

Sugar transport in *Trypanosoma brucei*: a suitable kinetic probe

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A transport assay has been developed for use in the investigation of 1-deoxy-D-glucose uptake in trypanosomes. 1-Deoxy-D-glucose has high affinity for the trypanosome sugar transport system (net influx $K_m = 4.03 \pm 0.42$ mM; $V = 0.052 \pm 0.005$ mM \cdot s $^{-1}$). D-Glucose oxidation is competitively inhibited by 1-deoxy-D-glucose. However, we show that 1-deoxy-D-glucose is not a substrate for metabolism and that the competition occurs because of interaction at the transport system. D-Glucose competitively inhibits 1-deoxy-D-glucose influx.

(*Trypanosoma brucei*) Sugar transport D-Glucose 1,5-Anhydro-D-glucitol 1-Deoxy-D-glucose

1. INTRODUCTION

The long slender bloodstream form of African trypanosomes of the *brucei* group is totally dependent on glycolysis for ATP production, and consumes D-glucose at rates which are up to 10-times those of the most glycolytically active mammalian cells (review [1]). This high flux is favoured by the localisation of most of the glycolytic enzymes in the glycosome [2] and the possible further compartmentation of these enzymes in a multi-enzyme complex [3]. Glycolytic flux in *brucei* trypanosomes appears to be uncontrolled in the sense that little evidence has emerged for modulation at the enzyme level. Studies of the classical glycolytic pacemakers, hexokinase [4,5], pyruvate kinase [6] and phosphofructokinase [4,7] have shown that these enzymes are unlikely to play a regulatory role through interactions with metabolic intermediates acting as positive or negative effectors.

Although much work has been published on the

nature and function of glycolysis in these organisms, the initial step, i.e. the transport of D-glucose across the plasma membrane, has received relatively little attention. Early studies of sugar transport in *Trypanosoma gambiense* [8,9] were interpreted as providing evidence for separate transporters for D-fructose and for D-glucose. More recently, Gruenberg et al. [10] have reported kinetic studies of D-glucose and 2-deoxy-D-glucose transport in *T. brucei* and concluded that a D-glucose transporter exists in this organism and that the uptake process is the rate-limiting step of D-glucose metabolism. However, as pointed out by Visser et al. [11] the high glycolytic flux poses a problem in the interpretation of uptake experiments involving metabolised sugars since most of the results published represent steady-state rather than initial rate measurements. It is clear that meaningful transport kinetics can only be obtained with intact cells by using D-glucose analogues which are non-metabolised and not phosphorylated. Such analogues would allow the dissection of effects of potential trypanocidal agents on transport from those on metabolism. We have found that 3-O-methyl-D-glucose, the analogue most often used for such studies in mam-

Dedicated to S.P. Datta upon his retirement as Managing Editor of FEBS Letters

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malian cells, is a very poor substrate for the trypanosome transporter and we have looked for a suitable alternative. We now report kinetics of uptake of 1,5-anhydro-D-glucitol and we show that this analogue is a good non-metabolised substrate for the trypanosome transporter.

2. EXPERIMENTAL

2.1. Materials

Enzymes and cofactors were purchased from Sigma. 1,5-Anhydro-D-glucitol (1-deoxy-D-glucose) was synthesised as described by Barnett et al. [12]. Radiolabelled 1-deoxy-D-glucose was prepared similarly except that D-[U- 14 C]glucose (275 mCi/mmol) was used. All other reagents were of analytical grade.

Cells of the long slender form of *T. brucei* were isolated from Wistar or Sprague-Dawley rats (250–400 g) infected with $1-3 \times 10^7$ cells of strain MITat 1.1 as described [13], and purified on a DEAE-cellulose column [14]. After preparation the cells were kept at 0°C in storage buffer (98 mM NaCl, 22 mM KH_2PO_4 , 1 mM MgSO_4 , 2 mM KCl; pH 8.0) containing 10 mM D-glucose.

2.2. Transport measurements

Immediately before the uptake experiment D-glucose was removed by centrifuging a cell suspension, suspending the pellet in cold storage buffer, recentrifuging and resuspending the pellet in an appropriate volume of buffer for the transport experiment. All centrifugations were carried out in an Ole-Dich refrigerated microfuge (15000 \times g, 20 s, 0°C) to ensure rapid processing of the cells.

The cells were preincubated at 37°C for 30 s and then 0.1–0.3 μCi 1-deoxy-D-[U- 14 C]glucose, diluted with an appropriate concentration of unlabelled sugar was added. At appropriate intervals duplicate 200- μl samples were removed, using a multi-channel pipette, and dispersed into vials containing 1 ml ice-cold storage buffer. The vials were immediately centrifuged and the supernatants removed. The pellets were resuspended and the wash stage was repeated; the supernatants were carefully removed. Carry-over of radiolabel or zero time uptake was estimated from samples which were added directly to ice-cold buffer. The cell pellets were lysed in distilled water and dispersed into scintillant for liquid scintillation coun-

ting. For the estimation of K_m generally just a single time point was used (30 s). In addition, for the lowest substrate concentration used, samples were taken after cellular equilibration with substrate had been reached (2–3 min). This provided an estimate of the volume of the cells accessible to the substrate so that rates could be calculated in units of concentration \cdot time $^{-1}$. For the D-glucose inhibition experiment, cells were suspended in buffer containing the appropriate concentration of D-glucose before the addition of 1-deoxy-D-glucose. Kinetic constants were calculated using the direct linear plot [15] or by least squares.

2.3. Hexokinase assay

Hexokinase activity was determined by measuring the production of ADP in a coupled system containing pyruvate kinase and lactate dehydrogenase at 25°C. The assay buffer (pH 7.4) contained 0.4 M triethanolamine, 2 mM EDTA, 4 mM MgCl_2 , 4 mM KCl, 0.5 mM ATP, 0.5 mM phosphoenolpyruvate, 0.5 mM NADH, 5 units pyruvate kinase, 5 units lactate dehydrogenase. Hexokinase was assayed in a sonicated cell lysate or a disrupted glycosomal preparation prepared essentially as described by Oduro et al. [16]. A correction was made for NADH oxidase activity. NADH oxidase activity was determined by omission of ATP and phosphoenolpyruvate and was found to be less than 0.5% of maximal hexokinase activity. Trypanosome respiration rates were estimated at 37°C using a Clark oxygen electrode.

3. RESULTS AND DISCUSSION

1-Deoxy-D-glucose (10 mM) showed no detectable ADP production in an assay for hexokinase activity (<0.1% of the rate with D-glucose under assay conditions) in either sonicated cell lysates or in a disrupted glycosome preparation. 1-Deoxy-D-glucose, at concentrations up to 10 mM, did not inhibit hexokinase activity, even at the lowest D-glucose concentration tested (0.25 mM).

Oxidation of D-glucose in trypanosomes is however inhibited by 1-deoxy-D-glucose. The inhibition was substrate-antagonised as shown in fig.1. Here the fractional inhibition at fixed inhibitor concentration decreases with increasing D-

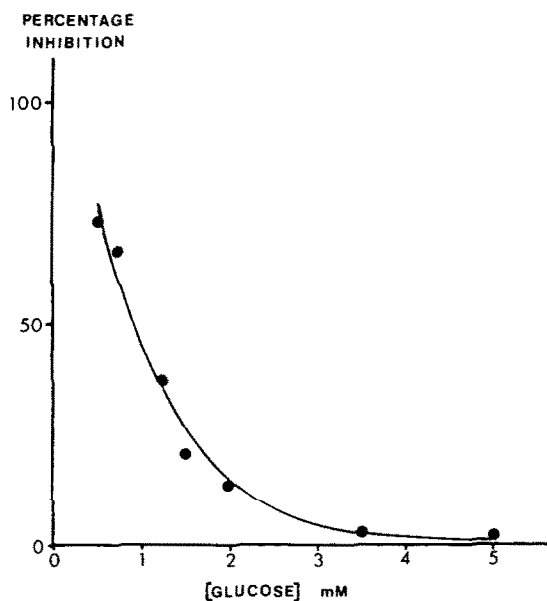


Fig. 1. Inhibition of rate of D-glucose respiration in *T. brucei* by 40 mM 1-deoxy-D-glucose. Percentage inhibition is given by $100 \times (1 - (v_i/v_o))$ where v_i and v_o are the rates of oxygen utilisation in the presence and absence, respectively, of 1-deoxy-D-glucose.

glucose concentration. Because of the lack of interaction of D-glucose and 1-deoxy-D-glucose at the hexokinase level the observed effect of 1-deoxy-D-glucose on respiration is likely to be mediated at the membrane transporter.

In order to set up procedures for investigating sugar interactions with their transport system a number of agents were tested as stoppers in uptake experiments. At 37°C neither *p*-hydroxy-mercuribenzoate, nor mercuric chloride, nor cytochalasin B was totally effective. However, a 5-fold dilution with ice-cold buffer was sufficient to prevent efflux of label. In the experiment shown in fig. 2 cells were equilibrated with 50 μ M 1-deoxy-D-glucose and then diluted into ice-cold buffer. No efflux was observed over a time course which was much longer than the cell processing time required for estimating trapped label (see section 2).

The time course for 50 μ M 1-deoxy-D-glucose uptake is shown in fig. 3. From the equilibrium level of substrate it is calculated that the available cell volume is 5.9 μ l/ 10^8 cells. This value is midway between those reported by other workers [17,18]. The approach to equilibrium shown is typical for

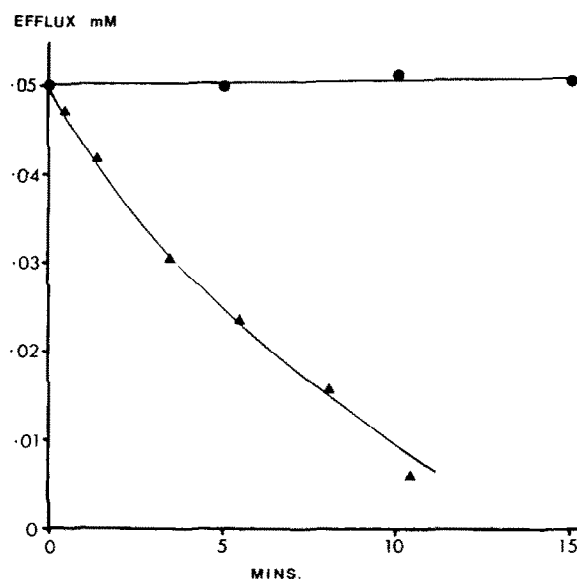


Fig. 2. Efflux of 1-deoxy-D-glucose from *T. brucei* (equilibrated with 50 μ M 1-deoxy-D-glucose) by dilution with 5 vols buffer at 0°C (●) and 37°C (▲). For details see text.

the flux of a substrate concentration below the K_m . The data fitted the integrated rate equation [19] from which an initial rate was calculated (fig. 3, inset). This showed that a 30 s time point gave an accurate estimate of the initial rate. In experiments in which K_m estimates for 1-deoxy-D-glucose were made a 30 s time point was generally used. A 45 s time point gave similar results. Variation of initial rates with 1-deoxy-D-glucose concentrations followed Michaelis-Menten kinetics. From the direct linear plot shown in fig. 4 $K_m = 4.03 \pm 0.42$ mM and $V = 0.052 \pm 0.005$ mM \cdot s $^{-1}$ were calculated. The clustering of the intersections of this plot gives a visual estimate of the error in the kinetic parameters. Uptake rates were competitively inhibited in the presence of added D-glucose (fig. 4, inset). In these experiments the conditions were adjusted to ensure that the D-glucose concentration outside the cells did not change by more than 10% during the incubation. The estimated K_i is 0.33 ± 0.05 mM but since the D-glucose concentration inside the cells is unknown the extent to which this value represents a true transport inhibition constant is unclear. However, this operational

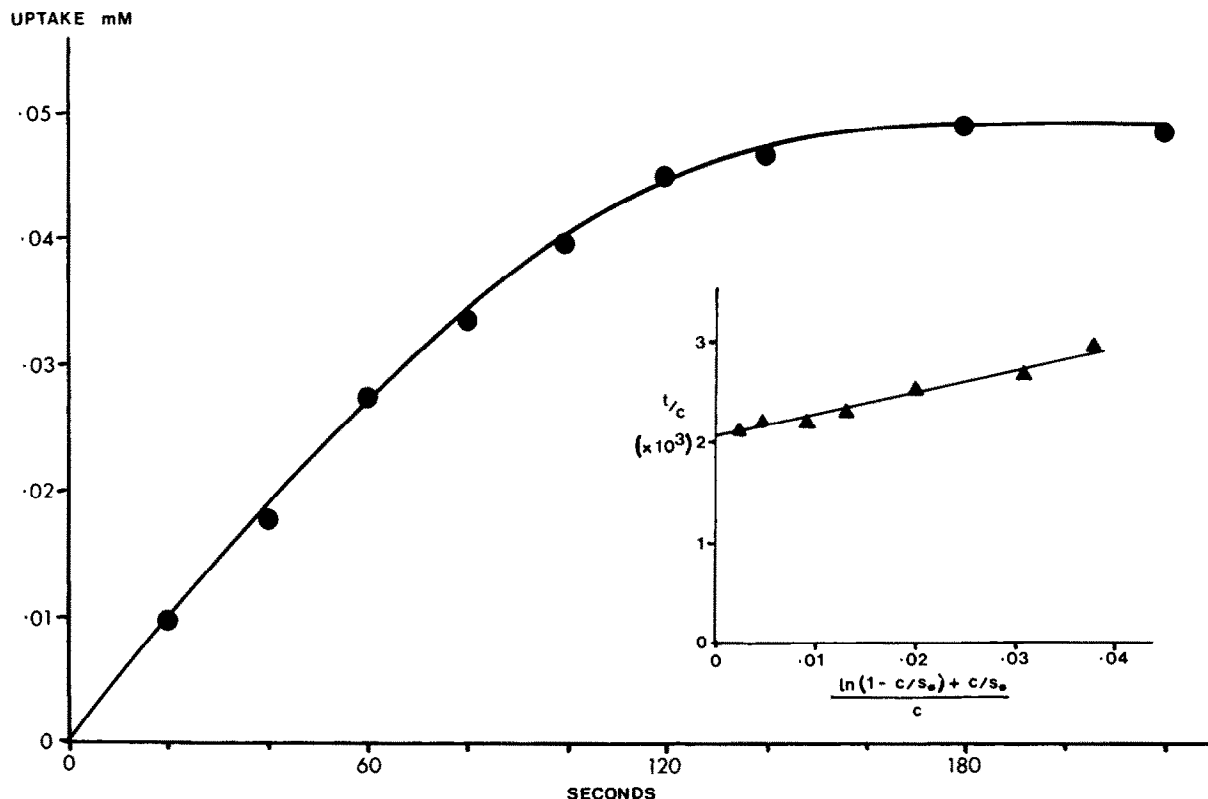


Fig.3. Time course of 1-deoxy-D-glucose uptake ($50 \mu\text{M}$) by *T. brucei* at 37°C . Inset: data fitted to integrated rate equation [19].

K_i is 10-fold lower than the half-maximal saturation constant for D-glucose oxidation and 3-fold lower than the K_i for D-glucose inhibition of 2-deoxy-D-glucose uptake and than the K_m for D-glucose uptake [10].

Further investigations of the specificity of this transport system are required. However, it is clear that this system is dissimilar to both bacterial and mammalian transport systems for sugars. *E. coli* GalP and MG phosphotransferase systems do not transport 1-deoxy-D-glucose [20] (P.J.F. Henderson, personal communication). Mammalian sugar transport systems will transport 1-deoxy-D-glucose but removal of the hydroxyl at carbon 1 results in a large affinity loss. The K_m in mammalian systems is 80–90 mM [12,21] which is a considerably lower affinity than that which we now report for the trypanosome system. Another marked difference between the trypanosome and mammalian systems is the difference in affinity for 3-O-methyl-D-

glucose. In the mammalian system the addition of the methyl group at C-3 has no effect on affinity but in trypanosomes a huge loss of affinity occurs. The rates of 3-O-methyl-D-glucose uptake in trypanosomes are less than 10% of the rates of 1-deoxy-D-glucose uptake. However, 6-deoxy-D-glucose (quinovose) was found to be an effective inhibitor of 1-deoxy-D-glucose uptake, and this analogue may also be suitable for studies of sugar transport in these cells.

The influx V for trypanosome sugar transport is slower than that for mammalian systems. In view of the much higher glycolytic rate in trypanosomes it seems likely that in these organisms overall D-glucose oxidation rates are more rate limited by membrane transport than in mammalian cells.

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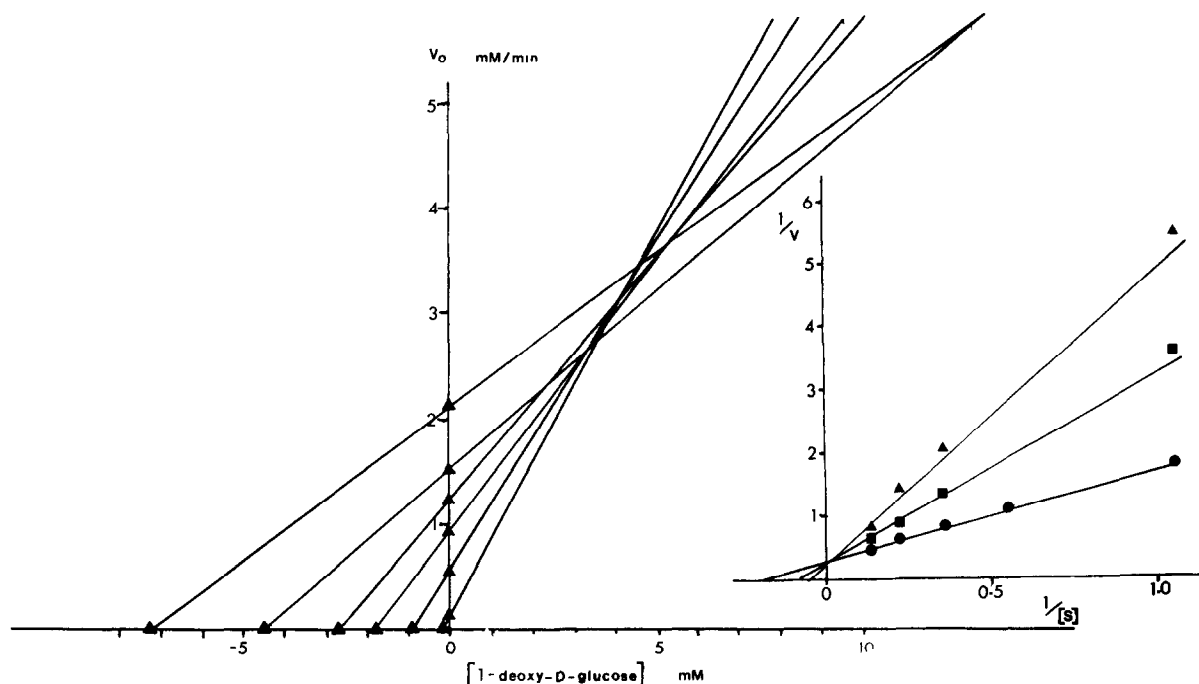


Fig.4. Direct linear plot of variation of uptake rate with initial 1-deoxy-D-glucose concentration. Inset: double-reciprocal plots of uptake rates at different fixed D-glucose concentrations: no glucose (●), 0.5 mM (■), 1 mM (▲).

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