Lloyd, K. O., Kabat, E. A., & Licerio, E. (1968) *Biochemistry 7*, 2976–2990.
Lundblad, A., Hammarström, S., Licerio, E., & Kabat, E. A. (1972) *Arch. Biochem. Biophys. 148*, 291–303.
Maisonrouge-McAuliffe, F., & Kabat, E. A. (1976) *Arch. Biochem. Biophys. 175*, 1090–1113.
Moreno, C., Lundblad, A., & Kabat, E. A. (1971) *J. Exp. Med. 134*, 439–457.
Nicholson, G. L. (1974) *Int. Rev. Cytol. 39*, 89–190.
Ouchterlony, Ö. (1948) *Acta Pathol. Microbiol. Scand. 25*, 186–191.
Pereira, M. E. A., & Kabat, E. A. (1974) *Biochemistry 13*, 3184–3192.
Pereira, M. E. A., & Kabat, E. A. (1979) *Crit. Rev. Immunol. 1*, 33–78.
Pereira, M. E. A., Kabat, E. A., Lotan, R., & Sharon, N. (1976) *Carbohydr. Res. 51*, 107–118.
Phillips, S. G., Bretting, H., & Kabat, E. A. (1976) *J. Immunol. 117*, 1226–1232.
Poretz, R. D., & Goldstein, I. J. (1970) *Biochemistry 9*, 2890–2896.
Poretz, R. D., Riss, H., Timberlake, J. W., & Chen, S. M. (1974) *Biochemistry 13*, 250–256.
Reisfeld, R. A., Lewis, U. J., & Williams, D. E. (1962) *Nature (London) 195*, 281–283.
Righetti, P., & Drysdale, J. (1971) *Biochim. Biophys. Acta 236*, 17–28.
Sarkar, M., & Kabat, E. A. (1979) *Carbohydr. Res. 69*, 143–149.
Sarkar, M., Liao, J., Kabat, E. A., Tanabe, T., & Ashwell, G. (1979) *J. Biol. Chem. 254*, 3170–3174.
Schiffman, G., Kabat, E. A., & Leskowitz, S. (1962) *J. Am. Chem. Soc. 84*, 73–77.
Schiffman, G., Kabat, E. A., & Thompson, W. (1964) *Biochemistry 3*, 113–120.
Sharon, N., & Lis, H. (1975) *Methods Membr. Biol. 3*, 147–200.
Toyoshima, S., Akiyama, Y., Nakano, K., Tonomura, S., & Osawa, T. (1971) *Biochemistry 10*, 4457–4463.
Tsuyuki, H., von Kley, H., & Stahmann, M. A. (1956) *J. Am. Chem. Soc. 78*, 764–767.
Vicari, G., & Kabat, E. A. (1969) *J. Immunol. 102*, 821–825.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.
Wood, C., Kabat, E. A., Ebisu, S., & Goldstein, I. J. (1978) *Ann. Immunol. (Paris) 129*, 143–158.

Maltosyl Isothiocyanate: An Affinity Label for the Glucose Transporter of the Human Erythrocyte Membrane. 1. Inhibition of Glucose Transport†

Richard E. Mullins‡ and Robert G. Langdon*

ABSTRACT: Maltosyl isothiocyanate (MITC) has been synthesized from maltose with an overall yield of 88%. It has been found to be a potent irreversible inhibitor of zero trans influx of glucose with human erythrocytes. Kinetic analysis of glucose transport after treatment of erythrocytes with MITC revealed that V_T was diminished while K_T was unchanged. Transportable sugars and competitive inhibitors of monosaccharide transport protected against MITC inhibition, while carbohydrates which do not interact with the transporter gave no protection. Covalent inhibitors of anion transport were without effect on glucose transport. MITC fulfilled the kinetic requirements for an affinity label of the glucose transporter of human erythrocytes [Groman, E. V., Schultz, R. M., & Engel, L. L. (1977) *Methods Enzymol. 46*, 54].

Monosaccharide transport through the plasma membranes of mammalian cells has been studied vigorously during the past 30 years, and an extensive body of kinetic evidence supports the conclusion that passive entry into cells such as erythrocytes, adipocytes, and muscle cells is a facilitated diffusion process (LeFevre, 1961; Stein, 1967; Deuticke, 1977; Wilson, 1978). However, the answers to the key questions of the identity of the membrane transporter and the mechanism by which translocation is effected still remain uncertain.

Two general approaches have been used to identify the glucose transporter of human erythrocytes and rat adipocytes. The first of these has employed transport inhibitors to label the transporter. The types of compounds used have been substrate analogue covalent affinity labels (Taverna & Langdon, 1973b; Trosper & Levy, 1977), nonspecific covalent inhibitors such as maleimides (Batt et al., 1976) and fluorodinitrobenzene (Jung & Carlson, 1975), and a bound reversible inhibitor, cytochalasin B, in conjunction with differential membrane extraction and fluorodinitrobenzene labeling (Lienhard et al., 1977; Zoccoli et al., 1978; Jung & Rampal, 1977; Pinkovsky et al., 1978). The second general approach has been to extract, purify, and isolate a specific membrane protein which, when reassociated with a lipid bilayer, conferred upon it stereospecific glucose transport (Kasahara & Hinckle, 1976, 1977; Kahlenberg, 1976; Zala & Kahlenberg, 1976; Jones & Nickson, 1978; Goldin & Rhoden, 1978; Shanahan & Czech, 1977a,b; Phutrakul & Jones, 1979).

Although some investigators have reported evidence which suggests that other proteins are involved, most evidence has supported the view that the transporter is an integral membrane protein having a M_r of either 90 000–100 000 [band 3 of the erythrocyte membrane in the terminology of Fairbanks

† From the Department of Biochemistry, University of Virginia School of Medicine, Charlottesville, Virginia 22908. *Received July 20, 1979.* This work was supported by Research Grant GM-19319 from the National Institutes of Health.

‡ Material for this publication is from a dissertation submitted by R.E.M. in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School of Arts and Sciences, University of Virginia.

0006-2960/80/0419-1199$01.00/0

et al. (1971)] or 50 000–60 000 (band 4.5 of the erythrocyte membrane). In general, those investigators who have used covalent inhibitors which are substrate analogues as affinity labels have concluded that band 3 is the more likely candidate, while those who have employed nonspecific inhibitors and extractions of erythrocyte membranes with nonionic detergents to purify the transporter and demonstrate reconstitution have concluded that the transporter is a component of band 4.5. However, Jones & Nickson (1978) have reported that a band 3 preparation of human erythrocytes confers glucose transport activity upon planar lipid bilayers, and Shanahan & Czech (1977a,b) have observed reconstitution of glucose transport with a 94 000 M_r protein extracted from adipocyte membranes.

Extraction of erythrocyte membranes with nondenaturing, nonionic detergents is known to activate their endogenous proteases (Bernacki & Bosmann, 1972; Tokes & Chambers, 1975), and this raises the possibility that protein components isolated from such extracts may have suffered degradation during extraction and purification. On the other hand, the substrate analogue glucosyl isothiocyanate, which had been used previously as an affinity label for the erythrocyte glucose transporter (Taverna & Langdon, 1973b), suffered from the defect that it was transported through the membrane by the monosaccharide transporter and nonspecifically labeled intracellular hemoglobin as well as most membrane proteins disposed exclusively on the cytoplasmic surface of the membrane.

Since affinity labels offer the possibility of labeling the transporter with minimal perturbation of other membrane components, we have approached the question of transporter identity by attempting to synthesize a specific affinity label which could be free of the deficiencies exhibited by glucosyl isothiocyanate. In designing such a compound, we required a nonpermeant glucose analogue which inhibited monosaccharide transport by forming a stable covalent bond with the transporter. In addition, we required that this compound react in a stoichiometric manner with a single membrane protein. Its reaction with this protein should be inhibited by transported monosaccharides and by reversible competitive inhibitors of monosaccharide transport, but it should not be affected by compounds such as L-glucose which do not interact with the transporter.

Here we report the synthesis of maltosyl isothiocyanate and evidence that this compound, which we have found to act as a powerful inhibitor of glucose transport, satisfies the kinetic criteria (Groman et al., 1977) for an affinity label of the glucose transporter of the human erythrocyte.

Experimental Procedures

Materials

Maltose, grade II, glucose oxidase, Type 5, Triton X-100, D-mannose, ethylenediaminetetraacetic acid (EDTA), and bovine catalase were purchased from Sigma Chemical Co. Anhydrous ammonia was obtained from Matheson. Thiophosgene was obtained from Aldrich. [^{14}C]Maltose, uniformly labeled, 360 Ci/mol, was purchased from ICN.

Dextrose was purchased from Pfanstiehl Laboratories. L-Glucose was supplied by Calbiochem. Cytochalasin B was from I.C.I. Research Laboratories. 4,4′-Diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) was purchased from Polyscience, Inc. *p*-Sulfophenyl isothiocyanate was supplied by Aldrich. All other chemicals were reagent grade. Polygram Sil-G thin-layer chromatography plates were supplied by Brinkman Instruments Co. Components on thin-layer plates were routinely detected by spraying with 1% $NaIO_4$, followed by 1% $KMnO_4$ (Wolfrom & Miller, 1956). Ninhydrin-reactive substances were detected by spraying with 0.5% ninhydrin in 50% ethanol. High-pressure liquid chromatography was performed on a Waters Associates ALC 202/401 with a Whatman PSX-1025 PAC column using water–acetonitrile azeotrope as the solvent. Ultraviolet absorption spectra were obtained by using a Beckman DB spectrophotometer. Natural abundance ^{13}C NMR was performed on a JEOL FX 60 Q spectrophotometer using enriched sodium [^{13}C]acetate as an internal standard. The positions of resonance peaks are relative to that of tetramethylsilane.

Methods

Synthesis of Maltosylamine. Commercial maltose was purified by conversion to the octaacetate, recrystallization of this derivative, deacylation, and crystallization of the maltose as described by Wolfrom & Thompson (1962). Maltosylamine was synthesized by modification of a method described by Muskat (1934). Dry maltose (15–45 mg) was dissolved in anhydrous methanol and introduced into a tube which was attached to a high-vacuum manifold. After the methanol had been evaporated under reduced pressure, anhydrous NH_3 gas was introduced into the manifold and condensed at −78 °C in the tube containing maltose until 2–4 mL of liquid NH_3 was present. The tube was sealed and allowed to stand at room temperature for 24–48 h; it was then stored at −20 °C until used. To recover maltosylamine, we cooled the tube to −78 °C, reattached it to the vacuum line, and removed the ammonia in vacuo. Maltosylamine was recovered as a white powder. It was usually used within 24 h for synthesis of maltosyl isothiocyanate.

Synthesis of Maltosyl Isothiocyanate. Sufficient 0.3 M $NaHCO_3$ in water was added to a tube containing dry maltosylamine to give a final concentration of 50 mM carbohydrate. A threefold excess of thiophosgene was added, and reaction was allowed to proceed under vigorous stirring for 15–20 min at 20 °C. When CO_2 evolution had ceased, excess thiophosgene was removed by extracting the reaction mixture twice with 3 volumes of dichloromethane, leaving a pale yellow aqueous phase containg maltosyl isothiocyanate. This solution was used immediately for analysis or in reactions with erythrocytes.

Measurement of the Rate of Glucose Transport. Fresh human blood was used in all experiments; it was drawn from healthy male donors, and EDTA, 1 mg/mL of blood, was used as an anticoagulant. Erythrocytes were sedimented from blood by centrifugation for 10 min at 3500*g*. The collected cells were washed 5 times by resuspension in 150 mM NaCl–5 mM sodium phosphate, pH 8.0 [phosphate-buffered saline (PBS)],[1] and the buffy coat containing leukocytes was carefully removed by aspiration after each centrifugation. The washed cells were always used within several hours for transport experiments. All transport studies were performed on resealed glucose oxidase-catalase containing pink ghosts (GOC ghosts) using an oxygen electrode as described by Taverna & Langdon (1973a) and measured the rate of glucose entry under zero trans conditions.

Reaction of MITC with Erythrocytes or Sealed Erythrocyte Ghosts. MITC was reacted with either GOC ghosts or intact erythrocytes with identical results. To react MITC with erythrocytes or GOC ghosts, we made a 50% suspension of

[1] Abbreviations used: MITC, maltosyl isothiocyanate; PBS, phosphate-buffered saline; glycine–PBS, 100 mM glycine dissolved in PBS, pH 8; GOC ghosts, glucose oxidase-catalase containing ghosts; EDTA, ethylenediaminetetraacetic acid; KRP, Krebs Ringer's phosphate.

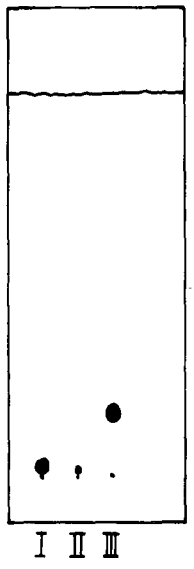

FIGURE 1: Thin-layer chromatogram of the products of reacting maltose with liquid ammonia. Approximately 50 mg of purified maltose was dissolved in approximately 2 mL of anhydrous ammonia as described under Experimental Procedures. The reaction was allowed to proceed 48 h when the ammonia was allowed to evaporate under anhydrous conditions, leaving maltosylamine. Developing solvent was the upper phase of 1-butanol–30% ammonia in water, 1:1. Spots were detected by IO_4^- spray as described in the text. I, glucose; II, maltose; III, maltosylamine.

erythrocytes or GOC ghosts in PBS at 37 °C 0.9, 4.5, 9.0, 27, or 41 mM in MITC by the addition of an aliquot of freshly prepared 45 mM MITC. The reaction was allowed to proceed in a 37 °C bath with occasional mixing for 1–30 min when the suspension was diluted 50-fold by the addition of a solution containing 100 mM glycine, 150 mM NaCl, and 5 mM sodium phosphate, pH 8.0 (glycine–PBS), to consume the unreacted MITC. MITC-treated erythrocytes or GOC ghosts were then collected by centrifugation and washed one additional time in glycine–PBS, followed by three washes in PBS. MITC-treated erythrocytes were converted immediately to GOC ghosts for measurements of glucose transport. If GOC ghosts had been treated with MITC, they were washed and used without delay in transport studies. It was determined that the GOC ghosts were stable for at least 24 h when stored at 4 °C. However, in all of the experiments reported here, less than 12 h elapsed from the time blood was drawn until the end of the experiment. When protecting agents were used, they were included at the designated concentration during the incubation with MITC.

Results

Commercial maltose is usually contaminated with 2–5% glucose and other carbohydrates. Since glucosyl isothiocyanate has been shown to be a potent inhibitor of glucose transport in erythrocytes and since it also permeates the membrane (Taverna & Langdon, 1973b), it was imperative that glucose be removed from the maltose preparation. Thin-layer chromatography and LC demonstrated that glucose was indeed present in the maltose which we obtained commercially. However, no glucose was detected by either means in the maltose purified via the octaacetate. This procedure yielded a product which was composed of 99.85% maltose as determined by LC.

Completeness of conversion of maltose to maltosylamine was assessed by thin-layer chromatography of the products and by quantitative analysis of ammonia released upon hydrolysis in 0.6 N HCl. In Figure 1 is shown a silica gel thin-layer chromatogram of glucose (channel I), maltose (channel II), and the products of reaction of maltose with liquid NH_3 (channel III) after the plate had been sprayed with IO_4^-/MnO_4^-. It is evident that a single major product (R_f 0.16) resulted from synthesis and that the only visible contaminant was a trace of unreacted maltose (R_f 0.01). The major product was ninhydrin positive, and it was the only component which stained with ninhydrin. In order to quantitatively evaluate the yield, aliquots of a maltosylamine solution of 80 μM in 0.6 N HCl were heated for 3 min at 100 °C to completely hydrolyze maltosylamine to ammonia and maltose (Pigman et al., 1951). Aliquots containing 20 nmol of maltosyl equivalent were injected into the reaction coil of a Beckman Model 119 amino acid analyzer. The areas under the emergent peaks were measured, and the quantity of ammonia was calculated. It was found that from 20 nmol of synthesized maltosylamine, 20.95 ± 0.21 nmol of ammonia was released by acid hydrolysis.

Maltosylamine was converted to MITC by reaction with thiophosgene. The product of this reaction was examined by thin-layer chromatography, ultraviolet spectroscopy, and natural abundance ^{13}C NMR. The NMR spectrum was identical with that of maltose except that the resonance intensities were greatly reduced at 92.58 and 96.45 ppm, which correspond to the resonance positions of C-1 α and C-1 β carbons having hydroxyl substitutions (Dorman & Roberts, 1971); in addition, there was a new peak at 140.85 ppm which corresponds to an –NCS carbon (Maciel & Beatty, 1965). The reaction product had an ultraviolet absorption maximum at 245 nM which increased in intensity upon addition of ammonia or glycine. This is typical of alkyl isothiocyanates (Schmid & Karrer, 1948; Koch, 1949).

In order to quantitate the overall yield of MITC and evaluate its purity, we synthesized this compound from [^{14}C]maltose and chromatographed the final product in triplicate on silica gel thin-layer plates before and after addition of ammonia to convert MITC to maltosylthiourea. Staining for carbohydrates revealed one major spot (R_f 0.35) before addition of ammonia and one major spot (R_f 0.42) after its additon (Figure 2, channels I and II, respectively). Radioautography of a duplicate plate revealed that the major radioactive components coincided exactly in mobility with the stained ones. From each channel of the third plate, segments of silica gel 5 mm in length were scraped from the plate and analyzed for radioactivity by liquid scintillation counting. The distribution of isotope is shown in the bottom panel of Figure 2. In each channel there was a small peak of radioactivity (R_f 0.64) which corresponded to maltose. From these experiments it could be calculated that the overall yield of MITC from maltose was 88% of theoretical, and the only impurity detected was a small amount of maltose.

Kinetics of Inhibition of Glucose Transport by MITC. Preliminary experiments revealed that pretreatment of erythrocytes or pink ghosts with MITC resulted in substantial irreversible inhibition of glucose translocation through their membranes when this was measured subsequently. An absolute requirement for the kinetic demonstration of an affinity label is that equilibrium binding of the specific ligand precede irreversible inactivation, which can be demonstrated by the kinetics of the inactivation process. For a process such as

$$T + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} T{\cdot}A \xrightarrow{k_2} T{-}A^*$$

where T = transporter, A = affinity label, T·A = a reversible complex between the transporter and affinity label and T–A* = an inactive covalent compound between affinity label and

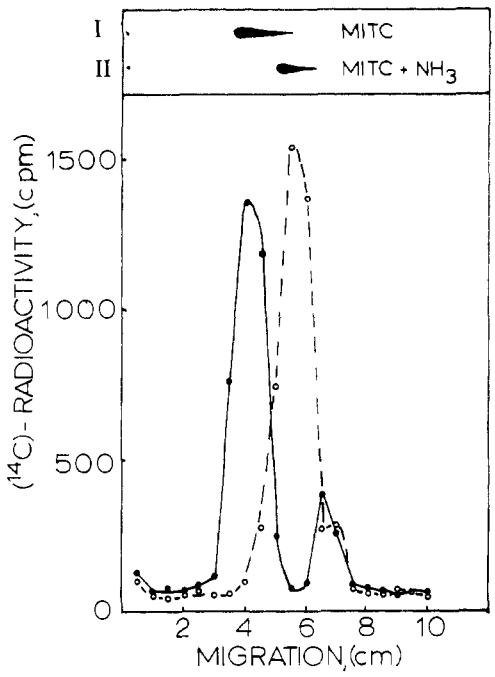

FIGURE 2: Distribution of ^{14}C on a thin-layer chromatograph of [^{14}C]MITC and [^{14}C]maltosylthiourea. [^{14}C]MITC was prepared as described and divided into two portions. To one portion of the [^{14}C]MITC was added 0.1 volume of 10% NH_4OH, and the reaction was allowed to proceed for 2 h at room temperature to produce maltosylthiourea from the isothiocyanate. The other portion of [^{14}C]MITC was kept at 4 °C. Duplicate chromatograms were run. Carbohydrates were detected on one plate by spraying with 1% $NaIO_4$, followed 5 min later by spraying with 1% $KMnO_4$, and allowing the plates to develop 20 min before washing with tap water. The other plate was analyzed for radioactivity by scraping 5-mm sections of the silica gel adsorbent into scintillation bags, adding scintillation cocktail, and counting in a liquid scintillation counter.

transporter, let $T_a = T + T{\cdot}A$ = active transporter, T_0 = total transporter, and $K_A = k_{-1}/k_1$. Then, the appropriate equation describing this process is

$$\ln \frac{T_a}{T_0} = -\frac{k_2 t}{1 + K_A/(A)} = -k_{obsd} t \qquad (1)$$

It therefore became of interest to examine the kinetics of the inhibitory reaction. For this purpose sealed GOC pink ghosts were treated with 4.5, 9.0, or 27 mM MITC for 2, 5, 10, and 20 min at 37 °C. The reaction was quenched by addition of glycine–PBS as described under Experimental Procedures. The rate of glucose entry after each period of MITC pretreatment was then measured from a solution containing 100 mM glucose. The results of a typical experiment are presented in Figure 3. It is clear that there was a progressive decrease in maximal transport rate which was pseudo first order in transport activity. However, it is apparent that, as predicted by eq 1, the slopes of these lines (k_{obsd}) were not linearly related to the concentrations of MITC; instead, the increment in inhibition decreased as the concentration of MITC was increased. This is shown more clearly in Figure 4, where the rate of inactivation (k_{obsd}) is plotted vs. MITC concentrations. It is evident that k_{obsd} approached a limiting value, k_2. When these data were treated by a nonlinear regression analysis (Wilkinson, 1961), values for K_A and k_2 were obtained. These were $K_A = 8.8 \pm 3.3$ mM and $k_2 = 0.071 \pm 0.007$ min^{-1}. A linear regression analysis of the same data fitted to a linear transformation of eq 1 gave values of $K_A = 15.6$ mM and $k_2 = 0.09$ min^{-1} with a correlation coefficient of 0.996. To examine the possibility that the apparent inhibition of transport might actually result from inhibition of intracellular glucose oxidase, we lysed aliquots of both untreated and MITC-treated GOC ghosts from each experiment by addition of sufficient Triton X-100 to give a final detergent concentration of 0.5%, and the released glucose oxidase activity was measured in the oxygen electrode assembly. No differences in activity were detected between the enzyme released from control and that from MITC-treated ghosts. This concentration of Triton X-100 had been shown previously (Taverna & Langdon, 1973a) to completely release trapped glucose oxidase from GOC ghosts. They also reported that the glucose oxidase-catalase system was fully active in 0.5% Triton X-100.

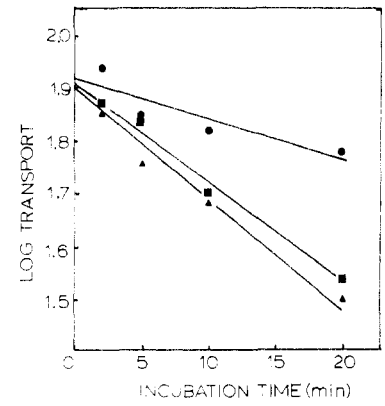

FIGURE 3: Time dependence of inhibition of glucose influx into erythrocyte ghosts incubated with three concentrations of MITC. 50% suspensions of GOC ghosts in PBS were made 4.5 (●), 9.0 (■), or 27 mM (▲) in MITC by the addition of a freshly prepared solution of 45 mM MITC. The reaction was allowed to proceed for 1–20 min when it was terminated by the addition of 50 volumes of glycine–PBS, and the ghosts were washed as described in the text. The rate of zero trans glucose influx into control and MITC-treated GOC ghosts was then determined at an ambient glucose concentration of 100 mM.

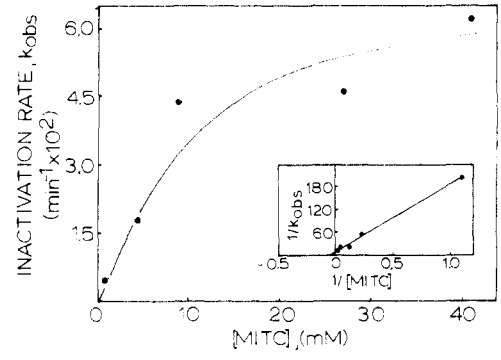

FIGURE 4: Dependence of the rate of glucose transport inactivation upon MITC concentration. Five experiments of the type illustrated in Figure 3 were carried out. The slopes of the resulting straight lines, k_{obsd}, were calculated by linear regression and plotted on the ordinate vs. the MITC concentrations which were utilized.

Effect of MITC Pretreatment upon the Kinetics of Glucose Transport into Erythrocyte Ghosts. The preceding experiments measured the rate of glucose influx into sealed ghosts from a solution containing 100 mM glucose, a concentration which is saturating for influx into erythrocytes and erythrocyte ghosts (Taverna & Langdon, 1973a). They therefore measured the maximal rate of influx V_T. To determine whether MITC treatment also affected the half-saturation constant K_T, we treated GOC pink ghosts with 9 mM MITC for 5 min at 37 °C, conditions which were shown in Figure 3 to lead to 30% inhibition. Dependence of the rate of glucose influx upon ambient glucose concentration was measured for MITC-treated and control ghosts. The results of several experiments are summarized in Table I. It is evident that MITC treatment

Table I: Effects of MITC Pretreatment on the Kinetic Parameters of Zero Trans Glucose Influx into GOC Ghosts[a]

calculation	K_T (mM)	V_T (mM/min)	r^2
linear regression[b]			
control	9.4	49	0.995
MITC-treated	8.1	36	0.991
nonlinear regression[c]			
control	8.6 ± 0.7	47 ± 1	
MITC-treated	8.5 ± 0.7	36 ± 1	

[a] A suspension of GOC ghosts was made 9 mM in MITC and incubated at 37 °C for 5 min. The reaction was terminated as described under Experimental Procedures, and the glucose uptake of the control and MITC-pretreated GOC ghosts was measured at different concentrations of external glucose by the oxygen electrode technique. [b] Linear regression analysis of a double-reciprocal plot of the data from three experiments. [c] Nonlinear regression analysis of three identical experiments by the method of Wilkinson (1961).

affected only V_T while K_T was unchanged.

Effect of Other Isothiocyanates on Glucose Transport. The preceding experiments showed that MITC pretreatment inhibited the maximal rate of glucose influx into GOC ghosts. However, isothiocyanates are reactive compounds, and such a result might have been obtained if MITC had reacted nonspecifically with the transporter. To test if this was a general effect of isothiocyanates, we treated GOC ghosts with two water-soluble isothiocyanates, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) and *p*-sulfophenyl isothiocyanate, which are potent inhibitors of anion transport in erythrocytes (Cabantchik & Rothstein, 1974; Ho & Guidotti, 1975). When GOC ghosts were treated with 20 μM DIDS for 30 min at 37 °C, conditions more severe than those which have been shown to completely inhibit anion transport (Ship et al., 1977), there was no effect on glucose influx as monitored by the oxygen electrode technique. Since this concentration of DIDS was approaching its maximum water solubility and it was lower than the concentration of MITC found to inhibit glucose transport, GOC ghosts were treated with 20 mM *p*-sulfophenyl isothiocyanate for 30 min at 37 °C in PBS. Under these severe conditions, glucose uptake as measured by the oxygen electrode was inhibited less than 10%. MITC, under these same conditions (20 mM; 30 min; 37 °C), was found to inhibit influx of glucose into GOC ghosts ~80%. Taverna & Langdon (1973b) also reported that L-glucosyl isothiocyanate had no effect on glucose uptake in erythrocytes. It would appear that MITC is capable of reacting with the glucose transporter in a way different from that of the other isothiocyanates tested.

Protection of Transport from MITC Inhibition by Substrates and Reversible Inhibitors of Transport. If inhibition by MITC resulted from a specific reaction at a glucose binding site, it might be expected that transportable substrates or reversible competitive inhibitors of transport might afford some degree of protection against MITC inhibition. On the other hand, sugars such as L-glucose and sucrose which do not interact with the transporter should afford no protection. These predictions were tested by including the compounds shown in Table II during preincubation with MITC. These include mannose, a transported monosaccharide, L-glucose, a noninteracting monosaccharide, maltose, a nontransported disaccharide which acts as a competitive inhibitor of glucose transport, sucrose, a disaccharide which has no effect on glucose influx, and cytochalasin B, a powerful reversible competitive inhibitor of glucose transport (Taverna & Langdon, 1973c; Block, 1973) which may interact directly with the transporter (Lienhard et al., 1977; Zoccoli et al., 1978) at the glucose binding site. It is evident that only those compounds which appear to compete with glucose for binding to the monosaccharide transporter were effective in preventing MITC inactivation of transport. Furthermore, the extent of protection afforded by these compounds agreed well with that predicted from their reported half-saturation constants. This suggests that the MITC reaction site is a site which interacts specifically with transported sugars or that access of MITC to its reaction site is specifically inhibited only by compounds which compete with glucose for its interaction with the transporter.

These experiments were carried out at single fixed times and concentrations of MITC and protectors. In order to examine the time dependence of the protective effect of substrates and reversible inhibitors, we performed experiments of the type illustrated in Figure 5 where GOC ghosts were incubated with MITC in the presence or absence of protectors for variable

Table II: Protective Effect of Substrates and Reversible Competitive Inhibitors against MITC Inhibition of Glucose Influx into Erythrocyte Ghosts[a]

pretreatment	V_T (% of control)[b]
none	100
MITC	52 (±2.7)
MITC plus 150 mM maltose	91 (±4.7)
MITC plus 150 mM mannose	79
MITC plus 10 μM cytochalasin B	97 (±3.8)
MITC plus 150 mM L-glucose	51 (±1.5)
MITC plus 150 mM sucrose	53 (±2.1)

[a] GOC ghosts were incubated for 5 min at 37 °C alone or in the presence of the protective agent to be tested. MITC was then added to give a final concentration of 9 mM, and incubation was continued for 10 min. The reaction was terminated by addition of glycine–PBS, and the cells were washed free of MITC and added protecting agents as described under Experimental Procedures prior to measurement of the rates of glucose influx from 100 mM ambient glucose. [b] Average of three experiments except for mannose, which was a single experiment. The values in parentheses are standard deviations.

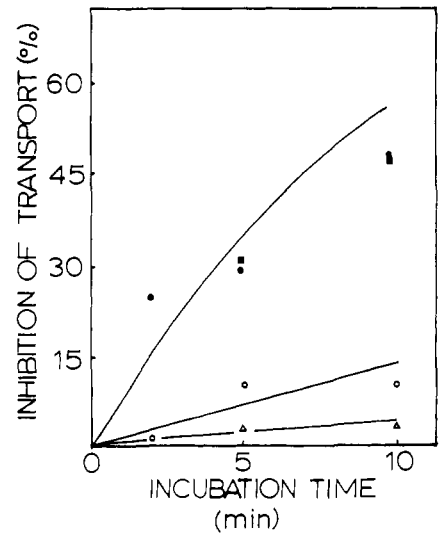

FIGURE 5: Time dependence of the protection against MITC inhibition afforded by maltose and cytochalasin B. GOC ghosts were incubated at 37 °C in the presence of 9 mM MITC (●), 9 mM MITC plus 150 mM L-glucose (■), 9 mM MITC plus 150 mM maltose (○), and 9 mM MITC plus 10 μM cytochalasin B (△) for the times shown on the abscissa. Incubations were terminated by addition of 100 mM glycine in PBS and washing as described in Figure 1. The rates of influx of glucose from a 100 mM external solution were then measured and compared to the influx rates into control ghosts, which were incubated at 37 °C without MITC or any protecting agents.

periods. It is evident that maltose and cytochalasin B protected at all times, while inclusion of L-glucose in the incubation mixture had no effect upon MITC inhibition at any time tested.

Discussion

Several criteria have been established to determine if a particular enzyme inhibitor is indeed an affinity label. Groman et al. (1977) have suggested four criteria for the definition of affinity labels. The first criterion is that the putative affinity label be a substrate analogue. Second, the rate of inactivation of the enzyme should be pseudo first order, and saturation of the rate of inactivation at high inhibitor concentrations should be demonstrated. A third criterion is that inclusion of a real substrate or a competitive inhibitor of the enzyme should provide a degree of protection from inactivation by the affinity label. Finally, the affinity label must react with the enzyme in a stoichiometric fashion.

The data presented in this communication demonstrate that MITC fulfills the first three criteria, the kinetic criteria, for a true affinity label of the erythrocyte glucose transport system. Maltose has long been known to be a competitive inhibitor of glucose transport (Lacko & Berger, 1962; Chen & LeFevre, 1965). It successfully competes with glucose for the active site of the transporter, since the K_I for maltose (14 mM) is very near the K_T for glucose transport (2–10 mM) (Krupka, 1971; Taverna & Langdon, 1973a). The introduction of the small uncharged isothiocyanate group into the maltosyl residue only slightly changed the structure, and the reversible dissociation constant of the MITC–transporter complex (9–16 mM) was found to be very near that of the reported K_I of maltose (14 mM). We conclude that MITC sufficiently resembles a true substrate to satisfy the first criterion.

It was shown in Figures 3 and 4 that the rate of inactivation was indeed pseudo first order and that a high concentration of MITC produced saturation of the rate of inactivation. It should also be noted that upon treatment of erythrocytes or GOC ghosts with MITC the V_T was diminished while K_T remained unchanged. This indicates that when MITC reacts with a particular protein of the transport system, it completely abolishes that carrier's ability to function, as opposed to altering its affinity for substrate. This argues for the hypothesis that MITC reacts with the active site of the transporter.

The most convincing evidence that MITC is an affinity label is the data shown in Table II and Figure 5. Compounds which are known to interact with the glucose transporter as either substrates or competitive inhibitors offer considerable protection from MITC inactivation, while carbohydrates not recognized by the transporter have no effect on MITC inactivation. Cytochalasin B in particular has been shown to interact with the glucose binding site of the transport system (Lienhard et al., 1977; Lin & Spudich, 1974) and was very effective in preventing MITC inactivation of the transport system.

The fact that the anion transport inhibitors DIDS and *p*-sulfophenyl isothiocyanate had little or no effect on glucose transport indicates that MITC and these other reagents are directed at different proteins of the erythrocyte membrane and that monosaccharide transport and anion transport are independent functions. From the evidence given in this paper, it is evident that the kinetic criteria for an affinity label of the glucose transport system are satisfied by MITC. By use of radioactively labeled MITC, it could be determined if MITC satisfied the fourth criterion, that of reacting stoichiometrically with the transporter. This final criterion and the identification of the MITC target protein, and thus the glucose transporter, are discussed in the following paper (Mullins & Langdon, 1980).

Acknowledgments

We thank Dr. Ross Brown of the Virginia Polytechnic Institute and State University, Department of Biochemistry, for performing LC analyses and Dr. Charles Schmidt of the University of Virginia, Department of Biochemistry, for carbon-13 magnetic resonance studies.

References

Batt, E. R., Abbot, R. E., & Schachter, D. (1976) *J. Biol. Chem. 251*, 7184.
Bernacki, R. J., & Bosmann, H. B. (1972) *J. Membr. Biol. 7*, 1.
Block, R. (1973) *Biochemistry 12*, 4799.
Cabantchik, Z. I., & Rothstein, A. (1974) *J. Membr. Biol. 15*, 207.
Chen, L., & LeFevre, P. G. (1965) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 24*, 465.
Deuticke, B. (1977) *Rev. Physiol. Biochem. Pharmacol. 78*, 1.
Dorman, D. E., & Roberts, J. D. (1971) *J. Am. Chem. Soc. 93*, 4463.
Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606.
Goldin, S. M., & Rhoden, V. (1978) *J. Biol. Chem. 253*, 2575.
Groman, E. V., Schultz, R. M., & Engel, L. L. (1977) *Methods Enzymol. 46*, 54.
Ho, M. K., & Guidotti, G. (1975) *J. Biol. Chem. 250*, 675.
Jones, M. N., & Nickson, J. K. (1978) *Biochim. Biophys. Acta 509*, 260.
Jung, C. Y., & Carlson, L. M. (1975) *J. Biol. Chem. 250*, 3217.
Jung, C. Y., & Rampal, A. L. (1977) *J. Biol. Chem. 252*, 5456.
Kahlenberg, A. (1976) *J. Biol. Chem. 251*, 1582.
Kasahara, M., & Hinkle, P. C. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 396.
Kasahara, M., & Hinkle, P. C. (1977) *J. Biol. Chem. 252*, 7384.
Koch, H. P. (1949) *J. Chem. Soc.*, 394.
Krupka, R. M. (1971) *Biochemistry 10*, 1143.
Lacko, L., & Burger, M. (1962) *Biochem. J. 83*, 622.
LeFevre, P. G. (1961) *Pharmacol. Rev. 13*, 39.
Lienhard, G. E., Gorga, F. R., Orasky, J. E., Jr., & Zoccoli, M. A. (1977) *Biochemistry 16*, 4921.
Lin, S., & Spudich, J. A. (1974) *J. Biol. Chem. 249*, 5778.
Maciel, G. E., & Beatty, D. A. (1965) *J. Phys. Chem. 69*, 3920.
Mullins, R. E., & Langdon, R. G. (1980) *Biochemistry* (following paper in this issue).
Muskat, I. E. (1934) *J. Am. Chem. Soc. 56*, 693.
Phutrakul, S., & Jones, M. N. (1979) *Biochim. Biophys. Acta 550*, 188.
Pigman, W., Cleveland, E. A., Couch, D. H., & Cleveland, J. H. (1951) *J. Am. Chem. Soc. 73*, 1976.
Pinkofsky, H. B., Rampal, A. L., Cowden, M. A., Jung, C. Y. (1978) *J. Biol. Chem. 253*, 4930.
Schmid, H., & Karrer, P. (1948) *Helv. Chim. Acta 31*, 1017.
Shanahan, M. F., & Czech, M. P. (1977a) *J. Biol. Chem. 252*, 8341.
Shanahan, M. F., & Czech, M. P. (1977b) *J. Biol. Chem. 252*, 6554.
Ship, S., Shami, Y., Breuer, W., & Rothstein, A. (1977) *J. Membr. Biol. 33*, 311.

Stein, W. D. (1967) *The Movement of Molecules Across Cell Membranes*, Academic Press, New York.
Taverna, R. D., & Langdon, R. G. (1973a) *Biochim. Biophys. Acta 298*, 412.
Taverna, R. D., & Langdon, R. G. (1973b) *Biochim. Biophys. Acta 323*, 207.
Taverna, R. D., Langdon, R. G. (1973c) *Biochem. Biophys. Res. Commun. 54*, 593.
Tokes, Z. A., & Chambers, S. M. (1975) *Biochim. Biophys. Acta 389*, 325.
Trosper, T., & Levy, D. (1977) *J. Biol. Chem. 252*, 181.
Wilkinson, G. N. (1961) *Biochem. J. 80*, 324.
Wilson, D. B. (1978) *Annu. Rev. Biochem. 47*, 933.
Wolfrom, M. L., & Miller, J. B. (1956) *Anal. Chem. 28*, 1037.
Wolfrom, M. L., & Thompson, A. (1962) *Methods Carbohydr. Chem. 1*, 334.
Zala, C. A., & Kahlenberg, A. (1976) *Biochem. Biophys. Res. Commun. 72*, 866.
Zoccoli, M. A., Baldwin, S. A., & Lienhard, G. E. (1978) *J. Biol. Chem. 253*, 6923.

Maltosyl Isothiocyanate: An Affinity Label for the Glucose Transporter of the Human Erythrocyte Membrane. 2. Identification of the Transporter†

Richard E. Mullins‡ and Robert G. Langdon*

ABSTRACT: Maltosyl isothiocyanate (MITC), a potent irreversible inhibitor of glucose transport in human erythrocytes [Mullins, R. E., & Langdon, R. G. (1980) *Biochemistry* (preceding paper in this issue)], has been found to react almost exclusively with band 3 of the human erythrocyte membrane. The incorporation of [^{14}C]MITC into band 3 was found to be antagonized by transportable sugars or competitive inhibitors of transport. On the basis of [^{14}C]MITC incorporation into band 3 and MITC inhibition of transport, it is estimated that there are 3×10^5 glucose transporters present in the erythrocyte membrane. It was found that [^{14}C]MITC-labeled band 3 could be converted into ^{14}C-labeled band 4.5 during the Triton X-100 extraction procedure described by Kasahara & Hinkle [Kasahara, M., & Hinkel, P. C. (1977) *J. Biol. Chem. 252*, 7384]. On the basis of the evidence presented here and in the preceding paper, it is suggested that in the native erythrocyte membrane a component of band 3 is the glucose transport protein and that during purification with nonionic detergents the transport protein may be enzymatically degraded with some retention of activity.

During recent years there have been several efforts to identify the glucose transporter in the human erythrocyte membrane. There have been two general approaches to this question of carrier identity. One approach has been to isolate and purify different membrane protein components and incorporate them into synthetic membranes in an attempt to reconstitute D-glucose-specific translocation. Another approach has been to devise an affinity label for the glucose transport protein and to label the carrier while in its native state in the membrane. Investigators using these techniques have been led to different conclusions on the question of carrier identity.

Taverna & Langdon (1973b) reported that the glucose transporter was probably a component of band 3 [nomenclature of Fairbanks et al. (1971)] based on their data using D-glucosyl isothiocyanate as an affinity label. Kasahara & Hinkle (1977) have reported that band 4.5, a 55 000 M_r protein component of the membrane, is the apparent carrier from their reconstitution experiments. Jones & Nickson (1978), on the other hand, using reconstitution into planar black lipid membranes, have reported that band 3 is the most likely candidate to be the glucose carrier. Other investigators have presented evidence supporting either band 3 (Trosper & Levy, 1977; Phutrakul & Jones, 1979) or band 4.5 (Kahlenberg & Zala, 1977; Goldin & Rhoden, 1978; Batt et al., 1976; Abbot & Schachter, 1976; Zoccoli et al., 1978) as the carrier. In this paper we present evidence that may reconcile the observations of all investigators.

In the preceding paper (Mullins & Langdon, 1980) a kinetic analysis of maltosyl isothiocyanate (MITC)[1] inhibition of glucose transport in human erythrocytes was reported. MITC fulfilled the kinetic criteria for an affinity label of the glucose transport system proposed by Groman et al. (1977).

In this communication we show that [^{14}C]MITC combines covalently in a stoichiometric reaction with a component of band 3, and this combination is specifically antagonized by transported sugars or competitive inhibitors of transport; this fulfills the final requirement of an affinity label. This label can therefore be used to identify the glucose carrier.

We propose on the basis of evidence presented here and in the preceding paper that band 3 in the native erythrocyte membrane contains the glucose transport protein and that during the nonionic detergent extractions used by some investigators to purify the putative membrane transporter a 55 000-dalton fragment which migrates with band 4.5 is generated from band 3 by the action of neutral proteases associated with erythrocyte membranes. This band 4.5

† From the Department of Biochemistry, University of Virginia School of Medicine, Charlottesville, Virginia 22908. *Received July 20, 1979.* This work was supported by Research Grant GM-19319 from the National Institutes of Health.

‡ Material for this publication is from a dissertation submitted by R.E.M. in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School of Arts and Sciences, University of Virginia.

[1] Abbreviations used: MITC, maltosyl isothiocyanate; PBS, phosphate-buffered saline; glycine–PBS, 100 mM glycine dissolved in PBS, pH 8; GOC ghosts, glucose oxidase-catalase containing ghosts; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; FDNB, fluorodinitrobenzene; D-GITC, glucosyl isothiocyanate; PMSF, phenylmethanesulfonyl fluoride.

0006-2960/80/0419-1205$01.00/0