Diffusional Solute Flux During Osmotic Water Flow Across the Human Red Cell Membrane

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ABSTRACT The effect of solvent drag on the unidirectional efflux of labeled water, urea, and chloride from human red cells was studied by means of the continuous flow tube method under conditions of osmotic equilibrium and net volume flow. Solvent (water) flow out of cells was created by mixing cells equilibrated in 100 mM salt solution with a 200-mM or 250-mM salt solution, while flow of water into cells was obtained by equilibrating the cells in the higher concentration and mixing them with the 100-mM solution. Control experiments constitute measurements of efflux of [14C]ethanol in normal cells and 3H2O in cells treated with p-chloromercuribenzenesulfonate under the conditions described above. In both instances, the solute is known to penetrate the membrane through nonporous pathways. As anticipated, the tracer flux of neither urea nor chloride showed any dependence on net solvent flow, regardless of the direction. If one assumes the recently reported reflection coefficient for urea of 0.7, the urea tracer flux should change by at least 24% under volume flow conditions. Since such changes would be easily detected with our method, we conclude that the pathways for water, for urea, and for chloride are functionally separated.

INTRODUCTION

30 years ago, it was reported that osmotic water permeability (Pf) was greater than diffusive water permeability (Pd) in human red cells (Sidell and Solomon, 1957; Paganelli and Solomon, 1957). The difference between Pf and Pd led Solomon (1968) to suggest, by analogy with studies of porous artificial membranes, that the human red cell membrane contains pores. He calculated a pore radius for these membranes of 4.5 Å. A porous nature for the human erythrocyte membrane also appeared to be consistent with the finding that small hydrophilic solutes, such as urea, permeate the membrane more readily than expected from the simple relation between sol-
ute permeability and lipid solubility originally suggested by Overton (1895). Thus, the human red cell membrane was considered inhomogeneous, with both hydrophilic and lipophilic pathways being present. The hydrophilic pores provided a pathway for water and small hydrophilic solutes, with radii of <4.5 Å, whereas larger hydrophilic solutes, such as sucrose, were hindered in entering the pore because of their greater molecular size.

The inhomogeneity of the erythrocyte membrane has indeed been verified within the last decade. Increasing evidence confirms the "fluid mosaic membrane" model of Singer and Nicolson (1972), with integral membrane proteins spanning the membrane from the interior to the exterior side. A major fraction of the integral proteins, designated as band 3 because of its location in sodium dodecyl sulfate gel electrophoresis, has been named capnophorin because of its involvement in the transport of HCO₃⁻ and Cl⁻ across the red cell membrane.

Recently, Solomon et al. (1983) have advanced the suggestion that dimers of capnophorin form pores with a diameter of 9 Å, which function as a common pathway for not only anions, but also cations, nonelectrolytes, and water. If a common pathway of that size transports solutes and water, solute transport should show a dependence on solvent drag.

To test their hypothesis, we investigated the tracer efflux of transport of water, urea, and chloride under osmotic equilibrium and osmotic flow of water into and out of cells, where a solvent drag should be present.

**MATERIALS, METHODS, AND CALCULATIONS**

**Media**
The suspension media were 100–250 mM KCl or NaCl buffered with 0.5 mM KH₂PO₄. In urea experiments, the media contained 100 mM urea, and in the ethanol experiments, the ethanol concentration was 1 mM. The media used for treatment of red cells contained 1 mM p-chloromercuribenzenesulfonate (PCMBS; Sigma Chemical Co., St. Louis, MO). All media were titrated with 1 N KOH to pH 7.2 at 25°C, which was the pH used in all experiments.

**Labeling and Packing of Red Cells**
Freshly drawn, heparinized blood from either of the authors was washed once in the appropriate incubation medium, titrated to pH 7.2 (25°C) with either CO₂ or 150 mM KHCO₃, and subsequently washed three additional times in the pH-adjusted medium. After the washes, the cells were resuspended to a hematocrit of ~60%, and the radioactively labeled molecules of the solute under study were added to obtain 0.2–1 μCi/ml of cell suspension (³H₂O and ³⁵Cl were obtained from AEK, Risø, Denmark; [¹⁴C]urea was from The Radiochemical Centre, Amersham Corp., Buckinghamshire, England; [¹⁴C]-ethanol was from New England Nuclear, Boston, MA). Isolation of cell samples for flux experiments and for determinations of intracellular radioactivity and cell water content was carried out as previously described (Brahm, 1977, 1983a).

**Modification of the Red Cell Membrane with PCMBS**
Red cells were treated with the sulfhydryl-reacting PCMBS by incubation at 25°C for 80 min, with 1 mM PCMBS at a hematocrit of ~30%. 

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**TABLE I**

Determination of Radioactivity and Cell Water Content

Cells and supernates were isolated by centrifugation in nylon tubes and precipitated with perchloric acid as described by Dalmark and Wieth (1972). The radioactivity of the cellular water phase, the supernate, and the filtrates was determined by beta scintillation spectrometry. Cell water content was determined by drying packed cell samples to a constant weight. In all calculations, a correction was carried out for the extracellular medium trapped between the cells, which is ~2% (Brahm, 1977). In the urea experiments, the cell solid content was corrected for the contribution of urea to the cell dry weight.

The labeled water experiments were all performed at 25°C, urea experiments were carried out both at 25 and 38°C, and chloride experiments were performed at 38°C. The distributions of tracer \( r = C_i/C_0 \) and cell water volume, which at fixed pH and temperature vary only in relation to changes of the extracellular salt concentration, are shown in Tables I and II, respectively. The volume-to-area ratio (\( V/A \) in centimeters), which is used for calculation of the permeability coefficient (see below), was calculated from the wet and dry weight determinations and by assuming that 1 g of cell solids equals

| KCl or NaCl | 100 mM | 200 mM | 250 mM |
|-------------|--------|--------|--------|
| 25°C        |        |        |        |
| \( r^{1H2O} \) | 1.005  | 1.061  | 1.073  |
| SD          | ±0.030 | ±0.045 | ±0.018 |
| n           | 5      | 5      | 3      |
| \( r^{NaCl} \) | 0.988  |        |        |
| SD          | ±0.024 |        |        |
| n           | 4      |        |        |
| 38°C        |        |        |        |
| \( r^{NaCl} \) | 0.954  | 1.050  | 0.986  |
| SD          | ±0.057 | ±0.004 |        |
| n           | 3      | 2      |        |
| \( r^{Cl} \) | 0.731  | 0.678  |        |
| SD          | ±0.023 | ±0.033 |        |
| n           | 3      | 3      |        |

**TABLE II**

Cell Water Volume at Different Tonicities, pH 7.2

| NaCl or KCl | V ± SD | n |
|-------------|--------|---|
| mM          | \( \times 10^{12} \) |   |
| 25°C        |        |   |
| 100         | 86.58±1.68 | 10 |
| 200         | 49.95±1.13  | 6  |
| 250         | 41.56±0.60  | 3  |
| 38°C        |        |   |
| 100         | 81.98±1.35  | 7  |
| 200         | 47.52±0.89  | 5  |
| 250         | 40.39±0.69  | 2  |
3.1 \times 10^{10} \text{ normal erythrocytes with a total membrane area of } 4.4 \times 10^4 \text{ cm}^2 (A = 1.42 \times 10^{10} \text{ cm}^2/\text{cell}; \text{see Brahms, 1982}).

**Determination of the Rate of Efflux**

The rate of tracer efflux from labeled cells into an isotope-free medium in a suspension of a low hematocrit (\(\sim 0.6\%\)) was determined with the continuous flow tube method as previously described (Brahms, 1977, 1982, 1983a). In each experiment, the collection of serial filtrates for determination of the increase of extracellular radioactivity with time was done within the first 25–50 ms after the tracer efflux was initiated in the mixing chamber.

The rate of tracer efflux was determined at osmotic equilibrium and during an osmotic flow of water into or out of cells. At osmotic equilibrium, one can assume a two-compartment model with constant compartment volumes, in which the kinetics of tracer efflux into a large compartment (\(\sim 0.6\%\) hematocrit) follow the equation:

\[
\ln(a_t - a_{0t})(a_0 - a_{0t})^{-1} = -k \cdot t,
\]

where \(a_t\) and \(a_{0t}\) are extracellular specific activities in samples taken at time \(t\) and after isotopic equilibrium has been achieved, respectively, and \(a_0\) is the extracellular activity at time zero. Graphically, \(a_0\) is the point of intersection of the straight line with the ordinate (for a discussion of the intersection with the ordinate, see Brahms, 1982). Eq. 1 expresses the fraction of tracer that remains intracellularly at time \(t\), and \(k\) (units per second), the slope of the line, represents the rate coefficient, which is determined by linear regression analysis.

**Calculation of Permeability Coefficient and Flux**

The permeability coefficient \(P\) (centimeters per second) is calculated from

\[
P = k \cdot V \cdot A^{-1},
\]

where \(V \cdot A^{-1}\) is the ratio of cell water volume to cell membrane area.

During an osmotic flow of water across the cell membrane, cell volume changes, and one cannot a priori apply a simple two-compartment model with a constant volume for the description of the kinetics of the concomitant tracer efflux. However, if one restricts the observation period of tracer efflux to a short interval compared with the time it takes for cells to gain their new volume, the cell volume change during the experiment is small. Under this experimental condition, the semilogarithmic plots of tracer efflux are linear (cf. Fig. 1), which suggests that the two-compartment model is still valid.

The tracer efflux permeability coefficients were calculated from Eq. 2 after an adjustment of the initial cell water volume to the volume at the midpoint of the experimental period. The change in volume at the midpoint of the experimental period (\(\Delta V_i\)) was calculated from:

\[
\Delta V_i = \Delta V_{out}(1 - e^{-K \cdot t}) \quad (\text{cm}^3),
\]

where \(\Delta V_{out}\) is the total change in the water volume of the cells resulting from a change of the extracellular salt concentration, \(K\) (units per second) is the rate constant for the cell volume change, and \(t\) is the midpoint of the experimental period.

We used a \(K\) value of 5.36 s\(^{-1}\), calculated from Galey (1978), for osmotic water flow in either direction, being aware of a possible rectification of osmotic water transport, in which case the \(K\) for flow into cells would be larger (Galey, 1978). The volume change so calculated was \(6-8 \times 10^{-12} \text{ cm}^3\) and was always <\(10 \times 10^{-12} \text{ cm}^3\).
The unidirectional efflux across the red cell membrane is defined as:

\[ J_{\text{uni}} = P \cdot C_i \quad (\text{mol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}) \]

where \( C_i \) is the intracellular solute concentration (moles per cubic centimeter).

**RESULTS**

**Control Experiments**

* Determination of diffusional water efflux. Fig. 1 shows two tracer water efflux curves obtained in experiments with a concomitant osmotic flow of water across the membrane. In one experiment (filled circles), shrunken cells, equilibrated in 250 mM KCl, were mixed in the flow tube apparatus (see Methods) with a 100 mM KCl solution; i.e., water flowed into the cells against the tracer efflux. In the other experiment (open circles), cells washed in 100 mM KCl were mixed with a 250 mM KCl solution, which caused cell shrinkage when water moved out of the cells in the same direction as the tracer. The linearity of the semilogarithmic plots in the figure indicates that one can assume a monoexponential course of tracer efflux in both experiments, where the single exponent, the rate coefficient \([k = \ln 2 \cdot (T_{1/2})^{-1} (\text{s}^{-1})]\), equals the slope of the lines in the figure. The rate coefficients were used to calculate the fluxes after an adjustment of the small volume change during the experiments as described above.

Table IIIA summarizes the results obtained with cells equilibrated in media
with different tonicities. At each tonicity, tracer efflux experiments were carried out both at osmotic equilibrium and during an osmotic flow of water across the membrane created by mixing cells equilibrated at one tonicity with efflux media of a different tonicity. This experimental procedure ensures that any possible dependence of tracer efflux on tonicity or cell volume is exposed. The table shows that, as expected, the diffusional tracer efflux from swollen cells increased if a superimposed bulk flow of water out of cells was present. With the most shrunken cells, it was found that an osmotic water flow into cells decreased the unidirectional efflux of $^3$H$_2$O as compared with the flux obtained at osmotic equilibrium. However, it is surprising that the efflux from cells shrunken in a 200 mM KCl medium was larger during swelling than was the efflux at osmotic equilibrium. It should be noted that when no net water transport is taking place, the tracer flux of water-swollen cells (100 mM KCl) is also smaller than that from shrunken cells (at both 200 and 250 mM KCl).

### TABLE III A

**Tracer Water Flux in Human Red Cells at 25°C, pH 7.2**

| KCl | Cell wash medium | Efflux medium | $j_{\text{H}_2\text{O}}$ | Range | $\times 10^4$ |
|-----|------------------|---------------|-------------------------|-------|-------------|
| mM  | mM               | mol·cm$^{-2}$·s$^{-1}$ |                     |       |             |
| 100 | 100              | 1.10          | 1.01-1.19               |       |             |
| 100 | 200              | 1.34          | 1.30-1.37               |       |             |
| 100 | 250              | 1.31          | 1.21-1.36               |       |             |
| 200 | 200              | 1.16          | 1.06-1.28               |       |             |
| 200 | 100              | 1.58          | 1.23-1.61               |       |             |
| 250 | 250              | 1.17          | 1.15-1.19               |       |             |
| 250 | 100              | 1.11          | 0.91-1.21               |       |             |

Each flux value is the average of two to four experiments.

### TABLE III B

**Tracer Water Flux in PCMBS-treated Red Cells at 25°C, pH 7.2**

| KCl | Cell wash medium | Efflux medium | $j_{\text{H}_2\text{O}}$ | Range | $\times 10^4$ |
|-----|------------------|---------------|-------------------------|-------|-------------|
| mM  | mM               | mol·cm$^{-2}$·s$^{-1}$ |                     |       |             |
| 100 | 100              | 1.10          | 1.01-1.19               |       |             |
| 100 | 200              | 1.34          | 1.30-1.37               |       |             |
| 200 | 200              | 1.16          | 1.06-1.28               |       |             |
| 200 | 150              | 1.58          | 1.23-1.61               |       |             |
| 250 | 250              | 1.17          | 1.15-1.19               |       |             |
| 250 | 100              | 1.11          | 0.91-1.21               |       |             |

The asterisks denote cells that were incubated with 1 mM PCMBS in a 150 mM KCl medium. Otherwise, the incubation was carried out in the media containing 1 mM PCMBS. The incubation time was 30 min (25°C). Each flux value is the average of two experiments.
**Diffusional $^3$H$_2$O efflux in PCMBS-treated red cells.** The sulphydryl-reacting agent PCMBS inhibits both diffusional and osmotic water transport to a level that can be obtained in nonporous artificial lipid bilayer membranes, where osmotic water transport equals diffusional water flux (Cass and Finkelstein, 1967). Table IIIB demonstrates that no solvent drag effect on diffusional water efflux could be detected in red cells treated with 1 mM PCMBS. Since PCMBS inhibits osmotic water transport by ~90% (Macey et al., 1972), the volume change during the experimental observation period can be neglected. Therefore, no adjustment of cell volume was carried out in the calculation of diffusional water flux. We can also see from the table that diffusional water flux through the remaining pathway, presumably the lipid matrix, decreased with increasing tonicity (decreasing cell water volume).

**Determination of ethanol efflux.** Solutesthat permeate the lipid phase of the membrane by simple diffusion should not experience a solvent drag effect. This was tested with the lipophilic alcohol ethanol, which has been shown to diffuse predominantly via the lipid pathway (Brahm, 1983a). Table IV shows that tracer ethanol efflux from swollen cells was constant whether or not a bulk flow of water out of cells took place. As predicted, identical fluxes were also obtained in shrunken cells with and without a water flow into the cells. It should be noted that ethanol efflux differs in the two sets of experiments performed at osmotic equilibrium, which indicates a dependence of ethanol efflux on tonicity and/or cell volume.

### Table IV

**Tracer Ethanol Flux in Human Red Cells at 25°C, pH 7.2**

| Cell wash medium (mM) | Efflux medium (mM) | $J_{ethanol}$ (mol·cm$^{-2}$·s$^{-1}$ × 10°) | Range |
|-----------------------|-------------------|-----------------|-------|
| 100                   | 100               | 2.72            | 2.58–2.85 |
| 200                   | 200               | 2.65            | 2.63–2.67 |
| 200                   | 200               | 2.20            | 2.17–2.23 |
| 200                   | 100               | 2.23            | 2.16–2.30 |

The ethanol concentration was 1 mM in the experiments. Each flux value is the average of two experiments.

At room temperature, the rate of tracer efflux of urea, at an intracellular and extracellular urea concentration of 100 mM, is ~3–4 s$^{-1}$; i.e., the half-time ($T_{1/2} = \ln2 \cdot k^{-1}$) is ~200 ms. The rate coefficient for water flow used to adjust the cell water volume in experiments at osmotic disequilibrium (see Methods) is 5.36 s$^{-1}$,
which corresponds to a half-time of 130 ms. Although the experimental observation period was only a fraction of the half-time for urea efflux, the period still lasted for 40–50 ms, so that there was a sufficient increase in the radioactivity in the extracellular fluid with time. By raising the temperature to 38°C, the rate of urea tracer efflux was increased, and the observation period could be reduced to ~30 ms. The rate of osmotic water flow also increased with a rise in temperature. This temperature effect, however, was not taken into account in the adjustment of cell water volume. Therefore, the urea fluxes in the table tend to be slightly overestimated when water flows out of cells, and a little underestimated when water flows into cells. The results obtained at body temperature show no increase of urea flux with osmotic water efflux, and no decrease in urea efflux with water moving against the tracer urea concentration gradient. It should also be noted that urea efflux at osmotic equilibrium decreased with an increase of medium tonicity, which causes cell shrinkage.

**Chloride.** The chloride experiments were done exclusively at body temperature, where the half-time of chloride self-exchange is 50–60 ms. The steep

### Table V

**Urea Flux in Human Red Cells (C\text{urea} = C_{\text{urea}}^\text{extr} = 100 mM, pH 7.2)**

| NaCl or KCl | Cell wash medium | Efflux medium | $J_{\text{urea}}$ | Range   |
|-------------|------------------|--------------|-----------------|---------|
| mM          | mM               | mol·cm$^{-2}$·s$^{-1}×10^8$ |                 |         |
| 25°C        | 100              | 100          | 1.97            | 1.65-2.43 |
|             | 100              | 200          | 1.80            | 1.72-1.88 |
|             | 100              | 250          | 1.84            | 1.81-1.88 |
| 38°C        | 100              | 100          | 2.50            | 2.44-2.57 |
|             | 100              | 200          | 2.13            | 2.11-2.15 |
|             | 100              | 250          | 2.42            | 2.13-2.64 |
|             | 200              | 200          | 2.38            | 2.34-2.42 |
|             | 200              | 100          | 2.27            | 2.20-2.33 |
|             | 250              | 250          | 1.98            | 1.93-2.02 |
|             | 250              | 100          | 1.93            | 1.86-1.99 |

Each flux value is the average of two to four experiments.

### Table VI

**Chloride Flux in Human Red Cells at 38°C, pH 7.2**

| KCl | Cell wash medium | Efflux medium | $J_{\text{Cl}}$ | Range   |
|-----|------------------|--------------|-----------------|---------|
| mM  | mM               | mol·cm$^{-2}$·s$^{-1}×10^8$ |                 |         |
| 100 | 100              | 4.40         | 4.10-4.72       |         |
| 100 | 200              | 3.93         | 3.82-3.99       |         |
| 200 | 200              | 4.96         | 4.65-5.16       |         |
| 200 | 100              | 4.96         | 4.69-5.28       |         |

Each flux value is the average of four experiments.
temperature dependence of chloride exchange implies a fivefold increase of the half-time by lowering the temperature to 25°C (Brahm, 1977), which would require an inconveniently long observation period during the experiments. Table VI shows that chloride efflux decreased during cell shrinkage, whereas chloride efflux from shrunken cells was unchanged whether or not cell swelling took place. The table also shows that chloride efflux at osmotic equilibrium was larger in shrunken cells than in swollen cells.

**DISCUSSION**

Osmotic and diffusional water transport have in common the characteristic of being inhibited by PCMBS. Inhibition of osmotic and diffusional water transport differs in that 1 mM PCMBS, which ensures a maximum inhibitory effect, inhibits osmotic water flow by ~90% and diffusional water transport by ~60% (Macey et al., 1972; Brahm, 1982). Upon maximal inhibition, both the osmotic and the diffusional water permeabilities of human red cells are reduced to the same permeability, $1 - 2 \times 10^{-5}$ cm·s⁻¹, which is as low as the water permeability of nonporous artificial bilayer membranes and chicken erythrocyte membranes, which have equal osmotic and diffusional water permeability coefficients (Cass and Finkelstein, 1967; Farmer and Macey, 1970; Brahm and Wieth, 1977). The inhibitory effect of PCMBS on the osmotic and the diffusional water permeability in human red cells to a “ground” permeability has been interpreted as a closure of water-transporting channels, so that water can permeate only through the lipid moiety of the membrane.

Attempts have been made to determine the size, specificity, and location of the presumed pores in the membrane. Solomon (1968) formally presented the “equivalent pore theory” to calculate a pore radius of 4.5 Å from the ratio of 3.3 of osmotic water permeability ($P_f$) determined by Rich et al. (1968) to the diffusive water permeability ($P_d$) determined by Paganelli and Solomon (1957). Although the calculation of pore size was based only on the $P_f/P_d$ ratio, it was assumed that the pores also transported hydrophilic nonelectrolytes (e.g., urea, formamide, acetamide) with molecular radii smaller than the pore radius, whereas solutes with greater molecular radii were sterically hindered from entering the pores. Thus, the relatively larger permeability of the human red cell membrane to the small nonelectrolytes was considered to be due to transport of the solutes through the pores.

Although protein labeling and water transport inhibition studies using 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) have implicated band 3 in the transport of water (Brown et al., 1975), more recent studies by Macey (1979) and Brahm (1982) have seriously questioned these results. Several studies from the Solomon laboratory have implicated band 3 in the transport of not only anions, which is well established, but also other solutes and water (Solomon et al., 1983; Chasan et al., 1984; Lukacovic et al., 1984a, b; Yoon et al., 1984; Dorogi and Solomon, 1985; Toon and Solomon, 1985). However, the issue our study addresses is whether the solutes and water have a common pathway. If we cannot demonstrate interactions between the flows of solutes and water, we must conclude that the transported substances do not functionally share a channel.

Solomon et al. (1983) have suggested that band 3 creates a 9-Å-diam aqueous
pore that functions as a common pathway for anions, cations, nonelectrolytes, and water. Their conclusion is based on analyses of complicated interactions between transport inhibitors, inferences from protein labeling studies, and interpretations of the hydrodynamics of water transport. We have recently questioned the interpretation of the hydrodynamic measurements and have suggested that the pore through which water moves may be too small to accommodate solutes such as urea. Indeed, we believe that the most appropriate model of the continuous aqueous pathway may be that in which water molecules must traverse the membrane in a single-file fashion (Galey and Brahm, 1985).

Furthermore, a comparative study of anion, urea, and water transport in human, chicken, duck, and salamander (Amphiuma means) red cells (Wieth and Brahm, 1977) demonstrates the four possible combinations of high and low permeabilities to water and urea of the four red cell species, whereas chloride self-exchange flux is the same for all. Finally, we have recently shown (Brahm and Galey, 1984) that the urea permeability of human red cells from different donors may vary >100%, whereas the permeabilities to water and chloride are identical. The results of the two studies are not consistent with the view that capnophorin offers a nonspecific pathway to water and the two solutes, as suggested by Solomon et al. (1983).

The present study was performed to test whether a net flow of water changes the transport of urea and chloride (solvent drag effect). If solvent and solute are transported through a common pathway, solute flux should increase when the viscous bulk flow of water has the same direction as the solute flux, and it should be decreased by a bulk flow of water in the opposite direction. Although the solvent drag effect is well established theoretically and experimentally (Andersen and Ussing 1957; Forster, 1971), the phenomenon has not been demonstrated experimentally in red blood cells.

Our measurements were all done with the improved version of the continuous flow tube apparatus, which has a time resolution of a few milliseconds and in which unstirred layers can be minimized to <2 μm. Any reduction in the flux of the tracers studied here brought about by an unstirred layer of this thickness is negligible (Brahm, 1983a). Furthermore, the excellent time resolution of the apparatus makes it possible to determine the rate of tracer efflux within a period of time in which the change of cell volume is <10%.

The reliability of the method was investigated in control experiments with ethanol, which permeates the red cell membrane almost as rapidly as water. Since ethanol permeation proceeds by simple diffusion predominantly, if not exclusively, through the lipid phase of the membrane (Brahm, 1983a), one can predict that ethanol should not be dragged by a net water flow. The results of Table IV confirm this prediction and demonstrate that a methodological error caused by the disruption of unstirred layers can be excluded as contributing to any apparent increase in tracer flux due to bulk flow.

In another series of experiments, the unidirectional efflux of labeled water was increased by an osmotically induced flow of water out of cells and was decreased by a net water flow into cells (Table IIIA). The results are consonant with the generally accepted view that water moves across the human red cell
membrane at least partially by transport through pores in which an interaction between the water molecules makes a drag of volume flow on diffusing water molecules possible.

Although the results of Table III B show a considerable spread, the unidirectional efflux of labeled water is neither increased by cell shrinkage nor decreased by cell swelling when the pores are "closed" by PCMBS. It is not likely that the spread in the results is caused by an error in determining the rate of tracer efflux by the applied method, since the faster efflux rates of ethanol and \(^3\)H\(_2\)O in unmodified red cells were determined with less variation, as seen in Tables III A and IV. It therefore appears most likely that the results of Table III B can be attributed to properties of the cell membranes, after the chemical modifications by PCMBS, that are yet to be understood.

At this point, it should be interjected that the flux values determined at osmotic equilibrium, both in the control experiments and in those examining urea and chloride transport (Tables V and VI), show a dependence on cell volume and tonicity. In the \(^3\)H\(_2\)O experiments with unmodified red cell membranes (Table III A) and in experiments with \(^36\)Cl (Table VI), the fluxes increased slightly with increased tonicity, which caused a decrease of the cell water volume. In contrast, the equilibrium fluxes were decreased by a raised tonicity (i.e., decreased cell solvent volume) in the experiments with \(^3\)H\(_2\)O efflux in PCMBS-treated red cells (Table III B), ethanol efflux (Table IV), and urea efflux (Table V). The solute fluxes thus show a dependence on the extracellular salt concentration and cell volume. Whatever the causes, the results emphasize the importance of comparing the solute flux obtained both at osmotic equilibrium and at a net flow of water at a given tonicity.

We see from Table V that urea flux, under conditions where the intracellular urea concentration was at equilibrium with an extracellular urea concentration of 100 mM, was nearly constant whether or not a net flow of water took place. The same picture appears from Table VI, which shows that chloride transport was not susceptible to solvent drag either. The data are particularly relevant in light of the fact that the efflux of tracer urea and chloride should be significantly influenced by the bulk water flow if the efflux and water share the same pathway. For example, the contribution to tracer urea flux by solvent drag can be expressed by the equation

\[
J_{\text{urea}}^{\text{sol}} = C_u (1 - \sigma_u) J_v,
\]

where \(J_{\text{urea}}^{\text{sol}}\) is the unidirectional flux of urea due to solvent drag, \(C_u\) is the concentration of urea in the "feed side" solution, \(\sigma_u\) is the reflection coefficient of the red cell membrane for urea, and \(J_v\) is the volume flow across the membrane. Under the experimental conditions we used in the experiments presented in Table V, \(C_u = 100\) mM. \(\sigma\) has recently been reported to be 0.7 (Chasan and Solomon, 1985). The \(J_v\) during the tracer flux can be estimated in two ways. If one assumes that \(^3\)H\(_2\)O acts as a perfect tracer of water (i.e., accurately reports H\(_2\)O movement), the value of \(J_v\) can be calculated from the \(^3\)H\(_2\)O fluxes during the cell shrinking or swelling experiments presented in Table III A. The net flow of water out of the cell during cell shrinking can be calculated by subtracting the
diffusive water flux at equilibrium (wash and efflux medium both being 100 mM) from the $^3$H$_2$O flux taking place as the cells shrink from their wash volume in 100 mM KCl to their volume in 250 mM KCl efflux medium. The water flux calculated in this way is $0.21 \times 10^{-4}$ mol·cm$^{-2}$·s$^{-1}$, and multiplying by the molar volume of water (18 cm$^3$·mol$^{-1}$) yields a $J_v$ of $3.8 \times 10^{-4}$ cm$^3$·cm$^{-2}$·s$^{-1}$. Substituting these values into the equation above, we obtain a theoretical solvent-drag contributed flux of urea, $J'_u$, of $1.1 \times 10^{-8}$ mol·cm$^{-2}$·s$^{-1}$. A contribution to the flux of this magnitude should be easily detectable in our experiments.

An alternative way of estimating the $J_v$ during the solute urea efflux is to calculate the volume change that occurs in the red cells when they are subjected to the hypertonic 250 mM efflux medium, with a known rate constant for the osmotic water flow. Using the rate coefficient of 5.36 s$^{-1}$ from experiments at 25°C (Galey, 1978), we find the $J_v$ during the tracer efflux to be $0.2 \times 10^{-3}$ cm$^3$·cm$^{-2}$·s$^{-1}$ and the contribution to urea flux ($J'_u$) to be $0.6 \times 10^{-8}$ mol·cm$^{-2}$·s$^{-1}$, which is in fairly good agreement with the value of $1.1 \times 10^{-8}$ mol·cm$^{-2}$·s$^{-1}$ calculated above. Hence, the tracer efflux for urea during cell shrinking should be increased by at least 24% and possibly as much as 44% if urea and water share the same pathway. The observation that there is no such increase in tracer urea or chloride fluxes (there is actually a decrease of 3% for urea and a 9% decrease in chloride flux) as they move in the same direction as the bulk water flow strongly argues against water and either of these two solutes sharing the same pore or channel. We conclude that bulk transport of water and solutes is not coupled and that water is transported through a pathway functionally separate from the pathways of urea and chloride. It is possible that the pathways for water and the two solutes are all formed by band 3 protein. At least for chloride, the lines of evidence for band 3 being the protein that mediates transport are so well established that the question can be considered settled. If water and urea are also transported by a band 3 protein-mediated process, one can conclude from the present transport studies that it cannot be the same structural units of the protein that form the transporting channels for the two solutes. It may be that different parts of the molecules create the different pathways. Such a topological separation of the pathways is possible because each monomer of the band 3 protein traverses the membrane several times (Kopito and Lodish, 1985).

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