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Studies on the Structure-Bound Sedimentability of some Rat Liver Lysosome Hydrolases

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1. Lysosome-rich fractions from rat liver were subjected to several disruptive procedures: osmotic lysis or freezing and thawing in different media, shearing forces in a high-speed blender, treatment with Triton X-100. 2. The soluble and particulate phases were then separated by high-speed centrifugation and assayed for their content of acid phosphatase, β-galactosidase, β-*N*-acetylglucosaminidase, acid proteinase, acid ribonuclease, acid deoxyribonuclease and protein. 3. The degree of elution of these hydrolases appeared to depend on both the enzyme species and the treatment. The resulting patterns of solubilization were rather complex, so that a clear-cut discrimination between soluble and structure-bound enzymes could not always be traced. 4. Although only β-galactosidase was readily solubilizable after all treatments, acid proteinase could also be extensively eluted from the sedimentable material in the presence of EDTA and acid phosphatase was fully extracted by Triton X-100. On the other hand, considerable proportions of the other activities could not be solubilized by any of the procedures used. 5. In other experiments, the adsorbability of hydrolases on subcellular structures was investigated by measuring the partition between sedimentable particles and soluble fraction of solubilized enzymes added to 'intact' liver homogenates. 6. Large proportions of acid proteinase, ribonuclease and deoxyribonuclease, and almost all of β-*N*-acetylglucosaminidase, were found to be adsorbed on the particulate material.

Lysosomes are endowed with a rich complement of hydrolases, most of which exhibit acid pH optima and the phenomenon of latency. Loss of latency or loss of binding to sedimentable particles or both have been considered to be the result of damage to the lysosome-limiting membrane. Accordingly, structure-linked latency and sedimentability of their contained enzymes are the two parameters most commonly used for evaluating the extent of the damage caused to lysosomes by treatments *in vitro* or resulting from changes *in vivo*. In spite of their wide application, relatively little work has been devoted to understanding the underlying mechanisms and to assessing the reliability of such parameters in expressing lysosome alterations.

Differences in the solubilization *in vitro* of lysosomal hydrolases have been reported (Beaufay & de Duve, 1959; Koenig & Jibril, 1962; Ugazio & Pani, 1963; Sawant, Shibko, Kumta & Tappel, 1964; Shibko & Tappel, 1964; Bohley, Kirschke, Langner & Ansorge, 1969). Differences in the relative ease with which hydrolases may be extracted in the soluble form from particles by repeated freezing and thawing, sonic disintegration, osmotic disruption or treatment with detergents have been utilized to discriminate 'soluble' (i.e. freely dispersed in the lysosome interior) from 'membrane-bound' enzymes (Mellors, Tappel, Sawant & Desai, 1964; Mellors & Tappel, 1967; Weissmann, Rowin, Marshall & Friederici, 1967; Beck & Tappel, 1968; Patel & Tappel, 1969; Fisher & Kent, 1969; Wattiaux-de Coninck & Wattiaux, 1969; Rahman, Verhagen & Wiel, 1970). However, the relative solubilization of lysosomal enzymes depends largely on the method of disruption. Verity, Caper & Brown (1968) showed that the association of different hydrolases with sedimentable material, after repeated freezing and thawing of lysosome-rich fractions from rat liver, is a function of ionic strength as well as of cation species in the suspension medium.

The present work is an attempt to investigate the factors, other than entrapment by the particle-limiting membrane, responsible for the association of lysosomal hydrolases with sedimentable structures in lysosome-rich fractions from rat liver. A preliminary account of this work has been given (Baccino & Rita, 1970).

EXPERIMENTAL

Tissue fractionation. Male Wistar rats (Morini, S.Polo d'Enza, Italy) weighing 200–250g and fed on a balanced semi-synthetic diet were killed by decapitation. Homogenates of their livers were prepared with a Potter–Elvehjem-type apparatus and fractionated by differential centrifugation by the method of de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955) in 0.25 M-sucrose containing 1 mM-EDTA, pH 7.4. The fractionation procedure was checked by determining the distribution among subcellular fractions of protein, cytochrome oxidase, glucose 6-phosphatase, and six acid hydrolases: β-*N*-acetylglucosaminidase, β-galactosidase, acid phosphatase, acid ribonuclease, acid deoxyribonuclease and acid proteinase.

Solubilization of lysosomal enzymes. In the first set of experiments the L (light mitochondrial) fraction, rich in lysosomes, was resuspended in 0.25M-sucrose (without EDTA). Portions of this suspension were each subjected to one of the following procedures: treatment with 0.1% (w/v) Triton X-100 for 30 min at 0°C (plus about 60 min for centrifugation); freeze–thaw cycles in iso-osmotic (0.25M) sucrose, with or without added ions (0.1M-KCl, 3mM- or 50mM-$MgCl_2$); homogenization with a Lourdes Multi-Mix MM-1 blender equipped with a 15 ATT assembly and operated at maximum speed for 5 min; and suspension in hypo-osmotic (0.125M) sucrose for 30 min, after which the medium was brought back to iso-osmoticity by the addition of suitable volumes of a concentrated sucrose solution. In other experiments the L fraction was resuspended in water or in one of the following solutions: 3mM-EDTA (adjusted to pH 7.4 with NaOH), 20mM-sodium acetate buffer, pH 5, and 21 mM-imidazole–HCl buffer, pH 6 or 7. Cycles of freezing and thawing were further applied to portions suspended in water or in 3mM-EDTA.

All of the above treatments were carried out at 0°C; freezing and thawing was performed in test-tubes that were alternately immersed and gently swirled in baths at −40°C or 37°C. Portions of the L suspension were then spun at $9\times10^6 g$-min (computed on the average radius between $r_{max.}$ 7.1 cm and $r_{min.}$ 4.8 cm) to separate the soluble phase (Ls) from sedimentable material. The supernatant was pipetted off; after accurate wiping of the tube with filter paper, the pellet was resuspended with a micro Potter device. The distribution of protein and of the six hydrolases listed above was then evaluated.

Adsorption of lysosomal enzymes. To study the degree of adsorption of solubilized lysosomal enzymes on subcellular structures, homogenates in 0.25M-sucrose were prepared from rat livers that had been perfused *in situ*, until bleached, with cold iso-osmotic (0.145M) NaCl via the thoracic aorta. Portions of these homogenates were mixed with Ls preparations obtained from L fractions resuspended in iso-osmotic sucrose and subjected to three freeze–thaw cycles. In these mixtures, the homogenate had a final concentration of 10% (w/v) and the Ls preparation of 8% (on a tissue-weight equivalent basis). The medium was iso-osmotic sucrose, alone or with added 3mM-EDTA or 3mM-$MgCl_2$ or 0.1M-KCl. After being left for 30 min at 0°C, the mixtures were spun at $9\times10^6 g$-min; the supernatant and the pellet thus obtained were assayed for protein and the six hydrolase activities. Appropriate controls with either homogenate or Ls preparation alone were run in parallel.

Enzyme assays. Cytochrome oxidase (EC 1.9.3.1), glucose 6-phosphatase (EC 3.1.3.9), acid phosphatase (EC 3.1.3.2), acid proteinase (EC 3.4.4.23), acid deoxyribonuclease (EC 3.1.4.6) and acid ribonuclease (EC 2.7.7.16) activities were measured as indicated by de Duve *et al.* (1955); β-galactosidase (EC 3.2.1.23) and β-*N*-acetylglucosaminidase (EC 3.2.1.30) activities were determined by the method of Sellinger, Beaufay, Jacques, Doyen & de Duve (1960), but modified by the use of *p*-nitrophenyl derivatives as substrates. All assays for hydrolases were performed in the presence of 0.1% (w/v) Triton X-100. Units of enzyme activities are those defined by de Duve *et al.* (1955) and Sellinger *et al.* (1960).

Enzyme assays were modified, where needed, to take into account the presence of the ions previously tested for their influence on enzyme solubilization. For example, the dependence of β-galactosidase activity on ionic strength (F. M. Baccino & M. F. Zuretti, unpublished work) was minimized by adding 0.1M-KCl to the incubation medium; the effect of Mg^{2+} on nuclease activities was counteracted with appropriate amounts of EDTA or by equalizing the Mg^{2+} concentration in all assays.

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as standard.

Materials. Cytochrome *c* (type III), glucose 6-phosphate, dipotassium salt (grade I), β-glycerophosphate (grade I), bovine haemoglobin (type II), calf thymus DNA (type V), *Torula* yeast RNA (type VI), *p*-nitrophenyl β-D-galactopyranoside, *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide and bovine serum albumin (crystallized and freeze-dried) were all purchased from Sigma Chemical Co., St Louis, Mo., U.S.A.

RESULTS

Table 1 illustrates the distribution of protein and three reference enzymes (cytochrome oxidase, glucose 6-phosphatase and acid phosphatase), as well as that of five acid hydrolases, among the subcellular fractions obtained after fractionation of liver homogenates. As expected, the cytochrome oxidase activity is primarily associated with the heavy mitochondrial fraction and the glucose 6-phosphatase activity with the microsomal plus soluble fraction. The activities of acid phosphatase and the other hydrolases are consistently associated with the L fraction. These results indicate that the fractionation results in an adequate separation of subcellular particles, consistent with reported findings (de Duve *et al.* 1955; Sellinger *et al.* 1960). The L fraction obtained was therefore utilized for further experiments as to the structure-bound sedimentability of the hydrolases.

The distribution of the protein and the six hydrolases between the soluble and particulate phases of L suspensions subjected to different treatments *in vitro* is shown in Table 2. The results indicate that Triton X-100 is the most disruptive agent of those used, in that acid phosphatase, acid

Table 1. *Intracellular distribution of enzymes*

Results are given as means ± S.D. Absolute values are mg of protein and units of enzyme activity/g wet wt. of tissue (for the definition of units see de Duve *et al.* 1955). The percentage distribution among fractions, is computed with respect to the sum of the values recovered in all the fractions. The relative specific activities (percentage of enzyme activity/percentage of protein) are given, in parentheses, since the numbers of determinations for each enzyme are different. Percentage recoveries are computed for the fractions M, L, and P+S with respect to the values obtained with the homogenate after sedimentation of the fraction N. Fractions: N, nuclear; M, heavy mitochondrial; L, light mitochondrial; P, microsomal; S, soluble (usually the latter two were not separated).

	No. of expts.	Absolute values	Percentage values				Recovery (%)
			N	M	L	P+S	
Protein	9	237 ± 19.8	19.1 ± 2.69	22.4 ± 1.60	3.7 ± 0.888	54.8 ± 2.76	101.8 ± 4.45
Cytochrome oxidase	8	16.8 ± 3.78	21.1 ± 5.68 (1.11)	69.7 ± 6.72 (3.08)	7.6 ± 3.47 (2.05)	1.6 ± 1.48 (0.03)	83.5 ± 13.2
Glucose 6-phosphatase	8	22.3 ± 2.09	13.8 ± 5.09 (0.73)	6.3 ± 1.02 (0.28)	3.5 ± 0.860 (0.95)	76.4 ± 5.59 (1.40)	101.1 ± 4.64
Acid phosphatase	9	6.89 ± 0.597	9.8 ± 2.37 (0.51)	20.6 ± 5.54 (0.92)	37.0 ± 6.51 (10.00)	32.6 ± 3.09 (0.59)	109.8 ± 5.07
β-Galactosidase	3	0.309 ± 0.0400	16.1 ± 6.69 (0.75)	24.9 ± 1.92 (1.13)	33.8 ± 7.02 (7.60)	25.2 ± 1.01 (0.49)	99.6 ± 2.91
β-*N*-Acetylglucosaminidase	3	1.77 ± 0.158	15.2 ± 6.25 (0.71)	21.3 ± 3.95 (0.96)	43.7 ± 8.76 (9.85)	19.8 ± 1.75 (0.38)	103.5 ± 1.81
Acid proteinase	3	0.782 ± 0.131	14.6 ± 6.14 (0.67)	21.3 ± 5.00 (0.96)	34.7 ± 12.5 (7.63)	29.4 ± 7.50 (0.56)	94.1 ± 4.21
Acid ribonuclease	3	1.88 ± 0.125	16.5 ± 5.43 (0.76)	22.1 ± 3.30 (1.00)	25.9 ± 4.00 (5.82)	35.6 ± 6.27 (0.69)	95.1 ± 11.0
Acid deoxyribonuclease	3	0.967 ± 0.247	16.0 ± 7.66 (0.74)	26.8 ± 4.14 (1.21)	33.6 ± 10.6 (7.58)	23.6 ± 0.200 (0.45)	98.2 ± 9.19

proteinase and β-galactosidase are almost completely solubilized. However, a large proportion of acid ribonuclease and deoxyribonuclease, and most of β-*N*-acetylglucosaminidase, are still sedimentable. The effectiveness of freezing and thawing in extracting acid hydrolases is slightly influenced by the number of cycles. Seven cycles, appear to generally parallel the effectiveness of Triton X-100, except that it is less efficient in solubilizing acid phosphatase and more efficient in solubilizing β-*N*-acetylglucosaminidase.

Considerable importance has to be attached to the medium in which particles are suspended during the procedure. When the L fraction is frozen and thawed in 0.25 M-sucrose, a low proportion of protein is brought into solution as compared with the much higher amount extracted in water. The enzymic activity released from particles in isoosmotic sucrose is markedly increased for acid deoxyribonuclease and β-*N*-acetylglucosaminidase, but decreased for β-galactosidase. The addition of 0.1 M-potassium chloride to the suspension to be frozen and thawed results in a more pronounced solubilization for most hydrolases except β-*N*-acetylglucosaminidase and acid deoxyribonuclease; a comparable pattern is obtained with 50 mM-magnesium chloride. In contrast, the addition of 3 mM-magnesium chloride results either in no appreciable effects or in an increased retention of enzymes, such as acid deoxyribonuclease and β-*N*-acetylglucosaminidase, by sedimentable material. The addition of 3 mM-EDTA to L fractions resuspended in water results in a greater detachment of acid phosphatase, acid ribonuclease and particularly acid proteinase activities from sedimentable material. Moreover, repeated freezing and thawing in 3 mM-EDTA usually brings about a further solubilization of enzymes.

Suspension in water or 125 mM-sucrose as well as mechanical disruption in the blender of the L fraction result in relatively moderate degrees of solubilization for all enzymes, with the exception of β-galactosidase, in water. When buffers of different pH are added to suspensions prepared in water, the solubility of enzymes usually increases with increasing pH; however, the point at which this phenomenon occurs, with respect to the solubility in water alone, varies depending on the enzyme species; at the extremes, acid phosphatase is most easily solubilized with the three buffers, whereas β-galactosidase and β-*N*-acetylglucosaminidase are less readily solubilized.

Table 3 reports the results of the experiments aimed to evaluate the adsorption of soluble hydrolases on the whole liver homogenate. For this purpose 'intact' homogenates were enriched with

Table 2. *Solubilization of acid hydrolases from lysosome-rich fractions*

Results are given as means+S.D. of three experiments. Unsedimentable protein and enzyme activities are expressed as percentages of the sum of the amounts recovered in the soluble and sedimentable phases. In experiments of group I the L fraction was originally resuspended in 0.25M-sucrose; in those of group II the suspension medium was water. Other details are given in the Experimental section. Recoveries refer to all the data of each group (22 for group I, 24 for group II).

Treatment	Protein	β-Galacto-sidase	Acid phosphatase	Acid proteinase	Acid deoxy-ribonuclease	Acid ribonuclease	β-*N*-acetyl-glucosamini-dase
Group I: 0.25M-sucrose							
(1) Triton X-100 (0.1%)	61.0±5.99	96.2±2.40	95.6±2.06	88.3±0.663	63.5±3.64	64.6±5.04	27.0±2.69
(2) Three freeze–thaw cycles	11.7±2.57	80.4±5.31	37.8±6.07	51.7±5.92	67.0±5.89	48.5±5.19	58.8±4.53
(3) Seven freeze–thaw cycles	16.6±2.47	88.3±1.76	39.8±0.500	61.1±0.500	77.5±5.94	51.7±3.60	61.0±5.37
(4) 0.1M-KCl+three freeze–thaw cycles	24.5±0.990	74.9±4.95	45.5±7.49	70.3±0.565	64.3±2.54	70.1±3.11	67.7±7.49
(5) 3mM-$MgCl_2$+three freeze–thaw cycles	21.6±2.19	74.4±2.90	47.5±0.849	64.5±2.83	50.5±0.781	60.7±2.61	20.6±2.26
(6) 50mM-$MgCl_2$+three freeze–thaw cycles	32.2±4.73	72.3±3.81	43.3±2.90	71.0±0.921	70.9±3.60	69.8±3.11	72.9±3.96
(7) 125mM-sucrose	6.7±0.640	39.3±5.58	17.1±1.48	21.9±0.00	26.1±1.48	20.5±3.89	26.8±3.04
(8) Blender, 5min*	23.4	64.0	27.5	42.9	44.5	24.1	19.4
Recovery (%)	98.5±6.82	100.4±5.13	100.1±5.28	102.1±6.11	95.6±7.88	93.3±9.98	95.7±5.89
Group II: water							
(1) None	27.3±2.90	90.2±2.33	38.3±1.76	46.0±0.291	59.6±6.15	49.4±1.90	36.8±6.93
(2) One freeze–thaw cycle	32.2±3.39	91.6±0.848	39.4±2.26	47.2±0.353	44.2±5.09	34.2±3.60	18.7±3.60
(3) Seven freeze–thaw cycles	40.7±2.33	91.5±0.500	44.3±2.54	50.3±2.47	47.6±1.98	35.2±2.89	11.7±1.55
(4) 3mM-EDTA	40.7±1.91	88.0±2.61	60.9±1.62	85.7±7.42	59.4±0.565	67.7±5.23	32.3±5.87
(5) 3mM-EDTA+seven freeze–thaw cycles	55.3±1.27	93.5±1.48	66.0±1.83	87.3±1.34	79.4±0.778	72.6±5.72	46.3±1.41
(6) pH5	27.8±5.94	52.2±3.18	66.1±8.62	31.5±1.55	27.4±7.21	26.8±6.43	7.1±3.39
(7) pH6	28.8±6.85	87.1±2.05	57.2±2.82	46.9±3.67	40.4±1.34	38.9±5.94	6.8±1.70
(8) pH7	34.3±1.13	85.0±0.640	57.7±3.53	70.9±11.1	59.4±0.989	73.2±0.849	19.3±0.424
Recovery (%)	96.1±3.08	97.1±2.40	97.0±3.64	97.8±4.50	101.4±5.45	99.4±7.79	97.9±3.46

* One experiment.

Table 3. *Adsorption of solubilized acid hydrolases on sedimentable particles in rat liver homogenates*

Results are given as means±S.D. of three experiments. Total activities are given as m-units displayed by the fraction from 1g wet wt. of tissue. The percentage gain of activity in recipient homogenates corresponds to: 100×(enzyme activity in L extract)/(enzyme activity in recipient homogenate). The percentage of non-adsorbed activity is obtained with respect to the sum of activities recovered in the soluble and sedimentable fractions of the homogenate. Recovery refers to the percentage of the added soluble activity found in the soluble and particulate phases of the homogenate; it is computed on all the data for each enzyme activity. Details are given in the Experimental section.

	β-Galactosidase	Acid phosphatase	Acid proteinase	Acid deoxyribonuclease	Acid ribonuclease	β-*N*-Acetylglucosaminidase
Total activities in L fractions*	102±6.63	2740±414	260±55.2	318±46.8	487±90.5	767±89.0
Activities recovered in soluble L extracts† (%)	79.6±3.26	36.0±4.57	47.0±1.60	66.0±4.31	48.6±6.60	60.0±1.18
Gain of activity in recipient homogenates (%)	29.2±2.35	13.1±1.21	14.5±2.15	16.1±2.66	13.8±0.49	21.8±4.87
Soluble activity in recipient homogenates (%)						
(1) No additions	8.2±1.13	6.1±1.20	8.2±1.25	4.3±0.848	9.7±0.100	1.4±0.400
(2) 3mM-EDTA	7.9±1.86	6.2±1.35	9.2±1.23	3.9±1.55	9.7±0.212	1.6±0.141
(3) 3mM-$MgCl_2$	8.2±0.851	6.8±1.00	8.4±2.72	5.1±1.12	10.2±0.324	1.5±0.200
(4) 0.1M-KCl	8.2±1.07	5.9±0.380	9.6±2.29	5.1±0.848	10.8±0.764	2.8±0.173
Non-adsorbed activity in homogenate-L extract mixtures (%)						
(1) No additions	79.6±15.6	90.4±0.587	63.2±10.2	47.8±11.1	72.8±6.03	10.0±2.44
(2) 3mM-EDTA	81.9±6.78	91.5±2.22	66.8±20.3	51.2±9.62	69.2±8.91	17.2±4.50
(3) 3mM-$MgCl_2$	86.3±5.87	95.9±0.474	67.2±20.0	62.2±14.7	87.3±5.27	11.0±2.12
(4) 0.1M-KCl	85.9±4.41	97.3±3.30	76.3±11.4	59.8±15.6	93.5±4.33	34.6±6.74
Recovery (%)	102.6±1.30	104.3±6.76	95.0±2.66	103.8±5.00	102.2±6.25	94.6±3.81

* Protein was 11.2±2.44mg/fraction from 1g wet wt. of tissue.
† Protein was 12.7±1.47%.

soluble acid hydrolases in the form of Ls extracts obtained from L fractions resuspended in 0.25M-sucrose, and frozen and thawed three times; these extracts proved to be the most suitable for this purpose because of their low content of protein and high activity of acid hydrolases, including β-*N*-acetylglucosaminidase. After incubation and centrifugation of enriched homogenates, the degree of adsorption of added hydrolases was evaluated from the amount of added enzymic activity left unsedimentable. The results show that 90% of the added acid phosphatase activity is not adsorbed on the homogenate, but that the extent of adsorption of the other enzymes increases in the order β-galactosidase, acid ribonuclease, acid proteinase, acid deoxyribonuclease and β-*N*-acetylglucosaminidase, whose unsedimentable activity is only 10% of the added enzyme. The addition to the enriched homogenate of 3mM-EDTA does noticeably influence this general pattern. On the other hand, the presence of 3mM-magnesium chloride and especially of 0.1M-potassium chloride clearly influence the partition of enzymes by keeping in solution a larger proportion of all the hydrolases examined. This is particularly true for acid ribonuclease.

DISCUSSION

Verity *et al.* (1968) suggested that there exists 'a different pattern of enzyme binding, unique for each enzyme, in the lysosomal population' from rat liver, and that 'membrane binding of lysosomal hydrolases depends on individual enzyme characteristics as well as general non-specific forces'. We consider that our present results provide substantial new evidence to support these suggestions.

In principle, procedures such as freezing and thawing, sonication or osmotic disruption should be the most suitable for discriminating soluble from membrane-bound enzymes in particles displaying as simple a structural organization as lysosomes do. The action of detergents is more complex, since they not only disrupt particles, by dissociating structural constituents of the membranes, but they are also able to solubilize membrane-bound enzymes (see, e.g., Sloat & Allen, 1969; Sołtysiak & Kaniuga, 1970). As suggested by Weissmann *et al.* (1967), the distribution pattern of enzymes can be obscured or complicated by the presence within lysosomes of some organizational principle (stroma?, internal membranes?) that could remain sedimentable even after particles have been subjected to

disruptive treatments and carry with it enzymes primarily or secondarily bound. Baudhuin, Beaufay & de Duve (1965) and de Duve (1965), for instance, report that small coacervates of non-homogeneous material, resembling lysosomes stripped of their limiting membrane, can still be sedimented from lysosome-rich fractions resuspended in water. Moreover, fragments of membranes, not necessarily derived from lysosomes, possibly recombine to form small vesicles in which enzymes could be trapped. The geometry and the extent of such recombination are likely to be influenced by the properties of the suspending medium. However, this entrapment is not normally important, as shown by the very high degree of solubilization of β-galactosidase. Further, secondary adsorption of enzymes could take place on particle fragments of any of the kinds listed above. Since enzymes can be held by particle fragments of variable physical properties, the measured apparent solubilization also depends on the centrifugal field applied to sediment the particulate material, as already stressed by Verity *et al.* (1968). This factor should be taken into account when results from different laboratories are compared. For instance, the high proportion of acid phosphatase and deoxyribonuclease extracted with water from the large-granule fraction of rat liver, as reported by Baudhuin *et al.* (1965), could well depend on the relatively low centrifugal field used ($2.5 \times 10^6 g$-min). Finally, the contamination of lysosome-rich fractions with different proportions of other subcellular particles (mitochondria, microsomes, peroxisomes and possibly others) adds a further source for variability of results.

With all of these reservations in mind, we can now discuss the present results here.

β-Galactosidase exhibits the definite pattern of a readily solubilizable enzyme. Its nature appears that of an enzyme freely dispersed in the lysosomal matrix, in agreement with previous observations (Beck & Tappel, 1968; Fisher & Kent, 1969; Bohley *et al.* 1969).

With regard to acid phosphatase, the extensive solubilization afforded by Triton X-100 (as well as by other surface-active agents: Sawant *et al.* 1964; Bertini, Mego & McQueen, 1967; Weissmann *et al.* 1967) suggests a localization within particles; however, this is not entirely supported by the results obtained with other treatments, particularly freezing and thawing and suspension in water (see also Sawant *et al.* 1964; Shibko & Tappel, 1964; Tappel, 1966; Bertini *et al.* 1967; Weissmann *et al.* 1967; Beck & Tappel, 1968; Verity *et al.* 1968; Bohley *et al.* 1969; Arsenis, Gordon & Touster, 1970). With the latter treatments no less than one-third of the activity remains structure-bound. On the other hand, only low proportions of solubilized acid phosphatase added to 'intact' liver homogenates are retained on sedimentable structures. This behaviour, at first sight contradictory, could be explained on the assumption that there exist at least two varieties of acid phosphatase associated with the L fraction from rat liver, the first easily and the other not readily dissociable from structures. This interpretation fits with the observations made by Sloat & Allen (1969). Since the solubilized acid phosphatase added to homogenates is extracted from lysosomes by freezing and thawing, it could be the soluble variety that exhibits low adsorbability. Verity *et al.* (1968) reported a marked biphasic effect of Mg^{2+} and Ca^{2+} on the association of acid phosphatase with sedimentable material: adsorption at concentrations below 10mM, desorption at 50mM. We failed to confirm this result. However, it should be pointed out that Verity *et al.* (1968) assayed an α-naphthylphosphatase activity, which has been shown by Verity & Brown (1968) to be largely distinguishable from β-glycerophosphatase activity (that assayed in our present work) on differential inhibition or inactivation.

Even for acid proteinase the discrepancy between the results with Triton X-100 and those with freezing and thawing or suspension in water is quite pronounced (see also Tappel, 1966). Nevertheless, the addition of 3mM-EDTA to the water, with or without further freezing and thawing, leaves bound to the sedimentable material only a small proportion of enzyme, comparable with that obtained with Triton X-100. This implies that bivalent cations are concerned with the binding of acid proteinase to sedimentable structures. EDTA is present in the suspending medium throughout the fractionation of the liver homogenate; on the other hand, EDTA does not appreciably influence the adsorption of solubilized acid proteinase added on 'intact' liver homogenates. Therefore it seems safe to infer that this binding involves sites, probably inside lysosomes, unmasked to the action of EDTA after the disruptive treatment.

A closer correspondence between the extractability with Triton X-100 and that with freezing and thawing or water is observed for acid ribonuclease and deoxyribonuclease, a large proportion of which none the less remaining sedimentable. Part of these bound enzyme activities are dissociable by EDTA, particularly when the latter is followed by freezing and thawing. However, even in this situation no less than 25% of the ribonuclease and 20% of the deoxyribonuclease remained structurally bound; these values are comparable with those reported by Sawant *et al.* (1964), Shibko & Tappel (1964) and Arsenis *et al.* (1970).

Triton X-100 affords a poor solubilization of β-*N*-acetylglucosaminidase, similar to that obtained by freezing and thawing L fractions suspended in water. By contrast, about 60% of the activity

becomes unsedimentable when the L fractions are frozen and thawed in iso-osmotic sucrose (under these conditions only β-galactosidase and acid deoxyribonuclease show extents of solubilization higher than those for β-*N*-acetylglucosaminidase); this value is somewhat higher than that (40%) obtained by Verity *et al.* (1968) with a lysosome-rich fraction suspended in 0.35M-sucrose and subjected to seven freeze–thaw cycles or that (48%) reported by Mahadevan & Tappel (1968) after freezing and thawing a purified particle suspension ten times. In contrast almost negligible β-*N*-acetylglucosaminidase activity was found by Weissmann *et al.* (1967) to be solubilized by Triton X-100 or freezing and thawing; since the L fraction they used was previously stored at 0°C in 0.25M-sucrose, an effect of this pretreatment on the solubilizability of β-*N*-acetylglucosaminidase cannot be ruled out. A further increase in the percentage of this enzyme recovered in the soluble phase after freezing and thawing is obtained by enriching the suspension medium with 50mM-magnesium chloride or 0.1M-potassium chloride. On the other hand, magnesium chloride at a low concentration (3mM) promotes a marked retention of the activity on particulate material. There is a good correspondence between these findings and those reported by Verity *et al.* (1968). When solubilized β-*N*-acetylglucosaminidase is mixed with 'intact' homogenates, it is almost completely removed from solution by the sedimentable material; although 3 mM-magnesium chloride or EDTA is almost uneffective in modifying this binding, an appreciable proportion of enzyme is kept in solution in the presence of 0.1M-potassium chloride. Conchie & Hay (1963) have shown that after homogenization of rat livers in water 70% of β-galactosidase activity but only 7% of β-*N*-acetylglucosaminidase was released in the soluble form. Therefore the present evidence, together with other findings (F. M. Baccino & M. F. Zuretti, unpublished work), rather than supporting the view of a localization in the lysosomal membrane suggests that β-*N*-acetylglucosaminidase is strongly adsorbable on both intact and broken subcellular particles. According to Robinson & Stirling (1969) rat liver lysosomes contain two forms (A and B) of β-*N*-acetylglucosaminidase, distinguishable by their electrophoretic mobility; the A variety displays a rapid anodic mobility that has been attributed to sialic acid residues. It has been suggested that the A form could derive from the plasmalemma (which also contains an enzyme of the A type) and the B form from primary lysosomes. It is not yet possible to establish a correlation between the distinguishing properties reported by Robinson & Stirling (1969) and the pattern of solubilization observed here.

On the whole, there appears to be no direct correlation among the relative degrees of solubilization of hydrolases obtainable with different procedures, such as treatment with the non-ionic detergent Triton X-100, osmotic lysis, or freezing and thawing. Moreover, the partition of enzymes between the soluble and sedimentable fractions largely depends on the osmolarity, ionic strength and pH of the suspension medium, suggesting that this may be the reason for at least some of the conflicting observations on the intralysosomal distribution of enzymes. Thus the precise internal localization of hydrolases within lysosomes seems to be hardly predictable on the basis of the results obtained with a single particle-disrupting procedure and can also fail to emerge clearly from combined experiments. Indeed, enzymes should be classified according to the relative ease with which they can be extracted from particles into the suspension medium. However, the hydrolase activities examined here usually do not exhibit a clear-cut behaviour allowing them to be attributed to the internal, soluble phase of lysosomes or to their limiting membrane; they instead display variable degrees of association to structures depending on the kind of procedure used to disrupt particles. Moreover, it remains to be established in most cases to what extent the observed distribution patterns of enzyme activities depend on the existence of isoenzymes with different properties or intralysosomal localization. Parenthetically, knowledge of this behaviour of enzymes can be advantageous in the preliminary steps of the purification of enzymes, as already suggested by Bohley *et al.* (1969).

The determination of the unsedimentable activity of acid hydrolases in tissue homogenates or particle suspensions is commonly used as a measure of the extent of lysosome disruption not only due to procedures *in vitro* but also occurring after changes *in vivo* (see, e.g., de Duve & Beaufay, 1959; Ugazio, Artizzu, Pani & Dianzani, 1964; Baccino, Rita & Dianzani, 1965; Slater & Greenbaum, 1965; Dianzani, Baccino & Comporti, 1966). The present results suggest that the observed variability in the solubilization of enzymes does not merely reflect possible differences in the intralysosomal location of enzymes or in the strength of their binding to particles. For example, in liver homogenates from rats poisoned with a crude extract of *Amanita phalloides* there occurs a conspicuous shift in the compartmentation of acid phosphatase and β-galactosidase activities from particles to the soluble fraction; in contrast, the solubilization of β-*N*-acetylglucosaminidase is almost negligible (F. M. Baccino & M. F. Zuretti, unpublished work). The present evidence suggests that the latter finding could depend on secondary adsorption on particulate matter of enzyme released from lysosomes.

Esterase (Shibko & Tappel, 1964), β-*N*-acetylglucosaminidase (Weissmann *et al.* 1967), neuraminidase (Mahadevan, Nduaguba & Tappel, 1967; Tulsiani & Carubelli, 1970), lipase (Mahadevan & Tappel, 1968), β-glucosidase (Beck & Tappel, 1968; Lloyd, 1969*a*), β-xylosidase (Beck & Tappel, 1968; Fisher & Kent, 1969) (the two last enzyme activities probably belong to a single enzyme species; Patel & Tappel, 1969), nucleoside diphosphatase (Wattiaux-de Coninck & Wattiaux, 1969), phospholipase A_2 (Rahman *et al.* 1970) and an NADH dehydrogenase (quoted by Tappel, 1968) have all been suspected to be membrane-bound lysosomal enzymes. However, the evidence supporting this view is controversial: when the evidence lies in the distribution of the activity between soluble and insoluble phases from lysosome-rich fractions, adsorption artifacts cannot be ruled out completely; when the evidence is the lack of latency for the enzyme activity measured with intact lysosomes, rapid permeation of the substrate through the lysosomal membrane has to be excluded (Lloyd, 1969*b*). There is one possible exception, since Lloyd (1969*a*) has been able to demonstrate that, although the β-glucosidase of rat liver lysosomes does not display the phenomenon of latency (in contrast with most lysosomal hydrolases), exogenous β-glucosidase segregated into liver lysosomes by endocytosis is latent. Thus the possibility of a rapid permeation of the substrate through the limiting membrane of lysosomes should be ruled out. Accordingly, Lloyd (1969*a*) suggested not only that endogenous β-glucosidase is located in the lysosome membrane, but that it occurs on its external aspect. Nevertheless, this conclusion is tenable only when the exogenous enzyme is taken up by the very particles that contain the endogenous enzyme. This remains to be proved. Lloyd (1969*a*) reported that the administered β-galactosidase is found associated with granules sedimentable from liver homogenates; however, the particles bearing the endogenous or exogenous activity could either derive from different (parenchymal or sinusoidal) liver cell populations or even correspond to different structures among those that are included in the concept of lysosomal or vacuolar system (phagosomes, primary or secondary lysosomes, for instance; de Duve & Wattiaux, 1966).

For the reasons outlined above, knowledge of the intralysosomal distribution of enzymes is as yet meagre and cannot be rationalized in terms of their function or pathophysiological role. As de Duve (1969) points out, the functional significance of membrane-bound acid hydrolases in the lysosomes is unclear, since soluble enzymes can more easily attack the substrates sequestered in these particles. Sloat & Allen (1969) adduced evidence showing that lysosomes in regenerating liver contain a larger proportion of bound to soluble acid phosphatase than do particles from control animals; they suggested that the bound form of the enzyme derives either from the membrane of Golgi vesicles or from the membrane of the endoplasmic reticulum.

Another possibility is that lysosomes derive their membrane-bound enzymes from the pieces of plasma membrane that encircle endocytotic vesicles. This possibility has been raised by Robinson & Stirling (1969) for the A variety of β-*N*-acetylglucosaminidase, as mentioned above. It stems also from data of Wattiaux-de Coninck & Wattiaux (1969), who detected a nucleoside diphosphatase activity mainly associated with the membrane fraction from rat liver lysosomes filled with Triton WR-1339. Rahman *et al.* (1970) suggested a possible role for the membrane-bound Ca^{2+}-activated phospholipase A in the adhesion and coalescence of lysosomes with other subcellular particles. It seems likely that a better knowledge of the enzymes associated with lysosomes could also help in understanding the life-cycle of these particles.

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