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Specificity and kinetics of hexose transport in *Trypanosoma brucei*

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Transport of 6-deoxy-D-glucose was studied in *Trypanosoma brucei* in order to characterise the kinetics of hexose transport in this organism using a nonphosphorylated sugar. Kinetic parameters for efflux and entry, measured using zero-trans and equilibrium exchange protocols, indicate that the transporter is probably kinetically symmetrical. Comparison of the kinetic constants of D-glucose metabolism with those for 6-deoxy-D-glucose transport shows that transport across the plasma membrane is likely to be the rate-limiting step of glucose utilisation. The transport rate is nevertheless very fast and 6-deoxy-D-glucose, at concentrations below K_m, enters the cells with a half filling time of less than 2 s at 20°C. Thus the high metabolic capacity of these organisms is matched by a high transport rate. The structural requirements for the trypanosome hexose transporter were explored by measuring inhibition constants (K_i) for a range of D-glucose analogues including fluoro and deoxy sugars as well as epimeric hexoses. The relative affinities shown by these analogues indicated H-bonds from the carrier to the C-3, C-4 and C-5 hydroxyl oxygens and from the C-1 and C-3 hydroxyl hydrogens to the binding site. Hydrophobic interactions are likely at the C-2 and C-6 regions of the glucose molecule. Spatial constraints appear to occur around C-4 indicating that the transport site at this position is not freely open to the external solution as is the case with the mammalian hexose transporter. However, the trypanosome transporter appears to accept D-fructose but the common mammalian (erythrocyte type) hexose transporter does not.

Introduction

The bloodstream form of *Trypanosoma brucei* is totally dependent upon D-glucose uptake and metabolism for its energy supply [1]. While there has been considerable interest in the glycolytic pathway and in particular the glycosome and its associated functions as a potential site for antitrypanosomal drugs [2], relatively little attention has been paid to the transport of metabolic substrates. Evidence from previous work [3] indicated that a facilitated diffusion system operates for the uptake of D-glucose into trypanosomes. It is likely that the transport system for D-glucose might provide an attractive target for selective trypanocidal agents, provided that there were sufficient differences in specificity between the parasite and host systems. The common inhibitors of mammalian hexose transport such as phloretin and cytochalasin B are relatively ineffective in trypanosomes [4] indicating that some major differences in the structure of the transporter in the region of its hexose binding site are likely.

In earlier work [4] we used the poorly metabolised D-glucose analogue, 1-deoxy-D-glucose (1,5-anhydro-D-glucitol). Although we succeeded in obtaining useful kinetic parameters for influx using this analogue, our attempts to characterise efflux and exchange kinetics for 1-deoxy-D-glucose were complicated by a slow but significant phosphorylation of the analogue [5]. Game et al. [4] also reported that 6-deoxy-D-glucose was an effective inhibitor of 1-deoxy-D-glucose uptake. As 6-deoxy-D-glucose is not metabolised at all by trypanosomes, we have now used this analogue in preference to 1-deoxy-D-glucose to obtain a full kinetic characterisation of influx, efflux and exchange transport parameters. In addition we have used 6-deoxy-D-glucose as a substrate in an investigation of the specificity requirements of the trypanosome sugar transport system. By using deoxy and deoxy-fluoro analogues and D-glucose epimers we have identified the likely positions of hydrogen bonding between the sugars and the transporter binding site.

Materials

Phloridzin was from Sigma. Other sugars were either purchased from Sigma or Koch-Light or were prepared according to previously described methods [6].

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6-Deoxy-D-glucose. Unlabelled 6-deoxy-D-glucose was either obtained from Koch-Light or was prepared following the method of Evans and Parrish [7]. Labelled 6-deoxy-D-[6-^{3}H]glucose was prepared by a modification of the method of Evans and Parrish. Their method involves the synthesis of methyl 6-deoxy-6-chloro-α-D-glucoside. In the preparation of the nonlabelled material this compound is then reduced with lithium aluminium hydride to obtain methyl 6-deoxy-α-D-glucoside. An attempt at reduction with lithium aluminium tritiide was unsuccessful [8] so instead we used an alternative route to the labelled methyl 6-deoxy-α-D-glucoside. We converted the 6-chloro intermediate to the 6-iodo compound by halide exchange. Methyl 6-deoxy-6-chloro-α-D-glucoside (500 mg) plus 8 g of sodium iodide were dissolved in 25 ml of dry acetone and heated in a sealed tube at 120 °C for 2 h. Acetone was removed by rotary evaporation and the solid residue resuspended in 10 ml of water. The product was deionised with Amberlite MB1 and purified on a silica gel column eluting with ethyl acetate/ethanol/water (90 : 10 : 6, v/v) Mass spectrometry confirmed that the isolated product was methyl 6-deoxy-6-iodo-α-D-glucoside, yield 25 mg (3.5%) of purified product: $R_f = 0.41$; R_f of corresponding chloro-sugar = 0.37. This material (approx. 20 mg) was reduced with tritium gas by Amersham (procedure TR3) using palladium as catalyst. This gave 630 mCi of impure methyl 6-deoxy-α-D-glucoside. The radiochemical purity was very high however (> 98%). 10 mCi was treated with 2 ml 1 M HCl for 4 h at 100 °C. This solution was then cooled and neutralised with Amberlite IRA-93. The product, 6-deoxy-D-[6-^{3}H]glucose was then separated from its impurities by paper chromatography in butanol/ethanol/water (52 : 33 : 15, v/v) giving 6.4 mCi of purified material.

Methods

Cell isolation. Cells of the long slender form of *Trypanosoma brucei* were isolated from Wistar rats (250–400 g) infected with $(1–3) \cdot 10^7$ cells of strain MITat 1.1 as described [9]. The cells were purified on a DEAE-cellulose column [10]. 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.

Metabolic rates. These were determined by measuring the rate of O_2 consumption in a thermostatted (37 °C or 20 °C) Clark oxygen electrode. Trypanosomes were washed in Krebs-Ringer phosphate (KRP) buffer [11] (pH 8.0) to remove extracellular D-glucose. The cells were resuspended in 300 μl of buffer and added to the electrode setup which contained 2.6 ml of air-saturated buffer (final cell concentration 10^8 cells/ml). The appropriate concentration of substrate in 100 μl water was then added. For inhibition experiments 100 μl of the inhibitor solution was added immediately prior to the substrate, the volume of buffer being adjusted to maintain a final volume of 3.0 ml. Initial O_2 concentration was assumed to be 240 μM.

Transport protocols

Zero-trans uptake. 90 μl of trypanosome suspension, washed free of exogenous glucose, containing $5 \cdot 10^7$ cells in KRP buffer were rapidly pipetted onto a 10 μl aliquot of 1.2 μCi of 6-deoxy-D-[6-^{3}H]glucose at the indicated concentrations in 3.5-ml tubes. Uptake was stopped by rapid addition of 2 ml of an ice-cold stopping solution (2 mM phloridzin in KRP at 0 °C). For incubation times of less than 10 s a metronome set at two beats per second was used to time the additions. Cell suspensions in the stoppping solution were immediately centrifuged at 15 000 × g for 20 s in a refrigerated microcentrifuge (Ole Dich). The pellets were resuspended in 1 ml of stopping solution and the centrifugation repeated. Pellets were resuspended in 0.5 ml of distilled water and the resulting cell lysate was added to 5 ml of scintillant for counting of radioactivity. Carry-over of extracellular radiolabel was estimated by direct addition of 90 μl of cell suspension to the stopping solution containing the radiolabelled 6-deoxy-D-glucose. The amount of radioactivity associated with the cells at equilibrium was obtained by incubation of the cells for 3 min with the labelled 6-deoxy-D-glucose. Uptake was calculated from the internal concentration, $C = f \cdot S_o$, where S_o is the extracellular concentration and f is the calculated fraction of the equilibrium level of radioactivity observed at each incubation time.

Zero-trans exit. 25 μl of trypanosome suspensions in KRP buffer containing $5 \cdot 10^7$ cells (20% cytocrit) previously equilibrated for 5 min with 2.4 μCi of 6-deoxy-D-[6-^{3}H]glucose at 1, 2, 5, or 10 mM were rapidly diluted into 1.225 ml of vigorously stirred KRP buffer. Aliquots (125 μl) were removed at the indicated times (3 to 18 s) and immediately dispersed into 2.5 ml of stopping solution. These cell suspensions were processed as described in the zero-trans entry protocol. A zero-time point for efflux was obtained by rapid addition of preincubated cells to 1.225 ml of ice-cold stopping solution followed by immediate removal of 125 μl to a further 2.5 ml of stopping solution. This reproduced the dilutions for all other time points. A time point obtained 90 s after efflux had commenced was used to account for trapped extracellular radiolabel.

Equilibrium exchange. This protocol was essentially identical to that for zero-trans uptake except that the cells were pre-equilibrated with the appropriate concentration of unlabelled substrate for 3–5 min prior to the addition of the same concentration of labelled substrate. As there was no net flux of 6-deoxy-D-glucose into the cells under this protocol the uptake of radiolabel closely approximated to a simple exponential rate,

calculated as $v = -\ln(1-f) \cdot S_0/t$, where S_0 is the substrate concentration, f is the fractional filling and t is the incubation time.

K_i determinations. K_i values were obtained using the zero-trans uptake protocol except that a single substrate concentration of 100 μM 6-deoxy-D-glucose and a range of inhibitor concentrations were mixed with the cells. In some cases (see Results and Discussion) the effect of preincubation with inhibitor on the apparent K_i was determined but appeared to have little effect on the observed inhibition. Since the substrate concentration is low compared to its K_m and the transport system is symmetrical, K_i can be calculated using $v_o/v = 1 + I/K_i$ where v_o and v are the uninhibited and inhibited rate, respectively, and I is the inhibitor concentration. In using this relationship we assume that because we have used short uptake times which give low fractional fillings, the inhibitor concentration inside the cell is low compared to its internal K_i. Thus the external K_i is likely to reflect the zero-trans K_m for those inhibitors that may themselves be transported. Support for this assumption is the observation that the K_m for 1-deoxy-D-glucose is identical to its K_i.

Results and Discussion

Earlier work on sugar transport in African trypanosomes by Read and his co-workers was based on the use of metabolised sugars or partially metabolised analogues. Southworth and Read [12,13], working with *Trypanosoma gambiense*, suggested the existence of a 'glucose site' through which glucose, mannose, fructose and glycerol were absorbed by the parasite, and a separate 'fructose site' through which only fructose and glucosamine were transported. Similar conclusions were drawn by Sanchez and Read [14] using *Trypanosoma lewisi*. Results from specificity studies of sugar transport in *Trypanosoma equiperdum* [15], using over 40 potential inhibitors, suggested a still more complex system: a 'hexose site' transporting glucose, mannose and fructose, and two 'glycerol sites', one specific only for glycerol and glyceraldehyde, and one which can also interact with glucose. Later work by Gruenberg et al. [16] on *T. brucei* showed that inhibition of glycerol permeation by D-glucose displayed kinetics different from those shown when 2-deoxy-D-glucose was the inhibitor. This is indicative of the problems of measuring true transport rates when using metabolised substrates. These workers concluded that glycerol inhibition was occurring at the metabolic level rather than at the carrier site, and went on to propose a separate asymmetric glycerol permeation process.

We have shown that glycerol does not significantly inhibit 6-deoxy-D-glucose uptake indicating that the hexose transporter has no ability to transport glycerol and that its uptake must be via a totally separate transport system. Thus the present study, using non-metabolised 6-deoxy-D-glucose, has clearly confirmed that glycerol does not interact with the hexose transporter [3].

Transport kinetics of 1- and 6-deoxy-D-glucoses

Neither 1-deoxy-D-glucose nor 6-deoxy-D-glucose showed any evidence of metabolism by intact cells as measured using the oxygen electrode, nor did either analogue appear to be a substrate for trypanosomal hexokinase. The estimated rates of hexokinase activity, assayed as described by Game et al. [4] using these analogues with cell-free extracts or glycosomal preparations, were $< 0.1\%$ of the D-glucose rate and were not significantly different from background rates.

In spite of the obviously poor phosphorylation of 1-deoxy-D-glucose by cell-free trypanosomal preparations, the metabolism of labelled 1-deoxy-D-glucose (50 μM) by intact cells is not negligible. TLC analysis of the fate of intracellularly trapped 1-deoxy-D-glucose showed high proportions of radiolabelled phosphorylated sugar following long incubations with the cells. This accumulation of the sugar phosphate thus prohibited the use of 1-deoxy-D-glucose in transport experiments (such as efflux and exchange protocols) that required long preincubations with the cells. However, it was possible to obtain an estimate of zero-trans uptake kinetic parameters by using very short incubation times. Using a 3 second time point gave a V_{max} of 0.32 ± 0.02 mM $\cdot$ s^{-1} ((20 °C) which is considerably faster than our previously reported value [4]. However, the K_m values from the two studies were similar (3.41 ± 0.26 mM, present study; 4.03 ± 0.42 mM, Ref. 4).

In contrast, TLC analysis showed no evidence of labelled 6-deoxy-D-glucose phosphorylation even following long (20 min) incubation times at 37 °C. Therefore this analogue was used in further transport system characterisation. The transport of 6-deoxy-D-glucose was found to be extremely fast at 37 °C and so the kinetic characterisation was carried out at 20 °C. Because of the fast transport rate cold buffer alone was found to be inadequate to stop the efflux of trapped radiolabel. However, 2.2 mM phloridzin in buffer at 0–4 °C was shown to stop efflux for up to 2 min (Fig. 1). This stopping solution was therefore routinely used and all samples were processed well within 90 s.

From the equilibrium values obtained for 6-deoxy-D-glucose uptake, the water space available to 6-deoxy-D-glucose was found to be 1.2 ± 0.13 μl per 10^8 cells (6 determinations). This value is comparable with the value of 1.7 μl per 10^8 cells calculated by Voorheis and Martin [17] but is much less than the value of 9.5 μl/10^8 cells determined by Damper and Patton [18] and our own value of 5.9 μl/10^8 cells determined using 1-deoxy-D-glucose [4]. This last estimate is in error due

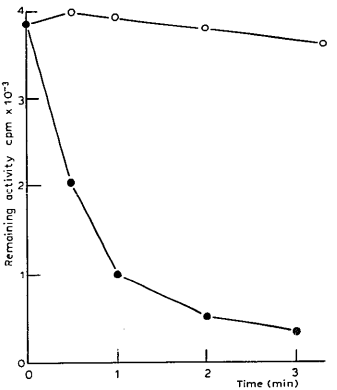

Fig. 1. Efflux of 100 mM 6-deoxy-D-glucose from preloaded trypanosomes; 'stopped' by addition of 5 vols. of ice-cold buffer (●), or ice-cold buffer containing phloridzin (○).

to trapping of some of the radiolabel as 1-deoxy-D-glucose phosphate.

Zero-trans uptake (nomenclature according to Eilam and Stein [19]) of 6-deoxy-D-glucose was determined at concentrations up to 20 mM by using 3–5 time points at each concentration. Plots of $-[\ln(1 - C/S_o) + C/S_o]/C$ vs. t/C were used to calculate the initial rate at each concentration. From the Hanes plot (Fig. 2) $K_m = 1.54 \pm 0.28$ mM with $V_{max} = 0.40 \pm 0.04$ mM · s^{-1}. Using a single early time point (4 s) as an approximation of the initial rate considerably underestimates V_{max} and overestimates K_m ($K_m = 2.16 \pm 0.07$ mM; $V_{max} = 0.28 \pm 0.01$ mM · s^{-1}) (Fig. 2). Thus K_m is 29% higher and V_{max} is 31% lower than the estimates obtained using the integrated rate equation. The largest underestimates in rate occur at low substrate concentrations since the K_m/V_{max} ratio is 50% lower when estimated using the integrated rate equation.

The determination of the kinetic parameters for the inside site of the transporter (to give the zero-trans efflux parameters) involved the measurement of a series of efflux time courses at 1–10 mM initial cellular concentrations. The concentration of sugar inside the cells falls very rapidly from these initial concentrations so that any estimation of initial efflux rate from the slopes of efflux time courses was considered to be likely to give rise to errors in K_m and V_{max}. Instead, the data from several time courses were pooled and collectively analysed using the integrated rate equation equivalent to a Lineweaver-Burk plot. Thus $-(\ln S_t/S_o)/(S_o - S_t)$, the equivalent of $1/S$ (mM^{-1}), was plotted against $t/(S_o - S_t)$, the equivalent of $1/v_o$ (s · mM^{-1}) (Fig. 3). From these data the efflux parameters are $K_m = 2.76 \pm 0.33$ mM and $V_{max} = 0.28 \pm 0.02$ mM · s^{-1}. The value for K_m is slightly higher than that for influx. However, there are several reasons why the efflux experiment is technically more difficult. The cells are equilibrated with the 6-deoxy-D-glucose and then diluted 50-fold in efflux buffer. The cell concentration in the equilibration solution could not be increased above 20% cytocrit because of decreased cell stability. Thus small numbers of cells are diluted in a large volume and care is needed to recover the small pellet after centrifugation and washing steps. In view of these difficulties and the similarity of the estimated K_m for entry and exit it seems reasonable to conclude that the transport system is kinetically symmetric. It may be noted that kinetic symmetry is a feature of certain mammalian transport systems (rat adipocyte [20] and rat hepatocyte [21]) but not of the human erythrocyte [22] nor the rat thymocyte [23].

The kinetics of the transport system were further investigated using the equilibrium exchange procedure. Cells were preloaded with a range of concentrations of unlabelled 6-deoxy-D-glucose. To these cell suspensions was added 6-deoxy-D-glucose at the same concentration but also containing the radiolabelled analogue. The log plot of the time course is linear (Fig. 4, inset) and shows that a single time point can be used to estimate the

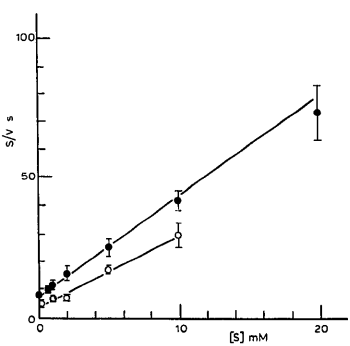

Fig. 2. Hanes plot of 6-deoxy-D-glucose uptake under zero-trans conditions at 20°C: using a 4-s single time point assay (●), points representing means of three independent experiments, each in duplicate; using initial rates calculated from integrated rate plots of uptake time course (four time points each) (○). For details see text.

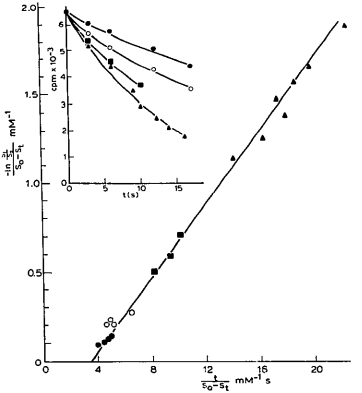

Fig. 3. Integrated rate replot of efflux time-courses for 6-deoxy-D-glucose at 20°C: 10 mM (●), 5 mM (○), 2 mM (■), 1 mM (▲). Inset: Time courses at the indicated concentrations.

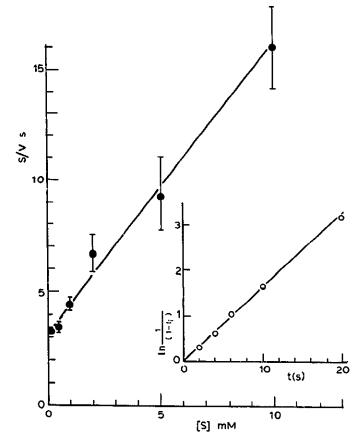

Fig. 4. Hanes plot of 6-deoxy-D-glucose uptake at 20°C under equilibrium exchange conditions using a 4-s single time point assay. Each point represents the mean of three independent experiments, each in duplicate. Inset: log plot of 1 mM 6-deoxy-D-glucose uptake under equilibrium exchange conditions.

exchange rate v since $v = -\ln(1-f) \cdot S_o/t$ where f is the fractional filling and S_o is the sugar concentration. Thus the exchange uptake of radiolabel at 4 s at a range of 6-deoxy-D-glucose concentrations up to 10 mM gave the Hanes plot shown in Fig. 4. This plot is approximately linear but may be slightly curved downward at high concentrations. S/v_i values at low but not at high concentrations are similar to those estimated for zero-trans influx (Fig. 2). From linear regression the estimated values for exchange are $K_m = 2.39 \pm 0.41$ mM and $V_{max} = 0.75 \pm 0.08$ mM · s^{-1}. Thus the V_{max} is significantly higher than that estimated from zero-trans efflux or from zero-trans influx experiments and suggests that the transport system shows slight accelerated exchange. However, the accelerated exchange is only clearly evident at high concentrations.

The relationship between transport and metabolism

Gruenberg et al. [3] carried out a detailed kinetic study of glucose permeation in *T. brucei* using labelled D-glucose and 2-deoxy-D-glucose as substrates. From their results a V_{max} of 0.29 mM · s^{-1} can be calculated for D-glucose entry at 37°C. This is in contrast to our value of 0.40 mM · s^{-1} for V_{max} of 6-deoxy-D-glucose entry at 20°C. The apparent underestimate for D-glucose uptake is not surprising however, as Gruenberg et al.'s results represent steady-state rather than initial rate measurements. Thus backflux may reduce the internal substrate concentration. Also the efflux of radiolabelled pyruvate, the metabolic product, may occur.

We have investigated the oxidation of substrates by intact cells using a Clark oxygen electrode. The kinetic parameters of D-glucose metabolism were $K_m = 0.32 \pm 0.07$ mM and $V_{max} = 62.7 \pm 7.5$ nmol/min per 10^8 cells at 37°C. These values are within the range of values previously reported (Refs. 24 and 25; see also Ref. 26). The kinetic parameters of D-glucose metabolism were also measured at 20°C to allow comparison with transport assay rates measured at 20°C. K_m was 0.39 ± 0.11 mM with $V_{max} = 20.3 \pm 0.9$ nmoles/min per 10^8 cells. Thus lowering temperature mainly reduces maximum velocity of D-glucose metabolism. Since the cell volume is approximately 1.2 μl/10^8 cells and V_{max} is for D-glucose metabolism (0.28 mM · s^{-1}). Thus the K_m is only slightly lower than the K_i for D-glucose inhibition of 6-deoxy-D-glucose transport and the V_{max} for D-glucose is only slightly lower than that for 6-deoxy-D-glucose transport. If allowances are made for some sugar backflux across the symmetrical transport system which may reduce the net supply of D-glucose for metabolism then it seems clear that transport of sugars into *T. brucei* is rate-limited by the membrane at physiological concentrations. Physiological concentrations of D-glucose available to trypanosomes are the plasma D-glucose concentrations of the host which are approx. 5 mM [27]. Hence transport is operating at V_{max}. This comparison

of transport and metabolism is based on measurements made at 20 °C but is likely to apply also at 37 °C. Although the overall rate of D-glucose metabolism is rate-limited by the membrane neither the transport step nor the subsequent metabolism can be considered to be slow. If the somewhat higher affinity for D-glucose than for 6-deoxy-D-glucose is taken into account then D-glucose at concentrations below K_m enters the cell with a $t_{1/2}$ equal to $\ln 2/(V_{max}/K_i) = 0.69/0.44 = 1.6$ s at 20 °C. At 37 °C this cell filling time will be faster. Thus the membrane of *T. brucei* has a large capacity for D-glucose transport which parellels the large metabolic rate of this organism. This large transport capacity is presumably due to a large copy number of transporters and may mean that *T. brucei* is a rich source of this type of hexose transporter.

Specificity of hexose transport in T. brucei

The specificity of hexose transport in *T. brucei* was analysed using a series of inhibitors of 6-deoxy-D-glucose transport. We have obtained half-maximal inhibition constants (K_i values) for a range of analogues substituted at each hexose carbon position thus investigating both the spatial and hydrogen bonding requirements of the transport binding site. These are listed in Table I. K_i values were calculated using a range of inhibitor concentrations at a constant (100 μM) 6-deoxy-D-glucose concentration. This concentration of substrate is well below the K_m so that a simple v_o/v vs. I plot gives the $-K_i$ as the intercept on the x axis (see Methods). When percentage inhibition at the highest concentration (20–40 mM) was less than 10% then the K_i was described as being > 250 mM.

Inhibition by carbon-1 analogues

The carbon-1 analogues used were 1-deoxy-D-glucose, fluoro-β-D-glucoside and methyl α-D-glucoside. As shown in Fig. 5 the deoxy and fluoro analogues have similar affinity. The K_i for 1-deoxy-D-glucose of 3.65 ± 0.43 mM is similar to the K_m (zero-trans entry) for this compound (3.41 ± 0.26 mM). This indicates that 1-deoxy-D-glucose and 6-deoxy-D-glucose probably share the same transport system. This view is further supported by the similarity of percentage inhibitions of zero-trans uptake of 1- and 6-deoxy-D-glucose by glucose analogues with a wide range of structural differences (Table II).

The similarity of the K_i values for the deoxy and the fluoro analogue indicate that there is no hydrogen bond directed toward the oxygen of the carbon-1 hydroxyl of glucose. However, the affinity for the deoxy compound is low compared with D-glucose ($K_i = 0.90 \pm 0.04$ mM) and this probably indicates a hydrogen bond from the hydrogen at carbon-1 OH to an electronegative group on the binding site. This would be similar to the hydrogen bonding shown for the arabinose transporter in *Escherichia coli* [28]. The lack of inhibition by methyl α-D-glucoside suggests that there is a close approach of the transport binding site to carbon-1 and thus very

TABLE I

Inhibition constants (K_i) for a range of D-glucose analogues.

The K_i values were determined by inhibition of 6-deoxy-D-glucose uptake (zero-trans) using a 3-s time point assay at 20 °C. For details see text.

	Name	K_i (mM)
Substrates of metabolism	D-glucose	0.90 ± 0.04
	D-mannose	0.67 ± 0.10
	D-fructose	2.56 ± 0.40
	glycerol	> 250
C-1 analogues	1-deoxy-D-glucose	3.65 ± 0.43
	1-fluoro-1-deoxy-D-glucose	2.89 ± 0.63
	methyl α-D-glucoside	> 250
C-2 analogues	2-deoxy-D-glucose	0.53 ± 0.08
	2-fluoro-2-deoxy-D-glucose	0.44 ± 0.07
	D-glucosamine	21.34 ± 3.68
	N-acetyl-D-glucosamine	11.11 ± 1.78
	mannitol	> 250
C-3 analogues	3-deoxy-D-glucose	> 250
	3-fluoro-3-deoxy-D-glucose	2.31 ± 0.24
	D-allose	> 250
	3-*O*-methyl-D-glucose	15.38 ± 0.86
C-4 analogues	D-galactose	> 250
	4,6-ethylidine-D-glucose	> 250
C-5 analogues	5-thio-D-glucose	11.67 ± 0.94
C-6 analogues	6-deoxy-D-glucose	1.54 ± 0.28 *
	6-deoxy-6-chloro-D-glucose	0.68 ± 0.09
	D-xylose	> 250

* K_m, not K_i.

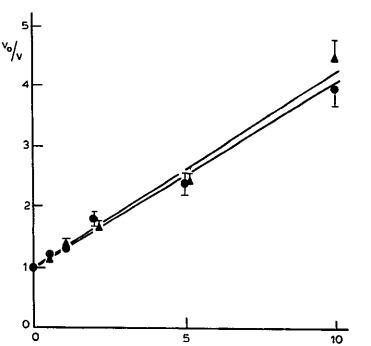

Fig. 5. Inhibition of 100 μM 6-deoxy-D-glucose uptake by C-1 analogues at 20 °C: fluoro-β-D-glucoside (▲), 1-deoxy-D-glucose (●). Points are the mean of two independent determinations.

TABLE II

Percentage inhibition of 10 μM 6-deoxy-D-glucose and 1-deoxy-D-glucose uptake by a range of D-glucose analogues

Inhibition was measured using zero-trans conditions in a single time point assay (3 s) assay at 20 °C.

20 mM inhibitor	Percentage inhibition	
	100 μM 1-deoxy-D-glucose	100 μM 6-deoxy-D-glucose
D-Glucose	88.2	87.7
D-Fructose	72.2	68.4
D-Mannose	91.75	90.4
Glycerol	11.75	7.0
Methyl α-D-glucosdide	0.0	0.0
1-Deoxy-D-glucose	87.5	86.3
2-Deoxy-D-glucose	96.3	96.2
2-Fluoro-2-deoxy-D-glucose	99.5	96.9
D-Glucosamine	62.3	45.4
N-Acetyl-D-glucosamine	73.7	60.9
3-*O*-Methyl-D-glucose	66.9	60.3
3-Fluoro-3-deoxy-D-glucose	99.0	97.5
5-Thio-D-glucose	65.8	48.7
6-Deoxy-D-glucose	86.9	92.0
6-Chloro-6-deoxy-D-glucose	95.4	95.2

little available space for accomodating bulky substituents here. The inhibition observed for the glucoside phloridzin (see Methods) is probably due to interaction between the phloretin moiety and the transport system rather than the sugar moiety. Phloretin itself could not be tested as an inhibitor at the concentrations used for phloridzin because it is only poorly soluble.

1-Deoxy-D-glucose and fluoro-β-D-glucoside only occur as pyranose ring forms. Thus the demonstrated interaction between these compounds and the transport system suggests that the site accepts sugars in the pyranose ring form. It probably also accepts any open-chain conformation that resembles the pyranose ring form since D-fructose is accepted reasonably well by this transporter ($K_i = 2.56 \pm 0.40$ mM). This contrasts quite markedly with the mammalian hexose transporter where the difference in affinity between D-glucose and D-fructose is greater than 1000-fold.

Inhibition by carbon-2 analogues

The K_i for 2-deoxy-D-glucose was determined with and without a 3 min incubation with inhibitor. As shown in Fig. 6 the K_i is not significantly altered by this preincubation. This result confirms the symmetrical nature of the transporter but also suggests that intracellular metabolism of the 2-deoxy-D-glucose has no effect on the transport inhibition kinetics. It was anticipated that preincubations for longer than 3 min with 2-deoxy-D-glucose would deplete intracellular ATP [30] and result in cell loss. Surprisingly, Gruenberg et al. [3] found no inhibition of 2-deoxy-D-glucose uptake by 6-deoxy-D-glucose. The K_i for 2-deoxy-D-glucose is not greatly different from those measured for D-glucose or the carbon-2 epimer, D-mannose. This suggests that there is no H-bond to the C-2 hydroxyl of glucose. This is confirmed by the result obtained using 2-fluoro-2-deoxy-D-glucose which has a K_i of 0.44 ± 0.09 mM, which does not differ significantly from the K_i for 2-deoxy-D-glucose (0.53 ± 0.08 mM). The transporter has only moderate affinity for D-glucosamine ($K_i = 21.34 \pm 3.68$ mM) though this may be due to poor acceptance of the charged amino group. Thus affinity is partially restored for *N*-acetyl-D-glucosamine ($K_i = 11.11 \pm 1.78$ mM).

Inhibition by carbon-3 analogues

Neither 3-deoxy-D-glucose nor the carbon-3 epimer D-allose significantly inhibited 6-deoxy-D-glucose uptake. This suggests that there is hydrogen bonding to the carbon-3 hydroxyl oxygen. This is confirmed by the K_i of 2.31 ± 0.34 mM (Fig. 7) for 3-fluoro-3-deoxy-D-glucose indicating that a fluorine at this position restores H-bonding when compared with the deoxy derivative. H-bonding is not completely restored when compared with D-glucose which has 2–3-fold higher affinity. Thus additional H-bonding to the hydrogen of the carbon-3 hydroxyl is a possibility. 3-*O*-Methyl-D-glucose, which retains the oxygen (but not the hydrogen) of the carbon-3 hydroxyl, has relatively poor affinity, though it does bind more strongly than the deoxy analogue; the K_i is 15.38 ± 0.86 mM (Fig. 7). We have

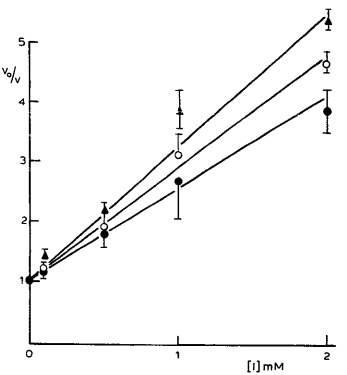

Fig. 6. Inhibition of 100 μM 6-deoxy-D-glucose uptake by C-2 analogues at 20 °C: 2-deoxy-D-glucose with no preincubation (○), and with 3 min preincubation at 20 °C (●); 2-fluoro-2-deoxy-D-glucose (▲).

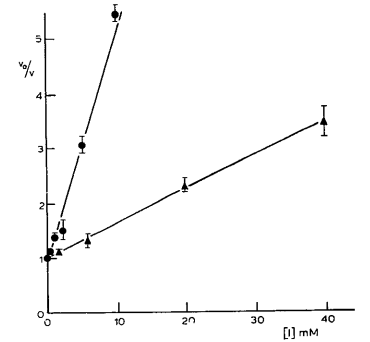

Fig. 7. Inhibition of 100 μM 6-deoxy-D-glucose uptake by C-3 analogues at 20 °C: 3-*O*-methyl-D-glucose (▲), 3-fluoro-3-deoxy-D-glucose (●).

found that radiolabelled 3-*O*-methyl-D-glucose is only poorly transported into trypanosomes (see also Gruenberg et al. [3]). This poor affinity for 3-*O*-methyl-D-glucose probably results from spatial interference by the methyl group of binding to the carbon-3 oxygen group, but poor affinity could also be partly a consequence of the absence of the hydrogen of the carbon-3 glucosyl hydroxyl.

Inhibition by carbon-4, -5 and -6 analogues

The carbon-4 hydroxyl in a glucose conformation is clearly essential for interaction with the transporter since D-galactose neither inhibited transport, nor was D-[1-^{14}C]galactose itself transported by *T. brucei* [8]. Also 4,6-*O*-ethylidene-D-glucose showed negligible interaction. This lack of interaction is probably due to steric interference by the ethylidene group of any approach of a binding group to the C-4 position. In contrast, a carbon-6 hydroxyl is not essential since 6-deoxy-D-glucose has only 1.5–2-fold less affinity than the parent compound D-glucose. Some hydrophobic bonding at this position is likely since 6-chloro-6-deoxy-D-glucose has higher affinity than 6-deoxy-D-glucose and the carbon-5 hydroxymethyl group gives D-glucose higher affinity than is observed for D-xylose which has a glucopyranose ring but lacks the carbon-5 hydroxymethyl group. 5-Thio-D-glucose shows a K_i of 11.67 ± 0.94 mM which indicates that the ring oxygen is not an essential requirement. However, if a ring oxygen is present it may be involved in some hydrogen bonding since the 5-thio derivative has a 10-fold lower affinity than does D-glucose.

The conclusions based on the values of the inhibitor constants of the various sugar analogues determined in this study may be used to construct a model for structural requirements of glucose binding to the trypanosomal carrier. Shown in Fig. 8a, this model illustrates the hydrogen bonds postulated above and is consistent with the relative affinities of the analogues. There appears to be more emphasis on hydrogen bonding involving the hydroxyl hydrogen atom. In mammalian hexose transporters fluoro analogues are generally better substitutes for D-glucose; this indicates that in the mammalian system H-bonding mainly involves the electronegative oxygen atoms of the hydroxyls. The model differs from that proposed by Barnett et al. [6] for the erythrocyte sugar transport system in that the specificity requirements of the trypanosomal carrier appear to be more stringent. In particular there appears to be a closer approach of the substrate to most of the hydrogen-bonding positions in the carrier. This includes C-4 which in the mammalian system is not restricted and is open to the extracellular space so that 4,6-*O*-ethylidene-D-glucose can bind. However, hydrophobic substitutions do appear to be tolerated at C-2 and C-6; ana-

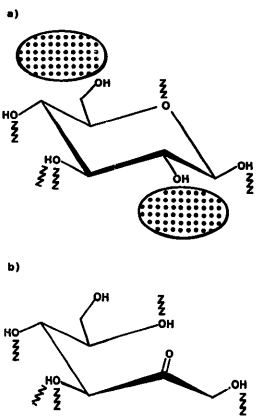

Fig. 8. Schematic model of structural requirements for glucose binding to the hexose transporter of *T. brucei*. (a) Glucose molecule: zig-zag lines indicate H-bonding positions required for effective binding; dotted portions are suggested hydrophobic binding positions. (b) Fructose molecule: drawn in a 'glucose-like' conformation for binding to the trypanosomal hexose transporter.

logues based on alterations at these positions thus offer the promise of acting as selective transport inhibitors. The ability of the trypanosomal system to accept D-fructose also constitutes a significant difference between this system and that of the erythrocyte. This ability suggests that fructose may interact with the carrier in a glucopyranose conformation of its open-chain form (Fig. 8b). Host-parasite differences such as these may provide a rationale upon which the design of selective trypanocidal agents may be based.

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