Urea and Ethylene Glycol-facilitated Transport Systems in the Human Red Cell Membrane

Saturation, Competition, and Asymmetry

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ABSTRACT   The equilibrium exchange of [^{14}C]urea and ethylene glycol was measured using a new type of fast flow system. Approximately equal volumes of saline and air were mixed to form a segmented fluid stream into which ^{14}C-loaded red cells are injected. The stream flows through three filter chambers which allow sampling of the ^{14}C in the extracellular fluid at three time points. The chambers are designed so that they do not disrupt the segmented bubble pattern. The alternating air and saline segments prevent laminar dispersion in the flowing stream and ensure good mixing at the injection and sampling sites. The equilibrium exchange of both urea and ethylene glycol showed saturation kinetics. The maximum permeability (P_o) measured in the limit of zero solute concentration is 1.6×10^{-3} cm/s for urea and 4.8×10^{-4} cm/s for ethylene glycol ($T = 23°C$). The apparent dissociation constant (K_m) was 218 mM for urea and 175 mM for ethylene glycol. The P_o for thiourea is 2.3×10^{-6} cm/s and the K_m is 19 mM. Urea and thiourea inhibit the transport of each other and the inhibition constant (K_I) is approximately equal to the K_m for both compounds. 53 other analogues of urea were screened for their inhibition of urea or thiourea transport. Several analogues [e.g., 1-(3,4-dichloro-phenyl)-2-thiourea] had a K_I in the range of 0.03 mM. The affinity of the inhibitor increased as it was made more hydrophobic. The urea analogues did not significantly inhibit the ethylene glycol or osmotic permeability. Glycerol inhibited ethylene glycol permeability with a K_I of 1,200 mM.

INTRODUCTION

In 1970, Hunter obtained evidence for saturation of urea transport in the human red cell, and Macey and Farmer showed that phloretin markedly inhibited the urea permeability without significantly altering the water permeability. Wieth et al. (1974) later demonstrated competition between thiourea

and urea and saturation of thiourea transport. These results established nearly conclusively that urea transport in the human red cell is associated with a facilitated transport system.

The main purpose of this paper is to investigate this transport system in more detail. Its saturation, asymmetry, and affinity for a large series of structural analogues will be presented. The results were obtained using a new type of fast flow system that requires <1 ml of blood for the determination of the time constant for tracer exchange. In addition, some studies of the glycerol-ethylene glycol red cell transport system will be presented. Some of these results have been reported in abstract form (Mayrand and Levitt, 1980).

In the standard "fast flow" system, cells equilibrated with a radioactive tracer of the test solute are mixed with a cell-free solution and the efflux of tracer is measured as a function of time by filtering the fluid at different distances from the mixing chamber. From the flow velocity one can determine the time it takes the cells to move from the mixing chamber to the sample site. The critical requirements of this technique are efficient mixing and minimal dispersion of the cells and associated fluid during the passage down the tube. In all previous experiments, this has required turbulent flow rates. This condition limits the permeability range that can be studied because, as the half-time of the process being studied increases, longer flow tubes and higher driving pressures are required. The system used in this paper eliminates the requirement for turbulent flow and therefore is well suited for solutes that have an intermediate permeability—too slow to be studied by the standard fast flow system and too fast to use the manual sampling technique (Dalmark and Wieth, 1972).

MATERIALS AND METHODS

Urea analogues were obtained from Pierce Chemical Co., Rockford, IL, Aldrich Chemical Co., Inc., Milwaukee, WI, and Eastman Organic, Eastman Kodak Co., Rochester, NY; ^{14}C-labeled urea, thiourea, ethylene glycol, and D-glucose were purchased from New England Nuclear, Boston, MA. The thiourea derivative of SITS was synthesized for us by Paul K. Smith of Pierce Chemical Co. by reacting the isothiocyanate derivative with ammonia. On the day of an experiment, 8 ml of fresh blood was obtained by venipuncture into heparinized tubes at the University of Minnesota Medical Center.

Standard-buffer "saline" contained 103 mM NaCl, 4 mM KCl, and 10 mM Tris titrated to pH 7.28 with HCl. When SITS or its thiourea derivative was used as an inhibitor, phosphate-buffered saline (3.9 mM Na_2HPO_4 titrated to pH 7.28) was used to avoid reaction between Tris and inhibitor. All the solutions that the cells were equilibrated or challenged with used this standard saline to which the test solute was added. This solution is hypotonic (220 mosmol) so that the results would be directly comparable to the light-scattering measurements which used the same solution (Levitt and Mlekoday, 1983). In addition, the half-time is increased in these swollen cells (larger volume to surface area ratio), which is an advantage for this fast flow system.

Cells were prepared by thrice washing 2 ml of whole blood at ~14-fold dilution (vol/vol) in saline with appropriate concentrations of nonelectrolyte. Cells were incubated with ^{14}C-labeled tracer for >10 half-lives to ensure complete equilibration before packing by a final centrifugation at 30,000 g for 7 min. Extracellular water

("trapped") was 10% as determined from $^{22}Na^+$ distribution. Most experiments were carried out at room temperature (22–26°C).

The urea and ethylene glycol exchange was determined using the fast flow system. The thiourea and D-glucose exchange was measured using the manual sampling technique of Dalmark and Wieth (1972). To slow the glucose exchange to a rate that could be conveniently measured by this technique, the exchange was measured at 10°C in the presence of 20 mM cold D-glucose. The control half-time under these conditions was 18 s.

Fast Flow System

A schematic diagram of the fast flow system is shown in Fig. 1. Dispersion of the cells is prevented by segmenting the fluid stream with air bubbles. This is achieved by mixing, at roughly equal flow rates, a saline stream and an air stream, similar to the procedure used in the "Autoanalyser" (Technicon Corp. Terrytown, NY). The streams
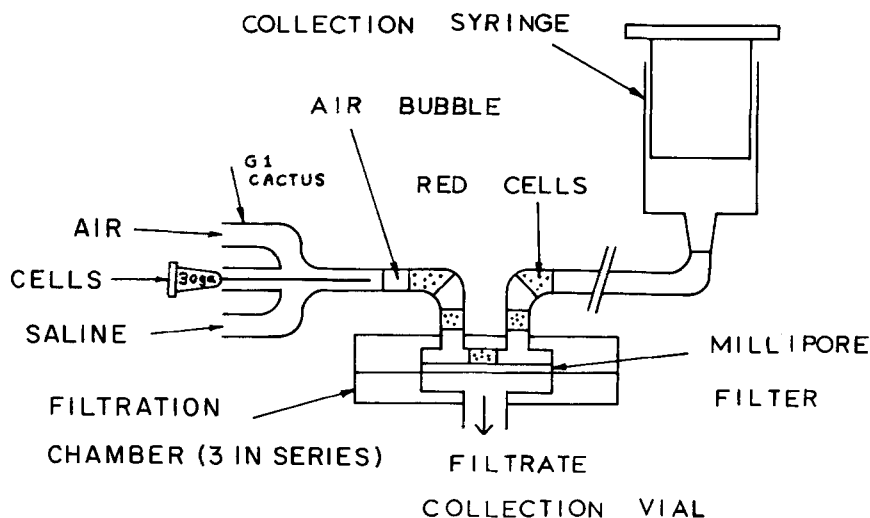

FIGURE 1.   Schematic diagram of fast flow system.

spontaneously divide into an air and fluid phase. Each fluid segment has the typical "tank tread" type of circulation that is associated with bolus flow (Prothero and Burton, 1961). The saline flow is driven by two 100-ml syringe pumps (Harvard Apparatus Co., Millis, MA) and the air flow is from a pressure tank with a flow regulator. Using a 1-ml syringe pump, packed red cells, pre-equilibrated with the test tracer, are slowly injected into the rapidly flowing segmented stream. The air bubbles not only prevent dispersion in the tubing, but also assure good mixing of the cells at the injection site, in each segment of fluid, and in the sampling chambers.

The rate of efflux of tracer from the cells is determined by the usual procedure of sampling the plasma by ultrafiltration through filters located in the wall of the fluid channel. The sampling chamber was designed to prevent a breakup of the regular bubble pattern established by the mixing chamber. Usually, three sampling chambers were connected in series. The time point associated with each sample site is determined by the flow rate and volume of tubing between the sample site and the point of addition of the red cells.

Fig. 2 is a detailed drawing of one of the filtration chambers. The filtration channel is a semicircle of radius 1.5 cm milled out of a lucite block such that the outside circumference (20 mm) is less than the diameter of the millipore filter. Three chambers are connected in series with autoanalyzer tubing (ID 0.15 cm) connected to male-male adaptors which insert into the threaded 20-ga. needles. Rubber O rings are necessary to prevent leakage. The filter holder was constructed from a 25-mm Millipore-Swinnex filter holder (SX 00 025 00; Millipore Corp., Bedford, MA).
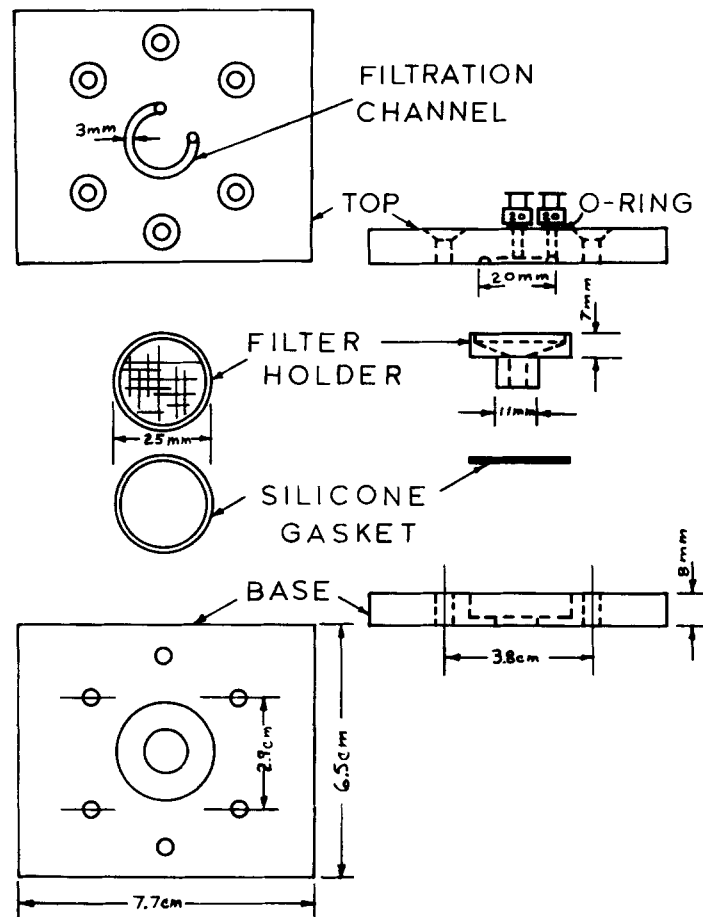
FIGURE 2.   Detailed diagram of filtration chamber.

A three-way stopcock was in the line just after the mixing chamber (not shown in Fig. 1). This made it possible to start the air flow (220–250 ml/min for ethylene glycol; 400–500 ml/min for urea), the saline flow (100 ml/min), and the blood flow (0.25 ml/min) and wait for a steady flow state to be established before switching the flow so that it passed through the sampling chambers. The duration of the filtration run was 20–30 s. The total output (air plus saline) was collected in the collection syringe to determine the total flow rate. Small upstream corrections were made for the filtered fluid (<2% of the flow). There was no detectable loss of air through the filters.

In the calculation of the transit time it was necessary to correct for the change in the volume flow of the air that resulted from the pressure fall in the system. The side (wall) pressure was measured at the inlet and outlet of each filter chamber. (This is the correct pressure to use for determining the volume of the gas.) For example, in a typical urea experiment, the total flow (determined by collection in the 200-ml outlet syringe) was 568 ml/min, of which 468 ml/min was air (atmospheric pressure = 15.7 psi). The pressure at the outlet of the first sampling chamber was 29 psi, so the air flow past this point is $468 \times 15.7/29 = 253$ ml/min. The air pressure at the entrance of the second chamber was 26 psi, which corresponds to an air flow of 282 ml/min. The transit time between these two points was determined by adding the saline flow (100 ml/min) to the average air flow and dividing by the known tubing volume. Similarly, from the pressure drop across the filters, the transit time for the filters could be determined. The flow rate up to the first filter was determined by extrapolation. In this example, the calculated sample times were 21.6, 81.3, and 128.3 ms for the three filters, respectively.

The permeability (P) for tracer exchange was determined from the equation:

$$\ln[(1 - S[t])/(1 - S[t_1])] = -(PA/V)(t - t_1) \tag{1}$$

where $S(t)$ and $S(t_1)$ are the counts per minute of filtrate/counts per minute at equilibrium (determined from the supernate in the collection syringe) for an arbitrary transit time (t) and for the transit time for the first sample (t_1), A is the cell surface area (137 μm^2), and V is the volume of cell water (81 μm^3) for cells in 220 mosmol impermeant (Levitt and Mlekoday, 1983).

The measurements were interpreted in terms of the kinetic equation derived by Regen and Tarpley (1974) for a general facilitated transport system. This equation reduces to the simple Michaelis-Menten form for the following special cases.

(a) For equilibrium exchange, where the concentration (C) of the test solute is the same on both sides, the tracer flux (J) is described by:

$$J = PC = P_oC/(1 + C/K_m) \rightarrow P = P_o/(1 + C/K_m), \tag{2}$$

where K_m is the apparent Michaelis-Menten constant, and P_o and P are the maximum and observed permeabilities, respectively.

(b) The flux (J) of a solute present in trace amounts (C) on one side of the membrane with a competitive solute present in equal concentrations (C') on both sides of the membrane is:

$$J = P'C = P_oC/(1 + C'/K_I) = >P' = P_o/(1 + C'/K_I), \tag{3}$$

where K_I is the inhibition constant for solute C'. It can be shown that $K_I = K_m$ for any competing solute.

(c) The asymmetry of the system was determined by measuring the K_I of thiourea when it was present on just one side of the membrane. Since the thiourea can be assumed to be impermeable relative to urea, the tracer flux of urea (J) can be described by:

$$J = P_oC/(1 + C^i/K_I^i); \qquad J = P_oC/(1 + C^o/K_I^o), \tag{4}$$

where K_I^i and K_I^o are the inhibition constants when thiourea is on the inside (at concentration C^i) and outside (at concentration C^o) of the cell, respectively. It can also be shown that:

$$1/K_I^i + 1/K_I^o = 1/K_I. \tag{5}$$

The K_m in Eq. 2 (or the K_I in Eqs. 3 and 4) was determined from a Hanes-Woolf plot:

$$1/P = 1/P_o + C/(P_oK_m). \qquad (6)$$

In a plot of $1/P$ vs. C, the intercept is equal to $1/P_o$ and K_m is equal to the intercept/slope. The interpretation of the thiourea data is complicated by the presence of a significant component of the permeability that does not saturate and is presumably due to simple diffusion through the lipid membrane. Thus, the flux is described by an equation of the form:

$$J = PC = P_oC/(1 + C/K_m) + P_LC, \qquad (7)$$

where P_L is the lipid membrane permeability coefficient. This equation can be
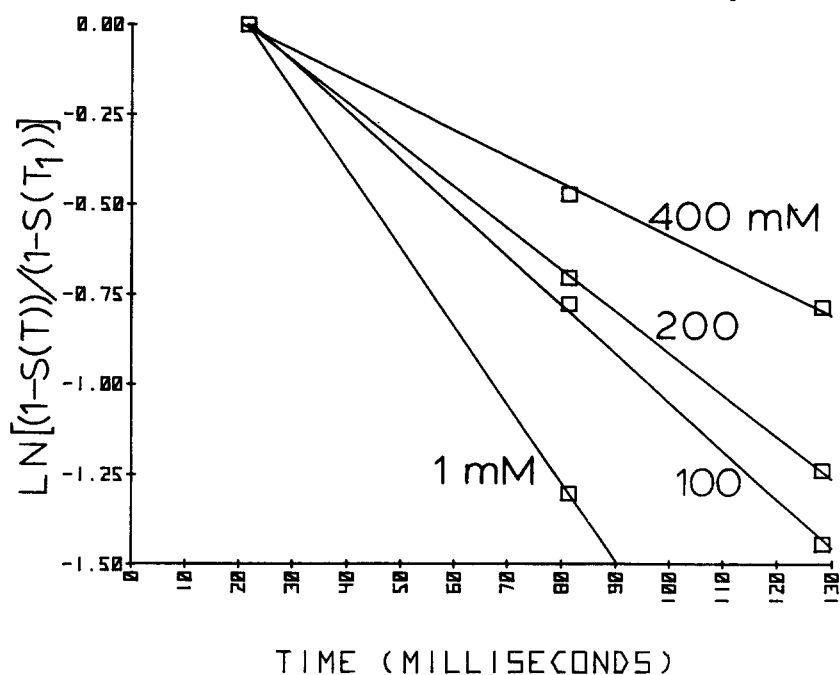
FIGURE 3.   Plot of the left-hand side of Eq. 1 vs. time. This shows the rate of efflux of [14C]urea from red cells that have been pre-equilibrated with 1, 100, 200, or 400 mM urea (in standard saline) and then mixed at $t = 0$ with a cell-free solution with the same urea concentration. The urea permeability is determined from the slope of the line.

rewritten in the form:

$$C/[P(0) - P] = K_m/P_o + C/P_o, \qquad (8)$$

where $P(0)$ is the permeability in the limit of zero thiourea concentration $(P_o + P_L)$. The value of K_m was determined from a plot of the left-hand side of Eq. 8 vs. C.

RESULTS

Urea System

Fig. 3 shows a typical plot of the left-hand side of Eq. 1 vs. time for the [14C]-urea efflux experiments. The permeability is determined from the slope of

the line. The average urea permeability in the limit of zero urea concentration (P_o) was 1.16×10^{-3} cm/s (Table I). Fig. 4 shows a Hanes-Woolf plot of P^{-1} vs. C (Eq. 6). The data fit a straight line with a Michaelis-Menten constant (K_m) of 218 mM. There is no net flux of urea in these experiments because the cells were equilibrated in the same urea solution that was used for the saline stream of the fast flow system.

The accurate fit of the data to the Michaelis-Menten equation provides a good test of the fast flow system. For example, poor initial mixing of the cells should produce an error whose relative importance is a maximum at the highest permeability and should produce deviations from a straight line at
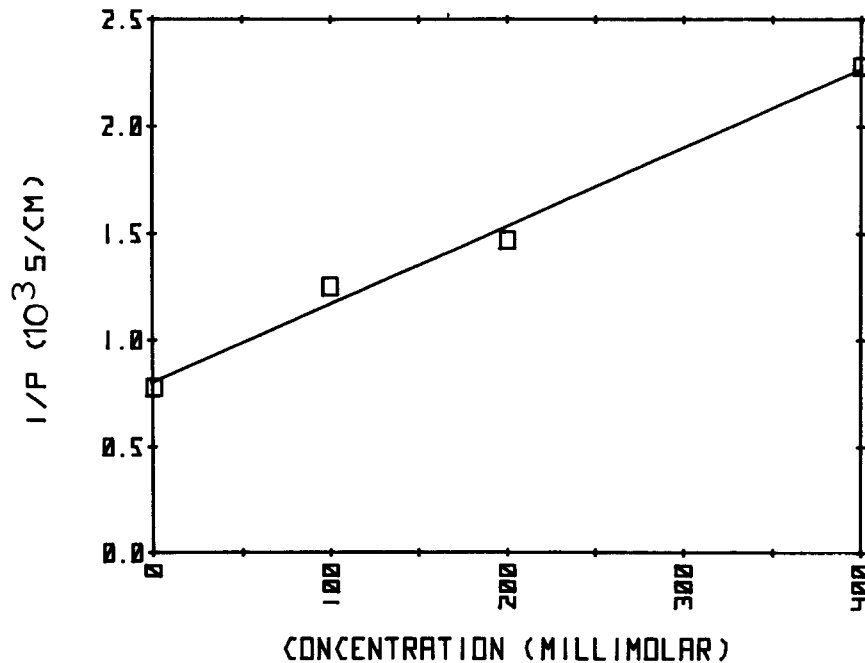
FIGURE 4.   Hanes-Woolf plot of $1/P$ vs. concentration. The intercept is $1/P_o$ and K_m is the intercept/slope.

the low concentrations in Fig. 4. Similarly, regions of poor mixing in the sampling chamber or errors in determination of the sampling time should also produce deviations from the straight line in Figs. 3 and 4. As another test of the system, the ethylene glycol permeability was measured at two different saline flow rates (45 and 100 ml/min). If there were problems with mixing or sampling, the error might vary with the saline flow rate. The permeability was not significantly different at the two flows.

Fig. 5 shows the urea flux in the presence and absence of a high-affinity inhibitor (5 mM phenylthiourea). The absolute value of the log $(1 - S)$ is plotted in Fig. 5 (not normalized for the value of the first sample point as in Fig. 3). It can be seen that over the time scale of Fig. 5 (125 ms) the urea flux is almost completely inhibited by the phenylthiourea. From the zero-time

intercept of the inhibited data, the value of S at $t = 0$ can be obtained. This should correspond to the fraction of tracer that is extracellular in the packed cells. The experimental value is 8%, in good agreement with the value of 10% estimated from ^{22}Na trapping in the packed cells. The times of the sample points in Fig. 5 were determined from the volume between the injection sample site and the flow rate (corrected for variations in pressure). If these times are correct, the uninhibited urea data should intercept the inhibited data at $t = 0$. The curves actually intercept at -3.5 ms. This error is negligible relative to the time constant of the urea exchange (minimum half-times of
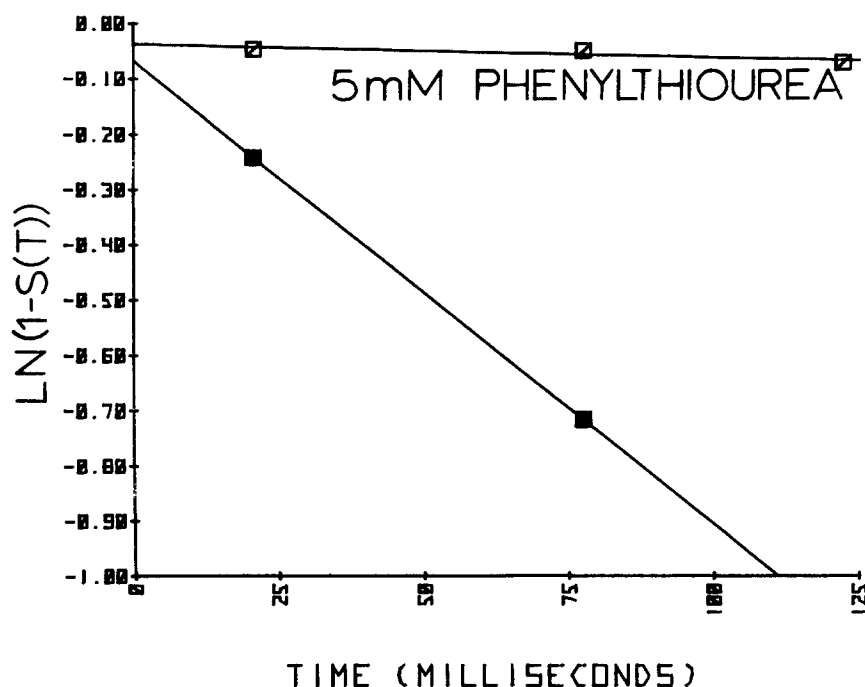
FIGURE 5. Comparison of [^{14}C]urea efflux in presence (□) and absence (■) of an inhibitor of red cell urea transport (5 mM phenylthiourea).

~37 ms). The agreement between these two times shows that the initial mixing is fast. For example, if it took 5 ms for the cells to become dispersed in the saline, the curves should intercept at +5 ms.

The permeability and Michaelis-Menten constant for [^{14}C]thiourea was determined using the manual filtration system of Dalmark and Wieth (1972). The value of P_0 was 2.3 × 10^{-6} cm/s and K_m was 19 mM (Table I). The nonsaturable thiourea permeability (P_L; Eq. 7) was 1.1 × 10^{-6} cm/s.

The mutual inhibition (K_I) of thiourea and urea was also measured. If thiourea and urea are using the same transport system, then K_m should be equal to K_I (Regen and Tarpley, 1974). The results are summarized in Table I. As predicted, the K_m and K_I are not significantly different ($P > 0.05$). These

values of K_m and K_I for thiourea are similar to the value of 15 mM recently reported by Solomon and Chasan (1980).

The asymmetry of the urea system was studied by measuring the K_I of thiourea for the two sides of the membrane. Cells were equilibrated with various concentrations of thiourea and the tracer [^{14}C]urea efflux was measured when the cells were added to a stream of saline containing zero thiourea. The thiourea permeability is low enough that there was no significant change in the cell thiourea concentration during the urea efflux measurements. This experimental arrangement provides a measure of the K_I of thiourea on the inside of the cell membrane. Similarly, the K_I of thiourea on the outside of the cell was determined from experiments where cells containing zero thiourea were mixed with saline containing various thiourea concentrations. The results show that the urea system is asymmetric, with a K_I of 12 mM on the outside and 35 mM on the inside. As described by Eq. 5, one should be able to predict the K_I from these values when there are equal concentrations of thiourea on the two sides of the membrane. The predicted value is 9 mM,

TABLE I

PERMEABILITY (P_o) AND DISSOCIATION CONSTANT $(K_m$ AND $K_I)$ OF THE UREA AND ETHYLENE GLYCOL TRANSPORT SYSTEMS

	P_o	K_m	K_I
	cm/s	*mM*	*mM*
Urea	1.16 ± 0.05 (SEM) $\times 10^{-3}$	218 ± 29	175
	$(N = 28)$	$(N = 28)$	$(N = 1)$
Thiourea	$2.3 \pm 0.16 \times 10^{-6}$	19 ± 5.3	12 ± 1
	$(N = 4)$	$(N = 4)$	$(N = 3)$
Ethylene glycol	$4.8 \pm 0.2 \times 10^{-4}$	175 ± 22	
	$(N = 6)$	$(N = 6)$	

which is not significantly different from the experimental value of 12 mM (Table I).

A large series of urea analogues were screened for their ability to inhibit the urea transport system. One purpose of these experiments was to try to find a high-affinity analogue that could be used as a specific inhibitor of urea transport and possibly as an affinity label for the transport protein. The results are summarized in Table II.

Ethylene Glycol Transport System

The rate of ethylene glycol tracer exchange was measured using the fast flow system. The permeability in the low concentration limit was 4.8×10^{-4} cm/s and the K_m was 175 mM (Table I). Glycerol inhibited ethylene glycol exchange with a K_I of 1,200 mM.

Specificity of the Urea Analogues

The high-affinity urea analogues of Table II were tested for their effects on other transport systems. Table III shows the effect of these analogues on the

TABLE II

INHIBITION OF THE UREA TRANSPORT SYSTEM BY UREA ANALOGUES

Name	Structure	K_i
		mM
(1) Formamide	$$\underset{\text{H—C—NH}_2}{\overset{\displaystyle O}{\overset{\|}{}}}$$	100
(2) Acetamide	$$\underset{\text{H}_3\text{C—C—NH}_2}{\overset{\displaystyle O}{\overset{\|}{}}}$$	100
(3) Proprionamide	$$\underset{\text{H}_3\text{C—CH}_2\text{—C—NH}_2}{\overset{\displaystyle O}{\overset{\|}{}}}$$	30
(4) Valeramide	$$\underset{\text{H}_3\text{C—CH}_2\text{—CH}_2\text{—CH}_2\text{—C—NH}_2}{\overset{\displaystyle O}{\overset{\|}{}}}$$	20
(5) Hexanamide	$$\underset{\text{H}_3\text{C—CH}_2\text{—CH}_2\text{—CH}_2\text{—CH}_2\text{—C—NH}_2}{\overset{\displaystyle O}{\overset{\|}{}}}$$	5
(6) N-methylformamide	$$\underset{\text{H}_3\text{C—NH—C—H}}{\overset{\displaystyle O}{\overset{\|}{}}}$$	100
(7) Methylurea	$$\underset{\text{H}_3\text{C—NH—C—NH}_2}{\overset{\displaystyle O}{\overset{\|}{}}}$$	100
(8) N,N'-dimethylurea	$$\underset{\text{H}_3\text{C—NH—C—NH—CH}_3}{\overset{\displaystyle O}{\overset{\|}{}}}$$	0.8
(9) N,N'-dimethylthiourea	$$\underset{\text{H}_3\text{C—NH—C—NH—CH}_3}{\overset{\displaystyle S}{\overset{\|}{}}}$$	0.3
(10) Butylurea	$$\underset{\text{H}_3\text{C—CH}_2\text{—CH}_2\text{—CH}_2\text{—NH—C—NH}_2}{\overset{\displaystyle O}{\overset{\|}{}}}$$	25
(11) Acrylamide	$$\underset{\text{H}_2\text{C=CH—C—NH}_2}{\overset{\displaystyle O}{\overset{\|}{}}}$$	100
(12) Malonamide	$$\underset{\text{H}_2\text{N—C—CH}_2\text{—C—NH}_2}{\overset{\displaystyle O \quad\quad O}{\overset{\| \quad\quad \|}{}}}$$	500
(13) Biuret	$$\underset{\text{H}_2\text{N—C—N—C—NH}_2}{\overset{\displaystyle O \;\; H \;\; O}{\overset{\| \quad\quad \|}{}}}$$	20
(14) Chloroacetamide	$$\underset{\text{Cl—CH}_2\text{—C—NH}_2}{\overset{\displaystyle O}{\overset{\|}{}}}$$	3
(15) Dichloracetamide	$$\underset{\underset{\text{Cl}}{\overset{\|}{\text{H—C—C—NH}_2}}}{\overset{\displaystyle \text{Cl} \;\; O}{\overset{\| \;\; \|}{}}}$$	15
(16) Fluoroacetamide	$$\underset{\text{F—CH}_2\text{—C—NH}_2}{\overset{\displaystyle O}{\overset{\|}{}}}$$	15
(17) Iodoacetamide	$$\underset{\text{I—CH}_2\text{—C—NH}_2}{\overset{\displaystyle O}{\overset{\|}{}}}$$	12
(18) Cyanoacetamide	$$\underset{\text{N≡C—C—NH}_2}{\overset{\displaystyle O}{\overset{\|}{}}}$$	2
(19) Thioacetamide	$$\underset{\text{H}_3\text{C—C—NH}_2}{\overset{\displaystyle S}{\overset{\|}{}}}$$	7
(20) Acetylthiourea	$$\underset{\text{H}_3\text{C—C—NH—C—NH}_2}{\overset{\displaystyle O \quad\quad S}{\overset{\| \quad\quad \|}{}}}$$	20

TABLE II—*continued*
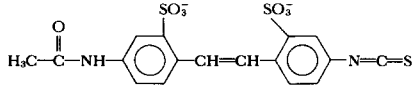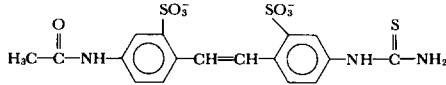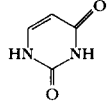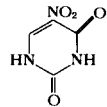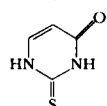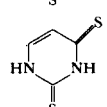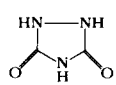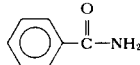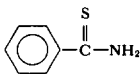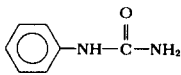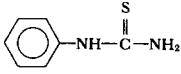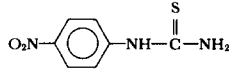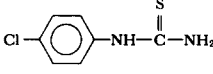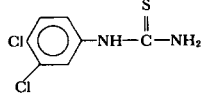
Name	Structure	K_1
(21) SITS		3
(22) Thio-SITS		4
(23) Uracil		No effect at 10 mM
(24) Nitrouracil		90
(25) Thiouracil		2
(26) Dithiouracil		5
(27) Urazole		30
(28) Benzamide		10
(29) Thiobenzamide		1
(30) Phenylurea		1
(31) Phenylthiourea		0.4
(32) 1-(*p*-Nitrophenyl)-2-thiourea		0.03
(33) *p*-Chloro-phenyl-thiourea		0.1
(34) 1-(3,4-Dichloro-phenyl)-2-thiourea		0.01

T A B L E I I—*continued*
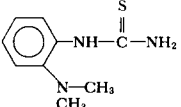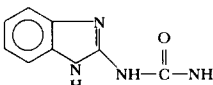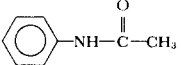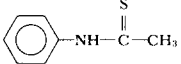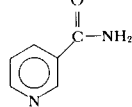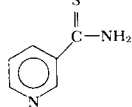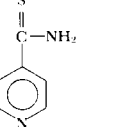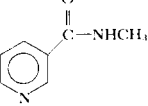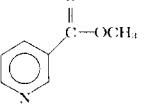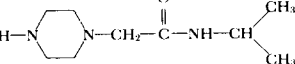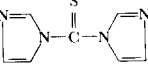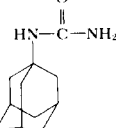
Name	Structure	K_1
(35) Dimethylamino-phenylthiourea		0.1
(36) 2-Benzimidazole urea		0.05
(37) Acetanilide		4
(38) Thioacetanilide		1
(39) Nicotinamide		2
(40) Thionicotinamide		0.1
(41) Thioisonicotinamide		0.3
(42) N-Methyl-nicotinamide		3
(43) Methylnicotinate		20
(44) N-Isopropyl-1-piperazineacetamide		70
(45) 1,1'-thiocarbonyl-diimidazole		3
(46) Adamantyl urea		10

TABLE II—*continued*

Name	Structure	K_I
(47) Ethylene glycol	HO—CH₂—CH₂—OH	No effect at 300 mM
(48) Carbamyl phosphate	H₂NC—O— P —O⁻ (with O and O double bonds, O⁻)	No effect at 35 mM
(49) ʟ-Alanine	H₃C—C—COO⁻ with H and +NH₃	No effect at 100 mM
(50) Oxamic acid	H₂N—C—COO⁻ (O double bond)	No effect at 100 mM
(51) ACES	H₂N—C—CH₂—NH—CH₂—CH₂—SO₃⁻ (O double bond)	No effect at 100 mM
(52) Allylthiourea	H₂C=CH—CH₂—NH—C—NH₂ (S double bond)	30
(53) Methylallyl-methylthiourea	H₂C=C—CH₂—NH—C—N with CH₃, CH₃, S, H	0.5

The inhibition constant (K_I) was determined from the inhibition of either urea or thiourea transport. For urea, K_I was estimated from Eq. 3. For thiourea, K_I was estimated either from Eq. 8 or by estimating the lipid permeability from the residual flux after the addition of 2 mM of the inhibitor thionicotinamide. All three approaches gave similar results.

TABLE III

INHIBITION OF GLUCOSE EXCHANGE BY UREA TRANSPORT
INHIBITORS

Compound	Concentration	Percent inhibition
	mM	
Methylallyl-methylthiourea	10	44.4
Benzimidazole urea	1	83.8
Dichloro-phenyl-thiourea	0.1	24.3
Thionicotinamide	1	16.8
Phenyl thiourea	5	40
p-Nitrophenyl thiourea	0.5	10
Dimethyl thiourea	10	0
Thiourea	200	40

D-glucose exchange rate. Most of the analogues were inhibitory. The most potent was 1 mM benzimidazole urea, which inhibited glucose exchange by 83%. Since this represents a concentration that is 30 times the K_I for urea transport, this substance can still be regarded as a relatively specific inhibitor of urea transport. This inhibition of glucose exchange is probably a nonspecific effect similar to that previously shown for benzyl alcohol (Lacko et al., 1978) and local anesthetics with similar structure (Lacko et al., 1977).

The effect of thionicotinamide on the ethylene glycol permeability and the hydraulic permeability of water was measured using the light-scattering technique (Mlekoday et al., 1983; Levitt and Mlekoday, 1983). Thionicotinamide at a concentration of 1 mM had no detectable effect on either the ethylene glycol or hydraulic water permeability.

DISCUSSION

The fast flow system described here is simple, accurate, and requires <1 ml of blood to determine the time course of tracer efflux. Its major advantage is that it does not depend on turbulence to prevent axial dispersion. This means that it is not necessary to maintain high linear flow rates and the sampling time can be adjusted simply by adjusting the rates of flow of the saline and air. The major disadvantage of this system is that the maximum flow rate is limited by the Harvard syringe pump used to deliver the saline. In our system, the uninhibited urea flux is about the maximum permeability that can be measured. The system is not fast enough, for example, to conveniently measure the tracer permeability of water. This limitation could be overcome by using higher-capacity pumps or a pressure reservoir system as is commonly used in fast flow systems (Brahm and Wieth, 1977).

Thiourea and urea are probably using the same transport system and competing for the same transport site since they mutually inhibit each other and $K_I = K_m$ (Table I). The maximum permeability of thiourea is ~500 times smaller than that of urea, whereas the affinity of thiourea for the binding site (K_m) is ~10 times greater than that of urea. This reciprocal relationship is suggestive of the "destabilization" effect in enzyme catalysis (Jencks, 1975), where part of the binding energy is used to increase the rate of enzyme turnover. For example, if urea binding forced a conformational change in the transport protein that increased the rate of transition to the state in which the urea was exposed to the opposite side of the membrane (Bowman and Levitt, 1977), then urea would have a low affinity and high transport rate. In contrast, if thiourea bound to the unperturbed site, it would have a higher affinity, but a lower transport rate.

Because of the relatively low permeability of thiourea, it can be measured using the simple manual sampling procedure of Dalmark and Wieth (1972). Because of the simplicity of this measurement, it is the method of choice for screening a series of analogues for their affinity for the urea transport system. Although the accuracy of the thiourea measurement is somewhat limited because it is necessary to correct for the relatively large non-inhibitable lipid component, this error is not important for screening studies.

These results confirm the existence of a facilitated transport system for urea. It becomes saturated at high urea concentrations and is competitively inhibited by a large number of urea analogues. Although Hunter (1970) and Canelli et al. (1974) were able to show saturation of net urea flux using a light-scattering technique, the data could not be accurately quantitated. Wieth et al. (1974) found a K_I of urea for thiourea exchange of 100 mM at 0°C. This is similar to the K_I (175 mM) and K_m (218 mM) at 25°C given in this paper. As has been shown previously for the glucose (Bloch, 1974) and uridine (Cabantchik and Ginsburg, 1977) red cell transport systems, the urea transport system is asymmetric.

Glycerol is transported by a facilitated transport system whose kinetics are quite complicated and not well understood (Stein and Danielli, 1956; Carlsen and Wieth, 1976; Deuticke, 1977). Although the kinetics may not be rigorously

described by the Michaelis-Menten equation, the data can be empirically described in terms of a K_m or a K_I. For the human red cell, Stein and Danielli found a K_m of ~1 M for net glycerol flux using light-scattering measurements. Carlsen and Wieth (1976) and Deuticke (1977) found an "equivalent" K_m of ~1 M for the tracer exchange of glycerol. Although ethylene glycol and 1,3-propanediol inhibit the glycerol permeability, it has not been clear whether this represents competition for the transport site or some nonspecific effect (Deuticke, 1977). It is shown in this paper that glycerol inhibits ethylene glycol transport with a K_I of ~1.2 M. The good agreement between this K_I and the previously determined value of K_m for glycerol suggests that glycerol and ethylene glycol are competing for the same transport site. This conclusion is also supported by the observation that Cu^{++}, which is known to be a specific inhibitor of glycerol transport, also inhibits ethylene glycol permeability (Levitt and Mlekoday, 1983). This system is clearly distinct from the urea transport system because thionicotinamide, a high-affinity inhibitor of urea transport, had no significant effect on the ethylene glycol permeability.

An analysis of the relation between structure and affinity of the analogues listed in Table II suggests the following generalizations about the urea binding site.

(a) The affinity increases as the side groups become more hydrophobic. This is illustrated by the effect of increasing the length of the hydrocarbon chain (analogues 1-5) or by adding a hydrocarbon ring structure. In general, the affinity of the analogue increased as its water solubility decreased. This suggests that there is a hydrophobic region at the binding site.

(b) The substitution of a sulfur for an oxygen in the amide group increases the affinity by a factor ranging from ~3 (e.g., analogues 8 and 9, 30 and 31, and 37 and 38) up to 20 (analogues 39 and 40).

(c) The analogues with the highest affinity (32-36) have the following structure:

$$\overset{\displaystyle H}{\underset{\displaystyle |}{}} \quad \overset{\displaystyle S}{\underset{\displaystyle \|}{}}$$
$$R\text{—}N\text{—}C\text{—}NH_2,$$
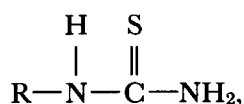
where R is an electron withdrawing group. As is illustrated by analogues 31, 33, and 34, the affinity increases as the electron withdrawing potential of the R group increases. This suggests that it is the N-H group proximal to R (rather than the terminal NH_2) that is responsible for the high affinity, possibly through the association of the hydrogen with a basic group at the binding site. The relative high affinity of dimethylthiourea, thiouracil, and thioacetanilide indicate that the terminal NH_2 group is not required for binding. The affinity of analogue 45 shows that even the NH group is not required.

(d) Charged analogues (48-52), in general, have a very low affinity. SITS and its thio derivative (21 and 22) are exceptions.

The permeability of urea and ethylene glycol obtained with this system is significantly larger than the previously reported values determined with a fast

flow system. Savitz and Soloman (1971) found a P_o for urea of 3.9×10^{-4} cm/ s for urea and 1.8×10^{-4} cm/s for ethylene glycol. In a preliminary report, Wieth et al. (1974) found a P_o of 3.5×10^{-4} cm/s for urea. (These values have been corrected for the slightly different surface area to volume ratio used in this paper.) Our values are about three times larger. The explanation for this difference is not clear. The earlier measurements were obtained using the standard fast flow system and it is possible that the flow rates used for these solutes were not high enough to establish turbulent flow. If, instead, the flow is laminar, then the cells at different positions in the flow tube will have different transit times and calculations made using the fluid sampled at the wall of the flow tube will be erroneous. Recently, Oberghaus et al. (1982) used a different procedure for determining P_f based on the measurement of tracer diffusion through a packed cell column. Their value for ethylene glycol is 3.3×10^{-4} cm/s, which is close to our value of 4.8×10^{-4}.

About one-third of the thiourea permeability is not inhibitable by urea analogues. This non-inhibitable permeability (1.1×10^{-6} cm/s) is within the range of the lipid bilayer permeability (0.9–50×10^{-6} cm/s, depending on the membrane lipid) reported by Gallucci et al. (1972). This confirms the suggestion by Wieth et al. (1974) that this residual permeability represents the basal permeability of the red cell membrane lipids.

Macey (1982) has pointed out that red cells need a facilitated transport system for urea because they must pass through regions of very high urea concentration in the kidney medulla. If they did not have this system they would tend to become loaded with urea in the medulla (because of their residual lipid permeability) and then could undergo osmotic hemolysis when they left the medulla and came in contact with normal plasma. In addition, the red cells would tend to wash out the urea gradient in the kidney, reducing its concentrating ability. To test these ideas, it would be desirable to have a specific inhibitor of red cell urea transport that could be infused into the whole animal. Unfortunately, although some of the urea analogues have a K_I in the range of 0.02 mM, it is difficult to raise the blood concentration to an inhibitory level because the analogues cannot be infused at a high enough concentration due to their limited water solubility. To be pharmacologically useful, these inhibitors must be made more water soluble.

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