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Differential labeling of the erythrocyte hexose carrier by *N*-ethylmaleimide: correlation of transport inhibition with reactive carrier sulfhydryl groups

James M. May

Diabetes Research and Training Center, Department of Medicine, Vanderbilt University School of Medicine, Nashville, TN (U.S.A.)

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Inhibition of hexose transport by *N*-ethylmaleimide was studied with regard to alkylation of different types of sulfhydryl group on the hexose carrier of the human erythrocyte. Uptake of 3-*O*-methylglucose was progressively and irreversibly inhibited by *N*-ethylmaleimide, with a half-maximal effect at 10–13 mM. A sulfhydryl group known to exist on the exofacial carrier was not involved in transport inhibition by *N*-ethylmaleimide, since reversible protection of this group by the impermeant sulfhydryl reagent 5,5′-dithiobis(2-nitrobenzoic acid) had no effect on the ability of *N*-ethylmaleimide to inhibit transport, or on its ability to decrease the affinity of the exofacial carrier for maltose. Nevertheless, the exofacial sulfhydryl was quite reactive with *N*-ethylmaleimide, since it was possible using a differential labeling technique to specifically label this group in protein-depleted ghosts with a half-maximal effect at 0.3 mM *N*-[^{3}H]ethylmaleimide, and to localize it to the M_r 19000 tryptic carrier fragment. Transport inhibition by *N*-ethylmaleimide correlated best with labeling of a single cytochalasin B-sensitive internal sulfhydryl group on the glycosylated M_r 23000–40000 tryptic fragment of the carrier, which was half-maximally labeled at about 4 mM reagent. Whereas *N*-ethylmaleimide readily alkylates the exofacial carrier sulfhydryl, it inhibits transport by reacting with at least one internal carrier sulfhydryl located on the glycosylated tryptic carrier fragment.

Introduction

Sulfhydryl groups are potentially important sites for protein modification, since they are often involved in the function of the protein, and since they react in a highly specific manner with a variety of sulfhydryl agents [1,2]. The human erythrocyte glucose carrier has six sulfhydryl groups [3], five of which are potentially reactive in the solubilized protein [4,5]. One group has been identified as an exofacial or Type I sulfhydryl [6], because it is accessible to impermeant sulfhydryl reagents in intact cells [6–11], and because its alkylation results in stoichiometric carrier labeling and inhibition of transport [12,13]. It is exposed only when the carrier is oriented with the substrate binding site facing outward [10–13], according to the one-site or alternating-conformation model of transport. In this model sugar may bind to either the inward- or the outward-facing carrier substrate binding site, but not to both simultaneously [14,15].

Less is known about the remaining internal carrier sulfhydryls. Abbott et al. [6] have classified as Type II those groups reactive with the impermeant glutathione-maleimide-I (GS-Mal) when it has access to both sides of the membrane, and as Type III those reactive only with highly permeant reagents such as *N*-ethylmaleimide (MalNEt) or 2,2′-dipyridyl disulfide. However, since the exofacial sulfhydryl is potentially also alkylated by agents having access to internal carrier sulfhydryls, it is difficult to define independent roles for these different types of sulfhydryl in the carrier mechanism.

A related issue is the location of reactive sulfhydryls within the known primary amino acid sequence of the carrier. Of the six carrier cysteines [3], three are in each of the two major membrane-bound tryptic fragments

Abbreviations: GS-Mal, glutathione-maleimide-I; MalNEt, *N*-ethylmaleimide; PCMBS, *p*-chloromercuribenzenesulfonate; Cys-Mal, *S*-(bismaleimidomethyl ether)-L-cysteine; PBS, phosphate-buffered saline; 5P8, 5 mM phosphate buffer, pH 8.0; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); K_D, equilibrium dissociation constant; B_0, total specific binding; K_i, inhibitory constant.

Correspondence: J.M. May, Vanderbilt University School of Medicine, Diabetes Research/Training Center, A-5105 Medical Center North, Nashville, TN 37232, U.S.A.

[3,16,17]. In a purified reconstituted carrier preparation, Deziel et al. [5] have estimated that two sulfhydryl groups in the broad glycosylated M_r 23000–40000 tryptic carrier fragment [16,17] are labeled by high concentrations of MalNEt, whereas only one group in the M_r 19000 tryptic fragment is labeled. The latter probably corresponds to the exofacial sulfhydryl, since its labeling by MalNEt was blocked by pretreatment of intact cells with the poorly permeant and sulfhydryl-specific *p*-chloromercuribenzenesulfonic acid (PCMBS) [5], and since a single group in the M_r 19000 tryptic fragment was selectively labeled by an impermeant maleimide derivative of glucosamine [13]. Thus, the reactive internal sulfhydryls appear to be confined to the M_r 23000–40000 tryptic fragment of the carrier.

The present studies were performed to correlate the inhibitory effects of MalNEt on transport with labeling of sulfhydryls in the two major membrane-bound tryptic fragments of the carrier. It was found that the exofacial sulfhydryl, although it can be differentially labeled with high selectivity at low concentrations of MalNEt in protein-depleted ghosts, nonetheless is not involved in the inhibition of transport by MalNEt. Rather, the transport inhibition by MalNEt correlates best with labeling of one or more internal sulfhydryls in the M_r 23000–40000 tryptic carrier fragment.

Materials and Methods

Materials

Radionuclides were obtained as follows: 3-*O*-[*methyl*-^{14}C]methylglucose (40 Ci/mol) from ICN, and *N*-[2-^{3}H]ethylmaleimide (45 Ci/mmol) and [4-^{3}H]cytochalasin B (15 Ci/mmol) were from New England Nuclear. GS-Mal was synthesized as described by Abbott and Schachter [7]. *S*-(Bismaleimidomethyl ether)-L-cysteine (Cys-Mal) was prepared in the same fashion as GS-Mal (May, J.M., unpublished observations).

Erythrocyte preparation and assays

Erythrocytes were prepared and uptake of 3-*O*-methylglucose (5.2 μM, 40 Ci/mol) was measured in 12.5 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl (PBS) as previously described [12]. The binding of [^{3}H]cytochalasin B to intact erythrocytes and the calculation of binding parameters was measured as previously described [11]. Briefly, erythrocytes at a 10% hematocrit were incubated with 63 nCi of [^{3}H]cytochalasin B, 10 μM cytochalasin E and various concentrations of unlabeled cytochalasin B (10, 63, 125, 250 and 3000 nM) for 15 min at 37°C until termination of the assay by centrifugation. For calculation of the equilibrium dissociation constant (K_D) and total specific binding (B_0), the binding data were subjected to Scatchard analysis according to Rosenthal [18]. Inhibitory constants (K_i) for transport inhibitors were measured at 10 nM [^{3}H]cytochalasin B using five concentrations of each inhibitor in the range of the K_i value calculated according to Gorga and Lienhard [15].

Erythrocyte lysis and membrane protein electrophoresis

Hypotonic cell lysis and preparation of leaky white ghosts was performed according to the method of Fairbanks et al. [19]. Protein-depleted ghosts were prepared as described by Gorga and Lienhard [15] and resuspended in 5 mM phosphate buffer, pH 8.0 (5P8). The membranes were stored at −20°C until sodium dodecyl sulfate–polyacrylamide gel electrophoresis, which was performed as previously described [20] with the following modifications: a mixture of C_{12}, C_{14} and C_{16} alkyl sulfates was used in the buffer ('lauryl' sulfate, Pierce Chemical Co.), samples were not boiled prior to electrophoresis, and 1.5-mm-thick slab gels were used. In all experiments 50 μg of membrane protein were applied to each gel lane, and upon completion of electrophoresis 1.9-mm gel slices were cut across three gel lanes, solubilized overnight in 0.4 ml of Soluene-350 and counted in 5 ml of Hionic Fluor (Packard Instruments, Inc). Samples were counted in a Packard 2000CA liquid scintillation spectrometer until at least 1000 disintegrations had accumulated in the gel slices of interest. Pre-stained molecular weight markers (Bethesda Research Laboratories, Bethesda, MD, USA) were run in lanes adjacent to the samples.

Calculations of labeled sites per cell were based on the measured specific activity of *N*-[^{3}H]ethylmaleimide, a value of 1.39 million cells/μg of leaky ghost membrane protein [21], and a doubling of carrier specific activity in the protein-depleted ghost membrane applied to gels (not shown). The number of sites per cell in membranes subjected to tryptic digestion was calculated following correction for protein loss based on the volume and concentration of recovered membrane protein, assuming no loss of carriers.

Differential labeling studies

The double differential labeling protocol was usually performed as follows. In the first carrier orientation step, protein-depleted ghosts (3.5–4 mg/ml) were incubated for 5 min at 37°C with an agent (usually 100 μM cytochalasin B), unlabeled MalNEt was added to a concentration of 4 mM, the membranes were incubated for another 30 min at the same temperature, and washed three times by centrifugation at 18000 × *g* in 80 volumes of ice-cold 5P8. Ghosts were resuspended to the original volume in 5P8 and the second orientation step of the protocol was performed, usually by adding 100 μM phloretin in 5P8 containing 1% ethanol for 5 min at 37°C. The indicated concentration of radioactive MalNEt was added and the ghosts were incubated for another 30 min at the same temperature. Labeling was terminated by two washes in 50 volumes of ice-cold 5P8

and the ghosts were stored at −20°C until electrophoresis.

Tryptic digestion of protein-depleted ghosts

Labeled protein-depleted ghost membranes (3.5–4 mg/ml) were divided into equal fractions, one aliquot incubated with 5P8 alone, and the other with trypsin (117 μg/ml, 1200 units/ml) for 30 min at 37°C. Soybean trypsin inhibitor was added to both samples to a concentration of 131 μg/ml (2300 units/ml). After another 5 min the ghosts were washed twice by centrifugation in 40 ml of ice-cold 5P8 and frozen until electrophoresis.

Data analysis

Except as noted, data are shown as means ± standard error from the indicated number of experiments. Statistical comparisons were made with Student's '*t*' test for paired values.

Results

Transport inhibition by MalNEt

Incubation of erythrocytes with increasing concentrations of MalNEt for 30 min at 37°C resulted in progressive and irreversible inhibition of 3-*O*-methylglucose uptake, with a half-maximal effect at about 10–13 mM (Fig. 1). In a time-course study not shown, the extent of irreversible transport inhibition by 10 mM MalNEt plateaued at about 50% inhibition at 30 min, the usual time for termination of the incubation.

The ability of several reversible transport inhibitors to modify the extent of transport inhibition by a submaximal exposure to MalNEt (10 mM for 20 min) was evaluated, with the results shown in Table IA. Both cytochalasin B and maltose afforded significant protection against irreversible transport inhibition by MalNEt, whereas phloretin did not. It was necessary to use relatively low concentrations of phloretin and cytochalasin B in order to ensure their complete removal before the transport assay. This and the irreversible nature of transport inactivation by MalNEt probably accounts for the less than complete protective effects observed with cytochalasin B.

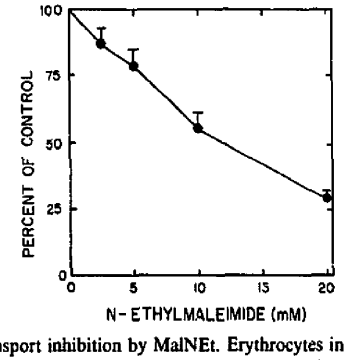

Fig. 1. Transport inhibition by MalNEt. Erythrocytes in a volume of 0.8 ml at a 20% hematocrit were incubated for 30 min at 37°C with the indicated concentration of MalNEt before three washes by centrifugation in 5 ml of PBS at 37°C. Following the last wash, the initial volume was restored and the transport assay performed in triplicate. Data from four experiments are expressed as percentages of an untreated control carried through the same steps.

TABLE I

Modification of MalNEt-induced transport inhibition by competitive transport inhibitors

A. In the experiments using reversible transport inhibitors, erythrocytes (0.8 ml) at a 20% hematocrit were incubated for 10 min with the indicated inhibitor (First treatment) followed by addition of 10 mM MalNEt for 20 min (Second treatment). The cells were washed three times with 5 ml of PBS containing 20 mg/ml bovine serum albumin (to aid in the removal of the cytochalasins and phloretin) and twice more in PBS alone before readjustment to the original volume and assay of hexose transport. B. Erythrocytes as in A were incubated 30 min in the presence or absence of DTNB at an extracellular concentration of 4 mM, followed where indicated by addition of 10 mM MalNEt for 30 min. Dithiothreitol was added to a final concentration of 10 mM for another 10 min, the cells were pelleted, and the supernatant was removed. The thiol incubation and wash was repeated, followed by a final wash in PBS alone prior to adjustment to the original volume and the hexose transport assay. All incubations and washes in A and B were performed at 37°C. Data are from '*N*' experiments expressed as percentages of an untreated control sample from the same experiment.

First treatment	Second treatment	Transport (% of control)	*P*	*n*
A. Buffer washes				
Sucrose (100 mM)	MalNEt	46±5	< 0.01	6
Maltose (100 mM)	MalNEt	69±5		
Cytochalsin E (6 μM)	MalNEt	55±4	< 0.01	6
Cytochalasin B (6 μM)	MalNEt	82±6		
None	MalNEt	54±4	n.s.	4
Phloretin (6 μM)	MalNEt	59±3		
B. Thiol washes				
DTNB	None	90±9		
None	MalNEt	54±9	< 0.01 [a]	
DTNB	MalNEt	49±7	< 0.01 [a]	
DTNB	MalNEt	49±7	< 0.01 [a]	

[a] Comparison between this treatment and DTNB alone.

In order to determine whether the exofacial carrier sulfhydryl is necessary for the inhibitory effects of MalNEt on transport, this group was protected by 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) before and during incubation with MalNEt. In this protocol cells were treated with 4 mM DTNB to block the exofacial sulfhydryl [22], allowed to react with 10 mM MalNEt, and the protective disulfide-coupled 2-nitro-5-thiobenzoate and excess unreacted MalNEt were removed by incubation and washes in a buffer containing 10 mM dithiothreitol before assay of transport. This procedure has been shown to protect the exofacial sulfhydryl group against alkylation by impermeant maleimides [22],

TABLE II

Effects of MalNEt on cytochalasin B binding in intact cells

Erythrocytes (0.8 ml) at a 10% hematocrit were incubated for 30 min at 37 °C in the presence or absence of 10 mM MalNEt, washed three times by centrifugation in 9 volumes of PBS,and resuspended to the original volume for assay of cytochalasin B binding as described under Methods. Units are as follows: equilibrium dissociation constant for cytochalasin B (K_D) = nM, total specific cytochalasin B binding (B_0) = nmol/ml packed cells, inhibitory constant (K_i) = mM for sugars and μM for phloretin. The data shown are from '*n*' experiments for each assay.

Assay	Control	MalNEt-treated	*P*	*n*
Binding analysis				4
K_D	107 ±6	132 ±5	< 0.01	
B_0	4.7±0.2	4.0±0.2	< 0.05	
D-Glucose	13 ±1	7 ±1	< 0.01	3
3-*O*-Methylglucose	23 ±3	12 ±1	< 0.01	7
N-Propyl-β-gluco-pyranoside	27 ±3	11 ±2	< 0.01	4
Maltose	36 ±2	154 ±8	< 0.01	3
Phloretin	5 ±1	28 ±4	< 0.01	4

and should leave it free on carriers having the internal sulfhydryls alkylated by MalNEt. In a previous study [22] 4 mM DTNB inhibited transport by 50–60%, an effect not reversed by subsequent washes in PBS. On the other hand, treatment with 4 mM DTNB followed by the thiol washes did not significantly affect transport (Table IB), presumably because the inhibitory 2-nitro-5-thiobenzoate had been removed by dithiothreitol. In cells treated sequentially with DTNB, MalNEt and thiol incubation and washes, transport was inhibited to the same extent as with MalNEt treatment alone (Table IB). This lack of protection by DTNB indicates that alkylation of the exofacial carrier sulfhydryl by MalNEt is not necessary for its inhibition of transport.

Effects of MalNEt on cytochalasin B binding in cells

Modification of the transport mechanism by MalNEt was assessed by studying its effects on the binding of cytochalasin B and transport inhibitors to the carrier. Treatment of cells with 10 mM MalNEt, which inhibits transport by about 50% (Fig. 1), caused small but significant decreases in both the affinity of cytochalasin B for the carrier, and the total number of specific binding sites (Table II). The affinity of the carrier for transport inhibitors and substrates was estimated by their ability to displace cytochalasin B from its binding site on the inward-facing carrier [15,23]. Pretreatment with 10 mM MalNEt decreased the affinity of the carrier for both maltose and phloretin by 4–5-fold (Table II). Since these agents bind to different sites on the outward-facing exofacial carirer [10,24], the fall in their ability to inhibit cytochalasin B binding reflects decreased availability or affinity of that form of the carrier.

When the exofacial sulfhydryl was protected by initial reaction with 4 mM DTNB and later freed by thiol treatment, according to the protocol used in the studies of Table IB, the ability of MalNEt to increase the K_i value for maltose was unchanged (maltose $K_i = 37 \pm 2$ mM; MalNEt-treated, maltose $K_i = 104 \pm 10$ mM; DTNB- and MalNEt-treated maltose $K_i = 135 \pm 23$, $n = 4$). Thus alkylation of the exofacial sulfhydryl by MalNEt is not required for its inhibition of maltose binding to the carrier.

On the other hand, the affinities of D-glucose, 3-*O*-methylglucose and *N*-propyl-β-D-glucopyranoside were increased 2–3-fold by pretreatment with MalNEt (Table II). Incubations with the *N*-propyl derivative were extended to 2 h to ensure equilibration by diffusion of this non-transported sugar across the membrane [25]. *N*-Propyl-β-D-glucopyranoside outside the cells did not inhibit cytochalasin B binding (not shown), and so was not removed during the binding studies. The conformational change induced by reaction of MalNEt thus increases the affinity of the carrier for sugars having access to the inward-facing substrate binding site, an effect not restricted to transported sugars alone.

These results suggest that the inhibition of transport induced by MalNEt is affected by reversible transport inhibitors, that it occurs independently of the exofacial sulfhydryl, and that it induces a conformational change in the carrier favoring binding of internal substrate.

Carrier labeling with N-[^{3}H]ethylmaleimide

In order to correlate transport effects with reaction of specific sulfhydryl groups on the carrier, labeling studies with radioactive MalNEt were performed. Protein-depleted erythrocyte ghosts were used to minimize unwanted labeling of peripheral membrane proteins. Even so, at a low concentration of *N*-[^{3}H]ethylmaleimide labeling of the M_r 43000–68000 band 4.5 carrier region was not prominent (Fig. 2), accounting for only 52 ± 13% (S.D.) of band 3 labeling in three experiments. Moreover, there was only a 5–10% inhibition of band 4.5 labeling by pretreatment with 100 μM cytochalasin B in these experiments. As shown in Fig. 2B, only a small fraction of the original radioactivity remained in labeled membranes following exhaustive tryptic digestion. This labeling was present in sharp peaks of M_r 21000, 19000 and 14000. Significantly, labeling of the M_r 19000 peak was inhibited 52 ± 2% (S.D., $n = 2$) by pretreatment with cytochalasin B, suggesting that it was derived from the glucose carrier.

Differential carrier labeling studies

In order to improve the specificity of carrier labeling with MalNEt, high concentrations of cytochalasin B and phloretin were used in a differential labeling protocol. As shown in Fig. 3, pretreatment of cells with cytochalasin E before addition of unlabeled MalNEt,

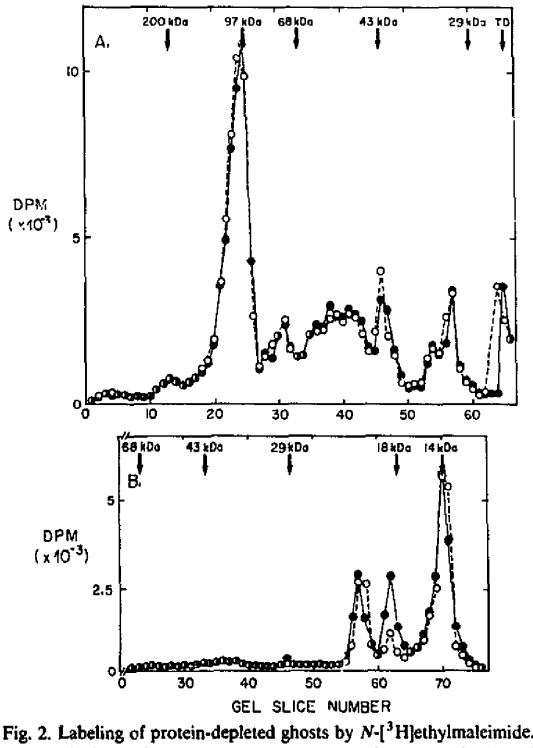

Fig. 2. Labeling of protein-depleted ghosts by *N*-[^{3}H]ethylmaleimide. Protein-depleted ghosts (4 mg/ml) in a 1-ml volume were incubated for 5 min at 37°C with 100 μM cytochalasin E (●) or cytochalasin B (○), followed by addition of *N*-[^{3}H]ethylmaleimide (49 Ci/mol) to a final concentration of 0.5 mM. The incubation was continued for an additional 30 min, followed by two washes by centrifugation in 40 ml of PBS at 5°C at 18000×*g*. Equal aliquots of labeled ghosts were treated without or with trypsin followed by electrophoresis as described under Methods. Panel A: membranes not exposed to trypsin (8% acrylamide gel). Panel B: membranes treated with trypsin (12% acrylamide gel).

followed by washes to remove reagents, and then by treatment with radioactive MalNEt resulted in a marked decrease in total labeling on the gel (compare Figs. 2A and 3), but little improvement in selectivity for band 4.5 compared to band 3. On the other hand, preincubation with cytochalasin B increased the labeling of band 4.5 to an average of twice that of band 3 in three experiments. Pretreatment with 100 μM phloretin alone had little effect on band 4.5 labeling in this protocol (not shown). However, in ghosts subjected to the differential labeling procedure using cytochalasin B in the first step and a final treatment with 100 μM phloretin just before labeling with radioactive MalNEt, band 4.5 labeling was increased compared to treatment with cytochalasin B alone (Fig. 3). This enhancement of the specificity of band 4.5 labeling contrasts with the lack of protective effect of phloretin on transport inhibition by MalNEt (Table IA). In six experiments band 3 contained only 5–20% of the radioactivity in band 4.5 at radioactive MalNEt concentrations ranging from 0.2 to 0.5 mM, with the lower concentration corresponding to the higher selectivity for band 4.5.

The results of several types of labeling study suggest that the exofacial sulfhydryl is the only group labeled in the band 4.5 carrier region at low radioactive MalNEt concentrations using the double differential labeling procedure. First, when labeling was performed according to this protocol (Fig. 4A), tryptic digestion of the labeled membranes resulted in a single M_r 19000 peak on electrophoretic gels (Fig. 4B). As noted in the Introduction, this tryptic fragment is known to contain the exofacial sulfhydryl. Reversal of the order of treatment with cytochalasin B and phloretin caused almost complete suppression of the labeling in both the band 4.5 peak (Fig. 4A) and in the M_r 19000 tryptic fragment (Fig. 4B), again indicating the specificity of the procedure.

Second, the experiment shown in Fig. 5 indicates that labeling under the double differential protocol involves one or more sulfhydryls located relatively superficially in the membrane, although not necessarily restricted to one side or the other. Ghosts were first carried through the double differential protocol, but just before labeling with radioactive MalNEt one aliquot was treated with 4 mM DTNP and the other with buffer alone. It is evident that labeling of all peaks in both intact (Fig. 5) and tryptic-digested membranes (Fig. 5B) was suppressed by DTNB, including that of band 4.5 and the M_r 19000 tryptic fragment.

Third, specific blockade of the exofacial sulfhydryl also resulted in nearly complete suppression of labeling of band 4.5 and its M_r 19000 tryptic fragment (Fig. 6). Intact cells were first treated with 5 mM unlabeled

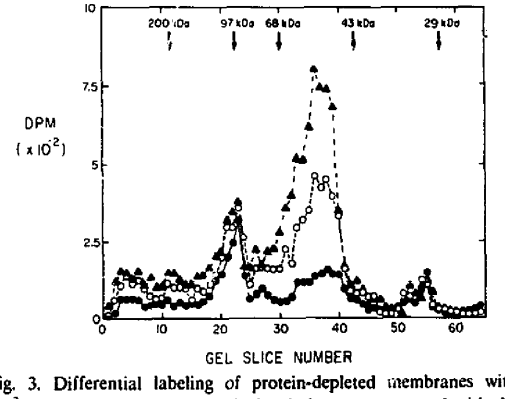

Fig. 3. Differential labeling of protein-depleted membranes with *N*-[^{3}H]ethylmaleimide. Protein-depleted ghosts were treated with the differential labeling protocol as described in Methods using either 50 μM cytochalasin E (●) or cytochalasin B (○,▲) in the first orientation step, and 1% (v/v) ethanol in 5P8 (●,○) or 100 μM phloretin (▲) in the second step. A concentration of 0.45 mM *N*-[^{3}H]ethylmaleimide (30 Ci/mol) was used in the labeling step. Electrophoresis was performed on an 8% acrylamide gel.

Cys-Mal, which under these conditions reacts with the exofacial sulfhydryl and inhibits transport, but does not significantly affect the ability of the carrier to reorient inwardly (May, J.M., unpublished observations). Protein-depleted ghosts were prepared from Cys-Mal-treated cells and carried through the double differential labeling protocol. The electrophoretic labeling pattern showed substantial suppression of labeling by *N*-[^{3}H]-ethylmaleimide in the band 4.5 region (Fig. 6A) as well as in the M_r 19000 tryptic fragment (Fig. 6B), compared to the usual labeling pattern in ghosts prepared from cells not treated with Cys-Mal.

Fourth, total radioactivity in the M_r 19000 tryptic fragment corrected for loss of protein during the tryptic digestion was 96 ± 17% (S.D.) of that in band 4.5 in eight experiments. This suggests that all of the labeling of band 4.5 was converted to the M_r 19000 tryptic fragment. It should be noted that the validity of this stoichiometry assumes that there is no loss of carriers during tryptic digestion and membrane recovery, and that the correction for changes in carrier specific activity due to removal of protein during trypsin treatment is accurate.

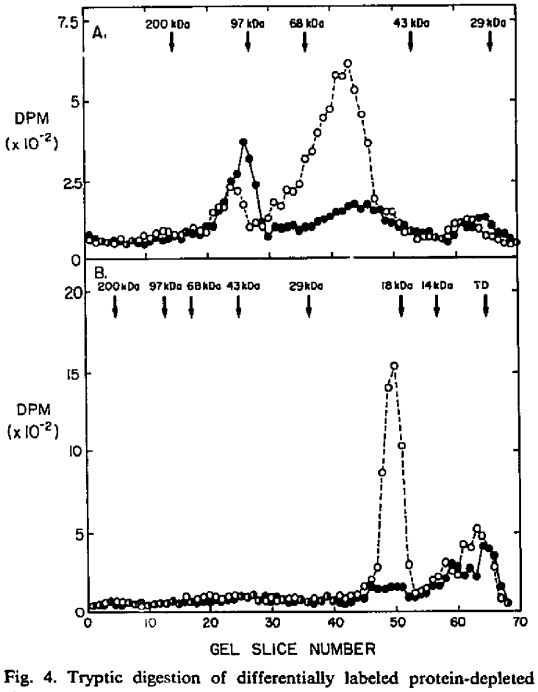

Fig. 4. Tryptic digestion of differentially labeled protein-depleted ghosts. Protein-depleted ghosts were subjected to the double differential labeling protocol as described under Methods using either 100 μM phloretin (●) or 100 μM cytochalasin B (○) in the first orientation step and either 100 μM cytochalasin B (●) or 100 μM phloretin (○) in the second orientation step. Labeling was performed at 0.24 mM *N*-[^{3}H]ethylmaleimide (19 Ci/mol) followed by the tryptic digestion protocol as described under Methods. Panel A: ghosts not exposed to trypsin were subjected to gel electrophoresis on an 8% acrylamide gel. Panel B: trypsin-treated ghosts were subjected to electrophoresis on a 12% acrylamide gel.

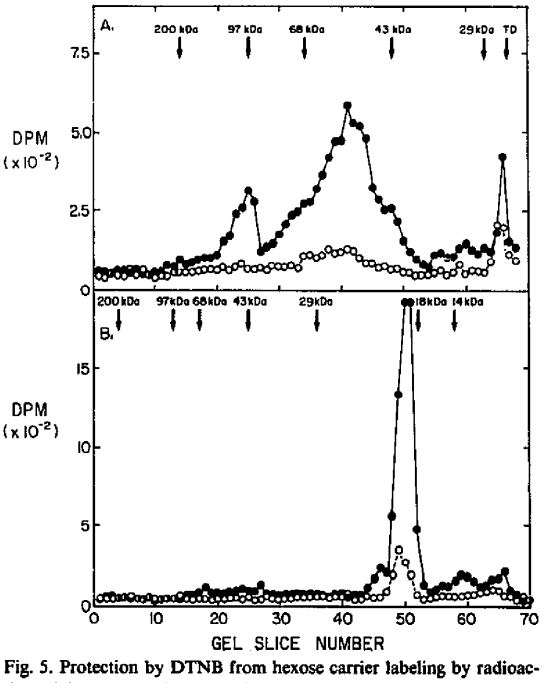

Fig. 5. Protection by DTNB from hexose carrier labeling by radioactive MalNEt. The double differential labeling procedure was performed with protein-depleted ghosts using 100 μM cytochalasin B in the first orientation step and 100 μM phloretin in the second step as described under Methods. After the 5 min incubation with phloretin, the ghosts were split into two equal fractions, one of which was incubated with 4 mM DTNB (○), and the other with buffer alone (●) for an additional 30 min at 37 °C, followed by labeling with 0.2 mM *N*-[^{3}H]ethylmaleimide (30 Ci/mol) for 30 min at the same temperature. The ghosts were washed by centrifugation in 33 volumes of ice-cold 5P8 and resuspended to the original concentration for the tryptic digestion procedure as described under Methods. Panel A: electrophoresis on an 8% acrylamide gel of ghosts not exposed to trypsin. Panel B: electrophoresis on a 12% acrylamide gel of ghosts exposed to trypsin.

The possibility that the M_r 23000–40000 glycosylated carrier fragment was labeled, but that the label was removed during exhaustive tryptic digestion was investigated by studying the effects of much lower concentrations of trypsin on the labeling pattern of MalNEt under double differential conditions. In Fig. 7 it is evident that the M_r, 23000–40000 fragment was not labeled even when radioactivity in the M_r 19000 fragment was decreased to 25% of that present in band 4.5 at the lowest concentration of trypsin used. Moreover, band 4.5 was progressively and stoichiometrically converted to the M_r 19000 fragment by increasing trypsin concentrations (Fig. 7, inset).

Finally, increasing concentrations of labeled MalNEt in the same protocol resulted in saturable labeling of

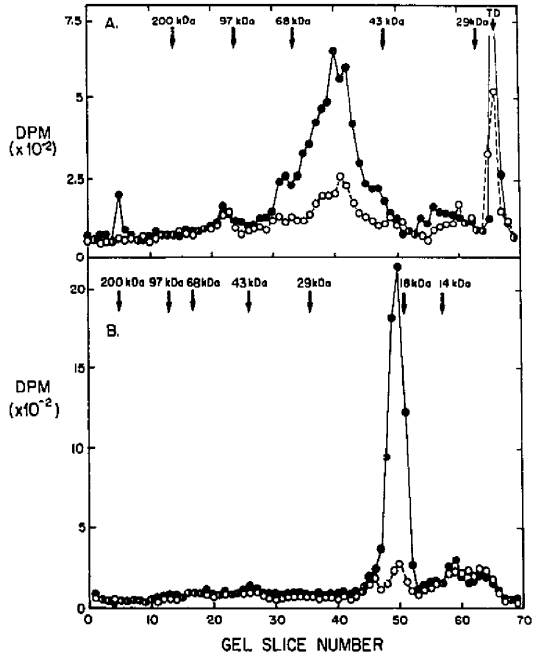

Fig. 6. Differential labeling with *N*-[^{3}H]ethylmaleimide of protein-depleted membranes prepared from cells treated with Cys-Mal. Erythrocytes at a 50% hematocrit in PBS were incubated for 30 min at 37°C in the absence (●) or presence (○) of 5 mM Cys-Mal, washed twice by centrifugation in 10 volumes of PBS at the same temperature, and subjected to hypotonic lysis and protein depletion. The protein-depleted ghosts were differentially labeled using 100 μM cytochalasin B in the first orientation step, 100 μM phloretin in the second step, and 0.2 mM *N*-[^{3}H]ethylmaleimide (24 Ci/mol) in the labeling step. Tryptic digestion was performed as described under Methods. Panel A: electrophoretic pattern on an 8% acrylamide gel of ghosts not exposed to trypsin. Panel B: electrophoretic pattern on a 12% acrylamide gel of ghosts treated with trypsin.

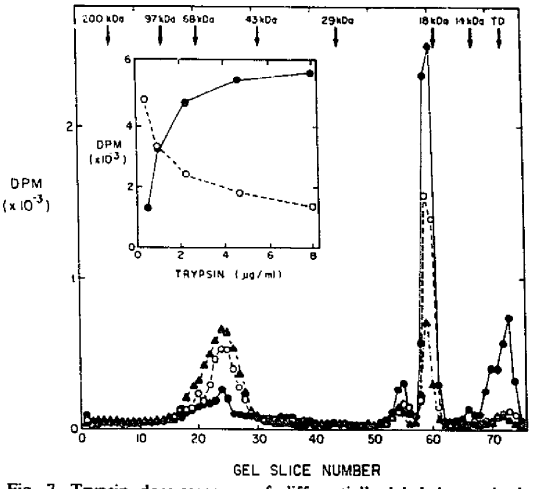

Fig. 7. Trypsin dose-response of differentially labeled protein-depleted ghosts. Protein-depleted ghosts were subjected to the double differential labeling protocol described under Methods using 100 μM cytochalasin B in the first orientation step, 100 μM phloretin in the second step, and 0.3 mM *N*-[^{3}H]ethylmaleimide (22 Ci/mol) in the labeling step. Tryptic digestion was performed at the indicated trypsin concentration on aliquots of these membranes as also described under Methods. Electrophoretic patterns on 12% gels are shown for trypsin concentrations of 0.6 (▲), 1 (○) and 8 μg/ml (●). Inset. Total radioactivity in band 4.5 (○) and the M_r 19000 tryptic fragment (●) from the gels is shown as a function of the trypsin concentration in the digestion.

the M_r 19000 tryptic fragment (Fig. 8). When the data were subjected to nonlinear least-squares analysis [26], the curve plateaued at about 300000 sites per cell, in agreement with other estimates [12,13,27,28]. The labeling of the M_r 19000 tryptic fragment was half-maximal at 0.34 ± 0.07 mM labeled MalNEt. Overall, these data suggest that the exofacial sulfhydryl is the only group on the carrier labeled under these conditions at low radioactive MalNEt concentrations.

At higher concentrations of radioactive MalNEt, labeling of the M_r = 23000–40000 tryptic carrier fragment also occurred (Fig. 9). The gel pattern shown was observed after differential labeling with 10 mM radioactive MalNEt using protection with cytochalasin B alone. Again, band 4.5 was prominently labeled compared to band 3 (Fig. 9A). Following tryptic digestion, labeling of both a broad M_r 23000–40000 fragment and of the M_r 19000 tryptic fragment was observed (Fig. 9B). With increasing concentrations of radioactive MalNEt labeling in the M_r 23000–40000 tryptic fragment showed saturation (Fig. 9B, inset), with a calculated half-maximal effect [26] at 3.6 ± 0.2 mM and a total of 200000 ± 4000 sites per cell. Given the error in estimating the amount of radioactivity in this broad fragment, the

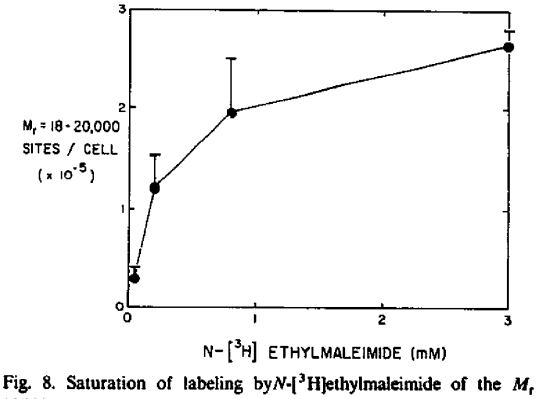

Fig. 8. Saturation of labeling by *N*-[^{3}H]ethylmaleimide of the M_r 19000 tryptic fragment. Protein-depleted ghosts were treated with the double differential labeling protocol as described under Methods using 100 μM cytochalasin B in the first orientation step and 100 μM phloretin in the second step. Labeling was performed with increasing concentrations of *N*-[^{3}H]ethylmaleimide as shown, followed by tryptic digestion as described under Methods. Label in the M_r 19000 tryptic fragment is shown as mean ± S.D. from two experiments.

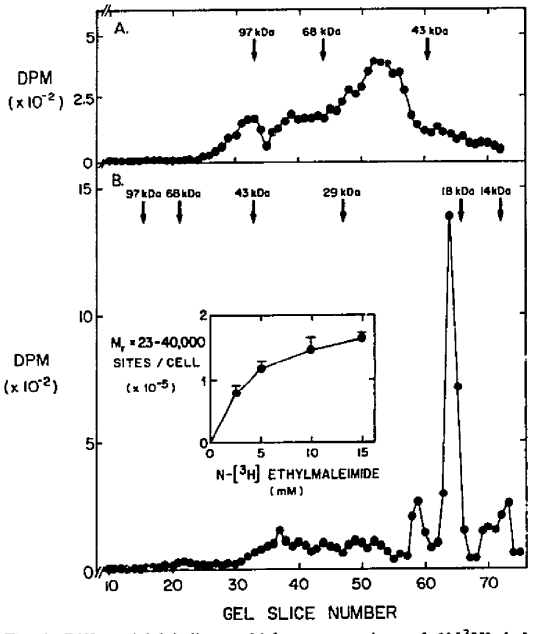

Fig. 9. Differential labeling at high concentrations of *N*-[^{3}H]ethylmaleimide of protein-depleted ghosts and the effects of tryptic digestion. Protein-depleted ghosts were incubated for 5 min at 37°C with 100 μM cytochalasin B, unlabeled MalNEt was added to a concentration of 4 mM for 20 min, the ghosts were washed three times by centrifugation in 32 volumes of ice-cold 5P8, and aliquots were labeled with 10 mM *N*-[^{3}H]ethylmaleimide of the same specific activity (5 Ci/mol) for 30 min at 37°C. The ghosts were washed and subjected to the tryptic digestion as described under Methods using a trypsin concentration of 32 μg/ml. Panel A: electrophoresis on an 8% acrylamide gel of ghosts not exposed to trypsin. Panel B: electrophoresis on a 12% acrylamide gel of trypsin-treated ghosts. Inset. Labeling of the M_r 23000–40000 tryptic fragment by increasing concentrations of radioactive MalNEt at theh same specific activity (5 Ci/mol).

observed labeling corresponds fairly well to one site per carrier. As expected from the results of Fig. 8, labeling of the M_r 19000 tryptic fragment remained constant over this range of radioactive MalNEt concentrations at 230000 sites per cell.

Discussion

Given its small size, lack of charge, and hydrophobicity, MalNEt should alkylate carrier sulfhydryls more extensively than impermeant sulfhydryl reagents in intact cells. As discussed by Abbott et al. [6], this concept is supported by the results of several types of experiment wherein the effects of MalNEt differ from those of impermeant sulfhydryl reagents, which in intact cells react only with an exofacial carrier sulfhydryl [7–9,12,13]. The present results indicate that the exofacial sulfhydryl is quite reactive with MalNEt. In fact, at concentrations of radiolabeled MalNEt below 3 mM, it was the only carrier group labeled (Figs. 2–7), an effect which was half-maximal at 0.34 mM MalNEt. Considering the lack of transport inhibition at such low concentrations of MalNEt (Fig. 1), alkylation of the exofacial sulfhydryl does not appear to be involved in transport inhibition by MalNEt. This conclusion is further supported by the results of the DTNB protection studies, which showed that alkylation of the exofacial sulfhydryl by MalNEt was not required for its ability either to inhibit transport (Table IB) or to decrease the affinity of the exofacial carrier for maltose. These effects are presumably related to modification of internal sulfhydryls by MalNEt.

This behavior contrasts with that of larger more hydrophilic impermeant sulfhydryl reagents, which inhibit transport by reacting exclusively with the exofacial sulfhydryl [7–13]. Significantly, even these reagents have differing effects on the ability of the carrier to change conformation. In reacting with the exofacial sulfhydryl, GS-Mal [11] and a maleimide derivative of glucosamine [13] lock the carrier in an outward-facing conformation unable to bind cytochalasin B inwardly, whereas DTNB [22] and Cys-Mal (May, J.M., unpublished observations) do not. MalNEt represents an extreme of this spectrum, since its alkylation of the exofacial sulfhydryl does not affect either transport or cytochalasin B binding. These data suggest that alkylation of the exofacial sulfhydryl per se does not cause the observed effects of impermeant reagents on the carrier mechanism, rather that the exofacial sulfhydryl is in a region in which either carrier conformational changes or substrate translocation are subject to steric hindrance by a bulky substituent.

The results of the cytochalasin B binding experiments indicate that MalNEt may inhibit hexose entry (Ref. 7 and Fig. 1) and exit [7,29,30] by different mechanisms. MalNEt treatment decreased the affinity of the carrier for maltose (Table II), as previously shown by Abbott et al. [6], as well as for phloretin (Table II), both of which bind only to the outward-facing carrier form [10,24]. The MalNEt-induced decrease in total binding and affinity for cytochalasin B itself (Table II) suggests additional effects on the availability and conformation of the inward-facing carrier. This contrasts with a MalNEt-induced increase in carrier affinity for sugars which have access to or preference for the inward-facing binding site (Ref. 6 and Table II). Abbott and Schachter [31] suggested that this increase in affinity related to trapping of transported sugars within the carrier pore. However, the finding that the affinity of the inward-facing carrier was also increased for the nontransported *N*-propyl β D-glucopyranoside in MalNEt-treated cells (Table II) tends to localize the alteration close to or at the inward-facing substrate-binding site itself. These data suggest that a decrease in

carrier affinity at the outward-facing substrate binding site may contribute to the inhibition of hexose entry by MalNEt. On the other hand, MalNEt inhibits exit in the face of increased affinity for substrate, implying a decrease in substrate translocation across the membrane as the major defect.

The specificity of radiolabeled MalNEt for the carrier is low, even in protein-depleted ghosts (Fig. 2A). In fact, at low concentrations of radioactive MalNEt, little inhibition of band 4.5 labeling by cytochalasin B was evident (Fig. 2A). This probably reflects labeling of non-carrier sites in this region. Fortuitously, exhaustive tryptic digestion of labeled membranes removed most of the radioactivity associated with other membrane proteins and revealed that labeling of the M_r 19000 carrier fragment (containing the exofacial sulfhydryl [5,13]) was sensitive to cytochalasin B (Fig. 2B). The labeled M_r 21000 fragment observed in some experiments may well correspond to a tryptic fragment of band 3 which was also evident in the studies of Rao and Reithmeier [32].

It was possible to improve the specificity of carrier labeling by MalNEt using differential labeling techniques, particularly with a two-step or double procedure (Figs. 3 and 4). This method involves initially protecting carrier sulfhydryl(s) by orienting the carrier inwardly with cytochalasin B, alkylating nonspecific sulfhydryls with 4 mM unlabeled MalNEt, washing to remove both agents, and treating with phloretin before labeling with radioactive MalNEt. The behavior of phloretin deserves further comment, since it had no effects on MalNEt-induced transport inhibition (Table I) or on band 4.5 labeling by low *N*-[^{3}H]ethylmaleimide concentrations, whereas it enhanced band 4.5 labeling in the double differential labeling protocol (Fig. 3). These discrepancies are best explained by an effect of phloretin to displace residual inhibitory cytochalasin B not removed by the buffer washes. Although phloretin is known to cause the carrier to reorient outwardly [10,24], this does not appear to directly modify the reaction of MalNEt with the carrier. Nonetheless, the improved specificity of labeling with this approach allowed localization of the sites of MalNEt labeling within the carrier structure.

In fact, the exofacial carrier sulfhydryl appears to be the only group labeled by low concentrations of MalNEt in the double differential procedure. Differentially labeled band 4.5 was quantitatively converted by exhaustive tryptic digestion to a single labeled M_r 19000 fragment (Fig. 4) known to contain the exofacial sulfhydryl from labeling studies using PCMBS [5] and an impermeant sugar-maleimide [13]. The labeling of both band 4.5 and the M_r 19000 tryptic fragment was blocked by pretreatment of ghosts with DTNB (Fig. 5), indicating the specificity of labeling for sulfhydryl(s) located relatively superficially in the membrane. Similar protection by treatment of intact cells with Cys-Mal before ghost preparation and double differential labeling with MalNEt confirmed that the exofacial sulfhydryl was in fact labeled. Moreover, dose-response studies showed that a single sulfhydryl was labeled in the M_r 19000 tryptic fragment (Fig. 8). Labeling of the M_r 23000–40000 tryptic carrier fragment was not evident over a range of trypsin concentrations (Fig. 7). This also rules out the possibility that trypsin-sensitive labeling on the latter fragment was removed at high concentrations of the enzyme. At a concentration of 0.2 mM radioactive MalNEt, which alkylates about 40% of exofacial carrier sulfhydryls (Fig. 8), labeling of band 3 in protein-depleted ghosts was only 5–10% of that in band 4.5 (Figs. 5 and 6). This may allow other hydrophobic probes, such as spin labels or fluorescent derivatives, to be attached to the exofacial carrier sulfhydryl with high selectivity, permitting study of its environment during changes in carrier conformation.

At high concentrations of labeled MalNEt during protection by cytochalasin B alone, both the M_r 19000 and the M_r 23000–40000 tryptic fragments of the carrier were labeled (Fig. 9). From the dose-response curves of labeling for each fragment it was estimated that only one site in each was specifically labeled. These results may be compared to those of Deziel et al. [5], who used a single high concentration of *N*-[^{3}H]ethylmaleimide in labeling purified reconstituted band 4.5. They also found one site labeled in the M_r 19000 tryptic carrier fragment corresponding to an externally exposed sulfhydryl, but two labeled sites in the M_r 23000–40000 fragment. However, labeling of only one of these groups was suppressed by cytochalasin B [5]. Under the present conditions of differential labeling, a sulfhydryl not protected by cytochalasin B would be alkylated by non-radioactive MalNEt and thus not available for subsequent labeling by radioactive MalNEt. Thus studies performed under different conditions and with different carrier preparations are in agreement regarding the number of cytochalasin B-sensitive internal sulfhydryls on the carrier. The role of a second cytochalasin B-insensitive internal sulfhydryl in transport inhibition by MalNEt is less clear. Labeling of the cytochalasin B-sensitive sulfhydryl in the M_r 23000–40000 tryptic fragment (Fig. 9, inset) occurred at lower concentrations of MalNEt than transport inhibition in intact cells (Fig. 1). This could mean that another group is involved, or simply that the effective concentration of MalNEt is somewhat greater in ghosts than in intact cells. Nevertheless, since cytochalasin B affords strong protection against transport inhibition by MalNEt (Table I and Refs. 8, 29 and 30), a single cytochalasin B-sensitive internal sulfhydryl may be crucial for transport inhibition by this agent.

In conclusion, the exofacial carrier sulfhydryl is quite reactive with the highly permeant MalNEt. However, its alkylation does not affect transport, rather this effect

requires alkylation of at least one cytochalasin-B-sensitive internal carrier sulfhydryl at higher reagent concentrations. The exofacial sulfhydryl can be selectively labeled in protein-depleted ghosts at low concentrations of MalNEt using a double differential labeling procedure. This may provide an important technique for attachment of other similarly hydrophobic molecules to this group, allowing study of the carrier mechanism with regard to changes in the local environment of the exofacial sulfhydryl.

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