EXHIBIT 26

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PATENT
ATTORNEY DOCKET NO. 11009/32685

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	)	
	)	For: PRODUCTION OF
Fu-Kuen Lin	)	ERYTHROPOIETIN
	)	
Serial No: 08/468,556	)	Group Art Unit: 1804
	)	
Filed: June 6, 1995	)	Examiner: James Martinell, Ph.D.

THIRD PRELIMINARY AMENDMENT
AND TERMINAL DISCLAIMER PURSUANT TO 37 C.F.R. §1.321

Assistant Commissioner for Patents
Washington, DC 20231

Sirs:

Please enter the following amendments.

In the Specification

At page 1, first paragraph please delete and insert

--This is a continuation of my co-pending U.S. Patent Application 08/202,874 filed February 28, 1994, now abandoned, which was a continuation of U.S. Patent Application 07/113,178, filed October 23, 1987, now abandoned, which was a continuation of U.S. Patent Application 06/675,298 filed November 30, 1984 and issued as U.S. Patent No. 4,703,008 on October 27, 1987, which was a continuation-in-part of U.S. Patent Application 06/655,841, filed September 28, 1984, now abandoned, which was a continuation-in-part of U.S. Patent Application 06/582,185, filed February 21, 1984, now abandoned, and which was a continuation-in-part of U.S. Patent Application 06/561,024, filed December 13, 1983, now abandoned.--

C1

At page 7, line 27, please delete [32 member] and insert in place thereof --32-member--.

At page 8, line 22, please delete the second occurrence of [the].

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At page 11, line 3, please delete [Expt. Hematol.] and insert in place thereof --Exp. Hematol.--.

At page 11, line 4, please delete [(1980:] and insert in place thereof --(1980)--.

At page 11, line 6, please insert a space before "1832".

At page 13, line 13, please insert --"-- after "effects".

At page 13, lines 20-21, please insert --)-- after "propagation".

At page 22, line 4, please delete [Tables V and VI] and insert in place thereof --FIGURES 5 and 6--.

At page 22, line 22, please delete [Table VI] and insert in place thereof --FIGURE 6--.

At page 22, line 24, please delete [Example] and insert in place thereof --Examples--.

At page 25, following line 5 of the original text, please insert the following:

--Reference is made to FIGURES 1 through 21, wherein: FIGURE 1 is a graphic representation of a radioimmunoassay analysis of products of the invention;

Figure 2 shows vector pDSVL-MkE.

Figure 3 shows vector pSVgHuEPO.

Figure 4 shows vector pDSVL-gHuEPO.

Figure 5A, 5B and 5C (collectively referred to as Figure 5) show the sequence of monkey EPO cDNA and the encoded EPO.

Figures 6A, 6B, 6C, 6D and 6E (collectively referred to as Figure 6) show the sequence of human genomic EPO DNA and the encoded EPO.

Figure 7 shows the sequence of the ECEPO gene.

Figure 8 shows the sequence of the SCEPO gene.

Figure 9 shows a comparison of the human and monkey EPO polypeptides.

Figure 10 shows the ECEPO section 1 oligonucleotides.

Figure 11 shows section 1 of the ECEPO gene.

Figure 12 shows the ECEPO section 2 oligonucleotides.

Figure 13 shows section 2 of the ECEPO gene.

Figure 14 shows the ECEPO section 3 oligonucleotides.

Figure 15 shows section 3 of the ECEPO gene.

Figure 16 shows the SCEPO section 1 oligonucleotides.

Figure 17 shows section 1 of the SCEPO gene.

Figure 18 shows the SCEPO section 2 oligonucleotides.

Figure 19 shows section 2 of the SCEPO gene.

Figure 20 shows the SCEPO section 3 oligonucleotides.

Figure 21 shows the section 3 of the SCEPO gene.--

C2 cont.

At page 27, line 24, please delete [Example] and insert in place thereof --Examples--.

At page 30, lines 21, please delete [Asn] and insert --Asn-- in place thereof.

At page 31, line 5, please delete [and RIA Analysis].

At page 32, line 35, please delete the comma[,] after "Springs".

At page 34, line 32, after "83" please insert --deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, under deposit accession No. A.T.C.C. 67545 on October 20, 1987--.

C3

At page 37, line 6, please delete [Table V] and insert --FIGURE 5, comprising portions 5A, 5B and 5C.--.

At page 37, line 6, please delete [Table] and insert --FIGURE--.

Please delete the entire text of pages 38 through 40.

At page 41, line 1, please delete [Table V] and insert in place thereof --FIGURE 5--.

At page 41, line 20, please delete [18, pp. 533-543 (1979] and insert --supra--.

At page 41, line 29, please delete [NEF-976] and insert --NEF-972--.

At page 42, line 24, after "λhE1]" please insert --,deposited with the American Type Culture Collection, 12301 Parklawn drive, Rockville, Maryland, under deposit accession No. A.T.C.C. 40381 on October 20, 1987--.

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At page 42, line 25, please delete [Table VI] and insert in place thereof --FIGURE 6, comprising portions 6A, 6B, 6C, 6D and 6E--.

Please delete the entire text of pages 43 through 47.

At page 48, line 1, please delete [Table VI] and insert in place thereof --FIGURE 6--.

At page 48, line 15, please delete [glutamine] and insert in place thereof --glutamic acid--.

At page 48, line 29, please delete [Table VI] and insert in place thereof --FIGURE 6--.

At page 48, line 34, please delete [Table VI] and insert in place thereof --FIGURE 6--.

At page 49, line 1, please delete [Table] and insert in place thereof --FIGURE--.

At page 49, line 6, please delete [Table] and insert in place thereof --FIGURE--.

At page 49, line 8, please delete [Table VI] and insert in place thereof --FIGURE 6--.

At page 49, line 13, please delete [therin] and insert in place thereof --therein--.

At page 49, line 15, please delete [Table] and insert in place thereof --FIGURE--.

At page 49, line 16, please delete [Table VII, below] and insert in place thereof --FIGURE 9--.

At page 49, line 18, please delete [Table] and insert in place thereof --FIGURE--.

At page 49, line 27, please delete [Table VI] and insert in place thereof --FIGURE 6--.

At page 49, line 29, please delete [Table VI] and insert in place thereof --FIGURE 6--.

Please delete the entire text of page 50.

Page 53, line 13, after "orientation" please insert --(vectors F, X and G).--.

At page 54, line 36, please delete [EcoR1] and insert in place thereof --EcoRI--.

At page 55, line 1, please delete [SalI] and insert in place thereof --SalI--.

At page 55, line 4, please delete [SalI] and insert in place thereof --SalI--.

At page 55, line 13, please delete [BamH1] and insert in place thereof --BamHI--.

At page 55, line 15, please delete [BamH1] and insert in place thereof --BamHI--.

At page 61, line 25, please delete [hemogeneous] and insert in place thereof --homogeneous--.

At page 63, line 35, please delete [Table 6] and insert in place thereof --FIGURE 6--.

At page 64, line 30, please correct the spelling of "recombinant.

At page 65, line 34, please delete [Table 6] and insert in place thereof --FIGURE 6--.

At page 66, line 12, please delete [Tables VIII through XIV below] and insert in place thereof --FIGURES 10 through 15 and 7--.

Please delete the entire text of pages 67 through 72.

At page 73, line 1, please delete [Table VIII] and insert in place thereof --FIGURE 10--.

At page 73, lines 6 and 7, please delete [Table IX] and insert in place thereof --FIGURE 11--.

At page 73, line 21, please delete [(Tables XI and XIII)] and insert in place thereof --(FIGURES 13 and 15)--.

At page 73, line 23, please delete [Tables X and XII] and insert in place thereof --FIGURES 12 and 14--.

At page 73, line 26, please delete [Table XI] and insert in place thereof --FIGURE 13--.

At page 73, line 32, please delete [Table XIV] and insert in place thereof --FIGURE 7--.

At page 74, line 9, after "1984", insert --(Published EPO Application No. 136,490)--.

At page 74, line 29, please delete [Table XIV] and insert in place thereof --FIGURE 7--.

At page 75, line 28, please delete [Tables XV through XXI] and insert in place thereof --FIGURES 16 trough 21 and 8--.

At page 75, lines 30 and 31, please delete [Tables XV, XVII and XIX] and insert in place thereof --FIGURES 16, 18 and 20--.

At page 75, line 32, please delete [Tables XVI, XVIII and XX] and insert in place thereof --FIGURES 17, 19 and 21--.

Please delete the entire text of pages 77 through 82.

At page 83, line 21, please delete [Table XXI] and insert in place thereof --FIGURE 8--.

At page 86, line 2, please delete [33932, 33934 and 33933] and insert in place thereof --39932, 39934 and 39933--.

At page 88, line 36, please delete [lablled] and insert in place thereof --labelled--.

At page 89, line 16, please delete [Table VI] and insert in place thereof --FIGURE 6--.

At page 89, line 21, please delete [118] and insert --128-- in place thereof.

At page 90, line 15, please delete [Table V] and insert in place thereof --FIGURE 5--.

At page 90, line 16, please delete [Table VI] and insert in place thereof --FIGURE 6--.

At page 90, lines 29 and 30, please delete [Table V and VI] and insert in place thereof --FIGURES 5 and 6--.

At page 91, line 29, please delete [a].

At page 92, line 10, please delete [Table VI] and insert in place thereof --FIGURE 6--.

At page 94, line 6, please delete [Tables V and VI] and insert in place thereof --FIGURES 5 and 6--.

At page 94, line 14, please delete [Tables V and VI] and insert in place thereof --FIGURES 5 and 6--.

At page 94, line 33, please delete [mammlain] and insert in place thereof --mammalian--.

At page 95, line 10, please delete [membrances] and insert in place thereof --membranes--.

In the Drawings

Please add the enclosed formal drawings FIGURES 1 through 21.

In the Claims

Please cancel claims 64 through 68 and insert new claims 69 through 75.

--69. An isolated erythropoietin glycoprotein having the *in vivo* biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells, wherein said erythropoietin glycoprotein comprises the mature erythropoietin amino acid sequence of Figure 6 and has glycosylation which differs from that of human urinary erythropoietin.

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70. An isolated erythropoietin glycoprotein having the *in vivo* biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells, wherein said erythropoietin glycoprotein comprises the mature erythropoietin amino acid sequence of Figure 6 and is not isolated from human urine.

71. A non-naturally occurring erythropoietin glycoprotein having the *in vivo* biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells, wherein said erythropoietin glycoprotein comprises the mature erythropoietin amino acid sequence of Figure 6.

72. A pharmaceutical composition comprising a therapeutically effective amount of an erythropoietin glycoprotein product according to claim 69, 70 or 71.

73. A method for providing erythropoietin therapy to a mammal comprising administering an effective amount of a pharmaceutical composition of claim 72.

74. A method for treating a kidney dialysis patient which comprises administering a pharmaceutical composition of claim 72 in an amount effective to increase the hematocrit level of said patient.

75. An isolated polypeptide product characterized by being the product of the expression by a procaryotic host cell of an exogenous DNA sequence encoding the mature erythropoietin amino acid sequence of Figure 6.

REMARKS

Upon entry of the above-requested amendments, claims 69 through 75 will be pending in the application. The requested amendments to the specification bring its text in line with the text of U.S. Patent No. 5,547,933 and do not introduce any new matter.

Applicant acknowledges with thanks the interview kindly granted by Examiner Martinell to the undersigned counsel of record and Mr. Stuart Watt on December 11, 1996. As reflected in the Interview Summary (PTO-413, Paper No. 4), agreement on allowability was not reached. Examiner Martinell did indicate, however, that he was favorably impressed with the Applicant's proposal to prosecute claims identical to independent claims 69 and 70 set out above, along with dependent pharmaceutical composition claims corresponding to canceled claims 65 through 67.

Applicant notes that claims 69, 70 and 71 all differ in scope from glycoprotein claim 1 of U.S. 5,547,933 in specifying that the claimed subject matter comprises the mature[1] human erythropoietin sequence of Figure 6. Claim 69 (like glycoprotein claim 1) recites carbohydrate differences in comparison to human urinary erythropoietin and claim 70 recites a negative limitation with respect to isolation from human urine. No discussion was had during the interview concerning the specific subject matter of newly-submitted claims 71 and 75.

Applicant attaches hereto a Terminal Disclaimer pursuant to 37 C.F.R. §1.321 which, *inter alia*, disclaims the terminal portion of any patent issuing on the present application which extends beyond the term of U.S. Patent 5,547,933.

Submitted concurrently herewith is an Information Disclosure Statement and associated PTO-1449, along with copies of all prior art of record in parent application Serial No. 08/487,774 (which issued as U.S. Patent No. 5,547,933) and in related patent application Serial No. 07/113,179 (which issued as U.S. Patent No. 5,441,868).

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[1] Support for reference to the "mature" sequence is found in the specification at page 48, lines 33-35.

The following remarks address the patentability of the procaryotic host cell expression products which constitute the subject matter of new claim 75.

Applicant notes at the outset that this claimed subject matter has its origins in great-grandparent application U.S. Serial No. 06675,298. Illustrative claims of that application include the following:

> 1. A purified and isolated polypeptide having part or all of the primary structural conformation and one or more of the biological properties of naturally-occurring erythropoietin and characterized by being the product of procaryotic or eucaryotic expression of an exogenous DNA sequence.
>
> 7. A polypeptide according to claim 1 possessing part or all of the primary structural conformation of human erythropoietin as set forth in Table VI [Figure 6] or any naturally occurring allelic variant thereof.
>
> 10. A polypeptide according to claim 1 which has the in vitro biological activity of naturally-occurring erythropoietin.
>
> 58. A purified and isolated DNA sequence as set out in Table V or VI [Figure 6] or a fragment thereof or the complementary strand of such a sequence or fragment.
>
> 59. A polypeptide product of the expression of a DNA sequence according to claim 58 in a procaryotic or eucaryotic host cell.

In the Office Action mailed July 3, 1986 in Serial No. 06/675,298, a six-way restriction requirement was imposed wherein groups I and II were set out as follows:

> I. Claims 1-13, 16, 39-41, 47-54 and 59, drawn to polypeptide, classified in Class 260, subclass 112.
>
> II. Claims 14, 15, 17-36, 58 and 61-72, drawn to DNA, classified in Class '536, subclass 27.

It was the Examiner's position with respect to invention groups I and II that:

> In this case, the product as claimed may be made by a materially different product, such as isolation from a naturally occurring source.

Clearly embraced by the original claims of group I were the procaryotic cell expression products now recited in new claim 75, shown in Example 12 of the specification to possess the in vitro biological activity of naturally-occurring human urinary erythropoietin.

Consistent with the restriction requirement, prosecution in Serial No. 06/675,298 was limited to the DNA subject matter eventually resulting in issuance of U.S. 4,703,008. The polypeptide subject matter of Group I was the subject of application Serial No. 07/113,178. Separate Interferences were declared by the Patent Office concerning the subject matter of U.S. 4,703,008 and Serial No. 07/113,178 and the present inventor was awarded priority in each case. Continuation of prosecution of Serial No. 07/113,178 led to issuance of product claims in U.S. Patent 5,547,933 and to prosecution of the present application.

Against this background, Applicant respectfully submits that the above-noted Terminal Disclaimer beyond the term of U.S. Patent 5,547,933 is appropriate to moot any possible obviousness-type double patenting consideration for any of the claims now pending, and particularly procaryotic expression product claim 75.

Applicant submits that the subject matter of the pending claims is conspicuously patentable over the prior art of record. Applicant specifically notes that, in the European counterpart of the present application, the assertion was made that the human urinary erythropoietin product subjected to de-glycosylation processing as described in a 1982 abstract authored by Dordal *et al.*, ***Experimental Hematology***, **10**, Supp. 11, p. 133 Abstract No. 222 (1982) (1449 Reference C-60, a copy of which is attached as Exhibit I hereto) was anticipatory of non-glycosylated erythropoietin products of expression in procaryotic host cells (which cells, of course, are incapable of effecting glycosylation). Applicant submits that this position is clearly incorrect. The Dordal *et al.* abstract, while disclosing 1982 attempts to remove carbohydrate from urinary erythropoietin, fails to disclose any product that is free of carbohydrate (*i.e.* a non-glycosylated product) such as is produced by procaryotic host cells.

Briefly summarized, the Abstract describes application of two different methods directed to removal of carbohydrate: (1) treatment with mixed glycosidases

of *Streptococcus pneumoniae*; and (2) treatment with 70% hydrogen fluoride. The mixed glycosidase treatment resulted in a molecular weight reduction from 39,000 to 28,500, clearly reflecting an incomplete removal of carbohydrate. Compare specification page 48, line 33 through page 49, line 1 wherein the mature amino acid sequence of human erythropoietin was noted to provide an estimated molecular weight of about 18,400 for the polypeptide alone. Had all carbohydrate been removed, the molecular weight of the treated product would be expected to approach 18,000. That the mixed glycosidase treatment was incapable of removing all carbohydrate is confirmed by consideration of existing knowledge of the mode of action of the bacterial glycosidases revealed in Glasgow *et al.*, *J. Biol. Chem.*, *252(23)*:8615-8623 (1977) attached as Exhibit 2 hereto. As noted in the right hand column of page 8615, the enzymes are not capable of removing an N-acetylglucosamine sugar which attaches to asparagine residues in N-linked glycosylation.

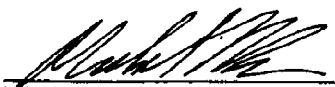
Turning next to the treatment of human urinary erythropoietin with hydrogen fluoride, the Dordal *et al.* abstract, on its face, states that such processing effected removal of "...75% of the carbohydrate found in the original material..."

Because the Dordal *et al.* abstract does not reveal the complete de-glycosylation of urinary erythropoietin, it cannot be maintained to provide a disclosure or suggestion of the generation of non-glycosylated products of procaryotic host cell expression recited in claim 75.

Applicant respectfully submits that claims 69 through 75 are in condition for allowance and an early notice thereof is respectfully solicited.

Respectfully Submitted,

MARSHALL, O'TOOLE, GERSTEIN,
MURRAY & BORUN

BY: 

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Chicago, Illinois
December 20, 1996

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EXHIBIT 1

SATURDAY 1330 - 1515

FUNCTION AND COMPOSITION OF THE CARBOHYDRATE PORTION OF HUMAN URINARY ERYTHROPOIETIN. Dordal, MS and Goldwasser, E, Dept. of Biochemistry. University of Chicago. 920 E 58th Street, Chicago Ill 60637, USA. Purified human urinary erythropoietin (epo) (Miyake, et al, 1977) was deglycosylated using protease-free mixed glycosidases from STREPTOMYCES SAMANENSIS. The resulting aglyco-epo had an apparent molecular weight of 28,500 by SDS-polyacrylamide gel electrophoresis compared to an apparent molecular weight of 39,000 for untreated epo and showed a decrease of 13,300 in apparent molecular weight on an HPLC sizing column. The aglyco-epo retained up to 66% of control activity in the in vitro bone marrow assay, but was consistently less active in the radioimmunoassay. These findings would suggest that, while deglycosylation does not have a major effect on the activity of epo, it does cause a change in some antigenic sites which appear to be distinct from the active site. When epo was deglycosylated by treatment with 70% hydrogen fluoride in pyridine for 8 hours, the aglyco-epo appeared as a diffuse band with a molecular weight range of 35,800 to 29,400. The aglyco-epo prepared by hydrogen fluoride deglycosylation had lost 75% of the carbohydrate found in the original material; 80% of the carbohydrate that remained was N-acetylglucosamine. Carbohydrate composition was determined by gas-liquid chromatography of the trifluoroacetate derivatives of methyl glycosides. Purified α-epo contains 4.9 ± 0.93 moles of fucose, 13.4 ± 1.55 moles of galactose, 9.8 ± 1.46 moles of mannose, 13.3 ± 0.21 moles of N-acetylglucosamine, and 18.1 ± 2.48 moles of N-acetylneuraminic acid per mole of epo. Purified β-epo contains 4.4 ± 0.10 moles of fucose, 11.6 ± 0.15 moles of galactose, 8.2 ± 1.01 moles of mannose, 9.3 ± 0.65 moles of N-acetylglucosamine, and 13.2 ± 1.05 moles of N-acetylneuraminic acid per mole of epo. The difference between the two forms with respect to N-acetylglucosamine and N-acetylneuraminic acid content were statistically significant. The mole ratio calculations are based upon the apparent molecular weight of 39,000. 222

References: Miyake T, Kung CK-H, and Goldwasser, E. J. Biol. Chem. 1977; 252:5558.
Supported in part by Grant No. HL 21676 from the National Institutes of Health. MSD is a trainee supported by Grant No. PHS 5-T32GM07281.

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EXHIBIT 2

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 252, No. 23, Issue of December 10, pp. 8615–8623, 1977
Printed in U.S.A.

Systematic Purification of Five Glycosidases from *Streptococcus (Diplococcus) pneumoniae**

(Received for publication, July 25, 1977)

LOWRIE R. GLASGOW,‡ JAMES C. PAULSON, AND ROBERT L. HILL

From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

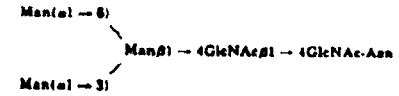
Five of the six known glycosidases in the culture medium of *Streptococcus pneumoniae* have been purified from 600- to 55,000-fold by a systematic procedure of ion exchange and affinity chromatography. Following partial separation of the glycosidases on DEAE-Sephadex, the neuraminidase, the endo-α-*N*-acetylgalactosaminidase, the β-galactosidase, and the β-*N*-acetylglucosaminidase were further purified on agarose affinity adsorbents with ligands derived, respectively, from ovine submaxillary mucin glycopeptides, antifreeze glycoprotein, *p*-aminophenyl-1-thio-β-D-galactoside, or *p*-aminophenyl-1-thio-β-D-*N*-acetylglucosaminide. The purified enzymes had specific activities from 25 to 48 μmol/min/mg. The endo-β-*N*-acetylglucosaminidase was also purified further by gel filtration and ion exchange chromatography and a persistent contaminant of β-*N*-acetylglucosaminidase was removed by adsorption on *p*-aminophenyl-1-thio-β-D-*N*-acetylglucosaminide-agarose. Each glycosidase preparation was substantially free of contaminating glycosidic, hemolytic, and proteolytic activities. Sodium dodecyl sulfate polyacrylamide gel electrophoresis showed a single polypeptide species for the β-galactosidase, the endo-α-*N*-acetylgalactosaminidase, and for the β-*N*-acetylglucosaminidase, corresponding to apparent molecular weights of 350,000, 190,000, and 180,000, respectively.

Rapid assay procedures for three of the glycosidases were developed. Substrates for neuraminidase and endo-β-galactosidase were synthesized by treatment of asialo-α_1-acid glycoprotein with specific glycosyltransferases to produce either [^{14}C]NeuAcα2 → 6Gal, or [^{14}C]GalNAcα1 → 3 (Fucα1 → 2)Gal at the nonreducing termini of the oligosaccharide chains. The only ovalbumin glycopeptide, (Asn(GlcNAc)$_2$(Man)$_3$), that served as a substrate for the *S. pneumoniae* endo-β-*N*-acetylglucosaminidase was labeled in the terminal mannose residues by reduction with NaB^{3}H$_4$ after mild periodate treatment.

Glycosidases are useful tools for the structural and functional analysis of oligosaccharides associated with glycoproteins and cell membranes. However, many glycosidases that have been described are of limited value because of low pH optima (1), activity only with low molecular weight substrates (2), or contaminating activities (3).

Streptococcus (Diplococcus) pneumoniae type I[1] is a rich source for six extracellular glycosidases which are active at neutral pH on both low and high molecular weight substrates. Neuraminidase (5), endo-β-galactosidase (6), β-galactosidase (5), endo-α-*N*-acetylgalactosaminidase (7, 8), β-*N*-acetylglucosaminidase (9), and endo-β-*N*-acetylglucosaminidase (10), have been partially purified and characterized from this source although none has been purified to homogeneity.

The neuraminidase, the β-galactosidase, and the β-*N*-acetylglucosaminidase are exoglycosidases which hydrolyze glycosidic bonds formed by sialic acid, galactose, and *N*-acetylglucosamine, respectively, when these unsubstituted monosaccharides are at the nonreducing end of oligosaccharides (5, 9). The endo-α-*N*-acetylgalactosaminidase hydrolyzes glycosidic bonds formed by *N*-acetylgalactosamine and the hydroxyl group of either serine or threonine when in the sequence Galβ1 → 3GalNAc-α-O-Ser/Thr (7, 8). The endo-β-*N*-acetylglucosaminidase hydrolyzes the glycosidic bond between 2 *N*-acetylglucosamine residues in the oligosaccharide with the following structure (11, 12).



Other sugars may be substituted on the mannose linked α1 → 6 to mannose but the mannose linked α1 → 3 to mannose cannot be substituted (11, 12). Moreover, the *N*-acetylglucosamine linked to asparagine may be substituted in α1 → 6 linkage with fucose. The endo-β-galactosidases hydrolyze oligosaccharides and A and B blood group types to release trisaccharides with the structures GalNAcα1 → 3 (Fucα1 → 2)Gal (A type) and Galα1 → 3 (Fucα1 → 2)Gal (B type) when the galactose is in a β1 → 4 linkage but not in a β1 → 3 linkage with *N*-acetylglucosamine or *N*-acetylgalactosamine (6).

Following published procedures it has been difficult to purify each enzyme free of residual contamination of the

* These studies were supported by Research Grant HL-06400 from the National Heart and Lung Institute, National Institutes of Health, and Research Grant GB-29334 from the National Science Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Research Fellow, National Institutes of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, 1975 to 1977.

[1] *Streptococcus pneumoniae* type I is often designated in the literature as *Diplococcus pneumoniae*. Although the organism was earlier classified by the latter name, *S. pneumoniae* is now preferred (4).

other glycosidases (3). This paper presents a systematic method of purification for five of these glycosidases free of contaminating glycosidic, proteolytic, or hemolytic activities. Four of the enzymes have specific activities from 75 to 2000 times those reported previously. The β-galactosidase, the endo-α-*N*-acetylgalactosaminidase, and the β-*N*-acetylglucosaminidase appear homogeneous as judged by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. A preliminary report of this work has been presented (13).

EXPERIMENTAL PROCEDURES

Materials

p-Nitrophenyl β-D-galactoside and *p*-nitrophenyl β-D-*N*-acetylglucosaminide were obtained from Sigma Chemical Co. *p*-Aminophenyl-1-thio-β-D-galactoside was obtained from Vega Fox (Tucson, Ariz.) and *p*-aminophenyl-1-thio-β-D-*N*-acetylglucosaminide from Bachem Inc. (Marina Del Rey, Calif.). CMP-[4-^{14}C]NeuAc, UDP-[1-^{14}C]GalNAc, GDP-[U-^{14}C]Fuc, and NaB^{3}H, were obtained from New England Nuclear. Ovalbumin was purchased from Sigma Chemical Co. GDP-fucose (14) and ovine submaxillary mucin (15) were prepared as previously described. Porcine submaxillary mucin was prepared from mixed glands by the methods of Plantner (16). Antifreeze glycoprotein was prepared from *Dissostichus mawsoni* serum kindly supplied by Dr. A. L. DeVries (University of Illinois) (17). Dr. K. Schmid (Boston University School of Medicine) supplied a generous gift of α_1-acid glycoprotein. *Streptococcus pneumoniae* type I was a gift of Dr. G. Ashwell (National Institutes of Health).

Analytical Methods

Free sialic acid was estimated by the thiobarbituric acid procedure (18) and total sialic acid by the Svennerholm (19) or the periodate resorcinol methods (20). *N*-Acetylgalactosamine and Galβ1 → 3GalNAc were assayed by the Morgan-Elson reaction (21). The galactose content in acid hydrolysates of oligosaccharides was determined with galactose dehydrogenase (22–24). Protein was determined by the Lowry method (25) using bovine serum albumin as standard. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was performed as previously described (26) and molecular weights were estimated by this means employing a cross-linked protein with known molecular weights (53,000 to 318,000) obtained from Gallard-Schlesinger Chemical Mfg. Corp. Hemolytic activity was determined by incubation of the sample (20 μl) with 0.5 ml of human erythrocytes (50% v/v) in 25 mM sodium barbital, pH 7.1, and 0.9% sodium chloride at 37° for 30 min. Cells were removed by centrifugation and the absorbance of the diluted supernatants determined at 560 nm. Each sample was compared to a control in which the cells were lysed with deionized water. Proteolytic activity was assayed by the method of Lin (27).

Preparation of Enzymes

α-*N*-Acetylgalactosaminidase was obtained from *Clostridium perfringens* (18). Partially purified α-fucosidase from *C. perfringens* was prepared by a procedure employing chromatography of the cell-free growth medium on Bio-Gel P-100 and DEAE-cellulose as described for the α-*N*-acetylgalactosaminidase. *C. perfringens* neuraminidase from Worthington Biochemical Co. was freed of protease activity as described previously (28). β-D-Galactoside α2 → 6 sialyltransferase (29) and β-D-(fucosyl α1 → 2)galactoside α1 → 3 *N*-acetylgalactosaminyltransferase (30) were prepared as previously described. β-Galactoside α1 → 2 fucosyltransferase was prepared from a Triton X-100 extract of porcine submaxillary glands by chromatography on SP-Sephadex and GDP-agarose.[1]

Preparation of O-β-D-Galactopyranosyl-(1 → 3)-N-acetylgalactosamine – The disaccharide Galβ1 → 3GalNAc was obtained by digestion of antifreeze glycoproteins with pure endo-α-*N*-acetylgalactosaminidase and isolated by gel filtration on a column (1.5 × 23 cm) of Sephadex G-25 (superfine) equilibrated with water. The disaccharide contained only galactose and *N*-acetylgalactosamine in a 1:1 molar ratio and gave a single reducing sugar spot (31) on descending paper chromatography (Whatman No. 3MM, ethyl acetate/pyridine/water, 2:1:2).

[1] T. A. Bayer and R. L. Hill, unpublished observations.

Preparation of (Galβ1 → 3GalNAc)-Porcine Submaxillary Mucin[2] – Porcine submaxillary mucin (174 mg) in 20 ml of 0.01 M cacodylate, pH 6, was incubated with 0.2 unit of α-*N*-acetylgalactosaminidase, 1 unit of neuraminidase, and 0.1 unit of fucosidase for 3 days at 37°; the same amount of each glycosidase was then added and the mixture incubated for an additional 24 h. The solution was dialyzed extensively against water and lyophilized. This procedure removed >95% of the sialic acid, fucose, and nonreducing terminal *N*-acetylgalactosamine to leave Galβ1 → 3GalNAc as the only carbohydrate prosthetic group.

Preparation of ([^{14}C]NeuAc)-α$_1$-acid Glycoprotein – The reaction mixture (0.65 ml) contained 50 μmol of sodium phosphate, pH 6.8, 8.5 mg of asialo-α$_1$-acid glycoprotein, bovine serum albumin (0.5 mg), 2 μCi of CMP-[^{14}C]NeuAc (200 μCi/μmol), and 25 milliunits of β-galactoside α2 → 6 sialyltransferase. The reaction was allowed to proceed for 16 h at 37° and the ([^{14}C]NeuAc)-α$_1$-acid glycoprotein was isolated by gel filtration on a column (1.5 × 23 cm) of Sephadex G-25 (superfine) equilibrated with 50 mM sodium cacodylate, pH 6.0. Over 90% of the labeled sialic acid was transferred to the asialo-α$_1$ acid glycoprotein.

Preparation of ([^{14}C]GalNAcα1 → 3(Fucα1 → 2Gal)-α$_1$-acid Glycoprotein[3] – A mixture (1.85 ml) containing 50 μmol of sodium cacodylate, pH 6.8, 1 mg of bovine serum albumin, 13 mg of asialo-α$_1$-acid glycoprotein, 1 μmol of GDP-[^{14}C]fucose (250 cpm/nmol), and 15 milliunits of β-D-galactoside α1 → 2 fucosyltransferase was incubated at 37° for 13.5 h and the (fucosylated product isolated by gel filtration on a column (1.5 × 23 cm) of Sephadex G-25 (superfine) equilibrated with 50 mM sodium cacodylate, pH 6.0. The (Fucα1 → 2Gal)-α$_1$-acid glycoprotein[3] was collected in ~5.0 ml and concentrated to 1 ml by ultrafiltration (Minicon B-15, Amicon Corp). The product, which contained 350 nmol of Fucα1 → 2Gal . . . sites, was dissolved in a solution (final volume, 1.2 ml) containing 50 μmol of sodium cacodylate, pH 6.0, 10 mg of Triton X-100, 20 μmol of $MnCl_2$, 40 milliunits of β-D-(fucosyl α1 → 2)galactoside α1 → 3 *N*-acetylgalactosaminyltransferase, and 40 nmol of UDP-[^{14}C]GalNAc (1 × 10^6 cpm/nmol). The solution was incubated for 4 h at 37° and the ^{14}C-labeled product was isolated by gel filtration as described in the previous step. The transfer of [^{14}C]GalNAc to (Fucα1 → 2Galβ1-α$_1$-acid glycoprotein was virtually quantitative and yielded about 40 nmol of the sequence [^{14}C]GalNAcα1 → 3(Fucα1 → 2)Gal at the nonreducing termini of the oligosaccharides of the asialo-α$_1$-acid glycoprotein.

Preparation of Dansyl-[^{3}H]Asn(GlcNAc)$_2$(Man)$_3$[3] – Glycopeptides were obtained from a pronase digest of ovalbumin by gel filtration as previously reported (32), and further fractionated with Dowex 50 as described by Huang *et al.* (33). The glycopeptides with the composition Asn(GlcNAc)$_2$(Man)$_3$ were labeled with tritium as described earlier (34) after periodate oxidation of the terminal mannose residues followed by reduction with NaB^{3}H$_4$. Asn(GlcNAc)$_2$(Man)$_3$ (2 μmol) in 2 ml of 10 mM acetic acid was treated with 1.6 mmol of periodic acid for 2.5 h at 4° in the dark, and 200 μl of 0.7 M potassium borate and 1.9 mg of NaB^{3}H$_4$ (300 mCi/mmol) were added before further incubation at 4° overnight. Excess NaBH$_4$ was destroyed with several drops of glacial acetic acid, and the [^{3}H]Asn(GlcNAc)$_2$(Man)$_3$ was isolated on a column (1.5 × 23 cm) of Sephadex G-25 (superfine) equilibrated in 0.1 M acetic acid. The ^{3}H-glycopeptides were dansylated as described previously (34) to give the final product, dansyl-[^{3}H]Asn(GlcNAc)$_2$(Man)$_3$.

[3] All glycans are of the D configuration except for fucose which is of the L configuration. Abbreviations for glycoprotein derivatives prepared as described under "Experimental Procedures" are as follows: (Galβ1 → 3 GalNAc) – porcine submaxillary mucin is used to designate the product of porcine submaxillary mucin which had been treated with neuraminidase, α-fucosidase, and α-*N*-acetylgalactosaminidase to leave Galβ1 → 3 GalNAc as the only oligosaccharide prosthetic group. Asialo-α$_1$-acid glycoprotein after treatment with CMP-[^{14}C]NeuAc and β-galactoside α2 → 6 sialyltransferase is referred to as ([^{14}C]NeuAc)-α$_1$ acid glycoprotein, and after treatment with β-galactoside α1 → 2 fucosyltransferase, GDP-fucose, β-(fucosyl α1 → 2)galactoside α1 → 3-*N*-acetylgalactosaminyltransferase, and UDP-[^{14}C]GalNAc is referred to as ([^{14}C]GalNAcα1 → 3 (Fucα1 → 2Gal)-α$_1$ acid glycoprotein. Dansyl-[^{3}H]Asn (GlcNAc)$_2$(Man)$_3$ is used to indicate the derivative of the ovalbumin glycopeptide of composition Asn(GlcNAc)$_2$(Man)$_3$ which had been periodate treated, reduced with NaB^{3}H$_4$, and dansylated.

Glycosidase Assays

Neuraminidase – Assay mixtures (75 μl) contained 2 μmol of sodium cacodylate, pH 6.0, 1 mg of α_1-acid glycoprotein, and 7.5 μg of bovine serum albumin. Reactions were initiated by the addition of enzyme and were incubated at 37° for 10 min. The sialic acid released was estimated by the thiobarbituric acid procedure (18). One unit of activity will release 1 μmol of sialic acid/min under these assay conditions.

An alternate assay used to locate neuraminidase in column eluates employed [^{14}C]NeuAc-α_1-acid glycoprotein. Incubation mixtures (55 μl) contained [^{14}C]NeuAc-α_1-acid glycoprotein (12,000 cpm), 0.55 μmol of sodium cacodylate, pH 6.0, and 7.5 μg of bovine serum albumin. Enzyme (2 μl) was added and the reaction incubated for 6 min at 37° and stopped with 1 ml of 50 mM sodium citrate, pH 3.0. The mixture was applied to a column (1 ml) of Dowex 50-X8 (H^+, 200 to 400 mesh) in a Pasteur pipette followed by 1.5 ml of 50 mM sodium citrate, pH 3.0. The eluate, which contained the [^{14}C]sialic acid, was collected directly into scintillation vials and counted. The assay was linear with time and enzyme concentration when less than 25% of the total counts were released.

Endo-β-galactosidase – Assay mixtures (50 μl) contained ([^{14}C]GalNAcol — 3(Fucol — 2)Gal)-α_1-acid glycoprotein (13,200 cpm), 0.5 μmol of sodium cacodylate, pH 6.0, and 7.5 μg of bovine serum albumin. Enzyme was added to initiate the reaction and the mixtures were incubated at 37° for 20 min. Reaction was stopped with 1.0 ml of sodium citrate, pH 3.0, and the free [^{14}C]GalNAcol — 3(Fucol — 2)Col was separated from the ^{14}C-protein substrate on a column (1 ml) of Dowex 50-X8 (H^+, 200 to 400 mesh) as described above for the neuraminidase assay. Activity is expressed as counts per min released under the assay condition.

β-Galactosidase – Assay mixtures (50 μl) contained 0.2 μmol of *p*-aminophenyl-β-D-galactoside, 2.5 μmol of sodium cacodylate, pH 6.0, and 25 μg of bovine serum albumin. Enzyme (2 to 10 μl) was added to initiate the reaction, and after incubation at 37° for 5 to 15 min, the reaction was stopped by adding 1 ml of 0.5 M sodium carbonate and the absorbance at 400 nm was measured (ϵ^{400}_{mM} = 17.7). One unit of activity will hydrolyze 1 μmol/min under these assay conditions.

Endo-α-N-acetylgalactosaminidase – Either (Galβ1 — 3GalNAc)-porcine submaxillary mucin or antifreeze glycoprotein was used as substrate for the endo-α-*N*-acetylgalactosaminidase. Enzyme (2 μl) was added to 100 μl of substrate (0.2 μmol in Galβ1 — 3GalNAc sites) in 20 mM sodium cacodylate, pH 6.0, and incubated at 37° for 20 min. The reaction was stopped with the addition of 50 μl of 0.8 M potassium borate, pH 9.1, and the released disaccharide measured with the Morgan-Elson method for *N*-acetylamino sugars (21). Galβ1 — 3GalNAc gives 110% of the color yield of free GalNAc. Units of activity of the enzyme are defined as micromoles of Galβ1 — 3GalNAc released per min under these assay conditions. The assay with antifreeze glycoprotein was linear with time and enzyme concentration up to a final absorbance at 585 nm of 0.25.

β-N-Acetylglucosaminidase – β-*N*-Acetylglucosaminidase was assayed as described for the β-galactosidase with *p*-nitrophenyl-β-D-*N*-acetylglucosaminidase as substrate.

Endo-β-N-acetylglucosaminidase – The enzyme was assayed by a modification of the method of Tarentino and Maley (34). Enzyme (2 μl) was added to 50 μl of 20 mM sodium cacodylate, pH 6.0, containing ^{3}H-labeled dansyl-Asn(GlcNAc)$_2$(Man)$_3$ (20,000 cpm) and 25 μg of bovine serum albumin and the mixture was incubated at 37° for 5 to 10 min. The reaction was terminated by addition of 30 mM ammonium hydroxide (1 ml) and the released ^{3}H-oligosaccharide was separated from the ^{3}H-glycopeptides on a column (1 ml) of Dowex 1-X2 (acetate cycle, 200 to 400 mesh) in a Pasteur pipette followed by an additional 1.5 ml of 30 mM ammonium hydroxide. Eluates were collected directly into scintillation vials and counted. The assay was linear with up to 20% of the total counts released and about 50% of the total counts in the assay were susceptible to enzymatic cleavage. One unit of activity represents the amount of enzyme that will release 1 μmol of oligosaccharide/min at saturating substrate concentration. Since the final substrate concentration (15 μM) was below the K_m (0.25 mM) of the enzyme (10), the value obtained under these assay conditions was multiplied by 17.5.

Preparation of Affinity Adsorbents

All adsorbents were prepared by reacting cyanogen bromide-activated Sepharose 4B (29, 35) with the appropriate ligand. Adsorbents were stored in 0.1% sodium azide.

Ovine Submaxillary Mucin-Sepharose 4B – Glycopeptides of ovine submaxillary mucin were prepared by digestion with thermolysin. Mucin (10 mg/ml) dissolved in 0.1 M Tris/HCl, pH 8.0, containing 10 mM calcium chloride, was incubated with thermolysin (30 μg/ml) overnight at 37°, dialyzed against deionized water, and lyophilized. Cyanogen bromide-activated Sepharose-4B was washed with 10 volumes of 0.2 M sodium pyrophosphate, pH 8.5 at 4°, and then added to an equal volume of the same buffer containing ovine submaxillary mucin glycopeptides (10 mg/ml). After 2 h at room temperature, the gel was washed in a sintered glass funnel, and the filtrate retained for quantitation of uncoupled protein (ϵ^{280}_{mM} = 3.6). Under these conditions about 60 to 80% of the total ligand was coupled to give ~ 7 μmol of sialic acid/ml of settled gel.

Antifreeze Glycoprotein-Sepharose 4B – Cyanogen bromide-activated gel was washed at 4° with 0.2 M sodium pyrophosphate, pH 8.5, and added to an equal volume of the same buffer containing antifreeze glycoprotein (14 mg/ml). The reaction was allowed to proceed at 4° for 5 min and then for an additional 2 h at room temperature. The gel was washed with 1.0 M NaCl in a sintered glass funnel and the filtrate was retained for quantitation of uncoupled protein (ϵ^{280}_{mM} = 1.3). About 50% of the antifreeze glycoprotein was coupled giving 12 μmol of Galβ1 — 3GalNAc/ml of settled gel.

p-Aminophenyl-1-thio-β-D-galactoside-Sepharose 4B – Cyanogen bromide-activated gel was added to 2 volumes of 0.1 M sodium bicarbonate, pH 10.0, containing approximately 6.7 mM *p*-aminophenyl-1-thio-β-D-galactoside. Coupling was allowed to proceed overnight at 4° with gentle shaking. The gel was washed with deionized water in a sintered glass funnel with filtrate retained for quantitation of uncoupled ligand (ϵ^{280}_{mM} = 2.8). About 30% of the total ligand was coupled to give 4 μmol/ml of settled gel.

p-Aminophenyl-1-thio-β-D-N-acetylglucosaminide-Sepharose 4B – The ligand was coupled as for the *p*-aminophenyl-1-thio-β-D-galactoside to yield 3 μmol of *p*-aminophenyl-1-thio-β-D-*N*-acetylglucosaminide/ml of settled gel. Before use the gel was treated with an equal volume of 1 M ethanolamine, pH 8.6, at room temperature for 2 h and washed exhaustively with deionized water.

Purification of S. pneumoniae Glycosidases

Step 1: Culture Supernatant – A strain of *S. pneumoniae*, type I, was grown for 72 h at 37° in a 15-liter culture medium as described earlier (5). The cells were collected by centrifugation at 7300 × *g* for 20 min and discarded. Further procedures were performed at 4° unless otherwise noted.

Step 2: First Ammonium Sulfate Precipitation – Ammonium sulfate (510 g/liter) was added with stirring to the cell-free growth medium, the solution stirred overnight, and then centrifuged at 7200 × *g* for 30 min. The resulting sticky brown precipitate was dissolved in a minimum volume of water and dialyzed overnight against distilled water.

Step 3: Second Ammonium Sulfate Precipitation – Ammonium sulfate (175 g/liter) was added to the dialyzed fraction from Step 2, stirred for 2 h, and the precipitate removed by centrifugation at 7200 × *g* for 40 min and discarded. Additional ammonium sulfate (310 g/liter) was added to the supernatant and after 1 h, the precipitate containing the glycosidases was collected by centrifugation (7200 × *g* for 40 min), dissolved in a minimum amount of water, and dialyzed against 10 mM Tris·HCl, pH 7.5.

Step 4: Chromatography on DEAE-Sephadex A-25 – The dialyzed fraction was applied to a column of DEAE-Sephadex A-25 (5.0 × 120 cm) equilibrated in 10 mM Tris·HCl, pH 7.5 (10). The column was washed with 3 liters of equilibration buffer followed by a linear gradient of sodium chloride formed with 12.5 liters of 10 mM Tris·HCl and 12.5 liters of 10 mM Tris·HCl containing 0.35 M sodium chloride. Fractions (15 ml) were collected at a flow rate of 5.5 ml/min. Peaks of glycosidase activity were pooled as they eluted from the column to give fractions enriched in neuraminidase and endo-β-galactosidase (A), β-galactosidase (B), endo-α-*N*-acetyllactosaminidase (C), β-*N*-acetylglucosaminidase (D), and endo-β-*N*-acetylglucosaminidase (E).

Step 5 (A to E): Concentration of Glycosidase Fractions – Each fraction (A to E) was stirred with ammonium sulfate (510 g/liter) for 1 h, centrifuged at 7200 × *g* for 30 min, and the precipitate containing the glycosidases was dissolved in a minimum amount of water and dialyzed against 0.01 M cacodylate, pH 6. Fraction A (60 ml) was further concentrated to ~10 ml by ultrafiltration over a PM-30 membrane (Amicon Corp).

Step 6A: Chromatography of Neuraminidase on Ovine Submaxillary Mucin-Sepharose-4B – Concentrated neuraminidase from Step 5 (Fraction A, 10 ml) was applied to a column (9 × 4 cm) of ovine

submaxillary mucin-Sepharose 4B equilibrated with 0.1 M sodium cacodylate, pH 6.0 at 4°, and the column was then washed with 400 ml of 20 mM sodium cacodylate, pH 6.0, containing 2.0 M sodium chloride. The column was then immersed in a constant temperature bath (37°) and neuraminidase was eluted by the immediate application of 50 mM sodium borate, pH 9.0, containing 1.0 M sodium chloride. Fractions were collected in an ice bath, adjusted to pH ~ 7.5 with 1 M cacodylic acid, and those containing neuraminidase were pooled. A flow rate of 12 ml/min was maintained throughout the column development.

Step 6B: Chromatography of β-Galactosidase on p-Aminophenyl-thio-β-D-galactoside-Sepharose 4B – The concentrated galactosidase solution from Step 5 (Fraction B, 61 ml in 10 mM sodium cacodylate, pH 6.0) was made 0.15 M in sodium chloride by addition of the solid salt and applied to a column (1.5 × 4 cm) of *p*-aminophenylthio-β-D-galactoside-agarose equilibrated with 0.1 M sodium cacodylate, pH 6.0. A flow rate of about 1.5 ml/min was maintained throughout the column development. The column was washed with 80 ml of 1 M sodium chloride in 0.1 M sodium cacodylate, pH 6.0. Elution of the β-galactosidase was achieved with 15 mM *p*-aminophenyl-1-thio-β-D-galactoside in 50 mM sodium cacodylate, pH 6.0, in the following manner. One bed volume (8 ml) was applied at 4°, the column was immersed in a 37° constant temperature bath and elution immediately resumed. Fractions were moved to an ice bath soon after collection. Active fractions were pooled and dialyzed exhaustively against 25 mM sodium cacodylate, pH 6.0. Elution of the β-galactosidase could also be achieved by using 20% ethylene glycol (v/v) instead of the 15 mM *p*-aminophenyl-1-thio-β-D-galactoside in the elution buffer.

Step 6C: Chromatography of Endo-α-N-acetylgalactosaminidase on Antifreeze Glycopeptide-Sepharose 4B – The concentrated solution of endo-α-*N*-acetylgalactosaminidase from Step 5 (Fraction C, 85 ml) in 0.01 M sodium cacodylate, pH 6.0, was made 300 mM in NaCl by adding the solid salt, and applied at 4° to a column (0.6 × 3 cm) of antifreeze glycoprotein-agarose at a flow rate of 1 ml/min. The column was washed with 60 ml of 2 M NaCl in 20 mM sodium cacodylate, pH 6.0. The column was brought to 37° and elution continued with the same buffer. Fractions of 2 ml were collected and those containing the endo-α-*N*-acetylgalactosaminidase were pooled.

Step 6D: Chromatography of β-N-Acetylglucosaminidase on p-Aminophenylthio-β-D-N-acetylglucosaminide-Sepharose 4B – The concentrated *N*-acetylglucosaminidase from Step 5 (Fraction D, 60 ml) was mixed with 6 ml of 1 M sodium chloride containing 0.1 M sodium cacodylate, pH 6.0, and applied to a column (1.5 × 8.5 cm) of *p*-aminophenylthio-β-D-*N*-acetylglucosaminide-agarose equilibrated with 0.15 M NaCl in 0.1 M sodium cacodylate, pH 6.0. The column was washed with 100 ml of 0.5 M NaCl, 0.05 M sodium cacodylate, pH 6.0, and the β-*N*-acetylglucosaminidase then eluted with 1 M NaCl, 50 mM Tris·HCl, pH 8.6, at a flow rate of 1 ml/min. Pooled active fractions were adjusted to pH 6.5 to 7.0 with 1 M cacodylic acid and dialyzed overnight against 50 mM sodium cacodylate, pH 6.0.

Step 6E: Chromatography of Endo-β-N-acetylglucosaminidase on Sephacryl G-200 – A column (5 × 84 cm) of Sephacryl G-200 was equilibrated with 0.01 M Tris·HCl, pH 7.5, containing 0.1 M NaCl. Endo-β-*N*-acetylglucosaminidase concentrated to 85 ml in Step 5E was applied to the column. The exclusion volume of the column containing the enzyme activity was pooled.

Step 7E: Chromatography of Endo-β-N-acetylglucosaminidase on DEAE-Sephadex G-25 and p-Aminophenyl-1-thio-β-N-acetylglucosaminide-agarose – The endo-β-*N*-acetylglucosaminidase was further purified on DEAE-Sephadex G-25 as previously described (10). To remove the substantial β-*N*-acetylglucosaminidase activity that remained, the enzyme was adjusted to pH 6.0 with 1 M cacodylic acid and applied to an 8-ml column of *p*-aminophenyl-1-thio-β-D-acetylglucosaminide-agarose. The unretarded endo-β-*N*-acetylglucosaminidase activity was pooled and stored at −20°.

RESULTS

Purification of Streptococcus pneumoniae Glycosidases – Glycosidases were prepared from 15 liters of *S. pneumoniae* culture filtrate. The enzymes were initially fractionated on DEAE-Sephadex A-25 as shown in Fig. 1, and each partially pure glycosidase was purified further to remove contaminating glycosidases. A summary of the purification is given in Table I, which lists the steps common for all enzymes through chromatography on DEAE-Sephadex A-25, and the further purification of each enzyme separately.

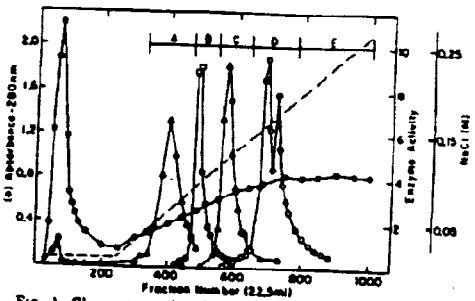


FIG. 1. Chromatography of the partially purified glycosidases on DEAE-Sephadex A-25. Glycosidases from Step 3 (578 ml) were applied to a column of DEAE-Sephadex A-25 (5.0 × 120 cm) in 10 mM Tris·HCl, pH 7.5. Fractions were collected as the column was developed with the same buffer followed by a linear gradient of sodium chloride (0 to 0.35 M). Details of the column conditions are given in Step 4 of "Experimental Procedures." *Symbols* refer to protein (●), NaCl concentration (——), neuraminidase by the thiobarbituric assay (Δ, 1 = 0.05 unit/ml), β-galactosidase (□, 1 = 0.005 unit/ml), endo-β-*N*-acetylgalactosaminidase by the (Galβ1 → 3GalNAcα)-porcine submaxillary mucin assay (▲, 1 = 0.01 unit/ml), and β-*N*-acetylglucosaminidase (○, 1 = 0.1 unit/ml). Endo-β-galactosidase elution at 0.05 M NaCl (E) and the endo-β-*N*-acetylglucosaminidase elution at 0.25 M NaCl (10) are not shown. The major peaks of activity (*A* to *E*) were pooled as indicated.

Purification of Neuraminidase – The neuraminidase from Step 5 was contaminated with endo-β-galactosidase and β-galactosidase. Two affinity adsorbents that have been used in the purification of other neuraminidases, *N*-(*p*-aminophenyl)-oxamic acid-agarose (36) and α_1-acid glycoprotein-agarose (28), were found unsuitable for further purification since the other enzymatic activities also bound these adsorbents and eluted with the neuraminidase. One problem with the α_1-acid glycoprotein-agarose is that degradation during chromatography may produce terminal galactose residues that serve as potential binding sites for the β-galactosidase. For this reason, ovine submaxillary mucin-agarose containing the disaccharide NeuAcα2 → 6GalNAc was examined and found to be effective.

An example of the purification of 145 units of neuraminidase obtained on ovine submaxillary mucin-agarose is shown in Fig. 2. The neuraminidase was eluted substantially free of other glycosidases with variable yields of 27 to 80%. The final specific activity (45 units/mg of protein) is about 400 times higher than reported previously for this enzyme (5). The purified enzyme was found to be unstable, but with the addition of 0.25 mg of bovine serum albumin/ml, no loss of activity was observed over 2 months when stored at −20°.

The column size and the time spent on the affinity adsorbent were critical factors for optimal purification. Loading of excess enzyme or prolonging the time of development of the column allowed for enzymatic cleavage of the sialic acid and premature elution of the enzyme. Several test columns showed that a minimum of 2 μmol of sialic acid bound to the adsorbent was necessary for application of 1 unit of neuraminidase when the total chromatography time was 30 min or less.

Purification of β-D-Galactosidase – The purification of the β-galactosidase is summarized in Table I. An example of the purification of β-galactosidase obtained on the affinity adsorb-

TABLE I

Purification of glycosidases from Streptococcus pneumoniae

Results are shown for the preparation of five glycosidases from 15 liters of cell-free supernatant. Details of the purification scheme are given under "Experimental Procedures." Examples of the purification at different stages are given in Fig. 1 (Step 4A-E), Fig. 2 (Step 6A), Fig. 3 (Step 6B), Fig. 4 (Step 6C), and Fig. 5 (Step 6D).

Step	Volume	Total Protein	Total Activity					Specific Activity	Yield	Purification
			α-NeuAc-ase	β-Gal-ase	Endo-α-GalNAc-ase	β-GlcNAc-ase	Endo-β-GlcNAc-ase			
	(ml)	(mg)	(units)					(units/mg)	(%)	
1. Cell Free Supernatant	15,300	228,200	1,130	105	351	1,910	1,100		100	1
2. 1st $(NH_4)_2SO_4$ Precipitation	915	22,420	709	77	100	940	1,120			
3. 2nd $(NH_4)_2SO_4$ Precipitation	578	13,900	189	68	94	838	846			
Neuraminidase										
4A. DEAE-A2S	3,200	2,016	563					0.28	50	56
5A. Concentration	10	811	328					0.4	29	80
6A. OSM-agarose	185	3.24	185					45	13	9,000
β-Galactosidase										
4B. DEAE-A2S	2,100	651		49				0.075	47	163
5B. Concentration	61	503		59				0.098	56	213
6B. Gal-agarose	17	0.5		12.4				25	12	55,000
Endo-α-N-Acetylgalactosaminidase										
4C. DEAE-A2S	2,250	945			105			0.12	30	80
5C. Concentration	85	935			59			0.06	16	42
6C. Antifreeze-Glycoprotein-agarose	18	1.5			40			27	11	18,000
β-N-Acetylglucosaminidase										
4D. DEAE-A2S	3,300	1,683				383		0.216	19	27
5D. Concentration	64	995				254		0.261	13	33
6D. GlcNAc-agarose	34	3.6				774		48.0	9	6,000
Endo-β-N-Acetylglucosaminidase										
5E. DEAE-25 and Concentration	85	1,138					682	0.42	44	88
6E. G-200 Sephacryl		80					352	4.4	32	913
7E. DEAE-A2S, GlcNAc-agarose 9		10					30	3.1	3	633

Abbreviations used are α-NeuAc-ase for neuraminidase, β-Gal-ase for β-galactosidase, Endo-α-GalNAc-ase for endo-α-N-acetylgalactosaminidase β-GlcNAc-ase for β-N-acetylglucosaminidase and Endo-β-GlcNAc-ase for endo-β-N-acetylglucosaminidase. A unit of activity is defined as 1 μmol of product formed/min determined by the standard assay.

A μmol of product formed/min/mg of protein

α1-Acid glycoprotein was used as substrate in the standard assay.

Antifreeze glycoprotein was used as substrate in the standard assay.

OSM-agarose refers to ovine submaxillary mucin-Sepharose-4B.

Gal-agarose refers to p-aminophenyl-1-thio-β-D-galactoside-Sepharose-4B.

GlcNAc-agarose refers to p aminophenyl-1-thio-β-D-N-acetylglucosaminide-Sepharose-4B.

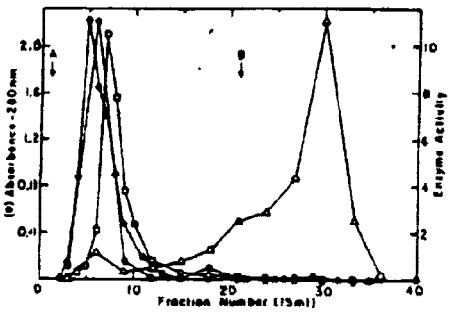


FIG. 2. Purification of neuraminidase on ovine submaxillary mucin-agarose. The concentrated neuraminidase fraction (10 ml in 10 mM sodium cacodylate) from Step 6A was applied to a column of ovine submaxillary mucin-agarose (9 × 4 cm). The column was washed at 4° with 2.0 M sodium chloride in 0.02 M sodium cacodylate, pH 6.0 (*A*) and elution of the neuraminidase was initiated by bringing the column to 37° and washing with 1.0 M sodium chloride in 0.05 M sodium borate, pH 9.0 (*B*). Details of the column conditions are given in Step 6A of "Experimental Procedures." *Symbols* refer to protein (●), neuraminidase (△, 1 = 200 cpm in standard ([^{14}C]NeuAc-α_2 acid glycoprotein assay), endo-β-galactosidase (○, 1 = 200 cpm in standard ([^{14}C]GalNAcα1 → 3(Fucα1 → 2)Galβ-α_1-acid glycoprotein assay) and β-galactosidase (□, 1 = 0.001 unit/ml).

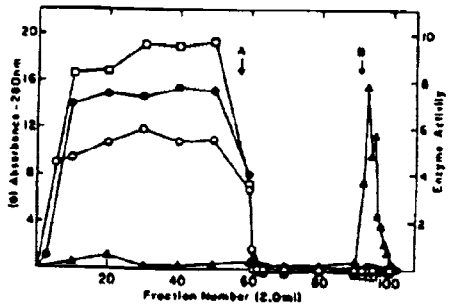


FIG. 4. Purification of endo-α-*N*-acetylgalactosaminidase on antifreeze glycoprotein-agarose. The concentrated endo-α-*N*-acetylgalactosaminidase (85 ml in 10 mM sodium cacodylate, pH 6.0, 0.15 M sodium chloride) obtained from Step 5C was applied (1 ml/min) at 4° to a column of antifreeze glycoprotein-agarose (0.6 × 3 cm) equilibrated in the same buffer. The column was washed with 2.0 M sodium chloride in 20 mM sodium cacodylate, pH 6.0 (*A*), and the enzyme eluted by bringing the column to 37° and continuing the wash (*B*). Details of the column conditions are given in Step 6C of "Experimental Procedures." Symbols refer to protein (●), β-galactosidase (□, 1 = 0.01 unit/ml), endo-α-*N*-acetylgalactosaminidase (▲, 1 = 1 unit/ml by (Galβ1 → 3 GalNAc)-porcine submaxillary mucin assay), β-*N*-acetylglucosaminidase (○, 1 = 0.01 unit/ml).

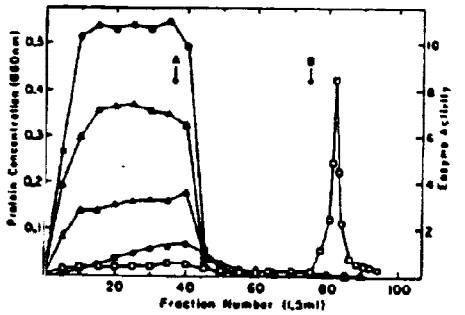


FIG. 3. Purification of β-galactosidase on *p*-aminophenyl 1-thio-β-D-galactoside-agarose. The concentrated β-galactosidase fraction obtained from Step 5B (61 ml in 10 mM sodium cacodylate, pH 6.0, 0.15 M sodium chloride) was applied to a column (1.5 × 4 cm) of *p*-aminophenyl-1-thio-β-D-galactoside-agarose. The column was washed with 0.1 M sodium cacodylate, pH 6.0, 1.0 M sodium chloride (*A*) and the enzyme eluted with 15 mM *p*-aminophenyl-1-thio-β-D-galactoside in 50 mM sodium cacodylate, pH 6.0 (*B*). Details of column conditions are given in Step 6B of "Experimental Procedures." *Symbols* denote protein concentration (●, Lowry assay), β-galactosidase (□, 1 = 0.25 unit/ml), neuraminidase (△, 1 = 360 cpm in standard [^{14}C]NeuAc-α_2-acid glycoprotein assay), endo-α-*N*-acetylgalactosaminidase (▲, 1 = 0.025 unit/ml), and endo-β-galactosidase (○, 1 = 40 cpm in standard assay).

ent, *p*-aminophenyl-1-thio-β-D-galactoside-agarose, is shown in Fig. 3. The majority of the protein and the contaminating glycosidases present in the galactosidase (Fraction B) from the DEAE-step are unretarded on the column to yield a highly purified β-galactosidase substantially free of other glycosidases. The specific activity (25 units/mg of protein) was about 75 times that reported previously (5). Variable yields of 20 to 80% were obtained at this step. Although the basis for the occasional low yields has not been systematically examined, better yields were obtained when 20% ethylene glycol was used to elute the enzyme instead of *p*-aminophenyl-1-thio-β-D-galactoside. The enzyme lost 10 to 15% activity in 30 days when stored at either 4° or −20° at a concentration of 30 μg/ml in 50 mM sodium cacodylate, pH 6.0. No loss of activity was observed when the enzyme was stored at 4° or −20° in bovine serum albumin (0.5 mg/ml).

Purification of Endo-α-N-acetylgalactosaminidase – The purification of the endo-α-*N*-acetylgalactosaminidase on antifreeze glycoprotein-agarose is shown in Fig. 4. The enzyme was eluted free of other enzymatic activities with a 68% step yield to give a specific activity (27 units/mg of protein) over 2000 times that obtained by conventional purification procedures (7). The β-galactosidase does not bind presumably because of its strict substrate specificity for Galβ1 → 4R linkage.[*] The enzyme can be stored for at least 2 months at −20° with no loss of activity. Limitations on column capacity and chromatography time have not been observed but significant changes in the method used could cause hydrolysis of the affinity ligand from the agarose.

Purification of β-N-Acetylglucosaminidase – Chromatography of the β-*N*-acetylglucosaminidase on *p*-aminophenyl-1-thio-β-D-*N*-acetylglucosaminide-agarose is shown in Fig. 5. The enzyme is obtained substantially free of glycosidases with a high specific activity (48 μmol/min/mg of protein) which is about 200 times that obtained previously (9). Although this enzyme was obtained in low overall yield, it is relatively abundant in the culture filtrate. As with the endo-α-*N*-acetylgalactosaminidase, 50% of the enzyme was lost in the first ammonium sulfate precipitation. By completing this step rapidly, however, virtually quantitative yields have been obtained (9).

Purification of Endo-β-N-acetylglucosaminidase – The endo-β-*N*-acetylglucosaminidase (Fraction 5E) was rechromato-

[*] J. C. Paulson and R. L. Hill, unpublished observations.

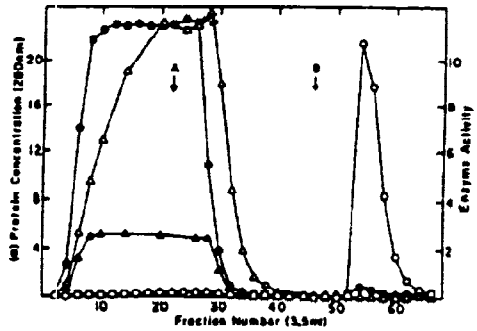


FIG. 5. Purification of β-*N*-acetylglucosaminidase on *p*-aminophenyl-1-thio-β-D-*N*-acetylglucosaminide-agarose. The concentrated β-*N*-acetylglucosaminidase (85 ml) in 10 mM sodium cacodylate, pH 6.0, 0.1 M sodium chloride) from Step 5D was applied to a column (1.5 × 8.5 cm) of *p*-aminophenyl-1-thio-β-D-*N*-acetylglucosaminide-agarose. Elution was continued with 50 mM sodium cacodylate, pH 6.0, 0.5 M sodium chloride (*A*) and the β-*N*-acetylglucosaminidase was then eluted from the column with 50 mM Tris·HCl, pH 8.6, 1 M sodium chloride (*B*). Details of the procedure are given under "Experimental Procedures." Symbols denote protein concentration (●), β-*N*-acetylglucosaminidase (○, 1 = 1.1 units/ml), β-galactosidase (△, 1 = 0.006 unit/ml), and endo-α-*N*-acetylgalactosaminidase (▲, 1 = 0.2 unit/ml).

TABLE II

Contaminating activities in purified glycosidase preparation

Contaminating enzyme activities were estimated by incubation of the purified enzyme with appropriate substrates. The aliquot of enzyme and time of incubation were increased in the standard assay to detect activities of neuraminidase (10 μl for 180 min), endo-β-galactosidase (10 μl for 20 h), β-galactosidase (10 μl for 120 min), endo-α-*N*-acetylgalactosaminidase (10 μl for 310 min), β-*N*-acetylglucosaminidase (10 μl for 180 min), and endo-β-*N*-acetylglucosaminidase (10 μl for 185 min). The results are shown as units of contaminating activity present in 1000 units of the major activity. Endo-β-galactosidase activity was not detected in any purified glycosidase.

Purified enzyme	Activity				
	Neuraminidase	β-Galactosidase	Endo-α-*N*-acetylgalactosaminidase	β-*N*-Acetylglucosaminidase	Endo-β-*N*-acetylglucosaminidase
Neuraminidase	1,000	<0.1	<0.1	<0.1	<0.1
β-Galactosidase	0.3	1,000	28	<0.3	0.3
Endo-α-*N*-Acetylgalactosaminidase	<0.1	<0.1	1,000	<0.1	<0.2
β-*N*-Acetylglucosaminidase	<0.1	0.2	<0.1	1,000	<0.1
Endo-β-*N*-Acetylglucosaminidase	1.2	0.78	0.16	0.27	1,000

graphed on a second DEAE-Sephadex column (10) removing most of the contaminating enzymatic activities except for β-*N*-acetylglucosaminidase. This activity was removed by chromatography on *p*-aminophenyl-1-thio-β-*N*-acetylglucosaminide-agarose (Step 7E). The column bound 99% of the remaining β-*N*-acetylglucosaminidase while 90% of the endo-β-*N*-acetylglucosaminidase was unretarded.

Contaminating Activities in Purified Glycosidase Preparations—The contaminating glycosidase activities were estimated with the standard assays described under "Experimental Procedures," except that the amount of enzyme added and the incubation times were increased to detect levels of activity 1/10,000th that of the major activity. The levels of neuraminidase, β-galactosidase, endo-α-*N*-acetylgalactosaminidase, β-*N*-acetylglucosaminidase, and endo-β-*N*-acetylglucosaminidase found in each preparation are listed in Table II. Most contaminating activities were found to be present at <0.05% the major activity. Endo-α-*N*-acetylgalactosaminidase activity was found as 2.8% contaminant of the β-galactosidase, but it could be removed by passing the β-galactosidase preparation through an antifreeze glycoprotein-agarose column as described for Step 6C in the purification procedure. As depicted in Fig. 4, the β-galactosidase flows through this column unretarded while the endo-α-*N*-acetylgalactosaminidase is adsorbed. In addition to the activities listed in Table II, no endo-β-galactosidase was detected when ([^{14}C]GalNAcα1 → 3(Fucα1 → 2)Galβ1-α,-acid glycoprotein was incubated with 10 μl of each enzyme for 20 h. Neither proteolytic nor hemolytic activities were detected in the purified enzymes or in the crude ammonium sulfate concentrate of the culture medium (Step 3).

Purity and Molecular Weights of Glycosidases—The purified glycosidases were analyzed by electrophoresis on 3.3% polyacrylamide gels in the presence of sodium dodecyl sulfate and 2-mercaptoethanol (26). The β-galactosidase (15 μg), the endo-α-*N*-acetylgalactosaminidase (10 μg), and the β-*N*-acetylglucosaminidase (15 μg) each gave a single Coomassie blue-staining band with apparent molecular weights of 150,000, 190,000, and 180,000, respectively. The neuraminidase (10 μg) and the endo-β-*N*-acetylglucosaminidase (35 μg) did not give any bands on gels when analyzed by the same electrophoretic methods.

DISCUSSION

Following the procedure reported in this paper, five of the six glycosidases from *S. pneumoniae* have been purified free of other glycolytic, hemolytic, and proteolytic activities. The enzymes were separated on DEAE-Sephadex (Fig. 1) as reported by others (6, 7, 10) but all had significant activities of each of the other enzymes at this stage. Affinity chromatographic techniques were used to purify the β-galactosidase, the endo-α-*N*-acetylgalactosaminidase, and the β-*N*-acetylglucosaminidase to homogeneity and to purify all five of the enzymes free of contaminating activities.

The neuraminidase and the endo-α-*N*-acetylgalactosaminidase were purified using degradable affinity adsorbents prepared from their glycoprotein substrates, ovine submaxillary mucin, and antifreeze glycoprotein, respectively. Particular attention to the amount of enzyme applied to a column and the total time of chromatography were important factors in obtaining optimal purification. Under the conditions employed here, the ovine submaxillary mucin-agarose was extensively degraded during chromatography and no attempt was made to use the column a second time. This is not a practical problem since gram quantities of ovine mucin are easily prepared. The antifreeze glycoprotein-agarose was much more stable, however, since the endo-α-*N*-acetylgalactosaminidase has very little activity at 4° and it is severely inhibited by the 2 M sodium chloride present during elution at 37°. When 1 ml of the adsorbent containing 10 units of the endo-α-*N*-acetylgalactosaminidase was kept overnight at 4°, the adsorption and elution properties of the adsorbent remained unchanged.

The *S. pneumoniae* β-galactosidase and β-*N*-acetylglucosaminidase have been purified to homogeneity on affinity adsorbents substituted with *p*-aminophenyl-1-thio-β-D-galactoside or *p*-aminophenyl-1-thio-β-D-*N*-acetylglucosaminide, respectively. The fact that these two enzymes can be purified free of each other on these adsorbents attests to the specificity with which the glycosidases bind the appropriate substrate analog. *p*-Aminophenyl-1-thioglycoside substituted adsorbents have been widely used as potential affinity adsorbents for glycosidases with variable results (37–43). In most studies a spacer arm between the ligand and the gel matrix was employed and frequently, an adsorbent was found to adsorb several glycosidases in addition to the one which is expected to bind (39–41). In one case, *Escherichia coli* β-galactosidase has been shown to adsorb equally well to the *p*-aminophenyl-1-thio-β-D-galactoside adsorbent and to a column containing only the spacer arm (44). In the present report, the *p*-aminophenyl-1-thioglycosides were coupled directly to cyanogen bromide-activated Sepharose 4B. Since spacer arms provide potential sites for nonspecific adsorption of inert protein or unwanted enzyme activities, it is noteworthy that in this case they were not required. Thus spacer arms need not be considered a necessary component for the design of ligands for all glycosidase affinity adsorbents.

Specific glycosyltransferases have been utilized to synthesize radiolabeled substrates for neuraminidase and endo-β-galactosidase. For the neuraminidase, resialylation of asialo-α_1-acid glycoprotein by incubation with pure β-galactoside α2 → 6 sialyltransferase and CMP-[^{14}C]NeuAc produces oligosaccharides with the terminal sequence [^{14}C]NeuAcα2 → 6Galβ1 → 4GlcNAc (24). The endo-β-galactosidase is known to act on substrates that contain the blood group A positive sequence GalNAcα1 → 3(Fucα1 → 2)Galβ1 → 4GlcNAc . . . (6). While the oligosaccharides of α_1-acid glycoprotein do not normally carry this structure, it can be synthesized with the aid of two glycosyltransferases. The β-galactoside α1 → 2 fucosyltransferase incubated with GDP-fucose and asialo-α_1-acid glycoprotein yields a product with the terminal sequence Fucα1 → 2Galβ1 → 4GlcNAc. This product is an acceptor for the β-(fucosyl α1 → 2) galactoside α1 → 3 *N*-acetylgalactosaminyltransferase (45) which on incubation with UDP-[^{14}C]GalNAc gives the final product [^{14}C]GalNAcα1 → 3(Fucα1 → 2)Galβ1 → 4GlcNAc. In addition to the trisaccharide [^{14}C]GalNAcα1 → 3(Fucα1 → 2)Gal released by the endo-β-galactosidase, α-*N*-acetylgalactosaminidase would also release a radiolabeled product as free [^{14}C]GalNAc. This activity, however, is not found in the culture medium of *S. pneumoniae* when incubated with *p*-nitrophenyl-α-*N*-acetylgalactosaminide.[5]

A third radiolabeled substrate was prepared from the ovalbumin glycopeptide with composition Asn(GlcNAc)$_2$(Man)$_5$. Tai and Kobata (11) have shown that of the five ovalbumin glycopeptides hydrolyzed by the endo-β-*N*-acetylglucosaminidase from *Streptomyces griseus*, only this one is susceptible to cleavage by the *S. pneumoniae* enzyme. The procedure used to label Asn(GlcNAc)$_2$(Man)$_5$ introduces tritium by NaB^{3}H$_4$ reduction of periodate-oxidized mannose residues (1 mol of periodate/mol of glycopeptide (34)). At least 50% of the labeled glycopeptides were susceptible to cleavage by the *S. pneumoniae* endo-β-*N*-acetylglucosaminidase. Thus, of the 3 terminal mannose residues susceptible to periodate oxidation at least one of these may be modified without destroying the ability of the glycopeptide to serve as a substrate.

[5] L. R. Glasgow and R. L. Hill, unpublished observations.

The substrate specificities for the *S. pneumoniae* endo-β-galactosidase (6), the endo-α-*N*-acetylgalactosaminidase (7, 8), and the endo-β-*N*-acetylglucosaminide (11, 12) are well documented. For the neuraminidase, the β-galactosidase and the β-*N*-acetylglucosaminidase, however, little has been reported about their specificity toward the penultimate sugar or the glycosidic linkage. The β-galactosidase appears to be quite specific toward galactose in a β1 → 4 linkage since Galβ1 → 4GlcNAc is a good substrate and Galβ1 → 4Glc is also a substrate, but no hydrolysis of Galβ1 → 3GlcNAc, Galβ1 → 6GlcNAc, or methyl β-galactoside can be detected.[6] Thus, until the substrate specificities of these exoglycosidases are better defined, it should not be assumed that the appropriate unsubstituted monosaccharide will be hydrolyzed when found at the nonreducing end of oligosaccharides.

The glycosidases of *S. pneumoniae* are active at neutral pH values and act on oligosaccharides of intact glycoproteins. These features make them particularly suitable for investigating the role of oligosaccharides on glycoproteins with a labile function of biological interest. Since these enzymes can be obtained virtually free of contaminating activities, they can be used in large excess to ensure complete digestion of the desired moiety without producing partial digestions from unexpected activities. In principle, the enzymes can remove most of the carbohydrate from a wide variety of carbohydrate groups linked to the polypeptide chain through asparagine or threonine/serine residues. The neuraminidase, β-galactosidase, β-*N*-acetylglucosaminidase, and endo-β-*N*-acetylglucosaminidase have been used together to remove >85% of the asparagine linked carbohydrate from rabbit IgG (46) and α_1-acid glycoprotein.[6] The endo-α-*N*-acetylgalactosaminidase removed about 90% of the Galβ1 → 3GalNAc units linked to the threonine residues of antifreeze glycoprotein[7] and in conjunction with a neuraminidase, α-fucosidase, and α-*N*-acetylgalactosaminidase from *Clostridium perfringens* virtually all of the carbohydrate from porcine submaxillary mucin.[8]

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[7] J. I. Rearick, L. R. Glasgow, and R. L. Hill, unpublished observations.
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