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Effect of Mercurial Compounds on Structure-Linked Latency of Lysosomal Hydrolases

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1. A partially purified lysosomal preparation was obtained from adult mouse livers by sucrose-density-gradient centrifugation of a large-granule fraction. 2. This lysosome-enriched subfraction was contaminated approx. 10% by mitochondrial cytochrome *c* oxidase and malate dehydrogenase. 3. Free acid phosphohydrolase and β-glucuronidase contributed less than 10% of the total (Triton X-100-solubilized) activity in contrast with approx. 30% free *N*-acetyl-β-D-glucosaminidase when assayed in an iso-osmotic incubation system. 4. Exposure of the lysosomal preparation to inorganic Hg^{2+} ions and organic mercurials (*p*-chloromercuribenzoate, phenylmercuric acetate) induced an irreversible loss of structure-linked latency with resulting enzyme activation. 5. Maximal activation was related to log [Hg^{2+}] and pH. The response was all-or-none for individual particles; the dose–response curve portrayed the variation in particle resistance within the lysosomal population. 6. L-Cysteine and GSH totally prevented Hg^{2+} ion-induced hydrolase activation. Ascorbate provided approx. 50% protection. 7. The three lysosomal hydrolases were differentially activated at constant [Hg^{2+}], suggesting a different pattern of binding, unique for each enzyme studied.

Studies of the action of heavy-metal ions on membrane function may provide a means for the investigation of membrane properties as well as for seeking the nature of the toxic effect of the metal at an ultrastructural level. Physiological functions associated with the interaction of cell membranes with heavy-metal ions have included changes in erythrocyte K^+ permeability produced by lead, mercury gold and silver (Joyce, Moore & Weatherall, 1954; Vincent, 1959), copper- and mercury-induced changes in erythrocyte permeability to glycerol (Willbrandt, 1941; Jacobs, 1950), inhibition of surface-bound membrane invertase and β-glucosidase of yeast cells by uranium, silver and mercury ions (Myrbäck, 1957; Passow, Rothstein & Clarkson, 1961; Kaplan & Tacreiter, 1966), inhibition of transfer of sugars through the yeast-cell membrane by uranium (Rothstein & Larrabee, 1948), activation of 'cryptic' catalase from intact yeast cells by non-penetrating heavy metals, uranium and mercury (Kaplan, 1963, 1965), and glucose-uptake inhibition in the excised rat diaphragm by mercury and copper (Demis & Rothstein, 1955). Less well documented are studies on the interaction of heavy-metal ions with the membrane interface of subcellular organelles. Changes in mitochondrial oxidative phosphorylation and K^+ ion binding after exposure to Hg^{2+} ions (Gamble, 1957; Scott & Gamble, 1961) and mitochondrial swelling induced by copper, mercury and calcium (Lehninger, 1962) have been related to membrane phenomena. The identification of a further subcellular organelle, the lysosome (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955), has been established from subcellular fractionation of liver homogenates, electron microscopy of these fractions and applications of cytochemical and electron histochemical staining procedures for acid phosphohydrolase. These organelles are characterized by a single limiting membrane, thought to confer the property of structure-linked latency on the associated acid hydrolases.

In view of the ligand-binding properties of mercury in various membrane systems (see above) it seemed appropriate to investigate the effect of mercury on lysosomal membrane integrity and structure-linked latency of lysosomal enzymes, especially acid phosphohydrolase. The studies reported here, with a partially purified lysosomal fraction, demonstrate the loss of enzyme latency in the presence of low concentrations of organic and inorganic mercurials as a result of irreversible damage of the membrane after the formation of mercaptide ligands.

MATERIALS AND METHODS

Adult male Swiss albino mice, weighing approx. 25g., were used. Substrates 1-naphthyl β-D-glucuronide and 1-naphthyl 2-acetamido-2-deoxy-β-D-glucopyranoside were obtained from Pierce Chemical Co., Rockford, Ill., U.S.A. Sodium hydrogen 1-naphthyl phosphate and cytochrome *c* (amorphous) were purchased from Mann Research Laboratories Inc., New York, N.Y., U.S.A. NADH, oxaloacetic acid, sodium *p*-chloromercuribenzoate, 2-mercaptoethylamine hydrochloride, 2-cysteine hydrochloride hydrate, 2,2′-dithiobisethylamine dihydrochloride and GSH were obtained from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. Phenylmercuric acetate and *N*-ethylmaleimide were procured from K & K Laboratories Inc., Plainview, N.Y., U.S.A. Dithiothreitol was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Disodium EDTA was used without further purification. Other chemicals including $HgCl_2$ were of analytical reagent grade.

Preparation of partially purified lysosomal fraction. Whole livers were rapidly removed, placed in 10ml. of ice-cold 0·25M-sucrose and homogenized in a smooth-walled Potter–Elvehjem homogenizer. The homogenate was spun in a Spinco model L ultracentrifuge (rotor 50) at 3000*g*-min. (forces expressed as time integrals of the maximum field where $R_{max.}=7·1$cm.; de Duve & Berthet, 1953). The resulting supernatant was spun at 110000*g*-min. The 'large-granule' pellet obtained was washed once in 0·3M-sucrose and respun at 110000*g*-min. The resulting pellet was resuspended in 4ml. of 0·3M-sucrose of which 1·6ml. was layered over a discontinuous gradient of equal volumes of 0·6M- and 1·2M-sucrose (Fig. 1). The gradient was centrifuged at 107000*g*-min. ($R_{max.}=9·8$cm., rotor SW 39). The resultant layers and band were removed separately and labelled as subfractions *A*, *B*, *C* and *D* (Fig. 1).

Enzyme assays. Lysosomal and mitochondrial enzyme activities were assayed in buffered systems made iso-osmotic with respect to 0·15M-KCl. Total enzyme activity refers to enzyme measured in the presence of Triton X-100 (0·1–0·3%, v/v), whereas free activity was measured in untreated samples of the subfractions.

Malate dehydrogenase (EC 1.1.1.37) was determined by the spectrophotometric method of Bergmeyer & Bernt (1963). Mitochondrial cytochrome *c* oxidase (EC 1.9.3.1) was measured by a slight modification of the procedure of Cooperstein & Lazarow (1951). The activities of the lysosomal enzymes acid phosphohydrolase (EC 3.1.3.2), β-glucuronidase (EC 3.2.1.31) and *N*-acetyl-β-D-glucosaminidase (EC 3.2.1.29) were determined by the sensitive fluorimetric procedures of Verity & Brown (1964*a*), Verity, Caper & Brown (1964) and Verity, Gambell & Brown (1967) respectively. In experiments on Hg^{2+} ion activation, the assay system contained mM-EDTA. Protein was measured on non-Triton X-100-treated samples by the method of Lowry, Rosebrough, Farr & Randall (1951). A statistical degree of significance between means of small populations was calculated from Student's *t* test, and inferred when $P<0·05$ (Bernstein & Weatherall, 1952).

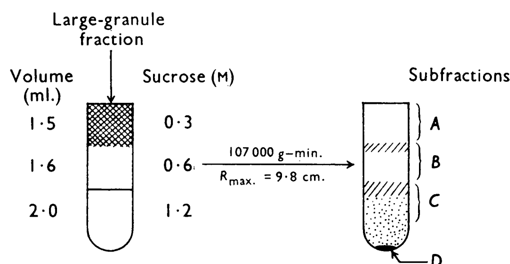

Fig. 1. Preparation of subfractions on discontinuous sucrose density gradient from adult mouse liver large-granule fraction.

RESULTS

Distribution of hepatic lysosomal and mitochondrial enzymes after density-gradient centrifugation of the large-granule fraction

The large-granule fraction obtained at 110000*g*-min. was submitted to sucrose-density-gradient centrifugation (Fig. 1). The resultant subfractions and pellet were labelled *A–D*. Representative lysosomal and mitochondrial enzyme analyses of the subfractions revealed a predominant localization of lysosomal acid phosphohydrolase and β-glucuronidase activity in subfraction *B*, whereas the mitochondrial enzymes cytochrome *c* oxidase and malate dehydrogenase were recovered principally in subfraction *C* and the resuspended pellet, subfraction *D* (Table 1). The relative specific activities for acid phosphohydrolase (3·0) and β-glucuronidase (2·03) in subfraction *B* indicated enrichment of this fraction in lysosomes. The relative specific activity of the lysosomal enzymes and malate dehydrogenase is high in subfraction *A* whereas that of cytochrome *c* oxidase is very low. Such activities are in large part represented by solubilized enzymes, liberated during resuspension of the large-granule pellet before it was placed on the gradient. Malate dehydrogenase is located within the mitochondrial matrix from which it is easily solubilized (Bendall & de Duve, 1960) and hence remained in subfraction *A*. Cytochrome *c* oxidase is membrane-bound and will migrate through the gradient depending on the degree of structural integrity of the mitochondrial population. Though subfraction *B* is enriched in lysosomal enzyme activity, there is approx. 10% mitochondrial contamination as determined from cytochrome *c* oxidase and malate dehydrogenase values. The method provided for an increase in specific activity of lysosomal enzymes in subfraction *B*. Direct assays on this subfraction of free and total acid phosphohydrolase, β-glucuronidase and *N*-acetyl-β-D-glucosaminidase revealed low percentage free activities (100 × free/total) for acid phosphohydrolase and β-glucuronidase (Table 2) but a significantly greater value for *N*-acetyl-β-D-glucosaminidase (9·2 : 6·6 : 27·9 respectively). Subfraction *B* was used in all subsequent experiments.

Table 1. *Distribution of hepatic acid phosphohydrolase, β-glucuronidase, cytochrome* c *oxidase and malate dehydrogenase in subfractions from discontinuous sucrose-density-gradient centrifugation of a large-granule fraction*

Large-granule fraction was obtained from sedimentation at 110000g-min. after removal of unbroken cells with nuclei. Subfractions *A*, *B*, *C* and *D* were obtained as described in the Materials and Methods section. Units of activity were: acid phosphohydrolase, μmoles of α-naphthol released/min. at 37°; β-glucuronidase, μmoles of α-naphthol released/hr. at 37°; cytochrome *c* oxidase, $\Delta\log E_{550}$/min. at 24°; malate dehydrogenase, ΔE_{340}/min. at 24°; protein, mg. Values are means of four experiments except those for malate dehydrogenase, which represent the mean of three experiments. Values in parentheses represent relative specific activities on a protein basis (% of total enzyme activity/% of total protein).

Fraction	Absolute protein (mg.)	Total enzyme activity: Acid phosphohydrolase	β-Glucuronidase	Cytochrome *c* oxidase	Malate dehydrogenase
Large-granule fraction	15·8	1·18	1·55	27·90	49·80
Subfraction *A*	2·4	0·18 (1·00)	0·29 (1·20)	0·65 (0·14)	6·00 (0·79)
Subfraction *B*	2·1	0·47 (3·00)	0·42 (2·03)	2·30 (0·58)	4·40 (0·66)
Subfraction *C*	3·2	0·14 (0·58)	0·29 (0·91)	8·00 (1·30)	10·90 (1·07)
Subfraction *D*	6·8	0·22 (0·43)	0·37 (0·55)	17·30 (1·35)	23·70 (1·10)
Recovery (%)	91·6	85·60	87·80	95·00	90·10

Table 2. *Activation of lysosomal acid phosphohydrolase, β-glucuronidase and* N*-acetyl-β-*D*-glucosaminidase by* Hg^{2+} *ions*

Samples of subfraction *B* were diluted with equivolumes of iso-osmotic 0·15M-KCl containing Hg^{2+} ions to give a final concentration of 30μM. These particulate subfractions were preincubated at 2° for 3min. Assays were performed on samples in a modified system (see the Materials and Methods section). Values represent means ± S.E.M. (*n* = no. of observations). Values in parentheses are values expressed as % of total.

Enzyme	Acid phosphohydrolase (μmoles/min./100mg. of protein) (*n*=7)	β-Glucuronidase (μmoles/hr./100mg. of protein) (*n*=5)	*N*-Acetyl-β-D-glucosaminidase (μmole/min./100mg. of protein) (*n*=4)
Free	1·21 ± 0·17 (9·2)	1·08 ± 0·21 (6·6)	0·081 ± 0·00 (27·9)
Total (Triton X-100 activation)	13·07 ± 1·30	16·20 ± 0·98	0·29 ± 0·04
Hg^{2+} (30μM)	8·10 ± 1·1 (61·9)	5·88 ± 0·80 (36·2)	0·23 ± 0·03 (78·6)

Effect of Hg^{2+} *ions on structure-linked latency of hepatic lysosomal acid phosphohydrolase*

During preliminary experiments on the stability and activation of lysosomal acid phosphohydrolase it was noticed that addition of Hg^{2+} ions to a particulate fraction increased the free activity. This result appeared paradoxical in view of the known inhibition of acid phosphohydrolase by heavy-metal ions, including Hg^{2+} ions (MacDonald, 1961; Tsuboi & Hudson, 1955; Verity & Brown, 1964*a*). This observation was confirmed with subfraction *B* and clarified when Triton X-100-activated acid phosphohydrolase showed marked inhibition (11% of the original activity remaining) in the presence of 50μM-Hg^{2+} ion in the assay system (Table 3). This inhibition was reversed in the presence of mM-EDTA. Incubation of the particulate preparation with Hg^{2+} ions at 2° before transference of a portion to the assay system revealed reversible

Table 3. *Effect of* Hg^{2+} *ions on Triton* X-100*-solubilized (total) acid phosphohydrolase activity*

Samples (1ml.) of activated subfraction *B* were incubated at 2° for 5min. in the presence of the inhibitory ion (final concn. in parentheses) in 0·2M-acetate–KCl buffer, pH 6·1: 0·1ml. was removed for assay at 37° in 0·2M-acetate–KCl with or without mM-EDTA as indicated. Values represent means of two experiments.

Hg^{2+} ions present in: Preincubated large-granule fraction	Final assay system	Remaining activity (% of control)
—	—	100
—	Hg^{2+} (50μM)	11
—	EDTA (1mM)	108
—	Hg^{2+} (50μM) + EDTA (1mM)	91
Hg^{2+} (100μM)	—	78
Hg^{2+} (100μM)	EDTA (1mM)	98

inhibition on dilution. Transference to an assay system containing mM-EDTA restored the activity to its original control value. These experiments confirmed the feasibility of measuring activated lysosomal acid phosphohydrolase, β-glucuronidase and N-acetyl-β-D-glucosaminidase in the presence of Hg^{2+} ions.

Effect of Hg^{2+} *ion concentration.* Activation of lysosomal acid phosphohydrolase in subfraction B was a function of Hg^{2+} ion concentration. Preincubation at 2° with concentrations in the range 10–100 μM-Hg^{2+} revealed a rapid activation to maximal values within the first minute of preincubation. Maximum activation was a function of Hg^{2+} ion concentration. At low concentrations of Hg^{2+} ions, there was no decline in the activity of the liberated enzyme, but slow inactivation was apparent at 80 μM-Hg^{2+} and may account for failure to obtain degrees of activation approaching 100%. The relationship between Hg^{2+} ion concentration and response (percentage enzyme activation) is an asymmetric sigmoid curve when expressed as a logarithmic function of Hg^{2+} ion concentration (Fig. 2). These observations indicate an 'all-or-none' effect of Hg^{2+} ions on individual lysosomal particles. On this basis, the maximal values represent the proportion of particles in which the threshold has been surpassed, and the sigmoid logarithmic function (Fig. 2) demonstrates the distribution of thresholds in the lysosomal population.

The loss of structure-linked latency (activation) in the lysosomal suspension occurred also in the presence of the thiol-characterizing reagents, p-chloromercuribenzoate and phenylmercuric acetate (Fig. 2). There was a significant shift to the right of the log(concentration)–response curve. In general, a threefold higher concentration of organic mercurial, as compared with the inorganic salt, was needed to effect an equivalent degree of enzyme activation at the 50% effective dose level.

Effect of pH *and time of preincubation with* Hg^{2+} *ions.* Previous studies (Wattiaux & de Duve, 1956) indicated that preincubation of the lysosomal particle at below pH 5 induced activation of the hydrolytic enzymes. Though amply demonstrated in the liver, this sensitivity to pH is not a feature of spleen particles (Rahman, 1964). In view of this 'labilizing' effect at acidic pH values it was necessary to examine the role of the preincubation pH on the activation of acid phosphohydrolase in the presence of Hg^{2+} ions (Fig. 3). There was an increased percentage enzyme activation at Hg^{2+} ion concentrations of 10 μM and 30 μM at acidic pH values. The pH–activation curves with Hg^{2+} ions are sigmoid although some decreased activation (or enzyme inhibition) occurred at below pH 4·5 with 30 μM-Hg^{2+} ion.

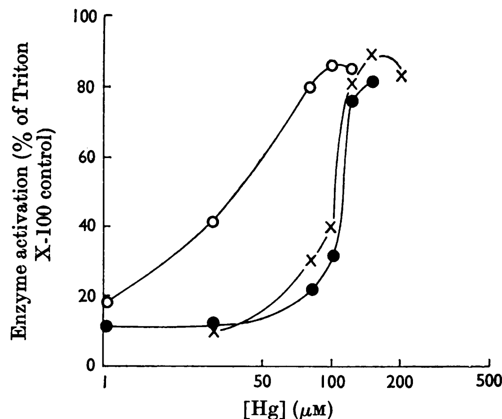

Fig. 2. Dose–response curves of acid phosphohydrolase activation as a function of $HgCl_2$ (○), p-chloromercuribenzoate (×) and phenylmercuric acetate (●) concentrations. Reagent was present in 0·5 ml. portions of buffered subfraction B; incubation was at 2° for 3 min. before assay in EDTA-containing system.

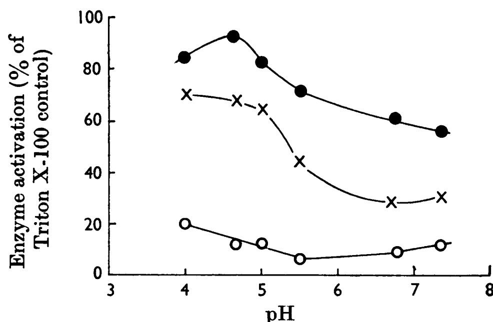

Fig. 3. Effect of pH on Hg^{2+} ion activation of lysosomal acid phosphohydrolase: ○, control (no Hg^{2+}); ×, 10 μM-Hg^{2+}; ●, 30 μM-Hg^{2+}. The procedure was similar to that in Fig. 2.

The rapid maximal activation that occurred during the first minute of preincubation at 2° suggested that the rate of enzyme activation had a low temperature coefficient. This proved to be the case when the percentage activation of acid phosphohydrolase was determined at preincubation temperatures of 2°, 24° and 37°. Moreover, there was rapid inactivation of liberated enzyme at 37°, but less so at 24°. This thermolability of activated acid phosphohydrolase has been shown to be due to oxidation of reactive thiol groups (Verity & Brown, 1964*b*). Such thermolability is not seen when the enzyme is in the bound or 'cryptic' state. Because of this inactivation, and the absence of any appreciable change in activity during the first minute of preincubation at 24° or 37°, all preincubations were performed at 2°.

Activation of lysosomal β-glucuronidase and N-acetyl-β-D-glucosaminidase by Hg^{2+} *ions*

The mode of preparation of subfraction *B* allowed for low free enzyme activities. It was therefore surprising that *N*-acetyl-β-D-glucosaminidase showed a significantly greater percentage free activity than did acid phosphohydrolase and β-glucuronidase (Table 2). This exceptional value suggested that the property conferring structure-linked latency on *N*-acetyl-β-D-glucosaminidase differed fundamentally from acid phosphohydrolase and β-glucuronidase and that the enzyme, although particulate-linked, is more available to substrate interaction. The generality of Hg^{2+} ion activation of the lysosomal hydrolases is appreciated from studies with β-glucuronidase and *N*-acetyl-β-D-glucosaminidase, which show activation comparable with that of acid phosphohydrolase (Table 2). However, the percentage activation at constant Hg^{2+} ion concentration (30 μM) varied with the three enzymes.

Inhibition of enzyme activation by Hg^{2+} *ions in the presence of thiol compounds*

GSH and L-cysteine in moderate excess prevented lysosomal acid phosphohydrolase activation by Hg^{2+} ions (Table 4). In these experiments the thiol agent was preincubated simultaneously with Hg^{2+} ions. In one experiment 2 mM-GSH protected against enzyme activation by 0·12 mM-*p*-chloromercuribenzoate. Slight protection was conferred by buffered ascorbate and 2,2′-dithiobisethylamine. The former agent maintained an excess of membrane mercaptide and thiol groups in the reduced state, and the latter afforded a surplus of disulphide groups, some of which existed in a reduced form, thus allowing Hg^{2+} ion chelation and diminished access to effective lysosomal membrane thiol groups.

Table 4. *Activation of lysosomal acid phosophohydrolase by* Hg^{2+} *ions in the presence of thiol and reducing agents*

Samples of subfraction *B* were preincubated with Hg^{2+} ions (30 μM) at 2° for 3 min. in the presence of equimolar concentrations (2 mM) of reagents. Values represent means of two experiments.

Reagent	Activation (% of Triton X-100 value)
Control	9·4
Hg^{2+}	67·4
Hg^{2+} + L-cysteine	8·1
Hg^{2+} + β-mercaptoethylamine	7·4
Hg^{2+} + dithiothreitol	5·3
Hg^{2+} + glutathione (GSH)	6·3
Hg^{2+} + 2,2′-dithiobisethylamine	49·8
Hg^{2+} + ascorbate	35·1

DISCUSSION

The preparation of purified lysosomes has become necessary in order to study their composition, structure and properties *in vitro*. Such purified preparations have been obtained from different tissues by Beaufay, Van Campenhout & de Duve (1959), Cohn & Hirsch (1960), Sawant, Shibko, Kumta & Tappel (1964) and Shibko & Tappel (1965). The method presented above used density-gradient centrifugation of a large-granule fraction and was adequate for the preparation of small amounts of intact particles with minimal mechanical release, low free enzyme activity and less than 10% mitochondrial contamination.

The phenomenon of lysosomal acid phosphohydrolase activation in the presence of Hg^{2+} ions represents a breakdown in structure-linked latency of the enzyme. Such activation also occurs with freezing and thawing, with osmotic stress and with Triton X-100, providing evidence for the sac-like representation with a membrane impermeable to internal enzymes and external substrates (de Duve, 1963). In a preliminary analysis of lysosomal composition (Tappel, Sawant & Shibko, 1963) approx. 60% of the total protein and 100% of lipid phosphorus were located in the membrane. The phospholipid nature of the membrane has also been confirmed from studies with phospholipase C (Verity, Caper & Brown, 1965). Mercury forms stable mercaptides with thiol groups, in preference to other ligand groups that may be present (Gurd & Willcox, 1956). The presence of thiol and disulphide groups in membrane systems is well established (Riley & Lehninger, 1964; Passow *et al.* 1961; Jacob & Jandl, 1962) and such functional groups may play a role in the integrity and permeability properties of the membrane. The experiments described above indicate the essential nature of such groups to lysosomal membrane integrity and the phenomenon of structure-linked enzyme latency. All agents irreversibly damaged the limiting membrane and produced an 'all-or-none' response with the formation of –S·Hg·S– bridges.

The 'all-or-none' nature of the activation has been interpreted in terms of individual particle sensitivities. The distribution of thresholds followed a characteristic log (dose) pattern, the lowest threshold corresponding to approx. 6·0 μM-Hg^{2+}/mg. of protein. Hence enzyme activation was a function of the proportion of lysosomes whose threshold has been exceeded. Shibko, Pangborn & Tappel (1965) in studies of the release of kidney lysosomal enzymes showed that at no stage during a graded release did the particles lose only part of their

electron-opaque matrix. The demonstrated interaction of mercury with membrane thiol groups suggests that no single ligand plays a significant functional role in membrane integrity, but that a critical number of ligands, if cross-linked, will disrupt membrane contiguity with resultant permeability. Moreover, differences in individual lysosome responses can be attributed to morphological and biochemical parameters that may decide the relative availability of thiol groups, assuming a constant stoicheiometric ratio and mass-law distribution of Hg^{2+} ions in the preincubation system (Clarkson & Magos, 1966).

The percentage free activity (100 × free/total) of acid phosphohydrolase, β-glucuronidase and *N*-acetyl-β-D-glucosaminidase were not comparable in this subfraction. The high percentage free activity (27·9) for *N*-acetyl-β-D-glucosaminidase was not explainable on the basis of smaller substrate molecular weight. Moreover, raising the substrate concentration by several multiples of the Michaelis constant failed to increase the free activity of *N*-acetyl-β-D-glucosaminidase in cytoplasmic extracts from bone-tissue homogenates (Vaes, 1965). The presence of significant free activity must reflect differential enzyme adsorption, extrinsic localization of the enzyme in the membrane and/or passage into the *B* fraction of morphologically intact but permeable particles. Though evidence for the localization of *N*-acetyl-β-D-glucosaminidase in a specific lysosomal particle species is not available, this cannot be excluded. The possibility that there is an extrinsic localization of the enzyme in the membrane would appear also to be negated by the above data referable to substrate concentration. A high specific adsorption to lipoprotein may be a feature of the enzyme, as Vaes (1965) has demonstrated that half of the total activity of *N*-acetyl-β-D-glucosaminidase is unsedimentable, whereas total recovery was obtained in the unsedimentable phase for acid phenylphosphatase and β-glucuronidase. Sawant *et al.* (1964) demonstrated a differential release of enzymes into the suspending media, although there was parallelism in their percentage activation measured before centrifugation. In this respect Shibko *et al.* (1965) consider that kidney lysosomal enzymes become totally available to substrate after osmotic activation but remain sedimentable to be followed by a phase of increased solubilization and unsedimentability.

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