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Water Exchange Through Erythrocyte Membranes: Nuclear Magnetic Resonance Studies on the Effects of Inhibitors and of Chemical Modification of Human Membranes

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Summary. The changes in water diffusion across human erythrocyte membranes following exposure to various inhibitors and proteolytic enzymes have been studied on isolated erythrocytes suspended in isotonic buffered solutions. An important issue was to investigate whether the sulfhydryl reacting reagents that have been applied in osmotic experiments showed similar effects on diffusional permeability. It was found that mercurials, including mersalyl, were the only sulfhydryl reacting reagents that were efficient inhibitors. Under optimal conditions a similar degree of inhibition (around 45%) was found with all mercury-containing sulfhydryl reagents. Other reagents, including the sulfhydryl reagent DTNB, phloretin, or H_2DIDS, the specific inhibitor of the anion transport system in erythrocyte membrane, did not appear to inhibit significantly the diffusional permeability. No changes in water diffusion were noticed after exposure of erythrocytes to trypsin and chymotrypsin. A new kind of experiment was that in which the effects of exposure of erythrocytes to two or more agents were studied. It was found that none of the chemical manipulations of membranes that did not affect water diffusion hampered the inhibitory action of mercurials. These findings show that the SH groups involved in water diffusion across erythrocyte membranes do not react with any of the other SH reagents aside from mercurials and that the molecular mechanism of water transport is not affected by chymotryptic cleavage of band 3 protein into the 60 and 35 kD fragments. The NMR method appears as a useful tool for studying changes in water diffusion in erythrocyte membranes following various chemical manipulations of the membranes with the aim of locating the water channel.

Key Words water exchange · erythrocyte membranes · NMR studies · inhibitors

Introduction

The process of water transport across biological membranes is of considerable importance for many physiological processes, ranging from regulation of cell volume to the protection of cells undergoing cryogenic preservation. Although various hypotheses have been proposed and investigated, the mechanism controlling the water movement across biological membranes has not been fully elucidated.

Because of its simple structure, lacking internal membrane systems, the red blood cell has been a favorite object for investigating water permeability. The methods for measuring the water exchange erythrocyte membranes fall into two categories: (a) nonstationary methods, and (b) stationary methods. The methods in category (a) involve subjecting the cells to an osmotic gradient and consequently there is a net flux of water in one or the other direction, depending on whether the cells swell or shrink. The membrane is subjected to a stress and the cell will eventually hemolyze. The information about osmotic permeability of erythrocyte membranes obtained using these methods has been recently reviewed by Sha'afi (1981).

In case of stationary methods the diffusional movement of water is measured and therefore there is no net flux of water through the membrane. The cells remain in their normal state and this is often considered as an advantage over the methods in group (a). The stationary methods can be classified in two groups: (i) radio-tracer method (Paganelli & Solomon, 1957; Vieira, Sha'afi & Solomon, 1970) and (ii) NMR[1] method (Conlon & Outhred, 1972; Fabry & Eisenstadt, 1975; Morariu & Benga, 1977; Pirkle, Ashley & Goldstein, 1979).

The NMR method has already been shown to be a valuable tool in studies of the physiology and pathology of erythrocyte water permeability (Benga & Morariu, 1977; Morariu et al., 1981). However, in contrast to many studies on osmotic permeability, there are few NMR data on the changes in water permeability following exposure

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[1] *Abbreviations:* DTNB = 5,5′-dithiobis-2-nitrobenzoate; EDTA = ethylene-diaminetetraacetic acid; FMA = fluorescein-mercuric acetate; H_2DIDS = dihydro-4,4′-diisothiocyano-stilbene-2,2′disulfonate; IAM = iodoacetamide; NEM = N-ethylmaleimide; NMR = nuclear magnetic resonance; 5P8 = 5 mM sodium phosphate buffer, pH 8.0; PCMB = *p*-chloromercuribenzoate; PCMBS = *p*-chloromercuribenzene sulfonate; SDS = sodium dodecylsulfate.

to inhibitors of this transport process across the erythrocyte membrane (Ashley & Goldstein, 1981; Benga et al., 1982).

A new approach in the study of transport processes through erythrocyte membrane has been the introduction of chemical probes (Cabantchik, Knauf & Rothstein, 1978) as well as the exposure of erythrocyte membranes to proteolytic enzymes. These chemical manipulations have proven useful not only in identifying transport proteins, but also in determining their general arrangement in the membrane, for example in case of the anion transport system in erythrocytes (Jennings & Passow, 1979; Rothstein, Ramjeesingh & Grinstein, 1980).

So far no studies of water diffusion through erythrocyte membranes following proteolytic digestion have been reported. On the other hand the suggestion that band 3 is involved in water transport (Brown, Feinstein & Sha'afi, 1975; Sha'afi & Feinstein, 1977) was based on the labeling experiments with DTNB and mercurials and on studies of the inhibitory effects of such compounds on osmotic permeability. Therefore investigations of the effects of DTNB and mercurials on water diffusion are of great interest.

The aim of our paper was to present NMR studies of changes in water diffusion through the human erythrocyte membranes following the exposure to various inhibitors and proteolytic enzymes. This will give us a better understanding of the molecular mechanisms of erythrocyte water transport. Significant differences were found regarding the effects of DTNB and mersalyl on water diffusion compared to data of other authors on osmotic permeability. No changes in water diffusion were noticed after exposure of erythrocytes to trypsin and chymotrypsin. A new kind of experiment reported here was that in which the effects of exposure of erythrocytes to two or more agents were studied. The findings are discussed in relation to the molecular mechanisms proposed for water transport.

Materials and Methods

Blood Sample Preparations

Human blood was obtained by venipuncture in heparinized tubes and used within 4 hr. The donors were healthy male subjects, 20 to 40 years old. The erythrocytes were isolated by centrifugation, and washed three times in 150 mM NaCl, 5 mM HEPES (pH 7.4) and 0.1% glucose.

The incubations of erythrocytes with various reagents were performed as indicated in the legend to the Figure and in Tables. After incubation three washings of the erythrocytes in 150 mM NaCl, 5 mM HEPES, each followed by a centrifugation, were performed to remove the reagent.

NMR Measurements

The erythrocytes were suspended in a wash solution containing 0.1% glucose and 1% defatted bovine serum albumin, at a hematocrit of 50%. Samples for NMR measurements were prepared by carefully mixing 0.2 ml cell suspension and 0.1 ml doping solution (40 mM $MnCl_2$, 100 mM NaCl).

The water proton relaxation time of the erythrocytes (T'_{2a}) was measured by the spin-echo method as previously described (Morariu & Benga, 1977; Morariu et al., 1981). The T'_{2a} is dominated by the exchange process through the membrane and the water exchange time through erythrocyte membranes is inversely related to the water permeability of erythrocytes as shown by Conlon and Outhred (1972). The control value of T'_{2a} was 6.80 ± 0.27 msec (mean and standard error of the mean for 27 determinations).

The inhibition of water diffusion across human red blood cell membranes was calculated assuming that the permeability coefficient is inversely related to T'_{2a}, according to the formula:

$$\% \text{ Inhibition} = \frac{\frac{1}{T'_{2a\,(\text{control})}} - \frac{1}{T'_{2a\,(\text{sample})}}}{\frac{1}{T'_{2a\,(\text{control})}}}.$$

The measurements were performed with an Aremi-78 spectrometer (Institute of Physics and Nuclear Engineering, Bucharest-Magurele, Roumania). The temperature was controlled to 37 ± 0.2 °C by air flow over an electrical resistance using the variable temperature unit of the spectrometer.

Preparation of Erythrocyte Ghosts and Polyacrylamide Gel Electrophoresis

The red blood cells 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 by Dodge, Mitchell and Hanahan (1963) and modified by Fairbanks, Steck and Wallach (1971). After three or four washes in 5P8 followed each time by centrifugation at 20,000 *g* for 20 min the pellets were homogenous and white. After washing, the ghost pellets were suspended in 5P8 to about 4 mg protein/ml. The protein concentration was estimated according to Lowry et al. (1951).

One vol. (usually 25 µl of ghosts) was added to 3 vol of a solution containing 4% sodium dodecylsulfate (SDS), 30% sucrose, 2% *beta*-mercaptoethanol, 4 mM sodium EDTA, 60 mM Tris-HCl (pH 8.8) and 0.02 mg/ml bromophenol blue. The mixture was heated for 3 min in a 100 °C bath. Membrane peptides were separated using the discontinuous SDS polyacrylamide gel system designed by Laemmli (1970). The slab gels used throughout this work 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 the stacking and running gel. Samples of 25 µl (25 µg protein) were applied, and the gels were run 1 hr at 70 V and then 3 hr at 125 V (18 to 25 °C) in the running buffer (25 mM Tris, 190 mM glycine, 0.1% SDS). Following electrophoresis the slab gels were dried, then reswollen in a mixture of 5% methanol and 10% acetic acid. The gels were stained for 1 hr with a solution containing 0.075% Coomassie Brilliant Blue R250, 45% methanol and 10% acetic acid. Destaining was performed with a mixture of 5% methanol and 10% acetic acid, followed by 15% trichloroacetic acid. After the destaining was completed the gel was extensively washed in water and restained as mentioned before. Destaining was performed with a mixture of 5% methanol and 10% acetic acid. In this way glycophorin could be noticed aside from other polypeptides (*see* Figure).

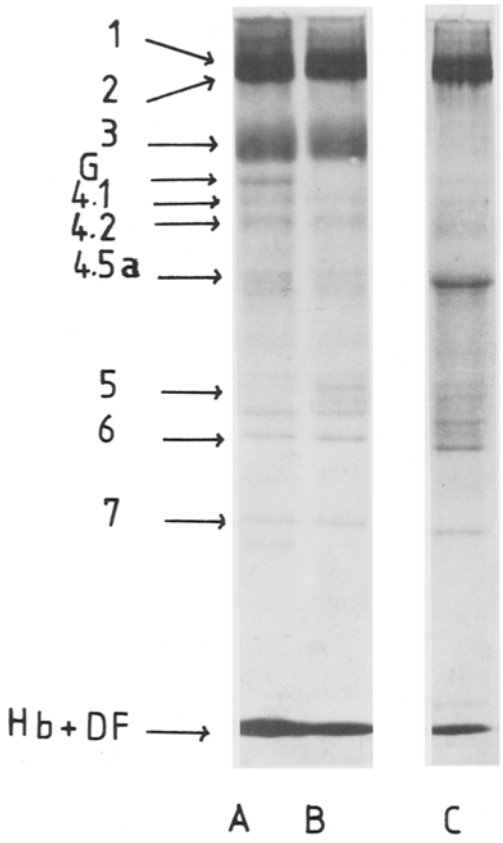

Figure. Electrophorograms of erythrocyte ghosts prepared from: (*A*) control red blood cells; (*B*) cells treated with trypsin; (*C*) cells treated with chymotrypsin. Details of sample preparation are given in Materials and Methods. Polypeptides are numbered following the system of Fairbanks et al. (1971) with minor changes. *G*=glycophorin; *Hb*=hemoglobin; *DF*=dye front

Table 1. Effect of mercury-containing reagents on water diffusion across human red blood cell membranes

Compound	No. of determinations	Conditions of Incubation		% Inhibition[a]
		Time (min)	Temperature (°C)	
10 μM $HgCl_2$	2	60	37	13.1
25 μM $HgCl_2$	2	60	37	29.2
100 μM $HgCl_2$	2	60	37	42.0
0.5 mM Mersalyl	2	15	22	15.7
1 mM Mersalyl	1	15	22	30.0
1 mM Mersalyl	2	15	37	35.2
1 mM PCMB	8	60	37	44.1±1.0[b]
0.5 mM PCMBS	2	10	37	35.3
1 mM PCMBS	2	300	22	35.0
1 mM PCMBS	2	45	22	38.7
1 mM PCMBS	12	60	37	43.6±1.1
2 mM PCMBS	8	30	37	49.0±0.6
50 μM FMA	2	60	37	19.3
250 μM FMA	7	60	37	46.0±1.3
250 μM FMA	2	90	37	45.0

[a] The inhibition was calculated as described in Materials and Methods.
[b] Mean and standard error of the mean.

Results

A wide variety of reagents and chemical treatments of erythrocyte membranes have been tested for their effect on the water exchange time. An important issue was to investigate whether the sulfhydryl reacting reagents (SH reagents) that have been applied in osmotic experiments showed similar effects on diffusional permeability. As can be seen from Table 1 the mercury containing compounds substantially inhibited the water exchange time through erythrocyte membranes. The degree of inhibition appeared to be dependent on the temperature and time of exposure of erythrocytes to the mercurial as well as on the reagent concentration. Under optimal conditions a similar degree of inhibition was found with all reagents, ranging between 42 and 49%. However, an important difference in the behavior of FMA compared to other mercurials became apparent when the reversibility of inhibition by cysteine was investigated (Table 2).

FMA was the only mercurial which appeared to inhibit irreversibly the water diffusion through erythrocyte membranes. This finding, reported previously (Benga et al., 1982) has now been confirmed by a greater number of measurements under different conditions of incubation.

A new kind of experiment reported here was that in which the effects of two mercurials upon the water exchange time were studied. From Table 2 it can be seen that maximal inhibition is obtained with either FMA or PCMBS. No further inhibition could be obtained after incubation with both reagents, regardless of the order in which these agents were used. However, in all cases where FMA was present in the incubation media the inhibition of water diffusion could not be reversed, even by a large excess of cysteine.

Other SH reagents, aside from mercurials, did not appear to significantly inhibit the diffusional permeability (Table 3). It should be emphasized that this also applies to DTNB, for which a degree of inhibition of only 10% was noticed, in contrast to a 60% inhibition of osmotic water permeability reported by Naccache and Sha'afi (1974).

From Table 4 it can be seen that none of the SH reagents which do not contain mercury either prevented or potentiated the inhibitory effect of

Table 2. Effect of cysteine on the inhibition of water exchange through erythrocyte membranes by mercurials

Compound	No. of determinations	Conditions of Incubation		Cysteine	% Inhibition[a]
		Time (min)	Temperature (°C)		
1 mM PCMB	22	60	37	10 mM, 15 min, 37 °C	5.2
2 mM PCMBS	8	30	37	10 mM, 15 min, 37 °C	5.0±0.2[b]
250 μM FMA	4	60	37	10 mM, 15 min, 37 °C	43.2±0.3
250 μM FMA, followed by		30	22		
1 mM PCMBS	2	30	22	–	42.1
1 mM PCMBS, followed by		45	22		
125 μM FMA	2	15	22	–	42.2
1 mM PCMBS, followed by		45	22		
125 μM FMA	2	15	22	10 mM, 30 min, 22 °C	46.0
1 mM PCMBS	2	45	22	10 mM, 30 min, 22 °C	0
125 μM FMA	1	15	22	10 mM, 30 min, 22 °C	48.0
125 μM FMA	1	15	22	100 mM, 30 min, 22 °C	47.4

[a] Inhibition was calculated as described in Materials and Methods.
[b] Mean and standard error of the mean.

Table 3. Effect of some sulfhydryl reacting reagents on water diffusion through erythrocyte membranes

Compound	No. of determinations	Conditions of Incubation		% Inhibition[a]
		Time (min)	Temperature (°C)	
1 mM DTNB	14	60	37	10.1±3.7[c]
1 mM DTNB[B]	2	120	37	10.5
2 mM DTNB	2	60	37	18
1 mM IAM	7	60	37	6.9±1.0
1 mM NEM	5	60	37	6.5±1.2
1 mM NEM, followed by 1 mM DTNB	2	60	37	12.7
1 mM DTNB, followed by 1 mM NEM	4	60	37	7.9±3.9

[a] % Inhibition was calculated as described in Materials and Methods.
[b] DTNB was present during washes of erythrocytes.
[c] Mean and standard error of the mean

a mercurial. This suggests that the SH group involved in the water transport does not react with any of these SH reagents.

Table 5 lists the degrees of inhibition noticed with a variety of reagents. None of these markedly increased water exchange time. It should be emphasized that H_2DIDS, a specific inhibitor of the anion transport sytem in erythrocyte membranes does not inhibit the diffusional water permeability. H_2DIDS does not prevent the inhibitory effect of PCMBS. Phloretin, that also inhibits anion transport, as well as other facilitated transport processes in the red blood cell membrane also did not change the water permeability. Other compounds that react with amino groups, such as N-succinimidyl-3-(2-pyridylthio) propionate (Carlsson, Drevin & Axen, 1978) or methoxy-nitro-tropone had no significant inhibitory effect on water exchange.

Water diffusion appears not to be energy-dependent since blocking the glycolysis with NaF did not affect the water exchange time.

The possible relation between water diffusion and integral membrane proteins, particularly band 3 was investigated by following the effect of

Table 4. Effect of incubation with both mercurials and other sulfhydryl reacting reagents on water diffusion through erythrocyte membranes

Compound	No. of determinations	Conditions of Incubation		% Inhibition[a]
		Time (min)	Temperature (°C)	
1 mM DTNB, plus 1 mM PCMB	2	60	37	48.0
2 mM DTNB, plus 1 mM PCMB	4	60	37	42.6
1 mM IAM, followed by 1 mM PCMB	2	15 105	37 37	39.7
1 mM NEM, followed by 1 mM PCMB	2	60 60	37 37	45.2
1 mM NEM, followed by 1 mM PCMBS	4	60 60	37 37	46.0±8.1[c]
1 mM NEM, plus 250 μM FMA	1	60	37	38.6[b]
1 mM IAM, plus 1 mM NEM, followed by 1 mM Mersalyl	2	45 15	37 37	50.2
1 mM IAM, plus 1 mM NEM, followed by 1 mM Mersalyl and 1 mM PCMBS	2	15 15 30	22 22 22	34.2[b]
1 mM IAM, plus 1 mM NEM, followed by 1 mM PCMBS	2	30 30	22 22	46.0
1 mM IAM, plus 1 mM NEM, followed by 1 mM PCMBS and 250 μM FMA	2	30 30 30	22 22 22	45.5

[a] % Inhibition was calculated as described in Table 1.
[b] Slight hemolysis.
[c] Mean and standard error of the mean.

Table 5. Effect of various compounds on the water diffusion across human red blood cell membranes

Compound	No. of determinations	Conditions of Incubation		% Inhibition[a]
		Time (min)	Temperature (°C)	
10 μM H_2DIDS	4	15	37	10.8±7.0[b]
10 μM H_2DIDS, followed by 1 mM PCMBS	2	15 15	37 37	30.5
10 μM H_2DIDS, followed by 1 mM PCMBS	2	60 60	37 37	39.5
1 mM Phloretin	8	60	37	11.2±3.0
0.5 mM Fluorescein	4	60	37	15.3±2.7
125 μM Fluorescein isothiocyanate	4	60	37	19.7±1.2
0.5 mM Eosin	4	60	37	14.9±2.3
0.5 mM Eosinmaleimide	1	60	37	9.9
0.5 mM N-Succinimidyl-3-(2-pyridylthio) propionate	6	60	37	6.6±1.2
Methoxy nitro-tropone (1.25 mg/ml)	2	60	37	15.5
40 mM NaF	2	60	37	9.2

[a] % Inhibition was calculated as described in Materials and Methods.
[b] Mean and standard error of the mean.

Table 6. Effect of proteolytic enzymes on water diffusion across human erythrocyte membranes

Compound	No. of determinations	Conditions of Incubation		% Inhibition[a]
		Time (min)	Temperature (°C)	
Trypsin[b]	12	60	37	13.5±2.8[c]
Trypsin, followed by		60	37	
1 mM IAM	4	30	37	7.8±4.3
Trypsin, followed by		60	37	
1 mM PCMB	2	60	37	41.2
Trypsin, followed by		60	37	
1 mM PCMBS	1	60	37	51.2
Trypsin, followed by		60	37	
1 mM NEM and		60	37	
1 mM PCMBS	2	60	37	55.2
Trypsin, followed by		60	37	
250 μM FMA	4	30	37	46.2
Chymotrypsin[b]	14	60	37	9.7±2.4
Chymotrypsin, followed by				
1 mM IAM	4	30	37	6.8±1.1
Chymotrypsin, followed by		60	37	
1 mM PCMB	2	60	37	44.6
Chymotrypsin, followed by		60	37	
1 mM PCMBS	2	60	37	54.4
Chymotrypsin, followed by				
1 mM NEM and				
1 mM PCMBS	2	60	37	56.5
Chymotrypsin, followed by				
1 mM IAM plus 1 mM NEM		60	37	
and 1 mM PCMBS	2	30	37	37.0
Chymotrypsin plus trypsin[b]	1	60	37	13.1

[a] % Inhibition calculated as described in Materials and Methods.
[b] The final concentration of enzymes was 0.4 mg/ml.
[c] Mean and standard error of the mean.

enzymic treatments of erythrocytes on the diffusional permeability.

As illustrated in the Figure in the conditions of incubation used in these studies trypsin digested glycophorin without significantly changing the pattern of other polypeptides in erythrocyte membrane. In contrast, with chymotrypsin an extensive digestion of the band 3 protein occurred. This is in agreement with Passow et al. (1977), who showed that only chymotrypsin and not trypsin digests band 3 protein in intact red blood cells. However, neither trypsin nor chymotrypsin treatment significantly inhibited water diffusion through erythrocyte membranes (Table 6). At the same time the enzymic treatment of membranes did not prevent the inhibition induced by mercurials. In contrast, the effect of mercurials appeared to be slightly potentiated by the enzymic treatment.

Discussion

Two mechanisms for the permeation of water through erythrocyte membranes have been proposed (Sha'afi, 1981). One model is based on the concept that the molecular motion of the hydrocarbon chains of membrane lipids generates structural defects through which water permeates (Traüble, 1972). The second model assumes the presence of aqueous membrane channels or "pores" assembled from membrane integral proteins which span the human red cell membrane (Sha'afi, 1981). The first model is thought to represent water flux

through the lipid bilayer accounting for about 10% of the total flux observed in red cell membranes (Sha'afi, 1981). The second model is compatible with the results observed with sulfhydryl reagents which suggest that proteins are involved. These reagents react with amino acid SH groups thereby blocking the channel or pore. The flux observed in their presence would represent permeability through the bilayer. Band 3, band 4.5 and glycophorin are the major proteins which span the erythrocyte membrane (Yu & Steck, 1975; Rothstein, 1981) raising the possibility that one of these proteins is involved in the formation of the hydrophilic pathway. The inhibition of water permeation by SH reagents would favor band 3 or band 4.5 as the protein forming the water channel, as glycophorin has no SH group (Tomita, Furthmayr & Marchesi, 1978). However, the possibility that a glycophorin-band 3 complex participates in water transport has to be considered as such a complex has been detected by antibody binding (Cherry & Nigg, 1980). The aggregation of band 3 into dimers or tetramers for which there is some evidence (Cherry & Nigg, 1980) could also create more than one pathway. In support of the role of band 3 in water transport, studies have been shown that liposomes prepared from egg lecithin and containing band 3 can be inhibited by PCMBS (Sha'afi & Feinstein, 1977). Liposomes reconstituted with glycophorin are also more permeable to water but the specificity of this effect has not been examined (Sha'afi, 1981).

As shown in the Figure the incubation of erythrocytes with trypsin digested the glycophorin (probably the outer hydrophilic fragment) and the incubation with chymotrypsin digested the band 3. However, the water diffusion was not inhibited and the inhibitory effect of mercurials was not hampered by proteolytic digestion. This suggests that if band 3 associated with glycophorin participate in the formation of the water channel this must be contained in the part of these proteins embedded in the lipid bilayer and therefore not accessible to proteolytic digestion of intact erythrocytes.

Studies on the effects of various SH reagents on water transport in human red blood cells can aid in the characterization of the SH groups involved, and in providing information on the site containing the SH groups. Mercurials were the only SH reagents that were efficient inhibitors. When the optimal conditions of inhibition were found (time and temperature of incubation, concentration of inhibitor) all mercurials produced the same degree of inhibition, around 45%. This is in agreement with the results of other authors (Macey, Karan & Farmer, 1972; Fabry & Eisenstadt, 1975; Ashley & Goldstein, 1981) who also have reported similar degrees of inhibition of diffusional permeability induced by mercurials. However, this is in contrast with a 90% inhibition of osmotic permeability induced by the same reagents (Macey & Farmer, 1970).

The irreversible inhibition of water diffusion induced by FMA recently described by us (Benga et al., 1982) has been substantiated by the results reported here. This feature seems to be a difference between the osmotic and diffusional permeability of human erythrocytes, since for the former the inhibition by FMA could be reversed by cysteine (R.I. Sha'afi, *personal communication*).

We could not detect a substantial inhibitory effect of DTNB on water diffusion across erythrocyte membranes. This is in contrast to the data of Naccache and Sha'afi (1974) who found that DTNB inhibited osmotic water transport by 60%. The 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 band 3 protein was involved in water transport. Some authors (Macey, 1979; Brahm, 1982; A.H. Solomon, *personal communication*) recently questioned the inhibitory effect of DTNB on water transport and the present results show that DTNB had a small effect on diffusional water permeability.

This finding and the differences in the reversibility by cysteine of the inhibition induced by FMA in diffusional and osmotic permeability may reflect differences in the molecular mechanisms and might even suggest that different protein components may be involved in the two kinds of water permeability.

As far as diffusional water permeability is concerned the results reported here are of considerable interest in relation with the suggestion (Brown et al., 1975) that band 3 protein is involved in water transport. It is known that band 3 contains the anion channel (Cabantchik & Rothstein, 1974). The SH groups of band 3 protein are now quite well defined (Steck et al., 1978; Rao, 1979; Ramjeesingh, Gaarn & Rothstein, 1980). Of six cysteine residues, five are exposed on the cytoplasmic side and are reactive with NEM. However, NEM does not inhibit diffusional permeability (Table 3). The sixth cysteine residue, cryptic to NEM, is in one of the transmembrane elements of band 3 protein. This SH group might be the target for PCMBS. On the other hand, the finding that DIDS does

not inhibit water permeability nor does it influence the inhibitory effect of PCMBS (Table 5) is relevant. It has been reported that DIDS blocks 98% of the flow of PCMBS through the anion channel of band 3 protein (Cabantchik et al., 1978). These observations might be taken as indicating that band 3 SH groups are not involved in the PCMBS-inhibition of diffusional water transport. However, blocking the flow of PCMBS with DIDS does not reduce the effect of the mercurial on K^+ permeability either in timing or extent; this was interpreted as indicating that the small fraction (1 to 2%) of PCMBS passing into DIDS-insensitive channels reaches the cation controlling groups (Rothstein, 1981). On a similar line of thinking it is possible that this fraction (1 to 2%) of PCMBS could be responsible for the effects on water permeability.

The role of band 4.5 protein in water permeability seems not to have been considered so far. This transmembrane protein is relatively abundant in the erythrocyte membrane, about 1/3 as abundant as band 3 protein (Jones & Nickson, 1982).

PCMBS reacts rapidly with a superficial SH group in this peptide to block sugar transport. In case of water permeability for the maximal inhibitory effect of PCMBS to occur an incubation period was necessary which was dependent upon the temperature of incubation and the concentration of the mercurial (Table 1). However, a relatively rapid component of the inhibition of water permeability by mercurials has also been reported (Naccache & Sha'afi, 1974; Ashley & Goldstein, 1981). It appears therefore possible for band 4.5 protein to be involved in water permeability.

The results reported here do not allow us to locate the water channel in a particular peptide of erythrocyte membrane. Nevertheless we may conclude that the NMR method is a useful tool for studying changes in water diffusion in erythrocyte membrane following various chemical manipulations of the membranes with the aim of locating the water channel.

The advantage of the NMR method include relative technical simplicity, speed of data collection and reproducible results. Pirkle, Ashley and Goldstein (1979) have recently questioned the validity of the NMR technique employing free manganese ions. They suggested that these ions in concentrations above 20 mM induce a 35 to 45% systematic decrease of the exchange time measurements (which they erroneously call an inhibition, though the exchange time decreased as the manganese ion concentration was increased). Shporer and Civan (1975) found the same permeability in the presence and the absence of 6 to 7 mM Mn^{2+}, a concentration that increased the permeability in the work of Pirkle et al. (1979) by 20%. Fabry and Eisenstadt (1975) determined an exchange time of 15 msec at an extracellular manganous ion concentration of 5 mM, i.e. more than 30% faster than the value obtained by Pirkle et al. (1979) at the same Mn^{2+} concentration. The Mn^{2+} concentration used in our procedure is 13.3 mM. Recent determinations by the tracer method (Brahm, 1982) showed that diffusional water permeability was 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%.

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