D-GLUCOSYL ISOTHIOCYANATE, AN AFFINITY LABEL FOR THE GLUCOSE TRANSPORT PROTEINS OF THE HUMAN ERYTHROCYTE MEMBRANE

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SUMMARY

D-glucosyl isothiocyanate has been found to be a potent irreversible inhibitor of glucose translocation in the human erythrocyte. [^{14}C]-D-glucosyl isothiocyanate was incorporated into two major proteins of the erythrocyte membrane, and this incorporation was inhibited by D-glucose. These proteins may be components of the glucose transport system.

INTRODUCTION

It has been recognized for many years that glucose enters most mammalian cells by carrier-mediated facilitated diffusion. However, little is known concerning the molecular constituents of the membrane which are involved in the transport process. We wish to report evidence which strongly suggests that D-glucosyl isothiocyanate is an effective irreversible inhibitor of membrane glucose transport in the human erythrocyte, and that [^{14}C]-D-glucosyl isothiocyanate may serve as an affinity label for the protein components of the transport system.

EXPERIMENTAL

Crystalline D-glucosylamine (MP 130°C) was synthesized by two methods, (1,2) and the products gave essentially equivalent results in the experiments to be reported. Infrared spectroscopy, as well as optical rotation (3), showed that the crystalline isomer, obtained in approximately 30% yield, was of the β configuration. This was converted to GITC by reaction with thiophosgene (4). Excess

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Abbreviation: GITC, D-glucosyl isothiocyanate.

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thiophosgene was removed by extraction with dichloromethane. Because the isothiocyanate is somewhat unstable, it was utilized immediately for reaction with the red cell membrane.

Human erythrocytes from freshly drawn blood were washed repeatedly by centrifugation in isotonic saline followed by KRP. Glucose transport was measured both by measuring the rate of influx of [^{14}C]-glucose into intact erythrocytes (5) and by utilizing an enzymatic method which we have recently described (6). To demonstrate inhibition of glucose transport into intact red cells by prior treatment with GITC a 50% erythrocyte suspension at 37°C was made 20 mM in GITC; after incubation at 37°C for 5 min., reaction was terminated by addition of 20 volumes of ice-cold KRP. The cells were washed repeatedly, and aliquots were then incubated with 30 mM [^{14}C]-glucose for 0 to 300 seconds at 22°C; transport was terminated by addition of ice-cold isotonic saline containing 10 mM $HgCl_2$ (5), the cells were harvested by centrifugation, and the [^{14}C]-glucose which they contained was assayed by liquid scintillation counting. GOC-ghosts were treated with GITC in essentially the same manner, and glucose transport was measured as previously described (6).

To demonstrate incorporation of [^{14}C]-GITC into membrane proteins, intact erythrocytes were incubated in KRP for 5 min. at 37°C with 20 mM [^{14}C]-GITC. Reaction was terminated by addition of 20 volumes of ice-cold KRP. From the washed [^{14}C]-labelled erythrocytes, hemoglobin-free ghosts were prepared (7). The membrane suspension was solubilized by making it 2% in sodium dodecyl sulfate and heating for 2 minutes in a steam bath (8). After cooling to room temperature, to each ml of membrane solution was added .05 ml of N-ethylmorpholine (89.9%, w/v) and 2.5 mg of dansyl chloride in 0.1 dimethyl formamide. After 1 hr. of reaction, the solution was subjected to disc gel electrophoresis on 5% polyacrylamide, 3% crosslinked as described by Weber and Osborn (9). The fluorescent protein bands were detected by excitation at 360 nanometers with an ultraviolet source,

Abbreviations: KRP, Krebs-Ringer-Phosphate buffer, pH 7.4; GOC-ghosts, Glucose Oxidase-containing pink erythrocyte ghosts.

TABLE I

INHIBITION OF [^{14}C]-GLUCOSE INFLUX INTO HUMAN ERYTHROCYTES

	Treatment*	% Initial Velocity†
I	Control	100
II	GITC	52
III	GITC plus Maltose	70
IV	GITC plus L-Glucose	52

* See Methods

† Initial velocity for control cells was 180 millimoles/liter cells/min

TABLE II

INCORPORATION OF [^{14}C]-GITC INTO SOLUBLE FRACTION OF HUMAN ERYTHROCYTES

Initial concentration external [^{14}C]-GITC	dpm in soluble fraction	
	without Glucose	100 mM Glucose
0.075 mM	251	23
0.375 mM	1353	79
0.750 mM	2350	190
3.75 mM	7582	927
7.50 mM	9739	1400

The soluble cellular contents upon hypotonic lysis of cells treated with [^{14}C]-GITC as in Figure 2 were examined for [^{14}C] content.

and their fluorescence was measured more quantitatively in a linear transport fluorescence gel scanner of our design. The gels were sliced transversely, and each 1.25 mm disc was rendered soluble by warming to 50°C with NCS (Amersham-

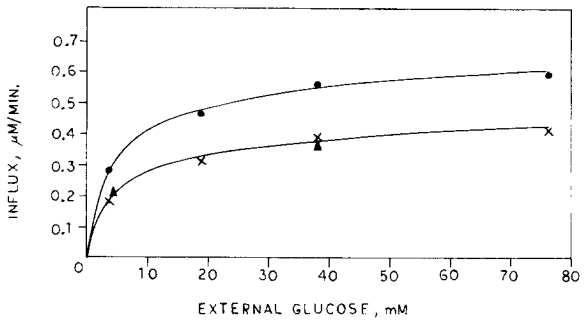

Fig. 1. Inhibition by 10mM GITC of D-glucose influx into GOC-erythrocyte ghosts(6). Where ●-● control; x-x GITC-treated; △-△ GITC plus 100 mM L-glucose treated.

Searle) in a scintillation vial. After the addition of scintillation cocktail each sample was counted in a Beckman LS-233 liquid scintillation counter.

RESULTS

The data in Table I show clearly that pretreatment of erythrocytes with 20 mM GITC resulted in substantial inhibition of [^{14}C]-D-glucose influx. Inclusion of 100 mM L-glucose during the incubation with GITC had no effect upon the extent of inhibition. On the other hand the presence of 100 mM maltose during treatment with GITC afforded substantial protection. Results very similar to those depicted in Table I were obtained in a number of essentially identical experiments.

The data in Figure 1 show that pretreatment with 10 mM GITC also effectively inhibited glucose influx into GOC-ghosts. L-glucose afforded no protection against its inhibitory action.

Figure 2a illustrates the incorporation of [^{14}C]-GITC into the membrane proteins of intact erythrocytes. It is apparent that extensive incorporation of the labelled compound occurred. It would be expected if GITC by virtue of its structural similarity to glucose were combining with a protein component of the membrane involved in glucose transport, that its incorporation should be inhibited by glucose. In Figure 2b it is clear that in the presence of 100 mM D-glucose, incorporation of the affinity label into components III (MW 100,000) and IV (MW 75,000) was substantially reduced. These experiments have been repeated many times with essentially the same results.

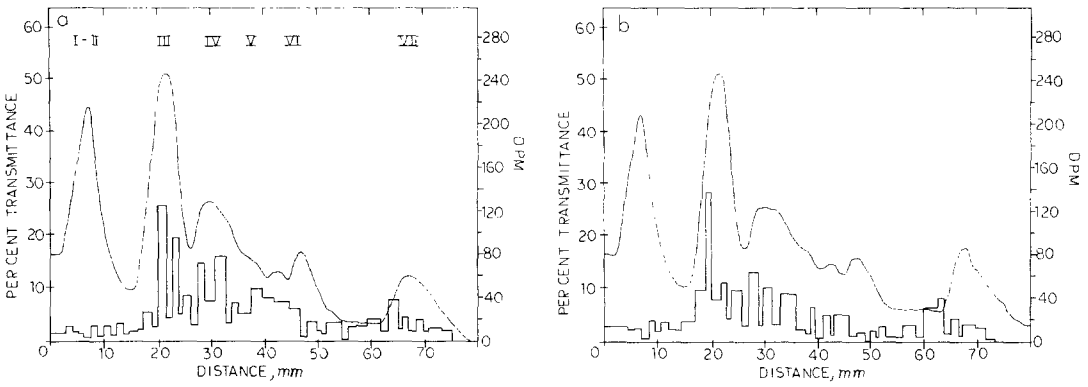

Fig. 2. Incorporation of [^{14}C]-GITC into membrane proteins of the human erythrocyte. (a)Membrane proteins of cells treated with [^{14}C]-GITC alone. (b)Membrane proteins of cells treated with [^{14}C]-GITC plus 100 mM D-glucose.

In experiments of the type illustrated in Figure 2, the soluble supernatent which was released upon hypotonic lysis from cells which had been treated with [^{14}C]-GITC and washed extensively was also examined. As shown in Table II, this fraction was also highly radioactive, which demonstrates that GITC penetrates the membrane. On the other hand, very little isotope was present in this fraction when the reaction was carried out in the presence of 100 mM D-glucose, which strongly suggests that GITC penetrates the membrane via the glucose transport system and competes with glucose for entry.

DISCUSSION

The data presented here indicate that GITC, a structural analogue of D-glucose which contains a reactive isothiocyanate group, may be a potent tool both for identification of the membrane components responsible for facilitated diffusion of glucose through the erythrocyte membrane, and for elucidating the mechanistic details of the transport process.

The data in Table II demonstrate that GITC penetrates the erythrocyte membrane, and probably enters via the glucose transport system. Furthermore, the results shown in Table I and Figures 1 and 2 show that GITC is a potent irreversible inhibitor of D-glucose transport; on the other hand we have found that L-glucosyl isothiocyanate is not an inhibitor. Additional evidence for the structural

specificity of GITC is provided by the observations that while L-glucose does not prevent inhibition by GITC of the initial rate of glucose influx, D-maltose is effective in this regard (Table I, Figure 1).

Examination of the pattern of labelling of membrane proteins by [^{14}C]-GITC reveals that a number of proteins were labelled by the reagent, with a preponderance of isotope in peaks III, IV, and VI. Of these, incorporation of [^{14}C] into components III and IV was substantially reduced in the presence of D-glucose. Two possibilities exist to explain these data. In the first place, reactive sites on these proteins may exist only upon the interior surface of the membrane to which access of GITC might be prevented by the ability of glucose to prevent translocation of GITC (Table II). The second possibility is that these two protein components of the membrane participate in D-glucose transport, and that glucose directly competes with GITC for association with them. Although the first possibility cannot be excluded, several facts argue for the second alternative. In the first place, inhibition of transport by GITC is largely prevented by D-maltose, which has been found to act as a non-transported competitive inhibitor of glucose transport. In addition, if it is assumed that inhibition of transport is directly proportional to the number of GITC molecules which have reacted, it can be calculated that there are approximately 300,000 GITC reactive sites per cell. This is also the number of transport sites which we have estimated to be present based upon cytochalasin B binding (10).

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