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Effect of Cations on Structure-Linked Sedimentability of Lysosomal Hydrolases

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1. A partially purified lysosomal preparation was obtained from mouse liver sucrose homogenates by differential and discontinuous gradient centrifugation. 2. Triton X-100 or repeated freezing and thawing of the lysosomal suspension (subfraction B) allowed comparison of free and activated values for acid phosphohydrolase, β-glucuronidase and *N*-acetylglucosaminidase in the presence and absence of ascorbate. 3. The distribution of hydrolase activities between supernatant and pellet after high-speed centrifugation was measured and the percentages of total enzyme found in the supernatant were: acid phosphohydrolase, 40·7; β-glucuronidase, 51; *N*-acetylglucosaminidase, 39·4. 4. Differential rates of elution of the three hydrolases from the membrane fraction occurred with increasing Na^+ and K^+ concentrations, whereas complex biphasic elution curves were obtained as a function of bivalent cation concentration with Ca^{2+} and Mg^{2+}. 5. Sucrose-density-gradient centrifugation of frozen-and-thawed subfraction B demonstrated highly significant changes in the protein gradient profile in the presence of a low concentration of bivalent cation, indicating membrane aggregation and enzyme–membrane association. 6. The data provide further evidence for the nature of lysosomal enzyme binding and indicate the presence of different enzyme–membrane bonds conferring structure-linked latency upon individual lysosomal enzymes.

Lysosomes are membrane-limited cytoplasmic particles containing hydrolytic enzymes. Such enzymes are further characterized by their latent (cryptic) activity, loss of which results in activation and solubilization of the hydrolases. Organization and control of such structure-linked latency is a fundamental function of the regulation of lysosomal activity in cells. However, knowledge of the underlying mechanism of structure-linked enzyme activity is meagre.

In a previous study (Verity & Reith, 1967) evidence was presented to indicate that lysosomal hydrolases were differentially activated at constant Hg^{2+} concentration, suggesting a different pattern of enzyme binding, unique for each enzyme, in the lysosomal population. Ugazio & Pani (1963) have demonstrated the differential release of bound hydrolases from rat liver lysosomes treated with Triton X-100. Also, differences in behaviour have been found to exist between several lysosomal enzymes as a function of age (Ugazio, 1960; Franklin, 1962; Verity & Brown, 1968*a*).

These observations suggest that the hydrolases are located either in different subclasses of lysosomal particles or in different compartments of the same particle. Though an unequivocal answer to this problem is still lacking, recent experiments on the sedimentability of enzymically active lysosomal membranes after activation by freezing and thawing have redefined the problem. These studies have demonstrated the usefulness of freezing and thawing in revealing different degrees of enzyme–membrane binding in the presence of univalent and bivalent cations.

MATERIALS AND METHODS

Adult male Swiss albino mice weighing approx. 25g. were used. The lysosomal enzyme 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. 1-Naphthyl phosphate was purchased from Mann Research Laboratories Inc., New York, N.Y., U.S.A. All other chemicals were of A.R. grade.

Preparation of partially purified lysosomal fraction. Whole livers were removed, placed in 10ml. of ice-cold 0·25M-sucrose and homogenized in a smooth-walled Potter–Elvehjem homogenizer. Differential centrifugation of the homogenate and preparation of subfraction B (enriched lysosomal fraction) from the large-granule fraction followed the technique of Verity & Reith (1967). Activation of

Table 1. *Comparison of lysosomal hydrolase activation by Triton X-100 and repeated freezing and thawing of subfraction B obtained by density-gradient centrifugation*

Assays were performed in non-iso-osmotic incubation systems for 10 min. at 37°. Acid phosphohydrolase assay (1·0 ml.) contained: 0·2 M-acetate buffer, pH 6·1; 2·6 mM-1-naphthyl acid phosphate; 0·1 ml. of enzyme sample. β-Glucuronidase assay (0·7 ml.) contained: 0·2 M-acetate buffer, pH 5·1; 3·1 mM substrate; 0·1 ml. of sample. *N*-Acetylglucosaminidase was assayed at pH 5·1 in 0·1 M-citrate buffer containing 5·7 mM-1-naphthyl 2-acetamido-2-deoxy-β-D-glucopyranoside. Values are means ± S.E.M. (n = 4–6).

Procedure	Acid phosphohydrolase (μmoles/min./100 mg. of protein)	*N*-Acetylglucosaminidase (μmole/min./100 mg. of protein)	β-Glucuronidase (μmoles/hr./100 mg. of protein)
No treatment (free enzyme) (*a*)	3·4 ± 0·20	0·14 ± 0·05	7·2 ± 0·55
Triton X-100 (0·2%)	9·3 ± 1·1	0·57 ± 0·06	19·3 ± 2·3
Freezing and thawing seven times (*b*)	10·1 ± 0·90	0·62 ± 0·08	17·8 ± 1·6
Ratio 100 × (*a*)/(*b*)	33·6	22·5	40·4

samples of subfraction B (approx. 0·6 mg. of protein/ml.) was obtained in the presence of 0·2% (v/v) Triton X-100 or freezing and thawing seven times in acetone–solid CO_2. Sedimentable (pellet) and non-sedimentable enzyme activities of the frozen and thawed activated subfraction B were obtained on 0·5 ml. samples, centrifuged at 30000*g* for 15 min. in the presence of 1 mM-ascorbate in a Misco micro-ultracentrifuge.

Lysosomal enzyme assays. Free activity was measured in unactivated samples of lysosomal subfraction. Activities of acid phosphohydrolase (EC 3.1.3.2), β-glucuronidase (EC 3.2.1.31) and *N*-acetyl-β-D-glucosaminidase (EC 3.2.1.30) were determined in the fluorimetric procedures of Verity & Brown (1964), Verity, Caper & Brown (1964) and Verity, Gambell & Brown (1967) respectively. Protein was measured on samples that had not been treated by Triton X-100 by the method of Lowry, Rosebrough, Farr & Randall (1951). Bovine serum albumin was used as a standard in the protein assay.

Continuous sucrose-density-gradient centrifugation. Portions (1 ml.) of sucrose of descending molarities 1·4 M, 1·2 M, 1·0 M, 0·8 M and 0·6 M were layered stepwise in a 6 ml. centrifuge tube and stored at 2° for 24 hr. before use. Subfraction B was frozen and thawed seven times in the presence of 5 mM bivalent cation or 10 mM-Na^+. Control mixtures contained no added ions. Similar concentrations of individual cations were added to the various sucrose molarities. Then 1 ml. of subfraction B (mean protein content 1·97 mg.) was placed on the gradient, and centrifuged at 7000 rev./min. for 15 min. in the SW 39 rotor of a Spinco model L ultracentrifuge.

RESULTS

Free and total activities of acid phosphohydrolase, N-acetylglucosaminidase and β-glucuronidase in subfraction B. Verity & Reith (1967) showed that a high specific activity for Triton X-100-activated lysosomal hydrolases existed in subfraction B obtained by density-gradient centrifugation of mouse liver large-granule fraction. In this previous study, with iso-osmotic buffered incubation systems, acid phosphohydrolase free activity was less than 10% of the total Triton X-100-activated activity. The incubation system was not made iso-osmotic with respect to 0·15 M-potassium chloride in the present studies and, though comparable activated specific activities are found, the free activities of acid phosphohydrolase, *N*-acetylglucosaminidase and β-glucuronidase are significantly higher than in the previous study (Table 1). Comparison between the activating ability of Triton X-100 and repeated freezing and thawing procedures revealed no significant difference in the magnitude of the activation. These assays were performed without further centrifugation of the activated subfraction B.

Distribution of acid phosphohydrolase, β-glucuronidase and N-acetylglucosaminidase activity in supernatant and membranous pellet fractions after repeated freezing and thawing. Samples of subfraction B in 0·35 M-sucrose were submitted to repeated freezing and thawing and centrifuged at 100000*g* for 30 min. A small tightly packed membranous pellet and clear supernatant were recovered. Table 2 indicates the activities in the original subfraction B, the supernatant and the pellet and the percentage recoveries of protein and enzyme activities. Over half the protein appeared in the pellet. In the absence of ascorbate in subfraction B before activation by freezing and thawing, poor recoveries of acid phosphohydrolase were obtained, though adequate recoveries of β-glucuronidase and *N*-acetylglucosaminidase are noted. In the presence of mM-ascorbate, an increased recovery of acid phosphohydrolase is obtained, especially in the supernatant phase (see also Verity & Brown, 1968*b*). The presence of ascorbate (1 mM) slightly inhibited β-glucuronidase and *N*-acetylglucosaminidase, but did not influence the percentage distribution of these hydrolase activities between the supernatant and pellet.

Effect of univalent and bivalent cations on the distribution of lysosomal enzyme activity between supernatant and pellet. Univalent ions, K^+ and Na^+,

Table 2. *Distribution of lysosomal hydrolase activity between supernatant and membranous pellet after centrifugation at* $3 \times 10^6 g$*-min. or* $4{\cdot}5 \times 10^5 g$*-min.*

Samples of subfraction B were frozen and thawed in the presence (+) and absence (−) of 1 mM-ascorbate. A portion was assayed and the remainder centrifuged into supernatant and pellet. The pellet was resuspended in 0·35 M-sucrose before assay. Values are means of three experiments.

Fraction	Total protein (mg.)	Acid phosphohydrolase (μmole/min.)		β-Glucuronidase (μmole/hr.)		N-Acetylglucosaminidase (μmole/hr.)	
Ascorbate (1 mM)		−	+	−	+	−	+
Subfraction B	2·93	0·39	0·44	0·38	0·30	0·78	0·69
Supernatant (S)	0·61	0·07	0·18	0·21	0·18	0·35	0·30
Membranous pellet	2·27	0·19	0·24	0·18	0·16	0·40	0·35
Recovery (%)	93	66	95	103	113	97	94
Percent S of total ($3 \times 10^6 g$-min.)	20·6	18·0	40·7	55·2	51·0	44·8	39·4
Percent S of total ($4{\cdot}5 \times 10^5 g$-min.)	26·8		45·8		57·1		43·7

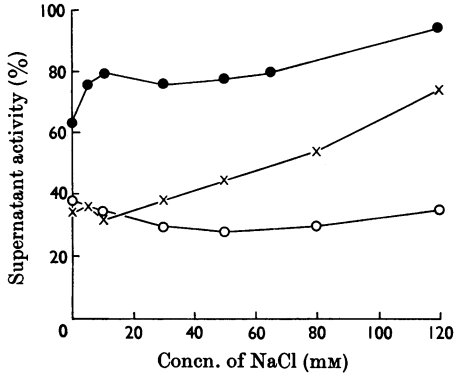

Fig. 1. Effect of increasing Na^+ concentration in 0·5 ml. samples of subfraction B (in 0·35 M-sucrose, 1 mM-ascorbate), frozen and thawed and centrifuged at 30000*g* for 15 min. Ordinate represents percentage of lysosomal enzyme activity after activation by freezing and thawing that remained in the supernatant. ○, Acid phosphohydrolase; ×, *N*-acetylglucosaminidase; ●, β-glucuronidase. Values represent means of three experiments.

were added singly to subfraction B in a range of concentrations (0–120 mM) before activation by freezing and thawing. The activity of each tube was assayed before and after centrifugation at 30000*g* for 15 min. The supernatant activity was expressed as a percentage of the activity in subfraction B, thus allowing for any direct effects of the individual cations on the total enzyme activity. No activation or inhibition of fraction B activity was noted with K^+ or Na^+ at concentrations of 120 mM. Fig. 1 indicates the change in supernatant activity as a function of Na^+ concentration. A similar profile was obtained in the presence of K^+. Comparable percentage supernatant activities for acid phosphohydrolase and *N*-acetylglucosaminidase were noted

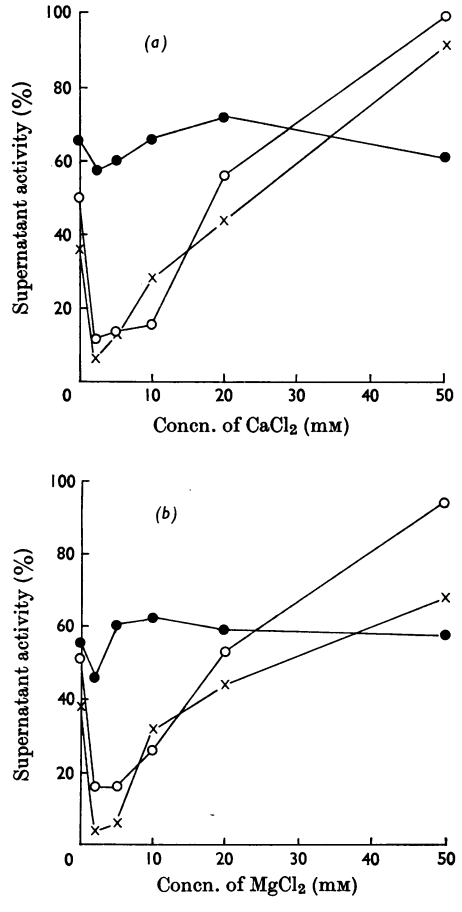

Fig. 2. Change in percentage supernatant enzyme activity in the presence of increasing bivalent cation concentration: (*a*) Ca^{2+}; (*b*) Mg^{2+}. Parameters are similar to those in Fig. 1. ○, Acid phosphohydrolase; ×, *N*-acetylglucosaminidase; ●, β-glucuronidase.

in the absence of cation. However, a significantly higher percentage supernatant activity for β-glucuronidase was always obtained. Elution of β-glucuronidase and *N*-acetylglucosaminidase into the supernatant phase occurred with increasing Na^+ concentration. No change in the distribution between sedimentable and unsedimentable activities of acid phosphohydrolase was noted.

With the bivalent cations Mg^{2+} and Ca^{2+} a more complex elution pattern was obtained (Fig. 2). No significant change in the distribution of β-glucuronidase was noted between sedimentable and supernatant phases with increasing cation concentration. A biphasic phenomenon was seen with acid phosphohydrolase and *N*-acetylglucosaminidase. At low concentrations of bivalent cation, 2–10mM, there was significant retention of hydrolase activity in the pellet with parallel loss from the supernatant. Elution from the sedimentable phase was obtained between 10mM- and 50mM-bivalent cation concentration. At 50mM, Ca^{2+} and Mg^{2+} had a slight activating effect on acid phosphohydrolase and β-glucuronidase activity (18% and 36% respectively). No activation or inhibition of *N*-acetylglucosaminidase was noted at comparable concentrations. There was no evidence that the cations differentially affected the activated fraction B or the supernatant activity, or both, thus substantiating the validity of expressing supernatant activity as a percentage of its corresponding activated fraction B at each concentration of cation.

Effect of cations on distribution of lysosomal membrane fragments in sucrose density gradient. The above biphasic effect in the distribution of hydrolase activity in the presence of bivalent cations suggested that membrane fragments associated with enzyme protein are aggregated with increasing binding or trapping of lysosomal enzymes. If membrane aggregation occurred and was responsible for the initial portion of the biphasic phenomenon, then a change in the sedimentation parameter of the aggregated particles should be seen. To investigate this possibility, samples of subfraction B were placed on a discontinuous sucrose gradient containing Na^+ or Mg^{2+} (see the Materials and Methods section) and centrifuged at 7000rev./min. for 15min. The distribution of protein throughout the gradient was determined (Fig. 3). Comparable mean protein recoveries of 90·8% ($n=3$) and 87·7% ($n=2$) were obtained in control and Na^+ experiments respectively. In two experiments, the mean protein recovery in the Mg^{2+} gradient was 74·4%, a low value reflecting the difficulty in resuspension of the tightly packed protein pellet found with this gradient. No protein was found at the bottom of the gradient from controls containing no cations. Significant amounts of protein were present in all

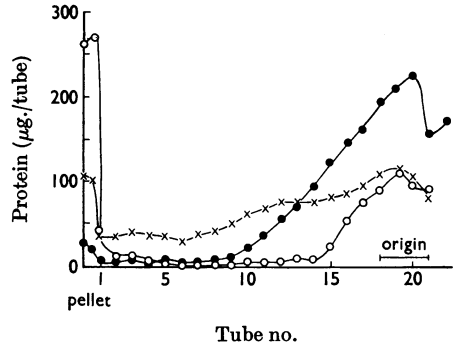

Fig. 3. Sedimentation of hepatic lysosomal membrane fragments obtained by repeated freezing and thawing of subfraction B (see the Materials and Methods section) in a discontinuous sucrose density gradient, 0·6–1·4M, containing cations, at 7000rev./min. for 15min. in a swinging rotor. ●, Control; ×, 10mM-NaCl; ○, 5mM-$MgCl_2$.

tubes, especially those from the upper third of the gradient containing univalent cations. In marked contrast (Fig. 3) was the distribution of protein in the Mg^{2+} gradient, in which little protein remained at the top of the gradient but considerable amounts were recoverable in the pellet. Such observations serve to demonstrate the increased sedimentability of membrane fragments in the presence of bivalent cations and provide a partial answer to the mechanism of the decreased lysosomal enzyme activities remaining in the supernatant after centrifugation in the presence of low concentrations of bivalent cations.

DISCUSSION

Hepatic naphthyl acid phosphohydrolase activity is dependent on the presence of fully reduced thiol groups (Verity & Brown, 1968*b*). The poor recovery of acid phosphohydrolase activity in these studies after 100000*g* centrifugation of the frozen and thawed subfraction B is due to thiol group oxidation, reversed or prevented by the addition of 1mM-ascorbate to the subfraction B before centrifugation. Moreover, the major increase appears in the supernatant fraction (Table 2), suggesting that the solubilized enzyme is more susceptible to thiol group oxidation than is enzyme adsorbed or linked to membrane fragments. Ascorbate added to fraction B slightly inhibited the activity of β-glucuronidase and *N*-acetylglucosaminidase, the former competitively and the latter non-competitively (Levvy & Marsh, 1959; Verity *et al.* 1967).

Though the phenomenon of enzyme latency and structure-linked activity of the lysosomal hydrolases is not in question, the nature of this phenomenon

has received little study (Gianetto & de Duve, 1955; Appelmans & de Duve, 1955; Romeo & de Bernard, 1966). Analogous activation and release of mitochondrial dehydrogenases has been studied by Chappell & Greville (1963), Egger & Rapoport (1961, 1963) and Lehninger (1965). Holowach, Howe, Laatsch & McDougal (1966) studied the effect of various anions and cations on the release of dehydrogenases from brain mitochondria. Univalent cations enhanced the release of NAD- and NADP-linked isocitrate dehydrogenases, but partially prevented the release of glutamate dehydrogenase. Both Ca^{2+} and Mg^{2+} inhibited the release of NAD-linked isocitrate dehydrogenase and glutamate dehydrogenase. Our studies revealed an enhanced release of lysosomal β-glucuronidase and *N*-acetylglucosaminidase into the supernatant with increasing univalent cation concentration. No such increased supernatant activity was evident with acid phosphohydrolase. Conversely, in the presence of Ca^{2+} or Mg^{2+}, acid phosphohydrolase and *N*-acetylglucosaminidase were released in parallel whereas β-glucuronidase appeared unaffected. Though the fundamental nature of the effect of univalent and bivalent cations on the release of enzymes from membrane fragments is little understood, the above observations suggest that membrane binding of lysosomal hydrolases depends on individual enzyme characteristics as well as general non-specific forces.

Egger & Rapoport (1963) have suggested that release and activation of mitochondrial enzymes follows the breaking of non-covalent bonds. They showed that the release of NAD-linked cytochrome *c* reductase depended on the breaking of two imidazole bonds, a conclusion based on pH-dependence and heat-of-inactivation studies. The known dependence of acid hydrolase activation at acidic pH suggests the presence of similar non-covalent bonds in lysosomes. Of note in this respect and our own observations are the studies of Romeo & de Bernard (1966), who demonstrated masking of lysozyme activity during the formation of a lipoprotein membrane *in vitro*. The 'bound' lysozyme was not removed by washing in 0·01 M-tris–acetate buffer, but 85% detachment was achieved by 0·9% potassium chloride, revealing a binding of basic enzyme protein through electrostatic forces.

Release of lysosomal hydrolases into the supernatant phase is a function of ionic strength and cation species. Paradoxically, increased sedimentability of enzyme is noted at low bivalent cation concentrations. This latter phenomenon may be due to: (*a*) bivalent cation-induced membrane contraction with increase in sedimentation velocity, (*b*) binding of substantial amounts of bivalent cation to membrane fragments, increasing the density of the fragment, (*c*) aggregation of membrane fragments, (*d*) aggregation of membrane fragments with increased trapping of enzyme, or (*e*) cation-induced enzyme adsorption on membrane fragments with no change in membrane aggregatability. Dallner & Nilsson (1966) demonstrated aggregation of some, but not all, of smooth microsomal membranes in the presence of Ca^{2+} and Mg^{2+}. No specific binding of substantial amounts of cations was found [see (*b*) above], and, though definitive experiments have not been performed with lysosomal membrane fragments, evidence for specific cation binding is unlikely. The increased sedimentability of some hydrolases at low bivalent cation concentration can be explained satisfactorily by an increased aggregation of vesicular membrane fragments with accompanying adsorption or trapping of solubilized enzyme [see (*d*) above]. That aggregation occurs is evident from the sucrose-density-gradient data (Fig. 3). That enzyme adsorption or trapping occurs is seen during centrifugation at low bivalent cation strength, to be followed by elution at high ionic strength. Lysosomes are known to be negatively charged, as are microsomal vesicles (Wallach & Kamat, 1964), and it is probable that the cations change the net charge on the lysosomal membrane fragments.

The failure of β-glucuronidase profile to parallel the acid phosphohydrolase and *N*-acetylglucosaminidase profiles appears as a serious criticism of the hypothesis. The dual localization of β-glucuronidase in lysosome and endoplasmic reticulum fractions (Van Lancker & Holtzer, 1963; Fishman, Goldman & DeLellis, 1967) may provide a clue to this discrepancy. The increased sedimentability of the hydrolase at low bivalent cation concentration is the result of membrane aggregation plus selective enzyme–membrane adsorption. This latter phenomenon appears as a characteristic for each enzyme, but apparently does not occur with β-glucuronidase.

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