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ON THE STRUCTURE-LINKED SEDIMENTABILITY OF RAT LIVER β-*N*-ACETYLGLUCOSAMINIDASE

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SUMMARY

The solubilization of β-*N*-acetylglucosaminidase (β-2-acetamido-2-deoxy-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30) has been investigated in subcellular preparations from rat liver and compared with that for other lysosomal hydrolases. The degree of retention of the enzyme activity by sedimentable material in lysosome-rich fractions largely varies depending on the disruptive procedure adopted. Solubilized enzyme added to "intact" homogenates is mostly adsorbed by sedimentable structures. After *in vivo* changes of lysosomes by *Amanita phalloides* poisoning, the activity recovered in the soluble fraction of the homogenate is almost negligible. In contrast, other lysosomal hydrolases display a pronounced shift from the particulate to the soluble phase. It is suggested that marked adsorption phenomena are likely to affect the distribution pattern of this enzyme activity in the situations examined.

Lysosomal hydrolases are assumed to be either contained in the matrix of lysosomes or to be associated with the limiting membrane of these granules, depending on the degree of their retention by particulate sedimentable material after disruption of lysosomes by such means as the use of surface-active agents, freezing and thawing and others. On the basis of its distribution pattern, β-*N*-acetylglucosaminidase (β-2-acetamido-2-deoxy-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30) has been regarded as a membrane enzyme, at least for liver lysosomes[1,2]. In the course of investigations on the structure-linked sedimentability of lysosomal enzymes and its modifications after *in vivo* and *in vitro* damage to these particles, we have obtained evidence which casts serious doubts on this inference.

In a first group of experiments, the solubilization of β-*N*-acetylglucosaminidase from lysosome-rich fractions induced by several *in vitro* procedures was studied in comparison with that for other lysosomal hydrolases. The light-mitochondrial fraction was prepared by differential centrifugation[3] from the liver of male Wistar rats weighing about 200 g. This fraction contained, on the average, 38% of the acid

TABLE I

DIFFERENTIAL SOLUBILIZATION OF HYDROLASE ACTIVITIES AND PROTEIN FROM LIGHT-MITOCHONDRIAL FRACTIONS SUBJECTED TO DIFFERENT TREATMENTS

These were :(*a*) 0.1% (w/v) Triton X-100; (*b*) suspension in distilled water; (*c*) three freeze–thaw cycles in 0.25 M sucrose; (*d*) the same with added 0.1 M KCl. Unsedimentable activities or protein are expressed as percentages computed with respect to the sum of the amounts found in the soluble and particulate phases. Recoveries were in the range of 96 to 103%. The values given are means of four experiments which gave very similar results. References refer to assay methods.

	a	*b*	*c*	*d*
Protein[4]	63.6	27.8	12.3	25.6
Acid phosphatase[3]	94.3	37.3	35 5	46.7
β-Galactosidase[5]	96.8	91.3	79.3	74.0
β-*N*-Acetylglucosaminidase[5]	23.6	35.6	57.0	68.2

phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2; a marker for lysosomes) of the homogenate at a relative specific activity of approx. 11 (*cf.* ref. 3). Aliquots of the light-mitochondrial preparations were subjected to different disruptive treatments and then centrifuged at $9 \cdot 10^6\, g \cdot \min$ (computed on the average radius between $r_{max} = 7.1$ and $r_{min} = 4.8$ cm) in order to separate the soluble phase from the particulate material. As shown in Table I, the pattern of solubilization of β-*N*-acetylglucosaminidase differs markedly from that observed for acid phosphatase and β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23); the latter was found to be the most easily solubilizable from particles when a group of hydrolases was examined[6]. Triton X-100, which is very effective in solubilizing β-galactosidase and acid phosphatase, leaves almost all β-*N*-acetylglucosaminidase bound to sedimentable material; this is in agreement with previous findings[1]. The retention of β-*N*-acetylglucosaminidase by sedimentable material occurs in spite of the extensive disruption of particles brought about by the surface-active agent, as evidenced by the large proportion of protein recovered in the soluble phase. After resuspending the light-mitochondrial fraction in distilled water, the greatest part of β-*N*-acetylglucosaminidase remains sedimentable, in a proportion similar to that for acid phosphatase (see also ref. 7); in contrast β-galactosidase again appears to be easily solubilizable. Finally, β-*N*-acetylglucosaminidase becomes mostly unsedimentable when light-mitochondrial fractions are frozen and thawed in 0.25 M sucrose, particularly in the presence of 0.1 M KCl. It is worth pointing out that the proportion of activity which remains structurally bound is higher for acid phosphatase than for β-*N*-acetylglucosaminidase. Even in this situation β-galactosidase is extensively extracted.

In a second set of experiments we studied the degree of adsorption of the above hydrolases onto structures present in liver homogenates. For this purpose, the soluble phase recovered from light-mitochondrial fractions frozen and thawed thrice in 0.25 M sucrose (thus comparatively rich in β-*N*-acetylglucosaminidase activity, as shown before) was added to fresh homogenates prepared in 0.25 M sucrose from livers perfused *in situ* with cold isotonic saline until bleached. The resulting mixtures were maintained at 0° for 30 min and then spun at $9 \cdot 10^6 \times g \cdot \min$. In parallel controls, either the soluble extract or the homogenate were omitted. As shown in Fig. 1, almost all of the acid phosphatase or β-galactosidase activities remain in

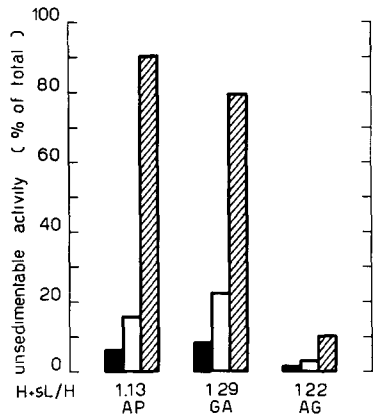

Fig. 1. Adsorption of solubilized hydrolases added to "intact" liver homogenates onto sedimentable structures. Unsedimentable hydrolase activities in homogenates before (black columns) and after (open columns) the addition of enzymes solubilized from light-mitochondrial fractions are expressed as percentages of the total activities present in each preparation. Total activities were assayed in the presence of 0.1% (w/v) Triton X-100, which makes enzymes completely available to substrates. The final concentration of homogenates was 10% (w/v). The ratio (H + sL)/H gives the relative proportions of total activities in homogenates (H) and soluble extracts (sL). The third (hatched) column refers to the proportions of solubilized hydrolases remaining in solution after addition to homogenates, this amount is expressed as percentages of the added activities recovered in the soluble and particulate phases of the homogenate. Mean recoveries were respectively 103, 103, and 95% for acid phosphatase, β-galactosidase, and β-*N*-acetylglucosaminidase, respectively. The values given are means of four experiments. Abbreviations: AP, acid phosphatase; GA, β-galactosidase; AG, β-*N*-acetylglucosaminidase.

solution; in contrast, a very large proportion of β-*N*-acetylglucosaminidase is retained by sedimentable structures.

In a third group of experiments we examined the compartmentation of the above hydrolases in liver homogenates from rats given a lethal dose of a crude aqueous extract of *Amanita phalloides* intraperitoneally. This causes prominent vacuolar changes of hepatocytes associated with a conspicuous enlargement of their lysosomal structures[8,9]. Livers were perfused *in situ* with cold isotonic saline and homogenized in 0.25 M sucrose. The enzyme assays were performed: (i) with aliquots of the "intact" homogenate; (ii) in the presence of 0.1% (w/v) Triton X-100; (iii) with the supernatant obtained after centrifuging the homogenate at $9 \cdot 10^6\, g \cdot \text{min}$. These measurements correspond to the so-called "free", "total", and "soluble" activities of lysosomal hydrolases[3]. Low ratios of free and soluble to total activities are usually taken as an indication of the integrity of lysosomes. As depicted in Fig. 2, after poisoning the free activity shows a progressive increase, which is roughly comparable for the three hydrolases. However, only acid phosphatase and β-galactosidase activities display a pronounced shift from the particulate into the soluble phase of the homogenate. The level of soluble activity, which is already extremely low in controls, still remains very low in homogenates from poisoned animals. Total activities are not significantly affected. The increase of free and soluble activities in liver homogenates is accompanied by a marked increase in the level of the three hydrolases in blood plasma.

The pattern of solubilization of the enzyme activities in the homogenates of

livers that exhibit changes of lysosomes *in vivo* is strictly comparable with that observed after adding solubilized hydrolases to "intact" homogenates: in both cases large proportions of acid phosphatase and β-galactosidase but almost negligible amounts of β-*N*-acetylglucosaminidase activity are found in the soluble phase. Therefore, it is very tempting to speculate that in the first situation β-*N*-acetylglucosaminidase could be secondarily adsorbed onto sedimentable structures after its extrusion from lysosomes. This possibility is not contradicted by the parallel increase of β-*N*-acetylglucosaminidase as well as of the two other hydrolases in blood plasma after *A. phalloides* poisoning; indeed, the extracellular release of lysosomal enzymes would appear to require[10] that the extruded enzymes are not firmly bound to the

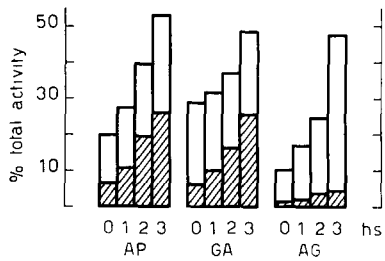

Fig. 2. Free (whole columns) and soluble (hatched columns) activities of three acid hydrolases in liver homogenates from control rats (0) and from animals at different times after poisoning with *A. phalloides* (1, 2, and 3 h). Values are given as percentages of total activities and are means of four experiments. Total activities were assayed in the presence of 0.1% (w/v) Triton X-100. Abbreviations: see Fig. 1.

lysosomal membrane. At any rate, the unsedimentable β-*N*-acetylglucosaminidase activity seems to be neither a suitable nor a sensitive index of lysosomal rupture in liver homogenates.

What kind of structures in the homogenate are responsible for the adsorption of β-*N*-acetylglucosaminidase is not clarified by the present investigation. On the one hand, β-*N*-acetylglucosaminidase displays a marked binding affinity for structures present in the light-mitochondrial fraction, as shown by its considerable retention by sedimentable material after different *in vitro* treatments. However, in these experiments the particles were extensively damaged, with exposure of internal sites.

Work is now in progress in our laboratory in order to find possible correlations between our present findings and the observation[11] that two forms of β-*N*-acetylglucosaminidase exist in rat liver lysosomes, distinguishable by their electrophoretic mobility. This seems important in view of the demonstration that the variety of β-*N*-acetylhexosaminidase activity that is absent in Tay–Sachs disease[12,13] corresponds to the enzyme assumed to be associated with the membrane of rat liver lysosomes[11]. However, the functional significance of membrane-bound hydrolases in lysosomes is unclear[14] in terms of the degradative action that lysosomal hydrolases admittedly perform on substrates segregated in these particles. On the other hand, impairment of this function is assumed[15] to be responsible for the accumulation of Tay–Sachs ganglioside in this genetically determined disease.

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