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EFFECTS OF SULFHYDRYL AND OTHER REAGENTS ON THE DIFFUSIONAL PERMEABILITY OF DOG ERYTHROCYTES TO SMALL SOLUTES

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The effects of *p*-chloromericuriphenylsulfonic acid (PCMBS), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), phloretin and thiourea on the diffusional permeability of dog erythrocytes to tritiated water and to small ^{14}C-labeled lipophilic and hydrophilic solutes were measured at 37°C by means of the linear diffusion technique. Permeability to ^{3}HHO was significantly decreased by PCMBS but was not affected by the other reagents. The permeability to the small hydrophilic solutes acetamide and urea was decreased by phloretin and thiourea but only the permeability to acetamide was reduced to a statistically significant extent by PCMBS. The permeability to the lipophilic solutes methanol, ethanol and antipyrine was not affected by any of these agents. We interpret these results as an indication that the small lipophilic solutes probably move through lipid areas, that the small hydrophilic solutes probably move through protein associated areas in the erythrocyte membrane and that pathways for the small hydrophilic solutes are distinct from those for water. While the pathways for water may be associated with membrane protein they do not appear to be associated specifically with band 3 protein as has been suggested for human erythrocytes. Diffusional water movement through the dog erythrocyte occurs by two distinct pathways.

Introduction

The permeability of mammalian erythrocytes to water and nonelectrolytes has been studied extensively in an attempt to describe the processess by which permeation occurs. In our studies of the diffusional permeability of lung cells [1,2] and dog erythrocytes [3,4] to a variety of small nonelectrolytes emphasis has been placed on the permeation characteristics of water and of lipophilic molecules. We now report changes in permeation due to alteration of the cell membrane by various reagents. Substances referred to as inhibitors (*p*-chloromercuriphenylsulfonic acid (PCMBS), 5,5′-dithiobis (2-nitrobenzoic acid)(DTNB), phloretin and thiourea) are known to affect the osmotic permeability and mediated transport of various solutes in human erythrocytes [5–8]. The processes involved in diffusional (steady state) and osmotic (bulk flow) permeation are considered to differ [8] but the effects of inhibitors on diffusional permeability at 37°C have not yet been widely explored. In addition, differences between water and solute permeation in dog and human erythrocytes may provide new insights into the permeation processes.

The linear diffusion method has been used suc-

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Abbreviations: PCMBS, *p*-chloromercuriphenylsulfonic acid; DTNB, 5,5-dithiobis (2-nitrobenzoic acid).

cessfully to measure cellular diffusional permeability under various conditions in a relatively simple manner [4,9,10]. With this technique the effects of inhibitors that are known to interact with specific membrane components were tested on the diffusional permeability of dog erythrocytes to a group of test solutes of different sizes and solubilities. Test solutes with different permeation characteristics were selected: small hydrophilic solutes, i.e. tritiated water, [^{14}C]acetamide and [^{14}C]urea; small lipophilic solutes i.e. [^{14}C]methanol and [^{14}C]ethanol; and a larger lipophilic solute, [^{14}C]antipyrine. The inhibitors selected were PCMBS, DTNB, thiourea and phloretin. Ethanol and 2,4-dinitrophenol were also tested in a limited study.

We have analyzed our results and compared them to those available in the literature in terms of: the permeability of small lipophilic solutes and of small hydrophilic solutes; the permeation patterns in dog erythrocytes and in human erythrocytes; the different effects of inhibitor effects on osmotic and diffusional permeabilities; and the differences between results obtained at 20°C and those obtained at 37°C. The straightforward design of our studies provides results that demonstrate the variety possible in membrane-solute interactions and the complexity required in the interpretation of these results. A preliminary account of this work appeared in Microvasc. Res. (1980) 20, 110.

Materials and Methods

Blood was drawn as required from a series of 40 different mongrel dogs of either sex into Vacutainer® tubes with EDTA as anticoagulant. The whole blood was saturated with carbon monoxide to avoid oxidation of the hemoglobin. Subsequent treatment of the blood and incubation with the inhibitors differed somewhat and is described below (Inhibitor treatment).

1. Diffusion experiments. After incubation with the inhibitors all blood samples were centrifuged at $450 \times g$ for 15 min to separate the cells. The plasma or supernatant fluid from this centrifugation was saved for diffusion experiments. Subsequent treatment of the cells, plasma, supernatant fluid and preparation of hemoglobin solutions have been described [3,9]. Briefly, an aliquot of the cells is mixed with an extracellular marker (^{125}I-human serum albumin), drawn into polyethylene tubing, sealed at one end, packed by centrifugation, pulsed with a radiolabelled solute (1 μCi/μl), the excess pulse removed and the cells incubated at 37°C. After a set time the diffusion tube is frozen and sliced (0.5–1.0 mm) from the pulsed end and radioactivity in the sections is determined in a liquid scintillation system. Diffusion coefficients for plasma, supernatant fluid or hemoglobin are measured in the same way [9].

The diffusion coefficient is calculated from the solution given by Crank [11] for one-dimensional semi-infinite diffusion through a homogeneous medium with impulse deposition of tracer amount m_0 (cpm/cm^2) at $x = 0$, $t = 0$

$$c(x,t) = m_0(\pi Dt)^{-1/2} \exp(-x^2/4Dt)$$

A plot of the radioactivity as ln$c$ (cpm) against x^2 (x = distance from the pulsing point) is a straight line of slope $1/(4Dt)$ which yields D since t is known [9]. Examples of the type of data obtained are reported in Ref. 9 on p. 713 by Redwood et al. and our results are similar to these. For all of the results reported in this paper the plots of ln$c$ against x^2 had $r = 0.98$–1.00.

Permeability coefficients were calculated from the diffusion coefficients with the series-parallel pathway model as developed by Perl in Redwood et al. [9]. The cells are assumed to be packed in a regular manner with the local steady-state diffusional flux idealized as a one-dimensional intracellular pathway in parallel with a one-dimensional extracellular pathway with solute exchange occurring within the series pathway and between pathways. The membrane permeability is calculated by separating the resistance to diffusion provided by the intact plasma membrane of the cells packed in the tubing from the resistance to diffusion provided by other components of the system. The resistance provided by the other components, including the unstirred layer effects, is determined by measuring diffusion through intracellular (D_2) and extracellular (D_1) material, hemoglobin and plasma, respectively, for erythrocytes. Numerical values of P_0 were obtained from the experimental values obtained for D, D_1, and D_2, the relative extracellular volume and the cell length in the

diffusion direction and the calculated values for tortuosity, cell surface exchange area and the cross-sectional area for diffusion as described previously [2,9]. Statistical significance was estimated by analysis of variance [12].

2. Inhibitor treatment. (a) The incubation of the blood was the same with PCMBS and thiocarbamide (thiourea). After treatment with carbon monoxide the blood was centrifuged at 450 × *g* for 15 min and divided into two portions, a control and a treatment sample. An aliquot of plasma was removed from the treatment sample, the inhibitor dissolved in the plasma and the aliquot mixed again with the remaining blood. The control sample was mixed also. Both blood samples were incubated at room temperature for 50 min and the pH and osmolality measured. The blood was then separated as described for the diffusion experiments.

(b) Phloretin and DTNB were dissolved in alcohol before being added to the erythrocytes. The cells were washed with buffer before being exposed to these inhibitors. After treatment with carbon monoxide the whole blood was mixed with an equal volume of phosphate buffer (4.7 mM KH_2PO_4, 12 mM Na_2HPO_4, 143.5 mM NaCl, pH 7.4, 300 mosM) and centrifuged at 450 × *g* for 10 min. The supernatant fluid and buffy coat were removed and the cells mixed with an equal volume of buffer and centrifuged at 450 × *g* for 10 min. The inhibitors were added to the cells with mixing; an equal volume of alcohol was added to the control cells (0.75% with DTNB and 0.50% with phloretin) with mixing. The cells were incubated for 50 min at room temperature and pH and osmolality measured. The cells were then separated as described for the diffusion experiments.

Tritiated water (25 μCi/μl), [^{14}C]methanol, [^{14}C]ethanol and [^{14}C]urea were purchased from New England Nuclear. [*N-methyl*-^{14}C]Antipyrine and [^{14}C]acetamide were purchased from Mallinckrodt. Each solute (except tritiated water) was brought to 1 μCi/μl with buffer, pH 7.4, 300 mosM.

Results

Diffusion coefficients and permeability coefficients for ^{3}HHO in PCMBS-treated cells

The mean (± S.E.) of the diffusion coefficients for ^{3}HHO in plasma (D_1), 33% hemoglobin solution (D_2) and packed erythrocytes (D) with PCMBS-treated blood are listed in Table I. At the pH used in these studies PCMBS is an anion which can react with sulfhydryl groups in the plasma membrane [13,14]. Organic mercurials such as *p*-chloromercuribenzoate (PCMB) and PCMBS interact with the protein sulfhydryl groups and stay attached to the protein [15]. They can be dislodged by other compounds, such as cysteine, which contain more 'reactive' sulfhydryl groups [14,15]. In the presence of PCMBS there is no significant added variance among the diffusion coefficients in plasma and hemoglobin.

There is a significant added variance ($p < 0.001$) among the diffusion coefficients for the treated

TABLE I

DIFFUSION COEFFICIENTS AND PERMEABILITY COEFFICIENTS AT 37°C FOR ^{3}HHO IN PCMBS-TREATED BLOOD

The values reported are mean ± S.E. with the number of determinations given in parenthesis. D is diffusion coefficient through packed erythrocytes, D_1 is diffusion coefficient through plasma, D_2 is diffusion coefficient through 33% hemoglobin solution. P_0 is calculated from the series-parallel pathway model [9].

PCMBS [a] (mM)	Diffusion coefficient ($cm^2 \cdot s^{-1}$), ($\times 10^5$)			Permeability coefficient ($cm \cdot s^{-1}$), ($\times 10^5$)
	D_1	D_2	D [b]	P_0 [b]
0	2.13 ± 0.10 (8)	1.01 ± 0.02 (20)	0.351 ± 0.009 (10)	800 ± 35 (10)
1	2.21 ± 0.06 (23)	1.13 ± 0.02 (8)	0.323 ± 0.009 (26)	651 ± 29 (26)
2	2.18 ± 0.05 (8)	1.01 ± 0.07 (6)	0.245 ± 0.005 (8)	428 ± 16 (8)
4	1.93 ± 0.06 (4)	0.96 ± 0.02 (4)	0.201 ± 0.006 (4)	302 ± 15 (4)

[a] Cells with PCMBS pH 7.5, 300 mosM.

[b] $p < 0.001$ for 1, 2 and 4 mM PCMBS compared to control.

and the control samples of the erythrocytes (D). This indicates that the decrease in D after treatment with PCMBS can be ascribed to a specific effect on the erythrocyte membrane by PCMBS. Similar comparions of diffusion coefficients in plasma and hemoglobin solutions for the other solutes and agents we used also indicated no significant differences in the plasma and hemoglobin diffusion coefficients. Use of analysis of variance followed by a priori tests of significance [12] enable us to state that there is a significant effect due to PCMBS at 1, 2 and 4 mM compared to the control value of P_0 ($p < 0.001$). In addition the permeability coefficients calculated at 2 and 4 mM are not significantly different from each other though they are significantly different from the control value. The concentrations 1, 2 and 4 mM correspond to 15.9, 31.8 and $63.6 \cdot 10^{-17}$ mol PCMBS/cell available for binding. The lowest of these concentrations is sufficient to saturate the PCMBS binding sites available both on the membrane proteins and on hemoglobin [13,16]. Similar results were obtained at 20°C for ^{3}HHO with P_0 of $415 \cdot 10^{-5}$ cm $\cdot$ s^{-1} in control cells and P_0 of $239 \cdot 10^{-5}$ cm $\cdot$ s^{-1} with 2 mM PCMBS.

Cysteine reversed the decreased permeability of ^{3}HHO that had occurred following treatment with PCMBS. For a particular blood sample the P_0 for ^{3}HHO in control cells at 37°C was $692 \cdot 10^{-5}$ cm $\cdot$ s^{-1}, for PCMBS-treated cells the P_0 was $515 \cdot 10^{-5}$ cm $\cdot$ s^{-1} and it was $665 \cdot 10^{-5}$ cm $\cdot$ s^{-1} for these PCMBS-treated cells after they were incubated with 10 mM cysteine for 10 min. There is no significant difference between the untreated cells and the cells treated with cysteine after exposure to PCMBS.

Permeability coefficients for small solutes at 37°C in PCMBS-treated erythrocytes

The diffusional permeability coefficients (mean ± S.E.) for ^{14}C-labeled acetamide, urea, methanol, ethanol and antipyrine in erythrocytes after PCMBS treatment are listed in Table II. There is a significant decrease in the permeability to acetamide after 1 and 2 mM PCMBS treatment. The permeability coefficients for acetamide at 1 and 2 mM PCMBS are not significantly different from each other. The mean permeability to urea, another small hydrophilic solute, is decreased compared to that in the control cells, however the decreases of 17% at 1 mM and of 25% at 2 mM are not statistically significant at the 5% level ($0.10 > p > 0.05$). The permeability to the three lipophilic solutes methanol, ethanol and antipyrine is not affected by PCMBS.

Permeability coefficients for hydrophilic solutes at 37°C in DTNB-treated erythrocytes

The disulfide compound DTNB reacts with a smaller percentage of the erythrocyte membrane sulfhydryl groups than does PCMBS [17]. It forms disulfide bonds in the membrane protein and subsequently dissociates from the protein [15]. The effects of DTNB on the diffusional permeability of the dog erythrocyte to the hydrophilic solutes water, acetamide and urea are listed in Table III. The addition of DTNB in solid form to the plasma caused precipitation of plasma proteins and of the

TABLE II

PERMEABILITY COEFFIENCIENTS AT 37°C FOR ERYTHROCYTES TREATED WITH PCMBS

The values reported are mean ± S.E. with the number of determinations given in parenthesis.

Solute	Permeability coefficient (cm $\cdot$ s^{-1}), ($\times 10^5$)		
PCMBS (mM)	0	1	2
Acetamide	42 ± 3 (8)	23 ± 5 (4)	20 ± 1 (5) [a]
Urea	114 ± 9 (6) [b]	95 ± 7 (4)	86 ± 9 (4)
Methanol	1425 ± 37 (3)	1298 ± 89 (5)	
Ethanol	1256 ± 106 (3)	1203 ± 99 (5)	
Antipyrine	263 ± 29 (4)		272 ± 17 (8)

[a] $p < 0.001$ for 1, 2 mM PCMBS compared to control.

[b] The value for urea found in this series agrees closely with that of 117 ± 11 reported previously [29]. The decrease of P_0 for urea from 0 to 2 mM PCMBS is not statistically significant at the 5% level ($0.10 > p > 0.05$).

TABLE III

PERMEABILITY COEFFICIENTS AT 37°C FOR ERYTHROCYTES TREATED WITH DTNB

The values reported are mean ±S.E. with the number of determinations given in parenthesis. DTNB in 0.75% ethanol.

Solute	Permeability coefficient ($cm \cdot s^{-1}$), ($\times 10^5$)		
DTNB (mM) [a]	0	0.5	1.0
^{3}HHO	741 ± 43 (7)	691 ± 45 (4)	763 ± 44 (4)
Acetamide	45 ± 1 (4)	54 ± 4 (4)	
Urea	83 ± 2 (3)	88 ± 4 (4)	

[a] Cells with DTNB pH 7.21, 297 mosM.

DTNB since it is sparingly soluble in water. To avoid these problems, the erythrocytes were washed with buffer before the DTNB incubation and the DTNB was dissolved in a minimum amount of alcohol before being added to the buffer. The control cells were treated in the same way, except that alcohol without DTNB was added to the cells.

There is no significant effect of the removal of plasma ($P_0 = 741 \cdot 10^{-5}$ cm · s^{-1}) or of 0.5 or 1.0 mM DTNB on the permeability of the erythrocyte to ^{3}HHO. The mean permeability to acetamide and urea appear to be higher in the DTNB-treated cells but the increase to P_0 is not statistically significant ($p > 0.05$).

Permeability coefficients for small solutes at 37°C in thiourea-treated erythrocytes

Because thiourea has been reported [8,15] to be a competitive inhibitor for urea uptake we examined the effect of thiourea on the permeability of the erythrocyte to the other hydrophilic solutes, water and acetamide. The cells were incubated with thiourea and separated for the diffusion studies with thiourea present in the plasma surrounding the cells. The results, listed in Table IV, show that there is no significant change in erythrocyte permeability to ^{3}HHO in the presence of thiourea. There is, however, a significant decrease in the diffusional permeability to acetamide and urea in the presence of thiourea. This decrease becomes more pronounced at higher thiourea concentrations for urea. The permeability coefficients for acetamide are not significantly different from each other at 30 and 60 mM thiourea. Thiourea had no effect on the permeability of the erythrocyte to the lipophilic solute antipyrine.

Permeability coefficients for small solutes at 37°C in phloretin-treated erythrocytes

Phloretin interacts with lipids of the plasma membrane [18] and is reported to have varying effects on the osmotic permeability of the eythrocyte membrane [7]. At the pH of these studies, much of the phloretin is un-ionized and interacts with the membrane constituents in this form [18]. The permeability coefficients for some small hydrophilic and lipophilic solutes after treatment with phloretin are listed in Table V. Because the phloretin was dissolved in 0.5% ethanol, the control preparations included 0.5% alcohol. There is a significant decrease in permeability to acetamide

TABLE IV

PERMEABILITY COEFFICIENTS AT 37°C FOR ERYTHROCYTES INCUBATED WITH THIOUREA

The value reported are mean ±S.E. with the number of determinations given in parenthesis.

Solute	Permeability coefficient ($cm \cdot s^{-1}$), ($\times 10^5$)		
Thiourea (mM) [a]	0	30	60
^{3}HHO	679 ± 4 (2)	640 ± 39 (4)	
Acetamide	41 ± 3 (6)	27 ± 3 (3)	22 ± 1 (4) [b]
Urea	100 ± 3 (4)	60 ± 7 (4)	48 ± 5 (2) [c]
Antipyrine	263 ± 29 (4)	243 ± 18 (4)	

[a] Cells with thiourea pH 7.4, 318 mosM.
[b] $p < 0.001$ for 30 and 60 mM thiourea compared to control.
[c] $p < 0.01$ for 30 and 60 mM thiourea compared to control.

TABLE V

PERMEABILITY COEFFICIENTS AT 37°C FOR ERYTHROCYTE TREATED WITH PHLORETIN

The values reported are mean ±S.E. with the number of determinations given in parenthesis. Phloretin in 0.5% ethanol.

Solute	Permeability coefficients ($cm \cdot s^{-1}$), ($\times 10^5$)	
Phloretin (mM) [a]	0	0.5
^{3}HHO	774 ± 64 (4)	648 ± 28 (8)
Acetamide	30 ± 3 (4)	8 ± 1 (4) [b]
Urea	101 ± 9 (4)	44 ± 5 (4) [b]
Methanol	1015 ± 76 (4)	1128 ± 149 (4)

[a] Cells with phloretin pH 7.3, 305 mosM.
[b] $p < 0.001$ for 0.5 mM phloretin compared to control.

and urea in the presence of phloretin but there is no effect on permeability to methanol or to ^{3}HHO.

Additional studies of ^{3}HHO permeability

The known respiratory inhibitor 2,4-dinitrophenol was tested for effects on the erythrocyte permeability to ^{3}HHO. The 2,4-dinitrophenol was added to the plasma in the same way as PCMBS but dissolved in ethanol. The studies were carried out at 20°C. A similar amount of alcohol was added to the control cells. Concentrations of 1.0 mM 2,4-dinitrophenol in 0.6% (0.13 M) ethanol had no effect on ^{3}HHO permeability ($400 \cdot 10^{-5}$ cm $\cdot$ s^{-1} for both control and 2,4-dinitrophenol-treated cells). At 10 mM 2,4-dinitrophenol and 6% ethanol P_0 for ^{3}HHO was $233 \cdot 10^{-5}$ cm $\cdot$ s^{-1} however, with 6% ethanol present and no 2,4-dinitrophenol, the P_0 was $328 \cdot 10^{-5}$ cm $\cdot$ s^{-1}. Therefore, while the values of P_0 were significantly lower in the presence of 2,4-dinitrophenol than in its absence, ethanol alone also decreased ^{3}HHO permeability: the role of 2,4-dinitrophenol itself is not easily defined.

Tests at higher concentrations of 2,4-dinitrophenol in ethanol were precluded by the ensuing cell lysis.

Discussion

Transport inhibitors which interact with proteins (PCMBS, DTNB, thiourea) or with phospholipids (phloretin, ethanol) in erythrocyte membranes decrease the diffusional permeability of hydrophilic nonelectrolytes but have no significant effect on the permeabilities of lipophilic nonelectrolytes. Within the group of hydrophilic solutes the effects on permeability of the inhibitors differs for each solute. The significance of these results can be considered in reference to: the differences between hydrophilic and lipophilic solute permeation and among different hydrophilic solutes; the effects of inhibitors on osmotic permeability; and the differences between human and dog erythrocytes.

We had proposed previously that the small hydrophilic solutes methanol and ethanol penetrated the erythrocyte membrane by pathways different from those for water and for acetamide under the conditions which obtain in the measurement of diffusional permeabilities [4]. The interpretation of our data agrees with the results of the present study since none of the protein reagents (PCMBS, DTNB) that decrease the permeability of small hydrophilic solutes interfere with methanol and ethanol permeation (Table II). Methanol and ethanol permeability, under an osmotic gradient at 20°C, are also not affected by PCMBS [5]. It is interesting that phloretin, which interacts with a specific membrane phospholipid in vesicles [19] and with phospholipid associated with protein in erythrocytes [20], does not affect the diffusional permeability of these small lipophilic solutes. Phloretin is reported to increase the permeability to small lipophilic solutes but solutes as small as methanol and ethanol have not been tested before the present study.

The combination of results from diffusional permeability coefficients [3], temperature dependence [4] and the inhibitor effects reported above point to the penetration of methanol and ethanol by diffusion through the membrane lipid matrix. The main barrier to resistance would then correspond to the tightly packed regions of the phospholipid chains and cholesterol of the type described recently in a review by Stein [21]. This same membrane area provides the barrier to antipyrine penetration which also is not affected by the inhibitors of hydrophilic solute permeation. The large size of antipyrine explains its lower diffusional permeability and higher temperature coefficients than those for the alcohols reported earlier [4].

The patterns for inhibition of the permeation of hydrophilic nonelectrolytes are more complex. The diffusional permeability to tritiated water is reduced by PCMBS but not by DTNB, thiourea or phloretin. In human erythrocytes PCMBS inhibits osmotic and diffusional transport at 20 and 37°C [5]. The temperature coefficients (E_a) between 20 and 37°C for water diffusional permeation in human erythrocytes is 6.8 kcal $\cdot$ mol^{-1} in control cells and 9.7 kcal $\cdot$ mol^{-1} in PCMB-treated cells [22]. In the dog cells E_a for diffusional water permeability is 5.3 kcal $\cdot$ mol^{-1} in control cells and 6.2 kcal $\cdot$ mol^{-1} in PCMBS-treated cells.

Differences in water permeation between dog and human erythrocytes were discussed by Viera et al. [23]. In addition, in human erythrocytes

PCMBS binds primarily to band 3 protein [24] while it binds primarily to band 4.9 and less to bands 5 and 7 in dogs [25]. Granted that caution is appropriate in any use of E_a in interpretations of membrane permeation [4], these data could also account for the difference in the effect of PCMBS on the E_a for water permeation in dog and human cells.

In both dog and human cells the diffusional water transport at 2 mM PCMBS is 50–60% of the control value (Table I; Fig. 1; Refs. 6, 22, 24) while in human cells the osmotic permeability is decreased in relation to the control value to 50% at 0.5 mM PCMBS and to 10% after treatment with 1 or 2 mM PCMBS [5,6]. The relationship between P_0 and the concentration of PCMBS present in the incubation medium is linear over the range 0–2 mM (evaluated by analysis of variance for linear regression [12]) but from 2 to 4 mM PCMBS the decrease of P_0 is not significantly different (Fig. 1). A pertinent comparison to make is the amount of PCMBS available per cell in these different studies. Unfortunately this is difficult to calculate from the limited data available in some of the reports. It does appear that most investigators used a more dilute solution of erythrocyte than ours during the exposure to PCMBS; in addition, at 1 mM all of the PCMBS reactive groups on proteins should be saturated. Therefore, we cannot draw any conclusion about the different degree of reduction in osmotic and diffusional permeability that is directly related to the amount of PCMBS bound to the cells.

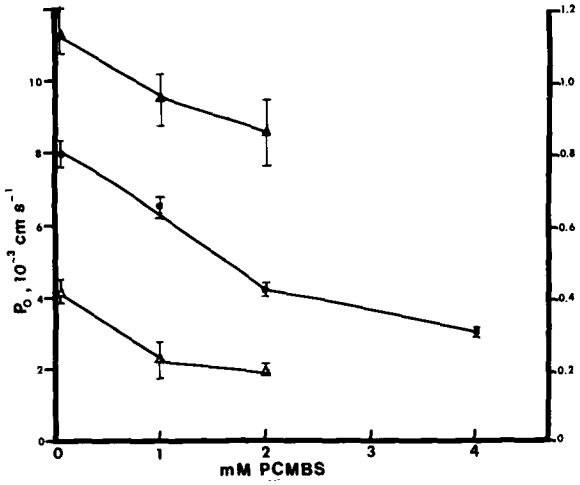

Fig. 1. Permeability coefficients (P_0) (mean ±S.E.) plotted against the concentration of PCMBS present in the incubation medium. The ordinate on the left is for P_0 for ^{3}HHO (●) and the ordinate on the right is for P_0 for [^{14}C]acetamide (▲) and [^{14}C]urea (▲).

The data from the osmotic studies were interpreted as demonstrating that most hydraulic water movement is through protein pores and 10% through membrane lipid areas [5,6]. If we use the value reported by Sha'afi [8] for osmotic (P_f) and diffusional (P_d) permeability coefficients in human erythrocytes (1.73 and $5.3 \cdot 10^{-3}$ cm · s^{-1}, respectively, at 20°C) a 90% reduction in P_f and a 60% reduction in P_d yield the same value of $2.0 \cdot 10^{-3}$ cm · s^{-1} for the permeability coefficients to water in 2 mM PCMBS-treated cells, a value similar to the P_0 we report at 20°C for dog erythrocytes after 2 mM PCMBS treatment. This indicates that diffusional water movement occurs in the presence of an osmotic gradient and that under both diffusional and osmotic gradients water movement is occurring by two pathways. A similar calculation and conclusion based on slightly different values were reported very recently by Brahm [30] in a study of the diffusional water permeability of human cells at 25°C.

The pathway inhibited by PCMBS is protein associated and accounts for 90% of osmotic water movement and for 40–50% of diffusional water movement. The difference in PCMBS distribution in the human and dog erythrocyte membrane combined with its consistent effect on water diffusion implies that it is the attachment of PCMBS to available protein sulfhydryl groups, possibly through a steric hindrance effect, and not alteration of a specific sulfhydryl bond that leads to inhibition of water transport. The remainder of water movement is either through protein areas which are not affected by PCMBS or through membrane lipid areas. Thus, in the dog erythrocyte diffusional water movement appears to occur by two distinct pathways.

Phloretin interacts with membrane phospholipids that seem to be intimately associated with band 3 protein [20]. It is reported to increase osmotic water permeability in human cells [7] but has no effect on diffusional permeability in dog cells (Table V). The data from phloretin treatment presents a similar pattern to that for PCMBS in

comparison of human and dog cells.

The other agents which were tested that interact with membrane lipids are ethanol and 2,4-dinitrophenol. As explained previously the studies with these agents were limited but a definite inhibition of water transport was observed after ethanol treatment.

We have presented evidence above that ethanol diffuses through lipid areas of the membrane. The alteration by 1 M ethanol of the interaction of fluorescent molecules with membrane protein has provided a basis for the interpretation that the effect of ethanol is on protein-associated membrane lipid [26]. In a more recent study De Bruijne and Van Steveninck [27] reported both a protein-associated ethanol-membrane interaction and a separate lipid-associated ethanol interaction at 1 M or higher. We observed decreased water movement at about the same ethanol concentration (1.3 M) but not at a lower concentration. Inhibition of osmotic water transport in human erythrocytes by another alcohol, hexanol, was reported recently and the data were interpreted as providing additional evidence of water transport through protein associated areas [28]. In addition Sha'afi [8] has interpreted work from his laboratory to indicate that the protein SH groups involved in water transport are located in a hydrophobic environment. Thus the distinction between protein and lipid pathways that we might have expected to see with 2,4-dinitrophenol and ethanol treatment of erythrocytes is not possible. Therefore, whether water movement that is not inhibited by PCMBS is through other protein areas or through lipid areas cannot be definitely stated.

The separation of water and the small hydrophilic solutes acetamide and ureas in the permeation of human erythrocytes that has been observed under osmotic gradients [6] is observed in our data under a diffusional gradient (Table II). The diffusional permeabilities of acetamide and urea are reduced by thiourea and phloretin while that of acetamide is reduced by PCMBS. However, the permeability of tritiated water is reduced only by PCMBS. Both acetamide and water permeation are reduced by 50% at 2 mM PCMBS but at 1 mM PCMBS water permeation is reduced 19% while that of acetamide is reduced 46% (Tables I and II, Fig. 1). The different patterns of PCMBS reduction of the permeation of water and acetamide indicate either different pathways of permeation or a pathway in which acetamide permeation is more easily affected than is that of water. We do not compare the actual permeability coefficients reported in the literature to our results because most of the published data are expressed as percentage of change in permeability and are reflection coefficients or permeability coefficients determined under osmotic rather than diffusional gradients.

Urea and acetamide differ structurally in the presence of a methyl group on acetamide and of an amino group on urea. They are almost of the same molecular weight although the molar volume of urea is about one-third smaller and the oil/water partition coefficient of urea is about one-half that of acetamide. Urea penetrates dog erythrocytes about three times faster than acetamide. For urea E_a is 8.1 $kcal \cdot mol^{-1}$ [29] while E_a for acetamide is 12 $kcal \cdot mol^{-1}$ [3]. A similar pattern in human cells at 20°C has led to the suggestion that urea enters erythrocytes by facilitated diffusion [8,15].

In human erythrocytes the competitive inhibition of urea transport by thiourea under an osmotic gradient and its inhibition by phloretin, a substance known to inhibit other facilitated diffusion systems, have been offered as additional evidence for the facilitated diffusion of urea. In our studies we found inhibition of diffusional permeability of both urea and acetamide by both of these agents. However, while PCMBS decreased the permeability to acetamide its effects on the average urea permeability even at a concentration of 2 mM was statistically significant at the 10% but not at the 5% level ($0.10 > p > 0.05$) by parametric (analysis of variance) or non-parametric (Wilcoxon U) tests [12]. Neither solute is affected by the band 3 specific agent DTNB.

Comparison of the results for acetamide and urea under a diffusional gradient at 37°C indicates that both solutes penetrate the membrane through areas affected by agents which interact with protein or protein associated phospholipids. Acetamide and urea probably share at least some 'pathways' through the membrane since thiourea competes with both (their structural similarity makes this resonable). Acetamide and urea also

have some different 'pathways' throught the membrane since PCMBS affects their permeation differently. Phloretin inhibition of urea permeation could reflect either inhibition of facilitated diffusion or less specific inhibition of small hydrophilic solutes that has been reported for phloretin. Our results do not allow separation of the two proposed mechanisms for urea permeation in dog erythrocytes.

We can summarize the interpretation of our results in reference to our original objectives as follows: (1) small lipophilic nonelectrolytes penetrate the erythrocyte membrane lipid; (2) the small hydrophilic nonelectrolyte tested penetrate the membrane by some pathways that are shared and some that are different but which seem to be associated with membrane protein; (3) we cannot distinguish conclusively between facilitated diffusion and passive diffusion for urea permeation; (4) diffusional water movement across the dog erythrocyte membrane occurs through two distinct pathways under both osmotic and diffusional gradients; (5) convergence of P_f and P_d.

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