

# Inhibition of hexose transport and labelling of the hexose carrier in human erythrocytes by an impermeant maleimide derivative of maltose

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Maltose-maleimide was synthesized as a potential affinity label for the facilitative hexose carrier with selectivity for exofacial sulphhydryl groups. This reagent, although probably a mixture of isomers, did not significantly penetrate the plasma membrane of human erythrocytes at concentrations below 5 mM at 37 °C. When allowed to react to completion, it irreversibly inhibited the uptake of 3-*O*-methylglucose, with a half-maximal response at about 1.5–2.0 mM-reagent. The rate of transport inactivation was a saturable function of the maltose-maleimide concentration. Studies of reaction kinetics and effects of known transport inhibitors demonstrated that irreversible reaction occurred on the exofacial outward-facing carrier, although not at a site involved in substrate binding. Reaction of intact erythrocytes with [<sup>14</sup>C]maltose-maleimide resulted in labelling of a broad band 4.5 protein of  $M_r$  (average) 45000–66000 in electrophoretic gels. This protein was very likely the hexose carrier, since its labelling was inhibited by cytochalasin B. Exofacial band 4.5 labelling was stoichiometric with respect to transport inhibition, yielding an estimated 300000 carriers/cell. These results suggest that the exofacial sulphhydryl which reacts with maltose-maleimide is distinct from the substrate binding site on the hexose carrier, but that it confers substantial labelling selectivity to impermeant maleimides. Additionally, the high efficiency of carrier labelling obtained with maltose-maleimide is useful in quantifying numbers of carriers in whole cells.

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## INTRODUCTION

Facilitative hexose transport in human erythrocytes is sensitive to irreversible inhibition by cell-impermeant thiol reagents [1–4]. Of these reagents, glutathione-maleimide [2,4] and *N*-maleoylmethionine sulphone [3] appear to react directly with the carrier, since they label a membrane protein in intact cells which has an electrophoretic mobility the same as that of the hexose carrier in the band 4.5 region (45–66 kDa), and since their inhibition of transport and band 4.5 labelling can be modified by carrier substrates and cytochalasin B [2–4]. Based on the relative specificity of maleimides for sulphhydryls, these reagents probably react with an exofacial sulphhydryl group required for activity of the transport mechanism [5,6]. However, their use in quantifying carrier numbers in whole cells and in structure-function studies of the transport mechanism is limited by relatively low affinity and specificity. Concentrations in excess of 10 mM of either reagent are required for half-maximal irreversible inhibition of hexose influx [2,3]. At higher concentrations they may produce osmotic effects or begin to enter cells [3], making them less useful for studies of the exofacial sulphhydryl group. With regard to specificity, reaction of erythrocytes with these reagents labels several membrane proteins in addition to the hexose carrier [3,4]. This lack of specificity contrasts with the observation that an impermeant sugar photolabel developed by Holman *et al.* [7] labels proteins other than the hexose carrier to only a minor extent in intact erythrocytes.

Availability of an impermeant maleimide with

improved specificity and affinity would also help to define the relationship of the exofacial sulphhydryl to the state of carrier conformation and to the location of the substrate binding site. Both cytochalasin B [2–4] and D-glucose [2,4] have been shown to protect against transport inhibition induced by impermeant maleimides. Although these effects could indicate competition at the substrate-binding site, a similar result would be obtained if a conformational change induced by glucose and cytochalasin B removed the reactive exofacial sulphhydryl group. As discussed by Krupka & Devés [8], the obligate asymmetry and irreversibility of reaction of impermeant maleimides makes them potentially useful tools in evaluating carrier orientation.

Given the limitations of available impermeant sulphhydryl reagents for carrier quantification in intact cells, and in order to characterize the reactive exofacial sulphhydryl with respect to the orientation of the substrate-binding site of the hexose carrier, I have synthesized maltose-maleimide. This reagent does not enter cells under the conditions employed, inhibits hexose transport irreversibly and reacts with an exofacial sulphhydryl which is not required for substrate binding. Transport inhibition is stoichiometric with respect to labelling of a single protein in intact cells possessing characteristics of the hexose carrier.

## EXPERIMENTAL

### Synthesis of maltose-maleimide

Maltose-maleimide was synthesized and initially purified as described for dextran-maleimide by Abbott &

Schachter [4]. In a typical reaction 3.0 g of *N*-chloromethylmaleimide [9] and 2.4 g of maltose were stirred overnight in 4.5 ml of water at room temperature. Undissolved material was removed by filtration and the supernatant was chromatographed at room temperature in water on a column of Sephadex G-10 (2.5 cm × 100 cm).

Two major peaks were detected, the first, or void-volume fraction, containing maltose-maleimide and unreacted maltose, the second mostly methylolmaleimide (the hydrolytic product of *N*-chloromethylmaleimide). The void volume peak was pooled and lyophilized. The hygroscopic residue was dissolved in methanol and chromatographed on two preparative silica gel G-60 plates (2 mm × 20 cm × 20 cm) in isopropanol/ethyl acetate/water (7:2:1, by vol.). The  $R_f$  of maltose was 0.25, while that of maltose-maleimide was 0.4. The maltose-maleimide band was identified by the presence of both carbohydrate and sulphhydryl reactivity. The former was detected by spraying an adjacent area with 5%  $H_2SO_4$  and heating at 110 °C for 10 min and confirmed by using radioactive maltose. The latter was detected and quantified in an assay using 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) as described below. The maltose-maleimide band was removed by scraping, eluted with methanol, and concentrated to dryness or saved as a methanolic solution (in which it was stable for at least 1 month). Based on maleimide content and weight this material contained a 1:1 ratio of maltose:maleimide. Yields were generally 5–10% of starting maltose. Traces of faster migrating material by t.l.c. had more than one maleimide/maltose molecule. Maltose-maleimide prepared in this fashion was probably a mixture of isomers, although this possibility was not further explored.

#### Assay of maleimide activity

The sulphhydryl reactivity of maltose-maleimide was measured using an assay involving DTNB, as suggested by Abbott & Schachter [4]. A sample of 0.1 ml of maltose-maleimide (< 0.2 μmol) in methanol or phosphate buffer was incubated for 30 min at room temperature with 0.1 ml of 3.26 mM-glutathione in water, followed by addition of 0.1 ml of 5 mM-DTNB in 50 mM-phosphate buffer (pH 7.4). After 10 min at room temperature, the samples were diluted with 3 ml of water and the absorbance at 412 nm was read on a Gilford Model 250 spectrophotometer. Maleimide reactivity (μmol/assay) was calculated by taking the absolute value of the difference between the sample reading and the blank reading, divided by the blank reading, and multiplied by 0.326.

#### Stability of maltose-maleimide in PBS

The decomposition of 2.5 mM-maltose-maleimide at 37 °C in PBS was assessed by its ability to react with an excess of glutathione as in the DTNB assay. Disappearance of maleimide reactivity was linear at a rate of about 0.01 min<sup>-1</sup> with a calculated half-time of 47 min.

#### Synthesis of radioactive maltose-maleimide

Labelled maltose-maleimide was typically synthesized by dissolving 50 μCi of [<sup>14</sup>C]-α-D-maltose (440 Ci/mol, ICN) and 3.5 mg of non-radioactive maltose (final specific activity 5 Ci/mol) in 150 μl of dry dimethyl sulphoxide followed by addition of 14.5 mg of *N*-

chloromethylmaleimide (100 μmol). The reaction was incubated at 55 °C for 45 min, evaporated to near dryness under  $N_2$ , dissolved in methanol, and subjected to t.l.c. as described for the non-radioactive material, except that 250 μm thick plates were used, and the peaks detected by scraping 0.75 cm sections and counting the radioactivity. Only one radioactive peak in advance of maltose was found, containing from 15 to 50% of total radioactivity applied. Under these conditions the product was cleanly separated from *N*-chloromethylmaleimide and methylolmaleimide. The maltose-maleimide band was scraped from the plate, eluted in methanol and used within 24 h. Unreacted maltose was reutilized.

Sulphydryl reactivity of the maltose-maleimide peak was determined by reaction with thiopropyl-Sepharose 6B (Pharmacia). Thiopropyl-Sepharose beads (100 mg) were swelled and deaerated in 20 ml of deionized water for 15 min at room temperature, followed by centrifugation at 1000 *g* for 3 min and removal of the supernatant. The beads were washed once in 0.4 ml of deaerated buffer containing 65 mM-dithiothreitol, 0.3 M-NaHCO<sub>3</sub>, and 1 mM-disodium EDTA (pH 8.4), and incubated for 40 min at room temperature in 0.4 ml of the same buffer. The beads were then washed five times by centrifugation in 8 ml of deionized water containing 100 mM-acetic acid, 0.5 M-NaCl, and 1 mM-disodium EDTA, followed by three more washes in 5 ml of 100 mM-NaH<sub>2</sub>PO<sub>4</sub> buffer (pH 7.5). The beads were brought to a total volume of 1.2 ml and 0.2 ml aliquots (1 μmol of sulphhydryl groups) were used in the assay of sulphhydryl reactivity. Radioactive maltose-maleimide (usually 110000 d.p.m., 10 nmol) in phosphate buffer was incubated for 1 h at room temperature with the beads, and the assay terminated by three washes by centrifugation at 3000 *g* for 5 min with 1 ml of phosphate buffer. The beads were resuspended in 0.2 ml of phosphate buffer, washed into a scintillation vial with 5 ml of ACS (Amersham), and radioactivity counted at 90% efficiency in a Packard Model 2000CA liquid scintillation spectrometer. Counting efficiency was not significantly affected by settling of the beads in the vials. The amount of radioactivity bound was calculated as a percentage of the total amount added. Using this procedure the sulphhydryl-coupling efficiency of different preparations of [<sup>14</sup>C]maltose-maleimide was usually in excess of 60% after a 30 min incubation at 37 °C in PBS.

#### Erythrocyte preparation

Blood was drawn from volunteers and anti-coagulated with heparin (16.7 units/ml of blood). Freshly drawn or stored erythrocytes were washed five times by centrifugation in equal volumes of 12.5 mM-NaH<sub>2</sub>PO<sub>4</sub> buffer, containing 150 mM-NaCl, pH 7.4 (PBS). After the first and second washes the cells were incubated for 5 min at 37 °C to remove intracellular glucose [10]. Following the washes the erythrocytes were brought to a 20% haematocrit for all transport studies. Unused but washed erythrocytes were stored up to 3 days at a 40% haematocrit in the presence of 11 mM-citrate, 22 mM-D-glucose, 0.25 mM-adenine, 12.8 mM-phosphate, and 131.6 mM-chloride with sodium as the cation, pH 7.4.

#### Hexose transport

The zero-*trans* uptake of 3-*O*-methylglucose was measured as follows. Erythrocytes were suspended to a

20% haematocrit at 5 °C in PBS containing other agents as noted. Uptake was initiated by pipetting 50 µl of swirled cells on to 0.02 ml of ice-cold PBS containing 3-*O*-methyl-[<sup>14</sup>C]methylglucose [except where noted, 0.125 µCi at a specific activity of 40 Ci/mol (ICN)]. The cells were swirled and incubated for 30 s in an ice bath. The assay was terminated by addition of 1.2 ml of ice-cold PBS 'stop' solution containing 10 µM-cytochalasin B. The suspension was transferred to a microfuge tube and centrifuged for about 5 s in a Beckman Model B microfuge. The supernatant was aspirated and the pellet was resuspended in another 1.2 ml of cold 'stop' solution followed by centrifugation. The second wash was aspirated, the pellet was resuspended in 0.2 ml of cold PBS, and 1 ml of 6% trichloroacetic acid was added with vortexing. The suspension was again centrifuged and 0.5 ml of the clear supernatant was removed for scintillation counting in 5 ml of ACS. Correction for trapped extracellular label was made by subtraction of a 'zero time' value (cells plus stop solution followed by labelled sugar). The transport rate was expressed as a percentage of equilibrium values obtained by incubating a separate aliquot of cells for 30 min at 37 °C. The basal rate of 3-*O*-methylglucose uptake was linear for 30–40 s (not shown). Except where noted, rates of transport are expressed as the fractional filling of the equilibrium 3-*O*-methylglucose space for each cell aliquot in 30 s. In experiments in which inhibition of transport by maltose-maleimide was measured without its removal by washing, the final concentration of the agent was taken as that after addition of radioactive 3-*O*-methylglucose. Evaluation of transport kinetics was performed according to the method of Wilkinson [11]. The concentration of inhibitor resulting in half-maximal inhibition of transport (EC<sub>50</sub>) was calculated according to the method of Holman & Rees [12].

#### Erythrocyte labelling with [<sup>14</sup>C]maltose-maleimide

Intact erythrocytes (1 ml) at a 50% haematocrit were incubated for 10 min at 37 °C with the indicated agents, followed by addition of [<sup>14</sup>C]maltose-maleimide and further incubation at the same temperature for 30 min. The cells were washed three times by centrifugation in 10 volumes of PBS and subjected to hypo-osmotic lysis and preparation of white ghosts according to the method of Fairbanks *et al.* [13]. The membranes were stored at –20 °C until electrophoresis.

#### SDS/polyacrylamide-gel electrophoresis

Electrophoresis of membrane protein was performed by the method of Laemmli [14] as previously detailed [15], except that a mixture of C-12, C-14 and C-16 alkyl sulphates was used in the buffer ('lauryl' sulphate, Pierce Chemical Co.), samples were not boiled prior to electrophoresis, and 1.5 mm thick slab gels were used. Gel sections of 1.9 mm were cut from the gels, solubilized overnight in 0.5 ml of TS-1 solubilizer (Research Products International), and prepared for liquid scintillation counting in 5 ml of ACS. The radioactivity of the samples was determined after 48 h at room temperature using a Packard 2000CA liquid-scintillation spectrometer with d.p.m. calculations and correction for a small amount of residual chemiluminescence. Samples were counted until at least 1000 disintegrations had accumulated. Environmental backgrounds were subtracted from all samples. Pre-stained molecular mass

markers (Bethesda Research Laboratories, Bethesda, MD, U.S.A.) were run in lanes adjacent to the samples.

Quantification of [<sup>14</sup>C]maltose-maleimide binding sites on erythrocytes was performed by determining the number of molecules of maltose-maleimide bound to band 4.5 in an electrophoretic gel from the total radioactivity on the band, minus the environmental background. The sites of maltose-maleimide binding/cell were calculated with the assumptions that there were 5 × 10<sup>9</sup> cells/ml of blood at a 50% haematocrit and 1.39 × 10<sup>6</sup> cells/µg of membrane protein applied to the gel [16].

#### Other methods

Syntheses of dextran-maleimide and glutathione-maleimide-I were carried out as described by Abbot & Schachter [4]. Measurement of erythrocyte glutathione content was performed according to the method of Hissin & Hilf [17]. Protein content of erythrocyte membranes was measured by the method of Lowry *et al.* [18]. Except as noted, data are expressed as means ± S.E.M. and compared by the Student *t* test for paired values.

## RESULTS

#### Penetration of the erythrocyte plasma membrane by maltose-maleimide

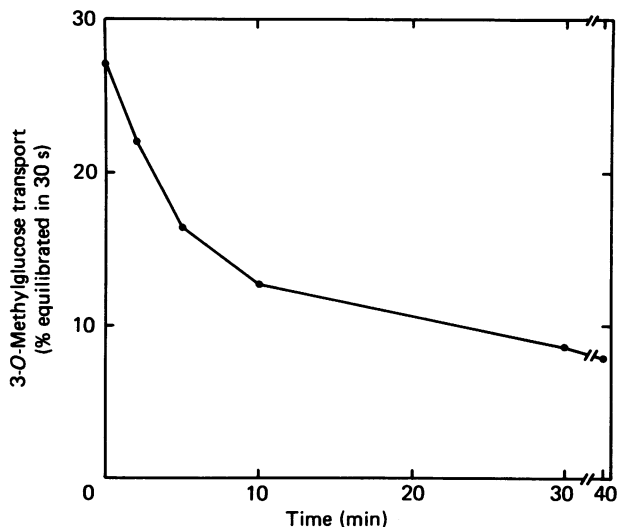
The ability of maltose-maleimide to penetrate the plasma membrane of erythrocytes was assessed by the measurement of intracellular glutathione following incubation with increasing concentrations of maltose-maleimide.

A significant fall (10%) in intracellular glutathione was not apparent until cells were incubated with an initial concentration of 10 mM-maltose-maleimide for 30 min at 37 °C. On the other hand, incubation with *N*-ethylmaleimide at 1 mM under similar conditions completely depleted intracellular glutathione (not shown). Therefore, the reagent was considered 'impermeant' at concentrations < 5 mM, as used in most studies.

#### Inhibition of hexose transport by maltose-maleimide

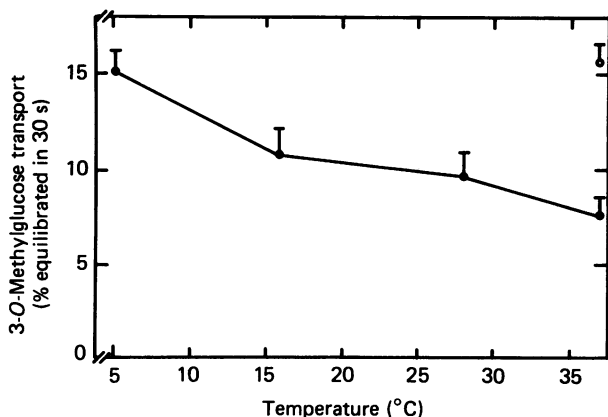
The irreversible inhibition of hexose transport by maltose-maleimide was evaluated as shown in Fig. 1. An initial concentration of 2.5 mM-maltose-maleimide was incubated with cells for varying times at 37 °C followed by several washes to remove unreacted reagent and then by the transport assay. Transport inhibition was rapid and essentially complete by 30 min. At low temperatures, however, maltose-maleimide showed little irreversible inhibition of transport. In fact, at 5 °C, irreversible transport inhibition was not evident after even 30 min of reaction (Fig. 2). The lower rate of uninhibited transport in the experiment of Fig. 2 compared with those shown in Figs. 1 and 3 probably reflects the use of different blood donors.

When incubations with varying concentrations of maltose-maleimide were performed for 30 min at 37 °C, the curve of residual transport activity shown in Fig. 3 was obtained. Half-maximal transport inhibition occurred at about 1.5 mM-maltose-maleimide, and transport inhibition was maximal at 10 mM-reagent (Fig. 3). The lack of complete transport inhibition could have been due in part to destruction of the reagent,



**Fig. 1. Time course of irreversible transport inhibition by maltose-maleimide**

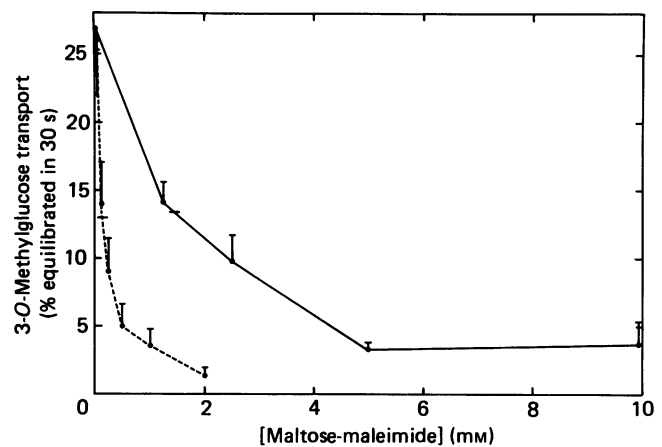
Erythrocytes at a 20% haematocrit were preincubated with an initial extracellular concentration of 2.5 mM-maltose-maleimide at 37 °C for the times noted, followed by three washes by centrifugation in 5 ml of ice-cold PBS, and by resuspension to the same cell concentration for the assay of hexose transport at 80  $\mu$ M-3-*O*-methylglucose as described under Experimental. Results are triplicate values from a representative experiment.



**Fig. 2. Effects of temperature on irreversible transport inhibition by maltose-maleimide**

Erythrocytes at a 20% haematocrit were incubated with an initial concentration of 1.25 mM-maltose-maleimide for 30 min at the temperatures indicated, washed three times in 5 ml of ice-cold PBS, and resuspended to the initial cell concentration for assay of hexose transport using 80  $\mu$ M-3-*O*-methylglucose. The control rate of transport at 37 °C is indicated by the open circle. Results are from a single experiment,  $\pm$  S.D.

but it could not be attributed to non-specific diffusion of 3-*O*-methylglucose into cells, since L-glucose space was no different from that obtained using the zero-time stop method (not shown). A change in carrier affinity also could not account for these results, since 1.25 mM-



**Fig. 3. Reversible and irreversible transport inhibition by maltose-maleimide**

For measurement of irreversible transport inhibition (●), erythrocytes at a 20% haematocrit were incubated with increasing concentrations of maltose-maleimide for 30 min at 37 °C, washed as described in the legend to Fig. 1, and subjected to the hexose transport assay at 80  $\mu$ M-3-*O*-methylglucose ( $n = 6$ ). For measurement of reversible acute transport inhibition (○), the indicated concentration of maltose-maleimide was added to 20% erythrocytes incubated on ice, the suspension swirled, and the transport assay performed immediately ( $n = 4$ ). The approximate concentration of half-maximal inhibition is noted by a horizontal line on each curve.

maltose-maleimide irreversibly lowered the  $V_{max}$  of zero-*trans* 3-*O*-methylglucose uptake by 54% without an effect on the  $K_m$  (1.5–2.0 mM) (not shown). The impermeant glutathione-maleimide I and dextran-maleimide at concentrations of 10 mM were markedly less potent under the same conditions, inhibiting transport by only 29 and 26% of control values, respectively.

If maltose-maleimide was added to the cells at 5 °C and the transport assay begun immediately without the wash steps, a greater inhibition was obtained than at higher temperatures following washes (Fig. 3). Under these conditions, which were chosen to minimize irreversible reaction with the carrier, transport was inhibited half-maximally at 0.125 mM, and was nearly completely blocked by 2 mM-maltose-maleimide. Hydrolysis of the reagent to maltose and methylol-maleimide with reversible transport inhibition by maltose does not seem to be a likely explanation for increased reversible affinity, since even if breakdown had occurred, in this system the  $EC_{50}$  for transport inhibition by maltose was 36 mM (results not shown). Methylol-maleimide similarly did not inhibit transport below 5 mM at 5 °C (results not shown). A plausible explanation is that maltose-maleimide may bind to a strongly inhibitory site, but that subsequent covalent reaction occurs at another site, which may be unrelated to the acute inhibition of transport. This possibility is supported by the following studies.

#### Rate of transport inactivation by maltose-maleimide

By performing time courses similar to those in Fig. 1 at various initial maltose-maleimide concentrations and taking the inactivation rate as the slope of the best-fit line

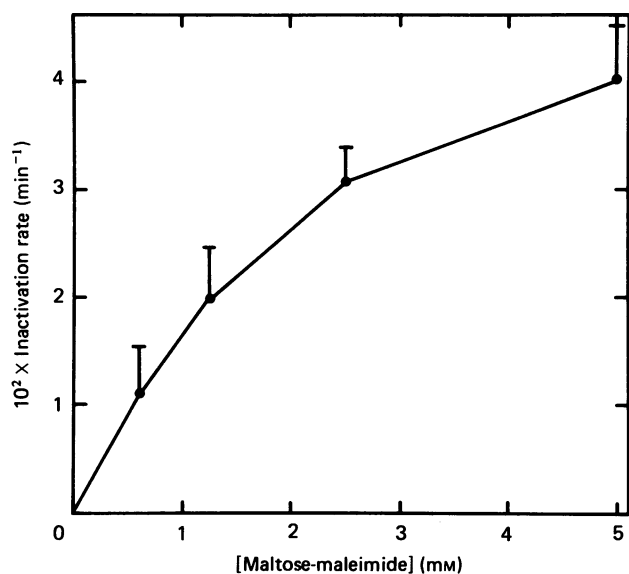


Fig. 4. Rate of inactivation of transport by maltose-maleimide

Assay of the time course of irreversible transport inhibition was performed as described in the legend to Fig. 1 for 0, 2, 5, and 10 min at the indicated initial extracellular concentration of maltose-maleimide. The logarithm of the fraction of space occupied by 3-*O*-methylglucose at equilibrium was used to calculate the rate of inactivation by linear regression analysis [11,19]. Results are from at least four experiments at each agent concentration.

of the logarithm of the early transport points (10 min or less), it was possible to derive the curve shown in Fig. 4. As the concentration of maltose-maleimide was increased, there was a plateau in the transport inactivation rate. Assuming an initial reversible interaction between ligand and binding site in the fashion of Mullins & Langdon [19], the dissociation constant of this step (calculated by nonlinear regression analysis [11]) was  $2.67 \pm 0.26$  mM, and the maximal rate of inactivation was  $0.062 \pm 0.003$   $\text{min}^{-1}$ . The dissociation constant calculated in this manner is appropriate for the slightly lower concentration of half-maximal irreversible transport inhibition ( $\sim 1.5$  mM) noted above, however it is over 10-fold greater than the half-maximal concentration for transport inhibition without washing obtained in the experiments of Fig. 3. This discrepancy between the measured  $\text{EC}_{50}$  for acute transport inhibition and a calculated estimate of the affinity of the ligand for the irreversible reaction site suggests the presence of two different sites.

#### Effects of maltose-maleimide on transport kinetics

When cells were exposed to maltose-maleimide under conditions designed to minimize irreversible reaction with the carrier (low temperatures and short incubation times), zero-*trans* 3-*O*-methylglucose uptake kinetics were modified such that the  $K_m$  was doubled and the  $V_{\text{max}}$  halved (Fig. 5). An effect of maltose-maleimide on the affinity of 3-*O*-methylglucose for the carrier differs from that observed following irreversible reaction, in which only the maximal rate of transport was decreased.

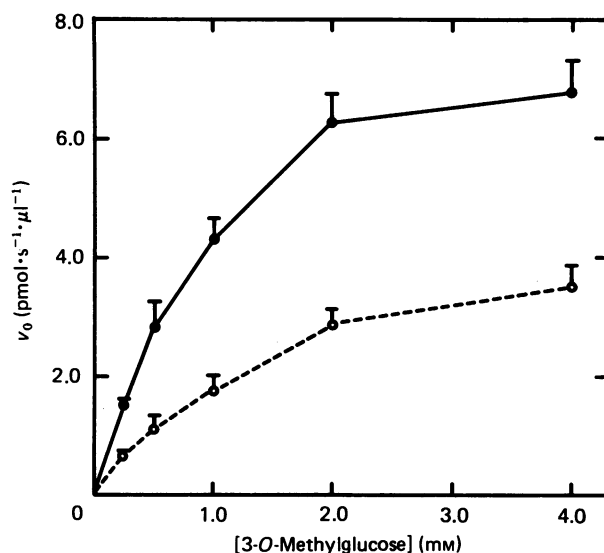


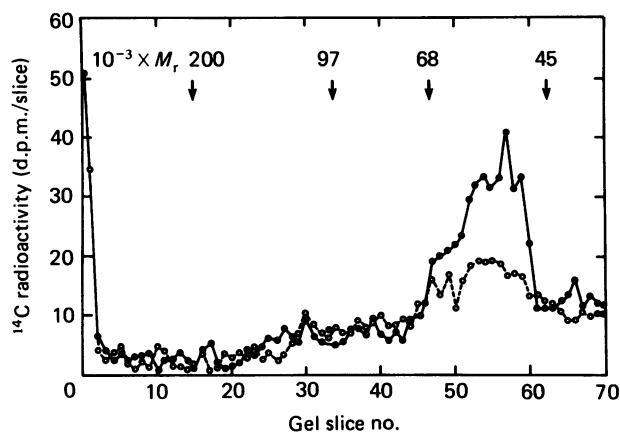
Fig. 5. Effects of maltose-maleimide on the kinetics of 3-*O*-methylglucose uptake

Maltose-maleimide (final concentration, 0.15 mM) was added to erythrocytes (final haematocrit, 20%) incubated on ice, followed immediately by swirling and the transport assay at the indicated concentration of labelled 3-*O*-methylglucose. For each hexose concentration, the specific activity of radiolabelled 3-*O*-methylglucose and time of transport at 5 °C was as follows: 0.25 mM, 6.2 Ci/mol, 30 s uptake; 0.5 mM, 3.12 Ci/mol, 30 s uptake; 1.0 mM, 2.0 Ci/mol, 45 s uptake; 2.0 mM, 1.0 Ci/mol, 45 s uptake; and 4.0 mM, 0.52 Ci/mol, 60 s uptake. Results are from five experiments and expressed as a function of intracellular 3-*O*-methylglucose space occupied at equilibrium (30 min at 37 °C). Calculated kinetic constants [11] for untreated cells (●) were:  $K_m$ ,  $1.2 \pm 0.4$  mM,  $V_{\text{max}}$ ,  $9.4 \pm 1.0$   $\mu\text{mol} \cdot \text{s}^{-1} \cdot \text{l}^{-1}$ ; and for maltose-maleimide-treated cells (○),  $K_m$ ,  $2.1 \pm 0.6$  mM,  $V_{\text{max}}$ ,  $5.5 \pm 0.9$   $\mu\text{mol} \cdot \text{s}^{-1} \cdot \text{l}^{-1}$ . Both kinetic parameters differed by  $P < 0.05$  between the two treatments.

These data also support the hypothesis that there are different sites of initial reversible interaction and ultimate covalent reaction.

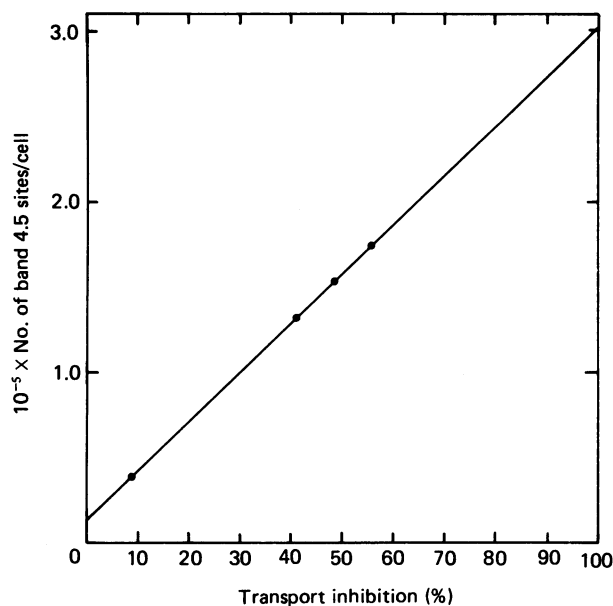
#### Modification of maltose-maleimide-induced irreversible transport inhibition by glucose and inhibitors of hexose transport

If maltose-maleimide reacts with the hexose carrier, its irreversible inhibition of transport should be affected by agents which are known to bind to the carrier. Erythrocytes at a 20% haematocrit were incubated for 10 min at 37 °C with 100 mM-maltose, 100 mM-sucrose, 100 mM-D-glucose, 100 mM-L-glucose, 0.5  $\mu\text{M}$ -cytochalasin B, or 0.5  $\mu\text{M}$ -cytochalasin E. Maltose-maleimide was then added to all samples to a final concentration of 2 mM, and the cells incubated for a further 10–15 min at 37 °C. The suspensions were washed three times by centrifugation with five volumes of PBS, brought back to the initial haematocrit, and the hexose transport assay performed in the usual fashion. Crucial to the design of these competition experiments is the use of transport inhibitors which can be removed by washing, as well as incubation with maltose-maleimide



**Fig. 6. Gel electrophoresis of ghost membranes from cells treated with [ $^{14}\text{C}$ ]maltose-maleimide.**

Intact erythrocytes were preincubated for 10 min at 37 °C with 10  $\mu\text{M}$  cytochalasin E (●) or 10  $\mu\text{M}$ -cytochalasin B (○), labelled with [ $^{14}\text{C}$ ]maltose-maleimide (1.1 mM), and subjected to electrophoresis performed as described under Experimental. For each treatment 120  $\mu\text{g}$  of membrane protein was applied to the gel.



**Fig. 7. Correlation of transport inhibition and band 4.5 labelling by maltose-maleimide**

The total number of band 4.5 sites labelled as described in the legend to Fig. 6 in each of experiments at different maltose-maleimide concentrations (1.5 mM, 1.1 mM, 0.9 mM, and 0.25 mM) was determined as described under Experimental and expressed as a function of the percentage irreversible decrease in transport rates caused by treatment of cells with the same maltose-maleimide concentrations under similar conditions of buffer, cell number, and temperature.

for times less than required for its complete reaction. In each instance it was confirmed that the washing procedure was effective in returning transport to basal rates (20–25 % of 3-*O*-methylglucose equilibrium space in a 30 s transport) in a sample treated only with the reversible inhibitor (not shown). Preincubation for 10 min at 37 °C with the impermeant maltose at 100 mM, followed by addition of maltose-maleimide for another 10–15 min prior to washing, caused a 20 % potentiation of the inhibitory effect of maltose-maleimide compared to cells pretreated with sucrose followed by maltose-maleimide ( $P < 0.02$ , 12 experiments). On the other hand, the same concentration of glucose, which should have equilibrated across the plasma membrane during the 10 min preincubation, protected transport from inhibition by maltose-maleimide by 36 % compared with cells pretreated with L-glucose ( $P = 0.02$ ,  $n = 8$ ). Cytochalasin B also had a significant protective effect of 32 % when compared with the same concentration of cytochalasin E in maltose-maleimide-treated cells ( $P < 0.05$ ,  $n = 4$ ).

#### Labelling of intact erythrocytes and erythrocyte ghosts with radioactive maltose-maleimide

Erythrocytes were incubated with radiolabelled maltose-maleimide under conditions similar to those of the transport assay, washed by centrifugation to remove unreacted reagent, and membranes prepared by hypo-osmotic lysis. Polyacrylamide-gel electrophoresis of solubilized membranes showed labelling of a broad band 4.5 peak depicted in Fig. 6. Although the amount of radioactivity in the individual gel fractions is low, the effect was consistent in four such experiments. While some label was found at the top of the gels, no other labelled peaks could be detected, nor were there counts in the stacking gels (not shown). Significantly, preincubation of intact cells with 100  $\mu\text{M}$ -cytochalasin B resulted in a 35 % decline in the radioactivity incorporated into the band 4.5 protein compared with similar treatment with cytochalasin E (Fig. 6). This

suggests that the hexose carrier in band 4.5 was indeed labelled under these conditions. Preincubation of erythrocytes with 100 mM-maltose before addition of radiolabelled maltose-maleimide resulted in a 8–35 % increase in the radioactivity associated with band 4.5 in three experiments (not shown), consistent with the observation that maltose potentiated transport inhibition by maltose-maleimide.

The total radioactivity present in band 4.5 in four experiments was expressed as the number of sites bound/cell and plotted as a function of the percentage of irreversible transport inhibition obtained at the same concentration of the reagent under similar incubation conditions, (Fig. 7). Even though the transport experiments were not performed on cells used in the labelling experiments, a linear relationship is evident. From this relationship it can be calculated that complete transport inhibition will occur at approx. 300 000 carriers/cell. Moreover, the line extrapolates to near zero in the absence of transport inhibition, suggesting that few sites unrelated to transport inhibition are labelled under these conditions. On the other hand, if leaky ghost membranes rather than intact cells were incubated with maltose-maleimide, most of the major cytoplasmic membrane proteins showed substantial labelling, there was relatively little label in the band 4.5 region, and suppression of labelling by 100  $\mu\text{M}$ -cytochalasin B was not evident in any of the bands (not shown). It is evident that the selectivity of maltose-maleimide for band 4.5 is lost under these conditions.

## DISCUSSION

Maltose-maleimide fits several of the criteria for an affinity label for the hexose carrier [20,21]. It is a substrate analogue of maltose, containing a disaccharide which binds to, but which is not transported by, the carrier. The rate of transport inactivation was found to plateau with increasing reagent concentrations (Fig. 4), and at least at low maltose-maleimide concentrations, there was a 1:1 stoichiometry between irreversible transport inhibition and labelling of band 4.5 protein (Fig. 7). Moreover, the number of carrier molecules/cell derived from extrapolation to the number of sites labelled at 100% transport inhibition (about 300 000) agrees very well with estimates of the number of carriers/cell based on cytochalasin B binding experiments [22–24]. It could also be demonstrated that high concentrations of both D-glucose and cytochalasin B protected against irreversible transport inhibition by maltose-maleimide, and cytochalasin B decreased band 4.5 labelling with maltose-maleimide (Fig. 6), consistent with interaction of the reagent with the exofacial substrate site [8].

However, two experimental results suggest that maltose-maleimide is not an affinity label for the hexose carrier in the strict sense. First, if maltose-maleimide had reacted with the exofacial substrate-binding site, maltose itself should have protected against transport inhibition and labelling of band 4.5. In fact, the opposite was true, since maltose potentiated both irreversible transport inhibition and band 4.5 labelling by maltose-maleimide.

Second, kinetic studies suggested a complex interaction of maltose-maleimide with the carrier protein. The reagent appeared to bind at low temperatures to a strongly inhibitory site different from the one involved in irreversible transport inhibition. This conclusion derives from the finding that different methods of estimating the affinity of maltose-maleimide for the carrier yielded discordant results (Figs. 3 and 4), and from the observation that incubation of cells with maltose-maleimide at low temperatures and for short time periods without subsequent removal by washing, lowered both the affinity of 3-O methylglucose for the carrier and the maximal influx rate of the hexose (Fig. 5). On the other hand, allowing the irreversible reaction to proceed to near completion lowered only the maximal rate of transport.

It is likely that maltose-maleimide reacts with the single sulphhydryl group said to be exposed on the exofacial carrier [5,6]. The present results suggest that this group is not directly involved in substrate binding. It therefore becomes necessary to account for the effects of maltose, glucose and cytochalasin B on transport inhibition and on the labelling of band 4.5 by maltose-maleimide. The alternating conformation carrier model, as recently reviewed by Krupka & Devés [8], appears to account for the results. In this model hexose can bind to one, but not both, sides of the carrier at the same time. The binding site of the carrier faces either inward or outward, which in turn determines the effects on transport of agents which bind asymmetrically to the carrier [8]. Although derived from the kinetics of transport inhibition by various agents, the model is supported by studies of cytochalasin B binding kinetics [25], and more recently by results of  $^1\text{H}$  n.m.r. studies of glucose binding to erythrocyte membranes [26]. The data involving effects of inhibitors of hexose transport can be accounted for by

this model if maltose-maleimide reacts with the exofacial outward-facing carrier at a non-substrate site. At concentrations below 5 mM the reagent is restricted to the outside of cells, and thus must react with the exofacial carrier. Preincubation with maltose, which would be expected to pull the carrier to its outward-facing position, should therefore potentiate irreversible transport inhibition induced by the reaction of maltose-maleimide with a non-substrate site. Cytochalasin B, on the other hand, binds to the inward-facing carrier [27–29], and should have the opposite effect on transport inhibition and labelling. These were the observed findings. The effect of the permeant glucose would depend upon the orientation of substrate-bound carrier. Kinetic and thermodynamic studies of Lowe & Walmsley [30] have suggested that at 37 °C the glucose-bound form of the carrier should be orientated about 70% outwardly, in which case irreversible transport inhibition by maltose-maleimide should be potentiated. However, in studies with both maltose-maleimide (present work) and glutathione-maleimide [2], D-glucose had a small protective effect against irreversible transport inhibition, suggesting a slight preponderance of inward- rather than outward-facing substrate-bound carriers at this temperature. Taken as a whole, these data are consistent with the alternating conformation model of hexose transport.

Maltose-maleimide had two major advantages compared with other impermeant maleimides [2–4]. Irreversible transport inhibition was half-maximal at a maleimide concentration of 1.5 mM (Fig. 3), which is at least five-fold lower than that observed with impermeant maleimides [3, 4, 6, and present work]. This increase in irreversible transport inhibition by maltose-maleimide could have been due to initial binding with a high affinity site followed by diffusion to a nearby site of irreversible reaction. It is also possible that it could have been related to other factors, such as a smaller molecular size, or lack of charge.

Maltose-maleimide also labelled predominantly the band 4.5 carrier protein on electrophoretic gels (Fig. 6), whereas other impermeant maleimides were less specific, labelling several membrane proteins [3,4]. It should be noted that additional proteins might also have been labelled by maltose-maleimide had it been possible to prepare it with a higher specific activity. Nonetheless, the labelling of band 4.5 by maltose-maleimide in whole cells contrasts dramatically with its more extensive labelling of the cytoplasmic portions of most membrane proteins in leaky ghosts. It also contrasts with predominant labelling of band 3 by maltosylisothiocyanate in intact erythrocytes [19]. Although both maltose-maleimide and maltosylisothiocyanate contain maltose, the former reacts with the hexose and the latter with the anion carrier [31]. The differential specificity relates therefore not to the sugar, but to the reactive preference of the coupling groups under physiological conditions: isothiocyanates with primary amines prominent on the anion transporter, and maleimides with at least one exofacial sulphhydryl on the hexose carrier.

In addition to the specificity of reaction provided by a probe confined to the extracellular space, use of a chemical rather than photolytic coupling reaction markedly increases labelling efficiency. When allowed to react to near completion, maltose-maleimide had an efficiency of carrier labelling of about 50% at 1 mM.



This compares with a maximal photolabelling efficiency of only a few per cent with the far more potent cytochalasin B [32,33], which is also accompanied by loss of cytochalasin B binding activity of the remaining carriers, probably due to denaturation by the short-wave u.v. light required for photolysis [33].

In conclusion, the impermeant nature and sulphhydryl reactivity of maltose-maleimide results in high specificity and efficiency of labelling of the hexose carrier in spite of relatively low affinity. These features make it potentially useful in the study of the exofacial sulphhydryl group on the carrier, and in quantifying the numbers of hexose carriers in intact cells other than erythrocytes under various conditions.

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