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RELATION BETWEEN RED CELL ANION EXCHANGE AND WATER TRANSPORT

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A new distilbene compound, 4',4'-dichloromercuric-2,2,2',2'-bistilbene tetrasulfonic acid (DCMBT), has been synthesized for use in studies of anion and water transport in the human red cell. DCMBT combines features of both the specific stilbene anion transport inhibitor, DIDS, and the mercurial water transport inhibitor, pCMBS. This new compound inhibits anion transport almost completely with a K_i of 15 μM. DCMBT also inhibits water transport by about 15–20% with a K_i of about 8 μM. Treatment of red cells with DIDS inhibits the effect of DCMBT on water transport, suggesting that anion transport and water transport are mediated by the same protein.

The sulfhydryl reagent, pCMBS (*p*-chloromercuribenzene sulfonate) has been shown to inhibit water transport in the human red cell [1] and it has been suggested that this transport is mediated by band 3, the red cell anion transport protein [2]. Anion transport can be inhibited specifically by stilbene anion transport inhibitors such as DIDS (4,4'-diisothiocyano-2,2'-disulfonic stilbene). When added to the outside of sealed red cells, DIDS binds primarily to band 3; the fractional inhibition of anion transport is linearly related to the fraction of band 3 molecules to which DIDS is bound [3]. DIDS initially binds reversibly by Coulombic interaction, followed by irreversible interaction of the isothiocyanate, presumably to a lysine. We have provided further evidence that the pCMBS site for water transport inhibition is located [4] on band 3 and have confirmed the observation [5] that pCMBS binding does not inhibit anion transport; furthermore DIDS binding does not alter the pCMBS inhibition of water transport. In order to study the pCMBS/band 3 relationship further, we have synthesized a distilbene molecule which contains groupings analogous to pCMBS. This new compound binds to the red cell membrane and inhibits both anion transport and water transport. We have found that DIDS treatment reverses water transport inhibition by this new compound which suggests that both processes are mediated by the same protein.

The starting material for the synthesis of our new compound, DCMBT (4',4'-dichloromercuric-2,2,2',2'-bistilbene tetrasulfonic acid) was the diamino stilbene compound DADS (4,4'-diamino-2,2'-stilbene disulfonic acid) (Eastman Kodak Co., 98% pure) which was diazotized with nitrite in a fluoboric acid/water mixture according to the reaction shown in scheme I.

The legend to Fig. 1 gives the details of the synthesis. In order to determine the purity of compound I, we first determined the mercury content as 34.3% by iodometric titration [6] which agrees very well with the theoretical figure of 34.9%. To determine whether compound I has an azo group, we obtained ultraviolet spectra in the

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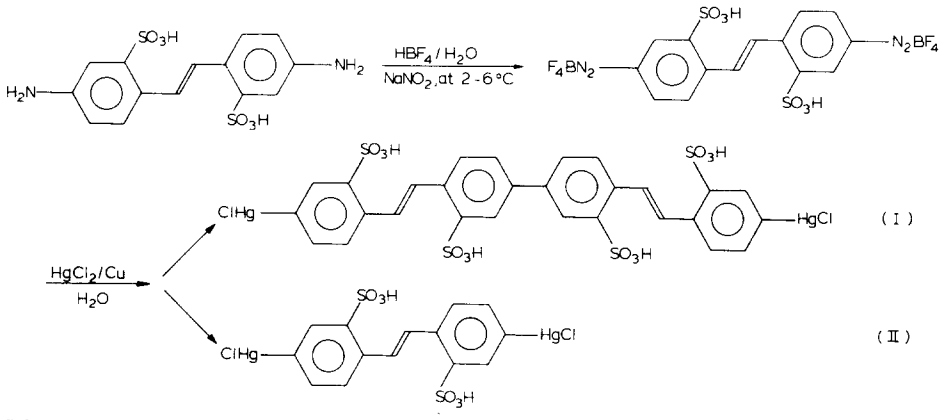

Schema 1.

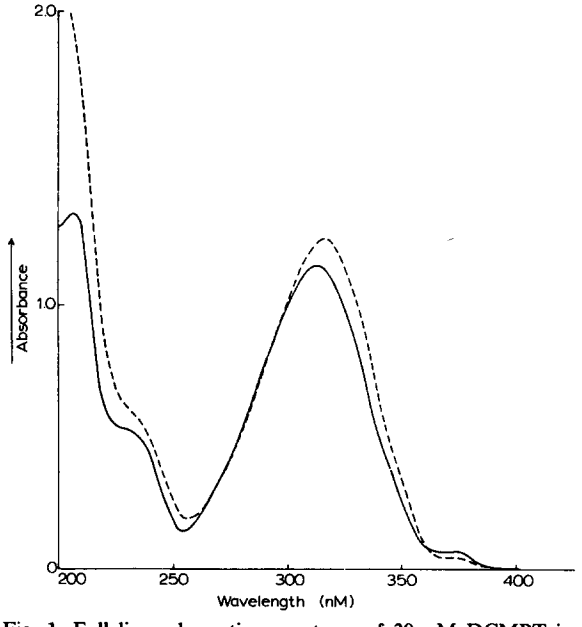

Fig. 1. Full line: absorption spectrum of 30 μM DCMBT in phosphate buffer (pH 7.4). Dashed line: absorption spectrum after 100 μmoles of cysteine were added to 1 ml of DCMBT solution. Methods. 0.11 mole of DADS was suspended in 500 ml of water and solubilized with 0.12 mole of Na_2CO_3 followed by addition of 0.25 mole of $NaNO_2$. This solution was added dropwise, with vigorous stirring, to 350 ml of 50% fluoboric acid in an ice bath. The deep yellow diazonium compound was collected on a Buchner funnel and washed three times with cold water, twice with alcohol and then with diethyl ether. The chloromercuration [14–16] was carried out as follows: a dry mixture of 0.17 mole of $HgCl_2$ and 0.086 mole of the diazonium compound was added slowly to a suspension of 0.42 mole of precipitated copper powder and 0.17 mole of $HgCl_2$ in 1.2 l of water. The mixture was stirred vigorously at room temperature until effervescence ceased. The solid was filtered out and extracted with 1.5 l of 5% NaOH solution at 50°C. The remaining solid was the starting material for compound II

presence of chromous ion, a reducing agent, and acid [7] and found no change in λ_{max} (312 nm, pH 7.4, phosphate buffer). This indication that there is no azo group is supported by the fact [8] that, when diazonium salts containing electron-withdrawing groups are treated with cuprous ion (or with copper and acid), the main product is the biaryl compound, whereas the presence of electron-donating groups leads to the azo compound. When protein sulfhydryl groups form mercaptides, there is a small shift of the absorption spectrum to longer wavelength and increased absorption [9–11].

described below. The filtered extract was precipitated with HCl and the precipitate was redissolved in alkaline solution and reprecipitated. The precipitate was dissolved in pure water and the water was partially evaporated. After centrifugation, the centrifugate was washed twice with ethanol and then with diethyl ether. All of the processes mentioned above were conducted under dark conditions. The yield of this deep blue crystalline powder, compound I, was 7.1 g. The solubility of the compound in water is a function of ionic strength and pH. Lower ionic strength and higher pH increase the solubility. In order to obtain compound II, the disubstituted compound, the solid remaining after extraction of compound I was extensively washed with hot dilute acid to remove metal hydroxides. The brownish solid suspension was decanted from copper metal and mercurous salt. The separated solid was repeatedly digested with acid and dried. The mercury was determined by iodometric titration [6] and found to be 47.8%. Since mercury does not add to the olefin of a trans-stilbene [17,18] we concluded that the insoluble product is compound II whose theoretical mercury content is 49.5%. When acetone was used as a solvent instead of water, and stannous chloride was used as a reducing agent instead of powdered copper, the reactions gave compound II and the mono-substituted compound, but no compound I was formed.

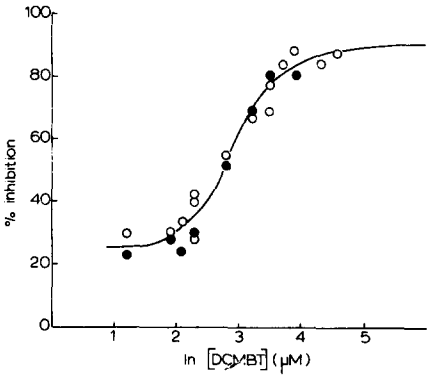

Fig. 2. Inhibition of sulfate exchange by DCMBT in *N*-ethylmaleimide-treated red cells. Data from duplicate experiments (○, ●). DCMBT concentration given in μM. Methods. Sulfate exchange was measured by the method of Jennings [19] which depends upon the volume change induced in red cells by SO_4^{2-}-Cl^- exchange at pH 6.3. Cells were washed three times with 0.15 M NaCl and then incubated at 50% hematocrit in isosmolar NaCl with 12 mM *N*-ethylmaleimide (Sigma Chemical Co., St. Louis, MO) for 30 min at 37°C as described by Rao [20]. Cells were then titrated to pH 6.3 with HCl. The SO_4^{2-}-Cl^- exchange was measured in 0.1 M Na_2SO_4, 0.5 mM Na_2HPO_4 (pH 6.3).

Similar absorption changes with compound I can be seen in Fig. 1 following cysteine addition to the DCMBT solution. At pH 7.4 the absorption maximum is shifted from 312 nm ($E_{max} = 4.3 \cdot 10^4$ $cm^{-1} \cdot mol^{-1}$) to 316 nm ($E_{max} = 5.1 \cdot 10^4$ $cm^{-1} \cdot mol^{-1}$). The purity of the compound was checked by thin-layer chromatography which showed a single spot on silica gel with water as solvent, and also a single spot on cellulose with ethanol/water (7 : 3, v/v) as solvent.

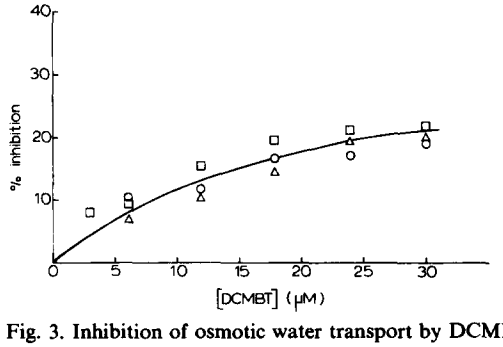

Fig. 3. Inhibition of osmotic water transport by DCMBT after 20 min incubation with *N*-ethylmaleimide-treated red cells in three experiments. Methods. Water transport was measured by the stopped-flow method, as described by Terwilliger and Solomon [21]. *N*-Ethylmaleimide treatment as in Fig. 2 but in the following buffer: NaCl, 125 mM; KCl, 4.4 mM; $NaHCO_3$, 24.9 mM; $MgCl_2$, 0.5 mM; Na_2HPO_4,5.9 mM; pH 7.4.

As Fig. 2 shows, DCMBT is a very effective inhibitor of anion exchange. The concentration for 50% inhibition is 15 μM which is about an order of magnitude higher than the 1.2 μM I_{50} of DIDS [12]. No incubation period is required to produce the inhibitory effect.

Solomon et al. [4] have shown that the SH group with which pCMBS interacts to inhibit red cell water transport does not react with *N*-ethylmaleimide and they have suggested that this SH group is the one located on the same 17 kDa membrane bound fragment of band 3 that Ramjeesingh et al. [13] have shown to contain the DIDS-binding site. When pCMBS is bound to *N*-ethylmaleimide-treated cells, the predominant peak in gel electrophoresis [4] is found on band 3. We have therefore studied the effect of DCMBT on osmotic water transport in *N*-ethylmaleimide-treated human red cells. It takes about 25–30 min for pCMBS to reach its maximum inhibition of 80% of osmotic water flux at [pCMBS] = 1–2 mM; the K_i in normal red cells (no *N*-ethylmaleimide) is 150 μM. As Fig. 3 shows, DCMBT also inhibits water transport in *N*-ethylmaleimide-treated cells with K_i about 8 μM; the induction period is similar to that for pCMBS. Since there is no delay in the onset of anion-exchange inhibition, binding of DCMBT to the stilbene site must be essentially instantaneous. Hence, the induction period for water transport inhibition appears to result from a slow reaction process that intervenes between the binding event and the inhibition event.

DCMBT is a bifunctional molecule. If the pCMBS-like moiety and the DIDS-like moiety could react independently of one another. there would be no coupling between them and the K_i values for the two processes would bear no relation to one another. If the two moieties were coupled rigidly and reacted with a single protein, there would be a single K_i. DCMBT is a relatively stiff linear molecule about 24 Å long; the two stilbenes can rotate relative to one another and there are several isomers. The maximum distance between one Hg and the furthest stilbene is about 12 Å. Our experiments show that the affinity for DCMBT binding to produce water inhibition is more than one order of magnitude tighter than

that for pCMBS. The K_i for the anion transport inhibition of DCMBT is about one order of magnitude looser than that for DIDS. Thus the two K_i values have been shifted in opposite directions and brought very close to one another, 8 μM for water inhibition and 15 μM for anion transport inhibition, consistent with tight coupling. It is significant that DCMBT binds so much more tightly than pCMBS, notwithstanding the fact that the pCMBS moiety has been joined to a large lipophobic sulfonated distilbene. Normally, we would expect that the additional steric hindrance introduced by this large chain would increase K_i substantially. Instead, K_i is reduced by a factor of twenty, which we ascribe to binding of the stilbene-moiety to the anion inhibitor site, thereby increasing the concentration of the pCMBS-like moiety locally in the vicinity of the water inhibition site. This observation is consistent with coupling between the two functions and provides strong support for the view that the two transport sites are located on the same protein.

The observation that the maximum inhibition of 22% produced by DCMBT after 20 min incubation is much less than the 80% inhibition produced by pCMBS could mean either that DCMBT is reacting with only 22% of the sites or that DCMBT is reacting with all the sites but that the stilbene moiety introduces a steric restraint that interferes with the full development of the conformational change responsible for the water transport inhibition. We have studied the concentration dependence and kinetics of pCMBS inhibition (Toon, M.R. and Solomon, A.K., personal communication) and have found that the inhibition is described by a single exponential time course and a single site binding curve over the range from 10 μM to 10 mM. Since there is no evidence for heterogeneity in the SH water inhibition site, it appears that the steric constraints on the pCMBS-like moiety of DCMBT make it significantly less effective in inhibiting water transport than pCMBS.

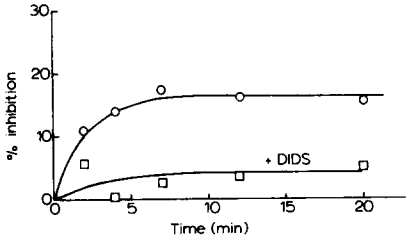

Fig. 4. Effect of DIDS on the time-course of water transport inhibition by DCMBT in one of three experiments. Cells were treated with *N*-ethylmaleimide as described in the legend to Fig. 3, 12 μM DCMBT: control (○); DIDS treated (□). Methods. Washed red cells were incubated at 37°C in the dark at 50% hematocrit in isosmolal buffer, pH 7.4 (see Fig. 3 legend) with 100 μM DIDS (US Biochemical Corp, Cleveland, OH) for 30 min. Cells were then washed three times with 10 volumes each of isosmolal buffer and then resuspended in buffer for *N*-ethylmaleimide treatment (see Fig. 3 legend).

Since the two inhibitory sites in DCMBT are closely coupled, we reasoned that blocking the stilbene site by irreversible reaction with DIDS would also inhibit the pCMBS-like action of DCMBT. Although the primary site for DIDS binding is band 3, the experiments of Lepke et al. [3] show that some 10% of the binding sites are on other unspecified membrane proteins (in addition to about 10% on glycophorin which is not involved in anion transport and has no SH group). As Fig. 4 shows, blocking the stilbene site also blocks the DCMBT (12 μM) inhibition of water transport. In one other experiment, 12 μM DCMBT inhibition was 100% blocked by DIDS; in another 24 μM DCMBT inhibition was 89% blocked. In experiments similar to that in Fig. 4 we found that 50 μM DCMBT could override the DIDS blockage, showing that the pCMBS moiety of DCMBT can reach the water inhibition site even when the stilbene site is occupied by DIDS. Thus, DIDS blocks the high affinity coupled reaction, while leaving other lower affinity pathways open. These DIDS experiments provide further evidence strongly supporting the view that water transport and anion transport are both mediated by the same protein, band 3.

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