Isolation of β-*N*-Acetylhexosaminidase, β-*N*-Acetylglucosaminidase, and β-*N*-Acetylgalactosaminidase from Calf Brain*

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ABSTRACT: Three enzymes which hydrolyze the β-glycosidic linkages of β-*N*-acetylglucosaminides and β-*N*-acetylgalactosaminides were isolated from calf brain. One enzyme (β-*N*-acetylhexosaminidase) hydrolyzed the *p*-nitrophenyl derivatives of both β-*N*-acetylglucosamine (pH optimum 4.2, K_m = 0.8 mM, V_{max} = 20 μmoles/mg per hr) and β-*N*-acetylgalactosamine (pH optimum 3.8, K_m = 0.5 mM, V_{max} = 3 μmoles/mg per hr). It also hydrolyzed several oligosaccharides. This enzyme is present mostly in particles sedimenting between 1000*g* and 25,000*g* and was purified about 65-fold. It was inhibited by acetate, free and *N*-acetylated glucosamine or galactosamine, *p*-hydroxymercuribenzoate and silver nitrate. The effects of these inhibitors on the hydrolysis of the glucosaminide and galactosaminide varied both in the degree and the type of inhibition. Attempts to separate the enzyme into two entities, specific for *N*-acetylglucosaminides and galactosaminides, were unsuccessful. The two other enzymes were obtained from the 100,000*g* supernatant. One (*N*-acetylglucosaminidase) hydrolyzed *p*-nitrophenyl β-*N*-acetylglucosaminide 80 times faster than the corresponding galactosaminide. It had an optimal pH at 5.2 and was inhibited by *N*-acetylglucosamine but not by *N*-acetylgalactosamine. It was also inhibited by *p*-hydroxymercuribenzoate, silver nitrate, and *N*-ethylmaleimide. The second enzyme obtained from the 100,000*g* supernatant (*N*-acetylgalactosaminidase) hydrolyzed *p*-nitrophenyl β-*N*-acetylgalactosaminide 11 times faster than the corresponding glucosaminide.

It was inhibited by β-*N*-acetylgalactosamine, but not by *N*-acetylglucosamine. It was very sensitive to *p*-hydroxymercuribenzoate and silver nitrate. *N*-Ethylmaleimide activated this enzyme. A millimolar solution of *N*-ethylmaleimide increased the reaction rates two- to threefold.

β-*N*-Acetylhexosaminidases (β-2-acetylamino-2-deoxy-D-glucoside acetylaminodeoxyglucohydrolase, EC 3.2.1.30) occur in many tissues (Watanabe, 1936; Pugh *et al.*, 1957; Conchie *et al.*, 1959; Woollen *et al.*, 1961a,b). Sellinger *et al.* (1964) have demonstrated the presence of β-*N*-acetylhexosaminidase activity in each of the fractions obtained by differential centrifugation of cerebral cortex homogenates. The *N*-acetylhexosaminidases tested hydrolyzed the β-glycosidic linkage of both *N*-acetylglucosaminides and *N*-acetylgalactosaminides. Woollen *et al.* (1961a,b) and Walker *et al.* (1961) attempted, unsuccessfully, to separate the β-*N*-acetylglucosaminidase and β-*N*-acetylgalactosaminidase from each other. These authors concluded that one protein is responsible for both activities. However, the possibility of two independent active sites on this protein was not precluded. Weissmann *et al.* (1964) and Buddecke and Werries (1964) have purified liver and spleen β-*N*-acetylhexosaminidases several thousandfold, each, but even then obtained no separation of the β-*N*-acetylglucosaminidase and -galactosaminidase activities.

Gatt and Rapport (1966) showed that brain β-glycosidases could be separated into a β-galactosidase and a β-glucosidase. In the present work brain β-glycosaminidases were investigated. Three separate enzymes were isolated: (a) β-*N*-acetylhexosaminidase, which hydrolyzes both β-*N*-acetylglucosaminides and β-*N*-acetylgalactosaminides; (b) β-*N*-acetylglucosaminidase; and (c) β-*N*-acetylgalactosaminidase. Preliminary communications have appeared (Frohwein and Gatt, 1966a,b).

Experimental Procedure

Substrates. *p*-Nitrophenyl *O*-β-(2-acetamido-2-deoxy)-D-glucopyranoside (GLU)[1] was prepared according to Leaback and Weissmann (1963). *p*-Nitrophenyl *O*-β-(2-acetamido-2-deoxy)-D-galactopyranoside (GAL) was synthesized by a modification of the method of Heyworth *et al.* (1959). Chitobiose (2-acetamido-2-deoxyglucopyranosyl-β-1,4-(2-acetamido-2-deoxy)glucopyranoside) and a disaccharide (2-acetamido-2-deoxyglucopyranosyl-β-1,4-muramic acid) (Sharon *et al.*, 1966) were gifts of Professor N. Sharon of the Weizmann Institute for Science, Rehovoth; lacto-*N*-triose (2-acetamido-2-deoxyglucopyranosyl-β-1,3-galactopyranosyl-β-1,4-glucosopyranose) (Kuhn *et al.*, 1956) was a gift of Dr. A. Gauhe of the Max-Planck

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[1] Abbreviations used: GLU, *p*-nitrophenyl β-*N*-acetylglucosaminide; GAL, *p*-nitrophenyl β-*N*-acetylgalactosaminide; NEM, *N*-ethylmaleimide; PHMB, *p*-hydroxymercuribenzoate.

Institut fur Chemie, Heidelberg. Phenyl *O*-β-(2-acetamido-2-deoxy)-D-glucopyranoside was a gift of Professor S. Roseman, McCollum-Pratt Institute, Johns Hopkins University, Baltimore, Md.

Chemicals were purchased from the following firms: *N*-ethylmaleimide (NEM) from British Drug House Ltd. and Aldrich Chemical Comp. Inc.; dithiothreitol from Calbiochem; *p*-hydroxymercuribenzoate and protamine sulfate (grade II) from Sigma Chemical Co.

Assay of Products of Hydrolysis. The *N*-acetylglucosamine and *N*-acetylgalactosamine released were determined by the following adaptation of the method of Reissig *et al.* (1955). Incubation mixture (0.1 ml), containing no more than 3 μmoles of buffer, was mixed with 0.05 ml of 0.8 M potassium borate (pH 9.3) and placed in a boiling water bath for exactly 3 min. The mixture was cooled and 0.9 ml of freshly prepared Ehrlich's reagent (1 g of *p*-dimethylaminobenzaldehyde dissolved in 0.5 ml of concentrated hydrochloric acid and 49.5 ml of glacial acetic acid) was added. After 20 min at 37°, the absorbance at 585 mμ was read.

The hydrolyses of GLU and GAL were routinely determined by measuring the absorbance of the *p*-nitrophenol (PNP) released. Incubation mixture (0.5 ml) containing crude enzyme preparations were deproteinized with 1 ml of 2.7% trichloroacetic acid and centrifuged. NaOH (0.2 ml of 1 N) and 1.3 ml of 0.125 M sodium tetraborate were added to the supernatant and the color was read at 420 mμ. With purified enzyme preparations, 0.8 ml of 0.125 M sodium tetraborate was added directly to the incubation mixtures (0.2 ml) and the color was read at 420 mμ. The protein content of the enzymes was determined according to Lowry *et al.* (1951) or Warburg and Christian (1941).

Results

Purification of the Enzymes. Rat brain, excised after cervical dislocation, was homogenized with nine volumes of 0.25 M sucrose–10^{-3} M EDTA (pH 7.0) and fractionated using a modification of the method of Schneider and Hogeboom (1950). The hydrolytic activity of each of the subcellular fractions was tested at pH 3.6 and 7.0 with GLU and GAL and substrates. Table I shows that at pH 3.6, the specific activity of the particles which sedimented between 1000*g* and 20,000*g* exceeded that of the 100,000*g* supernatant about threefold. At pH 7.0, however, the specific activity of the supernatant was higher than that of the particles. This suggested that there might be two different enzymes in these two subcellular fractions; these fractions were therefore treated separately.

The particles were suspended in sucrose–EDTA (4 ml/g of brain) and the enzymatic activity was tested with GLU or GAL. At pH 3.6, this suspension had about one-half of the total activity of the homogenate. Attempts were made to extract the enzymes from this fraction by the addition of 0.1% sodium cholate, or by repeated freezing and rethawing, or by incubation with or without substrate, for 18 hr at 37°. In an alternate procedure, hypotonic extraction was attempted by suspending the particles in water (20 ml/g of brain) and incubating for 90 min at 0°. None of these procedures solubilized more than 50% of the enzymatic activity of the particles. A higher yield was obtained by sonic disintegration of a suspension of particles in sucrose–EDTA (6 ml/g of brain), in a Raytheon sonic oscillator, cooled with ice water. The activity (74%) (*i.e.*, 37% of the total activity of the homogenate) was solubilized by sonic irradiation for 8 min at 10 kcycles. To obtain an even higher yield, the enzyme was subsequently prepared either from total brain material or from the precipitate obtained by centrifuging brain homogenates for 1 hr at 100,000*g*.

TABLE I: β-*N*-Acetylhexosaminidase Activity of Subcellular Fractions of Rat Brain.[a]

Substrate	pH	Sp Act. (μmoles of PNP/mg of protein per hr) Particles (1000–20,000*g* sediment)	100,000*g* Supernatant	Ratio Particles: Supernatant
GLU	3.6	0.32	0.12	2.70
	7.0	0.02	0.09	0.22
GAL	3.6	0.045	0.02	2.30
	7.0	0.004	0.006	0.67

[a] Incubation mixtures, in volumes of 0.4 ml, contained 50 μmoles each of sodium phosphate and citrate buffers at pH 3.6 or 7.0, 0.8 μmole of substrate, and the subcellular fractions in quantities sufficient to give a color yield of at least 0.1 OD unit. The mixtures were incubated for 1 hr at 37° and the reaction was terminated with 1 ml of 2.7% trichloroacetic acid. *p*-Nitrophenol (PNP) and protein were determined as described in the Experimental Procedure. The results are averages of three experiments.

Purification of "Particulate" Enzyme (N-Acetylhexosaminidase) from Calf Brain. Table II summarizes the procedure employed for the purification of the enzyme which is present mainly in the particles. Calf brain (100 g), fresh from the slaughter house, was cooled and homogenized for 30 sec in a Waring Blendor with 400 ml of acetone previously cooled to −20°. After centrifuging at −10° for 10 min at 10,000*g*, the precipitate was again homogenized with 300 ml of cold acetone and centrifuged. The precipitate obtained was dried over P_2O_5 *in vacuo* at 4°. The 16.2 g of dried powder thus obtained was blended with 200 ml of 0.04 M sodium phosphate (pH 7.4) and left overnight at 4°. The suspension was centrifuged for 10 min at 25,000*g*, the supernatant was retained, and the precipitate was again blended with 130 ml of the same buffer. After 2.5 hr at 4°, the suspension was centri-

TABLE II: Purification of the Particulate β-*N*-Acetylhexosaminidase from Calf Brain.[a]

Fraction	Protein (mg)	GAL		GLU		
		Act. (units)	Sp Act. (units/mg)	Act. (units)	Sp Act. (units/mg)	Purification (-fold)
Acetone powder	15,000	660	0.044	3,200	0.21	
Phosphate extract	2,150	486	0.23	2,560	1.19	5.7
pH 3.8 supernatant	860	402	0.47	2,476	2.87	13.6
Ammonium sulfate (25–50% saturation)	222	398	1.78	2,086	9.3	44
Same, after storage at −20°	122	229	1.88	1,600	13.1	63

[a] Incubation mixtures, in volumes of 0.5 ml, contained 50 μmoles each of sodium citrate and phosphate (pH 4.2), 0.8 μmole of substrate, and appropriate volumes of enzyme. They were incubated for 30 min (with GLU), or 2 hr (with GAL), at 37°. PNP and the protein content were determined as described in the Experimental Procedure. One unit is defined as the amount of enzyme which hydrolyzes 1 μmole of substrate/hr.

fuged as before, and the two supernatants were combined. The pH of the supernatant was adjusted, at 4°, to 3.8 with 1 M phosphoric acid. After standing overnight at 4°, the pH increased somewhat and was again adjusted to pH 3.8 with 1 M phosphoric acid. After centrifugation for 20 min at 25,000*g*, the supernatant was adjusted to pH 8.3 with 1 N NaOH. Ammonium sulfate was added to 25% saturation (144 g/l.), the suspension was centrifuged, the precipitate was discarded, and ammonium sulfate was further added to the supernatant to give 50% saturation (additional 158 g/l.). The precipitate was collected, dissolved in 0.002 M sodium phosphate buffer (pH 7.0), dialyzed overnight against the same buffer, centrifuged, and stored at −20°. Upon storage for several weeks, a precipitate devoid of enzymatic activity formed in some preparations and was removed by centrifugation.

This procedure (Table II) resulted in a specific activity 63 times that of the homogenate or acetone powder. The enzyme could be further purified two- to threefold by acetone fractionation (30–40%, v/v); this preparation, however, lost its activity within several days, at −20°.

As is evident from Table II, the ratio of activities using GLU or GAL was fairly constant (about 4). Attempts were made to separate the activities toward GLU and GAL from each other. These included fractionation with ammonium sulfate or acetone; absorption and desorption on calcium phosphate gel; and chromatography on columns of TEAE-cellulose, chitin, chitosan hydrochloride (pH 3.8), and β-1-*p*-nitrophenyl tetraacetylglucosaminide. None of these procedures changed the ratio of activities on the two respective substrates. Similarly, heating enzyme solutions (at 45–65°) in the absence or presence of *N*-acetylglucosamine or *N*-acetylgalactosamine did not change this ratio.

Preparation of Enzymes from 100,000g Supernatant. Rat or calf brain 100,000*g* supernatant showed activity toward both GLU and GAL. The effect of pH of the reaction on the hydrolysis of GLU by calf brain supernatant is shown in Figure 1, curve A. The optimal activity was obtained at pH 5.0; the activity of pH 7.0 was more than 50% of the optimal. This preparation was then fractionally precipitated with acetone; the fraction which precipitated between 40 and 50% acetone (v/v) was collected, dialyzed, and found to have most of the residual activity. The pH–activity curve of this preparation is presented in Figure 1, curve B. The optimal pH was about 4.3; at pH 7, only about 10% of the optimal activity was found. This suggested that the 100,000*g* supernatant had at least two separate hexosaminidases, one stable and other labile to acetone. The enzyme stable to acetone treatment showed a pH–activity curve similar in shape to that of the particulate *N*-acetylhexosaminidase (Figure 2, curves A and B). It may therefore be presumed that these two enzymes may be identical. Both the particulate enzyme subjected to acetone treatment and the preparation obtained by acetone fractionation of the 100,000*g* supernatant lost their activity after storage for several days at −20°.

Addition of neutralized protamine sulfate to the 100,000*g* supernatant precipitated part of the *N*-acetylhexosaminidase activity. The activity which remained in the supernatant exhibited properties similar to those of the particulate enzyme. This is most probably the "acetone-stable" component described before. The enzyme which precipitated with protamine sulfate could be extracted from this precipitate with phosphate buffer; it hydrolyzed both GLU and GAL. This preparation was subsequently separated into two fractions. One (*N*-acetylglucosaminidase), which could be inhibited by 10^{-3} M NEM, hydrolyzed GLU 40–80 times faster than GAL. The second (*N*-acetylgalactosaminidase), whose activity toward GAL was stimulated by NEM, hydrolyzed GAL 10–20 times faster than GLU. The preparation of those two enzymes has been described by Frohwein and Gatt (1966b). In that procedure, the supernatant obtained by centrifuging calf

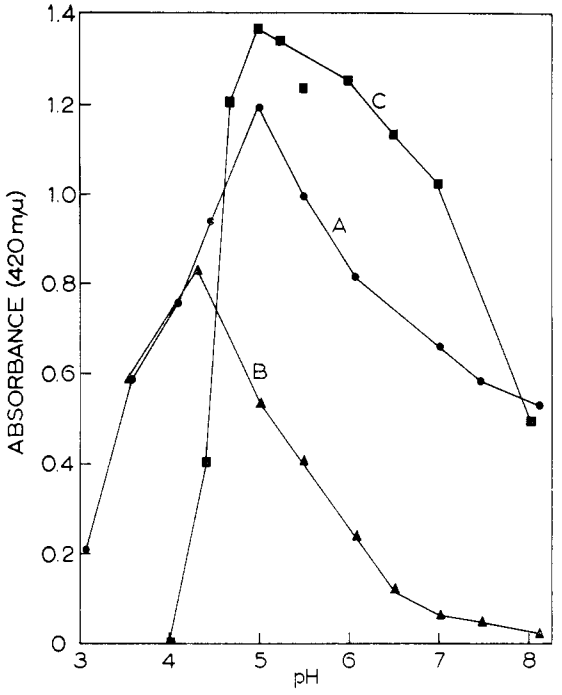

FIGURE 1: Effect of pH on the hydrolysis of GLU by enzymes from the 100,000*g* supernatant of calf brain. Curves A and B: Incubation mixtures, in volumes of 0.5 ml, contained 50 μmoles each of sodium phosphate and citrate buffers, 0.8 μmole of GLU, and enzyme. Curve A: The preparation (920 μg) obtained by centrifuging a 1:3 calf brain homogenate in 0.25 M sucrose–10^{-3} M EDTA (pH 7.0) for 1 hr at 100,000*g*. Curve B: The same preparation (107 μg), after acetone fractionation (40–50% fraction). The amounts of both preparations are equivalent to 83 mg of brain tissue. After 1 hr at 37°, 1 ml of trichloroacetic acid was added and the PNP was determined as in Experimental Procedure. Curve C: Incubation mixtures, in volumes of 0.2 ml, contained 10 μmoles, each, of sodium phosphate and citrate buffers; 0.2 μmole of GLU, and 18 μg of purified β-*N*-acetylglucosaminidase. After 30 min at 37°, 0.8 ml of 0.125 M borate buffer was added and the color was read at 420 mμ.

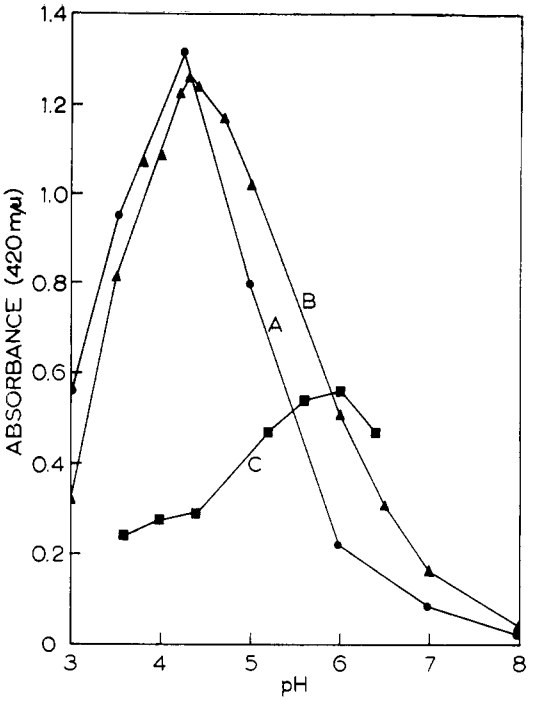

FIGURE 2: Effect of pH on the hydrolysis of GLU by the particulate *N*-acetylhexosaminidase. Curve A: Incubation mixtures, in volumes of 0.5 ml, contained 20 μmoles, each, of sodium citrate and phosphate buffers, 0.8 μmole of GLU, and 420 μg of rat brain particles, sedimenting at 1000–20,000*g*. After 1 hr at 37°, 1 ml of 2.7% trichloroacetic acid was added and the PNP was determined as described in Experimental Procedure. Curves B and C: Incubation mixtures, in volumes of 0.2 ml, contained 10 μmoles each of sodium citrate and phosphate (curve B) or 10 μmoles of potassium acetate buffers (curve C), 0.12 μmole of GLU, and 8 μg of enzyme, purified according to Table II. After 1 hr at 37°, 0.8 ml of 0.125 M sodium borate was added and the color was read at 420 mμ.

brain homogenate at 100,000*g* was precipitated with neutralized protamine sulfate. The precipitate was extracted with phosphate and the extract was fractionally precipitated with ammonium sulfate. *N*-Acetylglucosaminidase was purified in the presence of dithiothreitol, while *N*-acetylgalactosaminidase was prepared in the presence of *N*-ethylmaleimide. If this procedure was followed, except that the fraction precipitating between 25 and 50% saturation with ammonium sulfate was collected, a more active preparation of β-*N*-acetylgalactosaminidase was obtained.

Properties of the Enzymes. The *N*-acetylhexosaminidase of rat or calf brain particles exhibited a pH optimum of 4.2, with GLU as substrate (Figure 2, curve A). The enzyme obtained from acetone powders of whole brain and purified by the procedure described in Table II showed a similar pH–activity curve (Figure 2, curve B). This (together with the fact that at least half of the total enzymatic activity resides in the particles) suggests that the purified *N*-acetylhexosaminidase is derived mostly from the particles. The *N*-acetylglucosaminidase and -galactosaminidase present in the 100,000*g* supernatant are eliminated during the acetone treatment and fractionation procedure.

Other pH optima observed were as follows: using *N*-acetylhexosaminidase and GAL as substrate (pH 3.8) (Figure 3, curve A), *N*-acetylglucosaminidase and GLU as substrate (pH 5.2) (Figure 1, curve C), and *N*-acetylgalactosaminidase (prepared in the presence of NEM) and GAL as substrate (pH 5.5) (Figure 3, curve B).

The hydrolyses of both GLU and GAL by the respective enzymes were directly proportional to enzyme

TABLE III: K_m and V_{max} of Hydrolysis of GLU and GAL.[a]

Enzyme	Substrate	K_m (mM)	V_{max} (μmoles/mg per hr)
N-Acetylhexosaminidase	GLU	0.8	19
N-Acetylhexosaminidase	GAL	0.54	3
N-Acetylglucosaminidase	GLU	0.72	2.5
N-Acetylgalactosaminidase	GAL	0.35	0.67

[a] Incubation mixtures, in volumes of 0.2 ml, contained varying quantities of substrates (0.04–0.4 μmole) and 10 μmoles each of sodium phosphate and citrate buffers, at the optimal pH values of the corresponding enzymes: pH 4.2 with *N*-acetylhexosaminidase (8 μg); pH 5.2 with *N*-acetylglucosaminidase (130 μg); pH 5.5 with *N*-acetylgalactosaminidase (125 μg). After incubation at 37° (30 min with GLU and 2 hr with GAL), 0.8 ml of 0.125 M borate was added and the PNP color was read at 420 mμ. The results were calculated from Lineweaver and Burke plots.

TABLE IV: Substrate Specificities of β-*N*-Acetylhexosaminidases.[e]

Substrate	β-*N*-Acetylhexosaminidase (%)	β-*N*-Acetylglucosaminidase (%)	β-*N*-Acetylgalactosaminidase (%)
GLU	100	100	100
GAL	22	4	1100
β-Phenyl *N*-acetylglucosaminide	54	100	85
Lacto-*N*-triose[a]	7	5	6.5
Chitobiose[b]	0.25	0.3	0.7
Disaccharide[c]	0.13	0.1	0.17
Trihexosylceramide[d]	3.7		

[a] GlcNAc-β-1,3-Gal-β-1,4-Glc. [b] GlcNAc-β-1,4-GlcNAc (this substrate yields 2 equiv of GlcNAc on hydrolysis). [c] GlcNAc-β-1,4-muramic acid. [d] The activity was determined as described in the accompanying paper (Frohwein and Gatt, 1967). [e] Incubation mixtures, in volumes of 0.1 ml, contained 0.2 μmole of substrate and 2.5 μmoles each of sodium phosphate and citrate buffers at the pH values 4.2, 5.2, and 5.5, for the three corresponding enzymes. The amounts of the enzymes varied considerably, depending on the substrates employed. After 0.5–2 hr at 37°, *N*-acetylglucosamine or *N*-acetylgalactosamine were determined as described in Experimental Procedure.

concentrations up to at least 15 μg of the *N*-acetylhexosaminidase and 125 μg of the *N*-acetylglucosaminidase or -galactosaminidase, in 0.2 ml of reaction mixtures. They were also directly proportional to the time of incubation, up to at least 2.5 hr.

The K_m and V_{max} values of the corresponding enzymes and substrates are summarized in Table III. All K_m values were of a similar order of magnitude; however the V_{max} values differed considerably.

The action of the three enzymes on several *N*-acetylglycosaminides was compared. Table IV summarizes these results. The hydrolysis of each of the substrates tested is compared to that of *p*-nitrophenyl *N*-acetylglucosaminide; a value of 100 is assigned to the hydrolysis of GLU with each of the enzymes tested. The hydrolysis of GAL was 20 and 4% that of GLU with the hexosaminidase and glucosaminidase, respectively. With the *N*-acetylgalactosaminidase, however, it exceeded that of GLU about 11-fold. The rate of hydrolysis of phenyl *N*-acetylglucosaminide was similar to that of the *p*-nitrophenyl derivative. The di- and trisaccharides were, however, hydrolyzed at a much slower rate. The low rate of hydrolysis of chitobiose is especially noteworthy, as this substrate is a dimer of *N*-acetylglucosamine. The *N*-acetylhexosaminidase also hydrolyzed *N*-acetylgalactosamine-containing glycosphingolipids, as described in detail in the accompanying paper (Frohwein and Gatt, 1967).

Effect of Inhibitors. Table V summarizes the constants of inhibition obtained using acetate, *N*-acetylglucosamine, *N*-acetylgalactosamine, and free glucosamine or galactosamine.

There was a marked dissimilarity in the effect of acetate on the hydrolysis of GLU and GAL by the *N*-acetylhexosaminidase. While the hydrolysis of GLU was strongly inhibited by the addition of acetate, that of GAL was only little affected; the K_i with GAL as substrate was about 50 times higher than the corresponding value using GLU. Concomitant with the inhibitory effect of acetate on the hydrolysis of GLU there was a shift of the optimal pH from pH 4.2 (when measured in the presence of phosphate citrate) to about pH 6 (Figure 2, curve C). When GAL was used as substrate, the shift of the pH optimum was less marked and amounted to 0.3–0.5 pH unit at most. With the two enzymes isolated from the 100,000*g* supernatant, acetate somewhat inhibited the glucosaminidase but had practically no effect on the galactosaminidase.

The inhibitory effects of the free and acetylated hexosamines (Table V) provide further evidence for the presence of at least four enzymes or four separate active sites hydrolyzing *N*-acetylhexosaminides. *N*-Acetylglucosamine competitively inhibited the glucosaminidase, but not the galactosaminidase. Conversely, *N*-acetylgalactosamine competitively inhibited the galactosaminidase, but not the glucosaminidase. Both compounds inhibited the *N*-acetylhexosaminidase, irrespective of whether GLU or GAL was the substrate. Two differences were noted when the effects of *N*-acetylglucosamine and *N*-acetylgalactosamine on the latter enzyme were compared. The inhibition by *N*-

TABLE V: Inhibitory Effects of Acetate, *N*-Acetylated Hexosamines, and Free Hexosamines on the Hydrolysis of *p*-Nitrophenyl *N*-Acetylglycosaminides.[f]

Inhibitor	Constant of Inhibition (mM)			
	N-Acetylhexosaminidase		*N*-Acetyl-glucosaminidase	*N*-Acetyl-galactosaminidase
	GLU	GAL	GLU	GAL
Acetate	5.0 (C)[a]	250 (C)	25 (C)	*b*
N-Acetylglucosamine	5.5 (C)	20 (C)	3 (C)	*c*
N-Acetylgalactosamine	0.5 (NC)	2 (NC)	*d*	3.5 (C)
Glucosamine	20 (C)	15 (NC)	9 (C)	50 (NC)
Galactosamine	70 (C)	30 (NC)	27 (C)	6[e]

[a] C, competitive inhibition; NC, noncompetitive inhibition. [b] No inhibition at acetate concentration of 250 mM. [c] No inhibition at *N*-acetylglucosamine concentration of 40 mM. [d] No inhibition at *N*-acetylgalactosamine concentration of 50 mM. [e] The type of inhibition was not determined with certainty. [f] Conditions were the same as those in Table III except that the reactions were performed in the absence and presence of inhibitors. All values were calculated from Lineweaver and Burk plots.

acetylglucosamine was competitive, while that of the *N*-acetylgalactosamine was noncompetitive (Figure 4). Furthermore, *N*-acetylgalactosamine was a more potent inhibitor; the constants of inhibition obtained in the presence of this compound were about ten times lower than in the presence of *N*-acetylglucosamine.

The inhibition by glucosamine and galactosamine did not follow the pattern of the *N*-acetylated compounds. They both were inhibitory to all enzymes: competitive to the hydrolysis of GLU, but not to that of GAL.

The inhibitory effects of several reagents binding sulfhydryl groups were tested. The *N*-acetylhexosaminidase was strongly inhibited by *p*-hydroxymercuribenzoate ($K_i = 10^{-5}$ M with GLU and 2×10^{-5} M with GAL) and silver nitrate ($K_i = 4 \times 10^{-6}$ M with GLU and 6×10^{-6} M with GAL). Iodoacetate and *N*-ethyl-

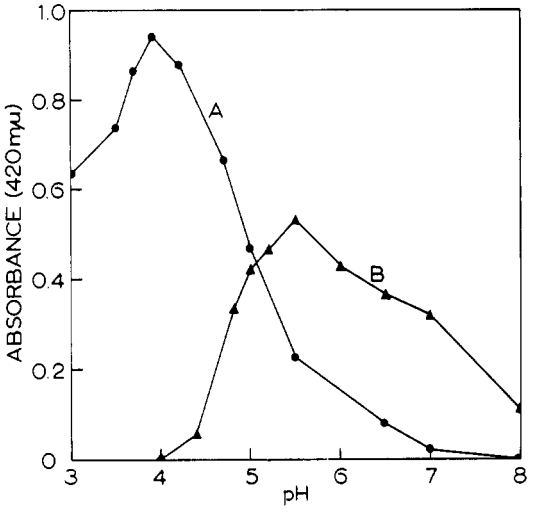

FIGURE 3: Effect of pH on the hydrolysis of GAL by β-*N*-acetylhexosaminidase and β-*N*-acetylgalactosaminidase. Incubation mixtures, in volumes of 0.2 ml, contained 10 μmoles each of sodium phosphate and citrate buffers, 0.2 μmole of GAL, and enzyme (curve A: 8 μg of β-*N*-acetylhexosaminidase, purified according to Table II; curve B: 58 μg of β-*N*-acetylgalactosaminidase). After 90 min at 37°, 0.8 ml of 0.125 M borate was added and the color was read at 420 mμ.

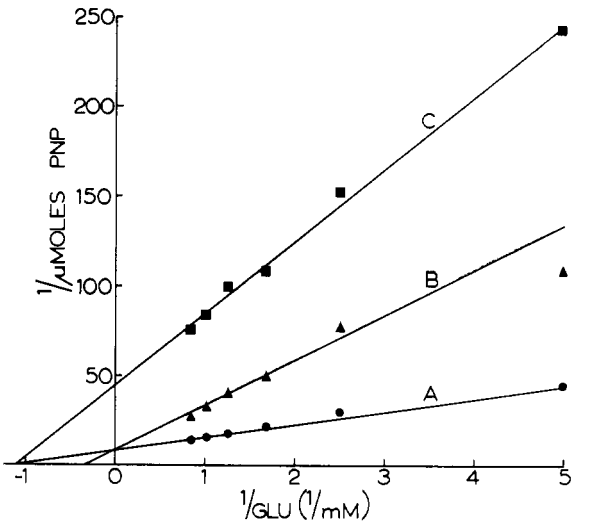

FIGURE 4: Inhibition of *N*-acetylhexosaminidase by *N*-acetylglucosamine and *N*-acetylgalactosamine. Conditions were the same as in Table V, using *N*-acetylhexosaminidase (8 μg) and GLU as substrate. The concentration of *N*-acetylglucosamine (curve B) was 1.2×10^{-2} M and of *N*-acetylgalactosamine (curve C) 2×10^{-3} M. Curve A was without inhibitor.

maleimide inhibited slightly and only at very high concentrations (K_i about 0.1–0.2 M). *N*-Acetylglucosaminidase was inhibited to a similar extent by PHMB, silver nitrate, and iodoacetate. *N*-Ethylmaleimide also inhibited this enzyme; however, straight lines were not obtained when $1/V$ was plotted against $1/S$ or against concentration of inhibitor (Webb, 1963). The estimated K_i value is about $2–4 \times 10^{-3}$ M.

When NEM ($0.5–1.5 \times 10^{-3}$ M) was added to *N*-acetylgalactosaminidase prepared in the absence of this reagent, enzyme activity increased two- to three-fold. NEM concentrations above $0.5–1 \times 10^{-2}$ M inhibited this enzyme. The estimated K_i value, obtained by extrapolation of the curve of $1/V$ against I, is about 2×10^{-2} M.

The inhibitory effect of PHMB and silver nitrate on the β-*N*-acetylgalactosaminidase was very pronounced; the K_i values were 3 and 1×10^{-6} M with these two inhibitors. This might be due to previous blocking of some SH groups by the *N*-ethylmaleimide which is added during the preparation of this enzyme. Iodoacetate caused only very little inhibition (the K_i was about 0.1 M).

Discussion

β-*N*-Acetylhexosaminidases have been studied by various investigators. Weissmann *et al.* (1964) and Buddecke and Werries (1964) have purified these enzymes from bovine liver and spleen several thousand-fold. In the present work, studies were made on β-*N*-acetylhexosaminidases of brain. This tissue is rich in *N*-acetylgalactosamine-containing glycolipids, in particular gangliosides which are sphingolipids containing glucose, galactose, *N*-acetylneuraminic acid, and *N*-acetylgalactosamine. It was presumed that a β-*N*-acetylgalactosaminidase hydrolyzing these compounds is present in brain. Several enzymes which hydrolyze sphingolipids have been isolated from this tissue (Gatt, 1966a; Hajra *et al.*, 1966). Gatt and Rapport (1966) have succeeded in separating the brain β-glycosidases into a β-glucosidase and a β-galactosidase. In the current work, a similar approach was used, *i.e.*, emphasis was put on the separation of brain *N*-acetylhexosaminidase into a β-*N*-acetylglucosaminidase and a β-*N*-acetylgalactosaminidase. Three enzymes hydrolyzing *N*-acetylhexosaminides have been isolated from calf brain. They were obtained from different subcellular fractions and differed in substrate specificity and several other properties.

The "particulate *N*-acetylhexosaminidase" of calf brain could not be separated into *N*-acetylglucosaminidase and *N*-acetylgalactosaminidase activities. In this respect it is similar to *N*-acetylhexosaminidase preparations from other organs, *i.e.*, bovine liver (Weissmann *et al.*, 1964), spleen (Buddecke and Werries, 1964), aorta (Buddecke and Werries, 1965), ram testis (Woollen *et al.*, 1961a), rat kidney (Pugh *et al.*, 1957; Walker *et al.*, 1961), and numerous other sources (Woollen *et al.*, 1961b). This raises the question whether this preparation is one enzyme which hydrolyzes both *N*-acetylglucosaminides and -galactosaminides, or whether it is a mixture of two individual enzymes which have not yet been separated from each other.

Woollen *et al.* (1961a) have studied extracts of ram testis. Kinetic experiments suggested that in this preparation only one enzyme is responsible for the hydrolysis of both *N*-acetylglucosaminides and *N*-acetylgalactosaminides. However, further experiments with preparations from other tissues did not yield the expected one-enzyme kinetic behavior (Walker *et al.*, 1961; Woollen *et al.*, 1961b). Deviations from one-enzyme kinetic behavior were also observed by Weissmann *et al.* (1964), using a highly purified bovine liver enzyme, but the *N*-acetylglucosaminidase and *N*-acetylgalactosaminidase activities could not be separated from each other under a variety of conditions. Furthermore, the respective substrates (*N*-acetylglucosaminides and *N*-acetylgalactosaminides) inhibited each other's hydrolysis. These authors therefore concluded that one enzyme was responsible for the hydrolysis of both substrates. Walker *et al.* (1961) suggested two alternate possibilities: (a) that there are two independent active sites on one enzyme protein or (b) one site on which the two substrates compete "with a direct displacement mechanism."

Attempts to fractionate the calf brain *N*-acetylhexosaminidase into two separate entities were unsuccessful. However, the hydrolyses of *N*-acetylglucosaminide and *N*-acetylgalactosaminide by this enzyme differed from each other in several respects, thus supporting the assumption that there are two separate active sites. The activities on the two substrates at various pH values differed from each other, and so did the K_m and V_{max} values. More striking differences were obtained in the presence of inhibitors. Acetate inhibited preferentially the hydrolysis of *p*-nitrophenyl *N*-acetylglucosaminide, but had only little effect on that of *p*-nitrophenyl *N*-acetylgalactosaminide. Depending on the concentration of acetate used, the ratio of hydrolysis of these two substrates varied from 5:1 to 0.7:1. Addition of acetate also changed the optimal pH of hydrolysis. With GLU, the optimal pH in the presence of acetate was about 1.5–2 pH units higher than that obtained with phosphate–citrate buffer; with GAL, the maximal change in the optimal pH amounted to 0.3–0.5 pH unit.

The products of the reaction (*N*-acetylglucosamine and galactosamine) as well as free glucosamine and galactosamine inhibited the hydrolyses of both GLU and GAL. However, both the type and magnitude of the effects differed with the two substrates. *p*-Hydroxymercuribenzoate and silver nitrate also inhibited the hydrolysis of both substrates, but here again there were quantitative differences in the K_i values.

The two enzymes which were obtained from the 100,000*g* supernatant differ from the enzymes previously described. They differ in the following aspects from the particulate *N*-acetylhexosaminidase. They are precipitated with protamine sulfate and poorly adsorbed on CM-Sephadex but avidly on DEAE- or TEAE-cellulose. They are also less stable than the *N*-acetylhexosaminidase. The activities of both the *N*-acetyl-

glucosaminidase and -galactosaminidase are lost by treatment with acetone and by adjusting the pH to 4.0 or less. The latter feature is a striking difference between these two enzymes and the particulate *N*-acetylhexosaminidase, whose optimal pH is about 4. The two supernatant enzymes lose activity upon storage for several weeks at −20°. This inactivation can be prevented by the addition of dithiothreitol or cysteine.

The β-*N*-acetylglucosaminidase and β-*N*-acetylgalactosaminidase which were obtained from the cell supernatant seem to be two distinct entities. The following data support this assumption. Upon purification, the ratio of the rates of hydrolyses of GLU and GAL changes. Ratios up to 80:1 were obtained by chromatography on DEAE-cellulose or CM-Sephadex; most of the *N*-acetylgalactosamine activity which was adsorbed on these columns could not be recovered. *N*-Ethylmaleimide had opposing effects on the two enzymes. NEM (10^{-3} M) inhibited about 90% of the *N*-acetylglucosaminidase activity. This same concentration increased the activity of the *N*-acetylgalactosaminidase two- to threefold. (The *N*-acetylhexosaminidase, with both GLU and GAL as substrate, was hardly affected by NEM concentrations up to 10^{-2} M.)

As might be expected for two separate enzymes, the products of hydrolysis inhibited specifically only the breakdown of their corresponding substrates. Thus, *N*-acetylglucosamine inhibited the hydrolysis of GLU by the *N*-acetylglucosaminidase, but not that of GAL by the *N*-acetylgalactosaminidase. Conversely, *N*-acetylgalactosamine inhibited the hydrolysis of GAL by *N*-acetylgalactosaminidase but not of GLU by its corresponding enzyme.

The three enzymes hydrolyzed both *p*-nitrophenyl *N*-acetylhexosaminides and oligosaccharides having a terminal *N*-acetylhexosamine group. The latter substrates were hydrolyzed at a much slower rate. Similarly, brain β-glucosidase (Gatt and Rapport, 1966) hydrolyzed nitrophenyl β-glucoside, but not cellobiose or gentiobiose. Both the β-glucosidase (Gatt, 1966b) and the β-*N*-acetylhexosaminidase (Frohwein and Gatt, 1967) also acted on the oligosaccharide moiety of the glycosphingolipids. These glycolipids which are abundant in brain tissue may be the native substrates for these enzymes.

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