Neutral-sugar transport by rat liver lysosomes

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Transport of D-glucose was studied in Percoll-gradient-purified rat liver lysosomes. D-Glucose uptake had a K_m of 22 mM and a $t_{1/2}$ of approx. 30 s. D-Fucose, 2-deoxyglucose and methyl α -glucoside were the most effective competitors for uptake of D-glucose, although D-galactose, D-mannose, D-xylose and L-fucose also appeared to compete for uptake. L-Glucose was a poor competitor for uptake. No competition was observed with *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, D-glucuronic acid, *N*-acetylneuraminic acid, D-glucosamine or the amino acids L-glycine, L-lysine and L-proline. Uptake was unaffected by *N*-ethylmaleimide, dithiothreitol, KCl, NaCl, ATP/Mg or alteration of buffer pH. D-Glucose efflux from lysosomes was temperature-dependent, with a Q_{10} of 2.3, and was inhibited by cytochalasin B. Counter-transport could not be demonstrated. In contrast, L-fucose uptake had a K_m of 65 mM and was largely unaffected by 5 M excess of neutral D-sugars. Both uptake and efflux of L-fucose were inhibited by cytochalasin B. It appears that lysosomes possess a facilitated transport system for D-glucose and perhaps other neutral D-sugars that is discrete from transport systems for acetylated and acidic sugars.

INTRODUCTION

The degradation of macromolecules within lysosomes results in the production of smaller-molecular-mass substances, including a variety of sugars. These products of intracellular digestion are then released to the cytoplasm, thus avoiding the generation of large osmotic gradients across the lysosomal membrane and allowing for re-utilization and more efficient metabolism. Transport systems for two classes of sugars have been identified and characterized in lysosomes [1–3]. One of these systems is specific for acidic sugars such as sialic and glucuronic acids [1]. It has a K_t of 0.24 mM for sialic acid, and transport is dependent on the lysosomal proton gradient. Dysfunction of this system has been implicated in a group of inherited human disorders which are characterized by lysosomal storage of free sialic acid [4–6]. A second transport system that recognizes the acetylated sugars *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine has also been described [2,3]. This carrier has a K_m of approx. 4 mM for either sugar, is Na^+ -independent and is inhibited by cytochalasin B. Unlike the acidic-sugar system, transport appears to be independent of the lysosomal proton gradient. Both the acidic- and acetylated-sugar systems exhibit counter-transport and are active in lysosomal membrane vesicle preparations.

Previous studies, largely based on indirect techniques such as osmotic protection, have suggested the existence of a third lysosomal transport system that is specific for neutral sugars [7–11]. These studies have been limited to examination of uptake of sugars, and little information is available about efflux of sugars from lysosomes. Argument persists over the existence of this transport system, and it has been suggested that neutral sugars merely diffuse across the lysosomal membrane [12–14]. In addition, transport of L-fucose, a glycoprotein constituent, has not been well studied. In view of new information available on lysosomal acidic- and acetylated-sugar transport systems and improved techniques for lysosome isolation, we have re-examined neutral-sugar transport by rat liver lysosomes.

MATERIALS AND METHODS

Materials

D-[U- ^{14}C]Glucose and L-[6- ^3H]fucose were obtained from ICN Biomedicals. Percoll was obtained from Pharmacia LKB Biotechnology Inc. All other chemicals were of the highest

purity available and were obtained from Sigma. Female Sprague–Dawley rats (150–200 g) were obtained from Harlan. GFC glass-fibre filters were obtained from Whatman.

Lysosome purification

Lysosomes were purified from homogenized rat liver by differential centrifugation in buffered solutions of iso-osmotic sucrose, pH 7.0, followed by Percoll-density-gradient centrifugation as previously described [15]. Purification was determined by measurement of the specific activity of β -hexosaminidase by a fluorimetric assay [16]; a unit of β -hexosaminidase activity was defined as 1 μmol of product formed/min at 37 °C. Protein was determined spectrophotometrically [17]. Lysosomes were purified approx. 90-fold by this method.

T.l.c.

Sugars were separated by t.l.c. on cellulose plates with butanol/pyridine/0.1 M-HCl (5:3:2, by vol.) as the solvent [18]. Lysosomal extracts containing radiolabelled sugars were chromatographed with 50 μg of the appropriate sugar standards. Iodine vapour was employed for detection.

Sugar-uptake studies

Lysosomes suspended in Percoll were diluted 10-fold with 0.25 M-sucrose/20 mM-Hepes (pH 7.0) at 4 °C and collected by centrifugation at 13000 g for 10 min. The pellet (0.6–1.0 mg of protein) was resuspended in 100 μl of buffer at 25 °C and then mixed with 100 μl of an iso-osmotic solution containing 40 μCi of the radiolabelled sugars of interest. At various times, a sample was removed and diluted 100-fold in cold buffer (20 μl in 2 ml). Portions (0.5 ml) were filtered through GFC filters and washed with 5 ml of Dulbecco's phosphate-buffered saline without Ca^{2+} , pH 7.4 at 4 °C. Filters were dried and subjected to scintillation counting in Scintifluor II (Fisher).

Lysosomal sugar content

Lysosomes were prepared as for uptake experiments, but were incubated with radiolabelled sugar for 30 min at 25 °C. They were then diluted 50-fold with cold buffer and collected by centrifugation at 13000 g for 10 min. The pellet was resuspended and washed again with cold buffer. The final pellet was broken by sonication in 150 μl of distilled water and subjected to analysis by t.l.c. To determine the percentage of soluble radiolabel, distilled water was substituted for buffer in the final wash step.

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Fig. 1. Uptake of D-glucose (●) and L-fucose (■) by rat liver lysosomes

Lysosomes were incubated with 10 mM sugar (2 μ Ci/50 μ l) at 25 °C in 0.25 M-sucrose/20 mM-Hepes, pH 7.0, and were collected by filtration as described in the Materials and methods section. Values are means $\pm$ S.D. ($n = 3$). K_m for D-glucose is 22 mM, and that for L-fucose is 75 mM. Data are from separate lysosomal preparations for each sugar.

Lysosomal sugar efflux

Percoll-free lysosomes (1 mg of protein) were incubated for 30 min at 25 °C in 200 μ l of 0.15 M-sucrose in 20 mM-Hepes, pH 7.0, with 20 μ Ci of radiolabelled D-glucose or L-fucose. Lysosomes were diluted 100-fold in 0.25 M-sucrose/20 mM-Hepes, pH 7.0 at 25 °C, and 0.5 ml samples were filtered through GFC filters. Filters were immediately washed with 5 ml of Dulbecco's phosphate-buffered saline at 4 °C. They were then dried and subjected to scintillation counting.

Lysosomal latency

Latency was determined by measurement of β -hexosaminidase activity with *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide substrate in iso-osmotic buffer with (total activity) and without (latent fraction) 0.1 % Triton X-100 [19].

RESULTS

Incubation of lysosomes with 10 mM-D-[14 C]glucose or -L-[3 H]fucose resulted in the uptake of radiolabel. Uptake at 25 °C was rapid for the first 30 s of incubation and appeared to reach equilibrium by 2 min (Fig. 1). Different lysosomal preparations were used for each uptake experiment. Relatively small differences in the determination of β -hexosaminidase activity may account for different equilibrium values for each sugar. Lysosomal latency was unchanged during uptake of either sugar. The K_m for the uptake of D-glucose was 22 mM, whereas that for L-fucose was 65 mM (Fig. 2). Uptakes of D-glucose and L-fucose exhibited saturation kinetics on Michaelis-Menten plots. The contents of lysosomes which had been incubated for 30 min at 25 °C with 100 mM radiolabelled sugar were analysed. Lysosomes were collected by centrifugation and washed free of external label as described in the Materials and methods section. They were then broken by hypo-osmotic treatment and centrifuged to sediment membranes. Approx. 90 % of the accumulated label was found in the soluble fraction, and corresponded to the appropriate free sugar on analysis by t.l.c. Thus only free sugar accumulated during uptake, and this sugar was apparently not subject to further metabolism.

Competition experiments were performed to determine the specificity of sugar uptake. Results were consistent with a carrier-mediated process. D-Fucose, 2-deoxyglucose and methyl α -

Fig. 2. Hanes plots of D-glucose (a) and L-fucose (b) uptake by rat liver lysosomes

Lysosomes were incubated for 1 min at 25 °C in 0.25 M-sucrose/20 mM-Hepes, pH 7.0, with 2 μ Ci of radiolabelled sugar per incubation. After incubation, they were collected by filtration. Units: v , μ mol/30 s per unit of β -hexosaminidase; s , mM. Data are pooled for each sugar from separate preparations.

glucoside were the most effective competitors for D-glucose uptake. D-Glucose uptake was decreased similarly by 5 M excess of D-glucose, D-galactose, D-mannose, D-xylose, L-fucose or 3-*O*-methylglucose. L-Glucose was the least effective competitor for uptake (Table 1). In view of the apparent stereospecificity of D-glucose uptake, the specificity of L-fucose uptake was also examined. In contrast with the observations for D-glucose, inhibition of L-fucose uptake by L- and D-fucose was equivalent. Neutral D-sugars had much less effect on L-fucose than on D-glucose uptake, consistent with the high K_m for L-fucose uptake. Uptake of either D-glucose or L-fucose was unaffected by 5 M excess of acetylated sugars, acidic sugars, amino sugars or amino acids. Cytochalasin B inhibited the uptake of both sugars (Table 2).

Uptake of D-glucose and D-fucose was essentially unchanged in the presence of dithiothreitol or the univalent cations Na⁺ and K⁺. A modest increase in D-glucose uptake occurred in the

Table 1. Competition for D-glucose and L-fucose uptake by neutral sugars

Percoll-purified rat liver lysosomes were incubated for 30 s in 50 μ l of 0.25 M-sucrose/20 mM-Hepes, pH 7.0, at 25 °C with 2 μ Ci of the appropriate radiolabelled sugar and any additions as indicated. Molarity of the buffer was kept constant. Samples were diluted 100-fold with ice-cold buffer and collected by filtration as in the Materials and methods section. Values are calculated as percentages of control uptake (pmol/min per unit of β -hexosaminidase) and are expressed as means $\pm$ S.D. ($n = 6$).

Competitor	Uptake (%)	
	10 mM-D-glucose	10 mM-L-fucose
None (control)	100	100
50 mM-D-glucose	58 $\pm$ 8	83 $\pm$ 15
50 mM-L-glucose	90 $\pm$ 8	71 $\pm$ 8
50 mM-D-fucose	42 $\pm$ 5	59 $\pm$ 2
50 mM-L-fucose	70 $\pm$ 9	64 $\pm$ 6
50 mM-D-galactose	66 $\pm$ 7	—
50 mM-D-mannose	68 $\pm$ 7	96 $\pm$ 5
50 mM-D-ribose	79 $\pm$ 10	90 $\pm$ 6
50 mM-D-xylose	65 $\pm$ 6	87 $\pm$ 8
50 mM-2-deoxyglucose	47 $\pm$ 5	72 $\pm$ 7
50 mM-3-O-methylglucose	66 $\pm$ 5	66 $\pm$ 4
50 mM-methyl α -glucoside	36 $\pm$ 2	62 $\pm$ 16

Table 2. Competition for neutral-sugar uptake by acetylated sugars, acidic sugars and amino acids

Conditions for uptake were as described for Table 1, with the additions as indicated. Sugar solutions were adjusted to pH 7.0 with NaOH when necessary. Values are calculated as for Table 1 and are expressed as means $\pm$ S.D. ($n = 3$).

Competitor	Uptake (%)	
	10 mM-D-glucose	10 mM-L-fucose
None (control)	100	100
50 mM-N-acetylglucosamine	91 $\pm$ 2	98 $\pm$ 9
50 mM-N-acetylgalactosamine	100 $\pm$ 4	90 $\pm$ 7
50 mM-D-glucosamine	104 $\pm$ 8	95 $\pm$ 22
50 mM-D-glucuronic acid	99 $\pm$ 3	93 $\pm$ 13
50 mM-N-acetylneuraminic acid	96 $\pm$ 10	—
50 mM-L-proline	95 $\pm$ 9	105 $\pm$ 9
50 mM-L-glycine	91 $\pm$ 4	103 $\pm$ 5
50 mM-L-lysine	101 $\pm$ 8	103 $\pm$ 12
50 μ M-cytochalasin B	30	39

Table 3. Effect of cations, thiol agents and ATP on neutral-sugar uptake

Conditions for uptake were as described as for Table 1. Values are calculated as for Table 1 and are expressed as means $\pm$ S.D. ($n = 3$).

Addition	Uptake (%)	
	D-Glucose	L-Fucose
None (control)	100	100
5 mM-dithiothreitol	93 $\pm$ 6	98 $\pm$ 10
5 mM-N-ethylmaleimide	103 $\pm$ 20	—
25 mM-KCl	107 $\pm$ 15	99 $\pm$ 12
25 mM-NaCl	118 $\pm$ 13	100 $\pm$ 7
5 mM-MgCl ₂	144 $\pm$ 14	107 $\pm$ 5
5 mM-MgCl ₂ /2 mM-ATP	99 $\pm$ 7	96 $\pm$ 10

Fig. 3. Efflux of D-glucose (●) and D-fucose (■) from rat liver lysosomes

Lysosomes were equilibrated in 100 mM sugar and then diluted in buffer as described in the Materials and methods section. Lysosomes were collected by filtration. Data are from separate lysosomal preparations for each sugar.

Fig. 4. Cytochalasin B inhibition of D-glucose (●) and L-fucose (■) efflux from rat liver lysosomes

Lysosomal sugar efflux (nmol/min per unit of β -hexosaminidase) was determined as described in Fig. 3. Cytochalasin B in methanol was added to incubation tubes.

presence of the bivalent cation Mg^{2+} . ATP, which acidifies lysosomes through the action of a proton-translocating ATPase [20], had no effect on sugar uptake (Table 3). Variation of pH between 6.0 and 8.0 with 50 mM-citrate/phosphate buffer had no effect on uptake. The pH of lysosomes loaded with fluoresceinated dextran was unchanged by incubation in 10–20 mM-D-glucose (results not shown).

Efflux of D-glucose or L-fucose from lysosomes loaded with 100 mM sugar by equilibration was time-dependent (Fig. 3), with a Q_{10} of approx. 2.3 for either sugar. Lysosomal latency decreased by 8% over the period of efflux. Sugar efflux was similar when lysosomes were suspended in either iso-osmotic sucrose buffer or iso-osmotic Dulbecco's phosphate-buffered saline without Ca^{2+} (results not shown). Similar to its effects on uptake, cytochalasin B inhibited efflux of both D-glucose and L-fucose (Fig. 4). Attempts to demonstrate counter-transport of either D-glucose or L-fucose by using lysosomes that had been equilibrated with either 10 mM or 100 mM sugar were unsuccessful. Efflux of 1 mM-D-[3H]glucose from lysosomes which had been loaded by equilibration was similar either with (5.5 pmol of β -hexosaminidase/30 s) or without (7.5 pmol/30 s) addition of 50 mM-D-glucose to the buffer.

DISCUSSION

By using direct methods and highly purified rat liver lysosomes, a Na⁺-independent transport system for the facilitated diffusion of D-glucose across the lysosomal membrane has been characterized. Transport is stereospecific, inhibited by cytochalasin B, and has a Q_{10} of 2.3 and a K_m of 22 mM. Competition studies indicate that this system is discrete from systems for acetylated and acidic sugars. D-Glucosamine did not compete for uptake of D-glucose, although lysosomal uptake of this amino sugar has been demonstrated [21]. Our studies are consistent with a single transport system for D-glucose and perhaps other neutral D-sugars that can function bidirectionally. Presumably, under physiological conditions the major direction of sugar flow is from lysosomes into the cytoplasm. Counter-transport of D-glucose and L-fucose could not be demonstrated, although this characteristic has been observed for both the acidic- and acetylated-sugar transport systems. Additional studies with resealed membrane vesicles may be required to resolve this issue.

In general, our findings support the contention by Docherty *et al.* [7–9] and Maguire *et al.* [11], that lysosomes possess a facilitated transport system for neutral sugars. However, there are some differences between our findings and those of earlier studies. Our determination of the K_m of this system is less than half the previously reported value. D-Glucose, 2-deoxyglucose and D-fucose are preferred substrates for transport over many other D-sugars, particularly D-ribose. We could not demonstrate effects of pH, univalent cations or bivalent cations on transport, as had been previously suggested [7]. Many of these differences may be related to the use of tritosomes and the indirect technique of osmotic protection used in earlier studies. Since osmotic-protection measurements are dependent on lysosomal breakage, perhaps some of the previously noted effects are due to stabilization of lysosomes by cations and pH in a manner that is independent of sugar flux.

The lysosomal D-glucose transport system shares some similarities with the high-affinity hexose system that has been described in rat myoblasts [22]. Both systems preferentially transport D-glucose and 2-deoxyglucose, unlike the low-affinity myoblast system, which prefers 3-O-methyl-D-glucose as a substrate. Although the lysosomal system is inhibited by cytochalasin B, relatively large concentrations are required for effect. This is also a characteristic of the high-affinity myoblast system, which is only one-tenth as sensitive to cytochalasin B as the low-affinity system. The lysosomal and high-affinity myoblast systems differ in that the high-affinity system is sensitive to thiol reagents.

It has been suggested that simple diffusion may play a role in the lysosomal transport of neutral sugars [12,14]. This may be the case for L-fucose transport, accounting for the extremely large K_m . It is also possible that L-fucose crosses the lysosomal membrane by multiple mechanisms and, at physiological concentrations, can serve as a substrate for the D-glucose transporter. Evidence that cytochalasin B blocks efflux of 100 mM-D-glucose more effectively than that of 100 mM-L-fucose

may be consistent with two mechanisms for L-fucose transport at high concentrations. We have limited our studies of competition for L-fucose transport to 50 mM sugar concentrations. Although extremely high concentrations have been used in osmotic-protection studies, we consider it difficult to interpret the significance of results obtained under what are apparently non-physiological conditions.

Studies of glycosaminoglycan degradation have demonstrated that acetylated sugars are recycled by the cell after egress from lysosomes [23]. It seems reasonable to assume that glucose and other neutral sugars derived from the lysosomal degradation of glycogen, glycosphingolipids and glycoproteins are similarly reutilized. Our studies were not designed to measure the contribution of lysosomally derived glucose to cellular pools, and the significance of such recycling is unknown. However, it is conceivable that under conditions of glucose deprivation both plasma- and lysosomal-membrane transport systems may act in concert to regulate cytoplasmic glucose concentrations.

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