Studies on the Permeability of Rat Liver Lysosomes to Carbohydrates

By J. B. LLOYD*

Department of Biochemistry, University of Miami School of Medicine, Miami, Fla. 33152, U.S.A.

(Received 7 July 1969)

1. The latency of nitrocatechol sulphatase activity was measured in rat liver lysosomes before and after preincubation in 0.25M solutions of 25 different carbohydrates. 2. Preincubation in disaccharides, hexitols, gluconate, glucuronate or lactate gave little or no rise in 'free' sulphatase activity, indicating that these compounds do not easily penetrate the lysosomal membrane, but incubation in monosaccharides or the lower glycitols caused a progressive loss of latency. 3. Rates of increase in 'free' activity were taken as an indication of rates of solute penetration into lysosomes and were correlated with the structure and molecular weight of each sugar. 4. Additional evidence for non-penetration of maltose was obtained by demonstrating that the latency of lysosomal α -glucosidase is independent of substrate concentration employed. 5. The results are discussed in the light of published data on the latency of lysosomal enzymes.

It is implicit in the concept of lysosomes as sites for intracellular digestion that many small molecules can penetrate the lysosomal membrane, at least from the inside out (de Duve & Wattiaux, 1966, p. 459). Also, Hainsworth & Wynn (1966) have reported that isolated rat liver lysosomes are permeable to glycerol, sorbose and *p*-nitrophenol if incubated with these compounds for 30 min. at room temperature. On the other hand the ability of sucrose, β -glycerophosphate (Appelmans & de Duve, 1955) and mannitol (Lejeune, Thinès-Sempoux & Hers, 1963) to act as osmotic protectors for lysosomes implies that these materials do not pass the lysosomal membrane, at least in ice-cold solution. By the same reasoning glucose and glycerol may be supposed to penetrate lysosomes at this temperature (Berthet, Berthet, Appelmans & de Duve, 1951). Indirect evidence that sucrose and mannitol cannot easily pass out of lysosomes *in vivo* comes from the demonstration by Fell & Dingle (1966) that exposure to these sugars (but not to glucose in the same concentration) leads to lysosome engorgement after endocytosis into embryonic chick bones grown in organ culture. Experiments by Cohn & Ehrenreich (1969) have shown that a number of disaccharides and trisaccharides will induce vacuolation of cultured mouse macrophages but that monosaccharides and metabolizable disaccharides (e.g. maltose and lactose) will not.

The latency of lysosomal enzymes in tissue homogenates prepared in osmotically protected

media is believed to result from the lysosomal membrane acting as a barrier to added substrates (see de Duve, 1963, 1965). However, even in freshly prepared lysosome-rich centrifugal fractions, latency is never complete and the observed 'free' activity, determined by short incubations in sucrose-protected buffer, can be explained in two ways. It may reflect the rupture of a proportion of the lysosomes during the homogenization and resuspension operations or, alternatively, it may indicate that the lysosomal membrane does not provide a complete barrier to the entry of substrates.

To learn more about the permeability of the lysosomal membrane, the ability of various sugars to afford osmotic protection to rat liver lysosomes was studied. The free and total activities of a lysosomal hydrolase (arylsulphatase) was measured after preincubation of lysosomes in 0.25M solutions of the different compounds. In a second series of experiments the 'free' and total activity of a lysosomal enzyme (α -glucosidase) was measured over a range of substrate concentrations extending from below the K_m to several times the K_m . The rationale for this type of experiment was explained by de Duve (1965), and the method has been used to study the latency of rat spleen lysosomal β -glucuronidase (Bowers, Finkenstaedt & de Duve, 1967). If 'free' activity represents lysosomal permeability, the proportion of 'free' to total activity should rise with increasing substrate concentration. By contrast the proportion should not change with substrate concentration if 'free' activity is due to the presence of some broken

* Present address: Department of Biochemistry, University College, Cathays Park, Cardiff.

Table 1. 'Free' nitrocatechol sulphatase of rat liver lysosomes preincubated at 25° in various media

Each value (mean $\pm$ s.d. of three experiments) is expressed as a percentage of total activity. Details are given in the Methods section. Compounds are listed approximately in order of increasing penetration.

Medium (0.25M)	Prein- cubation ...	Activity (% of total activity)		
		0 min.	30 min.	60 min.
Maltose		8.3 $\pm$ 5.0	7.2 $\pm$ 2.5	7.8 $\pm$ 3.0
Trehalose		6.5 $\pm$ 1.4	7.0 $\pm$ 0.6	7.8 $\pm$ 1.9
Sucrose		8.5 $\pm$ 1.5	8.6 $\pm$ 0.8	8.5 $\pm$ 1.7
Cellobiose		6.9 $\pm$ 1.3	8.5 $\pm$ 0.2	9.0 $\pm$ 2.0
Lactose		11.0 $\pm$ 5.5	11.1 $\pm$ 6.1	10.3 $\pm$ 4.6
D-Sorbitol		6.3 $\pm$ 0.7	8.6 $\pm$ 1.8	10.3 $\pm$ 3.3
D-Gluconate (potassium)		8.8 $\pm$ 3.7	9.9 $\pm$ 2.7	11.3 $\pm$ 1.6
D-Mannitol		11.0 $\pm$ 2.3	11.2 $\pm$ 2.7	13.0 $\pm$ 3.5
Lactate (lithium)		8.3 $\pm$ 2.0	10.5 $\pm$ 1.1	14.0 $\pm$ 1.7
D-Glucuronate (potassium)		7.2 $\pm$ 1.6	10.4 $\pm$ 2.8	14.7 $\pm$ 3.0
Inositol (inactive)		9.3 $\pm$ 3.7	10.8 $\pm$ 1.4	14.8 $\pm$ 0.6
D-Xylitol		8.4 $\pm$ 3.1	13.8 $\pm$ 3.2	27.5 $\pm$ 8.2
D-Arabitol		5.8 $\pm$ 4.5	14.9 $\pm$ 3.3	28.7 $\pm$ 4.4
Ribitol		7.4 $\pm$ 1.0	19.8 $\pm$ 5.7	46.0 $\pm$ 11.7
Methyl α -D-glucoside		8.5 $\pm$ 1.9	24.1 $\pm$ 7.1	49.6 $\pm$ 10.6
D-Glucose		9.6 $\pm$ 2.0	28.5 $\pm$ 2.0	50.5 $\pm$ 6.6
D-Mannose*		8.2 $\pm$ 2.4	34.0 $\pm$ 7.3	58.8 $\pm$ 16.2
L-Sorbose		8.0 $\pm$ 1.4	35.0 $\pm$ 11.5	63.7 $\pm$ 9.8
D-Glucuronic γ -lactone		8.7 $\pm$ 1.1	46.1 $\pm$ 8.8	68.7 $\pm$ 5.8
D-Arabinose		7.3 $\pm$ 2.5	43.6 $\pm$ 2.8	68.9 $\pm$ 4.0
D-Galactose		7.5 $\pm$ 1.7	44.3 $\pm$ 13.2	70.5 $\pm$ 14.5
D-Xylose		6.5 $\pm$ 1.1	52.1 $\pm$ 14.4	75.0 $\pm$ 11.8
D-Ribose		8.4 $\pm$ 2.1	69.1 $\pm$ 8.5	81.8 $\pm$ 5.6
Erythritol		7.3 $\pm$ 1.8	65.9 $\pm$ 5.4	83.2 $\pm$ 12.5
Glycerol		19.7 $\pm$ 8.5	83.8 $\pm$ 6.4	86.9 $\pm$ 7.7

* Results refer to four experiments.

lysosomes. α -Glucosidase was chosen because one of its substrates, maltose, is inexpensive enough to have been tested as an osmotic protector (Table 1).

METHODS

Measurements of 'free' and total arylsulphatase after incubation of lysosomes in 0.25M solutions of different carbohydrates. Male Wistar rats (230–280g., obtained from Rockland, Gilbertsville, Pa., U.S.A.) were starved for 16hr. and killed by a blow on the head. The liver was removed, pushed through a metal sieve and the resultant pulp was homogenized in 2.5vol. of ice-cold 0.25M-sucrose in a Teflon-on-glass Potter-Elvehjem-type homogenizer. The homogenate was diluted fourfold with 0.25M-sucrose and subjected to differential centrifugation in a Sorvall RC2-B automatic refrigerated centrifuge. The fraction sedimenting between 1100g (10min.) and 22500g (10min.) was gently resuspended in a volume of 0.25M test solution (at 4°) equal to four times the original weight of liver pulp. The suspen-

sion was placed in an incubation bath at 25° and samples withdrawn for assay at 0, 30 and 60min. 'Free' and total arylsulphatase activity was assayed in 0.1M-sodium acetate buffer, pH5.0, with 10mM-nitrocatechol sulphate (Sigma Chemical Co., St Louis, Mo., U.S.A.) as substrate (total volume 1ml.). All assays contained 0.25M-carbohydrate (0.20M-sucrose plus 0.05M of the compound in which the granules had been suspended), and those for total activity also contained 0.1% of Triton X-100. Incubation was for 10min. at 25° and was terminated by the addition of 6ml. of alkaline quinol reagent (Roy, 1953). Liberated nitrocatechol was determined by measuring the extinction at 540nm.

Measurements of 'free' and total α -glucosidase at various substrate concentrations. A 'large-granule' suspension was prepared as before, except that 0.25M-D-mannitol replaced sucrose as medium for both homogenization and resuspension. Part of the suspension was preincubated at 37° for 30min. and cooled in crushed ice for 10min. The 'free' and total α -glucosidase of both fresh and preincubated granules was measured. All assays were performed in 0.1M-sodium acetate buffer, pH5.0, containing 0.25M-D-mannitol and maltose concentrations from 1 to 20mM. Assays for total activity also contained 0.1% of Triton X-100. After 10min. incubation the reaction was stopped by the addition of 0.3ml. of 0.16M-ZnSO₄ and 0.3ml. of 0.32M-NaOH, and the precipitate was removed by centrifugation. Glucose in the supernatant was determined by a modification (Lloyd & Whelan, 1969) of the method of Fleming & Pegler (1963).

RESULTS

Table 1 shows the changes in availability of nitrocatechol sulphatase that resulted when lysosomes were preincubated in 0.25M solutions of different carbohydrates. No rise was seen with gluconate, mannitol and sorbitol, or with any of the five disaccharides tested, suggesting that these compounds gave complete osmotic protection to lysosomes under the conditions of the experiment. With the exception of inositol, lactate and glucuronate, where the rise was so slight as to be questionable, all the other compounds tested gave transient protection only, as revealed by a progressive increase in 'free' activity. The rate of increase varied very considerably with chemical structure, and certain generalizations can be made. Free activity increased more rapidly in solutions of aldoses than in solutions of the corresponding glycitols. Within the homologous series of glycitols increase in molecular weight led to an increase in osmotic protection; similarly the rise in free activity was greater with pentoses than with hexoses. Potassium glucuronate gave better protection than glucuronic lactone. Gluconate gave good protection, but gluconic lactone at 0.25M has pH2.8–3.0 and incubation in this solution led to an immediate loss of latency and also to progressive enzyme inactivation as judged by total activity in the 30min. and 60min. samples. Suspension of the particles

Table 2. 'Free' maltase of fresh and preincubated rat liver lysosomes as estimated at different substrate concentrations

Each value (mean $\pm$ S.D. of three experiments) is expressed as a percentage of total activity. Details are given in the methods section.

Concn. of maltose (mM)	Activity (% of total activity)	
	Fresh granules	Granules preincubated for 30 min. in 0.25M-mannitol at 37°
1	10.3 $\pm$ 1.7	56.2 $\pm$ 9.8
2	11.4 $\pm$ 2.5	58.2 $\pm$ 8.0
5	11.1 $\pm$ 3.1	59.4 $\pm$ 3.6
10	11.9 $\pm$ 5.0	58.4 $\pm$ 7.1
20	13.3 $\pm$ 3.6	68.2 $\pm$ 4.0

in distilled water made 90% of the arylsulphatase immediately available.

The α -glucosidase activity of rat liver granules shows apparent inhibition at maltose concentrations beyond 10mM. This has been reported previously for a comparable enzymic activity in dog liver (Torres & Olavarria, 1964), but the nature of the inhibition has not been thoroughly investigated. The decreased glucose release from maltose could result from a transition at high maltose concentrations from hydrolytic to transferase activity. A number of attempts were made to estimate the K_m of the α -glucosidase activity in the granules, but on no occasion was a truly linear double-reciprocal plot obtained, even at maltose concentrations below 10mM. An approximate K_m of 1–3mM is indicated.

Table 2 shows how the 'free' acid maltase activity of rat liver granules changed with substrate concentration. The maltose concentrations used in the experiment extend from below the apparent K_m to values where inhibition by substrate was observed. In both freshly prepared granules and in granules preincubated in 0.25M-mannitol for 30min. at 37°, the percentage 'free' activity was independent of maltose concentration, except at the highest concentration, where some increase was seen. In further experiments on fresh granules it was found that all the 'free' acid maltase was non-sedimentable at 22500g for 10min. The preincubated granules had a high 'free' activity of which 50–65% was found to be non-sedimentable.

DISCUSSION

The experiments described above are, in essence, extensions of those carried out some years ago by de Duve and his group (Berthet *et al.* 1951; Appelmans & de Duve, 1955). The theoretical justification is given in those papers and in a later summary (de Duve, 1965).

The method used to assess the ability of different carbohydrates to afford osmotic protection was

designed to eliminate complications arising from thermal activation of lysosomes during experiments. It is known from the work of many authors that rat liver lysosomes are stable if incubated in 0.25M-sucrose at temperatures just above 0°, but that incubation in sucrose at 37° leads to a rise in the 'free' activity of lysosomal enzymes. This rise is more rapid if the sucrose is buffered to pH 5.0. However, measurements of enzyme activity are not readily made at 0°, and damage to lysosomes will inevitably accompany transfer from a cold preincubation mixture to a warmer assay mixture. In preliminary experiments in this investigation it was found that preincubation of rat liver granules for 1hr. in 0.25M-sucrose or -mannitol at 25° caused no rise in the 'free' activity of a number of acid hydrolases. This was found equally true if the sucrose solution was buffered at pH 5.0 with 0.1M-sodium acetate-acetic acid. In the method used, therefore, both preincubation and assay were performed at 25°. Arylsulphatase was chosen as marker enzyme activity in these experiments, for two reasons. The assay with nitrocatechol sulphate is sufficiently sensitive for short assays at 25°. Also, the activity is generally not inhibited by carbohydrates; of the 25 compounds tested as osmotic protectors, the only inhibition observed was with cellobiose, which at 50mM inhibited nitrocatechol sulphatase to the extent of 15–17% under the assay conditions described. However, one compound (glycerophosphate) gave such profound inhibition (approx. 90% at 50mM) that it had to be excluded from the list.

Table 1 shows clearly that all the solutions tested differ from pure water in that they initially provide complete osmotic protection. They differ from each other in the degree to which this protection continues on extended exposure. The findings are consistent with a first-approximation model of the lysosome as a bag-like structure whose membrane is semi-permeable and of limited tensile strength. Further, if it is assumed that the lysosome contains solutes unable to pass through its membrane (and therefore able to exert an osmotic pressure), this model accounts for the immediate rupture observed when lysosomes are placed in water and for their stability in iso-osmotic solutions of non-penetrating solutes. Solutes whose ability to stabilize lysosomes decreases with duration of exposure may be supposed to penetrate through the lysosomal membrane at a finite rate, carrying water molecules with them.

From the results given in Table 1 some tentative conclusions may be made about the relationship between the chemical structure of a solute and its ability to pass into lysosomes. Disaccharides, hexitols and gluconate (and perhaps lactate and glucuronate) pass into lysosomes either not at all or too slowly for the consequences of their entry to be

revealed by the experimental method used. The remaining compounds all penetrate rapidly enough to cause a clear decrease in latency within the time-limit of the experiment. The comparative rates of entry of the various compounds, as far as these may be assessed, are generally consistent with a theoretical treatment developed by Stein (1967) to account for non-facilitated diffusion of molecules through biological membranes. This relates the rate of diffusion of a solute both to its molecular weight and to the number of hydrogen bonds it forms with water, the latter term being a measure of the retardation a molecule experiences in passing the phase boundary on entering the membrane's lipid milieu. Thus lysosomal permeability decreases in ascending the homologous series of glycitols or aldoses. Also, as predicted on the basis of the number of hydroxyl groups in the molecule [used by Stein (1967) as an estimate of the hydrogen bonds formed], the apparent rates of penetration of erythritol and the pentoses are approximately equal. On the other hand, the hexoses seem to penetrate faster than the pentitols. This could be taken as evidence for a facilitated diffusion system, but, in the absence of any apparent specificity (glucose enters somewhat more slowly than other hexoses), it is impossible to sustain this argument. It seems rather more likely that, although counting hydroxyl groups is a useful method of assessing hydrogen-bond formation within a homologous series, it is inadequate for comparing molecules as different as the predominantly cyclic aldoses and the linear glycitols. However, the methods used here are inappropriate to settle this point. It is relevant to note, though, that in studies with artificial phospholipid liquid crystals (liposomes), a system where no facilitated diffusion can take place, glucose was excluded as well as mannitol or sucrose, whereas erythritol and glycerol penetrated (Bangham, de Gier & Greville, 1967).

The measured latency of acid maltase in fresh rat liver lysosomes is independent of the substrate concentration used, at least between 1 and 10 mM (Table 2). This suggests that the granules are impermeable to maltose, and thus supports the interpretation given above to the data in Table 1. The 'free' maltase activity of the fresh lysosomes must therefore represent enzyme from broken particles, and indeed the 'free' and non-sedimentable activities were in this case equal. The data in Table 2 for lysosomes preincubated at 37° for 30 min. in 0.25 M-mannitol are more difficult to interpret, because a part of the 'free' activity remained associated with sedimentable material. This is a common finding in studies of the availability of lysosomal enzymes under different conditions (Savant, Shibko, Kumta & Tappel, 1964; Sawant, Desai & Tappel, 1964; Shibko, Pangborn & Tappel, 1965) and one that some authors take as a clear

indication of substrate penetration into lysosomes (Shibko *et al.* 1965; Rosenberg & Janoff, 1968). However, it may simply be that lysosomes can rupture without complete desorption of their enzymes or that released enzyme may be secondarily adsorbed on particulate matter present in the suspension. This alternative explanation would seem favoured in the present experiments, because the failure of substrate concentration to affect the measured latency suggests that lysosomes that remain unbroken after preincubation are still impermeable to maltose.

The results presented in this paper are in complete harmony with those previously available on the permeability of lysosomes to different solutes (see the introduction). They also permit comment on some other published observations, chiefly about lysosome latency. Some lysosomal enzymes do not display latency. Shibko & Tappel (1964) found that the esterase of rat liver lysosomes was such an enzyme, and, on finding that the enzyme was associated with the lysosome membrane, surmised that its non-latency resulted from a localization on the external (i.e. cytoplasmic) surface of this membrane. Although this is perhaps the most likely explanation, it is also possible that the highly non-polar substrate used (α -naphthyl acetate) can penetrate rapidly into lysosomes. Rat-liver lysosomal β -glucosidase (β -xylosidase) is also a membrane enzyme (Beck & Tappel, 1968), and is non-latent when *p*-nitrophenyl β -glucoside is used as substrate (Lloyd, 1969). In this case, however, it was possible to eliminate the explanation based on substrate permeability, by demonstrating latency of an exogenous lysosomal β -glucosidase (Lloyd, 1969). In human neutrophils lactate dehydrogenase is associated with lysosomes and is not latent (John, Berger, Bonner & Schultz, 1967). In reporting this John *et al.* (1967) concluded that the enzyme, which is in most cells associated with the cytosol, in leucocytes adheres to the lysosomal membrane. The present finding that liver lysosomes are impermeable to lactate tends to support this interpretation. In experiments on liver lysosomes from rats injected with yeast invertase Jacques (1968) found the endocytosed enzyme to be latent, an expected result in view of the impermeability of lysosomes to the substrate of invertase, i.e. sucrose. In parallel experiments Jacques (1968) found endocytosized horseradish peroxidase to be non-latent and concluded that rat liver lysosomes must be freely permeable to the substrates used, namely hydrogen peroxide and *NN*-dimethyl-*p*-phenylenediamine. However, John *et al.* (1967), using guaiacol as hydrogen donor, found that the myeloperoxidase of human neutrophil lysosomes displayed a similar degree of latency to the acid hydrolases. It is not known whether these results reflect true

differences in permeability properties between human leucocyte and rat liver lysosomes or merely differences between the experimental procedures employed in the two laboratories.

I thank the Florida Division of the American Cancer Society for a grant and Mrs Gwenda Denner for excellent technical assistance. The work was carried out during the tenure of a Wellcome Travelling Fellowship, and I thank Dr W. J. Whelan for the hospitality of his Department and for his interest in the work. Dr Whelan also read this manuscript and I am grateful for his many helpful suggestions.

REFERENCES

- Appelmans, F. & de Duve, C. (1955). *Biochem. J.* **59**, 426.
- Bangham, A. D., de Gier, J. & Greville, G. D. (1967). *Chem. Phys. Lipids*, **1**, 225.
- Beck, C. & Tappel, A. L. (1968). *Biochim. biophys. Acta*, **151**, 159.
- Berthet, J., Berthet, L., Appelmans, F. & de Duve, C. (1951). *Biochem. J.* **50**, 182.
- Bowers, W. E., Finkenstaedt, J. T. & de Duve, C. (1967). *J. Cell Biol.* **32**, 325.
- Cohn, Z. A. & Ehrenreich, B. A. (1969). *J. exp. Med.* **129**, 201.
- de Duve, C. (1963). In *Ciba Found. Symp.: Lysosomes*, p. 1. Ed. by de Reuck, A. V. S. & Cameron, M. P. London: J. and A. Churchill, Ltd.
- de Duve, C. (1965). *Harvey Lect.* **59**, 49.
- de Duve, C. & Wattiaux, R. (1966). *Annu. Rev. Physiol.* **28**, 436.
- Fell, H. B. & Dingle, J. T. (1966). *Biochem. J.* **98**, 40P.
- Fleming, I. D. & Pegler, H. F. (1963). *Analyst*, **88**, 967.
- Gianetto, R. & de Duve, C. (1955). *Biochem. J.* **59**, 433.
- Hainsworth, B. & Wynn, C. H. (1966). *Biochem. J.* **101**, 9P.
- Jacques, P. J. (1968). *Épuration Plasmatique de Protéines Étrangères, leur Capture et leur Destinée dans l'Appareil Vacuolaire du Foie*. Louvain: Libraire Universitaire.
- John, S., Berger, N., Bonner, M. J. & Schultz, J. (1967). *Nature, Lond.*, **215**, 1483.
- Lejuene, N., Thinès-Sempoux, D. & Hers, H. G. (1963). *Biochem. J.* **86**, 16.
- Lloyd, J. B. (1969). *Biochem. J.* **115**, (in the Press).
- Lloyd, J. B. & Whelan, W. J. (1969). *Analyt. Biochem.* **30**, 467.
- Rosenberg, M. & Janoff, A. (1968). *Biochem. J.* **108**, 889.
- Roy, A. B. (1953). *Biochem. J.* **53**, 12.
- Sawant, P. L., Desai, I. D. & Tappel, A. L. (1964). *Arch. Biochem. Biophys.* **105**, 247.
- Sawant, P. L., Shibko, S., Kumta, U. S. & Tappel, A. L. (1964). *Biochim. biophys. Acta*, **85**, 82.
- Shibko, S., Pangborn, J. & Tappel, A. L. (1965). *J. Cell Biol.* **25**, 479.
- Shibko, S. & Tappel, A. L. (1964). *Arch. Biochem. Biophys.* **106**, 259.
- Stein, W. D. (1967). *The Movement of Molecules Across Cell Membranes*, pp. 72-85. New York and London: Academic Press Inc.
- Torres, H. N. & Olavarria, J. M. (1964). *J. biol. Chem.* **239**, 2427.