Isolation and Characterization of the Major β-*N*-Acetyl-D-glucosaminidase from Human Plasma†

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ABSTRACT: The major β-*N*-acetyl-D-glucosaminidase component in human blood plasma has been isolated. Final purification is 195-fold with 14% yield. Purity is confirmed by gel electrophoresis and isoelectric focusing. The enzyme has an isoelectric pH of 4.73 and apparent molecular weight of about 105,000 from sedimentation equilibrium centrifugation and gel chromatography. This value remains unchanged after reduction and complete carboxymethylation of the cysteine residues, even in 6 M guanidine hydrochloride. The amino acid composition is determined. No free sulfhydryl groups have been found in native enzyme. The enzyme contains small amounts of neutral carbohydrate, sialic acid, and glucosamine, but no galactosamine. Kinetic studies indicate both β-*N*-acetyl-D-glucosaminidase and β-*N*-acetyl-D-galactosaminidase activity but no esterase, β-glucosidase, β-galactosidase, or α-*N*-acetyl-D-glucosaminidase activity could be detected. The enzyme has low activity but bovine and human serum albumin enhance V_{max} without changing K_m. Maximum activity is observed at pH 4.5–5.0. Hg^{2+} and Ag^{+} strongly inhibit the enzyme, and this inhibition is completely prevented by cysteine. However, inhibition by either Hg^{2+} or Ag^{+} becomes partly irreversible after standing. Fe^{2+} also inhibits the enzyme in citrate buffer (pH 4.5), but not in succinate or acetate buffers of the same pH. Hydrolysis of glycosides by Fe^{2+} ions has been observed, and these reactions depend also on the buffer composition. Although Cu^{2+} or ascorbate ions do not affect the enzyme significantly, the presence of both these ions inhibits the enzymatic reaction.

Human tissues contain two enzymes with β-*N*-acetyl-D-glucosaminidase (EC 3.2.1.30) activity (Robinson and Stirling, 1968). These enzymes also possess β-*N*-acetyl-D-galactosaminidase activity (Dance *et al.*, 1969). Heating at pH 4.4 and 50° destroys the activity of the acid form, enzyme A, whereas the basic form, enzyme B, is stable (Okada and O'Brien, 1969). Goldstone *et al.* (1971), Srivastava and Beutler (1972), and Carrol and Robinson (1973) have demonstrated that enzymes A and B are related to each other, despite differences in heat stability and electrophoretic mobility.

Human serum contains one major form of activity, which is heat labile and has electrophoretic mobility similar to that of enzyme A from other tissues (Hultberg, 1964; Okada and O'Brien, 1969; Price *et al.*, 1970). However, small amounts of heat stable enzymes have also been found (Price and Dance, 1972) especially in maternal serum during pregnancy (Stirling, 1972). Serum tests are widely used in detecting heterozygotes for the gene for Tay-Sachs disease (O'Brien *et al.*, 1970; Kaback and Zeiger, 1972; Lowden *et al.*, 1972), because of their low levels of heat-labile enzyme. Despite the advantages of this test the serum enzymes have not yet been purified and characterized.

We report the purification of the heat-labile enzyme from human plasma and describe some of its physicochemical properties. Results of kinetic studies, including observations on the inhibition by metal ions, will also be presented.

Materials and Methods

Materials

Plasma was prepared from citrated human blood less than 5 days old. *p*-Nitrophenyl and 4-methylumbelliferyl glycosides were obtained from Koch-Light Laboratories. Whatman microgranular DEAE-cellulose and DEAE-Sephadex A-50 and Sephadex G-200 (Pharmacia) were used in chromatography; the columns were prepared according to manufacturers' instructions. Poly(ethylene glycol 6000) (mol wt 6000–7500) was a product of Baker Chemicals Co. Bovine serum albumin was obtained from Nutritional Biochemicals Co. and human serum albumin from Pentex Research Products; both these preparations were free of β-*N*-acetyl-D-hexosaminidase activity. Celite 503 filter aid was obtained from Johns-Manville. Guanidine hydrochloride was prepared from the carbonate by the method of Nozaki and Tanford (1967).

Methods

Enzyme Assays. β-*N*-Acetyl-D-glucosaminidase activity was assayed using 0.5 ml of a solution containing 0.1 mg of MeUmbGlcNAc[1] in 0.05 M citric acid titrated to pH 4.5 with NaOH. The substrate was incubated at 37° with 25 μl of enzyme solution and the reaction was stopped at 30 min by adding 3 ml of 0.1 M glycine adjusted to pH 10.5 with NaOH. Free 4-methylumbelliferone was measured with an Aminco-Bowman spectrofluorimeter using excitation at 360 nm and emission at 450 nm.

β-*N*-Acetyl-D-galactosaminidase activity was assayed in the same way using MeUmbGalNAc. In some instances we also used NphGlcNAc and NphGalNAc for the enzyme assays as described elsewhere (Verpoorte, 1972).

α-*N*-Acetyl-D-glucosaminidase activity was assayed in 0.01 M citrate buffer with pH ranging from 4.0 to 6.0. Enzyme solution (25 μl) was added to 0.5 ml of buffer containing 0.5 mg of methyl α-*N*-acetyl-D-glucosaminide which was prepared as

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[1] Abbreviations used are: MeUmbGlcNAc, 4-methylumbelliferyl 2-acetamido-2-deoxy-β-D-glucopyranoside; MeUmbGalNAc, 4-methylumbelliferyl 2-acetamido-2-deoxy-β-D-galactopyranoside; NphGlcNAc, *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside; NphGalNAc, *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside.

described by Zilliken *et al.* (1955) and Matsushima *et al.* (1963). At 60 min we added 0.1 ml of 0.8 M potassium tetraborate (pH 9.1), heated in a boiling-water bath for 3 min, and assayed for free *N*-acetylglucosamine (Reissig *et al.*, 1955).

Esterase activity was asssyed with *p*-nitrophenyl acetate (Sigma) as described elsewhere (Armstrong *et al.*, 1966) and β-glucosidase (Robinson, 1956) and β-galactosidase (Leaback, 1961) were assayed with 4-methylumbelliferyl glycosides.

Gel Electrophoresis. Electrophoresis was done in 0.1 M sodium phosphate buffer (pH 7.2) in the presence of 0.1% sodium dodecyl sulfate (Mann Research Laboratories, at least 99% pure) using 5% polyacrylamide gels. The gels (4-mm diameter) were loaded with approximately 50 μg of protein and 4 mA was applied per tube for 2 hr. The gels were fixed and stained in a solution of 0.025% Coomassie Brilliant Blue (Mann Research Laboratories) in 10% trichloroacetic acid in acetic acid–methanol–water (14:40:160, v/v) as described by Peterson (1972).

Electrofocusing. Isoelectric focusing experiments were done with a 110-ml column (LKB column 8101). A 1.0% solution of Ampholine carrier ampholytes pH 3–6 (LKB) was used in a gradient of sucrose containing 0.1% Triton X-100 (Calbiochem). Electrophoresis at 10° and fractionation were done as described by Haglund (1971) and 1.1-ml fractions were collected for measurement of enzyme activity and pH.

Gel Chromatography. A Sephadex G-200 column (1.6 × 60 cm) was used with upward flow for determining molecular weights (Andrews, 1964). The column was standardized with Blue Dextran 2000, aldolase, ovalbumin, and chymotrypsinogen A (all products of Pharmacia).

Carboxymethylation. An enzyme solution in 6 M guanidine hydrochloride was reduced with 5 mM dithiothreitol (pH 8.0) at room temperature for 2 hr. Solid iodoacetic acid was added to a final concentration of 11 mM, the pH was readjusted to 8.0, and the solution was placed in the dark. After 1 hr at room temperature the solution was exhaustively dialyzed against water and the protein was freeze-dried. Complete carboxymethylation was demonstrated with an amino acid analyzer after hydrolysis in 6 M HCl.

Analytical Methods. Amino acid analyses were carried out on a Spinco Model 120C analyzer. Proteins were hydrolyzed in 6 M HCl at 110° in evacuated sealed tubes for 24 or 72 hr. Separate samples were oxidized with performic acid and analyzed for cysteic acid and methionine sulfone (Hirs, 1967). Tryptophan was estimated spectrophotometrically (Beaven and Holiday, 1952), following heating in 0.1 M NaOH at 60° for 1 hr. Neutral sugars were determined with the phenol–H_2SO_4 method (Dubois *et al.*, 1956) using glucose as standard. Sialic acid was determined by the thiobarbituric acid method of Warren (1959). Amino sugars were determined by the technique of Walborg *et al.* (1963) with an amino acid analyzer, after hydrolysis in 3 M HCl at 100° for 8 hr. Free sulfhydryl groups were assayed with iodoacetic acid in 6 M guanidine hydrochloride (Hirs, 1967) and with *p*-chloromercuribenzoate (Sigma) as outlined by Riordán and Vallee (1972).

Ultracentrifugation. Studies were performed with a Spinco Model E ultracentrifuge equipped with electronic speed control and temperature control. The conventional sedimentation equilibrium method outlined by Chervenka (1969) and a modified meniscus depletion method (Chervenka, 1970) were used to determine apparent molecular weight. The sedimentation equilibrium experiments were done at 8000 rpm for 48 hr after a 3-hr period of centrifugation at 12,000 rpm. The meniscus depletion method was done at 11,000 rpm for 6 hr. A partial specific volume of $\bar{V}$ = 0.73 ml/g was used in all calculations. Sedimentation equilibrium studies of solutions in 5 M guanidine hydrochloride were carried out at 12,000 rpm, using the same value for partial specific volume in the calculations.

Absorbance was measured with a Zeiss PMQ II spectrophotometer. The protein concentration was determined from the fringe shift in an interference pattern of a synthetic boundary using the ultracentrifuge at low speeds. The double-sector cell of capillary type was standardized with bovine serum albumin (Pentex Laboratories).

Results

Enzyme Purification. The purification of enzyme from 25 ml of plasma is summarized in Table I. A small amount of protein precipitated in 3% poly(ethylene glycol) at 4° and this was discarded. Further addition of poly(ethylene glycol) to 25% at 4° gave a dense precipitate which was centrifuged after 1 hr. This material was redissolved in 20 ml of 0.01 M sodium phosphate (pH 6.0). Anhydrous Na_2SO_4 was added to a final concentration of 250 g/l. and the suspension was stirred at 37° for 1 hr. The precipitate was filtered on Celite and redissolved in 20 ml of cold water. Inactive protein was precipitated with 0.02 M $ZnSO_4$ and centrifuged. The clear supernatant fluid was dialyzed against 50 volumes of 0.01 M sodium phosphate (pH 7.0) for 8 hr, replacing the buffer every 2 hr. Following dialysis some precipitate was centrifuged and the clear supernatant fluid was brought on a 2.5 × 10 cm column of DEAE-Sephadex equilibrated with 0.01 M sodium phosphate (pH 7.0). About 15% of the activity did not bind to the column at pH 7.0, but at least 60% was eluted only, when 0.05 M sodium citrate buffer (pH 4.5) was used. The active fractions which were eluted at pH 4.5 were dialyzed against 10 volumes of cold water for 3 hr, the water being replaced every hour. This solution was diluted with 0.02 M sodium phosphate buffer (pH 7.0) until the absorbance at 280 nm was about 2.0. The pH was carefully adjusted to 7.0 and solid $(NH_4)_2SO_4$ was added at 0° to a final concentration of 310 g/l. The suspension was stirred overnight at 2° and the precipitate was collected.

TABLE I: Isolation and Purification of β-*N*-Acetyl-D-glucosaminidase from Human Plasma.

	Protein[a] (mg)	Yield (%)	Purifcn (-fold)
Plasma	1688	100	1.0
Precipitated with poly(ethylene glycol)	949	90	1.6
Precipitated with Na_2SO_4	591	84	2.4
Supernatant liquid with 0.02 M $ZnSO_4$	416	74	3.0
Active fraction from DEAE-Sephadex	83	49	10.0
Precipitated with $(NH_4)_2SO_4$	31	39	21.2
Active fraction from DEAE-cellulose	5.5	34	104.3
Active fraction from Sephadex G-200	1.2	14	195.4

[a] Dry weight/25 ml of plasma.

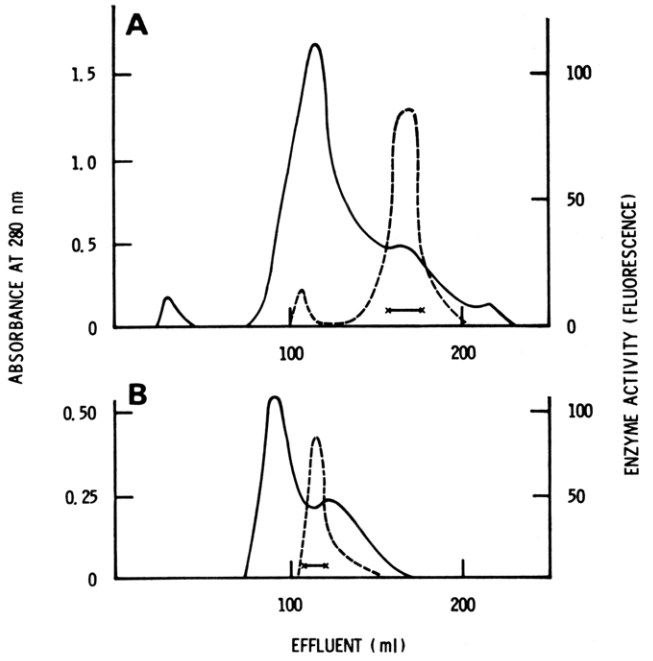

FIGURE 1: (A) DEAE-cellulose chromatography of isolated β-*N*-acetyl-D-glucosaminidase. Column, 2.0 × 15 cm; gradient, linear, using 100 ml of 0.01 M sodium phosphate and 100 ml of 0.01 M sodium phosphate with 0.25 M NaCl (pH 7.0); flow rate, *ca.* 19 ml/hr; fractions, 6 ml. (B) Pooled active fractions after DEAE-cellulose chromatography were dialyzed against solid $(NH_4)_2SO_4$. The precipitate was redissolved, briefly dialyzed against 0.01 M sodium phosphate (pH 7.0) and then chromatographed on Sephadex G-200. Column, 1.6 × 60 cm; flow rate, 9 ml/hr; buffer, 0.01 M sodium phosphate (pH 7.0); fractions, 3 ml. Absorbance at 280 nm (—) and activity (- - - -) were measured as described and active fractions were pooled (×—×).

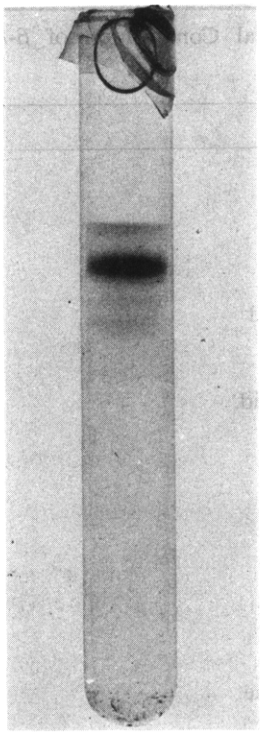

FIGURE 2: Gel electrophoresis pattern of pure β-*N*-acetyl-D-glucosaminidase at pH 7.2 in 0.1% sodium dodecyl sulfate.

This precipitate was first washed with a small volume of cold 50% saturated $(NH_4)_2SO_4$ in 0.01 M sodium phosphate buffer (pH 7.0), then redissolved. The solution was dialyzed against 50 volumes of 1 mM sodium phosphate buffer (pH 7.0) for 3 hr, the buffer being replaced every hour. Further purification was obtained by chromatography on DEAE-cellulose and then on Sephadex G-200, as illustrated in Figure 1. Only fractions with high activity were collected, dialyzed against water for 3 hr, and freeze-dried. Final purification was approximately 195-fold and yield was about 14%. Rechromatography of this material on Sephadex G-200 at pH 6.0 gave only one symmetrical peak.

Gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate gave only one band (Figure 2). However, older solutions did show several minor and slower moving components. Carboxymethylated enzyme also showed one band after gel electrophoresis in sodium dodecyl sulfate, and this band had the same mobility which indicates that no change in molecular weight occurred during carboxymethylation. It was concluded that the enzyme was free of contaminating proteins.

A typical example of electrofocusing is shown in Figure 3. Since the enzyme precipitated its solubility was increased by adding Triton X-100. Activity was maximal in a fraction at pH 4.73, and only one peak with absorbance at 280 nm was observed.

The molecular weight of the plasma enzyme estimated from Sephadex G-200 chromatography was 107,000. The same method gave a molecular weight of 140,000 for bovine spleen enzyme A, which was purified as described before (Verpoorte, 1972).

The amino acid composition of the enzyme is given in Table II. Identical values for half-cystine were obtained whether the enzyme was oxidized with performic acid or reduced with dithiothreitol in 6 M guanidine hydrochloride and then carboxymethylated with iodoacetate. No free sulfhydryl groups could be detected with *p*-chloromercuribenzoate, and no carboxymethylation was observed in 6 M guanidine hydrochloride without first reducing the enzyme with dithiothreitol. Values for neutral carbohydrate, sialic acid, and glucosamine are included in Table II. The enzyme did not contain galactosamine. Precipitation with 10% trichloroacetic acid did not change the glucosamine content and it is therefore likely that this component is part of a covalently bound carbohydrate moiety.

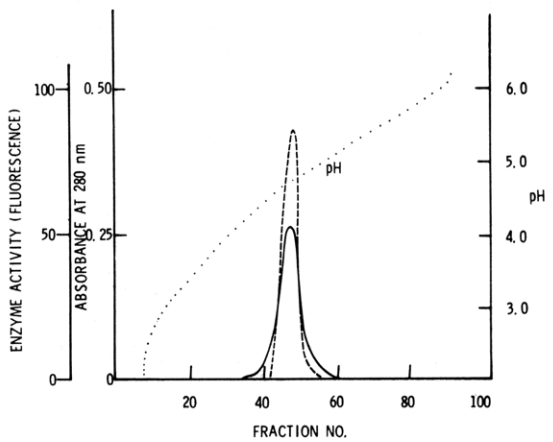

FIGURE 3: Isoelectric focusing of β-*N*-acetyl-D-glucosaminidase in a sucrose gradient with 1.0% ampholyte pH 3–6 and 0.1% Triton X-100, at 8° in a LKB 8101 column of 110 ml. Electrophoresis was done for 48 hr using 500 V. Fractions of 1.1 ml were collected and absorbance at 280 nm (—) and activity (- - - - -) measured.

TABLE II: Chemical Composition of β-*N*-Acetyl-D-glucosaminidase.

	Mol %[a]
Amino Acids	
Lysine	9.0
Histidine	2.3
Arginine	3.5
Ammonia[b]	10.3
Aspartic acid	9.5
Threonine[b]	6.3
Serine[b]	6.7
Glutamic acid	13.9
Proline	4.9
Glycine	5.2
Alanine	7.7
Half-cystine	2.1
Valine[c]	6.3
Methionine	1.1
Isoleucine[c]	2.4
Leucine	10.1
Tyrosine	2.8
Phenylalanine	4.4
Tryptophan	1.8
Carbohydrates	
Sialic acid	1.4[d]
Neutral carbohydrate[e]	30.0[d]
Glucosamine	3.5[d]
Galactosamine	Not detectable

[a] Average of four determinations. [b] Ammonia, threonine, and serine values were corrected by extrapolating the values obtained at 24- and 72-hr hydrolysis to zero time. [c] Valine and isoleucine obtained after 72-hr hydrolysis. [d] Number of residues per 100,000 g. [e] Galactose and glucose were detected after paper chromatography (Partridge, 1946); no fucose could be detected (Tsiganos and Muir, 1966).

Sedimentation velocity studies at pH 6.0 showed a single symmetrical peak with a sedimentation coefficient of about 4.0 S (Table III). At lower pH values, *e.g.*, pH 3.0, the sedimentation pattern revealed a second peak with higher sedimentation coefficient. This latter peak remained unchanged when pH was raised, even in the presence of 0.01 M β-mercaptoethanol and 0.5% Triton X-100. This indicated irreversible association.

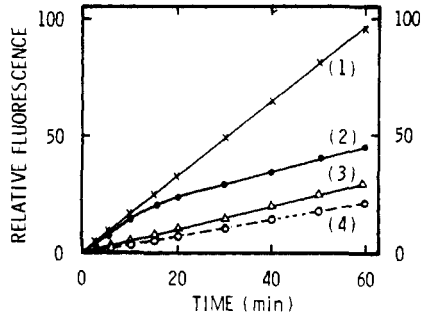

FIGURE 4: Hydrolysis of MeUmbGlcNAc (—) and MeUmGalNAc (- - - -) by β-*N*-acetyl-D-glucosaminidase. (1) No Hg^{2+}, (2) 2.5 μM $HgCl_2$ added together with substrate, (3) 2.5 μM $HgCl_2$ added to enzyme 1 hr before substrate, and (4) no Hg^{2+}.

TABLE III: Physical Properties of β-*N*-Acetyl-D-glucosaminidase.

Experimental Condition	Value
Isoelectric point 1% ampholine pH 3–6 and 0.1% Triton X-100	4.73
Sedimentation velocity coefficient (S)	
0.8% solution in 0.05 M citrate (pH 6.0)	3.9
0.8% solution in 0.05 M citrate (pH 2.5)[a]	3.8 and 6.6
Apparent weight-average molecular weight	
From sedimentation equilibrium in 0.02 M phosphate (pH 7.2)	107,000 ± 3500
From meniscus depletion in 0.02 M phosphate (pH 7.2)	105,000 ± 4000
From meniscus depletion in 0.05 M citrate (pH 2.5)[a]	260,000
From meniscus depletion in 0.05 M carbonate (pH 11.5)[a]	105,000 ± 3000
Apparent *z*-average molecular weight	
From sedimentation equilibrium in 6 M guanidine·HCl (pH 7.2)	118,000 ± 5000
From sedimentation equilibrium of carboxymethylated enzyme in 6 M guanidine·HCl (pH 7.2)	105,000 ± 4000
Absorbance at 279 nm 1.0% solution in 0.02 M phosphate (pH 7.2)	10.6

[a] Enzyme loses its activity instantly at this pH.

An apparent weight-average molecular weight of about 105,000 was obtained from sedimentation equilibrium and meniscus depletion experiments at pH 7.2. Data were plotted as described by Chervenka (1969, 1970) and gave linear plots throughout the cell. The apparent *z*-average molecular weight after exhaustive dialysis and freeze-drying was slightly higher, about 120,000, in 6 M guanidine hydrochloride at pH 7.2. The carboxymethylated enzyme had a molecular weight of about 105,000 in 6 M guanidine hydrochloride. From meniscus depletion experiments with fresh enzyme preparations at pH 2.5, an apparent molecular weight of about 260,000 was estimated. In this case the data did not yield a linear plot. The same enzyme at pH 11.5, on the other hand, had again a molecular weight of about 105,000 and the data gave a linear plot.

A 1.0% solution of enzyme in 0.02 M sodium phosphate buffer at pH 7.2 gave an absorbance of 10.6 at its maximum at 279 nm. The ratio of absorbances at 280 and 260 nm was 1.61.

Enzymatic Properties. The enzyme showed a linear release of 4-methylumbelliferone with either MeUmbGlcNac or MeUmbGalNAc at pH 4.5 and 37° for at least 30 min (Figure 4). Initial reaction velocities varied linearly with enzyme concentration to at least 50 μg/ml. Incubation with 0.1% Triton X-100 at room temperature for 16 hr did not reduce the activity of the enzyme. No esterase, α-*N*-acetyl-D-glucosaminidase, β-glucosidase, or β-galactosidase activity could be detected.

The pH dependence of β-*N*-acetyl-D-glucosaminidase activity at 37° showed maximum activity at pH 4.5–5.0. Sodium acetate buffers (0.05 M) gave similar pH dependence, but the activities were only about 20% of those in citrate.

The effect of substrate concentration on initial velocity at pH 4.5 gave linear Lineweaver–Burk plots and values of K_m and V_{max} are given in Table IV. The enzyme also showed

TABLE IV: Kinetic Parameters of β-*N*-Acetyl-D-glucosaminidase at pH 4.5 and 38°.

Substrate	K_m[a] (μM)	V_{max}[a] (μmol/min mg)
NphGalNAc	170 ± 40	0.019 ± 0.007
NphGlcNAc	830 ± 70	0.12 ± 0.02
MeUmbGalNAc	108 ± 22	0.006 ± 0.001
MeUmbGlcNAc	580 ± 37	0.078 ± 0.011
MeUmbGlcNAc in 0.01% BSA[b]	555 ± 41	0.098 ± 0.010
MeUmbGlcNAc in 0.01% HSA[b]	555 ± 29	0.094 ± 0.010

[a] Average of three determinations. [b] BSA and HSA are bovine and human serum albumin, respectively.

TABLE V: β-*N*-Acetyl-D-glucosaminidase Activity at pH 4.5 and 38°.

	Rel Fluorescence
0.05 M citrate	100
0.05 M citrate + 2.5 μM Hg^{2+} [a]	45
0.05 M citrate + 2.5 μM Hg^{2+} [b]	25
0.05 M citrate + 2.5 μM Ag^{+} [b]	18
0.05 M citrate + 1 mM cysteine	93
0.05 M citrate + 1 mM cysteine + 2.5 μM Hg^{2+} [c]	89
0.05 M citrate + 2.5 μM Hg^{2+} + 1 mM cysteine[d]	43
0.05 M citrate + 200 μM Fe^{2+}	28
0.05 M citrate + 200 μM Cu^{2+}	95
0.05 M citrate + 2 mM ascorbate	81
0.05 M citrate + 200 μM Cu^{2+} + 2 mM ascorbate	9
0.05 M succinate	94
0.05 M succinate + 200 μM Fe^{2+}	95
0.02 M acetate	49
0.02 M acetate + 200 μM Fe^{2+}	51

[a] Hg^{2+} added with substrate. [b] Enzyme incubated with Hg^{2+} or Ag^{+} at room temperature for 1 hr. [c] Enzyme incubated with cysteine and Hg^{2+} at room temperature for 1 hr. [d] Enzyme incubated with Hg^{2+} for 1 hr then another hour with cysteine.

β-*N*-acetyl-D-galactosaminidase activity, with K_m and V_{max} values different from those of the β-*N*-acetyl-D-glucosaminidase reaction. The enzyme was stimulated by 0.01% bovine serum albumin and to a lesser extent by 0.01% human serum albumin. Both albumins enhanced V_{max} but did not affect K_m (see Table IV). The glucosaminidase and galactosaminidase activities were equally stimulated by serum albumin.

The enzyme gave a nonlinear release of 4-methylumbelliferone when $HgCl_2$ was added together with substrate at pH 4.5 and 37°. This nonlinear reaction lasted for about 20 min as shown in Figure 4. In contrast no such nonlinear reaction was observed when $HgCl_2$ was added to the enzyme solution 1 hr before the glucosaminidase reaction was started. In the latter case the enzymatic reaction was strongly inhibited but linear. The inhibition by Hg^{2+} is independent of pH, at least between pH 3.5 and 6.5, and also seems to be noncompetitive. Cysteine prevents the inhibition by Hg^{2+} when added at the same time, but it does not completely reverse the effect of Hg^{2+} on the enzymatic reaction when added at a later stage; see Table V.

$AgNO_3$ inhibits the enzyme at lower concentrations than $HgCl_2$, and this inhibition is also partly irreversible (see Table V). Following the addition of $AgNO_3$ the reaction slows down instantly, and hydrolysis of substrate remains linear. It is believed that Ag^{+} and Hg^{2+} act similarly on the enzyme.

$FeSO_4$ inhibits less strongly (see Table V); this inhibition is also noncompetitive. The observed rates of the enzymatic reaction must be corrected for the catalytic effect of the ferrous salt. The corrected enzymatic reaction in the presence of Fe^{2+} in 0.05 M sodium citrate buffer (pH 4.5) remains linear.

Fresh solutions of ferrous must be used since the inhibition is less when older solutions are used. It has also been observed that the enzymatic activity of mixtures of enzyme with Fe^{2+} increase after standing in the presence of air. Since Fe^{3+} inhibits the enzyme also, one does not observe complete recovery of the enzymatic activity after standing.

$FeSO_4$ does not inhibit the enzyme when citrate buffer is replaced by succinate or acetate buffer of the same pH. Results of these experiments are shown in Table V from which it may also be noticed that the enzymatic activity decreases significantly when acetate buffer is used. Ferrous ions which catalyze the hydrolysis of *O*-glycosides in citrate buffer, with maximum rate at about pH 4.5, do not have any effect when acetate or succinate buffers are used unless EDTA is added to these latter buffers. Other buffers also stimulate the Fe^{2+}-catalyzed glycosidase reactions; among these are phosphate and carbonate which are both very effective at neutral and slightly alkaline pH, but are completely ineffective below pH 4.5.

Cleavage of glycosides in the presence of L-ascorbic acid has been reported by Caygill (1968). The velocities of these reactions are increased by addition of Cu^{2+}. It may be noticed from Table V that neither L-ascorbic acid nor Cu^{2+} inhibits but when these reagents are added together the enzymatic reaction is strongly inhibited.

Discussion

Serum and plasma prepared from freshly drawn blood have the same β-*N*-acetyl-D-glucosaminidase activity. Neither citrate nor EDTA has any effect, but storage at 2° and repeated freezing and thawing seem to reduce the activity.

Initial fractionation of serum with poly(ethylene glycol) at 4° leaves a large fraction of albumin in solution (Gambal, 1971). The first chromatographic step with DEAE-Sephadex at pH 7.0 leaves approximately 15% of the activity in solution. This activity is heat stable, whereas the activity which is bound to the column is heat labile.

Careful selection of active fractions after each chromatography ensures a purification of 195-fold. It has been demonstrated that several proteins, including human serum albumin, enhance enzymatic activity. Since the activities of samples were assayed in the absence of other proteins, the real purification must be higher than 195. The purified enzyme is not very stable in aqueous solution especially at low pH and low salt concentration. We therefore keep dialysis to a minimum and also avoid low pH values. It has been observed with the ultracentrifuge that the enzyme associates at low pH or ionic strength. This association does not seem to be completely reversed in 6 M guanidine hydrochloride. Enzyme previously dialyzed against water and freeze-dried has an apparent *z*-average molecular weight in 6 M guanidine hydrochloride higher than that of carboxymethylated enzyme. The carboxymethylated enzyme in 6 M guanidine hydrochloride has an apparent molecular weight similar to that of a fresh enzyme preparation in phosphate buffer.

The pH–rate profile of the enzymatic reaction showed a maximum value at pH 4.5–5.0. The decrease in activity at low pH could be due to the observed association. At alkaline pH, however, reduced activity must be attributed to some other reaction.

The enzyme does not contain free sulfhydryl groups. Amino acid analyses indicate that less than 1.0% of the half-cystine residues are carboxymethylated in 6 M guanidine hydrochloride unless the enzyme was first reduced with dithiothreitol. We also failed to detect any reaction with *p*-chloromercuribenzoate.

Inhibition of enzymatic activity by Hg^{2+} or Ag^{+} is partly irreversible. Kinetic data indicate a complex reaction which could be due to a change in conformation of the active site on the enzyme. However, Hg^{2+} also precipitates the enzyme. In contrast the inhibition by Fe^{2+} depends strongly on the buffer system. Taborsky (1973) has described an oxidative modification of proteins in the presence of ferrous ions and air; especially cytochrome *c* was very susceptible. Although a similar reaction could take place with the enzyme it is not believed to be responsible for the inhibition. Inhibition of the enzyme occurs at pH 4.5, which is different from that for the oxidative modification reaction, and is mostly reversible upon standing. Other experiments have also indicated that inhibition takes place in the absence of air, which is essential for the oxidative reaction. Fe^{2+} seems to inhibit the enzyme only in buffers which have been found to induce the Fe^{2+}-catalyzed hydrolysis of *O*-glycosides. These latter reactions are biphasic, that is an extremely fast reaction precedes a slow and linear one. The rates of the Fe^{2+}-catalyzed reactions are dependent on the aglycon part of the molecule, *e.g.*, *p*-nitrophenyl *O*-glycosides are hydrolyzed faster than 4-methylumbelliferyl *O*-glycosides. On the other hand the structure of the carbohydrate does not influence the rate of the Fe^{2+}-catalyzed reaction. It is therefore believed that formation of a complex between ferrous and buffer ions and *O*-glycosides reduces the concentration of free substrates for the enzyme. This possible explanation is supported by data obtained with Cu^{2+} and ascorbate ions which show similar effects.

The isoelectric point of the plasma enzyme is close to that of heat-labile enzyme A from human liver (Sandhoff and Wässle, 1971). Because of precipitation of the enzyme some Triton X-100 is added which does not alter the activity of the plasma enzyme nor that of enzyme A from human placenta (Johnson *et al.*, 1972). The plasma enzyme exhibits several features that have been observed with enzyme A from other human tissues, like pH optima, inhibition by metal ion, etc. Values of K_m for artificial substrates are also of the same order; however, the plasma enzyme gives lower V_{max} values (Sandhoff and Wässle, 1971; Wenger *et al.*, 1972; Johnson *et al.*, 1972).

β-*N*-Acetyl-D-glucosaminidases show enhanced activity when bovine serum albumin is added to the assay mixture (Wetmore and Verpoorte, 1972; Verpoorte, 1972). Bovine serum albumin is more effective than human serum albumin when the activity of human plasma enzyme is studied. Ovalbumin and ribonuclease also seem to stimulate the plasma enzyme although less than 10% increase is observed. It seems probable that the effect of other proteins is merely due to some protective role as has been suggested by Findlay and Levvy (1960).

Although the plasma enzyme shows lower activities for artificial substrates the same does not have to be the case when natural substrates are used. Studies with natural substrates, like gangliosides, are difficult to carry out because of the low sensitivity of detection of products. These studies are generally done with radioactive substrates (Sandhoff and Wässle, 1971; Wenger *et al.*, 1972; Tallman and Brady, 1972), which are not yet available to us.

The molecular weight of human plasma enzyme is lower than that reported for other human enzymes (Sandhoff and Wässle, 1972) or for enzymes of other species (Verpoorte, 1972; Wetmore and Verpoorte, 1972). Ultracentrifugation studies in the presence of 6 M guanidine hydrochloride with dithiothreitol indicated subunits for β-*N*-acetyl-D-glucosaminidases of beef spleen (Verpoorte, 1972) and pig kidney (Wetmore and Verpoorte, 1972). Subunits have been obtained from a β-*N*-acetyl-D-glucosaminidase of the limpet *Pastella vulgata* (Bannister and Phizackerley, 1973). Recent studies with antisera prepared against human liver enzymes also indicate the presence of subunits (Srivastava and Beutler, 1973; Robinson *et al.*, 1973). One of these subunits is enzymatically inactive but seems common to both enzymes A and B, and has a molecular weight of about 25,000 (Robinson *et al.*, 1973). This subunit is probably not present in the human plasma enzyme, although it could have been lost during the isolation. The absence of this subunit, however, does explain the lower molecular weight and possibly also the lower activity of the plasma enzyme.

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