Structural Equivalents of Latency for Lysosome Hydrolases

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1. Structure-linked latency, a trait for most lysosome hydrolase activities, is customarily ascribed to the permeability-barrier function performed by the particle-limiting membrane, which shields enzyme sites from externally added substrates. 2. The influence of various substrate concentrations on the reaction rate has been measured for both free (non-latent) and total (completely unmasked by Triton X-100) hydrolase activities in rat liver cell-free preparations. The substrates were: β -glycerophosphate, phenolphthalein mono- β -glucuronide, *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide and *p*-nitrophenyl β -D-galactopyranoside. The ratio (free activity/total activity) $\times 100$ is called fractional free activity at any given substrate concentration. 3. The fractional free activity of β -glucuronidase and β -*N*-acetylglucosaminidase were clearly independent of substrate concentration, over the range examined, in both homogenates and lysosome-rich fractions. The fractional free activity of acid phosphatase appeared to be either unaffected (homogenate) or even depressed (lysosome-rich fraction) by increasing the β -glycerophosphate concentration. The fractional free activity of β -galactosidase consistently showed a non-linear increase with increasing substrate concentration in both homogenates and lysosome-rich fractions. 4. Procedures such as treatment with digitonin, hypo-osmotic shock and acid autolysis, although effective in causing varying degrees of resolution of the latency of lysosome hydrolase activities, were unable to modify appreciably the pattern of dependence or independence of their fractional free activities on substrate concentration, as compared with that exhibited by control preparations. Ouabain did not affect the free β -*N*-acetylglucosaminidase activity of liver homogenates at all. 5. Preincubation of control preparations with β -glycerophosphate or *p*-nitrophenyl β -galactoside did not result in any significant stimulation of the free hydrolytic activity toward these substrates. 6. The results consistently support the view that the membrane of 'intact' lysosomes is virtually impermeable to all the substrates tested, except for *p*-nitrophenyl β -galactoside, for which the evidence is contradictory. Moreover the progressive unmasking of the hydrolase activities produced by these procedures *in vitro* reflects the increasing proportion of enzyme sites that are fully accessible to their substrates rather than a graded increase in the permeability of the lysosomal membrane.

Latency was early recognized as a distinguishing, albeit not exclusive, property of the hydrolytic activities associated with lysosomes, so much so that it revealed a landmark in the discovery of these particles and in the development of the concept of the lysosome [see de Duve (1969) for references]. A simple model suggested by de Duve (1965) to account for enzyme latency is based on the assumptions that (1) lysosomal hydrolases, at least most of them, are encapsulated by a membrane, (2) the lysosome-limiting membrane (henceforth also termed lysosome) acts as a permeability barrier, and (3) passage across the membrane is *in vitro* the major rate-limiting step in the supply of substrates to the enzymes contained within the lysosomes. Artificial models consonant with this view have been proposed to account for the structure-linked latency (Romeo & de Bernard, 1966; Sessa & Weissmann, 1970; Weissmann *et al.*, 1971).

The suggestion (de Duve, 1965) that the lysosome is essentially impermeable, possibly to all the substrates until then investigated, fits with several observations (Berthet *et al.*, 1951; Appelmans & de Duve, 1955; Ehrenreich & Cohn, 1969; Lloyd, 1969*a,b*, 1971, and personal communication). However, the possibility that at least some substrates are able to permeate the lysosome has been repeatedly suggested (Appelmans & de Duve, 1955; Conchie & Hay, 1963; Furth & Robinson, 1965; Rosenberg & Janoff, 1968; Koenig, 1969; Lucy, 1969; Robinson & Willcox, 1969; Rosenberg, 1970). Besides, the latency of lysosome hydrolases is never complete in subcellular preparations; even when the assay is performed after removal by centrifugation of the enzymes present in the suspending medium, non-latent activity can still be demonstrated in the sedimented fraction.

The degree of latency of lysosome hydrolases has been customarily used as an index for the integrity of

the lysosome after treatments *in vitro* as well as *in vivo*. A decrease in the proportion of latent enzyme activities has been often accounted for by the hypothesis that, possibly even before the actual disruption of the particles, a graded increase in permeability of the limiting membrane to substrates underlies the progressive unmasking of lysosome hydrolases by disruptive procedures (Sawant *et al.*, 1964; Shibko *et al.*, 1965; Shibko & Tappel, 1965; de Bernard, 1966; Misch & Misch, 1969; Wattiaux, 1971; Trigaux *et al.*, 1972).

The availability of a reliable model for the structural equivalents both of latency and of the unmasking of hydrolytic activities associated with lysosomes is essential to evaluate the possible mechanisms of regulatory or injurious effects exerted at the level of the lysosome. The aim of the present experiments [in part presented in a preliminary form in Baccino & Zuretti (1972)] is to obtain information on the permeability of the limiting membrane of rat liver lysosomes toward substrates for different enzymes. The observations were made not only on control lysosomes from normal animals, but also on particles exposed *in vitro* to damaging procedures or modified in some way as a consequence of treatments *in vivo* (the latter experiments are not reported here).

The theoretical background for the procedure used in most of the present experiments has been discussed by de Duve (1965). Briefly, the free and total activities (see the Experimental section for a definition) of lysosome hydrolases were assayed over a suitably wide range of substrate concentrations, extending whenever possible from below the apparent K_m to above the concentration at which the unmasked enzyme is saturated. Differences in free versus total activity in the relationship between rate of substrate hydrolysis and substrate concentration would imply the existence, in the assay for free activity, of a mechanism(s) or factor(s) interfering with the full expression of the catalytic activity of the enzyme sites, i.e. either a permeability barrier partially limiting the accessibility of substrate molecules or some intralysosomal inhibitory factor. An alternative hypothesis is that only the number of available sites, not the degree of their accessibility or activity, would determine the difference between free and total activity; in this case, free and total activities should be similarly dependent on substrate concentration. The results of these tests can be conveniently expressed in terms of fractional free activity, which is the percentage rate at 10 min of total rate in the presence of Triton X-100. The fractional free activity should either increase or be constant with increasing substrate concentration, according to whether the first or the second hypothesis is tenable.

An approach basically similar to that outlined above has already been applied to the study of lysosomes (Berthet *et al.*, 1951; Appelmans & de

Duve, 1955; Furth & Robinson, 1965; Vaes, 1965; Lloyd, 1969a). Likewise, Belpaire & Laduron (1968) showed, by this approach, that the membrane of adrenal medulla granules is essentially impermeable to tyramine, the substrate for the dopamine (3,4-dihydroxyphenethylamine) β -hydroxylase contained within.

Experimental

Tissue homogenates and subcellular preparations

Male Wistar-strain rats (Morini, S. Polo d'Enza, Italy) weighing 200–250 g were used. They were in the fed state when killed by decapitation. Homogenates in 0.25 M-sucrose were prepared with a Potter-Elvehjem-type homogenizer (A. H. Thomas, Philadelphia, Pa., U.S.A.) driven at 1500 rev./min. The lysosome-rich (light mitochondrial) fraction was prepared by the method of de Duve *et al.* (1955) and suspended in 0.25 M-sucrose; a few biochemical parameters useful for the characterization of this fraction were similar to those reported previously (Baccino *et al.*, 1971).

Enzyme assays

These were performed as follows: acid phosphatase (EC 3.1.3.2) and β -glucuronidase (EC 3.2.1.31) by the method of de Duve *et al.* (1955), β -N-acetylglucosaminidase (EC 3.2.1.30) and β -galactosidase (EC 3.2.1.23) by the method of Sellinger *et al.* (1960), but with *p*-nitrophenyl derivatives as substrates in the latter two assays. Moreover 0.2 M-KCl was added to the medium for β -galactosidase assay, to provide optimum conditions with respect to the ionic strength (F. M. Baccino & M. F. Zuretti, unpublished work). Substrate concentrations are specified in the Results section. Incubations were for 10 min at 37°C. Free (non-latent) activities were measured on tissue preparations osmotically protected with 0.25 M-sucrose. Total activities were assayed in the presence of 0.1% (w/v) Triton X-100, which causes complete disruption of the lysosomes.

Materials

p-Nitrophenyl β -D-galactopyranoside, *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide, β -glycerophosphate (disodium salt, grade I), and phenolphthalein mono- β -glucuronide were purchased from Sigma (St. Louis, Mo., U.S.A.). Digitonin (crystallized) was obtained from E. Merck A.G. (Darmstadt, Germany) and ouabain was from British Drug Houses Ltd. (Poole, Dorset, U.K.).

Table 1. Fractional free activity of four acid hydrolases in rat liver homogenates at various substrate concentrations

Total activities are expressed as percentages of the highest values obtained and free activities as percentages of the total activities at the corresponding concentration of substrate. The following substrates were used in that order: β -glycerophosphate, *p*-nitrophenyl *N*-acetyl- β -glucosaminide, phenolphthalein mono- β -glucuronide and *p*-nitrophenyl β -D-galactopyranoside. Results are given as means $\pm$ s.d. A standard analysis of variance was performed to test whether significant differences could be detected among fractional free activities; the levels of probability *P* are shown under each set of data.

	Substrate concentration (mM)	Total activity	Fractional free activity
Acid phosphatase (four expts.)	20	74.0 $\pm$ 6.22	20.8 $\pm$ 3.21
	50	87.9 $\pm$ 4.97	19.6 $\pm$ 2.33
	100	94.5 $\pm$ 3.97	19.8 $\pm$ 1.79
	150	98.0 $\pm$ 2.97	19.8 $\pm$ 2.18
	200	100	20.5 $\pm$ 3.75
			<i>P</i> > 0.05
β - <i>N</i> -Acetylglucosaminidase (six expts.)	1.0	44.2 $\pm$ 2.68	9.7 $\pm$ 3.43
	2.1	60.6 $\pm$ 5.67	10.8 $\pm$ 3.87
	4.0	80.2 $\pm$ 2.07	11.2 $\pm$ 3.01
	6.1	89.2 $\pm$ 5.18	11.2 $\pm$ 2.29
	8.0	93.9 $\pm$ 3.88	10.6 $\pm$ 3.28
	9.9	100	11.4 $\pm$ 3.54
β -Glucuronidase (two expts.)			<i>P</i> > 0.05
	0.025	29.0	40.5
	0.050	45.3	41.3
	0.100	67.8	40.9
	0.250	85.8	41.0
	0.500	95.6	40.3
	0.750	99.0	41.7
	1.000	100	40.8
	1.250	99.5	43.1
	1.500	98.5	41.5
β -Galactosidase (three expts.)	2.500	97.0	40.4
			<i>P</i> > 0.05
	0.2	56.1 $\pm$ 1.30	18.1 $\pm$ 3.68
	1.0	94.7 $\pm$ 0.41	22.5 $\pm$ 2.57
	2.5	100	27.0 $\pm$ 3.41
	5.0	98.7 $\pm$ 0.76	31.6 $\pm$ 3.72
	10.0	89.7 $\pm$ 0.49	40.9 $\pm$ 3.28
	15.0	84.4 $\pm$ 1.51	45.1 $\pm$ 4.99
			<i>P</i> < 0.01

Results

Fractional free activity versus substrate concentration

Four hydrolases were assayed for their free and total activities at various substrate concentrations in liver homogenates from normal rats (Table 1). The fractional free activities of acid phosphatase, β -*N*-acetylglucosaminidase and β -glucuronidase were clearly independent of substrate concentration. In contrast there was a marked effect of substrate concentration on the fractional free β -galactosidase activity.

The above experiments were carried out on homogenates, to minimize the number of preparative procedures that could result in some damage to the particles. This advantage is nonetheless counterbalanced by a loss in the sensitivity and accuracy of the assay because of the enzyme activity present in the soluble phase of the homogenate and, particularly for

β -glucuronidase, associated with particles other than lysosomes. To minimize such interference, free and total activities were also assayed on a particulate subcellular fraction enriched in lysosomes; it contained hydrolases purified 11–13-fold with respect to the homogenate. Table 2 shows that even when virtually all of the hydrolases are particle-bound, the fractional free activities of β -*N*-acetylglucosaminidase and β -glucuronidase are essentially independent of substrate concentration. The behaviour of β -galactosidase is again unusual, in that its free fractional activity is enhanced with increasing substrate concentration. Moreover a surprising effect is observed for acid phosphatase, the free fractional activity of which is gradually decreased as the substrate concentration is increased. Double-reciprocal plots by the method of Lineweaver & Burk (Dixon & Webb, 1966) of the free and total activities (both expressed as percentages of the highest value recorded

Table 2. Fractional free activity of four acid hydrolases in rat liver lysosome-rich fractions at various substrate concentrations

The results are expressed as in Table 1.

	Substrate concentration (mM)	Total activity	Fractional free activity
Acid phosphatase (five expts.)	10	62.3 ± 7.42	17.2 ± 2.70
	20	77.9 ± 6.39	16.5 ± 2.95
	50	91.0 ± 3.87	14.6 ± 2.57
	100	98.9 ± 2.64	11.5 ± 2.47
	150	98.5 ± 0.84	8.8 ± 1.66
	200	100	5.7 ± 1.34
β -N-Acetylglucosaminidase (one expt.)	1.0	40.5	18.8
	2.1	60.3	18.5
	4.0	77.6	19.5
	6.1	89.4	18.9
	8.0	96.9	19.9
	9.9	100	18.9
β -Glucuronidase (three expts.)	0.025	21.6 ± 2.35	19.4 ± 3.66
	0.050	39.6 ± 4.21	19.7 ± 4.33
	0.075	51.0 ± 5.53	19.8 ± 5.54
	0.100	60.5 ± 5.70	19.5 ± 4.80
	0.150	74.4 ± 4.96	19.6 ± 4.95
	0.200	80.3 ± 2.25	19.5 ± 4.41
	0.250	85.9 ± 1.89	19.7 ± 4.68
	0.500	96.7 ± 0.91	19.1 ± 5.29
	1.000	100	19.3 ± 4.33
β -Galactosidase (three expts.)	0.1	40.8 ± 5.15	19.4 ± 2.31
	0.2	61.3 ± 5.74	22.0 ± 2.45
	0.5	85.2 ± 2.33	23.4 ± 3.01
	1.0	95.4 ± 0.77	25.7 ± 3.54
	1.5	99.5 ± 0.18	28.1 ± 3.20
	2.0	100	28.8 ± 3.29
	2.5	99.4 ± 0.41	31.7 ± 3.33
	5.0	98.5 ± 1.15	32.7 ± 3.28
	10.0	87.3 ± 1.99	34.7 ± 3.51

for total activity) versus the substrate concentration are given in Fig. 1. Three of them fit a non-competitive inhibition model. For β -galactosidase, differences in both $V_{\max}$ and apparent K_m occur between total and free activity, accounting for a mixed-type inhibition model (Dixon & Webb, 1966).

The total β -galactosidase activity is apparently inhibited in a reversible way when concentrations of rat liver homogenate greater than 50 mg/ml are used for the assay (Fig. 2a). If the inhibitor(s) were of lysosomal origin, a relatively high inhibitor(s) concentration would occur within the intact particles when assayed for free activity. The differential effect of the substrate concentration on free and total β -galactosidase activity could then be viewed as the consequence of the progressive reversal of the inhibition of intralysosomal enzyme due to substrate molecules that easily permeate the lysollemma. However, even at concentrations comparatively much higher than those for homogenates, no inhibition could be demonstrated when lysosome-rich fractions were assayed for β -galactosidase activity (Fig. 2b).

Finally, the possibility has been investigated of whether the substrate itself brings about a labilization of lysosomes; the concentration-dependence of this eventual effect could account for the observed variation in the fractional free activity. However, no labilization could be detected either by measuring the free β -galactosidase activity in homogenates preincubated at pH 5 (cf. Marsh, 1972) with the galactoside for up to 60 min at 2°C or by assaying the free acid phosphatase activity after 10 min of preincubation at 37°C with the above substrate (Table 3).

Effect of labilizing procedures in vitro

It was decided to use the experimental approach outlined above to elucidate whether partial increase in the permeability of the lysollemma to substrates could contribute to the unmasking of acid hydrolase activities after treatment of lysosomes with injurious agents.

When rat liver homogenates were exposed to 50 or 100 μ g of digitonin/ml, the free acid phosphatase

Fig. 1. Double-reciprocal plots of free and total hydrolase activities in liver homogenates versus substrate concentrations

○, Free activity; ●, total activity. Both activities are expressed as fractions of the highest value obtained for the total activity. Slopes, intercepts and apparent K_m values were calculated by regression analysis. The K_m values are for (a) acid phosphatase $8.1 \times 10^{-3} M$ (free activity), $7.8 \times 10^{-3} M$ (total activity); for (b) β -N-acetylglucosaminidase $2.1 \times 10^{-3} M$ (free activity), $2.0 \times 10^{-3} M$ (total activity); for (c) β -glucuronidase $6.9 \times 10^{-3} M$ (free activity), $6.9 \times 10^{-3} M$ (total activity); for (d) β -galactosidase $3.5 \times 10^{-3} M$ (free activity), $2.1 \times 10^{-4} M$ (total activity).

activity, as measured at the usual substrate concentration (50mM), increased from 19.6% for control preparations to an intermediate (59.0%) and almost complete (92.9%) degree of unmasking respectively. Nevertheless, in both instances there were no appreciable signs of any discrepancy in the substrate-dependence of free versus total activity (Fig. 3). On the contrary, a definite decrease in free activity occurred at high substrate concentrations in the case of homogenates with partially 'activated' phosphatase activity (50 μ g of digitonin/ml).

Digitonin was also added to lysosome-rich fractions (Fig. 4). In the case of β -glucuronidase, it had not modified the ratio between free and total activity at increasing concentrations of phenolphthalein mono- β -glucuronic acid. In the case of β -galactosidase, the treatment with digitonin did not change the dependence of the fractional free activity on substrate concentration.

Labilization of lysosomes was also brought about by exposing lysosome-rich fractions to a hypo-osmotic medium or to acid autolysis. Neither of these

Fig. 2. Relationship between total β -galactosidase activity and concentration of rat liver homogenate (a) or lysosome-rich fraction (b)

The amount of tissue extracts is expressed on the abscissa as the original wet weight (mg equiv.) of the amount of tissue from which they were prepared. Incubation was at 37°C for: ●, 10 min; ○, 20 min; □, 40 min.

treatments conferred any appreciable slope on the curve of fractional free β -N-acetylglucosaminidase activity at various substrate concentrations (Fig. 5). Surprisingly enough, when acid phosphatase was assayed on these same lysosome-rich fractions (Fig. 6) the free activity appeared to be decreased, even in absolute terms, by increasing the β -glycerophosphate concentration, both in control and treated preparations. However, the usual substrate-activity curve was obtained for total activity. The effect observed with lysosome-rich fractions appears to be qualitatively, but not quantitatively, similar to that reported above for whole homogenates with partially 'activated' acid phosphatase (Fig. 3).

Table 3. Preincubation of rat liver homogenates with 2.5 mM-p-nitrophenyl β -D-galactoside (pNG): effect on the latency of β -galactosidase and acid phosphatase activities

The reaction medium contained 50 mM-sodium acetate-acetic acid buffer, pH 5.0. Total activities remained unchanged. The statistical significance of differences was estimated by a standard analysis of variance.

	Preincubation		pNG	Fractional free activity
	Temp-erature (°C)	Time (min)		
β -Galactosidase (three expts.)	2	0	+	28.4 $\pm$ 1.15
	2	10	+	29.3 $\pm$ 1.92
	2	20	+	30.0 $\pm$ 1.90
	2	30	+	29.8 $\pm$ 1.26
	2	60	+	28.1 $\pm$ 2.45
				$P > 0.05$
Acid phosphatase (two expts.)	37	10	-	25.4
	37	10	+	25.8

Fig. 3. Fractional free activity of acid phosphatase in digitonin-treated homogenates

Liver homogenates (2.5%, w/v) in 0.25 M-sucrose were exposed for 30 min at 2°C to 25 (Δ), 50 ($\square$) and 100 ($\bullet$) μ g of digitonin/ml; parallel controls ($\circ$) without digitonin were also run. Homogenates were then assayed for total and free activity.

In further experiments, liver homogenates were incubated for up to 30 min in the presence of 0.2 mM-ouabain (Table 4), in an attempt to evaluate whether an inhibitable adenosine triphosphatase activity is involved in the maintenance of the integrity of the lysosome. No effect was observed on the availability of β -N-acetylglucosaminidase activity, which is in keeping with the results of Guth & Amaro (1968), who failed to detect any influence of this glycoside on

Fig. 4. Effect of digitonin on the fractional free activity of β -glucuronidase and β -galactosidase in lysosome-rich fractions

Digitonin at a final concentration of 35 μ g/ml was added to suspensions that contained, in each ml, that amount of lysosome-rich fraction corresponding to 200mg of wet tissue. After 30 min at 2°C, the preparations were assayed for acid hydrolases. (a) β -Glucuronidase (three expts.); (b) β -galactosidase (one expt.). $\circ$, Controls; $\square$, digitonin.

Fig. 6. Fractional free activity of acid phosphatase in lysosome-rich fractions subjected to labilizing treatments

Each point corresponds to the mean of three experiments. Other details as in Fig. 5.

Fig. 5. Fractional free β -N-acetylglucosaminidase activity of lysosome-rich fractions subjected to labilizing treatments

$\circ$, Control preparations; $\square$, fractions exposed to hypo-osmotic shock (0.15M-sucrose for 30min at 2°C); Δ , fractions incubated for 10min at 37°C in the presence of 50mm-sodium acetate-acetic acid buffer, pH5.0. Each point corresponds to the mean of two experiments.

Table 4. Effect of ouabain on the availability of β -N-acetylglucosaminidase activity

Rat liver homogenate was preincubated at 25°C for the times indicated in a medium containing 10mm-imidazole-HCl buffer, pH7.4, and 0.25M-sucrose. Results of a single experiment are given.

	Time (min)	Fractional free activity	Total activity (arbitrary units)
Controls	0	13.5	100
	10	14.5	100
	20	14.0	99.2
	30	14.3	97.9
0.1 mM-Ouabain	0	13.5	100
	10	14.4	99.4
	20	14.8	97.9
	30	15.3	97.3

the release of acid phosphatase activity from lysosome-rich preparations.

Effect of preincubation with the substrate on fractional free activity

In order to investigate from a different standpoint the possibility that some substrates might slowly

permeate the lysoslemma, the free activity of two hydrolases was measured after preincubation of liver homogenates with their respective substrates for up to 60 min at 2°C. β -Galactosidase (Table 3) and acid phosphatase (Fig. 7) free activities were but minimally enhanced by preincubation, even when the particles were partially disrupted with digitonin (Fig. 7). Unless the lack of a definite effect depends on the low temperature of the preincubation, these data add to the view of the lysoslemma as being practically impermeable to β -glycerophosphate as well as to *p*-nitrophenyl β -D-galactopyranoside.

Fig. 7. Effect of the preincubation with β -glycerophosphate on the availability of acid phosphatase activity in liver homogenates

Control (○) or digitonin-treated (□) homogenates in 0.25 M-sucrose were preincubated at 2°C for the time shown on the abscissa in the presence of 50 or 100 mM- β -glycerophosphate, adjusted to pH 5.0. The concentration of digitonin was 50 μ g/ml. The results are expressed as fractional free activity (means of three separate experiments). The results obtained at the two concentrations of β -glycerophosphate were pooled because they were practically identical.

Discussion

As far as lysosomes are concerned, a major factor that most often limits the use of experimental procedures already successfully adopted for the study of the permeability of other subcellular particles (e.g. Slater *et al.*, 1969; Nilsson *et al.*, 1973; Palmieri *et al.*, 1972) is the extensive contamination of lysosome-rich preparations with structures other than lysosomes. This is the main reason why only essentially qualitative data have so far been obtained. The available evidence is discussed in detail by Lloyd (1973).

Recently, a quantitative approach has been used to measure the penetration into lysosomes of the weak base, methylamine, in order to make inferences on the internal pH of Triton WR-1339-filled (Reijngould & Tager, 1973) and normal (Goldman & Rottenberg, 1973) rat liver lysosomes. Although there is good agreement between the two reports in that they both suggest an acidic intralysosomal microenvironment, the pH value was possibly overestimated because of the limited accuracy of the measurement owing to the presence of contaminating non-lysosomal particles in the preparations under test, particularly in the experiments of Goldman & Rottenberg (1973). At any rate, the inference that uncharged methylamine easily permeates the lysosome appears to be well-grounded.

Permeability of the lysosome to small-molecular substrates

Four substrates have been assayed in the present work for their ability to permeate the membrane of rat liver lysosomes, namely, β -glycerophosphate, *p*-nitrophenyl *N*-acetyl- β -glucosaminide, phenolphthalein mono- β -glucuronide and β -D-galactopyranoside. Evidence has been consistently obtained that the lysosome is essentially impermeable to the first three, whereas the evidence for the last one is controversial.

The fractional free activities of acid phosphatase, β -*N*-acetylglucosaminidase and β -glucuronidase have been shown to be independent of substrate concentration in almost all of the experimental situations here examined. Lineweaver-Burk plots of the free and total activities of these hydrolases versus substrate concentration allow some useful considerations. If the translocation of substrate molecules across the lysosome were rate-limiting for the hydrolysis of substrate, a difference would be expected in the apparent K_m of free versus total activity. A similar result could also reflect the presence of competitive inhibitors maintaining the enzyme partially inactive towards the substrates. Clearly, however, only differences in V_{max} are observed; these are not coupled with differences in the apparent K_m for acid phosphatase, β -glucuronidase and β -*N*-acetylglucosaminidase activities. Therefore the only model left for the mechanism(s) underlying the structure-linked latency of these hydrolases is that of non-competitive inhibition. The latter could be accounted for either by the occurrence of non-competitive inhibitors within lysosomes or by clearcut discrimination between enzyme sites fully accessible and, alternatively, unavailable to substrates, owing to the presence of limiting membranes that are practically impermeable to substrates.

There is general agreement that β -glycerophosphate, in spite of its relatively small molecular weight, cannot penetrate lysosomes (Berthet *et al.*, 1951; Appelmans & de Duve, 1955; Lee, 1970, 1971, 1972); the charged group on the molecule probably explains this behaviour (Lee, 1970). Robinson & Willcox (1969) showed that the proportion of free acid phosphatase activity measurable in rat liver fractions enriched in lysosomes varies depending on the substrate; it is low with 4-methylumbelliferyl phosphate, but markedly higher with phenyl or *p*-nitrophenyl phosphate. These findings do not necessarily mean that the latter two substrates can cross the lysosome or that they are more permeant than the former two: enzymes that have different substrate specificity and that are adsorbed on sedimentable structures or carried by particles other than lysosomes could be responsible for the discrepancies observed by Robinson & Willcox (1969);

this is also suggested by the differences reported by the same authors to occur in the subcellular distribution of the activities towards the four substrates. It is worth mentioning that Vaes (1965) did not notice any significant effect of the concentration of one of the substrates used by Robinson & Willcox (1969), namely phenylphosphate, on the fractional free activity of acid phosphatase in lysosome-rich preparations from rat calvaria.

A further remark is important with regard to β -glycerophosphate. In some experiments (Figs. 3 and 6) increasing the substrate concentration resulted in a paradoxical fall in free acid phosphatase activity, even in absolute terms. Osborne *et al.* (1973) noticed a decline in total acid phosphatase activity in rat liver homogenates exposed to high concentrations of β -glycerophosphate. However, the possibility that a direct action of β -glycerophosphate on the enzyme molecules is responsible for the effect reported here should be ruled out, because for total activity the usual activity-substrate concentration relationship was observed. Hilderson *et al.* (1971) suggested that β -glycerophosphate is able to produce an aggregation of bovine thyroidal lysosomes. Whether the phenomenon observed by us depends on this or some other mechanism is not clear at present and is receiving further consideration.

Present evidence indicates the inability of phenolphthalein β -glucuronide to permeate the lysosomem, in agreement with the findings of Lloyd (quoted by Lloyd, 1973, and personal communication) and the investigations of Vaes (1965) on rat bone lysosomes and of Bowers *et al.* (1967) on rat spleen lysosomes. A conflicting view has been expressed by Rosenberg & Janoff (1968) and reiterated by Rosenberg (1970). The recovery of phenolphthalein sedimentable with structures at the end of an incubation of lysosome-rich preparations with phenolphthalein glucuronide is assumed by Rosenberg & Janoff (1968) to be evidence for the intragranular hydrolysis of the substrate and, hence, for its penetration through the lysosomem (by extrapolation, they envisaged a pathophysiological role for lysosome β -glucuronidase in the hydrolysis of glucuronides, of steroids for instance, diffusing from the cytoplasm). It is implicit in their reasoning that, in spite of a molecular size smaller than that of its precursor, the reaction product cannot readily leave the granules, possibly because of its very low solubility in water. For the same reason, however, one cannot disregard the possibility of adsorption artifacts, due to a distribution coefficient water/particles favourable to the latter because of the lipoprotein nature of their limiting membranes. Moreover, should the rate of outward diffusion of the reaction product be unable to keep pace with the reaction rate, the intralysosomal accumulation of the product would eventually result in osmotic rupture of the granules (Appelmans & de Duve, 1955;

Goldman & Kaplan, 1973), which was not noticed by Rosenberg & Janoff (1968). On the other hand, the present results are inconsistent with the view that enzyme sites responsible for the free activity are encapsulated by a membrane partially permeable to phenolphthalein glucuronide. Therefore the only possibility left seems to be that free activity is due to enzyme molecules either bound to the outer aspect of the lysosomem, primarily or secondarily (Baccino *et al.*, 1971; Baccino & Zuretti, 1971), or contained within particles fully accessible to the substrate.

Of the two *p*-nitrophenyl derivatives here investigated, the β -*N*-acetylglucosaminide is consistently shown to be essentially unable to permeate the lysosomem. The same conclusion was reached by Vaes (1965) for the *o*-nitrophenyl *N*-acetyl- β -D-glucosaminide with respect to rat bone lysosomes. By contrast, conflicting evidence has been produced for the β -galactoside. There seems to be more evidence in favour of its penetration through the lysosomem, because of the constancy with which, in various experimental situations, the fractional free activity of β -galactosidase is increased with an increase in substrate concentration, in agreement with previous findings (Conchie & Hay, 1963; Furth & Robinson, 1965). Moreover Furth & Robinson (1965) have noticed the occurrence of substrate inhibition of free activity at concentrations of *p*-nitrophenyl β -D-galactoside much higher than those for total activity, which would be strong evidence in favour of the permeability of the lysosomem to this substrate. However, we were unable to reproduce this result, in agreement with Conchie & Hay (1963). We do not have any good explanation for this discrepancy. It is worth noting that the enhancement of the fractional free activity obtained by increasing the substrate concentration is also observed at β -galactoside concentrations less than 2.5 mM, above which concentration the inhibitory effect on total activity becomes apparent. Therefore differential inhibition by an excess of substrate of total versus free activity cannot account for the particular behaviour of the fractional free β -galactosidase activity.

The double-reciprocal plot of free and total β -galactosidase activity fits a mixed-type inhibition model. This is compatible with the hypothesis of partial permeation of the lysosomem by *p*-nitrophenyl β -galactoside. The view that this substrate is able to penetrate the lysosomem is also favoured by Marsh (1972). Nonetheless, it is worth mentioning here the elegant experiment whereby Lloyd (1969*b*) showed that the membrane of rat liver lysosomes is impermeable to *p*-nitrophenyl β -glucoside; in contrast with the lysosome's own β -glucosidase, which is non-latent because it is externally bound to the lysosomem, exogenous enzyme administered intravenously and allowed to be endocytosed by liver cells is largely latent. Since the substrate used by Lloyd (1969*b*) is

very similar to that used in the present experiments for β -galactosidase, Lloyd's experiments provide some indirect evidence to support the view that even *p*-nitrophenyl β -galactoside is a non-penetrating solute.

A model for latency and its resolution

For three out of four substrates examined in the present investigations, and within the limits of the present experimental method, clearcut evidence is provided that the kinetics of their hydrolysis by liver homogenates or lysosome-rich fractions are merely related to the enzyme reaction for both free and total activities. Therefore the substrate must be immediately available (Webb, 1963) to all of the enzyme sites actually involved in the reaction. The conclusion is that the difference between total and free activities is a result of the differing proportions of fully active enzyme sites rather than the degree of their 'activation'. This same conclusion holds when lysosomes are subjected *in vitro* to injurious procedures that result in varying degrees of partial resolution of the latency, such as incubation with digitonin, hypo-osmotic shock and acid autolysis. It is worth noting that these three mechanisms of activation markedly differ in many respects. Digitonin acts instantaneously (de Duve *et al.*, 1963), owing to a combined chemical (binding of non-esterified cholesterol) and physicochemical (surface action) effect. The hypo-osmotic shock should result in a relatively graded activation, through partial or total osmotic lysis of particles. Finally, acid autolysis is thought to involve a hydrolytic breakdown of the lysosomemembrane (Beaufay & de Duve, 1959; Lauwerys & Buchet, 1972; Weglicki *et al.*, 1973), by necessity gradual. From the present experiments, the important inference can be drawn that the enhancement of free activity caused by the above procedures reflects the increased number of fully 'activated' particles, or at least enzyme sites, rather than a graded increase in permeability of the lysosomal membrane to the substrates.

Investigations on the permeability of liver lysosomes to substrates are currently being extended to situations in which lysosomes are modified as a result of treatments *in vivo*. The administration to rats of ethionine brings about an enlargement of liver lysosomes, possibly related to the depletion of ATP (Goldblatt & Williams, 1969; Goldblatt *et al.*, 1973), as well as a reduction in the latency of lysosomal hydrolases; correspondingly, their proportion in the soluble fraction of the homogenate is not or only slightly increased (Zuretti & Baccino, 1975). The intoxication of rats with a crude extract of *Amanita phalloides* results in a vacuolar degeneration of hepatocytes with conspicuous histochemical changes in lysosomes, followed by cell death (Dianzani *et al.*, 1969); a very marked increase in both free and

soluble activities of lysosomal hydrolases is observed in liver homogenates (Baccino & Zuretti, 1971 and unpublished work). In these two situations the results so far obtained are entirely consistent with the view expressed above, that the unmasking of lysosome hydrolase activities practically obeys an all-or-none rule. From a different standpoint, Verity & Reith (1967) reached the conclusion that the activation of rat liver lysosomes by Hg^{2+} ions is an all-or-none effect on individual particles; the apparent gradient of effect on lysosome suspensions exposed to various concentrations of Hg^{2+} perhaps reflects the distribution of different individual thresholds in the population. An all-or-none effect at the level of individual lysosomes, prepared from rat kidney and incubated at 37°C, has also been suggested by Shibko *et al.* (1965) for the loss of their electron-opaque matrices.

Therefore the free activity of lysosomal hydrolases appears to be the expression of enzyme sites fully accessible to the substrate, (1) because they are present in the soluble phase of the subcellular preparation or, when they are sedimentable with structures, (2) because they are either primarily or secondarily bound to the outer aspect of the particle membranes, or even (3) because they are contained within particles or fragments with fully permeable limiting membranes. The occurrence of situations where the penetration of substrates has become possible while the enzymes are still retained within the partially damaged lysosomes has been repeatedly suggested (de Duve *et al.*, 1963; Shibko & Tappel, 1965; Vaes, 1965; Verity & Reith, 1967; Lloyd, 1969a; Hilderson *et al.*, 1971; Osborne *et al.*, 1973). Brunk & Ericsson (1972) showed that, after improper fixation, leakage of even large molecules, enzymes included, can take place through the membrane of lysosomes 'ultrastructurally intact'.

If the model suggested by present observations is correct, it would be basically inconsistent with the hypothesis repeatedly set forth (Bitensky, 1963; de Bernard, 1966; Misch & Misch, 1969; Bitensky *et al.*, 1973) that the activation of lysosome hydrolases, and hence the labilization of lysosomemembrane, is reversible. Alternative explanations to the claimed reversibility could be found, for instance, in phenomena that are artifacts, such as adsorption of enzymes on to sedimentable structures followed by desorption, as Lee (1971) has already pointed out with regard to the experiments of Misch & Misch (1969).

A further remark is pertinent. The release from particle suspensions of hydrolases in a soluble form (not bound to sedimentable structures) is a very popular parameter used to evaluate the integrity of lysosomes (e.g., Wills & Wilkinson, 1966; Weissmann, 1969; Ignarro *et al.*, 1972), the solubilization of hydrolases being regarded as indicative of a degree of damage to the particles more severe than that

corresponding to a mere increase in permeability of the lysosomema to substrates. However, since the latter hypothetical stage has never been demonstrated and we too have failed to detect its occurrence in our present experiments, there seems to be no reason whatsoever for assaying the soluble rather than the free activity of hydrolases as a parameter for lysosome integrity. Further, the assay of soluble activities requires a supplementary centrifugation, and it raises problems of the eventual occurrence of adsorption or desorption artifacts (by a drug under test, for instance). Discrepancies in the observed solubilization of hydrolases can also reflect such phenomena (Baccino *et al.*, 1971; Baccino & Zuretti, 1971). The latter are always hard to rule out, particularly when single-point rather than kinetic experiments are performed.

The foregoing discussion has been founded on the assumption that the structure-linked latency of lysosome hydrolases *in vitro* reflects the barrier function of the lysosomema towards substrates. However, other interpretations of this phenomenon have been suggested (cf. Verity, 1973), the best known among which envisages a role for ionic interactions between acidic constituents within lysosomes and enzyme molecules in maintaining the latency of hydrolases (Koenig, 1969). Since the latency would reflect a real inhibition of the enzymes, an important implication of this hypothesis is that lysosome hydrolases would be similarly inactive *in vivo* too. Although a discussion of this 'matrix-binding theory' is beyond the scope of the present paper, we can state that the present results do not provide evidence for or against the argument. Moreover, clearcut evidence is provided that a competitive mechanism cannot account for the suggested intralysosomal inhibition of at least three enzyme activities, namely acid phosphatase, β -glucuronidase and β -N-acetylglucosaminidase.

Whether the tentative conclusions above can be extrapolated from rat liver lysosomes to particles from different tissues or species is hard to decide. However, the permeability of lysosomema to β -glycerophosphate does not appear to be basically different for particles from sources as different as rat liver and *Tetrahymena pyriformis* (Lee, 1971).

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