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Purification and Characterization of Rat Liver α-*N*-Acetylglucosaminyl Phosphodiesterase*

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Ajit Varki and Stuart Kornfeld

From the Washington University School of Medicine, Departments of Internal Medicine and Biological Chemistry, St. Louis, Missouri 63110

In an earlier report we described the identification of an α-*N*-acetylglucosaminyl phosphodiesterase that is capable of cleaving the outer phosphodiester-linked α-*N*-acetylglucosamine residues present on the high mannose oligosaccharides of newly synthesized lysosomal enzymes (Varki, A., and Kornfeld, S. (1980) *J. Biol. Chem.* 255, 8398-8401). We have now purified this enzyme 1800-fold with a 24% yield from rat liver, using subcellular fractionation, differential extraction with Triton X-100, DEAE-cellulose chromatography, heparin-Sepharose chromatography, concanavalin A-Sepharose affinity chromatography, and gel filtration on Sephacryl S-300. The purified preparation is free of lysosomal α-*N*-acetylglucosaminidase.

The enzyme exhibited a single form on both the ion exchange and gel filtration steps. It has a broad pH optimum between 6.0-8.0 and is unaffected by divalent cations or reducing agents. The enzyme cleaves α-*N*-acetylglucosamine residues from five different locations on the high mannose oligosaccharide. In the case of molecules with one phosphodiester, the rate of cleavage is not affected by the size of the underlying oligosaccharide or the presence or absence of an asparagine-linked peptide. Molecules with two phosphodiesters are cleaved in a nonrandom manner. The enzyme has no activity toward *p*-nitrophenyl-α-*N*-acetylglucosamine but is capable of cleaving phosphodiester-linked *N*-acetylglucosamine in molecules such as UDP-*N*-acetylglucosamine, indicating that it can only hydrolyze *N*-acetylglucosamine residues that are α-linked to a phosphate group.

Considerable evidence now indicates that the high mannose oligosaccharides of newly synthesized acid hydrolases are modified to generate a phosphomannosyl recognition marker that is necessary for the targeting of these enzymes to the lysosomes (1-11). Work from this laboratory has demonstrated that the early intracellular biosynthetic intermediates of β-glucuronidase contain phosphodiesters between high mannose oligosaccharides and outer α-linked GlcNAc residues (12). Similar findings have been reported by Hasilik *et al.* (13). We subsequently found that such phosphodiesters can be located at five different positions on these oligosaccharides, although the blocking residue is always α-*N*-acetylglucosamine (14). Since previous studies had shown that the mature recognition marker contains mannose 6-phosphate in a phosphomonoester form (5, 6), it appeared that an enzyme or enzymes must be responsible for processing these phosphodiesters to yield the phosphomonoesters of the mature targeting signal. We searched for and identified an α-*N*-acetylglucosaminyl phosphodiesterase activity capable of removing the outer "blocking" α-*N*-acetylglucosamine residues and demonstrated that this enzyme is distinct from the previously described lysosomal α-*N*-acetylglucosaminidase (15).

In this paper we describe the solubilization, partial purification, and characterization of the rat liver α-*N*-acetylglucosaminyl phosphodiesterase.

EXPERIMENTAL PROCEDURES

Materials

Male Sprague-Dawley rats (100–150 g) were obtained from Harlan Industries, Inc. The 3a70 liquid counting mixture and Triton X-100 were purchased from Research Products International. DE52 (DEAE-cellulose) was from Whatman. Amberlite MB-3 mixed bed resin was from Sigma. Concanavalin A-Sepharose (10 mg/ml) and Sephacryl S-300 superfine were from Pharmacia. Heparin-Sepharose (~2.5 × 10^3 units/ml of Sepharose) was kindly provided by Dr. Douglas Tollefsen (Washington University) and was prepared by coupling porcine intestinal heparin (Sigma) to cyanogen bromide-activated Sepharose after the method of March *et al.* (16). Homogenous *Escherichia coli* alkaline phosphatase (EC 3.1.3.1) was a kind gift of Dr. M. J. Schlesinger, Washington University. Endo-β-*N*-acetylglucosaminidase H was from Miles. α-Mannosidase from jack bean meal was prepared as described by Li and Li (17).

Substrates and Inhibitors

UDP-*N*-acetylglucosamine and *N*-acetyl-D-glucosamine 1-phosphate were from Sigma. *P*-Nitrophenyl-α-D-*N*-acetylglucosaminide was from Calbiochem. Purified Proteinase I from *D. discoideum* was kindly provided by Dr. Gary L. Gustafson, University of Montana (18, 19). Purified teichoic acid from the cell walls of *Micrococcus varians* (ATC 29750) (formerly *Staphylococcus lactis* 2102) was the generous gift of Sir James Baddiley, University of Newcastle-Upon-Tyne (20, 21). [6-^{3}H]GlcNAc-[^{32}P]Man$_{>4}$GlcNAc$_2$-Asn-peptide was kindly supplied by Marc L. Reitman of this laboratory and was enzymatically prepared using [β-^{32}P]UDP-[6-^{3}H]GlcNAc and cell extracts from the 15B Chinese hamster ovary cell line (22). GlcNAcα1 → ^{32}P → 6 Manα1-methyl(GlcNAc-P-α-methylmannoside) was prepared enzymatically using rat liver extracts, α-methylmannoside and [β-^{32}P] UDP-GlcNAc.[1] The product was isolated on quaternary aminoethyl (QAE) Sephadex and desalted on a Bio-Gel P-2 column. L-Glycan from group D streptococcus was kindly provided by Dr. John Pazur, Pennsylvania State University (23). Substrates IC_{10} (GlcNAc-P-[2-^{3}H]Man$_{6-9}$GlcNAc) and IAb_{50}((GlcNAc-P)$_2$-[2-^{3}H]Man$_{6-9}$GlcNAc) were prepared from [2-^{3}H]mannose-labeled mouse lymphoma cells as previously described (14). Man$_9$GlcNAc$_1$, Man$_8$GlcNAc$_1$, Man$_7$GlcNAc$_1$, Man$_6$GlcNAc$_1$, Man$_4$Glc$_{itol}$NAc,[2] mannotriose, mannobiose, Manα1 → 2 mannitol, and Manα1 → 3 mannitol were

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[1] M. L. Reitman, and S. Kornfeld, unpublished observations.

[2] The abbreviations used are: Glc$_{itol}$NAc, *N*-acetylglucosaminitol; ManNAc, *N*-acetylmannosamine; Rha, rhamnose. All sugars have the D configuration unless noted otherwise.

prepared as previously described (24). All other chemicals were of reagent grade and were obtained from commercial sources.

Analytical Methods

Protein was determined by the method of Lowry, in the presence of 0.5% sodium dodecyl sulfate to eliminate inteference by Triton X-100 (25). Free *N*-acetylglucosamine was measured by the method of Reissig *et al.* (26). Descending paper chromatography for separation of [^{3}H]mannose-labeled oligosaccharides was carried out in Solvent A: ethyl acetate/pyridine/acetic acid/H_2O (5:5:1:3). Mild acid hydrolysis for cleavage of phosphodiesters, $NaBH_4$ reduction, and acetolysis of phosphorylated oligosaccharides were carried out as previously described (14). The treatment of oligosaccharides with jack bean α-mannosidase and alkaline phosphatase and of glycopeptides with endo-β-*N*-acetylglucosaminidase H has been previously described (14).

Enzyme Assays

The rationale for the α-*N*-acetylglucosaminyl phosphodiesterase assay utilizing substrate IC_{10}, has been discussed (15). The assay was carried out as previously described (15) except for two modifications. First, *N*-acetylmannosamine (10 mM) and EDTA (5 mM) were added to the reaction mixtures to inhibit the lysosomal α-*N*-acetylglucosaminidase and the α1,2-specific mannosidase which were present in the crude fractions. Second, the phosphomonoester ("uncovered") product was eluted from the QAE-Sephadex column with 1 M NaCl containing 0.1 N HCl. This allowed all of the product to be eluted in 1.7 ml. One unit of activity is defined as cleavage of 1% of substrate IC_{10}/h under these conditions. α-*N*-Acetylglucosaminidase was assayed as previously described (15), using the *p*-nitrophenyl-α-D-*N*-acetylglucosaminide as substrate.

Buffers

The following buffers were used during the purification procedure: Buffer A (for all sucrose solutions), 50 mM sodium maleate, pH 6.5, with 5 mM $MgCl_2$; Buffer B, 5 mM sodium maleate, pH 6.8; Buffer C, 5 mM Tris maleate, pH 7.2, with 0.5% Triton X-100 (v/v); Buffer D, 20 mM Tris HCl (pH 8.0), 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ with 0.5% Triton X-100.

Purification of the α-GlcNAc Phosphodiesterase

All procedures were carried out at 4 °C, unless otherwise stated.

Steps 1-3: Homogenization and Subcellular Fractionation—The starting material was 115 g of fresh, finely minced liver from 16 male Sprague-Dawley rats. The homogenization was carried out with three 20-s bursts of a Polytron PCU-2-110 (Brinkmann Instruments) at setting 2 in Buffer A with 0.5 M sucrose (3 ml/g of liver). Subcellular fractionation was performed as previously described (27, 28) with some modifications. Nuclei and intact cells were removed from the homogenate by centrifugation at 600 × *g* for 10 min. The postnuclear supernatant was removed by aspiration and 20-ml aliquots were layered onto 10-ml cushions of 1.3 M sucrose in Buffer A, and centrifuged at 63,000 × *g* for 2 h (Spinco No. 30 rotor). The crude smooth membrane fraction that appeared as a white band above the 1.3 M sucrose layer was removed and adjusted to 0.25 M sucrose and 400 mM NaCl with ice-cold distilled water and 4 M NaCl. The membranes were then pelleted by centrifugation at 50,000 rpm in a 60 Ti (Spinco) rotor for 45 min. The pellet contained 65–70% of the α-GlcNAc phosphodiesterase activity from the original homogenate and was used for further purification.

Step 4: Differential Triton Solubilization—The pellet was resuspended in Buffer B with 0.1% Triton X-100 and 10 mM mannose 6-phosphate using 10 passes of a motor-driven Potter-Elvhejhem homogenization apparatus (final protein concentration, 25 mg/ml) and placed on ice for 2 h. The suspension was centrifuged at 160,000 × *g* for 45 min, and the supernatant was removed. The mannose 6-phosphate was included to ensure release of any receptor-bound lysosomal enzymes (29), especially the α-*N*-acetylglucosaminidase. The pellet was subjected to one more identical extraction except that the mannose 6-phosphate was omitted. Under these conditions greater than 95% of the α-*N*-acetylglucosaminyl phosphodiesterase remained in the pellet. The pellet was next resuspended as described above in 1.0% Triton X-100 (final protein concentration, 20 mg/ml) and placed on ice for 60 min. After centrifugation at 160,000 × *g* for 60 min, the supernatant was removed and the pellet was extracted once more under identical conditions. The supernatants from the last two steps were combined and contained 85% of the α-GlcNAc phosphodiesterase activity originally present in the crude smooth membranes.

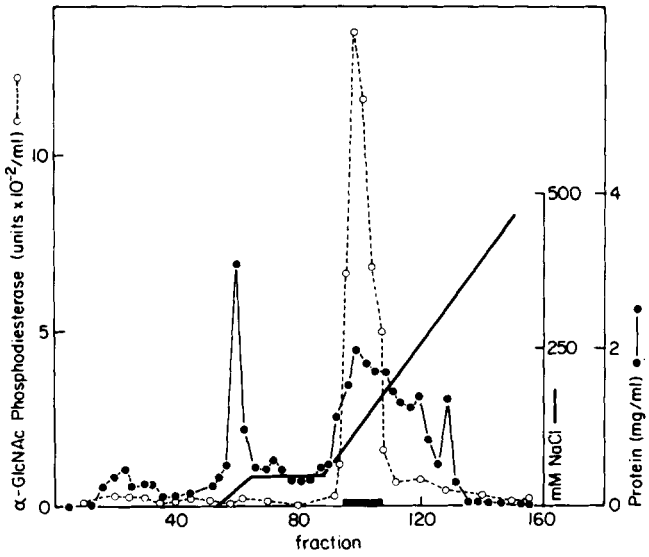

FIG. 1. **Chromatography of α-GlcNAc phosphodiesterase on DEAE-cellulose.** The details are described under "Experimental Procedures." The *black bar* indicates the fractions that were pooled for further purification.

Step 5: DEAE-Cellulose Chromatography—The Triton-solubilized material was dialyzed twice (4 h each) against 20 volumes of Buffer C and then loaded onto a column of DE52 equilibrated in Buffer C. The column (2.7 × 14 cm) was washed with 75 ml of this buffer, followed by 75 ml of the same buffer containing 50 mM NaCl. The column was then eluted with a linear gradient of NaCl (50–500 mM) in Buffer C, over a 150-ml volume. 2.25-ml fractions were collected and monitored for protein and the α-GlcNAc phosphodiesterase activity. As shown in Fig. 1 the enzymatic activity eluted as a single peak, which was pooled as indicated.

Step 6: Heparin-Sepharose—The pool material from the previous step was applied directly to a column of Heparin-Sepharose (0.9 × 15 cm) equilibrated in Buffer C with 150 mM NaCl, and the column was eluted with the same buffer. Under these conditions, half of the applied protein bound to the column, but all of the enzymatic activity passed through.

Step 7: Concanavalin A-Sepharose Affinity Chromatography—The run-through fraction from the previous step was pooled, adjusted to 1 mM $CaCl_2$ and 1 mM $MgCl_2$ using 1 M stock solutions, and applied to a column of concanavalin A-Sepharose (1.5 ml total volume) equilibrated in Buffer D. The eluate was reapplied twice to the column to ensure maximal protein binding.[3] The column was then washed with 30 ml of Buffer D, followed by 6 ml of Buffer D containing 5 mM EDTA and 0.5 M α-methylmannoside. Under these conditions, the α-GlcNAc phosphodiesterase remained bound to the column. The column was allowed to stand at 4 °C for 12 h, brought out to room temperature for 60 min, and then eluted with Buffer D (room temperature) containing 5 mM EDTA and 0.5 M α-methylmannoside. Most of the activity was recovered in the first 1.5 ml eluted from the column.

Step 8: Sephacryl S-300 Chromatography—The 1.5-ml fraction from the concanavalin A-Sepharose column (see above) was applied directly onto a column of Sephacryl S-300 (0.9 × 98 cm) equilibrated in Buffer C. The column was eluted with the same buffer. One-ml fractions were collected and monitored for protein and enzymatic activity. The α-GlcNAc phosphodiesterase activity eluted as a single peak of apparent molecular weight 500,000 (in Triton X-100). The fractions containing enzyme activity were pooled.

RESULTS

Purification of α-N-Acetylglucosaminyl Phosphodiesterase—A representative purification scheme is shown in Table

[3] The recovery at this step could be improved by using a larger concanavalin A column, to bind all of the activity. However, in order to keep the volume low for the next step, the column size was limited to 1.5 ml.

TABLE I

Purification of rat liver α-N-acetylglucosaminyl phosphodiesterase

Experimental details including the enzyme assays are described under "Experimental Procedures."

Step	Protein	Volume	α-N-Acetylglucosaminyl phosphodiesterase				α-N-Acetylglucosaminidase	
			Total activity	Specific activity	Yield	Purity	Yield	Purity
	mg	*ml*	*units*	*units/mg*	*%*	*-fold*	*%*	*-fold*
1. Homogenization	14,241	380	62,777	4.4	100	1	100	1
2. 600 × *g* spin	9,142	302	54,306	5.9	86.5	1.4	66	1.03
3. Sucrose gradient + 0.4 M NaCl wash	521	14	40,962	78.1	65.3	18	8.4	2.31
4. Differential Triton X-100 solubilization	157	22	35,019	223	55.8	51	1.3	1.2
5. DEAE-cellulose	53	28	26,965	509	43.2	116	0.29	0.78
6. Heparin-Sepharose	27	48	27,153	1,006	43.2	228	0.29	1.6
7. Concanavalin A-Sepharose	3.7	1.5	16,450	4,446	26.2	1,008	N.D.[a]	N.D.
8. Sephacryl S-300	1.89	7.3	14,892	7,879	23.7	1,787	<0.03	

[a] N.D., not done.

I. The phosphodiesterase was purified about 1800-fold with a 24% yield of the original activity. As shown in the table, the final preparation was totally free of lysosomal α-*N*-acetylglucosaminidase, an enzyme which is also capable of cleaving α-GlcNAc residues in phosphodiester-linkage to high mannose-type oligosaccharides (12–14). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified enzyme revealed several Coomassie blue-staining bands, suggesting that the preparation is not homogeneous.

Storage and Stability—The purified enzyme lost 50% of its activity when stored at 4 °C for 120 days. Over the same period of time, an identical sample which had been rapidly frozen using liquid nitrogen and stored at −80 °C lost 27% of its activity. The addition of bovine serum albumin (1 mg/ml), glycerol (10%), phenylmethylsulfonyl fluoride (2 mM), or EDTA (5 mM) did not affect this loss of activity. Repeated freezing and thawing caused considerable loss of activity. Repeated dialysis also caused significant loss of activity, even when the dialysate contained Triton X-100.

Effects of Triton X-100—Addition of Triton X-100 to crude membrane preparations resulted in considerable activation of enzyme activity, up to a maximum of 270% with 0.5% Triton X-100 at a protein concentration of 18 mg/ml. The enzyme remained bound to the membranes in the presence of 0.1% Triton X-100 and required a Triton/protein ratio of 1 μl/2 mg for solubilization. Thereafter, 0.5% Triton X-100 was included in all the buffers used for the purification because omission of the detergent appeared to cause a considerable loss of activity at some steps. However, when the purified enzyme was diluted to give a Triton X-100 concentration of 0.01% (critical micellar concentration of Triton X-100, 0.016% (30)), it still retained >90% of its activity.

General Properties—The enzyme was active over a broad range of pH with the optimum being between pH 6–8 (Fig. 2). Five millimolar concentrations of the cations Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Fe^{2+}, Cu^{2+}, and Hg^{2+} were without effect on enzyme activity whereas PO_4^{3} ions caused considerable inhibition. Thus, 5 mM phosphate buffer (pH 7.4) inhibited the enzyme 25%, whereas 33 mM phosphate buffer inhibited 82%. EDTA (5 mM) and dithiothreitol (0.2 mM) had no effect on the enzyme activity.

The Enzyme Cleaves Phosphodiester-linked α-GlcNAc Residues from Five Positions on the High Mannose Oligosaccharide—We have previously shown (14) that substrate IC_{10} consists of a mixture of isomers each of which contains a single phosphodiester-linked GlcNAc residue located at one of three different positions on the underlying oligosaccharide

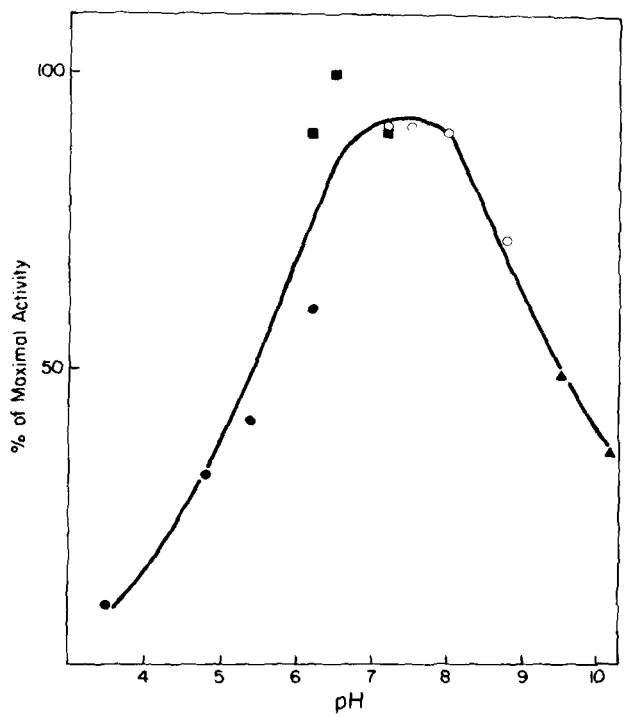

FIG. 2. **Effect of pH on the α-GlcNAc phosphodiesterase activity.** Reaction mixtures (30 μl) contained 1.3 μg (10 units) of the purified enzyme protein, 1000 cpm of substrate IC_{10}, 10 mM ManNAc, and 0.5% Triton X-100 in the appropriate buffer as indicated: ●, 0.05 M sodium citrate; ■, 0.05 M sodium cacodylate; ○, 0.05 M Tris HCl; ▲, 0.05 M sodium carbonate/bicarbonate. The reactions were incubated at 37 °C for 1 h and assayed as described under "Experimental Procedures."

(Fig. 3). Similarly, substrate IAb_{50} contains a mixture of isomers which contain two phosphodiesters at five different positions (Fig. 3). When these compounds were incubated with the purified enzyme for prolonged periods of time (8 h), there was complete conversion of all of the phosphodiesters to phosphomonoesters, demonstrating that the enzyme is capable of acting at all five positions.

To study the effect of the position of the phosphodiesters on the rate of uncovering, an aliquot of IAc_{10} (similar to IC_{10}; see Ref. 14) was treated with the enzyme until 25% of the material was uncovered. The unreacted material and the product were then separated on QAE-Sephadex and each was subjected to $NaBH_4$ reduction and acetolysis which selectively

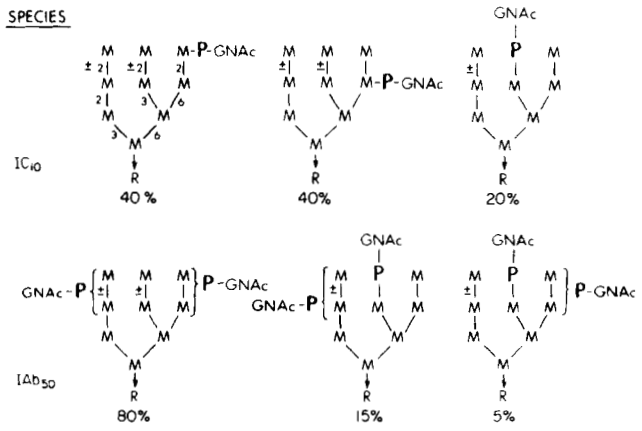

FIG. 3. **Structures of the phosphodiester-containing high mannose oligosaccharides used as substrates.** The various isomers present in IC_{10} (*top row*) and IAb_{50} (*bottom row*) are shown and the relative amounts of each in the mixture are indicated. *GNAc*, GlcNAc; *M*, mannose; *R*, GlcNAc.

cleaves the α1 → 6 linkages (14). The negatively charged fragments, which consisted exclusively of mannobiose (see Ref. 14 and Fig. 3), were isolated on QAE-Sephadex, reduced with $NaBH_4$, treated with alkaline phosphatase, and subjected to high voltage paper electrophoresis to separate Manα1 → 2mannitol from Manα1 → 3mannitol (31). The relative proportions of the α1 → 2- and the α1 → 3-linked mannobiose fragments were as follows: product, 68:32; remaining unreacted material, 82:18; original substrate, 74:26. Thus, under these conditions the isomer with the phosphodiester on the Manα1 → 3Man branch appeared to be uncovered at a slightly faster rate than isomers with the phosphodiester on the Manα1 → 2Man branch even though the former was only 26% of the starting mixture. However, a detailed kinetic analysis of these differences could not be performed because of the limited amount of the substrate and our inability to separate the isomers for individual study.

The time course of uncovering of substrate IAb_{50} is shown in Fig. 4. The intermediate product (one covered and one uncovered phosphate) appeared rapidly, whereas molecules with two uncovered phosphates were only detected after a lag period. In order to determine if the uncovering was proceeding in a random or a nonrandom fashion, a scaled-up reaction was allowed to proceed to partial completion (see *arrow* in Fig. 4) at which point 25% of the material was in the intermediate product. The entire reaction mixture was then treated with alkaline phosphatase to remove the uncovered phosphate residues, leaving the unreacted phosphodiesters intact. This treatment converted the intermediate to a species with one phosphodiester which was then separated from the totally uncovered material (now neutral) and the residual unreacted material by differential elution from QAE-Sephadex. The intermediate and the unreacted molecules were separately treated with mild acid to form phosphomonoesters and reisolated using QAE-Sephadex. The phosphorylated oligosaccharides were then reduced with $NaBH_4$ and subjected to acetolysis to selectively cleave the mannose residues linked α1 → 6, and the resulting fragments were separated into neutral and negatively charged species on QAE-Sephadex as previously described (14). The negatively charged fragments were converted to neutral species with alkaline phosphatase and analyzed by descending paper chromatography in Solvent A. As shown in Fig. 5, *panel B*, the negatively charged fragments arising from the residual, unreacted substrate consisted of $Man_4Glc_{itol}NAc$, $Man_3Glc_{itol}NAc$ and mannobiose. The pattern obtained was virtually identical to that observed with the starting fraction (see Fig. 12 of Ref. 14). If uncovering occurred randomly, then the pattern obtained with the intermediate would be identical with the pattern observed with the residual fraction. However, as shown in Fig. 5, *panel A*, the major phosphorylated fragment from the intermediate was mannobiose. This indicates that uncovering of the IAb_{50} species occurs in a nonrandom fashion with the phosphodiester on the "left side" of the molecule (Fig. 3) being the first to be converted to a phosphomonoester.

Structural Requirements for α-N-Acetylglucosamine Phosphodiesterase Activity—Since IC_{10} (one phosphodiester) contains core oligosaccharides ranging in size from $Man_9GlcNAc$ to $Man_6GlcNAc$ (14), we used this substrate to determine if the size of the underlying oligosaccharide influenced the activity of the phosphodiesterase. An aliquot of IC_{10} (10,000 cpm) was incubated with the enzyme until 10% of the phosphodiesters were cleaved. The reaction was then stopped and the molecules with the exposed phosphomonoesters were separated from the unreacted material by differential elution on QAE-Sephadex. A separate aliquot of IC_{10} was treated directly with mild acid to cleave all the phosphodiesters. The two samples were then converted to neutral species with alkaline phosphatase and the core oligosaccharides were sep-

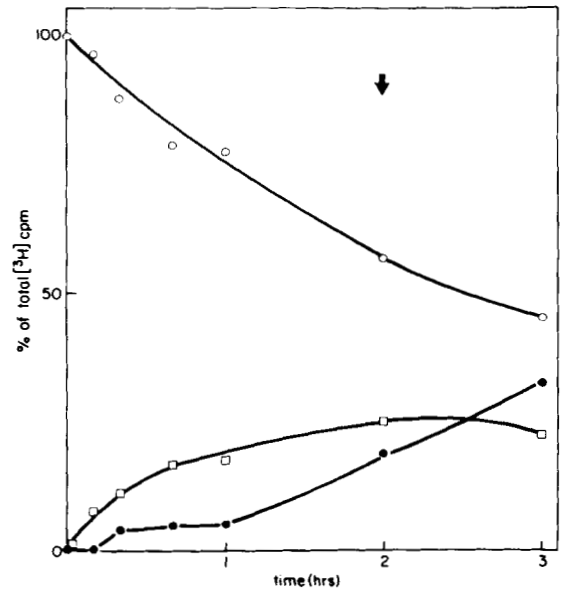

FIG. 4. **Time course of GlcNAc cleavage from oligosaccharides containing two phosphodiesters.** The reaction mixture (120 μl) contained 10.4 μg (80 units) of the purified enzyme protein and 4000 cpm of substrate IAb_{50} in 50 mM Tris HCl, pH 7.4, and 0.5% Triton X-100. The enzyme and rest of the mixture were prewarmed separately at 37 °C and then mixed. Fifteen-μl aliquots were removed at the times indicated and immediately heated to 100 °C for 5 min to inactivate the enzyme. After cooling, 1 μl of 1 M Tris base and 3 μl (0.6 units) of *E. coli* alkaline phosphatase were added to each (final pH 8.5) and incubated at 37 °C for 3 h to cleave all the exposed phosphomonoesters. The reaction mixtures were diluted to 1 ml with distilled water and applied to columns of QAE-Sephadex (0.5 × 3 cm) equilibrated in 2 mM Tris base. Each column was eluted successively with 6 ml of 2 mM Tris base, 8 ml of 20 mM NaCl in 2 mM Tris base, and 8 ml of 70 mM NaCl in 2 mM Tris base. The ^3H radioactivity in each fraction was determined and expressed as a percentage of the total radioactivity eluted from each column. The time course of the change in the percentage of the following species is shown: ●——●, molecules in which both phosphodiesters were cleaved (run-through of the QAE-Sephadex column); □——□, molecules in which one phosphodiester remained intact (eluting with 20 mM NaCl); and ○——○, material with both phosphodiesters remaining intact (eluting with 70 mM NaCl). The *arrow* indicates the time point at which another scaled up reaction was carried out, to provide material for detailed structural studies of the various fractions.

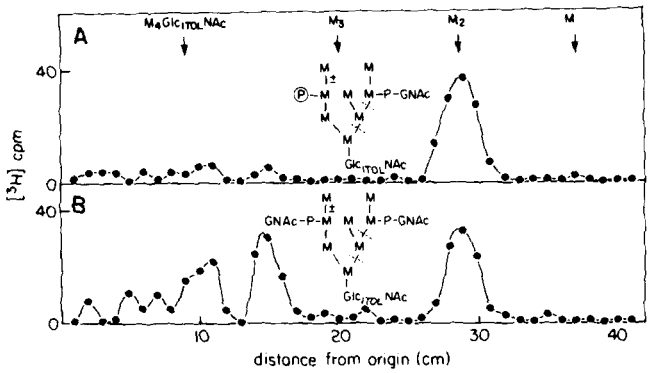

FIG. 5. **Paper chromatography of negatively charged acetolysis fragments.** A reaction containing $IIAb_{50}$ and the α-GlcNAc phosphodiesterase was allowed to proceed up to the point indicated by the arrow in Fig. 4. The reaction was stopped, the products were treated with alkaline phosphatase to destroy all "uncovered" phosphomonoesters and fractionated on QAE-Sephadex, and each fraction was subjected separately to acetolysis as described in the text. As indicated on the figure, acetolysis will cleave the molecules at the α1 → 6 linkages (---). The products of acetolysis were fractionated into neutral and negatively charged fragments on QAE-Sephadex as previously described (14). The negatively charged fragments from molecules with one remaining phosphodiester (*Panel A*) and those from molecules with both phosphodiesters intact (*Panel B*) were treated with alkaline phosphatase, desalted on Amberlite MB-3, and spotted on Whatman No. 1 paper. The chromatograms were developed in Solvent A for 18 h. One-cm strips were cut and the radioactivity was determined. The standards are: *$M_4Glc_{itol}NAc$*, Man$_4$-*N*-acetylglucosaminitol; *M_3*, Manα1 → 2Manα1 → 3Man; *M_2*, Manα1 → 2Man; and *M*, free mannose. Ⓟ, the alkaline phosphatase susceptible phosphomonoester of the intermediate that was removed prior to acetolysis.

arated by descending paper chromatography in solvent A for 4 days. The percent distribution of the label in the $Man_9GlcNAc$, $Man_8GlcNAc$, $Man_7GlcNAc$ and $Man_6GlcNAc$ species was as follows: 31:39:20:10 in the untreated substrate and 35:39:11:15 in the enzyme product. Since there was no significant enrichment of any species in the enzyme product, we conclude that the size of the underlying oligosaccharide in IC_{10} *has no significant effect on the activity of the phosphodiesterase.*

To modify the underlying oligosaccharide of IC_{10} further, the compound was incubated with jack bean α-mannosidase to remove all of the mannose residues except those blocked by phosphodiester groups (14). This treatment produces three isomers with the following structures: GlcNAc-P-Man1 → 2Man1 → 6Man1 → 6Man1 → 4GlcNAc, GlcNAc-P-Man1 → 6Man1 → 6Man1 → 4GlcNAc, and GlcNAc-P-Man1 → 3Man1 → 6Man1 → 4GlcNAc (see Fig. 3). These phosphorylated fragments were then separated from the released mannose by gel filtration on Bio-Gel P-2 and tested as substrates with the phosphodiesterase. The initial rate of cleavage of the phosphodiesters of these fragments was essentially identical with the rate of cleavage of untreated IC_{10} (data not shown), again indicating that the activity of the α-GlcNAc phosphodiesterase is not significantly affected by the size of the underlying oligosaccharide unit.

All of the above experiments were performed with phosphorylated oligosaccharides which had been released from glycopeptides by cleavage of the chitobiosyl core with endo-β-*N*-acetylglucosaminidase H. In order to study whether the phosphodiesterase activity was influenced by the presence of the peptide backbone, we utilized a glycopeptide substrate with the composition [6-^{3}H]GlcNAc-^{32}P-$Man_{>4}GlcNAc_2$-Asn-peptide. This substrate was purified from an *in vitro* reaction which utilized [β-^{32}P]UDP-[6-^{3}H]GlcNAc as donor and endogenous glycoproteins as acceptors (22). The phosphorylated

TABLE II

Comparison of the activity of the α-N-acetylglucosaminyl phosphodiesterase on various substrates

The assays contained 2–10 μl of the purified enzyme in a 30-μl final volume, with 50 mM Tris HCl, pH 7.5, and 0.5% Triton X-100, and various concentrations of the substrate. *N*-Acetylglucosamine release was assayed in one of three ways: 1) run-through of [6-^{3}H]GlcNAc through Dowex AG 1-X8 (substrates 1 and 2) 2) by the method of Reissig *et al.* (26) (substrates 3–6), and 3) by ΔA_{410} caused by the *p*-nitrophenol release (substrate 7). All assays were performed in duplicate and compared with similarly prepared blanks in which the enzyme was left out. Michaelis-Menten constants for substrates 1–3 were derived from double-reciprocal plots ([1/*v*] *versus* [1/*s*]) of multiple data points. These assays were performed at a time when the enzyme had lost 50% of its original activity.

Substrate	V_{max}	Apparent K_m
	nmol/μg/h	*mM*
1. GlcNAc-P-α-methylmannoside	3.17	0.19
2. UDP-GlcNAc	22.8	1.3
3. GlcNAc-1-P	0.34	1.6
	V^a	Concentration[b]
	nmol/μg/h	*mM*
4. *M. varians* teichoic acid (GlcNAc-P-GlcNAc)$_n$	4.13	1.25
5. Proteinase I (contains GlcNAc-P-serine)	0.85	0.75
6. L-Glycan (contains GlcNAc-β-P-Rha)	<0.19	0.4
7. *p*-Nitrophenyl-α-D-GlcNAc	<0.004	3.0

[a] For various reasons, it was not possible to obtain Michaelis-Menten constants for some of the substrates. In these cases the velocity obtained with the maximum concentration studied is reported.

[b] Indicates the concentration of terminal α-linked GlcNAc residues.

TABLE III

Effect of various compounds on the α-GlcNAc phosphodiesterase activity

The activity of the purified enzyme against the phosphodiester-linked GlcNAc in substrate IC_{10} (1000 cpm) was assayed as described in the presence or absence of the various compounds at the final concentrations noted. Results are expressed as the percentage inhibition of the initial rate of uncovering. Changes of <10% were not considered significant.

Compound	Concentration	Inhibition
	mM	%
N-Acetylglucosamine	2.5	39
	5	56
	10	67
N-Acetylmannosamine	10	<10
N-Acetylglucosamine 1-phosphate	2.5	73
	5	82
	10	98
UDP-*N*-Acetylglucosamine	5	78
	10	97
UDP-galactose	10	12
Mannose	5	20
	10	19
α-Methylmannoside	5	22
	10	18
Inorganic phosphate	5	25
	33	82
	66	91
Mannose 6-phosphate	10	38
p-Nitrophenyl-α-*N*-acetylglucosaminide	10	<10
M. varians teichoic acid (GlcNAc-P-GlcNAc)$_n$[a]	5[b]	10
	10[b]	12
	25[b]	47
Proteinase I (contains GlcNAc-P-serine)[a]	3[b]	<10

[a] See text for detailed description of these compounds.

[b] Measured in milligrams/ml.

glycopeptide could be converted to the oligosaccharide form with endo-β-*N*-acetylglucosaminidase H (22). Therefore, to compare the activity of the phosphodiesterase toward the glycopeptide versus the oligosaccharide, we determined the initial rate of release of [6-^{3}H]GlcNAc from this material with and without prior treatment with endo-β-*N*-acetylglucosaminidase H. The initial rate of [6-^{3}H]GlcNAc release was not significantly affected by prior treatment with endo H (mean values of 8.3%/h for glycopeptide *versus* 7.4%/h for oligosaccharide). Thus the presence of the peptide in this substrate did not affect the activity of the phosphodiesterase to any significant extent.

Activity toward Other Substrates—In order to understand the specificity of the enzyme better, we studied the activity of the phosphodiesterase towards a variety of substrates containing terminal *N*-acetylglucosamine residues (Table II). The enzyme displayed significant activity towards GlcNAc-P-α-methylmannoside, UDP-GlcNAc, GlcNAc 1-PO_4, *M. varians* teichoic acid (which contains GlcNAc-P-GlcNAc) and Proteinase I from *D. discoideum* (which contains GlcNAc-P-serine). However, it was totally inactive towards *p*-nitrophenyl-α-*N*-acetylglucosaminide and the L-glycan from group D streptococcus (which contains GlcNAc-β-P-Rha). This indicates that the enzyme can only hydrolyze GlcNAc residues that are α-linked to a phosphate group. The Michaelis-Menten constants obtained (see Table II) show that GlcNAc 1-PO_4 is a poorer substrate than UDP-GlcNAc or GlcNAc-P-α-methylmannoside, suggesting that there may be other factors involved in the the specificity of the enzyme.

The activity of the enzyme towards these substrates could not be compared directly to the activity towards substrate IC_{10} for two reasons. First, we were unable to separate the various isomers present in the mixture. Second, there was not enough of this substrate to determine its specific activity or to achieve saturating concentrations.

Inhibitors—*N*-Acetylglucosamine, *N*-acetylglucosamine 1-phosphate, and UDP-*N*-acetylglucosamine were effective inhibitors of enzyme activity, whereas mannose 6-phosphate, mannose, α-methylmannoside, and *M. varians* teichoic acid were weak inhibitors (Table III). The degree of inhibition seen with *N*-acetylglucosamine was somewhat lower than that obtained with crude enzyme preparations (15).

DISCUSSION

In our previous report, we demonstrated that in rat liver the α-*N*-acetylglucosaminyl phosphodiesterase activity was associated with the particulate fractions of the cell and was greatly enriched in preparations of light smooth membranes which correspond to the Golgi/GERL fractions (15). The finding that the enzyme remains membrane bound in the presence of high salt and low detergent concentrations and requires 1% Triton X-100 for solubilization suggests that it is an integral membrane protein. In addition, the strong binding to concanavalin A-Sepharose along with the elution by 0.5 M α-methylmannoside indicates that the phosphodiesterase is a glycoprotein. We had also suggested based on indirect evidence, that the enzyme is distinct from the nonspecific acid hydrolase, α-*N*-acetylglucosaminidase (15). In the purification scheme reported here the two activities are totally separated, providing conclusive evidence that the two enzymes are distinct proteins.

Our ability to perform detailed kinetic studies with the purified phosphodiesterase was severely limited by the lack of adequate amounts of the phosphorylated high mannose oligosaccharide substrates. These transient biosynthetic intermediates are present in cells in very low concentrations, and consequently we were unable to obtain enough material to be able to achieve saturating substrate concentrations in any assay. Furthermore, these substrates consisted of mixtures of isomers that we were unable to separate. In spite of these difficulties, we were able to elucidate a number of aspects of the specificity of the enzyme. The phosphodiesterase is capable of cleaving phosphodiesters at all of five known positions on the high mannose oligosaccharide, demonstrating that this enzyme could account for all of the uncovering reactions which are known to occur *in vivo* (14). In the case of oligosaccharides with two phosphodiesters, the enzyme showed a marked preference for the phosphodiesters located on the branch which contains the mannose residue linked α1,3 to the β-linked mannose (see Fig. 3). Consequently the uncovering of these molecules proceeded in a nonrandom fashion. The activity of the phosphodiesterase toward molecules with one phosphodiester was not influenced to any significant extent by the removal of up to 6 of the 9 mannose residues from the underlying high mannose oligosaccharide. However, the weak inhibition seen with α-methylmannoside and mannose 6-phosphate and the poor activity against *N*-acetylglucosamine 1-phosphate suggests that at least one, and perhaps more, of the mannose residues of the high mannose-type oligosaccharide may be required for optimal enzyme action. The enzyme appears to be specific for *N*-acetylglucosamine residues which are α-linked to a phosphate residue since it has no activity against *p*-nitrophenyl-α-*N*-acetylglucosaminide and L-glycan (GlcNAc-β-P-Rha) while having significant activity toward *N*-acetylglucosamine 1-phosphate and UDP-GlcNAc. Finally, the finding that the enzyme displayed approximately equivalent activity toward a phosphorylated glycopeptide and the phosphorylated oligosaccharide derived from the glycopeptide suggests that the peptide backbone of the glycopeptide may not have a major effect on the enzyme activity. However, this finding may not be relevant to the situation *in vivo* where the enzyme is assumed to act on intact proteins, *e.g.* newly phosphorylated acid hydrolases.

The α-*N*-acetylglucosaminyl phosphodiesterase is present in all the tissues that we have tested, including rat liver, human diploid fibroblasts, Chinese hamster ovary cells, murine lymphoma cells, and murine macrophages. Its wide distribution along with its apparent subcellular localization in the Golgi/GERL region of the cell and its activity toward phosphorylated high mannose-type oligosaccharides indicates that it is the enzyme which is responsible for the exposure of the phosphomannosyl recognition marker of newly synthesized acid hydrolases.

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Note Added in Proof—Waheed *et al.* (32) have recently described the partial purification and characterization of α-*N*-acetylglucosaminyl phosphodiesterase from human placenta.

REFERENCES

1. Kaplan, A., Achord, D. T., and Sly, W. S. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 2026–2030
2. Sando, G. N., and Neufeld, E. F. (1977) *Cell* **12,** 619–627
3. Kaplan, A., Fischer, D., and Sly, W. S. (1978) *J. Biol. Chem.* **253,** 647–650
4. Ullrich, K., Mersmann, G., Weber, E., and von Figura, K. (1978) *Biochem. J.* **170,** 643–650
5. Natowicz, M. R., Chi, M. M. Y., Lowry, O. H., and Sly, W. S. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4322–4326
6. Distler, J., Hieber, V., Sahagian, G., Schmickel, R., and Jourdian, G. W. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4235–4239
7. Von Figura, K., and Klein, U. (1979) *Eur. J. Biochem.* **94,** 347–354

8. Bach, G., Bargal, R., and Cantz, M. (1979) *Biochem. Biophys. Res. Commun.* **91,** 976–981
9. Fischer, H. D., Natowicz, M., Sly, W. S., and Brethauer, R. (1980) *J. Cell Biol.* **84,** 77–86
10. Hasilik, A., and Neufeld, E. F. (1980) *J. Biol. Chem.* **255,** 4937–4945
11. Hasilik, A., and Neufeld, E. F. (1980) *J. Biol. Chem.* **255,** 4946–4950
12. Tabas, I., and Kornfeld, S. (1980) *J. Biol. Chem.* **255,** 6633–6639
13. Hasilik, A., Klein, U., Waheed, A., Strecker, G., and von Figura, K. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 7074–7078
14. Varki, A., and Kornfeld, S. (1980) *J. Biol. Chem.* **255,** 10847–10858
15. Varki, A., and Kornfeld, S. (1980) *J. Biol. Chem.* **255,** 8398–8401
16. March, S. C., Parikh, I., Cautrecasas, P. (1976) *Anal. Biochem.* **60,** 149–152
17. Li, Y. T., and Li, S. C. (1972) *Methods Enzymol.* **28B,** 702–713
18. Gustafson, G. L., and Thon, L. A. (1979) *J. Biol. Chem.* **254,** 12471–12478
19. Gustafson, G. L., and Milner, L. A. (1980) *J. Biol. Chem.* **255,** 7208–7210
20. Brooks, D., and Baddilley, J. (1969) *Biochem. J.* **113,** 635–642
21. Archibald, A. R., and Stafford, G. H. (1972) *Biochem. J.* **130,** 681–690
22. Reitman, M. L., and Kornfeld, S. (1981) *J. Biol. Chem.* **256,** 4275–4281
23. Pazur, J., Capure, A., Kane, J. A., and Karakawa, W. W. (1971) *Biochem. Biophys. Res. Commun.* **43,** 1421–1428
24. Kornfeld, S., Li, E., and Tabas, I. (1978) *J. Biol. Chem.* **253,** 7771–7778
25. Wang, C.-S., and Smith, R. L. (1975) *Anal. Biochem.* **63,** 414
26. Reissig, J. L., Strominger, J. L., and Leloir, L. F. (1955) *J. Biol. Chem.* **217,** 959
27. Tabas, I., and Kornfeld, S. (1979) *J. Biol. Chem.* **254,** 11655–11663
28. Leelavathi, D. E., Esters, L. W., Feingold, D. C., and Lombardi, B. (1970) *Biochem. Biophys. Acta* **211,** 124–128
29. Fischer, H. D., Gonzalez-Noreiga, A., and Sly, W. S. (1980) *J. Biol. Chem.* **255,** 5069–5074
30. Helenius, A., and Simons, K. (1975) *Biochem. Biophys. Acta* **415,** 29–79
31. Verma, A. K., Raizada, M. K., and Schutzbach, J. S. (1977) *J. Biol. Chem.* **252,** 7235–7242
32. Waheed, A., Hasilik, A., and von Figura, K. (1981) *J. Biol. Chem.* **256,** 5717–5721