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Binding of DTNB to band 3 in the human red cell membrane

Michael R. Toon, Peter L. Dorogi *, Michael F. Lukacovic ** and A.K. Solomon

Biophysical Laboratory, Department of Physiology and Biophysics, Harvard Medical School, Boston MA 02115 (U.S.A.)

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Inhibition of red cell water transport by the sulfhydryl reagent 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) has been reported by Naccache and Sha'afi ((1974) J. Cell Physiol. 84, 449–456) but other investigators have not been able to confirm this observation. Brown et al. ((1975) Nature 254, 523–525) have shown that, under appropriate conditions, DTNB binds only to band 3 in the red cell membrane. We have made a detailed investigation of DTNB binding to red cell membranes that had been treated with the sulfhydryl reagent *N*-ethylmaleimide (NEM), and our results confirm the observation of Brown et al. Since this covalent binding site does not react with either *N*-ethylmaleimide or the sulfhydryl reagent pCMBS (*p*-chloromercuribenzenesulfonate), its presence has not previously been reported. This covalent site does not inhibit water transport nor does it affect any transport process we have studied. There is an additional low-affinity (non-covalent) DTNB site that Reithmeier ((1983) Biochim. Biophys. Acta 732, 122–125) has shown to inhibit anion transport. In *N*-ethylmaleimide-treated red cells, we have found that this binding site inhibits water transport and that the inhibition can be partially reversed by the specific stilbene anion exchange transport inhibitor 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS), thus linking water transport to anion exchange. DTNB binding to this low-affinity site also inhibits ethylene glycol and methyl urea transport with the same K_I as that for water inhibition, thus linking these transport systems to that for water and anions. These results support the view that band 3 is a principal constituent of the red cell aqueous channel, through which urea and ethylene glycol also enter the cell.

Introduction

It has been suggested by Brown et al. [1] that the human red cell membrane protein, band 3, provides the channel for aqueous transport across the membrane. These authors found that the sulfhydryl reagent DTNB bound almost exclusively to band 3 when other membrane sulfhydryl groups were protected by *N*-ethylmaleimide. Since Naccache and Sha'afi [2] had previously reported that DTNB inhibited 60% of the osmotic water flow across the membrane and that *N*-ethylmaleimide had no effect on water transport, Brown et al. concluded that band 3 was the probable site for water transport. However, Chien and Macey [3] were not able to confirm DTNB inhibition of osmotic water transport, though Levitt and Mlekoday [4] reported 30% inhibition. Brahm [5] studied

* Present address: Gillette Research Institute, 1413 Research Blvd., Rockville, MD 20850, U.S.A.

** Present address: Procter and Gamble, Sharon Woods Technical Center, 11520 Reed Hartman Highway, Cincinnati, OH 45241, U.S.A.

Abbreviations: BADS, 4-benzamido-4′-aminostilbene-2,2′-disulfonate; DBDS, 4,4′-dibenzamidostilbene-2,2′-disulfonate; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NTB, 2-nitro-5-thiobenzoic acid; NEM, *N*-ethylmaleimide; pCMBS, *p*-chloromercuribenzenesulfonate.

the effect of DTNB and found no inhibition of diffusional water permeability. Many authors (see for example, Refs. 2 and 6) have confirmed the observation of Macey and Farmer [7] that the sulfhydryl reagent pCMBS inhibits osmotic water transport by 80% or more and Solomon et al. [6] have used polyacrylamide gel electrophoresis to show that the predominant site for pCMBS binding to sealed red cell ghosts is on band 3, thus providing further evidence that the aqueous channel in the red cell is located on band 3.

Since we are particularly interested in the mechanism of water permeability, we have studied DTNB binding to *N*-ethylmaleimide-treated red cell membranes. There are two DTNB sites on band 3. DTNB reacts covalently with a sulfhydryl group that does not react with either *N*-ethylmaleimide or pCMBS and has not previously been reported. In addition there is a low-affinity binding site (non-covalent) which Reithmeier [8] has found to inhibit anion transport. Binding to this site also inhibits the transport of water, methyl urea and ethylene glycol. These results lead to the conclusion that band 3 is the principal constituent of the aqueous channel.

Materials and Methods

Ghost preparation. Red cell ghosts were prepared from human whole blood outdated less than 3 days, using an hemolysis procedure similar to that of Dodge et al. [9]. In general, each experiment reported in the paper was done on blood from a different donor. Whole blood was washed three times with phosphate-buffered saline (containing 150 mM NaCl and 5 mM Na_2HPO_4), pH 8.0, 4°C and was lysed in 5 mM Na_2HPO_4 under the same conditions with a buffer/cell volume ratio of 40 : 1. This lysing procedure was repeated three additional times, giving finally a white, hemoglobin-free pellet of red cell ghosts. The total protein in the final pellet was determined by the method of Lowry et al. [10] with bovine serum albumin as standard.

N-Ethylmaleimide treatment. Band 3 contains six sulfhydryl groups of which five are located on the cytoplasmic side of the membrane and react with *N*-ethylmaleimide [11–13]. The sixth, which is in the 17 kDa membrane-bound fragment of band 3 between the trypsin and chymotrypsin cuts [14], does not react with *N*-ethylmaleimide but does react with pCMBS [6,15,16]. Rao [15,16] has shown that reaction of the cytoplasmic sulfhydryl groups with *N*-ethylmaleimide blocks subsequent reaction with pCMBS. She found [16] that *N*-ethylmaleimide uptake in red cell ghosts saturated at 0.2–0.3 mM *N*-ethylmaleimide after 1 h incubation at 37°C. All of our fluorescence experiments have been done with *N*-ethylmaleimide-treated ghosts that have been incubated with one volume of 2 mM *N*-ethylmaleimide in phosphate-buffered saline (pH 7.4) for 1 h at ambient temperature (21–23°C) and subsequently washed three times with NEM-free phosphate-buffered saline at pH 7.4. Since we have used a higher NEM concentration and a lower temperature than Rao, we have carried out control experiments to see whether longer times of incubation or higher temperatures had any effect in our fluorescence experiments. Neither extending the incubation time to 2 h or raising the temperature to 37°C for a 1 h incubation caused any change in the effect of pCMBS on 4,4′-dibenzamidostilbene-2,2′-disulfonate (DBDS) fluorescence enhancement. This indicates that the pCMBS effects on fluorescence enhancement reported in this paper are not to be attributed to the cytoplasmic sulfhydryl groups.

Binding of sulfhydryl reagents. DTNB, which is used as Ellman's reagent [17] to determine sulfhydryl groups in proteins, forms its reaction product, NTB (2-nitro-5-thiobenzoic acid) when the disulfide bond between the two halves of DTNB is replaced by another disulfide bond formed from the sulfhydryl of the protein. Thus, measurement of the absorbance of NTB provides a positive indication that reaction has taken place with a sulfhydryl group. A stock solution of 1 mM DTNB (Sigma, St. Louis, MO) in phosphate-buffered saline (pH 7.4) was made up on the day of the experiment and diluted with phosphate-buffered saline to the desired concentrations. For measurements of DTNB binding to NEM-treated ghosts, 0.8 ml aliquots of the ghost pellet were mixed with 1 ml of the DTNB solutions in centrifuge tubes and the ghost suspensions were stirred intermittently for 30 min before centrifugation (40 000 × *g* for 40 min). About 1 ml of the supernatant was withdrawn with a Pasteur pipette and its ab-

sorbance was measured at 412 nm to determine the concentration of NTB.

Fluorescence studies of anion transport inhibitor binding. For investigation of the effects of sulfhydryl reagents on the binding of the anion transport inhibitor DBDS, NEM-treated ghosts were divided into two fractions. One fraction was incubated with 100 μM DTNB solution as described above; this concentration of DTNB is in at least 5-fold excess of total band 3 concentration in the suspension. After three washes with phosphate-buffered saline (pH 7.4), one half of the DTNB, NEM-treated ghost pellet and one half of the original NEM ghost pellet were suspended separately in 1 mM pCMBS. Ghosts were incubated for 30 min with pCMBS at room temperature before DBDS binding experiments were begun.

Kinetic fluorescence studies of DBDS binding to the four differently treated ghost preparations, NEM, DTNB/NEM, pCMBS/NEM and DTNB/pCMBS/NEM, were performed with the stopped-flow apparatus designed and built in our laboratory and described previously [18]. This device has a dead time of 60 ms and records optical signals on a Biomation 805 waveform recorder (Cupertino, CA); the data are then transferred on-line to a PDP 11/34 computer (Digital Equipment Co, Maynard, MA) for storage and analysis.

DBDS was synthesized by the method of Kotaki et al. [19] and checked for purity by thin-layer chromatography on Silica Gel G in pyridine/acetic acid/water (10 : 1 : 40, v/v). The concentration of DBDS in the stock solution was determined by absorbance at 336 nm. Since DBDS binding to ghost membranes enhances fluorescence by about two orders of magnitude [20], binding was monitored by the fluorescence enhancement (excitation, 356 nm; emission > 410 nm).

Osmotic permeability measurements in whole red cells. Osmotic permeability was measured using the stopped-flow light-scattering apparatus of Terwilliger and Solomon [21] to measure red cell volume. Whole blood, outdated by no more than three days, was centrifuged, plasma and buffy coat aspirated, and then washed three times with a red cell buffer of the following composition (in mM): NaCl, 142; KCl, 4.4; Na_2HPO_4, 6; $NaHCO_3$, 24.9; $MgCl_2$, 0.5; pH 7.4, 300 $\pm$ 5 mosM. Treatment of the red cells with NEM in the latter 75% of the light-scattering experiments consisted of incubation at 37°C for 1 h at 25% hematocrit at a final concentration of 12 mM NEM in the red cell buffer (in the earlier experiments the time was 30 min and the hematocrit was 25%). Cells were resuspended to a 2% hematocrit in the same buffer for further treatment with DTNB or pCMBS after washing three times in NEM-free buffer. DTNB treatment required an additional incubation at 37°C for 1 h. DTNB-treated cells were washed three times with 100 volumes each of red cell buffer to remove free NTB before resuspension at 2% hematocrit with 1 mM pCMBS.

Cells at 2% hematocrit were then mixed with an equal volume of solution made hyperosmolal by addition of NaCl to the red cell buffer; the response of the cells to a 250 mosM gradient was the basis of all L_p measurements. Nonelectrolyte permeability was determined by the method of Sha'afi et al. [22] and was based on the response of the cells to a 350–400 mosM nonelectrolyte gradient. All experiments were performed at 21–23°C. Permeability coefficients were calculated using a red cell volume, V, of $100 \cdot 10^{-12}$ cm^3, a cell area, A, of $1.35 \cdot 10^{-6}$ cm^2 and a fractional cell water content of 0.72 leading to $V/A = 5.33 \cdot 10^{-5}$ cm, as used by Dix and Solomon [23]. Osmolalities were measured using a Fiske model OS osmometer (Fiske Associates, Uxbridge, MA). DTNB and pCMBS were obtained from Sigma Chemical (St. Louis, MO). All other chemicals were of reagent grade and were obtained from Fisher Scientific (Medford, MA).

For experiments with DIDS, washed cells were incubated with 12 mM NEM $\pm$ 20 μM DIDS (U.S. Biochemical Corp., Cleveland, OH) for 1 h at 37°C at 25% hematocrit. Cells were then washed three times with NEM/DIDS-free buffer and resuspended to 2% hematocrit in buffer $\pm$ DTNB for an additional 1 h incubation at 37°C. This treatment routinely produced > 98% inhibition of anion flux.

$^{35}SO_4$ efflux experiments. Whole blood was obtained, washed and treated with NEM as for the osmotic permeability experiments, except that 10 mM Na_2SO_4 replaced 17 mM of NaCl in the buffer (sulfate flux buffer). Following NEM treatment, the cells were washed three times with 10

volumes each of the same buffer and then resuspended to 50% hematocrit for a 1 h incubation at 37°C with buffer containing $^{35}SO_4$ (New England Nuclear, Boston, MA) at a final concentration of 7.5 μCi/ml. The loaded cells were then washed with ice-cold sulfate flux buffer (2 volumes each) three times in a microcentrifuge (Eppendorf Model 3200, Brinkman Instruments, Westbury, NY) at 4°C. Cells were then resuspended to 35% hematocrit in ice cold buffer. Sulfate efflux was initiated by diluting 1 ml of the 35% suspension in 15 ml of sulfate flux buffer ± DTNB at 37°C. 1-ml aliquots of the solution were withdrawn at six set times, centrifuged for 30 s and 0.2 ml samples of supernatant were counted in 2.5 ml Ultrafluor (National Diagnostics, Somerville, NJ). An infinite time point was determined by solubilization (0.03 ml of 0.2% saponin) and trichloroacetic acid precipitation (0.11 ml of 50% ice-cold trichloroacetic acid) of 1 ml of each solution. Samples were counted to 1% in a Tracor Delta 300 liquid scintillation counter (Elk Grove Village, IL) and were corrected for dilution and quenching by use of the external standard ratio method.

Experiments with ^{3}H-NEM. Eight 1-ml aliquots of ghosts at a protein concentration of 4 mg/ml were incubated at 23–25°C for 1 h in 1 ml phosphate-buffered saline (pH 7.4) containing 2 mM NEM and 1 μCi/ml ^{3}H-NEM (New England Nuclear, Boston, MA) (final [NEM] = 1 mM). Ghosts were then washed three times in phosphate-buffered saline (15 vols) to remove unreacted NEM. Then four samples were incubated with 1 ml of 2 mM DTNB in phosphate-buffered saline at 23–25°C, while the remaining four samples were treated with 1 ml phosphate-buffered saline as a control. At the end of this period, all the ghosts were washed three more times with phosphate-buffered saline. Ghosts were solubilized in 5 ml of Ultrafluor and counted to 0.5%.

To correct for quenching due to bound DTNB, two sets of ghosts (in quadruplicate) were reacted and washed under identical conditions to those above except for the addition of ^{3}H-NEM. The resulting ghosts were then added to vials containing 0.02 μCi ^{3}H-NEM. The difference in counts was used to correct for the 5–8% DTNB quench effect.

Results and Discussion

Locus of DTNB binding site

Rao [13] reported that there are five intracellular sulfhydryl groups on band 3 in red cells; two of them form a disulfide when ghosts are prepared under non-reducing conditions such as we have used, so only three are found in ghosts. All five react with NEM or pCMBS; reaction with NEM blocks reaction with pCMBS and vice versa, as previously discussed under Materials and Methods. There is an additional, sixth sulfhydryl group that has been reported by Steck et al. [11] and Ramjeesingh et al. [12,14] which is located on the same 17 kDa fragment of band 3 that contains a covalent DIDS binding site. Since Rao and Reithmeier [15] have reported that there is no NEM-reactive group in the membrane bound fragment of band 3, the sixth sulfhydryl group does not react with NEM.

Since Naccache and Sha'afi [2] had found that neither NEM nor iodoacetamide inhibited water transport in the red cell, Brown et al. [1] treated their membranes with these reagents before determining the location of ^{14}C-DTNB in polyacrylamide gel electrophoresis of red cell ghosts. The gel that they published shows only a single significant peak which is located on band 3. We have measured the stoichiometry of DTNB binding to NEM-treated red cell ghost membranes by the optical absorbance of the DTNB reaction product which provides a positive indication that reaction has taken place with a sulfhydryl group (see Materials and Methods). Fig. 1 shows the results. In this experiment there were 9.0 μM of sites compared with 7.8 μM of band 3 (calculated on the basis that band 3 has a molecular weight of 95 000 and comprises 25% of the membrane proteins (see Ref. 6); the average ratio in three experiments was 1.1 ± 0.2. This observation of 1 : 1 stoichiometry is entirely consistent with the finding of Brown et al. that DTNB bound almost exclusively to band 3 in preparations that had been treated with NEM and iodoacetamide.

DBDS fluorescence enhancement studies

The stilbene anion transport inhibitor which has been characterized in most detail is DIDS; it

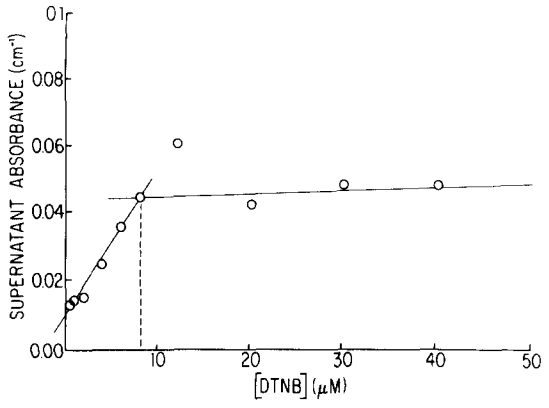

Fig. 1. DTNB reaction with NEM-treated ghosts as measured by supernatant absorption in one of three experiments. In this experiment there was a ratio of 1.1 DTNB sites per band 3; the average ratio was 1.1 ± 0.2.

has been convincingly shown that DIDS binds with high specificity to band 3 in intact red cells [24]. Rao et al. [25] have shown that DBDS, the fluorescent analog of DIDS used in our studies, binds only to the DIDS site in whole red cells. Verkman et al. [26] have shown that there is no fluorescence enhancement when DBDS interacts with ghosts to which DIDS has previously been covalently bound. Verkman et al. have also carried out experiments on inside out vesicles which show that there is no fluorescence enhancement that may be ascribed to DBDS sites on the inside face of the red cell membrane. These arguments lead to the conclusion that DBDS fluorescence enhancement is highly specific to band 3.

Dix et al. [27] and Verkman et al. [26] have provided evidence that the initial step of binding of the specific stilbene anion exchange inhibitor DBDS to the red cell membrane is a fast bimolecular association, followed by a conformational change, according to the following equation

$$\text{DBDS} + \text{band 3} \leftrightarrow \text{DBDS-band 3} \leftrightarrow \text{DBDS-band 3}^* \qquad (1)$$

The time-course of the band 3 conformational change (approx. 4 s^{-1}) is slow enough that the kinetics can be measured by the stopped-flow method. When a second ligand as, for example, pCMBS, reacts with band 3, kinetic methods can be used to derive a reaction scheme for the DBDS/pCMBS/band 3 system from the time-course of fluorescence enhancement, which incorporates the observation that pCMBS is a non-competitive inhibitor of DBDS binding [28]. On the basis of this conceptual framework, a less detailed analysis of the system can provide important information about the nature of interactions with band 3. In this study we have used changes in the kinetics of DBDS fluorescence enhancement to detect changes in band 3 conformation induced by treatment with DTNB.

The effect of DTNB on the average time-course of DBDS fluorescence enhancement in four experiments is shown in Fig. 2A. The time constant τ, is increased from 1.96 ± 0.31 s to 2.78 ± 0.70 s ($p < 0.01$, t-test). This result indicates that DTNB binding affects the conformation of band 3. Although DTNB alters the kinetics of DBDS fluorescence enhancement, it does not affect the equilibrium, as shown in the two left hand bars in Fig. 3A. In the absence of other chromophores, changes in the fluorescence enhancement of DBDS may be attributed exclusively to changes in the relationship between DBDS and band 3. However, free NTB absorbs at 412 nm and the emission peak of DBDS is at 427 nm, so the possibility exists that some of the emitted DBDS quanta are absorbed by NTB. The observation that DTNB does not change the equilibrium fluorescence of DBDS is consistent with the view that such an effect is not important; however, it does not entirely exclude any possible interaction since two opposing processes could cancel out. Thus the time dependence of DBDS fluorescence could possibly represent changes in the relative separation between DBDS and DTNB rather than changes in the environment of DBDS. Even if this were to be the case, it would not affect our conclusion that DTNB binds to a site on, or in apposition to, band 3.

As a preliminary step in determining whether the DTNB site is the pCMBS site in NEM-treated cells, we determined that 2 mM pCMBS had no effect on DTNB binding as measured by NTB absorbance. This result was to be expected since Sutherland et al. [29] had shown that pCMBS binding to the red cell membrane is labile, whereas the DTNB reaction is irreversible. We next looked for an effect of pCMBS on the DTNB/DBDS fluorescence enhancement and found that, following pre-incubation of NEM-treated ghosts with 1.0

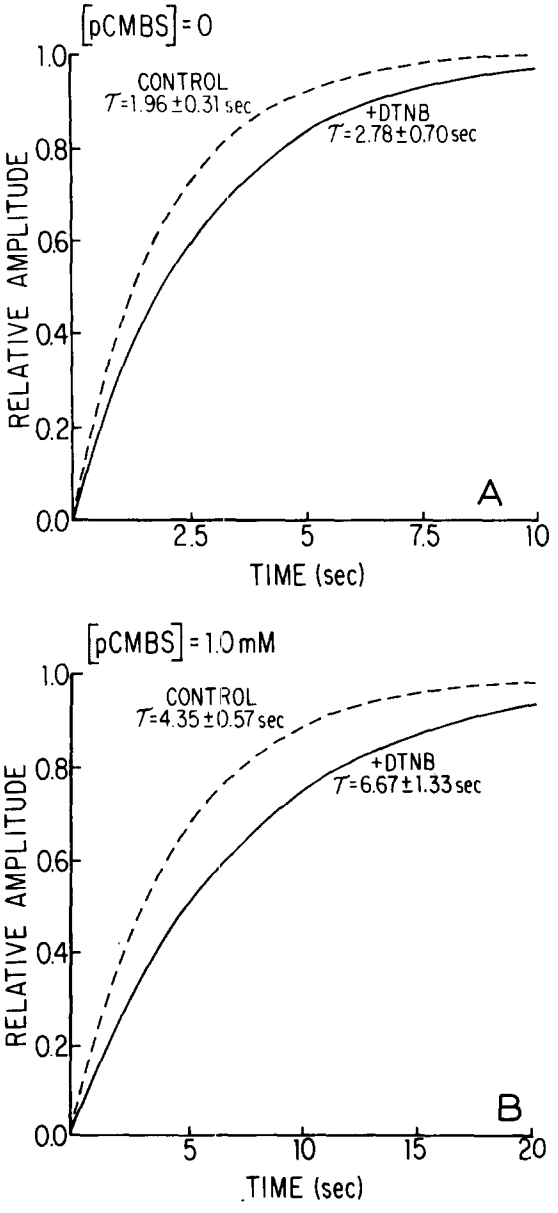

Fig. 2. Fluorescence enhancement kinetics following DBDS binding to NEM-treated ghosts measured by stopped-flow. (A) [pCMBS] = 0 mM. (B) [pCMBS] = 1.0 mM.

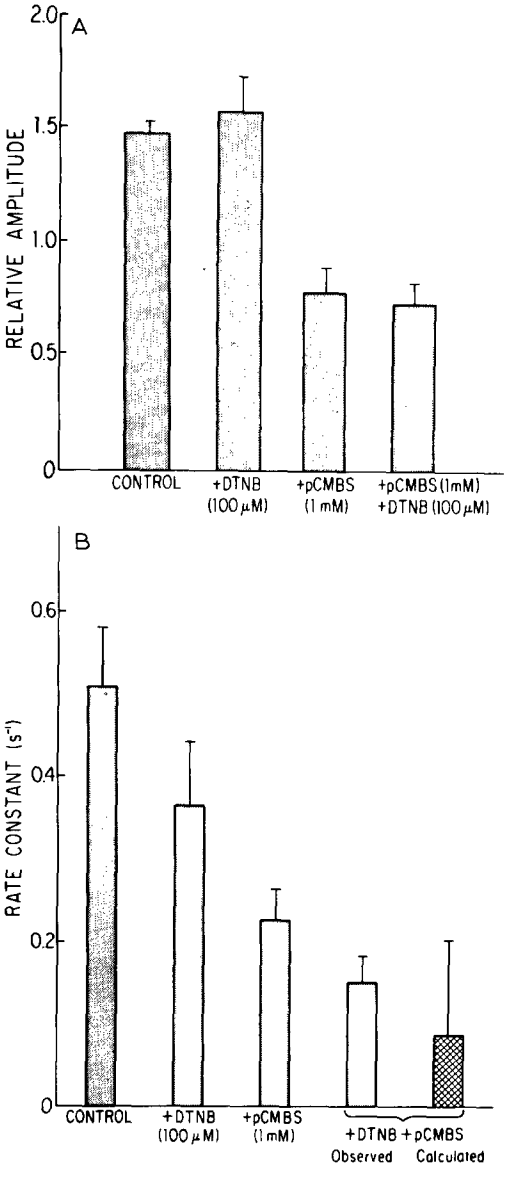

Fig. 3. Effect of DTNB and pCMBS on fluorescence enhancement in NEM-treated ghosts in four experiments. (A) Relative amplitude of equilibrium signal. (B) Rate constants. [pCMBS] = 1 mM; [DTNB] = 100 μM.

mM pCMBS, DTNB (0.1 mM) causes a shift in τ, from 4.4 ± 0.6 s in control ghosts to 6.7 ± 1.3 s ($p < 0.0025$) (Fig. 2B). The observation that pCMBS modulates the kinetics of fluorescence enhancement could be explained by competition for the same site, or by an allosteric interaction between separate sites. The data in Fig. 3A show that DTNB has no effect on the equilibrium amplitude of DBDS fluorescence enhancement, either in control cells or in those treated with pCMBS. This finding is consistent with separate pCMBS and DTNB sites. Furthermore, Fig. 3B shows that the effects of DTNB and pCMBS on the rate constants are additive, within the error of the measurements, which provides further evidence

TABLE I

EFFECT OF DTNB ON WATER PERMEABILITY OF RED CELLS

Number of experiments shown in parentheses. p has been determined by the t-test.

	$L_p(10^{-11}\,cm^3\cdot dyn^{-1}\cdot s^{-1})$		$\omega_{eth\ glycol}$ $(fmol\cdot dyn^{-1}\cdot s^{-1})$
	normal	+NEM	+NEM
Control	1.4±0.2 (14)	1.5 ±0.2 (16)	4.0±0.9 (7)
+5 mM DTNB	1.5±0.2 (8)	1.3 ±0.2 (16)	2.3±0.8 (7)
Ratio+DTNB/Control	1.1±0.1	0.86±0.09	0.6±0.1
p of ratio	< 0.1	< 0.0025	< 0.0005
+1 mM pCMBS	0.33±0.04 (2)	0.4±0.01 (2)	
+5 mM DTNB+ 1 mM pCMBS	0.37±0.02 (2)	0.4±0.05 (2)	

that DTNB and pCMBS occupy independent sites.

Although it was not expected that DTNB could displace NEM from any of the other five sulfhydryl groups with which NEM had reacted, we carried out control experiments to see if incubation with 5 mM DTNB could cause any loss of ^{3}H-NEM from red cells. In two experiments the ratio of ^{3}H-NEM radioactivity recovered (DTNB incubated cells/control) was 0.99 and 1.00, showing that DTNB caused no displacement of NEM. Since DTNB does not displace NEM from any of the five NEM sites and since the DTNB site is different from the pCMBS site, our experiments indicate that there is another sulfhydryl group on band 3 in addition to the six previously reported.

Effects of DTNB and pCMBS on water permeability in normal cells

We have carried out a number of experiments to look for evidence of DTNB inhibition of osmotic water transport. Finding no inhibition at a DTNB concentration of 1 mM, as used by Naccache and Sha'afi [2], the concentration was increased to 5 mM. There was still no evidence of inhibition at 5 mM DTNB in eight experiments in normal cells, as Table I shows *. In a further effort to produce a DTNB effect, we studied the effect of pH over the range from 5 to 9 and increased the time of incubation with DTNB from 1 to 3 h, but were still unable to inhibit osmotic water transport. On the other hand, when we turned to mercurial sulfhydryl reagents, we were able to confirm the results of Sha'afi and Feinstein [30] on all the compounds which we studied, including pCMB(*p*-chloromercuribenzoate), pAPMA (*p*-aminophenylmercuric acetate) and FMA (fluoresceinmercuric acetate) (Toon, M.R. and Solomon, A.K., personal communication). Our results on osmotic water permeability are in agreement with the findings of Chien and Macey [3], though in disagreement with those of Levitt and Mlekoday [4] who reported a 30% inhibition. Brahm [5] found no effect on diffusional permeability though Benga et al. [31] reported 10–18% inhibition of water diffusion.

Since DTNB has no effect, in our hands, on osmotic water permeability in normal cells, it is easy to determine whether DTNB has any effect on pCMBS inhibition of osmotic water transport. As the bottom lines in Table I show, pretreatment with 5 mM DTNB had no effect on pCMBS inhibition. Since, as stated above, the DTNB reaction with sulfhydryl groups is covalent, while the pCMBS reaction is much more labile, it is not to be expected that pCMBS could drive NTB off the sulfhydryl group. Consequently, these experiments

* The control value of L_p (in the absence of DTNB) in Table I of $(1.4\pm0.2)\cdot10^{-11}\,cm^3\cdot dyn^{-1}\cdot s^{-1}$ is smaller than the value of $(1.8\pm0.1)\cdot10^{-11}\,cm^3\cdot dyn^{-1}\cdot s^{-1}$ given previously by Terwilliger and Solomon [21], probably because the present experiments used red cells obtained from outdated bank blood while the earlier ones used fresh blood.

indicate that DTNB has no functional effect on pCMBS induced inhibition of osmotic water transport and provide strong support for our conclusion that DTNB does not react with the pCMBS site.

Effects of DTNB and pCMBS on water permeability in NEM-treated cells

The situation changes when the cells have been pretreated with NEM. In 16 experiments DTNB caused the osmotic permeability to decrease by 14%, as shown in Table I. Though the effect is not large, it is significant ($p < 0.0025$). The three NEM-sensitive sulfhydryl groups in ghosts are all in the cytoplasmic pole [13] and removal of this pole does not affect either anion transport [24] or the calorimetric profile of the membrane bound fragment [32]. Nonetheless, the fact that NEM treatment facilitates DTNB inhibition of osmotic water permeability is consistent with some coupling between the two segments of band 3. Our results are in agreement with Benga et al. [31] who reported 8–13% inhibition of water diffusion in NEM-treated cells. It is also possible to determine whether NEM treatment makes the pCMBS site available to DTNB, since NEM has only a small effect on L_p. As Table I shows, even after NEM treatment, DTNB does not interact with the pCMBS site.

The concentration of DTNB required to inhibit osmotic water flux in NEM-treated cells is about three orders of magnitude greater than the concentration needed to saturate the band 3 site. The red cell suspensions used in our measurements of permeability contain 2% red cells, equivalent to a band 3 concentration of 0.4 μmol/l of suspension (computed as above). Calculations from the data of Haest et al. [33] give 2.3 μmol of DTNB-reactive sulfhydryl sites in the red cell membrane per liter of suspension. The data of Benesch et al. [34] and of Garel et al. [35] give $3.4 \cdot 10^{-4}$ mol of DTNB-reactive sulfhydryl sites in the red cell hemoglobin in the suspension. Since DTNB binding to the sulfhydryl site on band 3 is irreversible, it is only necessary to have enough DTNB molecules to bind all the band 3 sites; 1 μM DTNB is much more than necessary but 5 mM DTNB was necessary to produce the inhibition shown in Table I.

We have studied the dose response characteristics of the DTNB inhibition of water permeability in NEM-treated cells in 13 experiments; the result of a typical experiment is shown as the control curve in Fig. 4. The data have been fitted to a single site binding curve with a K_I of 2.5 ± 0.3 mM and a maximum fractional inhibition of 0.29 $\pm$ 0.01 of the water flux. The shape of the curve is typical of a reversible binding site and does not exhibit the sharp break characteristics of irreversible reaction as shown in Fig. 1. These data show that the DTNB site responsible for inhibiting water permeation in NEM-treated red cells is not the sulfhydryl site on band 3 with which DTNB reacts covalently.

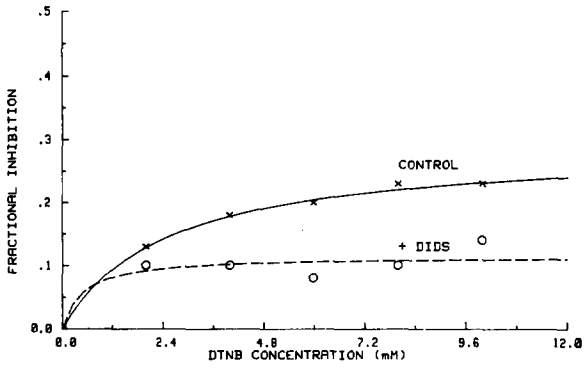

Fig. 4. Effect of DTNB on water flux. The curve in the control experiment has been drawn by non-linear least squares to fit a single site binding equation. $K_I = 2.5 \pm 0.3$ mM; maximum fractional inhibition $29 \pm 1\%$. After DIDS treatment, described under Materials and Methods, $K_I = 0.5 \pm 0.9$ mM; maximum fractional inhibition $= 12 \pm 2\%$. Data from a single sample of blood in one experiment (typical of three).

Low affinity DTNB site on band 3

Reithmeier [8] has shown that there is a lower affinity DTNB site on band 3 which inhibits red cell phosphate transport with $K_I = 0.6$ mM, 5 mM DTNB producing 90% inhibition. Reithmeier concluded that the DTNB binding responsible for this effect was not covalent, in part because the inhibition could be completely reversed by washing. He also found that DTNB competitively inhibited binding * of the stilbene anion transport inhibitor 4-benzamido-4′-aminostilbene-2,2′-disulfonate (BADS). After washing, BADS binding returned to normal.

* Please see footnote *, next page (p. 166).

In view of this evidence we carried out a series of experiments to determine whether this reversible DTNB site was also responsible for the DTNB inhibition of water permeability in NEM-treated red cells. We first carried out three experiments which showed that DTNB inhibited anion transport in NEM-treated cells but, as Fig. 5 shows, the K_I is increased to 1.7 ± 0.3 mM and the maximum inhibition is reduced ** to $76 \pm 3\%$. This K_I is in reasonable agreement with the K_I of 2.5 ± 0.3 mM for the inhibition of water flux, as shown in Fig. 4. However, the water flux inhibition can not be reversed by simple washing with buffer; the significance of this observation is discussed in a subsequent section. The effect of covalent binding of DIDS in one experiment (typical of three) is shown in Fig. 4, in which DIDS reduced the maximum inhibition of water permeability from $29 \pm 1\%$ to $12 \pm 2\%$. Since DIDS binding modulates water transport, there must be a common element in the mechanisms that govern anion transport and water permeability. We will call this common element a channel, consistent with previous conclusions [6] but alternative topological arrangements are possible.

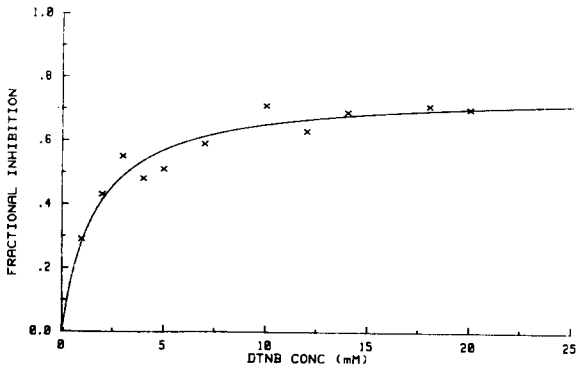

Fig. 5. Effect of DTNB on sulfate efflux. The curve has been drawn as above. $K_I = 1.7 \pm 0.3$ mM; maximum fractional inhibition = $76 \pm 3\%$.

Effect of DTNB on ethylene glycol permeability

Levitt and Mlekoday [4] had found that 0.5 mM DTNB inhibited ethylene glycol permeability by 80% in normal red cells. As Table I shows *, we

* Reithmeier [8] had used ^{14}C-labeled BADS to show that DTNB competitively inhibits the binding of stilbene anion transport inhibitors to red cells. In order to confirm this finding in NEM-treated cells we used ^{3}H-labeled H_2DIDS but found no significant effect of DTNB on ^{3}H-labeled H_2DIDS binding to NEM-treated cells. In a control experiment with untreated cells, we also were unable to find any significant effect of DTNB on binding of ^{3}H-labeled H_2DIDS. This may reflect differences between the binding of H_2DIDS and BADS, due to differences in the configuration of the two stilbenes or it may reflect some difference between our experimental protocol and that of Reithmeier.

** Reithmeier's studies [8] lead to the conclusion that anion exchange inhibition in normal red cells is caused by DTNB binding to a single class of sites, one for each band 3 monomer. One effect of NEM treatment is to reduce the efficiency of inhibition of anion exchange from about 90%, as observed by Reithmeier to 76%, which is at first sight inconsistent with one DTNB site per monomer. However, we have reported that NEM treatment makes red cells leaky to monovalent cations [36], so it is likely that the difference in anion exchange inhibition could be ascribed to a leak. As will be discussed below, DTNB inhibition of other transport processes is also not complete, ranging from 69% for ethylene glycol flux down to about 23% for water transport. Since each of these processes is characterized by the same K_I, they share a common mechanism. NEM treatment does not appear to produce a nonelectrolyte leak since we have found in seven paired experiments that NEM treatment reduces the ethylene glycol permeability coefficient from 4.2 ± 0.6 to 3.1 ± 0.8 fmol·dyn^{-1}·s^{-1}. The control experiments in Table I show that NEM treatment has no effect on water permeability. Thus, the maximal DTNB inhibition achieved appears to depend upon individual interactions between DTNB and the specific modulation mechanisms applicable to each nonelectrolyte.

* The control value of ω in Table I, 4.0 ± 0.9 fmol·dyn^{-1}·s^{-1} ($P_d = 1.0 \pm 0.2$ μm·s^{-1}) is about half of the value given earlier by Savitz and Solomon [37], whose corrected value is 8 ± 1 fmol·dyn^{-1}·s^{-1} based on the present value of V/A (see Materials and Methods). We attribute this difference (similar to the case for L_p) to the fact that bank blood was used in these experiments instead of fresh blood, as used by Savitz and Solomon. The present result is also smaller by a factor of about three than the P_d of 3.3 ± 0.3 μm·s^{-1} given by Osberghaus et al. [38] and almost five than the P_d of 4.8 ± 0.2 μm·s^{-1} given by Mayrand and Levitt [39]. We cannot understand the reason for these large differences; even though Osberghaus et al. used a different method for measuring P_d, the results they obtained by that method for water diffusion agree well with those of other investigators [23]. Though Mayrand and Levitt obtained their results by

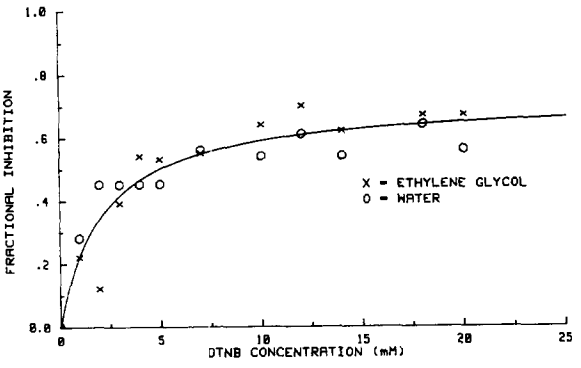

Fig. 6. Effect of DTNB on ethylene glycol flux (×) and hydraulic conductivity (○). The curve has been drawn as above. $K_I = 2.5 \pm 0.7$ mM; maximum fractional inhibition = 86 ± 6%. The hydraulic conductivity data has been scaled as described in the text.

found that 5 mM DTNB inhibited ethylene glycol permeability in NEM-treated cells by 43% in seven experiments ($p < 0.0005$). The results of one (of five) dose-response experiments, in which DTNB inhibition of both water and ethylene glycol transport was measured simultaneously in the same experiment using a single sample of blood, is shown in Fig. 6. In order to normalize the data, the ratio of the fractional inhibition of ethylene glycol flux to that of water was computed at each point. These ratios were then averaged and that average was used to multiply each water inhibition point to give the result shown as the open circles in Fig. 6. The value of $K_I = 2.5 \pm 0.7$ mM, determined by a non-linear least-squares fit to a single site binding model. When computed separately, the maximum inhibition of ethylene glycol flux was 69 ± 5%, while that for hydraulic conductivity was 49 ± 5%, higher than the other experiments which are typically around 30 ± 8% ($n = 13$). The results in Fig. 6, which are typical of the other experiments in this series, show clearly that there must be a common element governing the transport of water and ethylene glycol which we will call, as before, a channel.

It is important to discriminate between a channel, which provides a common route for the passage of Cl^-, nonelectrolytes and water, and a modulation mechanism which is specific to each of the transported species. A familiar example of a modulation mechanism is the modifier site in Cl^- transport. There are significant differences between the mechanisms that modulate ethylene glycol and water flux. The DTNB inhibition of ethylene glycol transport is readily reversible by washing, similar to Reithmeier's finding for anion transport, but different from the case for water. DIDS has no effect on the DTNB inhibition of ethylene glycol transport.

Reithmeier routinely allowed a 15 min incubation period at 30°C between addition of DTNB and measurement of the anion transport effect. Although we have not made a careful study of the incubation period in the ethylene glycol transport process, inhibition is not instantaneous but is well developed after 15 min at 37°C. In the case of water, 15 min incubation at 37°C produces no inhibition; the effects in Table I and Fig. 4 were produced by 60 min incubation at 37°C. This behavior is similar to that of the sulfhydryl reagent, pCMBS, which requires some 20 min to produce maximal inhibition of water transport [2]. Since pCMBS binding appears to be instantaneous [6], the delay may represent a conformational change in the transport protein. Thus, though the evidence that Cl^-, ethylene glycol and water use a common channel is persuasive, modulation is an idiosyncratic process which depends upon specific interactions between individual species of transported molecules and the transport protein.

Effect of DTNB on methyl urea permeability in NEM-treated cells

We have found that DTNB also inhibits methyl urea permeability in NEM-treated red cells. Our

the stopped-flow method, as we did, their measurements of the urea permeability coefficient by the same method are also larger by a factor of about three than ours [22] and those of Brahm [40]. Mayrand and Levitt determined the values of all three coefficients which describe solute flow through membranes (hydraulic conductivity, reflection and permeability coefficients) by simultaneously finding the values of all three coefficients which gave the best fit of the data to a single theoretical swell-shrink curve, a procedure which, in our hands, does not give reliable results. The absolute values of the permeability coefficients do not affect the results in the present paper since our conclusions depend only upon ratios with and without DTNB.

initial observations were made with urea but its permeability is so great that it was not possible to obtain reproducible dose-response curves. We have therefore used methyl urea as an analog for urea in three DTNB inhibition experiments since the transport properties of these two solutes are similar, although the permeability coefficient of methyl urea is about an order of magnitude smaller. Fig. 7 shows the results of one experiment, typical of three, in which the data were transformed the same way as for ethylene glycol. In the combined graph, $K_I = 0.6 \pm 0.2$ mM and the maximum fractional inhibition was $48 \pm 2\%$. Before transformation, the maximum fractional inhibition for water permeability was $19 \pm 1\%$. Similar results were obtained with dimethyl urea and the data for urea, though not accurate enough to obtain a K_I, also showed a DTNB effect. Furthermore, prior covalent reaction with DIDS completely suppressed the DTNB inhibition of methyl urea flux in three experiments and actually caused a small stimulation, as shown in Fig. 8. These experiments clearly show that a common element controls the permeability of methyl urea and water and indicate that urea and water use the same channel. As is the case for ethylene glycol, the modulation is quite specific to the urea family of solutes; this problem has been discussed in detail by Dorogi and Solomon [41]. As in the case for water, the DTNB inhibition of methyl urea permeability is not reversed by washing.

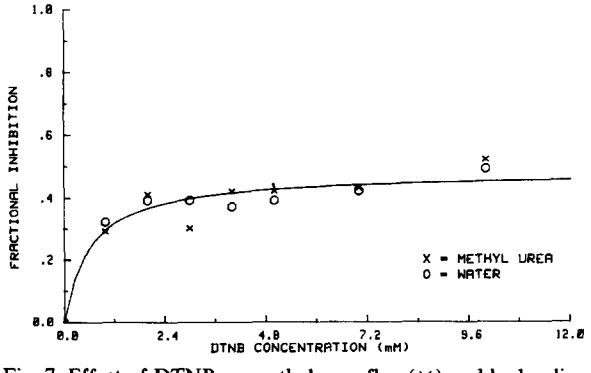

Fig. 7. Effect of DTNB on methyl urea flux (×) and hydraulic conductivity (○). The curve has been drawn as above. $K_I = 0.6 \pm 0.2$ mM; maximum fractional inhibition = $48 \pm 2\%$. The hydraulic conductivity data has been scaled as above.

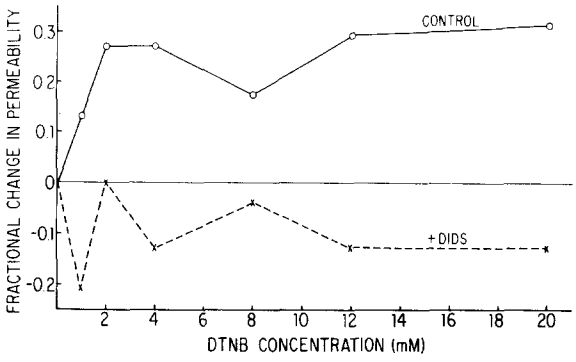

Fig. 8. Effect of DIDS on DTNB inhibition of methyl urea permeability. The positive scale shows DTNB inhibition, as in Fig. 7. DIDS reverses this effect and even produces a small stimulation, which is shown on the negative scale.

Locus of ethylene glycol permeability channel

The evidence given above indicates that ethylene glycol, urea and water share the same transport channel, which may be presumed to be band 3, since the specific anion inhibitor, DIDS, has been shown to interact with an inhibitor of water permeability. However, Macey and Farmer [7] and Levitt and Mlekoday [4] have suggested that both ethylene glycol and urea transport take place by a facilitated diffusion through individual channels, each different from the aqueous pore. It is possible to use the ethylene glycol permeability data to throw some light on this problem by computing the number of channels required to accomodate the observed ethylene glycol flux, which is very large. The value given in Table I for the ethylene glycol permeability coefficient, ω, is equivalent to $P_{d,eth\ gly}$ of 0.99 $\mu m \cdot s^{-1}$. This measurement was made at an initial concentration gradient of 0.4 M ethylene glycol so the flux per red cell (area = $1.35 \cdot 10^{-6}$ cm^2) is 47 fmol/s per cell. If this flux is mediated by an enzyme system, the number of copies required can be computed from the turnover number. Cantor and Schimmel [42] report a range of 10^2–10^6 s^{-1} for soluble enzymes. However, an enzyme responsible for solute transport must proceed by way of a conformational change and the time constants that Cantor and Schimmel give for unimolecular transitions range from 1 to 10^4 s^{-1}. In the case of the sarcoplasmic reticulum ATPase, Boyer et al. [43] have reported a turnover number of 2.9 s^{-1}. For our purposes we have used the

band 3 turnover number of $4 \cdot 10^4$ s^{-1} computed for chloride ions from Brahm's data on Cl^- exchange [44]. On this basis $7 \cdot 10^5$ copies of the putative enzyme would be required. There are two counterbalancing sources of error in this calculation. Since Mayrand and Levitt [39] report that K_m for ethylene glycol permeability is 175 mM, the ethylene glycol transport system can accomodate higher fluxes than those used in this calculation, which would require more enzyme. Also, although the turnover number of the enzyme is at the upper limit of reported configurational changes, we cannot be sure that this figure applies to the ethylene glycol system.

Alternatively, ethylene glycol might be transported through an aqueous channel. We can use the steric hindrance equations of Renkin [45] to compute the number of channels required, as was done by Solomon et al. [6] in their computation of the number of water channels required to accomodate the observed water flux in the red cell. This calculation * leads to a figure of $1.3 \cdot 10^5$ channels per cell, approximately half of the $2.7 \cdot 10^5$ water channels computed by the same method. Calculations of urea permeability give the same number of aqueous channels; for enzyme turnover, 10^7 proteins per cell would be required.

Since DTNB is an effective inhibitor of ethylene glycol, methyl urea and water permeability, DTNB must bind to a site on, or in association with, the locus of their transport channel. According to the calculations above 10^5–10^7 copies of the protein responsible are required per cell. The only three transmembrane proteins that satisfy this criterion are band 3, the band 4.5 region and glycophorin. The number of protein copies in the band 4.5 region is not yet determined but Haest [47] estimates that it comprises a family of six or seven different proteins whose number ranges from $0.7 \cdot 10^5$ to $2.3 \cdot 10^5$ copies per cell. Glycophorin, which is present in $2 \cdot 10^5$ to $3 \cdot 10^5$ copies per cell [47] has been sequenced and found to contain no sulfhydryl groups. Since pCMBS is a sulfhydryl reagent which inhibits the permeation of water, methyl urea and ethylene glycol and since pCMBS inhibition can be reversed by cysteine, it is clear that glycophorin, by itself, can not be the transport protein.

Our line of reasoning leads to the conclusion that the transport of anions, water, ethylene glycol and urea makes use of the same channel and that the primary channel protein is either band 3 or band 4.5. Since band 3 is the anion transport protein and since DIDS binds to band 3 with high specificity, the observation that DIDS also inhibits the DTNB effect on water transport, implicates band 3 as a major component of the water transport system. We conclude, therefore, that band 3 is the principal constituent of the aqueous channel either acting by itself, or possibly in a complex which could include band 4.5 and glycophorin.

* The computation is based on the following equation:

$$n = (P_d A \Delta x) / (D_{eg} \alpha \pi r^2)$$

in which n is the number of channels, P_d is the permeability coefficient of ethylene glycol, taken from Table I and converted to units of $cm \cdot s^{-1}$, A is the red cell area, taken as $1.35 \cdot 10^{-6}$ cm^2 [23] and Δx is the membrane thickness, taken as 4 nm. α is the steric hindrance computed from the equations of Renkin [45] using 0.309 nm ($3.09 \cdot 10^{-8}$ cm) as the pore radius parameter as described by Solomon et al. [6] and 0.281 nm ($2.81 \cdot 10^{-8}$ cm) as the ethylene glycol radius. D_{eg} is the diffusion coefficient for ethylene glycol in water of $1.153 \cdot 10^{-5}$ $cm^2 \cdot s^{-1}$ given by Longsworth [46]. α has a value of 0.012 as compared with a value of 0.064 for water, given previously by Solomon et al. [6].

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