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Structure of the Oligosaccharides of Three Glycopeptides from Calf Thymocyte Plasma Membranes†

Rosalind Kornfeld

ABSTRACT: The carbohydrate composition and oligosaccharide structure of three glycopeptides isolated from delipidated calf thymocyte plasma membranes following Pronase digestion have been determined. Five major glycopeptide fractions were separated using Bio-Gel P-6 gel filtration and diethylaminoethylcellulose chromatography. The structure of the oligosaccharide chains of three of these glycopeptides was determined by a combination of sequential degradation with glycosidases and methylation analysis. These oligosaccharide structures consist of complex, highly branched N-linked chains containing at their nonreducing termini the unusual sequence Gal(β1→3)Gal(β1→4)GlcNAc→ as well as the more usual sequence SA(α2→3)Gal(β1→4)GlcNAc→. In addition, one glycopeptide also contains short O-linked chains with the structure Gal(β1→3)GalNAc→Ser(Thr) which have receptor activity for the lectin from the mushroom *Agaricus bisporus.*

Exposed on the outer surface of animal cells are numerous oligosaccharide chains which are constituents of the cells' plasma membrane glycoproteins. These carbohydrate structures have been postulated to be involved in a number of physiologically important functions such as cell-cell interaction, adhesion of cells to the substratum, and migration of cells to particular organs (e.g., in the "homing" of lymphocytes to the spleen and metastasis of tumor cells to preferred sites). If, in fact, cell-surface oligosaccharides serve as the recognition sites for such interactions, it becomes important to know the detailed structure of these carbohydrate moieties. Membrane glycoproteins have been isolated and the structure of the oligosaccharide chains has been determined in only a few cases. The best studied has been the major sialoglycoprotein of the human erythrocyte membrane which has been shown to contain both N-glycosidically linked complex oligosaccharide chains and O-glycosidically linked oligosaccharide chains whose structures have been determined (Thomas and Winzler, 1969, 1971; Kornfeld and Kornfeld, 1971).

In the study described here, another cell type—the thymus lymphocyte—has been examined to determine how many different oligosaccharides are present in the membrane glycoproteins and what their structures are. In previous work (Kornfeld and Siemers, 1974), a procedure was devised for the large-scale isolation of purified plasma membranes from calf thymocytes which would provide the quantity of starting material required for this study. These plasma membranes contain at least eight glycoproteins which vary in molecular weight from 25 000 to 220 000, as revealed by periodic acid–Schiff reagent staining following electrophoresis of the membranes in sodium dodecyl sulfate–polyacrylamide gels. The initial plan of attack was to separate each of these glycoproteins, recognizing that there could well be more than eight. A partial separation of these glycoproteins was achieved by chromatography of sodium dodecyl sulfate solubilized membranes on hydroxylapatite columns followed by gel filtration on Bio-Gel P-300. The sugar composition and lectin-binding properties of glycopeptides derived from some of these partially purified glycoprotein fractions have been reported (Kornfeld, 1974). More recently, solubilization of the membranes in Triton X-100 followed by affinity chromatography on columns of lentil-lectin–Sepharose and *Ricinus communis* lectin–Sepharose revealed that the majority of the thymocyte membrane glycoproteins contain oligosaccharide chains with affinity for both lectins. The mixture of membrane glycoproteins which adsorbed to the lectin–Sepharose columns and could be eluted with methyl α-D-mannopyranoside and lactose, respectively, also had receptors for the erythro- and leuco-agglutinating lectins from red kidney beans and the lectin from the mushroom *Agaricus bisporus,* which have different sugar specificities than either the lentil or *Ricinus* lectins.

The similarity of lectin-binding properties of all the thymus membrane glycoproteins suggested that they might all contain a similar set of oligosaccharides. Therefore, a more efficient approach to determining the structure of the cell-surface oligosaccharides was to isolate these carbohydrate chains in the form of glycopeptides from the entire membrane pool of glycoproteins. Accordingly, in this study the glycopeptides isolated following delipidation and Pronase digestion of the purified plasma membranes have been fractionated and their carbohydrate structures analyzed.

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Experimental Procedure

Preparation of Delipidated Plasma Membrane. Plasma membranes from calf thymocytes were purified as previously described (Kornfeld and Siemers, 1974). The purified plasma membranes obtained in the "top" layer on sucrose step gradient centrifugation containing 113 mg of protein were dialyzed for 2 days in the cold against several changes of distilled water to remove any residual sucrose and then shell frozen and lyophilized. The membranes were suspended in 48 mL of chloroform–methanol (2:1) and transferred to four 15-mL Corex centrifuge tubes. The membrane suspension was kept at room temperature for 3 h with periodic stirring with a glass rod and then centrifuged for 45 min at 12 000 rpm in a Sorvall SS-34 rotor. The clear, slightly yellow supernatant was removed from the well-packed membrane pellets, and the pellets were each resuspended in 12 mL of chloroform–methanol (1:2) and extracted and centrifuged as before. The pelleted membranes were dried by lyophilization, combined, and resuspended in 2.5 mL of water.

Pronase Digestion of Delipidated Membranes. The delipidated membranes were digested with 2.4 mg of pronase (Calbiochem, Type B) in a final volume of 3.0 mL containing 2 mM $CaCl_2$ and 50 mM Tris[1]-HCl buffer, pH 7.9, for 24 h at 37 °C. To prevent bacterial contamination, a small vial of toluene was suspended by surgical thread into the stoppered reaction tube, thereby saturating the atmosphere with toluene vapor. At 24 and again at 48 h, 2.4 mg of fresh Pronase was added and the digestion terminated at 94 h by boiling the reaction mixture for 5 min. The Pronase digest was centrifuged to remove insoluble material which was washed with 1.0 mL of water, and the supernatant fluid and wash were combined and reduced to a volume of 1.0 mL. Ninety-five percent of the membrane sialic acid was recovered in this soluble fraction after Pronase digestion.

Chemical Analyses. Hexoses were measured on column eluates with the phenol–H_2SO_4 method scaled down to $^1/_5$ volume (Hodge and Hofreiter, 1962); protein was measured by the method of Lowry et al. (1951); and sialic acid was measured by the thiobarbituric acid method of Warren (1959). Individual sugars and amino sugars were measured by gas–liquid chromatography on 3% SE-30 of the trimethylsilyl derivatives of the methyl glycosides after methanolysis in 1.5 N methanolic HCl by a modification of the method of Reinhold (1972) as previously described (Baenziger et al., 1974). Amino acids and the amino sugars glucosamine and galactosamine of isolated glycopeptides were quantitated by an automated amino acid analyzer following hydrolysis in 6 N HCl for 18 h at 105 °C. Only the long column was used, so the content of basic amino acids was not determined. Periodate oxidation of glycopeptides was performed as described by Baenziger et al. (1974).

Methylation Analysis. The purified glycopeptides were methylated by the method of Hakomori (1964) using methylsulfinyl carbanion and methyl iodide. Thirty to fifty nanomoles of each glycopeptide was methylated in a reaction mixture of 0.3 mL, and the permethylated glycopeptides were subjected to acetolysis with 0.5 N H_2SO_4 in 95% acetic acid, hydrolysis, reduction, and acetylation as described by Stellner et al. (1973). The partially methylated alditol acetates and 2-deoxy-2-(*N*-methylacetamido)hexitol acetates were separated and identified by gas chromatography and mass spectrometry as described by Björndal et al. (1970) and Stellner et al. (1973) using a column of 3% ECNSS-M on Gas Chrom Q, 100–200 mesh (Applied Sciences Labs, State College, Pa.). The flame detector runs were carried out on an F&M 402 gas chromatograph and mass spectrometry was performed either on an LKB-9000 gas chromatograph–mass spectrometer or a Finnegan 3300 gas chromatograph–mass spectrometer. The latter instrument had the capability of monitoring the column effluent every 3 s and could thus produce a scan of the entire chromatogram for any selected *m/e* ion.

The methylated alditol acetate standards used were those described in a previous work (Kornfeld et al., 1971) as well as those derived from a highly purified IgG glycopeptide of known structure (Baenziger and Kornfeld, 1974) which upon methylation produces 2,3,4-tri-*O*-methylfucose, 2,3,4,6-tetra-*O*-methylgalactose, 3,4,6-tri-*O*-methylmannose, 2,4-di-*O*-methylmannose, and the 2-deoxy-3,4,6-tri-, 2-deoxy-3,6-di-, and 2-deoxy-3-*O*-methyl-2-(*N*-methylacetamido)glucose. The amounts of each methylated sugar derivative were determined by measurement of the areas of the peaks obtained on the tracing produced by the flame detector of the F&M gas chromatograph. Our experience and that of others (Björndal et al., 1970) indicate that the response of the flame detector is not significantly different for equimolar amounts of the various partially methylated alditol acetates compared with the variation or errors introduced by the methylation procedure and subsequent workup and derivatization. This assumption is probably less valid for the acetamido sugar derivatives, especially the mono-*O*-methyl species. Depending on the column packing material, the instrument used, and the amount of the sample, varying amounts of loss or destruction of the mono-*O*-methylacetamido sugar derivatives have been observed. Since these derivatives are eluted late in an isothermal run or at the higher temperature of a temperature programmed run on the ECNSS-M column, their peaks on the flame detector scan are either broad or occur on a rising baseline, making them difficult to measure accurately on small samples. To obtain more accurate ratios for the acetamido sugar derivatives, use has been made of selected ion scanning in the Finnegan GC–MS spectrometer. As shown by Stellner et al. (1973), all the methylated 2-deoxy-2-(*N*-methylacetamido)-hexitol acetates produce a fragment of *m/e* 158 containing carbon atoms 1 and 2, which is further degraded to a fragment of *m/e* 116 by loss of ketene. Tai et al. (1975) have shown that the amount of ion *m/e* 116 produced is proportional to the amount of acetamido sugar derivative present and that measurement of the peaks of the ion scan at *m/e* 116 can be used to quantitate the amounts of the various acetamido sugar derivatives. This technique has been used in the present study to complement the results obtained in the flame detector runs.

Degradation of Glycopeptides with Glycosidases. Glycopeptides were treated either sequentially or with combinations of glycosidases as previously described (Kornfeld et al., 1971). The enzymes used were *Vibrio cholera* neuraminidase (EC 3.2.1.18) from Calbiochem, highly purified Jack bean β-galactosidase (EC 3.2.1.23), β-*N*-acetylglucosaminidase (EC 3.2.1.30), and α-mannosidase (EC 3.2.1.24) prepared by the method of Li (1967) and hen oviduct β-mannosidase prepared by the method of Sukeno et al. (1972). Following digestion with glycosidases, the reaction mixtures were boiled and the residual glycopeptide was separated from the released sugars by gel filtration on calibrated columns of Sephadex G-25-80 (Pharmacia) or Bio-Gel P-2 (Bio-Rad Labs). Released *N*-acetylglucosamine was measured colorimetrically by the method of Reissig et al. (1955), and released galactose and mannose were measured enzymatically as previously described (Kornfeld et al., 1971).

[1] Abbreviations used are: DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane.

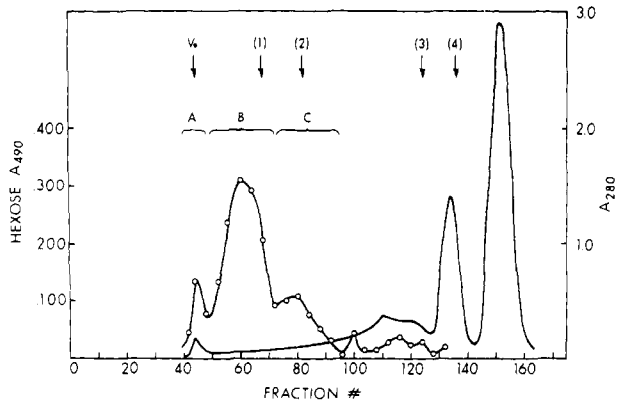


FIGURE 1: Gel filtration of the Pronase digest of thymocyte membranes on Bio-Gel P-6. A Bio-Gel P-6 column (1.4 × 88 cm), 100–200 mesh, equilibrated with 0.1 M NH_4HCO_3 was calibrated with the following markers: (V_0) human hemoglobin, (1) fetuin glycopeptide (3180 mol wt), (2) IgG glycopeptide (1900 mol wt), (3) the tetrasaccharide stachyose (666 mol wt), and (4) glucosamine. After loading the Pronase digest onto the column, it was eluted with 0.1 M NH_4HCO_3, and 1.0 mL fractions were collected. The absorbance at 280 nm (—) was measured and aliquots of the fractions were assayed for hexose (O–O). The column fractions were pooled as shown into A, B, and C glycopeptide fractions.

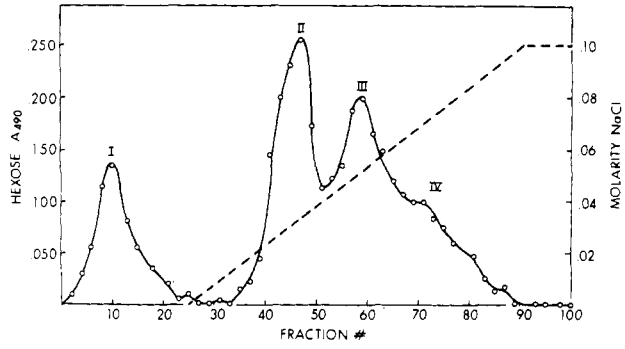


FIGURE 2: DEAE-cellulose chromatography of the B glycopeptide fraction from Bio-Gel P-6. Peak B from the Bio-Gel P-6 column was lyophilized to remove NH_4HCO_3, redissolved in 0.005 M Tris, pH 7, and adjusted to pH 7 with 0.1 N NaOH before it was loaded onto a column of DEAE-cellulose (Whatman DE-52), measuring 0.9 × 7 cm, and equilibrated in 0.005 M Tris-HCl, pH 7. The column was first eluted with 20 mL of 0.005 M Tris, pH 7, and then with a linear gradient formed from 40 mL of 0.005 M Tris, pH 7, in the mixing flask and 40 mL of 0.005 M Tris, pH 7, containing 0.1 M NaCl in the reservoir flask. Finally, the column was eluted with 0.005 M Tris, pH 7, containing 0.1 M NaCl. Fractions of 1.0 mL were collected and their conductivity was measured to determine the NaCl concentration (- - -) and aliquots were assayed for hexose (O—O). Fractions 1–20 were pooled as B-I, fractions 35–50 as B-II, fractions 51–66 as B-III, and fractions 67–90 as B-IV.

A. bisporus Lectin Receptor Activity. The amount of *A. bisporus* lectin receptor in the glycopeptides was measured by their ability to inhibit the binding of *A. bisporus* [^{125}I]lectin to purified thymocyte membranes. *A. bisporus* lectin was prepared and iodinated with ^{125}I as described by Presant and Kornfeld (1972), and the binding to membranes was carried out as previously described (Kornfeld and Siemers, 1974). Briefly, glycopeptide was incubated at room temperature in 0.9% NaCl–0.01 M $NaHCO_3$ containing 1 mg/mL bovine serum albumin and 0.7 μg of *A. bisporus* [^{125}I]lectin (440 000 cpm) in 0.25 mL for 10 min, then 0.05 mL of membrane suspension capable of binding 0.35 μg of *A. bisporus* lectin was added, and the reaction was allowed to proceed for another 45 min. The reaction mixture was diluted, filtered on Gelman 0.3-μm filters and the [^{125}I]lectin bound to the membranes retained on the filter was measured in a Packard γ counter. The amount of glycopeptide causing 50% inhibition of lectin binding to the membranes was considered to have 1 inhibitory unit (IU) of lectin receptor activity.

Results

Separation of the Glycopeptides in the Pronase Digest. The Pronase digest of delipidated thymocyte plasma membranes was fractionated into large, medium, and small molecular weight glycopeptides by gel filtration on a calibrated column of Bio-Gel P-6 (100–200 mesh) in 0.1 M NH_4HCO_3. As shown in Figure 1, a relatively small proportion of the total hexose of the digest eluted in the void volume designated fraction A and represented either incompletely degraded glycoprotein and/or glycopeptides having a molecular weight greater than ~4500. The majority of the hexose-containing material eluted in a broad peak (B) representing glycopeptides of ~2500 to ~4500 molecular weight and the remainder eluted in a third peak (C) representing glycopeptides of ~1500 to ~2500 molecular weight.

The peak B glycopeptides were further fractionated by chromatography on DEAE-cellulose (Whatman DE 52) as shown in Figure 2. The equilibrating buffer eluted a peak of hexose-containing material (peak I) and the NaCl gradient eluted in succession peaks II, III, and IV. Subsequent analysis showed that glycopeptide I contained no sialic acid, glycopeptide II contained about one residue of sialic acid per mole,

TABLE I: Carbohydrate Content of the Delipidated Thymocyte Plasma Membranes and Their Constituent Glycopeptides.

	Total nanomoles						
Sugar	Membranes	A	B-I	B-II	B-III	B-IV	C
Sialic acid	2530	404	0	252	539	635	170
Fucose	1270	61	102	260	295	235	95
Galactose	6000	695	590	1200	1280	1070	339
Mannose	4600	143	308	706	808	646	1203
N-Acetylglucosamine	4800	289	486	1030	1240	955	691
N-Acetylgalactosamine	965	190	19	36	69	111	375
IU[a]		1480	70	117	116	500	1000

[a] For *A. bisporus* lectin.

glycopeptide III contained about two residues per mole, and glycopeptide IV contained about three residues per mole. In Table I, the sugar composition of the delipidated plasma membranes and the glycopeptides derived from it are shown. The recovery in glycopeptides A, B-I, B-II, B-III, B-IV, and C of the various sugars in the delipidated membrane varied from 79 to 98% and, with the exception of *N*-acetylgalactosamine, the B series of glycopeptides accounted for between 54 and 77% of the various membrane sugars. The large molecular weight fraction A contained 3 to 20% of the various membrane sugars, being relatively enriched in sialic acid, galactose, and *N*-acetylgalactosamine. Since it was judged to be a heterogeneous mixture, it was not further analyzed. The smaller molecular weight fraction C, relatively enriched in mannose and *N*-acetylgalactosamine, and glycopeptide fraction B-II, having a composition similar to the other B glycopeptides, both proved to be mixtures upon further analysis. In contrast, glycopeptide fractions B-I and B-III proved to be practically homogeneous glycopeptides and fraction B-IV was shown to be a mixture of two glycopeptides with the same N-linked oligosaccharide, one of which also contained a short O-linked chain. The characterization of the oligosaccharide chains is described in the following sections.

TABLE II: Composition of Membrane Glycopeptides.

Constituent[a]	B-I (residues/mole)	B-III (residues/mole)	B-IV (residues/mole)
Sialic acid	0	2.0	2.9
Fucose	1.1	1.1	1.1
Galactose	5.7	5.2	5.3
Mannose	3.0	3.0	3.0
N-Acetylglucosamine	4.7	5.7	5.4
N-Acetylgalactosamine	0.2	0.2	0.5
Aspartic acid	1.0	1.1	1.5
Threonine	0.4	0.5	1.0
Serine	0.8	0.5	1.1
Glutamic acid	0.4	0.2	0.8
Proline	0	0.3	0.6
Glycine	0.9	0.5	1.1
Alanine	0.3	0.2	0.4
Min. mol wt	2927	3595	4146

[a] The values for the sugars are the average of three determinations and the values for the amino acids are from a single determination.

Table I also shows the receptor activity of each glycopeptide fraction for the *Agaricus bisporus* lectin which has been shown (Presant and Kornfeld, 1972) to bind to glycopeptides containing the O-linked oligosaccharide Gal(β1→3)-GalNAc(α→)(Ser, Thr). Fractions A and C, which are richest in *N*-acetylgalactosamine, also contain the most lectin receptor activity. The B series glycopeptides, which contain lesser amounts of *N*-acetylgalactosamine (less than a full residue), also have lower levels of *A. bisporus* lectin receptor activity, indicating the presence of small amounts of Gal(β1→3)GalNAc→(Ser, Thr) on the peptide chain. This most likely reflects some degree of incomplete Pronase digestion, since even with prolonged digestion times Pronase may not completely cleave between two oligosaccharide chains that are attached to closely spaced amino acid residues.

Composition of Glycopeptides B-I, B-III, and B-IV. In Table II the sugar and amino acid compositions of glycopeptides B-I, B-III, and B-IV are shown in terms of residues per mole of glycopeptide, setting mannose equal to 3.0 residues. Each glycopeptide contains at least 1 full residue of aspartic acid, from 0.4 to 1.1 residue of threonine, serine, and glycine, and lesser amounts of the other acidic and neutral amino acids. These are the amino acids commonly found in the N-linked glycopeptides from soluble glycoproteins and conform to the characteristic sequence around glycosylated asparagine residues, namely, Asn-X-Thr(Ser) (Marshall, 1972). The molecular weight of each glycopeptide has been calculated from the composition as shown in Table II. Since the glycopeptides were not analyzed for cysteine or basic amino acids, they may also contain some of these and therefore the calculated molecular weights must be considered minimum values. Nevertheless, the range of molecular weights (2900 to 4150) agrees well with the elution position of the B glycopeptides on the Bio-Gel P-6 column (ranges ~2500 to ~4500). The sugar composition of all these glycopeptides is very similar, differing primarily in the sialic acid content and showing small variations in the galactose and *N*-acetylgalactosamine content. They are characterized by a high galactose and *N*-acetylglucosamine content and the structural studies to be described indicate that the oligosaccharide chains of these glycopeptides constitute a series of structures having the same core arrangement of sugars and differing in the extent of branching and sialylation of the outer chains.

Structural Studies on Glycopeptide B-I. The results obtained when glycopeptide B-I was methylated and its constituent methylated sugars were separated and identified by gas–liquid chromatography and mass spectrometry are shown in Table III. Also shown are the methylated sugars contained in the "core" of B-I isolated after digestion of B-I with β-galactosidase and β-*N*-acetylglucosaminidase. Three of the galactose residues in B-I are terminal, giving rise to 2,3,4,6-tetra-*O*-methylgalactose and the remaining galactose residues are substituted at C-3. The single fucose residue is terminal and two of the three mannose residues are doubly substituted at C-3 and C-6, providing two branch points in glycopeptide B-I. The results obtained when glycopeptide B-I was digested with glycosidases are shown in Table IV. In experiment I, treatment of B-I with β-galactosidase released 5 out of 5.7 residues of galactose. Subsequent treatment with β-*N*-acetylglucosaminidase released 2.7 residues of *N*-acetylglucosamine and then α-mannosidase treatment released 1.7 residues of mannose. In Experiment II, combined β-galactosidase and β-*N*-acetylglucosaminidase treatment released 5.4 residues of galactose and 2.8 residues of *N*-acetylglucosamine from B-I, and the residual glycopeptide was then methylated. Since almost all of the galactose in B-I could be removed by

TABLE III: Methylated Sugars from Glycopeptide B-I.

	Intact B-I (residues/mole)	Core B-I[a] (residues/mole)
Fucose 2,3,4-triMe	1.1	1.0
Galactose		
2,3,4,6-tetraMe	3.0	0.3
2,4,6-triMe	2.8	
Mannose		
2,3,4,6-tetraMe		1.75
3,4,6-triMe	1.0	0.25
2,4-diMe	2.0	1.0
2-*N*-Methyl-*N*-acetyl-glucosamine		
3,6-diMe	4.0	1.2
3-Me	0.9	1.0

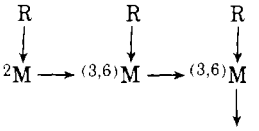
[a] Core glycopeptide produced by β-galactosidase and β-*N*-acetylglucosaminidase digestion of B-I (experiment II, Table IV).

TABLE IV: Glycosidase Digestion of Glycopeptide B-I.

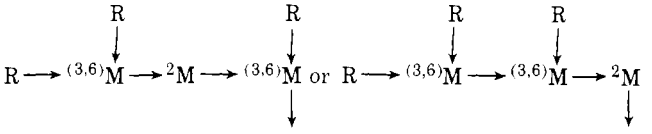
Glycosidase treatment	Residues released/mole of glycopeptide: Galactose	*N*-Acetylglucosamine	Mannose
Intact B-I[a]	5.7	4.7	3.0
(I) β-Galactosidase (3×);[b] then β-*N*-acetylglucosaminidase (2×);[b] then α-Mannosidase (2×)[b]	5.0	2.7	1.7
(II) β-Galactosidase and β-*N*-acetylglucosaminidase (2×)[b]	5.4	2.8	

[a] Total residues of each sugar, taken from Table II. [b] Indicates the number of sequential treatments with the same glycosidase. After each treatment, the reaction mixture was boiled and fractionated on Sephadex G-25 or Bio-Gel P-2, and the residual glycopeptide was retreated with the appropriate glycosidase.

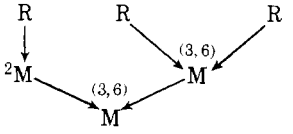
β-galactosidase alone, the terminal galactose residues must be linked to C-3 of underlying galactose residues (2,4,6-trimethyl derivatives in intact B-I). When the residual glycopeptide in experiment II was methylated, the only galactose species seen was 0.3 residue of terminal 2,3,4,6-tetra-*O*-methylgalactose, which indicates that the β-galactosidase treatment did not quite go to completion. Since almost three residues of *N*-acetylglucosamine substituted at C-4 could be released after removal of galactose, there are probably three outer chains with the structure Gal(β1→3)Gal(β1→4)-GlcNAc(β→). Although the average outer chain contains $(Gal)_2$→GlcNAc→, the data do not rule out combinations of $(Gal)_3GlcNAc + (Gal)_2GlcNAc + (Gal)_1GlcNAc$, or $(Gal)_4GlcNAc + 2(Gal)_1GlcNAc$, which would produce the same proportion of methylated galactose species. The structure of the inner region of B-I was deduced from comparing the methylated sugars derived from the residual glycopeptide from experiment II with the methylated sugars derived from intact B-I (Table III). Based on these studies, described below, the structure shown in Figure 3 is proposed for B-I where ±Gal is used to indicate the probable microheterogeneity in the outer-chain galactose content. Intact B-I, after methylation, gave 2.0 residues of 2,4-dimethylmannose and 1.0 residue of 3,4,6-trimethylmannose, but after the outer chains were removed from B-I (experiment II, Table IV) the residual glycopeptide upon methylation gave 1.0 residue of 2,4-dimethylmannose, 0.25 residue of 3,4,6-trimethylmannose, and 1.75 residues of 2,3,4,6-tetramethylmannose. In other words, 1.0 residue of 2,4-dimethyl- and 0.75 residue of 3,4,6-trimethylmannose have been converted to terminal tetramethylmannose. Accordingly, two outer chains must have been attached to C-3 and C-6 of one mannose in B-I and the third outer chain (all of which was not removed in experiment II) must have been attached to C-2 of another mannose. The finding in experiment I that 1.7 residues of mannose were removed by α-mannosidase indicates that the two outer mannose residues are in α linkage. The inner portion of degraded B-I from experiment II also gave rise to approximately one residue each of 2,3,4-trimethylfucose, 3,6-dimethyl-*N*-acetylglucosamine, and 3-methyl-*N*-acetylglucosamine. A linear arrangement of the mannose residues was ruled out by the methylated mannose species derived from the core of B-I after β-galactosidase and β-*N*-acetylglucosaminidase treatment (Table III). If the arrangement had been



where R represents the outer branches, removal of the R's would have given one tetramethylmannose and two new trimethyl derivatives (2,3,4 and/or 2,4,6). If the arrangement had been



one tetramethylmannose, one 3,4,6-trimethyl, and one new trimethyl derivative would have been seen. However, only the arrangement shown in Figure 3



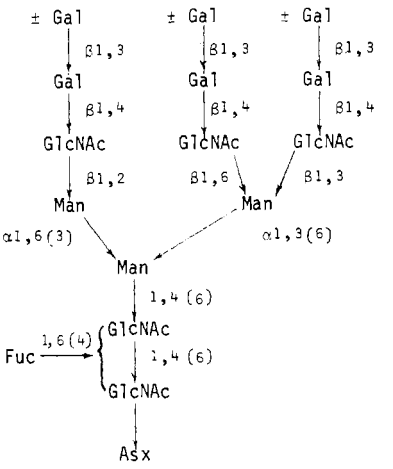


FIGURE 3: Proposed structure of glycopeptide B-I.

TABLE V: Methylated Sugars from Glycopeptide B-III.

	Intact B-III (residues/mole)	Partial digest of B-III[a] (residues/mole)
Fucose 2,3,4-triMe	0.7	0.8
Galactose		
2,3,4,6-tetraMe	2.0	
2,4,6-triMe	2.8	1.6
2,3,4-triMe	0.4	0.4
Mannose		
2,3,4,6-tetraMe		0.6
3,4,6-triMe	1.0	1.0
2,3,4-triMe		0.4
2,4-diMe	2.0	0.6
2-*N*-Methyl-*N*-acetylglucosamine		
3,6-diMe	4.0	2.0
6-Me	0.5	0.9
3-Me	0.3	0.7

[a] Product of β-galactosidase, β-*N*-acetylglucosaminidase, and α-mannosidase digestion of B-III (experiment I, Table VII).

would produce two tetramethylmannose residues and one 2,4-dimethylmannose, which is essentially what was found (1.75 tetra- and 1.0 2,4-dimethylmannose).

Structural Studies on Glycopeptide B-III. The methylated sugars derived from B-III and from glycosidase digested B-III are shown in Table V. In addition to the two sialic acid residues, B-III contains two terminal galactose residues, suggesting the presence of four outer chains. Two of the three mannose residues are doubly substituted at C-3 and C-6, providing two branch points. In agreement with the results of methylation, periodate oxidation of glycopeptide B-III destroyed all the fucose (1 residue), one third of the mannose (1 residue), and 46% of the galactose (2.4 residues) as shown in Table VI. All of the *N*-acetylglucosamine survived the oxidation as expected. Shown in Table VII are the results obtained when B-III was digested with glycosidases. In experiment I, sequential enzyme treatment beginning with β-galactosidase released 2.8 residues of galactose, 2 residues of *N*-acetylglucosamine, and 0.4 residue of α-mannose, suggesting the presence of two outer chains terminating in galactose with the sequence ±Gal(β→)-Gal(β→)GlcNAc(β→) and having an average of about 1.5

TABLE VI: Effects of Periodate Oxidation on Glycopeptides B-III and B-IV.

Glycopeptide	Composition (residues/mole of glycopeptide)				
	Fucose	Galactose	Mannose	*N*-Acetylglucosamine	*N*-Acetylgalactosamine
III-B					
Intact	1.1	5.2	3.0	5.7	0.2
$NaIO_4$	0	2.8	1.9	5.5	0.25
IV-B					
Intact	1.1	5.3	3.0	5.4	0.5
$NaIO_4$	0	3.7	2.1	5.7	0.6

TABLE VII: Glycosidase Digestion of Glycopeptide B-III.

Glycosidase treatment	Residues released/mole of glycopeptide			
	Sialic acid	Galactose	*N*-Acetylglucosamine	Mannose
Intact B-III[a]	2.0	5.2	5.7	3.0
(I) β-Galactosidase (3×);[b]		2.8		
then β-*N*-acetylglucosaminidase (2×);[b]			2.0	
then α-mannosidase (1×)[b]				0.4
(II) Neuraminidase (2×);[b]	1.5			
then β-galactosidase (2×);[b]		4.0		
then β-*N*-acetylglucosaminidase (1×);[b]			2.8	
then α-mannosidase (1×);[b]				1.2
then β-Mannosidase (1×)[b]				0.4

[a] Total residues of each sugar, taken from Table II. [b] Indicates the number of sequential treatments with the same glycosidase. After each treatment, the reaction mixture was boiled and fractionated on Sephadex G-25 or Bio-Gel P-2, and the residual glycopeptide was retreated with the appropriate glycosidase.

galactose residues per chain. The residual glycopeptide from experiment I was methylated. In experiment II, the digestion was begun with neuraminidase, and the difference in the amounts of sugars released in experiments II and I suggests that B-III contains two outer chains terminating in sialic acid with the sequence SA(α→)Gal(β→)GlcNAc. Based on the results obtained from the glycosidase digestions and methylation of intact and partially degraded B-III, the structure shown in Figure 4 is proposed for glycopeptide B-III. Methylation of the residual glycopeptide from experiment I after removal of 3 galactose, 2 *N*-acetylglucosamine, and 0.4 mannose residues from B-III (experiment I, Table VII) gave rise to the following amounts of methylated galactose: 1.6 residues of 2,4,6-trimethyl and 0.4 residue of 2,3,4-trimethyl. Thus, two residues of 2,3,4,6-tetramethylgalactose and approximately one residue of 2,4,6-trimethylgalactose present in methylated intact B-III (see Table V) were no longer present, establishing that in the galactose-terminated outer chains the terminal galactose had been linked to C-3 of an underlying galactose and that in the sialic acid terminated chains 0.4 residue of sialic acid was linked to C-6 of galactose and 1.6 residues of sialic acid were linked to C-3 of underlying galactose. As shown in Table V, a total of 2.6 residues of methylated mannose were found in the methylated residual glycopeptide, consistent with the removal of 0.4 residue of

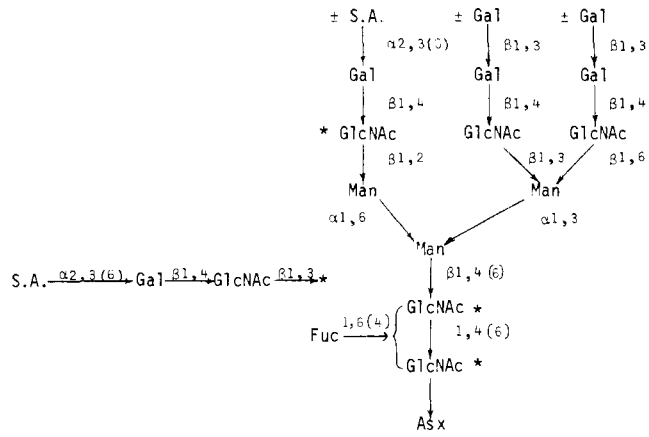


FIGURE 4: Proposed structure of glycopeptide B-III. SA is sialic acid. The side chain may attach to any of the starred (*) GlcNAc residues.

mannose in the glycosidase digestion. Two new species were formed upon removal of the galactose-terminated outer chains; tetramethylmannose (0.6 residue) and 2,3,4-trimethylmannose (0.4 residue). They must have arisen from the 2,4-dimethylmannose in methylated intact B-III and, with the removed mannose, account for the loss of 1.4 residues of 2,4-dimethylmannose. Thus, the galactose-terminated chains must be linked at C-3 and C-6 of one mannose residue, which in turn must be linked to C-3 of the core mannose. This indicates that the sialic acid containing branch must contain the C-2 substituted mannose which is attached to C-6 of the core mannose. The fact that in experiment II (Table VII) α-mannosidase treatment removed 1.2 mannose residues and subsequent β-mannosidase removed 0.4 mannose residue is the basis for deciding that the outer two mannoses are linked α and the core mannose linked β. The methylated *N*-acetylglucosamine derivatives formed by methylation of the experiment I product were present in the following amounts: 2 residues of 3,6-dimethyl, 0.9 residue of 6-methyl, and 0.7 residue of 3-methyl. This result, a loss of 2 residues of 3,6-dimethyl-*N*-acetylglucosamine from methylated intact B-III, shows that the galactose in the galactose-terminated outer chains had been linked to C-4 of *N*-acetylglucosamine. Furthermore, the recovery of almost four residues of methylated *N*-acetylglucosamine also suggests that B-III contains a total of six residues of *N*-acetylglucosamine with two having been removed by β-*N*-acetylglucosaminidase. If the partial residues of 3-methyl- and 6-methyl-*N*-acetylglucosamine seen in the methylation of intact B-III (Table V) really represent poor recovery of full residues, then the total residues of methylated *N*-acetylglucosamine would be six. The SA→Gal→GlcNAc→ side branch which may be attached to either the *N*-acetylglucosamine residue in the other sialic acid terminated chain or the *N*-acetylglucosamine residue in the core which is not substituted by fucose is probably linked at C-3 of the *N*-acetylglucosamine, giving rise to the 6-methyl derivative.

Structural Studies on Glycopeptide B-IV. The results obtained when glycopeptide B-IV was methylated are shown in Table VIII. In addition to 2.9 residues of sialic acid, B-IV contains 1.4 residues of terminal galactose, suggesting the presence of four outer chains plus 0.4 residue of another chain which could also give rise to 0.4 residue of 4,6-dimethyl-*N*-acetylgalactosamine. The two residues of 2,4-dimethylmannose provide two branch points and the 3-methyl- and 6-methyl-*N*-acetylglucosamine residues provide two more branch points. In agreement with the results of methylation, periodate oxidation of glycopeptide B-IV destroyed all of the fucose (1 residue), one third of the mannose (1 residue), and 30% of the

TABLE VIII: Methylated Sugars from Glycopeptide B-IV.

	Intact B-IV (residues/mole)	Partially degraded B-IV[a] (residues/mole)
Fucose 2,3,4-triMe	1.0	1.0
Galactose		
2,3,4,6-tetraMe	1.4	0.5
2,4,6-triMe	3.6	
2,3,4-triMe	0.4	0.3
Mannose		
2,3,4,6-tetraMe		1.6
3,4,6-triMe	1.0	0.4
2,4-diMe	2.0	1.0
2-*N*-Methyl-*N*-acetylglucosamine		
3,6-diMe	4.0	1.4
6-Me	0.8	0.0
3-Me	0.7	0.7
2-*N*-Methyl-*N*-acetylgalactosamine		
4,6-diMe	0.4	0.4

[a] Product of neuraminidase, β-galactosidase, and β-*N*-acetylglucosaminidase digestion of B-IV (experiment III, Table IX).

TABLE IX: Glycosidase Digestion of Glycopeptide B-IV.

Glycosidase treatment	Residues released/mole of glycopeptide: Sialic acid	Galactose	*N*-Acetylglucosamine	Mannose
Intact B-IV[a]	2.9	5.3	5.4	3.0
(I) β-Galactosidase (3×);[b]		1.9		
then β-*N*-acetylglucosaminidase (2×);			1.2	
then α-mannosidase (1×);				0.1
then neuraminidase (1×);	2.6			
then β-galactosidase and β-*N*-acetylglucosaminidase (1×);		3.2	2.9	
then α-mannosidase (1×)				1.7
(II) Neuraminidase (2×);	2.4			
then β-galactosidase (2×);		3.7		
then β-*N*-acetylglucosaminidase (2×);			2.8	
then α-mannosidase (1×);				0.4
then β-mannosidase (1×)				0.2
(III) Neuraminidase, β-galactosidase, and β-*N*-acetylglucosaminidase (2×)	2.6	4.5	3.5	

[a] Total residues of each sugar, taken from Table II. [b] Indicates the number of sequential treatments with the same glycosidase. After each treatment the reaction mixture was boiled, fractionated on Sephadex G-25 or Bio-Gel P-2 and the residual glycopeptide retreated with the appropriate glycosidase.

galactose (1.6 residue) as shown in Table VI. All of the *N*-acetylglucosamine and *N*-acetylgalactosamine survived the oxidation as expected. The results obtained when glycopeptide B-IV was digested with various sequences of glycosidases are shown in Table IX. In experiment I, treatment with β-galactosidase followed by β-*N*-acetylglucosaminidase released approximately two residues of galactose and one residue of *N*-acetylglucosamine, indicating the presence of an outer chain with the sequence Gal(β→)Gal(β→)GlcNAc(β→). Subsequent treatment with α-mannosidase released only trace amounts of mannose. When neuraminidase was added, 2.6 residues of sialic acid were removed, and subsequent treatment with a mixture of β-galactosidase and β-*N*-acetylglucosaminidase removed about 3 galactose and 3 *N*-acetylglucosamine residues. This finding and the results in experiment II where neuraminidase treatment was followed in sequence by first β-galactosidase and then β-*N*-acetylglucosaminidase indicate the presence of three outer chains with the sequence SA-(α→)Gal(β→)GlcNAc(β→). Then, in experiment I, α-mannosidase treatment released 1.7 residues of mannose. In experiment II, where removal of outer-chain sialic acid, galactose, and *N*-acetylglucosamine was less complete than in experiment I, treatment with α-mannosidase removed 0.4 residue of α-linked mannose, and subsequent treatment with β-mannosidase released 0.2 residue of β-linked mannose. In experiment III, glycopeptide B-IV was treated with neuraminidase for 3 days at pH 6.2, the pH of the reaction mixture was adjusted to pH 4.6, and β-galactosidase and β-*N*-acetylglucosaminidase were added for an additional 3 days. Two rounds of combined digestion released almost all of the outer-chain residues, and the product was then methylated. In Figure 5 is the structure proposed for glycopeptide B-IV based on the results obtained from the glycosidase digestions and the methylation of intact and partially degraded B-IV.

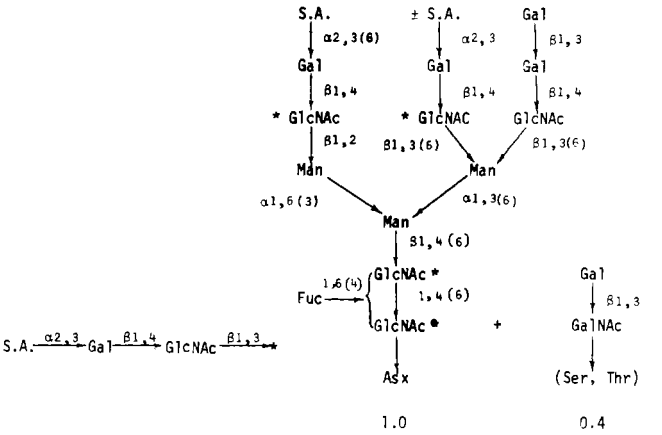
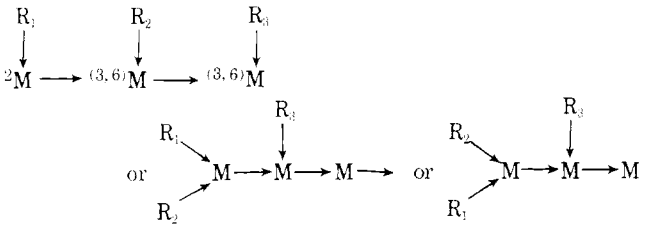


FIGURE 5: Proposed structure of glycopeptide B-IV. SA is sialic acid. The side chain may attach to any of the starred (*) GlcNAc residues.

In addition to the N-linked oligosaccharide chain, about 40% of the B-IV glycopeptide molecules also contain an O-linked disaccharide Gal(β1→3)GalNAc. The presence of this disaccharide was expected in view of the *A. bisporus* lectin receptor activity and *N*-acetylgalactosamine content of B-IV and confirmed by finding 0.4 residue of 4,6-dimethyl-*N*-acetylgalactosamine and an extra 0.4 residue of tetramethylgalactose upon methylation of intact B-IV. The linkage of galactose to *N*-acetylgalactosamine is presumed to be β, since that is the structure of the *A. bisporus* receptor. Jack bean β-galactosidase splits the galactose from Gal(β1→3)GalNAc extremely poorly, which accounts for the disaccharide surviving the glycosidase digestion and its methylated derivatives being present after methylation of the partially degraded B-IV (Table VIII). The N-linked oligosaccharide of B-IV resembles that of B-III, except that it contains one rather than two galactose-terminated outer chains. The methylated mannose species found after methylation of the partially degraded B-IV from experiment III were 1.6 residues of 2,3,4,6-tetramethyl-,

0.4 residue of 3,4,6-trimethyl-, and 1.0 residue of 2,4-dimethylmannose. Therefore, the residual undigested outer chain containing SA(0.3 residue)(2→6)Gal(0.4 residue)(1→4)-GlcNAc(0.4 residue)→ must be attached to C-2 of mannose and accounts for the 0.3 residue of 2,3,4-trimethylgalactose and 0.4 residue of 3,6-dimethyl-*N*-acetylglucosamine observed. The galactose-terminated outer chain and another sialic acid terminated chain must have been attached to C-3 and C-6 of mannose in intaet B-IV before their removal, thus accounting for the conversion of one 2,4-dimethylmannose derived from methylated intact V-IV to terminal tetramethylmannose in methylated degraded B-IV. The third SA→Gal→GlcNAc chain may be attached to C-3 of any of the starred (*) *N*-acetylglucosamine residues, and its removal by glycosidase digestion accounts for the loss of the 6-methyl-*N*-acetylglucosamine seen in methylated intact B-IV. The arrangement of the three mannose residues shown in Figure 5 is the only one compatible with the methylation pattern obtained on the partially degraded B-IV. A linear arrangement of the mannoses, no matter which of the outer chains is represented by R_1, R_2, and R_3, would have produced a different set of methylated mannose derivatives in the product from experiment III than that obtained.



In every case the methylation products would have been characterized by a significant proportion of a new species of mannose (2,4,6- or 2,3,4-trimethylmannose) derived from the 2,4-dimethylmannose in the original, and a maximum of 1.0 residue of 2,3,4,6-tetramethylmannose, in contrast to what was found (almost two tetramethylmannoses, one 2,4-dimethylmannose, and *no* 2,3,4- or 2,4,6-trimethylmannose).

Discussion

The structures of the oligosaccharide chains of glycopeptides B-I, B-III, and B-IV derived from calf thymocyte membrane glycoproteins contain some features common to other membrane glycoprotein oligosaccharides and serum glycoprotein oligosaccharides as well as some unique features. The nonreducing terminal sequence Gal(β1→3)Gal(β1→4)GlcNAc has apparently not been previously detected in other glycoproteins. However, preliminary structural data (deWaard et al., 1976) on the large O-linked oligosaccharides of the calf erythrocyte membrane glycoprotein indicate the presence of an oligosaccharide with the nonreducing terminal sequence SA(α2→3)-Gal(β1→3)Gal(β1→4)GlcNAc→. It is possible that the Gal(β1→3)Gal(β1→4)GlcNAc→ sequence is unique to bovine glycoproteins, but since the oligosaccharide structures of relatively few membrane glycoproteins of other species have been determined such a conclusion would be premature.

The nonreducing terminal sequence SA(α→)Gal(β→)-GlcNAc→ present in glycopeptides B-III and B-IV is commonly found in glycopeptides of both soluble and membrane glycoproteins. The linkage of the terminal sialic acid residue to C-3 of the underlying galactose residue also occurs in the bovine erythrocyte membrane oligosaccharides (De Waard et al., 1976), in fetuin (Spiro, 1964, 1973), a glycoprotein found in fetal calf serum, and in Chinese hamster ovary cell membrane oligosaccharides.[2] In contrast, the terminal sialic acid in human immunoglobulin glycopeptides (Baenziger and Kornfeld, 1974), transferrin glycopeptide (Jamieson et al., 1971), and the N-linked oligosaccharide of human erythrocyte membrane sialoglycoprotein (Kornfeld and Kornfeld, 1971) is linked to C-6 of galactose. The branching arrangement of the mannose residues in these glycopeptides occurs in other glycoproteins—especially soluble glycoproteins—but differs from that in the glycopeptide isolated from human erythrocyte membranes and in the glycopeptide I isolated by Kawasaki and Ashwell (1976) from rabbit liver membrane, both of which contain only two mannose residues. Another feature of the thymocyte membrane glycopeptides is the linkage of two outer chains to C-3 and C-6 of an outer mannose residue. Although other glycopeptides have been shown to contain two outer chains arising from an outer mannose residue, in those cases where the linkages are known they are to C-2 and C-4 of mannose (Schwarzman et al., 1974; Bayard and Montreuil, 1974; Wolfe et al., 1974; Ng Ying Kin and Wolfe, 1974). The SA→Gal→GlcNAc side chains that are linked to C-3 of *N*-acetylglucosamine residues in glycopeptides B-III and B-IV are similar to the Gal→GlcNAc→ side chain linked to C-3 of an *N*-acetylglucosamine residue in the glycopeptide of desialyzed α-1 acid glycoprotein (Schwarzman et al., 1974). It is of interest that all of the six *N*-acetylglucosamine residues in both B-III and B-IV are different from one another either because they are linked to different underlying sugars or to a different carbon atom of the same underlying sugar. It probably required six different *N*-acetylglucosaminyltransferase enzymes to attach these residues in the synthesis of the oligosaccharide.

The short O-linked Gal(β1→3)GalNAc→ chains found in glycopeptide B-IV occur very frequently in other membrane and soluble glycoproteins. Thomas and Winzler (1969) found that structure as well as its mono- and disialyl forms in human erythrocyte membrane glycoprotein, and Newman et al. (1976) have recently shown that the same disaccharide is present on desialyzed pig lymphocyte plasma membranes. The "antifreeze glycoprotein" of Antartic fish (Shier et al., 1975), human IgA_1 (Baenziger and Kornfeld, 1974), the β subunit of human chorionic gonadotropin (Bahl et al., 1972), cartilage keratin sulfate (Kieras, 1974), and epiglycanin of TA-3 cells (Codington et al., 1975) all contain this disaccharide linked to either serine or threonine in the peptide, and fetuin contains the tetrasaccharide in which sialic acid residues are attached to C-3 of galactose and C-6 of *N*-acetylgalactosamine (Spiro and Bhoyroo, 1974).

The finding that the oligosaccharide chains of glycopeptides B-I, B-III, and B-IV contain almost one-half of the total glycoprotein oligosaccharide material present in thymocyte plasma membranes supports the original supposition that oligosaccharide chains of the same structure occur on a number of different glycoproteins in these membranes.

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[2] Personal communication from Ellen Li.

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