Mammalian α-Acetylgalactosaminidase. Occurrence, Partial Purification, and Action on Linkages in Submaxillary Mucins*

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ABSTRACT: α-Acetylgalactosaminidase, a lysosomal enzyme, is demonstrable in mammalian tissues, using phenyl-*N*-acetyl-α-galactosaminide as a substrate. The enzyme has been purified 300-fold from pig liver extracts and 700-fold from beef liver extracts, and is distinct from α- or β-acetylglucosaminidase. Pig liver contains at least eight isozymes of α-acetylgalactosaminidase and at least four of β-acetylglucosaminidase. For beef liver α-acetylgalactosaminidase, apparent specific activity decreases on dilution. This abnormality is most pronounced at higher temperatures and lower substrate concentrations.

The purified α-acetylgalactosaminidase preparations catalyze release of *N*-acetylgalactosamine from desialized sheep and beef submaxillary mucins, but not from the untreated glycoproteins. Study of this action shows that most of the acetylgalactosamine residues in both glycoproteins are nonterminal (as already known for the sheep mucin), and that these residues are bound to the peptide core by α-glycosidic linkages. Results of similar experiments with purified β-acetylglucosaminidase suggest the presence of appreciable β-linked, nonterminal acetylglucosamine in beef submaxillary mucin. The purified α-acetylgalactosaminidase preparations also act on blood-group A substance from pig stomach.

Occurrence of α-acetylgalactosaminidase (α-2-acetamido-2-deoxy-D-galactoside acetamidodeoxygalactohydrolase) has been reported previously in extracts of *Trichomonas foetus* (Harrap and Watkins, 1964) and *Helix pomatia* (Tuppy and Staudenbauer, 1966). The terminal *N*-acetylgalactosamine residues of blood-group A substance could be liberated by action of the protozoan and snail enzymes, with concomitant loss of blood-group activity. In related work, Gottschalk and his colleagues (see Schauer and Gottschalk, 1968) found that enzymes from animal sources liberated substantially all of the bound acetylgalactosamine from desialized preparations of sheep submaxillary mucin (OSM[1]). These enzymes were distinct from β-acetylglucosaminidase (EC 3.2.1.30), but their steric specificity was otherwise unclear. Liberation of acetylgalactosamine from desialized OSM by an enzyme from *Clostridium perfringens* has also been reported (McGuire and Roseman, 1967). The bacterial enzyme was, however, found to be inactive with simple glycosides of acetylhexosamines. The configuration of the *N*-acetylgalactosamine residues in OSM, or in some other glycoproteins which contain galactosamine (see, Gottschalk, 1966), has accordingly not until now been determined. The present report documents the occurrence of α-acetylgalactosaminidase in mammalian tissues and describes the action of purified enzyme preparations on linkages in submaxillary gland glycoproteins.

Experimental Procedures

General Procedures. Some materials and methods used were described earlier (Weissmann *et al.*, 1967). All measurements of pH were made at 25°. Solutions used in enzyme purification were prepared in glass-distilled water containing neutralized 0.1 mM EDTA. Dialysis membranes were soaked in 10 mM EDTA and water before use. Dialyses were performed against 0.005 M sodium citrate buffer of nominal pH 6 (sodium citrate–citric acid in 5:1 molar ratio); dialysis precipitates were discarded. Collodion bags (Carl Schleicher & Schuell Co.) were used for concentration of protein solutions by ultrafiltration. Columns containing Whatman type DE-52 DEAE-cellulose (Reeve Angel Co., 100 g dry weight) were preequilibrated at 25° with 0.005 M sodium citrate buffer of pH 6 and washed with the same buffer at 4° after application of the load. The Sephadex G-150 columns (Pharmacia Fine Chemicals, Inc.), 5-cm i.d., were operated by upward flow at 4° using 0.05 M sodium citrate buffer of pH 6. In electrofocusing experiments (Vesterberg and Svensson, 1966), a column of 110-ml capacity and an ampholyte mixture of nominal pH range 5–8, both supplied by LKB Instruments, Inc., were used. The ampholytes (1%) were supplemented with 0.17% of *N*-tris(hydroxymethyl)methylglycine (tricine).

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[1] Abbreviations used: OSM and DOSM are undegraded and desialized preparations, respectively, of purified glycoprotein from submaxillary glands of sheep; BSM and DBSM are undegraded and desialized preparations of purified glycoprotein from submaxillary glands of cattle.

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

Fraction	Vol (ml)	Protein (mg)	α-Acetylgalactosaminidase Total (U/fraction)	α-Acetylgalactosaminidase Sp Act. (mU/mg of protein)	β-Acetylglucosaminidase (U)	α-Acetylglucosaminidase[a] (U)
Centrifuged homogenate	10,000	221,000	820	3.7	6,900	69
Acid-treated extract	8,900	88,000	640	7.3	5,200	9.2
0.30–0.55 saturated $(NH_4)_2SO_4$	1,000	24,100	460	19.3	4,400	4.7
0.38–0.53 saturated $(NH_4)_2SO_4$	208	11,400	370	27.0	3,300	1.8
DEAE-cellulose	34	2,360	250	106	520	
Sephadex G-150, I	17	260	189	730	139	
Sephadex G-150, II	6	108	123	1,150	58	<0.05

[a] α-2-Acetamido-2-deoxy-D-glucoside:acetamidodeoxyglycohydrolase (EC 3.2.1.-).

TABLE II: Partial Purification of α-Acetylgalactosaminidase from Beef Liver.

Fraction	Vol (ml)	Protein (mg)	α-Acetylgalactosaminidase[a] Total (U/fraction)	α-Acetylgalactosaminidase[a] Sp Act. (mU/mg of protein)	β-Acetylglucosaminidase (U)	α-Acetylglucosaminidase (U)
Centrifuged homogenate	2,500	69,000	860	12.5	4,900	5.4
0.50–0.62 saturated $(NH_4)_2SO_4$	240	10,600	370	34.6	420	<0.03
DEAE-cellulose	1,000	142	248	1,750	<6	
0–0.75 saturated $(NH_4)_2SO_4$	10	78	252	3,240	1.6	
Sephadex G-150	5	17.5	113	6,400	0.3	
0.50–0.65 saturated $(NH_4)_2SO_4$	2	9.0	83	9,100[a]	<0.05[b]	<0.01

[a] The assays of α-acetylgalactosaminidase in this table are only approximate, since the assays were performed at pH 4.3, the optimum for the pig liver enzyme, and at time of measurement the effect of enzyme concentration on apparent specific activity for the beef liver enzyme (see Text) had not yet been recognized. Reassay of the final preparation at pH 4.7, the optimum, at a concentration of 1 μg/ml gave a value for the specific activity of 11,900 mU/mg. [b] Using *p*-nitrophenyl *N*-acetyl-β-D-galactosaminide as a substrate, a value of <0.05 U was also measured.

Ammonium sulfate was an enzyme grade from Mann Research Laboratories. *N*-Acetylneuraminic acid was purchased from Pierce Chemical Co., Rockford, Ill. The other monosaccharides were C. P. materials from Pfanstiehl Laboratories, Waukegan, Ill. Crystalline bovine serum albumin (Sigma Chemical Co.) was used as a peptide color standard and as an enzyme diluent. Frozen submaxillary glands and anti-A blood-grouping serum were obtained from Pentex Co., Kankakee, Ill.

Enzyme Assays. The unit (U) of enzymic activity used corresponds to hydrolysis of 1 μmol/min of the appropriate aryl glycoside, under standard assay conditions or other conditions specified. Digests were regularly supplemented with 0.1–0.5 mg/ml of albumin and incubated at 38°. In standard α-acetylgalactosaminidase assays, 10 mM phenyl *N*-acetyl-α-galactosaminide in 0.05 M sodium citrate buffer was incubated at pH 4.7 with beef enzymes and at pH 4.3 with pig or rat enzymes. Enzymic hydrolysis of 10 mM *p*-nitrophenyl *N*-acetyl-β-galactosaminide (Heyworth *et al.*, 1959) was measured at pH 4.3. Other details are given elsewhere (Weissmann *et al.*, 1967).

Purified Pig Liver Enzyme (Table I). Centrifuged homogenate (3 ml of water/g of fresh liver, blender) was brought to pH 3.7 with 1 M citric acid, stored overnight at 4°, brought to pH 4.8 with 1 M sodium citrate, and centrifuged. The extract was fractionated twice with ammonium sulfate. The resulting fraction was exhaustively dialyzed and applied to a column of DEAE-cellulose. The column was washed overnight with 20 l. of starting buffer, and the activity was eluted with 1000 ml of buffer containing 0.6 M sodium chloride. Immediately following its collection,[2] this eluate was adjusted to 0.1 M with sodium citrate buffer of pH 6 and the activity was precipitated with ammonium sulfate (0.70 saturation). One-half of the dialyzed

[2] α-Acetylgalactosaminidase from pig liver or beef liver is inactivated rapidly at low protein concentration; losses of 60–80%/day at 4° and 0.1 mg/ml of protein concentration are not uncommon.

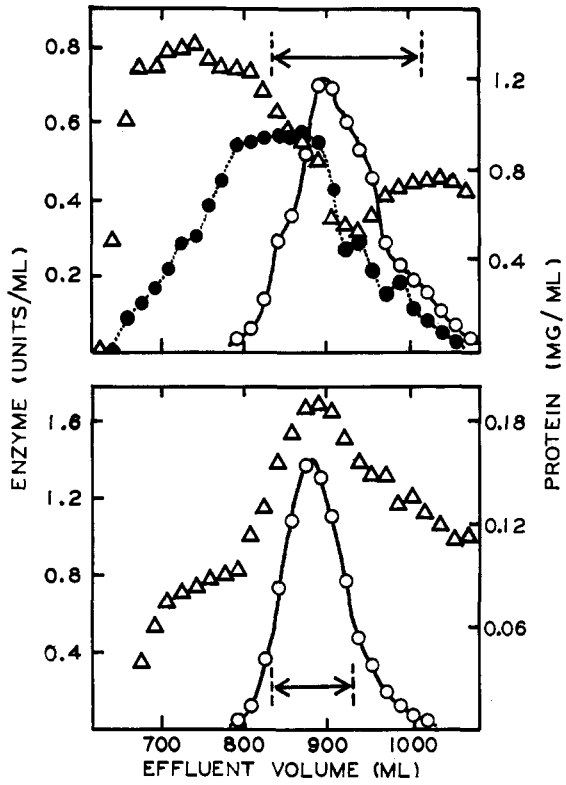

FIGURE 1: Chromatography of 1.2 g of an enzyme fraction from pig liver (upper chart) and of 75 mg of an enzyme fraction from beef liver (lower chart) on a column of Sephadex G-150 of bed volume 1740 ml and void volume 675 ml. The effluent was assayed for α-acetylgalactosaminidase (○), β-acetylglucosaminidase (●), and protein (△). Fractions pooled for further purification are indicated by the double arrows.

TABLE III: Assay of Enzymes Preparations.[a]

Enzyme Prepn	α-Acetyl-galactos-aminidase (mU/mg)	β-Acetyl-glucos-aminidase (mU/mg)	α-Acetyl-glucos-aminidase (mU/mg)
Purified pig	1,150	580	<0.05
Purified beef	11,900[b]	<0.05	<0.01
Crude beef	35[b]	42	<0.03
Crude calf	3	154	1.3
β-Acetyl-glucosaminidase	560	72,000	<0.15

[a] The purified pig and the crude and purified beef enzymes are the liver α-acetylgalactosaminidase preparations of the present report. β-Acetylglucosaminidase is a preparation purified from beef liver (Weissmann *et al.*, 1964). The crude calf enzyme is a dialyzed ammonium sulfate fraction (0.20–0.33 saturation) of calf liver extract. [b] As shown, the apparent specific activity of this enzyme from beef liver is dependent upon the enzyme concentration used in assays. The values designated were measured at a concentration equivalent to 1 μg/ml of the purified beef enzyme.

preparation was chromatographed on a column of Sephadex G-150, with the result shown in Figure 1. Selected fractions from two such columns were precipitated with ammonium sulfate (0.90 saturation), pooled, dialyzed, and rechromatographed on a similar column. The most active fractions, precipitated with ammonium sulfate, pooled, and dialyzed, were designated "purified enzyme." This has been stored at −18° for 15 months without significant loss of activity. Extensive attempts to refine the DEAE-cellulose chromatography or to improve the specific activity of the final preparation were unsuccessful.

Purified Beef Liver Enzyme (Table II). Centrifuged homogenate (3 ml of water at 25°/g of frozen fresh liver) was fractionated with ammonium sulfate. The exhaustively dialyzed preparation ("crude enzyme") was applied to a column of DEAE-cellulose. After washing the column with 1000 ml of starting buffer and with 1500 ml of 0.025 M sodium citrate buffer of pH 6, the activity was eluted with 1000 ml of 0.05 M sodium citrate buffer of pH 6 and immediately precipitated with ammonium sulfate (0.75 saturation). After 2 days, the precipitate was extracted with 10 ml of 0.05 M sodium citrate buffer of pH 6. The extract was chromatographed on a Sephadex G-150 column with the result shown in Figure 1. The effluent was assayed without delay, and the most active fractions were at once pooled and concentrated by ultrafiltration (45% inactivation in this step). A final ammonium sulfate precipitation and dialysis gave the purified preparation described, preserved for some months at −18° without significant loss of activity.

Experiments with Polymeric Substrates. The enzymes used and their assays are listed in Table III. Blood-group A substance from pig stomach, stated to contain about 7% galactosamine and 14% glucosamine, was a gift from Dr. David Aminoff. OSM-T (major) and BSM-T (major) were isolated as described by Tettamanti and Pigman (1968). Desialized preparations, DOSM and DBSM, were obtained from the purified mucins by mild acid treatment, dialysis, and lyophilization (Bhargava *et al.*, 1966). As seen in Table IV, negligible peptide or amino sugar was lost in this treatment, and some bound sialic acid remained.

Conditions found satisfactory for DOSM and beef liver α-acetylgalactosaminidase were applied to all digests of polymeric substrates: 38° (toluene), pH 4.5, 0.05 M sodium citrate buffer containing 0.15 M sodium chloride and 1 mg/ml of albumin. Enzymic action was usually terminated by addition of the borate used in colorimetry of the acetylhexosamine liberated (Reissig *et al.*, 1955). For the heating time of 2.5 min used, 2.5 μmol of acetylgalactosamine and 1.0 μmol of acetylglucosamine gave equivalent color. In digests of blood-group substance or digests with weak enzymic activity, the relatively large blank corrections necessitated a modified procedure. Here, digests and blanks were vigorously stirred with chloroform–amyl alcohol (5:1, v/v) and centrifuged. Monosaccharide fractions were isolated by chromatography in 0.05 M sodium

TABLE IV: Analysis of Polymeric Substrate Preparations.[a]

Prepn	Acetyl-hexosamine (%)	Sialic Acid %	Sialic Acid Molar Ratio[b]	Peptide (%)
OSM	22.9	26.8	0.84	31.1
DOSM	21.2	2.4	0.08	30.7
BSM	28.4	28.9	0.73	26.2
DBSM	27.2	6.4	0.17	26.4

[a] Analyses for OSM and BSM are corrected for loss of water on drying. Analyses for DOSM and DBSM are based on dry weight of the OSM and BSM from which they were prepared, taken as 100%. Hexosamine was estimated according to Boas (1953), after hydrolysis for 2 hr *in vacuo* at 100° with 6 M hydrochloric acid. Galactosamine hydrochloride was used as the standard, and values are reported as acetylhexosamine. Sialic acid was estimated by the Svennerholm resorcinol method (Miettinen and Takki-Luukainen, 1959), using *N*-acetylneuraminic acid as the standard. Peptide was estimated according to Lowry *et al.* (1951), using albumin as the standard. [b] These are molar ratios of sialic acid to hexosamine. Tettamanti and Pigman (1968) report values of 0.95 and 0.87 for their corresponding preparations of OSM and BSM. The apparent discrepancy may perhaps reflect partial enzymic or chemical desialization of the native mucins during storage of tissues or in isolation.

TABLE V: Effect of Various Parameters on the Velocity–Enzyme Concentration Relationship for Beef Liver α-Acetylgalactosaminidase.[a]

Temp (°C)	Substrate Concn (mM)	Exponent (*n*)	App K_m[d] (mM)
25	5	1.05	2.7
	10	1.00	
32	5	1.25	9.0
38	5	1.50	25.0
	10	1.34	
	10	1.39[b]	
	10	1.32[c]	
	30	1.16	

[a] The substrate used was phenyl α-acetylgalactosaminide. The exponent, *n*, was experimentally determined as the slope of log–log plots of initial velocity against enzyme concentration, and it defines the observed exponential relationship between these two variables. For other details, see text. [b] This value was obtained with crude enzyme; all others listed are for the purified enzyme. [c] This value was measured at pH 4.3; all other values were measured at pH 4.7, the optimum. [d] The values of K_m listed, and a value at 42° of about 90 mM, were measured at an enzyme concentration of 1 μg/ml. The corresponding values of maximal velocity were: at 25°, 8.7 U/mg; at 32°, 23 U/mg; at 38°, 29 U/mg; at 42°, about 87 U/mg.

chloride on small calibrated columns of Sephadex G-25, desalted with Dowex 50 (H^+) and Dowex 1 (phosphate) (Weissmann *et al.*, 1964), and concentrated on a rotary evaporator. Aliquots of the redissolved residues were analyzed. Recovery of added acetylgalactosamine was 97%.

Concentrates similarly prepared were chromatographed on borate-impregnated paper, which was developed with butanol–pyridine–water (Cardini and Leloir, 1957), stained with a silver reagent (Trevelyan *et al.*, 1950), and cleared with photographic fixer (Benson *et al.*, 1952). Standards for acetylglucosamine, acetylgalactosamine, fucose, and galactose were resolved.

Results

Enzymic Properties of Pig Liver α-Acetylgalactosaminidase. Activity with the phenyl glycoside for either crude or purified enzyme was optimal at pH 4.3 (Figure 2). The stability of the enzyme in acid solutions has been described (Weissmann *et al.*, 1967). Liberation of phenol at pH 4.3 progressed linearly for at least 3 hr. Initial velocities were strictly proportional to enzyme concentration and showed normal dependence upon substrate concentration (K_m = 6.4 mM, crude enzyme; K_m = 6.6 mM, purified enzyme). *N*-Acetylgalactosamine was a competitive inhibitor (K_i = 10.1 mM). Reaction velocities were unaffected by 0.15 M sodium chloride, 1 mM EDTA, or by the following substances (10 mM): *N*-acetylglucosamine, *N*-acetylmannosamine, *N*-acetylglucosaminolactone, acetamide, sodium acetate buffer of pH 4.3, or methyl *N*-acetyl-α-D-glucosaminide. Cupric sulfate (1 mM) or cysteine hydrochloride (1 mM) produced about 10% inhibition and ferric chloride (1 mM) produced 60% inhibition.

The rat liver enzyme displayed linearity of velocity as a function of incubation time or enzyme concentration under the conditions of assay used with the pig liver enzyme. Its enzymic properties were not otherwise investigated.

Enzymic Properties of Beef Liver α-Acetylgalactosaminidase. Optimal activity with the phenyl glycoside for the purified beef liver enzyme occurred at pH 4.7 (Figure 2). Liberation of phenol progressed linearly with time (Figure 3). However, velocity was not proportional to enzyme concentration, as illustrated in Figure 4. Despite this abnormality, at fixed enzyme concentration, Michaelis–Menten kinetics were obeyed, as shown in Figure 5. Values of K_m measured at various temperatures appear in Table V. Reaction velocity at 38° and 5 mM substrate concentration was unaffected by addition of 0.15 M sodium chloride, 2 mM calcium chloride, 0.5 mM EDTA, or boiled homogenate corresponding to 63 mg of beef liver/ml of digest.

As illustrated by the log–log plot of Figure 6, the empirical relationship regularly observed between

TABLE VI: Particulate Distribution of α-Acetylgalactosaminidase.[a]

Fraction	Activity (%)	
	α-Acetyl-galactos-aminidase	Acid Phos-phatase
Nuclear	47.5 ± 10.9	53.5 ± 10.4
Cytoplasmic extract	(100)	(100)
Mitochondrial	21.0 ± 3.1	19.0 ± 2.9
Lysosomal	49.3 ± 4.1	40.4 ± 3.5
Microsomal	15.5 ± 4.7	29.5 ± 9.4
Soluble	4.1 ± 1.3	5.9 ± 2.3

[a] Sucrose homogenates of rat liver were fractionated by differential centrifugation as described by de Duve *et al.* (1955). The numerical values designating activity are expressed relative to the cytoplasmic extract (supernatant fluid after removal of nuclei), taken as 100%, and indicate the mean and average deviation in seven experiments.

TABLE VII: Abundance of α-Acetylgalactosaminidase and of α- and β-Acetylglucosaminidase in Rat Tissues.[a]

Tissue	Enzyme		
	α-Acetyl-galactos-aminidase	α-Acetyl-glucosam-inidase	β-Acetyl-glucosam-inidase
Spleen	800	8.2	2600
Kidney	790	5.8	5900
Liver	740	6.3	3100
Intestine (jejunum)	480	4.3	2800
Lung	430	5.3	1400
Epididymus	190	4.3	2500
Pancreas	140	1.0	220
Skin	110	2.1	75
Brain	100	0.4	1600
Testis	84	6.1	790
Heart	53	3.6	100
Skeletal muscle	12	0.4	20

[a] Fresh tissues of two male rats (Sprague–Dawley strain, about 250 g) were frozen on Dry Ice, pooled, and subsequently homogenized with water in a Potter-Elvehjem tissue grinder. Assays with phenyl α-acetylgalactosaminide and with *p*-nitrophenyl α- or β-acetylglucosaminide are expressed as milliunits of activity per gram of moist tissue.

initial velocity and enzyme concentration was approximated by: initial velocity = [enzyme concentration]^n. No theoretical significance is attributed to the value of n, which may, however, be used as a measure of nonlinearity. Representative values of n measured under various conditions are shown in Table V. Similarity of the values of n for the crude and purified enzymes will be noted, as well as a marked dependence of n upon both substrate concentration and temperature.

In the typical progress curves of Figure 3, which are fully corrected for substrate, enzyme, and reagent blanks (all small), it is seen that the lines do not quite intersect the origin. A similar small and unexplained initial burst of reaction, complete in less than 25 sec, is also evident in progress curves recorded continuously at 278 mμ with a spectrophotometer.

Isozymes of α-Acetylgalactosaminidase and β-Acetylglucosaminidase from Pig Liver. The behavior in electrofocussing experiments (Vesterberg and Svensson, 1966) of the purified pig liver α-acetylgalactosaminidase preparation was studied. Eight peaks of α-acetylgalactosaminidase activity and four peaks of the β-acetylglucosaminidase activity also present were partially or completely resolved. Representative results are shown in Figure 7. It is seen that the isozymes of α-acetylgalactosaminidase differ in isoelectric point by as much as 2 pH units. The values of K_m with phenyl α-acetylgalactosaminide, measured for the enzyme species in peaks I, III, and VI, were 6.4, 7.1,

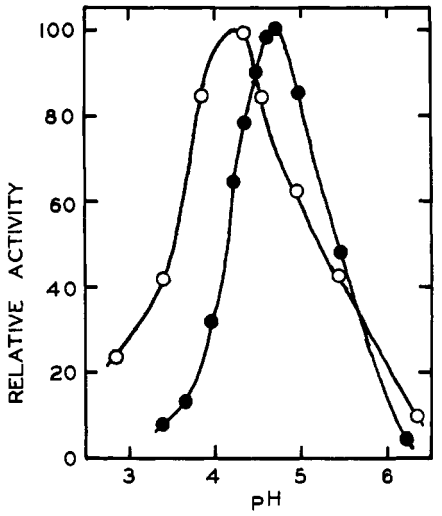

FIGURE 2. Relative activities at various values of pH of purified α-acetylgalactosaminidase preparations from pig liver (○) and beef liver (●). The enzymes were incubated with 10 mM phenyl α-acetylgalactosaminide at 38° for 1 hr in 0.05 M sodium citrate buffers containing 0.5 mg/ml of albumin.

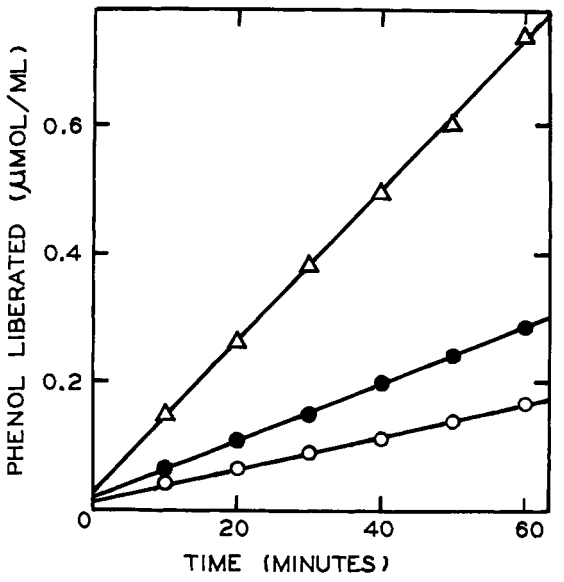

FIGURE 3. Progress of hydrolysis of phenyl α-acetylgalactosaminide (10 mM, pH 4.7, 38°) catalyzed by the purified beef liver enzyme (○, 0.33 μg/ml; ●, 0.50 μg/ml; Δ, 1.00 μg/ml).

and 6.8 mM; the pH values for optimal activity were 4.3, 4.4, and 4.3. These values correspond well with those for the unfractionated preparation.

Distribution of α-Acetylgalactosaminidase in Cells and Tissues. The sedimentation of activity in sucrose homogenates of rat liver parallels that of acid phosphatase (Table VI), and it is concluded that α-acetylgalactosaminidase occurs in lysosomes (de Duve, 1963). Confirming evidence comes from the acidic pH

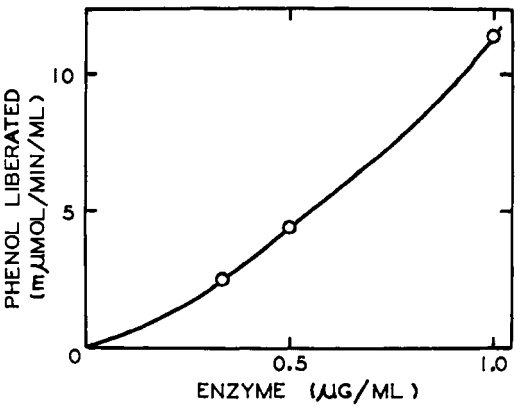

FIGURE 4: Hydrolysis of phenyl α-acetylgalactosaminide catalyzed by various concentrations of purified beef liver enzyme. The points represent the slopes of the lines shown in Figure 3.

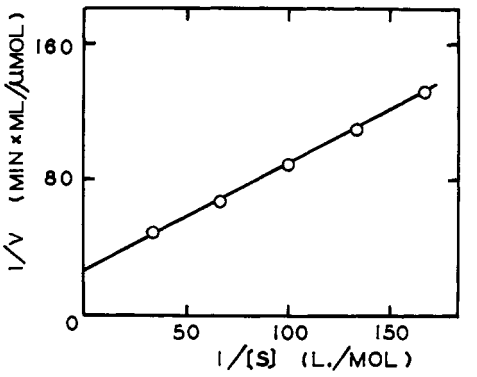

FIGURE 5: Hydrolysis of phenyl α-acetylgalactosaminide catalyzed by the purified beef liver enzyme, as a function of substrate concentration (1 μg/ml of enzyme, pH 4.7, 38°; Lineweaver–Burk plot).

optimum and from latency phenomena already reported (Weissmann *et al.*, 1967).

As illustrated in Table VII, the pattern of distribution of this enzyme in mammalian tissues is roughly similar to that of α- or β-acetylglucosaminidase. To illustrate individual variations in enzyme content, the range and median values for seven specimens of rat liver were 0.40–1.40 and 1.00 U per g of moist tissue; for five specimens of pig liver 0.41–0.56 and 0.55; for five specimens of beef liver 0.23–1.28 and 0.43. The enzyme was present in beef spleen (0.12 U/g, single specimen).

Optimal Conditions for Action of Purified Beef Liver α-Acetylgalactosaminidase on DOSM. In 0.05 M sodium citrate buffers containing 0.5 mg/ml of albumin, release of acetylgalactosamine was optimal at pH 4.5, when DOSM (2.3 μmol of hexosamine/ml) was incubated with enzyme (25 μg/ml) for 2 hr. Reaction velocities at pH 4.5 with 0.05 M sodium acetate buffer were about 50% less; with 0.05 M acetate or citrate buffers containing 0.15 M sodium chloride, some 10% greater than with citrate buffer alone. Liberation of acetylgalactosamine progressed approximately linearly

TABLE VIII: Rates of Action on Desialized Mucins of Various α-Acetylgalactosaminidase Preparations.[a]

Substrate	Enzyme in Digest: Preparation	Enzyme in Digest: Assay (mU/ml)	GalNAc Liberated (μmol/ml)	Relative Rate[b]
DOSM	Purified beef	292	0.314	8.9
DSBM	Purified beef	292	0.138	3.9
DOSM	Crude beef	386	0.376	8.1
DBSM	Crude beef	386	0.200	4.3
DOSM	Purified pig	413	0.910	18.2
DBSM	Purified pig	413	0.540	11.0

[a] The enzyme preparations, whose activity levels based on assays with phenyl α-acetylgalactosaminide are given, were incubated for 2 hr with DOSM (2.30 μmol of hexosamine/ml) and DBSM (2.74 μmol of hexosamine/ml). [b] Mol of acetylgalactosamine liberated from DOSM and DBSM per 1000 mol of phenol liberated from the phenyl glycoside by the same amount of each enzyme preparation in 2 hr under standard assay conditions.

TABLE IX: Action of α-Acetylgalactosaminidase on Blood-Group A Substance.[a]

Enzyme in Digest: Preparation	Enzyme in Digest: Concn (μg/ml)	Enzyme in Digest: Assay (mU/ml)	Incubation Time (hr)	GalNAc Liberated (μmol/ml)
Purified beef	25	292	2	0.003
Purified pig	350	413[b]	2	0.051
Purified beef	100	1170	18	0.038
Purified pig	350	413[b]	18	0.115[d]
β-Acetylglucosaminidase	8	5[c]	18	0.008

[a] Blood-group A substance from hog stomach (5.6 μmol of hexosamine/ml) was incubated with enzyme as indicated. The first two experiments are directly comparable with the experiments of Table VIII. [b] β-Acetylglucosaminidase assay, 206 mU/ml. [c] β-Acetylglucosaminidase assay, 620 mU/ml. [d] In tests suggested by a reviewer, inhibition of the agglutination of human erythrocytes by human A antiserum (Kabat, 1956) required digest corresponding to 32 μg of substrate, or 2 μg of untreated substrate. Similar loss of blood-group A activity accompanying the enzymic release of acetylgalactosamine was reported earlier for protozoan and snail enzymes (Harrap and Watkins, 1964; Tuppy and Staudenbauer, 1966).

with time and enzyme concentration. No consistent pattern was discernible in deviations from linearity observed in several runs.

Action of α-Acetylgalactosaminidase on DOSM and DBSM. The prolonged action of the purified beef liver preparation is illustrated in Figure 8. Only acetylgalactosamine was detectible in digest concentrates by paper chromatography. Acetylgalactosamine (1.15 μmol/ml) was also the sole monosaccharide product on digestion of DBSM (2.74 μmol of hexosamine/ml) with purified pig liver enzyme (700 μg/ml, 18 hr). Only minor amounts of acetylgalactosamine (about 0.1 μmol/ml) were liberated in similar digests of undegraded OSM or BSM with the purified beef and pig liver enzymes.

The briefer action on DOSM and DBSM of purified beef liver enzyme (25 μg/ml), crude beef liver enzyme (11 mg/ml), and purified pig liver enzyme (350 μg/ml) in digests containing roughly similar α-acetylgalactosaminidase activity is summarized in Table VIII.

α-Acetylgalactosaminidase and Blood-Group A Substance. As seen in Table IX, the action of purified beef liver α-acetylgalactosaminidase on porcine blood-group A substance was feeble under the conditions used with DOSM and DBSM; the purified pig liver enzyme was more effective. Acetylgalactosamine was the only reducing sugar liberated in significant amount by either enzyme in the 18-hr digests. The purpose and result of the experiment with purified β-acetylglucosaminidase from beef liver was to indicate that the acetylgalactosamine liberated in the other experiments did not possess a β linkage in the intact substrate.

Action of β-Acetylglucosaminidase on Linkages in BSM. As shown in Table X, at extremely high levels of activity based on aryl glycoside assays, purified beef liver β-acetylglucosaminidase had little action on BSM,

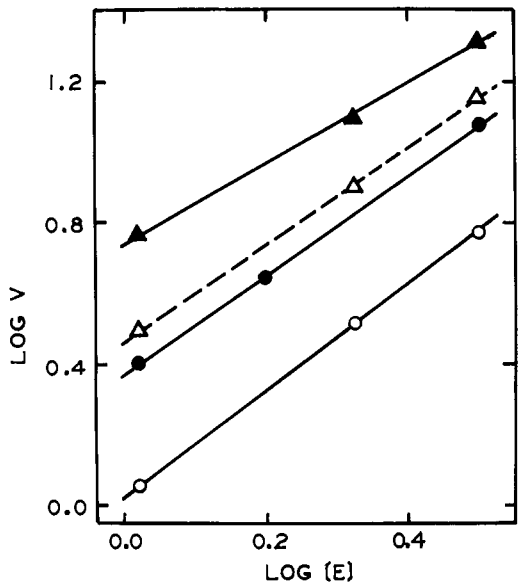

FIGURE 6: Logarithmic plot of initial velocity as a function of enzyme concentration for hydrolysis of phenyl α-acetylgalactosaminide catalyzed by beef liver enzymes at 38° and pH 4.7. The units of enzyme concentration and velocity are arbitrary, and different units are used for the purified and crude enzymes to permit representation of both on the plot (○, 5 mM substrate, purified enzyme; ●, 10 mM substrate, purified enzyme; ▲, 30 mM substrate, purified enzyme; △, 10 mM substrate, crude enzyme).

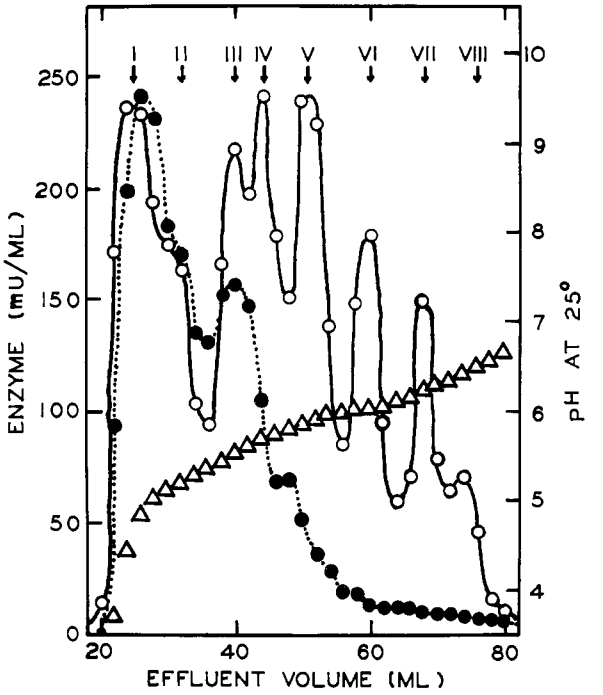

FIGURE 7: Isoelectric fractionation by electrophoresis in a stabilized pH gradient at 4° of the α-acetylgalactosaminidase (○) and the β-acetylglucosaminidase (●) in 10 mg of the purified pig liver preparation (△ = pH). After attainment of equilibrium (2 days) at a final potential of 700 V, the column was drained in fractions of 2 ml, starting from the anode end. The Roman numerals designate peaks referred to in the text.

TABLE X: Experiments with β-Acetylglucosaminidase.[a]

Substrate Prepn	Enzyme in Digest (U/ml)	GlcNAc Liberated (μmol/ml)
DBSM	1.24	0.016
DBSM	31.0	0.203
DBSM residue	31.0	0.118
BSM	31.0	0.008
DBSM residue	0.74[b]	0.018

[a] BSM (3.20 μmol of hexosamine/ml), DBSM (2.74 μmol of hexosamine/ml), or DBSM residue (1.45 μmol of hexosamine/ml) was incubated with enzyme for 18 hr at pH 4.5. Results of the colorimetry of the digests are reported as acetylglucosamine. DBSM residue is a preparation of the polymeric material remaining after exhaustive digestion of DBSM with purified beef liver α-acetylgalactosaminidase (Figure 8). It was recovered from the digest by gel filtration. [b] The enzyme used in this experiment was the crude calf liver enzyme (4.8 mg/ml), enriched in α-acetylglucosaminidase. The purified β-acetylglucosaminidase from beef liver was used in the other experiments.

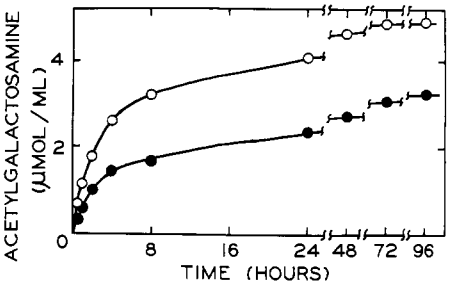

FIGURE 8: Liberation of acetylgalactosamine by purified beef liver α-acetylgalactosaminidase (100 μg/ml) from DOSM (○) and DBSM (●), 5.73 and 6.85 μmol of hexosamine per ml. Additions of fresh enzyme (100 μg/ml) were made after 24 and 48 hr. In 96 hr, 4.90 μmol/ml of acetylgalactosamine was liberated from DOSM, 3.22 μmol/ml from DBSM.

but liberated considerable acetylhexosamine from DBSM. This was found to be a mixture of acetylglucosamine and acetylgalactosamine, presumably resulting from presence in the enzyme preparation of traces of α-acetylgalactosaminidase. From DBSM which had been pretreated with this enzyme, acetylglucosamine was the sole monosaccharide produced by β-acetylglucosaminidase, while α-acetylglucosaminidase was without significant action, as seen in the table.

Discussion

Unlike mammalian β-acetylhexosaminidase (β-acetylglucosaminidase), a single enzyme which acts on glycosides of both acetylglucosamine and acetyl-

galactosamine (Walker *et al.*, 1961; Findlay and Levvy, 1960; Weissmann *et al.*, 1964), the mammalian α-acetylhexosaminidases, α-acetylglucosaminidase, and α-acetylgalactosaminidase, are distinct enzymes of mutually exclusive substrate specificities (*cf.* Weissmann *et al.*, 1967). Although the enzymic properties of α-acetylgalactosaminidase preparations from beef and pig liver are similar, some minor differences are observed, including differences in optimal pH, the abnormal kinetics of the beef enzyme, and the superior efficiency of the pig enzyme with natural substrates (Tables VIII and IX). Like the mammalian enzyme, the α-acetylgalactosaminidase of *H. pomatia* is reported to have no action on α- or β-acetylglucosaminides (Tuppy and Staudenbauer, 1966). Corresponding information is unavailable for the α-acetylgalactosaminidase of *T. foetus* (Harrap and Watkins, 1964).

The considerable difficulties encountered in purification of pig liver α-acetylgalactosaminidase are presumably attributable to the gradation in physical properties of the numerous isozymes demonstrated to be present. On the other hand, 50-fold purification of α-acetylgalactosaminidase from beef liver extracts in a single ion-exchange step (Table II) argues strongly against substantial polymorphism of this enzyme, which it has been impractical to examine electrophoretically. The isozymes of β-acetylglucosaminidase in pig liver may well be more numerous than the four detected in the present preparation, since this activity is selectively eliminated in the purification. At least two isozymes have previously been demonstrated by other means in human spleen, pig liver, and pig epididymus (Robinson and Stirling, 1966; Weissmann *et al.*, 1967; Leaback and Walker, 1967).

The most obvious explanation for the nonlinear relationship observed between initial velocity and enzyme concentration for beef liver α-acetylgalactosaminidase would be the presence of an activator in the enzyme preparation (Dixon and Webb, 1964). This appears unlikely, however, since the degree of nonlinearity is similar for preparations differing 340-fold in purity (Table V and Figure 6), and since reaction velocity is unaltered by boiled homogenate or EDTA. A possible explanation, currently being tested, ascribes the kinetic abnormality to rapidly reversible dissociation of active enzyme into less active or inactive subunits. This model is consistent with the observed decrease of apparent specific activity on dilution. Decreased nonlinearity at low temperature would be explained if low temperature favored association. Decreased nonlinearity at high substrate concentrations might be accounted for by tight binding of substrate only to the associated species, favoring association. Dissociation into inactive subunits has been authenticated for numerous enzymes (Hurlbert and Jakoby, 1965; Winstead and Wold, 1965; Maley and Maley, 1968; Cheng *et al.*, 1968; for additional literature, see Cheng and McKinley-McKee, 1968). Normal kinetics are reported in these cases, however, presumably reflecting the slow equilibration between these enzymes and their subunits, two points of difference from the model now considered.

Most of the carbohydrate in OSM can be accounted for in sialyl–acetylgalactosamine disaccharide moieties (see Pigman and Gottschalk, 1966; Murty and Horowitz, 1968), which are bound by *O*-glycosidic linkages to serine and threonine in the peptide core (Anderson *et al.*, 1964; Harbon *et al.*, 1968; Bertolini and Pigman, 1967; Bhargava and Gottschalk, 1967b). Assuming all the acetylgalactosamine in native OSM to occur in such disaccharide structures and allowing for residual-bound sialic acid in the present DOSM preparation, 92% of the total acetylgalactosamine in DOSM should be terminal and should be accessible to liberation by an appropriate exoglycosidase; 86% liberation is observed with the purified beef liver enzyme (Figure 8). This result agrees well with the earlier findings of Gottschalk and his colleagues with enzymes of incompletely determined specificity from beef spleen, snails, and earthworms (Bhargava *et al.*, 1966; Bhargava and Gottschalk, 1967a; Schauer and Gottschalk, 1968). These enzymes, designated *O*-seryl-*N*-acetylgalactosaminide glycohydrolases (Schauer and Gottschalk, 1968), are presumably similar to or identical with the α-acetylgalactosaminidase now considered; α-acetylgalactosaminidase activity is indeed present in beef spleen, as now shown, as well as in snail extracts (Tuppy and Staudenbauer, 1966).

Isolation of disaccharide derivatives and other evidence (Gottschalk and Graham, 1959; Bertolini and Pigman, 1967) suggests that BSM, which like OSM contains roughly equimolar quantities of sialic acid and acetylhexosamine, may have grossly similar structure. In BSM, however, about one-third of the acetylhexosamine is acetylglucosamine (Tettamanti and Pigman, 1968). As with OSM, the significant action of α-acetylgalactosaminidase on DBSM, but not on native BSM, is consistent with placement of the acetylgalactosamine in BSM in disaccharide moieties. If all of the acetylgalactosamine in native BSM were present in such disaccharide moieties and if residual bound sialic acid in DBSM were randomly distributed between acetylglucosamine and acetylgalactosamine, terminal acetylgalactosamine in DBSM would constitute roughly 55% of the total acetylhexosamine; 47% is liberated by digestion with purified beef liver α-acetylgalactosaminidase (Figure 8).

Hydrolysis of phenyl α-acetylgalactosaminide, DOSM, and DBSM must be catalyzed by the same enzyme in beef liver preparations, since the relative rates of action on these substrates show excellent correspondence for the crude and purified preparations (Table VIII), which differ 340-fold in purity. The extent of hydrolysis observed in exhaustive digests accordingly appears to establish conclusively that most, if not all, of the acetylgalactosamine residues in both OSM and BSM have an α-glycosidic linkage to peptide. In OSM, this linkage was earlier provisionally formulated as a β linkage (see Neuberger *et al.*, 1966; Pigman and Gottschalk, 1966).

The action of purified β-acetylglucosaminidase on desialized BSM derivatives, but not on BSM, indicates occurrence of appreciable β-linked acetylglucosamine in BSM, presumably in a disaccharide moiety or other

nonterminal positions. These conclusions are regarded as preliminary because of the large amounts of enzyme activity required. Whether the acetylglucosamine liberated in these experiments originates from macromolecules which also contain the α-linked acetylgalactosamine now demonstrated is uncertain in view of the incomplete homogeneity of current BSM preparations suggested by immunochemical criteria (Tettamanti and Pigman, 1968).

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