The permeability of red blood cells to chloride, urea, and water

Jesper Brahm

Department of Cellular and Molecular Medicine

The Faculty of Health, University of Copenhagen, DK-2200 Copenhagen N, Denmark

Corresponding address: jbrahm@sund.ku.dk; tel.: +45-35327568

SUMMARY

This study extends permeability (P, cm s^{-1}) data on chloride, urea, and water in red blood cells (RBC), and concludes that the urea transporter (UT-B) does not transport water. P of chick, duck, *Amphiuma means*, dog, and human RBC to $^{36}Cl^-$, ^{14}C-urea, and ^3H_2O was determined under self-exchange conditions. At 25°C, pH 7.2-7.5 $P_{Cl} \times 10^4$ is 0.94-2.15 (all RBC species, C_{Cl} =127-150 mM). $P_{urea} \times 10^6$ is 0.84 (chick) and 1.65 (duck) at C_{urea} 1-500 mM. In *Amphiuma*, dog, and human RBC P_{urea} is concentration-dependent (1-1000 mM, Michaelis-Menten-like kinetics; $K_{½}$ respectively 127, 173, 345 mM). $P_{urea} \times 10^6$ (C_{urea} =1 mM) is 29.5 (*Amphiuma*), 467 (dog), and 260 (human). Diffusional water permeability $P_d \times 10^3$ is 0.84 (chick), 5.95 (duck), 0.39 (*Amphiuma*), 3.13 (dog), and 2.35 (human). DIDS, DNDS, and phloretin inhibit P_{Cl} >99%. PCMBS, PCMB, and phloretin inhibit P_{urea} >99% in *Amphiuma*, dog, and human, but not in chick and duck RBC. PCMB and PCMBS inhibit P_d in duck, dog, and human, but not in chick and *Amphiuma* RBC. Temperature dependence, E_A, kJ mol^{-1}, of P_{Cl} is 117.8 (duck), 74.9 (*Amphiuma*), and 89.6 (dog). E_A of P_{urea} is 69.6 (duck), and 53.3 (*Amphiuma*). E_A of P_d is 34.9 (duck) and 32.1 (*Amphiuma*). Our present and previous RBC studies indicate that anion (AE1), urea (UT-B), and water (AQP1) transporters respectively only transport chloride (all species), water (duck, dog, human), and urea (*Amphiuma*, dog, human). Water does not share UT-B with urea, and the solute transport is not coupled under physiological conditions.

Keywords: erythrocytes, red cells, RBC, chloride, urea, water permeability, separate pathways

INTRODUCTION

Studies of red cell membrane transport have contributed considerably to different hypotheses of how water and small solutes cross the biological membrane. Sidel and Solomon (1957) reported that the osmotic water permeability, P_f cm s^{-1}, in human RBC was larger than the diffusional water permeability, P_d cm s^{-1} (Paganelli and Solomon, 1957). A P_f greater than P_d indicates that the membrane contains pores, and the ratio P_f :P_d was interpreted as a measure of the width of the pores in the so-called "Equivalent pore theory" (Solomon, 1968). The pores were assumed to accommodate transport of water, small nonelectrolytes such as urea, and even anions (Brown et al., 1975; Poznansky et al., 1976; Solomon et al., 1983). We questioned the concept in a study of chick RBC and a preliminary qualitative study of RBC from different species (Brahm and Wieth, 1977; Wieth and Brahm, 1977). Galey and Brahm (1985) showed that after a proper correction for both P_f and P_d related to the lipid phase of the red cell membrane, the pore according to the equivalent pore theory should accommodate even inulin that cannot permeate the membrane. Finkelstein (1987) suggested that the ratio P_f :P_d determines the length of the pore accommodating water molecules in support of Macey and Farmer's statement (1970) "It would appear that water channels transport water and very little else" in human RBC. It is generally accepted that the water transporting channel in the red cell membrane (aquaporin 1, AQP1) is a specific, or orthodox, water transporter. In other cells and cell systems other AQPs appear to create common pathways to water and several other solutes (see e.g. Borgnia et al., 1999; Verkman and Mitra, 2000; Wu and Beitz, 2007; Litman et al., 2009; Oliva et al., 2010; Zeuthen, 2010).

Yang and Verkman (1998) re-advanced that water and urea share a pathway in common in RBC and that the pathway is the abundant urea transporter UT-B (synonyms: UT3, UT11). They further concluded that UT-B was as efficient as AQP1 to transport water in RBC. Sidoux-Walter et al., (1999) questioned the conclusion of the expression studies in oocytes by Yang and Verkman (1998) because they found no increase in water permeability when UT-B was expressed at physiological levels. The critique was opposed in a study of RBC from double knockout mice (Yang and Verkman, 2002) and continued by Levin et al. (2007).

The present study has two goals: It extends the general characterisation of chloride, urea, and water transport in RBC from different species, and it uses a comparative

approach to elucidate whether UT-B in RBC creates a common pathway to urea and water. Inspired by earlier works (Jacobs, 1931; Jacobs et al., 1950) the present study, in combination with our previous studies (Brahm and Wieth, 1977; Brahm, 1977, 1982, 1983b) compares chloride, urea, and water permeability of RBC from chick, duck, salamander (*Amphiuma means*), dog, and human. Both previous and present results were obtained with the same techniques and are, therefore, directly comparable. The conclusion of the present study is that UT-B in intact RBC does not function as a common pathway that couples urea and water transport.

MATERIALS AND METHODS

Blood samples and reagents

Heparinised blood samples from chicks (white leghorn or white Plymouth Rock), ducks (mallards), salamanders (*Amphiuma means*, from Carolina Biological Supply Company, Burlington, N.C.), dogs (beagles), and humans were taken by venepuncture of a wing vein (birds), a foreleg (dogs), a forearm (humans), or by heart puncture (salamanders). Blood drawing was done according to the relevant ethical guidelines (all species) at the time of the experiment and after informed consent (humans). The blood was washed once in the proper medium to remove the plasma and buffy coat of white cells. Next, the cells were washed at least three additional times and titrated to the desired pH at the temperature of the experiments. After the last wash, the cells were suspended to a haematocrit of ~50 % and incubated at room temperature with radioactive isotopes (^3H_2O, [^{14}C]urea, $^{36}Cl^-$ or [^{3}H]inulin; Amersham Radiochemical Centre; ~18 kBq (0.5 µCi) pr. ml cell suspension). The cell suspension was gently stirred >6 half-times at room temperature to ensure equilibrium (except the extracellular marker [^{3}H]inulin) of ^3H_2O, [^{14}C]urea or $^{36}Cl^-$ across the cell membrane,.

Media

The media used were (mM): A. 145 NaCl (or KCl), 1.5 $CaCl_2$, 1 $MgCl_2$, 5 d-glucose, 27 glycyl-glycine. B. 150 KCl, 5 d-glucose, 27 glycyl-glycine. C. 150 KCl, 0.5 (or 2) KH_2PO_4. D. 118 NaCl, 2.5 KCl, 1.8 $CaCl_2$, 10 MOPS, 1 d-glucose, 0.1% albumen. Urea 1-1000 mM was added for urea flux experiments. The media were titrated to pH 7.2-7.5 at the temperature of the expewriments with 0.1 M of either NaOH, KOH or HCl. Me-

dia A-C were used for experiments with RBC from chicks, ducks, dogs, and humans. Medium D was used for experiments with salamander RBC.

Inhibitors

Phloretin (Sigma-Aldrich, Denmark) was dissolved in ethanol (25 mM) and added to the medium to give a final concentration of 0.5 mM. Incubation with 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS, Sigma-Aldrich, Denmark) was carried out analogous to the procedure used for complete (>99%) and irreversible inhibition of anion transport at room temperature (Brahm, 1977). Inhibition with the reversible anion transport inhibitor 4,4'-dinitrostilbene-2,2'-disulfonate (DNDS, Sigma-Aldrich, Denmark) was carried out as described by Fröhlich and Gunn (1987). RBC were also treated with 1 mM of the sulfhydryl-reacting reagents *p*-chloromercuribenzoate and *p*-chloromercuribenzosulfonate (PCMB and PCMBS, Sigma-Aldrich, Denmark) for 45 min at 38°C. During the incubation period the cell suspension was washed three times with the incubation medium. In all experiments with inhibitors the efflux medium contained the inhibitor at the concentration concerned.

Determination of radioactivity, cell surface area, cell volume, and cell water content

The radioactivity in cell samples, supernatants, and efflux media was determined by β-liquid scintillation counting. Solute transport in RBC is conveniently related to the amount of dried cell solids (if necessary corrected for extra solid contents at high solute concentrations as in the present study at urea concentrations >100 mM) and thus to the same number of RBC and a constant membrane area, if RBC are from the same species. Since cell surface area and volume vary among the species in the present study the measured transport rates are converted to permeability coefficients (see below). The cell volume, V, contains ~33% solids, primarily haemoglobin. Cell water volume, V_w, depends on pH and temperature and was determined as described previously (Brahm, 1977) by drying samples of packed RBC (haematocrit 97-98%) to constant weight at 105ºC for 24 hours and correcting for extracellular medium trapped between the cells by the centrifugation. The extracellular marker [^{3}H]inulin was used to determine the extracellular volume that amounted to 2-3% in all species.

Table 1 summarises "standard values" of *V*, *A*, and the ratio of cell water volume V_w to *A*, as determined in previous studies of chick, dog, and human RBC (Wieth et al., 1974; Brahm and Wieth, 1977). I calculated *A* of duck RBC using data from Gulliver (1875) as we did for chick RBC (Brahm and Wieth, 1977). The duck RBC is an oval nucleated cell with axes 13.1 µm × 7.4 µm and a thickness of 1 µm of the non-nucleated part of the cell. Duck RBC *V* was calculated to be ~175 femtoliter as 1 kg of cell dried solids equals 14.7×10^{12} cells (Lytle et al., 1998), and normal cells contain 1.55-1.57 litre cell water per kg cell dried solids (Lytle and McManus, 2002; the present study). *Amphiuma* RBC *V* and $V_w A^{-1}$ were calculated from $V_w = 0.682$ (w/w) (Siebens and Kregenow, 1985; the present study), $A = 5000$ µm^2 cell^{-1}, and 4.7×10^{11} cells (kg cell dried solids)$^{-1}$ (Cala, 1980).

Measurements of tracer efflux rates

The rate of tracer efflux under self-exchange conditions from the radioactive labelled RBC was determined by means of the Millipore-Swinnex filtering technique and the continuous flow tube method in the temperature range 0-40°C (Dalmark and Wieth, 1972; Brahm, 1977, 1989). By combining the two methods efflux rate coefficients as high as k ~230 s^{-1} ($T_{½}$ ~3 ms) can be determined (Brahm, 1983a). The principles of the two methods are the same. In short a small volume of packed and radioactive labelled RBC is suspended in a much larger volume of a non-labelled electrolyte medium, giving a suspension with a haematocrit <1%. At determined times cell-free filtrates are collected from the suspension. The increase of extracellular radioactivity with time in the series of filtrates is determined by β-liquid scintillation counting.

Calculations

The experimental setup is considered as a closed two-compartment model with constant volumes. The extracellular volume is >100 times larger than the intracellular volume, and the flow of tracer is very close to a unidirectional tracer efflux because the tracer flux back into the cells is ignorable. The kinetics of tracer efflux follows first order kinetics in accordance with the equation (Brahm, 1982):

$$\frac{a_t - a_\infty}{a_0 - a_\infty} = e^{-k\,t} \qquad (1)$$

a_t and a_∞ are the radioactive solute concentrations at time t and infinite respectively, and a_0 is the radioactivity in the dilute cell suspension at t=0. The rate coefficient k (s^{-1}) was determined by linear regression analysis as the numerical value of the slope of the curve in a semilogarithmic plot in which the logarithmic ordinate expresses the fraction of tracer in the cells at a given time (left hand side of Eqn. 1) and the abscissa is time (cf. Figs. 1, 2, and 4). The rate coefficient k is related to the halftime $T_{½}$ (s) of the efflux by:

$$T_{½} = \frac{\ln 2}{k} \tag{2}$$

k is set equal to the rate coefficient of the non-labelled compound, i.e. there is no isotope effect (see Brahm and Wieth, 1977). Hence, the higher k, the steeper is the efflux curve, and the shorter is $T_{½}$. The permeability P (cm s^{-1}) is related to k by:

$$P = k \times \frac{V_w}{A} \tag{3}$$

Chloride transport in RBC from human has been studied most extensively and shows complicated saturation kinetics. Both k and P depend in a complex manner on the intracellular and extracellular chloride concentrations and the asymmetric affinities of chloride to the transporter (see e.g. Knauf and Brahm, 1989; Gasbjerg et al., 1996; Knauf et al., 1996). The concentration dependent ("apparent") P_{Cl} was determined under equilibrium conditions with fixed extracellular chloride concentrations of 127 (*Amphiuma*) or 150 (chick, duck, dog, and human) mM where the transport system is almost completely saturated in human RBC. The narrow concentration interval allows comparing P_{Cl} of the different species.

Urea transport in *Amphiuma*, dog, and human RBC also shows saturation kinetics under self-exchange conditions. The saturation of urea transport, however, is kinetically less complicated and may be described in terms of Michaelis-Menten like kinetics where the apparent permeability coefficient P_{urea} is expressed by:

$$P_{urea} = \frac{J_{urea}^{max}}{K_{½} + C} \tag{4}$$

J_{urea}^{max} (mmol cm^{-2} s^{-1}) is the maximum urea flux, and $K_{½}$ (mM) is the half saturation constant (see e.g. Brahm, 1983b).

The apparent activation energy, E_A (cal mol^{-1}), of P in the temperature range 0-40°C was calculated by linear regression analysis of the relation:

$$\ln P = -\frac{E_A}{R} \times \frac{1}{T} + const. \quad (5)$$

R is the gas constant (1.99 cal $(mol\ K)^{-1}$), T is the absolute temperature (K), and E_A is determined from the slope of the curve.

RESULTS

Chloride transport

Fig. 1 shows the $^{36}Cl^-$ efflux curves under self-exchange conditions in RBC at an extracellular chloride concentration of 150 mM (chick, duck, dog, and human) and 127 mM (*Amphiuma*). For comparison, the figure shows efflux curves at 25°C that is the physiological temperature to *Amphiuma*, while the physiological temperatures are 37-40°C of the other four species. The rate coefficients were used to calculate P_{Cl} at the given chloride concentrations (cf. Eqns. 1-3, Table 2). The efflux curve of dog RBC (dashed line) was determined by interpolation of the data obtained at 38°C and 0°C and an E_A of 89.6 kJ mol^{-1}, cf. Table 3 that also summarises E_A of P_{Cl} of duck RBC (4-40°C) and *Amphiuma* RBC (5-30°C).

Urea transport

Urea transport in chick RBC is as low as in lipid bilayer membranes, while in human RBC it is high and saturates (Brahm and Wieth, 1977; Brahm, 1983b). The present study confirms and extends our earlier studies. The efflux curves in Fig. 2 further show that duck RBC transport urea almost as slowly as chick RBC, while *Amphiuma*, dog, and human RBC transport urea much faster. For comparison the efflux rates of urea were all determined at 1 mM urea and 25°C that is the physiological temperature for *Amphiuma*. Calculation of P_{urea} (cf. Eqn. 3) shows that P_{urea} of chick and duck is very low, P_{urea} of *Amphiuma means* is ~30 times higher, and P_{urea} of dog and human is ~300 times higher (Table 2).

Fig. 3 depicts P_{urea} dependence on C_{urea} in RBC of the five species at 25°C. P_{urea} of chick and duck RBC is concentration independent at C_{urea} =1-500 mM. In contrast, P_{urea} of *Amphiuma*, dog, and human RBC decreased with increasing C_{urea} to 1000 mM

in accordance with the concept of saturation kinetics. Urea transport in RBC of the three species is well described by a Michaelis-Menten like expression (Eqn. 4). Table 4 summarises J_{urea}^{max} (mmol cm^{-2} s^{-1}) and $K_{½}$ (mM).

Table 3 summarises that the temperature dependence of P_{urea} in duck RBC is 69.6 kJ mol^{-1}(4-40°C) and in *Amphiuma* RBC is 53.3 kJ mol^{-1}(0-25°C).

Water transport

Fig. 5 shows the diffusional efflux of ^3H_2O of the five species at 25°C and pH 7.2-7.5. $T_{½}$ of ^3H_2O efflux varies from 7 ms in duck to 154 ms in *Amphiuma* RBC. The P_d values of the RBC of the five species are summarised in Table 2. P_d was determined in RBC from two human donors whose P_{urea} varies >100% (Brahm, 1983b). Their P_{urea} and P_d values are summarised in Table 5. E_A of P_d in duck RBC with the highest P_d (4-40°C) and *Amphiuma* with lowest P_d (5-30°C) is similar, 32-35 kJ mol^{-1} (Table 3).

Inhibition of solute transport

Table 6 summarises the inhibitory effects of DIDS, DNDS, PCMBS, PCMB, and phloretin on chloride, urea, and water transport in the five red blood cell species as determined in the present and previous studies. The results (data not shown) of the present study are from double or triple determinations of efflux rate coefficients.

DISCUSSION

All procedures in the present study are well established in RBC transport studies of widely different solutes. The time resolution of the two methods suits quite well in the present study where $T_{½}$ ranges ~10^4 times (cf. Figs. 1, 2, and 4) from 40 s to ~4 ms, which is within the lower limit of the method (Brahm, 1983a). The time resolution of the two methods overlaps (Brahm, 1977) and the combined setup is, therefore, robust to detect even minor differences in the properties of the five red blood cell species.

All experiments were carried out under self-exchange conditions and osmotic equilibrium that ensures a constant cell volume during the tracer efflux measurements. The conditions prevent some sources of error. Firstly, as seen from Eqns. 2 and 3, $k \times V_w$ is proportional to P that is constant at a given solute concentration. If V_w changes, k changes inversely, and the efflux curves should show nonlinearity in the depictions in

Figs. 1, 2 and 4, bending downwards by cell shrinkage and upwards by cell swelling. Nonlinearity can be minimised if initial rates are determined by equilibrating only 10-15% of the intracellular tracer (Brahm and Galey, 1987; Gasbjerg and Brahm, 1991). In the present study the efflux curves show linearity up to 90% exit of the intracellular tracer, indicating that the tracer efflux follows a mono-exponential course. Secondly, the physiological V of the different species varies >40× (Table 1) and possible effects of volume changes on e.g. the mechanical properties of the RBC membranes from the different species are avoided. Thirdly, the constant V during the tracer efflux experiments prevents solvent drag effects as demonstrated for water in human RBC by Galey and Brahm (1987).

Chloride permeability

P_{Cl} of artificial bilayer membranes is of the order of 1×10^{-10} cm s^{-1} (Toyoshima and Thompson, 1975). P_{Cl} of RBC of all species so far investigated, except Lamprey erythrocytes (see Nikinmaa, 1990), is ~10^6 times higher (see e.g. Wieth et al., 1974; Jensen and Brahm, 1995; Jensen et al., 1998, 2001, 2003; Soegaard et al., 2012) and is due to a rapid anion exchange system that enhances the CO_2 transporting capacity of blood. Duck and *Amphiuma* RBC are no exception to that observation (Table 2). A comparison of anion transport in the different red cell species raises some issues to consider.

Firstly, the anion transport in human RBC is well characterised both structurally and kinetically as a saturable asymmetric transport system (AE1) with ~10^6 copies per cell that perform a tightly coupled anion exchange (see e.g. Knauf, 1989; Jennings, 1992a, 1992b; Knauf et al., 2002). The characterisation of the kinetics of anion transport in other RBC species is very incomplete. I assume that the saturation of the anion transporters of the different RBC species is comparable at the physiological concentrations of 127 and 150 mM used in the present study.

Secondly, the present study compares data obtained at 25ºC. An appropriate physiological approach is to compare the anion transport in RBC at the species respective "functional body temperature" (Jensen et al., 2001) that is 40ºC for duck and chick, 37ºC for human and dog, and 25ºC for *Amphiuma*. That approach gives very similar values of P_{Cl} of 3-4 × 10^{-4} cm s^{-1} at the functional temperatures of RBC of birds and mammals that is twice the value of *Amphiuma* RBC. However, the overall conclusion

still holds that the RBC under study all have a transport system that increases P_{Cl} ~10^6× above a "basic" P of lipid bilayer membranes and the lipid phase of the RBC membranes.

Thirdly, the anion transport by the RBC AE1 shows similar high E_A. At 0-40°C both chick and human RBC show a nonlinear E_A in an Arrhenius diagram. We (Brahm and Wieth, 1977; Brahm, 1977) simplified the findings by assuming two E_A values, 120-135 kJ mol^{-1} at low temperatures, and 80-94 kJ mol^{-1} in the physiological temperature range. If the nonlinearity is ignored the overall E_A is ~100-110 kJ mol^{-1}. The data of *Amphiuma*, dog, and human RBC (Table 3) does not allow the same distinction as for chick and human RBC. However, the overall E_A lies in the same narrow interval.

Fourthly, the specific inhibitors DIDS and DNDS and the non-specific inhibitor phloretin efficiently inhibit the anion transport in human RBC. A similar efficient inhibition of anion transport in the other red cell species is also obtainable by means of the inhibitors (Table 6).

The overall conclusion is that anion transport is similar in the selected RBC species. DIDS and DNDS inhibit neither urea nor water transport in the RBC, in agreement with that AE1 does not transport the two solutes. The conclusion from the comparative results of urea and water transport (see below) is also that this abundant transporter *per se* does not create a leak pathway to urea and water.

Urea permeability

P_{urea} and $P_{thiourea}$ is ~4×10^{-6} cm s^{-1} at 20-28°C in different lipid bilayer membrane systems with no built-in transporters (Vreeman, 1966; Galucci et al., 1971; Poznansky et al., 1976). Thiourea is ~10× more lipid soluble than urea (Collander and Bärlund, 1933) and the similar P_{urea} and $P_{thiourea}$ in artificial bilayer membrane systems underline that other factors than the partition coefficient, such as the entrance and exit rates of the solute in the membrane, are important. The P_{urea} and $P_{thiourea}$ are concentration independent, in agreement with a transport mode of simple diffusion through the lipid membrane phase.

Chick RBC have a low P_{urea}, $P_{thiourea}$, and $P_{methylurea}$ of ~1×10^{-7} cm s^{-1} in the concentration range 1-500 mM at 0°C (Brahm and Wieth, 1977) that is comparable to the permeability in the above-cited artificial systems. At 25°C the low P_{urea} in chick RBC is 0.84×10^{-6} cm s^{-1} ($T_{½}$ 40.8 s, Fig. 2; Table 2) that is concentration independent (C_{urea} = 1-

500 mM, Fig. 3) and agrees with a transport mode of simple diffusion through the lipid phase of the membrane. The same pattern was found in duck RBC where P_{urea} was 1.65 × 10^{-6} cm s^{-1} ($T_{½}$ 23.5 s, Fig. 2; Table 2; C_{urea} = 1-500 mM, Fig. 3). The present study does not reveal whether the twofold higher P_{urea} in duck RBC is due to a different lipid composition of the duck RBC membrane or an inter-individual variation as reported for chick RBC (Brahm and Wieth, 1977). Albeit P_{urea} is twice that of chick RBC the conclusion holds that urea is transported by simple diffusion in duck RBC. In accordance with the simple diffusion mode, P_{urea} of chick and duck RBC is inhibited by neither PCMBS nor PCMB that inhibit P_d and P_{urea} of human RBC, nor by phloretin that is a non-specific inhibitor of facilitated diffusion processes (Table 6; Brahm and Wieth, 1977; Brahm, 1982, 1983b). Further, E_A of P_{urea} is ~70 kJ mol^{-1} that is typical for solute transport through the lipid membrane phase (Table 3; Brahm and Wieth, 1977). E_A of UT-B-mediated P_{urea} is lower: 53 kJ mol^{-1} in *Amphiuma* and 12-35 kJ mol^{-1} in human RBC (Table 3; Brahm, 1983b). However, it should be emphasised that E_A is not a sensitive discriminator to specify which transport mode prevails.

Urea transport in RBC of *Amphiuma*, dog, and human shows the characteristic pattern of facilitated diffusion: A much higher transport than in lipid bilayer systems, saturation kinetics, both competitive and noncompetive, and reversible and irreversible inhibition, as well as temperature dependence different from that in bilayer systems.

P_{urea} of human RBC is 2-3 orders of magnitude higher than in chick and duck RBC. In the present study P_{urea} at 1 mM urea is 2.60 × 10^{-4} cm s^{-1} ($T_{½}$ 116 ms, Fig. 2), close to 2.67 × 10^{-4} cm s^{-1} in a previous study (Brahm, 1983b). The permeability is 4-5 times lower than the value of 1.16 × 10^{-3} cm s^{-1} reported by Mayrand and Levitt (1983) who determined P_{urea} from the slope of efflux curves with two points (Fig. 3 in Mayrand and Levitt, 1983). In the present study (Fig. 2) and Brahm (1983b) P_{urea} was determined from the slope of efflux curves with generally six points (regression coefficient r^2= 0.99).

P_{urea} decreases with increased urea concentration in accordance with saturation kinetics (Fig. 3; Eqn. 4) of the Michaelis-Menten type. Similar values of J_{urea}^{max} and $K_{½}$ were determined in the present and previous studies (cf. Table 4).

In dog RBC P_{urea} at 1 mM is almost twice as high (4.67 × 10^{-4} cm s^{-1}, $T_{½}$ 54 ms, Fig. 2) as is the apparent affinity, expressed by $K_{½}$, compared to human RBC, while J_{urea}^{max} is similar in the two species (Table 4).

Liu et al. (2011), using a stopped-flow light scattering methods, studied whether P_{urea} in RBC from selected mammals and birds is related to diet and urine concentrating ability, and reported a P_{urea} at 10°C and C_{urea} = 250 mM of dog and human RBC of respectively 5.3×10^{-5} and 1.1×10^{-5} cm s^{-1}. Extrapolated values to room temperature are one order lower than in the present study. P_{urea} in dog RBC shows extremely low activation energy of ~1 kJ mol^{-1} and suggests that e.g. unstirred layers may contribute significantly to the overall lower permeability.

Urea transport in *Amphiuma* RBC is also high and saturates (Fig. 3). The affinity to urea in these cells is even higher than in dog RBC as $K_{½}$ is 127 mM while J_{urea}^{max} is about 25 times lower than in dog and human RBC (Table 4). If J_{urea}^{max} is expressed per cell instead of per unit area, $J_{urea}^{max,cell}$ ($\times 10^{10}$ mmol $cell^{-1}$ s^{-1}) becomes similar: 1.6, 0.9, and 1.2 in respectively *Amphiuma*, dog, and human RBC. In human RBC UT-B is ascribed to be the Kidd antigen and the estimated number of transporters is between 14,000 and 32,000 (Masouredis et al., 1980; Fröhlich et al., 1991; Manuzzu et al., 1993; Neau et al., 1993; Olivés et al., 1995). Taking the 14,000 copies the turnover number in human RBC is ~5×10^6 urea molecules $site^{-1}$ s^{-1} at 25°C in agreement with previous estimates (Manuzzu et al., 1993; Sands et al., 1997). It is an open question whether the similar $J_{urea}^{max,cell}$ in the three species is due to the same number of transport sites per cell with the same turnover rate per site or different number of transport sites with different turnover rates per site. The turnover number indicates a channel-like mechanism (Manuzzu et al., 1993) but the term "facilitated diffusion" conveniently reflects the effect and not the mechanism of UT-B.

Diffusional water permeability

P_d in human, dog, and duck RBC was inhibited with either PCMB or PCMBS by 50%, 67%, and 81%, respectively (Table 6). The maximal inhibition leaves a residual P_d in all three RBC species of 1.1-1.3 $\times 10^{-3}$ cm s^{-1} that is as low as in chick RBC and artificial lipid bilayer membranes, and the same as the residual P_f in human RBC after PCMB or PCMBS treatment (Table 6; Cass and Finkelstein, 1967; Brahm and Wieth, 1977; Brahm, 1982; Finkelstein, 1987; Mathai et al., 2001, 2007). Most likely the two inhibitors close all water transporting channels completely (Finkelstein, 1987). In human red blood cell ghosts the complete inhibition increased E_A of P_d from 30 to 60 kJ mol^{-1}, which is a typical value

of artificial membranes and liposomes. E_A is, however, too crude to be a discriminator of transport modes: E_A of P_d is ~42 kJ mol^{-1} in chick RBC and 32 kJ mol^{-1} in *Amphiuma* RBC with no AQP1, and is of the same order of magnitude as in duck and unmodified human RBC with AQP1 (Table 3; Brahm and Wieth, 1977; Brahm, 1982).

Do urea and water share a pathway in common in red blood cells?

Yang and Verkman (1998) suggested "That the UT3 protein is associated with an aqueous channel that transports water and urea in a coupled manner". They further proposed that the UT-B was as efficient as AQP1 to transport water. Their conclusions were based upon expression studies in *Xenopus laevis* oocytes combined with volumetric measurements of water uptake at 10°C and [^{14}C]urea uptake at 1 mM at 23°C.

Sidoux-Walter et al. (1999) questioned the conclusion by Yang and Verkman (1998). They showed that expression at high levels of the human RBC UT-B in *Xenopus laevis* oocytes induced not only high P_{urea} and P_f as reported by Yang and Verkman (1998), but also an increased permeability to small solutes, such as formamide through propionamide, and to diols, such as ethylene glycol and propylene glycol. Further, neither phloretin nor PCMB inhibited P_{urea} as they do in RBC that transport urea by facilitated diffusion. The data indicates that the transport specificity disappeared at high level expression. In contrast, expression at physiological levels increased expectedly the phloretin-sensitive urea transport with no increase of P_f (Sidoux-Walter et al., 1999). The study by Lucien et al. (2002) also pointed out that expression of recombinant urea transporter (named hUT-B1) in *Xenopus* oocytes creates a P_{urea} that is efficiently inhibited by phloretin, but much less inhibited by PCMBS than the native P_{urea} in human RBC. The authors further concluded that hUT-B1 is not a water channel.

Yang and Verkman (2002) extended their expression studies by means of double knockout mice whose RBC lack AQP1 and UT-B, and concluded that UT-B is an efficient water transporter. According to their study P_f distributes at 37°C with 6% related to UT-B, 79% to AQP1, and 15% to the lipid phase (the numbers are not in harmony with Fig. 5 of their study where the respective numbers are 8%, 90%, and 2%). The UT-B mediated fraction of P_f had an E_A of <2 kcal mol^{-1} (8 kJ mol^{-1}). This is about half the values of E_A of self-diffusion of water in water and previously reported values of total P_f in RBC of which > 90% is ascribed to AQP1. Taking the 2 kcal mol^{-1} and the other re-

ported E_A values by Yang and Verkman (2002) for P_f of AQP1 (7.3 kcal mol^{-1}) and the lipid phase (19 kcal mol^{-1}), the 6% of P_f related to UT-B at 37°C increases to >14% at 10°C that is the experimental temperature of the study. The respective increase of P_f of AQP1 is from 79% to 84%, indicating that the lower the temperature the more P_f should be related to UT-B compared to AQP1.

The native and the modified mouse RBC were not tested for other functional properties than water and urea transport. Neither this study nor a later inhibition study from the same laboratory, using the same strategy (Levin et al., 2007) included the inhibitors PCMB and PCMBS that have been widely used by others to inhibit RBC water and urea transport (Macey, 1984).

The present study uses a different approach and compares P_{urea} and P_d of the native systems in intact RBC. The advantage is to avoid any major modification of cell membranes or expression in other cells that may modify the physiological pathway(s) or even give rise to artificial pathways. Earlier studies show that RBC from different species transport solutes differently (Jacobs, 1931; Jacobs et al., 1950), and a proper selection of RBC species may reveal whether urea and water share a pathway in common in RBC. The selection of RBC species reflects that chicks and ducks as other birds excrete uric acid and that their RBC have no UT-B. Humans and dogs, and to a lesser extent *Amphiuma means*, concentrate and excrete urea as the end product of their protein metabolism, and their RBC have UT-B. Chick and *Amphiuma* RBC have no AQP1, and hence, the four combinations of high/low P_{urea} and P_d are available.

Water and a solute are said to share a common pathway if: 1) Water and the solute experience the same structural environment as they cross the membrane, 2) they are able to interact or compete with one another to affect the permeability of one another, and 3) an inhibitor of water or solute permeating the pore also affects (inhibits or stimulates) the permeation of the other molecule in the pore (Brahm et al., 1993).

Whether water and urea share the AQP1 as suggested by some (Solomon, 1968; Solomon et al., 1983) and turned down by others (Macey, 1984; Galey and Brahm, 1985; Brahm and Galey, 1987; Finkelstein, 1987) or urea and water share UT-B (Yang and Verkman, 1998, 2002; Levin et al., 2007) makes no principal difference in the testing strategy of the hypothesis. Firstly, is P_{solute} above that of lipid bilayers that have no transporters inserted and if so does the transport saturate? Secondly, do the solutes interact?

Thirdly, is inhibition of both solutes present with the same inhibitor and with the same pattern? Fourthly, is the temperature dependence of solute transport through the proposed transporter different to that of diffusion through the lipid membrane phase?

Chick RBC show P_{urea} and both P_d and P_f as low as in lipid bilayer membranes (Table 2; Brahm and Wieth, 1977; Farmer and Macey, 1970). Urea shows no saturation kinetics (Fig. 3; Brahm and Wieth, 1977) and well-established inhibitors of water and urea transport in other RBC with UT-B and AQP1 (Table 6) inhibit neither P_{urea} nor P_d. E_A of P_{urea} is high 71.2 kJ mol^{-1} (Brahm and Wieth, 1977) and of the same magnitude as to other nonelectrolytes that permeate the lipid membrane phase (Wartiovaara, 1949; Macey et al., 1972; Galey et al., 1973; Brahm, 1983a). E_A of P_d is 41.9 kJ mol^{-1} (Brahm and Wieth, 1977).

The high anion self-exchange flux underlines that a proteinacous pathway that creates very high P_{anion} does not create a leak pathway to water and urea. The chick RBC results are in line with the concept of a basic P_{urea}, P_d, and P_f caused by simple diffusion through the lipid phase of the RBC membrane. Both E_A of P_{urea} and P_d and the lack of inhibition by means of phloretin, PCMB, and PCMBS support the concept.

The results of P_{Cl} and P_{urea} in duck RBC are in line with those of chick RBC. However, P_d of duck RBC is the highest of the five RBC species and supports the concept that the cell membrane contains AQP1 that transports water and no solutes.

The studies of *Amphiuma* RBC show that high P_{Cl} is combined with high P_{urea} and low P_d. In comparison with human RBC P_{Cl} is very close in the two RBC species (Table 2) while P_{urea} at 1 mM is about ten times lower in *Amphiuma* RBC. Our present knowledge of anion and urea transport in human RBC unquestionably calls for different pathways of the two solutes. No evidence points to a different concept in *Amphiuma* RBC. The other important finding is that P_{urea} is markedly higher in *Amphiuma* RBC than in chick and duck RBC while P_d is even lower than in chick RBC. Hence, UT-B in *Amphiuma* RBC neither increases P_d nor creates a common pathway to urea and water.

From the studies of P_{Cl}, P_{urea} , and P_d in intact human and dog RBC one might assume a coupling of water and urea transport because both cell types show higher values than in lipid bilayers. However, the ratio of P_d to P_{urea} differs considerably. The ratio is 6.7 in dog RBC, in pig RBC the ratio is 8.2 (J. Brahm, unpublished data), and in human McLeod erythrocytes with Kell antigen-null the ratio is 7 (Brahm et al., 1993). The range

of P_{urea} from different donors varies over 100% (Brahm, 1983b), and taking the highest and lowest values, the ratio shows a donor dependence between 9 and 4 (Table 5). These results and the comparative study of 11 different mammals by Liu et al. (2011) that showed a five-fold variation of P_{urea} and fairly similar P_f underline that the ratio is not fixed and support the conclusion that the transport of water and urea is not coupled. Brahm and Galey (1987) reached the same conclusion as they showed no solvent drag effect on urea transport in human RBC while the efflux of tritiated water increased with the osmotic flow of water and decreased against the osmotic flow of water.

The inhibition data (Table 6) neither supports a coupling of the two solutes. The nonspecific inhibitor of facilitated transport systems phloretin efficiently inhibits both anion and urea transport almost completely, but has no effect on P_d. According to the criteria listed above this argues against the common pathway. PCMB and PCMBS are also nonspecific inhibitors. Both compounds inhibit P_{urea} and P_d to values close to those of lipid bilayer systems and RBC of chick (basic P_{urea} and P_d), duck (basic P_{urea}), and *Amphiuma* (basic P_d). The tempting conclusion is that the two inhibitors close a common pathway. However, the time dependence of the PCMB/PCMBS inhibitory effect is different. Inhibition of P_{urea} appears much faster than P_f (Macey, 1984) and inhibition of P_f and P_d has the same time constant (W.R. Galey and J. Brahm, unpublished data).

The analogue compound thiourea inhibits urea transport. Thiourea is a competitive inhibitor that is also transported by UT-B in human RBC. Thiourea is transported ~100 × slower than urea (at 0ºC; Wieth et al., 1974). The half saturation constant $K_{½}^{thiourea}$ is 15-20 mM close to the half inhibition constant $K_{I,urea}^{thiourea}$ of 12-14 mM of urea transport (Wieth et al., 1974; Solomon and Chasan, 1980; Mayrand and Levitt, 1983; Brahm, 1983b) and the half inhibition constant of urea on thiourea transport $K_{I,thiourea}^{urea}$ is close to $K_{½}^{urea}$ (J. Brahm, unpublished data). The observation that $K_{½}$ and K_I of each solute are equal accords with the concept that urea and thiourea transport follows kinetics of simple Michaelis-Menten type with the two solutes competing for binding to one and the same site. Neither thiourea at 100 mM that inhibits P_{urea} >95% nor urea at 500 mM inhibits P_d (Brahm, 1982).

The overall conclusion of the present comparative study is that there is substantial evidence that urea and water do not share UT-B, and that transport of the two solutes is not coupled in intact RBC.

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FIGURE LEGENDS

Fig. 1. A semilogarithmic plot of representative examples of ^{36}Cl efflux under self-exchange conditions at pH 7.2-7.5 and 25ºC in RBC of chick, duck, *Amphiuma*, dog, and human. The logarithmic ordinate expresses the fraction of tracer that remains in the cells at a given time (abscissa). The efflux rate equals the numerical value of the slope of the curve. The chloride concentration was 127 mM in *Amphiuma* RBC experiments and 150 mM in the other RBC experiments. The efflux rate in dog RBC (dashed line) was estimated by interpolation of data obtained at 38ºC and 0ºC, and an E_A of 89.6 kJ mol^{-1} (cf. Table 3).

Fig. 2. A semilogarithmic depiction (see details in legend to Fig. 1) of representative examples of [^{14}C]urea efflux under self-exchange conditions in RBC from chick, duck, *Amphiuma*, dog, and human at 25ºC and pH 7.2-7.5. The experiments were performed at 1 mM urea.

Fig. 3. Concentration dependence of P_{urea} under self-exchange conditions in RBC from five species at 25ºC and pH 7.2-7.5. The decline of P_{urea} with increasing urea concentration in RBC of dog, human, and *Amphiuma* reflects saturation kinetics of urea transport. Each point on the curves is an average of 2-5 efflux experiments as those shown in Fig. 2. Standard deviations are shown in experiments where they exceed the size of the symbols (dog).

Fig. 4. A semilogarithmic plot (see details in legend to Fig. 1) of representative examples of diffusional efflux of ^3H_2O under self-exchange conditions in RBC from chick, duck, *Amphiuma*, dog, and human at 25ºC and pH 7.2-7.5.

TABLES

Table 1. RBC volume (V), water volume (V_w), and area (A) of chick, duck, *Amphiuma*, dog, and human at physiological conditions

	Chick[a]	Duck	*Amphiuma*[b]	Dog[c]	Human[c]
$V \times 10^{12}$ cm^3	128	175	6500	67	87
$\frac{V_w}{V}$ (%)	68	68	68	68	70
$A \times 10^8$ cm^2	175	190	5000	117	142
$\frac{V_w}{A} \times 10^5$ cm	5.0	6.3	8.9	3.9	4.3

[a]Brahm and Wieth (1977); [b]Cala (1980), [c]Wieth et al. (1974).

Table 2. Chloride, urea and diffusional water permeability of RBC from chick, duck, *Amphiuma*, dog, and human at 25°C and pH 7.2-7.4.

	P_{Cl} cm s^{-1} $\times 10^4$	P_{urea} cm s^{-1} $\times 10^6$	P_d cm s^{-1} $\times 10^3$
Chick	0.94 (0.03, n=2)	0.84 (0.02, n=3)	0.84 (0.19, n=5)
Duck	2.15 (0.06, n=2)	1.65 (0.33, n=6)	5.95 (1.17, n=11)*
Amphiuma	1.64 (0.06, n=2)	29.5 (2.9, n=4)*	0.39 (0.09, n=2)
Dog	1[a]	467 (37, n=3)*	3.13 (0.57, n=10)*
Human	1.42 (0.18, n=6)	260 (7, n=4)*	2.35 (0.09, n=4)*

The numbers are Mean (SD). P_{urea} was determined at 1 mM urea. [a]Calculated by interpolation of P_{Cl} values obtained at 0 and 38°C, and an E_A of 89.6 kJ mol^{-1}. P_{urea} and P_d values, respectively, were compared by means of one way ANOVA with multiple comparisons versus chick RBC as control group. *Indicates that the value is significantly different ($p<0.05$)

Table 3. Apparent activation energy of chloride and urea self-exchange and diffusive water transport in RBC from five species

	Chick	Duck	*Amphiuma*	Dog	Human
			E_A (kJ mol^{-1})		
Tp. (°C)	0-40	4-40	0-25	0-38	0-38
Chloride	96.4-138.5[a]	117.8 (3.9, n=14)	74.9 (3.8, n=8)*	89.6 (0.7, n=12)	83.8-125.7[b]
Urea	71.2[a]	69.6 (2.6, n=20)	53.3 (4.3, n=15)*	Not det.	12[c]
Water	41.9[a]	34.9 (4.2, n=26)	32.1 (6.2, n=6)	Not det.	21[d]

[a]Brahm and Wieth (1977), [b]Brahm (1977), [c]Brahm (1983b), [d]Brahm (1982) . The numbers are Mean (SD). *Significantly different from chick (Student's t-test, $p<0.05$)

Table 4. Half saturation constant ($K_{½}$) and maximum urea transport (J_{urea}^{max}) in RBC of *Amphiuma means*, dog, and human at 25°C and pH 7.2-7.5

	$K_{½}$ mM	J_{urea}^{max} mmol cm^{-2} s^{-1} $\times 10^6$
Amphiuma means	127	3.5
Dog	173	75
Human	345	83
Human[a]	334	82

[a]Brahm (1983b)

Table 5. Urea and diffusional water permeability of RBC from three human donors at 25°C and pH 7.2.

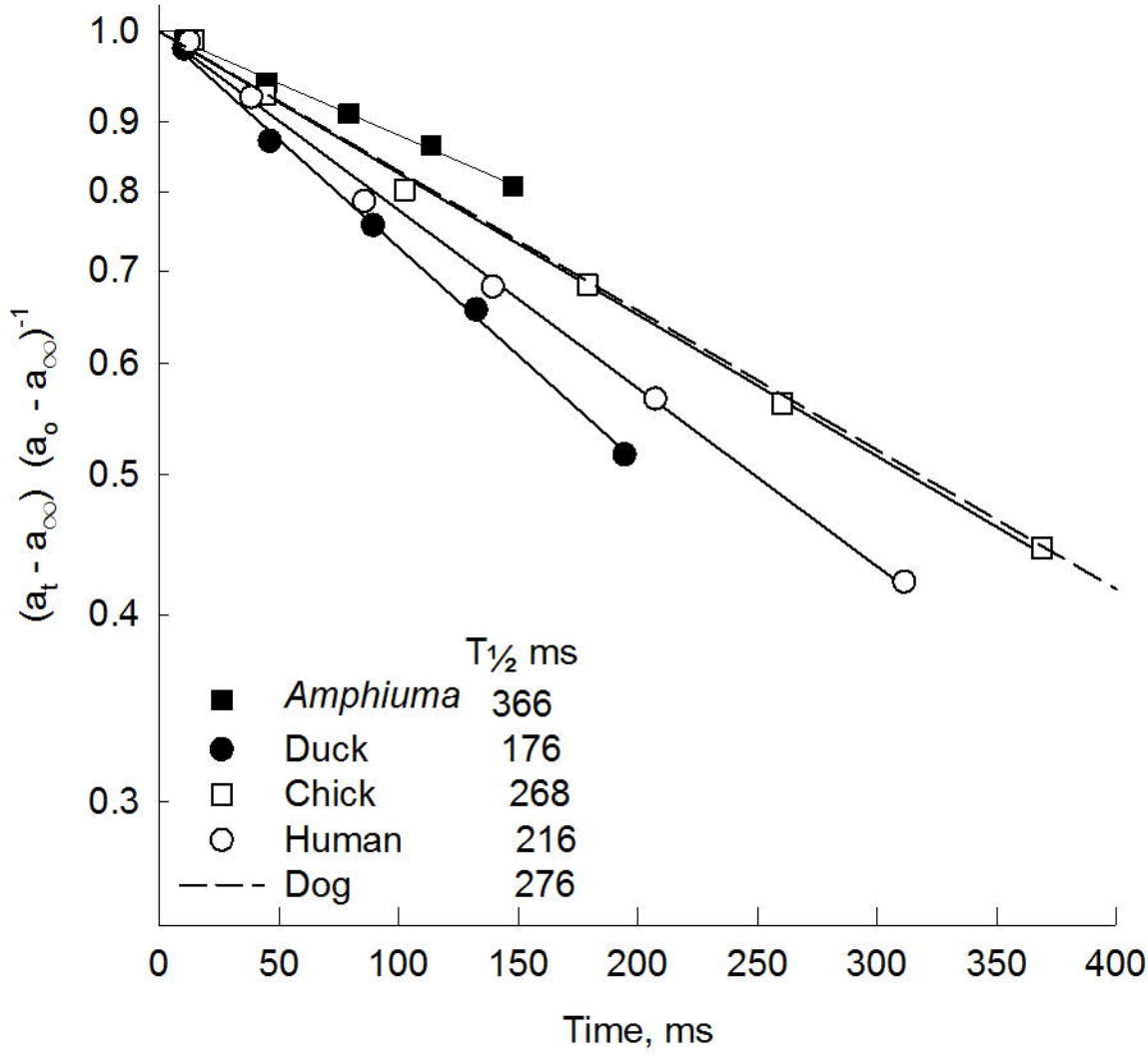
	P_{urea} cm s^{-1} $\times 10^4$	P_d cm s^{-1} $\times 10^3$	$P_d P_{urea}^{-1}$
J. B.	2.60 (0.07, n=4)	2.35 (0.09, n=4)	9.0
J. B.	2.67 (0.05, n=4)[a]	2.4 (0.2, n=18)[a]	8.9
J. S.	5.88 (0.26, n=4)[a*]	2.45 (0.16, n=6)	4.2

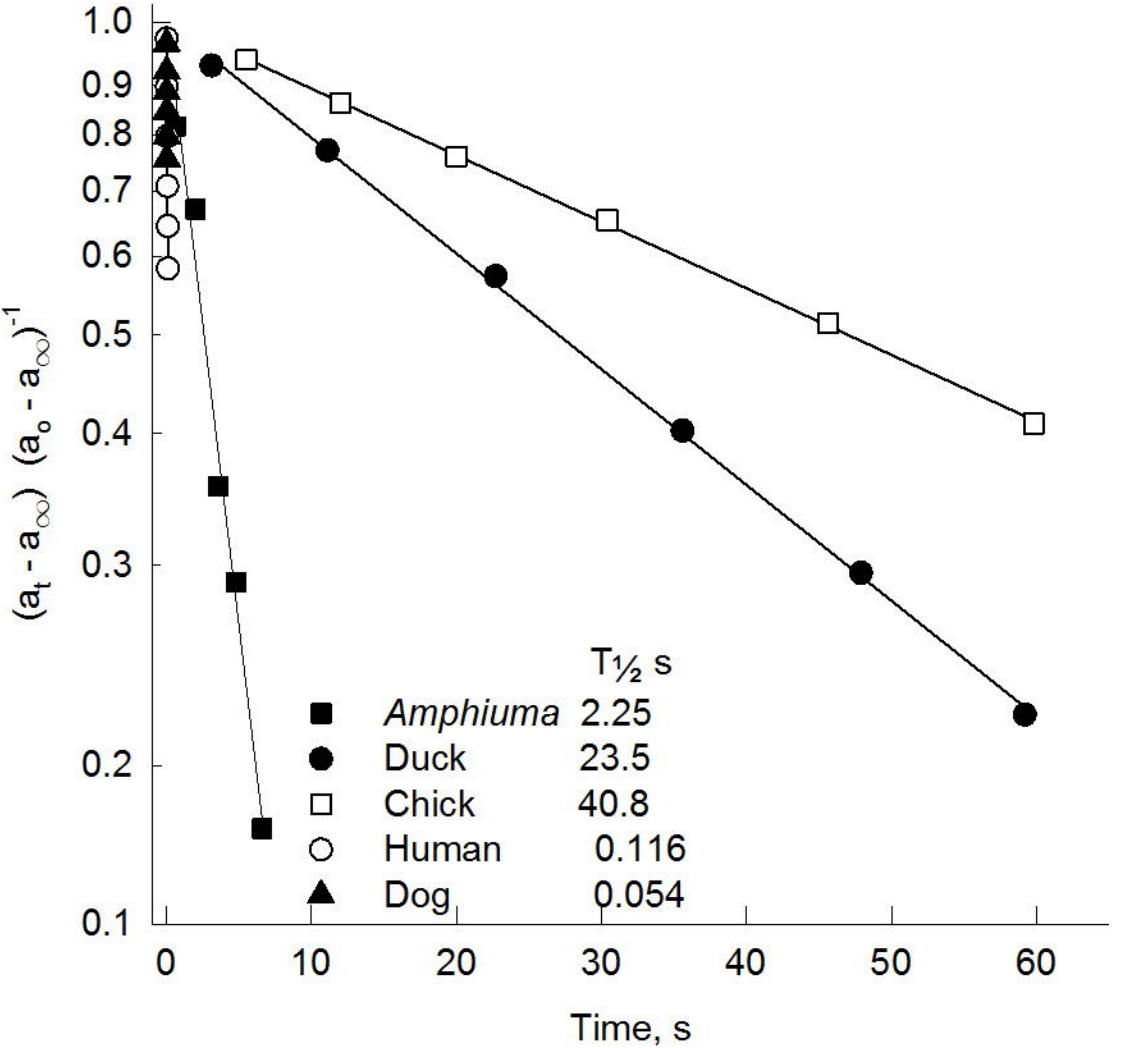
[a]Brahm (1983b). C_{urea} was 1 mM. The numbers are Mean (SD). *Significantly different from J.B. (Student's t-test, $p<0.01$)

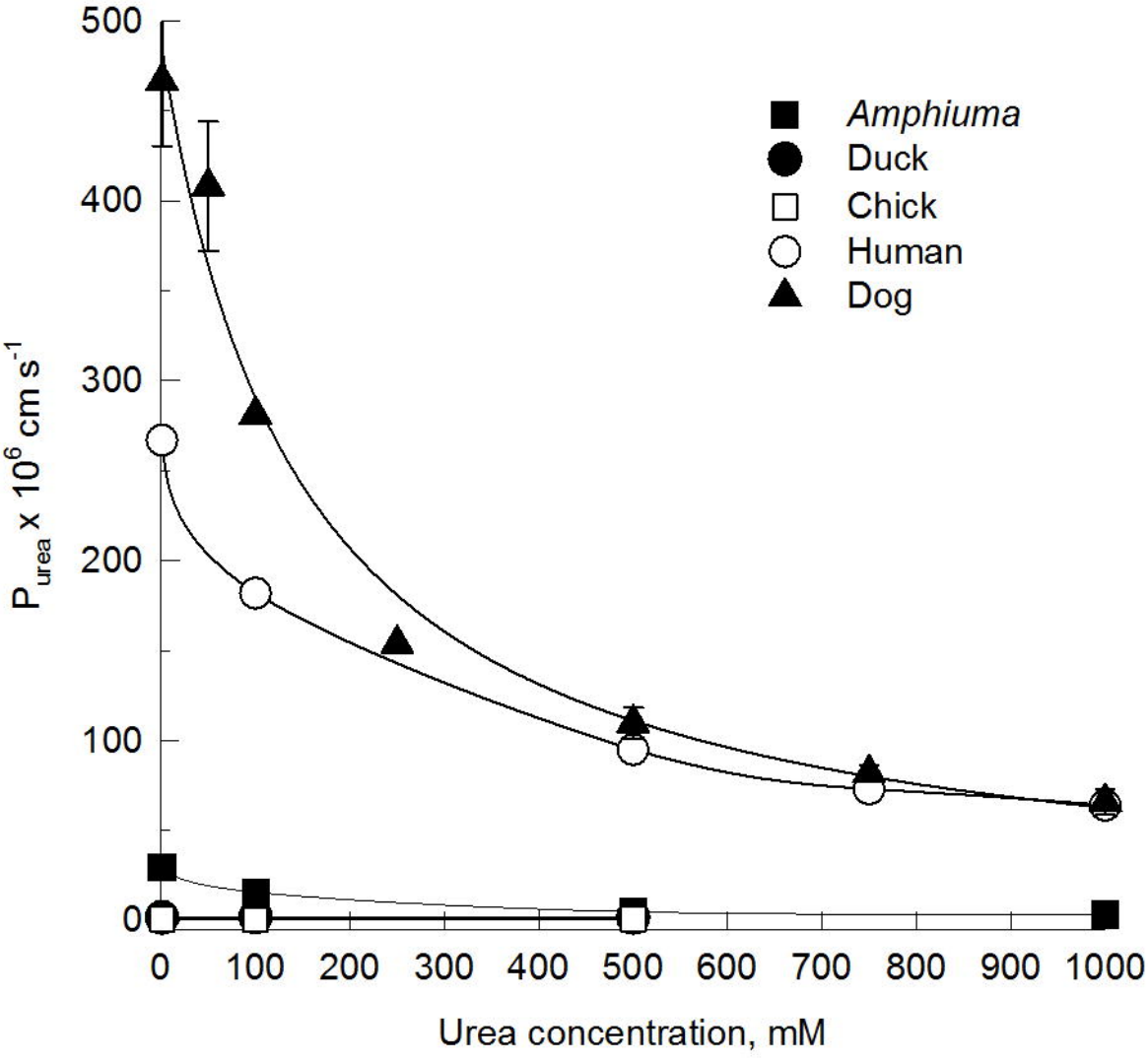
Table 6. Inhibition (%) of solute transport in RBC from five species

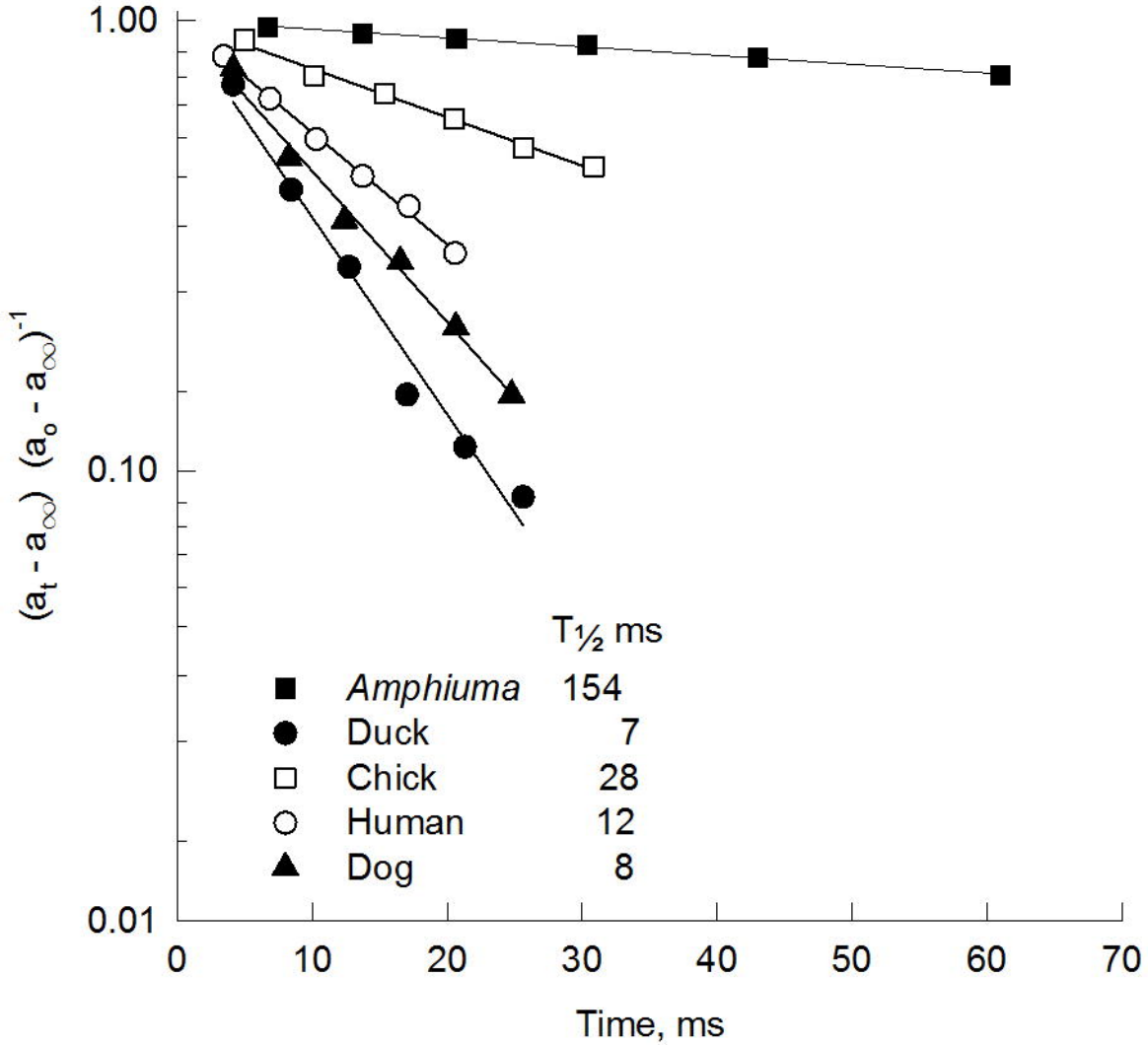
		Chick	Duck	*Amphiuma*	Dog	Human
Chloride	DIDS	>99[a]	99[b]	>99[d]	>99[e]	>99[h]
	DNDS				>99[f]	>99[h]
	Phloretin	>99[a]	>99[b]	>99[d]		>99[h]
Urea	PCMBS/PCMB		0[b]		>95[c]	>90[i]
	Phloretin	0[a]	0[b]	>99[c]		>99[i]
Water	DIDS/DNDS					0[j]
	PCMBS/PCMB	0[a]	~81[c]		~67[g]	~50[j]
	Phloretin	0[a]				0[j]

[a]Brahm and Wieth (1977). Experimental temperature was [b]0°C, [c]25°C, [d]10°C, and [e]37°C. [f]Estimated from a determination of the half inhibition constant K_I of 7 µM in the concentration range 0-50 µM. The value is close to a K_I = 6 µM as determined in human RBC by Gasbjerg et al. (1993). [g]Brahm et al. (1993). [h]Gasbjerg et al. (1993). [i]Brahm (1983b). [j]Brahm (1982). All experiments were carried out at pH 7.2-7.5.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
$(a_t - a_\infty)\ (a_o - a_\infty)^{-1}$
T½ ms
Amphiuma 366
Duck 176
Chick 268
Human 216
Dog 276
0
50
100
150
200
250
300
350
400
Time, ms


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
$(a_t - a_\infty)\ (a_o - a_\infty)^{-1}$
T½ s
Amphiuma 2.25
Duck 23.5
Chick 40.8
Human 0.116
Dog 0.054
0
10
20
30
40
50
60
Time, s


500
400
300
200
100
0
P_{urea} x 10^6 cm s^{-1}
0
100
200
300
400
500
600
700
800
900
1000
Urea concentration, mM
Amphiuma
Duck
Chick
Human
Dog


1.00
0.10
0.01
$(a_t - a_\infty)\ (a_o - a_\infty)^{-1}$
0
10
20
30
40
50
60
70
Time, ms
T½ ms
Amphiuma 154
Duck 7
Chick 28
Human 12
Dog 8