J. Membrane Biol. 108, 105–113 (1989)

The Journal of
Membrane Biology

Water Exchange Through Erythrocyte Membranes: Biochemical and Nuclear Magnetic Resonance Studies Re-evaluating the Effects of Sulfhydryl Reagents and of Proteolytic Enzymes on Human Membranes

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Summary. The water permeability of human red blood cell (RBC) membrane has been monitored by a doping nuclear magnetic resonance (NMR) technique on intact cells and resealed ghosts following exposure to various sulfydryl-reacting (SH) reagents and proteolytic enzymes. The main conclusions are the following: (i) When appropriate conditions for exposure of erythrocytes or ghosts to mercury-containing SH reagents (concentration, temperature and duration of incubation) were found, the maximal inhibition of water diffusion could be obtained with all mercurials (including $HgCl_2$ and mersalyl that failed to show their inhibitory action on RBC water permeability in some investigations). While previous studies claimed that long incubation times are required for the development of maximal inhibition of water diffusion by mercurials, the present results show that it can be induced in a much shorter time (5–15 min at 37°C) if relatively high concentrations of PCMBS (2–4 mM) are used and no washings of the inhibitor are performed after incubation. Higher than optimal concentrations of mercurials and/or longer incubation times result in lower values of inhibition, sometimes a loss of inhibition, or can even lead to higher values of permeability compared to control RBCs. (ii) The conditions for inhibition by mercurials are drastically changed by preincubation of erythrocytes with noninhibitory SH reagents (such as NEM or IAM) or by exposure to proteolytic enzymes. If the cells are digested with papain, the duration of incubation with PCMBS should be decreased in order for inhibition to occur. This explains the lack of inhibition reported previously, when a relatively long duration of incubation with PCMBS was used subsequent to papain digestion. (iii) The degree of inhibition of water diffusion induced by mercurials appeared to be dependent upon the temperature of which the water permeability was measured. The values of maximal inhibition ranged from 45–50% at 37°C, increased 10–15% at 20°C and further increased at lower temperatures, reaching values above 75% below 10°C; these results clarify the conflicting reports of various authors. (iv) The inhibition of water diffusion, either reversible, or irreversible, was not accompanied by significant changes in the pattern of RBC membrane polypeptides fractionated by polyacrylamide gel electrophoresis. (v) The mean value of the activation energy of water diffusion ($E_{a,d}$) obtained on 42 donors was 25.6 kJ/mol. The values of $E_{a,d}$ increased in parallel with the values of the inhibition of water diffusion induced by PCMBS until the maximal inhibition was reached (when $E_{a,d}$ = 41 kJ/mol) and then both sets of values decreased in parallel.

Key Words water exchange · erythrocyte membranes · NMR studies · sulfhydryl reagents · proteolytic enzymes

Introduction

Because of its availability and simple structure (lacking internal membranes), the red blood cell (RBC) is ideally suited for investigating water permeability. In fact, it has been for many years one of the most favored cells for such investigations (*see* recent reviews by Macey, 1984; Morariu & Benga, 1984; Solomon, 1986; Macey & Brahm, 1988; Benga, 1988). Studies of the effects of various reagents and of chemical modification of membranes can contribute to a better understanding of molecular mechanisms of water transport in red blood cells. The usefulness of this approach has already been documented by previous studies. It has been shown for example that mercury-containing sulfhydryl-reacting reagents (SH reagents) substantially inhibited the water exchange through erythrocyte membranes (Macey & Farmer, 1970; Naccache & Sha'afi, 1974; Benga et al., 1983*b*; Dix & Solomon, 1984). This had lead to the suggestion that aqueous channels accommodated in the membrane proteins play a major role in the water exchange and that the mercury-containing SH reagents act by closing the channels (Moura et al., 1984). Moreover, recent studies by using ^{203}Hg-labeled *p*-chloromercuribenzene sulfonate (PCMBS) has allowed us to identify the membrane proteins that are involved in the water exchange through the human red blood cells (Benga et al., 1986*b*,*c*).

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There are two basic strategies for measuring water exchange through the erythrocyte membrane: (i) nonstationary methods, by which the osmotic permeability of RBC is measured, and (ii) stationary methods, which can be classified into radio-tracer methods (Paganelli & Solomon, 1957; Vieira, Sha'afi & Solomon, 1970) and nuclear magnetic resonance (NMR) methods (Conlon & Outhred, 1972; Fabry & Eisenstadt, 1978; Morariu & Benga, 1977; Pirkle, Ashley & Goldstein, 1979). Studies on the effects of various reagents, performed by various methods have sometimes given conflicting results. One aim of this work was to find the real effects of various reagents by carefully studying all possible sources of divergent results. Consequently, we report here results of extensive studies with all mercury-containing SH reagents that have been previously found, at least in some studies, to be inhibitors of the RBC water permeability. In addition, the effects on this transport process of exposure of erythrocytes to proteolytic enzymes, followed by SH reagents, have been reevaluated. The sources of conflicting results have been found.

On the other hand, very few data have been published (regardless of the method used for measuring the water permeability) on the effects of temperature on water exchange of erythrocytes previously exposed to inhibitors, either alone, or subsequent to a previous treatment with proteolytic enzymes. Another aim of our paper was to present NMR studies on the activation energy of water diffusion ($E_{a,d}$) in erythrocytes following such chemical manipulations of membranes. A new kind of experiment reported here was that in which $E_{a,d}$ of erythrocytes exposed to two or more agents was determined. The findings are discussed in relation to the molecular mechanisms proposed for water transport in the red blood cell.

Materials and Methods

Human blood was obtained by venipuncture in heparinized tubes. The donors were healthy male or female subjects, 10–51 years old. The erythrocytes were isolated by centrifugation, and washed three times in medium S [150 mM NaCl, 5.5 mM glucose, 5 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.4]. For the preparation of resealed (pink) ghosts the procedure of Schwoch and Passow (1973) has been used.

The incubations of erythrocytes or ghosts with SH reagents and/or proteolytic enzymes were performed as indicated in the legend to the figures and tables. In some experiments, after incubation, three washings of the erythrocytes in medium S, each followed by a centrifugation, were performed to remove the reagent. In other experiments, after incubation, no washing was performed. Finally, the erythrocytes or the ghosts were suspended in medium S, supplemented with 0.5% bovine serum albumin at a cytocrit of 50%.

NMR Measurements

Samples for NMR measurements were prepared by carefully mixing 0.2-ml erythrocyte or ghost suspension and 0.1-ml doping solution (40 mM $MnCl_2$, 100 mM NaCl). The water proton relaxation time of the cells (T'_{2a}) was evaluated by the spin-echo method (Conlon & Outhred, 1972) as previously described (Morariu & Benga, 1977; Benga et al., 1986*a*). T'_{2a} is dominated by the exchange process through erythrocyte membrane and is related to the water diffusion exchange time (T_e) by the equation (Conlon & Outhred, 1972):

$$\frac{1}{T_e} = \frac{1}{T'_{2a}} - \frac{1}{T_{2i}} \tag{1}$$

where T_{2i} is the transverse relaxation time of the cell interior. T_{2i} was measured by the 90–180° method using the Carr-Purcell-Meiboom-Gill sequence (Farrar & Becker, 1971) on packed cells or ghosts from which the supernatant, with no added Mn, had been removed by centrifugation at 15,000 × *g* for 60 min.

The membrane permeability for water diffusion, P, is related to $1/T_e$, the cell water volume, V (= 0.72 times the cell volume) and the cell surface area, A, by:

$$P = \frac{V}{A} \cdot \frac{1}{T_e}. \tag{2}$$

Since different authors have used different values of V and A, we have used in the calculation of P three sets of values. On one hand, we have taken a value of 65 μm^3 for the intracellular solvent volume of erythrocytes and 86 μm^3 for that of resealed ghosts and a value of 1.42×10^{-6} cm^2 for the membrane area after Brahm (1982). These give V/A ratios of 4.58×10^{-5} and 6.06×10^{-5} cm for erythrocytes and ghosts, respectively. On the other hand, we have used a slightly higher V/A ratio, after Dix and Solomon (1984), e.g., 5.33×10^{-5} cm for erythrocytes and the corresponding value for ghosts. Finally, we have performed our own estimations of the cell volume based on cell counts and measurement of the cytocrit, with correction for extracellular medium trapped between the packed cells. The values of cell volume obtained in this way were within ranges reported in the literature (Canham & Burton, 1968; Weinstein, 1974). The value of A should be chosen from the values in the literature and the most accepted present value is 1.35×10^{-6} cm^2 (for discussion *see* Solomon, 1986). When calculated with this value of A and with values of V measured as described above, the values of P were not significantly different from the values calculated by using the V/A ratios after Dix and Solomon (1984). Consequently, in order to better compare our results with previous ones, we have given in the Tables the permeability values calculated using the V/A ratios given by Brahm (1982) and by Dix and Solomon (1984).

The inhibition of water diffusion across human red blood cell membranes was calculated in two ways. On one hand, assuming that the permeability coefficient is inversely related to T'_{2a}, we used the formula (Benga et al., 1983*b*,*c*, 1986*b*,*c*, 1987*b*):

$$\% \text{ Inhibition} = \frac{\left(\frac{1}{T'_{2a}\text{ (control)}} - \frac{1}{T'_{2a}\text{ (sample)}}\right)}{\left(\frac{1}{T'_{2a}\text{ (control)}}\right)} \times 100. \tag{3}$$

On the other hand, in order to better compare our results with those of other authors (Macey & Farmer, 1970; Naccache & Sha'afi, 1974; Dix & Solomon, 1984; Toon & Solomon, 1986) another formula was also used:

$$\% \text{ Inhibition} = \frac{P_{\text{control}} - P_{\text{sample}}}{P_{\text{control}}} \times 100. \quad (4)$$

The NMR measurements were performed with an AREMI-78 spectrometer (manufactured by the Institute of Physics and Nuclear Engineering Bucharest-Măgurele) at a frequency of 25 MHz. The temperature was controlled to ±0.2°C by air flow over an electrical resistance using the variable temperature unit attached to the spectrometer. The actual temperature in the sample was measured with a thermocouple connected to a microprocessor thermometer (Comark Electronics Limited, Rustington, Littlehampton, U.K.). The values of T_{2a} were calculated with a computer unit coupled on-line with the NMR spectrometer.

Polyacrylamide Gel Electrophoresis of Erythrocyte Membrane Proteins

The RBCs were lysed by mixing rapidly 1-ml portions into 20 ml of cold 5-mM sodium phosphate buffer, pH 8.0 (5P8), and the ghosts were prepared as described previously (Benga et al., 1983*a*). After three or four washes in 5P8, followed each time by centrifugation at 20,000 × *g* for 20 min, the homogenous and white ghost pellets were suspended in 5P8 to about 4 mg protein/ml and frozen at −20°C until analyzed (usually after 24–48 hr). Protein was determined by the procedure of Lowry et al. (1951).

One vol (usually 25 μl of ghosts) was added to 3 vol of a solution containing 1.3% sodium dodecylsulfate (SDS), 10% sucrose, 53.3 mM dithioerythritol, 1.3 mM sodium EDTA, 20 mM Tris-HCl (pH 6.8) and 0.007 mg/ml bromphenol blue. The mixture was heated for 3 min in a 100°C bath. Membrane peptides were separated using the SDS polyacrylamide system described by Laemmli (1970). The slab gel consisted of a running gel of 10% acrylamide and a 5% stacking gel. The acrylamide-to-bisacrylamide ratio was maintained at 36.5 : 1 in both gels. Samples of 25 μl/25 μg protein were applied and the electrophoresis was carried out at 15 mA (40 V) for 1 hr and at 20 mM (100 V) until the dye reached the bottom of the running gel (about 5 hr) in the running buffer (25 mM Tris, 190 mM glycine, 0.1% SDS). The gels were fixed for 1 hr in 45% (vol/vol) methanol/10% (vol/vol) acetic acid and then stained overnight in the same solution containing 0.07% (wt/vol) Coomassie brilliant blue R-250. Destaining was performed with 10% (vol/vol) acetic acid. The gels were dried according to a procedure previously described by Popescu (1983).

Other Procedures

The hemoglobin content of resealed ghosts (reduced to 4–7% of that of erythrocytes) was estimated spectrophotometrically (Antonini & Brunori, 1971). The calculations of the correlation coefficients of the lines obtained with the sets of data points in the Arrhenius plots were performed with an HP-41 CV computer (Hewlett-Packard).

Results

Factors Influencing the Inhibition by Mercurials of Water Diffusion Through Erythrocyte Membranes

New features of the development of mercurial-induced inhibition of water diffusion have been uncovered by our studies. Although it was generally agreed (Macey & Farmer, 1970; Sha'afi, 1981; Benga et al., 1983*b*; Dix & Solomon, 1984) that out of all SH reagents only mercury-containing compounds are powerful inhibitors of water permeability in human erythrocytes, studies by Sha'afi and Feinstein (1977) and Brahm (1982) failed to detect such effects for mersalyl and $HgCl_2$, respectively. As shown in Fig. 1, all mercurials markedly inhibited the water diffusion if appropriate conditions for exposure of erythrocytes to the reagent (concentration, temperature and duration of incubation) were found. The concentrations at which the inhibition was half maximum, ranged from 20 μM for $HgCl_2$, 60 μM for fluoresceinmercuric acetate (FMA), to 90 μM for PCMBS after a preincubation with N-ethylmaleimide (NEM) and 150 μM for PCMBS treatment alone, are in reasonable agreement with values reported elsewhere (Benga et al., 1982, 1983*b*, 1985*a*, Dix & Solomon, 1984; Toon & Solomon, 1986).

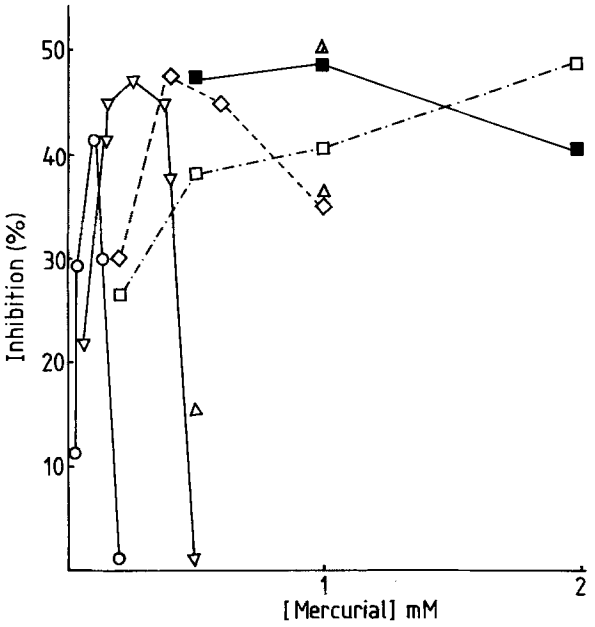

Fig. 1. Dependence of the inhibition of water diffusion in erythrocytes by various concentrations of mercurials. The inhibition was measured at 37°C and calculated according to Eq. (3) as described in Materials and Methods. Washed erythrocytes were suspended in medium S at a hematocrit of 10% and incubated at 37°C with mercurials (to give the final concentrations indicated) as follows: $HgCl_2$ (○) for 60 min; FMA (▽) for 60 min; mersalyl alone (△) for 15 min or subsequent (◭) to a preincubation of 45 min at 37°C with 1-mM IAM and 1-mM NEM; PCMBS alone (□, ■) for 30 min, or PCMBS for 15 min, subsequent (◇) to a preincubation of 60 min at 25°C with 2-mM NEM. After preincubations or after incubations, three washes of erythrocytes in the medium S were performed, except for samples marked ■ that were not washed.

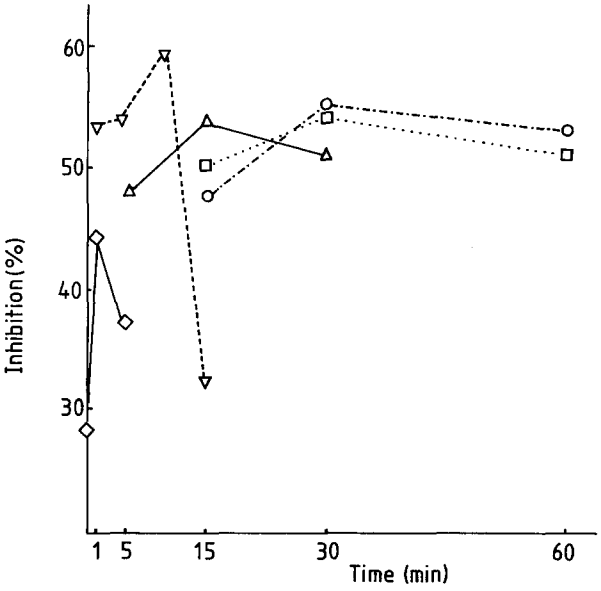

Fig. 2. The time course of inhibition of water diffusion in erythrocytes induced by PCMBS. Washed erythrocytes were suspended in medium S at a hematocrit of 10% and incubated at 37°C with PCMBS to give the final concentrations of 0.5 mM (○···○), 1.0 mM (□···□), 2 mM (△—△), 4 mM (▽---▽), and 10 mM (◇—◇). After incubation, the erythrocytes were sedimented by centrifugation and aliquots from the sediment used for NMR measurements. The inhibition was measured at 20°C and calculated according to Eq. (3), as described in Materials and Methods

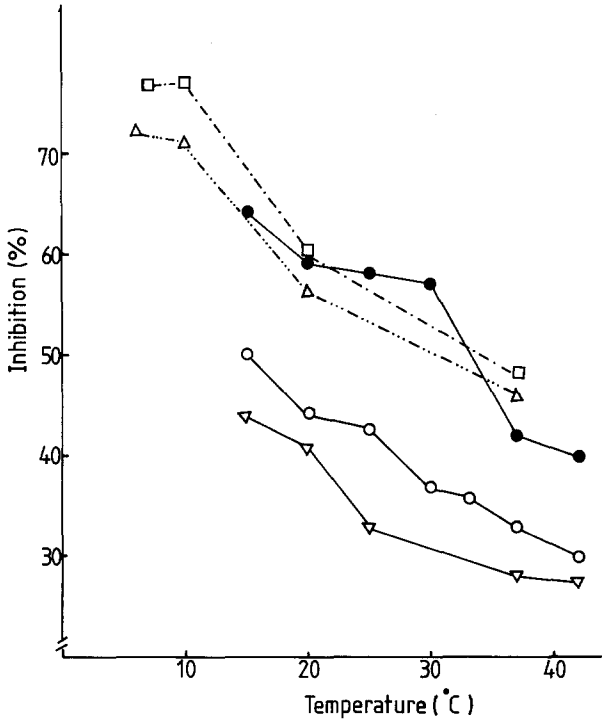

Fig. 3. The temperature dependence of inhibition of water diffusion in red blood cells induced by PCMBS. Washed erythrocytes were suspended in medium S at a hematocrit of 10% and incubated at 37°C with PCMBS as follows: 0.2 mM for 30 min (▽—▽), 0.5 mM for 15 min (○—○), 0.5 mM for 60 min (●—●), 1.0 mM for 30 min (□···□), 2.0 mM for 15 min (△·····△). After incubation, the red blood cells were sedimented by centrifugation and aliquots from sediment were used for NMR measurements. The inhibition was measured at the temperatures indicated, and calculated according to Eq. (4), as described in Materials and Methods

The concentration of PCMBS required to induce the inhibition of water diffusion in resealed ghosts was found to be 10 times lower compared to that required for intact cells.

The relationships between various conditions of incubation, some treatments of erythrocytes before and after incubation and the degree of inhibition of water diffusion have been carefully studied taking PCMBS as a typical inhibitor. As documented by Figs. 1 and 2, the maximal inhibition at 37°C could be obtained in a much shorter time (5–15 min) by using relatively high concentrations of PCMBS (e.g., 2–4 mM) compared to lower concentrations of inhibitor (0.2–0.5 mM) that required incubation times of 30–60 min at 37°C. Moreover, the incubation time and the concentration of PCMBS required for maximal inhibition could be decreased if no washings of the inhibitor were performed after incubation. These findings represent a reevaluation of previous studies (Macey & Farmer, 1970; Naccache & Sha'afi, 1974; Ashley & Goldstein, 1981; Dix & Solomon, 1984) claiming that long incubation times are required for the development of maximal inhibition induced by mercurials.

It should be emphasized that higher than optimal concentrations of inhibitor and/or longer incubation times result in lower values of inhibition, sometimes a loss of inhibition, or can even lead to higher values of permeability compared to control red blood cells.

The degree of inhibition appeared to be dependent upon the temperature at which the water permeability is measured, regardless of PCMBS concentration and incubation time (Fig. 3). The values of maximal inhibition ranged from 45–50% at 37°C, increased 10–15% at 20°C and further increased at lower temperatures, reaching values above 75% below 10°C.

The Activation Energy of Water Diffusion

Systematic studies were performed to determine in a large number of subjects the values of the activation energy of water diffusion in erythrocytes and ghosts, in the absence and in the presence of SH reagents. The values are given in Table 1. It should

Table 1. The activation energy of water diffusion in human erythrocytes[a]

Sample	Conditions of incubation			No. of donors	$E_{a,d}$ (kJ/mol)	Statistical significance compared to control samples
	conc. (mM)	time (min)	temp. (°C)			
Control				42	25.6 ± 3.2	
Stored for 24 hr at 4°C				20	26.8 ± 3.5	$P > 0.1$[b]
Preincubated		60	37	18	27.5 ± 3.6	$P > 0.1$[b]
PCMBS	0.5	15	37	1	32.7	
	0.5	30	37	1	32.9	
	0.5	60	37	3	41.2	$P < 0.001$[b]
	1.0	30	37	17	36.8 ± 3.8	$P < 0.001$[c]
Preincubated, followed by PCMBS		60	37			
	1.0	30	37	17	38.9 ± 5.0	$P < 0.001$[c]
	1.0	60	37	3	40.1 ± 5.2	$P < 0.001$[c]
	2.0	5	37	1	41.5	
	2.0	15	37	1	40.3	
	2.0	60	37	3	29.4	
	5.0	5	37	1	28.6	
Papain 1 mg/ml		60	37	4	24.7	$P > 0.1$[b]
Papain 1 mg/ml, followed by PCMBS	0.5	60	37	2	39.5	$P < 0.001$[b]

[a] The measurements were performed as described in the text. Results represent mean ± SD.
[b] Statistical significance calculated using the paired Student's *t*-test.
[c] Statistical significance calculated using the unpaired Student's *t*-test.

be noted that the values of $E_{a,d}$ increase in parallel with the values of the inhibition of water diffusion induced by PCMBS until the maximal inhibition is reached and then both sets of values decrease in parallel.

Effects of Proteolytic Enzymes and Sulfhydryl Group Reagents on Protein and Water Permeability in Human Erythrocyte Membranes

We have previously studied for the first time the effects of exposure of the human erythrocyte membranes to proteolytic enzymes on water diffusion (Benga et al., 1983*b*,*c*). It was found that none of the proteolytic enzymes (chymotrypsin, trypsin or papain) significantly inhibited water permeability. In contrast with trypsin or chymotrypsin treatment that did not prevent the inhibition induced by mercurials, after exposure of erythrocytes to papain no inhibitory effect of subsequent incubation with PCMBS could be noticed (Benga et al., 1983*c*).

However, having in mind that both chymotrypsin and papain digest band 3 protein in intact red blood cells (Fig. 4), we thought that these effects need to be further studied. We considered the possibility that after papain treatment the concentration of PCMBS and/or the incubation time necessary for inhibition should be decreased. Indeed, after exposure to papain, a subsequent incubation of erythrocytes with 1 mM PCMBS for 30 min at 37°C (instead of 60 min as used in our previous studies) induced maximal inhibition of water diffusion in human erythrocytes (Table 2). In parallel, the value of $E_{a,d}$ increased from 24.7 to 39.5 kJ/mol (Table 1).

When the proteins of erythrocyte membranes were fractionated by polyacrylamide gel electrophoresis, no significant changes could be detected under conditions of maximal inhibition of water diffusion (Fig. 4). It is known that cysteine reverses the inhibition induced by PCMBS (Naccache & Sha'afi, 1974), while it has no effect on the inhibition induced by FMA (Benga et al., 1982). In all conditions we have used, exposure of erythrocytes to mercurials did not induce significant changes in the RBC membrane polypeptide pattern, we may conclude that the inhibition of water diffusion, either reversible, or irreversible, is not accompanied by major alterations of proteins in the membrane. This is in agreement with our previous observations that SH reagents do not induce major morphological changes in the erythrocyte membrane under conditions of inhibition of water diffusion (Benga et al., 1986*c*, 1987*a*). In contrast, in open ghosts incubated with 1–2 mM PCMBS under conditions of low

Table 2. Parameters characterizing the effects of proteolytic enzymes and of sulfhydryl reagents on water diffusion in human red blood cells[a]

Compound	T'_{2a}	T_e (msec)	% Inhibition[b]		P^c (cm · sec^{-1} × 10^3)	
			A	B	I	II
Trypsin	8.1	8.6	12.0	12.6	5.34	6.21
Trypsin followed by 1 mM PCMBS	15.9	17.7	55.0	57.7	2.58	3.01
Chymotrypsin	7.9	8.4	10.0	10.5	5.47	6.37
Chymotrypsin followed by 1 mM PCMBS	15.5	17.3	54.0	56.6	2.65	3.08
Papain	7.8	8.3	9.0	9.3	5.54	6.78
Papain followed by 1 mM PCMBS	14.3	15.8	50.0	52.4	2.90	3.38

[a] Washed red blood cells were incubated for 60 min at 37°C with proteolytic enzymes (1 mg/ml), then washed three times in medium S. The incubation with 1-mM PCMBS was performed at a hematocrit of 10% for 60 min at 37°C in the case of chymotrypsin and trypsin, and for 30 min at 37°C in the case of papain. After incubation, three washings in medium S were performed and aliquots from the sediment were used for NMR measurements at 37°C as described in Materials and Methods. Results are the mean of 3–5 experiments.

[b] A, B: % inhibitions calculated according to Eqs. (3) and (4), respectively.

[c] I, II: The permeabilities calculated from T_e using V/A ratios of 4.58 × 10^{-5} cm and 5.33 × 10^{-5} cm, respectively.

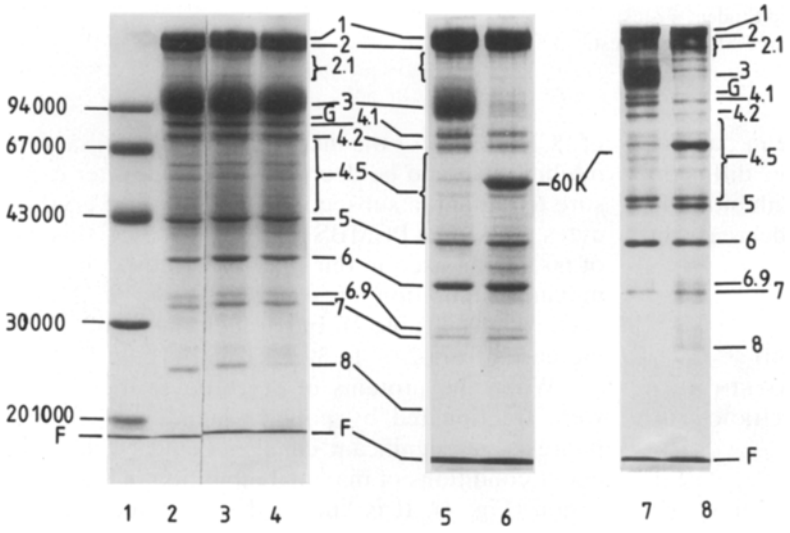

Fig. 4. Electrophoregrams of erythrocyte ghosts prepared from control red blood cells (lanes 2, 5 and 7); cells incubated with 1-mM PCMBS for 30 min at 37° (lane 3); cells incubated with 125-μM FMA for 60 min at 37°C (lane 4); cells treated with chymotrypsin (1 mg/ml) for 60 min at 37°C (lane 6); cells treated with papain 1 mg/ml for 60 min at 37°C (lane 8). After incubation, three washes of erythrocytes in medium S were performed and the cells were sedimented by centrifugation. The membrane samples were prepared as described in Materials and Methods

ionic strength, a solubilization of membrane proteins (especially of those from the cytoskeleton) has been noticed (Carter, 1973; Holdstock & Ralston, 1983).

Discussion

Our findings prompted us to reexamine images in the literature on the effects of SH reagents that have been applied in measurements of diffusional or osmotic water permeability of red blood cells. There is general agreement that SH reagents can be divided in two groups as far as their effects on water permeability are concerned: some reagents, such as NEM or iodoacetamide (IAM) have no effect, while other SH reagents are powerful inhibitors. While some mercurials (*p*-chloromercuribenzoate, PCMBS, FMA) have proved to have inhibitory effects in all studies (Macey & Farmer, 1970; Naccache & Sha'afi, 1974; Ashley & Goldstein, 1981; Benga et al., 1982, 1983*b,c*, 1986*a,b*, 1987*b*; Brahm, 1982; Dix & Solomon, 1984; Moura et al., 1984; Toon & Solomon, 1986) other mercurials ($HgCl_2$, mersalyl) failed to show their inhibitory action on RBC water permeability in some investigations (Sha'afi & Fein-

stein, 1977; Brahm, 1982). It is obvious from the experiments described in this paper that the conditions for exposure of erythrocytes to various compounds have to be carefully studied in order to prove or disprove their inhibitory effects on water permeability. If the duration of incubation and the concentration of inhibition are not chosen properly for each compound (*see* Fig. 1), one can get a whole range of effects, from lower than maximal inhibition values to permeabilities of the same magnitude with those of control cells. This explains the lack of inhibition of water permeability by mersalyl or $HgCl_2$ in studies of Sha'afi and Feinstein (1977) or Brahm (1982), respectively.

The conditions for inhibition by mercurials are drastically changed by preincubation of erythrocytes with noninhibitory SH reagents (such as NEM or IAM) or by exposure to proteolytic enzymes. The increased permeability of erythrocytes induced by mercurials on long incubation times has been noticed (Naccache & Sha'afi, 1974; Dix & Solomon, 1984) and it may ultimately lead to increased fragility (Toon & Solomon, 1986) and hemolysis. If the cells are digested with papain the duration of incubation with PCMBS should be decreased in order for inhibition to occur. This explains the lack of inhibition reported by us previously (Benga et al., 1983*c*), when a relatively long duration of incubation with PCMBS was used subsequent to papain digestion.

It is thus clear from our studies that all mercurials are powerful inhibitors of this transport process and the degree of inhibition that can be induced is quite similar. It appears that maximal inhibition depends upon mercurial binding to SH groups involved in water permeability. We have previously shown that such groups are located in bands 3 and 4.5 and that the binding of mercurial is facilitated by preincubation with NEM (Benga et al., 1986*b,c*). The binding also appears to be facilitated by a preincubation of erythrocytes for 60 min at 37°C or by the exposure of erythrocytes to proteolytic enzymes. The latter feature is in agreement with our previous observations (Benga et al., 1983*b*), when inhibitions around 55% (at 37°C) could be induced by PCMBS-treatment following a preincubation with chymotrypsin or trypsin, compared to inhibitions around 45% induced by PCMBS alone.

While previous studies (Macey & Farmer, 1970; Naccache & Sha'afi, 1974; Ashley & Goldstein, 1981; Dix & Solomon, 1984), claimed that long incubation times are required for the development of maximal inhibition induced by mercurials, the present results show that with higher concentrations than previously used, the inhibition can occur in several minutes. This shows that the SH groups located in bands 3 and 4.5 involved in water permeability can be reached in a relatively short time if concentrations above 2 mM PCMBS are used and no washing of the inhibitor after incubation is performed.

There were conflicting reports regarding the temperature dependence of the mercurial-induced inhibition of water permeability of human blood cells. While Naccache and Sha'afi (1974) reported an increase in the degree of inhibition when the temperature was increased (from 23% at 9°C to 40% at 20°C and 80% at 37°C), quite the contrary was predicted by Brahm (1982) on the basis of measurements of the inhibition at 25°C and calculations of the degree of inhibition at lower temperatures from the energy of water diffusion in PCMBS-treated RBCs. Based on our own observations on the development of the PCMBS-induced inhibition of water diffusion, the results of Naccache and Sha'afi (1974) can be explained by an incomplete inhibition at lower temperatures. In their experiments, PCMBS was added to erythrocytes (to give 1-mM final concentration) just prior to measurement of water permeability at various temperatures. Consequently, different rates of reaction of PCMBS with SH groups were obtained, with lower rates at lower temperatures. Therefore, for equal durations of incubation, lower degrees of inhibition are expected to occur at lower temperatures. On the contrary, when the measurements of water permeability at various temperatures were performed on red blood cells previously incubated with PCMBS, a higher degree of inhibition could be noticed at lower temperatures (Brahm, 1982; this paper).

The interpretation of our findings should take into account the mechanisms of water diffusion across erythrocyte membranes and of the action of mercurials. Two parallel pathways have been considered for water transport. One pathway, nonspecific, is the diffusion across the lipid region; the water molecules riding along with free-volume pockets created in the membrane interior by thermally generated mobile structural defects in the hydrocarbon chains, the so-called "kinks" (Traüble, 1971). The second pathway is represented by hydrophylic channels for water movement, the so-called "pores" localized in membrane proteins. An important issue is connected with the proportion of water permeating across the lipid and protein counterparts in RBC membranes. Macey (1984) is claiming that specific water channels in membrane proteins are responsible for 90% of water permeability, while only 10% of water is crossing through the lipid bilayer. These proportions are deduced from the 90% inhibition of osmotic permeability produced by PCMBS. There is general agreement that PCMBS inhibition reflects the closure of water channels in proteins. Macey, Karan and Farmer (1972) consid-

ered that water is entering the PCMBS-treated cells primarily by diffusion across the lipid areas of the membrane, i.e., that such cells are indistinguishable from lipid bilayers. This view was, however, questioned by Dix and Solomon (1984), who concludes that the primary route for water diffusion in PCMBS-inhibited cells is not through the membrane lipids, but rather through a membrane protein channel; the effect of PCMBS is to occlude the channel so that bulk flow is no longer possible, while not sealing it completely. Therefore, we agree with the designation (Moura et al., 1984) of the lipid pathway as "the PCMBS-insensitive pathway."

As shown above, a lower degree of inhibition can be induced in measurements of water diffusional permeability compared to measurements of osmotic permeability. This is in agreement with other studies (Conlon & Outhred, 1978; Brahm, 1982; Benga et al., 1983*b*, 1985*b*, 1987*b*; Dix & Solomon, 1984; Moura et al., 1984). While in previous studies the degree of inhibition was measured at one temperature and the values for other temperatures were calculated based on the $E_{a,d}$, in the present work we have measured the inhibition at various temperatures, which ranged from 5–42°. It is obvious that higher values of inhibition occur at lower temperatures. We suggest, therefore, that the proportion of water diffusing across the lipid pathway relative to the protein pathway decreases by decreasing the temperature. This can be explained by taking into account the effect of temperature upon the lipid counterpart of membrane. It is probable that by decreasing the temperature, the fluidity of the lipid bilayer decreases. Then, as only the protein pathway is inhibited by mercurials, a higher degree of inhibition of water diffusional permeability is expected to occur at lower temperatures.

The authors thank Rodica Gabor and Marieta Dan for technical assistance and to the Ministry of Education and Teaching, the Academy of Medical Science (Romania) and the Wellcome Trust for financial support.

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Received 9 June 1988; revised 17 October 1988