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Latency of Some Glycosidases of Rat Liver Lysosomes

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The latency of the α-glucosidase activity of intact rat liver lysosomes was studied by using four substrates (glycogen, maltose, *p*-nitrophenyl α-glucoside, α-fluoroglucoside) at a range of substrate concentrations. The results indicate that the entire lysosome population is impermeable to glycogen and maltose, but a proportion of lysosomes are permeable to α-fluoroglucoside and a still higher proportion permeable to *p*-nitrophenyl α-glucoside. Incubation at 37°C in an osmotically protected buffer of pH5.0 caused lysosomes to become permeable to previously impermeant substrates and ultimately to release their α-glucosidase into the medium. The latencies of lysosomal β-glucosidase and β-galactosidase were examined by using *p*-nitrophenyl β-glucoside and β-galactoside as substrates. The results indicate permeability properties to these substrates similar to that to *p*-nitrophenyl α-glucoside. On incubation in an osmotically protected buffer of pH5, lysosomes progressively released their β-galactosidase in soluble form, but β-glucosidase remained attached to sedimentable material. Lysosomal β-glucosidase was inhibited by 0.1% Triton X-100; α-glucosidase and β-galactosidase were not inhibited.

Lysosomes display little enzymic activity until their membranes are damaged or destroyed. This 'latency' of lysosomal enzymes, the phenomenon, that led de Duve (see de Duve, 1969) to discover the existence and many of the characteristics of lysosomes, results from the permeability properties of the lysosome membrane. This membrane is impermeable both to the enzymes within the lysosome and to the substrates commonly used for their assay.

Experimentally determined latency is never complete and in the case of some enzymes it is far from complete. These findings have been variously interpreted as indicating either damage to a proportion of lysosomes during preparation, or to contamination of lysosomes with non-lysosomal (and therefore non-latent) enzymes, or to a degree of permeability of undamaged lysosomes to at least some substrates. To test some of these hypotheses, experiments have been undertaken in which the latency of some lysosomal glycosidases of rat liver has been studied by using substrates of different molecular size over a range of substrate concentrations.

Methods

Preparation of a lysosome-enriched fraction of rat liver

Male Wistar rats (300–400*g*) were starved overnight and killed by a blow on the head. The liver was removed, washed in ice-cold 0.25M-mannitol, blotted dry, weighed, and pushed through a metal sieve. The resultant pulp was homogenized in 2.5 vol. of ice-cold 0.25M-mannitol in a Teflon-on-glass Potter–Elvehjem-type homogenizer (Tri-R Instruments Inc., Rockville Center, New York, NY 11570, U.S.A., model K41; diameter clearance 0.019cm), forcing the suspension past the pestle, which was rotating at approx. 3000 rev./min (speed setting 2.7), three times in 30s. The homogenate, after dilution with ice-cold 0.25M-mannitol to a final volume of 10ml per g of liver pulp, was subjected to differential centrifugation at 4°C in a MSE High-Speed 18 refrigerated centrifuge (rotor no. 69 181). The fraction sedimenting between 1100*g* (10min) and 22500*g* (10min) was gently resuspended in 2.5ml of ice-cold 0.25M-mannitol per g of original liver pulp. This lysosome-enriched fraction was freshly prepared immediately before enzyme assays.

Enzyme assays

p-Nitrophenyl glycosidases. The incubation mixture comprised 0.5ml of 0.1M-sodium acetate/acetic acid buffer (pH5.0) containing 0.25M-mannitol (subsequently referred to as acetate/mannitol buffer); 0.25ml of *p*-nitrophenyl α-D-glucoside, β-D-glucoside, β-D-galactoside or β-D-xyloside (Sigma London Chemical Co., London S.W.6, U.K.), in 0.25M-mannitol; and 0.25ml of liver fraction suspension. After incubation at 37°C, 0.5ml of aq. 3.3% (w/v) trichloroacetic acid was added, the precipitate removed by centrifugation at 1500*g* for 10min, and 1.0ml of the supernatant added to 2.0ml of 0.2M-Na_2CO_3. Liberated *p*-nitrophenol was determined spectrophotometrically at 420nm. Control experiments were performed in which substrate and enzyme

solutions were incubated separately and mixed only after the addition of trichloroacetic acid.

Maltase, α-fluoroglucosidase and glycogen hydrolase. The incubation mixture comprised 0.5ml of acetate/mannitol buffer; 0.25ml of maltose (Sigma, grade II), α-D-fluoroglucoside (supplied by Dr. J. E. G. Barnett, University of Southampton, U.K.) or rabbit liver glycogen (Sigma, type III), in 0.25M-mannitol; and 0.25ml of liver fraction suspension. After incubation at 37°C, 0.3ml of 0.16M-$ZnSO_4$ and 0.3ml of 0.32M-NaOH were added, the precipitate was removed by centrifugation at 1500*g* for 10min, and the glucose content of 1.0ml of supernatant determined by the method of Lloyd & Whelan (1969). Control experiments were performed as with *p*-nitrophenyl glycoside substrates.

Measurements of latency

The 'free activity' of enzymes was estimated by using the above assays and liver fraction suspensions were prepared as described above and otherwise untreated. 'Initial free activity' signifies the free

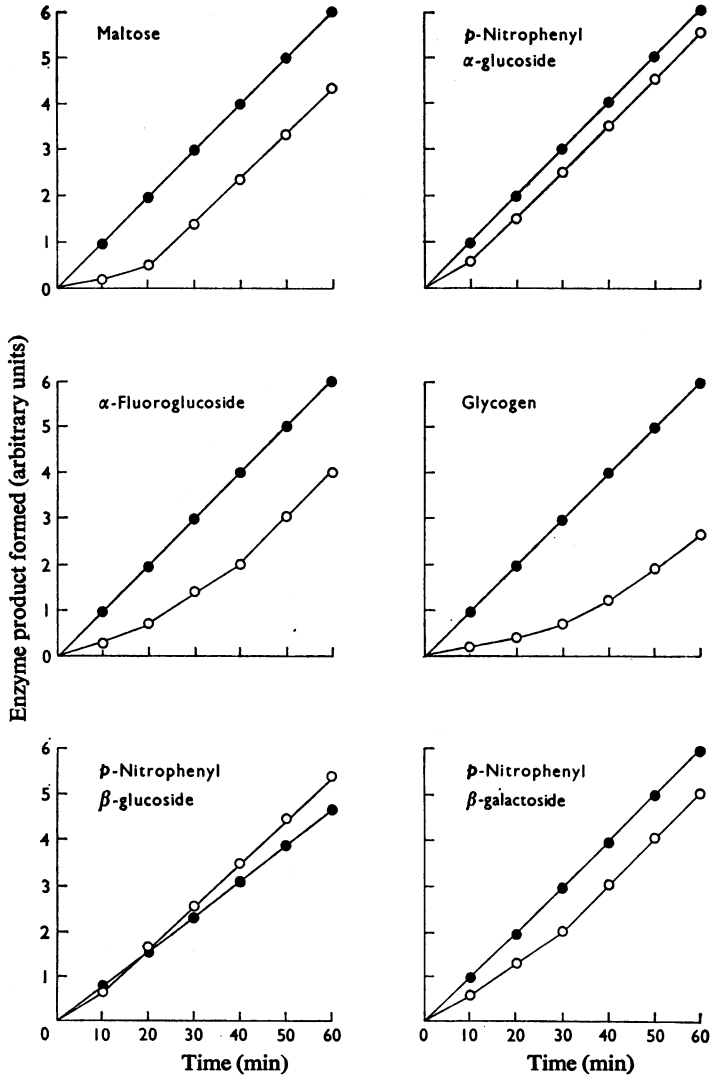

Fig. 1. *Time-course of hydrolysis of six glycosides by lysosome-enriched fraction of rat liver*

Incubations were at 37°C in buffered 0.25M-mannitol, pH5.0, in the absence (○) or presence (●) of 0.1% Triton X-100. Substrate concentrations were 20mg/ml for glycogen and 5mM for all other substrates.

activity as measured by a 10min assay. Two methods were used to measure 'total activity'. In the first method 0.1% of Triton X-100 (BDH Chemicals Ltd., Poole, Dorset, U.K.) was incorporated into the incubation mixtures. In the second method the free activity was measured for several incubation periods from 10 to 60min. It was found (see below) that the graph of free activity against time became linear when the glycosidases became fully available to substrate. Measurement of the slope of the graph after this point was reached gave a measure of the 'total activity'.

In some experiments free and total activities of glycosidases were measured in assays performed as above but at 25°C, after a period of preincubation of the liver fraction in acetate/mannitol buffer.

Sedimentability of glycosidases

Liver fraction suspension (0.5ml) was diluted with 1.5ml of acetate/mannitol, incubated at 37°C, and centrifuged at 4°C at 25000*g* for 15min. Enzyme determination on the supernatant yielded the non-sedimentable activity. Except for glycogen hydrolase, total activity was estimated on a similarly diluted sample, preincubated for 50min at 37°C but omitting the centrifugation. Total glycogen hydrolase activity was measured in the presence of Triton X-100, without preincubation.

Results

Fig. 1 shows the time-course of hydrolysis of six glycosides by the lysosome-enriched liver fraction in the presence and the absence of 0.1% Triton X-100. In the presence of Triton X-100 the rate of hydrolysis of each substrate was constant from the start of the incubation. In the absence of Triton X-100 the rate of hydrolysis increased from a low initial value until it became constant. The period of time taken to reach a constant value differed for each substrate and in

Table 1. *Free activities of some glycosidases in lysosome-enriched liver fractions incubated at 37°C in acetate/mannito buffer for periods up to 60min*

Free activity for each 10min time-interval is expressed as a percentage of the total activity. Total activity was defined as half the free activity measured over the 40–60min time-interval, except for glycogen hydrolase, where the activity as measured in the presence of Triton X-100 was used. Each value is the mean±S.D. for four experiments.

		Free activity					
Time-interval (min) ...		0–10	10–20	20–30	30–40	40–50	50–60
Substrate	Substrate concentration (mM)						
Maltose	1	16±4	56±14	82±12	91±21	101±19	127±23
	2	9±5	38±12	66±11	84±9	88±22	119±14
	4	16±3	65±12	79±18	85±21	100±18	109±19
	5	12±4	35±15	98±19	76±20	99±21	104±22
	10	14±5	46±14	72±10	74±19	87±13	114±18
p-Nitrophenyl α-glucoside	1	66±4	91±15	93±10	94±12	92±13	113±21
	2	77±8	78±16	100±15	92±6	99±9	96±17
	4	70±7	105±10	101±15	105±16	93±16	111±20
	5	69±11	72±19	105±8	90±13	114±11	85±24
	10	66±7	70±18	89±19	109±9	99±15	111±16
α-Fluoroglucoside	1	28±3	43±3	47±6	77±3	100±10	107±8
	2	24±2	41±4	60±3	71±8	100±1	94±9
	4	30±1	42±2	64±12	89±2	100±1	97±19
	5	28±1	43±1	62±2	78±8	101±2	106±4
	10	37±1	48±7	62±1	90±3	103±4	107±7
Glycogen	20mg/ml	13±7	22±15	25±13	62±16	68±12	79±3
p-Nitrophenyl β-glucoside	1	67±6	106±23	117±14	94±3	89±12	87±17
	2	70±11	75±9	105±14	108±19	98±7	95±17
	4	66±3	95±13	101±10	110±9	91±23	92±17
	5	72±5	74±23	100±14	96±3	102±12	97±20
	10	74±7	80±5	100±11	113±15	91±18	104±24
p-Nitrophenyl β-galactoside	1	52±7	59±12	94±8	85±15	106±10	95±22
	2	52±1	65±16	89±9	88±13	118±18	107±19
	4	59±3	64±11	93±7	94±7	112±23	85±24
	5	57±5	62±13	91±13	105±10	110±22	108±24
	10	51±5	59±5	110±21	108±9	108±10	85±22

the case of glycogen exceeded 60min. Except for glycogen and *p*-nitrophenyl β-glucoside, the rate of hydrolysis after 40min of incubation was equal to that in the presence of Triton X-100. Table 1 shows the free activity for each 10min interval, expressed as a percentage of the total activity, for each substrate at a range of substrate concentrations. Table 2 shows the apparent K_m values of the initial free and the total activities derived from the same data as used to compile Table 1.

Table 3 shows the initial free activity of glycosidases, expressed as a percentage of the total activity, measured at 25°C after preincubation of the lysosome-enriched fraction in acetate/mannitol buffer at 25°C for periods of time up to 120min.

Table 4 shows the non-sedimentable activity of glycosidases, expressed as a percentage of the total activity, after preincubation of the lysosome-enriched fraction in acetate/mannitol buffer for periods of time up to 60min.

Table 5 shows that decreasing the assay time from 10 to 5min has no significant effect on the initial percentage free activity of glycosidases.

It is clear from Fig. 1 that 0.1% Triton X-100 has no inhibitory effect on the action of lysosomal glycosidases on *p*-nitrophenyl α-glucoside, *p*-nitrophenyl β-galactoside, maltose, α-fluoroglucoside and glycogen. In the case of *p*-nitrophenyl β-glucoside, however, the activity in the presence of 0.1% Triton X-100 was lower than the maximum activity in its absence. Experiments were therefore performed in which a lysosome-enriched liver fraction, preincubated in acetate/mannitol buffer at 37°C for 40min to destroy latency, was assayed for *p*-nitrophenyl β-glucosidase activity at various substrate concentrations and in the presence of various concentrations of Triton X-100 (Table 6). Since it has been suggested that the lysosomal β-xylosidase and β-glucosidase activities may

Table 2. *Michaelis constants ($K_m$app.) of initial free and total glycosidase activities in lysosome-enriched liver fractions*

	$K_m$app. (mM)	
	Initial free activity	Total activity
Maltase	2.2	2.2
p-Nitrophenyl α-glucosidase	2.0	2.0
α-Fluoroglucosidase	3.2	3.0
p-Nitrophenyl β-glucosidase	1.0	1.0
p-Nitrophenyl β-galactosidase	1.1	1.1

Table 3. *Initial free activities of some glycosidases in lysosome-enriched liver fractions preincubated and assayed in acetate/mannitol buffer at 25°C*

Initial free activity is expressed as a percentage of the total activity. Total activity was measured by a 10min assay at 25°C after a 40min preincubation in acetate/mannitol buffer at 37°C to destroy latency. Each value is the mean±S.D. for four experiments.

	Initial free activity				
Preincubation (min) ... Substrate	0	30	60	90	120
Maltose (5mM)	15.5±1.9	25.7±5.5	47.3±10.6	69.0±13.8	72.2±12.2
p-Nitrophenyl α-glucoside (5mM)	57.5±5.7	47.0±2.7	56.5±4.4	64.0±6.3	75.0±9.6
p-Nitrophenyl β-glucoside (5mM)	47.5±3.3	54.5±3.3	72.3±9.8	96.2±18.7	107.3±17.6
p-Nitrophenyl β-galactoside (4mM)	38.7±5.4	61.1±8.3	73.5±11.7	81.3±9.7	89.0±10.8

Table 4. *Non-sedimentable activities of some glycosidases in lysosome-enriched liver fractions after incubation at 37°C in acetate/mannitol buffer*

Non-sedimentable activity is expressed as a percentage of total activity. Each value is the mean±S.D. for four experiments.

	Non-sedimentable activity					
Preincubation at 37°C (min) ... Substrate	0	10	20	30	40	60
Maltose (5mM)	5.5±0.5	12.4±3.9	44.2±18.2	55.3±13.6	66.3±8.7	71.5±9.0
p-Nitrophenyl α-glucoside (5mM)	6.1±1.6	11.2±6.4	45.3±22.2	53.9±14.4	67.2±12.9	68.8±6.9
α-Fluoroglucoside (5mM)	2.6±3.6	6.8±14.1	42.2±3.5	68.1±8.6	69.0±4.7	77.6±8.8
Glycogen (20mg/ml)	1.6±1.4	5.8±2.2	20.5±13.4	50.7±8.4	68.1±8.6	67.5±5.5
p-Nitrophenyl β-glucoside (5mM)	2.3±0.7	2.0±0.6	2.8±1.7	3.3±1.3	7.7±2.1	7.4±6.7
p-Nitrophenyl β-galactoside (4mM)	5.4±1.9	8.5±2.6	33.5±15.4	47.9±9.6	56.5±6.9	56.7±7.5

Table 5. *Initial free activities of some glycosidases in lysosome-enriched liver fractions, determined in assays at 37°C of either 5 or 10 min duration*

Initial free activity is expressed as a percentage of total activity. Total activity was measured after a 40 min preincubation in acetate/mannitol buffer to destroy latency. Each value is the mean±S.D. for four experiments.

Substrate	Substrate concentration (mM)	Initial free activity: Duration of assay (min) ... 5	10
p-Nitrophenyl α-glucoside	5	54.8±4.0	60.3±1.8
p-Nitrophenyl β-glucoside	5	50.8±1.6	52.5±5.7
p-Nitrophenyl β-galactoside	4	51.6±2.5	49.5±1.6
Maltose	5	14.7±2.5	14.0±1.6

Table 6. *Inhibitory effect of Triton X-100 on the p-nitrophenyl β-glucosidase activity of lysosome-enriched liver fractions*

Each result is the mean for two experiments. Inhibition is expressed as percentage loss of the activity found in the absence of Triton X-100.

Concentration of Triton X-100 (%, w/v)	Inhibition (%): Concentration of *p*-nitrophenyl β-glucoside (mM) ... 1	2	3	4	5
0.1	34	39	39	31	21
0.2	59	60	45	41	40
0.3	61	57	47	43	41

be due to a single enzyme (Robinson & Abrahams, 1967; Beck & Tappel, 1968), the effect of Triton X-100 on hydrolysis of *p*-nitrophenyl β-xyloside by the lysosome-enriched liver fraction was investigated. At a substrate concentration of 5 mM, β-xylosidase activity was inhibited approx. 25% by 0.1% and approx. 50% by 0.2% or 0.3% Triton X-100.

Discussion

α-Glucosidase

A major aim of this work was to examine the latency of a lysosomal enzyme towards substrates of a wide range of molecular weights. It was considered particularly interesting to use substrates known from other studies to be capable of penetrating the lysosomal membrane. α-Glucosidase was chosen for study because of its known ability to hydrolyse both maltose and glycogen. It was hoped originally to use α-methyl glucoside as a substrate, as there is experimental evidence (Lloyd, 1969*a*) that this substance like glucose itself can cross the membrane; however, α-methyl glucoside inhibits the action of α-glucosidase on glycogen (Rosenfeld & Belenki, 1968; Brown *et al.*, 1970; Palmer, 1971) and initial experiments showed that lysosomal enzymes fail to hydrolyse it. A good alternative was found in α-fluoroglucoside, whose molecular weight is very little higher than that of glucose itself, and which had been reported as a good substrate for a number of non-lysosomal α-glucosidases (Barnett *et al.*, 1967*a*,*b*). We found that lysosomal α-glucosidase hydrolysed α-fluoroglucoside at 4–5 times the rate seen with maltose.

Table 1 shows that the initial free activity of α-glucosidase in the lysosome-rich liver fraction is 10–15% of the total activity with maltose or glycogen as substrate, 25–30% with α-fluoroglucoside, and about 70% with *p*-nitrophenyl α-glucoside. These striking differences could reflect no more than the contamination of the lysosome-rich liver fraction with a non-lysosomal acid glycosidase able to hydrolyse *p*-nitrophenyl α-glucoside but not maltose or glycogen. This explanation is made exceedingly unlikely by the observations (Table 4) that, on incubating the liver fraction in acetate/mannitol buffer at 37°C, the four activities are released from the particulate into the soluble fraction at essentially the same rate and that this rate is different from the

rate of release of the two other enzymes studied. It would appear therefore that one enzyme only is responsible for the hydrolysis of the four α-glucosides in these experiments and that the differences in latency must reflect differences in the accessibility of this enzyme to the different substrates. Further evidence that liver lysosomes contain only one α-glucosidase of wide substrate specificity comes from studies on the purified enzyme (Jeffrey *et al.*, 1970*a*,*b*), subcellular fractionation studies (R. Burton & J. B. Lloyd, unpublished work) and from the observation that in patients with Pompe's (Type II) glycogen-storage disease, activities against glycogen, maltose, isomaltose (Brown *et al.*, 1970), *p*-nitrophenyl α-glucoside (Fluharty *et al.*, 1973) and 4-methylumbelliferyl α-glucoside (Salafsky & Nadler, 1973) are all absent.

The results of Tables 1 and 4 indicate that virtually all the α-glucosidase of intact liver lysosomes is inaccessible to glycogen and maltose. Much of the observed 10–15% free activity is non-sedimentable, indicating damage to the lysosomes during resuspension, and the remainder is readily explained as resulting from damage to lysosomes during a 10min incubation. In contrast, the lysosomes show a high initial free activity against α-fluoroglucoside and *p*-nitrophenyl α-glucoside. This could indicate that the lysosomes rapidly become permeable to these substrates during the 10min assay period. However, this explanation is made unlikely by experiments (Table 3) in which lysosomes suspended in acetate/mannitol buffer (pH5.0) at 25°C show only a slow increase in percentage free activity towards *p*-nitrophenyl α-glucoside over 2h, although demonstrating a high initial free activity to this substrate. It therefore appears that lysosomes impermeable to maltose and glycogen are permeable to these substrates of lower molecular weight. This conclusion is in keeping with the abilities of disaccharides, but not monosaccharides, to afford osmotic protection to lysosomes (Lloyd, 1969*a*) and to escape from the lysosomal interior after pinocytic uptake (Cohn & Ehrenreich, 1969).

If lysosomes are permeable to *p*-nitrophenyl α-glucoside, why is the initial free activity not 100%? One possible explanation is that lysosomes are indeed fully permeable to *p*-nitrophenyl α-glucoside but that the time taken to achieve an intralysosomal substrate concentration equal to that in the ambient assay medium is a significant fraction of the 10min assay period. If this were so, significantly lower values for the percentage free activity would be expected in assays of 5min duration. That this is not so (Table 5) indicates a rapid equilibration of *p*-nitrophenyl α-glucoside concentration across the membrane of lysosomes permeable to that substrate. A second possible explanation is that the passage of substrate across the lysosomal membrane is the rate-determining step, so that the substrate concentration encountered by the intralysosomal enzyme is lower than that in the external solution. de Duve (1965) has argued that, if such a situation obtained, the percentage free activity would increase towards 100% if the external substrate concentration were increased beyond the K_m value of the enzyme. Similarly the apparent K_m value of the free activity would be greater than that of the total activity. Tables 1 and 2 show that there is no evidence for membrane penetration being a rate-determining step in the hydrolysis of *p*-nitrophenyl α-glucoside, and only a hint of such evidence in the results with α-fluoroglucoside.

It is concluded therefore that rat liver lysosomes exhibit a degree of heterogeneity in respect of membrane permeability. The results are explicable if all lysosomes in the liver fraction used in these studies were initially impermeable to glycogen and maltose, but that some of them were permeable to α-fluoroglucoside and a still higher fraction also permeable to *p*-nitrophenyl α-glucoside. This heterogeneity could be intercellular in origin, representing differences between parenchymal and Kupffer cells, or intracellular, reflecting differences in lysosome size or the distinction between primary and secondary lysosomes. As pointed out elsewhere (Burton *et al.*, 1975), previous data on the permeability properties of rat-liver lysosomes (Lloyd, 1969*a*, 1971) are consistent with a degree of heterogeneity.

Our conclusion that some lysosomes are impermeable to α-fluoroglucoside probably implies that such lysosomes are impermeable to glucose, since there is little difference in molecular weight between the two substances and no evidence that permeability to hexoses is substrate-specific (Lloyd, 1969*a*). Presumably secondary lysosomes are permeable to glucose (Lloyd, 1973), indicating perhaps that primary lysosomes are the less permeable species. It is noteworthy too that more lysosomes appeared to be permeable to *p*-nitrophenyl α-glucoside (mol.wt. 285) than to α-fluoroglucoside (mol.wt. 182). It seems probable that the lipophilic aromatic residue on the former substrate aids penetration through the lysosomal membrane.

When the lysosome-rich liver fraction was incubated in acetate/mannitol buffer (pH5) at 37°C for periods up to 60min, the latency and sedimentability of its α-glucosidase were progressively lost. Non-sedimentable activity reached a maximum (approx. 70% of the total activity) after 30–40min incubation. The failure to reach 100% of the total activity probably reflects incomplete dissociation of the enzyme from broken lysosomes or secondary adsorption of released enzyme on components of the fraction. With glycogen as substrate, there was a good correspondence between percentage available (free) activity (Table 1) and percentage non-sedimentable activity (Table 4). This no doubt reflects

the size of the glycogen molecule, whose ability to traverse the lysosomal membrane is likely to be no greater than that of the α-glucosidase. With smaller substrates free activity of α-glucosidase rose on incubation more rapidly than non-sedimentable activity, indicating that on incubation lysosomes become accessible to substrates before losing their enzymes into the incubation medium. The percentage free activity is independent of substrate concentration for all times of incubation (Table 1). These data are consistent with a model in which lysosomes, on incubation, become progressively more leaky, permitting first the entrance of small and lipophilic substrates, later that of larger and hydrophilic substrates, and finally allowing the exit of the lysosomal enzymes.

β-Glucosidase

The results show that rat liver lysosomal β-glucosidase is inhibited, and that α-glucosidase and β-galactosidase are not inhibited, by Triton X-100 in concentrations widely used in assays of lysosomal enzymes to disrupt lysosomal membranes. This is the first report of inhibition of a lysosomal enzyme by Triton X-100, although lack of inhibitory properties has previously only been positively reported for a few enzymes (Wattiaux & de Duve, 1956). Quantitatively comparable inhibitions of β-xylosidase was discovered, providing further evidence for the postulate (Robinson & Abrahams, 1967; Beck & Tappel, 1968), that lysosomal β-glucosidase and β-xylosidase are the same enzyme.

Lloyd (1969*b*) claimed that rat liver lysosomal *p*-nitrophenyl β-glucosidase was wholly non-latent and deduced from this and other evidence that the enzyme was located on the cytoplasmic face of the lysosome. This conclusion, which was difficult to accept because of the postulated intralysosomal role of the enzyme, is now seen to be false and to result from the use of Triton X-100 in the assays for total activity. When total activity of *p*-nitrophenyl β-glucosidase was estimated by a method avoiding the inhibitory action of Triton X-100, latencies very similar to those found for *p*-nitrophenyl α-glucosidase were obtained. It may thus be concluded that both enzymes are intralysosomal in location and that the permeability of the lysosomal membrane to both anomers is similar. A striking difference is observed, however, in the degree to which α- and β-glucosidases are released from lysosomes on incubation at pH 5 and 37°C (Table 4). The failure of broken lysosomes to release their β-glucosidase in soluble form is consistent with an earlier report (Beck & Tappel, 1968) that the enzyme is firmly bound to the lysosomal membrane. The ability of Triton X-100 to inhibit β-glucosidase may indicate that enzyme activity is to some extent dependent on the integrity of the membrane.

β-Galactosidase

The initial percentage free activity of *p*-nitrophenyl β-galactosidase was similar to those of the *p*-nitrophenyl glucosidases, implying that the permeability properties of the membrane to all three *p*-nitrophenyl glycosides were similar. There was no increase in percentage free activity of β-galactosidase with increasing substrate concentrations (Table 1) and no difference between the apparent K_m of free and total activities (Table 2). Thus, in conflict with reports by Furth & Robinson (1965) and Baccino & Zuretti (1975), we find no evidence that penetration of *p*-nitrophenyl β-galactoside through the lysosomal membrane is a rate-limiting step in the hydrolysis of this substrate by intact lysosomes. As with the hydrolysis of *p*-nitrophenyl α- and β-glucosides, the data seem to favour an explanation in terms of a heterogeneity of lysosomal membrane permeability.

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