The Permeability of Rat Liver Lysosomes to Sugars

EVIDENCE FOR CARRIER-MEDIATED FACILITATED DIFFUSION

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1. By the osmotic-protection method, the penetration of sugars through the rat liver lysosomal membrane was studied with a view to determining whether sugar uptake was by facilitated diffusion. 2. The following criteria for this type of transport were established: sugar specificity, the order of uptake being 2-deoxy-D-glucose < D-glucose < D-mannose < D-galactose < D-ribose < 2-deoxy-D-ribose; stereospecificity, the uptake of L-glucose and L-ribose being 50% slower than their D-stereoisomers; inhibition by 1 mM-phlorrhizin and 1 M-cytochalasin B; competition between sugars for uptake, and a Q_{10} (rate difference over a 10°C temperature range) for uptake of approx. 2.8. 3. It is proposed that sugar uptake into lysosomes from rat liver is by facilitated diffusion.

The hypothesis has been put forward elsewhere (de Duve & Wattiaux, 1966; Hales, 1978) that polypeptide-hormone secretion has evolved from the processes of phagocytosis, degradation and defaecation in unicellular organisms. It has been suggested that the degradation of phagocytosed material in secondary lysosomes leads to the production of low-molecular-weight materials within that organelle, which are then transported through the lysosomal membrane into the cytoplasm. Such transport processes could then be subject to regulation by the contents of the secondary lysosome (Hales, 1978).

Lysosomes are known to be permeable to a number of sugars, amino acids and dipeptides, and most authors have concluded that non-electrolytes permeate the lysosomal membrane by simple diffusion with a molecular size limit of 200–300 daltons (Cohn & Ehrenreich, 1969; Ehrenreich & Cohn, 1969; Lloyd, 1969, 1971; Goldman, 1976).

The purpose of the present investigations was to determine whether, using the lysosome osmotic-protection method introduced by Berthet *et al.* (1951) and Appelmans & de Duve (1955), we could obtain evidence that sugar penetration of lysosomes was mediated by facilitated diffusion. Within the limitations of this method the criteria available were: sugar specificity, stereospecificity, inhibition by phlorrhizin and cytochalasin B, competition, and the presence of a characteristic Q_{10} (rate difference over a 10°C temperature range) for transport.

Experimental

Chemicals

Disodium *p*-nitrophenol phosphate, dipotassium *p*-nitrocatechol sulphate, β -methyl galactoside, β -

methyl D-glucoside, D-galactosamine hydrochloride, D-glucosamine hydrochloride, *N*-acetyl-D-glucosamine, 2-deoxy-D-glucose, 2-deoxy-D-ribose, D-fructose and cytochalasin B were all obtained from Sigma (London) Chemical Co. (Kingston-upon-Thames, Surrey, U.K.); D-ribose, L-ribose, L-glucose, D-galactose and D-mannose were from Calbiochem (Bishops Cleeve, Herts., U.K.); D-glucose, phlorrhizin, dimethyl sulphoxide and Triton X-100 were from British Drug Houses (Poole, Dorset, U.K.); sodium β -glycerophosphate was from Fisons Scientific Ltd. (Loughborough, Leics., U.K.); Lubrol was from ICI Ltd (Blackley, Manchester, U.K.).

Preparation of lysosomal suspension

Fed male Wistar rats (130–160 g) were stunned by a blow to the head and decapitated. The liver was removed and placed in ice-cold 0.25 M-sucrose, diced with a scalpel and homogenized in 3 vol. of 0.25 M-sucrose in a Teflon-on-glass Aldridge-type homogenizer (Aldridge *et al.*, 1960). The homogenate was diluted 1:10 and centrifuged at 1000 g_{av} for 10 min (MSE Mistral 6L, MSE Coolspin). The supernatant was centrifuged at 12000 g_{av} for 20 min and the pellet washed once in 0.25 M-sucrose (MSE Superspeed 50, Beckman L5 75). The resultant lysosome-enriched pellet was gently resuspended in a volume of 0.25 M-test sugar solution equal to 6 times the original liver weight. Up to this point all operations were carried out at 4°C.

Enzyme assays and determination of the percentage free activity

The lysosomes were suspended in 0.25 M-test sugar, and samples withdrawn for assay at the stated times

(usually 0, 15, 30 and 60 min). In initial experiments the sugar solutions were unbuffered, the pH being about 4.5–5.0. However, some sugar solutions, especially the L-isomers, caused clumping of the lysosomes, and it was found that the pH of these solutions was substantially lower (approx. 3.0) than that of other sugars. Consequently in some experiments the sugar solutions were buffered at pH 7.4 with 10 mM-Tris/acetate, and in these cases the results did not differ significantly from those obtained with unbuffered solutions.

The theory behind the osmotic-protection method is that permeant test sugars, moving down the concentration gradient, penetrate the membrane, and as a result water is drawn into the lysosomes. The resultant hydrostatic pressure ultimately disrupts the lysosomal membrane with liberation of intralysosomal enzymes. By following the release of these enzymes, an indication of the extent of lysosomal breakage, and as such an indirect measure of sugar uptake, is obtained.

The assay cocktails contained 0.25 M-sucrose, which being impermeant provided osmotic protection for the lysosomes (Berthet *et al.*, 1951), so that no further osmotic lysis occurred during the 10 or 20 min

assay period. The enzymes were measured in the presence (total activity) and the absence of detergent (free activity), and the results expressed in terms of free activity as a percentage of the total. Both the free and total activity were determined at each time point.

Free and total *p*-nitrophenol phosphatase were assayed by adding 0.1 ml of lysosomal suspension to 0.9 ml of assay medium so that the final concentrations were: 0.01 M-*p*-nitrophenol phosphate, 0.1 M-sodium acetate buffer, pH 5.0, 0.25 M-sucrose and 0.025 M of the suspending solute, and for total activity 0.1 % (v/v) Triton X-100. The reaction was stopped after 10 min by adding 4 ml of 0.1 M-NaOH and the liberated *p*-nitrophenol determined by measuring the A_{405} .

Free and total β -glycerophosphatase were assayed by adding 0.05 ml of lysosomal suspension to 0.45 ml of assay medium so that the final concentrations were: 0.02 M-sodium β -glycerophosphate, 0.1 M-sodium acetate buffer, pH 5.0, 0.25 M-sucrose and 0.025 M of the suspending solute, and for total activity 0.1 % (v/v) Triton X-100. The reaction was stopped after 20 min by the addition of 0.5 ml of 5 % (v/v) HClO_4 , and the liberated phosphate measured by the method of Itaya & Ui (1966).

The combined activities of arylsulphatases A and B

Fig. 1. Effect of temperature on the change in free (a) *p*-nitrophenol phosphatase and (b) β -glycerophosphatase of rat liver lysosomes incubated in 0.25 M-glucose

The values are the means $\pm$ S.E.M. (bars) of free activity (% of total) from three separate experiments in which the same lysosome suspension was incubated and assayed at 37°C (○) or 27°C (●).

Table 1. *Change in free p-nitrophenol phosphatase of rat liver lysosomes incubated in various sugars*

The incubation and assay were at 25°C and all sugars at 0.25 M. All values are means of the free activity (expressed as % of total) $\pm$ S.E.M. of the stated numbers of experiments.

	Incubation time (min)	Activity (%)			
		0	15	30	60
Sucrose	(12)	12.5 $\pm$ 0.2	10.8 $\pm$ 0.3	11.7 $\pm$ 0.4	12.8 $\pm$ 0.3
β -Methyl galactoside	(4)	13.9 $\pm$ 0.4	15.0 $\pm$ 0.7	18.9 $\pm$ 0.7	27.2 $\pm$ 1.5
β -Methyl glucoside	(5)	13.7 $\pm$ 0.6	17.2 $\pm$ 1.2	26.6 $\pm$ 2.7	44.0 $\pm$ 2.8
2-Deoxy-D-glucose	(3)	14.2 $\pm$ 0.7	24.1 $\pm$ 1.0	40.4 $\pm$ 3.7	57.0 $\pm$ 5.0
D-Fructose	(4)	13.7 $\pm$ 0.3	31.8 $\pm$ 1.1	58.4 $\pm$ 0.8	76.5 $\pm$ 2.3
D-Glucose	(10)	13.1 $\pm$ 0.3	32.2 $\pm$ 1.5	57.5 $\pm$ 1.5	75.0 $\pm$ 0.8
D-Mannose	(4)	13.7 $\pm$ 0.7	35.7 $\pm$ 3.1	60.9 $\pm$ 3.5	73.9 $\pm$ 3.2
D-Galactose	(8)	13.3 $\pm$ 0.5	48.4 $\pm$ 0.9	68.9 $\pm$ 1.3	79.7 $\pm$ 0.9
D-Ribose	(5)	13.4 $\pm$ 1.4	56.8 $\pm$ 3.4	74.5 $\pm$ 3.2	82.5 $\pm$ 2.3
2-Deoxy-D-ribose	(3)	16.2 $\pm$ 0.4	75.1 $\pm$ 4.7	81.6 $\pm$ 1.6	84.4 $\pm$ 2.0

were measured as described by Worwood *et al.* (1973), by adding 0.05 ml of lysosomal suspension to 0.45 ml of assay medium so that the final concentrations were: 0.02 M *p*-nitrocatechol sulphate, 0.1 M-sodium acetate buffer, pH 5.6, 0.25 M-sucrose and 0.025 M of the suspending solute, and for total activity 0.1% (w/v) Lubrol (Triton X-100 was found to interfere with the colour reaction). The reaction was stopped after 20 min by the addition of 0.5 ml of Roy's alkaline quinol reagent (Roy, 1953), and the liberated *p*-nitrocatechol determined by measuring the A_{515} .

Results

Fig. 1 shows the time course of osmotic lysis of lysosomes suspended in D-glucose, measured by the increase in free *p*-nitrophenol phosphatase and β -glycerophosphatase. For both enzymes a sigmoidal time course was obtained. At 27°C, 2.1% of *p*-nitrophenol phosphatase and 1.6% of β -glycerophosphatase became free per min, and at 37°C 5.8% of *p*-nitrophenol phosphatase and 4.4% of β -glycerophosphatase became free per min. This yielded a Q_{10} (27–37°C) for glucose of 2.8 and 2.75 respectively. At 37°C in 0.25 M-sucrose the amount of free enzymes at zero time was 9% and at 60 min was 14%. This difference was not significant.

The sugar specificity of protection was investigated. Table 1 shows the change in free *p*-nitrophenol phosphatase activity of lysosomes suspended in a number of sugars. In the course of 60 min only sucrose afforded complete osmotic protection, whereas the rate of increase in free activity in the other sugars was very different. β -Methyl galactoside and β -methyl glucoside protected much more than the parent hexoses; fructose, glucose and mannose protected similarly, but more than galactose, ribose and 2-deoxyribose.

As a result of the sigmoidal nature of the increase in free activity with time, the difference in free

Table 2. *Change in free β -glycerophosphatase and arylsulphatase of rat liver lysosomes incubated in various sugars*

The incubations and assay were at 25°C, and all sugars at 0.25 M. All values are means of the free activity (% of total) of two experiments.

Incubation time (min)	β -Glycerophosphatase			Arylsulphatase		
	0	15	30	0	15	30
Sucrose	13.0	12.8	12.9	10.7	9.3	11.2
D-Glucose	11.3	21.2	36.9	10.1	20.8	36.4
D-Mannose	10.7	30.2	45.9	—	—	—
D-Galactose	12.0	39.9	53.5	11.6	35.6	52.4
D-Ribose	12.7	56.1	73.8	13.3	57.8	69.6

activity between sugars was maximum at the 15 min and 30 min time points, but was not well resolved at the 60 min time point (cf. D-glucose and D-galactose: Table 1). Consequently in the subsequent results, although the experiments were carried out over a 60 min period, only the data from the 0, 15 and 30 min time points were included in the Tables.

A similar general pattern of protection to that shown in Table 1 is obtained by measuring the change in free β -glycerophosphatase and arylsulphatase (Table 2). The order of protection was glucose > mannose > galactose > ribose. The main difference between these and the results shown in Table 1 was that glucose provided a greater degree of protection, as measured by the release of β -glycerophosphatase and arylsulphatase, than that measured by the release of *p*-nitrophenol phosphatase.

The stereospecificity of protection is shown in Tables 3 and 4. L-Glucose and L-ribose protected to a greater degree than their D-stereoisomers, the rates of increase in free activity being about 50% slower in each case.

Two inhibitors of sugar transport, phlorrhizin and

Table 3. *Effect of stereoisomerism and phlorrhizin on the change in free p-nitrophenol phosphatase of rat liver lysosomes incubated in various sugars*

The incubation and assay were at 25°C, and the sugars at 0.25M. The values are means of free activity (% of total) $\pm$ s.e.m. for four experiments.

Sugar	Phlorrhizin (1 mM)	Incubation time (min)	Activity (%)		
			0	15	30
Sucrose	0		11.9 $\pm$ 0.5	12.2 $\pm$ 0.5	12.5 $\pm$ 1.0
	+		12.1 $\pm$ 0.7	12.9 $\pm$ 0.9	12.4 $\pm$ 0.8
D-Glucose	0		12.8 $\pm$ 0.5	34.0 $\pm$ 1.3	57.7 $\pm$ 2.0
	+		12.8 $\pm$ 0.5	17.0 $\pm$ 0.7	25.9 $\pm$ 2.3
L-Glucose	0		13.1 $\pm$ 1.2	24.2 $\pm$ 2.0	34.1 $\pm$ 2.4
	+		13.9 $\pm$ 0.2	14.3 $\pm$ 0.4	17.8 $\pm$ 0.2
D-Ribose	0		12.7 $\pm$ 1.7	55.9 $\pm$ 4.2	74.0 $\pm$ 4.1
	+		13.4 $\pm$ 1.8	36.4 $\pm$ 2.9	61.7 $\pm$ 1.2
L-Ribose	0		15.4 $\pm$ 1.4	38.9 $\pm$ 2.3	57.0 $\pm$ 1.8
	+		14.3 $\pm$ 2.3	26.8 $\pm$ 2.9	43.4 $\pm$ 3.7
D-Fructose	0		13.7 $\pm$ 0.3	31.8 $\pm$ 1.1	58.4 $\pm$ 0.8
	+		13.9 $\pm$ 0.4	18.8 $\pm$ 0.4	32.7 $\pm$ 0.6
D-Galactose	0		13.4 $\pm$ 0.4	47.3 $\pm$ 1.0	70.5 $\pm$ 1.6
	+		14.1 $\pm$ 0.5	25.9 $\pm$ 2.0	38.0 $\pm$ 0.7

Table 4. *Effect of stereoisomerism and of phlorrhizin on the change in free β -glycerophosphatase and arylsulphatase of rat liver lysosomes*

The incubation and assay were at 25°C, and the sugars at 0.25M. The values are means of the free activity (% of total) of two experiments.

Sugar	Phlorrhizin (1 mM)	Incubation time (min)	β -Glycerophosphatase			Arylsulphatase		
			0	15	30	0	15	30
D-Glucose	0		10.5	27.5	36.1	9.7	17.2	39.4
	+		7.5	16.4	27.2	9.2	15.6	26.6
L-Glucose	0		6.3	16.3	23.3	9.8	18.2	28.4
	+		5.7	8.6	14.1	10.2	15.8	23.0
D-Ribose	0		15.5	58.9	72.9			
	+		8.7	27.8	41.6			
L-Ribose	0		10.8	44.2	58.6			
	+		7.1	34.4	43.0			

Table 5. *Effect of cytochalasin B on the change in free p-nitrophenol phosphatase and β -glycerophosphatase of rat liver lysosomes incubated in 0.25M-glucose*

The values are means of the free activity (% of total) $\pm$ s.e.m. from three separate experiments for p-nitrophenol phosphatase. Dimethyl sulphoxide was present at 0.05% throughout the incubations.

Incubation time (min)	p-Nitrophenol phosphatase			β -Glycerophosphatase		
	0	15	30	0	15	30
Glucose	12.6 $\pm$ 0.3	27.5 $\pm$ 1.6	53.6 $\pm$ 2.5	13.8	26.2	38.2
+Cytochalasin B:						
10 μ M	12.7 $\pm$ 0.4	22.3 $\pm$ 1.2	48.4 $\pm$ 1.3	—	—	—
50 μ M	12.6 $\pm$ 0.6	18.3 $\pm$ 0.9	37.8 $\pm$ 1.2	—	—	—
100 μ M	12.8 $\pm$ 0.1	16.4 $\pm$ 0.9	31.5 $\pm$ 0.7	10.1	20.9	32.2

Fig. 2. Effect of sugar competition on the increase in free β -glycerophosphatase of rat liver lysosomes

Incubation and assays were at 25°C. The lysosomes were incubated in 0.25 M-D-ribose (▲), 0.24 M-D-ribose + 0.01 M-2-deoxy-D-glucose (●), 0.23 M-D-ribose + 0.02 M-2-deoxy-D-glucose (○) and 0.25 M-2-deoxy-D-glucose (■).

cytochalasin B, were also tested (Tables 3, 4 and 5). Phlorrhizin at a concentration of 1 mM had no effect on lysosome stability in sucrose, but greatly increased the protective effect of D- and L-glucose, D- and L-ribose, D-galactose and D-fructose (Tables 3 and 4). Cytochalasin B increased the degree of protection of D-glucose in a concentration-dependent fashion as measured by the release of *p*-nitrophenol phosphatase and β -glycerophosphatase (Table 5).

The final criterion to be demonstrated, competition between sugars, is shown in Fig. 2. When suspended in D-ribose, 72% of the lysosomal β -glycerophosphatase became free within 30 min. However, when 2-deoxy-D-glucose was present in concentrations of 0.01 M and 0.02 M along with D-ribose such that the final sugar concentration was 0.25 M, then the free β -glycerophosphatase was decreased to 68.5% and 66% respectively. With 0.25 M-2-deoxy-D-glucose the free activity was 36%.

Discussion

The osmotic-protection method itself limits the design and interpretation of investigations of sugar uptake. All studies are carried out at 0.25 M, a concentration which is very much higher than the affinity of a sugar for any physiologically significant transport process. The method itself reflects net uptake of the sugar rather than unidirectional flux. Finally the relationship between net uptake of sugar and access

of substrate to lysosomal enzymes is not clearly understood and is likely to be complex. Thus the time course of increased free enzyme activity has been shown not to be linear in all situations (Fig. 1).

An additional source of complexity is that assays of lysosomal enzymes differ in their specificity. Spurious results can be obtained when *p*-nitrophenol phosphatase is used as a lysosomal marker, since phosphatases originating elsewhere in the cell also hydrolyse its substrate (Beaufay, 1972). On the other hand acid β -glycerophosphatase is almost exclusively lysosomal in the rat liver (Neil & Horner, 1964), but its use may be limited by membrane association of the enzyme (Dobrota *et al.*, 1978). Consequently arylsulphatase was used as a third lysosomal marker enzyme in this work.

Despite its limitations, the osmotic-protection method has allowed us to establish that the process of sugar uptake is sugar-specific, stereospecific, inhibited by known inhibitors of glucose transport (phlorrhizin and cytochalasin B), shows competition, and has a Q_{10} of approx. 2.8.

Lloyd (1969) established an order for the rate of sugar uptake which the present work confirms and extends to provide the following series (shown in Table 1): β -methyl galactoside < β -methyl glucoside < 2-deoxy-D-glucose < D-fructose < D-glucose < D-mannose < D-galactose < D-ribose < 2-deoxy-D-ribose. This sugar specificity, together with the lack of permeation by hexitols (Lloyd, 1969), argues against any diffusion mechanism based solely on molecular size and degree of hydrophobicity. The ranking of relative rates of net uptake is not necessarily the order of permeability at physiological concentrations. If permeation is by facilitated diffusion, then at concentrations considerably in excess of the affinity for the carrier the net flow diminishes, since the rate of efflux rapidly approximates to the rate of influx (Wilbrandt & Rosenberg, 1961; Stein, 1967). Thus for a series of compounds the rate of net uptake may be the reverse of the relative affinities of the compounds for the carrier. The order of net uptake that we have established is in fact the reverse of the affinities of these sugars for transport in the human erythrocyte (LeFevre, 1961). If this kinetic explanation of the reversal of the apparent rate of net uptake is correct, some sugars with a low rate of accumulation should have a higher affinity for the carrier than more rapidly accumulating sugars. To test this and look for competition, the effect of lower concentrations of 2-deoxy-D-glucose on ribose uptake were investigated, and inhibition of ribose uptake was observed (Fig. 2).

Stereospecificity of the uptake process was demonstrated for both glucose and ribose, although the degree of difference between the uptake of D- and L-glucose was not as great as might have been expected from the differences in their affinity for the transport process in the human erythrocyte (LeFevre, 1961).

Part of the explanation for the poorer discrimination of the osmotic-protection method may reside in the differences inherent in the two methods of study as discussed above. The differences that we have observed are indeed very similar to those observed when the relative distribution spaces (a net uptake method) of D- and L-glucose were measured in the perfused rat liver (Williams *et al.*, 1968). It is therefore also possible that glucose transport in rat liver cells is less stereo-specific than that in human erythrocytes. Evidence has been obtained that D- and L-glucose compete for the same carrier process in human erythrocytes (Lin *et al.*, 1978).

Phlorrhizin inhibits D- and L-glucose transport in the perfused rat liver (Williams *et al.*, 1968) and in rat liver cells (Baur & Heldt, 1977). We have found that phlorrhizin also inhibits uptake of fructose, galactose and ribose by rat liver lysosomes. Cytochalasin B inhibits glucose uptake both by rat liver cells (Baur & Heldt, 1977) and by rat liver lysosomes.

In the perfused dog liver, in addition to demonstrating a stereospecific glucose- and galactose-transport system that is inhibited by phlorrhizin, Goresky & Nadeau (1974) and Goresky *et al.* (1973) also showed that β -methyl glucoside and β -methyl galactoside are nearly as impermeant as sucrose. In agreement with these observations, our results with rat liver lysosomes show that, although not excluded completely, these two derivatives permeate more slowly than the parent hexoses.

The Q_{10} of net glucose uptake by lysosomes (2.8) that we have observed may be compared with approx. 2 for the perfused liver (Park *et al.*, 1968), 2.5 for human erythrocytes (Bowyer, 1957) and 1.5 (above 18°C) or 3 (below 18°C) for rat liver cells (Baur & Heldt, 1977).

Thus we believe that by applying a number of criteria that may be used in conjunction with the osmotic-protection method for the study of sugar uptake by lysosomes, we have produced strong evidence in favour of this process being mediated by facilitated diffusion.

Having established good evidence of sugar transport in lysosomes, it is clear that a number of further studies will be of great interest. They include a search for other transport systems, a comparison of transport in primary and secondary lysosomes, a comparison of sugar transport in lysosomes from tissues such as muscle and adipose tissue where glucose uptake is rate-limiting for its utilization, and a search for possible control mechanisms.

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