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Mammalian α-Acetylglucosaminidase. Enzymic Properties, Tissue Distribution, and Intracellular Localization*

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ABSTRACT: A preparation of α-acetylglucosaminidase, purified some 80-fold in low yield from extracts of pig liver, was almost devoid of α-acetylgalactosaminidase or any other known mammalian glycosidase. Both this partially purified enzyme and a crude extract manifested simple enzyme kinetics with the aryl glycosides used as test substrates. Inhibition effects were investigated.

The enzyme occurred at low levels of activity in all rat tissues examined. Experiments with rat liver homogenates characterized α-acetylglucosaminidase as a typical lysosomal acid hydrolase. Drastic disruption of lysosomes rendered this and three other lysosomal enzymes nonsedimentable only in part. An endogenous inhibitor of lysosomal hydrolases appeared to be present in the disrupted lysosomes.

α-Acetylglucosaminidase (α-2-acetamido-2-deoxy-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.-)[1] activity was first demonstrated in extracts of snail hepatopancreas (Zechmeister *et al.*, 1939). A similar enzymic activity was subsequently found to be present in mammalian tissues and was characterized more fully (Roseman and Dorfman, 1951). The levels of activity in the sources described were extremely low, as measured with phenyl α-*N*-acetylglucosaminide, the test substrate used (see also, Findlay *et al.*, 1958; Watkins, 1959). Concentration of the mammalian enzyme has now been undertaken, with the intention of examining its specificity and of discovering the nature of its endogenous substrates, if possible. Reported here is some information regarding the localization and properties of the enzyme, which was gathered incidental to work on its purification, still in progress. For convenience of presentation, there have also been included certain parallel observations relating to α-acetylgalactosaminidase (α-2-acetamido-2-deoxy-D-galactoside acetamidodeoxygalactohydrolase, EC 3.2.1.-), a distinct mammalian enzyme, devoid of α-acetylglucosaminidase activity (Weissmann and Friederici, 1966), which was detected in the course of these experiments.

Experimental Procedures

Materials. *p*-Nitrophenyl α- and β-D-glucopyranosides, *p*-nitrophenyl β-D-galactopyranoside, *p*-nitrophenyl α-D-mannopyranoside, *p*-nitrophenyl α-L-fucopyranoside, and *p*-nitrophenyl β-D-xylopyranoside were obtained from the Pierce Chemical Co. Phenolphthalein glucuronide was obtained from the Sigma Chemical Co. The phenyl 2-acetamido-2-deoxy-α- and β-mannopyranosides were prepared by an extension, to be published, of methods used previously (Weissmann, 1966). The other glycosidase substrates used have been described (Weissmann, 1966). *N*-Acetylglucosaminolactone was prepared as an amorphous solid (Findlay *et al.*, 1958). Folin–Ciocalteau reagent (2 N) was purchased from the Fisher Scientific Co. and the nonionic detergent "Triton X-100" from the Rohm and Haas Co. Triethylaminoethylcellulose (TEAE-cellulose, 0.44 mequiv/g) was purchased from

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[1] Those enzymes not identified by IUB systematic nomenclature or number in the text are so identified in Table IV.

TABLE I: Partial Purification of α-Acetylglucosaminidase from Pig Liver.

Fraction	Vol. (ml)	Protein (mg)	α-Acetylglucosaminidase Total (U/fraction)	α-Acetylglucosaminidase Sp Act. (mU/mg of protein)	β-Acetyl-gluco-saminidase (U)	α-Acetyl-galacto-saminidase (U)
Centrifuged extract	2,400	57,600	18.7	0.33	1,600	266
0.20–0.40 saturated $(NH_4)_2SO_4$	505	6,460	7.2	1.1	243	65
0.25–0.35 saturated $(NH_4)_2SO_4$	150	590	3.33	5.7	10.6	6.2
0–0.31 saturated $(NH_4)_2SO_4$	300	222	1.42	6.4	2.1	0.07
Column, tube 28	20	5.7	0.152	27	0.02	<0.003

Bio-Rad Laboratories as Cellex-T. Albino rats of the Sprague–Dawley strain, usually males weighing 150–250 g, were used.

General Procedures. Preparative work was done at 4°. All measurements of pH were made at room temperature. Ammonium sulfate was added in solid form. Dialysis was performed against 0.01 M sodium citrate buffer of pH 6.0; dialysis precipitates were discarded. In preparative procedures, centrifugation was done at 12,000 rpm (23,000 g) for 20 min at 4°, except as specified. Protein was determined colorimetrically (Lowry *et al.*, 1951), using crystalline bovine albumin as the standard.

In the examination of the enzyme content of tissues, organs were removed rapidly from rats killed by decapitation and were frozen at once on Dry Ice. Subsequently, the tissues were extensively homogenized with water in a Potter–Elvehjem tissue grinder, and the centrifuged extracts were assayed. (It had previously been established that centrifugation of homogenates of liver prepared in this way removed none of the α-acetylglucosaminidase activity.)

Glycosidase Assays. Throughout this report, one unit (U) of enzymic activity is the amount of enzyme required for reaction of 1 μmole of substrate/min under the conditions of assay. With the exceptions specified, all glycosidase assays were conducted in 0.05 M sodium citrate buffers at 37°, with addition of toluene when incubation intervals exceeded 2 hr and with addition, when required, of crystalline bovine albumin to maintain a minimum protein concentration of 0.1 mg/ml. Enzyme concentrations were selected to limit maximal hydrolysis of substrates to 10%. The hydrolysis of *o*- and *p*-nitrophenyl glycosides was measured spectrophotometrically at 410 mμ following addition of 0.6 M potassium borate buffer of pH 10.4 (*cf.* Woollen *et al.*, 1961a) to a final volume of 2 or 2.5 ml; the turbidity frequently encountered at high concentrations of tissue or protein was removed effectively from digests and blanks, after addition of borate, by vigorous stirring with 1 ml of 1-pentanol–chloroform (1:5) and centrifugation. The hydrolysis of phenyl glycosides was terminated by addition of one volume of Folin–Ciocalteau reagent and centrifugation. Sodium carbonate (0.4 M, 2 ml) was added to a 0.2-ml aliquot of the supernatant solution and the optical density at 650 mμ was measured after 30 min. The conditions used for the assays of various enzymes follow (substrate concentration, pH, digest volume, and time). For assay of α-acetylglucosaminidase the substrate used except where otherwise specified was *p*-nitrophenyl α-*N*-acetylglucosaminide (1 mM, pH 4.8, 2 ml, 2 hr); also used were *o*-nitrophenyl α-*N*-acetylglucosaminide, under the same conditions, and phenyl α-*N*-acetylglucosaminide (10 mM, pH 4.8, 0.25 ml, 18 hr). *p*-Nitrophenyl β-*N*-acetylglucosaminide (5 mM, pH 4.3, 0.2 ml, 30 min) was used for assay of β-acetylglucosaminidase. Enzymic hydrolysis of *p*-nitrophenyl β-*N*-acetylgalactosaminide was measured under the same conditions. Phenyl α-*N*-acetylgalactosaminide (10 mM, pH 4.3, 0.2 ml, 2 hr) was used for assay of α-acetylgalactosaminidase. Phenyl α- and β-*N*-acetyl-D-mannosaminides (10 mM, pH 4.8, 0.25 ml, 2 hr) were used to test for possible existence of acetylmannosaminidases.

Fractionation of Particles. Fractions of subcellular particles were separated from sucrose homogenates of rat liver precisely as described by de Duve *et al.* (1955). The isolated fractions, as suspensions in 0.25 M sucrose, were treated with 0.1% Triton X-100 and 1 mM sodium ethylenediaminetetraacetate (adjusted to pH 4.5) and were stored for 24–48 hr at 4° before assay of *total* enzymic activity. Assays for cytochrome *c* oxidase (EC 1.9.3.1) and glucose 6-phosphatase (EC 3.1.3.9) were performed as described (de Duve *et al.*, 1955). In assays for acid phosphatase (EC 3.1.3.2), fractions were incubated for 1 hr in 0.2 ml of 0.05 M sodium acetate buffer of pH 5, containing 0.05 M disodium β-glycerophosphate. Following addition of 1 ml of 4% trichloroacetic acid and centrifugation, a 1-ml aliquot was analyzed colorimetrically (650 mμ; final volume, 2 ml) for inorganic phosphate (Fiske and Subbarow, 1925). The glycosidase assays have already been described.

Crude Enzyme Preparation. Frozen fresh pig liver (100 g) was homogenized in a blender with 450 ml of cold water (total time, 3 min). The homogenate was treated with ammonium sulfate and the precipitate collected between 0.25 and 0.60 saturation was dialyzed without addition of fluid. The resulting preparation

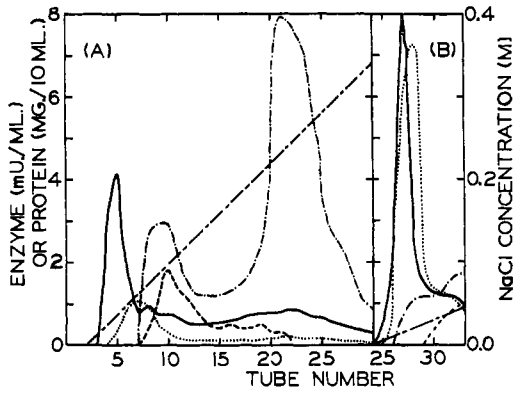

FIGURE 1: Chromatography of pig liver fractions on TEAE-cellulose columns. For application of the load and for development, 0.0056 M Tris buffer of pH 7.5 containing 0.01 M trisodium citrate was used. During development, sodium chloride, of concentration increasing as shown (— - — - —), was added to the developer. Tubes were assayed for protein (———), α-acetylglucosaminidase (· · · · · ·), α-acetylgalactosaminidase (------), and β-acetylglucosaminidase (— · — · —). (A) Trial chromatogram of a liver concentrate; 24 mg of protein, bed volume 12 ml (2 g dry wt), 6-ml fractions. (B) Chromatography of fraction A; 220 mg of protein, bed volume 150 ml (22 g dry wt), 20-ml fractions.

(105 ml after centrifugation) was designated "crude enzyme." It contained 35 mg of protein/ml and 8.5 mU of α-acetylglucosaminidase/ml.

Partially Purified α-Acetylglucosaminidase. Fresh pig liver (840 g) was homogenized in a blender in portions (30 sec) with 2520 ml of water. After centrifugation, the extract (Table I) was treated with ammonium sulfate. The precipitate collected at 0.20–0.40 saturation was dialyzed and fractionated with ammonium sulfate. The fraction collected at 0.25–0.35 saturation was selected for further purification and dialyzed. Because β-acetylglucosaminidase and α-acetylgalactosaminidase, both of which it was particularly desired to remove, precipitated at significantly higher degree of saturation than α-acetylglucosaminidase, a third ammonium sulfate fractionation was considered to be expedient. Fraction A, collected at 0–0.31 saturation, was dialyzed against 0.0056 M Tris hydrochloride buffer containing 0.01 M trisodium citrate, for use in chromatography.

A trial chromatogram on TEAE-cellulose of another ammonium sulfate fraction yielded a partial separation of α-acetylglucosaminidase from the other two enzymes assayed, as shown in Figure 1A. The same conditions were applied in abbreviated form to chromatography of fraction A, with the results shown in Figure 1B. The material in tube 28, the peak tube for α-acetylglucosaminidase, had the highest specific activity and was studied as "purified enzyme" in the present work. It was stable for some months at −18°.

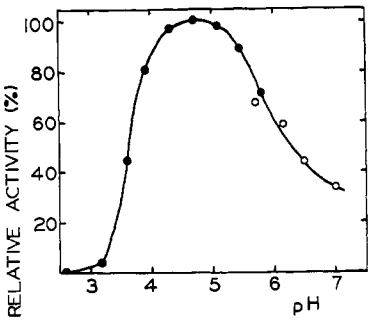

FIGURE 2: α-*N*-Acetylglucosaminidase activity of the crude pig liver enzyme with the *p*-nitrophenyl glycoside in 0.05 M sodium citrate (●) and sodium phosphate (○) buffers. Digests were incubated at 37° for 2 hr.

Results

Effects of pH Variation. As exemplified in Figure 2, optimal α-acetylglucosaminidase activity was exhibited in sodium citrate buffers at pH 4.7 by the crude enzyme with the *p*-nitrophenyl glycoside, at pH 4.7 by the purified enzyme with the *p*-nitrophenyl glycoside, and at pH 4.6 by the purified enzyme with the phenyl glycoside. Optimal activity at pH 4.5 was reported for this enzyme in extracts of rat testis with the phenyl glycoside (Roseman and Dorfman, 1951).

The crude enzyme was stable at 4° and pH 6 for several months. In tests of its sensitivity to acid, portions were acidified with citric acid, stored overnight at 4°, and centrifuged. The pH of the supernatant solutions was adjusted and they were assayed. Soluble α-acetylglucosaminidase activity declined sharply on storage below pH 5.0, as seen in Figure 3.

A limited study was made of this apparent insolubility of the enzyme at pH values in the region of its optimal activity. The amount of activity lost from solutions of the crude enzyme which had been brought to pH 4.9 and centrifuged was about 50%. Some 75% of the activity lost could be demonstrated by assaying suspensions of the precipitates. Precipitates collected at pH 3.8, where 96% of the activity disappeared from solution, were almost completely inactive. Digests containing the *p*-nitrophenyl glycoside were prepared in the cold at pH 4.8 and centrifuged for 10 min at 13,000 rpm before incubation. The rate of liberation of *p*-nitrophenol in the supernatant solutions at 37°, relative to that in uncentrifuged digests, was 82% for the crude enzyme and 90% for the purified enzyme. These results suggest aggregation of the enzyme near its optimal pH, reversed in part by substrate.

Substrate Specificity and Inhibition. With the *o*- and *p*-nitrophenyl α-*N*-acetylglucosaminides under standard conditions of assay, the extent of enzymic hydrolysis was linearly related to digestion time and enzyme concentration. With the phenyl glycoside as substrate, the rate of action decreased progressively after about 10 hr. Because of this circumstance and because of high enzyme blank values, meaningful kinetic

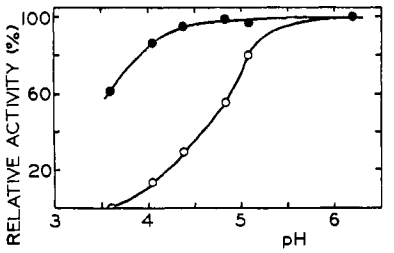

FIGURE 3: Proportion of α-acetylglucosaminidase activity (○) and α-acetylgalactosaminidase activity (●) remaining in solution after storage of crude pig liver enzyme at 4° for 18 hr at acid pH.

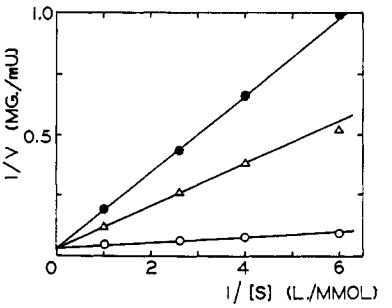

FIGURE 4: Liberation of nitrophenol from p-nitrophenyl α-*N*-acetylglucosaminide, catalyzed by the purified enzyme, as a function of substrate concentration (Lineweaver–Burk plot) in presence of: no inhibitor (○), 10 mM *N*-acetylglucosamine (●), and 1 mM *N*-acetylmannosamine (Δ).

TABLE II: Michaelis–Menten Constants of Pig Liver α-Acetylglucosaminidase.

Substrate	K_m (mM) Crude Enzyme	K_m (mM) Purified Enzyme
Phenyl glycoside		1.4[a]
p-Nitrophenyl glycoside	0.44	0.30
o-Nitrophenyl glycoside	0.10	0.08

[a] Incubation time, 2 hr.

TABLE III: Competitive Inhibitors of Pig Liver α-Acetylglucosaminidase.[a]

Compound	K_i (mM) Crude Enzyme	K_i (mM) Purified Enzyme
N-Acetylglucosamine	0.72	0.68
N-Acetylmannosamine	0.10	0.13
Methyl α-*N*-acetylglucosaminide[b]	8.2	
Phenyl α-*N*-acetylglucosaminide[c]	1.3	
N-Acetylglucosaminolactone	0.25	

[a] The substrate used was the *p*-nitrophenyl glycoside. [b] Trial of this compound as a substrate gave negative results. [c] This compound is itself a substrate but the products of its hydrolysis are not detected in the assay used.

measurements were impractical with the phenyl glycoside and crude extracts. Table II lists the values of Michaelis–Menten constants (K_m) which were measured. The values were calculated graphically from data exemplified in Figure 4.

A small activation (15% for the purified enzyme) was observed on addition of 0.1 M sodium chloride to standard digests. Competitive inhibition was observed with a number of substances, listed with their values of K_i in Table III. Compounds which were found to be noninhibitory (listed with the highest concentration tested) included: *N*-acetylgalactosamine (10 mM), phenyl α-*N*-acetylgalactosaminide (5 mM), acetamide (0.1 M), *N*-acetylglycine (50 mM), sucrose (0.2 M), and Triton X-100 (0.1%). Analogous to earlier findings for extracts of rat testis (Roseman and Dorfman, 1951) was a lower enzymic activity at pH 4.8 as measured in 0.05 M sodium acetate buffer than in sodium citrate buffer. The decrease in activity was 26% for one crude preparation but negligible for another, and was 17% for the purified enzyme. This irregularity and other aspects of acetate inhibition, omitted for brevity, are not understood. Tested in 0.05 M sodium acetate buffer at pH 4.8 for inhibition, 5 mM calcium chloride or 5 mM magnesium sulfate were without effect on the purified enzyme; an inhibition of 17% was caused by 1 mM EDTA, 82% by 1 mM cupric sulfate, and 76% by 1 mM ferric chloride.

Observations on the substrate specificity of α-acetylglucosaminidase are summarized in Table IV. Except for appreciable residual β-acetylglucosaminidase activity, the purified α-acetylglucosaminidase preparation (tube 28, Figure 1B) was nearly devoid of the glycosidase activities present in the extract from which it was prepared. An earlier fraction of the preparative chromatogram was almost free of β-acetylglucosaminidase (tube 26, less than 0.08 mU/mg; α-acetylglucosaminidase 1.6 mU/mg).

Tissue Distribution. Assays of homogenates of the pooled tissues of two rats with *p*-nitrophenyl α-*N*-acetylglucosaminide as the substrate gave the results which follow (milliunits of enzyme per gram of moist tissue): spleen, 8.3; liver, 6.4; testis, 6.1; kidney, 5.9; lung, 5.3; duodenum, 5.5; jejunum, 4.3; epididymus, 4.3; adrenals, 4.3; heart, 3.6; skin, 2.0; pancreas, 1.1;

TABLE IV: Specific Enzymic Activities of Crude Pig Liver Extract and of Purified Enzyme.[a]

Enzyme Assayed	Glycoside Substrate	Reference[b]	Specific Activities: Crude Extract (mU/mg)	ratio[c]	Specific Activities: Purified Enzyme (mU/mg)	ratio[c]
α-Acetylglucosaminidase (EC 3.2.1.-)	*p*-Nitrophenyl		0.33	(1.0)	27	(1.0)
α-Acetylglucosaminidase	*o*-Nitrophenyl		0.52	1.6	72	2.7
α-Acetylglucosaminidase	Phenyl	Roseman and Dorfman (1951)	0.50	1.5	39	1.4
α-Acetylgalactosaminidase (EC 3.2.1.-)	Phenyl	Weissmann and Friederici (1966)	4.8	15	<0.3	<0.01
β-Acetylglucosaminidase[d] (EC 3.2.1.30)	*p*-Nitrophenyl	Conchie *et al.* (1959)	36	109	3.7	0.14
β-Acetylgalactosaminidase[d]	*p*-Nitrophenyl	Woollen *et al.* (1961b)	4.5	14	1.0	0.04
α-Acetylmannosaminidase[e]	Phenyl		<0.8	<2.5	<0.8	<0.03
β-Acetylmannosaminidase[e]	Phenyl		<0.8	<2.5	<0.8	<0.03
α-Glucosidase (EC 3.2.1.20)	*p*-Nitrophenyl	Conchie *et al.* (1959)	0.48	1.5	<0.04	<0.001
β-Glucosidase (EC 3.2.1.21)	*p*-Nitrophenyl	Conchie *et al.* (1959)	6.3	19	0.5	0.02
α-Mannosidase (EC 3.2.1.24)	*p*-Nitrophenyl	Conchie *et al.* (1959)	2.1	6.4	1.3	0.05
β-Galactosidase (EC 3.2.1.23)	*p*-Nitrophenyl	Conchie *et al.* (1959)	0.3	0.9	<0.4	<0.02
β-Glucuronidase (EC 3.2.1.31)	Phenolphthalein	Conchie *et al.* (1959)	0.2	0.6	<1	<0.04
α-L-Fucosidase (EC 3.2.1.-)	*p*-Nitrophenyl	Levvy and McAllan (1961)	1.0	3.0	1.1	0.04
β-D-Xylosidase (EC 3.2.1.37)	*p*-Nitrophenyl	Fisher *et al.* (1966)	1.9	5.8	<0.4	<0.02

[a] See Table I for preparations tested. α-Acetylglucosaminidase, α-acetylgalactosaminidase, and β-acetylglucosaminidase were assayed in the crude extract soon after preparation; values for other enzymes of the crude extract may be too low, since these were measured after long storage at −18°. [b] References document occurrence of the enzyme in mammalian tissues; they also describe the method of assay used, except where this is described in the text. [c] Ratio of specific activity to specific α-acetylglucosaminidase activity, measured with the *p*-nitrophenyl glycoside. [d] These two enzymes have been shown to be identical (Woollen *et al.*, 1961a,b). [e] Hypothetical enzymes, not reported in any source.

brain, 0.5; and skeletal muscle, 0.4. (The results for skin were obtained with tissues from other rats.)

Subcellular Localization. The particulate distribution pattern of α-acetylglucosaminidase activity in rat liver was similar to that of acid phosphatase (de Duve *et al.*, 1955) and β-acetylglucosaminidase (Sellinger *et al.*, 1960), as shown in Table V. All three activities were most prominent in the particulate fraction characterized by de Duve *et al.* as rich in "light mitochondria" or lysosomes. The distribution pattern found for α-acetylgalactosaminidase was similar (Weissmann and Friederici, 1966). As in the earlier applications of this technique, glucose 6-phosphatase was used as a marker for the microsomal fraction and cytochrome *c* oxidase was used as a marker for the mitochondrial fraction. A persistently poor recovery of cytochrome oxidase activity in the separated particles was obtained, despite efforts at improvement. This circumstance, as well as a larger nuclear fraction (probably reflecting differences in initial homogenization), is the only significant difference in patterns from those reported earlier.

Release into the medium, following treatment with detergent or freezing, of activities from particles of the lysosomal fraction was studied. The results, shown in Table VI and discussed in a subsequent section, indicate a substantial but incomplete solubilization of α-acetylglucosaminidase, α-acetylgalactosaminidase, and acid phosphatase (but not of β-acetylglucosaminidase).

Endogenous Inhibitors in Tissues. In assays of the acid phosphatase, α-acetylglucosaminidase and α-acetylgalactosaminidase activities of rat liver homogenates, and of washed lysosomal fractions, inhibition phenomena were encountered regularly. Product formation increased linearly with time of incubation. However, the apparent specific enzymic activities observed declined with increased protein concentration. In preliminary experiments with α-acetylglucosaminidase, conducted at high concentrations of protein, the endogenous inhibition was partly reversed on increase

TABLE V: Particulate Distribution of Some Hydrolases and of Cytochrome *c* Oxidase in Homogenates of Rat Liver.[a]

	Activity (%)				
	Acid Phosphatase	α-Acetyl-glucosaminidase	β-Acetyl-glucosaminidase	Glucose 6-Phosphatase	Cytochrome *c* Oxidase
Nuclear	48 ± 13	50 ± 8	50 ± 11	34 ± 17	42 ± 3
Cytoplasmic extract	(100)	(100)	(100)	(100)	(100)
Heavy mitochondria	14 ± 2	23 ± 3	22 ± 3	9 ± 5	28 ± 10
Light mitochondria[b]	44 ± 7	44 ± 5	57 ± 5	14 ± 5	6 ± 2
Microsomes	28 ± 6	18 ± 2	23 ± 5	60 ± 7	6 ± 1
Soluble	6 ± 3	8 ± 3	2 ± 1	2 ± 2	0

[a] Rat liver was homogenized in 0.25 M sucrose, and particulate fractions were collected by centrifugation (de Duve *et al.*, 1955). Numerical values designating activity are expressed relative to the cytoplasmic extract (supernatant fluid after removal of nuclear fraction), taken as 100%, plus or minus average deviation (in four experiments). [b] Lysosomal fraction.

TABLE VI: Release of Lysosomal Hydrolases into a Soluble Fraction.[a]

	Activity (%)			
Treatment	Acid Phosphatase	α-Acetyl-glucosaminidase	α-Acetyl-galactosaminidase	β-Acetyl-glucosaminidase
Fresh,[b] solution	1	4	0	2
Fresh, precipitate	99	96	100	98
Control, solution	7	17	17	5
Control, precipitate	93	83	83	95
Frozen (three times), solution	39	33	49	2
Frozen (three times), precipitate	63	66	46	100
Frozen (nine times), solution	44	61	58	2
Frozen (nine times), precipitate	66	50	39	101
Triton, solution	92	87	84	7
Triton, precipitate	6	12	6	98

[a] A lysosomal fraction from rat liver (de Duve *et al.*, 1955) was suspended in 0.25 M sucrose. To an aliquot, 0.1% of Triton X-100 was added and the sample was stored for 18 hr. After storage of the untreated suspension for 18 hr, a sample (the control) was removed, and the remainder was repeatedly frozen and thawed; samples were removed after three and nine cycles. After treatment as indicated, all samples were centrifuged for 10 min at 13,000 rpm. The precipitates were resuspended in sucrose, and the solutions were stored for 24 hr in presence of 0.1% Triton X-100 and 1 mM EDTA, then assayed. All operations were performed at 4°. The activities of treated solutions and suspensions are expressed as per cent of the total activity of the control sample. [b] These are values for a similar lysosomal fraction which was examined immediately following its preparation.

of substrate concentration. Similar effects were observed in some limited experiments with acid phosphatase. Endogenous inhibitors of β-glucuronidase, apparently of uncompetitive type, were detected in rat liver by Walker and Levvy (1953). After ammonium sulfate precipitation and dialysis, the inhibition effects presently described could no longer be detected. Discrepancies in additivity of enzymic activities, resulting from inhibition effects with homogenates and particulate fractions, were decreased to a practical level (estimated at 10%) by selection of assay conditions which permitted use of minimal protein concentrations.

Discussion

The substrate specificity of mammalian α-acetylglucosaminidase was earlier investigated by Roseman and Dorfman (1951). They showed that this enzyme

in extracts of rat testis was considerably more stable to acid inactivation than, and therefore distinct from, some other carbohydrase activities also present. These included hyaluronidase (EC 3.2.1.35), β-acetylglucosaminidase, α-glucosidase, and β-glucosidase. The present partial purification of α-acetylglucosaminidase from pig liver eliminated the β-acetylglucosaminidase and α- and β-glucosidases present in extracts, in confirmation of the report cited (hyaluronidase was not assayed). Also eliminated were α-mannosidase, β-galactosidase, β-glucuronidase, α-L-fucosidase (α-L-fucoside fucohydrolase), and β-D-xylosidase, as well as α-acetylgalactosaminidase.[2] The apparently weak activity of the enzyme in tissues, as measured with aryl glycosides, is thus not explained by identity with any of the currently known mammalian glycosidases. Although *N*-acetylmannosamine was a potent inhibitor of α-acetylglucosaminidase, α- or β-phenyl glycosides of *N*-acetylmannosamine were not hydrolyzed by crude or purified extracts. Poor yields of activity have so far hampered investigation of the susceptibility to purified α-acetylglucosaminidase of natural products containing glucosamine. It is hoped that continuing efforts will make such an investigation possible. An obstacle is the unfavorably low activity level of α-acetylglucosaminidase relative to that of the other glycosidases from which it is to be separated.

The pH optima as well as those K_m and K_i values which were measured (Tables II and III) correspond for α-acetylglucosaminidase in the crude and the purified extracts. The purification factors (82 and 78, calculated from Table IV) measured with the *p*-nitrophenyl and phenyl glycosides are also in agreement. This would appear to dispose of the possibility, which arises from the low yield of activity in purification (about 1%), that the enzymes examined in the two preparations might be substantially different. The purification factor measured with the *o*-nitrophenyl glycoside (138) differs somewhat from that measured with the other two substrates. It is conjectured that this apparent minor change in specificity in course of purification (if not attributed to unsuspected artifacts of inhibition), may reflect a modification (*e.g.*, aggregation) of the native protein or perhaps a partial separation of isozymes. A perhaps related phenomenon is the resolution of pig liver β-acetylglucosaminidase into two discrete peaks by columns of TEAE-cellulose (Figure 1A). By the only criterion so far applied, identity of the values of K_m with *p*-nitrophenyl β-*N*-acetylglucosaminide, the enzymes in the minor and the major peaks appear to be similar in their catalytic properties.

Although the rates of enzymic hydrolysis of phenyl, *o*-nitrophenyl, and *p*-nitrophenyl α-*N*-acetylglucosaminides, are roughly comparable, *p*-nitrophenol liberated by hydrolysis can be estimated with some 20-fold greater sensitivity than phenol and about 2.5-fold greater sensitivity than *o*-nitrophenol. The shorter assay intervals possible with the *p*-nitrophenyl glycoside have permitted a more complete characterization of mammalian α-acetylglucosaminidase than possible with the phenyl glycoside used formerly (see Results). The lower enzyme blanks observed with tissue extracts and the new substrate have facilitated a study of the distribution of the enzyme in tissues and cells. All tissues of the rat which were examined in the present work contained α-acetylglucosaminidase in low and varying amounts. These results are consistent with the earlier findings of Roseman and Dorfman (1951). Reports of a lack of activity in most rat tissues by Findlay *et al.* (1958) are probably attributable to their use of an acid-extraction procedure. As mentioned, the soluble α-acetylglucosaminidase activity of pig liver extracts is greatly diminished below pH 5, and the enzyme is almost completely denatured below pH 4. This insolubility in acid medium may extend to the enzyme in other tissues. However, it stands in contrast to the reported stability in acid solutions of α-acetylglucosaminidase in extracts of rat testis (Roseman and Dorfman, 1951).

On the basis of their sedimentation behavior in sucrose homogenates of rat liver and their acidic pH optima, α-acetylglucosaminidase as well as α-acetylgalactosaminidase (Weissmann and Friederici, 1966) may be considered to occur in the class of subcellular particles classified as lysosomes (de Duve, 1963). These two enzymes apparently share this same behavior and particulate distribution with the other mammalian glycosidases which have so far been investigated in this regard (Walker, 1952; de Duve *et al.*, 1955; Sellinger *et al.*, 1960; Conchie and Hay, 1963; Weissmann and Friederici, 1966; Fisher *et al.*, 1966).

A property generally described for lysosomal hydrolases is an increase in the enzymic activity of particle suspensions following the application of disruptive conditions. This increase, designated the latent activity, is usually taken as the difference between the free activity, which is measured by brief assays in isotonic medium, and the total activity, which is measured by longer assays, following or concomitant with maximal disruption of particles by detergent, hypotonicity freezing, etc. (see, *e.g.*, Wattiaux and de Duve, 1956). Brief assays for measurement of free activity in suspensions were impractical, however, for the weak activities now studied. The latency phenomenon was accordingly demonstrated by the solubilization of activity by freezing and by detergent treatment, conditions previously found effective for release of total activity of other enzymes in suspensions of lysosomal particles. The significant fraction of the total activity which remained sedimentable following extreme disruptive treatment (Table VI) was unexpected, however. This discrepancy was particularly striking in the case of β-acetylglucosaminidase, which appeared to be especially tightly bound to sedimentable material. Related observations would appear to have been made by Conchie and Hay (1963), who mention that treatment of sucrose homogenates of tissues with Triton X-100 evoked full

[2] References to the occurrence in mammalian tissues of the glycosidases mentioned are listed in Table IV.

activity but not full solubility of glycosidases (see also, Tappel *et al.*, 1963).

These discrepancies between the activation and solubilization of lysosomal enzymes, as well as the endogenous inhibition phenomena noted in the present experiments, may have special significance. Perhaps they relate to a matrix or other organizational principle present in intact lysosomes, and present in suspensions of disrupted lysosomes as particulate material capable of binding enzymes.[3] Certainly, it would appear to be an oversimplification to regard lysosomes as mere bags of enzymes. The meaningfulness of the lysosomes as a distinct class of particles has been challenged by Conchie and Hay (1963). These workers emphasize the unequal rates of liberation of acid hydrolases following disruption of particles, differences of distribution of enzymes in particles from tissues other than liver, and the tenuous nature of the extrapolation to other enzymes of cytological correlations clearly demonstrable only with acid phosphatase. De Duve, on the other hand, has ascribed such discrepancies as exist to the heterogeneity of cell populations available for examination (de Duve, 1963). Should the speculation with regard to enzyme binding introduced here have any substance, the further experiments it suggests may perhaps contribute clarification to some of the controversial features of the lysosome concept.

[3] A reviewer suggests that unequal release of lysosomal enzymes might be referable to their possible native occurrence, in varying degree, as insoluble proenzymes requiring an enzymic step for activation. This proposal would be consistent with the observed solubilization of hydrolases during incubation of isotonic lysosomal suspensions (see, de Duve, 1963). Observations not mentioned in the text argue against such an interpretation. As shown, only minimal solubilization of β-acetylglucosaminidase occurs on cautious homogenization of rat liver in isotonic sucrose (Table V) or on subsequent storage of particles in the same medium at low temperature (Table VI). In contrast to this behavior, however, some 65–90% of the demonstrable β-acetylglucosaminidase activity is nonsedimentable in suspensions prepared by extensive homogenization in cold water. Under the more vigorous conditions, which were those used for examining the enzyme content of tissues, solubilization of α-acetylglucosaminidase and of α-acetylgalactosaminidase is apparently complete.

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