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# Modulation of water transport in human red cells: effect of urea

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**We have studied the effect of urea on water flux in the human red cell and have found that 500 mosmolal urea inhibits osmotic water transport by 39%. The $K_i$ for urea inhibition of water flux is 550 ± 80 mosmolal, higher than, but comparable with, the $K_m$ of urea transport into the red cell of 220–330 mM given by Mayrand and Levitt (J. Gen. Physiol. 55 (1983) 427) and Brahm (J. Gen. Physiol. 82 (1983) 1). Other amides, such as propionamide and valeramide, as well as methyl-substituted ureas, have similar effects, although an indifferent molecule, such as 0.5 M creatinine, has no effect. Urea can be washed off the inhibition site with buffer, and the effects of urea concentrations as high as 1.2 osmolal are entirely reversible. 500 mosmolal urea also significantly increases the reflection coefficient for ethylene glycol, $\sigma_{eth\ gly}$, from 0.71 ± 0.03 in control experiments to 0.86 ± 0.04 ($P < 0.0005$, $t$-test), and propionamide has a similar effect on $\sigma_{eth\ gly}$. These results show that urea can modulate ethylene glycol transport through the red cell membrane, and are consistent with, but not proof of, the presence of a single class of aqueous channels through which both ethylene glycol and urea enter the red cell. It is suggested that the physiological purpose of these low-affinity urea sites is to modulate water flow out of the red cell during passage through the regions of 0.5–0.6 M urea in the kidney.**

## Introduction

During the course of our experiments [1] to determine the reflection coefficients, $\sigma_i$, for the interactions of urea and ethylene glycol with the red cell membrane, we found that $\sigma_{urea} = 0.65 \pm 0.03$ and that $\sigma_{eth\ gly} = 0.71 \pm 0.03$. These results show, first, that the fluxes of urea and water across the red cell are coupled, and hence, that urea and water share a common pathway, and second, by the same argument, that ethylene glycol and water share a common pathway. However, these observations are inconclusive as to whether it is the same water pathway that both urea and ethylene glycol traverse.

One way to address this question is to look for cross-effects, that is, to determine whether urea affects $\sigma_{eth\ gly}$. Urea transport across the red cell membrane is a saturable process with a $K_m$ of 218–334 mM, as determined by Mayrand and Levitt [2] and Brahm [3], so we decided to look for urea effects on $\sigma_{eth\ gly}$ at concentrations about twice the $K_m$. In the ordinary course of events, such an experiment would be very difficult, because the irreversible thermodynamic equations we use are tractable when the system comprises a solvent and a single permeable solute, but are very

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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difficult to apply when there is more than a single permeable solute. However, the zero-time method of Chasan and Solomon [4] may also be applied to determinations of the hydraulic conductivity, $L_p$, in the presence of a second solute, providing the second solute is present at the same concentration on both faces of the membrane and is thus at equilibrium at zero time, to which all measurements are referred. We were therefore able to see whether the addition of 0.5 M urea on both sides of the membrane had any effect on $\sigma_{\text{eth gly}}$. We found that the addition of urea increased $\sigma_{\text{eth gly}}$ significantly and, more surprisingly, in the controls which were a necessary part of the experiment, we found that 0.5 M urea decreased the hydraulic conductivity by 39%. We have now carried out a series of experiments on urea and have found that the kinetics for the inhibition of osmotic water flux can be fitted to a single-site binding curve with a $K_i$ of 550 mM, and that the binding can be reversed by washing with buffer. The observation that binding of urea to a well-defined red cell site inhibits urea and water transport and affects the reflection coefficient for ethylene glycol shows that a common element is involved in all three transport processes, consistent with, but not proof of, a common aqueous channel for urea and ethylene glycol. It is suggested that the physiological purpose of these low-affinity urea sites is to control the water flux out of the red cells during their passage through the regions of 0.5–0.6 M urea in the kidney.

## Materials and Methods

### *Materials*

Valeramide, propionamide and 1,3-dimethylurea were obtained from Eastman Organic Chemicals (Rochester, NY), methylurea from Aldrich Chemical Co. (Milwaukee, WI), and Hepes and creatinine from Sigma (St. Louis, MO). Urea and ethylene glycol were supplied by Fisher Scientific (Fairlawn, NJ). All chemicals were of reagent grade. Outdated bank blood was kindly supplied by the Children's Hospital (Boston, MA).

### *Methods*

Outdated bank blood, after aspiration of plasma and buffy coat, was washed three times with a buffer of the following composition, in mM: NaCl, 150; Hepes-NaOH, 20 (pH 7.4), $300 \pm 5$ mosM. Osmolalities of all solutions were determined with a Fiske Model OS osmometer (Uxbridge, MA). Creatinine was equilibrated with the cells by incubation at 37 °C for 2 h, followed by cooling to 23–25 °C, the temperature for all stop-flow experiments.

The time-course of red cell volume changes was measured using the stop-flow apparatus of 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 hyperosmolar by the addition of NaCl. All permeant solutes were added at the same concentration, both to the red cell suspensions and to the solution with which the cells were to be mixed, so at zero time, the only gradient present was that imposed by the NaCl. For each mixing solution, 25 consecutive runs were averaged. Data were usually collected over the range of 4–200 ms. The averaged cell volume/time records were corrected for light-scattering intensity changes not associated with volume changes by subtraction of the same number of control runs in which the blood suspensions were mixed with isosmolal buffer solutions (with or without amides) as described by Levin et al. [6]. Since 1200 individual runs are required to measure the control flux and the inhibition at seven separate urea concentrations, this places an effective upper limit on the number of points in the solute inhibition curve in a single experiment.

Each difference curve was then fitted to a second degree polynomial $(a_1 + a_2 t + a_3 t^2)$ by nonlinear least squares from 4 ms to 120 ms. The slope of the curve at zero time (4 ms) is given directly by the coefficient for the first-order term of the polynomial (neglecting a small contribution of approx. 1–2% for $2a_3 t$ at 4 ms). A range of four or five initial NaCl gradients of 150–400 mosmolal (after mixing) was used to determine the hydraulic conductivity from the slope of the initial rate of volume change versus the osmotic pressure difference, as given by Eqn. 1 in the next section. All of our results are reported in practical units, which means that the red cell volume is reported in light scattering units (lsu), the unit of time is

seconds, and concentrations are measured in mosmolal units. Two sets of factors are required to convert practical units to classical ones: $\alpha$ is the coefficient which relates lsu to volume changes in $cm^3$, and $\beta$ and the other coefficient are primarily dependent upon manipulations of $RT$. Specifically, the rate of volume change is measured in $lsu \cdot s^{-1}$, $\alpha J_v$, rather than $J_v$ ($cm^3 \cdot cm^{-2} \cdot s^{-1}$) and $\alpha$ is the coefficient (in units of $lsu \cdot cm^2 \cdot cm^{-3}$) which transforms $J_v$ to $lsu \cdot s^{-1}$. $\alpha\beta$ ($\beta$ has units of $dyn \cdot cm^{-2}$) is the coefficient which turns $L_p$ into practical units ($lsu \cdot s^{-1}$). Since we only report ratios of permeability coefficients (fractional inhibition of hydraulic conductivity or $\sigma_i$), $\alpha$ does not have to be evaluated so long as it remains constant, as discussed in the following section.

## Results and Discussion

*Theory of the zero-time method*

The Kedem-Katchalsky equation for volume flow, $J_v$ ($cm^3 \cdot cm^{-2} \cdot s^{-1}$), in a system comprising a solvent and a single permeable solute 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 ($cm^3 \cdot dyn^{-1} \cdot s^{-1}$) and $\Delta\pi$ is the osmotic pressure difference ($dyn \cdot cm^{-2}$) across the red cell membrane, using classical units. Our stopped-flow experiments are carried out with 1% suspensions of red cells, so that the extracellular solute concentrations remain essentially constant, but as soon as an osmotic pressure gradient is produced, water moves out of the cell and solute diffuses in, so that $\Delta\pi_s$ and $\Delta\pi_i$ are time-dependent. As Goldstein and Solomon [7] pointed out, the permeable cell solute concentration is known exactly at zero time, so that extrapolation to zero time introduces a great simplification in the determination of $\sigma$. The version of the zero-time method we now use, which was developed by Chasan and Solomon [4], uses practical units. $L_p$ is determined by the slope of $(J_v)_{t=0}$ plotted against $\Delta\pi_i$ in experiments in the absence of any permeable solute, so that $\Delta\pi_s = 0$. This zero-time method of measuring $L_p$ has the practical advantage that it can also be used in the presence of a permeable solute, provided this solute is distributed at equilibrium across the membrane at zero-time, so that $(\Delta\pi_s)_{t=0} = 0$.

If a determination of $\sigma_s$ is desired, $\sigma_s L_p$ is then determined by a plot of $(J_v)_{t=0}$ against $\Delta\pi_s$ with $\Delta\pi_i = 0$ in a companion set of experiments, and $\sigma_s$ can then be obtained from the ratio of the two slopes.

Eqn. 1 is derived from the Onsager irreversible thermodynamic equations [8], and its validity depends upon the linearity of the dependence of $J_v$ upon $\Delta\pi_s$ and $\Delta\pi_i$. Although these equations are derived from consideration of systems close to equilibrium, they apply under conditions quite far from equilibrium, as discussed in Curran and Katchalsky (Ref. 8, Ch. 15). Miller [9] gives examples of such linear relationships in a wide variety of coupled systems away from equilibrium, so that Eqn. 1 should be expected to apply to our system.

We measure cell volume indirectly, by the intensity of scattered light (in lsu), so it is also necessary to determine whether the light scattering is a linear function of cell volume (that is, whether $\alpha$ is a constant). In general, as Levin et al. [6] and Terwilliger and Solomon [5] showed, the calibration curves relating light scattering intensity to red cell volume are not straight, but a linear relationship fits the data reasonably well up to a 20% shrinkage of red cell volume. In practice, the relationship between the initial slope (in $lsu \cdot s^{-1}$) and the initial osmotic pressure difference is linear, as shown in Figs. 1 and 2 in the paper by Chasan and Solomon [4] and in Figs. 2–5 in the paper by Toon and Solomon [1], and the correlation coefficients regularly are 0.98 or better. Toon and Solomon [1] have studied the effect of the changes in refractive index caused by addition of large concentrations of solutes, such as 0.5 M urea, and have concluded that these effects are small, and may be disregarded. However, it should be pointed out that the $y$-intercept of the least-squares-fitted line, which relates $(J_v)_{t=0}$ to $\Delta\pi$, is frequently less than zero, as in the ethylene glycol curve in Fig. 2A. The difference is never significant in these experiments, but it occurs so frequently that it must be real and represent some effect whose cause we do not know. Since the $y$-axis intercept is not used in the computation, we have made no allowance for this effect.

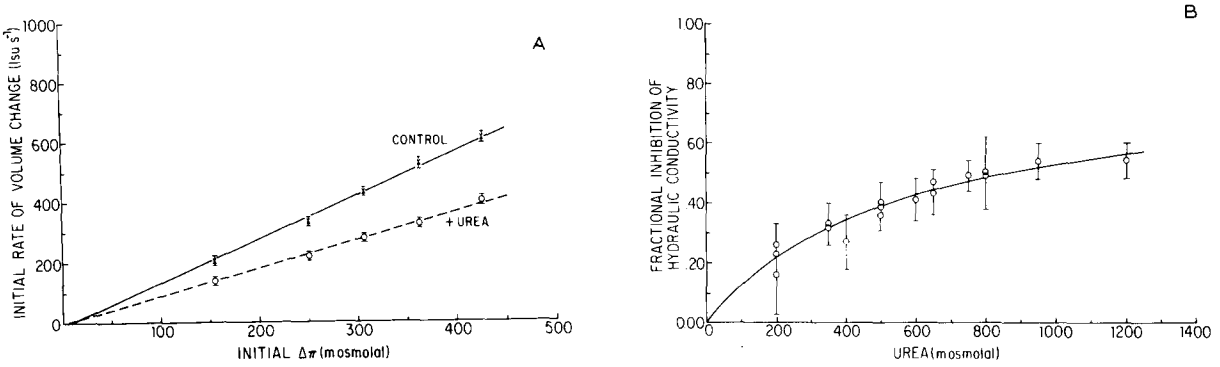


Fig. 1. (A) Inhibition of hydraulic conductivity by 0.5 osmolal urea, at equilibrium across the membrane at zero time. The slope of the control line without urea is $1.44 \pm 0.04$ lsu·mosmolal$^{-1}$·s$^{-1}$ and the intercept is $-13 \pm 12$ lsu·s$^{-1}$. The slope in the presence of 500 mosmolal urea is $0.92 \pm 0.02$ lsu·mosmolal$^{-1}$·s$^{-1}$ and the intercept is $-4 \pm 5$ lsu·s$^{-1}$. The ratio of the slopes is $0.64 \pm 0.05$, which shows that 500 mosmolal urea decreases the hydraulic conductivity by 36%. The average inhibition at this concentration, shown in Fig. 4, is 39%, which is the figure used in the remainder of the text. (B) Fractional inhibition of hydraulic conductivity as a function of urea concentration. The data from three experiments (Expts. 2, 3 and 5) have been pooled and fitted by non-linear least squares to a single-site binding curve with $K_i = 550 \pm 80$ mosmolal urea and maximum fractional inhibition $= 0.82 \pm 0.06$. Since this value has a much smaller error than the average of all five experiments in Table I ($560 \pm 160$ mosmolal), we have used it in the text.

*Modulation of osmotic water flux and $\sigma_{eth\,gly}$ by urea*

The rationale for our preliminary experiments was to see whether urea binding to the urea transport sites also affected water transport across the red cell membrane. In our preliminary experiments, we chose 500 mosmolal urea because this concentration is large compared to the $K_m$ of 200–300 mM for urea transport [2,3] and found that this concentration of urea inhibited water transport by 36%, as shown in Fig. 1A. We then carried out a number of experiments to see whether this effect could be ascribed to urea binding to the urea transport site.

Three experiments, of which the results are given in Table I (Expts. 2, 3 and 5) and Fig. 1B, show that the inhibition of water transport by urea can be described as a saturable process which is fitted by a single site binding curve with $K_i = 550 \pm 80$ mosmolal urea, and maximum fractional inhibition $= 0.82 \pm 0.06$. To obtain these data, we had to probe the membrane with urea concentrations as high as 1.2 M. It was necessary to show both that this high concentration did not damage the membrane and that the urea binding was reversible. In a control experiment, we treated the membrane with 1.2 M urea, and found that the water transport was inhibited by 68%. After washing these treated cells (and the control cells) four times with urea-free buffer, we found that the initial slope of the shrinking curve for the washed urea-treated cells was $1.27 \pm 0.09$ lsu · mosmolal$^{-1}$ · s$^{-1}$, in very good agreement with the control slope of $1.23 \pm 0.11$ lsu · mosmolal$^{-1}$ · s$^{-1}$. This experiment (which is supported by others at 0.5 and 0.8 M urea) shows that the urea binding responsible for inhibition of water transport is reversible and that treatment with 1.2 M urea does not permanently alter the water transport properties of the membrane.

In order to show that the effect depends upon the chemical identity of urea, we probed the membrane with an indifferent molecule. It is not possible to use either amides or alcohols for this purpose, since Mayrand and Levitt [2] have shown that there are specific binding sites for these classes of solutes. Creatinine, which permeates the membrane very slowly at room temperature, had been used by Rich et al. [10] to increase intracellular red cell osmolality, and they had found no deleterious effects after incubating red cells with 0.1 M creatinine. In three experiments, we found that exposing red cells to 0.5 M creatinine (distributed at equilibrium across the membrane) has no effect on water transport, because the ratio (initial $\text{slope}_{(+\text{creatinine})}$/initial $\text{slope}_{(\text{control})}$) $= 1.06 \pm 0.07$. These experiments show that inhibition of red cell osmotic water transport by addition of 0.5 M of a

TABLE I

CONSTANTS FOR INHIBITION OF WATER TRANSPORT BY UREA AND AMIDES

The two parameters, $K_i$ and the maximum fractional inhibition, have been determined by fitting the data to a single-site binding curve by non-linear least squares. In some cases, the fitted value of the maximum fractional inhibition was greater than 1.0, and the maximum fractional inhibition was chosen by eye to give a good fit. Since the least squares program fitted only a single parameter in these cases, the error is much less. There are two ways to decrease the error in the two-parameter fits: to include more points, or to go to very high concentrations to get a good experimental value of the asymptote. The first is excluded because of the practical difficulties in making more measurements, as discussed under Materials and Methods. The second is excluded because of our fear that high solute concentrations would damage the cells. In one experiment, we went up to 1.2 molal urea, which had no adverse effects, as discussed in the text, but in general, solute concentrations were limited to around 800 mosmolal.

| Expt. | $K_i$ (mosmolal) | Maximum fractional inhibition | $K_{i,\,urea}$ [a] (mosmolal) |
|---|---|---|---|
| Urea | | | |
| 1 | 330 ± 60 | 0.75 (set) | |
| 2 | 480 ± 170 | 0.8 ± 0.1 | |
| 3 | 620 ± 220 | 0.85 ± 0.15 | |
| 4 | 740 ± 60 | 0.75 (set) | |
| 5 | 630 ± 180 | 0.85 ± 0.1 | |
| Mean ± S.D. | 560 ± 160 | | 175 |
| Propionamide | | | |
| 6 | 310 ± 50 | 0.7 (set) | |
| 7 | 220 ± 60 | 0.65 ± 0.05 | |
| Mean ± S.D. | 270 ± 70 | | 30 |
| Methylurea | | | |
| 8 | 200 ± 120 | 0.65 ± 0.1 | |
| 9 | 400 ± 40 | 0.75 (set) | |
| Mean ± S.D. | 300 ± 140 | | 100 |
| 1,3-Dimethylurea | | | |
| 10 | 20 ± 30 | 0.1 ± 0.05 | 0.8 |
| Valeramide | | | |
| 11 | 160 ± 30 | 0.95 (set) | 20 |

[a] $K_i$ for urea transport inhibition, as given by Mayrand and Levitt [2].

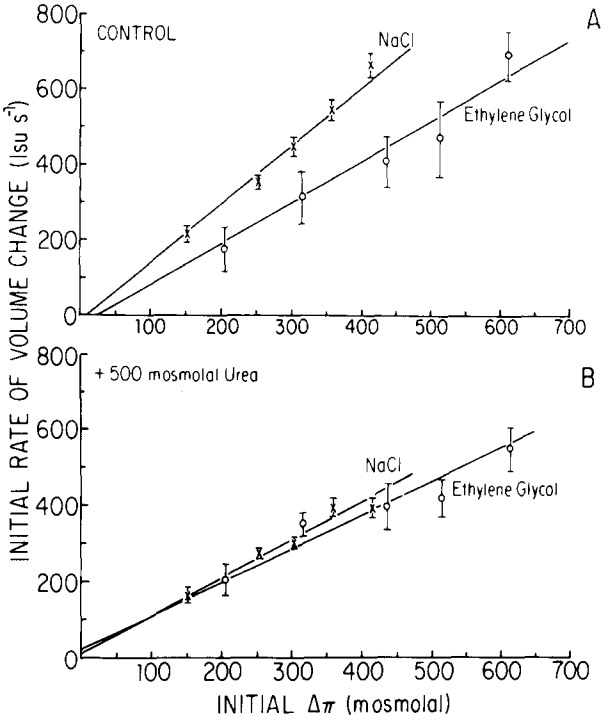


Fig. 2. (A) Determination of $\sigma_{eth\,gly}$ by the zero-time method in the absence of urea. The NaCl line has a slope of $1.52 \pm 0.07$ lsu·mosmolal$^{-1}$·s$^{-1}$ and an intercept of $-13 \pm 17$ lsu·s$^{-1}$. The ethylene glycol line has a slope of $1.07 \pm 0.08$ lsu·mosmolal$^{-1}$·s$^{-1}$ and an intercept of $-26 \pm 32$ lsu·s$^{-1}$, giving $\sigma_{eth\,gly} = 0.70 \pm 0.09$. (B) Determination of $\sigma_{eth\,gly}$ in a paired experiment (with (A)) in the presence of 500 mosmolal urea at equilibrium across the red cell membrane for both the NaCl and the ethylene glycol measurements. The slope of the NaCl control is $0.99 \pm 0.07$ lsu·mosmolal$^{-1}$·s$^{-1}$ and the intercept is $11 \pm 18$ lsu·s$^{-1}$. The slope of the ethylene glycol line is $0.88 \pm 0.09$ lsu·mosmolal$^{-1}$·s$^{-1}$ and the intercept is $23 \pm 30$ lsu·s$^{-1}$, giving $\sigma_{eth\,gly} = 0.88 \pm 0.09$.

nonelectrolyte depends upon the chemical identity of the nonelectrolyte.

The half-saturation constant for urea transport, $K_m$ is given as 218 ± 29 mM urea at 22–26 °C by Mayrand and Levitt [2] and as 334 mM at 25 °C by Brahm [3]. Our value for the $K_i$ for urea inhibition of water transport is 550 ± 80 mosmol/kg $H_2O$ at 23–25 °C, larger than the $K_m$ values. Our experiments were carried out on outdated bank blood, whereas Brahm, and probably Mayrand and Levitt, used fresh cells.

We also found that 0.5 M urea had a profound effect on the reflection coefficient for ethylene glycol, as shown in Figs. 2A and B. In four experiments with 0.5 osmolal urea, $\sigma_{eth\,gly,\,urea} = 0.86 \pm 0.04$. This figure may be compared to $\sigma_{eth\,gly} = 0.71 \pm 0.03$, in the absence of urea, as previously reported by Toon and Solomon [1] in a

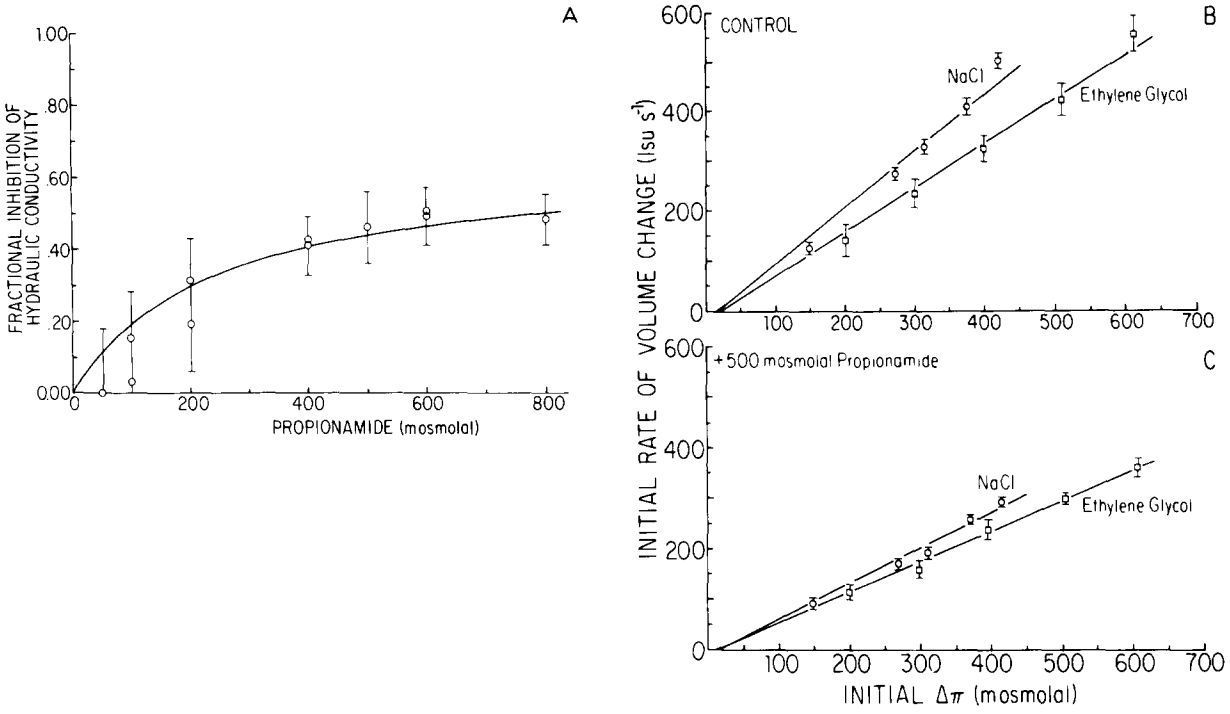


Fig. 3. (A) Fractional inhibition of hydraulic conductivity by propionamide in Expts. 6 and 7. The single parameter binding curve has been drawn with the maximum fractional inhibition set at 0.65, the fitted value for Expt. 7. This leads to $K_i = 240 \pm 20$ mosmolal, which we will use in the text, and which does not differ from the value in Table I ($270 \pm 70$ mosmolal). (B) The experiments shown in (B) and (C) show an increase of $\sigma_{eth\,gly}$ by 500 mosmolal propionamide in paired experiments similar to those with urea in Figs. 2A and B. Symbols: NaCl control, ○; ethylene glycol, □. In the absence of propionamide, the slope for the NaCl control is $1.15 \pm 0.08$ lsu·mosmolal$^{-1}$·s$^{-1}$ and the intercept is $-21 \pm 21$ lsu·s$^{-1}$. In the ethylene glycol experiment, the slope is $0.90 \pm 0.05$ lsu·mosmolal$^{-1}$·s$^{-1}$ and the intercept is $-21 \pm 17$ lsu·s$^{-1}$, leading to $\sigma_{eth\,gly} = 0.78 \pm 0.09$. (C) Results in the presence of 500 mosmolal propionamide at equilibrium across the membrane in both the NaCl control and the ethylene glycol experiment. The slope for the NaCl control is $0.69 \pm 0.04$ lsu·mosmolal$^{-1}$·s$^{-1}$ and the intercept is $-10 \pm 11$ lsu·s$^{-1}$. The slope for the ethylene glycol experiment is $0.59 \pm 0.01$ lsu·mosmolal$^{-1}$·s$^{-1}$ and the intercept is $-5 \pm 6$ lsu·s$^{-1}$, leading to $\sigma_{eth\,gly} = 0.85 \pm 0.06$. In the duplicate experiment in this series, the control value of $\sigma_{eth\,gly}$ was $0.75 \pm 0.09$ and the addition of 500 mosmolal propionamide raised $\sigma_{eth\,gly}$ to $0.91 \pm 0.2$.

series of nine experiments which included the four controls for the present determinations in the presence of 0.5 osmolal urea. The difference is highly significant ($P < 0.005$, $t$-test).

Propionamide also inhibits water transport and its binding to the inhibition site can also be described by a single-site binding curve, as shown in Fig. 3A. Furthermore, propionamide binding also causes an increase in $\sigma_{eth\,gly}$ comparable to that produced by urea. In the propionamide experiment (one of two) shown in Figs. 3B and C, $\sigma_{eth\,gly}$ increases from 0.75 in the control to 0.91 in the presence of 500 mM propionamide. Thus, the effects on water transport and $\sigma_{eth\,gly}$ are not limited to urea alone, but are to be attributed to a general class of amide sites.

The experiments with urea show that binding to a single well-characterized membrane site inhibits water fluxes by up to 80%. The only mechanism that can accomplish this is occlusion of the aqueous channel, either by narrowing it by an allosteric reaction or by blocking it sterically as urea is bound to its site. When urea occludes the channel, $\sigma_{eth\,gly}$ increases significantly. This means that there has been a change in at least one of the following three factors: the concentration of ethylene glycol in the aqueous channel; the frictional coefficient for the interaction between ethylene glycol and water; or the frictional coefficient for the interaction between ethylene glycol and the membrane. It follows that there is a linkage between urea binding and ethylene glycol transport,

just as there is between urea binding and water transport. Thus, these experiments show that there must be a common regulatory component which links the passage of ethylene glycol, urea and water across the red cell membrane.

*Characterization of the water transport inhibition site*

Inhibition of osmotic water flux in an aqueous channel is normally accompanied by an inhibition of diffusional flux. The best example of this coupling is in the case of pCMBS, which, as Macey et al. [11] have shown, inhibits both osmotic and diffusional permeability in the red cell, consistent with a site of action within the aqueous channel. If urea inhibits water flow by steric hindrance within an aqueous channel, we can compute the predicted inhibition to the diffusional flux on the basis of classical flow through right circular pores, which is governed by Fick's and Poiseuille's laws. 500 mosmolal urea, which inhibits osmotic flux by 39%, should inhibit diffusional flow (calculated on the assumption that diffusional flow is proportional to the square of the pore radius (Fick's law) and osmotic flow is proportional to the fourth power of the radius (Poiseuille's law) ($0.61 = 0.78^2$)) by 22%. No such inhibition has been reported by the several investigators who have studied the effect of urea on the diffusional flow of water in fresh blood. Brahm [12], who used the tracer method, gives a range of diffusional permeability coefficients of $(1.8–2.6) \cdot 10^{-3}$ cm $\cdot$ s$^{-1}$ for blood treated with 0.5 M urea, as compared to $(2.2–2.6) \cdot 10^{-3}$ cm $\cdot$ s$^{-1}$ in normal controls. Both Chien and Macey [13] and Fabry and Eisenstadt [14] used the nuclear magnetic resonance method, and neither reported any effect of urea, though Chien and Macey went up to 0.9 osmolal urea. The expected inhibition of diffusional water flow is large enough, particularly at the highest urea concentration used by Chien and Macey [13], to have been readily detectable. These arguments are independent of the mechanism of water transport, since they are also applicable to a single-file water channel, the mechanism suggested by Moura et al. [15] for red cell water transport. It follows either that the urea inhibition site is not within the aqueous channel, or that there is a difference between the permeability properties of bank blood, which we routinely use, and fresh blood, which the other investigators use *.

Fig. 4 shows that all the substituted urea molecules and the amides that we have studied inhibit water transport, whereas the one indifferent molecule, creatinine, does not. All of the amides that we have studied also inhibit urea transport, as shown by Mayrand and Levitt [2] and Solomon and Chasan [21]. The $K_{i,\,urea}$ values, determined by Mayrand and Levitt for inhibition of urea transport, are given in column 4 in Table I. Fig. 5 shows that there is a relationship between our $K_i$ values and the $K_{i,\,urea}$ values of Mayrand and Levitt. The correlation coefficient for the least-squares fit to the straight line is 0.87, very close to the coefficient of 0.88 for $P = 0.05$. The general trend demonstrates that there is some common element relating the binding that causes water flux

* It would be possible for the site for urea inhibition of water transport to be at the boundary layer, just at the entrance to the aqueous channel. The considerations which govern hindered diffusion and filtration through pores have been set out in a classical series of papers by Pappenheimer and his students [16–18]. Renkin [18] combined the equations of Ferry [19] with those of Faxen [20] to obtain the theoretical equations which describe the frictional resistance a small pore offers to the diffusion and filtration of small solutes. The frictional effect is given by the product of two terms: the friction within the membrane pore, which is the same for diffusion and filtration, and the steric hindrance to entrance to the pore, which is different. In the case of filtration, Ferry has calculated the steric hindrance at the entry to the pore by integrating the flow, of which the velocity varies according to the square of the distance from the axis, as in Poiseuille's law, over the area of the pore, to obtain the steric hindrance factor $(2(1-(a/r))^2-(1-(a/r))^4)$, in which $a$ is the radius of the solute molecule and $r$ that of the pore. Renkin [18] tested the restricted filtration equation, containing this factor, on filtration through Visking tubing of 15 Å pore radius, and found good agreement with the data. In the case of diffusion, there is no fourth power dependence, and the steric hindrance factor is $(1-(a/r))^2$; Renkin [18] also reported that the data for diffusion through the same tubing agreed with theory. These equations show that hindrance at the entrance to or exit from an aqueous channel can have different effects on osmotic flow than on diffusional flow. These considerations lead us to suggest that, if there is no difference between the effect of urea on bank blood and fresh cells, the water inhibition site could be located at, or near, the boundary layer between the aqueous channel and the adjacent solution at one face of the membrane.

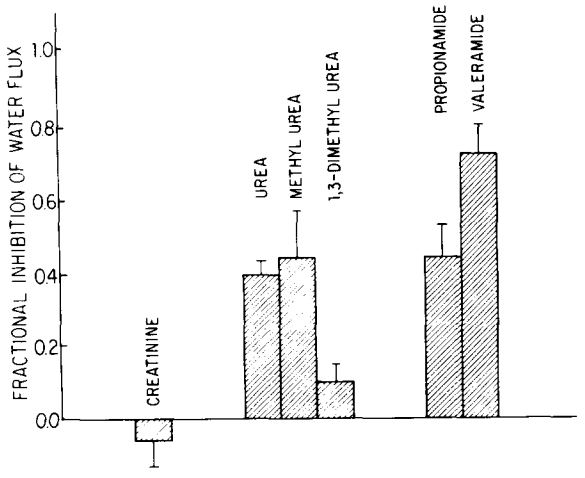


Fig. 4. Fractional inhibition of water flux by 500 mosmolal concentration of straight-chain amides, methyl-substituted ureas and creatinine. The data are the average of experimental points determined at 500 mosmolal concentration or, when experiments were not done at that concentration, the 500 mosmolal values are those computed from the fits given in Table I. The errors are estimated from the dispersion of the data.

inhibition to that responsible for urea flux inhibition, though the linearity of the relationship is of borderline significance. The slope of the line ($K_i/K_{i,\,urea}$) is $3.5 \pm 1.1$, which indicates that the binding affinity for inhibition of urea transport is tighter than for water transport inhibition. It is suggestive that the binding affinities for thiourea

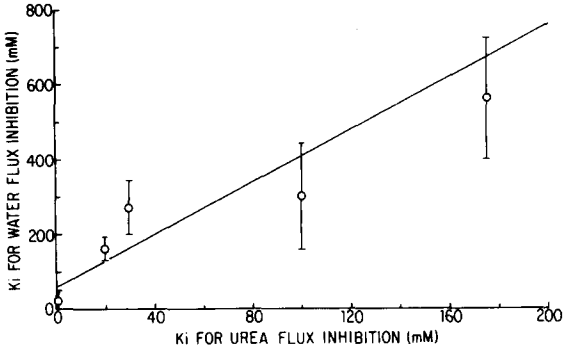


Fig. 5. Relation between $K_i$ for the effect of the solute on water flux inhibition and $K_{i,\,urea}$ for the effect on urea transport. The data are obtained from Table I and the line has been fitted by least squares with a slope of $3.5 \pm 1.1$ and an intercept of $59 \pm 35$ mosmolal. The correlation coefficient, $r = 0.87$. No errors were estimated by Mayrand and Levitt [2] for $K_{i,\,urea}$.

inhibition of urea transport are different on the two faces of the membrane (Mayrand and Levitt, Ref. 2), 35 mM on the inside face, as compared to 12 mM on the outside face.

Both polar and steric factors can be important in interactions between inhibitors and their receptors, and we have studied several amides in the hope of determining which factors play a role. For practical reasons, we have modified our inhibitors by addition of methylene groups, which makes it difficult to separate the two factors cleanly. Valeramide differs from propionamide by the addition of two methylene groups, which increase $k_{ether}$, the ether : water partition coefficient, by one order of magnitude [22] from 0.013 to 0.17 (the value for isovaleramide). The large increase in fractional inhibition shown in Fig. 4 suggests that decreases in polarity are correlated with increased affinity. Steric factors act in the opposite direction, since adding a single methyl group to form 1,3-dimethylurea from methylurea decreases the fractional inhibition by a factor of 3, even though it increases $k_{ether}$ by a factor of 3, from 0.0012 to 0.0031.

The picture that emerges is of a water transport inhibition site that is specialized for urea and amide binding and uses steric and polar constraints to discriminate among its ligands. The relative affinities for urea and amide binding to the water transport inhibition site are related to those for binding to the urea transport inhibition site, as shown in Fig. 5. The observation that there is a common regulatory element that controls the transport of urea, ethylene glycol and water, is consistent with, but not proof of, passage of the solutes through a common aqueous channel.

There is a sound physiological reason for the existence of such a site. As the human red cell traverses the kidney, it passes through regions containing 500–600 mM urea which create very large osmotic gradients to draw water rapidly out of the cells. As Fig. 4 shows, 500 mM urea reduces the osmotic permeability by 39%, thus conserving red cell water and limiting cell shrinkage in the short time that the red cells spend passing through these high urea regions. Since the $K_i$ of the site is set at 550 mosmolal, its regulatory ability is at a maximum at the concentrations the red cell encounters in its passage through the kidney.

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## References

1 Toon, M.R. and Solomon, A.K. (1987) Biochim. Biophys. Acta 898, 275–282.
2 Mayrand, R.R. and Levitt, D.G. (1983) J. Gen. Physiol. 55, 427–450.
3 Brahm, J. (1983) J. Gen. Physiol. 82, 1–23.
4 Chasan, B. and Solomon, A.K. (1985) Biochim. Biophys. Acta 821, 56–62.
5 Terwilliger, T.C. and Solomon, A.K. (1981) J. Gen. Physiol. 77, 549–570.
6 Levin, S.W., Levin, R.L. and Solomon, A.K. (1980) J. Biochem. Biophys. Methods 3, 255–272.
7 Goldstein, D.A. and Solomon, A.K. (1960) J. Gen. Physiol. 44, 1–17.
8 Katchalsky, A. and Curran, P.F. (1965) Nonequilibrium Thermodynamics in Biophysics, Ch. 15, Harvard University Press, Cambridge, MA.
9 Miller, D.G. (1960) Chem. Rev. 60, 15–37.
10 Rich, G.T., Sha'afi, R.I., Romualdez, A. and Solomon, A.K. (1968) J. Gen. Physiol. 52, 941–954.
11 Macey, R.I., Karan, D.M. and Farmer, R.E.L. (1972) in Biomembranes, Vol. 3; Passive Permeability of Cell Membranes (Kreuzer, F. and Slegers, J.F.G., eds.), pp. 331–339, Plenum Press, New York.
12 Brahm, J. (1982) J. Gen. Physiol. 79, 791–819.
13 Chien, D.Y. and Macey, R.I. (1977) Biochim. Biophys. Acta 464, 45–52.
14 Fabry, M.E. and Eisenstadt, M. (1978) J. Membrane Biol. 42, 375–398.
15 Moura, T.F., Macey, R.I., Chien, D.Y., Karan, D. and Santos, H. (1984) J. Membrane Biol. 81, 105–111.
16 Pappenheimer, J.R., Renkin, E.M. and Borrero, L.M. (1951) Am. J. Physiol. 167, 13–46.
17 Pappenheimer, J.R. (1953) Physiol. Rev. 33, 387–423.
18 Renkin, E.M. (1954) J. Gen. Physiol. 38, 225–243.
19 Ferry, J.D. (1936) J. Gen. Physiol. 20, 95–104.
20 Faxen, H. (1922) Arkiv. Mat. Astron. Fysik. 17 No. 27, 1–28.
21 Solomon, A.K. and Chasan, B. (1980) Fed. Proc. 39, 957a.
22 Collander, R. (1949) Acta Chem. Scand. 3; 717–747.