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A GLYCOPEPTIDE RESISTANT TO EXOGLYCOSIDASES*

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ABSTRACT

The carboxyl group of the terminal *N*-acetylneuraminic acid residue of the glycopeptide, prepared from α_1-acid glycoprotein by protease digestion, was esterified with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, and then reduced with sodium borohydride. The reduced glycopeptide, thus prepared, containing the reduced *N*-acetylneuraminic acid, was resistant to hydrolysis by neuraminidase, and consequently to other exoglycosidases. The penultimate β-D-galactosyl residue of the oligosaccharide chain of the reduced glycopeptide was hydrolyzed by β-D-galactosidase only after the removal of the terminal, reduced, sialic acid by mild hydrolysis with acid. The reduced glycopeptide should be a useful substrate for the assay of endoglycosidases in the presence of exoenzymes. It should also find use as a carbon source in the growth of endoglycosidase-elaborating bacteria.

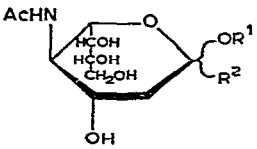
INTRODUCTION

Endoglycosidases of different anomeric and linkage specificities are needed for their potential utility for study of the carbohydrate structure of such biological macromolecules as glycoproteins and glycolipids. The availability of such enzymes would also permit precise modifications of the carbohydrate structures of biologically active macromolecules, making it possible to examine structure–activity relationships. Of the several known bacterial endoglycosidases, most are specific for *endo*-glycosyl linkages involving 2-acetamido-2-deoxy-β-D-glucose or its derivatives[1–8]. There is thus a need for other endoglycosidases capable of hydrolyzing the varied glycosidic linkages present in glycoproteins and glycolipids.

*Abbreviations: carboxyl-reduced NANA, 5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-nonulopyranose; reduced glycopeptide, glycopeptide in which NANA is reduced to carboxyl-reduced NANA. Glycopeptide preparations are designated as follows: Unreduced, A; reduced, B; partially reduced, C.

A possible approach to the isolation of endoglycosidases is the enrichment (microorganism capable of utilizing, as their sole source of carbon, glycopeptid(that are not susceptible to degradation by exoglycosidases without the prior inte vention of an endoglycosidase. Thus, modification of the sugar residue at the noi reducing terminus should protect the glycopeptide from the action of exoglycosidase

The glycopeptides derived from α_1-acid glycoprotein have, at the nonreducin termini of their oligosaccharide chains, glycosidically linked *N*-acetylneuraminic aci (NANA, **1**), whose carboxyl group can be altered, thus providing a convenient wa of modifying the terminal sugar-residue. Formation of the methyl ester of NAN considerably decreases the effectiveness of neuraminidase, suggesting that the carbox group is essential for neuraminidase action[9]. It was, therefore, expected that r(duction of the carboxyl group of NANA (**1**) in the glycopeptide to the primar hydroxyl analog would be equally effective in preventing neuraminidase actio



1 R^1 = H, R^2 = CO_2H
2 R^1 = Me, R^2 = CO_2Me
3 R^1 = Me, R^2 = CO_2H
4 R^1 = Me, R^2 = CH_2OH

Moreover, carboxyl-reduced NANA would provide more-durable protection unde conditions of bacterial growth, as it would not be adversely affected by the probabl presence of esterases as would, for example, the methyl ester of NANA. Simila susceptibility to proteases argues against modification of NANA (**1**) by amidatio of its carboxyl group.

We have utilized the glycopeptide(s) prepared from α_1-acid glycoprotein, a the ready accessibility and complex carbohydrate structure of this glycopeptid fulfills the requirements expected of a substrate for endoglycosidases. This pape describes the preparation of a glycopeptide that should be suitable as a substrat for the isolation of microorganisms capable of elaborating endoglycosidases, an as a substrate for *in vitro* studies with appropriate endoglycosidases.

RESULTS AND DISCUSSION

In preliminary studies, the carboxyl group of *N*-acetylneuraminic acid wa reduced with sodium borohydride following esterification with diazomethane. How ever, this procedure suffers from the disadvantage that, at the alkaline pH of th borohydride reduction, some esterified carboxyl groups are saponified and henc escape reduction. This factor necessitates repeated treatment with diazomethane an reduction with sodium borohydride. An undesirable consequence of the repeate exposure of the glycopeptide to diazomethane was that other sugar components o

the glycopeptide, notably 2-acetamido-2-deoxy-D-glucose, underwent methylation. The exact location of these methyl groups was not determined, but their presence was evident in the gas chromatograms of the samples prepared by methanolysis of the glycopeptides followed by trimethylsilylation. Several new peaks, in addition to those of the trimethylsilyl ethers of the methyl glycosides of the component sugars (galactose, mannose, fucose, *N*-acetylneuraminic acid, and 2-acetamido-2-deoxy-glucose), were observed. The preservation of the integrity of all sugars but the terminal NANA is mandatory for the reduced glycopeptide to be useful as a carbon source for the growth of endoglycosidase(s)-elaborating microorganisms. If other sugars were altered in any way, they might not be readily available for utilization by bacteria after the action of endoglycosidase. As a result of these considerations, the diazomethane–sodium borohydride method was abandoned in favor of the carbodiimide method.

The carbodiimide–sodium borohydride method for reduction of carboxyl groups offers a distinct advantage as regards the stability of the ester group during reduction. However, as the reduction with borohydride is performed at pH 7, excessively large amounts of the borohydride are required to compensate for its rapid decomposition at this pH. Taylor and Conrad[10] have employed this method for the reduction of the carboxyl group of the glucuronic acid in heparin, hyaluronic acid, the capsular polysaccharides from *Aerobacter aerogenes*, and chondroitin sulfate, as well as a galacturonan. When applied to the reduction of NANA-carboxyl groups in the α_1-acid glycoprotein–glycopeptide, two difficulties were encountered in employing this procedure. The free, unprotected, amino group of the *N*-terminal amino acid of the glycopeptide reacted with the carboxyl group, either of NANA or of the *C*-terminal amino acid, in the presence of EDC, resulting in the formation of a dimer and thus decreasing the number of carboxyl groups available for reduction. The dimer emerged from a column of Sephadex G-50 ahead of the monomeric, carboxyl-reduced glycopeptide, the basis on which assignment of the dimeric structure was made. The dimerization could be partially suppressed by *N*-acetylation of the *N*-terminal amino acids prior to reduction with the EDC–borohydride reagent. Under these conditions, dimerization still apparently occurs, possibly through the interaction of two carboxyl groups during the initial treatment of the glycopeptide at pH 4.7. This is particularly so as no nucleophile other than the hydroxyl and the ionized carboxyl groups is present to react with the carbodiimide-activated, carboxyl groups. That the isolation of the dimer from the reaction of the *N*-acetylated glycopeptide (**A**) with EDC–sodium borohydride was not due to partial reduction of the intermolecular ester initially formed was established by re-subjecting the dimer to the reduction procedure. There was no marked change in the elution pattern from a column of Sephadex G-50. The second difficulty was the apparent resistance of the carboxyl group of NANA to undergo reduction under these conditions. After two reductions, a 25% yield of the fully reduced glycopeptide (**B**) was obtained, in addition to another (glycopeptide **C**, 25%) in which 62% of the sialic acid had been reduced. It was possible to separate the completely reduced glycopeptide (**B**) from the partially

reduced one (**C**) on a column of DEAE-Sephadex. The former, being neutral, was eluted with water, whereas the latter was desorbed by employing 0.5% ammonium hydrogencarbonate as the eluant.

Two factors apparently contribute to the reluctance of the carboxyl group of NANA to undergo reduction, namely, (*1*) steric inaccessibility, and (*2*) relative paucity of the available, sterically accessible, hydroxyl groups for esterification, as compared to their abundance in the substrates of Taylor and Conrad[21]. Consequently, the incomplete reduction of NANA may be attributed to incomplete esterification of its carboxyl groups because of insufficiency of favorably positioned hydroxyl groups.

Reduced *N*-acetylneuraminic acid residues in the carboxyl-reduced glycopeptide (**B**) were characterized by g.l.c. For this purpose, the methyl glycoside (**4**) of carboxyl-reduced NANA was prepared by reduction of the methyl glycoside, methyl ester (**2**) of NANA with sodium borohydride[11]. The absence of unreduced **2** in the carboxyl-reduced methyl glycoside (**4**) of NANA was shown by treatment of both **2** and **4** with 0.1M sodium carbonate at room temperature. No anodal-migrating spot (corresponding to the acid **3**) was observed from **4** by paper electrophoresis at pH 4.7, whereas a fast-moving spot was observed from **2** that reflected a total conversion into **3**. G.l.c. of the per-*O*-trimethylsilyl derivative of **4** showed a single peak (Fig. 1B) that was distinct from any of the peaks of the *O*-trimethylsilyl derivative of **2** (Fig. 1A).

When the methyl glycoside (**4**) of carboxyl-reduced NANA was successively methanolyzed with 0.1M methanolic hydrogen chloride, *N*-acetylated, and trimethylsilylated, the g.l.c. peak shown in Fig. 1B had disappeared. In its place, two new peaks (shown in Fig. 1C) were observed. The nature and the extent of conversion of **4** into this new derivative(s) are not known. The same two peaks shown in Fig. 1C are also obtained when the carboxyl-reduced glycopeptide (**B**) is methanolyzed and

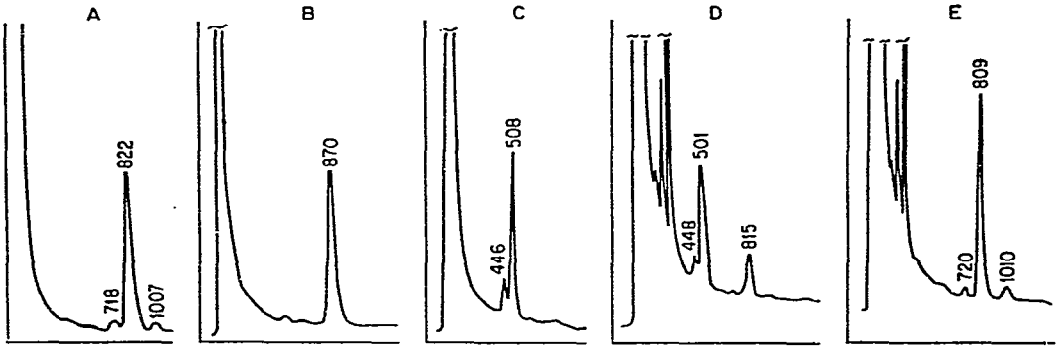


Fig. 1. Gas–liquid chromatography of trimethylsilyl ethers of *N*-acetylneuraminic acid derivatives. Runs were made isothermally at a column temperature of 190°. Chart speed was 10 min/in. Numbers on top of the peaks are retention times in sec. For further details, see "Methods". (*A*) Methyl ester, methyl glycoside (**2**) of NANA. (*B*) Methyl glycoside (**4**) of carboxyl-reduced NANA. (*C*) Product from the methanolysis of **4**. (*D*) Product from the methanolysis of the partially reduced glycopeptide *C*. (*E*) Product from the methanolysis of unreduced glycopeptide *A*.

trimethylsilylated, with no sign of the peak due to **4**, as shown in Fig. 1B. Fig. 1D displays the pattern of peaks obtained from the glycopeptide (**C**), which contains both the carboxyl-reduced and unreduced NANA. Unreduced glycopeptide (**A**), which contains only NANA (**1**) and no carboxyl-reduced NANA, gives the profile in Fig. 1E, resembling that of **2**, as shown in Fig. 1A.

The methyl glycoside (**4**) of carboxyl-reduced *N*-acetylneuraminic acid was hydrolyzed with 0.05M sulfuric acid for 1 h at 80°, and analyzed by the Warren procedure[12]. No perceptible color was observed. Onodera *et al.*[13] have made a similar observation from studies with carboxyl-reduced colominic acid. Under the same conditions, the methyl glycoside, methyl ester **2** gave rise to a color equivalent to approximately 20% release of *N*-acetylneuraminic acid (**1**). This incomplete hydrolysis quite probably reflects the low rate of hydrolysis of the methyl glycoside **2** as compared with the free acid **3**. The stability factor for the carboxyl-reduced NANA and its ability to form a chromophore under the conditions of the Warren assay[12] are not known. Svennerholm's resorcinol procedure[14] for determination of the total (free and combined) sialic acid gives a brown color with the methyl glycoside **4** of carboxyl-reduced NANA and a purple color with the methyl ester, methyl glycoside **2** of NANA. These chromophores have overlapping absorption profiles that make this procedure unsatisfactory for the determination of one in the presence of the other. In the resorcinol procedure[14], carboxyl-reduced colominic acid has been reported[13] to form a chromophore having an absorption maximum at 455 nm, instead of one having a maximum at 580 nm that is characteristic of sialic acid[13]. Therefore, both reduced and unreduced *N*-acetylneuraminic acids of the glycopeptide used in the study were determined by g.l.c. Two assumptions were made for this purpose: (*1*) The two peaks (Fig. 1C) formed on methanolysis of the reduced glycopeptide (**B**) truly reflect the amount of carboxyl-reduced NANA in the reduced glycopeptide, that is the conversion of carboxyl-reduced NANA glycoside to the unknown derivative represented by the two peaks is quantitative; (*2*) the

TABLE I

CARBOHYDRATE CONTENT[a] OF GLYCOPEPTIDES

Glycopeptide	*Carbohydrate (% by weight)*					
	Unreduced sialic acid	*Reduced sialic acid*	*2-Acetamido-2-deoxy-D-glucose*	D-*Mannose*	D-*Galactose*	L-*Fucose*
Unreduced (**A**)	32.1	[b]	28.4	15.8	19.6	1.1
Fully reduced (**B**)	[b]	27.2	30.2	14.5	20.1	0.6
Partially reduced (**C**)	10.9	18.4	28.4	14.6	21.0	0.7

[a]As determined by g.l.c. For details, see "Methods". [b]Undetectable.

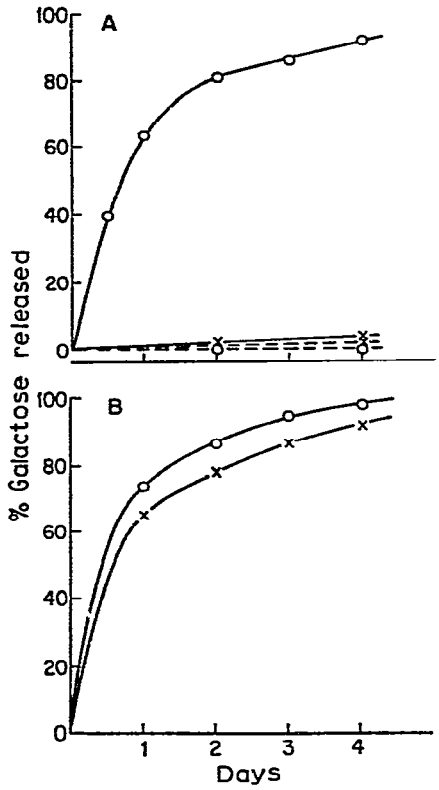


Fig. 2. Release of galactose from unreduced and reduced glycopeptides by *Aspergillus niger* β-D-galactosidase. (*A*) With and without treatment with *Vibrio cholerae* neuraminidase. ⊙—⊙, unreduced glycopeptide + neuraminidase + β-D-galactosidase; ○—○, unreduced glycopeptide + β-D-galactosidase; ×—×, reduced glycopeptide + neuraminidase + β-D-galactosidase; and ×—×, reduced glycopeptide + β-D-galactosidase. (*B*) After removal of NANA and carboxyl-reduced NANA with 0.1M hydrochloric acid. ⊙—⊙, unreduced glycopeptide; ×—×, reduced glycopeptide. For further details, see "Methods".

detector response in the gas chromatograph is the same for both the reduced and unreduced *N*-acetylneuraminic acid derivatives.

Table I summarizes the carbohydrate content of the glycopeptides **A, B,** and **C.** The observed differences of ~3–5% between the NANA (**1**) content of the unreduced glycopeptide (**A**) on the one hand and the combined (reduced and/or unreduced) NANA content of the reduced glycopeptide preparations **B** and **C** (Table I) on the other possibly result from lack of total compliance with the two assumptions stated here concerning determination of the methyl glycoside (**4**) of carboxyl-reduced NANA by g.l.c.

Reduction of the carboxyl group of *N*-acetylneuraminic acid (**1**) of the glycopeptide offers excellent protection from the action of neuraminidase. This may be seen by comparing the proportion of galactose released from the two glycopeptides (**A** and **B**) by β-D-galactosidase in the presence and absence of neuraminidase (Fig.

2A). Compared to the 91.5% of galactose released by the combined action of the two enzymes from the unreduced glycopeptide (A), only 4% is cleaved from the reduced glycopeptide (B). The 2% of galactose hydrolyzed from the reduced glycopeptide (B) by β-D-galactosidase alone suggests that, during reduction of the glycopeptide (A), some NANA (1) was lost by hydrolysis, thus promoting some galactose residues from the penultimate to the terminal position. This conclusion is warranted by the observed inactivity of β-D-galactosidase toward the unreduced glycopeptide (A) without prior removal of NANA by neuraminidase. Fig. 2B shows the removal of galactose by *Aspergillus niger* β-D-galactosidase from the unreduced glycopeptide A and the reduced glycopeptide B after hydrolysis of NANA (1) and carboxyl-reduced NANA, respectively, with 0.1M hydrochloric acid.

EXPERIMENTAL

Materials. — Enzymes used and their sources, were: pronase, Calbiochem; aminopeptidase M, CMBH Chemische Fabrik; carboxypeptidase A, Sigma Chemical Co.; and neuraminidase (*Vibrio cholerae*), Grand Island Biochemical Co. β-D-Galactosidase was purified from *Aspergillus niger* as previously described[15]. Sephadex G-50 and DEAE-Sephadex A-50 were purchased from Pharmacia Fine Chemicals, Piscataway, N.J. Iodoacetamide, 1,4-dithiothreitol, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) were products of Sigma Chemical Company, St. Louis, Missouri. Sodium borohydride was obtained from the Nutritional Biochemical Corporation, Cleveland, Ohio. α_1-Acid glycoprotein used was either isolated from human-blood serum according to the procedure of Weimer *et al.*[16] or was donated[17] from the National Fractionation Center, American Red Cross, Bethesda, Maryland.

Methods. — Gas–liquid chromatography was performed with a Packard Model 7821 Gas Chromatograph equipped with a flame-ionization detector and a disc integrator. The stationary phase was 3% SE-30 on Gas-chrom Q, 100–120 mesh, packed in a 1.9 m × 4 mm (i.d.) coiled-glass column through which nitrogen was passed at a flow rate of approximately 40 ml per min. Samples for g.l.c. analyses were prepared by methanolysis of the glycopeptides with methanolic hydrogen chloride. Glycopeptides were methanolyzed with 0.1M methanolic hydrogen chloride in sealed tubes in a nitrogen atmosphere for 6 h at 90° for the determination of NANA and carboxyl reduced NANA, whereas M methanolic hydrogen chloride for 24 h at 90° was employed for the analyses of other sugars. The methanolyzed samples were *N*-acetylated with acetic anhydride in methanol before conversion into per-*O*-trimethylsilyl derivatives[18]. Mannitol was used as an internal standard for these analyses. *N*-Acetylneuraminic acid (NANA) was determined either by the periodate–thiobarbituric acid procedure of Warren[12] or by g.l.c. Carboxyl-reduced NANA in the glycopeptides was determined by g.l.c. analysis of the per-*O*-trimethylsilyl derivative of the methanolyzed sample. Spectrophotometric measurements were made on a Zeiss Model PMQ II spectrophotometer. Absorbances in fractions from columns

were read at 230 nm for the detection of peptides, and at 490 nm for the detection of carbohydrates by the phenol–sulfuric acid method[19]. Two drops of toluene were added to tubes in incubations with enzymes extending beyond than 5 h. Concentrations were carried out at $<35°$ under diminished pressure with a rotary evaporator. Paper electrophoresis was performed on Whatman 3MM paper at pH 4.7 in pyridine–acetate buffer (2:2:96 pyridine–acetic acid–water).

Reduction and carboxamidomethylation of α_1-acid glycoprotein. — The procedure followed was essentially that of Bellisario *et al.*[20]. To a solution of 3.70 g of α_1-acid glycoprotein[16,17] in 148 ml of 0.5M Tris–HCl buffer, pH 8.5, containing urea (8M) and EDTA (2%) was added 1,4-dithiothreitol (0.67 g). The mixture was kept under nitrogen for 30 min at room temperature, and then iodoacetamide (1.63 g) was added. After incubation in the dark for a further 30 min, the mixture was dialyzed in the dark against 3 changes of distilled water (5 liters each) for 72 h at 4°; yield, 3.76 g.

Preparation of the glycopeptide by digestion of reduced, carboxamidomethylated α_1-acid glycoprotein with pronase, aminopeptidase M, and carboxypeptidase A. — Digestion of the reduced, carboxamidomethylated α_1-acid glycoprotein (3.76 g) with pronase was carried out essentially by the procedure of Wagh *et al.*[21]. The glycopeptide was isolated by passing the incubation mixture through a column of Sephadex G-50 (fine) packed in 0.5% ammonium hydrogencarbonate in 0.1% 1-butanol in water.

The foregoing glycopeptide (1.5 g) was dissolved in 38 ml of 0.025M Tris–HCl buffer, pH 7.5. Aminopeptidase M (8 mg) was added, and the mixture was incubated for 12 h at 37°. Carboxypeptidase A (0.4 mg, 20 units) dissolved in 1.3 ml of 10% lithium chloride was added to the mixture, followed by sodium chloride (230 mg) to attain a concentration of 0.5M. Incubation at 37° was then continued for an additional 12 h, after which time the mixture was lyophilized. The residue was dissolved in 20 ml of water and was then passed through a column (2.6 × 180 cm) of Sephadex G-50 that had been packed in and was eluted with 0.5% aqueous ammonium hydrogencarbonate. Fractions containing both the peptide and carbohydrate were pooled and lyophilized; yield, 1.47 g.

Acetylation of the N*-terminal amino acid of the glycopeptide.* — To a solution of the glycopeptide (1.47 g) in 100 ml of 10% aqueous sodium hydrogencarbonate, acetic anhydride (2.5 ml) was added dropwise. The mixture was kept overnight at room temperature, at which time the pH of the mixture was adjusted to 6.0, and it was then concentrated to 20 ml. The *N*-acetylated glycopeptide was isolated by gel filtration through a column (2.6 × 180 cm) of Sephadex G-50 (fine), packed in 0.5% aqueous ammonium hydrogencarbonate and eluted with the same buffer; yield, 1.45 g. This preparation was devoid of unsubstituted amino groups according to *N*-terminal amino acid analysis by the DNP-method[22–24]. The carbohydrate content of the glycopeptide is given in Table I.

Reduction of the N*-acetylated glycopeptide with EDC–sodium borohydride.* — The procedure of Taylor and Conrad[10], with modifications, was employed for the reduction of the carboxyl groups of sialic acid and the *C*-terminal amino acid of the

glycopeptide. A solution of the *N*-acetylated glycopeptide (0.400 g) in 52 ml of water was adjusted to pH 4.7, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (1.0 g) was added. The pH was maintained at 4.7–4.8 by titration with 0.1M hydrochloric acid for 3 h. A 2M solution of sodium borohydride (7.92 g) in 104 ml of water was then added slowly, while maintaining the pH at 7–7.2 by the addition of 4M hydrochloric acid. The mixture was concentrated to ~70 ml and applied to a column (2.6 × 180 cm) of Sephadex G-50 packed in 0.5% aqueous ammonium hydrogencarbonate. The column was eluted with the same buffer, and 5-ml fractions were collected. Fractions 90–122 (peak I) were pooled and lyophilized to obtain 0.093 g of solid residue that appeared to be the dimerized glycopeptide (see Discussion).

Fractions 125–156 (peak II) were pooled and lyophilized to obtain 0.255 g of the mixture of completely and partially reduced glycopeptides (**B** and **C**), which was fractionated on a column (2.3 × 17 cm) of DEAE-Sephadex A-50 packed in water. The fully reduced glycopeptide (**B**) was eluted with water. The partially reduced glycopeptide (**C**), eluted with 0.5% ammonium hydrogencarbonate, was resubjected to the foregoing reduction procedure. The overall yield of the fully reduced (Glycopeptide **B**) and partially reduced (Glycopeptide **C**) glycopeptides was 25% each. Table I records the carbohydrate content of glycopeptides **B** and **C**.

Methyl ester, methyl glycoside of N-*acetylneuraminic acid* [*methyl* (*methyl 5-acetamido-3,5-dideoxy*-D-glycero-D-galacto-*nonulopyranosid*)*onate*, **2**]. — *N*-Acetylneuraminic acid (**1**, 10 mg) was methanolyzed with 0.1M methanolic hydrochloric acid for 6 h at 90°. The acid was neutralized with silver carbonate (22 mg), and to the mixture was added acetic anhydride (0.1 ml) to reinstate the *N*-acetyl groups removed during methanolysis. After keeping overnight at room temperature, the inorganic solid was filtered off through Celite. Evaporation of the filtrate under diminished pressure left a light-yellow syrup of **2**. The g.l.c. pattern of the per(trimethylsilyl) derivative of **2** is shown in Fig. 1A. On paper electrophoresis at pH 4.7 at 2000 V for 45 min, compound **2** remained at the origin. Treatment of **2** with 0.1M sodium carbonate for 8 h at room temperature converted it into **3** which, on paper electrophoresis, migrated toward the anode at the same rate as NANA.

Methyl glycoside of carboxyl-reduced N-*acetylneuraminic acid* [*methyl 5-acetamido-3,5-dideoxy*-D-glycero-D-galacto-*nonulopyranoside*, **4**]. — The methyl ester, methyl glycoside (**2**, 4 mg) obtained (foregoing) was reduced with sodium borohydride to obtain **4**, essentially as described by Kuhn and Gauhe[11]. G.l.c. of trimethylsilylated **4** showed essentially one peak (Fig. 1B). However, methanolysis of **4** with 0.1M methanolic hydrogen chloride, followed by *N*-acetylation and trimethylsilylation, resulted in a pattern shown in Fig. 1C, which is different from that obtained prior to methanolysis of **4** (see Discussion). The methyl glycoside **4** did not migrate on paper electrophoresis at pH 4.7 at 2000 V. Treatment of **4** with 0.1M sodium carbonate for 8 h at room temperature showed the absence of **3** by paper electrophoresis, thus establishing the lack of contamination by **2**.

Enzymic studies with the reduced glycopeptide. — The protection of the reduced

glycopeptide (B) from neuraminidase by the carboxyl-reduced NANA groups was measured by the ability of *A. niger* β-D-galactosidase to release D-galactose in the presence of *V. cholerae* neuraminidase. Determinations were carried out with both the reduced glycopeptide (B) and the *N*-acetylated, unreduced glycopeptide (A). Assay mixtures contained: a 0.1% solution of the glycopeptide in water, 250 μl; 0.2M sodium acetate buffer, pH 5.4, 50 μl; 0.01M calcium chloride, 30 μl; neuraminidase (20 units), 40 μl, or water, 40 μl; β-D-galactosidase (4 μg, 80 μl); total volume, 450 μl. For each glycopeptide, 2 tubes were set up; one contained both the neuraminidase and β-D-galactosidase, and the other contained only β-D-galactosidase. The mixtures were layered with 2 drops of toluene and incubated for 24 h at 37°. At the end of incubation, 20 μg of mannitol (100 μl of 0.02% solution) was added. The mixtures were passed through columns (0.8 × 8 cm) of Amberlite MB-3, 10-ml effluents being collected by elution of the columns with water. After removal of water with a rotary evaporator, the residues were transferred to small vials with a small volume of 50% ethanol. The ethanol was evaporated and the samples were dried overnight under high vacuum. Trimethylsilylating reagent[18] (50 μl) was added, and 1-μl aliquots were injected into the g.l.c. column.

The unreduced glycopeptide (A) lost 91.5% of its galactose in the presence of the two enzymes after 4 days, whereas none was lost in the presence of β-D-galactosidase alone (Fig. 2A). The corresponding losses for the reduced glycopeptide (B) were 4% (both enzymes) and 2% (β-D-galactosidase alone) (Fig. 2A). Fig. 2B illustrates the action of β-D-galactosidase on the reduced and unreduced glycopeptides from which carboxyl-reduced NANA and NANA, respectively, had been removed by hydrolysis with 0.1M hydrochloric acid. Nearly identical amounts of galactose were hydrolyzed by β-D-galactosidase from the two glycopeptides.

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