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ARTICLE

Urea and water are transported through different pathways in the red blood cell membrane

Jesper Brahm[1], Morten Hanefeld Dziegiel[2,3], and Jonas Leifelt[1]

Several studies of the urea transporter UT-B expressed in *Xenopus* oocytes and in genetically modified red blood cells (RBC) have concluded that UT-B also transports water. In the present study, we use unmodified RBC to test that conclusion. We find that the permeability of urea, P_u (cm/s), has a 10-fold donor variation, while the diffusional water permeability, P_d (cm/s), remains unchanged. Additionally, we observe that phloretin inhibits P_u but not P_d, and that the time course of maximum *p*-chloromercuribenzosulfonate inhibition of P_u and P_d differs—P_u inhibition takes <2 min, whereas P_d inhibition requires ≥1 h of incubation. The findings in the present study are in line with a previous comparative study using unmodified RBC from four animals and a solvent drag study using human RBC, and they lead us to reject the conclusion that the UT-B transporter represents a common pathway for both solutes.

Introduction

It has been a long-lasting discussion whether water and some solutes may permeate the red blood cell (RBC) membrane by a common pathway, initiated by the equivalent pore theory (EPT) by Solomon (1968). The EPT was turned down in transport physiological studies (see, e.g., Macey, 1984; Galey and Brahm, 1985), and further support against EPT came from the identification of a series of transporters, including the anion exchanger AE1, the water transporting aquaporin AQP1, and the urea transporter UT-B. Several studies have settled that AQP1 in human RBC transports water and very little else of solutes, and probably CO_2 (see, e.g., Macey, 1984; Agre and Kozono, 2003; Kitchen et al., 2019; Endeward et al., 2006). Regarding UT-B, the specificity of the transporter has been challenged in several studies and the UT-B has even been suggested to be categorized as a water channel (Azouzi et al., 2013). A common basis for the conclusion that UT-B transports water is studies of genetically modified RBC or expression of the UT-B in *Xenopus* oocytes. We have taken another approach as we used unmodified RBC in the transport physiological studies of water and urea. Our results support neither the concept of UT-B as a common pathway for urea and water as suggested by Yang and Verkman (1998, 2002) nor that UT-B should be considered a water channel.

Materials and methods

Materials

Media

All media (in mM) were prepared from reagent grade chemicals: Medium A: 150 KCl and 2 KH_2PO_4, pH 7.2; Medium B: Medium A with 1 urea or thiourea, pH 7.2.

Inhibitors

Phloretin and *p*-chloromercuribenzosulfonate (PCMBS, Sigma-Aldrich Chemie, GmbH) were dissolved respectively in ethanol and water to obtain 50 mM stock solutions that were added to the media to obtain the final concentrations.

Preparation of RBCs

Blood was freshly drawn, obtained with consent either from healthy persons or from discharged, not outdated blood units from the Blood Bank, Copenhagen University Hospital. The bank blood was stored in a SAGM medium (salt, adenine, glucose, mannitol) with CPD (citrate, phosphate, dextrose) anticoagulant. The blood was washed once in Medium A or Medium B containing the urea or thiourea concentration concerned. The buffy coat was removed in the freshly drawn samples, and the cell suspension was next titrated to pH 7.2 at 0°C or 25°C. The cells were subsequently washed at least three times in the appropriate medium.

Cell water content and radioactivity

The cell's water content in RBC was determined by drying ≈300 mg packed RBCs at 105°C for 24 h. The packing was carried out in slender 0.8-ml nylon tubes that were centrifuged for 15 min at ≈40,000 *g* (Sorvall RC-5 centrifuge; DuPont Instruments-Sorvall Biomedical Division). A 2% correction was carried out for extracellularly trapped water. The determination of cell solids ensures the relationship that 1 g of cell solids equals 4.4×10^4 cm^2 membrane area (for details see, e.g., Brahm, 1982).

[1]Department of Cellular and Molecular Medicine, The Faculty of Health, University of Copenhagen, Copenhagen, Denmark; [2]Department of Clinical Medicine, Copenhagen University Hospital, Copenhagen, Denmark; [3]Department of Clinical Immunology, Copenhagen University Hospital (Rigshospitalet), Copenhagen, Denmark.

Correspondence to Jesper Brahm: jbrahm@sund.ku.dk.

The radioactivity of the cells and the supernatant was determined by liquid β-scintillation spectrometry after precipitation with 7% (vol/vol) perchloric acid. The radioactivity in cell-free filtrates and centrifuged samples from the efflux experiments was measured without preceding precipitation.

Cells for efflux experiments

After the last wash, the cells were resuspended to a hematocrit of ≈60%, and a radioactive isotope (^{14}C-urea, ^{14}C-thiourea, or $^{3}H_2O$, Perkin-Elmer) was added to obtain an activity of ≈18 kBq (0.5 μCurie) per ml cell suspension. Finally, the cells were centrifuged (see above) in slender 0.8 ml nylon tubes for efflux experiments with the Millipore-Swinnex filtering method (Dalmark and Wieth, 1972) or 8 ml tubes for efflux experiments with the continuous flow tube method (Brahm, 1977).

Flux experiments

All experiments were carried out under conditions of self-exchange (SE), where the net flux of the solute is zero. The distribution ratio, $r = C_{solute,i}/C_{solute,o}$, of urea, thiourea, and water is ≈1. Depending on the rate of solute tracer efflux, we used either the continuous flow method or the Millipore-Swinnex filtering method.

Continuous flow tube method

Uninhibited ^{14}C-urea, ^{14}C-thiourea, and $^{3}H_2O$ efflux have half times in the millisecond range. The continuous flow tube technique with a time resolution of 1–2 ms is suitable for use in these experiments. In short, 0.7–1 ml of packed RBCs with the labeled compound that is stored in 1 ml Manteaux syringes is continuously injected with constant velocity into a mixing chamber and mixed with ≈375 ml of efflux medium that is also injected at constant velocity. The mixing initiates an efflux of radioactive tracer from the cells to the medium that continues during the flow of the dilute suspension (hematocrit [hct] <0.3%) along a tube connected to the mixing chamber. Since the flow of the suspension along the tube is constant, any distance from the mixing chamber is equivalent to the time after initialization of the tracer efflux in the mixing chamber. Generally, six predetermined distances in the tube wall are replaced by Nucleopore filters with a pore diameter of 0.4–0.8 μm, which can withhold the cells and allow the collection of small-volume cell-free filtrates with increasing radioactivity with increasing distance from the mixing chamber (for details, see Brahm, 1977, 1983a, 1989).

Millipore-Swinnex filtering method

Inhibited ^{14}C-urea and ^{14}C-thiourea efflux rate is >10–100 times slower than non-inhibited urea and thiourea, and was determined by means of the Millipore-Swinnex filtering technique with a time resolution of ≈1 s. In short, ≈300 mg packed (hct ≈98%), labeled cells are injected into a temperature-regulated chamber containing 30 ml well-stirred non-radioactive medium. Subsequently, a series of cell-free filtrates is collected through Millipore-Swinnex filtering units at times that are registered with a pedal-activated timer (for details see Dalmark and Wieth, 1972).

Calculations

With both methods, the efflux of the tracer is well described as a closed two-compartment model with finite volumes:

$$C^*(t) = C^*(o) \times e^{-k^{*'}t}, \quad (1)$$

where $C^*(t)$ and $C^*(o)$ are the radioactive solute concentration at time t and zero, respectively, and $k^{*'}$ (s^{-1}) is the rate coefficient (the fraction of tracer removed per time unit).

The determined rate coefficient ($k^{*'}$) relates to k^*, and the rate coefficient for the unidirectional efflux is given by:

$$k^* = k^{*'} \times \frac{V_o}{V_i + V_o}, \quad (2)$$

where V_i and V_o are, respectively, the intracellular water (donor) volume and the extracellular (recipient) volume. It follows that $k^{*'} \rightarrow k^*$ for $V_o >> V_i$ is the condition in tracer efflux experiments with a hematocrit <1%. k^* is used to calculate the tracer permeability P^* (cm/s). As we assume that the rate coefficient and permeability of the tracer (k^*, P^*) and non-tracer (k, P), respectively, are indistinguishable, we have:

$$P = k \times \frac{V_i}{A} = \frac{\ln 2}{T_{1/2}} \times \frac{V_i}{A}, \quad (3)$$

where A (cm^2) is the cell membrane area available for the solute transport and $T_{½}$ is the half-time of the efflux process. A is related to the cell solid contents (corrected for any additional solute content >1 mM) as 1 g of cell solids equals 3.1×10^{10} "normal" cells with a total area of 4.4×10^4 cm^2 (see, e.g., Brahm, 1982).

Results

Donor variation

In a series of determinations of urea and water transport in human RBC (unpublished data), we selected two donors for the present study. Fig. 1 depicts two representative ^{14}C-urea efflux curves under SE conditions of the two donors. The logarithmic ordinate expresses the fraction of tracer that remains in the cells at a given time. The linearity of the efflux curves in the semi-logarithmic plot agrees with a monoexponential transport process with one rate coefficient. The urea permeability coefficient, P_u, is, respectively, 0.54 (SD ± 0.03, n = 4) × 10^{-4} cm/s (D13) and 3.3 (SD ± 0.6, n = 4) × 10^{-4} cm/s (D12).

Fig. 2 is the same setup as in Fig. 1 to determine the $^{3}H_2O$ diffusional efflux rate from the same donors of human RBC. P_d is, respectively, 2.3 (SD ± 0.2, n = 3) × 10^{-3} cm/s (D13) and 2.2 (SD ± 0.3, n = 3) × 10^{-3} cm/s (D12).

Table 1 combines new and previously published data from our laboratory of urea, thiourea, and water permeability from three donors (Brahm, 1982, 1983b). The three donors represent >100% variation of P_u, while the variation of P_{tu} is ≈ 60% and P_d is ≈ 0%.

Variation with age

With one of the donors (JBR), we have data back to 1973. Fig. 3 shows two urea efflux experiments performed under the same conditions with a 47-yr difference; in 2020 the donor's age was

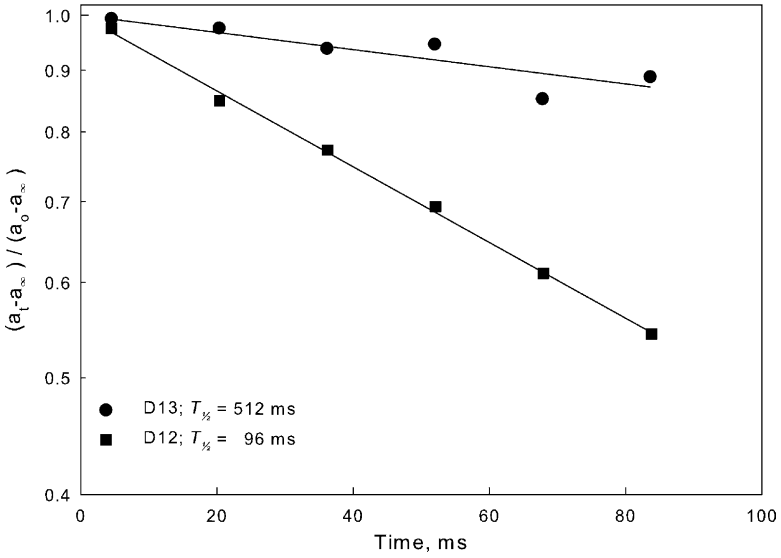

Figure 1. **Efflux of ^{14}C-urea from human RBC under *SE* conditions at 25°C, pH 7.2, 1 mM urea.** The logarithmic ordinate expresses the fraction of radioactive isotope that remains in the cells at a given time. The negative slope of the curves equals the rate coefficient k (s^{-1}) that is used to calculate the urea permeability P_u (cm/s). The two donors represent the variation of urea transport. The experiments were performed with the continuous flow tube technique. P_u is, respectively, 0.54 (SD ± 0.03, n = 4) × 10^{-4} cm/s (D13) and 3.2 (SD ± 0.6, n = 4) × 10^{-4} cm/s (D12).

75 yr. P_u has decreased by 82%, while P_d within almost the same range of years has not changed as shown in Fig. 4.

Inhibition

Urea and urea analogs

To further elucidate whether urea and water share a pathway in common, we studied the effects of urea and thiourea on P_u and P_d. Table 2 summarizes that 30 mM thiourea inhibits P_u by 72% and that urea self-inhibition amounts to 43% at 500 mM. Methylurea also inhibits P_u (results not shown) in accordance with competitive inhibition by urea analogs, but has not been tested on P_d. Both thiourea and urea at 500 mM show an insignificant inhibition of P_d.

Phloretin

Phloretin is an unspecific and very efficient inhibitor of several facilitated transport processes, including urea transport. In Table 3, we compare the effect of phloretin on P_u and P_d in two donors representing the low P_u (donor JBR) and the high P_u (D36) range in human RBCs (unpublished data). The uninhibited urea flux and all the diffusional water flux were determined by means of the continuous flow tube method, and the inhibited urea flux experiments were carried out with the Millipore-Swinnex filtering method. The table reveals a >100% variation of P_u in the control cells, while P_d is slightly lower in the "high-P_u-cells" compared with "low-P_u-cells."

PCMBS

It is well established that PCMBS is an efficient inhibitor of P_u, P_d, and osmotic water permeability, P_f. Fig. 5 shows that the inhibition pattern differs. Inhibition of urea permeability is rapid and reaches maximum in <1 min. In contrast, maximum inhibition of water permeability above the permeability of the lipid bilayer requires an incubation period of 1 h or more. The inhibition pattern of P_d appears also to apply to P_f (unpublished data).

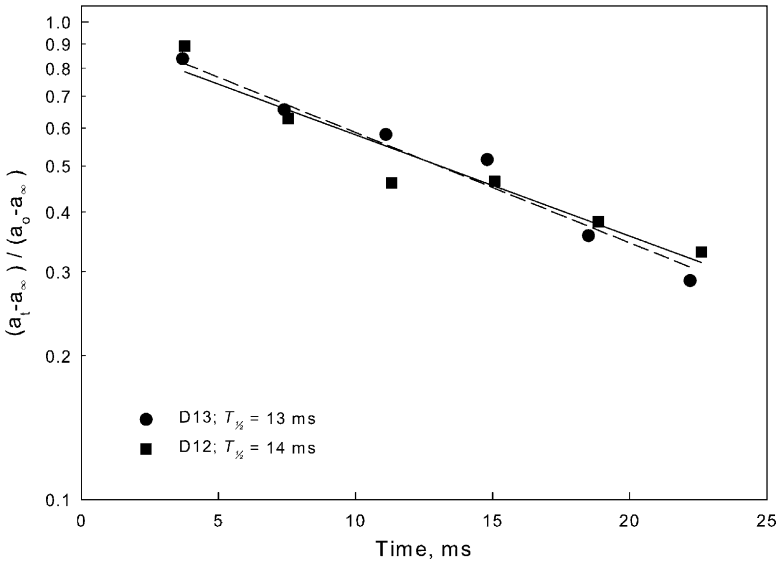

Figure 2. **This figure shows two representative ^3H_2O efflux curves with RBC from the same two donors.** Again, the linearity of the efflux curves in the semilogarithmic plot agrees with a monoexponentially transport process with one rate coefficient. The diffusional water permeability coefficient, P_d, is 2.3 (SD ± 0.2, n = 3) × 10^{-3} cm/s (D13) and 2.2 (SD ± 0.3, n = 3) × 10^{-3} cm/s (D12), respectively.

Table 1. **Urea, thiourea, and diffusional water permeability in human RBCs from three donors**

Donor	P_u	SD	n	P_{tu}	SD	n	P_d	SD	n	P_{tu}/P_u	P_d/P_u
	cm/s × 10^4			cm/s × 10^7			cm/s × 10^3			× 10^3	
JBR	2.67[a]			2.90	± 0.28	7	2.4[b]			1.1	9.0
D50	4.36	± 0.16	2	4.38	± 0.59	11	2.38	± 0.09	3	1.0	5.5
JSK	5.88[a]			4.78	± 0.21	3	2.45	± 0.16	6	0.8	4.2

[a]From Brahm (1983b).
[b]From Brahm (1982). P_u and P_d values are determined at 25°C, pH 7.2, 1 mM urea. P_{tu} values are determined at 0°C, pH 7.2 to minimize the fraction of thiourea that permeates the lipid phase of the membrane.

Discussion

Several studies of UT-B expression in *Xenopus* oocytes and genetically modified RBCs (Yang and Verkman, 1998, 2002; Levin et al., 2007; Azouzi et al., 2013; Geng et al., 2017) reach the conclusion that UT-B also transports water. The opposite position was presented in the key work by Fröhlich et al. (1991) which shows that osmotic water permeability was the same in Jk(a-,b+) and Jk(a-,b-) cells, the latter without UT-B. Some later studies of UT-B expression and hybridization (Olivès et al., 1994, 1995; Sidoux-Walter et al., 1999; Lucien et al., 2002) are in line with Fröhlich et al. (1991). Sidoux-Walter et al. (1999) found that expression of UT-B (termed HUT11A in the study) at high levels in *Xenopus* oocytes induced both a PCMBS and a phloretin insensitivity increased permeability to urea, water, and some small solutes. The authors further found that expression of UT-B at physiological levels only increased urea permeability and not water permeability (for mere details, see Bagnasco, 2006).

It appears from the literature that the expression of UT-B in *Xenopus* oocytes may not be so robust that a definitive conclusion can be reached. Further, some studies used stopped-flow light scattering technique, and we notice that in two studies, the urea permeability and the osmotic water permeability in RBCs respectively show a 10-fold variation (Yang and Verkman, 2002; Liu et al., 2011). We therefore turned to studying unmodified RBCs by means of methods that are well-established through many years and in several labs to study the transport of radioactive solutes and water. All experiments in the present study are carried out by measuring the efflux of the radioactive-labeled solute from a small volume of preloaded red cells into a large extracellular medium. We have also developed variations of the methods to measure influx, and the preliminary results are in accordance with the efflux experiments (unpublished data).

At this point, it may be relevant to recall how we interpret a common pathway: two solutes share a common pathway if (1) they experience the same structural environment as they cross the membrane, and (2) they are able to interact or compete with one another to affect the permeability of one another. Further, and consequently, an inhibitor of one solute permeating the pathway also affects (inhibits or stimulates) the permeation of the other solute in the pathway (Brahm et al., 1993). The criteria are well illustrated for urea and urea analogs, such as thiourea and methylurea, as transport of each one follows Michaelis–Menten kinetics in human RBCs, and their half-maximum constants ($K_{½}$, mM) are equal to their half inhibition constants (K_I, mM) on the analog solute.

Levin et al. (2012) used a stopped-flow method to study urea flux in reconstituted liposomes and *Xenopus* oocytes and concluded that osmotic stress modulates urea flux. Azouzi et al. (2013) criticized the use of oocytes because the water

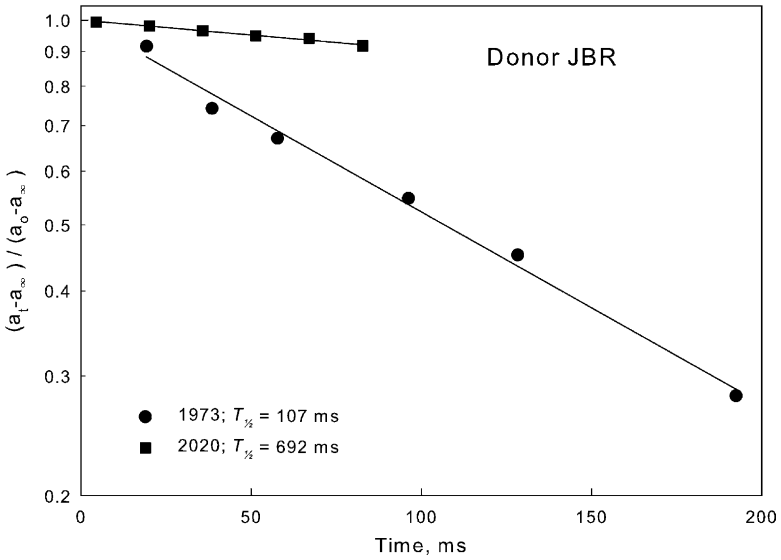

Figure 3. **Efflux of ^{14}C-urea under *SE* conditions at pH 7.2, 25°C, and 1 mM in RBC with a 47-yr interval from the same donor.** P_u was 2.8 (SD ± 0.2, n = 6) × 10^{-4} cm/s in 1973 and 0.5 (SD ± 0.2, n = 6) × 10^{-4} cm/s in 2020.

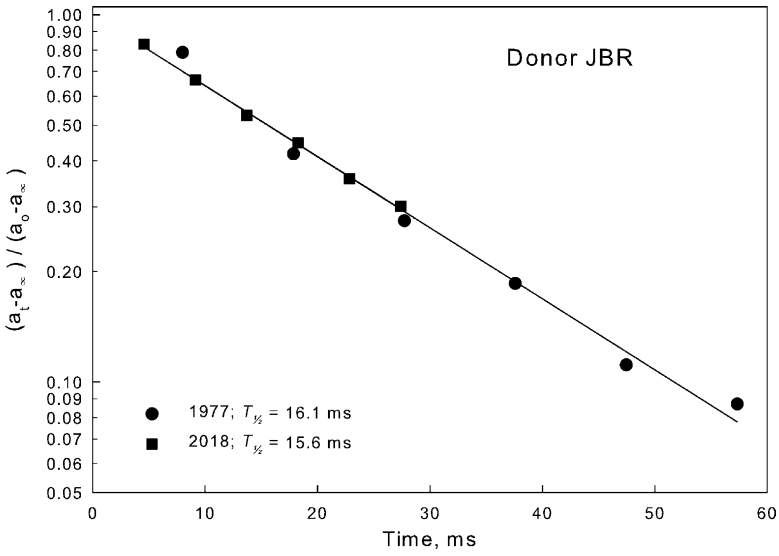

Figure 4. **Efflux of ^3H_2O under SE conditions at 25°C and pH 7.2 in RBC with a 41-yr interval from the same donor as in Fig. 3.** P_d was 2.3 (SD ± 0.14, n = 5) × 10^{-3} cm/s in 1977 and 2.4 (SD ± 0.12, n = 3) × 10^{-3} cm/s in 2018.

permeability was very low. Azouzi et al. (2013) also used a stopped-flow method to determine osmotic and diffusional water permeability in variants of cryopreserved RBCs and reached the conclusion that the osmotic water permeability of a UT-B unit is as high as a unit of AQP1. Since the estimated number per normal RBC of UT-B is 14,000 (Mannuzzu et al., 1993) and AQP1 is 200,000, the possible contribution of osmotic and diffusional water transport through UT-B is not detectable. This is illustrated in Fig. 5 in Fröhlich et al. (1991), where the curves of osmotic-induced cell shrinkage with time in normal and Jk(a-,b-) RBC coincide.

First, our argument against a common pathway for water and urea, as suggested by Yang and Verkman (1998), relates to any solvent drag effects. Levin et al. (2012) found that an inward osmotic gradient in oocytes expressing wild type UT-B increased urea uptake twofold, while an outward osmotic gradient had no effect on urea uptake. We observed no solvent drag effect on ^{14}C-urea efflux in RBCs whether the osmotic gradient was inward or outward (Brahm and Galey, 1987).

Second, a comparative study of RBCs from four species reveals the four possible combinations of high/low urea and water permeability (Brahm, 2013), where high refers to a permeability higher than in lipid bilayer membranes with no transporters and low refers to a permeability in lipid bilayer membranes. The combinations are high P_u and P_d (human RBC), low P_u and P_d (chick RBC), low P_u and high P_d (duck RBC), and high P_u and low P_d (giant salamander *Amphiuma means*). Either the urea transporter in *Amphiuma* differs from that in RBCs from other species or studies of systems with UT-B expression may not reflect the UT-B properties in intact unmodified RBCs.

Third, we have extended an earlier study regarding donor variation of urea permeability (Brahm, 1983a; and unpublished data) and confirmed a >10-fold donor variation that is even larger than that found in 1983. In the present study, we show a sixfold variation in two donors (Fig. 1). However, the diffusional water permeability in red cells from the same donors is equal, which we take as an argument against a common pathway for urea and water. At this point, we wish to emphasize that we disagree with Pagès et al. (2013) who claim that the membrane permeability to urea and water is comparable as we find at least a 10-fold difference. Their statement that RBCs are freely

Table 2. **Urea and diffusional water permeability in human RBCs: Effect of thiourea and urea**

	P_u	SD	n	Inhibition	P_d	SD	n	Inhibition
	cm/s × 10^4			%	%			%
Control	2.30	± 0.15	6	–	2.45	± 0.16	6	–
+Thiourea	0.64[a]	± 0.15	6	72.2	2.37[b]	± 0.10	4	3.2
+Urea	1.31[c]	± 0.03	2	43.0	2.29[c]	± 0.19	4	6.5

P_u and P_d values were determined at 25°C, pH 7.2. The urea concentration was 1 mM.
[a]30 mM thiourea.
[b]500 mM thiourea.
[c]500 M urea.

Table 3. **Phloretin effect on urea and diffusional water permeability in human RBCs**

	P_u	SD	n	Inhibition	P_d	SD	n	Inhibition
	cm/s × 10^4			%	cm/s × 10^3			%
Donor JBR control	2.69	± 0.05	4	–	2.35	± 0.01	3	–
Donor JBR phloretin	0.015	± 0.0002	4	99.4	2.42	± 0.29	4	n.s.
D36 control	5.62	± 0.26	4	–	2.18	± 0.27	4	–
D36 phloretin	0.014	± 0.0001	4	99.8	2.37	± 0.16	4	n.s.

P_u and P_d values were determined at 25°C, pH 7.2. The cells in the efflux experiments with phloretin were exposed to 0.5–0.8 mM phloretin both during the cell preparation and the efflux. The urea concentration was 1 mM. Not significant is marked n.s.

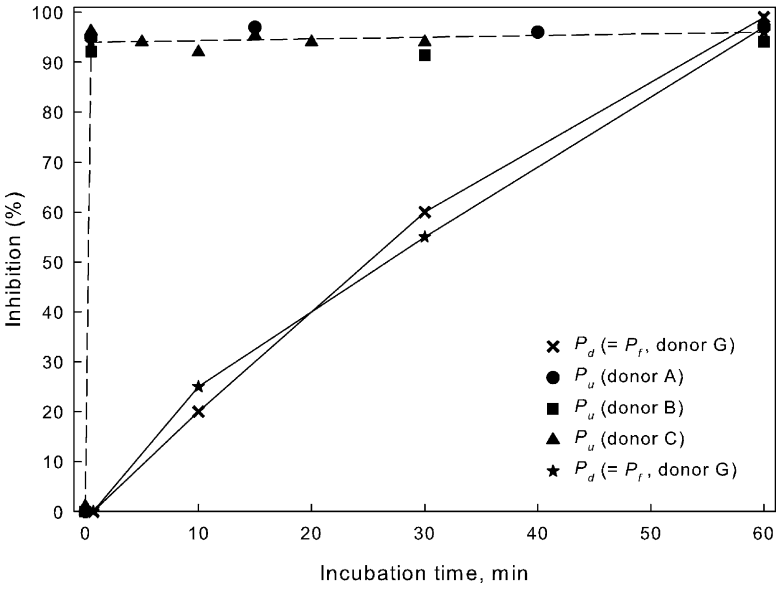

Figure 5. **Time dependence of PCMBS inhibition of urea and water permeability at 25°C, pH 7.2, in three P_u and two diffusional water permeability, P_d, determinations, that are calculated from results obtained with the continuous flow tube technique and the Millipore-Swinnex filtering method.** The inhibition of osmotic water permeability, P_f, refers to unpublished data.

permeable to urea is questionable. The diffusion coefficient of urea in water is ≈1.3 × 10^{-5} cm^2/s (Gosting and Akeley, 1952). Assuming a 10-nm thick membrane of water, the urea permeability would be 13 cm/s.

Fourth, we have had the extraordinary possibility to determine the urea permeability of RBCs with almost a 40-yr interval in two donors, one donor >65 yr and the other >75 yr old. Both donors' urea permeability is greatly reduced to 23 and even 10% of their values in 1983 (unpublished data). In the present study, we confirm the decrease in RBC urea permeability with the one donor, JBR, within an even greater time interval. It appears that the variation of urea permeability is a combination of an inborn donor variation and age. The results point to a general age-dependent decrease in RBC urea permeability and may have a side effect of reduced urinary concentrating ability, as described in Jk(a-,b-) persons (Sands et al., 1992). The age-dependent urea permeability decrease is not followed by a similar decrease in water permeability (Fig. 4) and supports our conclusion that urea and water do not share a pathway in common.

Fifth, the inhibition pattern differs with regard to urea and urea analogs compared with water. Transport of urea, thiourea, and methylurea follow Michaelis–Menten kinetics, and their half-saturation constants, $K_{½}$ mM, are respectively close to their half-inhibition constants, K_I mM (Wieth et al., 1974; Brahm, 1983a; and unpublished results). Table 3 shows that 30 mM thiourea inhibits urea permeability by 72% and that self-inhibition at 500 mM urea is 43% of urea permeability at 1 mM, in accordance with the Michaelis–Menten kinetics. Neither thiourea nor urea up to 500 mM inhibits water transport.

Phloretin is a nonspecific and very efficient inhibitor of most facilitated transport processes, including urea transport. Table 3 summarizes that urea permeability in RBCs exposed to phloretin is reduced to the same basic value of ≈1.5 × 10^{-6} cm/s in both high-P_u and low-P_u cells, while no inhibition was detected on P_d in the RBCs from the same donors, whose P_d is similar. The results are in support of the fact that urea and water do not share a pathway in common. Further, the higher uninhibited P_u in D36 compared with donor JBR is not caused by an additional leak pathway open to water (and urea).

PCMBS is a well-known inhibitor of the protein-mediated urea and water transport in RBCs. Diffusional water permeability at 25°C and pH 7.2 is ≈2.4 × 10^{-3} cm/s and the AQP1-related fraction is ≈58%. Osmotic water permeability amounts to ≈2 × 10^{-2} cm/s and the AQP1-related fraction is ≈95% (Brahm, 1983a; Moura et al, 1984; Mathai et al, 1996). Fig. 5 illustrates the very different time course of PCMBS inhibition of urea and water transport. Maximum inhibition of urea transport is reached within a very short time, while maximum PCMBS inhibition of water transport requires an incubation time of at least 1 h. The different time course indicates that different PCMBS binding sites are involved in urea and water transport in RBCs, respectively, which in accordance with the definition of a common pathway is consistent with our conclusion of separate pathways for urea and water in RBCs.

The UT-B as a common pathway for urea and water in RBCs was advanced and illustrated in Fig. 5 in Yang and Verkman (2002), which has been reproduced in later reviews (Li et al., 2012; Yu et al., 2019). According to the figure, UT-B accounts for 98% of urea transport and 2% diffuses through the lipid phase. We disagree because phloretin inhibits urea transport by ≥99.4%, leaving ≤0.6% to the lipid phase of the membrane. Further, the figure indicates that 2% of the total water permeability is through the lipid phase of the membrane. At room temperature and physiological pH, the water permeability of the lipid phase is 0.8–1.4 × 10^{-3} cm/s in chick RBCs with no water transporter, and in human RBCs after maximum inhibition with PCMB (*p*-chloromercuribenzoic acid) or PCMBS (Brahm and Wieth, 1977; Brahm, 1982). Hence, according to the figure, the protein-mediated osmotic permeability should be ≈5 × 10^{-2} cm/s, which is ≈2.5 times higher than published values in several studies. We also disagree with the distribution of water transport between AQP1 and UT-B, in part, because of the present results and, in particular, because of the comparative transport physiological study by Brahm (2013). Whether the trimeric

structure of UT-B (Levin et al., 2012; Azouzi et al., 2013) may create an independent, parallel pathway for water in intact RBCs from, for example, humans is difficult to settle because the possible contribution to the total water permeability is small compared with the contribution from AQP1. Referring to the comparative transport physiological study by Brahm (2013), such a contribution from AQP1 appears absent in *A. means* RBCs because the water permeability in those cells is as low as in lipid bilayer membranes, and PCMBS and PCMB have no inhibitory effect on water permeability. Both inhibitors as well as phloretin, on the other hand, inhibit urea permeability >99%. Unless the structure of the urea-transporting protein in *Amphiuma* RBCs differs from UT-B in other RBCs, the transport physiological results do not point to a parallel water pathway in UT-B.

The present study and earlier studies from our laboratory lead us to conclude that there is no interaction between urea and water in permeating the RBC membrane, the variation of urea permeability regarding donors and age of donors is not followed by any donor or age variation in water permeability, and the inhibition pattern of urea and water permeability differs clearly. According to the definition of a common pathway, our present and earlier results all point to the conclusion that urea and water share no pathway in common, and most likely UT-B is not a water-transporting protein.

Data availability

The data are available from the corresponding author upon reasonable request.

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Author contributions: J. Leifelt and J. Brahm performed experiments, analyzed results, and assembled figures. M.H. Dziegiel contributed to the design of the experimental strategy, discussed the results, and revised the manuscript. J. Leifelt and J. Brahm designed the project, planned the experiments, and wrote the manuscript. The three authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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