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MODIFICATION OF SIALYL RESIDUES OF GLYCOCONJUGATES BY REDUCTIVE AMINATION. CHARACTERIZATION OF THE MODIFIED SIALIC ACIDS*

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ABSTRACT

The sialic acid residues of α_1-acid glycoprotein and fetuin were modified by introduction of an amino residue, such as glycine and [^{3}H]glycine. This modification involved (a) the selective periodate oxidation of the exocyclic carbon atoms of the sialic acid residue generating an aldehyde group at C-7, and (b) the reduction of the Schiff base formed with an amino compound by use of sodium cyanoborohydride. Thin layer chromatography, high pressure liquid chromatography, and amino acid composition data of the modified glycoprotein showed that the conversion was essentially quantitative. The glycine-modified sialic acids were isolated by mild acid hydrolysis and identified by g.l.c.–m.s. and n.m.r. spectroscopy, thus confirming that the quantitative modification produced a glycine-aminated C-7 sialic acid analog. Strong acid hydrolysis of the glycine-modified sialic acid yielded a fragment that had chromatographic characteristics similar to those of glycine.

INTRODUCTION

Sialic acid is a nine carbon sugar containing an exocyclic chain and is found as a terminal nonreducing group linked, in glycoproteins and glycolipids, to D-galactosyl, 2-acetamido-2-deoxy-D-galactosyl, and on occasion to other sialyl residues[1]. Sialic acid has been implicated in many biological processes including receptor recognition by parasites[2] and toxins[3]. It is also one of the factors involved in determining the survival of glycoproteins in blood circulation. This role was

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initially described by Morell *et al.*[4], and subsequently has been under intensive investigation[5]. Removal of sialic acid from glycoproteins by sialidase treatment exposes D-galactosyl groups which are then recognized by receptors, thus signaling their uptake for degradation in the liver[5]. McLean *et al.*[6], and subsequently Gahmberg and Andersson[7] reported a procedure for the modification of the sialyl groups of glycoproteins, which consisted of a limited oxidation with periodate, followed by reduction with sodium borohydride. The modified sialoglycoproteins retained the same plasma half-life as their native counterparts[8].

The resultant aldehyde produced by periodate oxidation of sialic acid can also be subjected to various other modifications[9,10]. Recently, we described[11,12] a procedure wherein the 7-carbon aldehyde product reacted with various amino compounds to form a Schiff base adduct which, in turn, was selectively reduced with sodium cyanoborohydride; the modifications of the sialyl residues rendered them resistant to various sialidases, a property possessed in part by the C-7 analog[13]. We describe herein the isolation and structural characterization of the modified sialic acids. The biological properties of analogously modified gonadotropic hormones were also studied and will be reported elsewhere[14].

EXPERIMENTAL

Materials. — α_1-Acid glycoprotein (α_1-AGP) was purified in this laboratory[15] or obtained from Sigma Chemical Co., St. Louis, MO. Fetuin (obtained by Spiro method) was from Gibco, Grand Island, NY. Aspartic acid and alanine were purchased from Behring Diagnostics, San Diego, CA, and the alanine methyl ester from Sigma. Aldrich Chemical Company, Milwaukee, WI supplied 2-(2-aminoethyl)-1-methylpyrrolidine and 1-(2-aminoethyl)piperidine, and Bio-Rad Lab., Richmond, CA, glycine.

In all of the sialic acid modification experiments, the following chemicals were utilized: $NaIO_4$ (Matheson Coleman and Bell, Norwood, OH), $NaBH_3CN$ (Aldrich), $NaBH_4$, and 1,2-ethanediol (Fisher Scientific Company, Fairlawn, NJ). The methyl β-glycoside of neuraminic acid was obtained from Boehringer Mannheim Biochemicals, West Germany.

The column (300 × 7.8 mm) of liquid chromatography under pressure (l.c.), was filled with Aminex Ion Exclusion HPX-87H, and the t.l.c. plates (Silica Gel 60, layer thickness 0.25 mm) were purchased from Bio-Rad Lab. and MCB Manufacturing Chemists, Inc., Cincinnati, OH, respectively. Both Bio-Gel P-2 (200–400 and –400 mesh) and AG-50WX8, AG3-X4A, and AG I-X8 ion-exchange resins were obtained from Bio-Rad Lab. DEAE–Sephacel was from Pharmacia, Piscataway, NJ.

Modification of glycoproteins. — α_1-AGP and fetuin were dissolved in acetate buffer (0.1M sodium acetate buffer, pH 5.6 containing 0.15M NaCl) at a concentration of 2.12 mg/mL. Freshly prepared, ice-cold 12mM $NaIO_4$ (a ten-fold excess in relation to the amount of sialic acid present) was added to the glycoprotein

solution and the mixture stirred[8] for 10 min at 0°. The oxidation was terminated by adding 1,2-ethanediol (a five-fold excess) and the solution was stirred for another 10 min at 4°. Under the conditions utilized, no measurable side reactions were observed. The mixture was dialyzed against water to remove all the formaldehyde (from C-9 of sialic acid and from 1,2-ethanediol), and the oxidized glycoproteins were then lyophilized. The periodate-treated glycoproteins were resuspended in 0.2M borate buffer, pH 7.8. A fifty-fold excess (based on sialic acid) of one of the amino compounds (alanine, methyl ester of alanine, aspartic acid, glycine + 10 Meq of [^{3}H]glycine, the pyrrolidine derivative, or the piperidine analog) was added to each solution. Immediately following the addition of the amino compound, a ten-fold excess of $NaBH_3CN$ was added. Each solution was stirred at room temperature; after 10 and 20 min, a ten-fold excess of $NaBH_3CN$ was added and, after 45 min, a ten-fold excess of $NaBH_4$ was added[16,17]. The reduction was terminated after 1 h by neutralizing the solution with 0.04M acetic acid. The modified glycoproteins were dialyzed first against M NaCl and then water, and lyophilized. These conditions were determined to be optimal for preparation of the derivatives of interest[18].

Preparation of the methyl β-glycoside of N-*acetylneuraminic acid.* — *N*-Acetylation of the methyl glycoside of neuraminic acid was performed as described by Yu and Ledeen[19]. Examination by t.l.c. and l.c. showed that some *O*-acetylation had occurred. Therefore, the *O*-acetyl groups were hydrolyzed by resuspending the methyl glycoside in 4:1 methanol–aqueous ammonia and stirring for 24 h at room temperature in a glass-stoppered vessel. The solution was concentrated to dryness, the residue dissolved in water, the solution passed through a series of ion-exchange columns [AG 50W-X8 (H^+) and AG3-X4A (OH^-)], and the effluent lyophilized.

Modification of the methyl β-glycoside of N-*acetylneuraminic acid with amino compounds.* — The glycoside of **1** was modified by coupling either alanine, methyl ester of alanine, aspartic acid, or the pyrrolidine analog to the periodate-oxidized sialyl compound under conditions as described above for glycoproteins. The modified β-NeuAcOMe was purified by gel filtration in a column of Bio-Gel P-2.

Isolation of the modified sialic acid residues of glycoproteins. — The derivatized sialic acid was isolated by mild acid hydrolysis from the modified glycoproteins (50mM H_2SO_4, 1 h, 100°), followed by fractionation in a column of Bio-Gel P-2. The fractions were monitored for sialic acid by the periodate–resorcinol assay or for radioactivity.

Methanolysis of modified α_1-*AGP.* — The solution of modified α_1-AGP was transferred to an ampoule, lyophilized, and the residue dried overnight in a vacuum desiccator over P_2O_5 and NaOH. To the dry sample was added 1.5M methanolic HCl (0.5 mL), and the tube was flushed with N_2 and sealed. The ampoule was placed in an 85° oil bath for 24 h. Methanolysis was stopped by neutralization with Ag_2CO_3 and the sample was subjected to *N*-reacetylation by treatment with acetic anhydride (100 μL) for 6 h at room temperature. The content of the ampoule was transferred to a clean centrifuge tube and centrifuged at 2000 r.p.m. for 10 min.

The supernatant solution was collected, and the residue rinsed several times with dry methanol and recentrifuged. All supernatant solutions were combined and evaporated, under a stream of N_2, and a water solution of the residue was fractionated by gel filtration in a Bio-Gel P-2 column. The resulting methyl glycoside methyl ester of the modified sialic acid was dried overnight in a vacuum desiccator over P_2O_5 and NaOH.

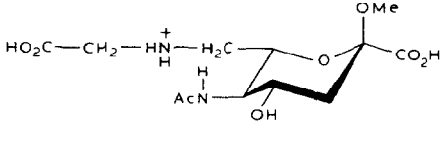
Preparative isolation of glycine-modified NeuAc (Gly-NeuAc, **1**). — For the isolation of large quantities of unlabeled Gly-NeuAc, glycine-modified fetuin (38 mg) was hydrolyzed with 50mM H_2SO_4 (4 mL) for 1 h at 80°. The hydrolyzate was made neutral by the addition of M NaOH (0.4 mL) and lyophilized. The residue was dissolved in 0.1M pyridine acetate (1 mL) and fractionated in a column (3 × 90 cm) of Bio-Gel P-2 (−400 mesh), eluted with pyridine acetate and previously calibrated with [^{3}H]glycine-NeuAc. The fractions corresponding to the elution position of [^{3}H]glycine-NeuAc were combined and lyophilized. The product was further purified by ion-exchange chromatography in a column (0.7 × 10 cm) of AG I-X8 (HCO_2^-). The sample was applied to the resin and the unbound material eluted with water; elution with 0.3M formic acid, followed by lyophilization yielded 2.7 mg of Gly-NeuAc (**1**; Scheme 1).

Thin-layer chromatography. — The modified sialic acids were rapidly screened by t.l.c. using pre-coated Silica Gel 60 plates without fluorescent indicator (5 × 20 cm, support glass, layer thickness 0.25 mm). The silica plates were activated for 1 h at 100° and developed with 7:3 propanol–water. The spots were detected with a periodate–resorcinol spray[21] and the R_F values calculated from the migration distances.

Mass spectrometry and gas chromatography–mass spectrometry. — The mass spectrometer was a Finnigan-MAT 312 with reverse geometry (magnetic sector preceding electric sector) fitted with a combined c.i.–e.i. ionization source. Fused silica columns containing a chemically bonded liquid phase (Durabond-1701, J & W Scientific, Inc.) were directly coupled to the mass spectrometer and the eluted peaks were ionized by c.i. The temperature program was 75–300° at 10°/min and the reagent gas used in the c.i. mode was ammonia.

Amino acid and radioactivity analysis. — Amino acid analysis was performed with a Dionex amino acid analyzer after hydrolysis of the material with 6M HCl *in vacuo* for 24 h at 110°.

Aqueous samples (0.2 or 1.0 mL) were mixed with ACS II counting scintillant



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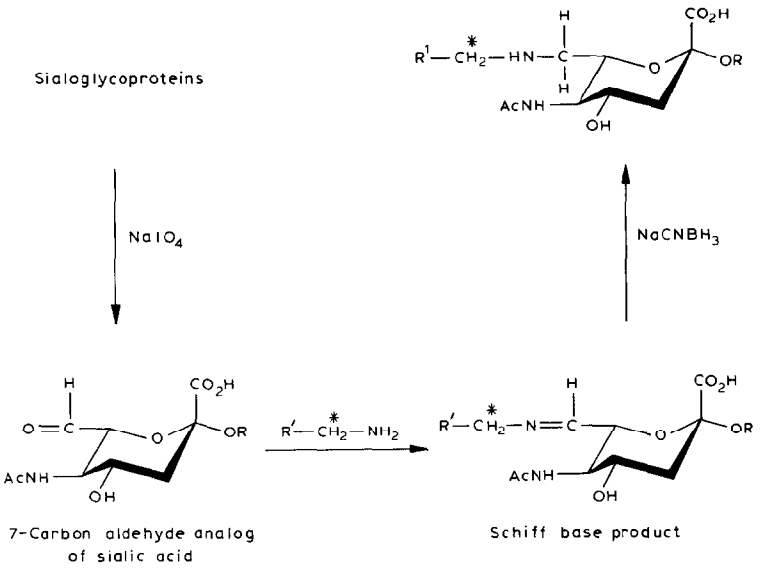
Scheme 1.

(Amersham) (2 or 10 mL) in plastic vials, and liquid scintillation counting was performed in an Intertechnique Model SL-4000 spectrometer equipped with a d.p.m. calculating module.

RESULTS AND DISCUSSION

Optimal reaction conditions for the modification of sialyl residues of glycoconjugates have been previously established[7,12,18]. The treatments described were shown to modify exclusively the sialyl residues of glycoproteins without altering the protein core or other sugars residues. The degree of modification of sialyl residues of glycoproteins was determined, as described previously, by mild acid hydrolysis followed by l.c. In all cases, the percent of incorporation of amino compounds was estimated to be between 90 and 95%, based on the levels of unmodified sialic acid or its C-7 and C-8 analogs detected by l.c. The modification was also examined by amino acid analysis of the samples modified with alanine and the methyl ester of alanine. An increase in material migrating as alanine was observed with no change in the other amino acids (data not shown). The hydrolysis conditions were such as to degrade the sialic acid, and the residual fragments migrating as alanine may contain some portion of the original structure (see below).

In the present study, the methyl β-glycoside of NeuAc was also modified with various amino compounds in order to determine the structure of the modified sialic acids (Scheme 2). The degree of modification of the methyl β-glycoside of NeuNAc by amino compounds was similarly examined by l.c. The decrease in the sialic acid peaks after modification is characteristic of the l.c. column, which irre-



Scheme 2. Proposed structure of the methyl β-glycoside of *N*-acetyl-D-neuraminic acid modified with glycine by reductive amination of its C-7 aldehyde; overall reaction scheme.

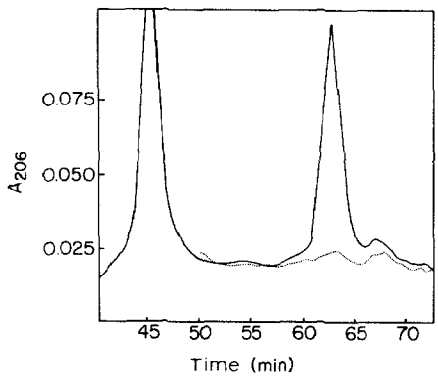


Fig. 1. Liquid chromatography of the methyl β-glycoside of NeuAc. After modification, the methyl glycoside derivatives were analyzed in an Aminex ion exclusion HPX-87H column with 3mM H_2SO_4 as mobile phase and a flow rate of 0.1 mL/min. The solvent and the samples were filtered through a 0.45-μm Millipore filter before use. The methyl glycosides of NeuAc analogs were detected by absorbance at 206 nm: (——) methyl β-glycoside of C-7-NeuAc; (·····) methyl β-glycoside of NeuAc after modification with glycine, L-alanine, methylester of L-alanine, L-aspartic acid, and 2-(2-aminoethyl)-2-methylpyrrolidine; these gave substantially identical curves. The peak eluted at ~63 min is β-NeuAcOMe (C-7 analog). The solvent peak was eluted at ~45 min and NeuAc at 51 min.

versibly binds amino acids and other cationic compounds. When modified sialic acids were analyzed, in all cases; neither native sialic acid nor any C-7 analog was detectable (Fig. 1). The derivatized sialic acids were also examined by t.l.c., typical R_F values are listed in Table I. The t.l.c. procedure provided a rapid screening for the completion of the modification of sialic acids. In the lanes where modified sialic acids were separated, neither the original sialic acid nor the C-7 analog was visible.

Acid hydrolysis of [^3H]glycine-modified glycoproteins. — We previously demonstrated that the modified sialic acids could be partially released from the glycoproteins by mild acid hydrolysis. In the present experiments, treatment of

TABLE I

T.L.C. OF MODIFIED SIALIC ACIDS

Compound	R_F *Value*
NeuAc	0.38
NeuAc C-7 analog	0.42
Aspartic acid-NeuAc[a]	0.30
Pyrrolidine-NeuAc[b]	0.23
Piperidine-NeuAc[c]	0.03
β-NeuAcOMe	0.38
β-NeuAcOMe C-7 analog	0.42
Gly-NeuAcOMe	0.50

[a]NeuAc modified with aspartic acid. [b]NeuAc modified with 2-(2-aminoethyl)-1-methylpyrrolidine. [c]NeuAc modified with 1-(2-aminoethyl)piperidine.

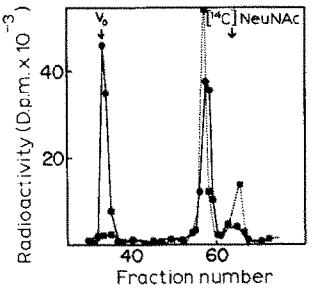


Fig. 2. α_1-Acid glycoprotein was modified with [^{3}H]glycine and hydrolyzed with 0.05M H_2SO_4 for 1 h at two different temperatures: 85° (—●—●—); and 100°, (····●····●····). Following hydrolysis, the [^{3}H]glycine-modified α_1-AGP samples (0.6 mL) were chromatographed on a Bio-Gel P-2 (0.9, 110 cm) column; 1.0-mL fractions were collected and analyzed for radioactivity. The material eluted in fractions 55–59 is Gly–NeuAc (**1**); the minor peak (fraction 64) is not NeuAc. The peak elution positions of Blue Dextran (V_0) and [^{14}C]NeuAc are indicated by arrows.

[^{3}H]glycine-modified α_1-AGP with 50mM sulfuric acid for 1 h at 85° resulted in the release of about 50% of the modified sialic acid ([^{3}H]Gly-NeuAc) (Fig. 2). More stringent conditions (4M hydrochloric acid, 2 h, 100°) resulted in the degradation of the released [^{3}H]Gly-NeuAc as previously reported[12].

[^{3}H]Gly-NeuAc (9300 d.p.m.) (**1**) was mixed with unlabeled NeuAc (1 μmol) and subjected to ion-exchange chromatography in a column of DEAE-Sephacel (Fig. 3). It can be seen that ~98% of the radioactive material was eluted in a single homogenous peak, slightly ahead of native NeuAc.

Thiobarbituric acid reaction of modified sialic acids. — Analysis of Gly-NeuAc (**1**) by the thiobarbituric acid method[22] showed that the chemical modification of NeuAc had a marked effect on the color yield. Gly-NeuAc (**1**) yielded only

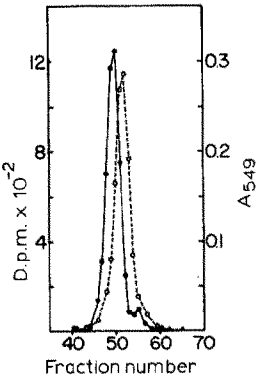


Fig. 3. Chromatography of [^{3}H]Gly-NeuAc (from the experiment illustrated in Fig. 2) and NeuAc on DEAE-Sephacel column. The column (1.5 × 25 cm) was eluted with a linear gradient (0.01–0.5M) of 0.1M pyridine–acetic acid (300 mL). Fractions (2.0 mL) were collected and aliquots analyzed for sialic acid by the thiobarbituric acid assay A_{549} (—●—●—) and for radioactivity (-O---O-)

65% of the color given by an equivalent amount of unmodified NeuAc. A molecular weight of 306 for Gly-NeuAc was used for the calculation on the basis that it was formed from the C-7 analog[12]. This decrease in color in the thiobarbituric acid reaction is in agreement with the previously observed 45–50% reduction in color formation of C-7 analog of NeuAc, and a 60% reduction for the cyanohydrin-modified NeuAc[8,13,23].

Structural studies of modified sialic acids. — The structures of the C-7 analog of the methyl β-glycoside of NeuAc and the methyl glycoside of Gly-NeuAc were analyzed by g.l.c.–m.s. The total ionization plot of the glycine-modified methyl β-glycoside methyl ester of NeuAc (Gly-NeuAcOMe) is shown in Fig. 4, (lower panel). The reaction products were per(trimethylsilyl)ated, fractionated by g.l.c. (Fig. 4, upper panel), and analyzed by c.i.m.s. (NH_3). The last peak eluted (scan number 251) had a retention time close to a neuraminyl derivative with the expected molecular weight. The molecular ion, at *m/z* 479 indicated a glycine-modified C-7 analog. There were no detectable peaks in the g.l.c. elution pattern for any higher analog, nor was there any unreacted neuraminyl starting material.

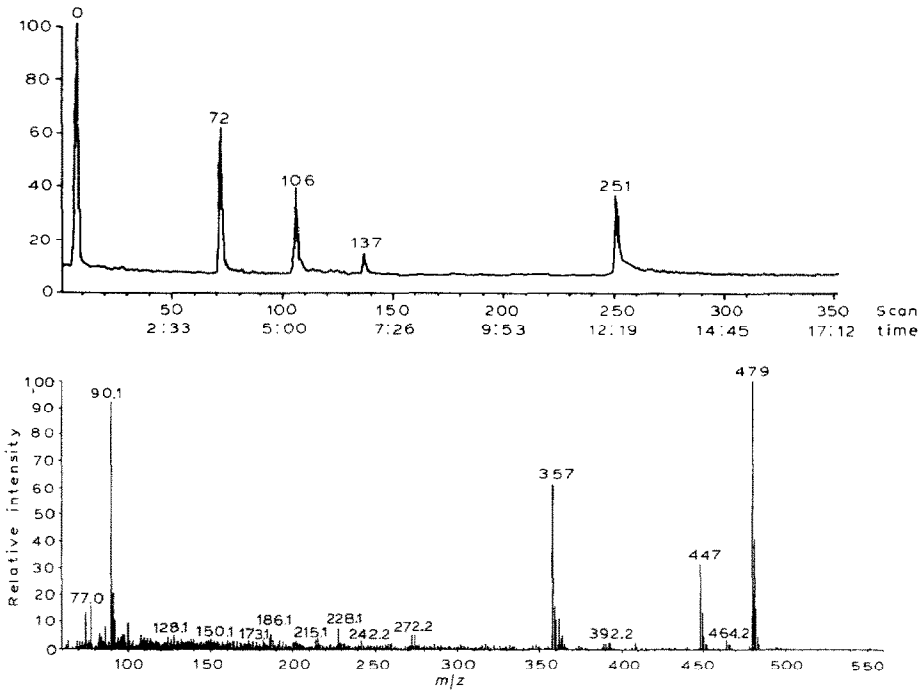


Fig. 4. G.l.c. and c.i.m.s. of the methyl β-glycoside of Gly-NeuAc. The samples were derivatized with a trimethylsilylating agent and subjected to g.l.c. The spectrum was recorded with a 3AB SE mass spectrometer (VG Analytical, Manchester, U.K.). The following samples were analyzed: methyl β-glycoside of analog of NeuAc C-7, methyl β-glycoside of Gly-NeuAc (spectrum shown here), and Gly-NeuAc isolated from modified α_1-AGP by methanolysis. The mass fragmentation pattern of all samples showed that the modified product was glycine coupled to the 7-carbon aldehyde analog of sialic acid.

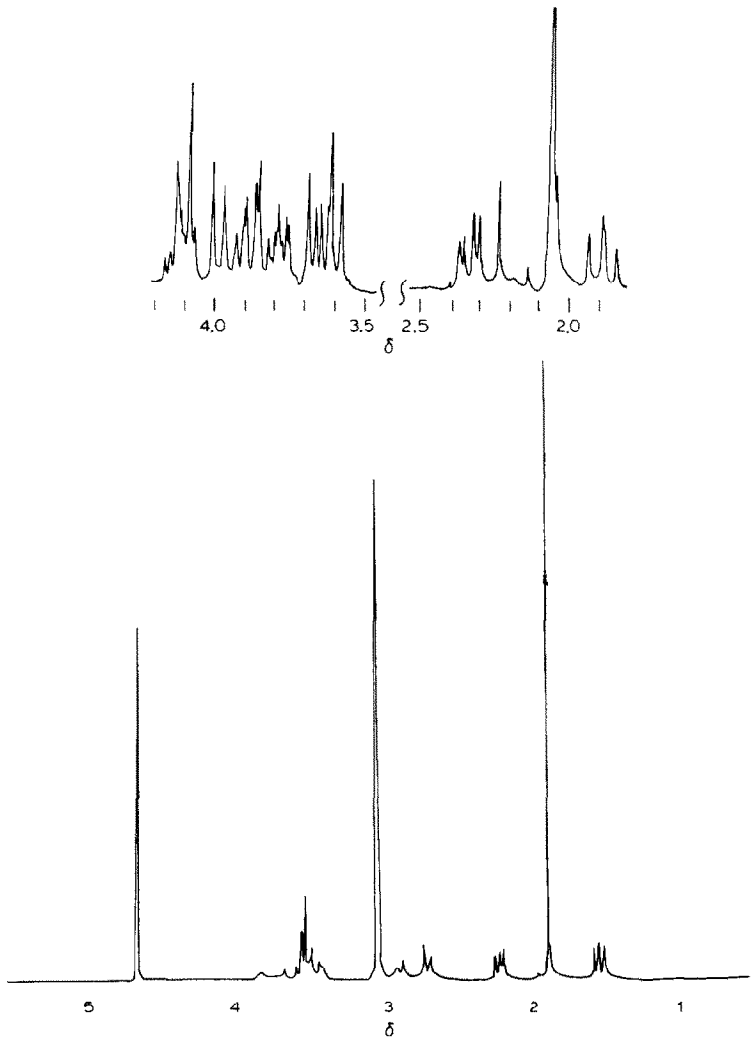


Fig. 5. ^{1}H-N.m.r. spectrum at 360 MHz of sialic acid (top panel) and of a modified methyl β-glycoside of NeuAc, obtained by $NaIO_4$ oxidation and $NaBH_4$ reduction (bottom panel). The modified product was purified by ion-exchange chromatography using a Dowex 1 (HCO_2^-) column. The spectrum was kindly recorded by Alan Freyer, Dept. of Chemistry, The Pennsylvania State University, State College, PA.

The fragment at *m/z* 447 was produced by methanol elimination and that at *m/z* 358 may be explained by the double loss of methanol plus a trimethylsilanol group. These data indicated that the modification produced the expected product, a Gly-NeuAc C-7 analog (**1**), and showed that the reactions were essentially quantitative. The mass spectrum of the periodate-oxidized, sodium borohydride-reduced methyl β-glycoside of NeuAc showed that only the C-7 analog was detected.

The ^{1}H-n.m.r. spectra for native sialic acid (top panel) and for the β-NeuAcOMe degradation product (bottom panel) are shown in Fig. 5. The splitting pattern observed in the spectrum for sialic acid analogs is typical for sialic acid. Splitting patterns of the methylene protons at C-3 (δ 1.9 and 2.4) can be seen in Fig. 5 (top and bottom panels). The proton, H-3*e*, is distinguished from H-3*a* by its lower resonance δ 2.4 and 1.9, respectively. The peak at δ 2.0 (top and bottom panels) was ascribed to the three methyl protons of the amide group. The loss of protons for C-8 and C-9 (in the range δ 3.8–4.2) of the modified sialic acid (bottom panel) confirms that the C-7 analog of sialic acid was produced by the present modification, and is in agreement with the results obtained by mass spectrometry.

Nature of the breakdown product of Gly-NeuAc. — In our previous studies[12], we observed that when the glycine-modified sialoglycoprotein or the isolated Gly-NeuAc was subjected to strong acid hydrolysis, a low-molecular-weight fragment was released. This breakdown product had a mobility identical to that of glycine in a Bio-Gel P-2 column and on the amino acid analyzer. To further investigate the nature of this breakdown product, [^{3}H]Gly-NeuAc was hydrolyzed with 6M hydrochloric acid for 24 h at 110°, and the products chromatographed together with various amino acid standards on Whatman No. 1 paper in 4:1:5 (upper layer) butyl alcohol–acetic acid–water for 24 h. The strip of paper containing the labeled sample was cut in 1-cm pieces, extracted with water (1 mL), and the radioactivity in the extract determined by scintillation spectrometry. The positions of the standard amino acids were determined by spraying with a solution of ninhydrin and heating the paper at 100°. It was found that ~91% of the radioactivity comigrated with standard glycine (data not illustrated). The release by acid hydrolysis of a component having a mobility identical with that of glycine in several chromatographic systems was unexpected. If the structure of the glycine-modified sialic acid is as expected (**1**), no release of glycine is expected, even under very strong acid hydrolysis conditions since the secondary amine (–C–N–) bond is not cleaved by acids. However, it is well known that when sialic acid is heated with mineral acids, it undergoes considerable decomposition, of a non-specified nature, to eventually form brown-black "humin" substances[24,25]. Therefore, we hypothesize that what is being released is a component, HO_2CH_2–NH–R, where R is a small fragment derived from the sialyl residue. Additional amounts of this compound were isolated by acid hydrolysis of unlabeled Gly-NeuAc, followed by gel filtration on a Bio-Gel P-2 column. Mass spectroscopic examination of the material failed to yield conclusive results as to its structure, but it is not glycine.

The present method makes available a simple procedure for specifically radiolabeling sialic acid groups of glycoproteins and makes this technique suitable for metabolic studies on circulating proteins. Advantages of this approach are that a wide variety of R groups can be used, ranging from simple to complex amino compounds, a variety of radiolabels may be introduced into this part of the macromolecule, and the modified sialyl residues are resistant to neuraminidase. Furthermore, the use of sodium cyanoborohydride allows the modification to be carried out at a neutral pH and in an aqueous environment.

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