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Synthesis of neoglycopeptides and analyses of their biodistribution *in vivo* to identify tissue specific uptake and novel putative membrane lectins

DIPTI GUPTA and AVADHESHA SUROLIA*
Molecular Biophysics Unit, Indian Institute of Science, Bangalore-560012, India

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Complex type *N*-linked oligosaccharides derived from fetuin, fibrinogen and thyroglobulin were coupled to acetyltyrosine affording a series of neoglycopeptides with retention of terminal structures and the β-anomeric configuration of their reducing end *N*-acetylglycosamine residue. The neoglycopeptides thus synthesized could be labelled to high specific activities with ^{125}I in the aromatic side chain of tyrosine. Analysis of the fate of these neoglycopeptides in conjunction with inhibition with asialofetuin and oligosaccharides of defined structure in mice *in vivo* revealed the uptake of galactosylated biantennary compound by kidneys, in addition to the known itinerary of triantennary galactosylated complex oligosaccharide from fetuin to liver and the galactosylated biantennary chain with fucosylation in the core to bone marrows. On the other hand, the agalacto, aglucosamino biantennary chains with and without fucosylation in the core region are taken up by submaxillary glands while the conserved trimannosyl core with fucose is primarily concentrated in stomach tissue. These studies thus define new routes for the uptake of complex *N*-linked glycans and also subserve to identify lectins presumably involved in their recognition.

Keywords: neoglycopeptides, biodistribution, membrane lectins, *in vivo* uptake

Introduction

Many biological recognition and adhesion processes involve interactions between carbohydrates and proteins [1, 2]. And indeed cells, organisms and soluble components exhibit information about themselves in the form of exquisitely defined saccharide structures displayed on their surfaces. This information is decoded by a group of highly stereospecific and specialized carbohydrate binding proteins, lectins, located at the surface of the cell [3–5]. Lectin-carbohydrate interactions, therefore, crucially determine cell growth and differentiation, immune response, metastatic distribution of malignancies as well as in the infectious cycle of viruses, bacteria, etc. [1, 6].

In vertebrates, lectins are distributed in a tissue specific manner and display remarkable specificities for a particular carbohydrate ligand [3, 4]. One of the best studied vertebrate lectins is the one discovered by Ashwell and Morell [7], the asialoglycoprotein receptor (ASGP-R) found at the surface of liver parenchymal cells [3, 7]. ASGP-R binds to exposed galactose and *N*-acetylgalactosamine residues on asialoglycoproteins and clears them from circulation. Therefore, liposomes or neoglycoproteins displaying galactose or glycolipids with exposed galactose residues have been used extensively for targeting them to liver [8–11]. Despite their ability of binding to simple saccharides, these lectins show exquisite specificities for the recognition of their complementary ligands [12]. The ASGP-R, for example, shows optimal binding with a cluster of three galactose terminals located at a triangular inter-distance of 15, 22 and 25 Å [12, 13]. Hence, the simple glycosides of galactose have been able to direct conjugated proteins and liposomes mostly to liver. Though carriers with unnatural sugars such as 6-deoxy-6-amino mannosamine have been able to ferry their contents to other organs such as lungs [14]. Being substantially different from their natural counterparts, such sugars are likely to elicit an immune response. Consequently, they are unlikely to find favour for use in the human [14]. On the other hand, usage of glycoproteins for targeting purposes is limited as a single glycoprotein often contains numerous glycan chains of closely related oligosaccharide structures [15, 16].

An alternative approach for the specific targeting of biomolecules proposed here is to conjugate various homogeneous *N*-linked oligosaccharide chains with an appropriate indicator molecule. Studies reported here show

* To whom correspondence should be addressed.

that complex glycans conjugated to an indicator molecule, acetyltyrosine, are able to reach specifically to tissues hitherto impregnable by other targeting molecules.

Materials and methods

Chloramine-T, sodium metabisulphite, human fibrinogen, *Arthrobacter ureafaciens* neuraminidase, bovine fetuin type V were obtained from Sigma Chemical Co., USA. Porcine thyroglobulin was purified as in [18]. Sephadex G-25 and Mono Q HR5/5 were obtained from Pharmacia, Sweden and AG 50WX-8 (H^+ form) and Biogel P-6 were from Bio-Rad, USA. A standard mixture of monosaccharides for compositional analysis of oligosaccharides was obtained from Dionex Corp., USA. Carrier free ^{125}I was obtained from the Bhabha Atomic Research Centre, Bombay, India. β-Galactosidase and β-*N*-acetylhexosaminidase were purified from jackbean meal as described in [18, 19]. A unit of the activities of these glycosidases is defined as the amount of enzyme required to hydrolyze 1 μmol of p-nitrophenyl glycoside per min at 37°C.

Asialogalactosylated triantennary oligosaccharide $g_3G_3M_3G_2$ (type I), asialogalactosylated biantennary oligosaccharide with ($g_2G_2M_3G_2F$) and without fucosylation ($g_2G_2M_3G_2$) in the core region, as well as their agalactosylated counterparts, i.e. $G_2M_3G_2F$ and $G_2M_3G_2$, the conserved trimannosyl core with (M_3G_2F) and without fucosylation (M_3G_2) were from Oxford Glycosystems, England (see Table 1 for oligosaccharide nomenclature).

Ricinus communis agglutinin (RCA_1), concanavalin A (Con A), *Lens culinaris* agglutinin (LCA) amd *Artocarpus integrifolia* agglutinin (jacalin) were available in the laboratory. Lectins were coupled to Sepharose-4B [20]. All other chemicals used were of highest purity available.

Preparation of oligosaccharides

Bovine fetuin (3 g), thyroglobulin (5 g) and human fibrinogen (3 g) were treated with anhydrous hydrazine as described in Takasaki *et al.* [21]. After the reacetylation of free amino groups with acetic anhydride, oligosaccharides were freed of protein degradation material and salts by ion-exchange chromatography on AG 50WX-8 (H^+ form) and paper chromatography as described in [21]. The oligosaccharides obtained from fetuin and fibrinogen were then desialylated by incubating them with 1 U of neuraminidase for 25–45 μmol of oligosaccharides in 2.5 ml of 15 mM sodium phosphate buffer pH 6.0 for 24 h at 37 °C. Extent of desialylation was assessed by measuring sialic acid content in aliquots drawn at 1, 3, 6, 10 and 24 h of incubation with the enzyme using High Performance Anion Exchange Chromotography with Pulsed Amperometric Detection (HPAEC-PAD) and eluted isocratically with 100 mM NaOH and 150 mM NaOAc at a flow rate of 1 ml min^{-1} for 15 min. The volume of sample injected was 1–1.5 μl. Subsequent to gel filtration on a Sephadex G-25 column (0.5 × 10 cm) equilibrated with water to remove free sialic acid, the contents of the sialic acid were also measured in oligosaccharide sample aliquots drawn at 3 and 24 h according to the method in [22]. Oligosaccharides from thyroglobulin were first fractionated by ion exchange chromatography on a Mono Q column to separate sialylated oligosaccharides from high mannose chains according to [23] prior to desialylation of the former.

Lectin affinity chromatography

Analytical (0.5 × 5.0 cm) and preparative (7.5 × 50 cm) affinity chromatography on RCA_1-Sepharose (lectin ≈ 10–12 mg per ml gel) column equilibrated with 20 mM phosphate buffer pH 7.2 was carried out as described in [23, 24]. For the preparative chromatography, oligosaccharides (approximately 6–7 μmol) dissolved in 10 ml of phosphate buffer were loaded on the column and washed with the same buffer. When the neutral sugar content in the effluent reached baseline, bound fractions were eluted with four column volumes of 0.1 M lactose in the above buffer. Preparative affinity chromatography on jacalin-Sepharose (7.5 × 50 cm) was carried out under identical conditions except that methyl-α-D-galactopyranoside was used for eluting the bound fraction. Analytical (0.5 × 5 cm) and preparative affinity chromatography with Con A-Sepharose and on LCA-Sepharose columns (7.5 × 50 cm) was also carried out as in [23, 25].

The eluate was concentrated by lyophilization and when the volume was close to 1.0 ml, the samples were subjected to gel filtration on Sephadex G-25 column (1.2 × 100 cm) equilibrated with water to purifying the oligosaccharides from salts and the respective eluting sugars. Fractions (2.0 ml) were monitored for neutral sugars.

High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

Analytical HPAEC-PAD for characterization of oligosaccharides was carried out on a Dionex-HPLC consisting of a Dionex Bio-LC pump, CarboPac PA-1 column (4.0 × 250 mm) and a PAD2 detector. Pulse potentials and durations used for detection were as follows: E_1 = 0.05 V(t_1 = 120 ms); E_2 = 0.70 V (t_2 = 120 ms); E_3 = –0.5 V (t_3 = 180 ms) and the response time of PAD was set to 3 s. Post-column addition of 0.3 M NaOH to the column effluent at 1 ml min^{-1} was done before the PAD cell to minimize baseline drift. The data were plotted and integrated using a Dionex Integrator (Model 4400). Samples were injected by a Rheodyne 7010 injection valve and eluted with either 150 mM NaOH containing 30 mM sodium acetate (isocratic mode; system A) at a flow rate of 1 ml min^{-1} or with 150 mM NaOH and a 0–100 mM gradient of sodium acetate for 30 min followed by reequilibration at a flow rate of 1 ml min^{-1} (system B) [26]. For semipreparative HPAEC-PAD (9 × 250 mm) a CarboPaC PA-1 column was used at a flow rate of 4.0 ml min^{-1} using the gradient system for elution, viz., system B.

Carbohydrate composition

Aliquots of the purified oligosaccharide samples were hydrolysed with 2 M TFA *in vacuo* at 100 °C for 5 h [27]. After removal of acid, samples were subjected to anion exchange chromatography on CarboPac PA1 column using Dionex-HPLC. Samples were eluted isocratically with 15 mM NaOH for 30 min at a flow rate of 1 ml min^{-1}. The following pulse potentials were used for detection $E_1 = 0.01$ V ($t_1 = 300$ ms); $E_2 = -0.70$ V ($t_2 = 120$ ms); $E_3 = 0.50$ V ($t_3 = 300$ ms). Post-column addition of 0.3 M NaOH at a flow rate of 1 ml min^{-1} was carried out prior to the detection. The data were analysed using an on-line Dionex Integrator.

Digestion with β-galactosidases and β-N-acetylhexosaminidases

Oligosaccharides were digested with β-galactosidase and β-N-acetylhexosaminidase [18, 19]. In a typical experiment, 5 μmol of oligosaccharide was incubated with 8 U of jackbean β-galactosidase (with or without 7.5 U of β-N-acetylhexosaminidase) in 1 ml of 20 mM sodium citrate/-phosphate buffer pH 6.0 containing 0.1% sodium azide and 0.5 mg of bovine serum albumin for 24 h. The release of galactose and *N*-acetylglucosamine was monitored every hour by injecting 2 nmol equivalent of oligosaccharide on HPAEC-PAD using the solvent system used for quantification of monosaccharides. Reactions were terminated by boiling the samples for 5 min and the samples speedvac concentrated to 0.1 ml and subjected to gel filtration on a Sephadex G-25 column (0.5 × 20 cm) equilibrated and eluted with water. Oligosaccharide fractions were pooled and freeze dried.

Preparation of oligosaccharide glycosylamines

To the dry sample of purified oligosaccharides (4.0–10 μmol), 1 g of ammonium bicarbonate was added and the mixture dissolved in 750 μl of water. The samples were then incubated at 25 °C for 5 days using the method described by Kallin *et al.* [28]. Glycosylamine formation was monitored every 12 h by subjecting aliquots containing 1–1.5 nmol of oligosaccharide of the reaction mixture to anion exchange chromatography on Dionex-HPLC. For M_3G_2F, M_3G_2, $G_2M_3G_2$ and $G_2M_3G_2F$ chromatograms were developed isocratically with 150 mM NaOH containing 30 mM of NaOAc. Glycosylamine formation for $g_2G_2M_3G_2$, $g_2G_2M_3G_2F$, $g_3G_3M_3G_2$ (a mixture of type I and II) was quantified by employing a gradient of 0–100 mM NaOAc in 30 min against 150 mM NaOH. Excess ammonium bicarbonate was removed by repeated freeze drying of the samples prior to HPAEC-PAD.

Separation of reducing oligosaccharides from oligosaccharide glycosylamines

Oligosaccharide glycosylamines were subjected to ion exchange chromatography on AG 50WX-8 (H^+ form; 3 × 25 cm). The column was initially washed with four bed volumes of water and then eluted with six bed volumes of 0.2 M triethylamine. After the removal of excess triethylamine by repeated freeze drying, an aliquot of the oligosaccharide glycosylamines was subjected to HPAEC-PAD to check their homogeneity as well as their separation from reducing end oligosaccharides.

Preparation of acylated tyrosine derivatives

Tyrosine (1 mmol) was treated with acetic anhydride (10 mmol) in a saturated solution (5 ml) of sodium acetate for 4 h at 25 °C. The reaction mixture was then dried and the acetylated tyrosine extracted twice with 10 ml of acidified ether, dried and treated with hydroxylamine prior to its ultimate purification by RP-HPLC. Acylation of tyrosine (20 μmol) with hexanoic, octanoic, myristic, palmitic and stearic acid (all about 200 μmol) was performed employing their p-nitrophenyl esters using p-dimethyl-aminopyridine as a catalyst in dimethylformamide (DMF) as described by Bodanski [29, 30]. After incubating the samples for 24 h at 37 °C, an equal volume of 7.5% methanol was added. They were then treated with an equal volume of 1 M NaOH for 30 min at 37 °C. Acylated tyrosine derivatives were then purified by RP-HPLC on a Waters HPLC equipped with Waters 460 E Variable Wavelength detector using a C-18 semipreparative column (0.78 × 30 cm). The following gradient was utilized: 0.1% TFA for 5 min after injection followed by a linear gradient of 0–55% acetonitrile in 0.1% TFA for 75 min, 55% acetonitrile in 0.1% TFA for the next 10 min and 55–65% acetonitrile from 85–100 min at a flow rate of 4 ml min^{-1}. Acetyl tyrosine residue, was for example, eluted at 25% acetonitrile. Data regarding the preparation of higher homologues of acylated tyrosine and their coupling with glycosylamines will be reported elsewhere. Acetyltyrosine thus purified was devoid of amino groups as judged by reaction with picryl sulfonate.

Coupling of oligosaccharide glycosylamines with acetyltyrosine

In a typical reaction, 100 μmol of acetyltyrosine was converted to its *N*-hydroxysuccinimide esters according to the method of Anderson *et al.* [31] by treating it with 100 μmol of *N*-hydroxysuccinimide and 120 μmol of di-cyclohexylcarbodiimide (DCC) in 1.0 ml of tetrahydrofuran at −18 °C for 3 h and then at 25 °C for 10 h. Following this it was treated with acetic acid to destroy DCC. After 30 min, the mixture was diluted with ethylacetate (5 ml) and the dicyclohexylurea filtered off. The residue was washed with 5 ml of ethylacetate and the combined filtrate and washing evaporated to dryness. The residue was dissolved in 3 ml of ethylacetate and recrystallized from it by addition of 500 μl of petroleum ether. Thus, starting with 100 μmol of acetyltyrosine, 24 mg of acetyltyrosine-*N*-hydroxysuccinimide ester was obtained.

Oligosaccharide glycosylamines (4 μmol) in 2 ml of DMF

were treated with a 20 M excess of acetyltyrosine-*N*-hydroxysuccinimide ester using p-dimethylaminopyridine (25 μg) as a catalyst at 25 °C for 24 h [30]. Progress of the reaction was monitored by the disappearance of oligosaccaride glycosylamines by HPAEC-PAD. NaOH (1 ml) was then added to the reaction mixture to a final concentration of 1 M and the sample incubated for 20 min at 37 °C and centrifuged to remove the residue. The pH of the supernatant was then adjusted to 7 by drop wise addition of 1 M acetic acid and the samples freeze dried. The samples were then dissolved in 1 ml of water and subjected to gel filtration on a Biogel P-6 column (1.2 × 100 cm) equilibrated and operated with 20 mM acetic acid to separate them from salts and unreacted acetyl tyrosine. Fractions were monitored by their absorbance at 280 nm.

Purification of glycosylated acyl-tyrosine derivatives on RP-HPLC

Synthetic glycopeptides (neoglycopeptides; acetyltyrosine-oligosaccharides) thus prepared did not require any further purification as on repurification on a Biogel P-6 column, no peak corresponding to free acetyltyrosine was observed. Moreover, no free oligosaccharide corresponding to either the reducing oligosaccharide or oligosaccharide glycosylamine was detected on HPAEC-PAD.

However, the purification of the triantennary oligosaccharides (type I and II, see Table 1 for nomenclature) from fetuin was achieved only after their coupling with acetyltyrosine. Purification was accomplished by RP-HPLC on a Waters C-18 column (7.8 × 30 cm). Samples were loaded in 0.02% TFA and the following gradient was used for elution: 0.02% TFA for 1 min and thereafter a gradient of 0–30% acetonitrile in 0.02% TFA applied at 5 ml min^{-1} for 1 h. Elution was monitored by absorbance at 280 nm.

Iodination of glycopeptides

To glycopeptides (0.5–2 μmol) in 100 μl of 20 mM sodium phosphate buffer (pH 7.2) 1–10 mCi of carrier-free Na[^{125}I] was added. Chloramine-T (500 ng in 100 μl) in the above buffer was added to it. After 3 min at room temperature, the reaction was stopped by the addition of 500 μl of sodium bisulphite solution (5 mg ml^{-1} in phosphate buffer pH 7.2). The radioactive neoglycopeptides were then separated from the reaction mixture by gel filtration on a Sephadex G-25 column (1.2 × 100 cm) equilibrated and eluted with 100 mM pyridine adjusted to pH 5.5 with TFA. Fractions corresponding to radiotyrosine were pooled and lyophilized.

Binding of acetyltyrosine oligosaccharides with mouse hepatocytes

Hepatocytes were isolated by a modification [32] of the collagenase perfusion method of Williams *et al.* [33]. Hepatocytes thus obtained excluded trypan blue (≥95%) and the contamination with non-parenchymal cells was less than 3–5%. Binding of $g_3G_3M_3G_2$ (I) acetyltyrosine to hepatocytes was measured in modified Dulbecco's Eagle's medium as follows. Stock solutions (0.25 ml) of $g_3G_3M_3G_2$ (I) acetyltyrosine were mixed with equal volume of 2% solution of radioiodinated $g_3G_3M_3G_2$ (I) acetyltyrosine and added to 0.5 ml of hepatocytes suspension (7.0×10^6 cells) in polystyrene test tubes (12 × 15 mm). Cells were maintained in suspension by slow gyration at 2–3 °C during all incubations and at 4 h, the aliquots were removed for measurement of total radioactivity. Cells (250 μl) were pelleted free of unbound ligand by centrifuging (10 000 × **g**, 1 min) through a silicon/mineral oil mixture (4:1) and then counted for the associated radioactivity. Cells (250 μl) preincubated with 5 mM EDTA for 10 min prior to pelleting and counting as above provided a measure of non-specific binding. The data were then fitted to obtain the number of receptor sites and their dissociation constant (K_d) according to Munson and Rodbard using a single site, single receptor model [34]. Dissociation constants (K_d) for the binding of other oligosaccharides were evaluated by measuring the concentrations required to inhibit the binding of a fixed amount of radioiodinated $g_3G_3M_3G_2$ to hepatocytes.

In vivo *experiments*

Each male Swiss mouse (about 20–21 g) was administered about 10 nmol of the radioiodinated oligosaccharide acetyltyrosine (about $8–19 \times 10^8$ cpm) intravenously through their portal vein. Typically four mice were used in each experiment unless otherwise indicated. The mice were killed and their organs carefully dissected. Bone marrow from both femurs was collected by washing with buffer, cells were centrifuged at 20 000 × **g** and washed twice with buffer before counting. Tissue distribution of injected radioactivity was determined by assaying for the major organs of the body after subtraction of the counts due to residual blood content in each tissue at 25, 50 and 500 min, and urinary losses. Residual blood content in various organs of the animal was determined in a separate set of experiments by injecting [^{125}I]acetyltyrosine ($1–2 \times 10^9$ cpm) intravenously and quantifying the radioactivity in various tissues at 10 min.

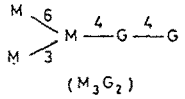
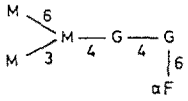
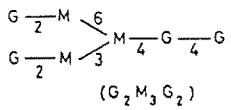
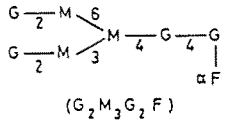
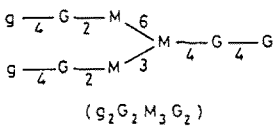
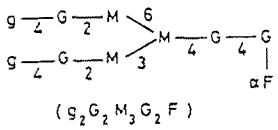
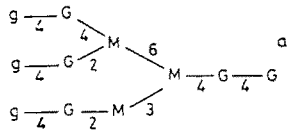
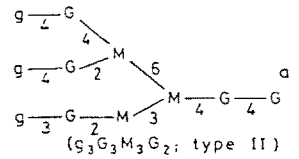
Inhibition experiments were carried out by preinjecting at least a 10-fold molar excess of asialofetuin or acetyltyrosine oligosaccharides 1 min prior to the injection of labelled derivatives into mice and the biodistribution analysed at 30 min as above. Inhibition experiments were conducted with a pair of mice.

Results

Oligosaccharide preparation

Oligosaccharides from glycoproteins were released by anhydrous hydrazine and were reacetylated three times after the removal of hydrazine. They were then separated from salts and the polypeptide degradation products by ion exchange

Table 1. Structures of oligosaccharides with their retention times in HPAEC-PAD on CarboPac PA-1 column and their manosaccharide compositions. Oligosaccharides were prepared as described in the text. An aliquot of oligosaccharide was subjected to HPEAC-PAD on CarboPac PA1 column. Solvent systems used for HPAEC-PAD are indicated. Monosaccharide compositions were determined as indicated in methods. The abbreviations used are: g, galactose; G, N-acetylglucosamine; M, mannose; F, fucose. Linkage between sugars are indicated by Arabic numerals. In all cases the core structure is Manα1, 6 [Manα1, 3] Manβ1, 4GlcNAc viz M_3G_2. All pheripheral Gal-GlcNAc linkages are β while all peripheral Man and Fuc linkages are α.

Oligosaccharide structures	*Solvent for HPAEC-PAD*	*Retention time (min) on HPAEC-PAD*	*Monosaccharide content mol per mol relative to mannose*			
			F	*g*	*M*	*G*
 (M_3G_2)	Isocratic: 150 mM NaOH and 30 mM NaOAc; flow rate: 1 ml min^{-1}	4.11	–	–	3.0	2.2 ± 0.1
 (M_3G_2F)	Isocratic: 150 mM NaOH and 30 mM NaOAc; flow rate: 1 ml min^{-1}	2.70	1.2 ± 0.11	–	3.0	2.1 ± 0.18
 ($G_2M_3G_2$)	Isocratic: 150 mM NaOH and 30 mM NaOAc; flow rate: 1 ml min^{-1}	11.52	–	–	3.0	4.2 ± 0.30
 ($G_2M_3G_2F$)	Isocratic: 150 mM NaOH and 30 mM NaOAc; flow rate: 1 ml min^{-1}	6.48	1.1 ± 0.15	–	4.0	4.05 ± 0.21
 ($g_2G_2M_3G_2$)	Gradient: 150 ml NaOH with 0–100 mM NaOAc for 30 min; flow rate: 1 ml min^{-1}	18.10	–	2.1 ± 0.12	3.0	4.2 ± 0.15
 ($g_2G_2M_3G_2F$)	Gradient: 150 ml NaOH with 0–100 mM NaOAc for 30 min; flow rate: 1 ml min^{-1}	16.66	1.05 ± 0.1	2.2 ± 0.18	3.0	4.3 ± 0.21
 ($g_3G_3M_3G_2$; type I)	Gradient: 150 ml NaOH with 0–100 mM NaOAc for 30 min; flow rate: 1 ml min^{-1}	18.75	–	3.2 ± 0.24	3.0	5.2 ± 0.33
 ($g_3G_3M_3G_2$; type II)	Gradient: 150 ml NaOH with 0–100 mM NaOAc for 30 min; flow rate: 1 ml min^{-1}	22.34	–	3.3 ± 0.28	3.0	5.1 ± 0.41

[a] Separation performed at analytical scale HPAEC-PAD on CarboPac PA1 column. Large scale separation performed between type I and type II structures after coupling to acetyltyrosine.

and paper chromatography. Oligosaccharides thus obtained were free of these impurities as judged by their negative reaction towards fluorescamine when 5 nmol of neutral sugars spotted on paper were tested. About 45 μmol of *N*-linked oligosaccharides from 3 g of bovine fetuin, 42 μmol from 3 g of human fibrinogen and 36 μmol from 5 g of bovine thyroglobulin were thus obtained. For thyroglobulin an additional step, viz., anion exchange on Mono Q was employed.

The oligosaccharides were then desialylated with *A. ureafaciens* neuraminidase which efficiently removed all the neuraminic acid residues from the oligosaccharides as judged by the HPAEC-PAD analyses of the reaction as well as by the quantification of *N*-acetylneuraminic acid in the end product. It was observed that by 3 h, almost all of the oligosaccharides were completely desialylated.

One of the main preparative steps utilized by us was the application of lectin affinity chromatography. For this purpose, 6–7 μmol of oligosaccharides were loaded on large capacity affinity matrices. Since fetuin contains both *N*- and *O*-linked oligosaccharide chains, as a first step in the purification of these saccharides, we employed jacalin-Sepharose which specifically recognizes *O*-linked chains with high affinity [35, 36]. The flow through contained *N*-linked chains exclusively, as evidenced by the absence of a galactosamine peak on HPAEC-PAD profiles of 2 M TFA hydrolysate of this fraction. The flow through was then passed through Con A-Sepharose which bound only 7–10% of oligosaccharide. Using the elution system B, the flow through fraction on HPAEC-PAD could be resolved into two peaks. These two peaks gave identical monosaccharide composition. The faster moving peak was assigned structure $g_3G_3M_3G_2$ (type I) as its elution position was coincident with the authentic oligosaccharide obtained from Oxford Glycosystems, while the latter peak was considered to contain structure $g_3G_3M_3G_2$ (type II) from its elution behaviour [26]. The flow through fraction of oligosaccharides from Con A-Sepharose column thus contained only the two triantennary structures.

As fibrinogen contains a single type of oligosaccharide chain of biantennary complex type, no additional purification step was employed [37]. However, authenticity of the oligosaccharide isolated was confirmed by retention of desialylated oligosaccharides on RCA_1-Sepharose and Con A-Sepharose columns and its failure to bind to jacalin-Sepharose. Moreover, the retention time and the monosaccharide composition of the desialylated oligosaccharide matched that of the authentic compound (Table 1).

Since bovine thyroglobulin oligosaccharide fraction depleted of high mannose chains by ion exchange chromatography contains both bi- and triantennary chains with fucosylation in the core region, as a first step we separated the latter saccharide from the former by employing a LCA-Sepharose column which binds tightly to the biantennary complex type oligosaccharide with the fucosylation in the core region.Oligosaccharide fraction bound to the lectin was eluted with 100 mM methyl-α-mannose. The oligosaccharide thus eluted was found to contain $g_2G_2M_3G_2F$ as judged by the isocratic elution at 2.7 min in HPAEC-PAD with 150 mM NaOH containing 30 mM NaOAc as well as by its monosaccharide composition. Thus, by employing LCA-Sepharose, the separation of the desialylated biantennary complex type oligosaccharide with fucosylation in the core ($g_2G_2M_3G_2F$) from those of triantennary type could be achieved.

Using these protocols, we were able to prepare 27 μmol of $g_3G_3M_3G_2$ (a mixture of type I and type II structures) from 3.0 g of bovine fetuin, 36 μmol of $g_2G_2M_3G_2$ from 3 g of human fibrinogen and 18 μmol of $g_2G_2M_3G_2F$ from 5 g of bovine thyroglobulin.

Preparation of agalacto- and agalacto-aglucosamino oligosaccharides from $g_2G_2M_3G_2F$ and $g_2G_2M_3G_2$

Purified biantennary asialo oligosaccharides derived from fibrinogen and thyroglobulin when subjected to either β-galactosidase (7.5 U) or both β-galactosidase and β-N-acetylhexosaminidase (8 and 7 U, respectively) treatments, were all degalactosylated or deglucosaminated by 3 h. The reactions were hence, terminated at 5 h. The oligosaccharides were freed of proteins and monosaccharides by gel filtration and the purity of oligosaccharides was checked with HPAEC-PAD as well as by their compositional analyses (Table 1). Purity of the oligosaccharides was also confirmed by their binding to lectin affinity matrices and the results were in accordance with their expected structures [23]. About 4.2–5.0 μmol of $g_2M_3G_2$, $g_2M_3G_2F$, M_3G_2 and M_3G_2F were thus available for glycosylamine formation.

Synthesis of oligosaccharide glycosylamine

Formation of oligosaccharide glycosylamine was assessed by HPAEC-PAD wherein it was observed that glycosylamines elute earlier than their reducing sugar counterparts, presumably due to an additional positive charge on them (Fig. 1). That the early eluting peak was that of glycosylamine was also confirmed by its reactivity with fluorescamine. More than 80% of oligosaccharides were converted to glycosylamine between 96 and 108 h of the reaction. Yields of the glycosylamine derivatives were independent of the complexity of the oligosaccharide chains. These yields are higher than reported earlier [28] probably due to the conversion of glycosylamine carbonate, an expected side product of the reaction during repeated freeze drying. The oligosaccharide glycosylamines were separated from free oligosaccharides by ion exchange chromatography on AG 50WX-8 wherein the reducing oligosaccharides were recovered in the breakthrough while the glycosylamines were eluted with 0.2 M triethylamine. Subsequent to the removal of triethylamine by repeated freeze drying, HPAEC-PAD analyses revealed the complete absence of a

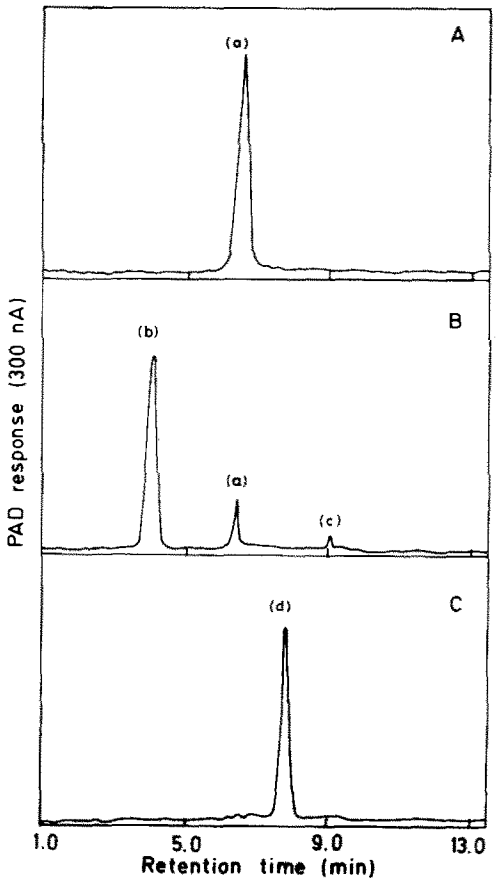


Figure 1. HPAEC-PAD analysis of $G_2M_3G_2F$ oligosaccharide, its glycosylamine and acetyltyrosine conjugate. [A] HPAEC-PAD analysis of $G_2M_3G_2F$ oligosaccharide where (a) refers to the position of the elution of this oligosaccharide with the reducing end. [B] HPAEC-PAD profile of glycosylamine sample subsequent to six cycles of freeze drying, (b) $G_2M_3G_2F$ oligosaccharide glycosylamine peak and (c) $G_2M_3G_2F$ oligosaccharide carbonate peak. [C] $G_2M_3G_2F$-acetyltyrosine peak (d). HPAEC-PAD was performed using the isocratic elution (system A) as described in the text.

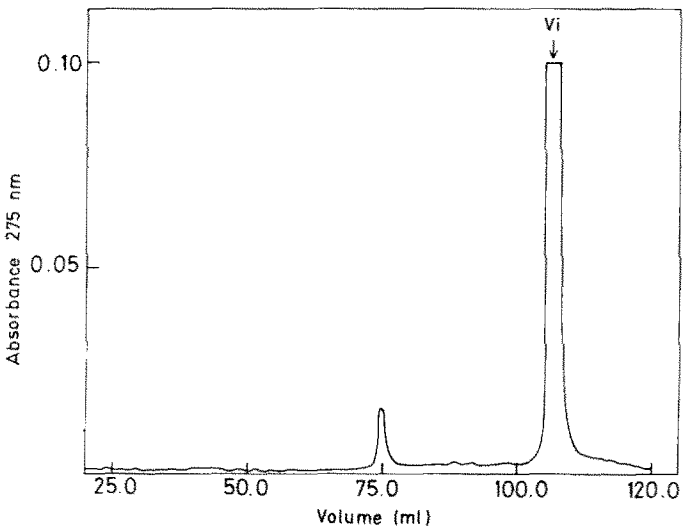


Figure 2. Gel filtration of acetyltyrosine oligosaccharide from acetyltyrosine on Sephadex G-25 column (1.2 × 100 cm). Gel permeation chromatography of $G_2M_3G_2F$-acetyltyrosine prepared with $G_2M_3G_2F$-NH_2 and acetyltyrosine-*N*-hydroxysuccinimide ester as described in the text. The early eluting peak corresponds to the neoglycopeptide while the peak eluting near the inner volume of the column corresponds with that of acetyltyrosine. On HPAEC analysis (see Fig. 1) of the early eluting peak, no reducing end oligosaccharide or oligosaccharide glycosylamine was detected.

reducing oligosaccharide. The oligosaccharide glycosylamines were then coupled with acetyltyrosine.

Coupling of acyltyrosine derivatives with oligosaccharide glycosylamines

The coupled derivatives were separated from the unreacted acyltyrosine(s) by gel filtration on a Biogel P-6 column where the latter compounds were eluted near the inner volume of the column (Fig. 2). Coupling of oligosaccharide glycosylamines with *N*-hydroxysuccinimide ester of acyltyrosines led to almost quantitative consumption of the former as revealed by its absence in HPAEC-PAD (Fig. 1).

Purification and the characterization of the glycosylated acyltyrosine derivatives

Because of the usage of an excess of the acetyltyrosyl-*N*-hydroxysuccinimide ester, all of the oligosaccharide glycosylamines were coupled at the end of the reaction. Further, purification of the glycosylated acyltyrosine(s) except the triantennary oligosaccharides from fetuin, was therefore, not necessary. Nonetheless, a small aliquot of each of these compounds was subjected to RP-HPLC on a C-18 column and lectin affinity chromatography as a test of its purity. RP-HPLC profile of $g_3G_3M_3G_2$ acetyltyrosine shows that the glycopeptide splits into two peaks (Fig. 3). The latter eluting peak was assigned to the type I structure of $g_3G_3M_3G_2$ as 3 M equivalents of galactose residues were released per mol glycopeptide upon treatment with jackbean β-galactosidase. The early eluting peak in RP-HPLC was assigned type II $g_3G_3M_3G_2$ structure as only 2 M equivalents of galactose residues were liberated on treatment with jackbean β-galactosidase [18, 19]. Glycosylated (acetyl)-tyrosine showed identical elution profiles on lectin-affinity matrices to that obtained for the native oligosaccharides (Table 2). Monosaccharide composition of these derivatives is also given in Table 2.

Iodination

Iodination of the glycosyl (acetyl)-tyrosine derivatives was quite facile and specific radioactivities in the range of 0.9–1.71 × 10^8 cpm $nmol^{-1}$ were obtained.

In vivo *studies*

Since the complete removal of blood from various organs is almost impossible, we have initially determined the blood

Table 2. Inferred structures of acetyltyrosine-oligosaccharides from their retention characteristics on lectin-affinity matrices and their monosaccharide composition.

Oligosaccharyl-[^{125}I] acetyltyrosine	*Retention on lectin matrices*				*Monosaccharide content mol per mol relative to mannose*				
	Con A	*RCA_1*	*Jacalin*	*LCA*	*F*	*g*	*M*	*G*	*MN*[a]
M_3G_2-Tyr-Ac	+	–	–	–	–	–	3.0	1.92	–
M_3G_2F-Tyr-Ac	+	–	–	+	1.11	–	3.0	2.01	–
$G_2M_3G_2$-Tyr-Ac	+	–	–	–	–	–	3.0	1.87	–
$G_2M_3G_2F$-Tyr-Ac	+	–	–	+	1.08	–	3.0	2.03	–
$g_2G_2M_3G_2$-Tyr-Ac	+	+	–	–	–	1.98	3.0	2.05	–
$g_2G_2M_3G_2F$-Tyr-Ac	+	+	–	+	0.96	2.07	3.0	2.12	–
$g_3G_3M_3G_2$-Tyr-Ac (type I)	–	+	–	–	–	3.06	3.0	5.01	–
$g_3G_3M_3G_2$-Tyr-Ac (type II)	–	+	–	–	–	3.15	3.0	5.11	–

Binding to lectin affinity matrices was carried out with 0.5 × 5 cm columns and 1 pmol of each of the neoglycopeptides was loaded for these studies.
[a] Mn stands for mannosamine, whereas, + and – corresponds to strong binding and no binding, respectively.

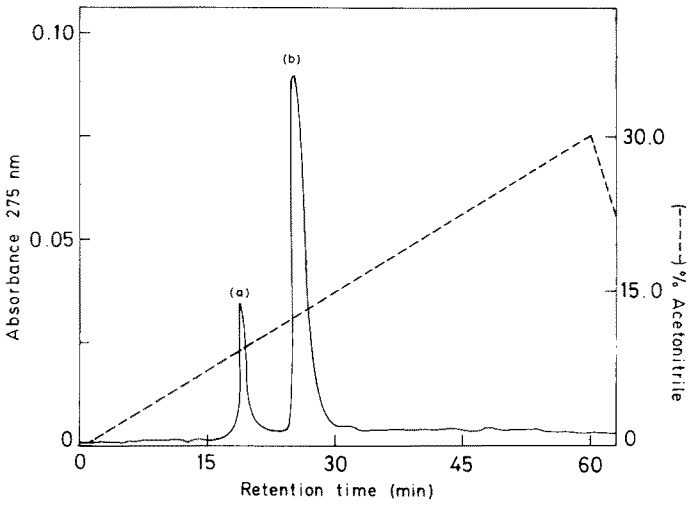


Figure 3. RP-HPLC for the purification of $g_3G_3M_3G_2$-acetyltyrosine derivatives on a Waters C-18 column (7.8 × 300 mm). (a) The early eluting peak is for the type II structure, while the late eluting peak (b) contains the neoglycopeptide with $g_3G_3M_3G_2$ type I structure.

volume of various tissues by monitoring the distribution of acetyl [^{125}I] tyrosine at 10 min after its intravenous injection. Blood is known to contribute to approximately 7.3% of the total weight of mice and we noted that 90% of the injected radioactivity was recovered in the blood at 10 min.

The distribution of radioactivity among the various tissues shown in Table 3 is therefore indicative of the blood content of various tissues. The values thus obtained are in agreement with those reported earlier using very different types of markers [14, 38]. It is to be noted that blood content for salivary glands and bone marrow are not listed as the former was difficult to determine accurately and the latter devoid of blood due to the procedure involved in the

Table 3. Distribution of acetyl [^{125}I] tyrosine at 10 min in various tissues.

Tissue	%	*Tissue*	%
Blood	39.3	Spleen	1.0
Liver	10.9	Stomach	0.6
Lungs	1.3	Intestine	2.1
Heart	1.4	Brain	0.7
Kidney	1.8		

Values reported are the mean of three experiments.

preparation. The tissue distribution quantified for various acetyltyrosine-oligosaccharides are corrected for the residual blood content for the respective tissues (Tables 4 and 5). As different organs/tissues differ in their relative mass, the results have been expressed per g wet weight of the tissue except for blood, stomach and intestinal fluids.

Analysis of the biodistribution of acetyltyrosine oligosaccharides (neoglycopeptides) reveals several interesting features. Neoglycopeptides with nearly identical structures are distributed at strikingly different locations in mice. The two triantennary oligosaccharide derivates from fetuin, for example, contain a similar type of antennary structure except that the type II chain contains one of its terminal galactose in β1–3 linkage to the subterminal *N*-acetylglucosamine residue [39]. Thus, while type I structure is taken up rapidly by liver, on an absolute basis, kidney appears to be the preferred site for the uptake of the type II structure. Likewise, the biantennary complex type oligosaccharide from bovine thyroglobulin differs marginally from the biantennary oligosaccharide from human fibrinogen in having a fucose attached to the reducing end *N*-acetylglucosamine in α1-6 linkage and yet their *in vivo* fates in

Table 4. Biodistribution of acetyl[^{125}I] tyrosine oligosaccharides in mice.

Tissue	$g_3G_3M_3G_2(I)$			$g_3G_3M_3G_2(II)$			$g_2G_2M_3G_2$			$G_2M_3G_2$		
	25	50	500 *min*	25	50	500 *min*	25	50	500 *min*	25	50	500 *min*
	(*cpm* × 10^8 *per g tissue*)			(*cpm* × 10^8 *per g tissue*)			(*cpm* × 10^8 *per g tissue*)			(*cpm* × 10^8 *per g tissue*)		
Blood[a]	3.60	2.40	1.90	3.88	2.70	2.60	3.70	3.06	2.71	7.80	3.98	2.40
Liver	14.22	12.60	9.14	7.20	6.63	5.31	1.80	1.22	1.05	0.63	0.53	0.43
Heart	0.07	0.073	0.081	0.18	0.21	0.25	0.16	0.21	0.24	*	*	*
Lungs	0.93	0.97	1.09	0.21	0.27	0.32	0.54	0.48	0.57	2.63	3.11	4.21
Kidney	0.96	1.03	1.12	8.42	10.10	8.60	24.10	21.21	15.21	7.26	8.76	10.23
Spleen	0.61	0.72	0.91	1.02	0.36	0.42	0.42	0.36	0.49	*	*	*
Stomach	*	*	*	*	*	*	*	*	*	*	*	*
Stomach fluid[b]	*	*	*	*	*	*	*	*	*	1.72	2.10	2.50
Intestine[b]	*	*	*	*	*	*	*	*	*	*	*	*
Intestinal fluid	*	*	*	*	*	*	*	*	*	0.42	0.63	0.72
Brain	*	*	*	*	*	*	*	*	*	*	*	*
Salivary glands	*	*	*	*	*	*	*	*	*	108.63	97.43	86.30
Bone marrow	*	*	*	*	*	*	1.0	1.81	2.1	*	*	*
Amount injected[c]	18.90	18.90	18.90	14.70	14.70	14.70	14.85	14.85	14.85	14.63	14.63	14.63
% Injected dose recovered	95	81	61	95	88	71	93	86	72	92	94	91

All values were corrected for the radioactivity in tissue blood.
* Values less than 1×10^6 cpm per g tissue.
[a] Total blood count; blood was considered to be comprised of 7.2% in the total weight of the animal.
[b] Values represent total fraction of the count in fluid.
[c] Total radioactivity injected.

Table 5. Biodistribution of acetyl[^{125}I] tyrosine oligosaccharides in mice.

Tissue	$g_2G_2M_3G_2F$			$G_2M_3G_2F$			M_3G_2F			M_3G_2		
	25	50	500 *min*	25	50	500 *min*	25	50	500 *min*	25	50	500 *min*
	(*cpm* × 10^8 *per g tissue*)			(*cpm* × 10^8 *per g tissue*)			(*cpm* × 10^8 *per g tissue*)			(*cpm* × 10^8 *per g tissue*)		
Blood[a]	5.70	4.62	3.60	5.71	4.30	3.42	4.12	2.12	1.80	5.40	4.21	3.51
Liver	0.72	0.61	0.40	0.39	0.51	0.42	1.01	1.22	0.96	0.36	0.45	0.21
Heart	0.39	0.45	0.70	*	*	*	0.064	0.072	0.075	*	*	*
Lungs	0.61	0.93	1.40	0.156	0.186	0.181	1.92	2.21	1.80	0.141	0.162	0.181
Kidney	3.01	4.51	3.30	0.054	0.072	0.110	0.09	1.21	1.32	0.066	0.088	0.110
Spleen	3.44	3.63	3.10	2.32	3.20	1.92	1.92	2.61	2.82	3.224	4.11	2.61
Stomach	*	*	*	*	*	*	9.60	10.71	8.10	*	*	*
Stomach fluid[b]	*	*	*	0.81	1.23	1.62	1.0	1.11	1.20	1.01	1.60	2.30
Intestine[b]	*	*	*	*	*	*	*	*	*	*	*	*
Intestinal fluid	*	*	*	0.36	0.60	0.84	0.60	0.67	0.72	0.41	0.63	0.31
Brain	*	*	*	*	*	*	*	*	*	*	*	*
Salivary glands	*	*	*	44.64	52.38	48.90	*	*	*	57.02	66.21	45.81
Bone marrow	54.03	51.30	42.00	*	*	*	*	*	*	*	*	*
Amount injected[c]	9.89	9.89	9.89	8.91	8.91	8.91	12.20	12.20	12.20	9.10	9.10	9.10
% Injected dose recovered	87	85	66	93	92	84	94	97	82	95	96	97

All values were corrected for the radioactivity in tissue blood.
* Values less than 1×10^6 cpm per g tissue.
[a] Total blood count; blood was considered to be comprised of 7.2% in the total weight of the animal.
[b] Values represent total fraction of the count in fluid.
[c] Total radioactivity injected.

mice are quite different [15, 37]. Thus, the synthetic glycopeptide containing the former glycan is located in bone marrow while the one with the latter mostly in kidney. All of the agalacto-glucosamino compounds were taken up almost exclusively by the salivary glands in the same way as the glycopeptide containing core pentasaccharide (M_3G_2). Core pentasaccharide with fucose is located mostly in the stomach tissue. Retention of these iodinated oligosaccharides in liver compares well with those observed for labelled glycoproteins until 50 min [11, 40–41]. However, the extrapolation of the turnover for ceruloplasmin at 500 min indicates that these neoglycopeptides are retained for a longer time [42]. This is perhaps related to the resistance of the amide linkage between the reducing end sugar moiety and the amino group of tyrosine as compared to the naturally occurring *N*-glycosidic bond.

That the transport of these synthetic glycopeptides is an attribute of their oligosaccharide chains alone is borne out by three different kinds of experiments. Acetyltyrosine by itself does not show any tissue specific distribution, oligosaccharides with different terminal structures display distinct *in vivo* distribution and as shown below glycoproteins or oligosaccharides are able to prevent their uptake in a specific tissue (Tables 6 and 7).

Asialofetuin blocked almost totally the uptake of the derivatives prepared with type I and II triantennary oligosaccharide from fetuin by the liver. Asialofetuin, on the other hand, did not prevent the entry of fetuin into the

Table 6. Effect of asialofetuin or oligosaccharides on the uptake of acetyl [^{125}I] tyrosine oligosaccharides in liver and kidney.

Experiment		*Radioactivity* ($\times 10^8$ *per g tissue*)	
Pre injection with	*Post injection with acetyl[^{125}I] saccharide*[a]	*Liver*	*Kidney*
–	$g_3G_3M_3G_2$ (I)	13.5	0.93
–	$g_3G_3M_3G_2$ (II)	7.2	10.90
Asialofetuin (45 μg)	$g_3G_3M_3G_2$ (I)	1.2	1.13
Asialofetuin (45 μg)	$g_3G_3M_3G_2$ (II)	0.9	10.12
$g_3G_3M_3G_2$ (I; 200 nmol)	$g_3G_3M_3G_2$ (I)	1.5	1.62
$g_3G_3M_3G_2$ (I; 200 nmol)	$g_3G_3M_3G_2$ (II)	4.2	13.62
$g_3G_3M_3G_2$ (II; 200 nmol)	$g_3G_3M_3G_2$ (I)	12.9	1.50
$g_3G_3M_3G_2$ (II; 200 nmol)	$g_3G_3M_3G_2$ (II)	2.4	0.81
$g_3G_3M_3G_2$ (I; 200 nmol) + $g_3G_3M_3G_2$ (I; 200 nmol)	$g_3G_3M_3G_2$ (I) + $g_3G_3M_3G_2$ (II)	0.91	1.11
–	$g_2G_2M_3G_2$	1.42	25.20
$g_3G_3M_3G_2$ (I; 200 nmol)	$g_2G_2M_3G_2$	1.24	24.30
$g_3G_3M_3G_2$ (II; 200 nmol)	$g_2G_2M_3G_2$	0.96	16.20
$g_2G_2M_3G_2$ (200 nmol)	$g_2G_2M_3G_2$	0.91	5.13

[a] Amount of radiactivity injected was in the range injected for experiments for which results have been summarized in Table 4.

Table 7. Effect of oligosaccharide on the uptake of acetyl [^{125}I] tyrosine oligosaccharides.

Experiment		*Radioactivity* ($\times 10^8$ *per g tissue*)		
Pre injection with	*Post injection with acetyl[^{125}I] saccharide*[a]	*Bone marrow*	*Stomach*	*Salivary glands*
–	$g_2G_2M_3G_2F$	51.30	ND[b]	ND
$g_2G_2M_3G_2$ (200 nmol)	$g_2G_2M_3G_2F$	42.23	ND	ND
$g_2G_2M_3G_2F$ (200 nmol)	$g_2G_2M_3G_2F$	4.32	ND	ND
$G_2M_3G_2F$ (200 nmol)	$g_2G_2M_3G_2F$	43.83	ND	ND
–	$G_2M_3G_2$	ND	ND	93.24
$g_2G_2M_3G_2$ (200 nmol)	$G_2M_3G_2$	ND	ND	88.11
$G_2M_3G_2$ (200 nmol)	$G_2M_3G_2$	ND	ND	10.22
M_3G_2 (200 nmol)	$G_2M_3G_2$	ND	ND	7.12
–	M_3G_2F	ND	9.33	–
M_3G_2 (200 nmol)	M_3G_2F	ND	8.34	–
M_3G_2F (200 nmol)	M_3G_2F	ND	0.73	–
$G_2M_3G_2F$ (200 nmol)	M_3G_2F	ND	8.75	–

[a] Amount of radioactivity injected was in the range injected for experiments for which results have been summarized in Tables 4 and 5.
[b] ND is not done.

kidney. In fact it enhanced the localization of this derivative in the kidney. While $g_3G_3M_3G_2$ (type I) failed to prevent the uptake of the galactosylated biantennary oligosaccharide derivative by the kidney, its type II counterpart was able to do so marginally (Table 5). On the other hand, the corresponding oligosaccharide was able to prevent its entry into kidney by almost 80%.

Uptake of agalactosylated biantennary oligosaccharide by salivary glands was inhibitable both by the corresponding oligosaccharide as well as by the core pentasaccharide of *N*-linked glycans. Presence of both the fucose in the core region and the galactose residue was found to be essential for entry into the bone marrow. Likewise, the uptake of M_3G_2F derivative was dependent on the presence of exposed mannose residues as well as the core fucose.

Binding of acetyltyrosine oligosaccharides to hepatocytes

Mouse hepatocytes were found to contain 1.8×10^6 receptor sites for $g_3G_3M_3G_2$ acetyltyrosine with a K_d of 5.1 nM (Fig. 4). These values are in the range of values reported for rat and rabbit hepatocytes [43–46]. Reversal of this interaction by non-radioactive oligosaccharide counterparts indicate retention of the carbohydrate receptor specific binding to ASGP-R on mouse parenchymal cells (Table 8). The values of K_d obtained for these saccharides indicate that $g_3G_3M_3G_2$ (I) is far superior a ligand to $g_3G_3M_3G_2$ (II) and a three orders of magnitude better ligand over the biantennary structure. Derivatives $G_2M_3G_2$, $G_2M_3G_2F$, M_3G_2, etc., were inactive. These binding data are thus consistent with the preferential localization of $g_3G_3M_3G_2$ in the liver.

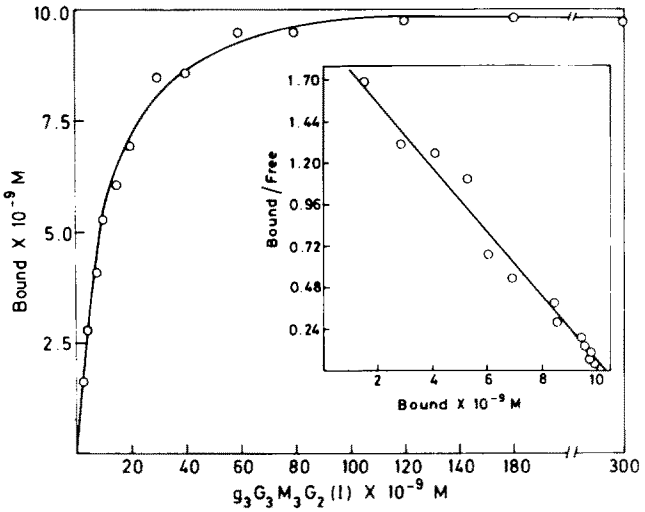


Figure 4. Scatchard analysis of the binding of $g_3G_3M_3G_2$ (I)-acetyl[^{125}I] tyrosine to mouse hepatoctyes. Amount of ligand bound to a constant quantity of hepatocytes (3.5×10^6) incubated with varying concentrations of $g_3G_3M_3G_2$ (I)-acetyl [^{125}I] tyrosine is shown and the Scatchard transformation of these data using the LIGAND program [34] is shown in the inset.

Table 8. Concentration of acetyltyrosine oligosaccharides required to achieve 50% inhibition (IC_{50}) for the binding of $g_3G_3M_3G_2$ (I)-acetyl [^{125}I] tyrosine with mouse hepatocytes.

Inhibitor	IC_{50} (μM)[a]
$g_3G_3M_3G_2$ (I)-acetyltyrosine	0.045
$g_3G_3M_3G_2$ (II)-acetyltyrosine	2.1
$g_2G_2M_3G_2$-acetyltyrosine	90.6
$g_2G_2M_3G_2F$-acetyltyrosine	86.4
$G_2M_3G_2$-acetyltyrosine	No inhibition at 1 mM
M_3G_2-acetyltyrosine	No inhibition at 1 mM
acetyltyrosine	No inhibition at 1 mM

[a] Average of two determinations.
Assays were done using 1.5×10^6 hepatocytes per ml and 2.5 mM of $g_3G_3M_3G_2$-acetyl[^{125}I] tyrosine.

Discussion

Oligosaccharide chains of glycoproteins are remarkable for an inherently large repertoire of chemical information that is crucial to their biological function [1]. In addition to their specific interactions with cells of the immune system, antibodies and lectins, the carbohydrate moieties also serve to determine their destination in an organism as well as their turnover [1, 3, 7]. However, their structural complexity and heterogeneity is an impediment to the elucidation of their biological function. Considering that availability of a homogeneous population of oligosaccharide chains with an appropriate label would facilitate such a task, we have undertaken a moderately large scale purification and their conjugation to acyltyrosine. By virtue of the presence of a tyrosine residue in these conjugates, we expected to have an oligosaccharide derivative with a chromophore. Since the absorptivity of tyrosine is independent of solvent polarity, it provides a convenient handle for the quantitation of glycopeptides during purification and subsequent handling. This apart, by virtue of the presence of a tyrosine in these glycopeptides, they could be labelled to high specific activities. Likewise, the incorporation of the acyl group at the amino terminus of tyrosine was expected to provide a non-polar component for the purification of these glycoconjugates, should that become necessary. Besides, by choosing the fatty acyl group of an appropriate chain length in the conjugate, it should be possible to produce either completely soluble derivatives of tyrosine as reported here for acetyltyrosine or compounds (myristoyl, palmitoyl, stearoyl, etc.) that could be incorporated stably in the liposomal membranes (A. Surolia, unpublished work). This should facilitate a precise elucidation of the homing specificities of glycosylated liposomes to different organs of an animal *in vivo* [47]. None of the other synthetic derivatives reported earlier provide this kind of versatility in the same molecule [48–50]. However, the biotinylated

diaminopyridine labelled oligosaccharides recently reported by Rothenberg *et al.* [51] possesses a high degree of versatility. In addition to its applicability as a sensitive tracer in structural studies, the above label by virtue of possessing a biotinyl group which forms multivalent and nearly irreversible interactions with streptavidin/avidin, has allowed not only raising of anti-oligosaccharyl IgG antibodies, but also should lead to the detection and isolation of oligosaccharide specific receptors from natural or combinatorially expressed sources. Despite its versatility, the method of Rothenberg *et al.* necessitates ring opening of the reducing end sugar, which would be disadvantageous if the reducing end sugar by itself was expected to play a biological role. An important consideration in these studies, therefore, has been that the carbohydrate moieties should suffer minimum perturbation during these manipulations. Hence, the reactions that led to the generation of open chain structures for the reducing end sugar, for example, the reductive amination etc., were precluded for the coupling of the saccharide with the indicator molecule [10, 52].

The yields reported here for the synthesis of glycosylamines is higher than that reported in the previous studies [28, 48, 49]. This is largely related to the repeated freeze drying step used in these studies for the removal of NH_4HCO_3 which probably results in the conversion of the glycosylamine carbonates to the glycosylamine. Moreover, the yields of the conjugation between the oligosaccharide glycosylamines and fatty acyltyrosine derivatives are also higher than those reported previously for the coupling of the glycosylamine of GlcNAc with dansyl chloride [48]. Poor yield in the previously reported reactions was considered to be due to the steric hindrance caused by the bulky dansyl group [48]. The poor yields in the coupling reactions could also be due to the regeneration of the reducing group from glycosylamine. This is thought to result from a decrease in pH as a consequence of the hydrolysis of the active ester coupling reagent utilized. It has been suggested that the addition of a base during the coupling reaction could overcome this side reaction. These studies, on the other hand, show that usage of anhydrous DMF for coupling stabilized the glycosylamine form, as the formation of traces of reducing oligosaccharides were not observed in HPAEC-PAD employed for monitoring of the progress of the reaction.

A related complication in the coupling reaction involves anomeric impurity in the product [48]. All of the oligosaccharides showed an anomeric resonance for the GlcNAc-β-glycosylamide close to 5.04 ppm with a coupling constant of 10.02 Hz indicating that most of the glycopeptides synthesized in this study contain the innermost GlcNAc residue in exclusively β-glycosylamide linkage. This is probably also facilitated by the coupling reaction carried out under the anhydrous conditions. Under the alkaline conditions of the reaction (pH >8.5), β-glycosylamine linkage of GlcNAc may also undergo base catalysed epimerization to ManNAc. Since ManNAc could not be detected in compositional analyses (Table 2), this side reaction did not occur probably due to the usage of milder conditions (i.e. temperature 20–25°C).

The fact that the lectin binding characteristics of neoglycopeptides thus synthesized were identical with those of parent oligosaccharides indicates that the structures of terminal residues are not altered as a consequence of chemical manipulations involved in their preparations (Table 2).

The neoglycopeptides thus synthesized could be radiolabelled to high specific activities with ^{125}I so that only minuscule quantities of them (about 1pmol) are required for elucidation of their lectin binding properties and structure as well as in targeting specificities which are discussed in greater detail below.

Since the pioneering discovery of Ashwell and Morell [7], numerous studies have very clearly established that mammalian hepatocytes contain a receptor protein which is able to recognize and endocytose a number of serum glycoproteins with exposed galactose residues. However, most of these studies examined this process using glycoproteins bearing multiple oligosaccharide moieties such as asialoorosomucoid, asialoceruloplasmin, etc. However, in the context of our studies, the work of Rogers and Kornfeld [8] which showed that a glycopeptide from fetuin when coupled to albumin is sufficient to mediate the rapid clearance of this neoglycoprotein from serum, and those of Baenziger and Fiete [44] which showed that glycopeptides and glycoproteins which differ in their association constants for the isolated receptor by as much as 750-fold are endocytosed by the hepatocytes at kinetically comparable rates, are of particular interest.

Moreover, studies of Baenziger and Fiete [44] have shown that a single oligosaccharide chain with appropriate structure is sufficient for the plasma clearance of glycoproteins. However, these studies were concerned primarily with the asialoglycoprotein receptor identified by Ashwell and Morell [3]. The present study, on the other hand, endeavours to elucidate the *in vivo* itinerancy of oligosaccharides of defined structures. Studies reported here have led to the identification of novel routes and sites for the carbohydrate ligand mediated uptake system *in vivo*. Interestingly, enough, our studies also suggests that differences in carbohydrate structures are responsible for dramatically different fates in the animal *in vivo*.

Amongst the triantennary complex type glycans, the oligosaccharide $g_3G_3M_3G_2$ (type I) has already been shown to be one of the most potent ligands both for binding and endocytosis and the results reported here are completely consistent with these studies (Tables 4 and 6) [12, 44, 45]. However, the poor uptake of $g_3G_3M_3G_2$ (type II structure) as compared to its type I counterpart by liver was unexpected. As liver is approximately three times larger in

mass than kidney in mice, in relative terms, liver still accounts for a greater percentage of uptake for this compound. Yet, a consideration of the uptake per g of tissue indicates that kidney is the preferred site for the uptake of this compound. This is also borne out by inhibition experiments. Asialofetuin, for example, due to the presence of type I oligosaccharide and several O-linked chains, is almost totally endocytosed by liver parenchymal cells [40]. Hence, it is able completely to inhibit the uptake of type II oligosaccharide structure by liver but fails to do so as far as its uptake in kidney is concerned. As a matter of fact, it increases the uptake of type II compound in kidney, presumably by increasing its pool in circulation consequent to the inhibition of its uptake by liver. This is also in agreement with inhibition studies with oligosaccharides, where the homologous structure (type II) is able to inhibit its uptake in kidney but not the type I oligosaccharide. Failure to observe the dichotomy of behaviour for the closely related oligosaccharide structure is presumably related to the differences in their overall conformational preferences [53] and have escaped the attention of earlier investigators as either only asialofetuin has been used or only the isolated hepatocyte has been examined in these studies [41, 45]. It is conceivable that the binding of the type II structure does not generate the appropriate receptor conformation optimal for endocytosis [45, 53].

A finding of considerable interest is the observation that the conjugate of galactosylated biantennary oligosaccharide is most specifically taken up by kidney. Though it is known that glycopeptides containing such oligosaccharides bind not only weakly with the hepatocyte receptor but are also poor substrates for endocytosis by liver, specific targeting of galactosylated biantennary complex type oligosaccharide by kidney has not been reported due to lack of *in vivo* studies [45]. Uptake of $g_2G_2M_3G_2$ is not inhibited by $g_3G_3M_3G_2$ (type I structure) and $g_2G_2M_3G_2F$, only partially by $g_3G_3M_3G_2$ (type II structure) and totally by the homologous oligosaccharide indicating that the latter two compounds share the same receptor. Non-inhibition by $g_2G_2M_3G_2F$ suggests that fucosylation in the core region alters the preference of the kidney lectin in a profound fashion, probably in a manner opposite to that observed for binding of such an oligosaccharide with plant lectins from pea and lentil [54].

Acetyltyrosyl-$g_2G_2M_3G_2F$, as expected is taken up by bone marrow cells as shown earlier with glycoproteins bearing this oligosaccharide chain [41]. Analyses of inhibition patterns reveals that both exposed galactosyl residues and an α-linked fucose are required for its productive interaction with the bone marrow cell-surface lectin.

Other remarkable observations in this study pertain to the specific uptake of acetyltyrosyl-$G_2M_3G_2$, $G_2M_3G_2F$ or M_3G_2 by salivary glands. This uptake appears principally to require exposure of *N*-acetylglucosamine or mannose. In other words, further extension of this chain by galactose has a negative influence on their interaction and uptake by the lectin displayed by the salivary gland tissue. The core fucose does not seem to play any role in the uptake process by salivary gland tissue as $G_2M_3G_2F$ and $G_2M_3G_2$ are equally potent. It may be noted that some radioactivity is also seen in the stomach and intestine much of which appears to be related to secretion in saliva. Thus, once absorbed in salivary glands, the compounds appear to be released slowly to saliva and from there reach stomach and intestinal spaces.

In contrast to salivary glands, exposure of both mannose and an α-linked fucose appears to be obligatory for uptake in the stomach tissue (Table 7). It is of interest that M_3G_2 and M_3G_2F possessing mannose residues at their non-reducing termini fail to concentrate in liver and spleen although these tissues contain considerable amounts of reticuloendothelial cells [55]. This is not as surprising as it might appear, as the reticuloendothelial cells are exquisitely specific for a β1–6 GlcNAc or an α1–6 Man substitution on the 1–6 branch of biantennary glycans [55]. In view of the above and the observations of Lee and coworkers about the short circuiting of endocytotic pathway for cluster glycosides [56], targeting studies with cluster glycosides should be interpreted with caution. In addition, as the routes of injection are also known to alter in a subtle fashion the ultimate fate of the administered molecules [14, 57], it would be of interest to investigate the role of the routes of injection on the itinerary of such oligosaccharide molecules.

In summary, usage of homogeneous oligosaccharides coupled to an indicator molecule such as acetyltyrosine has allowed us to define new sites for the routeing of glycoproteins and other biologically active molecules displaying such structures. How the different vertebrate membrane lectins displayed by various organs, for example the liver and kidney, discriminate between broadly similar oligosaccharides is an important question. Whether these abilities are encoded in a single binding site or are related to the spatial arrangement of subunits on the cell surface as in the case of the hepatocyte lectin [43] merits further studies.

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Note in Proof

Chiu *et al* (1994) *J Biol Chem* **269**:16195–202 and Tamura *et al* (1994) *Anal Biochem* **216**:335–44 are similar in intent but different in design. The results of Chiu *et al* are in broad agreement with those reported here.

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