

Isolation of a Human Milk Sialyloligosaccharide by Affinity Chromatography with Wheat Germ

Agglutinin (WGA)

by

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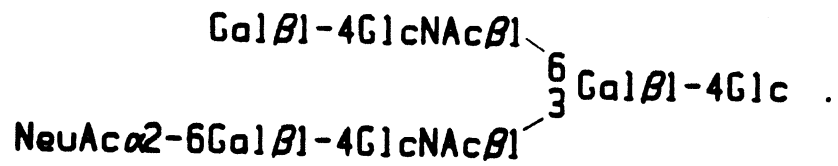
David F. Smith

Biochemistry

(ABSTRACT)

Lectin affinity chromatography has been applied to the separation of the sialyloligosaccharides of human milk. A human milk sialyloligosaccharide fraction was tritium labeled and applied to a highly substituted WGA-agarose column (20 mg/ml). Only a single component from the complete sialyloligosaccharide fraction was retarded in the WGA-agarose column. The WGA-bound fraction when applied to paper chromatography migrated with identical mobility as the sialylhexasaccharide fraction (S-5) of human milk, previously isolated and described by Kobata and Ginsburg in 1972 [Arch. Biochem. Biophys., 150:273-281]. A purified sialylhexasaccharide fraction (S-5), isolated according to the method of Kobata and Ginsburg, was radiolabeled and applied to the WGA-agarose column. The WGA-bound (60%) and WGA-unbound (40%) sialylhexasaccharide fractions were isolated. The WGA-bound sialylhexasaccharide fraction was subjected to neuraminidase digestion to remove sialic acid, and the resulting neutral oligosaccharide had more affinity for the WGA-agarose column. Sequential exoglycosidase digestion of the asialo derivative of the WGA-bound fraction with jack bean β -galactosidase and β -hexosaminidase demonstrated the presence of a lacto-N-neohexaose core. The position of sialic acid in the sialyllacto-N-neohexaose was determined by simultaneous digestion of the sialylhexaose with jack bean β -galactosidase and β -hexosaminidase, which removed the non-sialylated branch from the sialylhexaose and produced a sialyltetraose. The sialyltetraose was found to be sialyltetrasaccharide c as demonstrated by its elution time on HPLC and direct binding to monospecific anti-sialyltetrasaccharide c serum. The structural data indicated that the WGA-bound sialylhexaose is a sialyl derivative of lacto-N-neohexaose with sialic acid linked to the 3 branch of

this core structure which represents a previously undescribed sialyloligosaccharide in human milk.
The structure of the WGA-bound sialylhexaose is,



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List of Abbreviations

Gal	Galactose
Glc	Glucose
GlcNAc	N-Acetylglucosamine
Fuc	Fucose
NeuAc	N-Acetylneuraminic acid or sialic acid
MurNAc	N-Acetylmuramic acid
WGA	Wheat Germ Agglutinin
AcOH	Acetic acid
PBS	Phosphate-buffered saline
DEAE-cellulose	diethylaminoethyl-cellulose
HPLC	High Performance Liquid Chromatography
K_d	Distribution coefficient
U	Units of enzyme activity, μ mol/min

1.0 Literature Review

1.1 Introduction

Cell surface glycoconjugates are involved in many important biological functions such as cell growth and control [1,2], receptors of bacterial toxins and glycoprotein hormones, blood group antigens, cancer associated antigens, and determinants in the process of cell to cell recognition [2,3,4]. Most of the oligosaccharides found in human milk also occur in cell surface glycoproteins and glycolipids. This fact enables human milk oligosaccharides to be used as models in the study of the structure and functions of cell surface glycoconjugates.

Human milk oligosaccharides were first used in 1956 by Kuhn and Osman [5], when they utilized fucose containing oligosaccharides as haptens in inhibition studies to determine the structure of blood group antigens. Human milk oligosaccharides have also been used in the the determination of acceptor specificities of glycosyltransferase as well as substrate specificities of glycosidases [6,7]. In addition, human milk oligosaccharides have been used in the elucidation of carbohydrate specificity of carbohydrate binding antibodies and lectins. The oligosaccharides of human milk are found as sialyl and/or fucosyl derivatives of isomeric core structures (see table I). The core structures are only composed of one D-glucose residue always located at the reducing end,

and of D-galactose and D-N-acetylglucosamine residues in different proportions depending on the size of the oligosaccharide. The core structures that are larger than hexaoses seldomly occur by themselves, but with fucose and sialic acid residues attached to them in different linkages. For this reason, an enormous number of isomers of these sialylated and fucosylated core structures can occur. As a matter of fact, Egge and associates [8] have detected more than one hundred fucosyl derivatives of these core structures. These mixtures of isomeric human milk oligosaccharides, however, are very difficult to separate into pure components by conventional techniques.

A technique that has been very useful in the separation of complex mixtures of asparagin linked and mucin-derived oligosaccharides is lectin affinity chromatography [9,10,11,12]. This method has the advantage that it not only separates the oligosaccharides based on the elementary principles of liquid chromatography but also separates the sugars depending on stereospecific interactions between the carbohydrates and the immobilized lectin. Lectin affinity chromatography should therefore be applicable to the resolution of the complex mixture of human milk oligosaccharides. The work presented here, describes the isolation of a single component from the sialyloligosaccharide fraction of human milk by affinity chromatography on immobilized Wheat Germ Agglutinin (WGA).

1.2 Human milk oligosaccharides

Human milk is a rich source of oligosaccharides. In addition to lactose, it contains larger oligosaccharide structures [13]. In contrast, the oligosaccharide content of other mammal's milk is mainly limited to lactose, and more complex structures are scarcely found [13]. A liter of human milk contains about 70 grs of lactose and 3 to 4 grs of a mixture of complex oligosaccharides [13]. The first studies on the isolation and characterization of human milk oligosaccharides were carried out by Kuhn and coworkers in 1952 [13]. They determined the structures of fucosyllactose (1955), difucosyllactose (1958), lacto-N-tetraose (1956), lacto-N-neotetraose (1962), lacto-N-fucopentaose

I (1960), lacto-N-fucopentaose II (1960) and lacto-N-difucohexaose I (1962) [5,13] (see tables I and II). The use of these structures became important in 1957 [5], as they were used as haptens in inhibition tests to elucidate the structures of the *H*, *Lewis^a*, and *Lewis^b* blood group antigens [5,14]. Since then, oligosaccharides of human milk have served as models in other related studies including, the determination of acceptor specificities of glycosyltransferases, substrate specificities of glycosidases [6,7], and binding specificities of carbohydrate binding proteins or lectins [15].

As the interest in human milk oligosaccharides increased, other researchers such as Ginsburg and associates, isolated and characterized a number of novel structures [6,17, 18,19,20,21] in human milk. Results of these studies showed that human milk oligosaccharides are sialyl and/or fucosyl derivatives of isomeric core structures. Some of these core structures are represented in table I. The two basic structures besides lactose are, lacto-N-tetraose and lacto-N-neotetraose, which are synthesized by adding $Gal\beta 1 - 4(3)GlcNAc$ to lactose. The larger core structures are built by means of adding different numbers of $Gal\beta 1 - 4(3)GlcNAc$ residues (in a branched or linear array) to lacto-N-tetraose and lacto-N-neotetraose. As a result, the number of sugar units in these basic core structures is always even; e.g.: 2,4,6,8,... In addition, the monosaccharide composition is always Glc: Gal: GlcNAc in the proportion, 1 : n : n-1, where n equals the number of Gal residues and can take values n = 1,2,3,... For example, in lactose n = 1 (one Gal residue), 1 : n : n-1 = 1 : 1 : 1-1 = 1 : 1 : 0, or Glc : Gal = 1 : 1 which is the proportion of Gal and Glc in lactose. Note that only one glucose residue is found in these core structures which is invariably located at the reducing end of the oligosaccharide chain. Fucose can be attached to these core structures by the action of genetically controlled fucosyltransferases [8,14] that will add fucose in the following different linkages: 1) $Fuc\alpha 1 - 2$ to terminal Gal; 2) $Fuc\alpha 1 - 3$ to GlcNAc or Glc; 3) $Fuc\alpha 1 - 4$ to GlcNAc. Oligosaccharides containing these type of fucose bonds are represented in table II. In addition to fucose, sialic acid may be found linked to the core structures or to the fucosylated derivatives of them. Addition of sialic acid is a result of the action of specific sialyltransferases [22]. Sialic acid occurs in human milk oligosaccharides attached in three different linkages: 1) $NeuAc\alpha 2 - 3$ to terminal $Gal\beta 1 - 3GlcNAc$; 2) $NeuAc\alpha 2 - 6$ to terminal

$Gal\beta 1 - 4GlcNAc$; and 3) $NeuAc\ \alpha 2 - 6$ to internal GlcNAc residues. Some of these structures are represented in table II.

Some human milk oligosaccharides display H and Lewis blood group antigenic activity. The determinant for the H antigen is a $Fuc\ \alpha\ 1-2\ Gal\dots$, which is added by an $\alpha 1 - 2fucosyltransferase$ encoded by the H gene. The expression of the H gene product in non hematopoietic tissue depends on the presence of the secretor gene (Se), consequently only people that express the Se gene can show H specificity in secretory organs, and their secretions contain glycoproteins that exhibit H antigenicity. The $Lewis^a$ antigen consists of a $Fuca1 - 4GlcNAc$ which is synthesized by an $\alpha 1 - 4fucosyltransferase$ encoded by the Lewis gene (Le). The $Lewis^b$ antigen contains both the $Fuca1 - 2Gal$ and the $Fuca1 - 4GlcNAc$ formed by the action of the H gene product and the Lewis gene product. The expression of the $Lewis^b$ determinant in secretions requires the expression of the Se gene. Since human milk is a secretion, these specific fucosyltransferases are expressed in human milk depending on the Lewis blood type and secretor status of the donor [14,23,24,25]. For this reason, some human milk oligosaccharides present blood group antigenic activity. For example, 2-fucosyllactose and lacto-N-fucopentaose I contain H antigenic activity ($Fuca1 - 2Gal$) (see table II), and they are only expressed in milk of 'secretors' that have the H gene. The secretor status is required since the secretor gene (Se) controls the expression of H gene product, $\alpha 1 - 2fucosyltransferase$, in non hematopoietic tissue [14]. Another human milk oligosaccharide that manifests antigenic activity is lacto-N-fucopentaose II that contains the $Lewis^a$ immunodeterminant which corresponds to a Fuc linked $\alpha 1 - 4$ to an internal GlcNAc (see table II). The specific $\alpha 1 - 4fucosyltransferase$ encoded by the Lewis gene (Le) is only found in the milk of donors that express the Le gene. Another example is lacto-N-difucohexaose I which exhibits $Lewis^b$ antigenic activity since it contains a $Fuca1 - 2Gal$ linkage and also a $Fuca1 - 4GlcNAc$ linkage (see table II).

By reasons not well understood, oligosaccharides that are found in human milk are also expressed as cell surface glycoproteins or glycolipids. Moreover, some of them have been found to be tumor associated antigens such as the $sialyl - Lewis^a$ antigen found as a ganglioside in colorectal carcinoma cells [26,27,28], $NeuAc\ \alpha 2 - 3Gal\beta 1 - 3[Fuca1 - 4]GlcNAc\beta 1 - 3Gal\beta 1 - 4Glc$

Table 2. SOME HUMAN MILK OLIGOSACCHARIDES.

Fucosyloligosaccharides	
Trivial Name	Structure
2'Fucosyllactose	$Fuc \alpha 1 - 2Gal \beta 1 - 4Glc$
3'Fucosyllactose	$Gal \beta 1 - 4(Fuc \alpha 1 - 3)Glc$
Difucosyllactose	$Fuc \alpha 1 - 2Gal \beta 1 - 4(Fuc \alpha 1 - 3)Glc$
Lacto-N-Fucopentaose I	$Fuc \alpha 1 - 2Gal \beta 1 - 3GlcNAc \beta 1 - 3Gal \beta 1 - 4Glc$
Lacto-N-Fucopentaose II	$Gal \beta 1 - 3(Fuc \alpha 1 - 4)GlcNAc \beta 1 - 3Gal \beta 1 - 4Glc$
Lacto-N-Fucopentaose III	$Gal \beta 1 - 4(Fuc \alpha 1 - 3)GlcNAc \beta 1 - 3Gal \beta 1 - 4Glc$
Lacto-N-Difucohexaose I	$Fuc \alpha 1 - 2Gal \beta 1 - 4(Fuc \alpha 1 - 4)GlcNAc \beta 1 - 3Gal \beta 1 - 4Glc$

Sialyloligosaccharides	
Trivial Name	Structure
3'Sialyllactose	$NeuAc \alpha 2 - 3Gal \beta 1 - 4Glc$
6'Sialyllactose	$NeuAc \alpha 2 - 6Gal \beta 1 - 4Glc$
Sialyltetrasaccharide a	$NeuAc \alpha 2 - 3Gal \beta 1 - 3GlcNAc \beta 1 - 3Gal \beta 1 - 4Glc$
Sialyltetrasaccharide b	$Gal \beta 1 - 3(NeuAc \alpha 2 - 6)GlcNAc \beta 1 - 3Gal \beta 1 - 4Glc$
Sialyltetrasaccharide c	$NeuAc \alpha 2 - 6Gal \beta 1 - 4GlcNAc \beta 1 - 3Gal \beta 1 - 4Glc$

All monosaccharides are in the D configuration except fucose which is L

Ganglioside GM3 is composed of 3'-sialyllactose linked to a ceramide residue [29]. Sialyltetrasaccharides a and b attached to ceramide are also found as gangliosides. They have been detected in human meconium and also in a ganglioside fraction obtained from colorectal carcinoma cells [30,31,32].

1.3 Lectins

Lectins are carbohydrate binding proteins of non-immune origin that are widely distributed in nature. They occur, principally in the seeds of plants but can also be found in the roots, leaves and bark. Lectins also occur in vertebrate and invertebrate animals, bacteria, fungi, and seaweeds [33,34,35].

Lectins have the ability to agglutinate cells, to precipitate polysaccharides, or glycoconjugates and some induce mitogenesis in human lymphocytes [33,34,35,36,37]. Because certain lectins exhibit specific agglutination reactions with human blood groups (A,B,O), they have been employed as blood typing reagents. For example, *Lotus tetragonolobus* and *Ulex europaeus* lectins are specific for type O (H) blood; *Helix pomatia* and *Vicia cracca* lectins for type A blood; and *Griffonia simplicifolia* lectin is specific for type B [34,35].

The agglutination of cells by lectins is specifically inhibited by the presence of free sugars which indicates that lectins bind the carbohydrate moiety of cell surface glycoconjugates. The carbohydrate specificity of lectins has been determined by this principle, using the Landsteiner hapten-inhibition technique [5,35] in which several sugars are tested for inhibition of agglutination or precipitation reaction between the lectin and a reactive polysaccharide [35,37]. The specificity of the lectin is then established by the best sugar inhibitor [35,37]. Using this technique, lectins have been classified into a limited number of specificity groups, for example, the group of lectins specific for galactose, the group specific for fucose, the group specific for N-acetylglucosamine, and so on. Since specificities are restricted to the best monosaccharide inhibitor, specificities of these lectins for

more complex structures are not determined unless the oligosaccharides are available. One approach to determine these more complex specificities, is to apply mixtures of oligosaccharides to an affinity column containing the immobilized lectin, isolate the group of oligosaccharides specifically bound by the lectin and determine their structure(s).

The biological functions of lectins in nature are yet undetermined. The fact that lectins can bind certain carbohydrates indicates that the physiological role of lectins is related to sugar transport, storage, or immobilization [35]. Some theories propose that plant lectins may act as protective agents against pathogen infection, as well as mediate binding of nitrogen-fixing bacteria to the roots of leguminous plants [34,35]. In animals, lectins may function in the clearance of glycoproteins from the circulatory system and also in the intracellular transport and targeting of glycoproteins [34,35,38].

As a result of their unique properties, lectins have been very useful tools in many areas of biological and biochemical research. Some important applications of lectins include, isolation of glycoconjugates and polysaccharides, identification of cell surface receptors, study of cell surface architecture, detection of changes in cell surface due to differentiation, development or malignancy, selection of lectin resistant cell mutants, cell fractionation, and blood typing [34,37].

1.3.1 Wheat Germ Agglutinin (WGA)

The finding by Aub in 1963 [39] that wheat germ lipase was able to agglutinate tumor cells, led to the investigation of the wheat germ agglutinin principle. Wheat Germ Agglutinin (WGA) was first isolated by Nagata and Burger [40], and Le Vine et al [41], in 1972. Other investigators isolated the lectin either by conventional methods [42] or by affinity chromatography [43,44,45,46]. Initial studies on this lectin, indicated that WGA is not a blood group specific lectin since it agglutinates all types of red blood cells. Structural analysis demonstrated that WGA is not a glycoprotein because it does not contain covalently bound carbohydrate. It exists as four different isolectins, I, IIa, IIb, III [47]. Isoelectric focusing [47] of isolectins I, IIa, and III gives a sharp band

at pH, 8.7 while isoelectric focusing of isolectin IIb gives a sharp band at pH, 7.7. The amino acid compositions of the four lectin species are identical, except for isolectin I that does not contain histidine. The other three isolectins contain four histidine residues per subunit. Glycine is the most abundant amino acid in all four isolectins (25%) and every five amino acids is a half-cysteine, and no free sulfhydryl groups are found [47]. WGA (all four isolectins) is composed of two identical subunits of molecular weight 18,000. The crystallographic analysis of WGA [48,49] shows that each subunit is formed by four spatially distinct but structurally homologous domains, A, B, C, and D. Domains A, B, and C contain 43 aminoacids and domain D only contains 42 (171 aminoacids per subunit). The aminoacid chain in each domain is folded in an irregular manner, no secondary structure is observed (alpha-helices or beta-pleated sheets). Each subunit however, maintains a stable and compact structure by the presence of four interlocking disulfide bridges [50].

The carbohydrate specificity of WGA has been extensively studied [35]. Inhibition studies show that among simple sugars tested, only GlcNAc interacts with the lectin [42,51] and N-acetylgalactosamine binds weakly. The α and β glycosides of GlcNAc are bound equally well [42]. Glucosamine does not interact at all, indicating that the charged amino group in this sugar abolishes the binding to WGA. A methyl substituent at the hydroxyl group of C-4 or C-6 in GlcNAc does not prohibit binding but a substituted C-3 hydroxyl in GlcNAc blocks the interaction [42]. These findings suggested that the acetamido group at C-2 and the hydroxyl group at C-3 in GlcNAc, were necessary for binding to WGA. Allen and associates found that only oligosaccharides containing GlcNAc residues are able to bind WGA [42,51]. The best inhibitors were the N-acetylated chitin oligosaccharides. The order of inhibition was the following: chitotetraose > chitotriose >> chitobiose >> GlcNAc [42]. These findings suggested that the WGA combining site is complementary to a sequence of three or more β 1-4 linked GlcNAc residues. Also, a disaccharide containing GlcNAc linked β 1-4 to N-acetylmuramic acid (MurNAc), obtained from bacterial cell wall, was less inhibitory than chitobiose whereas the tetrasaccharide [*GlcNAc* β 1 - 4*MurNAc*]₂ was as good inhibitor as chitotriose [42]. These findings led to Allen and colleagues to propose a model in which the lectin binding site is composed of four adjacent subsites, a,b,c and, d, similar to the hen's ovalbumin lysozyme system [42] (note that subsites a,b,c,d are

different from domains A,B,C,D). In the model, subsites a,b and c will bind the GlcNAc residues while d will accommodate the aglycon of the glycoside. Subsite c will only bind GlcNAc and the C-3 hydroxyl group requires to be unsubstituted (see fig. 1).

Since the oligosaccharide [*GlcNAc*β1 – 4*MurNAc*]₂, was tightly bound by WGA, Allen et al [42] suggested that subsite b can accommodate other sugar residue that can be substituted at the C-3 hydroxyl group (*MurNAc* has a lactyl group at C-3). Keratan, a polysaccharide isolated from nasal cartilage, that contains alternate GlcNAc residues linked β1 – 4 was able to bind WGA [52]. Similarly, a polysaccharide from S14 capsular pneumococcus virus that contains GlcNAc residues substituted at C-4 and C-6 hydroxyl groups, was capable of binding WGA [53]. Recently, it was found that polylectosamine type glycans are able to interact with WGA [54].

The fact that neuraminidase treatment of cells decreased or completely suppressed binding to WGA, suggested that sialic acid could be implicated in binding to the lectin [55]. Inhibition studies related to binding of sialic acid to WGA have been carried out [56,57]. These studies showed that N-acetylneuraminic acid (sialic acid), the beta and alpha methyl ketosides of sialic acid, sialyllactose and the methyl ester of sialic acid could bind WGA but weaker than GlcNAc. Ovine submaxillary mucin, a glycoprotein that does not contain GlcNAc residues but contains sialic acid, could interact with WGA. However, N-glycolylneuraminic acid could not bind the lectin, indicating that an acetamido group is required for binding [56]. Vesicles containing embedded gangliosides were also able to react with WGA [57,58].

The succinylated derivative of WGA no longer interacts with sialic acid [59]. Since the succinylated lectin is negatively charged (pK, 4.0), the binding of WGA and sialic acid might be due to charge interactions between the positively charged lectin (pK, 8.7) and the negatively charged sugar. However, the methyl ester of sialic acid which is not charged, binds to the lectin [56], indicating that binding is not due only to electrostatic interactions. It has been proposed that sialic acid binding to WGA is determined by the similarity in configuration at C-2 (acetamido group) and C-3 (hydroxyl group) of GlcNAc and sialic acid (see fig. 2). Crystallographic analysis of WGA complexes containing chitobiose and sialic acid [48,49] showed that WGA binds two chitobiose molecules per subunit whereas it only binds one molecule of sialic acid per subunit. It also showed

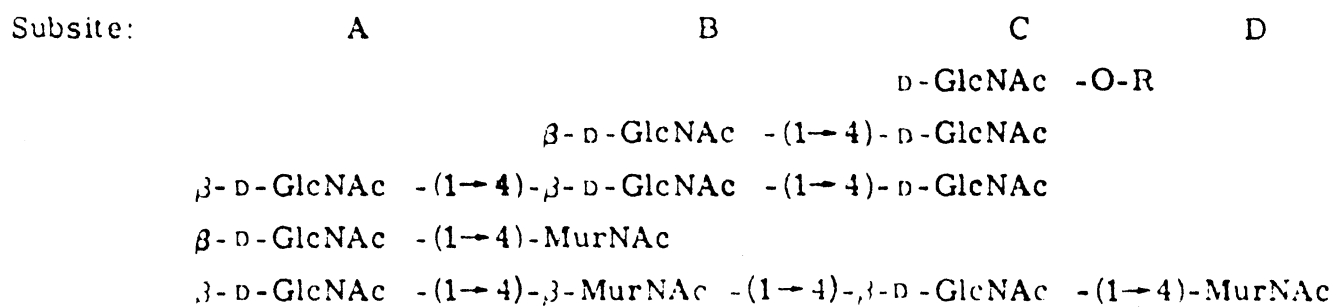


Figure 1. System of Subsites Proposed for the Binding Site of WGA.

that sialic acid and the non reducing GlcNAc in chitobiose bind to the same subsite in the primary binding site but that the orientation of both sugars with respect to the binding site is different. Kronis and Carver [60] have demonstrated by NMR analysis that in solution WGA can bind two molecules of 3'-sialyllactose per subunit, indicating the presence of two sialic acid binding sites/subunit, equivalent to the GlcNAc binding sites. According to Kronis and Carver, in solution WGA has equal number of binding sites for GlcNAc and sialic acid. Therefore difference in the binding of oligosaccharides containing GlcNAc and sialic acid will depend on the orientation of the rest of the molecule in the binding space and not in the number of binding sites on WGA.

In conclusion, WGA is able to interact with GlcNAc and sialic acid. However the affinity for GlcNAc is greater. WGA interacts better with oligosaccharides containing three or more GlcNAc residues linked β 1-4 like in the chitin oligomers. But oligosaccharides containing GlcNAc substituted at hydroxyl groups C-4 and/or C-6 by other glycosyl residues are also able to bind WGA. Sialic acid interacts with WGA in a similar pattern as GlcNAc does; yet, the interaction is weaker as compared with the chitin oligomers.

1.4 Statement of the problem

Since human milk oligosaccharides represent a great number of isomeric structures that are difficult to separate by classical methods, lectin affinity chromatography, a technique that has been very useful in the resolution of complex mixtures of asparagine linked oligosaccharides [9,10], was chosen as an alternative method for their separation. This thesis focuses on the application of this technique to the separation of a human milk sialyloligosaccharide fraction since many of these structures occur as oligosaccharides in human cell surface ganglioside antigens. Because of its specificity for GlcNAc and sialic acid, WGA was selected for these experiments. WGA-affinity chromatography of sialyloligosaccharides, will provide a useful method for the isolation of specific

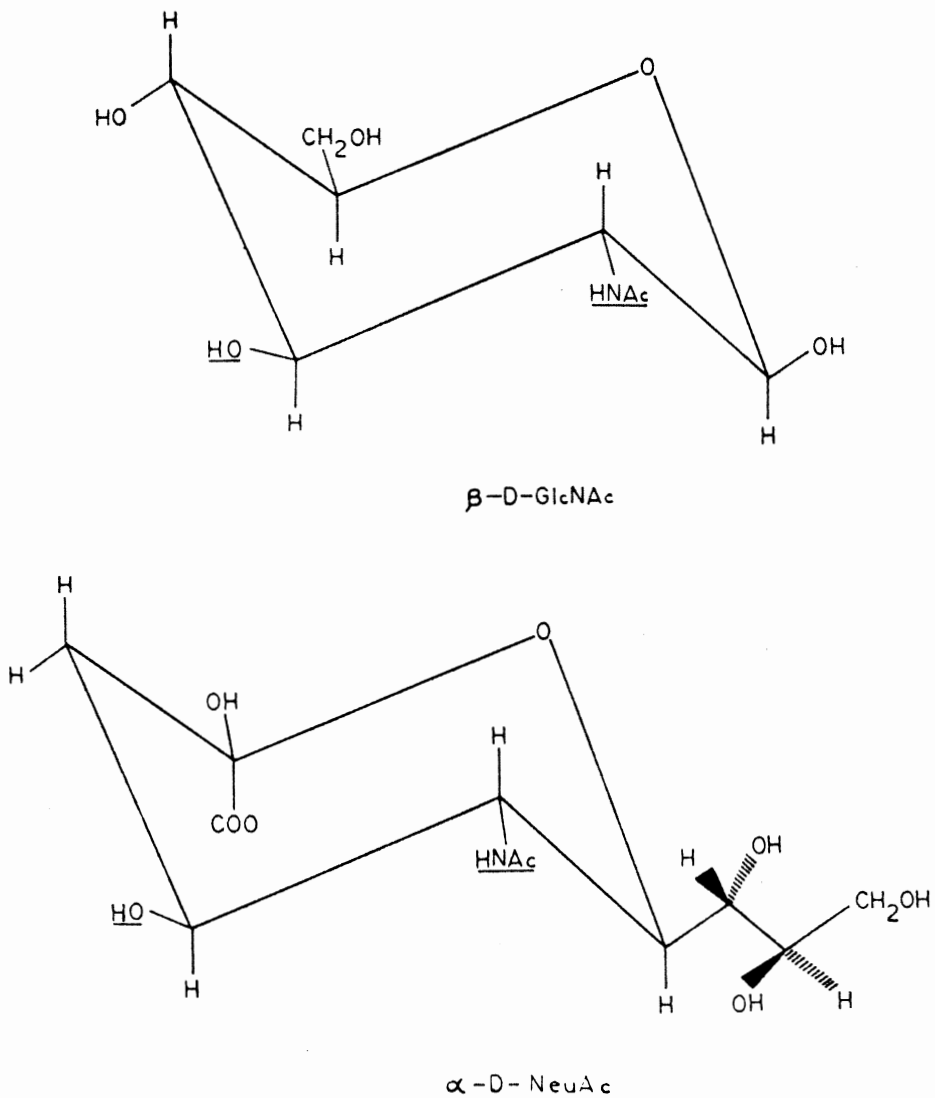


Figure 2. Similarity in Configuration of N-Acetylglucosamine (GlcNAc) and Sialic Acid.

oligosaccharides that bind WGA. Structural analysis of the specifically bound oligosaccharides will be helpful in defining detailed carbohydrate specificity of this lectin.

2.0 Materials and Methods

2.1 Materials

Raw wheat Germ was purchased from Annie's Kay Whole Foods Store (Blacksburg, VA). Purified wheat Germ Agglutinin lectin (WGA), used as standard, was from EY Laboratories (San Mateo, California). Human milk oligosaccharides were isolated as described [16]. Sialyl human milk oligosaccharides were separated by the method of Smith et al [17]. A sialylhexasaccharide fraction (S5) of human milk was isolated according to the method of Kobata & Ginsburg [21]. Sodium Borotritide, $NaB[{}^3H]_4$ (sp.act. 10-20 Ci/mmol) was purchased from Amersham (Arlington Heights, Ill). Affi-Gel 10, Bio-Rad Protein Reagent, Bio-Gel P6, and AG-50W-X8 ion exchange resin (hydrogen form) were from Bio-Rad (Richmond, CA). DEAE-cellulose (DE53) and Whatman No 1 paper were from Whatman (Clifton, NJ). *Clostridium perfringens* neuraminidase and GlcNAc-sepharose was from Sigma (St. Louis, MO). Nitrocellulose filters (No BH-85, 25 mm diameter) were from Schleicher & Scheull (Keene, NH). Jack Bean β -galactosidase, and β -N-acetylhexosaminidase, available in this laboratory, were isolated as described [61]. Antisera specific for sialyltetrasaccharide c was obtained as described by the method of Smith et al [62].

2.2 *Methods*

2.2.1 Analytical Methods

Protein was measured using Bio-Rad Protein Reagent which is based on Comassie brilliant blue method [63]; WGA from EY Laboratories was used as standard. Radioactivity as tritium, 3H , was quantified by liquid scintillation spectroscopy: aliquots of the samples were placed in plastic minivials (Denville Scientific, Denville NJ), 2-4 mls of Ready- Solve EP cocktail (Beckman, Fullerton, CA) were added, and measurements were done in a Beckman LS-7500 scintillation counter (Beckman, Fullerton, CA).

Agglutination activity was measured by testing the agglutination of a 1.5% solution (v/v) of packed guinea pig erythrocytes in PBS or Phosphate-buffered saline (0.14 M NaCl , $2.7 \times 10^{-3}\text{ KCl}$, $1.5 \times 10^{-3}\text{ M KH}_2\text{PO}_4$, and $8.1 \times 10^{-3}\text{ M Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$) with different concentrations of WGA in PBS. Briefly, ten two-fold dilutions of WGA/PBS were prepared, 0.50000 mg/ml, 0.25000 mg/ml, 0.12500 mg/ml, 0.06250 mg/ml, 0.03125 mg/ml, 0.01563 mg/ml, 0.00782 mg/ml, 0.00391 mg/ml, 0.00196 mg/ml, and 0.00098 mg/ml. Fresh guinea pig blood, collected in heparin, 10 mg/ml, was washed four times with PBS and then packed by centrifugation at 4000 rpm for 5 minutes. A 1.5 % solution, vol./vol., of packed red blood cells in PBS was made. An aliquot containing 10 μl of each dilution of WGA, was placed in the wells (20 μl vol.) of a tissue culture plate and then 10 μl of the 1.5 % solution of packed guinea pig erythrocytes was added to each well containing the lectin dilution. PBS, 10 μl , plus 1.5 % solution of packed guinea pig erythrocytes 10 μl , was used as control. Agglutination was observed after 45-60 minutes at room temperature.

2.2.2 Isolation of Wheat Germ Agglutinin

Wheat Germ Agglutinin was isolated from raw wheat germ according to the method of Allen et al [42] with some modifications. Briefly, one kilogram of wheat germ was defatted with light petroleum ether and air dried. Defatted wheat germ was stirred with 3 l of water over 18 hours in the cold room, and then centrifuged at 600 g for 15 minutes. The supernatant was kept and $(NH_4)_2SO_4$ was added to 55% saturation. The suspension was allowed to settle overnight in the cold room and then collected by centrifugation at 9000 g for 20 min. The precipitate was dissolved in water (approximately 1l) and dialyzed against 6 changes of distilled water (4l). The heavy precipitate formed during dialysis was removed by centrifugation at 9000g for 20 min. The resulting supernatant was adjusted to pH 8.6 with 1 M Tris and applied to a DEAE-cellulose column (30 X 3.5 cm), previously equilibrated with 0.05 M Tris, pH 8.6. The column was washed with approximately 1.5 l of the same buffer and the eluate which contained WGA as determined by agglutination of guinea pig erythrocytes, was collected. The eluate is then applied to a column made of GlcNAc covalently linked to sepharose (5 x 3 cm), equilibrated with 0.05 M Tris, pH 8.6. After application of a portion of eluate (500 ml), the column was washed with 0.05 Tris, pH 8.6 until A_{280} was less than 0.02. WGA that was specifically bound to the column was then eluted with 0.5 M acetic acid until. The acetic acid eluate was then concentrated by ultrafiltration to 25-30 mg of protein per ml. The protein concentrate was dialyzed against distilled water. The final protein concentration was determined with Bio-Rad Protein Reagent. WGA isolated by this procedure gave a single band on SDS-acrylamide slab gel electrophoresis (10% acrylamide), that comigrated with a standard of WGA from EY Laboratories, and had a a molecular weight of approximately 17k. The purified WGA, 0.06250 mg/ml (10 μ l), was able to agglutinate 10 μ l of 1.5 % of packed guinea pig erythrocytes in Phosphate-buffered saline (PBS). WGA purified by EY Laboratories, 0.03125 mg/ml (10 μ l), agglutinate 10 μ l of a 1.5% solution of packed guinea pig erythrocytes in PBS which corresponds to a twofold less amount of lectin necessary to agglutinate 10 μ l of 1.5%

of guinea pig packed erythrocytes, as compared to our WGA preparation. The yield of WGA was approximately 200 mg of lectin per Kg of raw wheat germ.

2.2.3 Coupling of WGA to derivatized agarose (Affi-Gel 10)

Pure WGA was coupled to Affi-Gel 10 according to the manufacturers's instructions. Briefly, 2 mls of Affi-Gel 10 were washed 3 times with 2 mls of cold water. To a solution of WGA, containing 30 mg of protein per ml, $NaHCO_3$ was added until the concentration was 0.1 M and the pH of this solution was adjusted to 8.5. The washed Affi-Gel 10 was added to 2 ml of the buffered protein solution and allowed to react in the cold room, agitating constantly in a Roto Torque rotator (Cole-Parmer Instrument Co, Chicago, Ill). After 24 hours, 0.2 ml of 0.1 M ethanolamine, pH 8.0, was added to block any active coupling sites, and allowed to react. After one hour, the resin was poured into a column (0.4 x 10 cm), and washed with Phosphate Buffered Saline (PBS). All washes were collected (approximately 20 mls) and the amount of protein coupled was estimated by determining the quantity of protein that remained uncoupled in the washes. In a typical coupling 80-85 % of the protein was covalently bound to the resin so that the final affinity column had approximately 20-26 mg of WGA per ml of gel.

2.2.4 Reductive labeling of oligosaccharides

Human milk oligosaccharides were labeled as described [62,66]. Approximately, 300 nmoles oligosaccharides were dissolved in 0.5 ml of 0.01 M NaOH and were reduced with a 4-fold molar excess of $NaB[^3H]_4$ dissolved in 0.025 ml of 0.01 M NaOH. After 3 to 4 hours, 30 μ l of a 1 mM solution of $NaBH_4$ (100 fold excess) was added to ensure complete reduction. This mixture was allowed to react for another hour and 0.1 ml of 1M acetic acid were added to destroy excess $NaB[^3H]_4$. The radiolabeled products were transferred to a "Y shaped" sublimation vessel [67], and

shell frozen. A vacuum was applied to the vessel and after sublimation, the volatile radioactive waste was collected and discarded according to the appropriate safety regulations. The sublimation was repeated 3 times with 1 ml of distilled water to ensure complete removal of tritiated water. The resulting radiolabeled oligosaccharides were dissolved in 1.0 ml of water and applied to an AG-50W (H^+ form) column (0.5 x 3 cm). Two additional washes, 1 ml each, of the sublimation vessel were applied to the column. The cation exchange column was then eluted with 4 mls of water, and the combined eluates were dried under reduced pressure. The dried sample was evaporated 9 times from methanol in order to remove boric acid as its methyl ester. The resulting labeled sample contained the tritiated oligosaccharide alditols free from the by-products of the radiolabeling reaction.

2.2.5 Chromatographic Methods

2.2.5.1 WGA affinity chromatography

The WGA affinity columns (0.4 x 10 cm), were eluted at room temperature, at a flow rate of 15 ml/h, with either PBS/0.02% NaN_3 or with PBS/0.02% NaN_3 , followed by 0.1 M acetic acid. Tritiated oligosaccharides were applied to the column in 0.1 ml of PBS and fractions of 0.5 or 1.0 ml were collected. After use, columns were stored in PBS/0.02% NaN_3 at 4° C.

2.2.5.2 Gel filtration chromatography

Bio-Gel P6 Chromatography was done by applying the tritiated oligosaccharides plus 0.2-0.3 mg of dextran and 30,000 cpm of ^{14}C in 0.1-0.2 ml of 0.1 M pyridine acetate buffer, pH 5.4, to a column (0.9 x 95 cm) equilibrated in the same buffer. Flow rate was 3.5 ml/hr and 0.5 ml fractions were collected. Fractions were assayed for total hexose by the phenol-sulfuric acid method [64] to

determine the elution volume of the dextran (void volume). Aliquots from fractions were assayed for radioactivity to determine the elution volume of the tritiated oligosaccharide alditols and the elution volume of ^{14}C -glucose (total volume). Mono-, di-, tri-, tetra-, penta-, and hexasaccharides were identified as a function of their distribution coefficient, K_d , defined as $K_d = \frac{\text{Elution vol sample} - \text{Void vol}}{\text{Total vol} - \text{Void vol}}$.

2.2.5.3 Paper chromatography

Descending paper chromatography was performed on Whatman No 1 paper with the following solvent system: ethyl acetate/pyridine/acetic acid/water (5/5/1/3). Tritiated oligosaccharides were spotted on 2.5 x 50 cm paper strips, placed on a previously equilibrated chromatography tank and allowed to run until separation was accomplished. Location of the sample was done by cutting the dried strips into 1 cm pieces. Each piece was extracted with 1 ml of 0.1 M pyridine acetate buffer, pH 5.4, by sonicating in a bath sonicator for 15 min. Aliquots of extracts were assayed for radioactivity. At various times during chromatography, the position of the sample was estimated by taking out separate guide strips containing the same tritiated sample (5,000 cpm) and assaying them for radioactivity as described.

2.2.5.4 High performance liquid chromatography (HPLC)

HPLC was carried out using a Beckman Model 332 gradient liquid chromatograph system. The mobile phase was composed of a mixture of acetonitrile, and deionized, distilled water containing 15 mM potassium phosphate, pH 5.1. Phosphate buffer content increased on a linear gradient at a rate of 0.2 %/min that started with a mixture of acetonitrile/phosphate buffer 80/20 (v/v). The flow rate was set at 2 ml/min. The immobile phase consisted of a 10 μ amino phase analysis column (Alltech Associates, Deerfield IL). Fractions were collected every 0.5 min (1 ml) and assayed for radioactivity.

2.2.6 Enzyme incubations

Tritiated sialyloligosaccharides were digested with *Clostridium perfringens* neuraminidase as described [65]. Briefly, radiolabeled oligosaccharides (100,000 cpm) were dried in 0.5 ml microcentrifuge tubes 0.05 ml of a solution containing 0.25 U of enzyme in 0.1 M potassium acetate buffer, pH 4.8, was added. The mixture was gently mixed and incubated for 18 hr at 37° C. The incubation mixture was diluted with 0.5 ml of water and applied to a small DEAE-cellulose column (0.5 x 3 cm), equilibrated with 0.002 M pyridine acetate buffer. Neutral oligosaccharides obtained after digestion (96 %) were eluted in starting buffer, non digested material remained bound to the column.

Jack bean β -galactosidase and β -N - acetylhexosaminidase were stored in 0.02 M potassium phosphate buffer, pH 8.0 with an specific activity of 1 U/ml. Digestions were carried out as described [31]. Briefly, incubations with β -galactosidase were performed at 37° C for 18 hr. Tritiated oligosaccharides (100,000-500,000 cpm) were dried in 0.5 ml microcentrifuge tubes, and an aliquot containing 0.02 U of the enzyme was added and brought to a final volume of 0.05 ml with 0.4 M citrate buffer, pH 3.2. Incubations with β -N - acetylhexosaminidase were performed at 37° C for 72 hr. Radiolabeled oligosaccharides (50,000-100,000 cpm) were dried in microcentrifuge tubes, and an aliquot containing 0.03-0.04 U of the enzyme were added and brought to a final volume of 0.05 ml with 0.4 M citrate buffer. Enzyme digestions were stopped by application to Bio-Gel P6. Simultaneous digestion of radiolabeled sialyloligosaccharides with β -galactosidase and β -N - acetylhexosaminidase was carried out at 37 o C for 72 hr. Tritiated sialyloligosaccharides (100,000 cpm) were dried in 0.5 ml microcentrifuge tubes, and aliquots containing 0.045 U of β -galactosidase and 0.04 U of β -N - acetylhexosaminidase were added and brought to a final volume of 0.10 ml. In order to remove any neutral products formed during digestion, the incubation mixture was applied to a small DEAE-cellulose column (0.5 x 3 cm), equilibrated with 0.002 M pyridine acetate buffer, sialyloligosaccharides hydrolyzed during digestion were eluted in the starting buffer. Intact sialyloligosaccharides (90 %) remained bound to the

column and were eluted with 0.1 M pyridine acetate buffer, pH 5.4. All enzyme incubations contained 2 μ l of toluene to prevent bacterial growth.

2.2.7 Binding of radiolabeled oligosaccharides to specific antiserum

Direct binding of labeled oligosaccharides was performed as described [18,66]. Aliquots from HPLC fractions were added to 12 x 75 mm tubes and dried in Speed Vac concentrator (Savant, Hicksville, NY). Dried samples were diluted in 0.110 ml of 0.01 M Tris-HCl buffer, pH 7.5, that contains 0.14 M NaCl, 0.5 mM MgSO₄ and 0.15 mM CaCl₂. A 1:2 dilution of anti-sialyltetrasaccharide c antisera was added (0.020 ml), and the mixture was incubated for 30 min at 37° C and overnight at 4° C. The incubation mixtures were filtered through nitrocellulose filters in a vacuum filtration apparatus (Hoffer Scientific Instruments, San Francisco, CA) and washed with 10 mls of the incubation buffer. The filters were cut in pieces and extracted with 1.5 ml of 0.1 M acetic acid by sonicating in bath sonicator for 15 min. Aliquots of the extracts are tested for radioactivity. Radioactivity represents the trapped antibody in the nitrocellulose filter binding the [³H] -oligosaccharide alditols that are its homologous haptens. Non specific counts were washed through the nitrocellulose filters.

3.0 Results and discussion

3.1 *WGA affinity chromatography of the human milk sialyloligosaccharides*

Human milk sialyloligosaccharides were partially separated by ion exchange chromatography on DEAE-cellulose according to the method of Smith et al [17]. Briefly, a sialyloligosaccharide fraction prepared as described [16], was applied to a DEAE-cellulose column previously equilibrated in 0.002 M pyridine acetate buffer, pH, 5.4. The different sialyloligosaccharides were partially resolved by increasing concentrations of the pyridine acetate buffer from 0.002 M to 0.06 M at the same pH (refer to fig. 3).

The monosialyloligosaccharides were partially separated by elution with with 0.02 M pyridine acetate buffer, pH 5.4, into three different fractions, a sialyl high molecular weight fraction that contains sialyl derivatives of hexaoses and larger core structures (fig. 3, fraction II); the sialyltetrasaccharide fraction (fig. 3, fraction III) and the sialyllactose fraction (fig. 3, fraction IV). Disialyloligosaccharides were eluted in 0.115 M pyridine acetate, pH 5.4 (fig. 3, fraction V).

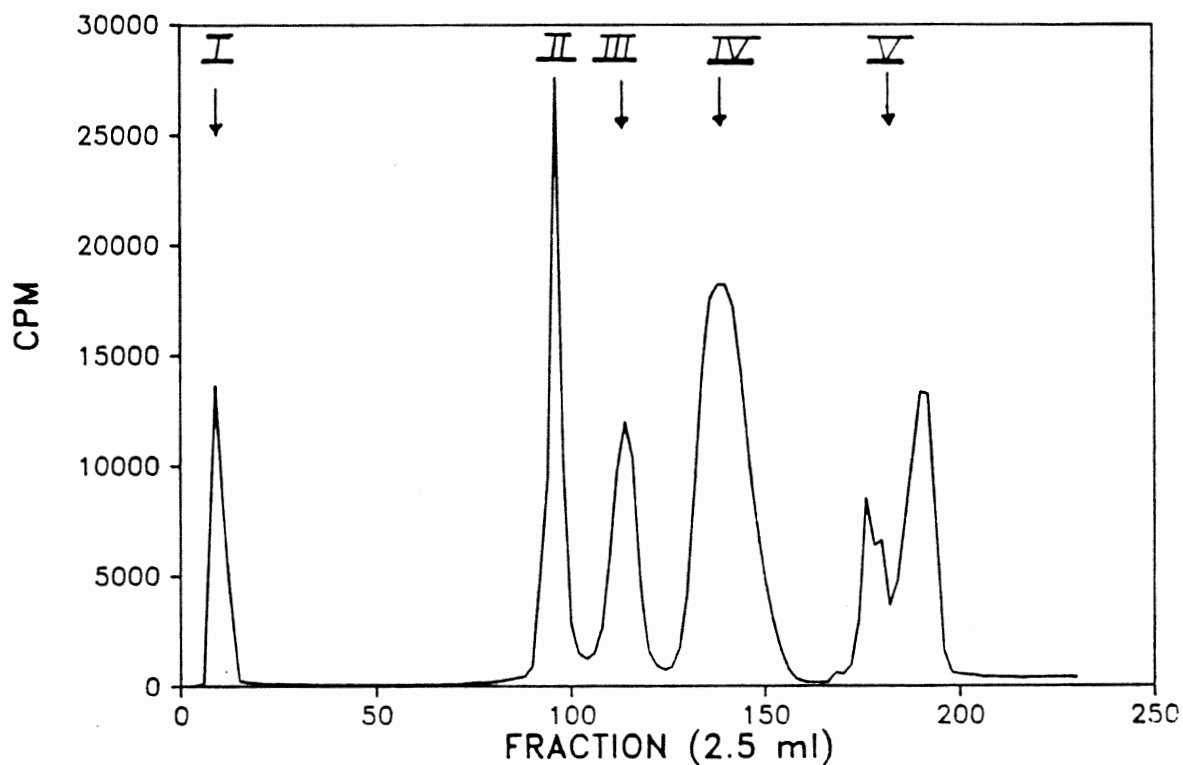


Figure 3. DEAE-cellulose Chromatography of Human Milk Sialyloligosaccharides [3H]-oligosaccharide alditols were applied to a DEAE-cellulose column previously equilibrated in 0.002 M pyridine acetate, pH 5.4. Neutral oligosaccharides were eluted with the same buffer (I); the monosialyloligosaccharides were partially separated by elution with 0.02 M pyridine acetate, pH 5.4, into three fractions, a high molecular weight fraction (II), sialyltetrasaccharide fraction (III), and sialyllactose fraction (IV); the disialyloligosaccharides (V) were eluted with 0.115 M pyridine acetate, pH 5.4. Each of the monosialyloligosaccharide fractions (II, III, IV) were tested for their ability to bind a WGA-agarose affinity column.

Tritiated monosialyloligosaccharide fractions III and IV were tested for their ability to bind a WGA-agarose column (20 mg/ml), none of them were able to bind WGA in the affinity column system (data not presented). The tritiated high molecular weight sialyloligosaccharide fraction II, containing sialyl derivatives of hexaoses and larger core structures, was also applied to the WGA-agarose column (20 mg/ml). The results showed that most of the counts (80%) were eluted in the void volume (counts not bound), but 20% of the counts were specifically retarded in the column (fig 4).

3.2 Paper Chromatography of the sialyloligosaccharide fraction

In order to determine what type of structures were present in the WGA-bound sialyloligosaccharide fraction, the complete, the WGA-unbound, and the WGA-bound sialyloligosaccharide fractions were subjected to descending paper chromatography in the solvent system, ethyl acetate/pyridine/acetic acid/water (5/5/1/3). The results (fig.5) showed 2 major peaks (a and b) in the paper chromatogram of the complete fraction (fig. 5A).

These 2 peaks are also observed in the chromatogram of the WGA-unbound fraction (fig. 5B) however, the amount of counts in peak b are decreased by 55% as compared to the complete fraction. The WGA-bound fraction (fig. 5C) moves in the chromatogram as a single peak that comigrated with peak b in the complete and WGA-unbound fraction, and the total number of counts under this peak corresponded to the amount of counts missing in peak b of the WGA-unbound fraction. The mobility of peak b during the paper chromatography corresponds to the sialylhexasaccharide fraction (S-5) of human milk that has already been isolated by Kobata and Ginsburg in 1972 [21]. These workers concluded that the sialylhexasaccharide fraction of human milk contained two isomeric hexaoses substituted with sialic acid. The structures designated

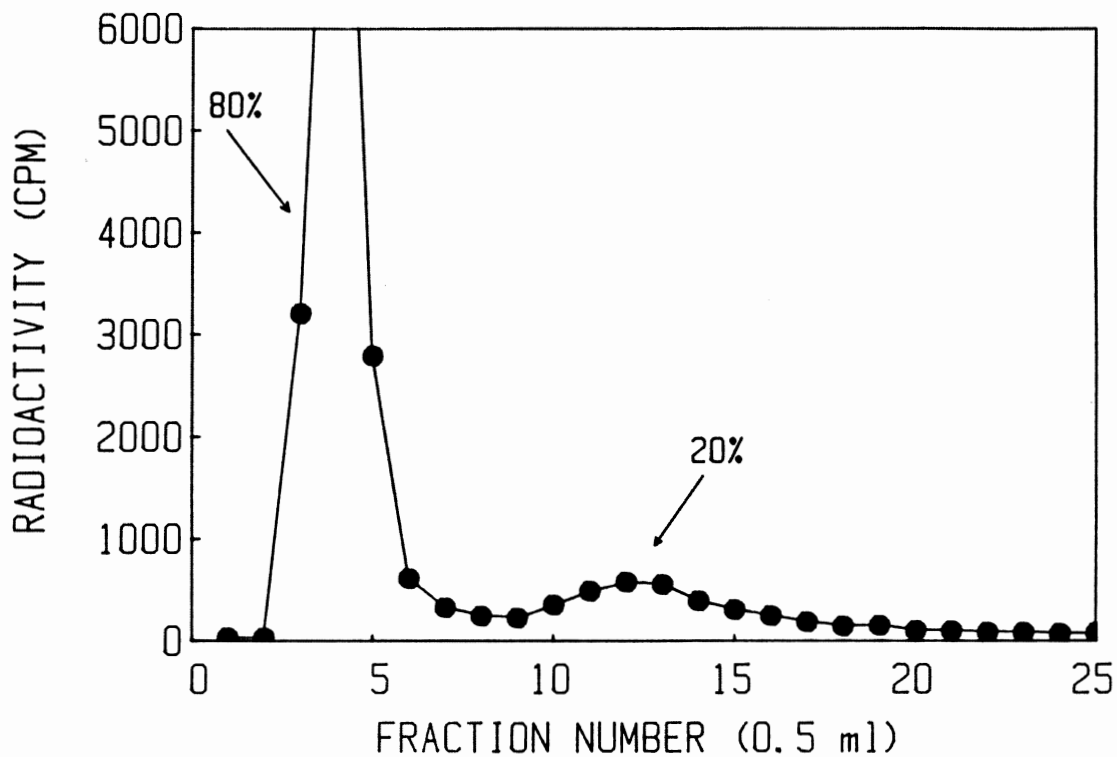


Figure 4. WGA Affinity Chromatography of Human Milk Sialyloligosaccharides. The tritiated sialyl high molecular weight fraction (fraction II in fig. 3) was applied in 0.1 ml of PBS in a 20 mg/ml WGA-agarose column (0.4 x 10 cm) equilibrated in the same buffer. The column was eluted with 15 ml of PBS and 0.5 ml fractions were collected. Aliquots of fractions were assayed for radioactivity.

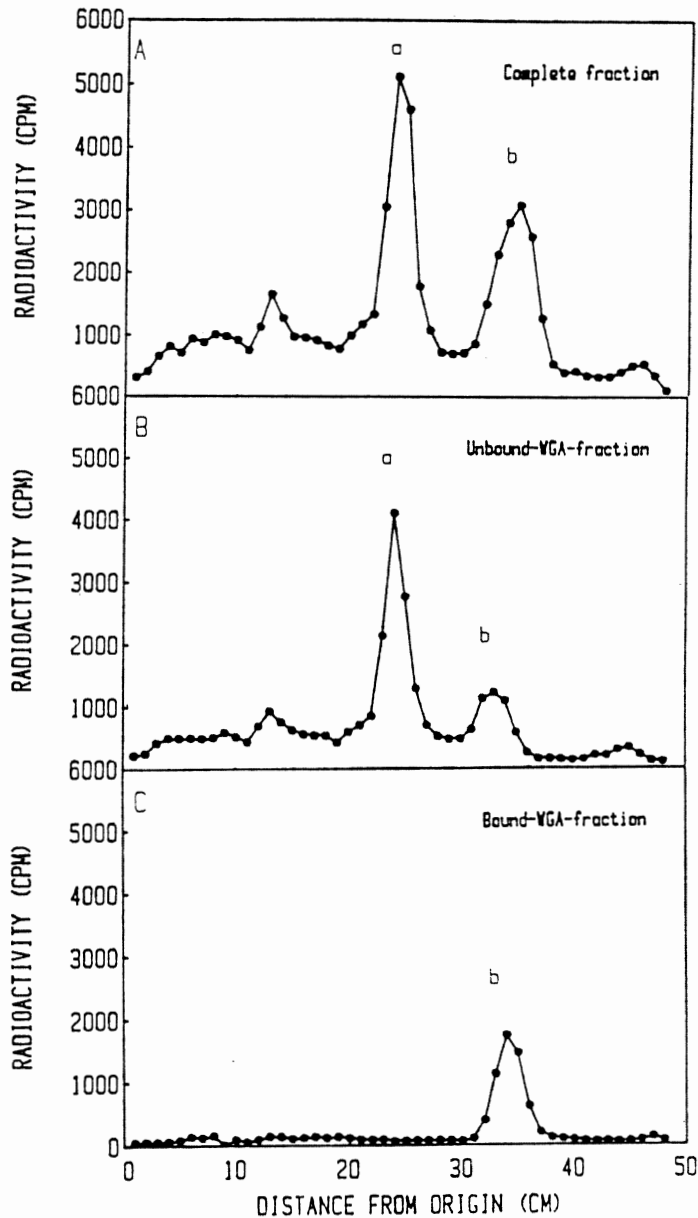


Figure 5. Paper Chromatography of Human Milk Sialyloligosaccharides. Paper chromatography of the tritiated high molecular weight fraction was carried out with the solvent system, ethyl acetate/pyridine acetate/acetic acid/water (5/5/1/3) for 25 days on Wathman no. 1 paper. Segments of the paper (1 cm) were eluted using 1 ml of 0.1 M pyridine acetate, pH 5.4, and aliquots were assayed for radioactivity. (A) Complete radiolabeled sialyloligosaccharide fraction. (B) WGA-unbound [3H] sialyloligosaccharide fraction (fig. 4, fractions 2-6). (C) WGA-bound [3H] sialyloligosaccharide fraction (fig. 4, fractions 7-18).

sialyllacto-N-hexaose and sialyllacto-N-neohexaose are represented by structures I and II respectively shown in fig. 6.

In discussing the two sialylhexaoses, the 3 branch refers to the branch that contains one of the GlcNAc residues linked β 1-3 to the branched galactose; similarly, the 6 branch refers to the branch that contains the other GlcNAc residue linked β 1-6 to the branched galactose. In lacto-N-hexaose, the terminal galactose is attached β 1-3 to the GlcNAc residue, whereas in lacto-N-neohexaose the terminal galactose in the 3 branch is linked β 1-4 to the GlcNAc residue. The 6 branch in both hexaoses contains the terminal galactose attached β 1-4 to the other GlcNAc residue. Sialic acid in the two isomeric hexaoses is linked α 2-6 to the 6 branch.

3.3 WGA-affinity chromatography of the sialylhexasaccharide fraction

In order to confirm that the WGA-bound oligosaccharide was a part of the previously described sialylhexasaccharide fraction, S-5, the human milk sialylhexasaccharide fraction (S-5) was isolated according to the method of Kobata and Ginsburg [21], radiolabeled, and then applied to the WGA-affinity column (25 mg/ml). The column was eluted first, with 4 ml of PBS and then with 20 ml of 0.1 M acetic acid in order to remove the counts specifically bound to the column. Since the WGA-affinity column used in this experiment was of higher capacity (25 mg/ml) as compared to the WGA-affinity column used to separate the sialyloligosaccharide fraction in the experiment mentioned before, specifically bound counts had to be removed either by eluting with a solution of GlcNAc/PBS (removal of the counts by competition with GlcNAc) or by lowering the pH using a 0.1 M solution of acetic acid since binding ability of WGA is pH dependent [35]. The results of this experiment showed that 60% of the counts were bound by the column (fig. 7). The amount bound, corresponded to the approximately 55% removed from peak b in the paper

chromatogram of the unbound sialyloligosaccharide fraction (refer to fig. 4B). These results indicate that the WGA-bound sialyloligosaccharide was present in the sialylhexaose fraction of human milk, originally characterized by Kobata and Ginsburg [21].

3.4 Contribution of sialic acid and N-acetylglucosamine in the binding to WGA

The sialylhexasaccharide fraction (S-5) of human milk, as it has been demonstrated by Kobata and Ginsburg [21], is composed of one sialic acid residue, three galactose residues, two N-acetylglucosamine residues and one glucose residue. Since it has been proved that N-acetylglucosamine and sialic acid are able to interact with WGA [34,35], the contribution of any of these two carbohydrate residues, present in the WGA-bound sialylhexaose, fraction was determined. Also, considering the number of sialic acid linkages that occur in milk (see table 2) and the fact that only one sialyloligosaccharide was bound by WGA, it was questioned if the binding of this sialyloligosaccharide to WGA was unique to the type of sialic acid linkage present in this molecule.

The approach taken was to remove sialic acid by treatment with *Clostridium perfringens* neuraminidase and then rechromatograph the resulting neutral derivative in the WGA-affinity column. The results in fig. 8, show that after removal of sialic acid, the resulting neutral hexaose was slightly more retarded (fig. 8, close circles) than the corresponding sialyl derivative (fig. 8, open squares). This indicates that the interaction of the WGA-bound sialylhexaose with WGA is not due to the presence of sialic acid; it seems more likely that sialic acid is preventing the interaction of the lectin with the WGA-bound sialylhexaose. Therefore, by an elimination process, this results led to the conclusion that there is nothing unique about the sialic acid linkage in the binding to WGA, that only the GlcNAc residues existing in the WGA-bound sialylhexaose are important in

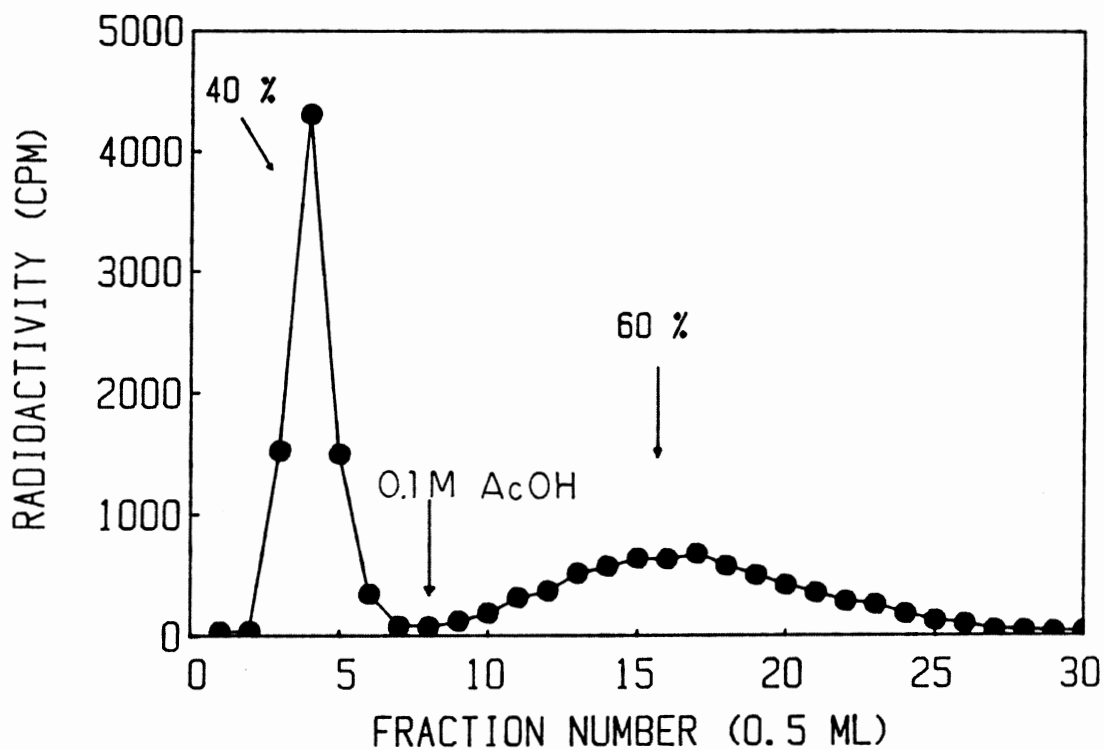


Figure 7. WGA Affinity Chromatography of the Sialylhexasaccharide Fraction (S-5) of Human Milk. [3H] sialylhexasaccharide fraction was applied to a 25 mg/ml WGA-agarose column (0.4 x 10 cm) equilibrated was eluted with 4 ml of PBS followed by 15 ml of 0.1 M acetic acid (AcOH). Fractions of 0.5 ml were collected and aliquots of them were assayed for radioactivity.

the binding to the lectin. Note that if removal of sialic acid had abolished binding of the neutral hexaose to WGA, it would have implied that the interaction was only due to the presence of sialic acid. In addition, if the absence of sialic acid had slightly decreased the binding but not abolished it, it would have indicated that binding was due to the contribution of both, sialic acid and N-acetylglucosamine. Also, if binding had not changed after sialic acid removal, it would have meant that the presence or absence of sialic acid in the oligosaccharide did not make any difference in the interaction with the lectin. In conclusion, the approach taken to determine what carbohydrate units in the WGA-bound sialylhexaose were contributing to the interaction with WGA, was valid. Because, any result obtained would have only explained one cause of binding and it would have eliminated any of the other possibilities.

3.5 Sequential exoglycosidase digestion of the WGA-bound hexaose

In order to determine the structure of the core hexasaccharide (either a lacto-N-hexaose or a lacto-N-neohexaose core according to previous studies [21]), the affinity purified oligosaccharide after treatment with neuraminidase was subjected to sequential exoglycosidase digestion. The sequential enzyme digestion is diagramed in fig. 9, and the results of this experiment are shown in fig. 10. The starting material had a distribution coefficient, $K_d = 0.61$, on Bio-Gel P6 identical to that of a [3H] hexaitol (fig. 10A). The complete asialo sialylhexasaccharide fraction of human milk was used as standard. The radiolabeled hexaitol was treated with 0.2 U/ml of jack bean β - galactosidase, at 37° C for 18 hours, conditions that will only cleave the Gal β 1-4 linkages (jack bean β - galactosidase is more specific for the linkage Gal β 1-4 GlcNAc than for the linkage Gal β 1-3 GlcNAc and as a consequence it cleaves the Gal β 1-4 linkage faster than the Gal β 1-3), see under Methods. After digestion, the incubation mixture was applied to Bio-Gel P6. As shown

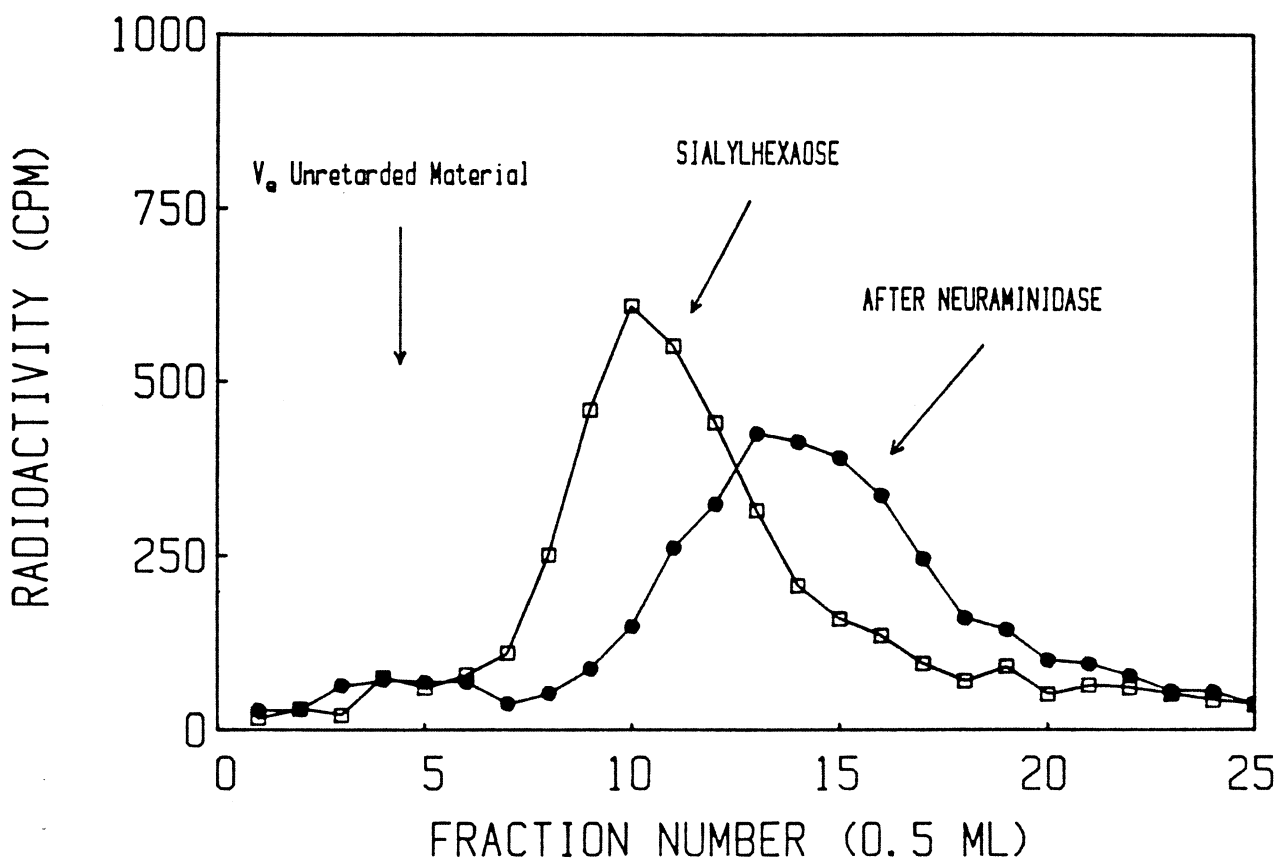


Figure 8. WGA Affinity Chromatography of the Sialylhexasaccharide Fraction Before and After Neuraminidase Digestion. [³H] WGA-bound sialylhexaose fraction (fig. 4, fractions 7-18) was subjected to neuraminidase digestion and the resulting neutral hexaose (●) was rechromatographed in the WGA-agarose column (20 mg/ml) as described in fig. 4. Intact WGA-bound sialylhexaose fraction (■) was used as control.

in fig. 10B, the [3H] hexaitol was completely converted to a compound with a $K_d = 0.71$, identical to that of a [3H] tetraitol. Authentic lacto-N-tetraose was used as standard (refer to table 1 for the structure).

Subsequently, the [3H] tetraitol was treated with 0.6 U/ml of jack bean β - *hexosaminidase* (see under Methods), at 37° C for 72 hours, and then applied to Bio-Gel P6. The product of the digestion eluted in identical position as [3H] lactitol (see fig. 10C). Results of the sequential exoglycosidase treatment indicated that the radiolabeled WGA-bound hexaitol was a branched structure, since β - *galactosidase* digestion released two hexose units, and subsequent β - *hexosaminidase* digestion also released two N-acetylhexosamine units yielding [3H] lactitol. If this were a linear structure the exoglycosidase digestion, would have released only one hexose unit at a time. These results also indicated that the branched hexaose contains both terminal galactoses linked β 1-4 to the N-acetylglucosamine residues, which is consistent with a lacto-N-neohexaose core. Therefore, the WGA-bound hexaose is a sialyl derivative of lacto-N-neohexaose. This structure would fulfill the requirements for binding to WGA, since the hydroxyl group at C-3 in both GlcNAc residues are unsubstituted [42].

3.6 Position of Sialic Acid in the

Sialyllacto-N-neohexaose

According to the studies of Kobata and Ginsburg [21], the sialylhexasaccharide fraction (S-5) of human milk only contains one type of sialic acid linkage, that is NeuAc α 2-6Gal....., which has only been found in human milk, linked to terminal Gal β 1-4GlcNAc....., and not to terminal Gal β 1-3GlcNAc.... This observation is explained by the specificity of the α 2-6 sialyltransferase which will only add sialic acid α 2-6 to terminal Gal β 1-4GlcNAc..... . Based on the specificity of the α 2-6 sialyltransferase, sialic acid could only be added in the 6 branch of lacto-N-hexaose (this

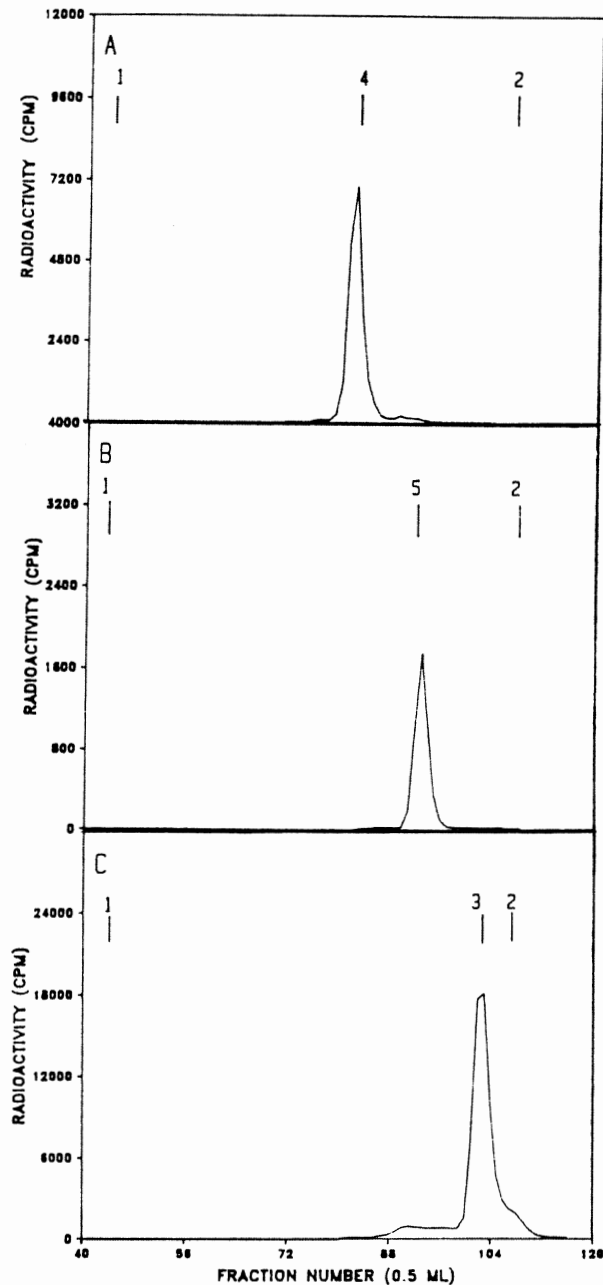


Figure 10. Bio-Gel P6 Chromatography of the WGA-bound Hexaose After Sequential Exoglycosidase Treatment. (A) Bio-Gel P6 chromatography of the asialo derivative of the [3H] WGA-bound sialylhexaose. (B) Bio-Gel chromatography of the hexaose after digestion with jack bean β -galactosidase (0.4 U/ml, 37 $^{\circ}$, 18 hr). (C) The tritiated oligosaccharide generated after β -galactosidase digestion was treated with jack bean β -hexosaminidase (0.6 U/ml, 37 $^{\circ}$, 72 hr) and applied to Bio-Gel P6 chromatography. Aliquots of the fractions were assayed for radioactivity. Void volume (1), and total volume (2) are indicated. Position of standards during chromatography are also indicated, (3) lactitol, (4) asialo derivative of sialylhexaitol, (5) lacto-N-tetraitol.

is the branch that contains the terminal galactose linked β 1-4 to N-acetylglucosamine, see fig. 6, but it could be added in both the 6 (structure I, fig. 11) as well as in the 3 branch (structure II, fig. 11) of the lacto-N-neohexaose core. However, Kobata and Ginsburg [21] determined that sialic acid was only linked to the terminal galactose located in the 6 branch of both, lacto-N-hexaose and lacto-N-neohexaose (see fig. 6). The procedure they used to determine the position of sialic acid in both hexaose cores, was based on acetolysis of the complete sialylhexasaccharide fraction (S5) and analysis of the acetolysis products by cochromatography in paper with known standards [21]. Briefly, the procedure was the following: purified sialylhexasaccharide fraction (S5) was subjected to acetolysis. Since sialic acid linkages are more resistant to acetolysis than hexosyl or N-acetylhexosaminyllinkages, two different fragments containing sialic acid were obtained. One of the fragments, based on the mobility on paper chromatography corresponded to a disaccharide that cochromatographed with NeuAc α 2-6Gal and on hydrolysis produced sialic acid and galactose.

These results demonstrated that sialic acid was only found linked α 2-6Gal in the human milk sialylhexaose fraction. The mobility of the second fragment on paper chromatography, corresponded to a tetraose containing sialic acid. The acid hydrolysis products of the tetraose produced sialic acid and a triaose that comigrated during paper chromatography with Gal β 1 - 3GlcNAc β 1 - 3Gal, but did not comigrate with Gal β 1 - 4GlcNAc β 1 - 3Gal. Since this triaose was cleaved by jack bean β - galactosidase under conditions that will only act upon Gal β 1 - 4GlcNAc linkages, and it did not comigrate with Gal β 1 - 4GlcNAc β 1 - 3Gal, they assumed that this triaose was distinct from Gal β 1 - 3GlcNAc β 1 - 3Gal and it had the probable structure of Gal β 1 - 4GlcNAc β 1 - 6Gal. These results suggested that sialic acid could only be found attached to the 6 branch of lacto-N-hexaose and lacto-N-neohexaose. However, these conclusions were made based on exoglycosidase digestion and lack of cochromatography of the triaose with known standards. The structure of the triaose was not confirmed by any independent method such as methylation analysis.

Since the position of sialic acid was not unequivocally proved by Kobata and Ginsburg [21], the possibility existed that sialic acid could be also attached to the 3 branch in the

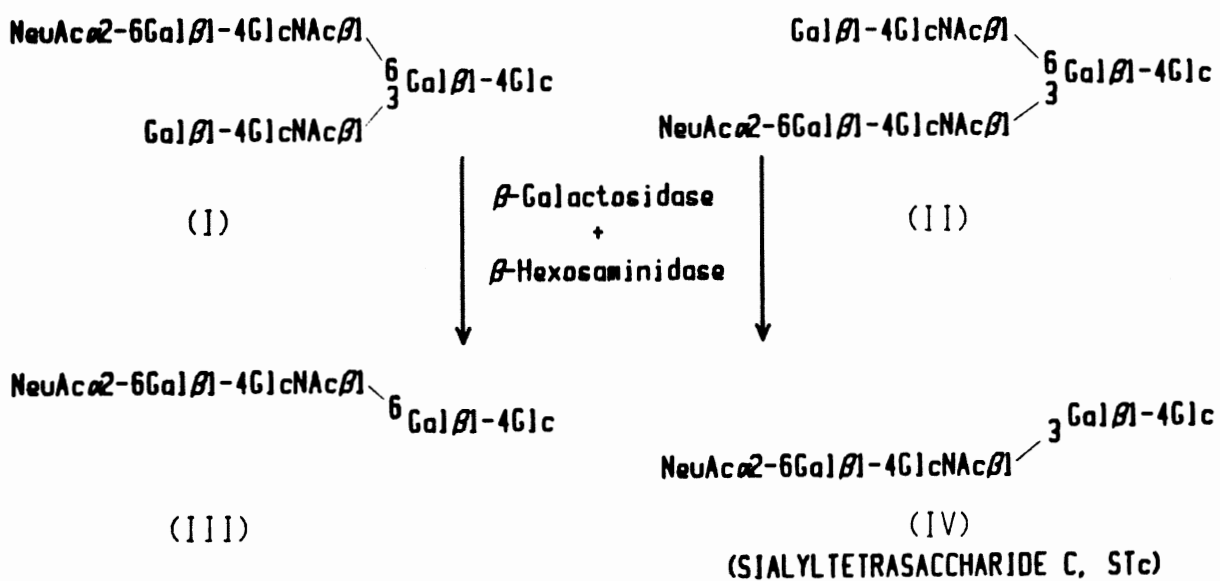


Figure 11. Sialyltetrasaccharides Generated From the Two Possible Sialyl-lacto-N-neohexaoses.

lacto-N-neohexaose core. For this reason, the position of sialic acid in the WGA-bound sialyllacto-N-neohexaose core had to be confirmed. In order to accomplish this, the WGA-bound sialyllacto-N-neohexaose was subjected to simultaneous digestion with β - galactosidase and β - hexosaminidase to remove the galactose and N-acetylglucosamine in the branch that was free from sialic acid. For example, if sialic acid is located in the 6 branch of the lacto-N-neohexaose core, hydrolysis of the galactose and N-acetylglucosamine contained in the 3 branch will be removed and a corresponding sialyltetraose will be formed (fig. 11, structure III). If sialic acid is located on the 3 branch, the combined action of the enzymes will remove the galactose and N-acetylglucosamine in the 6 branch and to generate another sialyltetraose that has the structure of sialyltetrasaccharide c, a known sialyloligosaccharide in human milk (fig. 11 structure IV).

The WGA-bound sialylhexaose was digested with high concentrations of β - galactosidase (0.9 U/ml) and β - hexosaminidase (0.8 U/ml), at 37° C for 72 hours, and then applied to Bio-Gel P6. Results of the Bio-Gel P6 showed that there was complete conversion of the sialylhexaose to a compound with a $K_d = 0.62$, identical to that of a sialyltetrasaccharide (see fig. 12). Authentic sialyltetrasaccharide c was used as standard. The fractions containing the sialyltetrasaccharide were pooled and applied to HPLC. The elution profile on the HPLC (fig. 13) showed the presence of a single peak that eluted with identical elution time as sialyltetrasaccharide c (see table II and structure IV in fig. 11). In order to confirm the presence of sialyltetrasaccharide c under the HPLC elution peak, aliquots of the fractions were tested for direct binding to an antisera raised against the reduced sialyltetrasaccharide c alditol. Radioactivity bound by the antibody (fig. 14, open triangles) correlated exactly with the HPLC elution profile (fig. 14, closed circles) of the unknown sialyltetraose.

These results indicated that the sialyltetrasaccharide generated from the bound sialylhexaose fraction is sialyltetrasaccharide c, suggesting that the WGA-bound sialylhexaose is a sialyl derivative of lacto-N-neohexaose where sialic acid is linked α 2-6 to the terminal galactose that is located in the 3 branch. This structure is not consistent with the sialyl derivative of lacto-N-neohexaose reported by Kobata and Ginsburg where sialic acid is attached to the terminal galactose in the 6 branch, and represents a previously undescribed sialyloligosaccharide in human milk (see fig. 15).

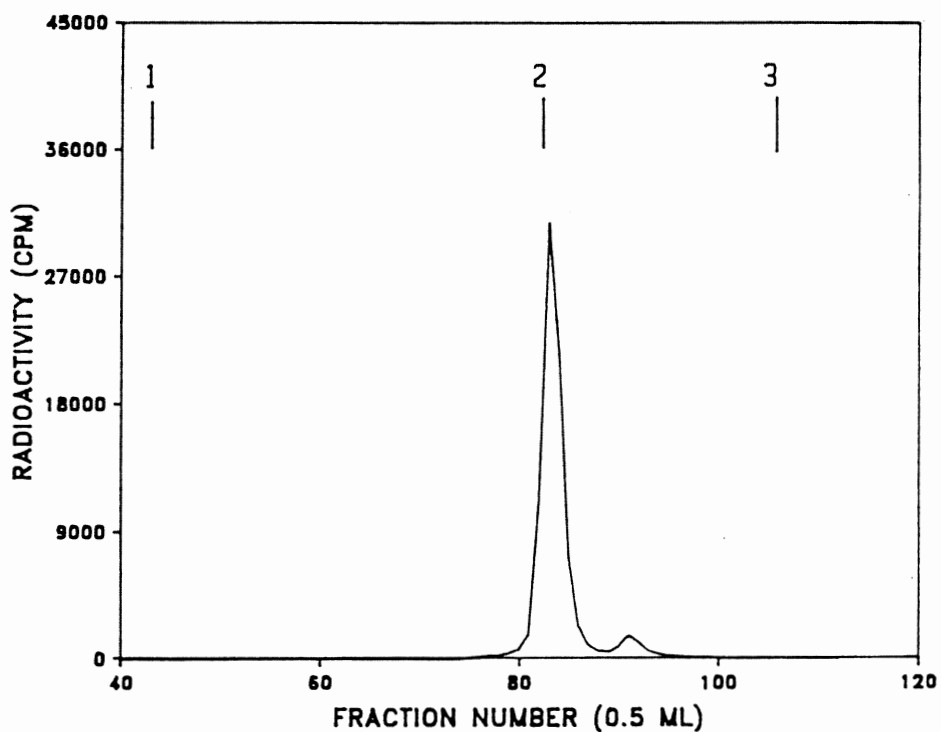


Figure 12. Bio-Gel P6 Chromatography of the the Digestion Products of the WGA-bound Sialylhexaose. [3H] WGA-bound sialylhexaose was subjected to simultaneous digestion with 0.9 U/ml of jack bean β -galactosidase and 0.8 U/ml of β -hexosaminidase (37° , 72 hr) and applied to Bio-Gel P6 chromatography. Aliquots of the fractions were assayed for radioactivity. Void volume (1) and total volume (3) are indicated. Position of authentic sialyltetrasaccharide c (2) during chromatography is also indicated.

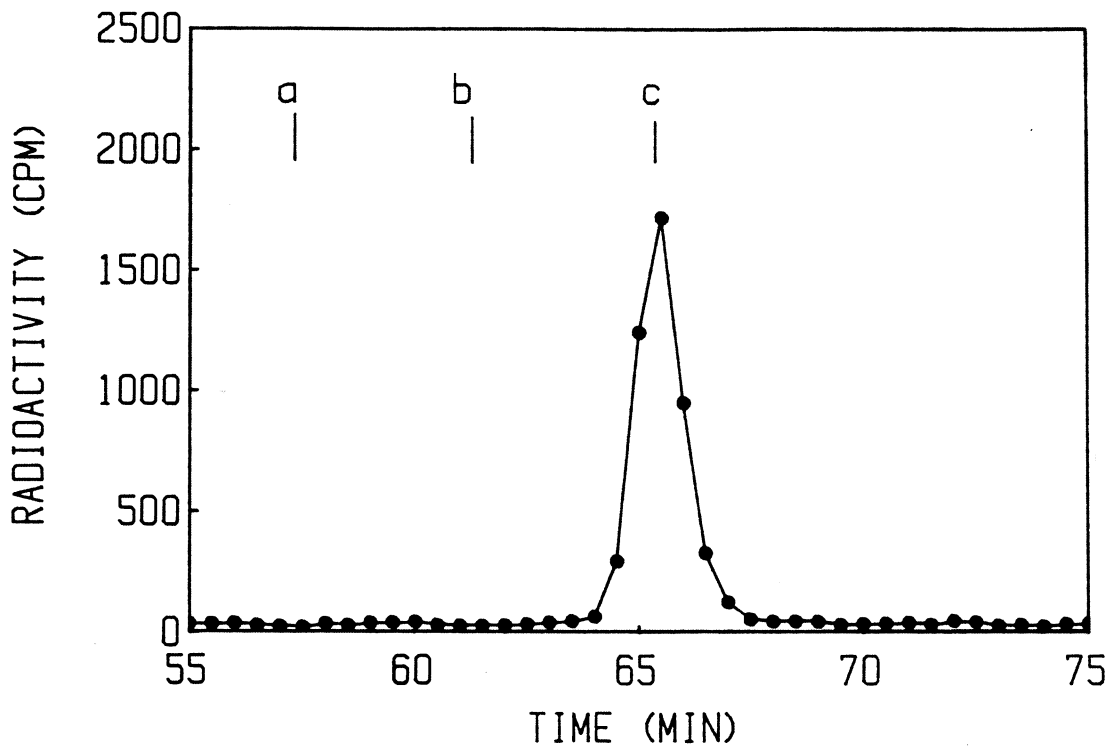


Figure 13. HPLC of the Sialyltetrasaccharide from the WGA-bound Sialylhexaose. The sialyltetrasaccharide derived from simultaneous digestion with β -galactosidase and β -hexosaminidase of the WGA-bound sialylhexaose was applied to HPLC after P6 Bio-Gel chrom. (fig. 12 fractions 81-85), as described under Material and Methods. Aliquots of the fractions were assayed for radioactivity. Position of standards during chromatography are indicated, (a) sialyltetrasaccharide a, (b) sialyltetrasaccharide b, (c) sialyltetrasaccharide c.

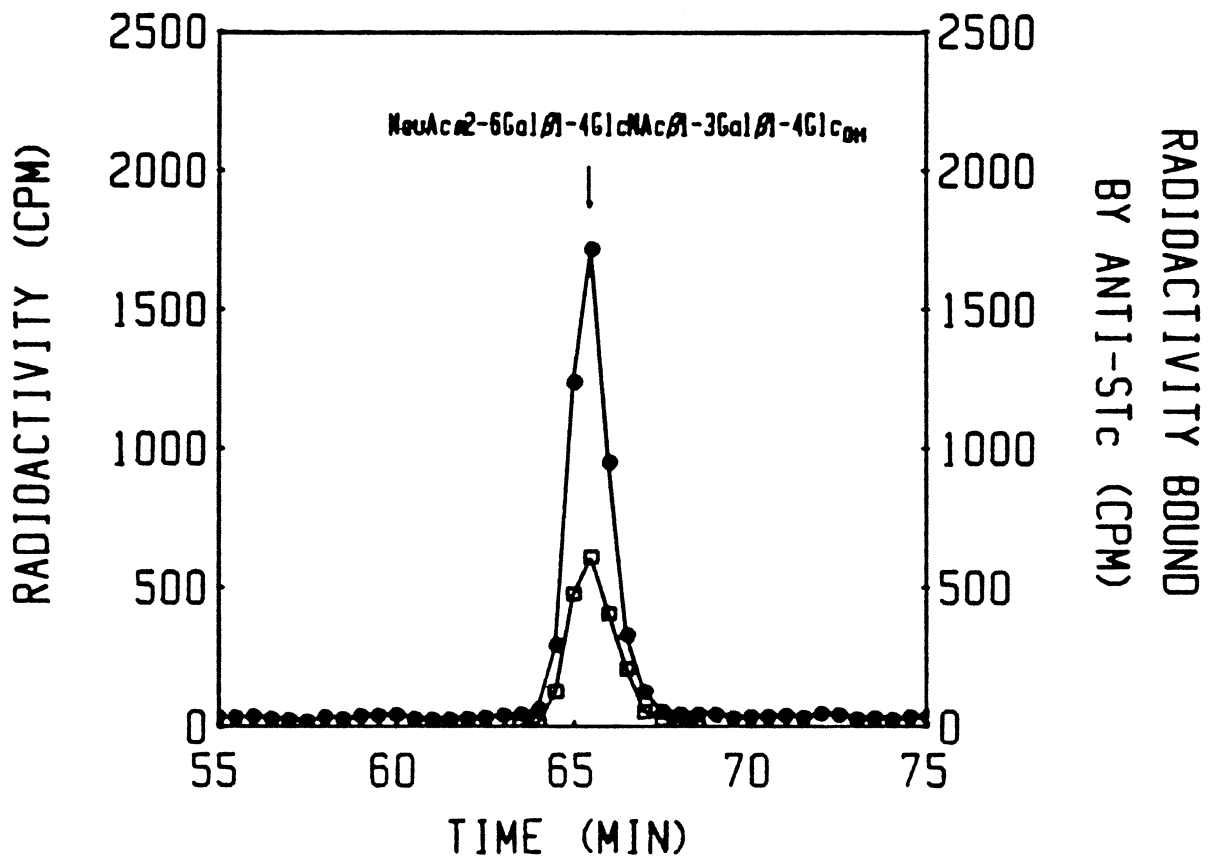


Figure 14. Identification of the Sialyltetrasaccharide Derived from the WGA-bound Sialylhexaose. Aliquots from the HPLC (●) of the sialyltetrasaccharide were assayed for binding to anti-sialyltetrasaccharide c (■) as described under Materials and Methods.

3.7 *The WGA-unbound sialylhexaose fraction*

Since the WGA-bound sialylhexaose fraction did not contain either of the structures (fig. 6) described by Kobata and Ginsburg [21], the WGA-unbound fraction presumably contained the sialyl derivatives of lacto-N-hexaose and lacto-N-neohexaose where sialic acid is linked α 2-6 to the 6 branch. To confirm the presence of these structures, the WGA-unbound fraction was subjected to simultaneous digestion with high concentrations of β -galactosidase (0.9 U/ml) and β -N-acetylhexosaminidase (0.8 U/ml), at 37° C for 72 hours, in order to generate the corresponding sialyltetrasaccharide(s). If the two structures described [21] are the only ones present in the WGA-unbound fraction, only one sialyltetrasaccharide will be produced since sialic acid is located in the 6 branch of lacto-N-hexaose and lacto-N-neohexaose (fig. 16). The enzymes were applied to a small column of DEAE-cellulose following the procedure under Material and Methods. The 0.1 M pyridine acetate eluate containing the sialyloligosaccharides was applied to a Bio-Gel P6 column. Results of Bio-Gel P6 chromatography (fig. 17) showed 65 % of counts applied had an elution volume corresponding to that of an undigested sialylhexaose, the rest of the counts were eluted at a volume identical to that of a sialyltetrasaccharide (sialyltetrasaccharide c was used as standard), see fig. 17. The fractions corresponding to the sialyltetrasaccharide were pooled, concentrated and a part of it was applied to HPLC (fig. 18A). The rest of the sample was mixed with authentic sialyltetrasaccharide c and applied to HPLC (fig. 18B).

The results showed a single sialyltetraose that was clearly separated from sialyltetrasaccharide c, (see fig. 18B). These data indicated that only one sialyltetraose was generated from the WGA-unbound fraction and that it was different from sialyltetrasaccharide c. Fractions from the HPLC eluate containing the unknown sialyltetraose were pooled, and desalted on a column of Bio-Gel P6. Increasing amounts of counts of the tritiated desalted sialyltetraose (ranging from 500 cpm to 8,000 cpm) were tested for binding in a direct binding assay with anti-sialyltetrasaccharide c serum. In this assay, authentic sialyltetrasaccharide c and the sialyltetrasaccharide c generated from the WGA-bound fraction were used as controls. The sialyltetrasaccharide obtained from the

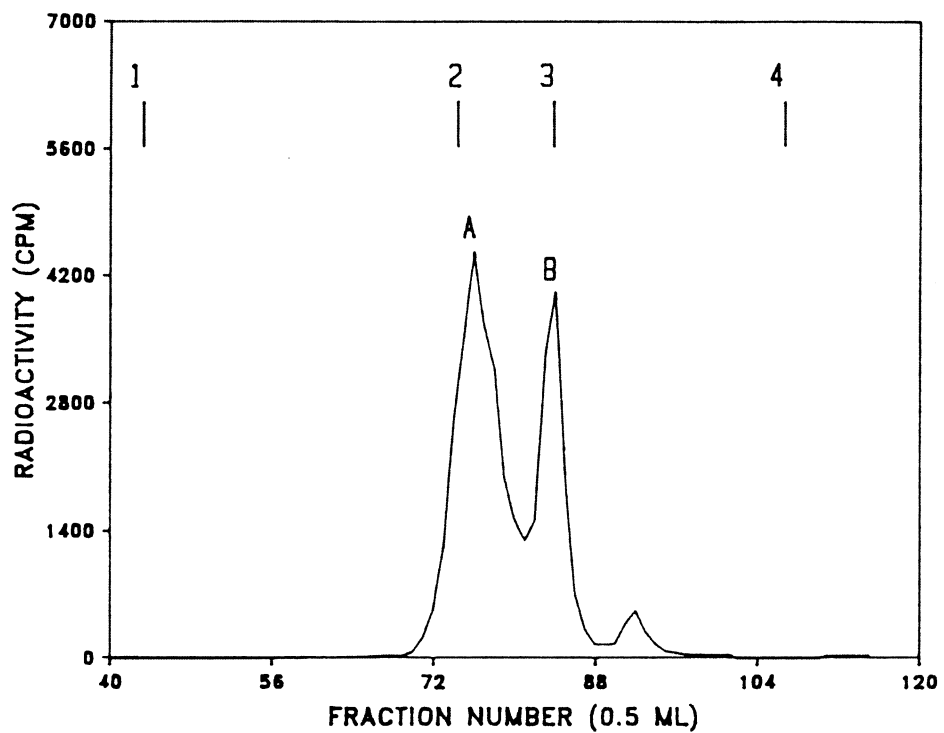


Figure 17. Bio-Gel P6 Chromatography of the Digestion Products of the WGA-unbound Sialylhexaose Fraction. The WGA-unbound sialylhexaose fraction was subjected to simultaneous digestion with β -galactosidase and β -hexosaminidase as described in the section of Materials and Methods, and applied to Bio-Gel P6. Void volume (1) and total volume (4) are indicated. Position sialyltetrasaccharide c (3) and sialylhexasaccharide fraction (2) during chromatography are indicated. (A) Undigested WGA-unbound sialylhexaose fraction. (B) Sialyltetrasaccharide generated after digestion.

WGA-unbound fraction showed no binding to the antibody, whereas sialyltetrasaccharide c and the sialyltetrasaccharide c from the WGA-unbound fraction were bound by the antibody (see fig. 14). These results indicated that the WGA-unbound fraction gives rise to a single sialyltetraose which is different from sialyltetrasaccharide c.

The sialyltetraose from the WGA-unbound sialylhexaose fraction most probably corresponds to the sialyltetraose generated from the sialyl derivatives of lacto-N-hexaose and lacto-N-neohexaose where sialic acid is linked to the 6 branch, which are the structures described by Kobata and Ginsburg [21].

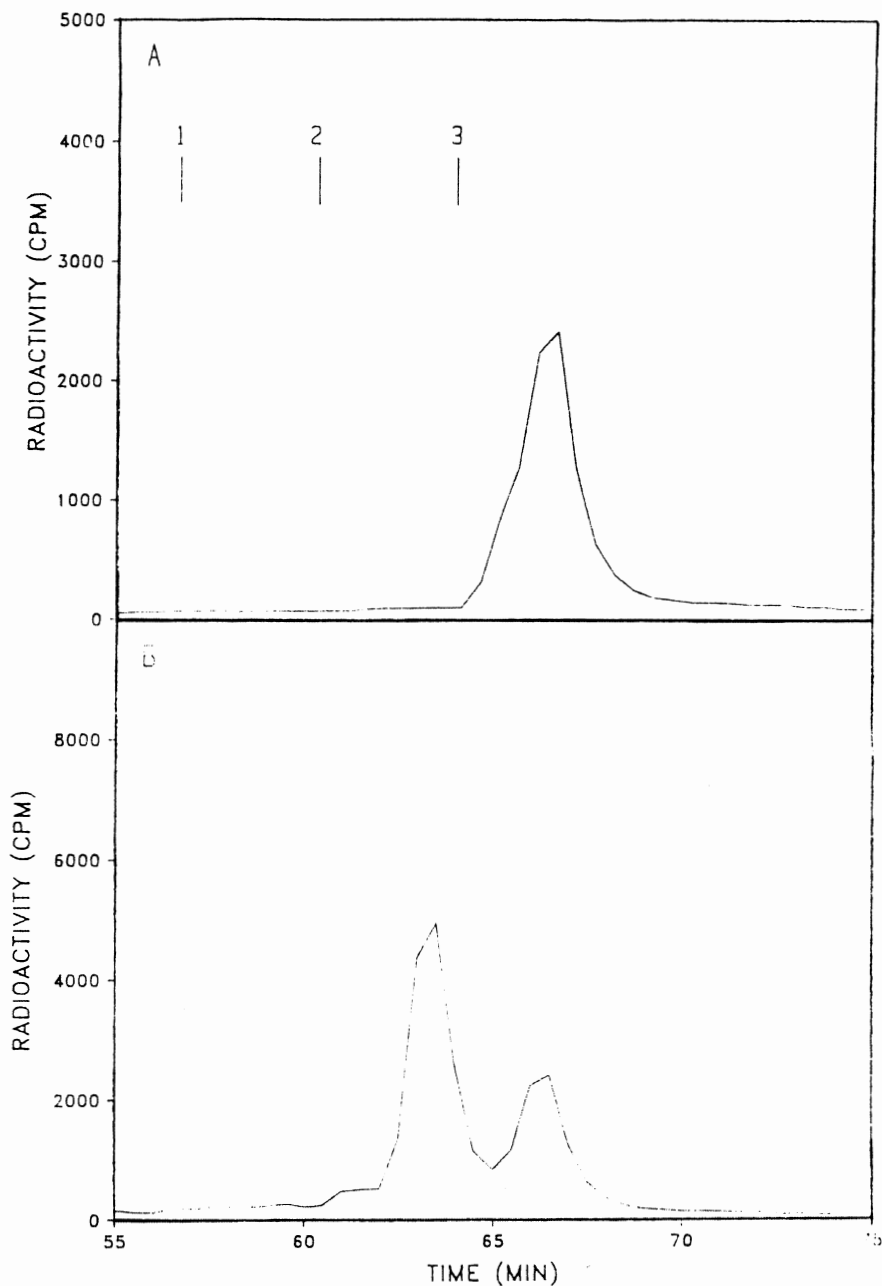


Figure 18. HPLC of the Sialyltetrasaccharide Derived from the WGA-unbound Sialylhexaose Fraction. The sialyltetrasaccharide derived from digestion with β -galactosidase and β -hexosaminidase of the WGA-unbound sialylhexaose fraction was applied to HPLC as described under Material and Methods. (A) HPLC of the sialyltetrasaccharide from the WGA-unbound hexaose fraction. (B) HPLC of the sialyltetrasaccharide from the WGA-unbound sialylhexaose fraction plus authentic sialyltetrasaccharide c. Position of the standards during chromatography are indicated, (1) sialyltetrasaccharide a, (2) sialyltetrasaccharide b, (3) sialyltetrasaccharide c.

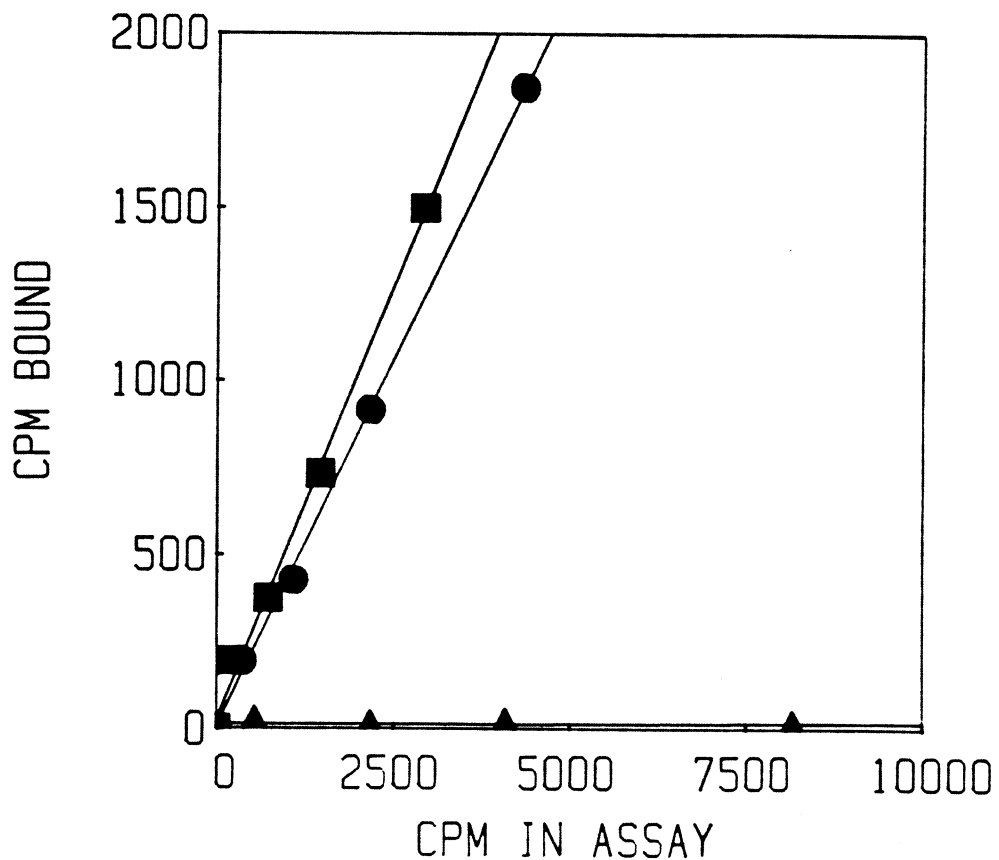


Figure 19. Direct Binding of the Sialyltetrasaccharide from the WGA-unbound Sialylhexaose Fraction With Anti-sialyltetrasaccharide c. The binding assay was performed as described under Material and Methods, using 20 μ l of the antibody and varying amounts of the radiolabeled oligosaccharides. (\blacktriangle) Sialyltetrasaccharide from the WGA-unbound sialylhexaose fraction; (\blacksquare) sialyltetrasaccharide c from WGA-bound sialylhexaose; (\bullet) authentic sialyltetrasaccharide c.

4.0 Conclusions

Lectin affinity chromatography represents a useful method for the separation of the isomeric oligosaccharides found in human milk. Columns substituted with high concentrations of WGA (> 20 mg/ml), were used to purify a single sialylhexasaccharide from the total mixture of human milk sialyloligosaccharides. The purified sialylhexasaccharide was a component of the sialylhexasaccharide fraction, S-5, originally described by Kobata and Ginsburg [21]. The structural analysis of this sialylhexaose indicated that is a sialyl derivative of lacto-N-neohexaose with sialic acid attached to the 3 branch of this structure. The presence of the lacto-N-neohexaose core in the WGA-affinity purified sialylhexaose was determined by sequential exoglycosidase digestion with jack bean β -galactosidase and β -hexosaminidase . The results of the sequential β -galactosidase and β -hexosaminidase digestion were consistent with a branched structure and the results of the β -galactosidase digestion, under the conditions of the assay, indicated the presence of a lacto-N-neohexaose core. The position of sialic acid in the sialylhexasaccharide was determined by simultaneous digestion of the sialyllacto-N-neohexaose with β -galactosidase and β -hexosaminidase, which removed the non-sialylated branch from the sialylhexaose and produced a sialyltetraose. The sialyltetraose was found to be sialyltetrasaccharide c as demonstrated by its elution time on HPLC and direct binding to sialyltetrasaccharide c antiserum. The structural data indicated that the sialylhexaose bound by WGA was different from the sialyllacto-N-hexaose and

sialyllacto-N-neohexaose already described in the sialylhexasaccharide fraction, S-5, of human milk [21]. The WGA-bound sialylhexaose corresponded to a previously undescribed sialyloligosaccharide in human milk which has been recently found as a ganglioside in bovine buttermilk [68]. This oligosaccharide is represented in figure 15.

According to a previous analysis of the sialylhexasaccharide fraction, S-5, of human milk [21], only one sialyltetrasaccharide could be generated by simultaneous digestion of the sialylhexasaccharide fraction with β - *galactosidase* and β - *hexosaminidase* (fig. 16). However, the WGA-bound sialylhexasaccharide fraction generates one sialyltetraose that is different from the sialyltetraose generated by the WGA-unbound sialylhexasaccharide fraction. These data indicate that at least two different sialyltetraoses can be generated from the sialylhexasaccharide fraction, which is contradictory to what has been reported before [21]. The structure of the sialyltetraose isolated from the WGA-bound sialylhexaose corresponded to sialyltetrasaccharide c. The structure of the sialyltetraose isolated from the WGA-unbound sialylhexaose fraction was not determined in this work. However, this sialyltetraose had an elution volume on HPLC different from sialyltetrasaccharide c and differed from any other milk sialyltetrasaccharides available as standards (sialyltetrasaccharides a and b). It most probably corresponds to the sialyltetraose that would be containing the 6 branch of the lacto-N-hexaose or lacto-N-neohexaose core (fig. 16), generated by simultaneous digestion of the sialylhexaose where sialic acid is located on the 6 branch.

The binding of the new sialyllacto-N-neohexaose to WGA was due to weak interactions since very highly concentrated affinity gels (20-25 WGA mg/ml) were required in order to detect binding. The interaction of this oligosaccharide is obviously not due to sialic acid. Based on the specificity of WGA for GlcNAc with free hydroxyl groups at the C-3 position, binding of this novel sialylhexaose by WGA may be explained by the presence in this structure, of two GlcNAc residues unsubstituted at the hydroxyl group of C-3. The observation that the sialyl derivative of lacto-N-neohexaose with sialic acid in the 6 branch [21], was not found in the WGA-bound sialylhexasaccharide fraction, suggests that in addition to the free hydroxyl groups at C-3 of the two GlcNAc residues, the 6 branch of the lacto-N-neohexaose core must be unsubstituted with sialic acid in order to bind WGA.

Although WGA has been shown to bind sialic acid and glycoproteins containing sialic acid, only one human milk sialyloligosaccharide was able to bind WGA in an affinity column system. Binding of the novel sialylhexaose to WGA however, was not due to the presence of sialic acid, moreover the presence of sialic acid prevented binding to WGA. Prevention of binding to WGA by sialic acid in glycopeptides has been reported [54] and it has been explained by the assumption that sialic acid was blocking the the access of internal GlcNAc residues to the WGA binding sites. It has been found that glycoproteins containing highly sialylated oligosaccharides bind very strongly to WGA, but when the oligosaccharides are released from the peptide, they no longer bind WGA [54]. The interaction of sialic acid and WGA has been explained by the similarity in configuration of sialic acid and GlcNAc. However, the tight binding of WGA to glycoproteins containing sialic acid may be due to the high density of oligosaccharide chains with terminal sialic acid residues that are attached to the glycoprotein. As a result, the exterior side of the protein may be envisioned as a polyanionic surface where sialic acid is outwardly directed. Since the concentration of sialic acid residues at the surface of the glycoprotein is high, the probability of productive binding will be increased when WGA is approaching the glycoprotein. In addition, since WGA is a basic protein (pK, 8.6) and sialic acid is negatively charged, it may be that charge attraction could help to stabilize the binding.

In summary, lectin affinity chromatography is a useful method in the separation of the isomeric oligosaccharides from human milk. WGA affinity chromatography was able to separate a previously undescribed sialyloligosaccharide from all human milk sialyloligosaccharides. Since WGA demonstrated more affinity for the neutral core of this sialyloligosaccharide than for the sialyl derivative, WGA affinity chromatography may be a more useful method in the separation of the larger, neutral human milk oligosaccharides which contain poly lactosamine type structures. In addition, other lectins specific for GlcNAc such as the tomato lectin and potato lectin, whose specificities are not well defined, may also be useful in the resolution of the larger neutral human milk oligosaccharides. Structural analysis of the specifically bound oligosaccharides will also provide additional information on the carbohydrate specificities of these lectins.

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