The mechanism of action of this enzyme has been studied in detail using a variety of substrate structure/activity and kinetic experiments. Rate data plotted versus pH depends on the following ionization constants, respectively: for $pK_{an}$, 2.95; for log $k_{cat}$, 7.8; and for log $k_{cat}/K_m$ 2.95 and 8.25. The $K_m$ value of H$_2$O/D$_2$O for p-nitrophenyl-$\beta$-N-acetylglucosaminide hydrolysis is 1.27 at pH 4.6 and 1.00 at pH 7.0. The $\rho$ value for the hydrolysis of para-substituted phenylglucosaminides is +0.36; $\rho$ for the hydrolysis of fluoro-substituted N-acetyl derivatives is ~1.41. Two sulfur-containing substrate analogues, the 1-thioglucosaminide, and the N-thioacetetyl derivative, exhibit either no or little substrate activity. The hydrolysis of the 2,4-dinitrophenylglucosaminide is not biphasic as indicated by stopped flow kinetic studies. These several results are interpreted to show that: 1) enzymatic nucleophilic catalysis is not employed by $\beta$-N-acetylglucosaminidase; 2) the glycosidic oxygen is protonated very early in the reaction, perhaps even in the Michaelis complex; 3) the acetaldehyde oxygen provides anchimeric assistance to hydrolysis via charge stabilization of the oxocarbonium ion (or via oxazoline formation); 4) additional charge stabilization is provided by an enzymic anion, perhaps a side chain carboxylic group. The role of the acetaldehyde group is discussed and comparisons are made between lysozyme, $\beta$-galactosidase, and $\beta$-N-acetylglucosaminidase.

The mechanism of action of glycosidases has been the subject of much research. This literature has been reviewed by Capon (1) and more recently by Legler (2) and Bruce (3). Since $C_1$-glycosidic oxygen bond fission is formally a nucleophilic substitution at $C_1$ of the glycoside, glycosidases can be divided into two classes based on whether catalysis proceeds with inversion or retention of configuration (1). Glycosidases catalyzing hydrolysis with retention of configuration utilize general acid catalysis which apparently promotes formation of a glycopyranosyl carbonium ion intermediate (1-3) via a typical SN1 process. The retention of configuration observed also has been associated with the classical double displacement (ping-pong) mechanism in which the oxocarbonium ion is trapped by an enzyme nucleophile generating a glycosylenzyme intermediate (4). However, whether the putative enzyme nucleophile is covalently linked to the glycosyl unit or is simply part of an ion pair appears to remain an unresolved question (2, 5, 6). Irrespective of this ambiguity, there is no evidence for the reactions catalyzed by glycosidases to be SN2 and single displacement (sequential) in nature.

An additional factor in the hydrolysis of 2-acetamido-2-deoxyglycosides is potential anchimeric assistance by the acyl side chain (1, 7-9). Such a role for the acetamido group is attractive for two reasons: 1) it can explain the large difference in reactivity between glycosides (which do not hydrolyze spontaneously) and the corresponding glycosaminides (9), and 2) it is consistent with the retention of configuration observed (1, 10). In parallel with mechanisms employing an enzyme nucleophile as above, proposals focusing on the acetamido group failed to distinguish between catalytic participation (an SN1 displacement process leading to an oxazoline intermediate) or simple ion pairing between the oxocarbonium ion and the partial negative charge on the acyl oxygen.

$\beta$-Glucosaminidase is an exoglycosidase which removes $N$-acetylgalactosamine from glycosides and polysaccharides with retention of configuration as has been shown to be the case for many other similar exoglycosidases (1, 11). The enzyme is specific for the $\beta$ configuration and is fairly specific for the sugar moiety; it exhibits very little specificity for the aglycone (12). Glycosyl transfer from the sugar moiety to hydroxylic acceptors other than water has been demonstrated (13), which suggests the presence of an enzyme-glycosyl intermediate of some type and would appear to exclude from consideration a single displacement, SN2-type reaction mechanism. However, while lysozyme, another glucosaminidase hydrolase, has been the subject of much research, $\beta$-glucosaminidase has been much less well characterized and less mechanistic information on this enzyme has been published. Available data (13-16), while useful, have enabled only qualitative aspects of the reaction mechanism to be established. Therefore, we undertook a detailed mechanistic study of $\beta$-N-acetylgalactosaminidase (from Aspergillus niger) in an attempt to establish the mechanism of action of this enzyme type.

**EXPERIMENTAL PROCEDURES**

**Materials**

- p-Nitrophenyl-$\alpha$ and $\beta$-galactoside, $p$-nitrophenyl-$\alpha$ and $\beta$-glucoside, $p$-nitrophenyl-$\alpha$ and $\beta$-mannoside, $p$-NP-$\beta$GlcNAc, $p$-NP-$\beta$GalNAc, $p$-NP-$\beta$Gal.

*The abbreviations used are: $p$-NP-$\beta$GalNAc, $p$-nitrophenyl-$N$-acetyl-$\beta$-D-galactosaminide; $p$-NP-$\beta$Glc, $p$-nitrophenyl-$\beta$-D-glucoside; $p$-NP-$\beta$GlcNAc, $p$-nitrophenyl-$\beta$-D-N-acetylglucosaminide.*
βGalNAc, phenyl-N-acetyl-β-D-glucosaminide, GlcN-HCl, GlcNac, monofluoracetate, and bovine serum albumin were all obtained from Sigma. p-Nitrophenyl-β-D-xyloside and p-Np-GlcNac-TAc were gifts from Dr. O. P. Bahl (State University of New York at Buffalo). p-Nitrophenol, p-methoxphenol, p-chlorophenol, 2,4-dinitrophenol, trifluoroacetic acid, difluoroacetic acid, and bromoacetyl bromide came from Aldrich. All other reagents were of the highest purity available from commercial sources.

Analytical Methods
Specific rotations obtained on a Hitachi-Perkin Elmer 140 automatic polarimeter. NMR spectra were recorded using either Varian A60 or XL100 instruments and IR spectra using a Beckman IR33 Spectrophotometer. Melting points, obtained on a Fisher-Johns Mel-Temp apparatus, are uncorrected. Thin layer chromatography was performed using Silica Gel 6 plates with fluorescent indicator (Eastman Kodak). Elemental analyses of substrates were done by Galbraith Laboratories to ensure purity and were satisfactory in all cases. Protein analytical methods are described in the figure legends. Unless noted otherwise, all operations were performed at ambient temperature (~25°C).

Synthesis

p-Np-β-D-GlcNAc was prepared as described (17).

2,4-Dinitrophenyl-2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-D-glucopyranoside—This compound was prepared by a method similar to that of Leaback and Walker (18). 2-Acetamido-2-deoxy-3,4,6-tri-O-acetyl-β-D-glucopyranosyl chloride (4) (19) and 2,4-dinitrophenol (5 g) were stirred together in 100 ml of acetone. To this mixture were slowly added 36 ml of 3.3% (w/v) aqueous NaOH, and the resultant solution was stirred for 6 h, then overnight at 4°C, washed with water and then with ether (2 x 25 ml). The off-white compound was recrystallized from acetone/ether, yielding 0.12 g (36%) of product, m.p. 167-168°C. TLC analysis in CHCl₃/methanol, 1:1, yielded one spot, Rₜ = 0.764. [α]D = -17.1°C (c, 0.46, dimethylformamide). IR spectra analysis (KBr pellet): 3340 (NH), 1750 (acetate, C=O), 1080 (aromatic-OCH₃), 1125 and 830 (p-disubstituted phenyl), 1400, 1385 (C-N), 880 (C-H). NMR 300 MHz (SO-D₄) 8 3.76 (3H, singlet, MeO), 4.00 (3H, singlet, OCH₃), 6.90 (6H, aromatic hydrogens at 5.0-8.0 ppm), 8.10 (1H, aromatic hydrogen atom centered at 8.1 ppm); of p-substituted aromatic hydrogens between 1.75 and 8.0 ppm.

2,4-diNp-β-GlcNAc—The deacetylation method of Hengstenberg and Wallenfels (21) was used. 2,4-Dinitrophenyl-2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-β-D-glucopyranoside was dissolved in MeOH/H₂O (1:1), and the resultant solution was thoroughly mixed, anhydrous ether (250 ml) was added. A flocculent precipitate immediately formed which was removed after 1 h by filtration ( suction). Further crystallization occurred during 24 h at 0°C. Recrystallization from ethanol/ether yielded 1.25 g (15%), m.p. 175-177°C. TLC analysis in CHCl₃/Methanol (9:1) yielded one spot, Rₜ = 0.655. IR spectra analysis (KBr pellet): 3340 (NH), 1750 (acetate, C=O), 1675 (amide type I), 1540, 1520 (NH), 1230 (C-N), 920 (C-N) and 1385 cm⁻¹. NMR (300 MHz in deuterated solvents) showed the material to be homogeneous.

2,4-dimethyl-2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-β-D-glucopyranoside—This compound was prepared from 2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-β-D-glucopyranosyl chloride (7.5 g) and p-methoxphenol (8 g) by the method (18) described above. The combined CHCl₃ extracts were dried over anhydrous MgSO₄ (5 g) and were evaporated to 50 ml at 37°C. As this solution was thoroughly mixed, anhydrous ether (250 ml) was added. The obtained precipitate was recrystallized from CHCl₃/methanol, 2:1, to yield 9.49 g of product, m.p. 238-240°C. TLC analysis (CHCl₃/methanol, 5:1) yielded one spot, Rₜ = 0.08. IR spectra analysis (KBr pellet): 3440 (NH), 1750 (acetate, C=O), 1675 (amide type I), 1540, 1520 (NH), 1230 (C=O-C), 1290 (shoulder), 1080 (aromatic-CH₂), 1125 and 830 (p-disubstituted phenyl), 1380, and 760 cm⁻¹. NMR (60 MHz in deuterated solvents) showed characteristic absorption of a C-H hydrogen centered at 6.5 ppm; aromatics and hydrogens at 7.5 ppm (methoxy hydrogens), and characteristic acetate and acetaldehyde hydrogens at 1.7 to 2.0 ppm.

Methoxyphenyl-2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-β-D-glucopyranoside—Recrystallization of p-methoxyphenyl-2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-β-D-glucopyranoside (0.5 g) with sodium methoxide followed by Dowex 50 H⁺ treatment proceed as described above. Recrystallization of the product from methanol/ether produced 0.19 g (52%) of product, m.p. 249-251°C. TLC analysis in CHCl₃/Methanol (5:1) yielded one spot, Rₜ = 0.81. [α]D = -91.9°C (c, 0.47, dimethylformamide). IR spectra analysis (KBr pellet): 3310, 3295, 3095, 3000 (NH), 1735 (acetate, C=O), 1675 (amide type I), 1540, 1520 (NH), 1230 (C=O-C), 1290 (shoulder), 1080 (aromatic-CH₂), 1125 and 835 (p-disubstituted phenyl); 1385, 1330, and 1050 cm⁻¹.

p-Nitrophenyl-2-amino-2-deoxy-3,4,6-tri-O-acetyl-β-D-glucosamine hydrochloride (23), p-nitrophenyl-2-benzoylalaminio-2-deoxy-3,4,6-tri-O-acetyl-β-D-glucopyranoside, and the corresponding deacetylated compound (24), p-nitrophenyl-2-difluorooacetamido-2-deoxy-3,4,6-tri-O-acetyl-β-D-glucopyranoside, and the corresponding deacetylated compound (25) were prepared using literature procedures.

Enzyme Assays

β-N-Acetylglucosaminidase, β-N-acetylglucosaminidase, β-galactosidase, α-galactosidase, β-N-acetylgalactosaminidase, β-glucosidase, α-glucosidase, β-mannosidase, α-mannosidase, and β-xylodidase specific activities were all determined by measuring the liberation of p-nitrophenol (as the phenolene anion) from the respective glycosides. In the standard assay, 100 μl of 50 mM sodium citrate (pH 4.8) and 100 μl of 5 mM solutions (2 the appropriate p-nitrophenol glycoside in the same buffer were mixed together and equilibrated at 37°C. To this was added 20 μl of an appropriate dilution of enzyme, and the reaction allowed to proceed at 37°C for either 5 or 10 min, then stopped by the addition of 1 ml of 0.2 M Na₂CO₃. The resulting phenolene anion was measured at 405 nm on an Hitachi/Perkin Elmer model 139 spectrophotometer equipped with digital readout. Enzyme assay using the para-substituted phenyl-2-acetamido-2-deoxy-β-D-glucopyranoside substrate analogs proceeded as above ex-
cept that the reaction was stopped by addition of 1.0 ml of a solution containing 98 ml of 0.5% NaClO in 0.1 N NaOH, 1 ml of 1% potassium tartrate, and 1 ml of 0.5% CuSO₄. It was allowed to stand for 10 min, and then was vortexed during the addition of 100 ml of 50% Folin-Ciocalteu reagent. After 90 min, the blue-colored Folin-phenol complex was measured spectrophotometrically at 740 nm. The Folin-Ciocalteu reagent has been used in assaying for a variety of phenols (20). Standard curves for each of the phenols were constructed as were color production versus time curves.

Kinetic Analysis

The kinetic constants (Kᵥ and Vᵥ) were determined by Lineweaver-Burk analysis and by computer analysis of the best fit hyperbola to the v versus 5 kinetic data (27). The computer programs were obtained from Professor W. W. Cleland, University of Wisconsin, and modified slightly to expedite usage with the Cyber 173 computer. The computer-determined kinetic constants did not differ significantly from those obtained by the Lineweaver-Burk method.

Inhibition studies were analyzed primarily by computer programs obtained from Dr. Cleland, but were also analyzed by the Lineweaver-Burk method; both methods yielded comparable results. The enzymatic rate data were normally obtained with two inhibitor concentrations.

pH-rate kinetic experiments were carried out in a phosphate-citrate McIlvaine constant ionic strength (0.1 M) buffer system (29). Enzyme stability was established over the pH range used. The kinetic constants, obtained as above, were plotted as log kᵥ, log Kᵥ, and log kᵥ/Kᵥ versus pH. On the basis of the observed variations of these parameters with pH, the data were fit to appropriate pH functions which yielded the pH-independent values of the kinetic constants and the residual least squares (variance) of the fit (27). The theoretical curves in the figures were subsequently constructed using these values.

Stopped flow studies were carried out on a modified Durrum instrument interfaced with a PDP-8 computer system. 2,4-DNP-N-acetylglucosaminidase was used as substrate for these studies since the production of 2,4-dinitrophenol (the anion) could be followed continuously at 380 nm. The hydrolysis was carried out in 50 mM citrate, pH 4.6, at 25°C. The stopped flow enzymatic rates were determined for the first 200 ms of the reaction, and were corrected for spontaneous hydrolysis of substrate.

RESULTS

Purification of β-N-acetylglucosaminidase

Crude Extract—Rhozyme HB-150 (Rohm and Haas), an acetone powder extract of A. niger (100 g), was suspended in 650 ml of distilled water, stirred for 1 h, then centrifuged at 32,000 × g. The supernatant was saved, the pellet was reextracted with an additional 200 ml of distilled water, and the supernatants were combined ("crude extract").

Ammonium Sulfate Precipitation—To 850 ml of crude extract were added 476 g of (NH₄)₂SO₄. The suspension was stirred for 15 min and centrifuged at 31,000 × g for 1 h. The resulting pellet was redisolved in 1.6 liters of 60 mM phosphate, pH 4.6. To this were added 1230 g of (NH₄)₂SO₄; the suspension was stirred for 30 min and then centrifuged as above. The supernatant was discarded and the pellet was redisolved in a minimum volume (~600 ml) of 50 mM citrate, pH 4.6 ("100% ammonium sulfate precipitation").

Batch DEAE—The 600 ml of 100% ammonium sulfate precipitation were diluted to 6 liters with 3 liters of distilled water and 2.4 liters of 50 mM phosphate, pH 4.6; the pH was adjusted to 4.8 with 5 N NaOH. Enough 20% NaCl was added to bring the conductivity from 7.8 to 12.0 mmho. To this solution were added 476 g (suction filtered wet weight) of DEAE-cellulose previously equilibrated with 50 mM phosphate, pH 4.8. The suspension was mixed thoroughly for 15 min, then suction filtered. The filter cake was resuspended in 500 ml of 50 mM phosphate, pH 4.8, which contained 0.15 M NaCl and was suction filtered again. The filtrates ("batch DEAE") were saved.

Batch Hydroxylapatite—The 8 liters of batch DEAE were diluted with distilled water until a conductivity of 2.0 mmho was attained (to 12 liters). To this were added 1.2 liters of swelled and settled hydroxylapatite HTP (~400 g of dry weight), the suspension stirred for 20 min, and then suction filtered. The filtrate was discarded and the filter cake was resuspended in 2 liters of 0.2 M phosphate, pH 4.8, stirred for 20 min, and suction filtered again. The filtrate was saved ("batch HA") following an additional washing (500 ml) of the hydroxylapatite.

PM10 Ultrafiltration—The batch HA solution was concentrated (Amicon PM10), washed with 1 liter of distilled water, and reconcentrated to a final volume of 57 ml ("PM10 ultrafiltrate").

Sephadex G-150—The column (2.5 × 100 cm) was packed and equilibrated with 50 mM phosphate, pH 4.8. A portion (25 ml) of PM10 ultrafiltrate was loaded and eluted with 50 mM phosphate, pH 4.8, at a flow rate of 9.5 ml/h. Fractions of 5 ml were collected after an initial 150 ml had eluted. Fractions 5 through 25, which contained the major peak of enzymatic activity, were pooled ("Sephadex G-150").

PM10 Ultrafiltration II—The Sephadex G-150 fraction was dialyzed against distilled water (4 liters for 2 h), and the pH of the solution was lowered to 3.9 by addition of 5 mM H₂PO₄. Following concentration to 50 ml (Amicon PM10), the solution obtained ("PM10 ultrafiltrate II") was in 2 mM phosphate, pH 3.9.

Sulfoethyl-Sephadex—The column (2.5 × 100 cm) was packed and equilibrated with 5 mM citrate, pH 3.9. The PM10 ultrafiltrate II (25 ml) was loaded and the column eluted with a stepwise pH gradient, as shown in Fig. 1. The fractions indicated in Fig. 1, which contained the most highly purified β-N-acetylglucosaminidase, were pooled and then dialyzed against 50 mM citrate, pH 4.6, for storage. This was the fraction used for the characterization and kinetic studies described in this paper. These purification steps are summarized in Table I. Overall purification was ~153-fold with a recovery of 14% of the original activity.

Homogeneity

Isoelectric focusing of purified enzyme resolved one major and one minor component, both of which exhibited enzymatic activity.

Fig. 1. Ion exchange chromatography of partially purified β-glucosaminidase on Sulfoethyl-Sephadex. Fifty milliliters of concentrate of the appropriate fractions from the Sephadex G-150 column (see text) were applied to the column (2.5 × 55 cm). The column was eluted stepwise with 5 mM sodium citrate buffers, pH 3.9, 4.55, and 4.75, at a flow rate of ~30 ml/h. Five-milliliter fractions were collected. ---, C, β-glucosaminidase activity; -○-, absorbance at 280 nm. The pooled fractions noted in the figure were used for the studies described herein.
activity (Fig. 2). The major component had an isoelectric point of 4.4. The $\beta$-glucosidase (and $\beta$-galactosaminidase) activity (see also Table I) comigrated with $\beta$-glucosaminidase activity (Fig. 2). The purified enzyme was tested for other glycosidase activity by assay with the appropriate $p$-nitrophenyl glycosides (Table II). These specific activities were not of photo-polymerized gels which contained 60% 4 to 6 ampholines, glycosidase activity by assaying with the appropriate p-nitroanilide with acrylamide. Enzyme, 150 pg, was applied to the top of photo-polymerized gels using a modification pH 4.6, at 37°C as described under "Experimental Procedures." H, and 30% 3 to 45% 1.62

**Table I**

Purification of $\beta$-N-acetylglucosaminidase from *A. niger*

| Step            | Total activity | Specific activity | Yield (%) in step | Glmase$^/$ \textit{G}aldmase |
|-----------------|----------------|-------------------|-------------------|-----------------------------|
| 1. Crude extract| 23,603         | 0.47              | 100               | 1.55                        |
| 2. 100% ASP     | 22,720         | 1.20              | 96                | 1.56                        |
| 3. Batch DEAE   | 22,185         | 2.20              | 94                | 1.46                        |
| 4. Batch HA     | 13,045         | 2.77              | 94                | 1.50                        |
| 5. PM10 ultrafiltrate | 9,915   | 2.96              | 55                | 1.50                        |
| 6. Sephadex G-150| 9,728          | 20.48             | 41                | 1.50                        |
| 7. PM10 ultrafiltrate II | 7,296 | 31                | 75                | 1.62                        |
| 8. Sulphopropyl-Sephadex | 3,261 | 72.18             | 14                | 1.50                        |

$^*$ $\beta$-Glucosaminidase to $\beta$-galactosaminidase activity ratio determined as described under "Experimental Procedures."

**Fig. 2**. Isoelectric focusing of purified $\beta$-glucosaminidase on polyacrylamide disc gels using a modification of the Baumann and Chrambach method (20) employing N,N'-diallyltartardiamide with acrylamide. Enzyme, 150 pg, was applied to the top of photo-polymerized gels which contained 60% 4 to 6 ampholines, and 30% 3 to 10 ampholines. Focusing proceeded at constant voltage (200 V) for 20 h. One gel was stained for protein after fixing in 10% trichloroacetic acid for 3 h, and the other was sliced and assayed for pH and enzymatic activity. $\bullet$, $\beta$-glucosaminidase activity; $\circ$, $\beta$-galactosaminidase activity; and $\square$, pH.

**Table II**

Glycosidase activity present in purified $\beta$-glucosaminidase

Assays were performed using 2.5 mM substrate in 50 mM citrate, pH 4.6, at 37°C as described under "Experimental Procedures."

| Enzyme activity | Specific activity |
|-----------------|------------------|
| $\beta$-Glucosaminidase | 72.20 |
| $\beta$-Galactosaminidase | 44.60 |
| $\alpha$-Galactosidase | 0.00 |
| $\alpha$-Glucosidase | 0.00 |
| $\beta$-Glucosidase | 17.00 |
| $\alpha$-Mannosidase | 0.00 |
| $\beta$-Mannosidase | 0.00 |
| $\gamma$-Xylosidase | 2.80 |

versus c intersected on the $1/\sigma_{\text{app}}$ axis, which is also consistent with homogeneity (Fig. 3B). The negative slopes of these plots suggest a self-associating system. Furthermore, in a two species plot (Fig. 3A), the data points fell on a straight line between the theoretical monomer-dimer and monomer-trimer behavior. Similar results were obtained at two enzyme concentrations (0.5 and 1.0 mg/ml).

**Composition**

The amino acid composition of purified $\beta$-N-acetylglucosaminidase is given in Table III. The analysis showed a low mole per cent of Lys, His, and Arg, and a high mole per cent of Asp and Glu. This is consistent with the low pI and pH optimum of this enzyme. No cysteine or cysteic acid was detected in any of the sample runs.

**Fig. 3**. Sedimentation equilibrium studies performed on a Beckman model E analytical ultracentrifuge using double sector cells and Raleigh interference optics at 12,000 rpm, for 27 h at 20°C, with 1.0 mg/ml of enzyme. The data were analyzed according to the method of Roark and Yphantis (31). In B, the two standard plots of concentration in the cell versus $1/\omega t$ are presented. The lines are least squares fit to the data points. In A, a two species plot (31, 32) is presented as $1/\omega t$ versus $c$. The theoretical 2 $\times$ M, and 3 $\times$ M are represented as (- -) with the data as closed circles. The experimental line is a least squares fit to the data points.

**Table III**

Amino acid composition of purified $\beta$-glucosaminidase

An average of 22-, 48-, and 72-h values unless otherwise noted. Hydrolyses in 6 N HCl and in 3 M methane sulfonic acid were both used. Analyses were performed on a Beckman/Spinco amino acid analyzer model MS.

| Amino acid | Mol % |
|------------|-------|
| Lysine     | 2.38  |
| Histidine  | 1.59  |
| Arginine   | 2.85  |
| Aspartic acid | 13.13* |
| Threonine  | 8.27* |
| Serine     | 8.04* |
| Glutamic acid | 9.04 |
| Proline    | 5.96  |
| Glycine    | 9.53  |
| Alanine    | 8.76  |
| Cystine    | <0.1  |
| Valine     | 7.29  |
| Methionine | 1.19  |
| Isoleucine | 4.44  |
| Leucine    | 7.35* |
| Tyrosine   | 5.02* |
| Phenylalanine | 3.81 |
| Tryptophan | 1.33  |

$^*$ Extrapolated to zero time.

$^*$ 72-h value.

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A. niger $\beta$-N-Acetylgalactosaminidase
percentage of Man and smaller, but significant, levels of GlcNAc. Small amounts of Gal and Fuc were present as well. Glc was detected, but was considered to be a contaminant arising from the Sephadex columns used in the purification. A $M_0 = 148,000 \pm 2,500$ was determined using the sedimentation data. A $M_0 = 149,000$ was calculated based on the amino acid composition.

**Effect of Metals and Other Inhibitors**

Sodium chloride up to 0.5 M showed no inhibitory effect on purified $\beta$-N-acetylglucosaminidase. At 100 mM, Zn$^{2+}$, Ca$^{2+}$, Cu$^{2+}$, Hg$^{2+}$, and Ag$^+$ all significantly inhibited the enzyme; Ag$^+$ and Hg$^{2+}$ were most effective. Neither iodoacetamide nor $p$-chloromercuribenzenesulfonic acid caused significant inhibition of enzymatic activity. Atomic absorption spectrophotometric analysis indicated that the purified enzyme contained neither zinc nor magnesium.

**Kinetic Analysis**

The kinetic behavior of $\beta$-N-acetylglucosaminidase toward a variety of substrates and substrate analogs is presented in Table V. The enzyme was inhibited by substrate concentrations $>3$ mM. Note that $p$-Np-$\beta$GlcNAc and $p$-Np-$\beta$GlcNBz were neither substrates nor inhibitors at the concentrations employed, while Glc was without effect on the hydrolysis of glycosaminides.

**pH Dependence**

Detailed pH-rate studies were performed using $p$-Np-$\beta$GlcNAc over a pH range, 2.5 to 8.0. The $K_m$ and $k_{cat}$ values for the enzymatic hydrolysis at these pH values were determined as described under "Experimental Procedures." These data, expressed as log $k_{cat}$, $pK_m$, and log $k_{cat}/K_m$ versus pH, are presented in Fig. 4.

The theoretical curves in Fig. 4 were derived using the following $pK_a$ values: for the $\beta$-glucosaminidase reaction.

**Table IV**

| Monosaccharide | Weight | Integral value |
|----------------|--------|----------------|
| GlcNAc         | 2.39   | 16             |
| GalNAc         | 0.00   | 0              |
| Man            | 7.00   | 57             |
| Gal            | 1.22   | 10             |
| Fuc            | 0.13   | 1              |
| Glc$^a$        | 1.89   | 15             |
| Sia            | 0.00   | 0              |

$^a$ Considered suspect because of possible contamination by columns used for purification of the protein.

**Table V**

| Compound | $k_{cat}$ | $K_m$ | $K_a$ |
|----------|-----------|-------|-------|
| $p$-Np-$\beta$GlcNAc | 1.12 x 10$^4$ | 0.34 |       |
| $p$-Np-$\beta$GlcNBz | 6.62 x 10$^4$ | 0.86 |       |
| $p$-Np-$\beta$GlcN-TAc | 1.7 x 10$^4$ | 1.3  |       |
| 2,4-di-Np-$\beta$GlcN | 2.3 x 10$^4$ | 0.27 |       |
| $p$-Np-$\gamma$-T-$\beta$GlcN | 1.14 |       |       |
| GlcNAc        | 0.41 |       |       |
| GlcN          | 16   |       |       |

The method of Spackman et al. (33) was used for amino sugar analysis while the alditol acetate method of Lehnhard and Winder (34) was employed, while Glc was without effect on the hydrolysis of glycosaminides. Small amounts of Gal and Fuc were present as well. The method of Spackman et al. (33) was used for amino sugar analysis while the alditol acetate method of Lehnhard and Winder (34) was employed, while Glc was without effect on the hydrolysis of glycosaminides. Small amounts of Gal and Fuc were present as well. Glc was detected, but was considered to be a contaminant arising from the Sephadex columns used in the purification. A $M_0 = 148,000 \pm 2,500$ was determined using the sedimentation data. A $M_0 = 149,000$ was calculated based on the amino acid composition.

**FIG. 4. pH-rate curves for $\beta$-glucosaminidase activity.** $\beta$-Glucosaminidase was assayed using $p$-Np-$\beta$GlcNAc as substrate in a McIlvaine constant ionic strength buffer system. The initial velocities were determined and used to calculate log $k_{cat}$, $pK_m$, and log $k_{cat}/K_m$, which are presented versus pH. The solid lines represent the theoretical curves calculated using the appropriate pH functions (28).

**FIG. 5. Substituent analysis of $\beta$-glucosaminidase reaction.** The reaction was measured using various para-substituted phenyl derivatives of GlcNAc (A) and fluoroacetamido derivatives (B). Initial velocity data were used to generate $K_m$ and $V_{max}$ values for the various derivatives. The parameter log $V_{max}/K_m$ calculated from these data, is plotted versus $\sigma^+$ for the various substituents (36). A, $\sigma^-$, $p$-NO$_2$, p-Cl; A, p-H; and $\times$, p-OCH$_3$. B, $\sigma^-$, acetamido; $\sigma^-$, difluoroacetamido; and A, trifluoroacetamido. Rates were obtained at 37°C in 50 mM citrate, pH 4.6.

**Kinetic Solvent Isotope Effect**

Rates of hydrolysis of $p$-Np-$\beta$GlcNAc were determined in D$_2$O at pH (pD) 4.6 and 7.0. The $k_{cat}$ values of H$_2$O/D$_2$O found were 1.27 and 1.00, respectively.

**Substituent Effects**

Hammett studies were carried out utilizing p-Cl, p-NO$_2$, p-H, and p-OCH$_3$-substituted phenyl glucoside derivatives of GlcNAc. The rate of enzymatic hydrolysis of these derivatives is plotted as log $V_{max}/K_m$ versus $\sigma^+$ (36) in Fig. 5A. The slope of the line, equivalent to the $p$ value for the reaction, is +0.36. Taft-type studies were carried out also utilizing p-nitrophenyl-2-deoxy-N-trifluoroacetyl, N-difluoroacetyl, and N-acetyl-$\beta$-N-glucosaminide substrate analogs. The rate of enzymatic hydrolysis of these derivatives is plotted as log $V_{max}/K_m$ versus $\sigma^+$ (36) in Fig. 5B. The slope of the line, the $p$ value for the reaction, is $-1.41$. There were only minor vari-
ations in the $K_m$ values for the members of these two series of substrates.

**Stopped Flow Studies**

In an effort to demonstrate a “burst” phenomenon in the enzymatic hydrolysis, stopped flow studies were conducted using the substrate analogue, 2,4-diNp-$\beta$GlcNAc. The hydrolysis of this substrate was followed continuously at pH 4.6 by measuring the production of the 2,4-dinitrophenolate anion. The $k_{\text{cat}}$ obtained from the stopped flow experiments (1958 ± 150 min$^{-1}$) was compared to the $k_{\text{cat}}$ determined in steady state studies (2300 ± 250 min$^{-1}$) with the same substrate analogue; the two rates were found to be indistinguishable within experimental error. Steady state kinetic studies established a $K_m$ value of 0.27 mM for this substrate (Table V), thus demonstrating a binding comparable to that observed for the $p$-nitrophenyl derivative.

**DISCUSSION**

The isolation of *A. niger* $\beta$-N-acetylglucosaminidase resulted in an overall purification similar to that reported for other fungal hexosaminidases (37-39). The purified protein exhibited apparent homogeneity on polyacrylamide gel electrophoresis, with the only protein band coinciding with enzyme activity. As noted, the ratio of $\beta$-glucosidase to $\beta$-N-acetylglucosaminidase activities co-purified, indicating that both were associated with the same protein. The absence of GalNAc suggests that the enzyme preparation also possessed $\alpha$-glucosidase as well as $\beta$-N-acetylglucosaminidase activity. Although other $\beta$-N-acetylglucosaminidases lack GalNAc, most contain Sia (42, 45); both are apparently absent in the *A. niger* protein. The enzyme does not contain either metal nor did it require either for activity. A catalytic role(s) for a carboxyl, imidazole, or tyrosyl side chain cannot be ruled out (see below).

The kinetic studies provide some insight into the mechanism of action of this enzyme. The two mechanistic questions posed by this enzyme type are: 1) is the reaction kinetically sequential or ping-pong (Schemes I and II below); and 2) is the reaction chemically SN1, SN2 or, perhaps, SN1 in nature?

The lack of a burst of 2,4-dinitrophenolate anion (aglycone) in the stopped flow studies using 2,4-diNp-$\beta$GlcNAc and the dependence of the rate of substrate hydrolysis on the nature of the aglycone ($\rho = 0.36$) shows that if Scheme II obtains, $k_s < k_b$. However, the interaction between the acetamido group and the C1 carbon indicated by this work would appear to preclude simple mechanisms involving an enzyme nucleophile. In such a mechanism, the side chain and enzymatic group would presumably approach the glycosidic carbon from the same a or axial direction in competition with one another. Consequently, these inferences most reasonably support Scheme I for the hydrolysis of N-acetylglucosaminides, but, by themselves, do not indicate whether the reaction is a SN1, SN2, or SN process.

That the acetamido group is involved in the reaction is indicated by: 1) $\rho^* = -1.41$ for the hydrolysis of the fluoro-substituted N-acyl derivatives; and 2) the weak substrate activity of the thioacetamido derivative. These substrates exhibited Michaelis constants comparable to that for P-Np-$\beta$GlcNAc, thus, their poor substrate activity was not due to large differences in binding. These data suggest that 1) the acetamido group is needed, and 2) the basicity of the carbonyl oxygen (or sulfur) modulates catalysis. Replacement of oxygen by sulfur diminishes the basicity of this structural atom as does the increasing electron withdrawal caused by fluoro substitution in the acetamido group. Based on relative velocities, Yamamoto has calculated a $\rho^*$ value of $\sim 0.6$ for the effect of acetamido side chain substitution in the substrates for Taka-$\alpha$-acyt-$\beta$-glucosaminidase (15). Steric factors may also reduce the activity of the thio-carbonyl derivative, although such a large effect is most easily rationalized on the basis of a geometrically precise function for the carbonyl group, e.g. oxazoline formation. Although oxazoline formation...
requires an unfavorable C1-C2 transdiastxial conformation of
the substrate, this conformation could be stabilized by the
effective ion pairing provided by the ring structure (1). Fur-
thermore, acid-catalyzed hydrolysis of derivatives of GlcNAc
has been shown to proceed through an oxazoline intermediate
(10). We propose, therefore, that the acetamido group pro-
vides anchimeric assistance to glycoside bond cleavage by
electrostatic stabilization of the oxocarbonium ion or, perhaps,
by facilitating intermediate oxazoline formation. Thus, the
overall reaction may be described as a kinetically sequential
process (Scheme I) which involves an SN1 or SN2 displacement
in one or a series of steps within a single central complex.

In contrast to the effects of acetamido group alteration,
aglycone substitution was without large effect ($\rho = +0.36$).
Also, the $k_{cat}$ value of H$_2$O/D$_2$O, 1.27, indicated that proton
transfer(s) did not appear to be an important component of
the rate-determining step. The interpretation of these data is
conceptually simple in the context of the general scheme for
acid-catalyzed acetal (ketal) hydrolysis outlined by Jencks
(48), Dunn (49), and Bruce (3) (Scheme 3).

The general acid-catalyzed hydrolysis of glycosides by gly-
cosaminides is described here as an SN2 reaction, apparently
proceeding via the path A $\rightarrow$ D generating the oxocarbonium
ion. This model represents a chemically concerted process
over a saddle point in the free energy contour linking the four
ground states. For the contour to have such a saddle point,$\Delta G^3_A \rightarrow B$ must be similar to $\Delta G^3_C \rightarrow B$ if this condition is not
fulfilled, the reaction will proceed exclusively through either
state B or C (3). Based on the evidence discussed above for
$\beta$-N-acetylgalactosaminidase, this condition is achieved by the
anchimeric assistance provided by the acetamido group. That
is, the stabilization by this group of the oxocarbonium ion,
state D, lowers the free energy of states B and D relative to C,
and thus generates a concerted path (via a saddle point)
between A and D. This model, by itself, does not deal with the
fate of the oxocarbonium ion, i.e. whether or not it is
trapped by H$_2$O, an enzyme nucleophile, or the acetamido
group.

The other data can be interpreted in terms of where this
saddle point is relative to states B and C (and A and D). For
example, the small effect of aglycone substitution ($\rho = +0.36$)
can be due to the opposing effects of electron withdrawal
(basicity) on protonation (C) and bond cleavage (B). The
substrate p-Np-1-Th-GlcNAC suffers a similar fate and con-
sequently is not hydrolyzed. The sulfur is a poorer base than
is oxygen and, thus, would not be protonated readily, desta-
bilizing state C. At the same time, the p-nitrothiophenolate
moiety is a poorer leaving group that the p-nitrophenolate
itself; thus, state B is also destabilized. Consequently, the free
energy of the saddle point is raised and no hydrolysis is
observed.

The solvent isotope effect at pH 4.6 was consistent with the
results of Hamnett studies. The latter indicate that the
transition state looks somewhat more like state C than B.
This suggestion is based on the fact that $\rho$ for specific acid
catalysis (through state C) is small and negative (~0.06 to
-0.6), while for alkaline and spontaneous hydrolysis, where
the phenolate anion is fully formed as in B, $\rho$ values range
from +2.5 to +2.8 (1, 10). Therefore, the proton transfer in
the concerted process as indicated by the data appears to be
nearly complete in the transition state. Under these circum-
cstances, a small solvency isotope effect, such as is observed
here, would be expected. The lack of a solvent isotope effect
at pH 7.0 may be explained on the basis of a shift in the saddle
point toward state B. This could be caused by the greater
stability of the conjugate base of the aglycone (the nitrophen-
olate anion) at higher pH which reduced the contribution of
proton transfer in the rate-determining step. Under these
conditions, proton transfer would appear to occur after this
step. This implies that the $\rho$ value for the hydrolysis of the
glucosaminides studied would be pH dependent and would
be larger at neutral and alkaline pH. This possible behavior was
not explored. Such dependence might also indicate whether
the values of $\rho$ reflected only one of a number of partially rate-
determining steps (1).

The pH-rate studies suggested the presence in the free
enzyme of a general acid group(s), $pK_a = 8.2$. This ionizing
function cold be either an imidazole, or a carboxyl with an
unusual $pK_a$; since amino acid analysis showed this enzyme
to lack cysteine, this $pK_a$ cannot be associated with sulphydryl
group dissociation. An attractive alternative is to assign this
$K_a$ to a Tyr residue as Sinnott has suggested for $\beta$-galacto-
sidase (6).

The kinetic $pK_a$ of this group appears to be 7.6 in the
Michaelis complex. If, as has been suggested, proton transfer
to the aglycone occurs early in the reaction, such a transfer is
likely to be reflected in the Michaelis complex to some extent.
The resulting group $pK_a$ in this complex might be taken as
some weighted average of the enzymic $pK_a$ and that of
the leaving group itself. It is tempting to relate the pH
dependence of $K_a$ to this model, too. The ionization of an
enzymic group ($pK_a = 2.5$) enhanced substrate binding. This
could be explained in terms of an electrostatic interaction
between this conjugate base (perhaps a carboxylate group)
and the protonated reaction center. Again, an analogy could
be drawn with $\beta$-galactosidase (6).

There are certain factors which indicate a greater level of
complexity in this enzyme's reaction mechanism than that
apparent in the previous discussion. In particular is the fact
that p-Np-Glc is hydrolyzed at ~20% of the rate of the N-
acetyl derivative (Table II). This suggests that the acetamido
group is not, in fact, involved in a rate-determining step.
However, other data indicate that this substrate may bind at
a different locus and be hydrolyzed by a somewhat different
mechanism than for glucosaminides. This is suggested by: 1)
the lack of inhibition by Glc of glucosaminidase hydrolysis; and 2) the substrate inhibition noted for GlcNAc substrates. That GlcNAc and not Glc is a inhibitor of N-acetylglucosaminidase activity suggests that for the hydrolysis of glucosides a different site is utilized. This site may be nonproductive for glucosaminide hydrolysis, thus causing the substrate inhibition observed with these substrates. The presence of such a nonproductive binding mode may also perturb the protein ionization in the Michaelis complex indicated by the log \( k_{\text{cat}} \) versus pH profile. Consequently, the difference in this \( k_{\text{cat}} \) from that in the free enzyme may not be related to the reaction mechanism per se.

Whether or not acetamido group participation could be important in glucosaminidase hydrolysis of oligosaccharides was not established by this work. In lysozyme catalysis, the relative importance of geometric strain, electrostatic stabilization (by Asp-52), and acetamido participation may vary with the nature of the substrate. Levitt has suggested that distortion is not a major factor in the activation of substrate oligosaccharides (50). In this regard, the \( K_r \) value for GlcNAc (0.41 mm), which was not different from the \( K_r \) value for pNp-βGlcNAc, suggests that this inhibitor is not a true transition state analog for β-glucosaminidase. On the other hand, the crystallographic evidence for lysozyme is that an oxazolinium ion by Asp-52 (51), or general base catalysis by direct measurement, the involvement of this group in the oxocarbenium ion by Asp-52 (51), or general base catalysis by Asp-52 of oxazoline formation (8). However, the model studies of Piszkiewicz and Bruce show clearly that the acetamido group can provide significant anchimeric assistance to glycoside hydrolysis and that this effect is not general-base assisted (8). Significantly, the pH versus \( \log k_{\text{cat}} \) profile for β-N-acetylglucosaminidase indicated a lack of general base catalysis in the enzyme reaction.

Furthermore, the binding of small substrates to lysozyme is a two-step process involving an isomerization of the ES complex (32). What the significance of this is to catalysis, or to differences in the binding of small sugars and oligosaccharides, is not clear. Inasmuch as the crystallographic studies have not yet to elucidate the nature of acetamido group–enzyme interactions by direct measurement, the involvement of this group in the hydrolysis of, at least, some substrates cannot be ruled out. Importantly, substrates lacking this side chain are hydrolyzed by lysozyme as well (53), thus, its presence is not obligatory; however, as with β-N-acetylglucosaminidase, substrate effectiveness is enhanced in the acetamido derivatives. Indeed, as shown in Table V, either the absence of or gross substitution in the acyl group effectively abolished binding of glucosaminides.

The significance of the acetamido group as a catalytic element becomes clearer upon comparing glucosaminidases (lysozyme, N-acetylglucosaminidase) with glycosidases (β-galactosidase). β-Galactosidase from *Escherichia coli* is thought to catalyze the formation of a galactosyl enzyme intermediate (6, 54). However, based on kinetic isotope experiments and an analysis of the β-galactosidase-catalyzed isomerization of lactose to allolactose, Sinnott (6) has suggested that this galactosyl enzyme is a mixture of two species. One is the galactosyl enzyme, *i.e.*, one in which a covalent bond exists between galactose and an enzyme nucleophile, while the other species is an ion pair consisting of the nucleophile and oxocarbenium ion. In the presence of good nucleophiles, e.g. methanol, the latter species can be trapped before it collapses into a covalent bond. In a sense, this behavior may be similar to that of β-N-acetylglucosaminidase in which the role of the putative enzyme anion (nucleophile) might be played by the substrate acetamidogroup. However, as in the β-galactosidase reaction, the degree to which this potentially nucleophilic group participates in covalent bond formation remains an open question.

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