Diffusional Water Permeability of Human Erythrocytes and Their Ghosts

J. BRAHM

From the Department of Biophysics, The Panum Institute, University of Copenhagen, DK-2200 Copenhagen N, Denmark

ABSTRACT The diffusional water permeability of human red cells and ghosts was determined by measuring the rate of tracer efflux by means of an improved version of the continuous flow tube method, having a time resolution of 2-3 ms. At 25°C, the permeability was $2.4 \times 10^{-3}$ and $2.9 \times 10^{-3}$ cm s$^{-1}$ for red cells and ghosts, respectively. Permeability was affected by neither a change in pH from 5.5 to 9.5, nor by osmolality up to 3.3 osmol. Manganous ions at an extracellular concentration of 19 mM did not change diffusional water permeability, as recently suggested by NMR measurements. A “ground” permeability of $1 \times 10^{-3}$ cm s$^{-1}$ was obtained by inhibition with 1 mM of either p-chloromercuribenzoate (PCMB) or p-chloromercuribenzenesulfonate (PCMBS). Inhibition increased temperature dependence of water permeability for red cells and ghosts from 21 to 30 kJ mol$^{-1}$ to 60 kJ mol$^{-1}$. Although diffusional water permeability is about one order of magnitude lower than osmotic permeability, inhibition with PCMB and PCMBS, temperature dependence both before and after inhibition, and independence of osmolality showed that diffusional water permeability has qualitative features similar to those reported for osmotic permeability, which indicates that the same properties of the membrane determine both types of transport. It is suggested that the PCMB(S)-sensitive permeability above the ground permeability takes place through the intermediate phase between integral membrane proteins and their surrounding lipids.

INTRODUCTION

The work of Sidel and Solomon (1957) and Paganelli and Solomon (1957) on osmotic and diffusional water permeability in human red blood cells showed that osmotic water transport was two to three times larger than transport by diffusion. Studies of nonhomogenous artificial membranes in which water transport by osmosis was larger than by diffusion revealed that the porous nature of the membranes determines the difference between the magnitude of the two types of transport. Thus Mauro (1960) clearly showed that the porosity of the membrane, indicated by the dimensionless ratio between the osmotic and the diffusional water permeability in artificial collodion membranes, increased as “tight” membranes were replaced by “coarse” mem-
branes. Later investigations have demonstrated that the apparent porosity of artificial lipid bilayer membranes was caused by insufficient mixing of layers of water adjacent to the two sides of the membrane because a correction for the operational "unstirred layers" in diffusion studies indicated that the diffusional water permeability was equal to osmotic permeability of lipid membranes (Cass and Finkelstein, 1967). Because the lower diffusional permeability in human red cells cannot be ascribed to an effect of unstirred layers (Sha'afi et al., 1976; Brahm, 1982) in the determinations of permeability by the applied continuous flow method, the difference in magnitude of the two permeability coefficients suggests the presence of pores in the human red cell membrane.

In the present study, diffusional water permeability was determined by measuring the efflux of tritiated water from labeled human red cells or ghosts by means of the continuous flow method. The permeability determined by efflux experiments is $2.4 \times 10^{-3}$ cm s$^{-1}$ at $25^\circ$C, which is lower than the diffusional permeability determined by Paganelli and Solomon (1957) by influx experiments, but agrees with results of permeability studies performed using the nuclear magnetic resonance technique. Because the diffusional permeability is lower and osmotic permeability is larger (Colombe and Macey, 1974) than previous determinations, the porosity of the human red cell membrane appears to be 7-8 at $25^\circ$C.

Treatment of red blood cell membranes or ghost membranes by the sulfhydryl (SH) reagents $p$-chloromercuribenzoate (PCMB) and $p$-chloromercuribenzenesulfonate (PCMBS) reduces both osmotic (Macey and Farmer, 1970) and diffusional water transport to a "ground" permeability of $1-2 \times 10^{-3}$ cm s$^{-1}$, similar to the permeability of artificial lipid membranes, in which it has been established that diffusional water permeability is equal to osmotic permeability (Cass and Finkelstein, 1967). The porosity of human red cell membranes thus can be reduced to a level close to that in artificial membranes, which indicates that the SH reagents close a water-transporting channel. In a binding study of 5,5'-dithiobis-2-nitrobenzoate (DTNB), it was suggested by Brown et al. (1975) that the integral band 3 protein of red cell membranes, in addition to its involvement in inorganic anion transport, inevitably offers a nonspecific leak pathway to water and small polar nonelectrolytes. However, the present study reveals no effect of DTNB on diffusional water permeability, thereby supporting the view that integral membrane proteins involved in anion transport do not necessarily induce leak pathways (Brahm and Wieth, 1977), as originally suggested by Brown et al.

**MATERIALS, METHODS, AND CALCULATIONS**

**Electrolyte Media**

The following media were used in the experiments with red cells: (a) 150 mM NaCl, 1.5 mM CaCl$_2$, 1 mM MgCl$_2$, 27 mM glycyl-glycine. (b) 150 mM KCl, 0.5 mM KH$_2$PO$_4$. (c) The experiments with varying tonicity were performed in NaCl media buffered with 0.5 mM KH$_2$PO$_4$. (d) Experiments with ghosts were all carried out in 165 mM KCl buffered with 2 mM Tris. The media were titrated to the desired pH at the temperature concerned with either 1 N HCl or 1 N KOH.
Labeling and Packing of Red Cells and Ghosts

Freshly drawn, heparinized human blood was centrifuged at room temperature, and the plasma and buffy coat were removed. The cells were washed once in one of the stock media. Red cell ghosts were prepared as described previously (Schwoch and Passow, 1973; Funder and Wieth, 1976). The red cells were incubated at the temperature of the experiment and titrated to the desired pH with either CO₂ or a 150 mM sodium or potassium bicarbonate solution. The subsequent washes removed the bicarbonate. After titration, the red cells or their ghosts were washed three times in the medium applied for the efflux experiments, resuspended to a cytocrit of 60%, and incubated with \(^{3}\)H₂O (18 mCi mol⁻¹, AEK, Riss, Denmark), the radioactivity being 0.5–1 μCi/ml cell suspension. The cells and the ghosts were centrifuged at 50,000 g for 15 min (Sorvall RC-5; Dupont Instruments, Sorvall operations, Newtown, CT) in 10-ml tubes and in nylon tubes as described previously (Brahm, 1977) to obtain samples of packed, labeled cells for the efflux experiments and determinations of intracellular radioactivity and cell water content, respectively.

In the present study it was also found that the extracellular medium trapped between the packed cells or their ghosts in the nylon tubes, determined by means of tritium-labeled inulin, was 2 and 8% (wt/wt⁻¹), respectively. In determinations of cellular radioactivity and of cell water content, corrections for extracellular trapped medium were carried out.

In the inhibition study, phloretin (K & K Laboratories, Inc., Plainview, NY) dissolved in ethanol and added to the medium to give a final concentration of 0.5 mM was used. Incubation with 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS) was carried out analogous to the procedure used for complete (>99%) and irreversible inhibition of anion transport at room temperature (Brahm, 1977).

Red cells or their ghosts were also treated with the sulfhydryl-reacting reagents parachloromercuribenzoate (PCMB), parachloromercuribenzenosulfonate (PCMBS; Sigma Chemical Co., St. Louis, MO), 5,5'-dithiobis-2-nitrobenzoate (DTNB; Sigma Chemical Co.), N-ethylmaleimide (NEM; Fluka AG, Buchs, Switzerland), and diamide (Calbiochem-Behring Corp., San Diego, CA), and the bifunctional arylating reagent 1,5-difluoro-2,4-dinitrobenzene (FFDNB; Sigma Chemical Co.). All reagents except diamide were applied at a concentration of 1 mM and incubated with red cells or ghosts for 45 min at 38°C. For ghosts, the rescaling medium contained PCMB, which gave a final intracellular concentration identical with that of the incubation medium of 1 mM. In all experiments with red cells medium B (ghosts: medium D) containing 1 mM of the reagent under study was used. During the incubation period the cell suspension was washed three times with the incubation medium. Diamide treatment of red cells was undertaken as described by Haest et al. (1977) with 1 and 5 mM diamide in medium B at 38°C for 1 h.

Red cells were fixed in 0.05 and 0.2% osmium tetroxide prepared from a 0.5% stock solution buffered with 25 mM KH₂PO₄ (pH 7.2). The fixation was performed on ice for 1 h, and the cells were washed after fixation as described above.

Determination of Radioactivity and Cell Water Content

Cell water content was determined by drying a sample of red cells to constant weight. Expressed as the ratio between the intracellular solvent volume \((V \text{ cm}^3)\), and the membrane area \((A \text{ cm}^2)\), which is assumed to have a constant value of \(1.42 \times 10^{-6}\) cm\(^2\) (Hoffman et al., 1958; Westerman et al., 1961; LaCelle, 1972)\(^1\) the ratio \(V/A\) at 25°C and pH 7.2 was 4.56 (SD ± 0.15, \(n = 11\)) \(\times 10^{-5}\) cm.

\(^1\) Brahm, J., and P. D. Wimberley. Transport of chloride and bicarbonate, and water in human fetal erythrocytes. Manuscript in preparation.
For ghosts, the cell water content was set equal to the cell volume calculated from cell counts (model DN Coulter Counter; Coulter Electronics, Inc., Hialeah, FL) and measurement of the cytocrit, disregarding a minor error due to the remaining cell solids (hemoglobin reduced by >97%). In both determinations of the cellular solvent volume, correction for extracellular medium trapped between the packed cells was carried out.

Radioactivity in the supernates and cells after precipitating with perchloric acid (Dalmark and Wieth, 1972), and in the filtrates was measured by liquid β-scintillation spectrometry (model 2450; Packard Instrument Co., Inc., Downers Grove, IL).

In erythrocytes the distribution of tritium-labeled water between the intracellular solvent volume and the extracellular medium, $H_2O = H_2O(C_{H_2O})$, deviated from unity, e.g., at 25°C and pH 7.2 $H_2O = 1.05$ (SD ± 0.03, n = 11). The deviation from unity is caused by isotope exchange with hemoglobin (Benson et al., 1973), which may account for a 5-11% increase of the intracellular tritium (Brahm and Wieth, 1977).

**Determination of the Rate of Efflux**

All experiments were performed under steady state conditions, where the intracellular and extracellular concentrations remained constant during the experiments, i.e., the net efflux of tritium-labeled water was counterbalanced by an equal net influx of nonradioactive water. The rate of tracer efflux was measured by means of the continuous flow method in the version described recently (Brahm, 1977, 1982), in which the diffusional water permeability ($<4 \times 10^{-5}$ cm s$^{-1}$) was within the accessible range of determinations.

In each run a dilute cell suspension was made by the continuous mixing of $\approx 1$ ml of packed, labeled cells and 270 ml of medium. The suspension was forced down the observation tube with a constant velocity of flow. Six cell-free filtrates were collected simultaneously at filtering units that replace the tube at well-determined distances from the mixing chamber. Distance is related to time of sampling because the velocity of flow was constant and it was thus possible to follow the increase of radioactivity in the filtrates as a function of time that is directly proportional to the distance from the mixing chamber.

**Permeability**

All experiments were performed at a very low cytocrit ($<0.6\%$), and the rate of efflux of tritium-labeled water was well described by a two-compartment model with compartments of constant volume. The kinetics of tracer efflux followed the equation

$$(a_t - a_w) (a_0 - a_w)^{-1} = e^{-kt}$$

where $a_t$ and $a_w$ are the radioactivity in the sample at time $t$ and in the equilibrium sample, respectively. $a_0$ denotes the extracellular radioactivity in the dilute cell suspension at time zero. The rate coefficient, $k$ (s$^{-1}$), was determined by linear regression analysis as the slope of the linear curve depicted in a semilogarithmic plot, where the ordinate expresses the fraction of tracer that remains in the cells at a given time, and the abscissa is time in milliseconds (cf. Fig. 1).

The rate coefficient $k$ (s$^{-1}$) is connected to the permeability coefficient $P$ (cm s$^{-1}$) by the equation

$$P = k V A^{-1}$$

$VA^{-1}$ (cm$^2$) is the ratio between the intracellular solvent volume ($V$, cm$^3$) and the membrane area ($A$, cm$^2$).
Eq. 1a can be rewritten as

$$1 - a_t a_m^{-1} = e^{-kt} (1 - a_0 a_m^{-1}).$$

The left side of the equation expresses the fraction of tracer that has left the intracellular phase at time $t$. The right-side term $(1 - a_0 a_m^{-1})$ determines the intercept of the curve with the ordinate and expresses the fraction of the total radioactivity present in the extracellular phase at zero time. Ideally, the curve intersects the ordinate at 1.0. However, it can be seen in Fig. 3 that the intercept deviates from the ideal value of 1.0, and that the intercept decreases from 0.82 and 0.39 by raising the temperature from 2.6 to 38°C. As will be discussed later, the influx measurements performed by means of the flow tube method by Paganelli and Solomon (1957) was criticized (Barton and Brown, 1964) because they included the theoretical intercept in their calculations whereby the rate of self-exchange of water was increased. In the present study the theoretical value was not included in the regression analysis. The deviation from the ideal value can be explained by (a) extracellular trapping of radioactivity in the cell sample used for efflux experiments, (b) a time lag due to the geometry of the mixing chamber, and (c) a temperature-dependent property of the human red cell membrane.

**Extracellular Trapping of Radioactivity** Some radioactivity is trapped with the extracellular medium between the cells during the packing procedure before the efflux experiments. The trapped volume in the cell sample for the efflux experiments amounts to 8–15% for red blood cells and ghosts, respectively (Brahm, 1977). The distribution ratio of tracer between the cellular and the extracellular water phases was constant and ~1 within the temperature range studied, the range of $r_{H_2O}$ being 1.05 (38°C)–1.07 (2.6°C). A trapped volume of 8–15% thus represents a similar fraction of extracellularly trapped radioactivity that decreases the intercept to 0.92–0.85.

**Time Lag in the Mixing Chamber** The mixing chamber constitutes the first 2 mm of the flow tube (cf. Fig. 1 of Brahm, 1977). Because of the geometry of the mixing chamber, which establishes a tangential introduction of cells and suspending medium, the diameter of the chamber is twice that of the subsequent sections of the flow tube. Consequently, the linear velocity of flow in the chamber is half the velocity of the other segments of the tube. A correction for this additional time displaces the wash-out curves to the right, whereby the intercept with the ordinate increases towards the ideal value. The magnitude of the correction depends on the flow velocity and amounts to 6–9% under the present experimental conditions with flow rates between 275 and 375 cm s$^{-1}$.

**Temperature-Dependent Property Related to Human Red Cells** It is evident from Fig. 3 that the deviation of the intercept from the ideal 1.0, which is caused by extracellular trapping of radioactivity and a time lag in the mixing chamber, constituting 14–25% in all, suffices to explain the graphically determined intercept at low temperatures only. The decrease of the intercept with increasing temperature (Fig. 3) may reflect a property of human red cell membranes by which the water permeability increases transitorily because of a change of the shear stress in the flow tube. It must be noted that a low intercept is not a constant finding of rapid transport processes studied at physiological temperatures. Thus, intercepts lowered solely by the time lag and the initial extracellular radioactivity were found in wash-out curves both for inorganic anions such as chloride (Brahm, 1977), and for hydrophilic nonelectrolytes such as urea and glucose (J. Brahm, in preparation), all having
exchange rates in the millisecond range at 38°C. Concomitantly determined wash-out curves of §Cl and §H2O from red cells labeled with both isotopes stressed that a different and lower intercept of water exchange curves was found under experimental conditions that exclude causes such as significant hemolysis as well as differences in, for instance, flow velocity, mixing, and shear stress. In fact, these findings exclude methodological errors. So far the temperature-dependent low intercept has been found only in exchange curves for water, aliphatic primary alcohols (Brahm, 1982), and phenylglyoxal (J. Brahm, unpublished results). The temperature-dependent variation of the intercept appears to be related to the nature of the transport process. It is not found for ions and polar molecules that permeate the membrane by specific, proteinaceous pathways. On the other hand, it is invariably found for molecules that are transported to a significant degree through the lipid core of the membrane. The low intercept of water self-exchange cannot be due to the protein-related fraction of water transport of ~50% at 38°C because a closure of the transport path of the proteins with PCMB or PCMBS did not raise the intercept. Nor did the intercept increase after a reduction of the elasticity of the membrane through modifications of the proteins by means of heat or protein fixatives (formaldehyde, glutaraldehyde, osmium tetroxide, diamide).

It appears most likely that the low intercept is caused by a transitory (<2–3 ms) increase of permeability to solutes permeating the lipid core of the membrane. The permeability increase may be caused by a "membrane thinning" effect, and the process of rearrangement of the lipids may be due to a sudden increase of shear stress (Chien et al., 1978). The underlying cause of the short-lived permeability increase may be the abrupt doubling of flow velocity of the cell suspension as it leaves the mixing chamber and enters the more narrow part of the tube. At 38°C the water permeability was determined to be 3.4 × 10^{-3} cm s^{-1} (T1/2 = 7.6 ms, cf. Fig. 3), of which ~50% is caused by water permeating the lipid core of the membrane. If the above interpretation is correct, the temperature-dependent decrease of the intercept to 0.4 is caused by a sevenfold increase of the permeability of the lipids from 1.7 × 10^{-3} cm s^{-1} to 12 × 10^{-3} cm s^{-1} during a period of 2 ms before the sampling of the first filtrate in the tube. The magnitude of the permeability increase may be related to the change of the membrane surface viscosity, which for a given shear stress decreases by a factor of 6 when the temperature is raised from 6 to 38°C (Hochmuth et al., 1980). Only a small fraction of chloride and glucose transport takes place through the membrane lipids, membrane transport being totally dominated by facilitated diffusion processes. A short-lasting increase of the minute fraction of the transport through the hydrophobic pathways will therefore be undetectable.

The question is whether a short-lasting permeability increase vitiates the determination of water self-exchange and of the temperature dependence of water permeability, which appeared to be low in intact red cells and resealed ghosts (cf. Fig. 4). However, the low temperature dependence of water transport cannot be due to the change of the intercept with temperature. PCMB-treated cells having similar low intercepts of their wash-out curves showed a higher temperature dependence of permeability (Fig. 4) similar to that obtained by quite different techniques in artificial nonporous lipid membranes and that obtained with the flow tube technique in chicken erythrocytes whose bilayer-like permeability to water and urea (Brahm and Wieth, 1977) is not complicated by the problems of the intercept. Temperature-insensitive intercepts of water and methanol exchange curves for chicken red cells (J. Brahm, unpublished results) suggest that the different lipid composition of human and chick red cell membranes (White, 1973) makes theavian red cell membrane resistant to the postulated shear-stress effects.
Determination of Temperature Dependence

The temperature dependence of the permeability coefficient was calculated by linear regression analysis of the function of $\ln P$ against the reciprocal of the absolute temperature according to the equation:

$$\ln P = -\frac{E_A (RT)}{R} + \text{constant}. \quad (3)$$

$E_A$ (J mol$^{-1}$) is the apparent activation energy of the transport process, $R$ is the gas constant (8.32 J [mol K]$^{-1}$), and $T$ is the absolute temperature (K).

**RESULTS**

**Dependence on pH**

The pH dependence on diffusional water permeability in erythrocytes was determined by measuring the rate of efflux of tritium-labeled water as described in the Methods section. In all experiments the rate of efflux followed first-order kinetics giving straight lines in a semilogarithmic plot of the fraction of radioactivity that remains in the cells at a given time against the time of sampling. This is illustrated in Fig. 1, which shows the rate of tracer wash-out from labeled cells in two experiments performed with red cells and their ghosts.
at 25°C and pH 7.2. The somewhat longer half-time of exchange found in ghosts \((T_{1/2} = \ln 2 \cdot k^{-1} = 16.3 \text{ ms})\) compared with a half-time of 13 ms of red cells is caused by the fact that the intracellular solvent volume was increased by the removal of hemoglobin, the cell volume of the resealed ghosts (in the actual case 86 \(\mu m^3\)) being only slightly different from that of the red cells, which had a solvent volume of 65 \(\mu m^3\).

In Fig. 2 the permeability coefficient \((P, \text{ cm s}^{-1})\) in erythrocytes, the rate coefficient \((k, s^{-1})\), and the ratio of intracellular solvent volume and membrane area \((VAg^{-1}, \text{ cm})\), which are yielded in the permeability coefficient (cf. Eq. 2), are depicted as a function of extracellular pH. Within the pH range from 5.5 to 9.5 the diffusional water permeability was constant, the average value obtained, e.g., at pH 7.2, being 2.4 (SD \(\pm 0.2\), \(n = 18\)) \(\times 10^{-5}\) cm s\(^{-1}\). The constant water permeability was found even though the rate coefficient increased by a factor of two within the pH range considered because the intracellular solvent volume decreased concomitantly, the product of \(k x (VAg^{-1})\) thus remaining constant. The independence of extracellular pH was also found to be a constant feature of diffusional water permeability at 38°C (results not shown), which showed a value of 3.4 \(\times 10^{-5}\) cm s\(^{-1}\).
Dependence on Osmolarity and Cell Volume

The diffusional water permeability was determined under conditions of varying osmolarity. The osmolarity of the incubation medium that was identical to the efflux medium was varied in two ways. In one set of experiments the tonicity of the medium was varied by a change in the extracellular sodium chloride concentration from a hypertonic concentration of 250 mM through isotonicity to a hypotonic concentration of 100 mM. Because of the low permeability of red cells to sodium, the cell volume was varied by a factor above two by the variation of the tonicity of the extracellular medium in the establishment of osmotic equilibrium. In Table I the permeabilities found at the various tonicities are presented relative to the permeability at isotonicity. The table shows that permeability increased only 6–8% as

tonicity of the extracellular medium was decreased to 100 mM. An increase of tonicity above isotonicity reduced permeability by 9% at an extracellular sodium chloride concentration of 210 mM. At 250 mM NaCl in the suspending medium permeability was reduced by 21%. This reduction may in part be due to experimental difficulties in mixing because cells at the very low cell volume tend to adhere more firmly to each other, thereby reducing the membrane area exposed for the transport process.

In the other series of experiments, osmolarity was increased above isotonicity by adding solutes that are permeable to the red cell membrane. Through repeated washes, the concentration of the solute was increased in the extracellular medium to obtain a step-wise equilibration of the solute across the cell membrane in order to minimize any osmotic damage of the cell membrane. Table II lists the solutes that were added to the medium, and the water permeability under these variations of osmolarity. It was possible to inhibit the diffusional water permeability by ~40% with all the solutes except urea listed in the table. It is, however, noteworthy that the decrease of permeability appears not to be related to the osmolarity of the medium. This can be illustrated by a comparison of the results with thiourea and glycerol.

### Table I

| [NaCl]o (mM) | Relative tonicity | Relative cellular solvent volume | Relative rate coefficient | Relative permeability |
|--------------|------------------|---------------------------------|--------------------------|----------------------|
| 100          | 1.00             | 1.00                            | 1.00                     | 1.00                 |
| 120          | 1.00             | 1.00                            | 1.00                     | 1.00                 |
| 150          | 1.00             | 1.00                            | 1.00                     | 1.00                 |
| 210          | 1.00             | 1.00                            | 1.00                     | 1.00                 |
| 250          | 1.00             | 1.00                            | 1.00                     | 1.00                 |

The results of the double experiments performed at hypo- and hypertonicity are presented relative to the average values obtained at isotonicity at which the rate coefficient, $k$, was $52 \text{ s}^{-1}$, the ratio of $VA^{-1}$ was $4.6 \times 10^{-5} \text{ cm}$, and the permeability, $P$, was $2.4 \times 10^{-5} \text{ cm s}^{-1}$.
reduction of permeability with thiourea was found at a solute concentration of 500 mM, whereas the same degree of inhibition with glycerol was not observed until the concentration was raised to 6 M.

Inhibition

Table III lists a wide variety of reagents that were tested for effect on the diffusional water permeability. The primary task was to investigate whether the sulfhydryl-reacting reagents that have been applied in osmotic experiments (PCMB, PCMBS, NEM, and DTNB) showed similar effects on diffusional permeability. As can be seen from the table, only PCMB and PCMBS showed a substantial degree of inhibition, as in osmotic permeability studies (Macey and Farmer, 1970; Macey et al., 1972; Naccache and Sha'afi, 1974), amounting to 60% at room temperature. In contrast, DTNB, which according to Naccache and Sha’afi (1974) inhibited osmotic water transport by 60%, showed no inhibition of diffusional water permeability. Diamide, an SH reagent that cross-links the intracellular located protein spectrin, but not the band 3 protein (Haest et al., 1977), had no effect on water permeability.

The possible relation between water transport and integral membrane proteins (Brown et al., 1975) was not revealed by treating the red cell membranes with protein fixatives (formaldehyde, glutaraldehyde, osmium tetraoxide) at concentrations applied for morphological studies. At most, the fixatives caused an increase of permeability by 15%. Neither did the efficient anion transport inhibitor DIDS, which binds to band 3 protein, cause any large change of water permeability that was only reduced by 15%. Phloretin, which also inhibits anion transport by almost 100%, as well as other facilitated

| Solute added | Concentration mol liter⁻¹ | Osmolarity osmol liter⁻¹ | Permeability range cm⁻¹ × 10³ |
|--------------|---------------------------|-------------------------|-------------------------------|
| Control      |                           | 0.3                     | 2.4                           |
| Urea         | 0.1                       | 0.4                     | 2.2                           |
| Urea         | 0.5                       | 0.8                     | 2.2                           |
| Thiourea     | 0.1                       | 0.4                     | 2.2                           |
| Thiourea     | 0.5                       | 0.8                     | 1.4                           |
| Glycerol     | 1                         | 1.3                     | 2.4                           |
| Glycerol     | 3                         | 3.3                     | 2.0                           |
| Glycerol     | 6                         | 6.3                     | 1.6                           |
| DMSO         | 3                         | 3.3                     | 1.5                           |
| DMSO         | 6                         | 6.3                     | 1.5                           |
| 1,3 Propandiol| 6                         | 6.3                     | 1.4                           |

The solutes were added in successive steps during the washing procedure of the red cells. After the last wash, the cells stood long enough to ensure that equilibrium across the membrane of the solute was obtained. The permeabilities are average values from two to four experiments.
transport processes in the red cell membrane, left water permeability unchanged. As discussed below, this could be caused by two opposing effects: inhibition of water transport through proteins, and an increase of permeability through the lipids by a liquefying effect. An increase of water permeability through the lipids of the membrane could not, however, be produced by aliphatic alcohols at concentrations that increase alcohol permeability in red cells (Brahm, 1982), and water and electrolyte permeability in artificial membrane systems (Jain et al., 1973; Hochster and Prestegard, 1977; Guttknecht and Tosteson, 1970), presumably by a fluidization of the membrane lipids.

The bifunctional arylating reagent 1,5-difluoro-2,4-dinitrobenzene (FFDNB) cross-links aminophospholipids in the red cell membranes (Marfey and Tsai, 1975). However, despite the cross-linking property, no inhibitory effect on water permeability was found.

It was also investigated whether MnCl₂ at an extracellular concentration of free ions of 19 mM changed water permeability, because Pirkle et al. (1979) recently questioned the validity of previous determinations of diffusional water permeability by means of the NMR technique at manganese ion
concentrations >5–10 mM. The present study did not confirm the postulated effect of the divalent cations on the diffusional water permeability when measured by means of the tracer efflux.

Dependence on Temperature
The temperature dependence of diffusional water permeability was determined in the temperature range from 2.5 to 38°C for both red cells and ghosts, and for ghosts pretreated with PCMBS as described in the Methods section. The results are shown in Fig. 3 and in the Arrhenius diagram of Fig. 4, depicting the logarithm of permeability as a function of the reciprocal of absolute temperature. The slope of the linear curves in Fig. 4 expresses the apparent activation energy of the transport process. It was found that both erythrocytes and control ghosts had a low temperature dependence, 21.1 (SD ± 1.7) and 29.6 (SD ± 2.4) kJ mol⁻¹, respectively. In contrast, the PCMBS-treated ghosts that were maximally inhibited, having a "ground" permeability at 25°C of 0.98 × 10⁻³ cm s⁻¹, exposed a significantly higher temperature dependence of 59.7 (SD ± 5.1) kJ mol⁻¹.

Fig. 4 shows that the degree of PCMBS inhibition was more pronounced at the low temperatures. According to the regression line, the inhibition was 87% at 6°C, being gradually reduced to 50% at 38°C.

DISCUSSION

³H₂O is Transported
The application of tritium-labeled water in permeability studies raises the question whether efflux of radioactive-labeled water or efflux of tritium ions
is measured. Recent investigations of pH equilibration across the red blood cell membrane (Jennings, 1978; Wieth et al., 1980) give neither qualitative nor quantitative grounds for the assumption that hydrogen ion transport was studied in the present investigation. Jennings (1978) and Wieth et al. (1980) showed that transport of hydrogen ions is highly temperature dependent and depends on pH and an intact anion transporting system, because DIDS inhibits bicarbonate transport and thereby "facilitated" transport of H⁺. In

![Figure 4](image)

**Figure 4.** The temperature dependence of water exchange in human red cells (●), ghosts (○), and in ghosts treated with 1 mM PCMBS (□). The PCMBS treatment reduced water permeability to a ground permeability that at 25°C was $1 \times 10^{-3}$ cm s⁻¹. PCMBS-treated ghosts had a considerably higher temperature dependence of water permeability, indicating that the closure of a parallel pathway to water changes the properties of the membrane, which becomes similar to an artificial bilayer membrane. All experiments were performed at an extracellular pH of 7.2.

the present study, tritium permeability was independent of a variation of pH from 5.5 to 9.5 (Fig. 2), by which the hydrogen ion concentration varies by a factor of $10^4$. Furthermore, the temperature dependence of permeability was low (Fig. 3), and was insensitive to 1 µM DIDS, which inhibits anion transport at room temperature by >99% (Brahm, 1977). There can be no doubt that diffusion of tritium-labeled water molecules across the red cell membrane was studied.

One must also consider whether the permeability to tritiated water repre-
resents a "true" water permeability or differs because of an isotope effect. This question was also considered by Paganelli and Solomon (1957), who concluded that the measured permeability should be increased by 14% to correct for isotope effects. Their conclusion was based on the study of the different isotopic forms of water by Wang et al. (1953), who found a 9% deviation between diffusion coefficients in ordinary water of $^3$H$_2$O and of H$_2^{18}$O, which is considered to be the best tracer of water. House (1974) has discussed this problem in the light of later diffusion studies, too and concluded that "It seems unlikely that an isotope effect is a serious source of error in determinations of diffusional permeability for biological membranes." In the present study it was therefore decided not to carry out any correction for isotope effect.

Comparison with Other Diffusion Studies

PERMEABILITY DETERMINED BY MEANS OF RADIOACTIVE-LABELED WATER

Diffusional permeability determined by means of radioactive-labeled water has hitherto only been measured by influx experiments. Paganelli and Solomon (1957), who were first to apply the flow tube technique for investigations of the diffusional water permeability of the red cell membrane, calculated a permeability of $5.3 \times 10^{-3}$ cm s$^{-1}$ from the experimental half-time of 4.2 ms. Barton and Brown (1964) under similar experimental conditions found a slower exchange time of 7.7 ms that equals a permeability of $3.4 \times 10^{-3}$ cm s$^{-1}$ (calculated from their Table II). They concomitantly raised a critique of the determination of the exchange rate by Paganelli and Solomon that included the application of a theoretical value of the intercept with the ordinate leading to an overestimate of the exchange rate (cf. Intercept with the Ordinate). If the theoretical point of Fig. 5 in the work of Paganelli and Solomon (1957) is neglected, the exchange rate decreases to a value similar to that published by Barton and Brown (1964). In both studies the calculated permeability was increased by 14% to compensate for isotope effect as discussed previously. A comparison of permeabilities of the influx experiments with the present value of $2.4 \times 10^{-3}$ cm s$^{-1}$ therefore implies a 14% reduction of their permeability coefficients from $3.4 \times 10^{-3}$ to $2.9 \times 10^{-3}$ cm s$^{-1}$, a value that must be considered as similar to the present value.

Vieira et al. (1970) applied the continuous flow method of Paganelli and Solomon (1957), modified by Barton and Brown (1964), to determine the rate coefficient of influx of $^3$H$_2$O. They concluded that their diffusional water permeability of human red cells was in excellent agreement with previous values. The agreement is, however, based on results showing a large variation from 0.5 to 1.3 of the normalized rate coefficient at 22°C in their Fig. 4 (in the present study the range of rate coefficients at 25°C and pH 7.2 similarly expressed relative to the mean of 52 s$^{-1}$ was 0.8–1.1). In the inhibition study

---

2 Conversion of experimentally determined exchange times to permeability involves a correction because influx experiments are performed as exchange between two compartments of finite size.
of Macey et al. (1972), a value of the permeability of the control cells was not published, but a value above $2.8 \times 10^{-3}$ cm s$^{-1}$ is indicated by Fig. 4 and Table I of their work.

Previous determinations of diffusional water permeability by influx experiments thus show results comparable to the permeability coefficient in the present study of $^{3}H_{2}O$ efflux. At this point it must be emphasized that the apparently somewhat smaller permeability in the present study is not the result of an error of unstirred layers, because determinations of the thickness of unstirred layers under similar experimental conditions indicated layers $<2 \mu$m thick (Brahm, 1982).

**Permeability determined by means of the NMR technique**

Within the last decade several studies have been published in which the diffusional water permeability was determined by means of the nuclear magnetic resonance (NMR) technique. A water molecule placed in a magnetic field absorbs energy, which during the subsequent liberation to the surroundings produces characteristic signals of the relaxation times $T_1$ (spin-lattice) and $T_2$ (spin-spin) of the energy loss from the activated nucleus (for review, see Mathur-De Vrè, 1979). Manganese ions increase the relaxation times above the observable limit. Because manganese ions have an extremely low permeability to the red cell membrane, manganese ions in the extracellular medium of a cell suspension eliminate signals from the excited nucleus of water molecules that have diffused from the intracellular to the extracellular phase. The NMR studies of water permeability were mainly performed by measuring the proton relaxation (Conlon and Outhred, 1972, 1978; Outhred and Conlon, 1973; Andraske, 1976; Morariu and Benga, 1977; Chien and Macey, 1977; Fabry and Eisenstadt, 1978; Pirkle et al., 1979), whereas few were carried out as $^{17}O$ resonance studies (Fabry and Eisenstadt, 1975; Shporer and Civan, 1975). In several of the studies only exchange times ($T = k^{-1}$, ms) are published. Assuming that pH was within a physiological range, and that the cell water fraction was similar in the different studies ($63 \times 10^{-12}$ cm$^3$ H$_2$O cell$^{-1}$, equalling a water fraction of 0.7), permeability varies from $2 \times 10^{-3}$ cm s$^{-1}$ (Pirkle et al., 1979) to $4 \times 10^{-3}$ cm s$^{-1}$ (Conlon and Outhred, 1978). Fabry and Eisenstadt (1978) published a permeability at 25°C of $2.1 \times 10^{-3}$ cm s$^{-1}$. The coefficient is, however, based on too large a membrane area (1.63 vs. $1.42 \times 10^{-8}$ cm$^2$; Hoffman et al., 1958; Westerman et al., 1961; LaCelle, 1972; Jay, 1975). Using the somewhat lower membrane area, the permeability coefficient of Fabry and Eisenstadt (1978) increases to $2.4 \times 10^{-3}$ cm s$^{-1}$, which is identical to the coefficient in the present study. The $^{17}O$ resonance study by Shporer and Civan (1975) gave a half-time ($T_{1/2}$) of the exchange of 11.6 ms at pH 7.4, which equals a permeability of $2.6 \times 10^{-3}$ cm s$^{-1}$. The study by Conlon and Outhred (1978) showed the fastest exchange time of 11 ms ($T_{1/2} = \ln 2 \times 11 = 7.2$ ms), which can be converted to a permeability of $3.9 \times 10^{-3}$ cm s$^{-1}$.

Pirkle et al. (1979) have recently questioned the validity of previous determinations by means of the NMR technique because their findings suggest that free manganese ions in the applied concentrations above 20 mM induce a
35-45% systematic decrease of the exchange time measurements (which they erroneously call an inhibition, although the exchange time decreased as the manganese ion concentration was increased). In their investigation the exchange time decreased from \( \sim 22 \) to \( \sim 12 \) ms by raising the extracellular \( \text{MnCl}_2 \) from 1.5 to \( \sim 50 \) mM. Shporer and Civan (1975) found the same permeability in the presence and the absence of \( 6-7 \) mM \( \text{Mn}^{2+} \), a concentration that increased the permeability in the work of Pirkle et al. by 20%. Fabry and Eisenstadt (1978) found that permeability increased at manganese ion concentrations above 10 mM, which has even raised the permeability obtained by Pirkle et al. by 25%. Pirkle et al. concluded that reasonable agreement was found with their data and the results of Andrasko (1975) obtained in the absence of manganese ions. However, they would have found the same close agreement by a comparison with the results of Chien and Macey (1977), who measured an exchange time of 18 ms in the presence of 20-53 mM free manganese ions. Furthermore, Fabry and Eisenstadt (1975) determined an exchange time of 15 ms at an extracellular manganous ion concentration of 5 mM, i.e., >30% faster than the value obtained by Pirkle et al. at the same \( \text{Mn}^{2+} \) concentration.

It is important to clarify whether diffusional water permeability depends on manganese ions in the extracellular phase. Table III shows that tracer determinations were not affected by the presence of 19 mM free manganese ions extracellularly, which, according to Pirkle et al. (1979), should have increased permeability by 50%. The present results indicate that the observation by Pirkle et al. is due to an artifact of the method and not to an effect of the divalent cation on the diffusion process across the erythrocyte membrane.

Dependence on Osmolality

Rich et al. (1968) found that the hydraulic water permeability \( (L_p, \text{cm}^3 \text{dyn}^{-1} \text{s}^{-1} \text{convertible to } P, \text{cm} \text{s}^{-1} \text{by multiplication with } 1.38 \times 10^9) \) decreased from \( 2.6 \times 10^{-2} \text{ cm s}^{-1} \) to \( \sim 1.1 \times 10^{-2} \text{ cm s}^{-1} \) by raising the osmotic concentration from \( \sim 200 \) mosmol to 500 mosmol (Fig. 2 in Rich et al., 1968, upper frame), and they concluded that \( L_p \) was independent of the direction of water movement and of the volume of the red cells, \( L_p \) being determined only by the final osmolality of the system. However, in a previous study of the entrance of water in red cells, Sidel and Solomon (1957) did not find a variation in hydraulic permeability within a similar range of osmolalities. Farmer and Macey (1970) questioned the interpretation by Rich et al. (1968), which was based on results obtained at influx experiments at hypotonic extracellular concentrations and efflux experiments at hypertonicity. Farmer and Macey (1970) measured osmotic water transport at hypertonicity as well as hypotonicity. They found that independent of tonicity and cell volume permeability determined by influx invariably was \( \sim 50\% \) larger than permeability determined by efflux, \( 2.28 \times 10^{-2} \) and \( 1.61 \times 10^{-2} \text{ cm s}^{-1} \), respectively. The rectification suggested by Farmer and Macey has recently been confirmed by the study of Galey (1978), who also found that water influx was 50% larger than efflux. Blum and Forster (1970) found both a rectification of flow and a
dependence of osmolality in human red cells. However, both phenomena were less pronounced than in previous studies, the ratio between influx and efflux being 1.2 compared with the 1.5 obtained by Farmer and Macey (1970), and the permeability decreased with a factor of 1.3, whereas Rich et al. (1968) found a factor of 2.4 within the same range of osmolalities.

In the above-mentioned water experiments, osmolality was changed by varying the extracellular sodium chloride concentration, which also changes the cell volume because of the low permeability of the erythrocyte membrane to sodium. To reduce effects of cell volume changes, Chien and Macey (1977) in a NMR study determined the diffusional water permeability after equilibration with different solutes (urea, methanol, ethanol, glycerol) that permeate the red cell membrane, which ensures that the cell volume remains normal. The permeability dependence of osmolality can thus be determined under conditions that eliminate factors involved in rectification. However, this assumption is based on the anticipation that the fraction above the ground permeability of the larger hydraulic and of the diffusional water permeability are both determined by the same interactions between the water molecules and the components of the membrane during the transport process. A justification for comparing the two types of transport may lie in the indirect evidence that qualitative features of the transport processes such as temperature dependence and inhibition with PCMBs are similar. Provided that diffusional permeability studies reveal properties of osmotic water transport, the independence of osmolality of the permeability in the study of Chien and Macey (1977) supports the suggestion of rectification of osmotic water transport advanced by Farmer and Macey (1970).

In the present study, diffusional permeability was determined with a technique different from that applied by Chien and Macey (1977), and within a considerably larger range (300–6,300 mosmol) of osmolalities. The results of Table II show that permeability was independent of osmolality within large variations. An increase of osmolality to 3.3 osmol by addition of glycerol only reduced permeability with 10–20% at the highest concentration. The >40% decrease of permeability in the presence of 500 mM thiourea must therefore represent a thiourea effect on membrane components that interact with the water molecules during the diffusion process, and cannot be ascribed to an osmotic effect as such on the diffusional water permeability.

Fabry and Eisenstadt (1978), by means of the NMR technique, found that the diffusional water permeability varied with changes in osmolality that were followed by changes in the cell volume. By changing the extracellular sodium chloride concentration, permeability increased both as the cells shrank below 70 μm³ and swelled above 105 μm³. This picture of permeability was not seen in the present investigation (cf. Table I). Cell volume was varied from 110 μm³ at an extracellular NaCl concentration of 100 mM to 64 μm³ at 250 mM NaCl in the extracellular medium, and thus was similar to the variation in the study of Fabry and Eisenstadt. In contrast to their results, diffusional tracer permeability remained almost constant until the cell volume was 64 μm³, at which permeability was reduced by 20%. It must be noted that experiments with such shrunken cells are difficult to perform because the
small cells tend to adhere to each other, thereby preventing an efficient mixing in the mixing chamber of the flow tube and reducing the membrane area available for the transport process. Furthermore, it cannot be excluded that the decrease of permeability is caused by a restricted intracellular diffusion of $^3$H$_2$O resulting from the increase of the hemoglobin concentration (Redwood et al., 1974).

It is not known what caused the permeability increase in the shrunken cells in the study of Fabry and Eisenstadt (1978), but it is noteworthy that an increase of permeability was not a constant feature of their shrunken cells. A permeability decrease was also found (their Fig. 6) in two series of experiments in which the suspending medium was either plasma or a sodium-potassium-serum albumin-fibrinogen medium. The permeability increase was found only in the fibrinogen-free sodium chloride medium.

The present results (Tables I and II) do not indicate a dependence of the logarithm of permeability to the reciprocal of the extracellular osmolality as suggested by Rich et al. (1968) for hydraulic permeability. The results of Table I further show that permeability was independent of variation of intracellular solvent volume. The same conclusion can be drawn from Fig. 2. As pH was raised from 5.5 to 9.5 cell volume changed from 100 $\mu$m$^3$ to 64 $\mu$m$^3$, i.e., a cell volume change similar to that obtained by variation of extracellular NaCl (Table I), without causing any permeability change in the pH range studied.

If water transport by diffusion and by osmosis depends on the same qualitative properties of the membrane, the present results of diffusional water permeability support the suggestion of rectification of osmotic water transport advanced by Farmer and Macey (1970). The cause of the 35% lower $L_p$ of efflux in human red blood cells ($L_p$ efflux = 0.67 $\times$ $L_p$ influx) originally observed by Farmer and Macey (1970) is not clear. One explanation might be that the intracellular components, mainly hemoglobin, in hydraulic efflux experiments by solvent drag are sucked toward the inside of the membrane, thereby contributing to the diffusion barrier of the membrane. If that is the case, this effect should be reduced in experiments with ghosts whose $L_p$ efflux is expected to be equal to $L_p$ influx, which in turn is equal to $L_p$ influx for red cells (Colombe and Macey, 1974). This may explain why Levin et al. (1980), in an osmotic efflux study of erythrocytes and ghosts, found a ratio of $L_p$ efflux between red cells and ghosts of 0.65, which indicates that the osmotic efflux permeability of ghosts is 50% larger than that of red cells.

**Inhibition of Diffusional Water Permeability**

Table III shows that the diffusional water permeability is indifferent to a large number of substances that modify the lipoidal and the proteinaceous components of the membrane. Substantial inhibition was only obtained by means of the SH reacting agents PCMB and PCMB. In the previous sections of permeability dependence and osmolality, it was also shown that 0.5 M thiourea and glycerol, DMSO, and 1,3-propanediol at high concentrations (3-6 M) inhibited water permeability 33-42% (cf. Table II). Before a more
detailed discussion of these inhibitors, the implications of the negative findings of the effect of some of the other solutes under study will be discussed. The 60% inhibition of osmotic water transport by DTNB reported by Naccache and Sha'afi (1974), and later binding studies with the radioactive-labeled compound led Brown et al. (1975) to conclude that the integral band 3 protein (Steck, 1974) was involved in water transport. Macey (1979) recently questioned the inhibitory effect of DTNB on osmotic water transport, and the present results show that DTNB had no effect on diffusional water permeability. An inhibition of 60% of diffusional permeability could not be missed, because the degree of inhibition would have increased the half-time of exchange at 25°C and pH 7.2 from 13 ms to 33 ms. The lack of effect could be due to the fact that a fraction of osmotic water transport proceeds via pathways that are not accessible for diffusional transport, or play a minor role in the diffusion process. A separation of transport pathways caused by different effects of DTNB on diffusional and osmotic water permeability appears unlikely because both osmotic and diffusional permeability may be inhibited by PCMB to a "ground" permeability of \(10^{-3} \text{ cm s}^{-1}\) (Macey et al., 1972; Table IV of the present study) like that of artificial lipid membranes (Cass and Finkelstein, 1967) and erythrocytes from some species (Farmer and Macey, 1970; Brahm and Wieth, 1977; Wieth and Brahm, 1977).

The importance of the integral membrane proteins for water transport above the ground permeability was not revealed by treating the erythrocyte membranes with protein fixatives that are applied for morphological studies. Formaldehyde, glutaraldehyde, and osmium tetraoxide form cross-links in proteins and interact with several reactive groups of proteins (Millonig and Marinozzi, 1968; Korn et al., 1972), inducing conformational changes in all the proteins of the red blood cell membrane (Lenard and Singer, 1968). The fixatives, however, apparently do not react exclusively with the membrane proteins, because glutaraldehyde also cross-links with phosphatidylethanolamine and phosphatidylserine, probably to proteins (Wood, 1973), and osmium tetraoxide reacts with the double bonds of unsaturated membrane lipids.

Despite extensive changes of the membrane protein structure, the protein fixation did not change the diffusion permeability. A similar treatment with formaldehyde did not change osmotic water permeability (Sirs, 1969). It is also unlikely that the anion transport pathway of the integral membrane proteins concomitantly offers a nonspecific leak pathway to water and small nonelectrolytes, as suggested by Brown et al. (1975), because the specific anion transport inhibitor DIDS and the nonspecific inhibitor of facilitated transport processes phloretin both inhibit anion transport \(>99\%\), whereas water permeability remained unaffected. At this point it must be interposed that these results do not exclude the possibility that other parts of the proteins, or even other proteins, than the apparently specific anion transport path(s) mediate water transport above the ground permeability. The lack of effect of, for instance, phloretin could be due to a dual effect of phloretin on water transport by an inhibition of transport through the proteins and an increase of water transport via the lipid phase by a liquefying effect on the membrane lipids.
Evidence for binding of phloretin both to membrane proteins and lipids was presented by Jennings and Solomon (1976), who showed high affinity binding sites (\(K_{d_{\text{high}}} 1.5 \mu\text{M}\)) of the proteins, and a low affinity (\(K_{d_{\text{low}}} 54 \mu\text{M}\)) to the lipids. The possible role of liquefaction of the lipids for water transport is still a matter of debate. Macey and Farmer (1970) found no effect of phloretin on the hydraulic permeability, whereas Owen and Solomon (1972) and Owen et al. (1974) found a 30% increase of osmotic water permeability at phloretin concentrations >0.1 mM. The latter two studies disagree with the present result of diffusional permeability. One might argue that a fluidization of the membrane lipids is not necessarily revealed by measuring the permeability by diffusion, and that the lack of effect both in human red blood cells and chicken erythrocytes, whose permeability is as low as in artificial lipid membranes (Brahm and Wieth, 1977), might be caused by different experimental conditions in measuring osmotic and diffusional water permeability. It must be noted that the lack of effect of phloretin on diffusional water permeability cannot be caused by an experimental artifact, because phloretin under similar conditions increased the diffusional methanol permeability in human red cells by 15–20% (Brahm, 1982). It must also be noted that if the osmotic permeability increased 30% because of the liquefying effect of phloretin on the lipids, the fraction of the total permeability that takes place through the lipids (1-2 \(\times 10^{-3}\) cm s\(^{-1}\) out of \(18 \times 10^{-3}\) cm s\(^{-1}\)) should increase to \(5 \times 10^{-3}\) cm s\(^{-1}\), i.e., \(\sim 300-500\%\).

The aliphatic alcohols also fluidify the membrane lipids (Seeman, 1972). However, in agreement with results found with phloretin, this treatment did not change the diffusional permeability. This is in contrast to a recent study of the effect of aliphatic alcohols on osmotic water permeability (Kutchai et al., 1980) in which the permeability decreased 40% by raising the hexanol concentration to 18 mM. Kutchai et al. (1980) concluded that the inhibitory effect on osmotic permeability is caused by an effect of the alcohol on the protein-mediated water transport. If the alcohol effect also applies to the diffusion permeability, the permeability coefficient should be lowered to 1.2 \(\times 10^{-3}\) cm s\(^{-1}\) at the hexanol concentration of 25 mM used in the present study. The fact that the diffusional water permeability remained unchanged in the presence of aliphatic alcohol indicates that inhibition of water transport by alcohols is observed only in osmotic experiments, and that the alcohol-inhibited fraction of osmotic permeability differs from the PCMB-inhibitable fraction of both osmotic and diffusional water permeability. Once again, the lack of effect of alcohols cannot be caused by an artifact of the method, because under similar experimental conditions the methanol permeability was increased 40–50% by hexanol and octanol at the same concentrations as applied in the present study (Brahm, 1982).

Similarity of inhibition of diffusional and osmotic water permeability could only be obtained by means of the two SH reagents PCMB and PCMB. Macey and Farmer (1970), Macey et al. (1972), and Naccache and Sha‘afi (1974) all found an identical inhibition of 80% of hydraulic permeability with
1 mM PCMB or PCMBS at room temperature. Previous inhibition studies of diffusional water permeability showed results between 54% obtained by Macey et al. (1972) and Conlon and Outhred (1978) and 65% obtained by Andrasko (1976), who used 20 mM PCMB. In the present study a similar degree of inhibition was obtained in ghosts whose permeability was reduced to 0.9 \times 10^{-3} \text{ cm s}^{-1} \text{ at } 25^\circ\text{C}. A further inhibition below this "ground" permeability appears not to be obtainable either for osmotic or for diffusional water permeability. The different degree of inhibition in the two different types of water transport, therefore, merely reflects the fact that the fraction of permeability above the ground permeability differs in magnitude in the two types of transport, because in both cases the permeability above the ground permeability can be inhibited completely by means of the SH reagents. (Table IV).

### Table IV

| WATER PERMEABILITY OF HUMAN RED CELL MEMBRANES AT 25°C |
|-----------------------------------------------------|
| **Total permeability** | **PCMBS-insensitive permeability** | **PCMBS-sensitive permeability** |
| \(P, \text{ cm s}^{-1} \times 10^3\) |
| Diffusion | 2.4 (100%) | 1.0 (42%) | 1.4 (58%) |
| Osmotic | 18 (100%)\* | 1.8 (95%)\* | 18.2 (95%) |

The osmotic data were taken from Colombe and Macey (1974),* and Macey et al. (1972).* The evaluation of the magnitude of the different permeabilities was based on the assumption of two parallel pathways to water transport, one sensitive and one insensitive to PCMB inhibition.

The apparently maximal inhibition obtained with PCMBs in experiments with ghosts may be caused by the incubation procedure, because the rescaling medium contained PCMBs, which ensured that both sides of the membrane were exposed to PCMBs at the same time. This may be crucial for a maximum inhibition because PCMBs permeates the membrane via at least two distinct pathways, of which the smaller one apparently leads to the functionally important populations of sulhydryl sites within the membrane (Knauf and Rothstein, 1971). If inhibition is facilitated by the addition of PCMBs to the inside of the membrane, it suggests that the reactive groups may be located deeper in the membrane, or possibly closer to the inside of the membrane, and that the reaction between the reagent and the membrane sites is a time-consuming process, as pointed out by Macey (1979).

The diffusional water permeability was also reduced by some nonelectrolytes (thiourea, glycerol, 1,3-propanediol, and DMSO; cf. Table II). As discussed in the preceding section, the inhibition is not due to the osmotic effect of the solutes, which is clearly seen by the same degree of inhibition by 0.5 M thiourea and 6 M glycerol of 1,3 propanediol. It is not clear how water
transport is inhibited by these nonelectrolytes, but it is noteworthy that all the solutes apparently were able to inhibit water permeability to the same value \( \sim 1.4 \times 10^{-3} \) cm s\(^{-1}\). The inhibitory effect of thiourea, glycerol, and 1,3 propanediol may be caused by their ability to form hydrogen bonds (Stein, 1969) with membrane components in competition with water. The degree of inhibition by each nonelectrolyte cannot, however, be determined by their relative hydrogen bonding ability, because thiourea whose hydrogen bonding ability expressed by an assigned \( N \) value of 4 (Stein, 1967) similar to that of water, was a considerably more efficient inhibitor than glycerol with an \( N \) value of 6, which indicates a larger hydrogen bonding ability. It is therefore most likely that the different efficiency of inhibition also depends on, for example, the concentration of the inhibitor in the membrane phase.

The inhibitory effect of DMSO whose hydrogen bonding ability is lower than that of glycerol may be due to the strong interaction with water molecules through the S-O dipoles (Frank, 1973) that probably cause the strong decrease of the self-diffusion coefficient of water (Zeidler, 1973). The inhibition by DMSO, therefore, does not necessarily involve interactions with membrane components because inhibition could be caused by reduced self-diffusion of water in the intra- and extracellular phases.

**Temperature Dependence**

Previous determinations of the temperature dependence of diffusional water permeability in human red cells by means of tracer (Vieira et al., 1970) and NMR techniques (Shporer and Civan, 1975; Morariu and Benga, 1977; Conlon and Outhred, 1978) gave values between 22 and 36 kJ mol\(^{-1}\). Conlon and Outhred (1978) found that an overall value of 22 kJ mol\(^{-1}\) was compounded of a high temperature dependence (26-29 kJ mol\(^{-1}\)) in the physiological temperature range, and a lower one (16-18 kJ mol\(^{-1}\)) below 20-30°C. A recent NMR study (Morariu et al., 1981) shows an even more pronounced change of activation energy of water exchange times from 6 kJ mol\(^{-1}\) below 26°C to 24 kJ mol\(^{-1}\) above that temperature.

The results shown in Fig. 4 do not indicate that two different temperature-dependent steps control water transport at high and low temperatures, as suggested by Conlon and Outhred (1978) and Morariu et al. (1981). The Arrhenius diagram reveals straight curves both for intact red cells and ghosts, as well as for PCMBS-inhibited ghosts. One can therefore conclude that one apparent activation energy of the transport process that differs in control cells and PCMBS-inhibited cells determines the rate of transport in the whole temperature range under study. The temperature dependence of diffusional water permeability in red blood cells of 21 kJ mol\(^{-1}\) between 2.5 and 38°C is identical with the temperature dependence of osmotic water transport determined by Macey et al. (1972), and of a similar magnitude as the activation energy of self-diffusion of water (19.3-20.1 kJ mol\(^{-1}\); Wang et al., 1953; Wang 1965). A temperature dependence of 21 kJ mol\(^{-1}\) for the diffusion process across the erythrocyte membrane indicates that the water molecules during the transport either interact little with the components of the membrane or
that the interaction with the membrane has an equally low, or even lower, temperature dependence than that of self-diffusion of water.

In the study of Vieira et al. (1970) the activation energy of osmotic water transport was also determined to be as low as 14 kJ mol\(^{-1}\). They interpreted the low temperature dependence within the frames of the assumption that water transport could be described by transport through equivalent pores in the membrane (Solomon, 1968), where the low activation energy “suggests some slippage at the water-membrane interface.” Naccache and Sha'afi (1974), who found a similar low temperature dependence of osmotic water transport, did not consider the problem. However, if slippage should explain the low activation energy of water transfer, the slippage itself as a part of the transport process must be almost insensitive to temperature. On the other hand, the low temperature dependence may be due to a methodological artifact that follows determinations of permeability by osmotic swelling of cells (Vieira et al., 1970) or by means of the hemolysis technique (Naccache and Sha'afi, 1974), which presumably increases the membrane area (Jay, 1978) and/or increases the permeability in general or locally. Both effects may cause results that were interpreted as slippage by Vieira et al. The fact that Macey et al. (1972) found a larger temperature dependence of osmotic permeability could thus be due to the different experimental procedure. Their perturbations of the extracellular medium in determinations of permeability must cause less damage of the mechanical properties of the cell membrane, which may reduce the phenomenon which Viera et al. called slippage.

The temperature dependence of osmotic and diffusional water permeability were both changed by inhibition of water transport with PCMB. The osmotic study by Macey et al. (1972) showed an increase of activation energy from 20 kJ mol\(^{-1}\) to 48 kJ mol\(^{-1}\), and in the present diffusional study the temperature dependence was raised to 60 kJ mol\(^{-1}\) in the PCMB-treated ghosts (Fig. 4). The present data and those of Macey et al. show that water transport in human red cells and their ghosts can be inhibited to the same degree at the different temperatures, leaving a residual or ground permeability as low as that of artificial lipid membranes (Cass and Finkelstein, 1967). This ground permeability, furthermore, displays a temperature dependence very similar to the theoretically and experimentally verified activation energy of 50–58 kJ mol\(^{-1}\) for water diffusion through the hydrocarbon phase of a lipid membrane (Redwood and Haydon, 1969; Price and Thomson, 1969). PCMB and PCMBS apparently close a pathway in the human red cell membranes that is characterized by having a low temperature dependence to water transport, and by mediating ~60% of diffusional and 80–90% of osmotic water transport at 25°C (cf. Table IV).

**Do Integral Membrane Proteins Transport Water?**

Because osmotic water transport in human (and dog) red cells is considerably larger than transport by diffusion (Table IV), Solomon (1968) suggested that the transport processes could be described by the presence of so-called equivalent pores, which for the human cells were calculated to be 4.5 Å. Direct
evidence for the pores has not been presented, but Brown et al. (1975) directed attention to the obvious possibility that the integral membrane proteins that span the red cell membrane (for review, including nomenclature, see Steck, 1974) provide nonspecific leak pathway to water and small nonelectrolytes. Brown et al. suggested that the band 3 protein, which is involved in anion transport, also transports water, because their binding studies with radioactive labeled DTNB showed binding exclusively to this fraction of membrane proteins by polyacrylamide sodium dodecyl sulphate gel electrophoresis. Similarly, Sha'afi and Feinstein (1977) determined an exclusive binding of PCMB to the band 3 protein fraction. It must be noted, however, that the apparently specific binding in the two studies was obtained only after pretreatment of the membranes with other SH-reacting reagents to reduce a significantly nonspecific binding of the radioactive labels to different protein components of the membrane. Recent binding studies with PCMB (Deuticke, 1979) and PCMBS (Holdstock and Ralston, 1980) suggest that binding of the SH reagents to other integral membrane proteins (bands 4.5, 4.9, and 7) may be quantitatively more important. Brown et al. suggested two alternative models of the supposed proteinaceous leak pathway. In one model the leak was formed by the hydrophilic path created by the dimerization of band 3 protein monomers. In the other model it was assumed that a third and different protein molecule should be placed close to one band 3 protein molecule, establishing the nonspecific path between the two different protein molecules. This model is interesting in light of the investigation by Cherry and Nigg (1980) of the rotational diffusion of band 3 proteins. They concluded that it is most likely that band 3 proteins appear as dimers, although higher oligomers may occur as well, forming a noncovalent complex with the integral glycophorin A protein in the erythrocyte membrane.

However, evidence indicates that integral membrane proteins do not necessarily induce nonspecific leaks to water and small hydrophilic nonelectrolytes, as suggested by Brown et al. The osmotic permeability of chicken erythrocytes (Blum and Forster, 1970; Farmer and Macey, 1970) is similar to the diffusional water permeability of 1.35 × 10⁻³ cm s⁻¹ (Brahm and Wieth, 1977), and thus as low as that of artificial bilayer membranes although integral membrane proteins are quantitatively indistinguishable from the integral proteins of human red cells. Red cells from the giant salamander *Amphiuma means*, which possess a chloride exchange mechanism as efficient as that of human and chicken red cells presumably also mediated by integral membrane proteins, have a very low diffusional water permeability of 0.7 × 10⁻³ cm s⁻¹ (Wieth and Brahmr, 1977). Fetal, human erythrocytes show the same morphological picture of the membrane proteins (Shapiro and Pasqualini, 1978) and have a physiological anion transport system with the same properties as in adult cells, whereas water permeability is reduced by 35% (Barton and Brown, 1964; Brahm and Wimberley, 1981).

Thus it is obvious that integral membrane proteins do not necessarily provide an indiscriminate leak pathway to water and small hydrophilic molecules. On the other hand, there is the fact that water permeability is considerably larger in erythrocytes from certain species (e.g., human, dog,
duck), and that the increase of permeability is followed by a difference in magnitude of osmotic and diffusional water permeability coefficients. The examples of low water permeability of the above-mentioned red cells obviously do not exclude the possibility that the larger permeability in, for instance, human red cells is caused by a water channel lined with proteins. Brown et al. (1975) based their conclusion on DTNB labeling of band 3 protein and a 60% inhibition of osmotic water transport by DTNB shown by Naccache and Sha’afi (1974). Macey (1979) has questioned the validity of DTNB both as an inhibitor of osmotic water transport, which appears to be minimal if present at all, and as a marker of the water transporting channels. In this connection it is noteworthy that DTNB did not inhibit diffusional water transport (Table III). However, the facts that PCMB and PCMBS inhibit both osmotic and diffusional water permeability to the same ground permeability, and that radioactive labeling of erythrocyte membranes is apparently linked to integral membrane proteins suggest that such proteins are involved in transport of the fraction of diffusional and osmotic water transport above the ground permeability. This fraction of water transport is not mediated by nonspecific transport pathways. On the contrary, the water-transporting channels should be characterized as specific to water transport because their presence in the membrane does not inevitably increase, for example, urea permeability (Wieth and Brahm, 1977). Furthermore, the inhibition study by Macey et al. (1972) clearly demonstrated the separation of transport paths to water and different nonelectrolytes, which led to their conclusion that the water channels transport water and very little else.

One may consider how these water transporting channels are related to the proteins. Brown et al. (1975) suggested an intrinsic channel lined with protein molecules. Alternatively, the channel may be formed between the integral proteins and the sealing lipids surrounding the intramembranaceuous part of the protein molecule. A channel created by lipoproteins may contain outer polar segments between the polar heads of the lipid molecules and the underside of the protein molecules protruding from the membrane phase, and a central segment with more nonpolar interactions between the lipids and the protein molecule (Finean, 1973). Such an arrangement would be in agreement with the experimental observation that the faster permeating lipophilic PCMB more readily inhibits osmotic water transport than does PCMBS (Fig. 1 of Macey and Farmer, 1970). Furthermore, a recent study by Macey et al. (1979) of the kinetics of channel closure by means of PCMBS suggests that hydrophobic interactions may also be involved in the channel closure.

I am grateful to Drs. J. O. Wieth and P. J. Bjerrum for stimulating and constructive discussions and criticism. I thank Mrs. Birgitte D. Olsen for expert technical assistance.

Received for publication 8 June 1981 and in revised form 16 November 1981.

REFERENCES

Andrasko, J. 1976. Water diffusion permeability of human erythrocytes studied by a pulsed gradient NMR technique. *Biochim. Biophys. Acta* **428:**304–311.
Barton, T. C., and D. A. J. Brown. 1964. Water permeability of the fetal erythrocyte. J. Gen. Physiol 47:839–849.

Benson, E. S., M. R. R. Fanelli, G. M. Giacometti, A. Rosenberg, and E. Antonini. 1973. Effects of ligand binding on the rates of hydrogen exchange in myoglobin and hemoglobin. Biochemistry. 12:2699–2706.

Blum, R. M., and R. E. Forster. 1970. The water permeability of erythrocytes. Biochim. Biophys. Acta. 203:410–423.

Brahm, J. 1977. Temperature-dependent changes of chloride transport kinetics in human red cells. J. Gen. Physiol. 70:283–306.

Brahm, J. 1982. Permeability of human red cells to a homologous series of aliphatic alcohols. Limitations of the continuous flow method. J. Gen. Physiol. In press.

Brahm, J., and J. O. Wieth. 1977. Separate pathways for urea and water, and for chloride in chicken erythrocytes. J. Physiol. (Lond.). 266:727–749.

Brown, P. A., M. B. Feinstein, and R. I. Sha'afi. 1975. Membrane proteins related to water transport in human erythrocytes. Nature (Lond.). 254:523–525.

Cass, A., and A. Finkelstein. 1967. Water permeability of thin lipid membranes. J. Gen. Physiol. 50:1765–1784.

Cherry, R. J., and E. A. Nigg. 1980. Molecular interactions involving band 3: information from rotational diffusion measurements. In Membrane Transport in Erythrocytes. Alfred Benzon Symposium 14. U.V. Lassen, H.H. Ussing, and J.O. Wieth, editors. Munksgaard, Copenhagen. 130–138.

Chien, D. Y., and R. I. Macey. 1977. Diffusional water permeability of red cells. Independence on osmolality. Biochim. Biophys. Acta. 464:45–52.

Chien, S., K. P. Sung, R. Skalak, S. Usami, and A. Tözeren. 1978. Theoretical and experimental studies on viscoelastic properties of erythrocyte membrane. Biophys. J. 24:463–487.

Colombe, B.W., and R. I. Macey. 1974. Effects of calcium on potassium and water transport in human erythrocyte ghosts. Biochim. Biophys. Acta. 363:226–239.

Conlon, T., and R. Outhred. 1972. Water diffusion permeability of erythrocytes using an NMR technique. Biochim. Biophys. Acta. 288:354–361.

Conlon, T., and R. Outhred. 1978. The temperature dependence of erythrocyte water diffusion permeability. Biochim. Biophys. Acta. 511:408–418.

Dalmark, M., and J. O. Wieth. 1972. Temperature dependence of chloride, bromide, iodide, thiocyanate and salicylate transport in human red cells. J. Physiol. (Lond.). 224:583–610.

Deuticke, B. 1979. The specific monocarboxylate carrier system in the erythrocyte membrane. In Biophysics of Membrane Transport. Fifth Winter School. J. Kuczera, J. Gabrielska, and S. Przestalski, editors. Publish. Dept. Agricult. Univ. Wroclaw, Wroclaw. 157–190.

Fabry, M. E., and M. Eisenstadt. 1975. Water exchange between red cells and plasma. Measurements by nuclear magnetic relaxation. Biophys. J. 15:1101–1110.

Fabry, M. E., and M. Eisenstadt. 1978. Water exchange across red cell membranes. II. Measurement by nuclear magnetic resonance $T_1$, $T_2$, and $T_{12}$ hybrid relaxation. The effects of osmolality, cell volume, and medium. J. Membr. Biol. 42:375–398.

Farmer, R. E. L., and R. I. Macey. 1970. Perturbation of red cell volume: rectification of osmotic flow. Biochim. Biophys. Acta. 196:53–65.

Finean, J. B. 1973. Phospholipids in biological membranes and the study of phospholipid-protein interactions. In Form and Function of Phospholipids. G.B. Ansell, J.N. Hawthorne, and R.M.C. Dawson, editors. Elsevier Scientific Publishing Company, Amsterdam. 171–203.

Frank, F. 1973. The solvent properties of water. In Water. A Comprehensive Treatise. F. Franks, editor. Plenum Press, New York. 21–54.
Funder, J., and J. O. Wieth. 1976. Chloride transport in human erythrocytes and ghosts. A quantitative comparison. J. Physiol. (Lond.). 262:679–698.

Galey, W. R. 1978. Determination of human erythrocyte membrane hydraulic conductivity. J. Membr. Sci. 4:41–49.

Gutknecht, J., and D. C. Tosteson. 1970. Ionic permeability of thin lipid membranes. Effects of n-alkyl alcohols, polyvalent cations, and a secondary amine. J. Gen. Physiol. 55:359–374.

Haest, C. W. M., D. KAMP, G. PLAAS, and B. DEUTICKE. 1977. Intra- and intermolecular cross-linking of membrane proteins in intact erythrocytes and ghosts by SH-oxidizing agents. Biochim. Biophys. Acta. 469:226–230.

Hochmuth, R. M., K. L. Buxbaum, and E. A. Evans. 1980. Temperature dependence of the viscoelastic recovery of red cell membrane. Biophys. J. 29:177–182.

Hochster, H. S., and J. H. Prestegard. 1977. Pulse NMR studies of water permeability in phosphatidylcholine vesicles containing general anesthetics. J. Membr. Biol. 35:303–307.

Hoffman, J. F., M. Eden, J. S. BARR, and R. H. S. Bedell. 1958. The hemolytic volume of human erythrocytes. J. Cell. Comp. Physiol. 51:405–414.

Holdstock, S. J., and G. B. Ralston. 1980. Disruption of triton shells of erythrocyte membranes by p-mercuribenzenesulphonate. IRCS Medical Science: Biochemistry; Cell and Membrane Biology; Hematology. 8:723–724.

House, C. R. 1974. Water transport in cells and tissues. Edward Arnold Publishers, Lond. 19–22, 152–162.

Jain, M. K., D. G. Touissaint, and E. H. Cordes. 1973. Kinetics of water penetration into unsaponified liposomes. J. Membr. Biol. 14:1–16.

Jay, A. W. L. 1975. Geometry of the human erythrocyte. I. Effect of albumin on cell geometry. Biophys. J. 15:205–222.

Jay, A. W. L. 1978. Hydraulic permeability coefficients of individual human erythrocytes. Can. J. Physiol. Pharmacol. 56:458–464.

Jennings, M. 1978. Characteristics of CO₂-independent pH equilibration in human red blood cells. J. Membr. Biol. 40:365–391.

Jennings, M. L., and A. K. Solomon. 1976. Interaction between phloretin and the red blood cell membrane. J. Gen. Physiol. 67:381–397.

Knauf, P. A., and A. Rothstein. 1971. Chemical modification of membranes. II. Permeation paths for sulphydryl agents. J. Gen. Physiol. 58:211–223.

Korn, A. H., S. H. Fearheller, and E. M. Filachione. 1972. Glutaraldehyde: nature of the reagent. J. Mol. Biol. 65:525–529.

Kutchai, H., R. A. Cooper, and R. E. Forster. 1980. Erythrocyte water permeability. The effects of anesthetic alcohols and alterations in the level of membrane cholesterol. Biochim. Biophys. Acta. 600:542–552.

LaCelle, P. L. 1972. Effect of spherling on erythrocyte deformability. Biochemistry. 9:51–59.

Lenard, J., and S. J. Singer. 1968. Alteration of the conformation of proteins in red blood cell membranes and in solution by fixatives used in electron microscopy. J. Cell. Biol. 37:117–121.

Levin, S. W., R. L. Levin, and A. K. Solomon. 1980. Improved stop-flow apparatus to measure permeability of human red cells and ghosts. J. Biochem. Biophys. Methods. 3:255–272.

Macey, R. I. 1979. Transport of water and nonelectrolytes across red cell membranes. In Transport Across Single Biological Membranes. D.C. Tosteson, editor. Springer-Verlag, Berlin. 1–57.

Macey, R. I., D. CHEN, T. Moura, and D. Karan. 1979. Closure of water channels in the red cells. Biophys. J. 25:102a.

Macey, R. I., and R. E. L. Farmer. 1970. Inhibition of water and solute permeability in human red cells. Biochim. Biophys. Acta. 211:104–106.
MACEY, R. I., D. M. KARAN, and R. E. L. FARMER. 1972. Properties of water channels in human red cells. In Biomembranes. F. Kreuzer and J. F. G. Slegers, editors. Plenum Press, New York. 333–340.

MARFEO, S. P., and K. H. TSAI. 1975. Cross-linking of phospholipids in human erythrocyte membrane. Biochem. Biophys. Res. Commun. 65:31–38.

MATHUR-DE VRÉ, R. 1979. The NMR studies of water in biological systems. Prog. Biophys. Mol. Biol. 35:103–134.

MAURO, A. 1960. Some properties of ionic and nonionic semipermeable membranes. Circulation. 21:845–854.

MILLONG, G., and V. MARINOZZI. 1968. Fixation and embedding in electron microscopy. In Advances in Optical and Electron Microscopy. R. Barer and V. E. Cosslett, editors. Academic Press Inc., London. 2:251–341.

MORARIU, V. V., and G. BENGA. 1977. Evaluation of a nuclear magnetic resonance technique for the study of water exchange through erythrocyte membranes in normal and pathological subjects. Biochim. Biophys. Acta. 469:301–310.

MORARIU, V. V., V. I. POP, O. POPESCU, and G. BENGA. 1981. Effects of temperature and pH on the water exchange through erythrocyte membranes: nuclear magnetic resonance studies. J. Membr. Biol. 62:1–5.

NACCACHE, P., and R. I. SHA’AFI. 1974. Effect of PCMBS on water transfer across biological membranes. J. Cell. Physiol. 83:449–456.

OUTHRED, R., and T. CONLON. 1973. The volume dependence of the erythrocyte water diffusion permeability. Biochim. Biophys. Acta. 318:446–450.

OWEN, J. D., and A. K. SOLOMON. 1972. Control of nonelectrolyte permeability in red cells. Biochim. Biophys. Acta. 290:414–418.

OWEN, J. D., M. STEGGALL, and E. M. EVRING. 1974. The effect of phloretin on red cell nonelectrolyte permeability. J. Membr. Biol. 19:79–92.

PAGANELLI, C. V., and A. K. SOLOMON. 1957. The rate of exchange of tritiated water across the human red cell membrane. J. Gen. Physiol. 41:259–277.

PARKER, J. L., D. L. ASHLEY, and J. H. GOLDSTEIN. 1979. Pulse nuclear magnetic resonance measurements of water exchange across the erythrocyte membrane employing a low Mn concentration. Biophys. J. 25:389–406.

PRICE, H. D., and T. E. THOMPSON. 1969. Properties of liquid bilayer membranes separating two aqueous phases: temperature dependence of water permeability. J. Mol. Biol. 41:443–457.

REDWOOD, W. R., and D. A. HAYDON. 1969. Influence of temperature and membrane composition on the water permeability of lipid bilayers. J. Theor. Biol. 22:1–8.

REDWOOD, W. R., E. RALL, and W. PERL. 1974. Red cell membrane permeability deduced from bulk diffusion coefficients. J. Gen. Physiol. 64:706–729.

RICH, G. T., R. I. SHA’AFI, A. ROMUALDEZ, and A. K. SOLOMON. 1968. Effect of osmolality on the hydraulic permeability coefficient of red cells. J. Gen. Physiol. 52:941–954.

SCHWOCH, G., and H. PASSOW. 1973. Preparation and properties of human erythrocyte ghosts. Mol. Cell. Biochem. 2:197–218.

SEEMAN, P. 1972. The membrane actions of anesthetics and tranquilizers. Pharmacol. Rev. 24:583–596.

SHA’AFI, R. I., and M. B. FEINSTEIN. 1977. Membrane water channels and SH-groups. In Advances in Experimental Medicine and Biology. Membrane Toxicity. M. W. Miller and A. E. Shamo, editors. 84:67–80.

SHA’AFI, R. I., G. T. RICH, V. W. SIDEL, W. BOSSERT, and A. K. SOLOMON. 1976. The effect of the unstirred layer on human red cell water permeability. J. Gen. Physiol. 50:1377–1399.
SHAPIRO, D. L., and P. PASQUALINI. 1978. Erythrocyte membrane proteins of premature and full-term newborn infants. Pediat. Res. 12:176–178.

SHPORER, M., and M. M. CIVAN. 1975. NMR study of $^{17}$O from H$_2^{17}$O in human erythrocytes. Biochim. Biophys. Acta. 385:81–87.

SIDEL, V. W., and A. K. SOLOMON. 1957. Entrance of water into human red cells under an osmotic pressure gradient. J. Gen. Physiol. 41:243–257.

SIRS, J. A. 1969. The rate of osmotic influx of water by flexible and inflexible erythrocytes. J. Physiol. (Lond.). 205:147–157.

SOLOMON, A. K. 1968. Characterization of biological membranes by equivalent pores. J. Gen. Physiol. 51:335s–364s.

STECK, T. L. 1974. The organization of proteins in the human red blood cell membrane. J. Cell. Biol. 62:1–19.

STEIN, W. D. 1967. The movement of molecules across cell membranes. Academic Press, Inc., New York. 65–90.

VIEIRA, F. L., R. I. SHAAFI, and A. K. SOLOMON. 1970. The state of water in human and dog red cell membranes. J. Gen. Physiol. 55:451–466.

WANG, J. H. 1965. Self-diffusion coefficients of water. J. Phys. Chem. 69:4412.

WANG, J. H., C. V. ROBINSON, and I. S. EDELMAN. 1953. Self-diffusion and structure of liquid water. III. Measurements of the self-diffusion of liquid water with H$^2$, H$^3$ and O$^{18}$, as tracers. J. Am. Chem. Soc. 75:466–470.

WESTERMAN, M. P., L. E. PIERCE, and W. N. JENSEN. 1961. A direct method for the quantitative measurement of red cell dimensions. J. Lab. Clin. Med. 57:819–824.

WHITE, D. A. 1973. The phospholipid composition of mammalian tissues. In Form and Function of Phospholipids. G.B. Ansell, J.N. Hawthorne, and R.M.C. Dawson, editors. Elsevier Scientific Publishing Company, Amsterdam. 441–482.

WIETH, J. O., and J. BRAHM. 1977. Separate pathways to water and urea in red blood cells? A comparative physiological approach. Proc. Intern. Cong. Physiol. Sci. Abstract 1.06.27.

WIETH, J. O., J. BRAHM, and J. FUNDER. 1980. Transport and interactions of anions and protons in the red blood cell membrane. Amn. N. Y. Acad. Sci. 341:394–418.

WOOD, J. G. 1973. The effects of glutaraldehyde and osmium on the proteins and lipids of myelin and mitochondria. Biochim. Biophys. Acta. 329:118–127.

ZEIDLER, M. D. 1975. NMR spectroscopic studies. In Water. A Comprehensive Treatise. F. Franks, editor. Plenum Press, New York. 2:529–584.