Levitt and Mlekoday observed that the final volume (as determined by the intensity of the scattered light) was markedly smaller than the initial volume measured the same way. They attributed this difference to changes in the cell refractive index as urea permeated the cytoplasm and then corrected their fitting procedure to remove this refractive index effect. We carried out our experiments on normal biconcave cells and minimized the noise by the averaging procedure described in the text. In our experiments, the light-scattering intensity, after exposure of the red cells to a large urea gradient and return to equilibrium, was very close to the initial light-scattering intensity so there was no reason to expect a large effect from the presence of cellular urea. The experimental results shown in Fig. 2 indicate that there is very little difference in the refractive index effect due to Dextran (in the external solution alone) and that of urea (on both sides of the membrane).

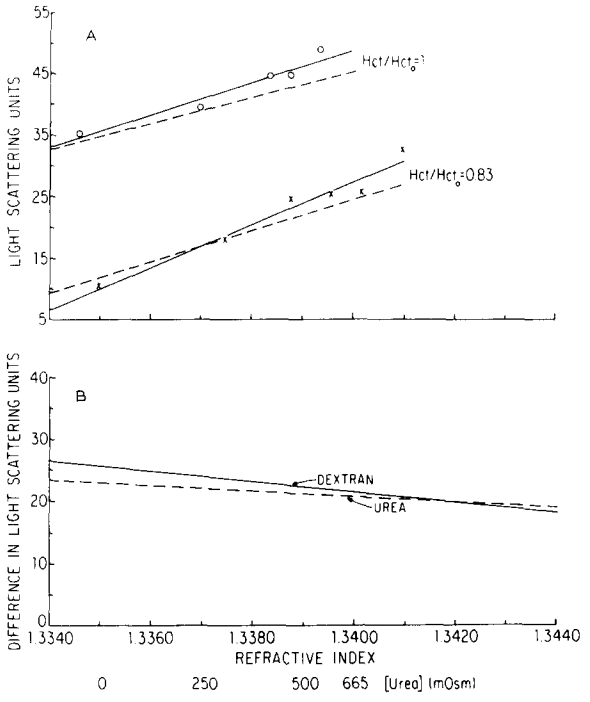

Fig. 2. (A) Light scattering intensity as a function of refractive index. The experiments were done as in Fig. 1B at relative Hct ratios of 1.0 and 0.83 but were plotted as a function of the refractive index of the suspending solution. The cells were equilibrated before mixing either with urea over the concentration range of 0 to 665 mosM or with dextran (average molecular weight, 73100) over the same refractive index range. The hematocrits were identical ± dextran indicating that the osmotic contribution of the dextran was negligible. The points and the full line are the results of the dextran experiments (isosmotic cell slope: 3.4 ± 0.3 lsu·(0.001 unit of n)$^{-1}$ and shrunken cell slope: 2.58 ± 0.03 lsu·(0.001 unit of n)$^{-1}$). The dashed line is obtained from a least-squares fit to urea data obtained in the same experiment (isosmotic slope: 2.1 ± 0.2 lsu·(0.001 unit of n)$^{-1}$ and shrunken cell slope: 2.58 ± 0.03 lsu·(0.001 units of n)$^{-1}$. (B) Differences in light scattering units (0.83 Hct/Hct$_o$ subtracted from Hct/Hct$_o$ = 1.0) for dextran and urea, obtained from the fitted lines in the top figure.

(Hct/Hct$_o$ = 1.0) and shrunken cells (Hct/Hct$_0$ = 0.83) and the data are plotted as a function of refractive index. In this experiment, the slopes of the dashed urea lines are not statistically different (as in Fig. 1B): 2.1 ± 0.2 lsu · (0.001 unit of n)$^{-1}$ for isosmotic as compared with 2.5 ± 0.4 lsu · (0.001 unit of n)$^{-1}$ for hyperosmotic. In the Dextran case there appears to be a real difference, 2.58 ± 0.03 lsu · (0.001 unit of n)$^{-1}$ as compared to 3.4 ± 0.3 lsu · (0.001 unit of n)$^{-1}$. As in Fig. 1, the major effect of the change in refractive index due to urea is an upward displacement of the curve with no significant change in calibration. However, when the index of refraction of the medium alone changes (the full line for dextran in Fig. 2A) there is a small additional effect which causes a difference in the slopes, as shown explicitly in the difference lines in Fig. 2, B. The size of the effect is given by the slope of the Dextran difference line which varies by 7.2% between 0 and 250 mosM urea. The zero-time method tends to minimize this correction because the method is based on observations over the time-course between 4 ms and 60 ms after mixing with the urea solution. At zero-time there is no urea in the red cell and at 60 ms the urea concentration is about 30% of the external concentration. In the case of ethylene glycol, the effects would be significantly reduced since the refractive index of ethylene glycol solutions is much closer to NaCl and the permeability to ethylene glycol is only about 25% of that of urea. Since the refractive index corrections are small, we have not applied them to our data.

Results and Discussion

Theory of the zero-time method

The zero-time method of Goldstein and Solomon [1] takes advantage of the fact that the intra- and extra-cellular concentrations of a permeating solute can be specified exactly at zero-time, before any solute permeation has taken place. The Kedem-Katchalsky equation for volume flow, J_v (cm^3 · cm^{-2} · s^{-1}) is:

$$J_v = -L_p \Delta\pi_i - \sigma_s L_p \Delta\pi_s \quad (1)$$

in which the subscripts i and s represent impermeable and permeable solutes. L_p is the hydraulic conductivity (cm^3 · dyn^{-1} · s^{-1}) and $\Delta\pi$ is the osmotic pressure (osmol · cm^{-3}). In the method of Chasan and Solomon [4] one set of experiments is carried out in which $\Delta\pi_i$ is varied, holding $\Delta\pi_s = 0$, so that L_p can be determined from the slope of J_v against $\Delta\pi_i$. A second analogous set of experiments is carried out to determine $\sigma_s L_p$ by varying $\Delta\pi_s$, holding $\Delta\pi_i = 0$. σ can then be determined directly from the ratio of the slopes. Since L_p does

not appear in the ratio, it need not be determined explicitly and J_v can be measured in light-scattering units rather than in cell volume units, thus eliminating the cumbersome calibration procedure.

This method of measuring L_p (in light scattering units) at zero-time has another advantage. If a permeant solute, say urea, is added at the same concentration to the red cell suspension and to the solution with which the cells are to be mixed, $\Delta\pi_s$ will be zero at $t = 0$ and the presence of the urea will have no effect on the applicability of the method for determining L_p from the slope of zero-time J_v against $\Delta\pi_i$. In the conventional method of determining L_p from the time-course of red cell volume change, both urea and water move across the cell membrane as the experiment proceeds and there is no simple and accurate way to determine L_p because it is necessary to make explicit allowance both for the volume flow of urea and the change in $\Delta\pi_s$ with cell volume.

The major difficulty of the zero-time method comes from the requirement to determine J_v at $t = 0$ ms. When the flowing motion of a suspension of red cells is suddenly arrested, as in the stopped-flow method, shock waves are propagated through the system. Since the red cells are discoidal and not spherical, the scattered light intensity depends upon the angle of the red cell axis to the illuminating beam. The shock wave causes a damped oscillation in the scattered light signal which dies down after about 100 ms. However, for rapidly permeating solutes, such as ethylene glycol and urea, all the information from which $J_{v,t=0}$ must be obtained is contained in the first 100 ms. Chasan and Solomon averaged 10–15 runs which led to a significant decrease in the noise. They then drew a smooth curve through the data from 20 to 200 ms, constructed the tangent at 20 ms and applied a correction to compensate for the 20 ms delay. The Levitt group [8] treated the cells with phosphatidylcholine to transform them into spherical echinocytes and Owen and Eyring [2] drew a tangent to the J_v curve over the range from 20 to 130 ms.

We have found that averaging 25 runs for each measurement reduces the noise much further, as shown in Fig. 3. All the random noise disappears and the remaining noise is composed of traces of

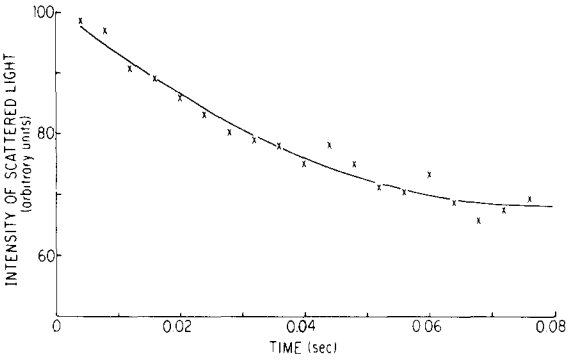

Fig. 3. Fit of second-order polynomial by non-linear least squares to experimental data. The lsu plotted for each time point is the average of 25 runs with an initial gradient of 514 mosM ethylene glycol after subtracting the average of 25 control runs without an ethylene glycol gradient. Data were fit from 4 to 80 ms. The best fit is given by: $(101 \pm 1) - (849 \pm 78)t + (5434 \pm 950)t^2$ and the initial slope is -849 lsu$\cdot$s^{-1}.

the damped oscillation caused by the sudden stop. The data can be fit very well to a second degree polynomial by non-linear least squares, beginning with our first point at 4 ms. We have terminated the fitting procedure at about the minimum volume (for permeant solutes) in order not to compromise the initial course of the polynomial by events which are dominated by the solute permeation. For the more permeable solute, urea, we terminated at 60 ms, for ethylene glycol we terminated at 80 ms and for NaCl at 120 ms. The fit is good, as illustrated in Fig. 3. The slope of the curve at zero time, which in this case is 4 ms, is given directly by the coefficient for the first-order term of the polynomial.

Our nominal time point of 4 ms after mixing must be adjusted for the effects of the dead-time and the unstirred layer. The dead-time has been found to be approx. 16 ms, as measured in our apparatus under similar conditions by Terwilliger and Solomon [5]. They have estimated that the thickness of the unstirred layer causes a delay of about 10 ms before the osmotic gradient is exercised at the membrane surface so that volume flow can begin. The net effect is a delay of about 6 ms so that our nominal 4 ms point is about 10 ms after mixing with an uncertainty of 3–5 ms. Chasan and Solomon [4] have derived an equation to correct for the underestimate in J_v which amounts to $5 \pm 3\%$ for a 10 ms delay for urea. The correc-

tion would be negligible for the more slowly permeating ethylene glycol. Since the uncertainties in the delay are large and the correction is small, we have not applied it to our data.

Reflection coefficients for ethylene glycol and urea

The results of one of nine experiments to determine σ_{ethgly} are given in Fig. 4 which shows that the ethylene glycol points fall on a different line than the NaCl control. Table I shows that the average σ_{ethgly} is 0.71 ± 0.03. The exact requirement for independent transfer of solvent and solute is that:

$$\sigma_{ethgly} = 1 - (\omega_{ethgly}\bar{V}_{ethgly}/L_p) \quad (2)$$

in which ω_{ethgly} is the permeability coefficient for ethylene glycol and $\bar{V}_{ethgly}$, its partial molar volume. The average of three values for ω_{ethgly} in the literature [2,3,9] is $5.7 \cdot 10^{-15}$ mol $\cdot$ dyn$^{-1} \cdot$ s^{-1} and our [5] best value for L_p is $1.8 \cdot 10^{-11}$ cm$^3 \cdot$ dyn$^{-1} \cdot$ s^{-1}. Taking $\bar{V}_{ethgly}$ as 55.9, the correction term = 0.02 so that the condition for coupling between ethylene glycol and water transport is that $\sigma_{ethgly} < 0.98$. Our value for σ_{ethgly} is significantly lower than this ($P < 0.0005$, t-test) so that these experiments show that a significant fraction of the ethylene glycol transport crosses the red cell membrane through an aqueous channel.

It is gratifying and indeed, surprising, that our present determination agrees so well with the figure of 0.63 ± 0.03 measured by Goldstein and Solomon [1] in 1960 using a continuous flow apparatus in which the only accessible time points were 45, 90, 145 and 190 ms after mixing. Owen and Eyring's value of 0.86 ± 0.03 is considerably higher than ours, though still < 0.98, probably because their measurement was based on a tangent drawn to the curve over the entire range from 20 to 150 ms while ours is based on the slope at 4 ms after mixing. Levitt and Mlekoday [3] devised a method to determine two red cell permeability coefficients (ω and σ) and the solute binding constant, K_m, from the entire time-course of a shrink-swell curve and an independent determination of L_p. They applied this method to determine that $\sigma_{ethgly} = 1$. Smith et al. [10] subsequently carried out a sensitivity analysis of a similar method and found that the two permeability coefficients were tightly coupled, so that the value found by the computer for σ depended upon the choice the computer had made for ω. Smith et al. concluded that precise determinations of σ by this method required independent and precise measurements

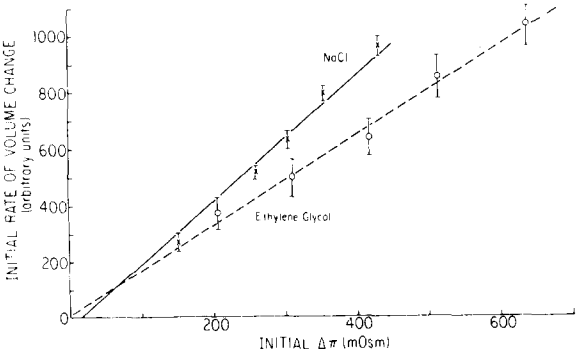

Fig. 4. Data showing determination of σ_{ethgly} in experiment 5. The NaCl line has a slope of 2.2 ± 0.1 lsu$\cdot$mosM^{-1} and an intercept of -36 ± 33 lsu. The ethylene glycol line has a slope of 1.60 ± 0.06 lsu$\cdot$mosM^{-1} and an intercept of 8 ± 20 lsu. $\sigma_{ethgly} = 0.72 \pm 0.07$.

TABLE I

DETERMINATION OF σ_{ethgly} AND σ_{urea} IN THE HUMAN RED CELL

Expt.	Slope (lsu$\cdot$mosM$^{-1} \cdot$s^{-1})		σ
	Expt.	Control	
Ethylene glycol			
1	1.52 ± 0.11	2.03 ± 0.07	0.75 ± 0.08
2	1.34 ± 0.10	1.83 ± 0.09	0.73 ± 0.09
3	1.65 ± 0.13	2.20 ± 0.14	0.75 ± 0.10
4	1.37 ± 0.06	2.15 ± 0.13	0.64 ± 0.07
5	1.60 ± 0.06	2.23 ± 0.12	0.72 ± 0.07
6	2.03 ± 0.21	2.72 ± 0.17	0.74 ± 0.12
7	1.52 ± 0.16	2.13 ± 0.11	0.71 ± 0.12
8	1.38 ± 0.06	1.89 ± 0.19	0.73 ± 0.11
9	1.07 ± 0.09	1.52 ± 0.07	0.70 ± 0.09
Av. $\pm$ S.D.			0.71 ± 0.03
Urea			
10	1.32 ± 0.01	2.46 ± 0.14	0.54 ± 0.05
11	1.19 ± 0.14	2.15 ± 0.13	0.55 ± 0.14
12	1.62 ± 0.14	2.23 ± 0.12	0.73 ± 0.10
13	1.31 ± 0.11	1.59 ± 0.04	0.82 ± 0.09
14	1.62 ± 0.07	2.29 ± 0.13	0.71 ± 0.07
15	1.39 ± 0.17	2.35 ± 0.13	0.59 ± 0.13
Av. $\pm$ S.D.			0.65 ± 0.03

not only of L_p, but also of ω. This explanation presumably accounts for Levitt and Mlekoday's finding that, though the best fit for σ_{urea} was 1.0, they also obtained a satisfactory fit for $\sigma_{urea} = 0.75$. The conclusion of Smith et al. [10] is also in conformity with our experience with total time course analysis (Levin, private communication) in which we have been unable to obtain satisfactory resolution of the three coefficients from a single shrink-swell curve.

We have also determined $\sigma_{urea} = 0.65 \pm 0.03$ in six experiments, as shown in Table I which agrees well with Chasan and Solomon's value [4] of 0.70 $\pm$ 0.02, though higher than Sha'afi et al.'s value [7] of 0.55 $\pm$ 0.02. All of these figures are smaller than Owen and Eyring's figure [2] of 0.79 $\pm$ 0.02, probably for the reason given above. The present value also agrees reasonably well with the lower bound of the 0.75–1.0 range given by Levitt and Mlekoday [3]. As was the case with ethylene glycol, our value for σ_{urea} agrees surprisingly well with the original value of 0.62 $\pm$ 0.02 of Goldstein and Solomon [1]. This agreement shows that the extrapolation procedure used by Goldstein and Solomon in determining σ_{urea} led to the correct answer and that Levitt's contrary conclusion [11], based on a computer simulation, was in error.

We have proposed that ethylene glycol, urea and water permeate the red cell through an aqueous channel whose radius, using recent values of the relevant parameters [12], is 6.5 Å. Steric hindrance in such a pore would discriminate sharply against solutes as their radius approached 6.5 Å. The equivalent pore model predicts that σ should increase with solute molar volume, approaching 1.0 as the solute radius approaches 6.5 Å. Fig. 5 gives the results of an experiment showing that the addition of first one, and then two symmetrical methyl groups to urea increases σ from 0.71 first to 0.93 and then to 0.97. From Table II, which gives the average σ in two experiments, it can be seen that the ether/water partition coefficient, k_{ether} for both solutes [13] is no greater than that for water [14] ($k_{ether} = 0.003$). By this criterion both methylated solutes are hydrophilic, so that transport by lipid diffusion can be neglected. Sha'afi et al. [14] have shown that the red cell permeability coefficient, ω, for these three solutes decreases linearly with the molar volume.

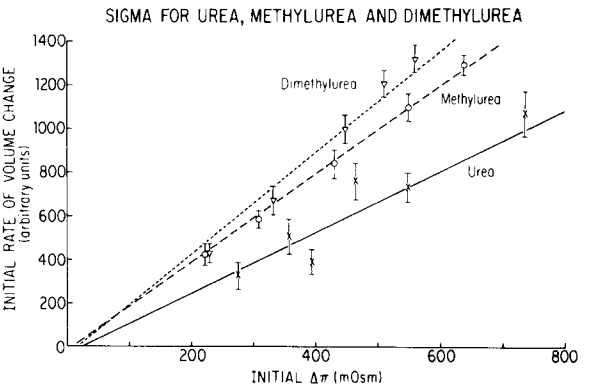

Fig. 5. The effect of molar volume on σ values in one of two experiments. For urea, the slope is 1.60 ± 0.06 lsu·mosM^{-1} and the intercept is -4 ± 20 lsu with $\sigma = 0.71$. For methyl urea, the slope is 2.14 ± 0.07 lsu·mosM^{-1} and the intercept is -26 ± 25 lsu with $\sigma = 0.93$. For 1,3-dimethylurea, the slope is 2.2 ± 0.1 lsu·mosM^{-1} and the intercept is -24 ± 45, with $\sigma = 0.97$. For the NaCl control (not shown), the slope is 2.3 ± 0.1 lsu·mosM^{-1} and the intercept is -23 ± 28 lsu.

This is in a direction contrary to that predicted by Overton's rule and is consistent with entry into the cell through an aqueous rather than a lipid pathway. In Table II, we have computed the radius of these solutes from the molar volume per molecule. Since urea has a large hydrogen bonding capacity, it is reasonable to assign a single hydration shell to these solutes, leading to a hydrated radius of 5.9 Å for methyl urea and 6.1 Å for dimethylurea which show the predicted increase in σ with solute volume ($\sigma_{methylurea} = 0.89$; $\sigma_{dimethylurea} = 0.98$). These figures are entirely consistent with an equivalent pore radius of 6.5 Å.

When a solute enters a cell through an aqueous channel, the flow velocity may be modulated either directly, as by an obstruction lodged in the channel, or indirectly by an allosteric effect arising from an interaction at a distant site. The transport of urea is governed by specific urea interactions with each face of the membrane, as shown by the individual K_m values determined by Levitt and Mlekoday [3], which means that the transport of urea can be modulated by allosteric interactions on either, or both, faces of the membrane. Both Levitt and Mlekoday [3] and Macey [15] have pointed out that the mercuric sulfhydryl reagent, pCMBS (p-chloromercuribenzene sulfonate), inhibits urea transport much more rapidly (and at a

TABLE II

PHYSICAL CHEMICAL PROPERTIES OF METHYLATED UREA MOLECULES

The values for $\bar{V}_s$ were computed from the crystalline density and the molar radius of the molecule was obtained by dividing by Avogadro's number. k_{ether} is the ether:water partition coefficient given by Collander [13]. k_{ether} for water was determined by Gary-Bobo and is given in Table II of Ref. 14 as 0.003.

Solute	k_{ether}	$\bar{V}_s$ ($cm^3 \cdot mol^{-1}$)	Molar radius (Å)	Hydrated molar radius (Å)	σ
Urea	0.00047	44.98	2.6	5.6	0.65 ± 0.03
Methylurea	0.0012	61.52	2.9	5.9	0.89 ± 0.04
1,3-Dimethylurea	0.0031	77.15	3.1	6.1	0.98 ± 0.05

lower concentration) than it inhibits water transport and we (Toon and Solomon [16]) have measured the binding constants and kinetics of these two separate pCMBS binding sites. These observations, as previously discussed [16], are consistent with modulation of urea transport by an allosteric mechanism.

Our present results do not address the question of how the transport of urea and ethylene glycol * is modulated, but rather provide thermodynamic evidence that the transport of each of these solutes is coupled to that of water. This shows that each solute must travel through an aqueous channel, whose radius, as shown by the methyl-substituted ureas, must have a sharp cutoff in the region of 6–7Å.

* Macey and Farmer [17] reported that 0.4 mM pCMBS did not inhibit ethylene glycol transport, though it did inhibit glycerol transport by about 50%. Likewise, Levitt and Mlekoday [3] did not find any pCMBS inhibition of ethylene glycol transport, though they did not specify the pCMBS concentration. However, in two unpublished experiments on red cells treated with *N*-ethylmaleimide, we (Toon and Solomon) have found pCMBS inhibition of ethylene glycol that fits a single site binding curve with K_i values in the range of 0.5 mM–0.7 mM, higher than the K_i of 0.16 mM given by Toon and Solomon [16] for pCMBS inhibition of water transport in *N*-ethylmaleimide-treated cells. Furthermore, Chasan, Balkanski and Solomon [18] reported that monothioethyleneglycol (β-mercaptoethanol) inhibits virtually the entire ethylene glycol flux with a K_i of 25–30 mM, as well as inhibiting water flux by 50% with a K_i of 70 mM. These data are consistent with an interaction between the transport of ethylene glycol and water, but the data are so fragmentary that it would be premature to suggest a mechanism.

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