Urea Permeability of Human Red Cells

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ABSTRACT The rate of unidirectional [^{14}C]urea efflux from human red cells was determined in the self-exchange and net efflux modes with the continuous flow tube method. Self-exchange flux was saturable and followed simple Michaelis-Menten kinetics. At 38°C the maximal self-exchange flux was 1.3×10^{-7} mol cm^{-2} s^{-1}, and the urea concentration for half-maximal flux, $K_{1/2}$, was 396 mM. At 25°C the maximal self-exchange flux decreased to 8.2×10^{-8} mol cm^{-2} s^{-1}, and $K_{1/2}$ to 334 mM. The concentration-dependent urea permeability coefficient was 3×10^{-4} cm s^{-1} at 1 mM and 8×10^{-5} cm s^{-1} at 800 mM (25°C). The latter value is consonant with previous volumetric determinations of urea permeability. Urea transport was inhibited competitively by thiourea; the half-inhibition constant, K_i, was 17 mM at 38°C and 13 mM at 25°C. Treatment with 1 mM *p*-chloromercuribenzosulfonate inhibited urea permeability by 92%. Phloretin reduced urea permeability further (>97%) to a "ground" permeability of $\sim 10^{-6}$ cm s^{-1} (25°C). This residual permeability is probably due to urea permeating the hydrophobic core of the membrane by simple diffusion. The apparent activation energy, E_A, of urea transport after maximal inhibition was 59 kJ mol^{-1}, whereas in control cells E_A was 34 kJ mol^{-1} at 1 M and 12 kJ mol^{-1} at 1 mM urea. In net efflux experiments with no extracellular urea, the permeability coefficient remained constantly high, independent of a variation of intracellular urea between 1 and 500 mM, which indicates that the urea transport system is asymmetric. It is concluded that urea permeability above the ground permeability is due to facilitated diffusion and not to diffusion through nonspecific leak pathways as suggested previously.

INTRODUCTION

Urea permeability of human red cells is larger than predicted from Overton's rule, which states that an increase of the membrane permeability coefficient can be correlated to the relative increase of the lipid solubility of the solutes. The relatively larger permeability of human red cells to small hydrophilic nonelectrolytes like urea, formamide, and acetamide has been taken as evidence of the presence of hydrophilic pathways (aqueous pores) in the cell membrane (Goldstein and Solomon, 1960; Solomon, 1968; Sha'afi et al., 1971; Sha'afi, 1977). Solomon (1968), reviewing the theoretical and experimental basis for pores in biological

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membranes, calculated an operational "equivalent" pore radius in the human erythrocyte membrane of 4.5 Å. Brown et al. (1975) later suggested that the integral membrane proteins of band 3, which mediate the tightly coupled anion exchange, induced nonspecific leaks to water and small hydrophilic nonelectrolytes by forming part of the postulated aqueous pores.

It has alternatively been suggested that urea is transported by facilitated diffusion in human red cells (Macey and Farmer, 1970; Macey and Wadzinsky, 1974; Kaplan et al., 1974; Wieth et al., 1974; Solomon and Chasan, 1980). The present study was undertaken to provide a more detailed characterization of urea transport kinetics in human red cells. The results show that urea transport has features that are commonly applied in characterizing a facilitated diffusion process: saturation kinetics of the Michaelis-Menten type, competitive inhibition with the urea analogue thiourea, and irreversible inhibition with *p*-chloromercuribenzosulfonate (PCMBS). Phloretin inhibited urea permeability of human red cells by two orders of magnitude to $\sim 1 \times 10^{6}$ cm s^{-1} (at room temperature). This is as low as urea permeability in artificial lipid membranes (Vreeman, 1966; Galucci et al., 1971; Poznansky et al., 1976; Finkelstein, 1976) and in the chicken erythrocyte membrane (Brahm and Wieth, 1977), systems where urea permeates by simple diffusion. The study further shows that the urea transport system has asymmetric properties. Urea permeability decreased with increasing urea in self-exchange experiments with equal urea concentrations on both sides of the membrane. In contrast, the urea permeability coefficient remained high in net efflux experiments where the extracellular urea concentration was kept at zero.

It is concluded that urea transport in human red cells proceeds by facilitated diffusion and that diffusion through nonspecific leaks, as suggested by Brown et al. (1975), is highly unlikely.

MATERIALS, METHODS, AND CALCULATIONS

Media

All experiments were performed in media containing (mM): 150 KCl, 0.5 KH_2PO_4, and 1–1,000 urea. The media were titrated to the desired pH at the temperature concerned with 1 N KOH.

Labeling and Packing of Red Cells

Freshly drawn, heparinized human blood was washed once in the incubation medium and titrated with either CO_2 or 150 mM potassium bicarbonate to the desired pH at the temperature of the subsequent experiment. After titration, the cells were washed three additional times with the titrated incubation medium, resuspended to a hematocrit of 50–60%, and incubated with [^{14}C]urea (The Radiochemical Centre, Amersham Corp., Buckinghamshire, England) to obtain a radioactivity of 0.5–1 μCi/ml cell suspension. Isolation of cell samples for flux experiments and for determinations of intracellular radioactivity and cell water content was carried out as described previously (Brahm, 1977).

Several potential inhibitors of urea permeability were employed. Phloretin (ICN K&K Laboratories Inc., Plainview, NY), 0.25 M dissolved in ethanol, was added both to the incubation and the efflux medium to give a final concentration between 0.1 and 1 mM (ethanol 0.04–0.4% vol/vol). The effects of PCMBS (Sigma Chemical Co., St. Louis, MO), *N*-ethyl-maleimide (NEM; Fluka, Buchs, Switzerland), and 5,5′-dithiobis-2-nitrobenzoate (DTNB; Sigma Chemical Co.) at 1 mM were studied in red cells incubated for 45 min at 38°C as described previously (Brahm, 1982). Treatment with 4,4′-diisothiocyano-2,2′-stilbenedisulfonate (DIDS) was carried out using the procedure for complete (>99%) and irreversible inhibition of anion transport as described elsewhere (Brahm, 1977).

Determination of Radioactivity and Cell Water Content

The radioactivity of [^{14}C]urea in the intracellular phase and in the supernate was determined by liquid β-scintillation spectrometry after precipitating with perchloric acid as described by Dalmark and Wieth (1972). Radioactivity of the filtrates of the flux experiments was measured by scintillation spectrometry without prior precipitation.

Cell water content was determined by drying cell samples to constant weight. The cell samples contained ~2% (wt/wt) extracellular medium trapped between the cells during the packing procedure, as measured by means of [^{3}H]inulin, which remains extracellular (Funder and Wieth, 1976). A correction for trapped medium was carried out in determinations of cell water content. By the drying procedure, the cellular solvent volume (V, cm^3) can be related to the cell membrane area (A, cm^2), assuming that 1 g of cell solids equals 3.1×10^{10} normal erythrocytes with a total area of 4.4×10^4 cm^2 (1.42×10^{-6} cm^2/cell; Brahm, 1982). In experiments with high urea concentrations, the cell solids content was corrected for the contribution by urea.

Determination of the Rate of Efflux

All experiments were performed at low hematocrits (<1%) to measure the rate of [^{14}C]urea going from the prelabeled cells into an isotope-free medium. With the exception of a series of net flux experiments, the rate of tracer efflux was determined as urea self-exchange under steady state conditions, at which the intra- and extracellular chemical concentrations remain constant during the experiment, i.e., the net flux of both solute and solvent is zero. In urea net flux experiments, "initial" rates were determined by following the efflux of ≤25% of intracellular tracer.

Depending on the rate of tracer efflux, two different methods were used for determinations. At rates above 0.3–0.7 s^{-1}, equal to half-times of 1–2 s ($T_{1/2}$ = ln2 k^{-1} s), the continuous flow method was used in the version described previously (Brahm, 1977, 1982, 1983*a*). At slower rates of urea efflux the Millipore-Swinnex filtering technique described by Dalmark and Wieth (1972) was employed.

Calculations

RATE COEFFICIENT, PERMEABILITY, AND UNIDIRECTIONAL FLUX The rate of [^{14}C]urea efflux was determined in cell suspensions with hematocrits below 1%. The tracer efflux was well described by a monoexponential function describ-

ing the time-dependent increase of extracellular radioactivity (or decrease of intracellular radioactivity) in a two-compartment model with compartments of constant volume. The kinetics were found to follow the equation

$$a_t = a_\infty(1 - e^{-kt}) + a_0, \quad (1)$$

where a_0, a_t, and a_∞ denote the extracellular radioactivity at zero time, at sampling time t, and at isotopic equilibrium, respectively. Ideally, $a_0 = 0$. However, extracellular medium trapped between the packed cells in the samples for efflux experiments with the flow tube amounts to ~10–15% (Brahm, 1977), and a similar fraction of extracellular radioactivity was thus found at the start of the experiments because the distribution of tracer between the intracellular and extracellular water phases is close to unity.

The exponent k has the dimension s^{-1} and is the rate coefficient of the transport process. The rate coefficient was determined by linear regression analysis of a plot of $\ln(1 - a_t a_\infty^{-1})$ vs. t, in which $(1 - a_t a_\infty^{-1})$ expresses the fraction of tracer that remains intracellular at time t.

The rate coefficient at a constant urea concentration is directly proportional to the permeability coefficient, P:

$$P = kVA^{-1} \quad (\text{cm s}^{-1}). \quad (2)$$

VA^{-1} is the ratio between the intracellular solvent volume (cm^3) and the cell membrane area, which was assumed to be constant (1.42×10^{-6} cm^2/cell). V depends, for instance, on pH (cf. Fig. 1) and temperature. At pH 7.2 and 25°C, VA^{-1} was 4.43 (SD ± 0.19; $n = 15$) $\times 10^{-5}$ cm.

The unidirectional efflux across the red cell membrane is defined as

$$J_{uni}^{urea} = kVA^{-1}C_i = PC_i \text{ mol cm}^{-2} \text{ s}^{-1}, \quad (3)$$

where C_i is the intracellular urea concentration (mol cm^{-3}).

TEMPERATURE DEPENDENCE The temperature dependence of urea transport was calculated by linear regression analysis of the relation:

$$\ln J_{uni}^{urea} = -E_A(RT)^{-1} + \text{constant}. \quad (4)$$

E_A(J mol^{-1}) is the Arrhenius activation energy of the transport process, R is the gas constant [8.32 J (mol K)$^{-1}$], and T is the absolute temperature in Kelvin (K).

RESULTS

Variation of Urea Permeability of Cells from Different Donors

Table I shows the results of determinations of urea permeability of different donors. The experiments were performed with a urea concentration of 1 mM at 25°C and pH 7.2. The permeability coefficients varied by a factor of >2. The difference was a constant finding, because no day-to-day variation could be detected. Care was therefore taken to ensure that each series of experiments was performed with blood from the same donor.

Dependence on pH

The urea permeability of human red cells determined at 38°C in the pH range from 5.8 to 9 at a constant urea concentration of 1 mM is shown

in Fig. 1. The figure demonstrates that the permeability remained constant in the pH range under study (upper graph). The permeability is calculated by multiplication of the rate coefficient, k (s^{-1}), by the ratio between intracellular solvent volume and the membrane area (VA^{-1} [cm]; cf. the Methods section). The constant permeability coefficient is due to the fact that the increase of the rate coefficient with increasing pH (the second graph of Fig. 1) is compensated by a decrease of the cellular solvent volume by raising pH (lower graph). Experiments performed within the same pH range at 1 M urea (results not shown) also showed a constant urea permeability, which averaged 8.7×10^{-5} cm s^{-1} (range 8.0–9.2×10^{-5} cm s^{-1}).

TABLE I

Urea Permeability of Human Red Cells from Different Donors

Donor	Rate coefficient of urea self-exchange	Cellular solvent volume/cell membrane area	Permeability coefficient
	k (s^{-1})	$VA^{-1} \times 10^5$ (cm)	$P \times 10^4$ (cm s^{-1})
J.B.	6.28 (±0.12)	4.25	2.67
J.J.H.	8.70 (±0.33)	4.16	3.62
S.L.	8.77 (±0.58)	4.52	3.97
N.P.	9.04 (±0.11)	4.46	4.03
O.S.A.	9.96 (±0.32)	4.58	4.56
E.H.	10.65 (±0.41)	4.73	5.04
P.B.N.	11.50 (±0.07)	4.50	5.17
G.O.	11.90 (±0.21)	4.58	5.45
J.S.	12.95 (±0.58)	4.54	5.88

The experiments were performed at 25°C, pH 7.2, and 1 mM urea. The permeability coefficients in the table are average values of two to four self-exchange experiments carried out with the continuous flow tube technique. Standard deviations of the rate coefficients are in parentheses.

Dependence on Concentration

Fig. 2 illustrates that the rate of urea self-exchange at 38°C and pH 7.2 decreased by raising the extracellular urea concentration from 1 to 1,000 mM. In Fig. 3*A*, the urea self-exchange flux at 38°C is depicted as a function of the extracellular urea concentration and demonstrates that urea transport is a saturable process. The curve was drawn according to the assumption that urea transport can be described by simple Michaelis-Menten kinetics [$J_{uni}^{urea} = J_{uni}^{urea}(max) \times C_0^{urea}(K_{½} + C_0^{urea})^{-1}$], with a maximal urea flux, $J_{uni}^{urea}(max)$, of 1.3×10^{-7} mol cm^{-2} s^{-1}, and a half-saturation constant, $K_{½}$, defined as the cellular urea concentration at which the flux is half-maximal, of 396 mM. The half-saturation constant and the maximal urea flux were determined in the plot of Fig. 3*B* of the reciprocal of permeability vs. urea concentration. The half-saturation constant was determined by the intersection of the curve with the abscissa, and the maximal urea flux was calculated as the reciprocal of the slope of the curve.

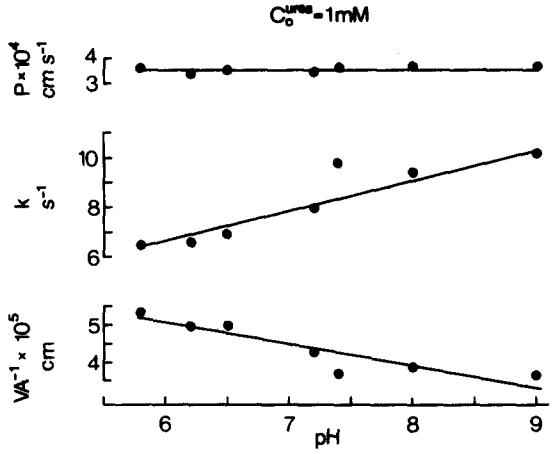

FIGURE 1. The pH dependence of urea permeability in human red cells from a single donor at 38°C and a constant urea concentration of 1 mM. The rate coefficient (k, s^{-1}, second graph), which was determined as the efflux rate of [^{14}C]urea from labeled cells into a large isotope-free extracellular medium, increased with increasing pH. The ratio between the cellular solvent volume (V, cm^3) and the membrane area (A, cm^2) decreased by raising pH (lower graph), because an increase of negative charges of intracellular buffers, mainly hemoglobin, causes a lowering of the cellular concentrations of osmotically active anions (chloride). Thus, the permeability coefficient, which equals the product $k \times VA^{-1}$, was found to be constant within the pH range studied.

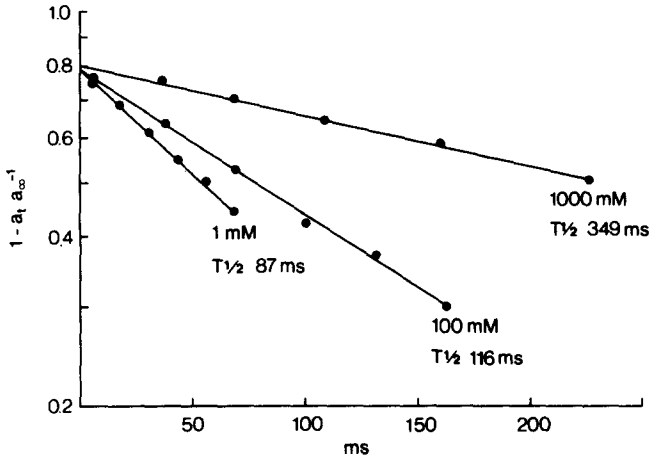

FIGURE 2. The efflux rate of labeled urea from prelabeled red cells into a large isotope-free extracellular medium comprising >99% of the total volume. By raising the urea concentration, the slope of the curves, which equals the rate coefficient (cf. the Methods section), decreased, and the half-time of urea self-exchange, $T_{½}$ (= $\ln 2 \times k^{-1}$, s), increased. The experiments were performed at 38°C and pH 7.2.

The similar treatment of results obtained at 25°C (Figs. 4*A* and *B*) showed a maximal urea flux of 8.2×10^{-8} mol cm^{-2} s^{-1} and a half-saturation constant of 334 mM. It should be noted that the data in Figs. 3 and 4 were obtained on red cells from a single donor.

Inhibition of Urea Permeability

THIOUREA It has previously been shown (Wieth et al., 1974) that urea is a competitive inhibitor of thiourea transport. Here, the inhibitory effect of thiourea on urea transport was studied. Two series of experiments were performed. At 38°C the dependence of urea permeability on

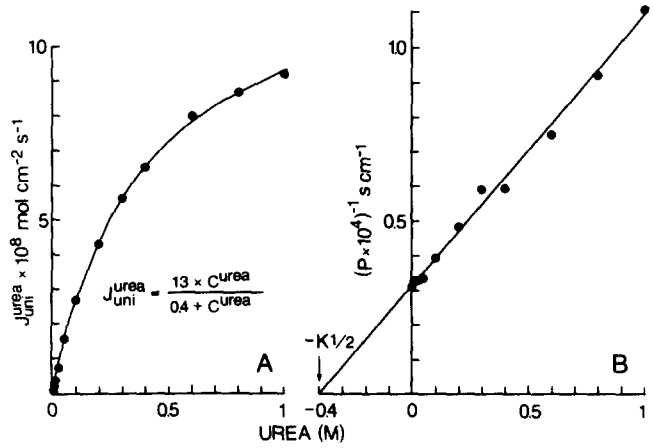

FIGURE 3. (*A*) The concentration dependence of urea transport and (*B*) the reciprocal permeability vs. urea concentration in a Hanes plot. Linear regression analysis of the results depicted in *B* gave (regression coefficient, 0.996): $(P \times 10^4)^{-1} = 0.780$ (SD ± 0.021) $C^{urea} + 0.309$ (SD ± 0.009). The half-saturation constant, $K_{½}$ (mM), defined as the cellular urea concentration at which urea transport is half-maximal, was determined from the intersection of the curve with the abscissa to be 396 mM. The maximal urea flux, which equals the reciprocal of the slope of the curve, was 1.3×10^{-7} mol cm^{-2} s^{-1}. The saturable urea transport follows Michaelis-Menten kinetics, and the curve of *A* was drawn according to the equation in the figure, using the constants obtained from *B*. All experiments were performed at 38°C and pH 7.2.

urea concentration was determined in the presence of 15 mM thiourea. The results are shown in Fig. 5. For comparison, the curve obtained in the absence of thiourea is also shown in the figure. The resulting plots of reciprocal permeability vs. urea concentration are parallel. In this type of plot, a parallel displacement of the curve by an inhibitor indicates competitive inhibition (Dixon and Webb, 1979). The intersection of the curve with the abscissa gives a value (K_p) of 800 mM, which is related to the inhibitor concentration, K_i (mM), of thiourea by: $K_p = K_{½} (C^{thiourea} K_i^{-1} +$

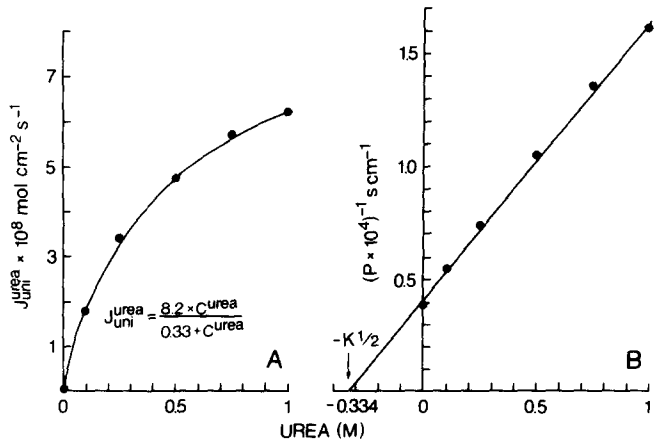

FIGURE 4. Results of urea transport determined at 25°C and pH 7.2, analyzed as described in the legend to Fig. 3. The regression analysis of results of *B* showed (regression coefficient, 0.998): $(P \times 10^4)^{-1} = 1.223$ (SD ± 0.050) $C^{urea} + 0.409$ (SD ± 0.028). $K_{½}$ was 334 mM, and the maximal urea flux was 8.2×10^{-8} mol cm^{-2} s^{-1}. The curve of *A* was drawn according to the inserted equation.

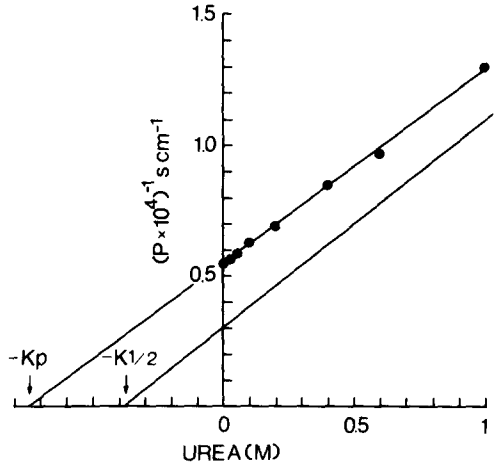

FIGURE 5. A Hanes plot depicting the reciprocal of urea permeability (38°C, pH 7.2) against the urea concentration in experiments with a fixed thiourea concentration of 15 mM. Regression analysis gave (regression coefficient, 0.999): $(P \times 10^4)^{-1} = 0.739$ (SD ± 0.015) $C^{urea} + 0.545$ (SD ± 0.007). The slope of the curve equals the slope of the curve of control experiments of Fig. 3*B*, which, for comparison, is also shown. The parallel displacement upward of the curve by the fixed thiourea concentration indicates competitive inhibition (Dixon and Webb, 1979) by thiourea on urea transport. The inhibitor constant, K_i (mM), of 17.4 mM thiourea, which is the concentration of inhibitor producing a twofold increase of the apparent $K_{½}$ for urea transport, was calculated from the relation: $K_i = C^{thiourea} (K_p K_{½}^{-1} - 1)^{-1}$, in which K_p represents the apparent increase of $K_{½}$ at a given concentration of inhibitor.

1). From this relation, the K_i of thiourea was found to be 17.4 mM. Thus, urea transport in the presence of thiourea follows the equation

$$J_{uni}^{urea} = \frac{J_{uni}^{urea}(max)}{1 + \frac{K_{1/2}}{C_0^{urea}}\left(1 + \frac{C_0^{thiourea}}{K_i}\right)} \text{ mol cm}^{-2}\text{ s}^{-1}, \quad (5)$$

where K_i is the thiourea concentration producing an apparent doubling of $K_{1/2}$ without affecting the maximal urea flux.

At 25°C the inhibition by thiourea of urea transport at a fixed urea concentration of 100 mM was measured. Fig. 6*A* shows the fractional

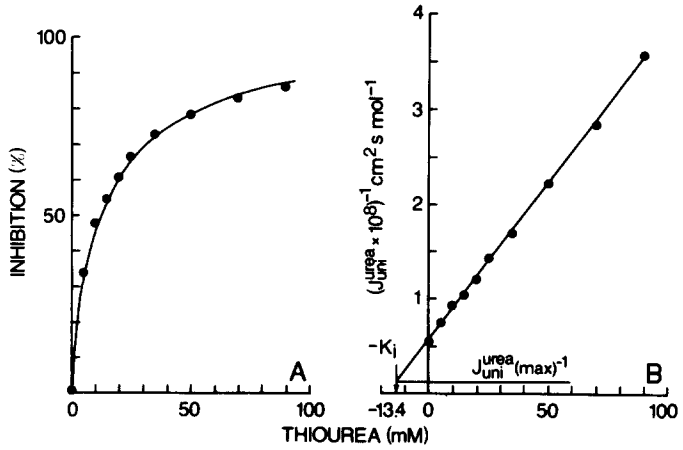

FIGURE 6. Thiourea inhibition of urea transport at 25°C (pH 7.2) and a constant urea concentration of 100 mM. *A* shows the fractional inhibition (in percent) of urea transport at 100 mM urea by increasing the thiourea concentration from 0 to 90 mM. *B* is a Dixon plot (Dixon, 1953) of the reciprocal urea transport against the thiourea concentration. Regression analysis of the curve showed: $(J_{uni}^{urea} \times 10^8)^{-1} = 0.033$ (SD ± 0.0003) $C^{thiourea}$ + 0.565 (SD ± 0.014) (regression coefficient 0.999). The inhibitor constant, K_i (mM), was determined as the intersection of the curve with the horizontal line equal to $J_{uni}^{urea}(max)^{-1}$ obtained from the slope of the curve in Fig. 4*B*. The analysis gave a K_i of 13.4 mM.

inhibition (in percent of control experiments at 100 mM urea) of urea transport as thiourea was varied from 0 to 90 mM. In Fig. 6*B* the reciprocal of urea transport is depicted as a function of the inhibitor concentration. In this plot, the inhibitor constant (K_i) is determined by the intersection point of the data curve and the horizontal curve representing the reciprocal of the maximal, uninhibited urea transport obtained from Fig. 4, in analogy with kinetic analysis applied in enzymology (Dixon, 1953; see also Dixon and Webb, 1979). The procedure revealed a K_i of 13.4 mM of thiourea.

PHLORETIN, SH-SPECIFIC REAGENTS, AND DIDS The effects of phlor-

etin, the sulfhydryl (SH)-specific reagents PCMBS, NEM, and DTNB, and the anion transport inhibitor DIDS are shown in Table II. Because urea permeability at a given concentration varies among different donors (cf. Table I), the table also includes the permeability coefficient of control experiments in each series of inhibition experiments. The inhibition by phloretin was studied at 1, 100, and 750 mM urea. Urea permeability

TABLE II

Urea Permeability in Human Red Cells at 25°C and pH 7.2

Inhibitor	$C_0^{inhibitor}$	C_0^{urea}	Permeability	Inhibition
	mM		*cm s^{-1}*	*%*
Control		1	$2.67(\pm0.05) \times 10^{-4}$	—
Phloretin	0.10	1	$8.87(\pm0.60) \times 10^{-6}$	97.7
—	0.25	1	$2.66(\pm0.13) \times 10^{-6}$	99.0
—	0.50	1	$1.48(\pm0.08) \times 10^{-6}$	99.4
Control		100	$2.06(\pm0.01) \times 10^{-4}$	—
Phloretin	0.10	100	$7.69(\pm0.87) \times 10^{-6}$	96.3
—	0.25	100	$2.93(\pm0.19) \times 10^{-6}$	98.6
—	0.50	100	$1.12(\pm0.05) \times 10^{-6}$	99.5
—	1	100	$0.77(\pm0.04) \times 10^{-6}$	99.6
Control		750	$0.73(\pm0.01) \times 10^{-4}$	—
Phloretin	0.10	750	$1.05(\pm0.01) \times 10^{-5}$	85.6
—	0.25	750	$2.22(\pm0.11) \times 10^{-6}$	97.0
—	0.50	750	$1.25(\pm0.04) \times 10^{-6}$	98.3
Control		1	$2.73(\pm0.14) \times 10^{-4}$	—
PCMBS*	1	1	$2.24(\pm0.21) \times 10^{-5}$	91.8
DIDS*	0.001	1	$2.86(\pm0.06) \times 10^{-4}$	5% increase
Control		1	$3.62(\pm0.14) \times 10^{-4}$	—
NEM*	1	1	$3.98(\pm0.02) \times 10^{-4}$	10% increase
DTNB*	1	1	$3.99(\pm0.08) \times 10^{-4}$	10% increase

In experiments with phloretin, both the incubation medium and the efflux medium contained phloretin at the concentration indicated. The asterisk denotes preincubation of the red cells with the compound for 45 min (38°C). Permeability coefficients below $\sim 2 \times 10^{-5}$ cm s^{-1} correspond to half-times of exchange >1 s, and the rate of [^{14}C]urea efflux was therefore determined by the Millipore-Swinnex filtering technique (Dalmark and Wieth, 1972). The permeability coefficients are average values of two or more experiments. Standard deviations are in parentheses.

was effectively inhibited by phloretin at all three urea concentrations, inhibition being >98% by 0.5 mM phloretin. In the presence of 1 mM phloretin, urea permeability at 100 mM urea was reduced to 8×10^{-7} cm s^{-1}, which most likely represents the "ground" permeability of human red cells to urea caused by simple diffusion through the lipid core of the membrane.

The effects of PCMBS, NEM, and DTNB, which react with SH groups in the membrane, were tested. Only PCMBS inhibited urea transport, the inhibition being ~92%, whereas the other SH-reacting compounds, NEM and DTNB, showed no inhibitory effect.

The stilbene derivative DIDS very efficiently inhibits anion transport by covalently binding to the integral membrane proteins of band 3 (nomenclature after Steck, 1974). Covalently binding of about one million DIDS molecules per cell inhibits anion transport by >99% (Wieth, 1979). Use of the same binding procedure described by Wieth (1979) did not, however, lead to any inhibitor effect on urea transport.

Self-Exchange and Net Efflux of Urea

Urea permeability, determined as the rate of tracer efflux (cf. the Methods section), was measured during self-exchange and net efflux In self-exchange experiments, the rate of tracer efflux is constant, because the intra- and extracellular chemical concentrations and the cell volume remain constant. In contrast, the osmotic disequilibrium and the progressive decrease of intracellular urea concentration during net efflux experiments induce a change of cell volume affecting the rate of tracer efflux. The experimental errors are, however, greatly reduced by measuring "initial" rates, determined by following the exit of the first 25% or less of labeled urea. This procedure gave linear washout curves in semilogarithmic plots, even at the most unfavorable experimental conditions, i.e., high intracellular urea concentrations (e.g., $T_{1/2}$ for net efflux at 500 mM [25°C] was 109.6 ms; the regression coefficient of the efflux curve was 0.999), because the time resolution of the present version of the continuous flow method is as low as 2–3 ms (Brahm, 1982, 1983*a*).

Table III shows the results of net efflux and self-exchange experiments at 25°C and pH 7.2. During both, the same permeability coefficient of $\sim 2.6 \times 10^{-4}$ cm s^{-1} was found at a urea concentration of 1 mM. In self-exchange experiments, urea permeability decreased by raising the urea concentration to 500 mM, as was also observed at 38°C. In contrast, urea permeability remained constantly high in net efflux experiments even at an initial intracellular urea concentration of 500 mM. The experiments thus show that urea permeability decreased by raising the extracellular urea concentration only.

Temperature Dependence of Urea Transport

The temperature dependence of urea transport was determined in the temperature range from 3 to 38°C (pH 7.2). The results of self-exchange experiments performed at urea concentrations of 1 mM and 1 M are shown in Fig. 7. Linear regression analysis of $\ln J_{uni}^{urea}$ vs. the reciprocal of absolute temperature (Eq. 4 in the Methods section) showed that the Arrhenius activation energy, E_A, of urea transport at 1 M was 34.3 (SD ± 0.7) kJ mol^{-1} in the whole temperature range. Experiments performed at 1 mM urea, a concentration at which the transport system is highly unsaturated, displayed an extremely low activation energy of 11.7 (SD ± 0.9) kJ mol^{-1} within the same temperature range. In four triplicate experiments performed between 15 and 38°C after maximal inhibition of urea transport by 1 mM phloretin, the activation energy of the residual

urea transport using 100 mM urea was 59.2 (SD ± 3.8) kJ mol^{-1} (data not shown).

DISCUSSION

Concentration Dependence of Urea Transport

Measurements of the unidirectional efflux of labeled urea allow determinations of urea transport over a broader concentration range than do optical measurements of cell volume change. In the present study urea transport was measured in the concentration range from 1 to 1,000 mM. The results of Fig. 3*A* show that urea transport at 38°C (pH 7.2) was not

TABLE III

Urea Permeability During Self-Exchange and Net Efflux in Human Red Cells (25°C, pH 7.2)

Type of experiment	Initial urea concentration of cells	Initial urea concentration of medium	Permeability	
			$P \times 10^4$	Range
	mM	*mM*	*cm* s^{-1}	
Exchange	1	1	2.67	2.63–2.70
Net efflux	1	0	2.70	2.69–2.70
Exchange	100	100	1.82	1.81–1.82
Net efflux*	100	0	2.56	2.50–2.62
Net efflux	100	0	2.55	2.50–2.61
Exchange	500	500	0.95	0.93–0.97
Net efflux*	500	0	2.91	2.88–2.94
Net efflux	500	0	2.43	2.27–2.59

The asterisk denotes that the net flux experiments were performed in efflux media in which urea has been replaced by sucrose. The permeability coefficients are average values of two to four experiments.

a linear function of urea concentration, as would be the case if urea were exclusively transported by simple diffusion. The curve suggests that a saturation process is operative. By using a Hanes plot (Fig. 3*B*), it was found that maximum urea flux was 1.3×10^{-7} mol cm^{-2} s^{-1} and that $K_{½}$, the urea concentration at which urea transport is half-maximal, was 396 mM. A similar kinetic analysis of results at 25°C gave a somewhat lower half-saturation constant of 334 mM and a maximal urea flux of 8.2×10^{-8} mol cm^{-2} s^{-1}. The half-saturation constant thus appears to be moderately sensitive to a temperature change from 38 to 25°C.

Comparison with Previous Studies

Previous net influx determinations by methods measuring volume changes accompanying a sudden increase of extracellular urea concentration (Sha'afi et al., 1970, 1971; Galey et al., 1973; Owen and Eyring,

1975) gave urea permeability coefficients at room temperature[1] from 2.3 to 3.1 × 10^{-4} cm s^{-1} at 0.3–0.8 M urea in the test solutions. Systematic investigations of permeability dependence on urea concentration have not been carried out, but Sha'afi et al. (1970) apparently found no difference between permeability coefficients obtained at 0.3 and 0.8 M urea in their influx studies. However, the low apparent affinity of urea for the transport system observed in the work presented here suggests

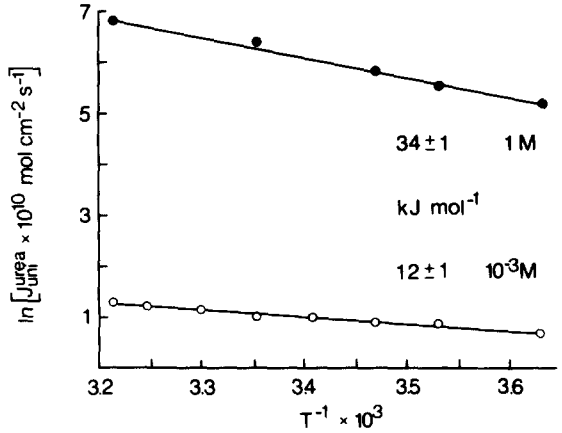

FIGURE 7. The temperature dependence of urea transport in human red cells at pH 7.2. The Arrhenius activation energy of 34 (±1) kJ mol^{-1}, using 1 M urea, decreased to 12 (±1) kJ mol^{-1} when the urea concentration was decreased to 1 mM. The extremely low apparent activation energy of urea transport at 1 mM is in part due to the fact that the relative saturation of the transport system at low urea concentrations decreased more by raising the temperature than did the relative saturation at high urea concentrations. At 1 mM the degree of saturation of the transport system decreased by 14% by raising the temperature from 25 to 38°C, whereas at 1 M the decrease was only 4% in the same temperature range.

that it is appropriate to study permeabilities over wider ranges of urea concentration. In the present study, urea permeability decreased only from 1.3 to 0.8 × 10^{-4} cm s^{-1} in the range from 0.3 to 0.8 M urea.

We note that caution should be taken in comparing absolute values of urea permeability coefficients, because interindividual differences of urea permeability were considerable, even under identical experimental conditions (cf. Table I). The somewhat lower permeability coefficient in the present self-exchange experiments therefore does not necessarily indicate that urea permeates the red cell membrane more readily in osmotic experiments. The difference may be due to the fact that the concentra-

[1] The published permeability coefficients (ω) in mol dyn^1 s^{-1} were converted to permeability coefficients in cm s^{-1} by multiplication by 2.48 × 10^{10} (erg mol^{-1}, 25°C). For comparison with the present results, the conversion also includes a correction for membrane area per cell from 1.67 to 1.42 × 10^{-6} cm^2, a value that is used in the present study.

tion-dependent self-exchange experiments in the present study were carried out with blood samples from the donor with the lowest red cell permeability to urea (donor J.B. in Table I) before it was realized that this individual had a permeability only 45% of that found for the fastest urea-transporting donor (cf. Table I).

In a tracer influx study of human red cell membrane permeability to some small nonelectrolytes, Savitz and Solomon (1971) found a urea self-exchange permeability of 2.7×10^{-4} cm s^{-1} at ~4 mM urea, which is consistent with the present findings. A variation of urea concentration was not done in their study. Recently, Mayrand and Levitt (1983) measured [^{14}C]urea efflux rates in the self-exchange mode at room temperature with a fast-flow system. They determined a $K_{½}$ of 218 mM and an extrapolated permeability coefficient at zero urea concentration of 1.16×10^{-3} cm s^{-1}. This gives a J_{max} of 2.5×10^{-7} mol cm^{-2} s^{-1}, using equations for simple Michaelis-Menten kinetics. Their $K_{½}$ of 218 mM is considerably lower than the present $K_{½}$ of 334 mM, and their J_{max} is ~3 times larger than the present result (cf. Fig. 4). It remains uncertain whether the different results of the two studies are due to variations of red cell urea permeability or to the different methods employed. The fast-flow system of Mayrand and Levitt has a lower time resolution than the continuous flow tube method, because the uninhibited efflux rates of urea are close to the upper detection limit of their fast-flow system (Mayrand and Levitt, 1983).

Inhibition of Urea Transport

THIOUREA Figs. 5 and 6*B* show that thiourea competitively inhibits urea transport and that saturation and inhibition kinetics of urea transport follow Eq. 5. At 38°C the inhibitor constant, K_i, was 17.4 mM. By lowering the temperature to 25°C, the K_i decreased to 13.4 mM. In agreement with the present findings, Solomon and Chasan (1980) recently reported that 15 mM thiourea caused half-inhibition of urea transport, and Mayrand and Levitt (1983), in urea tracer exchange experiments, found a K_i of 12 mM for thiourea.

The present study confirms and extends the observations of Wieth et al. (1974), who showed that thiourea transport in human red cells saturates ($K_{½}$ 15–20 mM at 0°C), and that the thiourea and urea compete for the same membrane site(s) involved in transport of urea and urea analogues. Solomon and Chasan (1980) have recently suggested that these same sites may also participate in the transport of the small hydrophilic amides formamide and acetamide.

PHLORETIN It has repeatedly been shown that phloretin inhibits urea permeability in human red cells (Macey and Farmer, 1970; Owen and Solomon, 1972; Kaplan et al., 1974; Owen et al., 1974; Wieth et al., 1974; Kaplan et al., 1975). Farmer and Macey (1970), Owen and Solomon (1972), and Owen et al. (1974) reported that phloretin, at concentrations above 0.25 mM, inhibited urea permeability by 66–75%. In the present

study, phloretin was much more efficient because 0.25 mM phloretin reduced urea permeability at urea concentrations between 1 and 750 mM by ≥97% (Table II). Thus, urea transport is inhibited by phloretin as efficiently as other facilitated transport processes in the red cell membrane, e.g., glucose transport and anion exchange. It is not clear why inhibition of urea permeability by 0.25 mM phloretin was lower in urea net influx experiments (Macey and Farmer, 1970; Owen and Solomon, 1972; Owen et al., 1974) than in tracer efflux studies (Wieth et al., 1974; Table II). The results of Table II show that inhibition by 0.25 mM phloretin only decreased from 99 to 97% by raising the urea concentration from 1 to 750 mM, which was the urea concentration used by Owen and Solomon (1972) and Owen et al. (1974). Hence, if phloretin is a competitive inhibitor of urea permeability, the affinity must be high and cannot account for the low degree of inhibition in the above-mentioned inhibition studies. It must be noted that a small decline of inhibition at a fixed phloretin concentration is expected because of the rise of urea concentration. The ground permeability to urea diffusing through the lipid core of the membrane remains constant and independent of variation in urea concentration, whereas the fraction of urea permeability caused by facilitated diffusion decreases with increasing urea concentration (cf. Figs. 3*B* and 4*B*). A constant ground permeability of $\sim 1 \times 10^{-6}$ cm s^{-1} therefore constitutes a larger fraction of the total permeability at the higher urea concentrations. At 1 mM urea the fraction is 0.4%, at 100 mM it is 0.5%, and at 750 mM urea the fraction is 1.4%.

It can also be ruled out that the large residual urea permeability of ~30% in volume change experiments after phloretin treatment is the result of a dual effect of phloretin, one by reducing the major fraction of urea permeability caused by facilitated diffusion, and the other by increasing the ground permeability caused by simple diffusion of urea through the lipid core of the membrane. In osmotic experiments Poznansky et al. (1976) showed that 0.25 mM phloretin enhanced the urea permeability of red cell lipid liposomes by a factor of 2–3. However, an increase of the urea permeability of the erythrocyte lipids from $\sim 1 \times 10^{-6}$ to $\sim 3 \times 10^{-6}$ cm s^{-1} is too small to account for a 30% residual urea permeability of $\sim 1 \times 10^{-4}$ cm s^{-1} in the volumetric determinations of urea permeability after phloretin inhibition. Both the low inhibition of urea permeability by >0.25 mM phloretin (Macey and Farmer, 1970; Owen and Solomon, 1972; Owen et al., 1974) and the increase of urea permeability by 0.1 mM phloretin reported by Owen et al. (1974) differ from the present results, which show a pronounced inhibition of urea permeability even at low phloretin concentrations.

SULFHYDRYL REAGENTS Urea self-exchange was inhibited ~92% by 1 mM PCMBS (Table II), a reagent reported to inhibit urea transport induced by osmotic gradients across the red cell membrane (Macey and Farmer, 1970). Both in their study and in the present investigation, the residual urea permeability after PCMBS treatment was about one order

of magnitude larger than the ground permeability obtained after maximal inhibition by phloretin. Hence, PCMBS appeared to be a less effective inhibitor than phloretin of the facilitated diffusion of urea. It is possible that the incomplete inhibition by PCMBS may be due to the incubation procedure (cf. the Methods section). In human red cells, 1 mM PCMBS added to the extracellular medium only reduced diffusional water permeability from 2.4×10^{-3} to 1.6×10^{-3} cm s^{-1}, whereas red cell ghosts exposed to PCMBS from both sides of the membrane during the incubation showed a further decrease of diffusional water permeability to a ground permeability of 1×10^{-3} cm s^{-1} (Brahm, 1982). This observation led me (Brahm, 1982) to suggest that the reactive SH groups involved in water transport may be located deeper in the membrane, or possibly closer to the inside of the membrane. In the present study, urea permeability was only determined in intact red cells, and it may be that PCMBS inhibition of urea permeability in red cell ghosts would improve on treatment from both sides of the membrane in analogy with the findings of inhibited diffusional water permeability.

It should be noted that inhibition by modification of membrane SH groups involved in urea and in water transport cannot be obtained with the SH reagents DTNB or the rapidly permeating NEM (Table II; Brahm, 1982). Therefore, it appears unlikely that SH groups are involved directly in transport of urea and of water, but that modification of SH groups with certain types of reagents, i.e., PCMBS, may lead to gross structural alterations that subsequently produce inactivation.

It must be emphasized that a similar inhibition pattern of water and urea permeability by PCMBS does not necessarily indicate that water and urea are transported by the same pathway. The fact that phloretin inhibits urea permeability efficiently while neither osmotic nor diffusional water permeability is affected by phloretin (Macey and Farmer, 1970; Table II; Brahm, 1982) clearly substantiates the concept of separate pathways for transport of water and urea (Farmer and Macey, 1970; Brahm and Wieth, 1977; Wieth and Brahm, 1977).

Temperature Dependence of Urea Permeation

It is currently accepted that hydrophilic and lipophilic nonelectrolytes permeate the human red cell membrane through different pathways. In accordance with this view, Galey et al. (1973) reported that the temperature dependence of permeation of lipophilic nonelectrolytes was >79 kJ mol^{-1}, and that of hydrophilic nonelectrolytes was <50 kJ mol^{-1}. Urea net influx permeability at 0.75 M urea showed an activation energy of 46 kJ mol^{-1} between 20 and 30°C. In the present study, the E_A of urea self-exchange at 1 M was 34 kJ mol^{-1} between 3 and 38°C (Fig. 7). At 1 mM urea the apparent activation energy of urea permeability was only 12 kJ mol^{-1}, which is even lower than the temperature dependence of urea diffusion in water of 19 kJ mol^{-1} (Horowitz and Fenichel, 1964). The extremely low E_A of urea permeability at 1 mM may in part be due to a more pronounced temperature-dependent desaturation of the urea trans-

port system at low than at high urea concentrations. The relative change of saturation by raising temperature from 25 to 38°C can be calculated from $(K_{½}^{38°C} + C^{urea})\ (K_{½}^{25°C} + C^{urea})^{-1}$. At 1 mM urea the ratio is 0.84, which indicates that the low saturation of the transport system decreases 16% by the rise of temperature. In contrast, at 1 M urea the ratio is 0.96, the degree of saturation being reduced by only 4%. Calculations show that the temperature dependence of urea permeability at 1 mM would be 23 kJ mol^{-1} if the degree of desaturation of the transport system were temperature independent. It is not clear what caused a further decrease of E_A of 11 kJ mol^{-1} by lowering the urea concentration from 1 M to 1 mM.

The concentration-independent residual urea permeability after phloretin inhibition was low (Table II) and possessed a high E_A of 59 kJ mol^{-1}, in conformity with properties of the phloretin-insensitive urea permeation through the lipid core of the membrane in red cells from duck (J. O. Wieth and J. Brahm, unpublished results) and chicken (Brahm and Wieth, 1977). Hence, the high E_A in human red cells for the small fraction of urea transport across the lipid phase of the membrane and the low E_A for the overall urea permeation dominated by transport through a different and probably hydrophilic path are consonant with the interpretation of a different activation energy for nonelectrolytes permeating hydrophilic and lipophilic pathways in human red cells (Galey et al., 1973).

It should be noted, however, that a high E_A for urea permeating a lipid phase is not a consistent finding. In spherical bilayers and liposomes of egg lecithin and liposomes of total lipid extract from human red cells, the activation energy of urea permeability varies between 39 and 60 kJ mol^{-1} (Cohen, 1975*a*; Poznansky et al., 1976), probably because of the different lipid composition of the membranes. The lower E_A of 39 kJ mol^{-1} for urea permeation in egg lecithin liposomes determined by Cohen (1975*a*) was reduced to 30 kJ mol^{-1} in dimyristoyl lecithin-dicetyl phosphate liposomes containing gramicidin A (Cohen, 1975*b*). Cohen (1975*b*) concluded that the decrease of E_A of urea permeability in liposome membranes containing gramicidin A channels indicates that the small urea molecule fits the size of the pore and that a substantial fraction of urea transport takes place through this pathway. However, the conclusion of Cohen can be questioned. Finkelstein (1974), investigating the permeability characteristics of egg lecithin-cholesterol membranes treated with gramicidin A, concluded that gramicidin A channels have a radius of 2 Å and are permeable to water but impermeable to the slightly larger urea molecule. Thus, the similar low E_A of urea permeability in artificial lipid membranes containing pores and of human red cells allows no conclusion as to the nature of the parallel and substantially more important pathway in human erythrocytes.

Asymmetry of Urea Permeation

Urea permeability under conditions of self-exchange decreased with increasing urea concentration on both sides of the membrane, whereas

urea permeability was constant in net efflux experiments with zero urea on the outside of the membrane (Table III). The constant urea permeability in net efflux cannot be caused by experimental errors leading to an increasing overestimation of the permeability coefficient by the gradual increase of the intracellular urea concentration. The continuous flow tube method has a time resolution of 2–3 ms (Brahm, 1982, 1983*a*) and is therefore particularly well suited for determination of initial rates by following the exit of [^{14}C]urea within a fraction of a half-time. Hence, the change of both the intracellular urea concentration and the cell volume was held at minimum during the short-lived observation period. The net efflux permeabilities of 100 mM urea-loaded red cells suspended in urea-free media with and without 100 mM sucrose were identical (Table III), which indicates that even the initial swelling of cells in the sucrose-free medium did not introduce any measurable error in determination of the permeability coefficient. At 500 mM intracellular urea, the swelling of cells suspended in the urea–sucrose-free electrolyte medium is significant (~15%). Accordingly, urea permeability was lower than in 500 mM urea-loaded cells suspended in the sucrose medium. However, the difference between the two sets of determinations is small and does not modify the conclusion that the concentration dependence of urea permeability is related to the urea concentration on the outside of the membrane only.

A detailed characterization of the asymmetric urea transport system in human red cells is difficult, because the affinity is low and the asymmetry is moderate. Thus, by an increase of urea concentration from 1 mM to 1 M, urea permeability decreased only four to five times to a value that is still about two orders of magnitude larger than the ground permeability. In further studies of the kinetics of asymmetry of the urea transport system, we have benefited from the fact that the facilitated transport of the urea analogue thiourea proceeds (much more slowly) by the urea transport system (Wieth et al., 1974) and shows a pronounced degree of asymmetry (J. O. Wieth and J. Brahm, unpublished results).

Is Urea Transported by Facilitated Diffusion or by Nonspecific Permeation?

It is widely accepted that the structurally inhomogenous erythrocyte membrane behaves operationally like a mosaic structure with hydrophilic and lipophilic pathways. For nonelectrolytes permeating the lipid core of the membrane, the relative membrane permeability coefficient has been related to the relative lipophilicity as a first approximation (Overton's rule; for a review, see, for example, Macey, 1979). Because small hydrophilic nonelectrolytes like urea, formamide, and acetamide show a relatively larger permeability in human red cells, it has been suggested that the deviation from Overton's rule was caused by sieving of the small hydrophilic nonelectrolytes through nonspecific pores (Goldstein and Solomon, 1960; Solomon, 1968; Sha'afi et al., 1971; Sha'afi, 1977). The existence of pores was also evidenced by the fact that osmotic water permeability (Sidel and Solomon, 1957; Rich et al., 1968) exceeded

diffusional water permeability (Paganelli and Solomon, 1957). From the ratio of 3.3 between osmotic and diffusional water permeability, Solomon (1968) calculated an operational "equivalent" pore radius of 4.5 Å. It was later suggested that the nonspecific leak pathways were formed by integral membrane proteins of band 3 (Brown et al., 1975), which by dimerization create channels with a radius of 4.5 Å, providing a common pathway in red cells for anions, cations, nonelectrolytes, and water (Solomon et al., 1982).

However, the assumption that urea is transported nonspecifically through pores that also mediate transport of water and ions is highly questionable. First, in human red cells the pathways for urea and for water that cause permeability coefficients above their respective ground permeability coefficients have been separated by means of inhibitors. PCMBS inhibits both urea net flux and self-exchange, as well as osmotic and diffusional water transport, whereas phloretin inhibits only urea transport (Macey and Farmer, 1970; Brahm, 1982; Table II). Macey and Farmer (1970), by separating transport paths for water and for a selected number of hydrophilic nonelectrolytes, including urea, concluded, "It would appear that water channels transport water and very little else." This conclusion is supported by the fact that recent determinations of osmotic and diffusional water permeability (Galey, 1978; Brahm, 1982) give a ratio of 9, which according to the equivalent pore theory gives an equivalent pore radius of 9.4 Å (Brahm, 1983*b*). From the point of view of transport physiology, this is indeed a highly unlikely average dimension of pore radius in the unmodified erythrocyte membrane. Bjerrum et al. (1980) have shown that titration of ghost membranes to pH 5.1 reversibly aggregates integral membrane proteins and increases the nonspecific leak permeability to ions and solutes, including sucrose. By applying the equivalent pore theory, Bjerrum et al. calculated an equivalent pore radius of 5.5 Å. Hence, a presumed radius of 9.4 Å in the unmodified erythrocyte membrane is not compatible with its extremely low sucrose permeability. I (Brahm, 1983*b*) alternatively suggested that the difference between osmotic and diffusional water transport might be due to single-file transport through narrow water-transporting channels that, according to the single-file theory, contain a row of 15 water molecules.

Second, a comparative study of anion, urea, and water transport in red cells from human, duck, chicken, and the giant salamander *Amphiuma means* (Wieth and Brahm, 1977) gives no support for the conclusion by Solomon et al. (1982) that there is a common pathway open for ions, nonelectrolytes, and water in red cells. All four species possess a similar high anion-exchange permeability. In human red cells, both urea and water permeability are larger than the respective ground permeability coefficients. In contrast, the permeability coefficients of chicken red cells to water and urea are as low as the permeability coefficients determined in artificial bilayer systems (Cass and Finkelstein, 1967; Finkelstein, 1976; Poznansky et al., 1976), even though the abundance of integral membrane

proteins is as large as that of human red cells, which indicates that integral membrane proteins do not necessarily induce nonspecific leaks to water and urea (Brahm and Wieth, 1977). Furthermore, duck red cells have a high water permeability and a low urea permeability, whereas *Amphiuma* erythrocytes show a high urea permeability, because of facilitated diffusion, and a low water permeability (Wieth and Brahm, 1977). These findings stress that the water-transporting channels in duck red cells are not accessible to the slightly larger urea molecule, and that the urea transport system in *Amphiuma* red cells is very tight to the smaller water molecule.

Third, the present results exclude the possibility that the relatively larger urea permeability in human red cells is caused by nonspecific diffusion through aqueous channels. Urea transport shows classic signs of facilitated diffusion. The transport process is saturable and follows simple Michaelis-Menten kinetics (Figs. 3 and 4). It can be inhibited competitively by thiourea (Figs. 5 and 6) and irreversibly by PCMBS. The efflux rate coefficient ($\propto$ permeability) decreases by raising extracellular urea concentration (decelerative *trans* effect; Table III). The temperature dependence of urea transport (Fig. 7) differs from the apparent activation energy for simple diffusion through the lipid phase of the membrane, which indicates specific interaction between the transported molecule and membrane components.

Sha'afi (1977) emphasized that saturation kinetics imply only that the number of urea molecules in the solution is larger than the number of the pores having sufficient size to permit transport of small hydrophilic molecules (and water). However, if saturation of the transport process for two analogue nonelectrolytes with similar radii and aqueous mobilities were due to a simple disparity between the number of transported molecules and the number of accessible pores, the apparent half-saturation constants would be nearly equal. This is not the case for urea and thiourea transport. Urea transport has a large $K_{1/2}$ of 334 mM (25°C), whereas the $K_{1/2}$ for thiourea transport is much lower, 15–20 mM (0°C; Wieth et al., 1974). Both nonelectrolytes are transported predominantly through the same transport pathways, as indicated by the competitive inhibition of thiourea on urea transport (Figs. 6*A* and *B*) and vice versa (Wieth et al., 1974), but at very different rates. At 0°C and 1 mM, the half-time of thiourea self-exchange was as long as 100 s (Fig. 9 of Wieth et al., 1974), whereas the half-time of urea self-exchange was only 0.2 s.

In conclusion, urea is transported by a specific facilitated diffusion process. The reactivity of the transport system to amino acid reagents suggests that the transport system, like many others, is provided by an integral membrane protein. The kinetic investigation has demonstrated a high degree of asymmetry. It is not possible to decide on the cause of this property. It could be due to single filing, to an effect of extracellular urea on a rate constant in the transport cycle, or, more likely, to the presence of a specific binding site, to which urea must bind on entering

or leaving the transport system at the extracellular side. To distinguish between these possibilities it will be necessary to perform influx experiments. It seems that the more slowly transported urea analogue thiourea will be suited to such experiments.

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