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Modification and Introduction of Various Radioactive Labels into the Sialic Acid Moiety of Sialoglycoconjugates

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A method for modifying and isotopic labeling the sialyl moiety of sialoglycoproteins is described. The basis of the procedure is the reductive amination of the exocyclic aldehyde group, generated on sialic acid by mild periodate oxidation, with a variety of amino compounds and sodium cyanoborohydride. Optimal conditions were selected to obtain maximum modification of sialic acid and minimal non-specific incorporation of the amino compound (glycine). The glycine modified model glycoproteins (α_1-acid glycoprotein, fetuin) yielded single homogenous peaks upon gel filtration and on ion exchange chromatography. On gel electrophoresis a major band accounting for 92-98% of the modified glycoprotein and two minor bands consisting of dimers and trimers of the glycoprotein were observed. The modification did not alter the ability of the sialoglycoproteins to bind to wheat germ agglutinin-Sepharose or to interact with antibodies.

The modified sialic acid was only partially released by mild acid hydrolysis suggesting that the introduction of an amino compound into the polyol chain of sialic acid has a stabilizing effect on the ketosidic linkage of the sugar. Interestingly, the modification rendered the sialic acid resistant to a variety of sialidases.

The potential uses of this modification procedure include 1) the introduction of different isotopic labels (^{3}H, ^{14}C, ^{35}S, ^{125}I) into the sialic acid moiety of glycoproteins; 2) the preparations of biologically active sialoglycoprotein (hormones, enzymes, co-factors) with increased circulating half-lives in animals; 3) preparation of substrates to search for endoglycosidases; 4) the direct comparison of sialoglycoprotein patterns obtained in small

Abbreviations: α_1-AGP, α_1-acid glycoprotein; C-8, C-7, refer to the 8-carbon and 7-carbon analogs of sialic acid; Gly-NeuNAc, *N*-acetylneuraminic acid modified with glycine; HPLC, High Performance Liquid Chromatography; NeuAc, *N*-acetylneuraminic acid; WGA, Wheat Germ Agglutinin.

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amounts from normal and pathological cells or tissues, and 5) the isolation and purification of cell surface sialoglycoproteins.

Sialoglycoconjugates which are widespread in animals have been implicated in an array of biological functions [1,*2]. Specifically, the terminal sialic acids or saccharides containing it have been implicated to play a role in the receptor sites of hormones, toxins, viruses, parasites; in the masking of recognition markers and antigenic sites; in the expression of antigenic determinants and in malignancy and metastasis. Procedures to modify and introduce different isotopes into sialoglycoconjugates specifically would greatly facilitate studies on the properties and biological roles of sialic acids in sialoglycoconjugates. Winzler and co-workers utilized the high periodate susceptiblity of the exocyclic polyol group of sialic acid to modify this sugar to its 8- and 7-carbon analogs (C-8 and C-7 sialic acids) and investigated the effect of the modification on its properties [3-5]. A general method for the introduction of tritium label into the sialic acid moiety of glycoproteins reported by van Lenten and Ashwell [6] has been extensively used. The procedure has also been extended to label gangliosides [7] and cell surface sialoglycoconjugates [8, 9].

A procedure for introducing a ^{14}C-label into sialyl residues by reacting the mild periodate-generated aldehyde groups with $Na^{14}CN$ by the Kiliani cyanohydrin reaction has also been reported [10]. In this report we describe a versatile method for modifying and labeling the sialyl moiety of sialoglycoproteins. The basis of the procedure is the reductive amination of the exocyclic aldehyde group, generated on sialic acid by mild periodate oxidation, with a variety of amino compounds and $NaCNBH_3$ as outlined in Fig. 1. This method allows the introduction of different isotopic labels (^3H, ^{14}C, ^{35}S, ^{32}P and ^{125}I) into the sialic acid moiety of glycoconjugates. Since NaB^3H_4 is not used, the problems with non-specific labeling and contamination by acid-resistant radioactive impurities present in preparations of NaB^3H_4 are avoided. The ability to introduce different isotopes (for example, ^3H or ^{14}C) makes this method ideal for the direct comparison of sialoglycoprotein patterns obtained in small amounts from normal and pathological cells or tissues. Further, the introduction of functional groups into sialoglycoproteins will aid in the subsequent purification. Finally, the amino-compound modified sialic acid was found to be resistant to the action of sialidases. This finding suggests that the modified sialoglycoproteins could serve as substrates in the search for endoglycosidases and should have increased circulating half-lives in animals [11]. An abstract covering part of this work has been published [12].

Materials and Methods

Materials

Preparations of α_1-acid glycoprotein available in this laboratory were used after further purification by chromatography on a column of Sephadex G-50. Fetuin (Spiro method) was obtained from GIBCO, Grand Isaland, NY, USA. Bovine lutropin was provided by the USDA Reproduction Laboratory, Beltsville, MD, USA. Sodium cyanoborohydride and sodium borohydride were purchased from Aldrich, Milwaukee, WI, USA. [2-^3H] glycine (15-30 Ci/

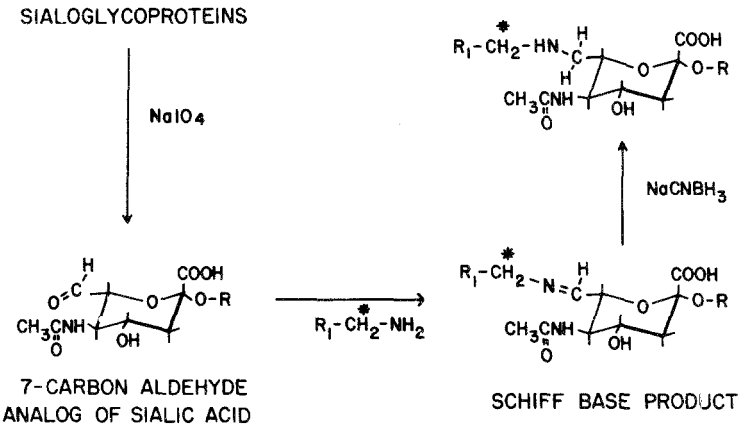

Figure 1. Scheme for the modification and *in vitro* labeling of sialoglycoproteins by mild periodate oxidation followed by reductive amination. The sialoglycoprotein is subjected to periodate oxidation (5 mM $NaIO_4$, 0°C, 10 min) and the sample is dialyzed against 0.2 M sodium borate buffer, pH 7.8. The oxidized sialoglycoprotein in the above buffer is incubated at room temperature with an excess of amino compound (R_1-CH_2-NH_2) and $NaBH_3CN$ to reductively aminate the aldehyde groups. The modified glycoprotein is recovered by dialysis followed by lyophilization.

mmole) was from New England Nuclear, Boston, MA, USA. Sodium cyanoboro[^{3}H]hydride (98.8 mCi/mg) and ^{14}C-NeuNAc (351 mCi/mmole) were obtained from Amersham, Arlington Heights, IL, USA. BioGel P-2, P-6 and P-100, and the HPX-87 HPLC column were obtained from Bio-Rad Laboratories, Richmond, CA, USA. DEAE-Sephacel, DEAE-Sepharose CL-6B, Sephadex G-50, Sepharose CL-6B, Sephacryl S-200 and WGA-Sepharose 6MB were purchased from Pharmacia, Piscataway, NJ, USA. The Lichrosorb-NH_2 column was from E. Merck, Cherry Hill, NJ, USA. *Vibrio cholerae*, influenze virus and *Arthrobacter ureafaciens* sialidases were purchased from Calbiochem-Behring, San Diego, CA, USA and *Diplococcus pneumoniae* sialidase was prepared in this laboratory [13]. Newcastle disease virus sialidase [14] was a gift from Dr. J.C. Paulson, University of California, Los Angeles, CA. Rabbit anti-α_1 acid glycoprotein antisera was obtained from Miles Laboratories, Elkart, IN, USA.

Column Chromatography

Columns of Bio-Gel P-2 (-400 mesh) and P-6 (200-400 mesh) were equilibrated and eluted with 0.1 M pyridine/0.1 M acetic acid, pH 5.0. Sepharose CL-6B and Bio-Gel P-100 were eluted with 50 mM Tris-HCl, pH 8.0. DEAE-Sephacel was generated in the acetate form, packed in column, and extensively washed with 0.01 M pyridine/0.1 M acetic acid, pH 5.0 and equilibrated in 0.01 M pyridine/0.01 M acetic acid, pH 5.0 before use. DEAE-Sepharose CL-6B was generated in the chloride form by washing with 1 M NaCl and then equilibrated with 50 mM Tris-HCl, pH 8.0 before use. Affinity chromatography on WGA-Sepharose was done as described [15].

Digestions with Enzymes

Vibrio cholera sialidase digestion was done in 0.1 M sodium acetate buffer, pH 5.6, containing 1 mM Ca^{2+} with 50 mU enzyme in a volume of 100 µl. *Arthrobacter ureafaciens* sialidase digestion was done in 50 mM sodium phosphate buffer, pH 6.8 with 0.1 unit enzyme in a total volume of 100 µl. Influenze virus neuraminidase digestion was carried out in 0.05 M Tris-maleate buffer, pH 6.5, containing 1 m M $CaCl_2$ with 0.1 unit enzyme in a volume of 200 µl. Treatment with Newcastle disease virus sialidase was in 0.1 M sodium cacodylate buffer, pH 6.5 with 0.1 unit enzyme in a total of 100 µl. All incubations were carried out at 37°C for 48 h unless otherwise stated. The treatments were terminated by heating at 100°C for 5 min, the digest centrifuged and the supernatant analyzed by thiobarbituric acid assay [16] or gel filtration for released sialic acid.

Modification of Sialic Acid Residues of Glycoproteins

In a typical experiment, α_1-acid glycoprotein (15 mg; about 6 µmol of sialic acid) was dissolved in 6.3 ml of ice cold buffer (0.1 M sodium acetate, pH 5.6 containing 0.15 M NaCl), 4.6 ml freshly prepared 0.012 M $NaIO_4$ added and the mixture stirred at 0°C (ice bath) for 10 min [17]. The oxidation was terminated by addition of ethylene glycol (50 µl) and stirring at 0°C for 5 min. The reaction mixture was dialyzed against 0.2 M sodium borate buffer, pH 7.8 to remove all formaldehyde (derived from sialic acid and ethylene glycol) and concentrated to 2.0 ml in an Amicon filter apparatus. To this solution of the oxidized glycoprotein in a glass vial was added, wtih stirring, 25 mg (about 330 µmol) of glycine in 150 µl of water followed immediately by 40 µl of a 10% aqueous solution of $NaBH_3CN$. The solution was stirred at 20°C and two more additions (30 µl each) of $NaBH_3CN$ were made after 20 and 40 min. After stirring for a further 20 min, 20 µl of a 1% aqueous solution of $NaBH_4$ was added (to reduce any unmodified aldehyde groups), the reaction vial flushed with N_2, capped and the contents stirred at 20°C for 30 min [18]. The reaction mixture was dialyzed first against 0.15 M NaCl for two days followed by distilled water for two days and lyophilized. The yield of the glycine modified glycoprotein was 13.8 mg. When necessary, additional purification of the modified glycoprotein was carried out by redissolving the sample in Tris HCl buffer, pH 8.0 containing 4 M guanidine HCl and chromatography on a column of Sephadex G-50 (see below). In other experiments, either α_1-acid glycoprotein or fetuin has been modified with norleucine, ethanolamine or cysteic acid.

In radiolabeling experiments, to 10 mg of periodate-oxidized α_1-acid glycoprotein (or fetuin) in 1.5 ml of 0.2 M sodium borate buffer, pH 7.8, 100 µCi of [2-^{3}H]glycine in 100 µl of 0.1 N HCl (+ 10 µl 1 M NaOH to neutralize the acid) was added and the mixture treated with $NaBH_3CN$ and $NaBH_4$ as described above.

The yield of [^{3}H]glycine modified glycoprotein was 8.7 mg and the product had a specific activity of 1.17×10^6 dpm per mg. In another set of experiments, periodate oxidized α_1-acid glycoprotein (15 mg) in 2.25 ml of 0.2 M sodium borate buffer, pH 7.8 was mixed with 22.5 mg of glycine in 150 µl and treated with 3 x 25 µl of sodium cyanoboro[^{3}H]hydride (750 µCi; 98.8 mCi/mg) in sodium borate buffer added at 0, 10 and 20 min. The reaction mixture was further treated with $NaBH_3CN$ and $NaBH_4$ as described. The yield of [^{3}H]glycine modified

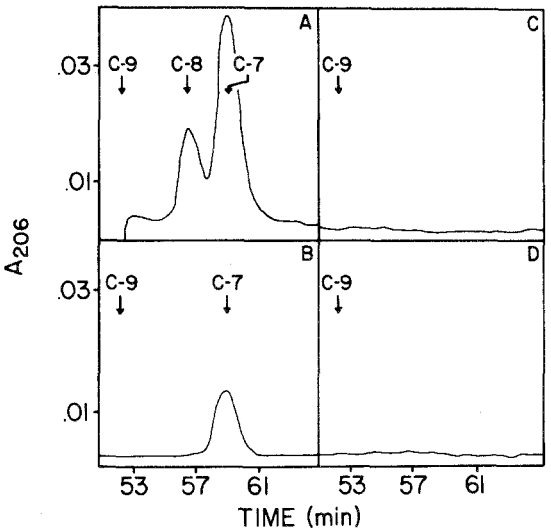

Figure 2. High performance liquid chromatogram of the acid hydrolysis products of α_1-acid glycoprotein which was treated with $NaIO_4$ and either reduced with $NaBH_4$ or reductively aminated with glycine and $NaBH_3CN$ as described in the text. The modified sialic acids were released by mild acid hydrolysis (0.1 N H_2SO_4, 100°C, 1 h) and examined by HPLC on an Aminex ion exclusion HPX-874 column (300 x 7.8 mm) using 0.006 N H_2SO_4 as eluant at a flow rate of 0.1 ml per min. The sialic acids were detected by absorbance at 206 nm. Positions of radioactive samples were determined by collecting the effluent in fractions (0.1 ml) and measuring radioactivity. The elution positions of sialic acid (C-9), 8-carbon analog of sialic acid (C-8) and 7-carbon analog of sialic acid (C-7) are indicated by arrows.
Panel A, α_1-AGP oxidized with $NaIO_4$ for 3 min and reduced with $NaBH_4$;
Panel B, α_1-AGP oxidized with $NaIO_4$ for 10 min and reduced with $NaBH_4$;
Panel C, α_1-AGP oxidized with $NaIO_4$ for 3 min and reductively aminated with glycine;
Panel D, α_1-AGP oxidized with $NaIO_4$ for 10 min and reductively aminated with glycine.

glycoprotein was 13 mg and the product had a specific activity of 5.70×10^6 dpm/mg. A control sample of α_1-acid glycoprotein (5 mg), not subjected to periodate oxidation, was also treated with glycine and NaB^3H_3CN followed by $NaBH_4$ as above. The isolated product (5 mg) had a specific activity of 0.41×10^6 dpm/mg. (see the Results section for further controls).

Results

Optimal Conditions to Maximize Sialyl Modification

The results of HPLC analysis of the sialic acids released by mild acid hydrolysis of periodate oxidized, sodium borohydride reduced α_1-AGP are illustrated in Fig. 2 (Panels A and B). It can be seen that periodate oxidation for 3 min gave rise to both C-8 and C-7 analogs; in addition, a small amount of the unoxidized C-9 analog was also detectable. In contract, oxidation for 10 min produced only the C-7 analog in agreement with the paper chromatographic results obtained by van Lenten and Ashwell [6].

In an attempt to optimize the reaction conditions and obtain maximum modification of the C7 analogs of sialic acid residues of sialoglycoproteins, we varied the concentrations of the amino compound and $NaBH_3CN$ used for reductive amination. Firstly, we tested the effect of varying the concentration of glycine on the extent of sialyl modification of periodate-oxidaized α_1-AGP using a borate buffer of pH 7.8 and a 10-fold excess of the reducing agent over the concentration of sialic acid. It was found that 0.007, 0.02, 0.4, 0.8 and 1.1 mol of glycine were incorporated per mol sialic acid when 1-, 2-, 5-, 10-, 20-, 50- and 100-fold excess of glycine in relation to the amount of sialic acid was used. In other experiments we found that varying the concentration of the reducing agent ($NaBH_3CN$) from 10- to 50-fold excess over the concentration of sialic acid, while maintaining a constant level of glycine, had no effect on the per cent sialyl modification. The reaction of an aldehyde with an amine compound in the presence of a reducing agent is pH dependent. While the Schiff base formation requires the uncharged amino group and is therefore favoured at pH's higher than the pK of the amino group of the amine compound (for glycine 9.6), the formation of the protonated form of the Schiff base (iminium ion) the species which is reduced by borohydrides, is optimal at about pH 6 [19]. The pH optimum for the reduction by $NaBH_3CN$ is between 6 and 7 [19]. Thus, we also tested, within small limits, the influence of pH on the reductive amination using buffers in the pH range of 7.4 to 8.7 but found no detectable effect. Jentoft and Dearborn [18] showed that the inclusion of transition metal ions in the reaction mixture led to a 20-30% increase in protein methylation by HCHO and $NaBH_3CH$. We therefore tested the influence of including nickel ions (10 nM of $NiCl_2$) during reductive amination of the sialyl aldehyde. However, no difference was found in the extent of sialyl modification when Ni^{++} was present in the reaction mixture.

Thus, the following standard conditions were used in all subsequent experiments involving sialyl modification: a 10 min periodate oxidation, a fifty-fold excess of the amino compound, ten-fold excess of $NaBH_3CN$ and borate buffer of pH 7.8 for the reductive amination.

Determination of the Extent of Sialyl Modification

Initially an attempt was made to separate and quantify the modified NeuAc by HPLC. Two systems reported in the literature for the analysis of sialic acid [20] and sialyl oligosaccharides [21] were tested using NeuAc, [^{14}C]-NeuAc, [^{3}H]-labeled 7-carbon analog of NeuAc [6], [^{3}H]glycine-NeuAc and glycine-NeuAc isolated by acid hydrolysis of glycine-modified α_1-AGP as described below. It was found that in both systems, the glycine modified NeuAc was retained by the column irreversibly and could not be eluted by the suggested eluants. NeuAc and the C-8 and C-7 analogs of NeuAc were well separated on the HPX-87 column (Fig. 2A and B) and their radioactive counterparts could be recovered quantitatively. Hence HPLC analysis could be used only for checking the presence of unmodified C-9, C-8 or C-7 analogs of sialic acid but not for the direct estimation of the modified sialic acid. Thus, when α_1-AGP modified under optimal conditions with glycine was subjected to mild acid hydrolysis (0.1 N H_2SO_4, 85°C, 1 h) and the products analyzed by HPLC the results illustrated in Fig. 2C and D were obtained. The absence of native NeuAc or its C-8 and C-7 analogs is indirect evidence for the quantitative modification of sialic acid by glycine.

To estimate directly the quantity of amino acid (glycine, norleucine, cysteic acid) incorporated into glycoproteins we took advantage of the release of a fragment very similar to it by

strong acid hydrolysis (discussed below). The amino acid modified fetuin and α_1-acid glycoprotein were hydrolyzed (6N HCl, *in vacuo*, 110°C 24 h) and the product analyzed on an amino acid analyzer. The results are summarized in Table 1. Assuming the introduction of one amino acid per sialyl residue, it can be seen that the modification of sialic acid in these glycoproteins ranged form 54 to 75%.

When modification was done with [^{3}H]glycine, in preliminary experiments using 100 µCi of [^{3}H]glycine (14.8 Ci/mmol) for 10 mg of α_1-acid glycoprotein only 9% of the sialic acid was modified, as calculated on the basis of the specific activity (1.17×10^6 dpm per mg) of the product. This is most probably due to the reason that Schiff base formation requires high concentrations of aldehyde and amine reactants and is therefore not favored at the very low concentration of [^{3}H]glycine used. When the reductive amination of 3 mg of periodate oxidized α_1-AGP (1.2 µmol sialic acid) was carried out using 10 µCi of [^{3}H]glycine mixed with 4.32 (58 µmol) of unlabeled glycine and optimal concentrations of $NaBH_3CN$, essentially quantitative modification of sialic acid was achieved. However, the specific activity of the product was only 6.5×10^4 dpm per mg. Thus, for the incorporation of radioactive amine compounds into sialoglycoproteins, the recommended procedure is to first carry out reductive amination with high specific activity radioactive compound followed by treatment with unlabeled compound (50 fold excess) and further $NaBH_3CN$ to achieve complete modification of sialyl residues. For preparing tritium labeled amine compound modified sialoglycoproteins, an alternative is to use sodium cyanoboro[^{3}H]hydride for the reductive amination. By this approach we were able to prepare [^{3}H]glycine α_1-AGP to higher specific activity (5.70×10^6 dpm per mg). Utilization of ^{35}S- methionine or ^{125}I-histamine allows for preparations of very high specific activity.

Non-specific Incorporation of Amine Compounds into Glycoproteins

In order to determine the degree of non-specific incorporations of glycine, i.e. the extent of modification (if any) on sugar residues other than sialic acid, we treated periodate oxidized asialo-α_1-acid glycoproteins and native α_1-acid glycoprotein without periodate oxidation with [^{3}H]glycine and $NaBH_3CN$. The dialyzed, lyophilized products contained 46 000 dpm and 88 000 dpm per mg, respectively. When these samples were redissolved in 50 mM Tris-HCl buffer, pH 8.0 containing 4 M guanidine HCl and dialyzed against the same buffer, about 38% of the radioactivity was found to be dialysable. These results indicate that non-specific incorporation of amine compound (^{3}H-glycine) is small and that dialysis versus 4 M guanidine HCl helps to remove unincorporated glycine more efficiently.

The specificity of the modification was also checked by using bovine serum albumin, and bovine lutropin (a glycoprotein devoid of sialic acids in its saccharides). The above protein and glycoprotein were subjected to periodate oxidation and reductive amination with [^{3}H]glycine under optimal conditions and the reaction mixture chromatographed on a column of Bio-Gel P-2. All radioactivity was included in the column and only insignificant amounts were detectable in the excluded material confirming that the modification was specific for sialic acid.

Gel Filtration of the Modified Glycoprotein

The native and [^{3}H]glycine modified fetuin samples were separately chromatographed on columns of Sepharose CL-6B and Bio-Gel P-100 using 50 mM Tris-HCl, pH 8.0 as eluant,

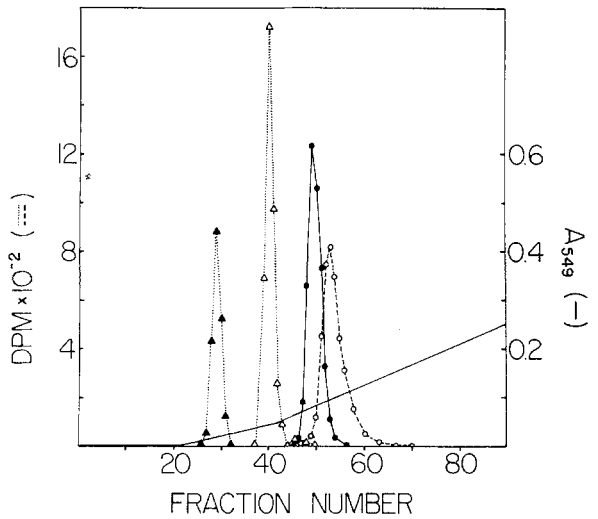

Figure 3. Ion-exchange chromatography of [^{3}H]glycine modified-fetuin (○) and unmodified fetuin(●)on DEAE-Sepharose CL-6B. The sample was mixed with [^{14}C]glucose(▲)and [^{14}C]NeuAc (△)applied on a DEAE-Sepharose column (1.5 x 27 cm) and eluted with a linear gradient (190 ml) of NaCl (0 to 1 M) in 50 mM Tris-HCl pH 8.0. Fractions of 1.5 ml were collected and after measuring conductivity (—) aliquots were analyzed for radioactivity and sialic acid by thiobarbituric acid assay (A_{549}). The results are presented by superimposing the elution patterns of two separate runs.

and on Sephacryl S-200 with the above buffer containing 4 M guanidine HCl as eluant. The elution profiles, determined by assaying protein or sialic acid for native fetuin and radioactivity for modified fetuin, were not significantly different. This suggests that the modification did not appreciably alter the hydrodynamic size and shape of the glycoprotein molecules.

Ion Exchange Chromatogrpahy of the Modified Glycoprotein

The elution profiles of the modified glycoprotein were compared with those of the native molecule on columns of DEAE-Sephacel or DEAE-Sepharose using linear gradients of pyridinium acetate or NaCl, respectively. In all cases the glycine modified glycoprotein eluted as a single peak and was partially separated from the native glycoprotein. Typical elution profiles are illustrated in Fig. 3. The retarded elution of the modified fetuin is consistent with its increased acidity resulting from the contribution of the glycine carboxyl group. Attempts to obtained complete separation of the modified and unmodified glycoproteins by using shallower or concave gradients of NaCl were unsuccessful.

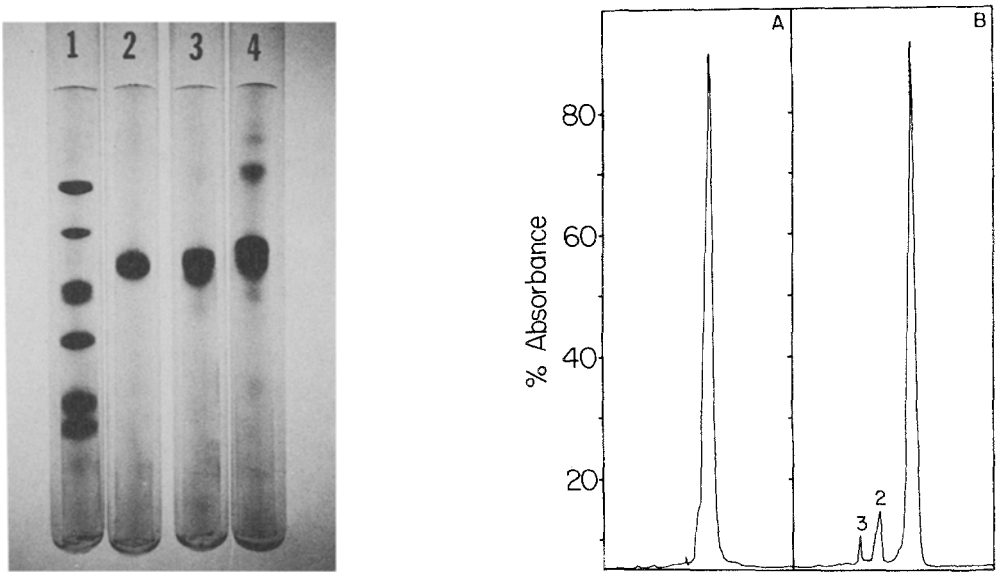

Figure 4. SDS-Polyacrylamide gel electrophoresis of fetuin (lane 3) and glycine modified fetuin (lane 4). The samples were electrophoresed in a 6% acrylamide gel and located by staining with Coomassie blue. Standards proteins shown in lane 1 are: phosphorylase (92 000); bovine serum albumin (66 000); ovalbumin (45 000); carbonic anhydrase (31 000); soy bean trypsin inhibitor (21 500); and lysozyme (14 400) and lane 2 is α_1-acid glycoprotein. The densitometric scans presented in the right panel are those of A, fetuin gel and B; glycine-modified fetuin. The areas of the peaks in B are 0.09 (peak 3) 0.40 (peak 2) and 9.45 (large peak) cm^2.

Polyacrylamide Electrophoresis

Fetuin and α_1-acid glycoprotein modified with various amino compounds (glycine, norleucine, cysteic acid) and unmodified controls were subjected to gel electrophoresis in the presence of sodium dodecyl sulfate. Typical results, illustrated in Fig. 4, show that about 95% of the modified fetuin is present in a major band, with a mobility slightly slower than that of native fetuin. The balance (5%) is distributed in two bands having apparent molecular weights of 101 000 and 160 000. These are probably the dimer and trimer of fetuin formed as a result of intermolecular cross-linking. Similar results were obtained with the other modified fetuins and with α_1-acid glycoprotein. The higher molecular weight dimer and trimer present, together, usually accounted for between 2 to 8% of the total. The [^{3}H]glycine modified fetuin was also electrophoresed on a gel and the gel cut into 2 mm slices, solubilized by incubation with perchloric acid and hydrogen peroxide and analyzed for radioactivity. Over 95% of the radioactivity was located in the area corresponding to the major Coomassie blue-stained band (data not illustrated).

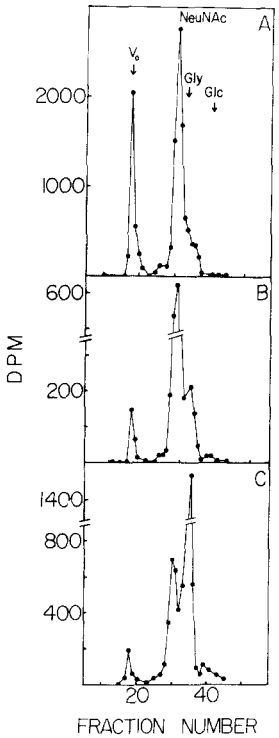

Figure 5. Gel filtration of [^{3}H]glycine-fetuin on a Bio-Gel P-2 column (0.9 x 70 cm) after acid hydrolysis. A. 0.1 N H_2SO_4 at 85°C for 1 h; B, 0.1 N H_2SO_4 at 85°C for 4 h; C, 1 N H_2SO_4 at 100°C for 2 h. The hydrolysates were neutralized with NaOH, applied to the column and eluted with 0.1 N pyridine/acetic acid. Fractions (1 ml) were collected and analyzed for radioactivity. The peak elution positions of fetuin (V_0), sialic acid (NeuAc), glycine (Gly) and glucose (Glc) are indicated.

Acid Hydrolysis of [^{3}H]Glycine-modified Fetuin

Sialic acid is readily cleaved by mild acid hydrolysis, thus [^{3}H]glycine-modified fetuin was treated with 0.1 N H_2SO_4 at 85°C for 1 h and the hydrolysate, after neutralization with NaOH, was chromatographed on a column of Bio-Gel P-2. The results, illustrated in Fig. 5A, indicate that about 72% of the modified sialic acid ([^{3}H]glycine NeuAc) was released. In control experiments, it was shown that under identical conditions, sialic acid was quantitatively released from unmodified fetuin. Thus, it appears that the introduction of amine compound (glycine) into the polyol chain of sialic acid has a stabilizing effect on the ketosidic linkage of the sugar. When hydrolysis was continued for four hours, an additional low molecular weight peak eluting in the area of standard glycine appeared (Fig. 5B). Under stronger hydrolysis conditions (1N H_2SO_4, 100°C, 2h) the proportion of this low molecualr weight peak increased at the expense of the [^{3}H]glycine NeuAc peak (Fig. 5C). To obtain further information on this low molecular weight breakdown product, we treated [^{3}H]glycine-

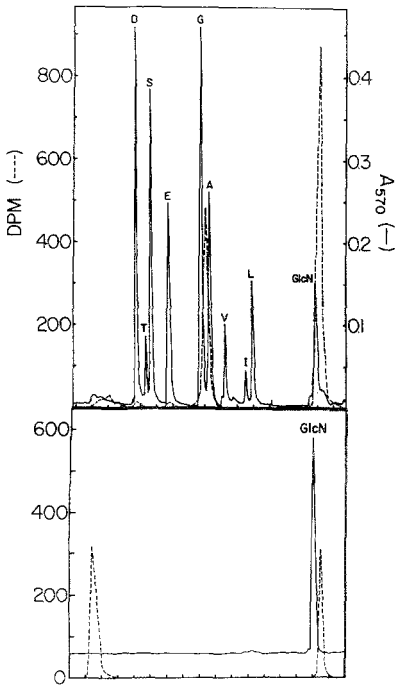

Figure 6. Top panel, analysis on an amino acid analyzer of a mixture of 6N HCl hydrolysate of [^{3}H]-glycine-α_1-acid glycoprotein and [^{3}H]glucosamine. The effluent from the analyzer was collected in fractions (0.3 ml) and assayed for radioactivity. The tritium activity (---) derived from the modified sialic acid elutes between the glycine (G) and alanine (A) peaks. In a similar experiment without the addition of [^{3}H]glycine α_1-AGP (Fig. 5A) and [^{3}H]glucosamine in the same system.

α_1-acid glycoprotein with 6 N HCl *in vacuo* at 110°C for 24 h and analyzed the dried hydrolysate on an amino acid analyzer. The results illustrated in Fig. 6 (top panel), show the elution of a labeled low molecular weight product between glycine and alanine. If corrected for the delay in elution, by reference to added [^{3}H]glucosamine, the elution position of the low molecular weight component is coincident with that of glycine. When [^{3}H]glycine-NeuAc was analyzed on the amino acid analyzer without hydrolysis, all the radioactivity eluted close to the position of cysteic acid (Fig. 6 - bottom panel). This observation of the release by acid hydrolysis of a component having a mobility identical to that of glycine on Bio-Gel P-2 (Fig. 5C), and cation exchange resin (Fig. 6) was unexpected but had a practical value. The quantitation of the released fragment on the amino acid analyzer enabled us to estimate easily the number of sialic acid moieties modified in a glycoprotein by unlabeled (non-radioactive) amino compound (Table 1).

The full characterization of glycine NeuAc and its breakdown product will be reported elsewhere.

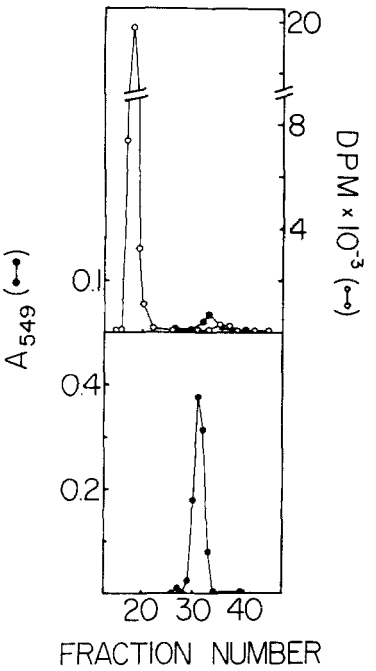

Figure 7. Gel filtration of *V. cholerae* sialidase digests of [^{3}H]glycine-modified fetuin (1 mg) [top panel] and fetuin (1 mg) [bottom panel]. Digestion was in sodium acetate buffer, pH 5.6 containing 1 mM Ca^{++}. The reaction mixture was applied to a Bio-Gel P-2 column (0.9 x 70 cm) and eluted with 0.1 M pyridine/acetic acid. Fractions (1 ml) were collected and analyzed for radioactivity (○)and thiobarbituric acid-reactive material, A_{549} (●). The peak in the bottom panel represents the position of NeuAc.

Treatment of [^{3}H]Glycine-modified Fetuin with Sialidase

The results illustrated in Fig. 7, indicate that [^{3}H]glycine-modified sialic acid is resistant to *Vibrio cholerae* sialidase under conditions (50 mU, 48 h at 37°C) which cause complete release of the unmodified sialic acid. In simialr experiments, we found that the modified sialic acid was also resistant to the action of sialidases from *Arthrobacter ureafacines, Diplococcus pneumoniae,* influenza virus and Newcastle disease virus. Equimolar amounts of fetuin and glycine-modified fetuin in 0.1 M sodium acetate buffer, pH 5.6 containing 1 mM Ca^{++} were treated with *V. cholerae* sialidase (50 mU) for 48 h and the reaction mixtures analyzed for released sialic acid by thiobarbituric acid assay. It was found that glycine-modified fetuin gave a colour yield of only 4.3% of that produced by fetuin. Further, on HPLC analysis of the reaction mixture no NeuAc or or C8- and C7-analogs of NeuAc peaks were detectable in the case of the modified fetuin, in agreement with the above results. To determine whether the modified sialoglycoproteins could be used as affinity ligands for sialidase we tested the action of *V. cholerae* sialidase on α_1-AGP in the absence and presence of the glycine-modified glycoprotein. The results indicate that glycine-modified α_1-acid glycoprotein produced only a slight inhibition of the action of *V. cholerae* sialidase on the unmodified glycoprotein (Fig. 8).

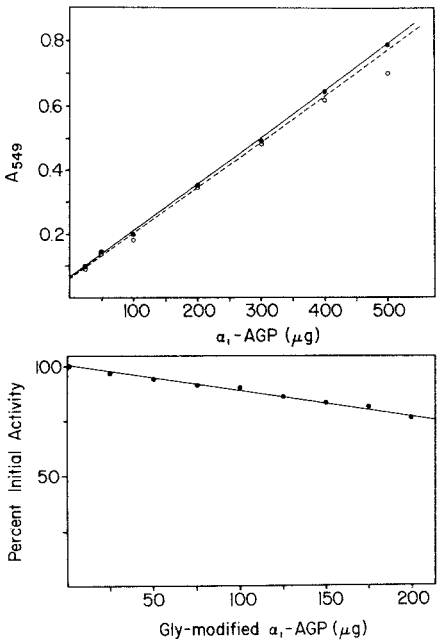

Figure 8. Effect of glycine-modified α_1-acid glycoprotein on the hydrolysis of unmodified α_1-acid glycoprotein by *V. cholerae* sialidase. The glycoproteins were incubated with 5 mU of the enzyme in 1 ml of 0.1 M sodium acetate buffer, pH 5.6 containing 1 m M Ca^{++} at 37°C for 30 min and aliquots analyzed for released sialic acid by the thiobarbituric acid assay (A_{549}). Top panel, the release of sialic acid from increasing amounts of α_1-acid glycoprotein (25 to 500 µg per ml) in the absence (●) and presence (○) of 50 µg glycine-modified α_1-acid glycoprotein. Bottom panel, the effect of adding increasing amounts of glycine-modified glycoprotein (50 to 200 µg) to the incubation mixture containing 300 µg α_1-acid glycoprotein per ml.

Table 1. Degree of modification of the sialic acid moiety of glycoproteins.

Glycoprotein	Sialic acid (mol/mol[a])	Increase in moles of amino acid per mol[b] after modification with:		
		Gly	Norleu	Cyst. Acid
α_1-Acid glycoprotein	14.3	10.4 (73)[c]	7.8 (54)	9.1 (64)
Fetuin	13.1	9.8 (75)		

[a] Sialic acid was determined by mild acid hydrolysis followed by thiobarbituric acid assay [16].

[b] Estimated by strong acid hydrolysis followed by analysis on a Dionex amino acid analyzer. The assumption is made that the fragment of unknown structure eluting in the position of the respective amino acid gives the same fluorescence yield as the parent amino acid in the fluorapa detection system.

[c] The figures in parentheses are percentages of sialyl residues modified assuming the introduction of one amino acid per residue.

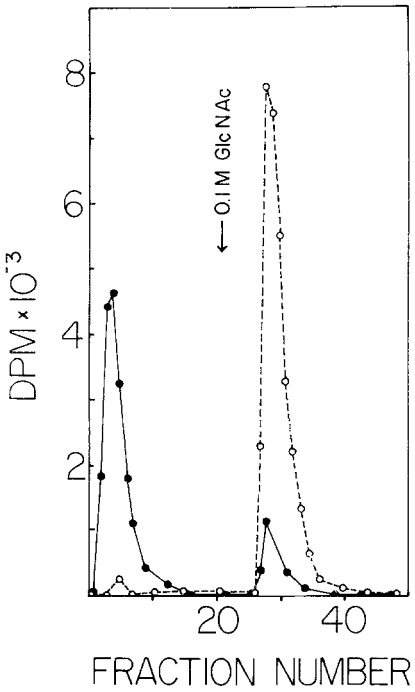

Figure 9. Affinity chromatography of sialidase-treated [^{14}C] dimethyl fetuin (●) and [^{3}H]glycine-modified fetuin (○) on WGA-Sepharose (0.7 x 12 cm) containing about 5 mg lectin per ml gel. The column was eluted with 50 mM Tris-HCl, pH 8.0, followed by 0.1 M *N*-acetylglucosamine in the same buffer and fractions analyzed for radioactivity.

Interaction of the Modified Sialoglycoproteins with WGA-Sepharose

Glycine-modified α_1-acid glycoprotein and fetuin, like their unmodified counterparts, were quantitatively bound and eluted with 0.1 M *N*-acetylglucosamine from a column of WGA-Sepharose (5 mg/ml gel) (not illustrated).

In another experiment, a mixture of [^{14}C]-dimethylfetuin (prepared by reductive methylation of fetuin with H^{14}CHO and $NaBH_3CN$ [18] and [^{3}H]glycine-modified fetuin were digested exhaustively with *Vibrio cholerae* sialidase and then chromatographed on a WGA-Sepharose column. The results, illustrated in Fig. 9, show that while the native glycoprotein after treatment with sialidase failed to bind to the lectin, the binding of the modified glycoprotein was not altered. These results are in agreement with the fact that fetuin interacts with WHA *via* its sialyl residues [15] and that the glycine modified sialyl residues are not susceptible to sialidase as described above.

Interaction of Modified α_1-Acid Glycoprotein with Antibody

To determine whether the modification of the sialyl residues had any influence on the interaction of the glycoprotein with its antibody, immunodiffusion experiments were done. We found that both native and glycine-modified α_1-acid glycprotein gave single bands of identity with rabbit anti-α_1-acid glycoprotein antisera (data not shown).

Distribution of the Modified Sialyl Residue on the Fetuin Oligosaccharides

In order to determine whether the sialic acid on the serine- and threonine-linked saccharides [22] as well as asparagine-linked saccharides were modified, [^{3}H]glycine-modified fetuin was treated with 1 M $NaBH_4$ in 0.1 N NaOH in a N_2 atmosphere in the dark, at 37°C for 72 h. Chromatography of ther reaction mixture (after neutralization) on a column of Bio-Gel P-6 showed that [^{3}H]glycine has been introduced into the serine- and threonine-linked saccharides (included peaks) in addition to the asparagine-linked saccharides (void peak).

Discussion

The current methods of the *in vitro* labeling of carbohydrates are limited largely to the introduction of tritium into the sialyl [17] or galactosyl [23] residues. These methods have been extensively used despite the above imitations and the additional problem of very high non-specific labeling caused by NaB^3H_4. The latter is particularly noticeable in experiments involving labeling of membrane or cell associated glycoconjugates [24-26]. In addition, we found that when plasma membrane sialoglycoconjugates were labeled by treatment with $NaIO_4$ and NaB^3H_4, about 40% of the non-dialysable radioactivity was in sialic acid-free components [27]. We are not aware of the origin of this high non-specific label, but possible sources are reduction of unsaturated lipids by NaB^3H_4, contamination by acid-resistant non-dialysable impurities in NaB^3H_4 [28, 29], reduction of -S-S-bridges and some peptide bonds by NaB^3H_4 [30], some periodate oxidation of other sugars and peptides [31] and reduction of naturally occurring Schiff's bases [32]. The present method enables the introduction of isotopes other than tritium (for example ^{32}P-phosphoryl ethanolamine, ^{125}I-tyrosine, ^{35}S-methionine) into sialic acids and also avoids the problems associated with the non-specific labelling caused by NaB^3H_4. However, the specific activity of labelled product obtainable is limited by the specific activity of the amino compound used. The [^{3}H]glycine α_1-acid glycoprotein we prepared had a specific activity of 1.2×10^6 dpm per mg compared to 11.5×10^6 dpm for [^{3}H-NeuAc] α_1-acid glycoprotein prepared by van Lenten and Ashwell [6].

It was established that the labelling occurred primarily on the sialyl residue as expected. A low degree of non-specific labelling was evident, but it appears this might be mainly of a non-covalent nature since it could be further reduced by either dialysis or gel filtration in 4 M guanindine HCl. It has been previously noted that non-specifically bound fluorescent labels could be removed from glycoproteins by gel filtration in 6 M urea [33]. Some intermolecular cross-linking of the glycoprotein occurred during the modification as evidenced by the

presence of oligomers (Fig. 4). This is most probably caused by the reactions of amino gorups (-NH_2 of lysine and α-amino terminal) interacting with the aldehydes generated by periodate treatment. When a high concentration of the amino compound was used (50-fold excess of glycine), the oligomers formed accounted for less 2% of the modified glycoprotein. The treatment of periodate-oxidized sialoglycoprotein with a 50-fold excess of the amino compound in borate buffer of pH 7.8 with an 10-fold excess of $NaBH_3CN$ also resulted in almost quantitative modification of sialyl residues as indicated by HPLC analysis. Direct estimation of amino compound incorporated by strong acid hydrolysis followed by amino acid analysis gave lower values for per cent modification (54 to 75%). This low estimate could be due to reasons such as incomplete release of the uncharacterized amino compound fragment, destruction during hydrolysis and unknown fluroescence yield of the adduct with Fluoropa.

The location of the amino compound was further established by the isolation and structural characterization of the glycine-modified sialic acid [34]. The glycine-modified sialoglycoprotein was not significantly altered in its size (gel filtration and SDS-PAGE), ability to interact with wheat germ agglutinin and antibody, but had an altered elution profile on anion exchange chromatography. The modified sialic acid was partially resistant to mild acid hydrolysis (0.1 N H_2SO_4, 80°C, 1 h) and totally resistant to all sialidases tested. The resistance to enzymic cleavage is not surprising in view of the bulky substitution introduced which is likely to interfere in the recognition of the substrate by the sialidases. This would also explain the lack of significant inhibition of the sialidase by the modified glycoprotein. Because of its resistance to sialidases, and therefore to other exoglycosidases, the modified sialoglycoproteins may be useful in a search for endoglycosidases. This method of sialyl modification can also be used for preparing biologically active sialoglycoproteins (for example, hormones and enzymes) having increased circulating half-lives in animals [35].

While our work was in progress, a similar modification of human α_1-protease inhibitor by ethanolamine was reported [36]. These workers carried out the reductive amination at pH 8.9 for 24 h which could cause release of *O*-glycosidically-linked saccharides and degradation of proteins. They obtained a heterogenous preparation with the degree of modification varying from 44 to 78%; further, a high degree of non-specific labeling was evident. It was found that the modified sialic acid was resistant to *Clostridium perfringens* sialidase in agreement with our data.

That the modified sialoglycoprotein is resistant to sialidases but still interacts with WGA-Sepharose can be utilized for the selective purification of cell surface sialoglycoproteins. Chromatography of extracts [^{3}H]glycine-modified cells on WGA-Sepharose would yield a mixture of both modified (cell surface) and unmodified (intracellular) sialoglycoproteins. Treatment of the mixture with sialidase to selectively desialylate intracellular glycoproteins followed by rechromatography on WGA-Sepharose should result in the binding of only the modified cell surface glycoproteins, which could be recovered by elution with 0.1 M *N*-acetylglucosamine followed by a dialysis and lyophilization. The method described in this paper can be extended to label glycoproteins with galactose/*N*-acetylgalactosamine terminals by treatment with galactose oxidase followed by reductive amination of the generated aldehyde [36].

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References

1 Gottschalk A (1960) in The Chemistry and Biology of Sialic Acids and Related Substances, Cambridge Univ Press, Cambridge.
2 Schauer R (1982) Adv Carbohydr Chem Biochem 40:131-234.
3 McLean RL, Suttajit M, Beidler J, Winzler RJ (1971) J Biol CHem 246:803-09.
4 Suttajit M, Winzler RJ (1971) J Biol Chem 246:3398-404.
5 Suttajit M, Reicher LE, Winzler RJ 8 1971) J Biol Chem 246:3405-8.
6 van Lenten L, Ashwell G (1971) J Biol Chem 246:1889-94.
7 Veh RW, Corfield AP, Sander M, Schauer R (1977) Biochim Biphys Acta 486:145-60.
8 Liao T-H, Gallop PM, Blumenfeld OO (1973) J Biol Chem 248:8247-53.
9 Gahmberg CG, Andersson LC (1977) J Biol Chem 252:5888-94.
10 Carubelli R, Wen G, McCaffree DR (1984) Anal Biochem 137:42936.
11 Ashwell G, Morell AG (1974) Adv Enzymol 41:99-128.
12 Bhavanandan VP (1984) Fed Proc 43:2037.
13 Umemoto J, Bhavanandan VP, Davidson EA (1977) J Biol Chem 252:8609-14.
14 Paulson JC, Weinstein J, Dorland L, van Halbeek H, Vliegenthart JFG (1982) J Biol Chem 257:12734-38.
15 Bhavanandan VP, Katlic AW (1979) J Biol Chem 254:4000-8.
16 Warren J (1959) J Biol Chem 234:1971-75.
17 van Lenten L, Ashwell G (1972) Methods Enzymol 28:290-311.
18 Jentoft N, Dearorn DG (1979) J Biol Chem 254:435965.
19 Borch RF, Bernstein MD, Durst HD (1971) J Am Chem Soc 93:2897-904.
20 Silver HKB, Karim KA, Gray MJ, Salinas FA (1981) J Chromatogr 224:38188.
21 Bergh ML, Koppen P, van den Eijnden DH (1981) Carbohydr Res 94:225-29.
22 Spiro R G, Bhoyroo VD (1974) J Biol Chem 249:5704-17.
23 Morell AG, van den Hamer CJA, Ccheinberg IH, Ashwell G (1966) J Biol Chem 241:3745-49.
24 Gahmberg CG, Hakomori S (1973) J Biol Chem 248:4311-17.
25 Steck TL, Dawson G (1974) J Biol Chem 249:2135-42.
26 Flowers H, Glick MC (1980) Cancer Res 40:1550-57.
27 Furukawa K, Mino JE, Hegarty JD, Bhavanandan VP (1986) J Biol Chem 261:7755-61.
28 McLean C, werner DA, Aminoff D (1973) Anal Biochem 55:72-84.
29 Bhavanandan VP, Yeh AK, Carubelli RC (1975) Anal Biochem 69:385-94.
30 Crestfield AM, Morre S, Stein WH (1963) J Biol Chem 238:622-27.
31 Cater WG (1982) J Biol Chem 257:3249-57.

32 Robins SP, Bailey AJ (1972) Biochem Biophys Res Commun 48:76-84.
33 Ingham K, Brew SA (1981) Biochim Biophys Acta 670:181-89.
34 Murray M, Bhavanandan VP, Reinhold V, Davidson EA (1988) Carbohydr Res, in press.
35 Murray M, Bhavanandan VP. Davidson EA (1988) Glycoconjugate J 5:485-98.
36 Omichi K, Savic G, Yoshida A (1983) Int J Biochem 15:1345-51.
37 Barsoum AL, Davidson EA (1981) Mol Immunol 5:367-72.