Glucose Transport in *Entamoeba histolytica*

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That the uptake of glucose by the parasitic amoeba *Entamoeba histolytica* occurs by an equilibrative transport system is supported by the following observations. 1. The rate of glucose uptake is several orders of magnitude greater than the uptake by pinocytosis. 2. The uptake of glucose exhibits saturation kinetics, with $K_m = 1.6$ mM and V_{max} ranging from 2 to 5 μ mol/min per ml of cells at 37°C. 3. The glucose analogues 2-deoxyglucose, 3-O-methylglucose and D-xylose are transported by the glucose system although with much less affinity. Competitive inhibition was observed between pairs of substrates, with K_i values for any sugar closely coincident with the corresponding K_m . 4. D-Xylose, a sugar not metabolized by the cells, equilibrated with 80% of the amoebal cell water. 5. Cells equilibrated with xylose exhibited countertransport of this sugar against its concentration gradient when another substrate was added to the medium. 6. Blocking of glycolysis by iodoacetate or F^- has no immediate effect on transport. The presence of a glucose-transport system in *E. histolytica* contrasts with the situation found in the non-parasitic amoeba, where pinocytosis seems to be the only mechanism of solute uptake.

Growth in culture of the parasitic amoeba *Entamoeba histolytica* requires glucose or a polymer that can be converted into glucose. No other substance appears to fulfil its energy requirements (Reeves, 1972). Starch granules are known to be ingested by phagocytosis (Nakamura, 1953), but nothing is known about the mechanism of glucose uptake.

Studies with free-living amoebae (Holter, 1959) and with a soil amoeba (Bowers & Olszewski, 1972) indicate that these cells lack specific transport systems for glucose, the slow uptake of this sugar being accomplished by pinocytosis. However, specific transport systems for sugars have been reported for several other protozoa, as in the parasitic flagellates of the genus *Trypanosoma* (Min, 1966; Sanchez & Read, 1969) and in the free-living ciliate *Tetrahymena pyriformis* (Cirillo, 1962).

The present work with *Entamoeba histolytica* was directed towards elucidating its mechanism of glucose uptake. For the first time the existence of a specific transport system is reported in an amoeba. Some properties of this system are described.

Experimental

Organism

The typical strain DKB of *Entamoeba histolytica* was used in these studies. It was grown at 37°C with penicillin-inhibited bacteria as described by Reeves & Ward (1965). The cells were harvested and washed

by centrifugation at room temperature. The wash fluid was phosphate-buffered saline, containing 0.1 M-NaCl, 20 mM- K_2HPO_4 , 0.5 mM- $MgCl_2$ and 0.1 mM- $Ca(NO_3)_2$, adjusted to pH 7.0 with HCl. The suspension of washed cells was layered above the buffered saline containing 12% (w/v) Ficoll and centrifuged for 5 min at 700g. Only healthy cells were pelleted through this barrier. Those remaining at the interface were inactive and dark under the anoptral phase microscope (Reichert). To determine the volume of packed cells they were resuspended in the buffered saline and centrifuged for 3 min at 700g. Under these conditions 1 ml of packed cells weighed 1.04 g and contained 70–80 mg of protein (Lowry *et al.*, 1951) and 0.15 g of dry weight. [^{14}C]Inulin was used to determine that 1 ml of cells contain 0.19 ml of extracellular fluid. A 10% (v/v) cell suspension in buffered saline was stored at 25°C for use within 2 h. No microscopically visible bacteria were present in this suspension.

Standard sugar uptake procedure

Packed cells (3–5 μ l) were placed in a tube with buffered saline and the sugar under investigation in a final volume of 0.1 ml. The tubes were incubated at 37°C with occasional gentle shaking by hand. Incubation was terminated by adding 5 ml of ice-cold phosphate-buffered saline which effectively stopped the transport (see below). The suspension was centrifuged for 70 s at 700g and the pellet was twice washed with 5 ml portions of the cold buffered

saline. The final pellet was dissolved in 0.4 ml of 0.3M-NaOH. After standing at room temperature for 10–30 min, six drops of 1M-acetic acid were added and the content of each tube was transferred to a scintillation vial by using 0.6 ml of water as a rinse. The scintillant (10 ml/vial) contained toluene-Triton X-100 (2:1, v/v), 5 g of 2,5-diphenyloxazole (PPO)/litre and 50 mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene (POPOP)/litre. Energetic shaking gave a clear solution, which was counted for radioactivity in a Beckman LS-100 liquid-scintillation counter. Up to 10 μ l of cells per vial could be used under these conditions without affecting the counting efficiency of internal standards.

D-[U- 14 C]Glucose, D-[U- 14 C]xylose and 3-O-[14 C]-methyl-D-glucose were used at 60–80 c.p.m./nmol and [14 C]inulin was used at 15000 c.p.m./mg.

When 2-deoxy-D-glucose was used, the uptake was followed by the sensitive and specific method of Waravdekar & Saslaw (1959). The washed amoebal pellet was dissolved in 1 ml of boiling water, centrifuged from debris and a sample of supernatant was taken for analysis. In this method deoxyglucose gives the same colour as deoxyglucose 6-phosphate.

Zero-time controls were determined by diluting the amoeba in ice-cold phosphate-buffered saline before addition of the sugar. These control values, which amounted to less than 20% of the measured uptakes, have been subtracted in all the presented data.

Chromatography of transported sugars

Packed cells (40 μ l) were incubated at 37°C in 0.4 ml of the buffered saline containing 50 mM-3-O [14 C]methyl-D-glucose (40 min), 60 mM-D-[U- 14 C]-xylose (40 min) or 20 mM-D-[U- 14 C]glucose (15 min). In the last case, the amoeba had been pretreated with 5 mM-iodoacetate for 10 min at room temperature and then washed free of the substance. After incubation the cells were washed with 4 $\times$ 5 ml of the ice-cold buffered saline, and 0.1 ml of boiling water was then added to the pellet. Extraction was continued for about 7 min in a boiling-water bath. Debris were sedimented and 50 μ l of the supernatant solution (2000–3000 c.p.m.) was applied to Whatman 3MM paper strips. The irrigating solvent was butanol-ethanol-water (13:8:4, by vol.) as described by Putman (1957). After about 9 h of descending chromatography, the strips were dried and cut in 2 cm portions, which were counted for radioactivity in the scintillant described above.

Chemicals

All the sugars used were of the D configuration. The non-radioactive sugars were from Pfanstiehl (Waukegan, Ill., U.S.A.). Methyl α -D-glucopyranoside, maltose and galactose were recrystallized before use. The radioactive sugars were obtained

from ICN (Cleveland, Ohio, U.S.A.). All other reagents were of analytical grade.

Results

Suitability of the standard washing procedure for uptake measurements

Cells were incubated for 10 min with 50 mM-2-deoxyglucose and then subjected to successive cold-wash cycles before determination of the sugar uptake. After subtraction of the appropriate control, no difference in the uptake values (15–16 μ mol/ml of cells) was found with one, two or three washes by centrifugation in ice-cold buffer. This indicated that there is no loss of transported sugar during this procedure.

Unsuccessful attempts were made to wash the cells by filtration techniques using Millipore, 0.45 μ m pore size, or glass-fibre paper. The values for sugar retained were less than 10% of those found with the standard wash procedure, indicating extensive leaching or cell damage during the filtration step.

Time-course of the uptake of monosaccharides

During the incubation of amoebal cells with glucose, 3-O-methylglucose, 2-deoxyglucose and xylose there was a progressive uptake of sugar (Fig. 1).

Fig. 1. Time-course of the uptake of monosaccharides by *E. histolytica*

The nature and concentration of the sugars were: ●, 5 mM-[U- 14 C]glucose; ■, 25 mM-3-O-[14 C]methylglucose; ▲, 50 mM-2-deoxyglucose; ▼, 15 mM-[U- 14 C]xylose. Uptake was measured by the standard procedure (see the Experimental section).

Fig. 2. Countertransport of xylose induced by 3-*O*-methylglucose

The amoebal cells were incubated with 10mM-[U-¹⁴C]-xylose and at the time indicated by the arrows 1/8 vol. of either 1M-3-*O*-methylglucose (a) or 1M-sucrose (b) was added. Amoebal concentration was 5% and 0.1ml samples were processed as in the standard procedure (see the Experimental section).

In each case the uptake was linear with time for at least 1min, and for 5min with 2-deoxyglucose. In subsequent studies initial rates were measured by 0.5 or 1min incubations except for 2-deoxyglucose, where 5min incubations were used.

Countertransport

The existence of carrier-mediated transport is suggested by the detection of countertransport (Stein, 1967). This is demonstrated in the experiment of Fig. 2. The amoeba were incubated with 10mM-[U-¹⁴C]xylose until equilibration was almost reached. Then 1/8 vol. of 1M-3-*O*-methylglucose was added. Xylose immediately began to exit from the cells against its concentration gradient. Controls that received sucrose, a non-transported sugar (see below) instead of 3-*O*-methylglucose maintained their content of cellular xylose during the duration of the experiment.

Kinetic parameters of transport

The initial rates of uptake of glucose, 2-deoxyglucose, 3-*O*-methylglucose and xylose were measured

Fig. 3. Lineweaver-Burk plot showing hyperbolic kinetics and competitive inhibition in the uptake of 2-deoxyglucose by *E. histolytica*

●, No inhibitor; ▲, 5mM-glucose; ■, 50mM-xylose; ○, 25mM-mannose. The standard uptake procedure was used (see the Experimental section). Initial rates were determined by 5min incubations.

Table 1. Apparent affinities of the transport system for several sugars

The K_m values are the combined results of three to five different experiments. The K_i values are the mean values of two determinations that differed by less than 20%.

Sugar	K_m (mM)	K_i versus glucose entry (mM)	K_i versus 2-deoxyglucose entry (mM)
Glucose	1.6	—	1.6
3- <i>O</i> -Methylglucose	19	21	15
Xylose	30	43	27
Mannose	—	13	12
2-Deoxyglucose	40	54	—

at different concentrations in the absence or in the presence of one of the other sugars. Mannose was studied as inhibitor, but not as a substrate. The kinetics were always hyperbolic and the inhibitions were perfectly competitive, as shown in Fig. 3 for a typical experiment. The calculated values for the apparent affinities appear in Table 1. A good correlation exists between the K_m and K_i values for a given sugar, with the inhibition constants having similar values when tested against glucose or 2-deoxyglucose as substrate. This is consistent with the presence of a single transport system common to the sugars tested. The affinities were constant from day to day, but wide variations were found in the V_{max} of transport.

Fig. 4. Final concentration of $[U-^{14}C]$ xylose inside the amoeba after incubation with the external xylose concentrations indicated on the abscissa

The incubation times were 30–60 min, when no further uptake was observed. The standard uptake procedure was used as described in the Experimental section.

These values may reflect unrecognized differences in the growth state of the amoeba cultures. For glucose and 2-deoxyglucose the usual range of V_{max} values was from 2 to 5 $\mu\text{mol}/\text{min}$ per ml of cells. When studies were made with glucose, 3-*O*-methylglucose and xylose on the same batch of cells, the V_{max} values for the latter two sugars were about double the value for glucose.

Several other sugars were tested at a concentration of 50 mM as possible inhibitors of the transport of 10 mM-3-*O*-methylglucose. Maltose, ribose, arabinose, sucrose, fructose and mannitol gave less than 5% inhibition, but methyl α -glucoside and galactose gave about 75% inhibition. From these data it can be estimated that the first six sugars had K_i values greater than 0.4 M, whereas the last two sugars had K_i of the order of 10 mM.

Nature of transport

To ascertain the nature of the transport system, a non-metabolized substrate of the transport was used. In principal we considered three likely substrates, 3-*O*- $[^{14}C]$ methylglucose, $[U-^{14}C]$ xylose, or $[U-^{14}C]$ glucose, the last in the presence of iodoacetate. The first of these was shown to be slowly converted by the amoeba into a product which migrated at a decreased rate in paper chromatography. Iodoacetate did not completely stop the conversion of glucose into other compounds. With xylose no evidence of transformation of the sugar was detected. This sugar was chosen for the further studies.

Fig. 5. Equality of entry and exit rates for xylose transport in *E. histolytica*

The amoebal cells were incubated with 12 mM- $[U-^{14}C]$ -xylose and at the point indicated by an arrow the suspension was diluted 20-fold with pre-warmed buffered saline. The initial rates of entry and exit were 1.0 $\mu\text{mol}/\text{min}$ per ml of cells. Before dilution, the concentration of amoebal cells was 5% and the sample volume 0.1 ml. After dilution, 2 ml samples were taken. Samples were processed by the standard procedure described in the Experimental section.

Cells were incubated with several different concentrations of $[U-^{14}C]$ xylose until no further uptake was observed. The intra- and extra-cellular concentrations of xylose at equilibrium are shown in Fig. 4. The transport is not concentrative. In fact, the xylose equilibrated with an intracellular space corresponding to about 55% of the packed cell volume or about 80% of the cell water. This equilibrative space remained almost unchanged when the medium was varied from 0.13 to 0.35 osmol/l with NaCl. At values greater than 0.4 a steep decrease occurred.

In accordance with the equilibrative nature of the transport, the initial rate of entry was the same as the initial rate of exit after dilution of the external xylose (Fig. 5).

Effect of temperature, pH and some reagents on the transport

The rate of transport at 1°C was 0.1% that at 37°C. This low value justifies the suitability of the cold washing procedure described in the Experimental section.

In phosphate-buffered saline the transport of 10 mM-3-*O*-methylglucose exhibited a pH optimum of 7. Deviation of 1 pH unit produced only a 10–20% decrease in the transport rate.

Phlorrhizin (1 mM), colchicine (0.25 mM), NaF (25 mM) and iodoacetate (5 mM) had no effect on the initial rate of transport of 10 mM-3-*O*-methylglucose. This sugar was added after a 10 min preincubation of the cells with one of the above compounds. Under similar conditions, 0.5 mM-*N*-ethylmaleimide blocked about 50% of transport. Replacement of the Na⁺ of the incubation medium by K⁺ had no effect on transport.

Rate of pinocytosis

Pinocytosis was measured by using ¹⁴C-labelled inulin (0.5 mg/ml) as a tracer not likely to be transported by a carrier system. The uptake of inulin was linear with time for about 20 min with rates corresponding to the pinocytosis of 0.6–0.9 μ l of medium/min per ml of cells. The rate of uptake of inulin was not affected by the presence of 50 mM-glucose, 0.5 mM-colchicine, 5 mM-iodoacetate or 0.2 mg/ml of [³H]dextran from Amersham/Searle (Arlington Heights, Ill., U.S.A.). The rate of glucose uptake that could be expected from this pinocytosis activity is about 0.8 nmol/min per ml of cells for a glucose concentration of 1 mM.

Discussion

The uptake of glucose by *Entamoeba histolytica* seems to fulfil all the requirements established by Stein (1967) to identify facilitated diffusion (equilibrative transport). In the first place, the observed rate of glucose uptake is much greater than that expected from non-mediated uptake. At 1 mM external glucose amoeba can take up about 1 μ mol of sugar/min per ml of cells. Our measurements indicate that at this concentration pinocytosis could only account for a glucose uptake of about 1 nmol of glucose/min per ml of cells. At the high glucose concentration present in the growth medium (1%) the observed uptake is still about two orders of magnitude greater than would be expected for pinocytosis. Passive diffusion need not be considered as being capable of supplying the measured rate of glucose uptake (Heredia *et al.*, 1968; Holter, 1959; Stein, 1967). The observed saturation kinetics and competitive inhibitions between analogues indicate that uptake is mediated by a single transport system. The demonstration of countertransport suggests a mobile carrier mechanism (Stein, 1967). Protein components of the membrane are implicated in the transport system by the inhibitory effect of *N*-ethylmaleimide, a typical reagent for thiol groups.

The equilibrative nature of the transport was proved with xylose, a substrate shown not to be metabolized by the amoebal cells. About 20% of total cell water seems to be inaccessible to this sugar. This may be due to membrane-enclosed vacuolar

spaces and nuclear spaces. The objection that a non-metabolizable sugar does not generate energy for concentrative transport cannot be raised in this instance because the amoebal cells had a high rate of endogenous glycogenolysis during the experiments (Montalvo *et al.*, 1971).

This is the first time that the existence of a specific transport system is reported in an amoeba. Studies with the large, free-living amoeba (Holter, 1959) and with the soil amoeba *Acanthamoeba castellanii* (Bowers & Olszewski, 1972) indicate that pinocytosis is the only mechanism available for glucose uptake in these organisms. The rate of pinocytosis in these organisms is not very different from that of *Entamoeba*. The fact that the former organisms have mitochondria, not present in *Entamoeba* (El-Hashimi & Pittman, 1970), may explain their lower requirement for glucose uptake in terms of energy production.

The specificity of glucose transport in *Entamoeba histolytica* presents some striking differences when compared with the two most studied facilitated glucose transport systems, those of erythrocytes (Stein, 1967) and of yeast (Heredia *et al.*, 1968). 2-Deoxyglucose has much less affinity than glucose for the amoebal system, whereas in erythrocytes and yeasts the substrate and its analogue have almost equal affinities. Maltose, although not transported, is a good inhibitor of glucose transport in yeast and erythrocytes but it has no detectable affinity for the transport system in amoeba. Methyl α -glucoside has a good affinity in amoeba but not in the other two organisms. Fructose is as good a substrate as is glucose for the yeast transport system, but with erythrocytes or amoeba the affinity for fructose is very low. A different pattern of structural requirements seems to be implicated in each organism.

Phlorrhizin is a general inhibitor of sugar transport in different animal tissues (Stein, 1967) but it has no effect in *E. histolytica*, nor in *Trypanosomas* (von Brand *et al.*, 1967). This indicates an important qualitative difference in transport systems between the protozoa and animal tissues.

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