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SEPARATE PATHWAYS FOR UREA AND WATER, AND FOR CHLORIDE IN CHICKEN ERYTHROCYTES

By JESPER BRAHM AND JENS OTTO WIETH
From the Department of Biophysics, University of Copenhagen, DK-2200 Copenhagen N, Denmark

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SUMMARY

1. Urea and water permeabilities of chicken erythrocytes are considerably lower than those of mammalian red cells.

2. The permeabilities to urea, thiourea and to *N*-methylurea (about 10^{-6} cm/sec at 25° C) were independent of concentration within a very broad range, and we found no evidence of interaction between transport of analogue molecules. The activation energies were between 17 and 19 kcal/mole, and urea transport was not inhibited by phloretin, which inhibits urea transport in mammalian red cells.

3. The water permeability of chicken red cells (as measured by the diffusion of tritiated water) was $1\cdot35 \times 10^{-3}$ cm/sec at 25° C. The activation energy was 10 kcal/mole, and the water permeability was not affected by phloretin or parachloromercuribenzoate.

4. It is concluded that the urea and water permeabilities of the chicken erythrocyte membrane are similar to those of a non-porous bimolecular phospholipid membrane.

5. Like the red cells of other animal species the chicken red cell membrane contains an anion transport system, mediating a rapid exchange of chloride across the cell membranes. The pH dependence, temperature dependence, and sensitivity to inhibitors were similar to the properties of the anion transport system found in mammalian red cells. Our study shows, therefore, that the transport system offers a highly specific pathway to the exchange of anions, without presenting an inspecific leak to the permeation of water and urea.

INTRODUCTION

Do the integral membrane proteins, which span the red cell membrane like a wick (Jenkins & Tanner, 1975), necessarily provide an indiscriminate leak-pathway for water and small hydrophilic molecules? Our negative answer to this question is based on evidence from studies of chloride, urea, and water transport in chicken red cell membranes.

A specific anion exchange mechanism with characteristic transport properties has so far been found in the membranes of erythrocytes from all animal species that have been studied (Wieth, Funder, Gunn & Brahm, 1974). Available evidence summarized by Rothstein, Cabantchik & Knauf (1976) and by Lepke, Fasold, Pring & Passow (1976) suggests that anion exchange is dependent on the integrity of an integral membrane protein with a molecular weight of 100,000, a protein which penetrates the hydrophobic core of the human red cell membrane in an S-shaped loop (Jenkins & Tanner, 1975). One might anticipate that this protein, which is involved in the anion exchange, inevitably will present an inspecific leak, open to the permeation of small polar molecules as, for example, water and urea. This view has already been posed by Brown, Feinstein & Shaáfi (1975), who suggested that the protein molecule 'forms part of the structure of aqueous channels which regulate non-carrier mediated transport in human erythrocyte membranes'.

Mammalian red cell membranes are considerably more permeable to urea and water than bimolecular phospholipid membranes (Poznansky, Tong, White, Milgram & Solomon, 1976). It has been suggested from studies of urea net fluxes (Macey & Farmer, 1970), and supported by studies of the exchange kinetics of thiourea and urea transport (Wieth *et al.* 1974), that the large urea permeability of human red cells is due to the existence of a specific passive transport mechanism rather than to an indiscriminate diffusion leak. This view was opposed by Poznansky *et al.* (1976), who concluded that urea transport in human red cells quantitatively fits to expectations for passive diffusion through aqueous channels, in which case the anion transport system might provide the leak channels as suggested by Brown *et al.* (1975).

We do not believe this is the case. Water and urea permeabilities of chicken red cells are known from the qualitative studies of Jacobs (summarized by Jacobs, Glassman & Parpart, 1950) to be very low. After having found that chicken red cells possess a chloride exchange mechanism as efficient and with the same properties as that of human red cells, we have, therefore, determined the urea and water permeabilities of chicken erythrocyte membranes. Both permeabilities were found to be as low as those of the cleanest bimolecular phospholipids studied. We can, therefore, conclude that the efficient anion exchange system does not offer a significant leak-pathway to the diffusion of small polar molecules through the chicken red cell membrane.

METHODS

All experiments were performed by measuring the rate of efflux of radioactive isotopes from packed labelled chicken erythrocytes suspended in a large extracellular compartment. In all experiments with chloride and water, $^{36}Cl^{-}$ and

^{3}HOH exchange were measured under steady-state conditions. A few experiments with urea and urea derivatives were performed as net flux experiments, measuring the net flux of, for example, urea into an initially urea free medium from cells loaded with the compound studied at a concentration of 100 mM.

Blood. Heparinized blood samples were taken from a wing vein of chicken (*Gallus domesticus* belonging to one of the two species: White Leghorn (single comb) and white Plymouth Rock). The animals were between 8 days and 2 yr of age. No permeability differences which could be attributed to age or race were observed during the study.

Mean corpuscular volume (MCV). This was determined on erythrocytes from ten animals by determining haematocrit, cell counts (Coulter Counter, Model DN) and the fraction of extracellular inulin trapped between the packed erythrocytes (centrifuged at 50,000 g for 15 min). The MCV was 128 μm^3 ($\pm$ 8·9 S.D.) in native plasma, and 134 μm^3 ($\pm$ 6·0 S.D.) in the glycyl–glycine buffered NaCl medium used for experiments. The fraction of extracellular medium trapped between the packed red cells (determined with ^{3}H-labelled inulin) was 1·7 % (w/w) (S.D. 0·1 %, $n = 10$).

Our value of MCV agrees well with the value of 127 μm^3 reported previously by Wintrobe (1933).

Surface area. The chicken red cell is a flat, oval nucleated cell with axes 12·1 and 7·3 μm long, and a thickness of about 1 μm of the non-nucleated part of the plasmalemma (Gulliver, 1875). Considering these values and a nucleus with a radius of 1·8 μm we calculated a surface area of 175 μm^2.

Radioactive isotopes. The following radioactive tracers were employed in the study: Radiochemical Centre, Amersham: [^{14}C]urea (60mc/m-mole), thiourea ([thio-^{14}C]urea, 60 mc/m-mole), [^{3}H]inulin (300 mc/m-mole), and $^{36}Cl^-$ (0·5 mc/m-mole). New England Nuclear Corporation: [*N*-methyl-^{14}C]urea (5 mc/m-mole). AEK RISØ, Denmark: ^{3}HOH (18 mc/mole). The amount of radioactivity employed for loading the red cells was in all cases between 0·5–1 μc/ml. cell suspension.

Determination of radioactivity. Radioactivity of cells and medium was determined by liquid scintillation after precipitating with perchloric acid as described by Dalmark & Wieth (1972). It was checked that the recovery of organic radioactive compounds was complete in the perchloric acid precipitates, and special precautions were only necessary for the quantitative recovery of ^{14}C-labelled thiourea. At low concentrations of thiourea (1 mM and below) up to 50 % of the radioactivity was lost following precipitation (presumably due to the formation of HSCN by oxidation). This loss could be completely prevented by adding 0·5 ml. 500 mM non-radioactive thiourea to the 200 mg samples of cells or medium before the addition of perchloric acid.

Equilibrium distribution of tracers between cells and medium. Before studying the rate of efflux chicken red cells were loaded with the isotope and with the chemical compound at the desired concentration by incubating them long enough to ensure complete equilibration across the cell membrane (i.e. longer than six half-times of the isotope exchange). Following equilibration in the appropriate medium, cells were isolated by centrifuging 15 min at 50,000 g, and the radioactivity of cells and medium was determined. The water content of the cells was determined by drying cell specimens to constant weight at 105° C, and correcting for the trapping of 2 % extracellular medium between the cells. The distribution ratio ($r = C_{in}/C_{out}$) where C_{in} and C_{out} are the concentrations of radioactivity in intra- and extracellular water phases was

Urea	1·07 (S.D. 0·027),	$n = 29$,
Thiourea	1·58 (S.D. 0·084),	$n = 16$,
Methylurea	1·00 (S.D. 0·024),	$n = 14$,
^{3}HOH	1·07 (S.D. 0·035),	$n = 13$.

Only the distribution of *N*-methyl-urea agrees with a simple distribution between the intra- and extracellular water phases. The deviation of r_{urea} from unity has been observed before by Murdaugh & Doyle (1961) and was ascribed to binding to haemoglobin. A similar explanation is likely to apply to the high value of $r_{thiourea}$. Values of the same magnitude are found in human red cells, and we have found that the distribution ratio in human erythrocyte ghosts where haemoglobin is absent is 1·0. The binding sites show an extremely low affinity both to urea and to thiourea, because the distribution ratios were constant within the range 10^{-6} to 1 M of the two compounds.

The distribution ratio for chloride is a function of pH, due to the chloride shift accompanying the titration of intracellular buffers. Our findings are reported in Fig. 6 of the Results section.

In the case of tritiated water the deviation of the distribution ratio from unity is due to isotope exchange with protons of haemoglobin (Benson, Fanelli, Giacometti, Rosenberg & Antonini, 1973). From the capacity of hydrogen exchange it can be calculated that the theoretical distribution ratio for tritiated water after complete equilibration of the hydrogen isotope will be 1·05–1·11 at an intracellular haemoglobin concentration of 6–7 mM.

We found that the binding of thiourea was not rate limiting for the tracer exchange, because the rate of exchange followed a perfect monoexponential course until at least 95 % of the tracer had been lost. In the case of tritiated water the results of Benson *et al.* (1973) show that the hydrogen exchange between water and haemoglobin is so slow (seconds to minutes) that it does not affect the measurement of the rapid water exchange across the membrane, which has a half-time considerably below 0·1 sec.

Electrolyte medium. The electrolyte medium had the following basic composition (mM): NaCl 145, $CaCl_2$ 1·5, $MgCl_2$ 1·0, D-glucose 5, and glycyl–glycine 27. The media were titrated to the appopriate pH with 1 M-NaOH.

The inhibitors used in the study were: phloretin (K and K Laboratories, Inc.) and 4,4-diisothiocyano-2,2-stilbene disulphonic acid (DIDS). The DIDS was kindly supplied by Dr Z. I. Cabantchik, The Hebrew University of Jerusalem. Phloretin was dissolved in ethanol and added to the medium to give a final concentration of 0·25 mM (ethanol 0·1 % v/v). The cells were equilibrated with 0·25 mM phloretin before the experiment was performed in a medium containing 0·25 mM phloretin. DIDS is an irreversible inhibitor of chloride transport in human red cells (Cabantchik & Rothstein, 1974). The chicken erythrocytes were treated with DIDS for 45 min at 38° C by adding 250 μl. 1 mM-DIDS solution to 5 ml. of a red cell suspension with a haematocrit of 30 %. After incubation the erythrocytes were isolated by centrifugation, and ^{36}Cl exchange was determined at 0° C in a DIDS-free medium.

The effect of parachloromercuribenzoate (PCMB, Sigma) on water transport (Fig. 3) was investigated after the cells had been incubated for 90 min at 25° C in a medium containing 1 mM-PCMB. Because PCMB induces a high cation permeability Na^+, Ca^{2+}, and Mg^{2+} were substituted by 150 mM-K^+ in these experiments.

Experimental procedures. The experimental procedure employed was chosen according to the rate of tracer exchange: when the rate coefficient of tracer efflux was less than 0·35 sec^{-1} (corresponding to a half-time of tracer exchange of more than two seconds) we employed the Swinnex–Millipore technique, described by Dalmark & Wieth (1972). Faster rates cannot be determined with a high degree of precision by this method, and such rates (all the water experiments and measurements of chloride exchange at temperatures above 15° C) were, therefore, measured by a flow-tube technique in the version described by Brahm (1976). In both cases the isotope efflux takes place into a very large extracellular compartment (haemato-

crit below 1 %). The rate of tracer efflux was well described in all experiments by a two compartment model with compartments of constant volume, and the kinetics were found to follow the equation

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

where a_t is the concentration of extracellular activity at the time of sampling (t), a_∞ the concentration after isotopic equilibrium has been achieved, k (sec^{-1}) the rate coefficient of tracer efflux, and a_0 is the concentration of radioactivity at time = 0, due to the introduction of extracellular medium trapped between the packed cells injected into the reaction vessel (Swinnex–Millipore method) or into the mixing chamber of the flow tube. The rate coefficient (k) was determined by linear regression analysis of a plot of ln $(1-a_t/a_\infty)$ *vs.* t, and converted to the permeability (cm/sec) as described in eqn. (2) below.

In a similar way the standard deviation of the permeability in individual experiments was calculated from the standard deviation of the rate coefficient (k), which was determined by the linear regression analysis.

Calculation of permeabilities. The permeabilities were calculated from the measured rate coefficients of tracer efflux according to the equation

$$J^* = k(V/A)\,(C^*_{in}-C^*_{out}), \quad (2)$$

where J^* is the tracer flux (c/cm^2.sec), k is the rate coefficient of tracer efflux (sec^{-1}), V is the intracellular solvent volume ($134 \times 0{\cdot}72 = 96{\cdot}5\ \mu m^3$) and C^*_{in} and C^*_{out} are the tracer concentrations in the intra- and extracellular water phases. The experiments were carried out at a haematocrit below 1 %, and the tracer efflux was found to follow first order kinetics. According to eqn. (2) the rate coefficient of tracer efflux (k, sec^{-1}) is converted into a permeability coefficient (cm/sec) by multiplication with $V/A = 5{\cdot}5 \times 10^{-5}$ cm (cells at 0° C, pH 7·2). In the case of urea transport the permeability was found to be independent of concentration, whereas the apparent permeability of a saturable transport, as the chloride transport system, will be a function of chloride concentration. Changes of volume with extracellular pH was determined by drying red cells to constant weight, and appropriate values of V/A were applied to calculate the results shown in Table 6 and Fig. 5.

RESULTS

Urea and urea-derivatives

Concentration dependence

The permeability of the chicken red cell membrane to urea, methyl-urea, and thiourea appeared to be independent of concentration within a very broad range (Table 1). The membrane tolerated concentrations of 0·5 M methyl-urea and 1 M urea without any sign of deterioration, whereas haemolysis (and increased permeability) was often seen when the thiourea concentration exceeded 300 mM.

Table 2 shows that the same permeabilities to urea were found in experiments where we measured the rate of self-exchange of urea, and in experiments where we determined the net flux of urea into a urea-free medium. Similar results were obtained with methyl-urea and thiourea.

TABLE 1. Permeability to urea, *N*-methyl-urea, and thiourea as a function of concentration. The self-exchange of [^{14}C]-labelled urea derivatives was measured on red cells which had been equilibrated previously at the concentrations of urea or urea derivatives stated in the Table (0° C, pH 7·20). A similar independence of urea permeability on concentration was found in experiments at 20° C. S.D. of the rate coefficients are stated in parentheses

Compound	Concentration (mM)	Rate coefficient of self-exchange k (min^{-1})	Permeability P (cm/sec × 10^7)
Urea	1	0·131 (±0·004)	1·21
	100	0·132 (±0·002)	1·22
	300	0·134 (±0·002)	1·23
	500	0·123 (±0·001)	1·13
	1000	0·114 (±0·001)	1·05
N-methyl urea	1	0·120 (±0·002)	1·11
	100	0·127 (±0·003)	1·17
	300	0·118 (±0·002)	1·09
	500	0·125 (±0·002)	1·15
Thiourea	1	0·099 (±0·001)	0·91
	100	0·080 (±0·001)	0·80
	300	0·095 (±0·004)	0·88
	500	Haemolysis	

TABLE 2. Comparison of the rates of [^{14}C]urea efflux during self-exchange and net-flux. Red cells from two animals were loaded with urea at concentrations of 1 and 100 mM and labelled with [^{14}C]urea. The rate of efflux was determined at a haematocrit of 0·5% in media with and without urea. S.D. of the rate coefficients are stated in parentheses. There was no detectable difference in urea permeability under the two sets of conditions. The experiments were carried out at 0° C, pH 7·20

Type of exp.	Initial urea conc. of cells (mM)	Initial urea conc. of medium (mM)	Rate coefficient of [^{14}C] urea efflux k (min^{-1})	Permeability P (cm/sec × 10^7)
Exchange	1	1	0·134 (±0·002)	1·23
Net-flux	1	0	0·122 (±0·001)	1·12
Exchange	100	100	0·128 (±0·002)	1·17
Net-flux	100	0	0·131 (±0·005)	1·20
Exchange	1	1	0·129 (±0·002)	1·18
Net-flux	1	0	0·138 (±0·003)	1·27
Exchange	100	100	0·126 (±0·003)	1·16
Net-flux	100	0	0·129 (±0·002)	1·18

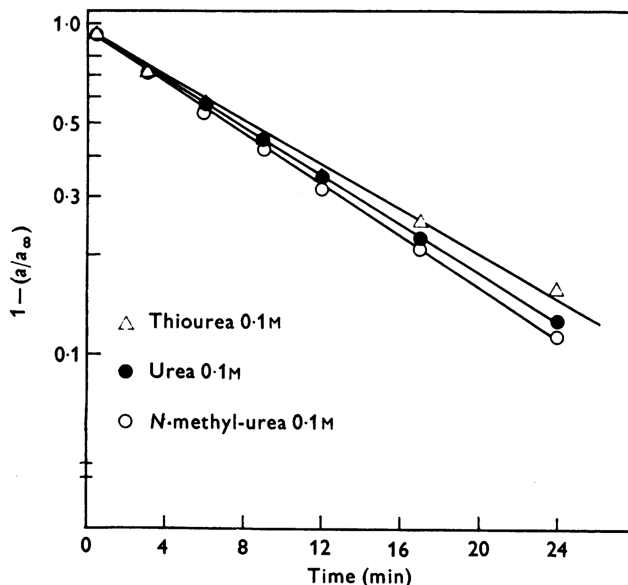

Fig. 1. The rate of urea, thiourea and of *N*-methyl-urea self-exchange in chicken red cells measured as the tracer wash-out from the labelled cells into a medium with the same composition as the incubation medium. The logarithmic ordinate shows the fraction of tracer remaining in the cells at a given time. The rate coefficients are equal to the negative slope of the curves because the exchange is well described by a closed two compartment system with an extracellular volume of 99 % of the total volume. The experiments were carried out at 0° C and pH 7·2. The permeability coefficients of the three compounds were calculated as described in the Methods section and are stated in Table 3.

TABLE 3. Comparison of the permeability of chicken red cells to urea, thiourea, and *N*-methyl urea. The permeabilities were determined at 0° C, pH 7·20, at a concentration of 100 mM. The experiments in the three series were performed with the three compounds on red cells from the same chicken, because permeabilities varied by a factor of 2–3 when the transport in red cells from different animals was compared. S.D. of the permeabilities in individual experiments are stated in parentheses. The total ranges of variation found at 0° C, pH 7·20, were: urea (n = 58): 0·5–1·4 $\times 10^{-7}$ cm/sec; thiourea (n = 20): 0·7–1·6 $\times 10^{-7}$ cm/sec; *N*-methyl urea (n = 8): 0·8–1·8 $\times 10^{-7}$ cm/sec

Animal no.	Permeability P (cm/sec $\times 10^7$) Urea	Thiourea	*N*-methyl-urea
1	0·77 (± 0·01)	0·69 (± 0·01)	0·81 (± 0·01)
2	1·41 (± 0·03)	0·91 (± 0·03)	1·00 (± 0·03)
3	1·80 (± 0·04)	1·58 (± 0·02)	1·43 (± 0·03)

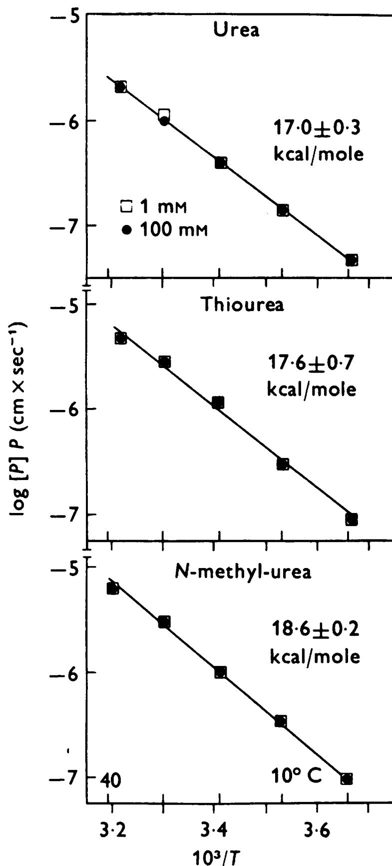

Fig. 2. The temperature dependence of permeability to urea, thiourea and to *N*-methyl-urea in chicken red cells at 0–40° C (pH 7·2). The logarithm of the permeability is displayed as a function of the reciprocal of the absolute temperature. The activation energies were calculated by linear regression analyses of the function: $\log P = (E_A/2{\cdot}3 . R)(1/T) + \text{const.}$, where E_A is the activation energy (cal/mole), R the gas constant (1·99 cal/mole.K) and T the absolute temperature. The transport of both urea and the two derivatives has an activation energy of 17–19 kcal/mole.

Comparison of permeabilities to urea, thiourea, and methyl-urea

Although repeated measurements of the permeabilities of red cells from a single animal showed a variation of less than 10% from day to day, we found variations by a factor of 2–3 of the permeabilities of cells from different chickens. A comparison of the permeabilities to urea and its two derivatives was, therefore, performed on red cells from three animals. The results, which are shown in Fig. 1 and Table 3, demonstrate that the

chicken red cell membrane shows no selectivity towards the three compounds.

Temperature dependence

The temperature dependence of the permeability was determined in the range between 0 and 38° C. Fig. 2 shows the three Arrhenius diagrams depicting the permeability as a logarithmic function of the reciprocal absolute temperature. In all three cases we found an Arrhenius activation energy between 17 and 19 kcal/mole. The permeabilities at 25° C (where permeability data from bimolecular lipid membranes are available for comparison) were: urea $0{\cdot}7 \times 10^{-6}$, thiourea $1{\cdot}5 \times 10^{-6}$, and methyl-urea $1{\cdot}7 \times 10^{-6}$ cm/sec.

The effect of thiourea on urea transport

In contrast to the findings in mammalian cells (Wieth *et al.* 1974) we found that transport of urea in chicken red cells is affected very little by

TABLE 4. The effect of thiourea on urea permeability at a constant urea concentration of 1 mM. The self-exchange of urea was measured at 20° C, pH 7·2, on red cells which had been equilibrated previously with a medium containing 1 mM urea and the thiourea concentrations stated in the Table

Thiourea conc. (mM)	Rate coefficient of urea self-exchange k (min^{-1})	Urea permeability P (cm/sec $\times 10^7$)
0	0·746	6·8
0	0·712	6·6
100	0·754	6·9
300	0·675	6·2
500	0·582	5·3
500	0·601	5·5

the presence of other urea-derivatives. The results in Table 4 show that urea permeability at a low urea concentration was not affected by the presence of 300 mM thiourea, but at a concentration of 500 mM thiourea – a concentration which is only tolerated by some erythrocyte samples without haemolysis (cf. Table 1) – we found a decrease of urea permeability by 20–25 %.

Effect of phloretin on urea transport

Phloretin inhibits a number of transport processes in the red cell membrane. The results of five experiments (Table 5) show that we found a small, insignificant increase of urea permeability in the presence of 0·25 mM phloretin, a concentration which as shown below inhibited chloride transport in chicken red cells by 99·5 %.

The pH dependence of urea transport

Urea, thiourea, and methyl-urea permeabilities were determined at pH 5·9, 7·2 and 8·1. The results are shown in Table 6. The volume variation of chicken red cells with pH was determined in order to be able to convert the rate coefficients of exchange into permeabilities. A decrease of permeability with increasing pH was found with all three compounds studied, most pronounced in the case of urea and thiourea.

TABLE 5. Urea permeability of chicken red cells in the absence and presence of phloretin (0·25 mM). The experiments were carried out at 0° C, pH 7·20. The slight increase of urea permeability is not statistically significant: $0 \cdot 2 < P < 0 \cdot 3$ (Students *t* test). S.D. of the rate coefficients are stated in parentheses

	Phloretin 0 mM		Phloretin 0·25 mM		
Urea conc. (mM)	Rate coefficient of exchange k (min^{-1})	Permeability P (cm/sec × 10^7)	Rate coefficient of exchange k (min^{-1})	Permeability P (cm/sec × 10^7)	Increase of permeability (%)
1	0·134 (±0·002)	1·23	0·148 (±0·001)	1·35	+10
100	0·164 (±0·003)	1·50	0·171 (±0·002)	1·56	+ 4
100	0·128 (±0·002)	1·17	0·131 (±0·005)	1·20	+ 3
100	0·131 (±0·001)	1·20	0·161 (±0·001)	1·48	+23
100	0·147 (±0·001)	1·34	0·158 (±0·002)	1·45	+ 8

TABLE 6. pH dependence of chicken red cell permeabilities to urea, thiourea, and *N*-methyl-urea at 0° C (100 mM). S.D. of the permeabilities are stated in parentheses. Volume variation of the red cells was determined by drying cells to constant weight after equilibration of the cells at appropriate pH. By linear regression analysis the water content was found to vary as a function of pH: cell water (kg/kg solids) = 3·57 (±0·10)–0·234 (±0·014) (pH). Assuming the solids to have a specific gravity of 1·3 cell volume decreased from 145 to 120 μm^3, when pH increased from 5·9 to 8·1

	Permeability P (cm/sec × 10^7)		
pH	Urea	Thiourea	*N*-methyl-urea
5·9	1·75 (±0·03)	1·66 (±0·03)	0·99 (±0·02)
7·2	1·20 (±0·01)	0·98 (±0·01)	0·93 (±0·02)
8·1	0·67 (±0·01)	0·98 (±0·02)	0·81 (±0·02)

Water

The osmotic water permeability of chicken red cells is much lower than that of mammalian erythrocytes (Macey & Farmer, 1970; Blum & Forster, 1970), but the permeability of chicken erythrocytes to water

diffusion has not been measured. Table 7 shows the results of our measurements of the self-exchange of tritiated water at 25° C. The average permeability was $1{\cdot}35 \times 10^{-3}$ cm/sec. This permeability was not altered by treating the cells with phloretin (Fig 3*A*), and neither was the water permeability affected by DIDS, which as shown in the following section are potent inhibitors of chloride exchange in chicken red cells.

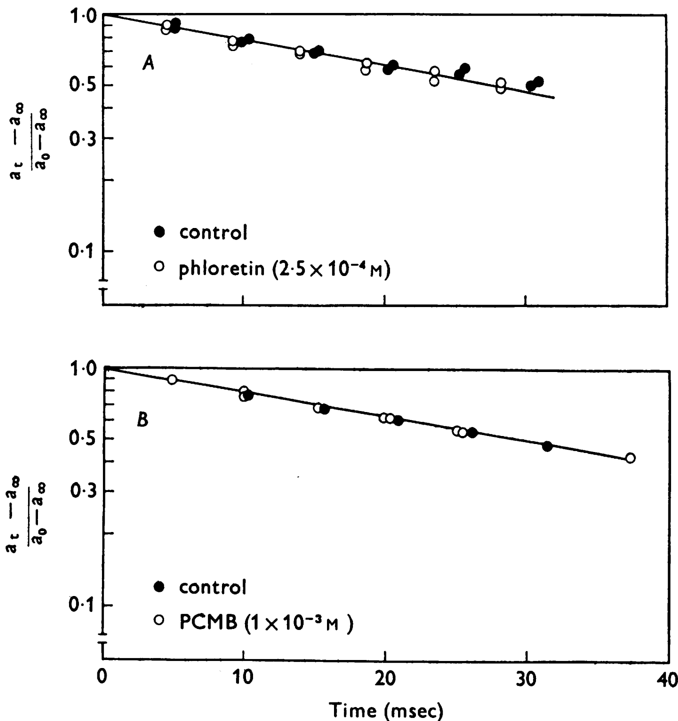

Fig. 3. The rate of ^3H_2O-exchange in chicken red cells at 25° C and pH 7·4. The results were obtained by means of the flow-tube method. Incubation of the cells with phloretin (*A*) or parachloromercuribenzoate (*B*) did not affect the permeability of water. The permeability coefficients are stated in Table 7.

As shown in the Discussion section, the water permeability of chicken erythrocytes is as low as that of bimolecular lipid membrane. The higher water permeability of human red cells can be reduced to the same low level by treating the cell membranes with PCMB (Macey, Karan & Farmer, 1972). PCMB is believed to block a permeation pathway by reacting with sulfhydryl groups in the membrane proteins. The water permeability of chicken red cells is so low that one might anticipate that permeation through the membrane proteins plays no role. Our finding

(Table 7, Fig. 3*B*) that PCMB did not alter the water permeability of chicken red cell membranes is in agreement with this possibility.

A study of the effect of temperature on the rate of water diffusion (Fig. 4) showed that the activation energy was 10 kcal/mole, the same magnitude as found for osmotic water transport by Macey & Farmer (1970), and considerably larger than that found in mammalian red cells.

TABLE 7. The water permeability of chicken red cells at 25° C, pH 7·4. The Table includes fifteen experiments performed with five batches of cells. The addition of phloretin* ($2 \cdot 5 \times 10^{-4}$ M), PCMB† (1×10^{-3} M) or DIDS‡ (500 μl. 1 mM-DIDS solution per 10 ml. cell suspension) showed no effect on the permeability

Expt. no.	Rate coefficient of water self-exchange k (sec^{-1})	Water permeability P (cm/sec × 10^3)
1	22·67	1·25
	30·95	1·71
2	21·67	1·19
	24·51	1·35
3	22·44	1·24
	25·14	1·39
	24·37*	1·34
	25·61*	1·41
4	22·30	1·23
	21·25†	1·19
	23·16†	1·28
5	27·04	1·49
	26·19	1·44
	25·16‡	1·39
	24·01‡	1·32
Mean	24·43	1·35
S.D. (n = 15)	2·48	0·14

Chloride

Chloride exchange through the chicken red cell membrane appeared to occur by a mechanism which is quite similar to that found in all other animal erythrocytes, which have been examined so far (Wieth *et al.* 1974). Fig. 5 shows the self-exchange as a function of extracellular pH at 0° C. Both the bell shape of the graph and the location of the acidic branch is similar to that found in human red cells, where the flux is also halved at a pH near 6 (Gunn, Dalmark, Tosteson & Wieth, 1973; Dalmark, 1975; Funder & Wieth, 1976). Fig. 6 shows the variation of the chloride distribution and of the rate coefficient of chloride exchange which was very

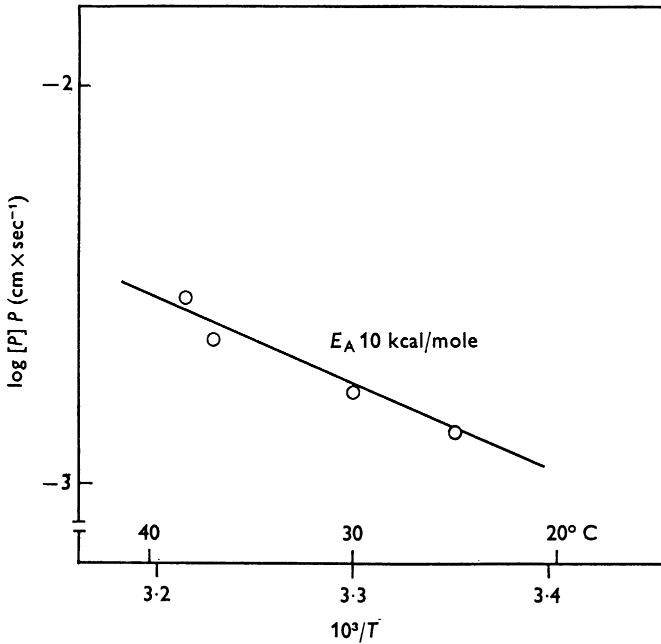

Fig. 4. The temperature dependence of water transport in chicken red cells between 25° and 38° C at pH 7·4.

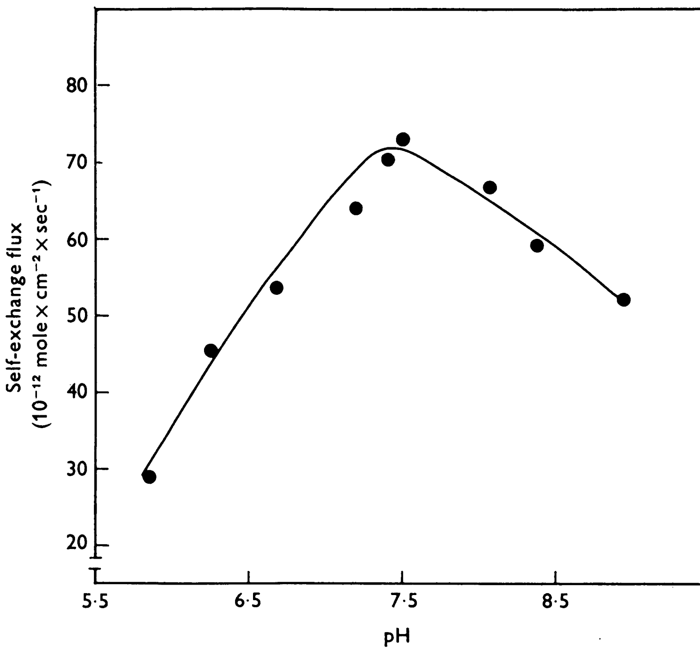

Fig. 5. Chloride self-exchange in chicken red cells at 0° C as a function of extracellular pH.

similar to the findings in human red cells (Gunn *et al.* 1973). We next examined the temperature dependence of chloride exchange in the range between 0 and 40° C (Fig. 7). The Figure shows that the apparent chloride permeability had the same high temperature dependence as found in

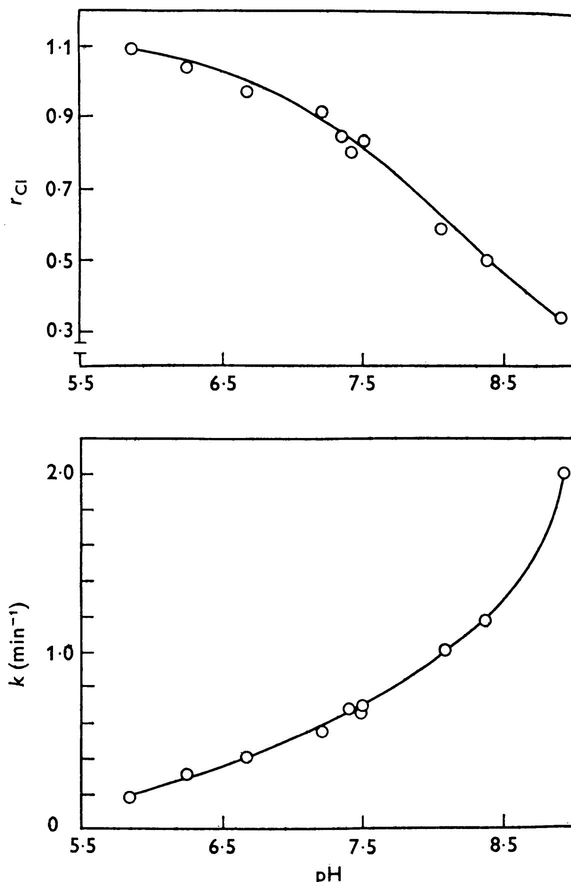

Fig. 6. Chloride distribution between extra- and intracellular water phases (r_{Cl}, upper frame) and the rate of chloride self-exchange (k min^{-1}, lower frame) as a function of extracellular pH at 0° C (same set of expts. as shown in Fig. 5).

human red cells (Brahm, 1976), increasing from 10^{-6} to 10^{-3} cm/sec between 0° C and 40° C. The activation energy decreases from 30 to 20 kcal/mol in human red cells when the unidirectional chloride flux exceeds a rate of 4×10^9 ions per second per cell. Fig. 7 shows that a similar decrease (from 33 to 23 kcal/mole) was found in chicken red cells at a temperature of about 20° C, where the transport rate was 8×10^9 ions

per cell per sec. The same decrease of activation energy was found regardless of whether the calculations were based on the apparent chloride permeabilities as shown in the Figure or on the magnitudes of unidirectional chloride fluxes.

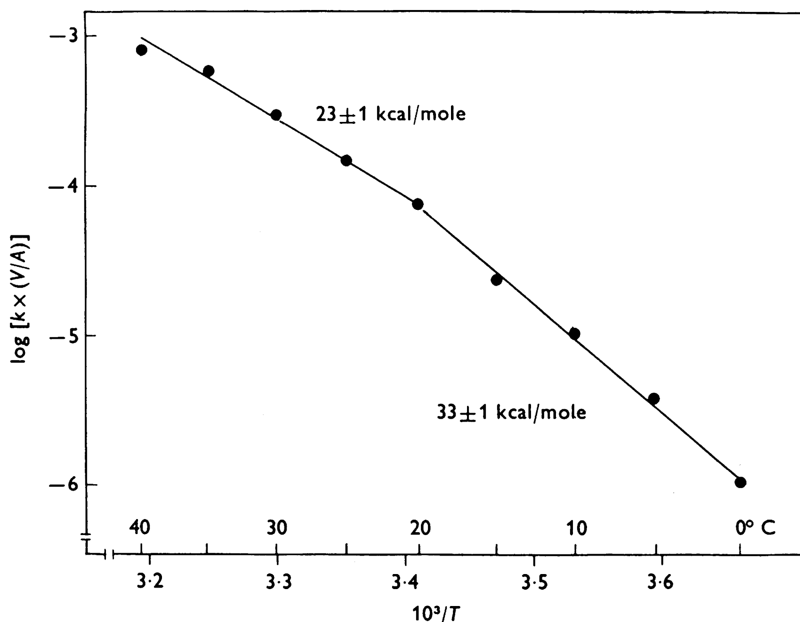

Fig. 7. The temperature dependence of chloride permeability in chicken red cells at 0–40° C (pH 7·4). From 0–15° C the results were obtained by means of the Millipore–Swinnex filtration technique. At the higher temperatures the flow-tube method was used. The results suggest that the activation energy decreases from 33 to 23 kcal/mole at temperatures above 20° C.

We have tested the effect of a potent non-specific inhibitor of chloride transport (phloretin) and of the most specific inhibitor known, the amino reagents DIDS (Cabantchik & Rothstein, 1974). Phloretin inhibits a multitude of facilitated transport processes in red cells (see Discussion section), whereas DIDS so far has been found to inhibit only the chloride transport system. The results shown in Table 8 demonstrate that both agents are potent inhibitors of chloride exchange in chicken red cells, although the 90 % inhibition caused by DIDS-treatment is smaller than the 99 % inhibition of chloride transport found in human erythrocyte membranes (Funder & Wieth, 1976).

It may be noted that the exchange flux in the control experiments of Table 8 is 1·6 times higher than the maximum found in the pH studies

(Fig. 5). We have observed such variations between different broods of animals, although the day to day reproducibility of results obtained with blood from animals of the same brood was excellent.

TABLE 8. Inhibition of chloride transport in chicken erythrocytes by phloretin and DIDS at 0° C, pH 7·4

Inhibitor	Rate coefficient of chloride exchange k (sec^{-1})	Unidirectional flux $J_{uni} \times 10^{12}$ (mole/cm^2 × sec)	Flux as % of control
None	$1·74 \times 10^{-2}$	124	100
None	$1·68 \times 10^{-2}$	120	
Phloretin	$7·3 \times 10^{-5}$	0·5	0·4
Phloretin	$5·5 \times 10^{-5}$	0·4	0·3
DIDS	$1·88 \times 10^{-3}$	13·4	10·8
DIDS	$1·85 \times 10^{-3}$	13·2	10·8

DISCUSSION

As discussed below our study shows that water and urea permeabilities of the chicken erythrocyte membrane are as low as those of a bimolecular phospholipid membrane. In spite of this indisputable evidence of a continuous hydrophobic barrier towards the permeation of small polar molecules the same membrane permits a rapid exchange of chloride ions between the intra- and extracellular water phases, an exchange which exceeds that found in phospholipid vesicles by five to six orders of magnitude (Toyoshima & Thompson, 1975). It seems, therefore, evident that the chloride transport system offers a highly specific pathway to anions, a path which does not present a leak to urea or to water molecules.

Urea permeability

At 25° C, where data for comparison with bimolecular lipid membranes are available, the urea permeability of chicken red cells (Fig. 2) is a little less than 10^{-6} cm/sec. In lecithin bilayers Vreeman (1966) obtained a value of $4·2 \times 10^{-6}$ cm/sec at 20° C, Galucci, Micelli & Lippe (1971) found a permeability of $3·7 \times 10^{-6}$ cm/sec at 28° C, and Poznansky *et al.* (1976) found an urea permeability across egg lecithin spherical bilayers of $4·1 \times 10^{-6}$ cm/sec at 25° C. The work of Galucci *et al.* (1971) also shows that the permeability to thiourea ($4·3 \times 10^{-6}$ cm/sec) was only slightly higher than the urea permeability, in spite of the fact that the partition coefficient of thiourea between olive oil and water ($1·2 \times 10^{-3}$, Collander & Bärlund, 1933) is almost tenfold higher than that of urea ($1·5 \times 10^{-4}$). This finding stresses the observation of Poznansky *et al.* (1976) that the permeation of

small amides is not just a simple function of partitioning, but depends also on the rate coefficient for entrance into the membrane from the aqueous environment.

The only kinetic study of urea transport in other slowly transporting red cells, the red cells of the spiny dogfish (Rabinowitz & Gunther, 1973), agrees well with our findings in avian cells. Urea permeability was found to be independent of concentration (except maybe at the highest concentrations of about 1 M). At a low urea concentration urea permeability was not affected by the presence of 1–700 mM methyl-urea, the activation energy calculated from two of their experiments at 3° and 9° C was 15–20 kcal/mole, and the urea permeability was as low as that found by us. The activation energy of urea transport in erythrocytes with a low urea permeability (Fig. 2) is somewhat higher than that found in human red cells of 11 kcal/mole (Galey, Owen & Solomon, 1973). In bimolecular phospholipid membranes values of 12–14 kcal/mole have been found by Poznansky *et al.* (1976).

Attempts to extend the observations of Jacobs (1931) of species differences in red cell urea transport by a simple volumetric technique has not given much new information, and the results of Hunter, George & Ospina (1965) show that the light scattering method is too crude to demonstrate facilitated diffusion of slowly transported urea derivatives as, for example, the facilitated diffusion of thiourea in human erythrocytes, which can easily be demonstrated by kinetic studies of the transport process itself (Wieth *et al.* 1974).

Kaplan, Hays & Hays (1974) concluded that a significant decrease of urea transport by phloretin indicates the presence of a saturable facilitated transport system for urea, whereas phloretin does not inhibit urea transport by simple diffusion. This is in agreement with the observations that phloretin did not reduce urea transport in chicken red cells (Table 5), where there is no evidence of a specialized urea transport system, whereas phloretin inhibition of the facilitated urea transport in human red cells has been reported repeatedly (Macey & Farmer, 1970; Wieth *et al.* 1974; Kaplan *et al.* 1974). Poznansky *et al.* (1976) reported a doubling of the urea permeability of lipid bilayers (red cell lipid liposomes) by 0·25 mM phloretin, and ascribed the permeability increase to a liquifying effect of phloretin on the lipids. We could not have missed an effect of this magnitude in chicken red cells, where the same phloretin concentration only caused an insignificant increase of urea permeability (Table 5). It is possible that the lipids in the red cell membrane are stabilized through their interaction with membrane proteins. Neither can it be excluded, as mentioned above, that entrance of urea into the lipid phase is a major rate limiting step for urea transport through the chicken red cell membrane,

reducing the importance of lipid viscosity for the over-all rate of urea transport.

Water permeability

The osmotic water permeability of chick red cell membranes has been determined by Blum & Forster (1970) who in two experiments found permeabilities of 1·3 and 4×10^{-3} cm/sec. The value found by Farmer & Macey (1970) was slightly lower ($0{\cdot}9 \times 10^{-3}$ cm/sec at 25° C). Farmer & Macey noted that the osmotic filtration coefficient of chicken red cells was lower than the one found in beef and human red cells by one order of magnitude, and also reported an activation energy of 11 kcal/mole for water transport through the chicken red cell membrane, in contrast to the values for mammalian red cells of 4–6 kcal/mole (see Forster, 1971).

The diffusional water permeability of chicken red cells of $1{\cdot}35 \times 10^{-3}$ cm/sec found by us (Table 7) is very close to the values reported for osmotic permeabilities by Blum & Forster (1970) and by Farmer & Macey (1970). Price & Thompson (1969) and Redwood & Haydon (1969) calculated that the theoretical activation energy of water diffusion through the hydrocarbon phase of a lipid membrane would be 12 kcal/mole. Their experimental values were of the same magnitude, and so were the values for the activation energies of water filtration (Macey & Farmer, 1970) and of water diffusion (Fig. 4) through the chicken red cell membrane. The fact that water transport by osmosis in mammalian red cells has a higher permeability than water transport by diffusion seems to indicate that water molecules may interact during filtration in a similar way to that described in artificial porous membranes (Mauro, 1960). It became clear from the work of Cass & Finkelstein (1967) and of Redwood & Haydon (1969) that osmotic and diffusional water permeabilities approach the same low value in non-porous bimolecular phospholipid membranes. The absolute value of this permeability varies with the composition of the lipid membrane, but the lowest permeabilities of 10^{-3} cm/sec have been found in cholesterol containing membranes (Cass & Finkelstein, 1967). It can, therefore, be concluded that the water permeability of the chicken red cell membrane is of the same magnitude as that found in artificial phospholipid membranes.

Chloride permeability

Following the above considerations the conclusion is that the diffusion of water, urea, and of urea derivatives through the red cell membrane can be accounted for by diffusion through the membrane lipids. Still the membrane contains an anion exchange transport system which can transfer chloride ions through the membrane at a rate which is several orders of magnitude higher than that found in bimolecular lipid mem-

branes. Toyoshima & Thompson (1975) have recently examined the electrically silent exchange of chloride ions through phospholipid membranes, an exchange which was ascribed to the ion-pairing of chloride ions with phospholipid molecules flip-flopping from one side of the membrane to the other. Chloride exchange by this mechanism has a permeability coefficient of $11{\cdot}3 \times 10^{-11}$ cm/sec at 20° C, about 10^6 times lower than the chloride exchange permeability of the chicken red cell (cf. Fig. 7).

Our study clearly shows that the chloride transport system in the chicken red cell membrane does not present an aqueous channel or a leak to small polar molecules as recently proposed by Brown *et al.* (1975). Of course the complete similarity between the chloride transport system in chicken red cells and that of mammalian cells does not prove that this consideration also applies to mammalian cells, although it is possible that it does. The pH dependence, temperature dependence, and the effect of inhibitors on chloride transport are all alike in human and chicken red cells. The 'kink' in the Arrhenius diagram (Fig. 7) is similar to that found in human red cells, where it has been ascribed to a change of activation energy occurring when a critical transport rate is reached (Brahm, 1976). This critical rate was of the same magnitude in cells from the two species: 4×10^9 ions per sec per cell in human red cells to be compared with 8×10^9 in the chicken cell. The difference is somewhat smaller if the comparison is based on the membrane areas of the two cell types (140 *vs.* 175 μm^2) and both cell types also present the same density of intra-membrane particles by freeze-fracture electron microscopy (Gazitt, Ohad & Loyer, 1976).

Pathways to chloride, urea, and water in human red cells

If it is accepted that the present results make it possible that the chloride transport system also in human red cells is tight to the permeation of urea and water, one may proceed to consider whether urea and water share a common pathway through the highly permeable human red cell membrane.

After treatment of human red cells with *p*-chloro-mercuribenzoate (PCMB) or *p*-chloro-mercuribenzosulphonic acid (PCMBS) urea and water permeabilities of human red cells can both be reduced to levels which are comparable to those of chicken red cells and of bimolecular phospholipid membranes (Macey & Farmer, 1970; Macey *et al.* 1972). Osmotic water permeability decreased from 20×10^{-3} to 2×10^{-3} cm/sec, the ratio of osmotic permeability to diffusional permeability (P_{osm}/P_{diff}) became close to unity, and the activation energy of water transport increased from 4 to 11·5 kcal/mole. At the same time urea permeability decreased by a factor of 10, so that the sulfhydryl reagents brought the permeability properties of the human red cell membrane quite close to those of a lipid bilayer.

However, urea permeability of human red cells could be decreased by a factor of 50 by phloretin with negligible effect on water permeability. This observation made Macey & Farmer (1970) conclude that 'it would appear that water channels transport water and very little else'.

This statement was based on an interpretation of the phloretin inhibition results. However, the effect of phloretin on the membrane is complicated. It has been demonstrated in permeability studies that phloretin affects the permeation of both hydrophilic and of lipophilic molecules (Owen & Solomon, 1972), and recent binding studies have shown that phloretin binds to both proteins and lipids in the membrane (Jennings & Solomon, 1976).

It is a general property of phloretin that it increases permeation processes taking place through the lipids of the membrane (Owen & Solomon, 1972; Wieth, Dalmark, Gunn & Tosteson, 1973; Andersen, Finkelstein, Katz & Cass, 1976), whereas it inhibits facilitated diffusion processes of hexoses (LeFevre & Marshall, 1959), erythritol (Wieth, 1971), glycerol (Macey & Farmer 1970; Carlsen & Wieth, 1976), chloride (Wieth *et al.* 1973), and urea and urea-derivates (Macey & Farmer, 1970; Wieth *et al.* 1974). It can, therefore, be argued that the finding by Macey & Farmer (1970) and by Owen & Solomon (1972) of an unchanged or slightly increased osmotic water permeability of phloretin treated human red cells in the presence of a decreased urea permeability could be the over-all result of two effects: (i) a decreased water flow through a protein pathway (maybe the urea pathway), and (ii) a compensating increase of water diffusion through a liquefied lipid membrane phase.

The results obtained on chicken red cells do not support the idea that water diffusion through the lipids is increased by phloretin (Table 7, Fig. 3). The possibility can also be tested in human red cells, where phloretin should cause a considerable increase on water diffusion, in contrast to the negligible effect on osmotic water transport, because the ratio P_{osm}/P_{diff} should approach unity if water transport after phloretin treatment took place through a non-porous lipid phase (Cass & Finkelstein, 1967). However, we have found that 0·25 mM phloretin only causes a 30 % increase of water diffusion through human red cell membranes (J. Brahm, unpublished results), an increase of exactly the same magnitude as found by Owen & Solomon for the osmotic flow of water.

Although the question cannot be finally settled yet, it appears likely to us that the interpretation of Macey & Farmer is essentially correct, namely that phloretin in human red cells closes a pathway to urea, which is not a diffusion pathway to water.

We have elsewhere presented our preliminary characterization of the facilitated urea transport in human red cells (Wieth *et al.* 1974). Further

experimental characterization of this transport system is still in progress. Another possible line of studying the specificity of facilitated transport systems appears from the aspects of comparative physiology, which in the present work has been used to separate the anion transport pathway from that (or those) of urea and water. It appears possible to find examples in nature of the existence of separate pathways to the permeation of urea and water: by examining the classical qualitative studies of Jacobs (1931) it appears that amphibian red cells have a high permeability to urea although the water permeability is low. We have confirmed that the red cells of *Amphiuma means* possess a saturable urea transport system with a high permeability together with a low water permeability of 7×10^{-4} cm/sec at 25° C (J. Brahm, U. V. Lassen & J. O. Wieth, unpublished observation). The data of Jacobs also predict that a relatively *high* water permeability together with a low urea permeability is found in the red cells from some birds (pigeon and duck) and from certain fish (mackerel, bonito and hickory shad). Therefore, it seems likely that further comparative studies may solve the problem of whether a channel which is accessible to the osmotic bulk flow of water can be closed to the permeation of urea, and whether a high urea permeability mediated by a specialized transport system can exist in a 'water tight' cell membrane, confirming the prediction of Jacobs (1931) that studies of red cells from different animal species may furnish 'to the cell physiologist the useful tool of comparative physiological knowledge for attacking such general problems as, for example, that of cell permeability'.

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