J. Membrane Biol. 108, 227–233 (1989)

The Journal of
Membrane Biology

Inhibition of Hexose Transport in the Human Erythrocyte by 5, 5′-Dithiobis(2-Nitrobenzoic Acid): Role of an Exofacial Carrier Sulfhydryl Group

James M. May
Diabetes Research and Training Center, Department of Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-2230

Summary. The sulfhydryl reagent 5, 5′-dithiobis (2-nitrobenzoic acid) (DTNB) was used to study the functional role of an exofacial sulfhydryl group on the human erythrocyte hexose carrier. Above 1 mM DTNB rapidly inhibited erythrocyte 3-O-methylglucose influx, but only to about half of control rates. Efflux was also inhibited, but to a lesser extent. Uptake inhibition was completely reversed by incubation and washing with 10 mM cysteine, whereas it was only partially reduced by washing in buffer alone, suggesting both covalent and noncovalent interactions. The covalent thiol-reversible reaction of DTNB occurred on the exofacial carrier, since (*i*) penetration of DTNB into cells was minimal, (*ii*) blockade of potential uptake via the anion transporter did not affect DTNB-induced hexose transport inhibition, and (*iii*) DTNB protected from transport inhibition by the impermeant sulfhydryl reagent glutathione-maleimide-I. Maltose at 120 mM accelerated the covalent transport inhibition induced by DTNB, whereas 6.5 μM cytochalasin B had the opposite effect, indicating under the one-site carrier model that the reactive sulfhydryl is on the outward-facing carrier but not in the substrate-binding site. In contrast to glutathione-maleimide-I, however, DTNB did not restrict the ability of the carrier to reorient inwardly, since it did not affect equilibrium cytochalasin B binding. Thus, carrier conformation determines exposure of the exofacial carrier sulfydryl, but reaction of this group may not always "lock" the carrier in an outward-facing conformation.

Key Words sugar transport · one-site carrier model · impermeant sulfhydryl reagents · cytochalasin B · 5, 5′-dithiobis(2-nitrobenzoic acid) · human erythrocytes

Introduction

The presence of a reactive exofacial sulfhydryl group on the human erythrocyte glucose carrier was first suggested by Van Steveninck et al. [23], who found that *p*-chloromercuribenzenesulfonic acid (PCMBS), which penetrated the plasma membrane in intact cells very poorly, nonetheless inhibited glucose transport to the same extent as the more penetrant *p*-chloromercuribenzoic acid. Subsequent studies using impermeant maleimides [1, 2, 19] provided further evidence that a sulfhydryl was located on the exofacial carrier, since the irreversible inhibition of transport caused by these agents was partially prevented by D-glucose [2] and cytochalasin B [2, 19], and since the maleimides specifically labeled a protein in intact cells in the band 4.5 region on electrophoretic gels [2, 19]. More recently, Krupka [10] showed that irreversible transport inhibition by the poorly permeant sulfhydryl reagent sodium tetrathionate was accelerated by reagents known to bind to the form of the carrier with the substrate-binding site facing outward, such as maltose [11] and phloretin [9], and inhibited by cytochalasin B, which binds only to the inward-facing form of the carrier [5]. These data support the hypothesis that the exofacial sulfhydryl is exposed only with the carrier in the outward-facing conformation [10, 11].

In view of these findings, it is surprising that Smith and Ellman [21] found that 5, 5′-dithiobis(2-nitrobenzoic acid) (DTNB) at concentrations up to 10 mM had no effect on erythrocyte glucose efflux. DTNB is specific for sulfhydryls, forming mixed disulfides, which can be cleaved by an excess of added thiol [8]. Additionally, Smith and Ellman [21] showed that DTNB penetrated erythrocytes poorly, if at all, making it otherwise an excellent impermeant sulfhydryl reagent with which to study the exofacial carrier sulfhydryl.

Because of the reported lack of expected inhibition of hexose transport by DTNB, and because of its potential use as a probe in studies of the exofacial carrier sulfhydryl group in relation to carrier conformation, I have evaluated the effect of DTNB on erythrocyte hexose transport. The results show that low millimolar concentrations of DTNB do in fact inhibit 3-O-methylglucose transport in a thiol-reversible manner, and that this inhibition probably occurs via direct interaction with an exofacial carrier sulfhydryl group.

Materials and Methods

Materials

Glutathione-maleimide-I (GS—Mal) was synthesized by the method of Abbott and Schachter [1]. S-nitrosoglutathione was synthesized as described by Park [17] and used immediately. Radionuclides were obtained as follows: 3-O-[methyl-^{14}C]methylglucose (40 Ci/mol) from ICN and [4-^{3}H]cytochalasin B (15 Ci/mmol) from New England Nuclear. Solutions of phosphate-buffered saline (PBS, composed of 12.5 mM sodium phosphate and 150 mM NaCl) containing up to 64 mM DTNB were prepared immediately before each experiment by carefully adjusting the pH up to 7.4 with an NaOH solution. All concentrations of DTNB are expressed relative to extracellular space. At the most frequently used concentration of DTNB (4 mM) and erythrocyte hematocrit (20%), the amount of DTNB present relative to cells was 20 μmol/ml of packed erythrocytes.

Erythrocyte Preparation

Freshly drawn human blood was anticoagulated with heparin (16.7 U/ml blood). Before use, either fresh or stored erythrocytes were washed five times by centrifugation in equal volumes of PBS (pH 7.4). Between the first two washes the cells were incubated 5 min at 37°C to allow exit of intracellular glucose. After the washes the erythrocytes were brought to the appropriate hematocrit for subsequent studies. Unused but washed erythrocytes were stored no more than 48 hr at 5°C at a 40% hematocrit in the presence of 11 mM citrate, 22 mM D-glucose, 0.25 mM adenine, 12.8 mM phosphate, and 131.6 mM choride with sodium as the cation, pH 7.0.

Hexose Transport

Uptake of 3-O-methylglucose (5.2 μM, 40 Ci/mol) was measured in triplicate as previously described [12] in PBS using 50-μl aliquots of cells, an incubation temperature of 4–5°C, an uptake time of 30 sec, correction for trapped extracellular label by subtraction of a "zero time" value (cells plus stop solution followed by labeled sugar), and expression of the transport rate (fraction of equilibrium sugar space occupied following a 30-sec uptake) as a percent of control values. Under these conditions, 3-O-methylglucose space at 30 sec was usually 10–15% of equilibrium values.

Measurements of 3-O-methylglucose efflux were performed by first equilibrating 0.8 ml of 20% erythrocytes for 30 min at 37°C with 0.5 mM labeled 3-O-methylglucose (5 Ci/mmol). The cells were then pelleted in a microfuge, the supernatant was removed, the tube containing the cell pellet was cooled on ice, and duplicate 10-μl aliquots of packed cells were removed to a culture tube also on ice. The efflux assay was started by adding 1 ml of ice-cold buffer and terminated by vigorously adding 3 ml of ice-cold PBS "stop" solution containing 10 μM cytochalasin B. The cells were pelleted and 0.5 ml of supernatant counted. The time of efflux until addition of "stop" solution was generally 15 sec, adequate to keep the net efflux below 30% of equilibrium values (*not shown*). Correction was made in each assay for label which was extracellular at the beginning of the assay by subtracting the extracellular radioactivity measured when "stop" solution was added to cells before diluent. Transport rates were normalized to the equilibrium space of 3-O-methylglucose for that sample.

[^{3}H]Cytochalasin B Binding Studies

Following the indicated preincubation, cytochalasin B binding in intact cells was determined by the following procedure. Erythrocytes in PBS at a 10% hematocrit were incubated with 62.5 nCi of [^{3}H]cytochalasin B, 10 μM cytochalasin E, and one of several concentrations of unlabeled cytochalasin B (10, 62.5, 125, 250 and 3000 nM) in a final volume of 0.4 ml in a microfuge tube. After 15 min at room temperature, the suspension was swirled, and a 50-μl aliquot removed for determination of total radioactivity. This aliquot was placed in a 7-ml plastic scintillation vial, diluted with 5 ml of ACS (Amersham), centrifuged for 5 min at 3000 $\times$ g to pellet denatured protein, and the radioactivity counted in a Packard 2000CA liquid scintillation counter with DPM correction. Preliminary studies showed that the decrease in counting efficiency under these conditions (usually <10%) could be corrected for by the DPM program. Furthermore, no significant loss of radioactive cytochalasin B in the precipitate could be detected in recovery studies (*not shown*).

The remaining cell suspension was centrifuged for 30 sec in a Beckman Model E microfuge, and an 0.2-ml aliquot of the supernatant containing unbound label was removed for scintillation counting. The amount of bound cytochalasin B was calculated as the difference between the total and the unbound radioactivity in each sample. The data for different concentrations of cytochalasin were subjected to Scatchard analysis as modified by Rosenthal [20]. Correction was made for nonspecific binding measured in the presence of 3000 nM cytochalasin B.

Maltose inhibition of the binding of a low concentration of cytochalasin B (10 nM) was performed according to the above procedure and analyzed as described by Gorga and Lienhard [6]. This method is appropriate for binding data in which the free ligand concentration is much lower than its dissociation constant. The K_i value is derived from the equation $F/B = K_D/[T] + K_D[I]/K_I[T]$, in which F/B is the ratio of unbound to bound ligand, K_D the equilibrium dissociation constant for cytochalasin B, $[T]$ the total number of binding sites, $[I]$ the concentration of inhibitor, and K_I the inhibitory constant. In this equation, the intercept on the x-axis equals $-K_I$ when F/B equals zero [6].

Other assays: Total glutathione (GSH) content was measured by the method of Hissin and Hilf [7]. Sulfate efflux was measured as previously described [13].

Data Analysis

Data are shown as mean ± SE from the indicated number of experiments. Statistical comparisons were made using the Student's t test for paired values.

Results

Hexose Transport Inhibition by DTNB

Incubation of erythrocytes with 4 mM DTNB for various times at 37°C followed by three washes in PBS to remove unreacted DTNB resulted in a pro-

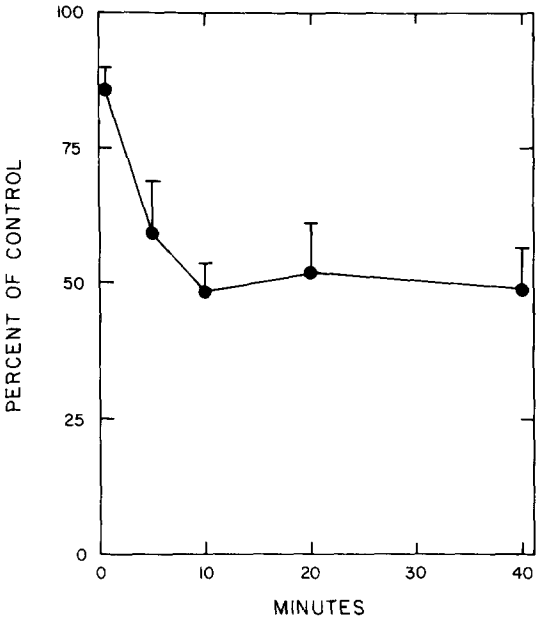

Fig. 1. Time course of irreversible transport inhibition by DTNB. Erythrocytes at a 20% hematocrit in 0.8 ml of PBS were incubated at 37°C with 4 mM DTNB for the indicated times, washed three times by centrifugation in 5 ml of PBS at the same temperature, readjusted to the original hematocrit, cooled on ice for 5 min, and their ability to take up 3-O-methylglucose assessed as described under Materials and Methods. The earliest wash was initiated just after the addition of DTNB. Data are from four experiments

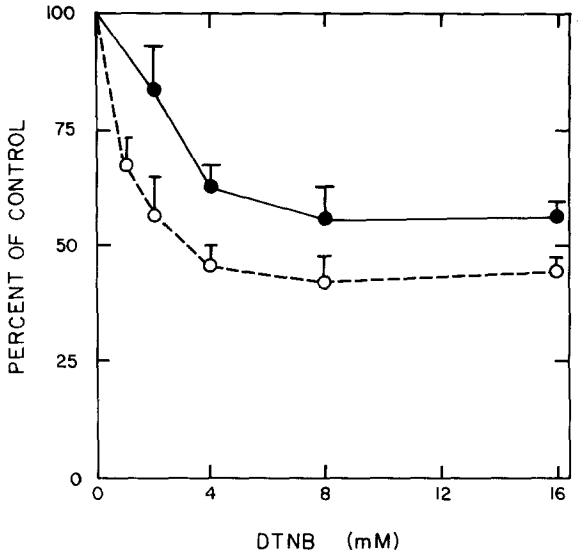

Fig. 2. Dose-response relationship of transport inhibition by DTNB. Irreversible transport inhibition (●) by the indicated DTNB concentration was measured following incubation of 20% erythrocytes in 0.8 ml of PBS for 30 min at 37°C, followed by the washing and transport procedures described in the legend to Fig. 1. Transport inhibition in the absence of washes (○) was measured by incubating 0.4 ml of 20% cells for 30 min at 37°C with DTNB at the concentration shown, cooling on ice for 5 min, and directly assaying hexose transport. The data are from eight wash experiments and four experiments performed without the wash step

gressive decrease in 3-O-methylglucose transport relative to control, which was evident even in cells washed immediately upon addition of DTNB, and which appeared to be complete by 10 min (Fig. 1). There was no effect of DTNB on 30-min 3-O-methylglucose equilibrium space, assessed in each sample as part of the transport assay (*not shown*). Treatment of cells at 37°C with increasing concentrations of DTNB produced irreversible transport inhibition to about one-half of control rates, with a half-maximal effect observed at about 2–3 mM DTNB (Fig. 2). Incubations performed without the wash step resulted in an inhibition curve, which was shifted to the left (half-maximal effect = 1–2 mM) and slightly downward compared to that seen in washed samples (Fig. 2). This suggests that DTNB has an inhibitory effect beyond that related to a covalent interaction.

In order to assess the effects of DTNB on hexose efflux, 20% erythrocytes were incubated with 5 mM DTNB for 30 min at 37°C, followed by three washes in six volumes of PBS, then by the transport assay as described under Materials and Methods. In five such experiments, DTNB irreversibly inhibited efflux by 22 ± 1.4% from control values ($P < 0.01$).

Penetration of DTNB Through the Cell Membrane

The ability of increasing concentrations of DTNB to penetrate the plasma membrane and react with intracellular GSH was measured and is shown in Fig. 3. Whereas 0.32 mM N-ethylmaleimide decreased GSH by 44%, even 16 mM DTNB decreased GSH content by only 12% (nonsignificant). When cells were lysed in the presence of 16 mM DTNB, GSH content was decreased to less than 10% of that in control samples, indicating that DTNB does not affect the measurement of GSH in the assay (*not shown*). These results suggest that under these conditions DTNB concentrations below 8 mM exert their effects on the exofacial cell surface.

The possibility that even small amounts of DTNB might enter the erythrocyte via the anion transporter and inhibit hexose transport by reacting with the endofacial carrier was explored and the results shown in Table 1. First, DTNB did not appear to covalently modify the anion transporter, since it did not irreversibly inhibit sulfate efflux under conditions in which 12.5 μM 4, 4′-diisothiocyanostilbene-2, 2′-disulfonate (DIDS) produced an

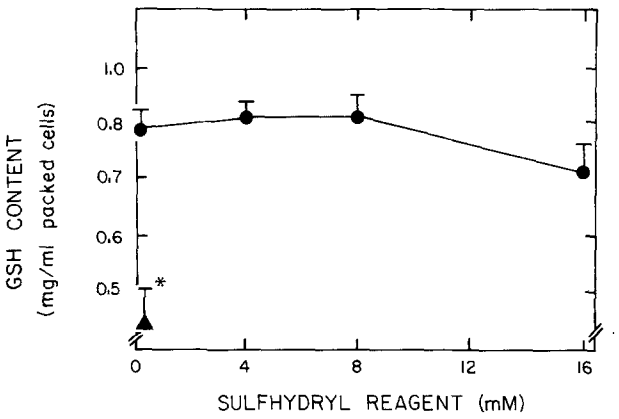

Fig. 3. Effects of DTNB on erythrocyte GSH content. Erythrocytes at a 20% hematocrit were incubated in 0.2 ml of PBS with the indicated concentration of sulfhydryl reagent for 30 min at 37°C, washed three times in 5 ml of PBS at the same temperature, readjusted to the original hematocrit, and their GSH content determined in duplicate. Results shown are from four to six experiments for DTNB treatment (●) and from six experiments for 0.32 mM N-ethylmaleimide treatment (▲)

Table 1. Effects of DTNB and DIDS on hexose transport and sulfate efflux[a]

Inhibitor	Sulfate efflux	Hexose transport
	Percent of untreated control	
DTNB (4 mM)	98 ± 4	59 ± 5[b]
DIDS (12.5 μM)	20 ± 2[b]	76 ± 4[b]
DIDS + DTNB	ND	37 ± 3[b]
	$N = 2$	$N = 5$

[a] Erythrocytes (0.8 ml) at a 20% hematocrit were incubated for 30 min at 37°C with the indicated concentrations of DTNB or 4, 4'-diisothiocyanostilbene-2, 2'-disulfonate (DIDS), washed three times with 5 ml of PBS, and the appropriate transport assay performed as described under Materials and Methods. In the experiments depicted in the third row, cells were incubated for 30 min at 37°C with 12.5 μM DIDS, followed by 4 mM DTNB for an additional 30 min, and then by washing and the hexose transport assay. ND indicates not determined.

[b] $P < 0.05$ *vs.* untreated control for the indicated number (N) of experiments.

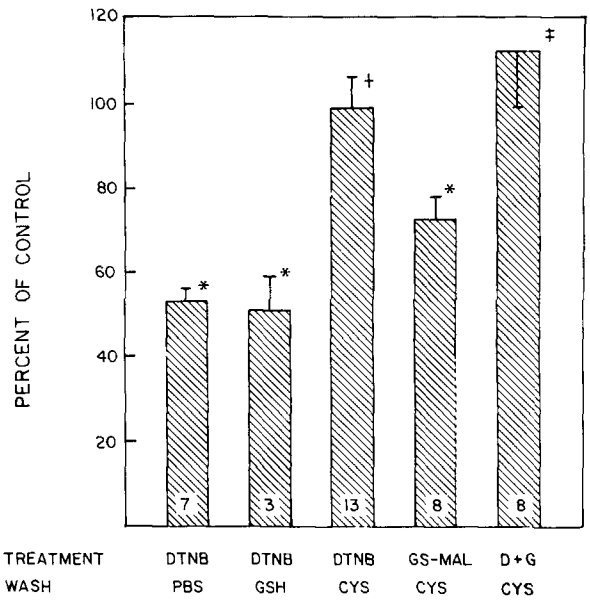

Fig. 4. Sulfhydryl specificity of transport inhibition by DTNB. In experiments shown by the three bars on the left, 20% erythrocytes in 0.8 ml of PBS were incubated for 30 min at 37°C with 8 mM DTNB (noted under *treatment*), followed by addition of 5 ml of PBS containing no thiol, 100 mM GSH, or 10 mM cysteine (noted under *wash*), and the incubation continued for an additional 20 min at 37°C. The cell suspensions were centrifuged and washed three additional times with 5 ml of thiol-free PBS prior to assay of hexose influx. In the experiments represented by the two bars on the right, the same cell concentrations and volumes were incubated with ("*D* + *G*") or without 4 mM DTNB for 30 min at 37°C, then incubated with 12.5 mM GS-Mal for an additional 30 min at 37°C. All samples were diluted with 5 ml of PBS containing 10 mM cysteine, incubated for 10 min at 37°C, centrifuged, and the dilution and incubation repeated once more. A further wash in 5 ml of PBS alone was performed prior to adjustment of the cell suspension to the original hematocrit and assay of transport. Data are expressed relative to transport rates in samples which were not treated with sulfhydryl reagent or thiols but carried through all the wash and incubation steps. The number of experiments is shown in the base of each bar. An asterisk (*) indicates $P < 0.01$ *vs.* untreated control; a † indicates $P < 0.05$ *vs.* treatment with DTNB alone; and an ‡ indicates $P < 0.01$ *vs.* treatment with both GS-Mal and cysteine

80% inhibition (Table 1). Therefore 12.5 μM DIDS was used to minimize potential uptake of DTNB via the anion transporter during treatment with DTNB in the usual manner. However, the resulting inhibition of hexose uptake was similar in extent to the added effects of each agent alone (Table 1). The lack of protection from the inhibitory effect of DTNB on hexose transport during blockade of anion transport with DIDS is consistent with the hypothesis that the DTNB effect is not due to entry into cells and subsequent reaction with the endofacial carrier.

Specificity of Hexose Transport Inhibition by DTNB

The sulfhydryl specificity of DTNB was confirmed with the observation that the inhibitory effect of 8 mM DTNB on hexose transport was completely reversed by incubating and washing cells in buffer containing 10 mM cysteine (Fig. 4). On the other hand, even 100 mM GSH did not reverse the inhibitory effect of DTNB on transport under the same conditions (Fig. 4). These findings indicate that the sulfhydryl reactive with DTNB is accessible to cys-

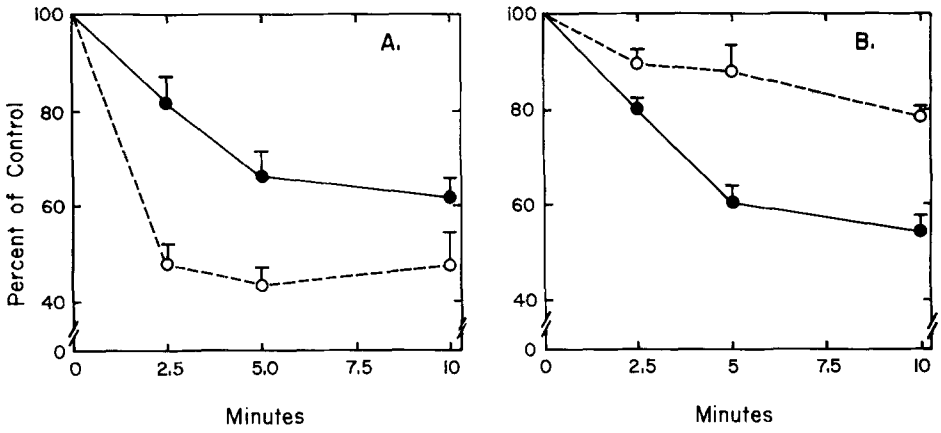

Fig. 5. Modification of DTNB-induced transport inhibition by competitive transport inhibitors. (*A*) Erythrocytes (0.8 ml) at a 20% hematocrit were incubated with 120 mM sucrose (●) or 120 mM maltose (○) for 10 min at 37°C, followed by addition of DTNB to a final concentration of 4 mM. The samples were incubated at the same temperature for the indicated times before dilution with 5 ml of PBS, three washes by centrifugation, restoration to the original volume, and assay of hexose uptake. Procedures in *B* were the same, except that cells were incubated with 6.5 μM cytochalasin E (●) or 6.5 μM cytochalasin B (○) for 10 min before addition of DTNB, and that the washes were performed with 1% (wt/vol) bovine serum albumin in PBS (to aid in removal of the cytochalasins). The data are from four experiments for each panel

teine but not to the tripeptide GSH. Similar treatment of cells with the sulfhydryl-specific S-nitrosoglutathione at concentrations up to 25 mM had no effects on rates of transport after washing (*not shown*). However, the more hydrophobic GS-Mal (which is also membrane impermeant) did cause irreversible transport inhibition, as shown in Fig. 4. Pretreatment of cells with DTNB followed by GS-Mal and lastly by incubation and washing in the 10 mM cysteine buffer to remove DTNB prevented the irreversible transport inhibition induced by GS-Mal compared to GS-Mal alone (Fig. 4). This supports the notion that the reagents react with the same exofacial sulfhydryl group on the carrier.

Carrier Conformation and DTNB Reactivity

Preincubation of cells with 120 mM maltose, which is known to orient the carrier to a conformation with the substrate-binding site facing outward [11], accelerated the rate of transport inhibition by DTNB (Fig. 5*A*). On the other hand, cytochalasin B, which orients the carrier with substrate-binding site facing inward [5], protected transport from inhibition by DTNB at early time points (Fig. 5*B*). These data favor the interpretation that DTNB reacts with the exofacial carrier in its outward-facing form, but at a site that does not overlap with the substrate-binding site.

As shown in Table 2, covalent reaction of DTNB in intact cells had no effect on total cytochalasin B binding (B_o), the affinity of cytochalasin B for the inward-facing carrier in those cells (K_D), or on the ability of extracellular maltose to inhibit tracer cytochalasin B binding (K_i). However, when cells were not washed free of DTNB prior to the binding assay, a small but significant rise in the K_i for maltose inhibition of cytochalasin B binding was observed (Table 2). Thus covalent reaction of DTNB does not affect the ability of the carrier to reorient to an inward-facing form capable of binding cytochalasin B, but a noncovalent interaction does slightly lower the affinity of the outward-facing carrier for substrate.

Table 2. Effects of DTNB on equilibrium binding of cytochalasin B to intact cells[a]

Treatment	K_D	B_o	K_i
		Cells not washed	
Control	124 ± 5.3	206 ± 15	27 ± 3.1
DTNB	124 ± 13	199 ± 6.1	39 ± 2.1[b]
		Cells washed	
Control	95 ± 1.5	173 ± 10	37 ± 3.6
DTNB	113 ± 16	213 ± 18	43 ± 4.9

[a] Erythrocytes at a 10% hematocrit were incubated with or without 4 mM DTNB and either not washed (four experiments) or washed three times in 11 volumes of PBS (three experiments) prior to determination of equilibrium cytochalasin B binding as described under Materials and Methods. The parameter units are K_d = nM; K_i = mM; B_o = pmol/3.2 × 10^8 cells.
[b] $P < 0.01$ *vs.* untreated controls.

Discussion

DTNB inhibited 3-O-methylglucose transport into intact human erythocytes in a time- (Fig. 1) and dose-dependent (Fig. 2) fashion. There appeared to be two components to this effect. The major component was not reversed by washing in fresh buffer and thus probably reflected a covalent reaction. The other component, which was probably noncovalent, was evident as an additional inhibitory effect when cells incubated with DTNB were not washed prior to the transport assay. This effect could be related to the DTNB-induced decrease in the apparent affinity of the exofacial carrier for substrate binding observed under similar conditions (Table 2).

In constrast to the previous study of Smith and Ellman [21], DTNB was also found to inhibit hexose efflux, albeit to a lesser extent than influx. The presence of an inhibitory effect of DTNB may in part be attributed to the use of different transport assays, but more likely to the fact that Smith and Ellman loaded cells with 167 mM D-glucose during the period of incubation with DTNB, whereas in the present work cells were treated with DTNB prior to equilibration with the transported sugar. It has been shown that high concentrations of D-glucose present on both sides of the carrier prevent irreversible transport inhibition by impermeant maleimides [2, 14]. The effects of glucose present during the period of treatment with DTNB could have prevented any transport inhibition in the study of Smith and Ellman [21]. In adipocytes, no inhibition of basal hexose uptake was detected at 4–5 mM DTNB in the absence of washes [3, 4]. However, in those experiments the bovine serum albumin required for cell incubation could have bound to or reacted with DTNB and decreased its effective concentration. It has also been shown that DTNB inhibits both anion exchange [18] and water transport [22] in a reversible and probably noncovalent manner in human erythrocytes. The covalent thiol-reversible effect of DTNB described herein, therefore, appears to be selective for the erythrocyte hexose carrier.

It appears that the reactive sulfhydryl responsible for the covalent thiol-reversible transport inhibition by DTNB is on the exofacial portion of the carrier, since no evidence of membrane penetration was detected in a previous study [21] or in the present work (Fig. 3, Table 1) at DTNB concentrations effective in transport inhibition. The exofacial sulfhydryl reactive with DTNB may well be the one shown to be involved in transport inhibition by other impermeant [1, 2, 19] or poorly permeant [10, 23] sulfhydryl reagents. In support of this conclusion are the observations that pretreatment with DTNB prevents irreversible inhibition of transport by the impermeant sulfhydryl reagent GS-Mal (Fig. 4), and that reactivity of the exofacial sulfhydryl relative to carrier conformation appeared to follow a pattern similar to that seen with other sulfhydryl reagents. The acceleration of the rate of transport inactivation by a high concentration of the impermeant transport inhibitor maltose, coupled with the protection from transport inhibition afforded by cytochalasin B (Fig. 5), reflects previous results obtained with tetrathionate [10] and GS-Mal [14], suggesting that DTNB reacts with the same sulfhydryl. As proposed by Krupka and Devés [11], such data also strongly support the one-site or alternating conformation model of transport [6, 24], in which substrate or inhibitor can bind only to one face of the carrier at a time.

The reaction with DTNB provides some indication regarding accessibility of the exofacial carrier sulfhydryl. Whereas cysteine reversed the transport inhibition caused by DTNB, there was no reversal by the larger and more negatively charged GSH (Fig. 4). It was also found that transport was unaffected by S-nitrosoglutathione, which in addition to having high selectivity for sulfhydryl-disulfide interchange [17], carries the same charge and is only slightly larger than GSH. These results contrast with the observation that GS-Mal, which also contains GSH, does react with the sulfhydryl in question (Fig. 4). The most likely explanation for this discrepancy is that the sulfhydryl, although accessible from the outside of the cell, nonetheless may be buried in a hydrophobic region of the carrier, which can be reached by the *bis*- maleimidomethyl ether bridge of GS-Mal or by DTNB. The lack of complete transport inhibition by DTNB (Fig. 2) as well as other agents known to react with the exofacial carrier sulfhydryl [14, 15] may also relate to incomplete accessibility of the sulfhydryl, as previously discussed [14]. Significantly, hydropathy analysis derived from the primary amino acid sequence of the carrier [16] indicates that only one of the six available cysteines, cys^{429}, is potentially exposed on the outside of the cell, and this is only one amino acid away from entering the membrane.

A major difference between the reaction of DTNB and other impermeant sulfhydryl reagents with what may be the same site is that covalent reaction with DTNB does not affect the ability of the carrier to reorient inwardly and bind cytochalasin B, nor does it affect the competition between extracellular maltose and cytochalasin B for the carrier (Table 2). Both GS-Mal [14] and sugar-maleimides [15] "lock" the carrier in an outward-facing conformation able to bind sugar but unable to bind cytochalasin B. The cause of this difference is

unknown, but may relate to size and charge differences between the reagents. Additionally, even though both GS-Mal [2, 14] and DTNB appear to inhibit transport by reaction with an exofacial carrier sulfhydryl, GS-Mal affects hexose efflux more strongly than influx [2], whereas DTNB has the opposite effect. Such differences make DTNB a useful probe for study of the role of the exofacial sulfhydryl in the carrier mechanism.

In conclusion, DTNB reacts with an exofacial carrier sulfhydryl and inhibits hexose transport in erythrocytes in a fashion similar to that observed with impermeant maleimides, but with the important differences that the effect is reversed by thiol treatment and that carrier orientation is not affected. This suggests that the transport inhibition caused by reaction of this sulfhydryl group relates at least in part to impedance of sugar translocation rather than solely to restriction of carrier conformational changes.

The technical assistance of A. Denise Due and Judy Y. Harris, as well as preparation of the manuscript by Jo Ann Allsbrooks is appreciated. This work was supported by Research Career Development Award DK-1781 and research Grant No. DK-38794 from the National Institutes of Health.

References

1. Abbott, R.E., Schachter, D. 1976. Impermeant maleimides. Oriented probes of erythrocyte membrane proteins. *J. Biol. Chem.* **251:**7176–7183
2. Batt, E.R., Abbott, R.E., Schachter, D. 1976. Impermeant maleimides. Identification of an exofacial component of the human erythrocyte hexose transport mechanism. *J. Biol. Chem.* **251:**7184–7194
3. Clark, S., Harrison, L.C. 1983. Disulfide exchange between insulin and its receptor. A possible post-binding step in insulin action. *J. Biol. Chem.* **258:**11434–11437
4. Czech, M.P. 1976. Differential effects of sulfhydryl reagents on activation and deactivation of the fat cell hexose transport system. *J. Biol. Chem.* **251:**1164–1170
5. Devés, R., Krupka, R.M. 1978. Cytochalasin B and the kinetics of inhibition of biological transport. A case of asymmetric binding to the glucose carrier. *Biochim. Biophys. Acta* **510:**339–348
6. Gorga, F.R., Lienhard, G.E. 1981. Equilibria and kinetics of ligand binding to the human erythrocyte glucose transporter. Evidence for an alternating conformation model for transport. *Biochemistry* **20:**5108–5113
7. Hissen, P.J. Hilf, R. 1976. A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Anal. Biochem.* **74:**214–226
8. Jocelyn, P.C. 1987. Chemical reduction of disulfides. *Methods Enzymol.* **143:**246–256
9. Krupka, R.M. 1985. Asymmetrical binding of phloretin to the glucose transport system of human erythrocytes. *J. Membrane Biol.* **83:**71–80
10. Krupka, R.M. 1985. Reaction of the glucose carrier of erythrocytes with sodium tetrathionate: Evidence for inward-facing and outward-facing carrier conformations. *J. Membrane Biol.* **84:**35–43
11. Krupka, R.M. Devés, R. 1986. Looking for probes of gated channels: Studies of the inhibition of glucose and choline transport in erythrocytes. *Biochem. Cell Biol.* **64:**1099–1107
12. May, J.M. 1986. Photoafffinity labeling of glyceraldehyde-3-phosphate dehydrogenase by an aryl azide derivative of glucosamine in human erythrocytes. *J. Biol. Chem.* **261:**2542–2547
13. May, J.M. 1987. Labeling of human erythrocyte band 3 with maltosylisothiocyanate. Interaction with the anion transporter. *J. Biol. Chem.* **262:**3140–3145
14. May, J.M. 1988. Reaction of an exofacial sulfhydryl group on the erythrocyte hexose carrier with an impermeant maleimide: Relevance to the mechanism of hexose transport. *J. Biol. Chem.* **263:**13635–13640
15. May, J.M. 1988. Selective labeling of the erythrocyte hexose carrier with a maleimide derivative of glucosamine: Relationship of an exofacial sulfhydryl to carrier conformation and structure *Biochemisty* **28:**1718–1725
16. Mueckler, M., Caruso, C., Baldwin, S.A., Panico, M., Blench, I., Morris, H.R., Allard, W.J., Lienhard, G.E., Lodish, H.F. 1985. Sequence and structure of a human glucose transporter. *Science* **229:**941–945
17. Park, J.-W. 1988. Reaction of s-nitrosoglutathione with sulfhydryl groups in protein. *Biochem. Biophys. Res. Commun.* **152:**916–920
18. Reithmeier, R.A.F. 1983. Inhibition of anion transport in human red blood cells by 5,5′-dithiobis(2-nitrobenzoic acid). *Biochim. Biophys. Acta* **732:**122–125
19. Roberts, S.J., Tanner, J.A., Denton, R.M. 1982. Properties of N-maleoylmethionine sulphone, a novel impermeant maleimide, and its use in the selective labelling of the erythrocyte glucose-transport system. *Biochem. J.* **205:**139–145
20. Rosenthal, H.E. 1967. A graphic method for the determination and presentation of binding parameters in complex systems. *Anal. Biochem.* **20:**525–532
21. Smith, R.P.P., Ellman, G.L. 1973. A study of the dependence of the human erythrocyte glucose transport system on membrane sulfhydryl groups. *J. Membrane Biol.* **12:**177–188
22. Toon, M.R., Dorogi, P.L., Lukacovic, M.F., Solomon, A.K. 1985. Binding of DTNB to band 3 in the human red cell membrane. *Biochim. Biophys. Acta* **818:**158–178
23. Van Steveninck, J., Weed, R.I., Rothstein, A. 1965. Localization of erythrocyte membrane sulfhydryl groups essential for glucose transport. *J. Gen. Physiol.* **48:**617–632
24. Vidaver, G.A. 1966. Inhibition of parallel flux and augmentation of counter flux shown by transport models not involving a mobile carrier. *J. Theor. Biol.* **10:**301–306

Received 6 July 1988; revised 23 November 1988