222
24

STRUCTURAL ANALYSIS OF GLYCOLIPID-DERIVED OLIGOSACCHARIDES FROM METABOLICALLY RADIOLABELLED COLORECTAL CARCINOMA SW1116 CELLS

by

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(ABSTRACT)

This dissertation describes the analysis of the carbohydrate portion of glycosphingolipids from colorectal carcinoma cells, SW1116, by metabolically labelling the cells with radioactive monosaccharide precursors. SW1116 cells (1 x 10^6) metabolically labelled with 222 μCi/ml of either 6-[^{3}H]-D-galactose (25 Ci/mMol) or 6-[^{3}H]-D-glucosamine (38 Ci/mMol) for 30 hours, incorporated 1%-3% of the radioactivity into their glycoconjugates. Approximately 63% of the radioactivity recovered in the glycoconjugates corresponded to glycolipids when cells were labelled with 6-[^{3}H]-D-galactose, and about 12% when cells were radiolabelled with 6-[^{3}H]-D-glucosamine. Metabolically radiolabelled glycolipids were separated into neutral (88-91% of the radioactivity recovered in glycolipids) and acidic (9-12% of the radioactivity in glycolipids) fractions by ion exchange chromatography. Glycolipids in these fractions were subjected to ozonolysis and alkali fragmentation to release the oligosaccharide chains from the ceramide portion. Oligosaccharides obtained from the neutral glycolipids were separated into single components by a combination of high performance liquid chromatography (HPLC) and *Ricinus communis* agglutinin I (RCA-I)-agarose affinity chromatography. Oligosaccharides were identified based on the monosaccharide composition, methylation

analysis, and exoglycosidase digestions. Major glycolipid components present in the neutral fraction were, glucosylceramide (Glc-Cer), galactosylceramide(Gal-Cer), galabiosylceramide (Galα1-4Gal-Cer), lacto-N-tetraosylceramide (Galβ1-3GlcNAcβ1-3Galβ1-4Glc-Cer), Le^a-pentaglycosylceramide (Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc-Cer), H1-pentaglycosylceramide (Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc-Cer), a difucosylated lacto-N-tetraosylceramide, and a fucosylated lacto-N-norhexaglycosylceramide. Minor components detected in this fraction corresponded to lactosylceramide (Galβ1-4Glc-Cer), lacto-N-neotetraosylceramide (Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer), and fucosylated and difucosylated lacto-N-neotetraosylceramides. The acidic fraction was separated into monosialylgangliosides and disialylgangliosides by ion exchange chromatography. Monosialyloligosaccharides were further purified on HPLC, and biochemically characterized by methylation analysis, exoglycosidase digestions, and monosaccharide composition. The major component of this fraction corresponded to the sialyl-Le^a glycolipid (NeuAcα2-3Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc-Cer) as previously reported by Magnani et al. [183]. GM3 (NeuAcα2-3Galβ1-4Glc-Cer) (0.42% of radioactivity recovered in glycolipids), sialyltetraosylceramide a (NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc-Cer) (0.46% of radioactivity in glycolipids), sialyltetraosylceramide b (Galβ1-3[NeuAcα2-6]GlcNAcβ1-3Galβ1-4Glc-Cer) (0.21% of radioactivity in glycolipids), and sialyllated fucosylhexaglycosylceramide, were present in minor quantities.

Results from this study demonstrate that metabolic radiolabelling provides a method for the structural analysis of glycolipids, as sensitive as

the immunostaining procedures, as unmistakable as physical techniques (Mass Spectrometry, and Nuclear Magnetic Resonance), and that permits the identification of the majority of glycolipids expressed by a cell line, using relatively small number of cells in culture (6×10^6). Application of this method could be extended to the study of changes in glycolipid accompanying oncogenic transformation and differentiation, glycolipid biosynthesis, intracellular sorting of glycolipids, recycling and turnover.

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DEDICATION

This dissertation is dedicated to my dear parents, Ramon Tarrago and Carmen Medina de Tarrago. And to my beloved husband, Antonio Trani as credit to his love.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Glucose	Glc
Galactose	Gal
Fucose	Fuc
Glucosamine	GlcN
Galactosamine	GalN
N-Acetylglucosamine	GlcNAc
N-acetylgalactosamine	GalNAc
N-Acetylneuraminic Acid	NeuAc
High Performance Liquid Chromatography	HPLC
Thin Layer Chromatography	TLC
Curies	Ci
Counts per Minute	CPM
Ricinus communis agglutinin I	RCA-I
Ceramide Monohexoside	CMH
Ceramide Dihexoside	CDH
Ceramide Trihexoside	CTH
Globoside	Gb_4
Lacto-N-tetraose	LNT
Lacto-N-neotetraose	LNnT
Sialyltetrasaccharide a	STa
Sialyltetrasaccharide b	STb
Sialyltetrasaccharide c	STc

I
INTRODUCTION

Glycosphingolipids are constituents of all eukaryotic cell-surface membranes. Their location in the plasma membrane, in general limited to the outer leaflet, and their potential for structural heterogeneity, determine some of the biological functions that have been ascribed to glycosphingolipids. Some of these functions include, receptors for viruses [31], bacteria [4], bacterial toxins [4, 12, 13], hormones [11], extracellular matrix proteins [32], interferon [15], interleukin-2 [14], and antibodies [16, 17]. One of best defined roles of cell surface glycoconjugates is that of blood group antigens which are specified by monosaccharide sequences at the non-reducing end of the glycoprotein and glycolipid carbohydrate chains [10, 33, 35]. In recent years, a number of studies, have demonstrated that the expression of cell-surface glycolipids is altered in association with embryogenesis, cell differentiation and oncogenic transformation. Some glycolipids such as GM3 have been found to be capable of inducing differentiation of certain leukemic cell lines [128, 129, 131], and arresting cell-growth of cancer cells and fibroblasts [23-26, 133-135]. Furthermore, some of the changes observed in glycolipid expression during mammalian embryogenesis seem to have a role in cell-cell recognition. This is supported by the finding that the stage specific embryonic antigen 1 (SSEA-1), represented by the Le^x carbohydrate determinant (Galβ1-4[Fucα1-3] GlcNAcβ1-R), and expressed at the morula stage (3rd-5th cell division stage) in mouse embryos, appears to be involved in the compaction of the cells at this

stage, since free oligosaccharides, glycolipids or synthetic conjugates carrying the Le^x epitope can inhibit morula compaction [112-116]. Similarly, a number of glycolipid structures that are expressed in cancer tissue, but are either absent or expressed in minor quantities in the normal tissue counterpart, have been defined by the aid of monoclonal antibodies [18, 27, 29]. The significance of the variations in glycolipid expression accompanying oncogenic transformation is still obscure, however, it is clear that the factors inducing malignancy in cells also affect the regulation of glycoconjugate biosynthesis. It is, therefore, important to identify the glycolipid structures expressed by cancer cells since they in turn reflect the synthetic reactions of the glycosyltransferases that are involved in their expression. This information will help to establish the biochemical basis for the synthesis of tumor-associated-glycolipid antigens. In addition, the identification of the glycosyltransferases that are associated in the synthesis of tumor-associated glycolipid antigens will lead to the cloning and localization of their corresponding genes.

The current physical methods for structural analysis of glycolipids include nuclear magnetic resonance (NMR), mass spectrometry (MS), and methylation analysis [199-202]. Although these methods are sensitive, large quantities of cells or tissues are required to obtain amounts of pure glycolipid that are within the levels of detection (micrograms) of these techniques. The immunological methods are much more sensitive, detecting nanogram amounts of glycolipids [67-78, 208, 209]. Since monoclonal antibodies recognize specific determinants wthin a carbohydrate sequence, separate methods are usually required to confirm the presence of an epitope and define

the complete structure. In addition, a battery of monoclonal antibodies is absolutely necessary to completely characterize a complex glycolipid mixture, and consequently detection of all the glycolipid structures in this mixture will require monoclonal antibodies against all possible structures. Since the production of monoclonal antibodies by mice is limited to those carbohydrate epitopes that are able to stimulate an immune response in that animal, those carbohydrate sequences that are not recognized by the immune system of the mouse will not be identified, by methods that rely exclusively on immunological techniques.

In order to establish a method for structural analysis of glycolipid-derived cabohydrates, that is as sensitive as the immunological methods, that provides unequivocal structural definition, and that permits the identification of the whole spectrum of glycolipids synthesized by an specific cell line, we have elected to use a new approach. This approach involves the metabolic labelling of cells in culture with radioactive monosaccharide precursors, isolation of the radiolabelled glycolipids, release of the oligosaccharide chain from the ceramide portion by chemical (ozonolysis and alkali fragmentation) or enzymatic methods (endoglycoceramidase), purification of the different species by serial lectin affinity chromatography, and structural analysis of pure glycolipid-derived oligosaccharides by a combination of classical methods such as methylation and exoglycosidase digestions. This technique has the advantage that small amounts of cells in culture are used to isolate sufficient quantities of radiolabelled oligosaccharide mixtures which can be separated into single species and structurally analyzed. Kornfeld and co-workers [212-220] who were the pioneers in the metabolically labelling of

cells in culture with radiolabelled monosaccharides, have used this approach to analyze the carbohydrate chains of biologically important glycoproteins such as the low density lipoprotein (LDL) receptor, and to delineate the biosynthesis and sorting of glycoproteins within cells. This is a powerful method for the analysis of the structure and biosynthesis of glycoconjugates in cultured cells, however, it has never been applied to the structural analysis of glycolipids. This dissertation describes the application of this technique to the structural analysis of the glycolipids expressed by the colorectal carcinoma cell line SW1116.

The colorectal carcinoma cell line SW1116 was selected for this study because it has been used to produce the monoclonal antibody NS-19-9 which defines an oncofetal antigen that is expressed by gastrointestinal and pancreatic carcinomas. The epitope of this antigen is a specific carbohydrate sequence, that is carried by glycolipids at the cell-surface, and by secreted mucin-type glycoproteins. The structure of the 19-9 antigen corresponds to a sialylated form of the human Le^a determinant (NeuAcα2-3Galβ1-3[Fucα1-4] GlcNAcβ1-3Gal-R). Due to the elevated levels of mucin-type glycoproteins bearing the sialyl Le^a determinant, in the blood serum of patients with gastrointestinal and pancreatic cancer, the NS-19-9 monoclonal antibody has been used as an immunodiagnostic tool to detect those malignancies. In spite of the usefulness of the NS-19-9 antigen in the diagnostic of gastrointestinal and pancreatic cancer, its application is limited to those patients that express the Lewis gene. Individuals who lack an active Lewis gene are unable to synthesize any Lewis antigens, including the sialyl Le^a, in normal or cancerous tissues. For this reason, identification of other tumor-associated

antigens whose expression does not depend on the blood group phenotype of the individual will be more helpful in the development of monoclonal antibodies used to detect and treat human cancer. For example, the precursor of the sialyl Le^a glycolipid, sialyllacto-N-tetraosylceramide a (STa-Cer) (NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc-Cer) which is also an oncofetal antigen, as it has been detected in human meconium [221], may be a potential candidate for the development of monoclonal antibodies specific for gastrointestinal malignancies. Another glycolipid, which is also an oncofetal antigen [222], and has a related structure, STb-Cer (Galβ1-3[NeuAcα2-6] GlcNAcβ1-3Galβ1-4Glc-Cer), may qualify too for the production of monoclonal antibodies with unique specificity for gastrointestinal cancer. Both STa- and STb-ceramide have been detected in the monosialylganglioside fraction of SW1116 cells [223]. In addition, the glycolipid disialyllactotetraosylceramide, which is an isomer formed by both STa and STb (NeuAcα2-3Galβ1-3[NeuAcα2-6] GlcNAcβ1-3Galβ1-4Glc-Cer) may also be useful in the development of monoclonal antibodies that selectively react with gastrointestinal cancer.

In this project, I used the colorectal carcinoma cell line SW1116 as a model to define the glycolipid pattern expressed on a colorectal carcinoma cell line, by the application of a metabolic labelling procedure for the structural analysis of oligosaccharides derived from glycolipids. Characterization of the glycolipids synthesized by SW1116 cells may serve to identify potential blood-group-independent tumor markers, which in the future may be targeted for the production of monoclonal antibodies that will be valuable in the diagnosis and therapy of cancer. Furthermore, knowledge of the glycolipid structures expressed by SW1116 cells will indicate which glycosyltransferases are

involved in the synthesis of these structures, and consequently it will help to delineate their biosynthesis. Ultimately, the data on the glycosyltransferases can provide the tools to clone the genes for these enzymes.

II

LITERATURE REVIEW

2.1 CELL-SURFACE GLYCOSPHINGOLIPIDS AND THEIR FUNCTIONS

Glycosphingolipids occur in nature as components of the cell membranes of eukaryotic organisms[1] . Glycosphingolipids are amphipathic molecules consisting of a lipophilic portion composed of sphingosine substituted at the amino group by a fatty acid (ceramide), and a hydrophilic moiety formed by a carbohydrate that is linked to the first hydroxy group of the sphingosine (see figure 1). The carbohydrate portion of glycolipids may be composed of glucose, galactose, N-acetylglucosamine, N-acetylgalactosamine, fucose and sialic acid; mannose and glucuronic acid occur in non-vertebrate glycolipids, glucuronic acid, although rare, has also been reported in glycoconjugates of human neural tissues [1]. Even though only six types of monosaccharides occur in glycolipids, a great diversity of structures may exist based on the monosaccharide sequence, types of anomeric linkages (α or β), positions of substitution of the linkages, and the length of the oligosaccharide chain. In fact, glycolipids have been classified into series [2, 3, 4] depending on their oligosaccharide backbone, some examples are showed in table I. The basic core structure in each of these series can be modified by addition of fucose, sialic acid or sulfate; by elongation of the chain, repeating certain sequences or adding new ones in a linear or branched fashion; and even by attachment of blood group-antigenic residues at the end [4].

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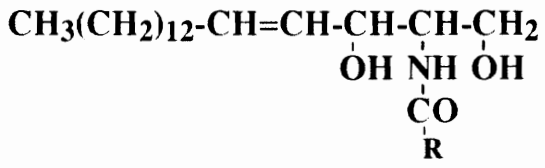
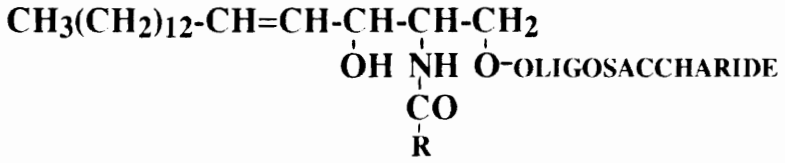
[1] This text will only deal with glycolipids from vertebrate animals.



Ceramide

R=FATTY ACID



Glycosphingolipid

Figure 1.- Chemical structures of a ceramide and a glycosphingolipid.

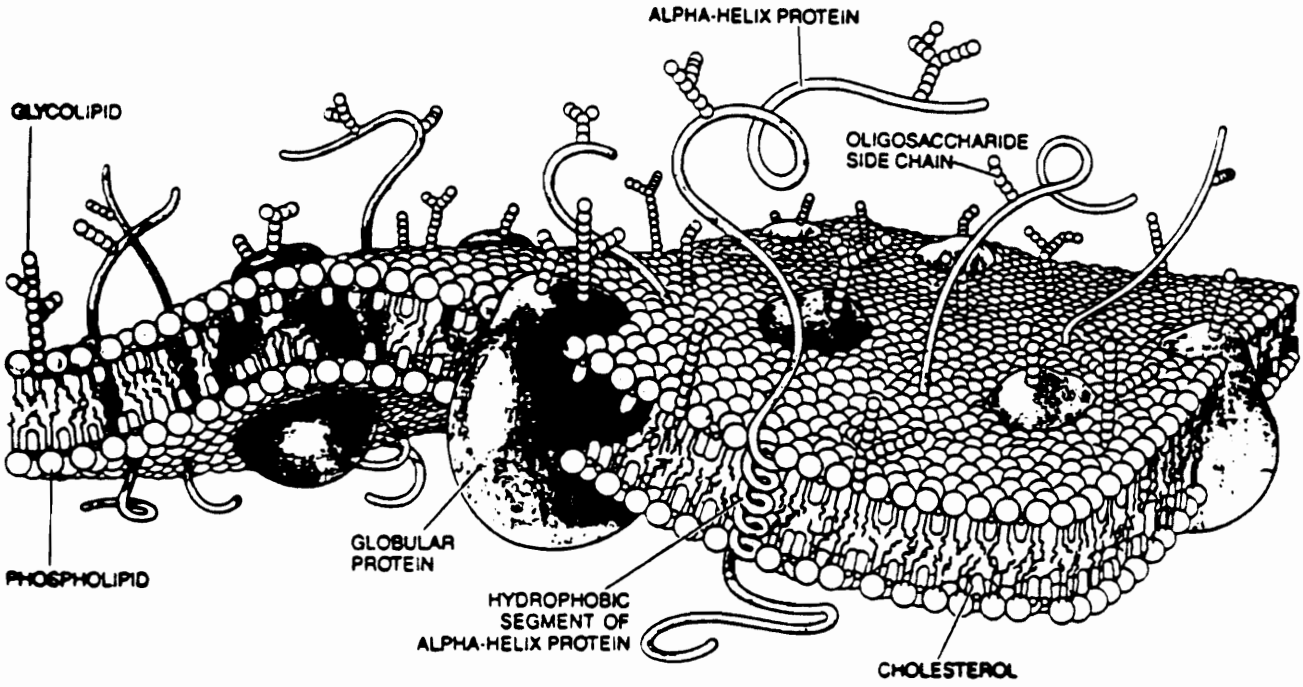


Figure 2.- Orientation of the oligosaccharide portions of glycoproteins and glycolipids in the plasma membrane.

TABLE 1

Examples of the Glycolipid Series

Structure	Name
Gala Series	
Gal-Cer	Galactosylceramide
Galα1-4Gal-Cer	Galabiosylceramide
Glucosylceramide and Lactosylceramide	
Glc-Cer	Glucosylceramide
Galβ1-4Glc-Cer	Lactosylceramide
Globo and Isoglobo Series	
Galα1-4Galβ1-4Glc-Cer	Globotriaosylceramide
Galα1-3Galβ1-4Glc-Cer	Isoglobotriaosylceramide
GalNAcβ1-3Galα1-3Galβ1-4Glc-Cer	Globoside
GalNAcα1-3GalNAcβ1-3Galα1-3Galβ1-4Glc-Cer	Forssman
Ganglio Series	
GalNAcβ1-4Galβ1-4Glc-Cer	Gangliotriaosylceramide
Galβ1-3GalNAcβ1-4Galβ1-4Glc-Cer	Asialo GM1
Lacto and Neolacto Series	
GlcNAcβ1-3Galβ1-4Glc-Cer	Lactotriaosylceramide
Galβ1-3GlcNAcβ1-3Galβ1-4Glc-Cer	Lacto-N-tetraosylceramide
Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer	Lacto-N-neotetraosylceramide

It is interesting to note that all the oligosaccharide chains in glycolipids contain lactose at the reducing end, which is linked to to the ceramide through the glucose, with the exception of those in galactosylceramide, and galabiose which contain only galactose, and are linked to the ceramide through the reducing galactose. Glucosylceramide which may be another exception, is obviously the precursor for lactosylceramide. The presence of lactosylceramide (Gal-Glc-Cer) in most glycolipids may be compared to the core Man_3-GlcNAc-GlcNAc-Asn that all asparagine-linked oligosaccharides contain, and also to the core GalNAc-Ser/Thr that all O-linked oligosaccharides share. These three distinct cores specific to each glycoconjugate specie probably reflect the separation of the three different pathways of biosynthesis of these glycoconjugate types.

The ceramide portion of glycolipids also may vary in the degree of hydroxylation, unsaturation and chain length of the fatty acid and the long chain base [3, 5].

It is generally believed that glycosphingolipids primarily are located in the plasma membrane, however, several investigators have found that some glycolipids are constituents of internal membranes [6, 7, 8]. For instance, Matyas and Morre' [8], observed that 76% of total gangliosides from rat liver were associated with the plasma membrane, and that the remaining gangliosides were detected in the endoplasmic reticulum, Golgi apparatus and the mitochondrial fractions. Moreover, they found that the ganglioside composition of the internal membranes was different from that of the plasma membrane. Symington et al. [9] have also shown that most of the

lactosylceramide in polymorphonuclear neutrophils is present in internal lysosomal granules.

The localization of glycolipids in the plasma membrane is limited to the external layer where the hydrophobic ceramide portion is inserted, while the carbohydrate chain is facing the aqueous extracellular space (see fig. 2). Due to the particular location of glycolipids in the cell membrane, their oligosaccharide chains are antigenic. For example, the human ABH and Lewis blood group systems are carried by carbohydrate determinants on glycolipids as well as glycoproteins in the erythrocyte plasma membrane [10]. Likewise, glycolipids may interact at the cell surface with different biological substances, operating as receptors for several hormones [11], bacterial toxins [12, 13], bacteria [4], lymphokines [14], interferon [15] and antibodies [16, 17]. In recent years, a large number of studies have indicated that the occurrence of glycosphingolipids and glycoproteins in eukaryotic cells is not accidental, but that they may have a relevant function in diverse physiological activities such as cell to cell communication [18]; cell adhesion [19, 20]; recognition markers during embryo development and cell differentiation [21, 22]; regulation of normal cell growth and during oncogenic transformation [23-26]; and finally as tumor-associated carbohydrate antigens [27-32].

Some of these cellular functions conferred by glycolipids will be discussed in more detail in this section.

2.1.1 Blood Group Antigens

The blood group antigens ABO, H, and Lewis are determined by specific carbohydrate structures carried by the glycoproteins and the glycolipids of

the human erythrocyte plasma membrane [10, 33-35]. Blood group antigens also are destributed in other human tissues such as the epithelial cells of the digestive, respiratory and urinary tracts [35-38]; and in body secretions like saliva, intestinal mucus, and human milk where they mainly exist as glycoproteins or as free oligosaccharides in human milk.

The blood group antigens ABO, H and Lewis are expressed at the nonreducing end of the oligosaccharide chains of glycoproteins and glycolipids. The synthesis of these antigens requires the availability of the appropriate precursor molecules. Those precursors are designated as type 1 chain (Galβ1-3GlcNAcβ1-R) [39], type 2 chain (Galβ1-4GlcNAcβ1-R) [39], type 3 chain (Galβ1-3GalNAcα1-R) [40, 42], and type 4 chain (Galβ1-3GalNAcβ1-R). R may be an N-linked oligosaccharide chain (.....Man_3-GlcNAc-GlcNAc-Asn), or and O-linked oligosaccharide chain (....GalNAcα1-Ser/Thr) in glycoproteins; and lactosylceramide (Galβ1-4Glc-Cer) in glycolipids [44]. Type 1 and type 2 chain-blood group active glycolipids are based on lacto-N-tetraosylceramide (Galβ1-3GlcNAcβ1-3Galβ1-4Glc-Cer) and lacto-N-neotetraosylceramide (Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer) respectively. Type 3 and 4 have been recently discovered and they have been found only in blood group A individuals [34, 48]. Also, type 4 chain is found only in glycolipids but not in glycoproteins since its structure is based on the glycolipid, globoside (GalNAcβ1-3Galα1-4Galβ1-4Glc-Cer) [43]. This text will be mostly concerned with type 1 and type 2 chains since most of the blood group active structures are based on this two classes of sequences.

The precursor oligosaccharide chains are synthesized by the sequential addition of monosaccharide residues, catalyzed by specific

glycosyltransferases. The glycosyltransferases are the primary products of the genes encoding their protein sequence, consequently, the resulting oligosaccharide chains are the secondary products of those genes. The expression of the final blood group antigens depends on the presence of the H, ABO, and Lewis genes which also encode specific glycosyltransferases. The H gene is considered a public gene since everybody expresses it, except for the rare Bombay blood group type. The H gene encodes an α1-2fucosyltransferase that transfers a fucose in an α1-2 linkage to the non reducing end galactose of any type of the precursor chains (see figure 3) The expression of the H blood group oligosaccharides in erythrocytes only depends on the presence of the H gene. However, the expression of H-active antigens in epithelial cells and in body secretions relies on the existence of another gene, the Secretor gene (Se). It is believed that the Se gene is a structural gene expressed in secretory and in epithelial tissues, that codes for another α1-2fucosyltransferase with different acceptor specificity from that of the one encoded by the H gene expressed in hematopoietic tissue [45]. A series of experiments have suggested that the Se gene fucosyltransferase acts preferentially on type 1 chain [33, 35], whereas, the H gene fucosyltransferase primarely fucosylates type 2 acceptor chains [33] (see section 2.2). The presence or absence of H-type substances in saliva or other body secretions has been used as an indicator of the expression of the Se gene. Individuals expressing H-type antigens in saliva are typified as secretors. In Europe, 80% of the population is secretor [33].

The expression of A, B, or AB antigens relies on the presence of active A and B genes. The A gene encodes an α1-3-N-acetylgalactosaminyltransferase and the B gene encodes an α1-3galactosyltransferase. Both A and B enzymes use as

precursor H-type antigens (see figure 3). The A and B transferases are expressed in hematopoietic tissue, epithelial tissue, and glandular tissue, regardless of the secretor status of the individual. However, the A or B type oligosaccharide chains will only appear in the body secretions or epithelial cells of secretor individuals, since their biosynthesis requires the H-type precursors (fig. 3).

The expression of the Lewis antigens, Le^a and Le^b, is dependent on the existence of an active Lewis gene, which encodes an α1-4fucosyltransferase. This enzyme catalyzes the transfer of an α1-4 fucose to the subterminal GlcNAc residues of either type 1 or H-type 1 precursors, producing the Le^a and Le^b antigens respectively (fig. 3). The Le^a antigens are expressed in all glandular and epithelial tissues of secretors and non-secretors. But the expression of Le^b antigens in those tissues is dependent on the secretor status of the individual [33, 46] since the formation of Le^b requires H-type 1 precursor structures. The Le^a and Le^b antigens are not endogenously expressed in the erythrocytes. However, the erythrocyte plasma membrane passively acquires Lewis active glycolipids from the blood where they are carried by plasma lipoproteins. The site of synthesis of these Lewis active glycolipids present in the blood is still unknown.

The A and B type 1 antigens also can serve as substrates for the Lewis gene fucosyltransferase, generating the ALe^b and BLe^b hybrid structures (fig. 3). Nevertheless, if the Le^b antigen is formed first, the A or B transferases are unable to use this structure as precursor to form the ALe^b and BLe^b antigens. Since no further elongation of the precursor chains occurs following addition of the α1-4 fucose to the GlcNAc by the Le gene fucosyltransferase, this step is

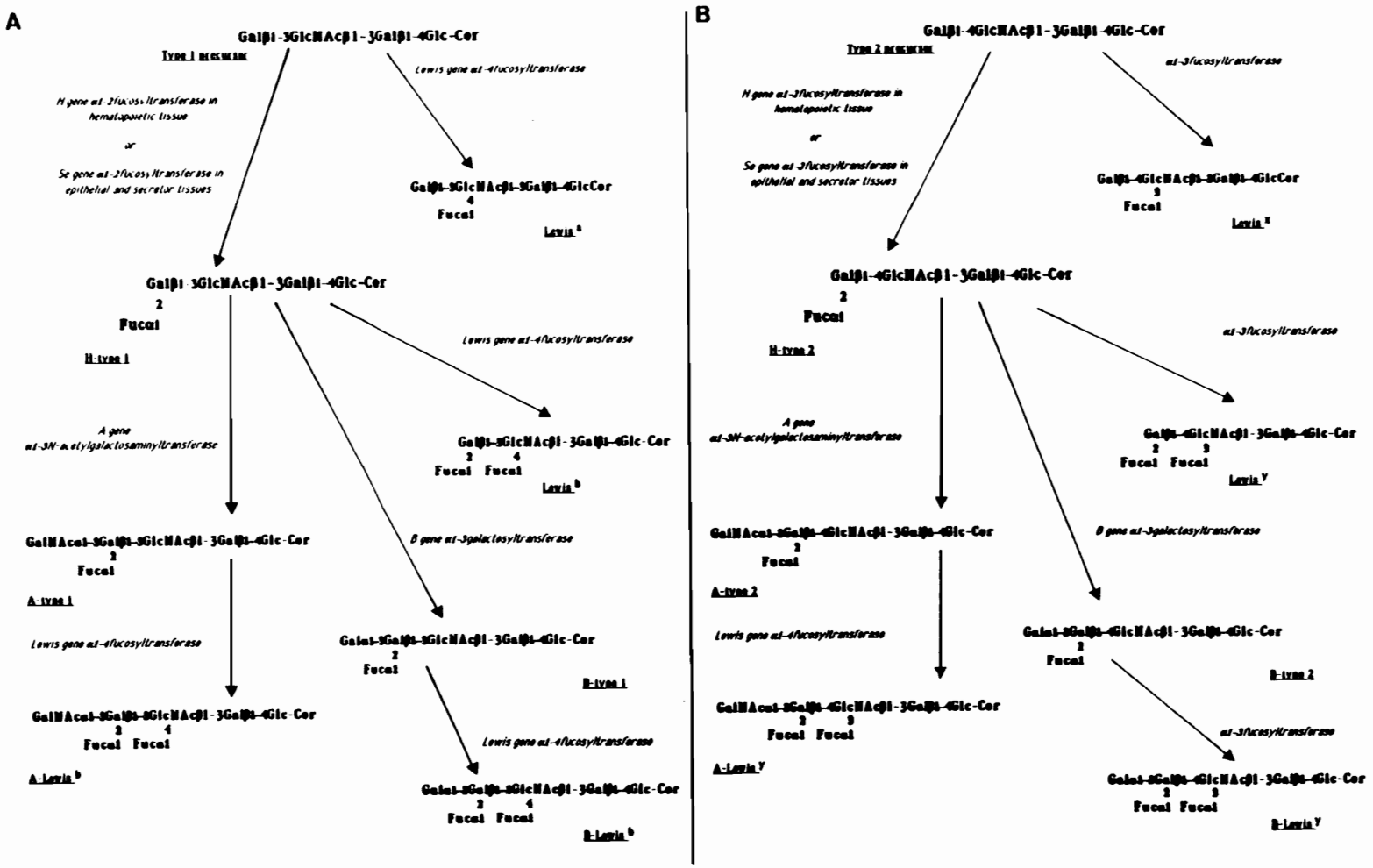


Figure 3.- **Biosynthesis of blood group antigens.-** Panel A, biosynthesis of blood group antigens using type 1 chain as precursor. Panel B, biosynthesis of blood group antigens using type 2 chain precursor.

considered to be a chain termination event. At some point in the synthesis of blood group active glyconjugates, there is going to be competition among the H, A, B, and Le glycosyltransferases for the same precursor molecules. For example, an individual who is blood type A, secretor and Le (a^-, b^+), expresses Le^a, Le^b and ALe^b antigens in body secretions and epithelial cells. The same individual, should have glycolipids in the erythrocyte membrane bearing these structures that were passively taken up from the blood, however, the glycoproteins should only carry H and A antigens.

The type 2 chain structures also can be fucosylated at the subterminal GlcNAc in a similar way as the type 1 chain is fucosylated by the Lewis enzyme. This enzyme is an α1-3fucosyltransferase that adds fucose to the 0-3 position of the subterminal GlcNAc in type 2 chain and H-type 2 chain (fig. 3). The corresponding structures are designated as Le^x and Le^y antigens, which are positional isomers of the Le^a and Le^b respectively. The α1-3fucosyltransferase is not a product of the Le gene, and it is expressed by all individual regardless of their secretor status [47, 48].

2.1.2 Cell Development and Differentiation

The expression of carbohydrates associated with glycolipids and glycoproteins is regulated during the different stages of embryo formation and development, and cell differentiation. For instance, the stage specific antigen 1 or SSEA-1, which is determined by the Le^x epitope (Galβ1-4[Fucα1-3]GlcNAcβ1-R) carried by both glycoproteins and glycolipids, is expressed at the third to fifth division stage or morula stage (8-32 cells) in mouse embryos [112-114]. At this stage the embryonic cells start the process of compaction, in

which they actively stick one to another. After morula compaction the concentration of SSEA-1 decreases markedly. It is believed that the SSEA-1 antigen is involved in the compaction of the embryonic cells, since addition of Le^x determinant as a free oligosaccharide or conjugated to lipid or protein, resulted in the inhibition of the adhesion process [114-116]. These findings inferred that the cohesion of the embryonic cells at the morula stage may be mediated by the interaction of SSEA-1 and a receptor protein. It has been hypothesized that the receptor could be a cell-surface glycosyltransferase [138], a glycosidase, or a lectin; recently, it has been proposed that the receptor is actually the SSEA-1 antigen itself, and that a carbohydrate-carbohydrate interaction may occur [116].

Due to the limited availability of mouse embryos, teratocarcinoma cells have been used as models to study the early embryonic stages. Teratocarcinoma cells or embryonal carcinoma (EC) cells as they are called, are very similar to the multipotential cells of the premature phases of embryo development [117]. These cells when treated with retinoic acid, a morphogen, differentiate into somatic cells such as neurons [118]. Fenderson et al. [119], when examining the glycolipids of the human EC cells TERA-2 and those of the retinoic acid differentiated equivalent, detected that differentiation is accompanied by a change in the glycolipid pattern of these cells. The stage specific embryonic antigen 3 (SSEA-3) (GalNAcβ1-3Galα1-4Gal...) is a globo-series glycolipid that appears in embryonic cells at the 1 cell stage. Fenderson et al. found, by using monoclonal antibodies, that SSEA-3 and other globo-series glycolipids were mainly expressed in TERA-2 cells. By contrast, when TERA-2 were induced to differentiate in the presence of retinoic acid, they found that most of the

glycolipids expressed switched from globo-series to lacto-series (Le^x, sialylparagloboside) and ganglio-series (GM3, GD3, GM2, GD2) glycolipids. The same group of investigators [120], recently demonstrated that the changes observed in the TERA-2 glycolipids after retinoic acid differentiation coincided with variations in the activities of the glycosyltransferases associated with their biosynthesis. For example, they showed that the α1-4galactosyltransferase required for the synthesis of globotriaosylceramide (Galα1-4Galβ1-4Glc-Cer), which is the precursor for all globo-series structures, declined 3-4-fold after differentiation of the TERA-2 cells; on the other hand, the activity of the β1-3N-acetylglucosaminyltransferase, necessary for the formation of lactotriaosylceramide (GlcNAcβ1-3Galβ1-4Glc-Cer), the precursor for the lacto-series glycolipids, increased 4-fold. Likewise, the α1-3fucosyltransferase, responsible for the synthesis of fucosylated lacto-series glycolipids such as Le^x, increased 2-fold after differentiation. In a similar way the activity of the α2-3sialyltransferase that synthesizes GM3 and other ganglio-series glycolipids, increased 4-fold as compared to the undifferentiated cells.

The pluripotent bone marrow-stem cells differentiate to become mature blood cells [121]. Bone marrow stem cells can develop through two types of pathways, hematopoietic and lymphopoietic. The hematopoietic pathway gives rise to erythrocytes, granulocytes, monocytes and natural killer cells; the lymphopoietic pathway generates B-lymphocytes, and T-cells. Leukemia cells which are representative of intermediate phases along the differentiation pathway, have been very useful in studying the differentiation and ontogeny of cells. Similarly they have also been helpful in examining the

transformations that occur in glycolipids in parallel with cell differentiation. For instance, the human promyelocytic cell line HL-60 can be induced to differentiate along different lineages depending on the chemical used. When HL-60 cells are treated with TPA (phorbol ester) the cells mature into macrophages, but when exposed to DMSO (dimethylsulphoxide) they differentiate into granulocytes [122]. Exposure to either one of these agents, besides producing differentiation of the cells, also inhibits growth at a certain point. The content of acidic glycolipids with a lacto-series core increases when HL-60 differentiate into granulocytes, whereas differentiation of these cells into macrophages is accompanied by a significant increase in ganglioside GM3 [123]. In the same way, the myeloid leukemia cell line M1, can be induced to differentiate into granulocytes or macrophages by exposure of the cells to various substances such as retinoids [124], glucocorticoids [125], and lymphokines [126]. M1 cells that differentiate into macrophages [127] show a marked increase in the quantity of globotriaosylceramide (Galα1-4Galβ1-4Glc-Cer), that corresponds with a 10-fold increment in the activity of the α1-4galactosyltransferase associated with the synthesis of this glycolipid. In contrast, the undifferentiated M1 leukemia cells mostly express gangliotriaosylceramide (GalNAcβ1-4Galβ1-4Glc-Cer), showing a shift from ganglio-series to globo-series glycolipids upon differentiation. This is similar to the shift discussed above for the differentiation of embryonal carcinoma cells TERA-2, with the exception that the switch in M1 cells was opposite to that observed in the TERA-2 cells (globo-series to ganglio- and lacto-series). In any case, all these findings strongly suggest that glycolipid metabolism and its regulation are intimately involved in the events that occur during cell

differentiation and in the somehow inverse process of oncogenic transformation in which cells revert to a more undifferentiated phenotype. However, the role that glycolipids play in these changes is not clear.

In recent investigations, some clues about the underestanding of the function of glycolipids in cell differentiation and oncogenic transformation have been found. For example, various research groups have reported that ganglioside GM3 and other glycolipids induce differentiation of leukemic cell lines as well as inhibition of cell growth [134, 135]. As previously mentioned, when HL-60 cells differentiate into macrophages by exposure to phorbol esters, the cells dramatically increased the synthesis of GM3 [123] which correlated with an increase on the levels of the α2-3sialyltransferase that synthesizes GM3 [128]. Surprisingly, HL-60 cells, grown in the presence of exogenously added ganglioside GM3, differentiated into macrophage cells in an analogous manner as when they were treated with phorbol esters [129]. Likewise, the myeloid leukemia cell line M1 was also induced to differentiate by addition of GM3 to the culture media [131]. Other neutral glycolipids like lactosylceramide and globotriaosylceramide also caused differentiation of M1 cells but to a lesser degree [131]. Moreover, neo-lacto series gangliosides, which increased during granulocytic differentiation of HL-60 cells induced by DMSO, also provoked granulocytic differentiation of HL-60 cells when exogenously added [130] to the culture media. These results indicate that the glycolipids that specifically increase during cell differentiation may actually prompt the differentiation process.

When glycolipids are exogenously added to cells they are incorporated into the surface membrane [131-135] as for example, Lewis active glycolipids are

adsorbed by the erythrocyte membrane [section 2.1.1]. It has been speculated that the insertion of glycolipids in the plasma membrane may affect the architecture of the membrane and consequently influence the functions of cell surface enzymes or receptor glycoproteins that are directly involved in differentiation of the cells [130-132]. Xia et al. [122] recently demonstrated that, associated with phorbol esters (TPA), DMSO or GM3 induced differentiation of HL-60 cells, there was a 2-fold increase in the activity of protein kinase C. These authors [122] indicated that TPA, DMSO or GM3 can be incorporated into the plasma membrane; and that the incorporation of any of these agents may cause a change in the membrane topography which in turn alters the conformation of protein kinase C by increasing its affinity for any of its activators such as Ca^{+2} and phosphatidylserine. However, phorbol esters bind directly to protein kinase C, increasing its activity and changing its cellular location [136]. Protein kinase C, which mediates many cellular processes, is believed to play a key role in the differentiation of HL-60 cells induced by phorbol esters. Stevens et al. [136] were able to block the phorbol ester-dependent differentiation of HL-60 cells by treating the cells with sphingosine and sphinganine, two potent inhibitors of protein kinase C [137]. Nevertheless, sphingosine or sphinganine were not able to block the differentiation of HL-60 cells induced by ganglioside GM3; indicating that the mechanisms by which phorbol esters and GM3 cause the macrophage maturation of HL-60 cells are different. These results can also be interpreted in another way; Xia et al. [122], also reported that treatment of HL-60 cells with phorbol esters, and DMSO, besides increasing the activity of protein kinase C, also increased the activity of the α2-3sialyltransferase responsible for the

synthesis of GM3; however, exposure of HL-60 cells to exogenously added ganglioside GM3 inhibited the α2-3sialyltransferase, probably by end-product inhibition. The authors suggested [122] that the increase in the synthesis of GM3 and the activity of protein kinase C are events that are linked to the differentiation of HL-60 cells. They also suggested that the activity of the α2-3sialyltransferase was regulated by protein kinse C by a phosphorylation mechanism [122]. This explanation, in conjunction with the results of Stevens et al. [136], leads one to think that the events occurring during the differentiation of HL-60 cells happen perhaps in the following order, first phorbol esters bind to protein kinase C and activate it, activated protein kinase C phosphorylates the sialyltransferase (most likely a membrane bound sialyltransferase), that will synthesize ganglioside GM3 from lactosylceramide present in the membrane, and finally GM3 will start the differentiation process by an unknown mechanism. This theory agrees with the fact that inhibitors of protein kinase C block differentiation by phorbol esters, probably due to the incapability of protein kinase C to turn-on the α2-3sialyltransferase by phosphorylation, which leads to no synthesis of GM3, which if this theory is correct, is the differentiation agent. Also supporting this theory is the fact that inhibitors of protein kinase C do not halt differentiation induced by exogenously added GM3 since endogenously synthesized GM3 is no longer required to start the differentiation process. A mechanism by which GM3 triggers the differentiation process could be by insertion of the exogenous GM3 in the membrane bilayer, internalization and then binding to an inner cytoplasmic receptor; the receptor-GM3 complex is then targeted to the nuclei where it recognizes a DNA sequence where it binds

and initiates the differentiation of the HL-60 cells, in a similar fashion as steroid hormones behave. On the other hand, glycolipid micelles [130] in the culture media may interact with some receptor at the cell surface and start differentiation by another mechanism. Or it may happen that the exogenous GM3 is inserted in the membrane but it can bind to a receptor protein in the plasma membrane which in turn can be endocytocized as a receptor-ganglioside complex, initializing the differentiation in another way.

Glycolipids, especially gangliosides which are abundant in neural tissues, also have been implicated in the differentiation and growth of neuronal cells. For example, ganglioside GM1 alters the growth of cultured astrocytes and prevents rat astroglial cells from changing morphology from flat- to star-shaped when induced with neurotansmitters and neuropeptides [139, 140]. Moreover, exogenously administered gangliosides provoke the extension of neuronal fibers of normal nerves in vivo and in vitro and also the extension of axons in neuronal cell lines in vitro [141]. Ariga et al have reported the accumulation of globo series glycolipids in a subclone (PC12h) of PC12 pheochromocytoma cells, derived from a rat adrenal medullary tumor [142-145]. PC12 cells, when treated with physiological concentrations of nerve growth factor (NGF), were converted into the subclone PC12h which displayed morphological and physiological characteristics of a sympathetic neuron-like phenotype. In these cells, globoside was 36% of the neutral glycolipids, however, they expressed other glycolipids with unique globo series core structures such as Galα1-3Galα1-4Galβ1-4Glc-Cer (40% of neutrals) [144] and Galα1-3Galα1-3Galα1-4Galβ1-4Glc-Cer [143]. Ariga et al have suggested that the presence of those glycolipids may be due to the induction of an α-

galactosyltransferase in the subcloned PC12h cells, since the parent cells PC12 only expressed globoside as their major neutral glycolipid and did not contain any of the other atypical globo series glycolipids. The same cells also contained particular gangliosides such as fucosyl-GM1 and fucosyl-GD1b and the α1-3 galactosylated derivatives of these gangliosides [145].

2.1.3 Tumor-Associated Carbohydrate Antigens

The expression and metabolism of the oligosaccharide structures in glycolipids and glycoproteins are greatly affected during oncogenic transformation. The notion of tumor-associated carbohydrate antigens has been established by the fact that monoclonal antibodies recognize specific glycolipid structures in the tumor cell surface which may be present in amounts in the corresponding plasma membrane of normal cells, below the limits of immunological detectection [27]. Most of the monoclonal antibodies that recognize cancer-related carbohydrate epitopes on glycolipids or glycoproteins have been produced by immunizing animals with tumor cells [31, 55] or their glycolipid extracts. The B-lymphocyte clones that produce the monoclonal antibodies have been chosen on the basis of their reactivity to the tumor cell-surface. The antigenicity of glycolipids at the cell surface depends on various factors [27, 29]. One of them is that the glycolipid antigen should be in high density at the cell surface to interact with low affinity immunoglobulins; and the other is that the antigen has to be exposed or accessible at the plasma membrane in order to be recognized by the antibody [56]. As a consequence glycolipids that are not expressed in high amounts or that are shielded by glycoproteins or longer-carbohydrate-chain glycolipids

at the plasma membrane would not be identified on the basis of antibody reactivity. Considering these facts, not all the the glycolipids present in the surface of tumor cells can induce an immune response in animals, and neither can they be detected at the cell surface by monoclonal antibodies previously made against other antigens. One way to circumvent these drawbacks, has been to immunostain glycolipids on thin layer plates, which, of course, eliminates the obstacle of glycolipids not being recognized by antibodies due to their crypticity at the cell surface. In addition, since the immunostaining technique is very sensitive, detecting nanogram amounts of glycolipids, and the glycolipid antigens will be concentrated in a band on the thin layer plate, the limitation of the glycolipid antigen of having to be in high density at the cell surface to react with the low affinity antibody is excluded. Both the immunostaining of tumor cells and that of their glycolipid extracts on thin layer plates have been used to define many tumor-associated-carbohydrate antigens. In any case, the two methods have been helpful, and the monoclonal antibodies have been very valuable tools to detect carbohydrate structures in tumor cells. However, the use of the word glycolipid "antigens" may not be entirely accurate in all the cases, and perhaps it may be more proper to call them tumor-associated carbohydrate structures or molecules rather than tumor-associated carbohydrate "antigens", since alterations in the glycolipid structures do take place as a consequence of the malignant state. Yet, those alterations might or might not be immunogenic at the tumor cell surface.

Hakomori has classified the changes observed in the glycolipids during malignancy into two categories, according to the carbohydrate structures expressed in the cancer tissue and that of the normal counterpart [18, 27, 29].

One is the accumulation of precursor carbohydrate molecules due possibly to the blocked expression of one or more glycosyltransferases required for the biosynthesis of the complete structure found in the normal tissue; and the other is the excessive expression of glycolipid structures by the tumor cells, that are not synthesized by the normal cells and if they are, they are synthesized in insignificant amounts. The latter type of change might be caused by the activation of a glycosyltransferase that is expressed by the normal cells in very low amounts, but after transformation it is highly synthesized, or, perhaps by the deletion or inhibition of a glycosidase that is required in the normal tissue to process the particular glycolipid that is present in high amounts in the corresponding transformed tissue.

Accumulation of precursor molecules has been observed in certain epithelial cancers, where the ABO and Lewis blood group molecules expressed by the normal epithelial cells are deleted in the malignant state. For example, individuals that are secretors and with blood group type A or B, normally express A or B antigens in the glycoconjugates of the "proximal" colon epithelia. However, during oncogenic transformation of the epithelial cells in this part of the colon, several investigators have found a deficit in the expression of carbohydrates containing A, B, or H antigenicity, and the corresponding accumulation of the precursor structures [57-62].

The appearance of novel carbohydrates structures in the glycoconjugates of many different cancer tissues is probably the most widespread phenomenon observed in the numerous studies done concerning tumor-associated carbohydrate antigens [63-90]. For instance, Hakomori and other investigators have identified a diversity of sialylated and/or fucosylated derivatives of lacto

and lacto-neo series glycolipids, with unusual oligosaccharide structures, in many human adenocarcinomas [63-78], such as the di- and trimeric Le^x glycolipids in gastrointestinal and lung carcinomas [63, 64, 68, 69]. Another example are human melanomas which synthesize large amounts of gangliosides, GM3 (NeuAcα2-3Galβ1-4Glc-Cer) and GD3 (NeuAcα2-8 NeuAcα2-3Galβ1-4Glc-Cer), and low amounts of GM2 (NeuAcα2-3[GalNAcβ1-4]Galβ1-4Glc-Cer) and GD2 (NeuAcα2-8NeuAcα2-3[GalNAcβ1-4]Galβ1-4Glc-Cer) [79-90]. In contrast, normal melanocytes contain mainly GM3, low amounts of GM2 and traces of GD3. The ratio of GM3:GD3 changes from 19:1 to 1:16 when neoplastic transformation of melanocytes occurs [79], suggesting that the α2-8sialyltransferase that synthesizes GD3 (NeuAcα2-8NeuAcα2-3Galβ1-4Glc-Cer) from GM3 (NeuAcα2-3Galβ1-4Glc-Cer) is activated after malignant transformation. Moreover, some of the sialic acid residues of GM3 and GD3 from melanomas have been found to be O-acetylated [79], or in the lactone conformation [57, 81]. Expression of particular gangliosides also has been reported in human neuroblastoma which synthesizes GD2 as the major ganglioside [91]; in hepatomas which accumulate ganglioside GM2 [92]; and in mouse and human T-lymphomas that contain ganglioside GD2 [93]. Other acidic glycolipids with unique structures, that are not composed of sialic acid, have been reported in hepatocellular cancer like bisulfated-asialo-GM1 (SO_3-3Galβ1-3GalNAcβ1-4[SO_3-3]Galβ1-4Glc-Cer) [94].

Alterations in the glycolipids during malignancy not only have been observed *in vivo* but also in experimentally induced tumors [49, 50]. For instance, Nakaishi et al [51-54] have demonstrated that the ganglioside composition of cells transformed by transfecting with oncogenes changes as

compared to the untransformed equivalent. When they transfected adenovirus E1 gene or *myc*-oncogene into the rat fibroblast 3Y1 cells, they found that the cells neosynthesized ganglioside GD3 [51, 52]. The normal 3Y1 cells did not synthesize GD3 (NeuAcα2-8NeuAcα2-3Galβ1-4Glc-Cer) whereas they did synthesize its precursor, GM3 (NeuAcα2-3Galβ1-4Glc-Cer), which is the major ganglioside of the 3Y1 cells. These findings indicated that transfection with the E1 or *myc* oncogenes resulted in the activation of the α2-8sialyltransferase responsible for the formation of GD3 from GM3, similar to the effect observed in human melanoma which is an *in vivo* tumor. The protein products of the E1 or *myc* oncogenes reside in the nuclei where they probably interact with DNA at a transcriptional level. Transfection with oncogenes with products that are expressed extranuclearly, like the *fes, fps, ras,* and *src* ended in the expression of other glycoplipid patterns by the same recipient cells (3Y1) [53, 54]. The 3Y1 cells transfected with the later class of oncogenes did not express GD3 but synthesized neolacto series gangliosides like sialylparagloboside (NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer), and the ganglio series glycolipids, GM1α and GM1b [53, 54].

The significance of the changes in the expression and metabolism of glycolipids in cancerous cells are not yet clear. Are the glycolipids expressed in the cancerous state just markers of the tumor cells, are they a consequence of the cancerous state, or do they play a causal role in inducing or maintaining malignancy?.

Recent studies have suggested the involvement of glycolipids in the modulation of the immune response of the host to the tumor [91, 95]. Tumor cells shed large amounts of glycolipids into the culture media or into the

serum and secretions of the host [96]. For instance, normal hepatocytes release 0.25% of the total gangliosides per hour into the culture media whereas hepatoma cells release 25-80% of their total gangliosides per hour into the medium [97]. Most of the glycolipids that are shed contain a higher ratio of gangliosides to neutral glycolipids as compared to the glycolipids in the plasma membrane where the concentration of neutral glycolipids is much more elevated than that of gangliosides [98]. This suggests that the shedding of glycolipids might be a controlled process in which particular regions of the plasma membrane containing higher concentrations of gangliosides are specifically detached [99].

The exfoliation of glycolipids from tumors also occurs in vivo; the serum of cancer patients contains higher amounts of lipid bound sialic acid than that of normal individuals [96, 100]. As a matter of fact, the concentration of gangliosides in serum has been used as a test to detect and monitor several types of cancer [100-102]. It has been proposed that shedding of glycolipids by tumors is possibly an immunoregulatory mechanism of the tumor to escape from the immunosurveillance of the host [91, 95, 99]. For example, the immune response to melanoma tumors is biphasic; the initial immune response is directed to the tumor, but as the tumor progresses the immune response is suppressed [103]. As stated above, melanomas synthesize large amounts of GM3 and GD3 and low amounts of GM2 and GD2. However, the two later gangliosides are autoimmunogenic in man [104]. It has been noticed that there is a correlation between the concentration of each of this four gangliosides, which differs in each specific melanoma, and the progression of the tumor [105]. Also, melanomas shed GM3 and GD3, and their concentration in the blood

of the patients bearing the tumor is unusually high. It has been suggested that the gangliosides shed by melanomas may influence the function of infiltrating lymphocytes near the tumor area [106]. Hoon et al. [80] have examined the effect of melanoma gangliosides on the response of lymphocytes to interleukin-2 (IL-2), a growth factor released by T-helper lymphocytes which promotes T cells proliferation and the continuation of an immune response by other T cells after antigen stimulation [107]. These authors found that GM3 and GD3 purified from melanoma tumors inhibited the IL-2-dependent growth of lymphocytes isolated from melanoma draining-lymph nodes. They showed that IL-2 pretreated with GM3 and GD3 decreased the capacity of IL-2 to induce lymphocyte proliferation by 80%; moreover, preincubation of the lymphocytes with GM3 and GD3 before adding IL-2 decreased growth profoundly. They did not find any evidence that GM3 and GD3 were able to bind the IL-2 receptor in T-lymphocytes, indicating that the inhibition of the mitogenic effect of IL-2 on lymphocytes by these gangliosides possibly is due to the binding of GM3 and GD3 to IL-2, whether they are free in the serum or inserted in the lymphocyte membrane. In the same study, Hoon et al. [80] found that increasing quantities of GM2 and GD2 enhanced IL-2-dependent lymphocyte growth, which parallels with the fact that GM2 and GD2 are autoimmunogenic. It may be that in vivo one could see the same phenomena, since the antitumor activity of the host is higher at the onset of the melanoma, which correlates with the fact that GM2 and GD2 in the tumor surface may generate an immune response and the amounts of GM3 and GD3 released by the melanoma may not be enough at this stage to neutralize the physiological concentrations of IL-2, but as the tumor grows, higher

quantities of GM3 and GD3 are shed by the tumor which are capable of neutralizing the effect of IL-2, and consequently the immune response of the host to the tumor is suppressed, as is observed in the progression of most melanomas [80].

Hoon et al. [87], also have examined the effect of melanoma gangliosides on the infiltrating macrophages near the tumor area. Macrophages can act as accessory cells by secreting interleukin-1, another growth factor that activates antigen stimulated-T and -B lymphocytes; or by directly killing the tumor cells [107]. Hoon et al. [87], found that gangliosides GM2 and GM3 inhibited Fc receptor synthesis in macrophages; furthermore, GD3 prevented the production of interleukin-1. These results extend the concept that gangliosides are potentially associated to the escape mode of the tumor from the immunosurveillance of the host. These results also infer that the mechanism of suppression of the immune response by gangliosides is not a simple one but one that happens by the participation of various factors.

Other investigators have demonstrated the involvement of gangliosides in the regulation of natural killer (NK) cell functions. Natural killer (NK) cells are a subpopulation of cytotoxic lymphocytes capable of killing virally infected cells and tumors without former antigen stimulation [107]. When NK cells were pretreated with gangliosides GM3 and GD3 the cytotoxicity towards lymphoma cells was impaired, nonetheless, when the target cells which in this experiment were the lymphoma cells, were preincubated with GM3 and GD3 the cytotoxicity of the NK cells was magnified [99]. These effects were specific since other gangliosides could not produce similar reactions; and the inhibition or increase in the activity of the NK cells was dose dependent, using

ganglioside levels in the range of those found circulating in the blood of cancer patients (50 nmol/ml) [99]. The mechanism by which NK cells act upon their targets is unclear; it seems that the NK cell needs to bind to the target cell, then the binding induces the secretion of granules from the NK cell that contain a lytic protein (cytolysin) that produces lethal lesions in the target cell surface [109]. For binding of the NK cell to the target, a receptor protein is probably required, however, no receptor in the NK cells has been identified [109]. The experiments described above suggest the implication of gangliosides GM3 and GD3 in the binding of the presumed NK receptor, and mediation of the NK cell lysis when those gangliosides are adsorbed onto the target cell. On the other hand, when the NK cells are pretreated with GM3 and GD3, these gangliosides may be saturating the receptor in the NK cells and therefore blocking further binding of the NK receptor to the GD3 and GM3 that may occur naturally in the tumor cell surface, and no lysis of the tumor cells could take place.

In another study by MacDougall et al. [108], a clone (K562-Clone I) of a leukemia cell line that was selected on the basis of its resistance to natural killer cells lysis, was found to incorporate more [^{3}H]-fucose into its glycolipids than the parent cell line (K562); also, other complex fucosylated glycolipids were expressed in the NK-resistant clone that were not present in the original leukemia cell line. In addition, a subclone (K562-Clone I-Con A1) of the NK-resistant clone, obtained by its insensitivity to Concanavalin A (Con-A), reverted its glycolipid pattern to that of the parental cell line (K562) and also became sensitive to NK cell-lysis. Although the results of both studies [99, 108] were obtained using in vitro experiments, they suggest a possible role of

gangliosides in modulating NK cell activity *in vivo*. It may well be that tumors secrete gangliosides into the vicinity of the tumor that might bind to the NK cell receptors present in the infiltrating NK lymphocytes and as a consequence the blocked receptors in the NK cells become uncapable of further binding to the tumor cell. On the other hand, malignant cells may be able to modify the structure of the surface glycolipids or glycoproteins involved in the binding of the NK cell, by increasing or decreasing a type of glycosylation, e.g. sialylation or fucosylation, and as a consequence rendering the tumor resistant to recognition by the NK cells [108].

Radin et al. have proposed that glycolipids might be causally involved in cancer [110, 111], based on the fact that Gaucher disease patients have a high incidence of leukemia and other diseases of B-cell proliferation. Gaucher disease is caused by a genetic defect in which the glucosylceramide (Glc-Cer) glucosidase activity is very low and glucosylceramide accumulates in large amounts. The liver and the spleen, which digest and process blood cells, accumulate high levels of glucosylceramide, and this results in hypertrophy of these organs in Gaucher disease patients. These authors believe that the elevated quantities of glucosylceramide in these patients stimulates B-cell growth that can produce malignant transformation. Inokuchi et al. [111] injected mice bearing Ehrlich ascites tumors with an inhibitor (1-phenyl-2-decanoylamino-3-morpholino-1-propanol) of the enzyme UDP-Glc-Ceramide glucosyltransferase. Inhibition of this transferase resulted in complete regression of the tumors in 30% of the mice and prolongation of the life in the other 70%. In contrast, when mice bearing Ehrlich ascite tumors were inoculated with glucosylceramide, tumor growth was stimulated in 50% of the

mice. This last effect was reversed by the inhibitor of glucosylceramide synthesis. They also demonstrated that injecting normal young mice with glucosylceramide [146] produced liver uptake of the glucosylceramide and rapid enlargement of the liver that coincided with increased DNA, protein and lipid synthesis. The growth of the liver was amplified by injecting mice with glucosylceramide together with conduritol B epoxide, an inhibitor of the glucosylceramide glucosidase. Radin et al. [110], have postulated that cancer is "glucosphingolipidosis of a single cell" [110], meaning that in cancer what may happen is that a specific glucosidase responsible for the hydrolysis of a glycolipid is deficient or defective in a single cell. Since this glucosidase is unsuited to hydrolyze that glycolipid, it will accumulate in extraordinary amounts, enough to promote the proliferation of this cell (by unknown mechanisms), and leading to malignant transformation.

2.2 COLON CANCER-ASSOCIATED CARBOHYDRATE ANTIGENS

Most of the cancers derived from tissues that embryologically originated from the endoderm such as the urinary, respiratory and digestive epithelia, are characterized by the accumulation of lacto- and lactoneo-series glycolipids [62]. These lacto/lactoneo-series glycolipids are usually sialylated and/or fucosylated and many of their structures represent the ABH and Lewis blood group antigens or modifications of them. The majority of these tumor-associated antigens are also oncofetal antigens since they are expressed at certain stages during embryo development but they are either repressed or

expressed in minor quantities throughout adulthood. Adenocarcinomas of the gastrointestinal tract typically express a variety of these lacto/lactoneo-series glycolipids. As a matter of fact, most of this class of tumor-associated antigens has been detected and characterized in gastrointestinal adenocarcinomas and derived cell lines.

The gastrointestinal epithelia is an abundant source of blood-group active glycolipids, for example, Bjork et al [147, 148] reported that a single adult small intestine contains 600 mg of glycolipids from which 300 mg corresponds to blood group glycolipids. Blood group active glycolipids are particularly distributed along the gastrointestinal tract; a decreasing gradient in the expression of these glycolipids is observed from the stomach and small intestine where they are maximally expressed, to the colon where their expression is limited only to the proximal part with almost no detectable amounts in the distal portion of the colon [57, 58, 62, 147-158]. In contrast, during fetal life, the appropriate blood-group active glycoconjugates are present throughout the whole gastrointestinal tract but progressively disappear from the distal segments of the colon from birth to maturity [57, 150, 152, 153, 156-158]. Karlsson et al. [159] have reported that human meconium which is the first feces of the newborn and it is mainly composed of extruded epithelial cells of the fetal digestive tract, contains 30% of the total weight of neutral glycolipids as blood group active glycolipids.

The expression of blood group active glycolipids by normal gastrointestinal epithelial cells is controlled by the Se, A, B, and Lewis genes. As stated in section 2.1.1, the formation of H, A, B, and Le^b substances in epithelial cells and in secretory tissues is controlled by the Se gene, which is believed [33] to

encode a different α1-2fucosyltransferase from that encoded by the H gene in hematopoietic tissues. This assumption is based on the fact that the α1-2fucosyltransferase present in the submaxillary glands (secretory tissue) of ABH [160] secretors showed a marked preference for type 1 chain precursor oligosaccharides whereas the same enzyme from non-secretors was barely detectable and showed no distinct preference for type 1 precursors. Other experiments supporting this theory are the ones carried out by Watkins et al. [35] in which epithelial cell scrapings from different regions of the gastrointestinal tract (stomach, small intestine and colon) from secretors and non-secretors were assayed for the activity and specificity of the α1-2fucosyltransferase responsible for the synthesis of H-type substances. They found that the the α1-2fucosyltransferase activity of secretors was high in the stomach and small intestine and decreased as it reached the colon. Moreover, the α1-2fucosyltransferase had a marked preference for type 1 acceptors in the upper portions of the gastrointestinal tract, but this preference was not observed in the colon enzyme, which displayed almost no difference in specificity for type 1 or type 2 acceptors. In samples from non-secretors there was a weak detectable amount of α1-2fucosyltransferase activity that had no distinguishable specificity for either type 1 or type 2 precursors, similar to the colon enzyme in secretors. The authors concluded that there was a constant small amount of α1-2fucosyltransferase activity throughout the gastrointestinal tract in secretors and non-secretors, that had acceptor specificity for both type 1 and 2 chains which probably corresponds to the H-gene fucosyltransferase; and that the other α1-2fucosyltransferase specific

for type 1 acceptors is probably the product of the Se gene which is highly expressed in the upper portion of the gut but to a lesser degree in the colon.

The results obtained by Watkins et al [35] also agree with numerous studies related to the expression of blood group active glycoconjugates in the gastrointestinal tract, most of which have been done by immuncytochemical localization of these antigens [57, 58, 62, 147-158]. These studies [57, 58, 62, 147-158] have proved that most of the blood group glycoconjugates are mainly expressed in the stomach and the small intestine, and that their expression gradually declines in the proximal region of the colon, with almost no expression in the distal colon. Most important is the fact that the majority of the A, B, H glycolipid antigens in the gastrointestinal epithelia are derived from type 1 acceptor structures. In contrast, the blood group active oligosaccharides attached to glycoproteins in the small intestinal epithelium are mostly constituted by type 2 chains [186]. This finding is in conflict with the results obtained by Watkins et al. [35] which clearly support the presence of type 1 chain based-blood group active glycolipids in the gastrointestinal mucosa, but they do not explain the occurrence of glycopeptides mainly composed of type 2 precursor oligosaccharides. However, this controversy in the distinct type of precursors used in the expression of blood group active glycolipids and glycoproteins, may suggest that the pathways for the synthesis of oligosaccharide chains of glycolipids and glycoproteins are different.

The pathways may be different in the glycosyltransferases used; for instance, some strictly may glycosylate glycoproteins and others only glycolipids. Alternatively, the separation in the pathways may be controlled by the different location of the set of glycosyltransferases involved in the

synthesis of either oligosaccharide chains of glycoproteins or glycolipids within the endoplasmic reticulum and the Golgi apparatus. Watkins et al. [35] observed that the supposed Se gene α1-2fucosyltransferase was able to fucosylate type 2 chain precursors at a low level, suggesting that the same enzyme is able to fucosylate both precursor chains for glycolipids (type 1) and glycoproteins (type 2). The fact that in glycoproteins the majority of the blood group active glycans are based on type 2 chain may not be determined by the specificity of the α1-2fucosyltransferase, but by the availability of the β1-4 to β1-3galactosyltransferase required for the synthesis of type 2 versus type 1 oligosaccharide precursors, at the site where glycoproteins are glycosylated. This topic will be discussed in more detail in section 2.2.5.

The activity of the α1-3-N-acetylgalactosyltransferase responsible for the formation of A antigens has also been measured in all the regions of the digestive tract [161]. It was found that this enzyme was equally distributed throughout the length of the gut, confirming that the lack of expression of A and also B active glycoconjugates in the distal part of the colon was not due to the unavailability of the A or B enzymes but to that of the α1-2fucosyltransferase that synthesizes the suitable H-precursor molecules.

The Le^a antigen is uniformly expressed in every segment of the digestive tract of secretors and non-secretors [57, 58, 62, 147-149, 153-154], and it is also expressed during embryonic development. The expression of Le^b antigens in the digestive tract is controlled by the Se gene, since synthesis of the Le^b requires the H-type 1 structure as precursor. Therefore, Le^b glycoconjugates only appear in the stomach, small intestine and in the proximal portion of the colon of secretors. The Le^b antigens are expressed throughout the fetal

gastrointestinal tract of secretors. The positional isomer of Le^a, Le^x, is present in small amounts in all the gastrointestinal epithelia of secretors and non-secretors [62,147, 149, 151-153, 158] , however, the Le^x antigen, also known as stage specific antigen 1 (SSEA-1) (see section 2.1.3), is maximally expressed during fetal life [159], specifically during the 5th and 19th week of gestation [150]. The Le^y antigen, which is the isomer of the Le^b antigen, according to Cordon-Cardo et al. [153], is not present at all in the normal colonic mucosa. Nevertheless, Brown et al. [151] reported that Le^y antigen was present in the proximal colonic mucosa and in the immature crypt cells of the distal portion of the colonic epithelium only of secretor individuals. Abe et al. [155] showed similar results but they found that the expression of Le^y antigens was increased in the proximal colon and that it was almost undetectable in the distal part. Holgersson et al. [149], reported that minor quantities of Le^y antigens were present in the large intestine only of secretor individuals; and Bjork et al. [147] detected, by immunostaining of glycolipids on thin layer plates, complex Le^y active glycolipids (more than 6-monosaccharide chains), from the small intestinal epithelia of secretors; the same Le^y glycolipids were weakly noticed in the sample from a non-secretor individual. Compared to the normal gastrointestinal epithelium, all portions of the fetal digestive mucosa strongly express the Le^y antigens, although Abe et al. observed that their expression decreased a little in the distant regions of the fetal colon [155].

Carcinomas of the gastrointestinal tract are characterized by anomalies in the expression of the A, B, H, Le^a, Le^b, Le^x, and Le^y antigens along the different segments of the digestive epithelium. Four different changes in the

pattern of expression of these antigens can be observed associated with malignant transformation. 1, Deletion of the A, B, H, Le^a, Le^b antigens; 2, reexpression of A, B, H, Le^b and Le^y antigens in the distal colon; 3, expression of A, B, H, Le^b, and Le^y antigens that are incompatible with the secretor and ABH phenotype of the individual; 4, expression of modified Le^a, Le^b, Le^x, and Le^y structures.

2.2.1 Deletion of the A, B, H, Le^a, Le^b Antigens

Deletion of A, B, and H antigens from regions of the digestive tract where they are normally expressed, has been reported in gastrointestinal cancers. For example, Yuan et al. [57] found that 2 of 10 patients with blood group A (one secretor, the other unknown secretor status), and 1 of 4 with AB (secretor) did not express A antigens in tumors from the ascending colon, as assayed by immunoperoxidase staining of the involved tissues. Moreover, 4 of 20 patients Le (a^-, b^+), failed to express Le^b antigens in tumors from the proximal colon [57]. Schoentag et al. [58] found diminished expression of Le^a antigens in colorectal cancers, which in normal colorectal epithelia are always present independent of the secretor status. Sakamoto et al. have also reported [62] a diminution of the Le^a antigens in poorly differentiated colonic carcinoma and in metastatic cancer.

The deletion of any of the blood group active molecules from the upper region of the digestive tract (stomach, small intestine and proximal colon) at the onset of cancer has been proposed to be caused by the inactivation of any of the glycosyltransferases required for their synthesis [162]. Since most of the studies concerning deletion of blood group antigens in gastrointestinal

carcinoma have been done by immunostaining techniques, it may actually be that the blood group active substances are expressed but they are camouflaged by the oligosaccharide chains of other glycoconjugates, or they are masked by other sugar residues attached to them, like sialic acid for example, which makes them unrecognizable by the antibodies used. It also has been suggested that the blood group antigens may have been degraded by bacterial glycosidases. Dahiya et al. [163] have analyzed several colon cancer cell lines for the expression of A, B, H active glycoconjugates by immunocytochemistry, and also by measuring the activities of the glycosyltransferases and glycosidases associated with the synthesis and degradation of these antigens. Two of the cell lines tested, H-498 and SW1417 showed blood group antigen deletion. The cell line H-498, which was derived from the colon tumor of a blood group A individual, failed to express A antigens, but accumulated the precursor H antigens as demonstrated by immunocytochemical detection. This cell line had almost no A transferase activity, indicating that lack of expression of A type molecules was caused by the absence of this enzyme. The cell line SW1417 originated from the tumor of a blood group B individual. In this cell line no B or H antigens were detected by immunocytochemistry, however, the activities of the B and H transferases were high. Also, the corresponding glycosidase activities were normal. Dahiya et al. [163] suggested that the lack of expression of the B antigens was due to competition by other glycosyltransferases, like sialyltransferases or fucosyltransferases, for the same precursor molecules; or maybe by masking of the B antigens with sialic acid so that the antibodies were unable to react with them.

2.2.2 Reexpression of A, B, H, Le^b, and Le^y Antigens

The reexpression of A, B, H, Le^b, and Le^y antigens in the distal part of the colon is commonly observed in colorectal carcinomas of secretors [57, 58, 62, 154, 155]. Since all of these antigens are present throughout the length of the digestive tract during fetal life, they vanish from the distal segments of the colon of mature individuals, and they reappear in the distal colon as a consequence of malignant transformation, they are considered to be oncofetal antigens. This phenomenon is most likely caused by the activation of the Se gene, which seems to be suppressed in the distal colon of adult secretor individuals [153]. The expression of the H gene glycosyltransferase, which is presumed to be present in very low quantities in the colon and probably accounts for part of the small levels of blood group active glycolipids based on type-2 chain in the normal colon, might also be triggered, since a high proportion of colorectal cancers express Le^y glycolipids which are based on H-type 2 precursors. Furthermore, H-type 2 antigens were found by Brown et al. [151], to be expressed in 86% of the colon adenocarcinomas studied, whilst they were absent from normal distal colonic mucosa. Another explanation for the presence of blood group glycolipids based on type 2 structures in colon cancer may be that, besides the enhanced activity of the Se gene and/or the H-gene fucosyltransferases in the distal colon, maybe by derepression of the Se gene or stimulation of the H-gene, the specificity of the Se gene fucosyltransferase also might be altered in such a way that it is able to fucosylate equally well type 1 or type 2 chains, in contrast to the normal Se gene enzyme which prefers type 1 chain.

2.2.3 Incompatible Blood Group Antigen Expression

The third transformation associated with colorectal cancer is the expression of blood group antigens that are not compatible with the phenotype of the individual [57, 62, 154, 164, 165]. Yuan et al. have reported that over one half of the colonic tumors assayed contained incompatible blood group antigens [57]; for example, blood group O individuals expressed in their tumors A and/or B like antigens as assessed by the immunoperoxidase method. They found that 5 out of 10 cancers from type A patients contained B like substances and 7 out of 13 cancers from type B individuals expressed A like molecules. They also found that three Le^a patients (non-secretors) expressed Le^b antigens in colonic tumors. In another study, Sakamoto et al [62] observed that a high proportion of colonic carcinomas strongly expressed Le^b and Le^y antigens, irrespective of whether the tumors were from secretor or non-secretor patients; Cordon-Cardo et al. [154] reported the expression of Le^b and Le^y antigens in colon cancers of non-secretors. When Sakamoto et al. [62] examined normal colonic mucosa near the tumor of a non-secretor that expressed Le^b antigens, they found that it did not have any Le^b antigenicity. These results led Sakamoto et al. to propose that the "Se gene and/or H genes are not absent in non-secretors, but that they are suppressed or inactive" [62].

Other investigators have examined incompatible expression of blood group antigens in colon cancer derived cell lines [61, 163]. Dahiya et al. [163], found that there was weak reactivity with anti-A monoclonal antibodies in a cell line from a blood type O and in another from blood type B. They also detected B antigenicity in a cell line from a blood type O individual and in another from a blood type A. They measured the activity of the A-gene and B-gene encoded

glycosyltransferases in the cell lines expressing unexpected antigens, and they found that there was little or no activity of the corresponding enzymes. It has been implied that these incompatible antigens are expressed in colonic cancer because the the A or B transferases lose specificity and they are able to form both A and B type antigens. However, this theory does not explain the incompatible expression of A or B antigens in colonic cancers of blood type O individuals [163]. An alternative explanation for the expression of incompatible B antigens in A individuals may be that the A antigen is converted into an "acquired" B antigen by deacetylation of the terminal α-N-acetylgalactosamine, as it has been observed in red blood cells [163]. Also, addition of α-galactosyl residues at the end of precursor glycolipids that are not the H-type precursor, can produce cross-reactivity with B antibodies [163].

Another hypothesis suggests that the incongruent A or B antigens detected by monoclonal antibodies, are not real A or B molecules, but that they are similar structures that cross-react with the specific antibodies. For example, the Forssman glycolipid (GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glc-Cer) which has a terminal α1-3GalNAc, can cross-react with anti-A monoclonal serum. However, the monoclonal antibodies employed by Dahiya et al. [163] did not cross-react with Forssman-positive sheep erythrocytes. Even more, Hattori et al. who reported incompatible expression of A like glycolipids in the gastric cancer of blood type O patient, did not find any Forssman activity in the cancer and uninvolved tissue from this person [165]. Clausen et al. [166] have studied the expression of A type glycolipids in colonic tumors from blood group O individuals by isolating and analyzing the structure of the A active glycolipids, and by measuring the activity of the A-gene glycosyltransferases

from extracts of the tumors. In this study, Clausen et al. [166] documented that the incidence of incompatible A in tumors of O individuals was 13% based on immunostainig of glycolipids on thin layer plates, and 10% based on immunofluorescent staining of the tumors. They determined, by using a panel of monoclonal antibodies with different reactivities for A active glycolipids, that the incompatible A like glycolipids in the tumors of O individuals corresponded to A-type1 and ALe^b glycolipids [166]. In addition, they detected A-transferase activities in extracts from one of the colonic tumors which supported the presence of these antigens in the involved tissue. This indicated that the incompatible A type glycolipids in the colon cancer of O individuals were real A antigens and not related antigens. These findings implied that the A or B genes are not absent in O type individuals, but that they are repressed, and they can become active at the onset of cancer [163].

2.2.4 Expression of Le^a, Le^x, and Le^y Antigens and Their Modifications

Modifications of the Le^a, Le^x, and Le^y antigens have been encountered in colorectal cancers. The Le^x antigen or SSEA-1, is ubiquitously but weakly expressed throughout the normal colon epithelium despite the secretor status of the individual. The Le^x antigen appears in the crypt cells which are the most proliferative cells in normal colonic mucosa and it is also present in much larger amounts in the fetal colon [152, 153]. It has been demonstrated that the Le^x antigen is strongly expressed in most colonic cancers, metastases and in the most invasive cancer cells [77, 152, 153], which places Le^x as an oncofetal antigen, and probably as a marker of the aggressiveness of the

cancer. The Le^x glycolipid antigen is a pentaglycosylceramide (Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4Glc-Cer), variations of this structure have been described by Hakomori et al. [150, 158, 167-173] such as the dimeric and trimeric forms of the Le^x glycolipid present in human lung, liver and colonic adenocarcinoma (these structures are shown in table 2). The distribution of the Le^x glycolipid and the related di- and trimeric forms in fetal, adult, and cancer tissues has been studied by Fukushi et al. [150] with the aid of monoclonal antibodies, one specific for the simple Le^x structure, and the other specific for both the di- and trimeric derivatives. They reported that Le^x and the di- and trimeric Le^x were intensely expressed in the fetal gastrointestinal epithelium; however, the dimeric and trimeric configurations appeared at a later embryonic stage than the Le^x structure, and the di-and trimeric forms also regressed rapidly after further development as compared to the simple Le^x antigen which was present for a longer period and prevailed at a low level in the gastrointestinal epithelium after parturition. The dimeric and trimeric Le^x were completely absent in the adult colonic epithelium and they were present only in the stomach parietal cells and pyloric glands, the intestinal Paneth cells and basal granular cells, and in the convoluted tubules of the kidney [150]. These multimeric forms of the Le^x glycolipid were highly expressed in well differentiated stomach cancer, colon cancer, and breast cancer, but not in poorly differentiated cancers of the stomach. It also was noticed that the multimeric Le^x antigens tended to stain the cytoplasm of the colon cancer cells [158], suggesting that part of them may accumulate intracellularly, or that they may be slowly processed. Since the multimeric forms of Le^x are maximally expressed at a certain stage of embryonic development (7-9 weeks

of gestation), Fukushi et al. [150] proposed that the presence of dimeric and trimeric Le^x in those cancers "can be regarded as a retrograde expression of these antigens to a certain stage of fetal development in which their expression was maximal" [150].

Other derivatives of the Le^x glycolipid are the sialyl Le^x and sialyl dimeric Le^x [158, 169-171] (table 2), both antigens are expressed during embryonic development, they are not expressed by normal colonic mucosa, but they appear in most colonic cancers (80% of specimen assayed [158]). The sialyl $Lewis^x$ antigen was also shown to be present in the blood serum of advanced colon cancer patients (44% of individuals assayed in [174]); likewise, the sialyl difucosyl $Lewis^x$ was detected in the blood serum of colon cancer patients, but the asialo derivative, the dimeric Le^x antigen, was not. [171]. According to results of Itzkowitz et al. [158], it seems that the sialyl $Lewis^x$, sialyl dimeric $Lewis^x$, and the di- and trimeric $Lewis^x$ antigens are more exclusive for cancer tissues than the simple $Lewis^x$ antigen (present at basal levels in normal colonic crypt cells). Moreover, monoclonal antibodies specific for these derivative structures react with most colonic cancers, but these are not expressed in the epithelium of normal colon [158].

Holmes et al. [172, 173] have studied the biosynthesis of the dimeric, trimeric and sialyl derivatives of the Le^x glycolipid antigens in human lung carcinoma cells, which are known to accumulate these structures. They discovered that elongation of lacto-N-neotetraosylceramide (nLc_4) to form lactonorhexaosylceramide (nLc_6) and lactonoroctaosylceramide (nLc_8) occurred before addition of the α1-3fucose to the penultimate GlcNAc residue of either the tetrasaccharide (nLc_4), the hexasaccharide (nLc_6), or the

octasaccharide (nLc8). After transfer of the fucose to the penultimate GlcNAc, one or two additional fucoses could then be transfered in successive steps to the internal GlcNAc residues to make the dimeric or trimeric Le^x configurations (this pathway is depicted in figure 4). Holmes et al. [172], based on several observations, proposed that the fucosyltransferase that transfered fucose to the penultimate GlcNAc residue was the same enzyme as the one that added fucose to the internal GlcNAc residues. Formation of the sialyl Le^x and the sialyl dimeric Le^x was shown to proceed [173] via sialylation of lactoneotetraosylceramide or lactonorhexaosylceramide prior to the addition of the fucose residue/s (fig. 4). The internal fucosylation of elongated lacto-neo type glycolipids may be a biosynthetic pathway associated with malignant transformation [167, 172, 173], since the Le^x α1-3fucosyltransferase expressed by normal tissues only fucosylates the penultimate GlcNAc of type 2 or elongated type 2 chains, but does not transfer fucose to the inner GlcNAc residues; except for normal small intestinal epithelium where the glycoprotein-N-linked oligosaccharide chains, (but not the glycolipid-oligosaccharide chains) are internally fucosylated [186]. Furthermore, the extension of lactoneotetraosylceramide (fig. 4) by the stepwise addition of GlcNAc and Gal, which represents the addition of an N-acetyllactosamine unit (Galβ1-4GlcNAcβ1), in a linear fashion and not in a branched configuration, appears to be a characteristic pathway in cancer cells [167]. Normal adult tissues usually elongate oligosaccharide chains by branching, as for example the I antigen, fucose and GalNAc or Gal can be added to the non-reducing end of the branched structures to form blood group active glycolipids [167].

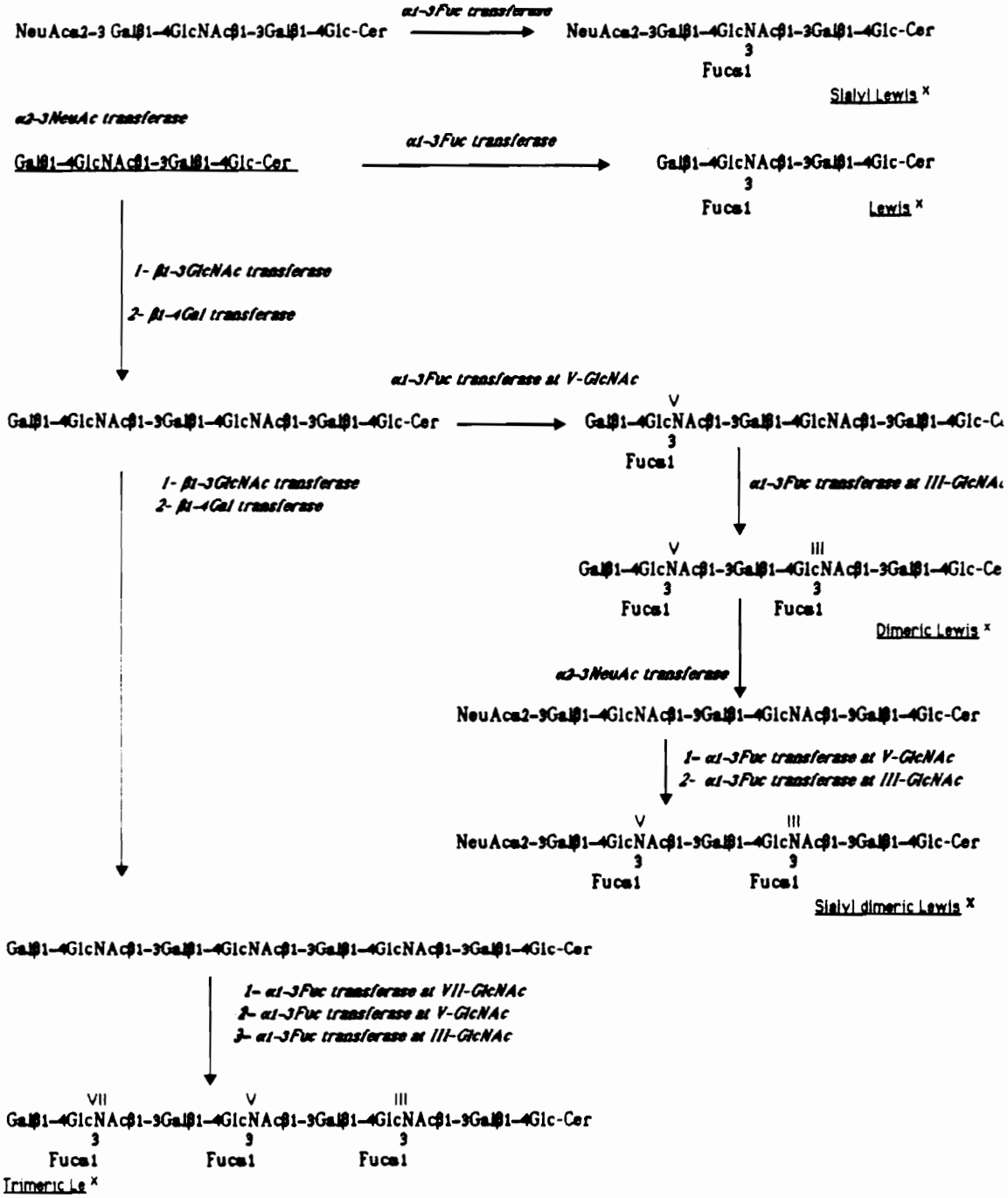


Figure 4.- Biosynthesis of dimeric, trimeric and sialyl Le^X antigens in lung carcinoma cells.

TABLE 2

Some Glycolipid Structures from Human Normal and Malignant Gastrointestinal Epithelial cells

Structure	Trivial Name	Source
NeuAcα1-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc-Cer	STa	Human meconium, SW1116 cells
Galβ1-3GlcNAcβ1-3Galβ1-4Glc-Cer 6 NeuAcα1	STb	Human meconium, SW1116 cells
NeuAcα1-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc-Cer 6 NeuAcα1	Disialyllactoteraosylceramide	Colonic cancer
Galβ1-3GlcNAcβ1-3Galβ1-4Glc-Cer 4 Fucα1	Lewis^a	Gastrointestinal tract
NeuAcα1-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc-Cer 4 Fucα1	Sialyl Lewis^a	Colonic cancer
NeuAcα1 6 NeuAcα1-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc-Cer 4 Fucα1	Disialyl Lewis^a	Colonic cancer
Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc-Cer 4 Fucα1	Lewis^b	Normal stomach, small intestine, proximal colon Distal colonic cancer
Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer 3 Fucα1	Lewis^x	Normal colonic crypt cells Colonic cancer
NeuAcα1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer 3 Fucα1	Sialyl Lewis^x	Colonic cancer
Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer 3 3 Fucα1 Fucα1	Difucosyl Lewis^x	Colonic cancer, lung cancer
Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer 3 3 3 Fucα1 Fucα1 Fucα1	Trifucosyl Lewis^x	Colonic cancer, lung cancer
NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer 3 3 Fucα1 Fucα1	Sialyl difucosyl Lewis^x	Colonic cancer, lung cancer
Fucα1-2Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer 3 Fucα1	Lewis^y	Normal stomach, small intestine, proximal colon Colonic cancer
Fucα1-2Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer 3 3 Fucα1 Fucα1	Difucosyl Lewis^y	Colonic cancer

Only a minor percentage of glycolipids is extended linearly in normal tissue, and usually they are converted into lacto-series gangliosides; very few of the linear chains are used to make blood group active glycolipids; and also insignificant amounts are fucosylated at the penultimate GlcNAc to become Le^x active [167]. Likewise, in normal tissues no fucosylation of the internal GlcNAc residues of either branched or linear lacto-series glycolipids occurs [167].

The Le^y glycolipid antigen is mildly expressed by the proximal colonic mucosa of normal secretor individuals, it is strongly expressed in the fetal colon, and it is present in most colonic adenocarcinomas regardless of the secretor status of the patient and the location of the tumor [62, 151, 153]. The pattern of expression of this antigen in fetal, normal and cancer tissue indicates that this is an oncofetal antigen, unique to the malignant state [155]. Modifications of the Le^y antigens, such as the extended version of this antigen, the trifucosyl Le^y structure (table 2) (which is the derivative of the difucosyl Le^x antigen), have been detected in colonic adenocarcinomas and in colonic polyps with malignant potential [156]. The trifucosyl Le^y antigen was also shown to be present in proximal segments of the fetal colonic mucosa [156], but was absent in most normal colonic mucosa tested [156].

The Le^a glycolipid antigen is expressed by the entire normal colonic mucosa irrespective of the secretor status of the individual. It is also present in the fetal gastrointestinal tract. However, modifications of this structure have been detected in colorectal carcinomas, such as the sialyl $Lewis^a$ antigen (table 2) which, will be described in more detail in section 2.3.

Another example is the disialyl Le^a antigen which is expressed as a minor component in colonic adenocarcinoma (5-10% of the amount of monosialyl Le^a) [175] (table 2). The precursor for the disialyl Le^a, which is disialyllactotetraosylceramide, is also a constituent of the disialosyl glycolipid fraction of many human colonic adenocarcinomas (table 2) [176].

2.2.5 Basis for the Expression of Type 2 Chain Derived Antigens in Colon Cancer

Normal colonic mucosa mostly synthesizes lacto-series glycolipids and blood group glycolipid antigens based on a type 1 chain [147-149]]. One of the reasons for the expression of blood group active glycolipids derived from type 1 chains is that the Se gene α1-2fucosyltransferase responsible for the synthesis of H-precursor structures in epithelial and secretor tissues has a marked preference for type 1 chain acceptor structures. If this were the only reason one should see an accumulation of precursor type 2 chain glycolipids such as lacto-N-neotetraosylceramide in the normal colonic epithelium. In addition, normal colonic mucosa [70] has significant activity of the α1-3fucosyltransferase that makes Le^x antigens, therefore one should expect that part of the type 2 chain glycolipids might be fucosylated by this enzyme to produce Le^x structures. The reality is that normal colonic mucosa has moderate amounts of the β1-4galactosyltransferase (0.5-0.33 the activity of the β1-3galactosyltransferase [73]), which provides discrete levels of type 2 glycolipids [70]. However, only a minor proportion of these type 2 chain based structures are converted into Le^x active glycolipids despite the considerable levels of the α1-3fucosyltransferase present in the normal colon epithelium

[70]. Instead, the majority of the type 2 chain glycolipids are found in normal colonic mucosa as sialyl derivatives [70]. Holmes et al. [70] found, by immunostaining analysis, that the neuraminidase-treated ganglioside fraction from normal colonic epithelium, did not contain any Le^x active glycolipids, suggesting that no sialyl Le^x was present in this fraction. Previous results with the human lung carcinoma cell line PC9 [173] showed that biosynthesis of sialyl Le^x occurred via sialylation prior to fucosylation of lacto-N-neotetraosylceramide; it is interesting to note that none of the sialylated type 2 glycolipids of the normal colonic mucosa were fucosylated. This finding might indicate that the α1-3fucosyltransferase present in normal colonic epithelium is unable to fucosylate previously sialylated structures, and that the corresponding enzyme in the malignant tissue has been altered in such a way that it has a broader specificity and it can fucosylate previously sialylated structures. The other possibility might be that in normal colonic epithelium the β1-4galactosyltransferase that makes type 2 chains is located in the Golgi apparatus next to the sialyltransferases in the trans-Golgi so that the newly synthesized type 2 oligosaccharide chain is immediately sialylated in the next step of the oligosaccharide-assembly sequence and then exported to the plasma membrane without coming in contact with the α1-3fucosyltransferase. Possibly, in the Golgi complex of malignant cells the topography of the glycosyltransferases involved may be changed in some way that is feasible to fucosylate the sialylated type 2 chain. Also, the fact that in normal colonic epithelium only sialylated type 2 glycolipids, or fucosylated type 2 glycolipids, but not both sialylated and fucosylated glycolipids exist, seems to indicate that these are two separate pathways, in other words, type 2 chains can go either

through the sialylation pathway, or through the fucosylation pathway and then each structure yielded from either route is exported to the plasma membrane. The separation of these two pathways may be due to the organization of the involved glycosyltransferases in the Golgi complex, or may be due to the biosynthesis of either structure in different organelles. For example, sialylation may occur in the Golgi apparatus, and fucosylation may occur in the endoplasmic reticulum. Another important fact is that glycopeptides derived from normal small intestinal mucosa, contain glycans mainly based on type 2 chains, and few of them are sialylated, but they are highly fucosylated even in the internal GlcNAc residues [186].

Holmes et al. [71, 72] have shown that the basis for the accumulation of lacto-series glycolipids in colonic adenocarcinomas is due to the enhanced activity of the β1-3N-acetylglucosaminyltransferase in the tumor as compared to that in the normal tissue counterpart. This enzyme is required for the synthesis of lacto-N-triaosylceramide (GlcNAcβ1-3Galβ1-4Glc-Cer) from lactosylceramide (Galβ1-4Glc-Cer) and UDP-GlcNAc. Lacto-N-triaosylceramide is the precursor for all type 1 and type 2 chain glycolipids. While the elevated activity of the β1-3N-acetylglucosaminyltransferase can explain the increased amounts of lacto-series glycolipids based on both type 1 and type 2 chains in colonic cancers, it does not explain why in normal colonic mucosa type 1 chains are preferentially synthesized over type 2 chains, and why colonic cancers seem to be able to produce both type 1 and type 2 chain glycolipids. In a recent publication, Holmes presented data that sheds some light on this eventuality [73]. In this study [73], he looked at the expression and characteristics of the β1-3galactosyltransferase and the β1-4galactosyltransferase required for the

synthesis of type 1 and type 2 chains, respectively, from the common precursor lacto-N-triosylceramide, in two colonic cancer cell lines. One of the cell lines, Colo 205 was regarded as similar to normal colonic epithelium since most of the glycolipids synthesized by this cells are based on a type 1 chain. The other cell line SW403, synthesized both type 1 and type 2 derived glycolipids. Holmes [73] determined that in Colo 205, the activity of the β1-3galactosyltransferase was 9-fold higher than that of the β1-4galactosyltransferase. The activity of the β1-3galactosyltransferase in normal colonic mucosa was found to be 2-3 fold higher than that of the β1-4galactosyltransferase under the same assay conditions. Moreover, the K_m of the β1-3galactosyltransferase was 20-times lower than that of the β1-4galactosyltransferase in Colo 205 cells. Holmes [73] pointed out that one of the reasons for the the preferential expression of type 1 glycolipids in Colo 205 cells and in normal colonic mucosa was due to the higher enzyme activity of the β1-3galactosyltransferase as compared to the β1-4galactosyltransferase; and to the higher affinity of the β1-3galactosyltransferase for the acceptor lacto-N-triaosylceramide.

Biosynthesis of type 1 or type 2 chains in Golgi membrane fractions from Colo 205 and SW403 were analyzed under two assay conditions [73]. One of the conditions was the *in vitro* synthesis of lacto-N-triaosylceramide from exogenously added UDP-[^{14}C]-N-acetylglucosamine and lactosylceramide, by endogenous membrane bound N-acetylglucosaminetransferase, followed by addition of unlabeled galactose. The other condition was synthesis of lacto-N-tetraosylceramide and/or lacto-N-neotetraosylceramide from exogenously added lacto-N-triaosylceramide and UDP-[^{14}C]-galactose. When biosynthesis

was carried under the first condition, Colo 205-Golgi membranes made mostly type 1 chain glycolipids which is consistent with what is observed in the intact Colo 205 cells. SW403-Golgi membranes made both type 1 and type 2 glycolipids, with preferential expression of type 1 chain glycolipids. By contrast, when exogenous lacto-N-triaosylceramide was supplied to the Golgi-rich membranes (second assay condition) from both cell lines, the two cell lines synthesized type 1 and type 2 chain glycolipids, eventhough Colo 205 almost exclusively synthesizes type 1 chains *in vivo*. These results indicated that lacto-N-triosylceramide exogenously added to the membranes and *in situ* generated lacto-N-triaosylceramide were used in separate biosynthetic pathways. Holmes [73] explained that this probably happened because exogenously administered lacto-N-triaosylceramide is taken up evenly by the Golgi membranes, whereas the *in situ* synthesized lacto-N-triaosylceramide could be localized to a certain region of the Golgi membrane, in the vicinity of the β1-3galactosyltransferase that can immediately use it to form lacto-N-tetraosylceramide. Holmes [73], also suggested that, in the Golgi membranes, the β1-3N-acetylglucosaminyltransferase and the β1-3galactosyltransferase may be found associated in an enzyme complex, so that the individual reactions from each glycosyltransferase are coupled due to the spatial proximity of these two enzymes. These results suggest that localization and compartmentalization of the substrates and glycosyltransferases within the Golgi membranes may contribute to the control of the biosynthesis of type 1 versus type 2 chain glycolipids. The transfer reactions that were carried out with detergent solubilized membranes produced both type 1 and type 2 chain glycolipids in the two cell lines, regardless of whether lacto-N-triaosylceramide was

exogenously supplied or was internally synthesized. These results further substantiate the importance of membrane organization in the biosynthesis of type 1 versus type 2 chains.

The distribution of the involved glycosyltransferases was measured in subcellular fractions from the two cell lines, separated in sucrose density gradients [73]. The β1-4galactosyltranferase was enriched in fractions of moderate density (Golgi) of Colo 205 and SW403 cells; in these fractions there was also β1-3galactosyltransferase activity; but a considerable amount of the β1-3galactosyltransferase activity was found in more dense fractions (endoplasmic reticulum) of Colo 205 cells. The activity of the β1-3-N-acetylglucosaminyltransferase was uniform throughout the density gradients from both cell lines. It was also found that the ratio of synthesis of type 1 chain versus type 2 chain was greater in the more dense fractions (20-30 fold) than in lighter membrane fractions (7-10 fold) of Colo 205 cells, indicating the enrichment of the β1-3galactosyltransferase in the more dense fractions. The more dense membrane fractions of SW403 cells contained a significant percentage of the activity of the β1-4galactosyltransferase. The ratio of β1-3- versus β1-4galactosyltransferase activity in these fractions was lower than in those from Colo 205, which was consistent with the almost equivalent quantities of type 1 over type 2 chain glycolipids generated by these fractions [73]. These results further support the hypothesis that membrane organization may be crucial in regulating the biosynthesis of either type 1 or type 2 chain glycolipids. To summarize, in Colo 205 cells the preferential synthesis of type 1 chain may occur by the enrichment of the β1-3galactosyltransferase in the more dense membrane fractions and probably by

the colocalization of this enzyme and the acceptor lacto-N-triaosylceramide in certain regions of the Golgi complex or in the endoplasmic reticulum. The synthesis of low levels of type 2 chain in Colo 205 may be explained by the lower activity and affinity for lacto-N-triaosylceramide of the β1-4galactosyltransferase, and perhaps by the inaccesibility of the precursor lacto-N-triaosylceramide to the Golgi-membrane region where this enzyme is localized. This type of mechanism proposed for the preferential synthesis of type 1 chain-based glycolipids in Colo 205, may actually occur in normal colonic mucosa. However, more detailed studies using normal colonic epithelial cells should be done to confirm this possibility.

In a recent study, Finne et al. [186] reported that most of the glycans attached to glycoproteins of normal small intestine epithelial cells were short N-linked chains, and only a small proportion of them were O-linked. Furthermore, they demonstrated [186] that the majority of the blood group active glycopeptides were based on type 2 chains, that were highly fucosylated, even in the internal GlcNAc residues. They also found that few of the glycans contained sialic acid, and that most of those containing sialic acid were O-linked oligosaccharide chains. These findings may indicate that the pathways for glycosylation of glycoproteins and glycolipid are different and that they may occur in different locations of the Golgi apparatus. Most of the studies done in the glycosylation of glycoconjugates have been done in the biosynthesis of N-linked oligosaccharide chains of glycoproteins. The pathway depicted and the site for the N-linked glycosylation of glycoproteins may not actually be the same for the biosynthesis of glycolipids. The addition of lacto/lacto-neo sequences to the glycoprotein- oligosaccharide chains is known to occur in

the trans-Golgi where β-galactosyltransferase activity exists. Addition of fucose, sialic acid and blood group active residues also occurs along the trans-Golgi. In Colo 205 cells [73], the more dense membrane fractions of the density gradient contained the highest β1-3galactosyltransferase activity, and those fractions were also associated with the synthesis of type 1 chain glycolipids. Nevertheless, there was β1-4galactosyltransferase activity in less dense fraction that corresponded to the trans-Golgi. Probably the β1-4galactosyltransferase activity in the trans-Golgi is associated with the biosynthesis of glycoproteins and the β1-3galactosyltransferase in the more dense fractions is related to the synthesis of glycolipids. When Colo 205 membranes were detergent-solubilized, the membrane organization of these enzymes was disrupted, therefore, Colo 205 membranes were able to form type 1 and type 2 chain glycolipids. Likewise, when the precursor lacto-N-triaosylceramide was supplied to the intact Colo 205 membranes, they could make type 1 and type 2 glycolipids. To further support the theory that the pathway for glycoproteins and glycolipids are different and the enzymes involved are localized in separate compartments of the Golgi, it will be interesting to check if the N-linked oligosaccharide chains of Colo 205 cells are mainly based on type 2 chains. It also will be interesting to localize these glycosyltransferases within the Golgi complex, by immunostaining with specific antibodies.

In a more general view, membrane organization may be important in modulating the correct sequence of monosaccharides that are added to growing oligosaccharide chains of an specific glycolipid or glycoprotein. This hypothesis also poses an alternative mechanism by which changes in the

oligosaccharide chains of glycoconjugates occur as a consequence of malignant transformation; it may actually be that rather than activation or deletion of glycosyltransferases, their different organization within the Golgi or the ER may be responsible for alterations in the expression of their products. For example, glycolipids with multimeric Le^x and Le^y structures are expressed by colonic cancer cells but not by normal cells. However, normal small intestinal epithelial cells express multimeric Le^y structures in their glycoprotein-N-linked oligosaccharides like those expressed in the glycolipids of malignant cells. This similarity may indicate that in cancer cells the organization of the Golgi complex has changed in some way, that the glycolipids instead of being glycosylated by their normal biosynthetic pathway, go through glycosylation using the pathway normally used by glycoproteins. In this way glycolipids from malignant cells express oligosaccharide structures that resemble those carried by glycoproteins in normal cells.

2.2.6 Other Tumor-Associated Carbohydrate Antigens in Colon Cancer

Other tumor-associated antigens that are not based in lacto- or lactoneo-series structures have been reported in colon cancer. For example, GM2 ganglioside composed of N-glycolylneuraminic acid instead of N-acetylneuraminic acid has been found in colonic carcinomas [177]. Also, ganglioside GM3 containing 4-O-acetyl-N-glycolylneuraminic acid has been detected in ganglioside fractions purified from human colon cancer tissues [178].

2.3 SW1116 CELLS AND THE SIALYL LEWIS^a ANTIGEN

2.3.1 SW1116 Cells

The colorectal carcinoma cell line SW1116 was established by Leibovitz et al. [179] from a grade II colon adenocarcinoma of a 73 year-old, white male donor, of blood group type O. SW1116 cells are columnar, resembling normal absorptive epithelium. Microvesicular bodies are often observed along the brush border membrane. The chromosome number of each cell is hypertriploid. SW1116 cells secrete large amounts of carcinoembryonic antigen (CEA) into the culture media (7 $\mu g/10^6$ cells/21 days).

Several investigators [17, 180-183] have used this cell line to immunize mice, producing several monoclonal antibodies. Some of these monoclonal antibodies are, CO-293, CO-294, CO-301, CO-431 [180], NS-33a, NS-38a, and NS-48a specific for Le^b antigens [17, 181]; NS-10-17 and CO-432 specific for Le^b and H-type 1 antigens [17, 180-182]; and NS-19-9, and NS-52a, specific for the sialyl Le^a antigen [17, 183]. All these monoclonal antibodies were found to have an apparent specificity for colorectal tumors cells [17, 180]. One of the most investigated of these hybridoma antibodies, is the NS-19-9 which has been extensively used in the sereologic detection of gastrointestinal cancer. The antigen recognized by this antibody was defined by Magnani et al. [183], as a ganglioside containing the sialyl Le^a epitope.

2.3.2 The Sialyl Lewis^a Antigen

The sialyl Le^a, 19-9, or gastrointestinal cancer antigen (GICA) defined by the monoclonal antibody NS-19-9, is one of the first identified and most extensively studied tumor-associated carbohydrate antigens [183, 184]. The

epitope of this antigen is a specific carbohydrate sequence that is expressed both as a glycolipid [183] at the cell surface and as a secreted high molecular weight mucin type glycoprotein [187, 188]. The carbohydrate structure of this antigen corresponds to a sialylated form of the human Le^a determinant [183] (table 2).

The selective binding of the hybridoma antibody NS-19-9 to colorectal tumor cells [17], catalogued the 19-9 antigen as a marker for colorectal cancer. For instance, Magnani et al. [183] using a solid phase radioimmunoassay, detected the 19-9 antigen in lipid extracts of 12 out of 21 colonic adenocarcinomas, 4 out of 5 gastric adenocarcinomas and 4 out of 7 pancreatic carcinomas. Similarly, Atkinson et al. [191] demonstrated, by the immunoperoxidase technique, that the NS-19-9 monoclonal antibody stained 59% of the colonic carcinoma, 86% of pancreatic adenocarcinoma, and 89% of all the gastric adenocarcinoma tissues assayed in their study. Haglund et al. [189], have also showed that the 19-9 antigen is well expressed in most pancreatic carcinomas. Futhermore, the 19-9 antigen was detected in blood from patients with colorectal carcinoma [185, 190], since serum from these patients inhibited the binding of the monoclonal antibody NS-19-9 to SW1116 cells [185]. The antigen also had a high incidence in the blood serum from patients with pancreatic cancer [189, 190] and gastric adenocarcinoma [190]. Further experiments demonstrated that the 19-9 antigen present in serum of cancer patients and in culture media from SW1116 cells, was carried not by a glycolipid, but by a high molecular weight mucin-glycoprotein [187, 188]. Those findings led to the use of the the NS-19-9 monoclonal antibody in the serologic detection of gastrointestinal and pancreatic carcinomas [185, 187, 190]. In spite of the usefulness of the NS-19-9

monoclonal antibody in the diagnosis of pancreatic and gastrointestinal malignancies, its application is limited to those patients that express the Le gene. Individuals that are Lewis negative, Le (a^-, b^-), are unable to synthesize any Le active determinants, including the sialyl Le^a, in normal or transformed tissues.

The distribution of the 19-9 antigen in fetal, normal and neoplastic tissue depends on whether the individual carries an active Lewis gene. The 19-9 antigen has been detected in lipid extracts from human meconium, which represents tissue from fetal origin. Likewise, Raux et al. [192] reported that the 19-9 antigen was present in all the gastrointestinal tracts of all the fetuses and newborns examined by immunohistology; the biliary and pancreatic ducts were also positively stained; but the rest of the fetal tissues were negative for the 19-9 antigen. Raux et al. [192] concluded from their data, that the 19-9 antigen was present in the fetal organs as mucin-type glycoproteins. By comparison, Olding et al. [193], found that the 19-9 antigen was only expressed in the fetal small intestinal mucosa but not in the colorectal epithelium; they also detected the antigen in other fetal organs such as the fetal larynx, trachea and main bronchi epithelium, in the conjunctiva, lacrimal and salivary glands, in the gall bladder and in pelvic renal epithelium. The appearance of the 19-9 antigen, in human meconium, and in the fetal gastrointestinal tract [192] or at least in a portion of it [193] have indicated that this is an oncofetal antigen.

The presence of the 19-9 antigen in normal adult tissues also has been examined. Magnani et al. [183] reported that the 19-9 antigen was absent from normal gastric and colonic mucosa, from the pancreas, liver, kidney and bone

marrow. However, other studies have demonstrated the presence of the 19-9 antigen in several normal gastrointestinal tissues from Le (a^+, b^-) and Le (a^-, b^+) individuals [191, 194, 195], such as goblet cells of the upper crypts, esophagus squamous epithelium, esophageal glands and ducts, and the pyloric surface mucosa. The 19-9 antigen was absent in the normal ileal and colonic absorptive epithelial cells [194], indicating that the 19-9 antigen is probably a differentiation marker for different subpopulations of gastrointestinal cells. The 19-9 antigen also has been detected in the gall bladder epithelium [191, 195], small ducts of the pancreas [189], and in seminal fluid [27].

The biosynthesis of the sialyl Le^a antigen has been studied by Hansson and Zopf [196], in the colorectal carcinoma cell line, SW1116. They found that sialylation of the type 1 core precedes the transfer of the α1-4 fucose to the GlcNAc residue, catalyzed by the Le^a-specific fucosyltransferase (fig. 5). Individuals lacking an active Lewis gene would not be able to express the sialyl Le^a determinant as a normal antigen in the tissues where it normally exists, or as a tumor-associated antigen when malignant transformation occurs. This implies that in the SW1116 cells and in the colorectal carcinoma the expression of the sialyl Le^a antigen is caused by the activation of a sialyltransferase that is not normally expressed by the colorectal epithelial cells. Indeed, Jolif and Liepkans [197] have reported the presence of an α2-3sialyltransferase, able to transfer sialic acid to the terminal galactose of lacto-N-tetraosylceramide, in subcellular membrane fractions of SW1116 cells. However, the synthesis of the sialyl Le^a antigen has not been studied in normal cells that express this epitope in order to check if its biosynthesis follows the same path as when this antigen is synthesized in cancerous tissues.

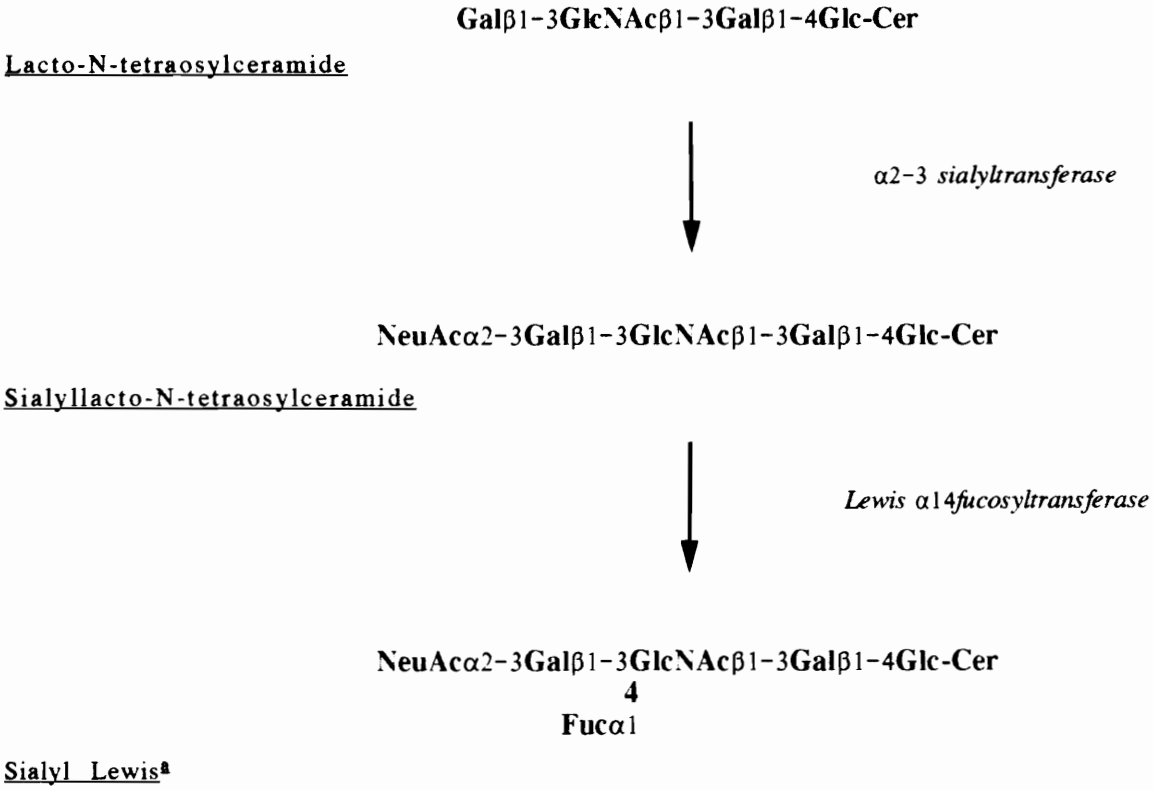


Figure 5.- Biosynthesis of the sialyl Le[a] antigen in SW1116 cells.

It could be the case that the Lewis-gene α1-4fucosyltransferase is unable to fucosylate previously sialylated type 1 chain precursors in the normal tissues where it is expressed, and that the biosynthesis proceeds via fucosylation first and then sialylation of the Le^a antigen in the normal tissues. If this is the normal pathway for the synthesis of this antigen, one should anticipate that in the malignant tissue what has really been altered is the specificity of the Lewis-gene α1-4fucosyltransferase which is now able to fucosylate previously sialylated type 1 chains as occurs in the synthesis of the sialyl Le^a antigen in SW1116 cells. On the other hand, it will be interesting to determine if the precursor sialyllacto-N-tetraose, STa (NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc-Cer) is present in normal colon epithelial cells, which will indicate if the α2-3sialyltransferase actually exists in the normal tissue counterpart, and it will eliminate the possibility that this sialyltransferase is activated as a consequence of malignant transformation. Moreover, it should be verified if the Lewis gene fucosyltransferase present in the normal colonic epithelial cells, where this antigen is not expressed, is able to fucosylate the precursor sialyllacto-N-tetraose, as the Lewis enzyme does in the SW1116 cells. Different routes of synthesis of Lewis antigens have been observed in cancer cells as compared to those in the equivalent normal cells [75]. As an example, the gastric carcinoma cell line KATO III, is able to form the Le^b glycolipid either by the classical pathway (see fig. 3) in which the H-precursor glycolipid is synthesized first and then the α1-4 fucose is added to the GlcNAc residue, or by an aberrant pathway, in which the Le^a glycolipid served as precursor for the H-specific α1-2fucosyltransferase, therefore forming the Le^b glycolipid from the Le^a precursor [75]. This unusual pathway can be explained either by an

alteration in the H-specific fucosyltransferase that now has a broader specificity or by the expression of a new α1-2fucosyltransferase with a different specificity than that of the H-fucosyltransferase. Liepkans and Larson [198], also have studied the biosynthesis of the Le^b glycolipid in SW1116 cells, and determined that synthesis of this antigen in SW1116 cells occurs by the normal classical route, with no aberrant formation of Le^b from Le^a.

2.4 ANALYSIS OF CELL SURFACE GLYCOLIPIDS BY METABOLIC LABELLING

Current methods for determination of carbohydrate structures in glycolipids include nuclear magnetic resonance (NMR) [199, 200], mass spectrometry (MS) [200-205], and methylation analysis [200, 202]. These methods are sensitive enough to permit complete structural identification of a single glycolipid using microgram quantities. For instance, 100-500 μg of a pure glycolipid are necessary for NMR analysis [69, 76, 200], 100 μg for methylation analysis [69], 1-5 μg for fast atom bombardment-mass spectrometry (FAB/MS) [200, 203], and 5-10 μg of purified glycolipid for electron impact-mass spectrometry [69, 76]. In spite of the sensitivity of these methods, the concentration of glycolipids in cells is very low[2], and in order to get purified glycolipid samples in the

[2] For example, blood group active glycolipids represent 0.04% of the erythrocyte plasma membrane [206]; the concentration of globoside, the major glycolipid of the erythrocyte plasma membrane, is 9.4 mg/100 ml of packed erythrocytes [207]; the concentration of lactotriaosylceramide is 47 μg/100 ml of packed erythrocytes [207]; sialyl Le^a represents 0.003% of the weight of wet SW1116 cells [183]; upper neutral glycolipids from colonic adenocarcinoma-metastatic liver tumors represent 30-40 mg/ 100 g of wet tissue [69]; total neutral glycolipids vary from 1.0-6.1 mg per gram of dry tissue from intestinal epithelial cells [149]; and a single human small intestine contains approximately 600 mg of neutral glycolipids [147, 148].

microgram range, large amounts of cells or tissues are required. For example, to obtain 300 μg of the sialyl Le^a glycolipid, 10 grams of SW1116 cells were required [183]; likewise, to determine the structures of a Le^y and a BLe^y glycolipids in KATO III cells, 100 grams of packed cells were used [76]. To identify the structure of the trifucosyl Le^y glycolipid, 100 grams of wet colonic adenocarcinoma-metastatic tumors in liver tissue were employed [69]. To biochemically characterize the 5 major glycolipids of the hepatoma cell line Hep-G2 [208], 12 ml of packed cells were utilized, which represents the use of approximately 700, 60-milimeter-diameter tissue culture plates of confluent cells.

In combination with the methods mentioned above, monoclonal antibodies and lectins can be used to detect glycolipids by staining on thin layer chromatograms [67-73, 74-78, 167-170, 175- 178, 183, 184, 196, 208-211]. The immunostaining technique is very sensitive, detecting nanogram amounts of glycolipids. However, monoclonal antibodies react with specific determinants in a carbohydrate sequence, therefore, a separate method is commonly required to determine the complete structure. Likewise, to completely characterize a complex mixture of glycolipids, a set of monoclonal antibodies specific for all possible carbohydrate sequences is absolutely necessary [208]. Another limitation of this method is that monoclonal antibodies, produced by immunizing mice with a particular cell type, are restricted to those carbohydrate epitopes that are able to induce an immune response in the animal. For instance, carbohydrate sequences used for immunization that are also native to the animal, probably will not provoke an immune response.

Also, oligosaccharide chains in glycolipids that are not expressed at a high concentration in the plasma membrane, that are masked by other longer oligosaccharide-chain-glycoconjugates, or that are not present at the cell surface at all stages, may not be capable of generating a strong immune response in immunized mice. As a consequence, those glycolipid structures unable to stimulate the immune system of the animal, will not be identified, if the methods used for their detection rely exclusively on the immunostaining technique.

A method that has been used for analysis of carbohydrate structures on glycoproteins involves the metabolic labelling of cells in culture with radioactive monosaccharide precursors and analysis of the labelled glycopeptides by several classical methods such as methylation analysis and exoglycosidase digestions. This technique has the advantage that minor quantities of complex oligosaccharides are sufficient to analyze the complete structure of individual species. This method was originally applied by Kornfeld et al. [212-214] to elucidate the carbohydrate structure of the pyrophosphoryldolichol-linked oligosaccharide intermediate used in the biosynthesis of the N-linked oligosaccharide chains of glycoproteins. They metabolic labelled the cells with [2-^{3}H]-mannose as the radioactive monosaccharide precursor, isolated the radiolabelled lipid-linked intermediate, and determine its structure by methylation analysis and exoglycosidase digestions. They found that the [2-^{3}H]-mannose was exclusively incorporated as mannose in the lipid-linked oligosaccharide. When they labelled the cells with [1-^{3}H]-glucosamine, the radiolabel was found only in N-acetylglucosamine. Labelling with [^{14}C]-mannose, resulted in

incorporation of the radiolabel into 80% of the mannose, while the rest was incorporated as glucose. When labelling with [^{3}H]-galactose, 90-95% of the incorporated counts were found as glucose [212]. The metabolic labelling method was further used by Kornfeld et al. in the identification of the carbohydrate structures of high mannose and complex N-linked oligosaccharides [215]. Moreover, Kornfeld and Cummings, in combination with metabolic labeling, established the separation of complex mixtures of glycopeptides by serial lectin affinity chromatography, and subsequently analyzed the separated single structures using classical biochemical and chemical methods [216]. This innovative approach has been extremely useful for the structural analyses and the study of the biosynthesis of the oligosaccharide chains of biologically important glycoproteins. Such as, the identification of the oligosaccharide chains of the murine Ia antigen [217], the N- and O-linked oligosaccharides of the low density lipoprotein (LDL) receptor [218], the oligosaccharide moieties of the epidermal growth factor (EGF) receptor [219], and the N-linked oligosaccharides from the mouse lymphoma cell lines BW5147, and PHA^R 2.1 [220].

While this is a powerful technique for the structural analysis and biosynthesis of glycoconjugates in cultured cells, its application to the structural characterization of glycolipids has been restricted to studies in this laboratory. Analysis of the carbohydrate sequences derived from glycolipids using this method, only requires small quantities of cells in culture and permits the identification of the whole spectrum of glycolipids synthesized by a specific cell line. Monoclonal antibodies and lectins, as well as biochemical (sequential exoglycosidase digestion) and chemical techniques (methylation

analysis), can be used to elucidate the structures of the metabolically labelled glycolipids. Indeed, the main goal of this project has been to apply this method to the study of the different glycolipids expressed by the colorectal carcinoma cell line SW1116.

III

EXPERIMENTAL PROCEDURES

3.1 MATERIALS

SW1116 cells were purchased from the American Type Culture Collection (Rockville, Maryland). Disposable culture plates (60 x 15 mm) were purchased from Hazleton (Denver, PA). Fetal calf serum, F-12 media, RPMI 1640, and penicillin/streptomycin were from Flow Laboratories Inc. (Philadelphia, PA). 6-[^{3}H]-galactose (25 Ci/mMol) and 6-[^{3}H]-glucosamine (38 Ci/mMol) were purchased from Amersham (Arlington Heights, IL). Pronase and *Arthrobacter ureafaciens* neuraminidase were purchased from Calbiochem (San Diego, CA). Beef kidney α-fucosidase, and coffee bean α-galactosidase were purchased from Boehringer Mannheim (Indianapolis, IN). Jack bean β-galactosidase was from Sigma Chemical Company (St. Louis, MO). *Ricinus communis* agglutinin I (RCA-I) covalently linked to agarose, *Wistaria floribunda* (WFA) agglutinin, and *Griffonia simplicifolia*-I (GS-I) were purchased from EY Laboratories, Inc. (San Mateo, CA). *Helix-pomatia* lectin and Concanavalin A-Sepharose were from Sigma Chemical Company (St. Louis, MO). *Maackia amurensis* leukoagglutinin (MAL) coupled to Concanavalin A agarose was a gift from Dr. R. D. Cummings, University of Georgia (Athens, GA). Eldeberry bark agglutinin or *Sambucus nigris* (SNA) lectin was purchased from Boehringer Mannheim (Indianapolis, IN). Globoside was purchased from Supelco (Bellefonte, PA). Bovine brain gangliosides, sulfatides, gluco- and galactocerebrosides were from Sigma Chemical Company (St. Louis, MO).

Ganglioside GM3 purified from cow erythrocytes, ganglioside GM2 from Tay-Sachs brain, ganglioside GM1 from cow brain, and CTH from pig heart were a gift from Dr. T. W. Keenan, Virginia Tech (Blacksburg, VA). Beta-vials (7 ml) were from Denville Scientific (Denville, NJ). Ecolume scintillation cocktail was purchased from ICN Radiochemicals (Los Angeles, Ca). Sep-Pak C_{18} cartridges were from Waters Associates (Milford, MA). DEAE-Sepharose and Sephadex G-25 were from Pharmacia (Uppsala, Sweden). AG 50 x 4-hydrogen form and AG 3 x 4-acetate form were from Bio-Rad (Richmond, CA). DE-52 cellulose, DE-53 cellulose and Whatman No 1 chromatographic paper were purchased from Whatman (Clifton, NJ). Precoated Merck silica gel-60 aluminum-backed thin layer plates were from Bodman Chemicals (Media, PA). Constant boiling 6 M HCL ampules (1 ml) were from Pierce (Rockford, IL). Chloroform, methanol, pyridine, sodium methoxide and dimethylsulfoxide (DMSO) were purchased from Fisher Scientific Company (Pittsburgh, PA). Iodomethane, and trifluoroacetic acid (1 ml ampules) were obtained from Sigma Chemical Company (St. Louis, MO). Reaction vials (1 ml), and acetonitrile were purchased from Baxter Healthcare Corporation (Philadelphia, PA).

3.2 METHODS

3.2.1 Metabolic Labelling of SW1116 Cells

SW1116 cells were cultured in 60 x 15 mm petri dishes, in F-12 Ham's nutrient medium, supplemented with 10% fetal calf serum, 10% RPMI 1640 medium and penicillin/streptomycin (500 iu/ml). Cells were grown at 37° C in a 10%

carbon dioxide/air atmosphere. When SW1116 cells were approximately 70% confluent ($1x10^6$ cells/dish), the medium was removed and new medium containing 222 μCi/ml of either 6-[^{3}H]-galactose (25 Ci/mmol), or 6-[^{3}H]-glucosamine (38 Ci/mmol) was added. After labelling the cells for 30 hours, the radioactive medium was removed and the cells were washed 3 times with cold phosphate-buffered saline (PBS) (0.14 M NaCl, $2.7x10^{-3}$ M KCl, $1.5x10^{-3}$ M KH_2PO_4, and $8.1x10^{-3}$ M Na_2HP0_4 . 7 H_2O) . Cells were scraped from the plates using a rubber policeman, then they were resuspended in cold PBS and transferred to 1.5 ml Eppendorf tubes. The cells were centrifuged, and the PBS was decanted.

3.2.2 Extraction of Glycolipids from Metabolically Labelled SW1116 Cells

The glycolipids and also glycoproteins from metabolically labelled cells were extracted according to the method of Finne and Krusius [230]. Briefly, wet cell pellets were resuspended in cold water (0.225 ml) and sonicated for 15 minutes at 4° C. Methanol (0.6 ml) was added and sonication was continued at room temperature for 15 minutes; chloroform (0.3 ml) was then added to the mixture and sonicated at room temperature for 15 min, so that the final concentration of chloroform/methanol/water was 4/8/3. The cell mixture was centrifuged, and the supernatant was removed. The pellets were extracted one more time with chloroform/methanol/water (4/8/3), then twice with chloroform/methanol (2/1), and finally with absolute ethanol. All organic extracts were combined and dried under reduced pressure.

The remaining pellets were subjected to pronase digestion (5 mg/ml pronase in 0.1 M Tris-HCl buffer, pH 8.0, containing 1 mM $CaCl_2$), at 60° for 24 hours to release soluble glycopeptides.

The crude glycolipid fraction was dissolved in chloroform/methanol (2/1) and subjected to partition with 20% water (v/v) (Folch's partition). Lower and upper phases were separated by centrifugation and the upper phase was removed. The lower phase was washed 4 times with methanol/water (1/1) (theoretical upper phase) and the washes were combined with the first upper phase. The lower phase obtained after partition contained small oligosaccharide chain glycolipids. The upper phase contained acidic glycolipids and larger neutral oligosaccharide chain glycolipids, as well as radiolabeled contaminants such as radiolabeled phosphorylated monosaccharides and their nucleotide derivatives. To separate these water soluble contaminants from the radiolabelled glycolipids, the upper phase was further purified on Sep-Pak C_{18} reverse phase cartridges [231]. Briefly, the upper phase was applied to the Sep-Pak cartridge and passed through it 3 times to ensure adsorption of all glycolipids. Then, the Sep-Pak column was washed with 50 ml of water, to remove the water soluble sugar derivatives; bound glycolipids were eluted from the cartridge with 10 ml of methanol, 10 ml of chloroform/methanol (1/1) and 10 ml of chloroform/methanol/water (4/8/3); these three organic fractions were combined and dried under reduced pressure. Usually, 33% of the radioactivity of the ^{3}H-galactose-labelled upper phase corresponded to radiolabelled water-soluble contaminants when SW1116 cells were labelled for 30 hours. In contrast, as much as 80% of the radioactivity of the ^{3}H-glucosamine-labelled upper phase corresponded to

water-soluble contaminants in 30-hour labellings. This high quantity of ^{3}H-glucosamine-derived, water-soluble radioactive components may be due to several factors. One of them is that ^{3}H-glucosamine is channeled through several biosynthetic pathways to make N-acetyl-glucosamine, N-acetylgalactosamine, N-acetylmannosamine and N-acetylneuraminic acid. These steps probably delay the incorporation of any of these radiolabelled derivatives into the glycolipids and glycoproteins, therefore at 30 hours of labelling most of the radiolabelled derivatives of ^{3}H-glucosamine are still in the cytosol. Another explanation may be that the transport system of the ER and Golgi for the nucleotide derivatives of GlcNAc, GalNAc and NeuAc works more slowly than the transport system for UDP-Gal and UDP-Glc.

In order to separate neutral from acidic glycolipids, upper phase glycolipids were combined with lower phase glycolipids and subjected to DEAE-Sephadex chromatography. The total glycolipids (both phases) were dissolved in chloroform/methanol/water (60/30/4.5) and applied to a DEAE-sephadex column (0.5 x 3 cm) equilibrated in the same solvent. Neutral glycolipids were eluted with 30 ml of the starting solvent, and 30 ml of chloroform/methanol/water (30/60/8). Both fractions were combined and dried under reduced pressure. Acidic glycolipids were eluted with 50 ml of 0.5 M sodium acetate in methanol. This fraction was dried and desalted on a Sep-Pak cartridge as described.

3.2.3 Ozonolysis and Alkali Fragmentation of Glycolipids

Unsaturated sphingosines in glycosphingolipids can be oxidized by ozone to form an ozonide intermediate that decomposes into two aldehyde forms. Base

hydrolysis catalyzes the elimination of the free oligosaccharide from the lipid aldehyde fragment [221, 222, 232]. Metabolic labelled acidic and neutral glycolipids were subjected to ozonolysis and alkali fragmentation as described [221, 222]. Briefly, neutral glycolipids dissolved in 1 ml of chloroform/methanol/water (60/30/4.5) and acidic glycolipids dissolved in 1 ml of chloroform/methanol/water (30/60/8), each containing 20 nmol of carrier glycolipid, were incubated at 4° C for 10 minutes with 0.1 ml of a 31 mM solution of ozone in methylene chloride. The reaction was stopped and excess ozone was destroyed by addition of 0.025 ml of dimethylsulfide. The reaction mixture was dried under nitrogen, dissolved in 1 ml of 0.077 M sodium methoxide in methanol, and incubated for one hour at room temperature. The reaction was terminated by addition of 0.080 ml of 1 M acetic acid and 0.920 ml of water and directly applied to a Sep-Pak cartridge to separate released oligosaccharides from glycolipids resistant to ozonolysis and alkali fragmentation.

3.2.4 Chromatographic Methods

3.2.4.1 Thin Layer Chromatography of Metabolically Labelled Glycolipids

The purified glycolipids prepared from metabolically labelled cells were analyzed on thin layer plates. Aliquots of the neutral and acidic glycolipids (5 x 10^3 cpm) were spotted on aluminum-backed plates and developed for 30-50 minutes in chloroform/methanol/0.25% KCl in water (5/4/1). Radioactivity on the thin layer plates was detected either by scanning the dry chromatograms with a Bio-Scan imaging scanning system (System 200); or by exposing the

thin layer plates to X-ray films, after spraying them with En^{3}Hance. Chromatograms were exposed to X-ray film for 3 to 15 days at -70° C.

3.2.4.2 High Performance Liquid Chromatography (HPLC)

HPLC was carried out using a Beckman Model 332 gradient liquid chromatograph system. A 5 μm amino phase analysis column (4.5 x 300 mm)(Alltech Carbohydrate Associates, Deerfield IL) was used for all liquid chromatograms. To separate neutral oligosaccharides by size program #1 was used. Briefly, the column was eluted with 70% acetonitrile/water as the starting buffer. The concentration of acetonitrile was decreased at a rate of 0.67%/min over 30 minutes, and then was changed to 0.5%/min over 30 minutes. The flow rate was 1 ml/min and 0.5 ml fractions were collected.

Acidic oligosaccharides were separated using program #2 [227], the HPLC column was initially eluted with a mixture of 80% acetonitrile/20% 15 mM KH_2PO_4, pH 5.1. Phosphate buffer concentration was increased linearly at a rate of 0.228%/min over 70 minutes, and then was changed to a rate of 0.300%/min over 50 minutes. Flow rate was 2 ml/min, and 1 ml fractions were collected. Alternatively, neutral oligosaccharides were separated on HPLC employing program #3. The gradient used in this program was the same as that of program #2, but instead of phosphate buffer, distilled-deionized water was used. Acidic samples purified by HPLC were desalted in a G-25 column (2 x 50 cm) equilibrated in 0.1 M pyridine acetate, pH 5.0. Radioactivity was measured by counting aliquots of fractions in 7 ml beta-vials with 3 to 4 ml of Ecolume scintillation fluid, in a Beckman liquid scintillation counter, model LS-5801.

3.2.4.3 DE-53-Cellulose Chromatography

Metabolically labelled acidic oligosaccharides were separated by DE-53 cellulose chromatography according to Smith et al. [233]. The acidic oligosaccharides were applied to a DE-53 cellulose column (0.7 x 20 cm) equilibrated in 2mM pyridine acetate buffer, pH 5.0, and neutral oligosaccharides were eluted with 25 ml of the same buffer. Monosialyloligosaccharides were eluted with 200 ml of 20 mM pyridine acetate, pH 5.0; disialyl and trisialyloligosaccharides were eluted with 60 ml of 500 mM pyridine acetate, pH 5.0. Aliquots of fractions were assayed for radioactivity as above.

3.2.4.4 Lectin Affinity Chromatography

Affinity chromatography on *Ricinus communis* I (RCA-I)-agarose was performed as described [232]. The column (0.7 x 50 cm) was equilibrated in PBS/0.02% sodium azide at room temperature. Samples were applied to the column and allowed to interact for 15 minutes before beginning elution with PBS/0.02% sodium azide. Fractions of 1 ml were collected and assayed for radioactivity as described. Pooled fractions were desalted by directly applying them to a mixed bed ion exchange column (1 x 10 cm) (AG 50 x 4-hydrogen form, and AG 3 x 4-acetate form). The column was eluted with 6 volumes of distilled, deionized water. The eluate was dried either, by lyophilization or evaporation under reduced pressure in a rotary evaporator. Affinity chromatography on *Griffonia simplicifolia* I (GS-I) linked to Con A-Sepharose was carried out in a 1 ml pipet column (0.3 x 14 cm) as described [237]. *Helix Pomatia* (HP)-agarose chromatography (column of 0.5 x 5 cm) and *Wisteria floribunda* (WFA)-agarose chromatography (column of 0.5 x 18 cm) were

carried out as described [236]. Affinity chromatography of acidic oligosacchrides on *Maackia amurensis* (MAL) linked to Con A-Sepharose (1 ml pipet column, 0.3 x 14 cm) was performed as described [244]. Affinity chromatography of acidic oligosaccharides on *Sambucus nigris* (SNA) linked to Con A-Sepharose (0.3 x 14 cm) was carried out as the MAL affinity chromatography.

3.2.5 Monosaccharide Composition of Metabolically Labelled Oligosaccharides

The monosaccharide composition of the ^{3}H-galactose- and ^{3}H-glucosamine-metabolically labelled oligosaccharides was determined by strong acid hydrolysis of each purified sample followed by descending paper chromatography of the hydrolyzate. The identity of radioactive monosaccharides was established by cochromatography with authentic unlabelled standards. Briefly, dried samples (oligosaccharides or glycolipids) were treated with 2N HCL for 4 hours at 100^{o} C. After hydrolysis, HCL was evaporated under a nitrogen stream, then the sample was dissolved in 50 µl of water and applied to Whatman No 1 chromatography paper (2.8 x 50 cm strips). Descending paper chromatograms were developed in the solvent system ethyl acetate/pyridine/water (12/5/4) for 24 hours for ^{3}H-galactose-labelled oligosaccharides, and for 48 hours for ^{3}H-glucosamine labelled samples. Unlabelled galactose, glucose, mannose and fucose (50 µg of each) were used as standards when determining the composition of ^{3}H-galactose-labelled oligosaccharides, and unlabelled glucosamine and galactosamine (50 µg of each) when ^{3}H-glucosamine oligosaccharides were analyzed. Unlabelled

standards were visualized by silver nitrate staining [234]. Radioactive monosaccharides were localized by cutting the paper chromatogram into 1 cm segments. Paper segments were placed in 7-ml beta vials, to which 0.5 ml of water were added. Ten minutes after addition of water, 4 ml of Ecolume scintillation fluid were added. Each vial was capped and mixed vigorously in a vortex mixer for 30 seconds, and radioactivity was determined.

3.2.6 Methylation Analysis

Methylation analysis was performed to determine the position of the glycosidic linkages. Oligosaccharides were reduced with $NaBH_4$ before methylation. The reduction was carried out by treating the samples with 1 ml of a 2 mg/ml solution of $NaBH_4$ in 0.01 M NaOH for 2 hours at 37° C. An additional 1 ml of the same solution was added after this period and incubation was continued for another hour. Unreacted $NaBH_4$ was destroyed by acidification with acetic acid. The resulting solution was applied to a cation exchange column, AG-50 x 4 W-hydrogen form (1 x 5 cm) to eliminate Na^+ ions. The column was washed with 5 volumes of distilled, deionized water, and the eluate was dried under reduced pressure in a rotary evaporator. Dry samples were resuspended in methanol and evaporated 7 times, in order to remove boric acid as its methyl ester. At this stage neutral oligosaccharides were ready for methylation. In order to remove desialylated oligosaccharides formed during the reduction, acidic samples were applied to a DE-52-cellulose column (1 x 2 cm), preequilibrated in 2 mM pyridine acetate, pH 5.0. The column was washed with 20 ml of the same buffer and then with 20 ml of 0.1 M pyridine acetate, pH 5.0 to elute retained acidic oligosaccharides.

Methylation of the reduced samples was carried out using the method of Ciucanu and Kerek [235] with some modifications. Briefly, samples were dried in 12 x 100 mm screw cap tubes, just before methylation was going to be started. They were resuspended in 1 ml of DMSO and sonicated for 1 hour, with intermitent vigorous mixing in a vortex mixer. Then, 50 μg of finely powdered, dry NaOH was added to samples, and they were sonicated for 30 min. At this point, 0.5 ml of methyl iodide was added to samples and they were sonicated for one hour. Then, 1 ml of chloroform and 2 ml of 2 M acetic acid were added, mixed vigorously, centrifuged to resolve phases, and the water layer decanted. The chloroform layer was washed 3 more times with 2 ml of 2 M acetic acid. The chloroform layer containing the permethylated oligosaccharides was dried under nitrogen, resuspended in 0.5 ml of methanol and transferred to a 1 ml reactivial. Using this procedure, 60-80% of the oligosaccharides were permethylated. The methanol was evaporated under nitrogen, the samples were dissolved in 0.5 ml of 2N trifluoroacetic acid and incubated for 4 hours at 100° C. The trifluoroacetic was evaporated under nitrogen, the partially methylated monosaccharides were dissolved in 50 μl of methanol and spotted on an aluminum backed thin layer plate (20 x 20). The thin layer plate was developed in the solvent system acetone/water/28% NH_4OH (100/1.2/0.6) for 70 minutes at room temperature. When the chromatogram was dry it was either scanned on a Bio-Scan scanner imaging system (model 200); or it was divided into 0.5 cm segments from bottom to top. The plate was sprayed lightly, with distilled water, the silica was scraped from each segment into a 7 ml scintillation vial, 0.5 ml of water were added and then

4 ml of scintillation fluid. Each vial was mixed vigorously in a vortex mixer for 30 seconds, and counted for 5 minutes in a liquid scintillation counter.

3.2.7 Exoglycosidase Digestions

3.2.7.1 Beef Kidney α-L-Fucosidase Digestion

Oligosaccharide samples were dried in 0.5 ml microcentrifuge tubes, and incubated with 0.05 ml of a solution of 2 U/ml of the α-fucosidase in 0.05 M sodium acetate, pH 5.4, with 5 μl of toluene to prevent bacterial growth, for 72 hours at 37^0 C. After digestion, samples were boiled for 3 minutes and then applied to a G-25 column (2 x 50) as described elsewhere. Desalted samples were then applied to HPLC and the digestion products separated.

3.2.7.2 Coffee Bean α-Galactosidase Digestion

Samples dried in 0.5 ml microcentrifuge tubes were incubated with 0.05 ml of a solution of 0.1 U/ml of α-galactosidase in 0.1 M NaH_2PO_4 buffer, pH 6.0, for 4.5 hours at 37^o C in a toluene atmosphere. After digestion samples were boiled for 3 minutes to denature the enzyme, then centrifuged and directly applied to HPLC to separate the digestion products.

3.2.7.3 Jack Bean β-Galactosidase Digestion

Dried samples in 0.5 ml microcentrifuge tubes were incubated with 0.05 ml of a solution of 0.8-1.6 U/ml of β-galactosidase in 0.05 sodium citrate buffer, pH 3.5, for 24 hours at 37^o C in a toluene atmosphere. After digestion samples were boiled for 3 minutes, centrifuged, and directly applied to HPLC to separate the digestion products. At the concentration of 0.8-1.6 U/ml of β-galactosidase, the Galβ1-4 linkage was totally digested while only 13-18% of the Galβ1-3 linkage was hydrolyzed. These conditions were determined using authentic human

milk ^{3}H-lacto-N-tetraitol and ^{3}H-lacto-N-neotetraitol and different concentrations of the enzyme.

3.2.7.4 *Arthrobacter ureafaciens* Neuraminidase Digestion

Samples dried in 0.5 ml microcentrifuge tubes were treated with 0.05 ml of 0.2 U/ml neuraminidase in 0.1 M pyridine acetate, pH 5.0, in a toluene atmosphere, for 18 hours at 37° C. After digestion, samples were boiled for 3 minutes, centrifuged, diluted to 0.5 ml and then applied to a DE-52 cellulose column (1 x 1 cm) equilibrated in 2 mM pyridine acetate, pH 5.0. Desialylated oligosaccharides were eluted with 15 ml of the same buffer, and undigested oligosaccharides were eluted with 15 ml of 0.1 M pyridine acetate. Usually, 95-98% of sialylated oligosaccharides were digested under these assay conditions. The neutral oligosaccharides were applied to HPLC to determine their sizes.

IV

RESULTS AND DISCUSSION

4.1 GLYCOLIPIDS AND GLYCOPROTEINS FROM METABOLICALLY LABELLED SW1116 CELLS

SW1116 cells were differentially labelled with ^{3}H-galactose and ^{3}H-glucosamine and the glycolipids from each labelling were extracted and separated into neutral and acidic fractions. The remaining pellets were digested with pronase in order to convert glycoproteins into soluble glycopeptides. The distribution of radioactivity incorporated into glycolipids and glycoproteins is shown in table 3. When cells were labelled with ^{3}H-galactose for 30 hours, most of the radioactivity was recovered in the neutral glycolipid fraction. The incorporation of radioactivity into ^{3}H-galactose-labelled acidic glycolipids was 9 times lower and that of ^{3}H-glucosamine was 7 times lower as compared to that of the respective neutral glycolipids which, suggests either that acidic glycolipids represent a very small fraction from the total glycolipids of SW1116 cells or that the turnover of acidic glycolipids is very slow. Glycolipids obtained from SW1116 cells labelled with ^{3}H-glucosamine represented only 12% of the radioactivity recovered while the glycoproteins represented 88% of the radioactivity. This may indicate that radiolabelled derivatives of ^{3}H-glucosamine (N-acetylglucosamine, N-acetylgalactosamine, and sialic acid) are incorporated more rapidly into glycoproteins than glycolipids, perhaps due to a more efficient transport of the nucleotide derivatives of these monosaccharides into the Golgi region

where their addition to the growing oligosaccharide chains of glycoproteins occurs. Since every asparagine-linked oligosaccharide molecule contains two residues of N-acetylglucosamine at its reducing end, therefore when labelling with ^{3}H-glucosamine all N-linked oligosaccharides should be radiolabelled. If these N-linked oligosaccharides also contain sialic acid, N-acetylglucosamine and N-acetylgalactosamine at their non-reducing ends, the radioactivity per N-linked oligosaccharide molecule will increase considerably. Likewise, all O-linked oligosaccharides always contain an N-acetylgalactosamine at their reducing ends and they can also contain sialic acid, N-acetylgalactosamine, and N-acetylglucosamine as part of their monosaccharide chains. As a consequence O-linked oligosaccharides metabolically labelled with ^{3}H-glucosamine should have a high ratio of radioactivity per O-linked chain.

Metabolically labelled neutral and acidic glycolipids were analyzed by thin layer chromatography (shown in figures 6 and 7). The major component present in the ^{3}H-galactose-labelled neutral glycolipids as well as in the ^{3}H-glucosamine-labelled neutral glycolipids migrated on the thin layer plate slightly slower than the tetraglycosylceramide standard (globoside), indicating that this fraction most likely contained other tetraglycosylceramides and also pentaglycosylceramides (fig. 6A, and 6C). The ^{3}H-glucosamine-labelled neutral glycolipids did not contain any of the smaller chain glycolipids such as monohexosylceramides and dihexosylceramides, consistent with the fact that mammalian glycolipids of this size do not contain amino-sugars. Trihexosylceramides were not observed either, indicating that neither gangliotriaosylceramide, nor lactotriaosylceramide (see table 1) were present in neutral glycolipids from SW1116 cells.

Thin layer chromatograms of acidic glycolipids metabolically labelled with ^{3}H-galactose and ^{3}H-glucosamine (fig. 7A and 7B) demonstrated that the major component of the acidic glycolipids migrated slightly slower than the sialyltetraglycosylceramide GM1; suggesting that this peak contained other sialyltetraglycosylceramides and also sialylpentaglycosylceramides.

4.2 OZONOLYSIS/ALKALI FRAGMENTION OF METABOLICALLY LABELLED GLYCOLIPIDS

Neutral and acidic glycolipids from each separate radiolabelling were subjected to ozonolysis and alkali fragmentation in order to release the oligosaccharide from the ceramide portion. The yield of free oligosaccharides obtained by this method ranged from 65%-90%. Analysis by thin layer chromatography of the metabolically labelled glycolipids and that of the corresponding glycolipids resistant to ozonolysis/alkali fragmentation is shown in figures 6 and 7. Representative oligosaccharide samples which had a distribution parallel to that of the original glycolipid mixture were obtained. The thin layer chromatogram profiles of metabolically labelled glycolipids and those of the glycolipids resistant to cleavage by ozonolysis/alkali fragmentation (fig. 6 and 7) showed that the distribution of radioactivity among the different peaks is approximately equivalent in the original glycolipid samples as compared to that of the corresponding glycolipids remaining after ozonolysis/alkali fragmentation.

TABLE 3

Distribution of Radioactivity Incorporated into the Glycolipids and Glycoproteins of Metabolically Labelled SW1116 Cells

^{3}H-Monosaccharide Precursor	Neutral Glycolipids (cpm/10^6 cells)	Acidic Glycolipids (cpm/10^6 cells)	Glycoproteins (cpm/10^6 cells)
GALACTOSE	3.73×10^6 (58%)	0.36×10^6 (6%)	2.30×10^6 (36%)
GLUCOSAMINE	0.36×10^6 (10.5%)	0.05×10^6 (1.5%)	3.03×10^6 (84%)

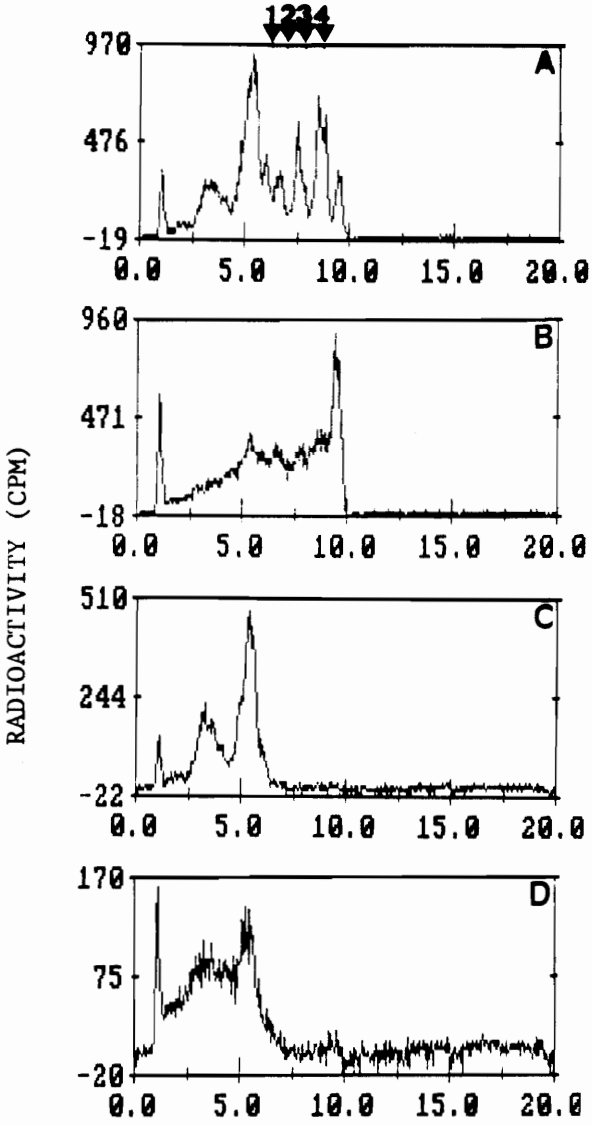


Figure 6.- Analysis of neutral glycolipids and the corresponding ozonolysis/alkali fragmentation resistant glycolipids from ^{3}H-galactose- and ^{3}H-glucosamine-metabolically labelled SW1116 cells by thin layer chromatography.- Neutral glycolipids isolated from ^{3}H-galactose (5,000 cpm = 1,351 cells) and ^{3}H-glucosamine (5,000 cpm = 13,889 cells) labelled SW1116 cells by the procedures described in chapter III, and the corresponding glycolipids from each fraction that were resistant to ozonolysis/alkali fragmentation were applied to thin layer chromatography. Aliquots containing 5,000 cpm of each glycolipid fraction were spotted on aluminum-backed TLC plates (10 x 10 cm) and developed for 40 minutes in chloroform/methanol/0.25% aqueous KCL (5/4/1). Dry chromatograms were scanned with a Bio-Scan imaging scanning system 200. A, lane 1, ^{3}H-galactose-neutral glycolipids; B, lane 2, ^{3}H-galactose-neutral-ozonolysis/alkali fragmentation resistant glycolipids; C, lane 3, ^{3}H-glucosamine-neutral glycolipids; D, lane 4, ^{3}H-glucosamine-neutral-ozonolysis/alkali fragmentation resistant glycolipids. Unlabeled glycolipid standards are indicated, 1, globoside; 2,CTH, globoside trihexoside; 3, CDH, lactosylceramide; 4, CMH, glucose- and galactocerebrosides. The standards were chemically detected with orcinol reagent.

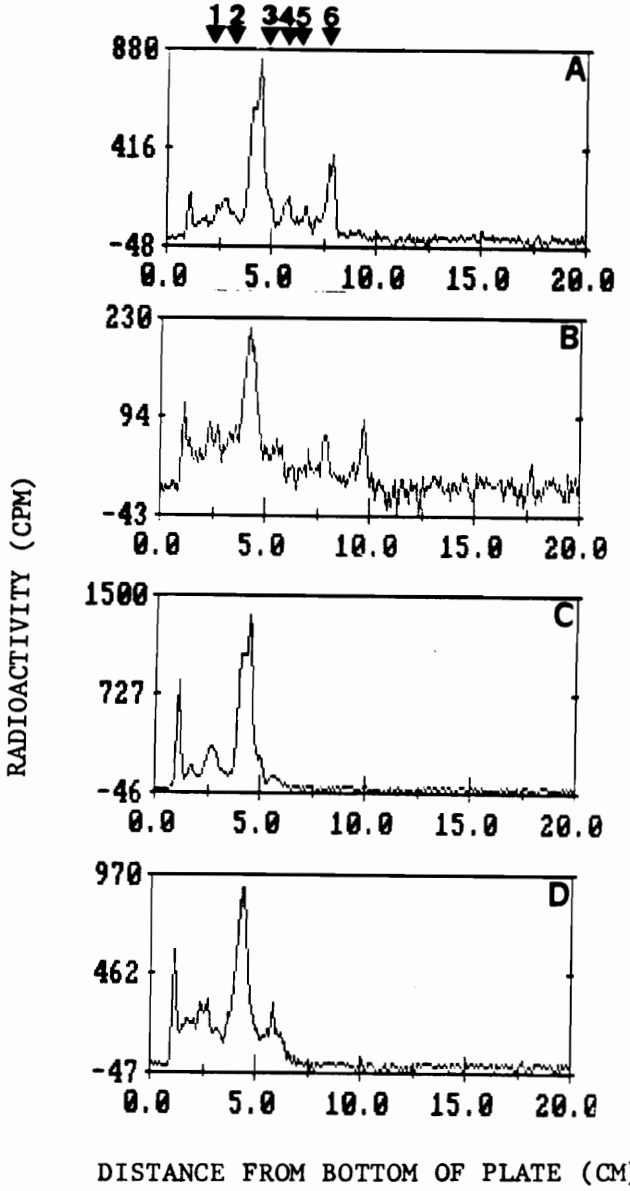


Figure 7.- **Analysis of acidic glycolipids and the corresponding ozonolysis/alkali fragmentation resistant glycolipids from ^{3}H-galactose- and ^{3}H-glucosamine-metabolically labelled SW1116 cells by thin layer chromatography.**- Acidic glycolipids isolated from ^{3}H-galactose (5,000 cpm = 13,889 cells) and ^{3}H-glucosamine (5,000 cpm = 98,039 cells) labelled SW1116 cells by the procedures described in chapter III, and the corresponding glycolipids from each fraction that were resistant to ozonolysis/alkali fragmentation were applied to thin layer chromatography. Aliquots containing 5,000 cpm of each glycolipid fraction were spotted on aluminum-backed TLC plates (10 x 10 cm) and developed for 40 minutes in chloroform/methanol/0.25% aqueous KCL (5/4/1). Dry chromatograms were scanned with a Bio-Scan imaging scanning system 200. A, lane 1, ^{3}H-galactose-acidic glycolipids; B, lane 2, ^{3}H-galactose-acidic-ozonolysis/alkali fragmentation resistant glycolipids; C, lane 3, ^{3}H-glucosamine-acidic glycolipids; D, lane 4, ^{3}H-glucosamine-acidic-ozonolysis/alkali fragmentation resistant glycolipids. Unlabeled glycolipid standards are indicated, 1, GD1b; 2, GD1a; 3, GM1; 4, GM2; 5, GM3;, 6, sulfatides. Glycolipid standards were chemicaly detected with orcinol reagent, the structures are shown in table 4.

TABLE 4

GANGLIOSIDE STANDARDS

SYMBOLS	STRUCTURE
GM3	NeuAcα2-3Galβ1-4Glc-Cer
GM2	GalNAcβ1-4Galβ1-4Glc-Cer NeuAcα2-3
GM1	Galβ1-3GalNAcβ1-4Galβ1-4Glc-Cer NeuAcα2-3
GD3	NeuAcα2-8NeuAcα2-3Galβ1-4Glc-Cer
GD1a	NeuAcα2-3Galβ1-3GalNAcβ1-4Galβ1-4Glc-Cer NeuAcα2-3
GD1b	Galβ1-3GalNAcβ1-4Galβ1-4Glc-Cer NeuAcα2-8NeuAcα2-3

4.3 SEPARATION AND STRUCTURAL ANALYSIS OF NEUTRAL OLIGOSACCHARIDES

After ozonolysis/alkali fragmentation of the neutral glycolipids, free oligosaccharides were separated from the lipid portion by Sep-Pak reverse phase chromatography. In preliminary studies, neutral oligosaccharides were subjected to serial lectin affinity chromatography using lectin-agarose affinity systems that have been developed in this laboratory [236, 237]. Lectins bind specific types of glycosidic linkages, generally at the non-reducing end of the oligosaccharide chain. The interactions between lectins and carbohydrates are stereospecific, allowing the separation of positional isomers. Lectin affinity chromatography has been used in the fractionation of human milk oligosaccharides [227, 238], glycolipids [239] and glycolipid-derived oligosaccharides [236, 237]. For example, we have employed *Ricinus communis* agglutinin-I (RCA-I) immobilized to agarose to separate human milk lacto-N-tetraose (Galβ1-3GlcNAcβ1-3Galβ1-4Glc) and lacto-N-neotetraose (Galβ1-4GlcNAcβ1-3Galβ1-4Glc) based on the capability of RCA-I to strongly bind terminal Galβ1-4GlcNAc residues and to weakly retard its positional isomer Galβ1-3GlcNAc. RCA-I is also able to interact to a lesser extent with lactose, Galβ1-4Glc, than with terminal Galβ1-4GlcNAc. Another lectin affinity column used in this laboratory has been *Helix Pomatia* -agarose (HP) which binds with high affinity terminal GalNAc residues linked α1-3 to Gal, which defines the blood group A antigen. Forssman glycolipid (GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glc-Cer) has been purified on HP-agarose using a tetrahydrofuran/water system [239], and Forssman-derived pentaose has also been purified in the same column using phosphate buffered saline [236].

Wisteria floribunda (WFA) lectin immobilized to agarose has a weak affinity for GalNAcβ1-3Gal and GalNAcα1-3Gal but is able to strongly bind GalNAcβ1-4Gal. WFA-agarose has been employed to separate globoside tetraose (GalNAcβ1-3Galα1-4Galβ1-4Glc) and asialo GM2 triose (GalNAcβ1-4Galβ1-4Glc) [236]. Another lectin affinity chromatography column developed in this laboratory is *Griffonia simplificifolia*-I(GS-I)-agarose that has specificity for terminal Galα1-3Gal residues, which corresponds to the blood group B antigen [237].

Oligosaccharides released from metabolically labelled SW1116 neutral glycolipids were subjected to affinity chromatography in each of the lectin-agarose systems described. Oligosaccharides were neither retarded nor bound to any of these lectin affinity columns except to the RCA-I-agarose column. These results, although largely negative, suggested that the neutral oligosaccharide structures did not contain any of the terminal sequences recognized by those lectins, and that most likely the neutral oligosaccharides were composed mainly of lacto- and lacto-neo series structures since they were able to bind RCA-I-agarose.

Neutral oligosaccharides were then applied to HPLC and separated into different peaks based on their size (figure 8 and in table 5). The major peaks from the ^{3}H-galactose-labelled oligosaccharides corresponded to a disaccharide (18%) and a pentasaccharide (14%) (fig. 8A). The main components from the ^{3}H-glucosamine-labelled neutral oligosaccharides, were the pentasaccharide (24%) and the hexasaccharide (19%) peaks (fig. 8B).

It also was found that the ^{3}H-glucosamine-labelled oligosaccharides contained barely detectable amounts of the smaller chain oligosaccharides

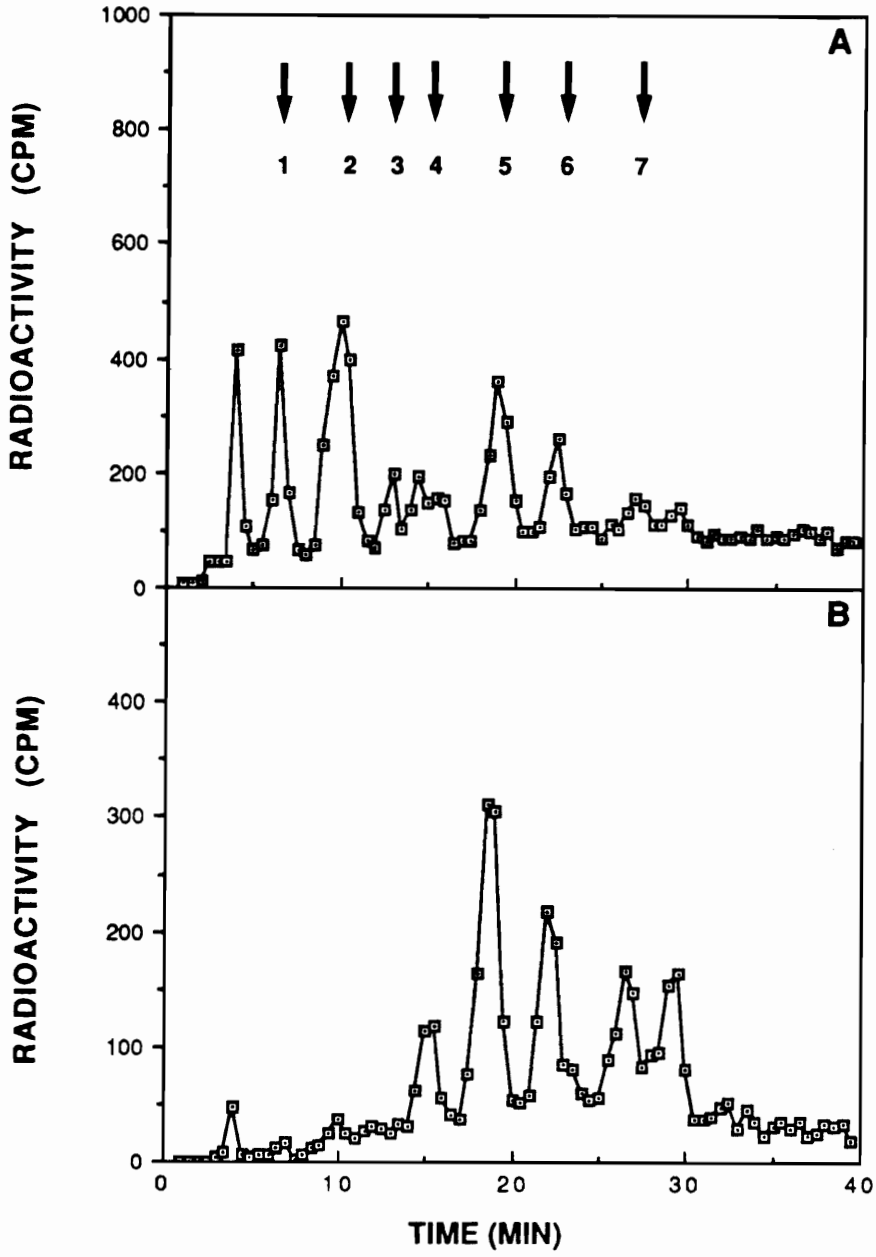


Figure 8.- HPLC of oligosaccharides released by ozonolysis/alkali fragmentation of SW1116-neutral glycolipids.- Oligosaccharides derived from neutral glycolipids were applied to HPLC in order to separate them by size. Aliquots (20µl) containing $2x10^6$ cpm of ^{3}H-Gal-neutral-oligosaccharides ($0.54x10^6$ cells) and $0.5x10^6$ cpm of ^{3}H-GlcN-neutral-oligosaccharides ($1.4x10^6$ cells) were separately applied to HPLC on an Alltech NH_2 column using the conditions described in chapter III. Aliquots of 0.5 ml fractions (30 seconds) were assayed for radioactivity. Elution times of 1, monosaccharide; 2, disaccharide; 3, trisaccharide; 4, tetrasaccharide; 5, pentasaccharide; 6, hexasaccharide; and 7, heptasaccharide are indicated. Panel A, HPLC profile of ^{3}H-galactose-neutral-oligosaccharides, and panel B, HPLC profile of ^{3}H-glucosamine-neutral-oligosaccharides.

TABLE 5

PERCENTAGES OF METABOLICALLY LABELLED NEUTRAL OLIGOSACCHARIDES

HPLC Peak from Fig. 8	^{3}H-Galactose (%)	^{3}H-Glucosamine (%)
1	10.0	1.0
2	18.0	3.0
3	5.0	4.0
4	10.0	9.0
5	14.0	24.0
6	13.0	19.0
7	9.0	14.0
8	6.0	14.0
>9	15.0	26.0

(monosaccharide, disaccharide and trisaccharide), consistent with the fact that ceramide monhexosides and ceramide dihexosides do not contain N-acetylglucosamine or N-acetylgalactosamine. Ceramide trihexosides like lacto-N-triaosylceramide and ganglio-N-triaosylceramide do contain N-acetylglucosamine and N-acetylgalactosamine respectively. However, the thin layer chromatogram of the ^{3}H-glucosamine-labelled neutral glycolipids (fig. 6C) did not show the presence of a ceramide trihexoside fraction that would account for any of these two triaosylceramides.

4.3.1 The Disaccharide Peak (Peak 2)

The disaccharide fractions from the ^{3}H-galactose and the ^{3}H-glucosamine labellings (peak 2 in figs. 8A and 8B) were purified further by affinity chromatography using a *Ricinus communis*-agglutinin-I-agarose (RCA-I) column (0.7 x 50 cm). The ^{3}H-galactose-disaccharide peak was separated into three fractions as shown in figure 9A. The first fraction, 2-I, eluted in the void volume (peak I in fig. 9A), and constituted 54% of the disaccharides; the second one, fraction 2-II, was only slightly retarded (peak II, fig. 9A), and it was 37% of the disaccharides; and the third one, fraction 2-III which only constituted 9% of the disccharides, was the most retarded (peak III, fig. 9A).

Fraction 2-III.- Fraction 2-III was the most retarded in the RCA-I column, indicating that it corresponded to lactose . When compositional analysis of this fraction was done, the radioactivity was recovered as 55% ^{3}H-galactose, and 45% ^{3}H-glucose (see fig. 10 A), which corresponded to a ratio of galactose/glucose of 1.22/1.00. This ratio should be 1 Gal/1 Glc in the ^{3}H-Gal-lactose (2-III), but it is 22% higher, probably due to differences in the specific

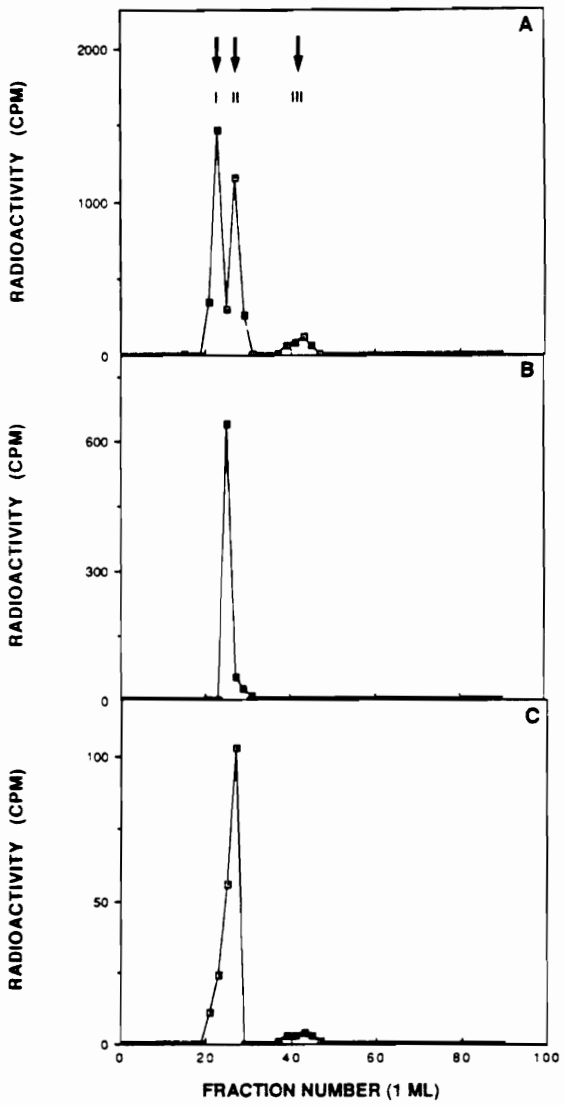


Figure 9.- Purification of the neutral disaccharide fraction (peak 2) by affinity chromatography on RCA-I-agarose.- ^{3}H-Gal-peak 2 and ^{3}H-GlcN-peak 2 were separately applied to a RCA-I column (0.7 x 50 cm), equilibrated in phosphate buffered saline at room temperature. Samples were incubated for 15 minutes before eluting them in the starting buffer at a flow rate of 8 ml/hr. Aliquots of 1ml fractions were assayed for radioactivity. The void volume, I; the elution position of authentic human milk LNTol, II; and the elution volume of lactose, III are indicated. Panel A, ^{3}H-Gal-peak 2, panel B, ^{3}H-GlcN-peak 2, panel C, ^{3}H-Gal-CDH-derived oligosaccharides.

activities (dpm/mol) of the intracellular UDP-^{3}H-galactose and UDP-^{3}H-glucose pools in SW1116 cells. Finding the real ratio of Gal/Glc in ^{3}H-Gal-lactose was important in establishing the ratios of Gal/Glc in the rest of the metabolically labelled oligosaccharides, which except for galabiose, contain lactose at their reducing end. For example, if the metabolically labelled tetrasaccharide contains 30% glucose and 70% galactose which is a ratio of 2.33 Gal/1 Glc, using the Gal/Glc ratio obtained from lactose, one can calculate the number of Gal units per Glc units by dividing 2.33 by 1.22, 2.33/1.22 = 1.91. This value, which is the corrected ratio of Gal/Glc, indicates that the tetrasaccharide, in this example, is composed of 1.91 Gal units and 1 Glc unit which is in close agreement with the expected value of 2/1 (Gal/Glc).

Methylation analysis of this fraction showed a terminal galactose at the non-reducing end (2,3,4,6-tetramethylgalactose, peak 1 in fig. 11), and glucose at the reducing end (1,2,3,5,6-pentamethylglucose, peak 2 in fig. 11). Treatment of fraction 2-III with jack bean β-galactosidase completely converted it into a monosaccharide (fig. 12C). This results, confirm that fraction 2-III actually corresponded to lactose, Galβ1-4Glc.

Fraction 2-II.- This fraction was only composed of ^{3}H-galactose as determined by 2N HCL hydrolysis and paper chromatography (see fig. 10B). This fraction was reduced with $NaBH_4$ and then subjected to methylation analysis. The partially methylated derivatives corresponded to 2,3,6-trimethylgalactose (18%) (peak 1, fig. 13A), 2,3,4,6-tetramethylgalactose (56%) (peak 2, fig. 13A) and 1,2,3,5,6-pentamethylgalactose (peak 3, fig. 13A) (26%). Since this structure was a disaccharide, its methylation analysis was expected to yield a non-reducing terminal galactose (2,3,4,6-tetramethylgalactose) and

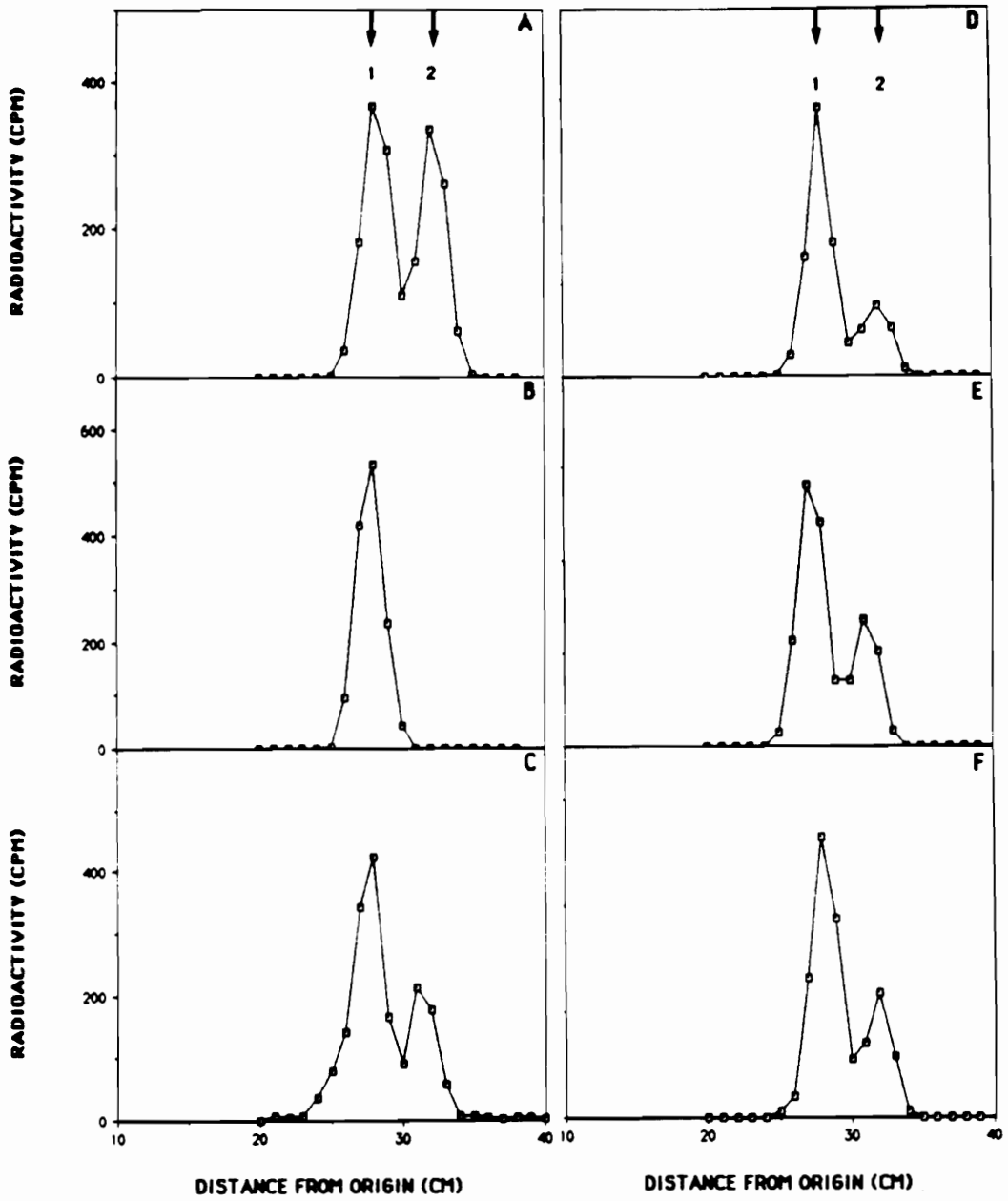


Figure 10.- Monosaccharide composition of ^{3}H-Gal-2-III, ^{3}H-Gal-2-II, ^{3}H-Gal-monsaccharides, ^{3}H-Gal-4-I, ^{3}H-Gal-4-II, and ^{3}H-Gal-4-III.- Samples subjected to 2N HCL hydrolysis for 24 h at 100°, were applied to descending paper chromatography and developed in the solvent system ethyl acetate/pyridine/water (12/5/4) for 24 h. Radioactive monosaccharides were localized by cutting the paper chromatogram into 1cm segments. Paper segments were placed in 7 ml beta-vials, to which 0.5 ml of water and 4 ml of scintillation cocktail were added, and then counted in a liquid scintillation counter for 5 min. Panel A, ^{3}H-Gal-2-III; panel B, ^{3}H-Gal-2-II; panel C, ^{3}H-Gal-monosaccharide fraction; panel D, ^{3}H-Gal-4-I; panel E, ^{3}H-Gal-4-II; and panel F, ^{3}H-Gal-4-III. The positions of unlabelled galactose, 1, and glucose, 2, are indicated by the arrows.

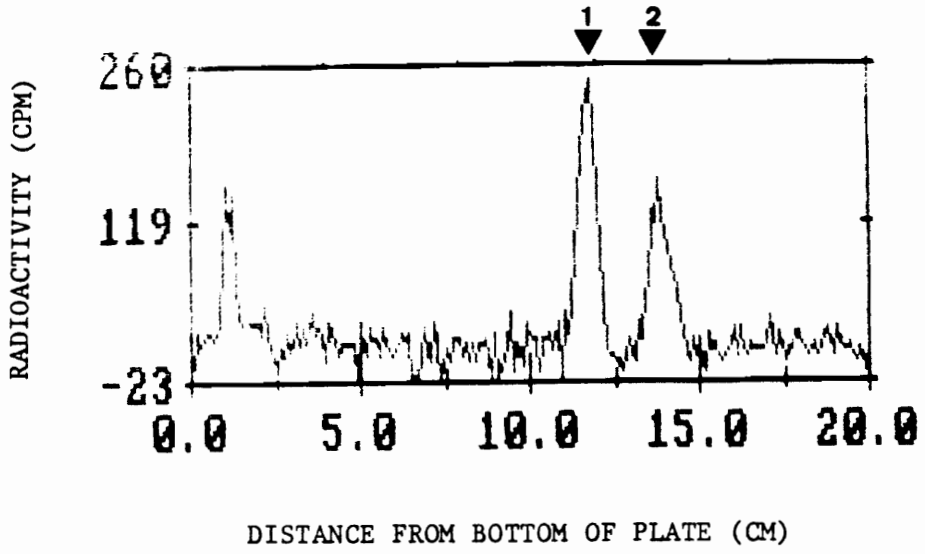


Figure 11.- Methylation analysis of ^{3}H-Gal-2-III.- ^{3}H-Gal-2-III was reduced, methylated and hydrolyzed according to the procedures described in chapter III. The partially methylated derivatives were analyzed by thin layer chromatography. Samples were spotted on aluminum backed plates (5 x 20 cm), and developed for 70 minutes in the solvent system acetone/water/ammonium hydroxide (250/3/1.5). Partially methylated standards are indicated, 1, 2,3,4,6-tetramethylgalactose; and 2, 1,2,3,5,6-pentamethylglucitol.

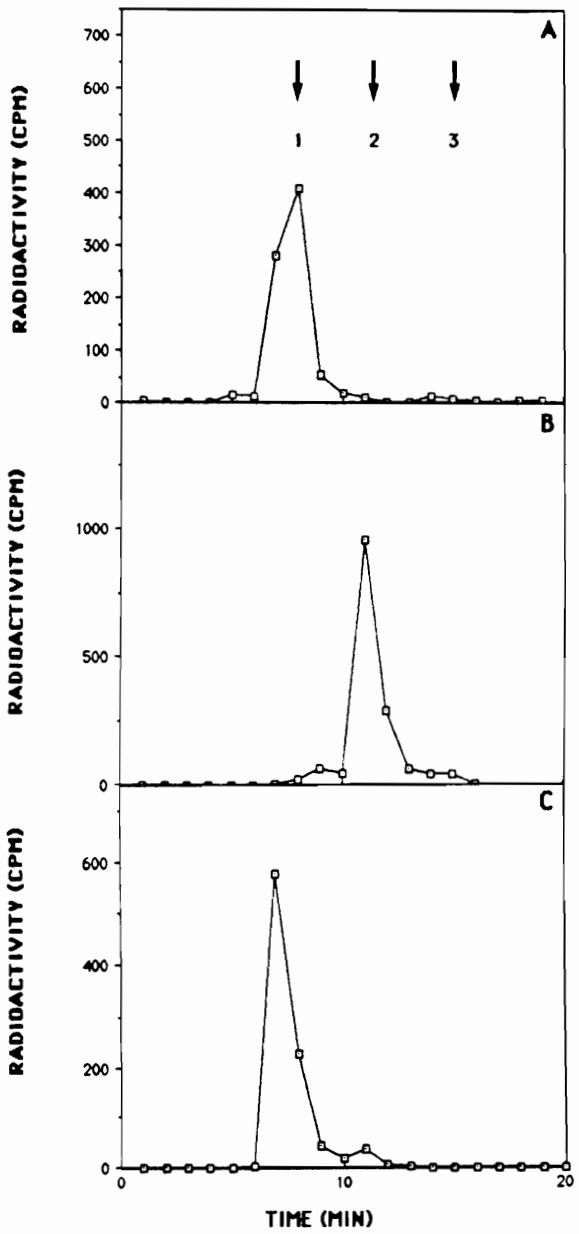


Figure 12.- HPLC of the jack bean β- and/or coffee bean α-galactosidase digests of ^{3}H-Gal-2-II.and ^{3}H-Gal-2-III.- Jack bean β- and/or coffee bean α-galactosidase digestions of samples were carried out as described in chapter III. The digestion products were analyzed by HPLC, on an Alltech carbohydrate NH_2 column using the conditions described in chapter III. Radioactivity was detected by counting aliquots of 0.5 ml fractions. The elution times of authentic, 1, ^{3}H-glucitol; and, 2, ^{3}H-lactitol are indicated. Panel A, coffee bean α-galactosidase digestion products of ^{3}H-Gal-2-II; panel B, jack bean β-galactosidase digestion products of ^{3}H-Gal-2-II; and panel C, jack bean β-galactosidase digestion products of ^{3}H-Gal-2-III.

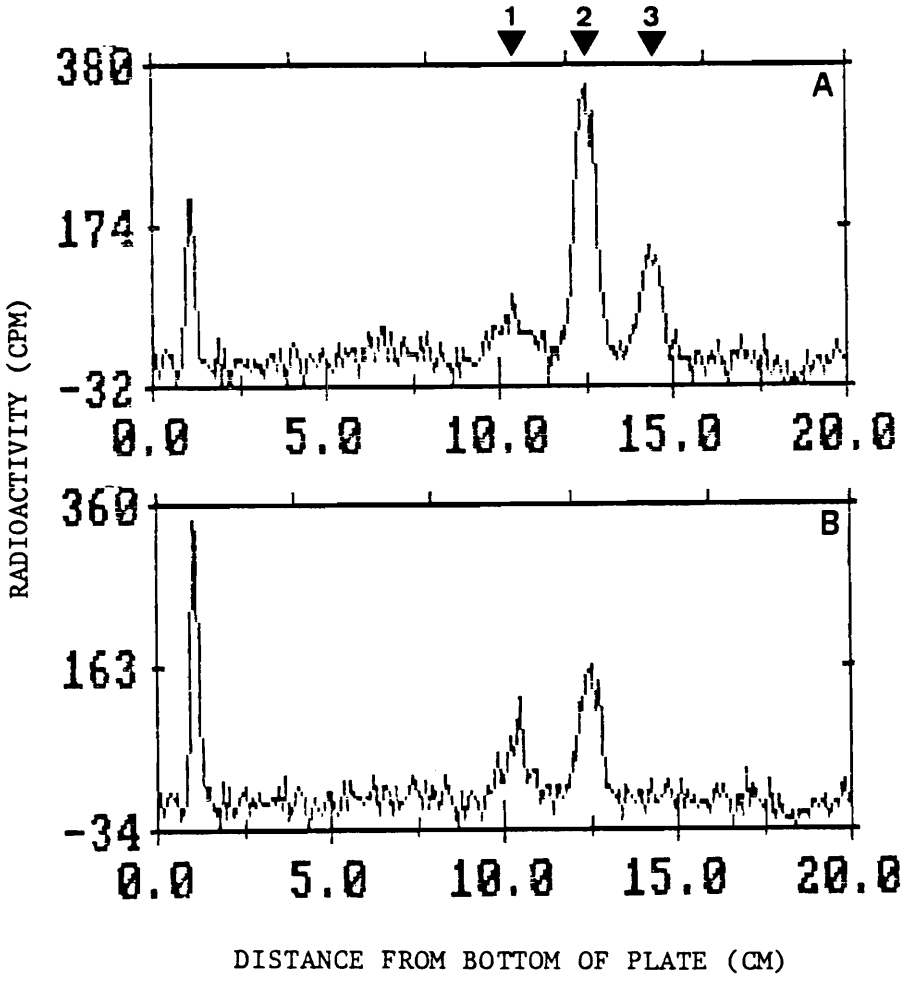


Figure 13.- Methylation analysis of ^{3}H-Gal-2-II.- ^{3}H-Gal-2-II was methylated and hydrolyzed according to the procedures described in chapter III. The partially methylated derivatives were analyzed by thin layer chromatography. Samples were spotted on aluminum backed plates (5 x 20 cm), and developed for 70 minutes in the solvent system acetone/water/ammonium hydroxide (250/3/1.5). Partially methylated standards are indicated, 1, 2,3,6-trimethylgalactose; 2, 2,3,4,6-tetramethylgalactose; and 3, 1,2,3,5,6-pentamethylglucitol. Panel A, ^{3}H-Gal-2-II reduced before methylation, panel B, unreduced ^{3}H-Gal-2-II.

the reducing galactose (1,2,3,5,6-pentamethylgalactose). The presence of the 2,3,6-trimethylgalactose indicated that perhaps a proportion of the disaccharide molecules were not reduced under the reaction conditions used. Therefore, another sample of 2-II was

reduced using a higher concentration of $NaBH_4$ (5 mg/ml of $NaBH_4$ in 0.01M NaOH at 37° C, for 2-3 hours), however the same results were obtained (not shown). We decided to methylate a sample of 2-II that was not previously reduced, and the partially methylated derivatives obtained were 2,3,6-trimethylgalactose (42%), and 2,3,4,6-tetramethylgalactose (58%) (fig. 13B). Also, the ceramide dihexoside (CDH) fraction, separated by preparative thin layer chromatography of the ^{3}H-galactose-labelled neutral glycolipids, was subjected to ozonolysis/alkali fragmentation, and the free oligosaccharides were applied to the RCA-I column to separate them into the different components (fig. 9C). The profile obtained from RCA-I chromatography of the CDH-derived oligosaccharides (fig. 9C), only showed peaks 2-II (94%), and 2-III (6%), no peak 2-I was present in the CDH-derived oligosaccharides. Compositional analysis of CDH-2-II, demonstrated that it only contained galactose, and that of CDH (glycolipid), showed that it contained 97% galactose and 3% glucose. The percentages of Gal and Glc in the ceramide dihexoside (CDH) corresponded to a ratio of 26.5 Gal/1 Glc. Methylation analysis of the reduced CDH-2-II-derived disaccharide produced the same partially methylated derivatives as that of 2-II, 2,3,6-trimethylgalactose (13%) (peak 1, fig. 14), 2,3,4,6-tetramethylgalactose (57%) (peak 2, fig. 14) and 1,2,3,5,6-pentamethylgalactose (peak 3, fig. 14) (30%).

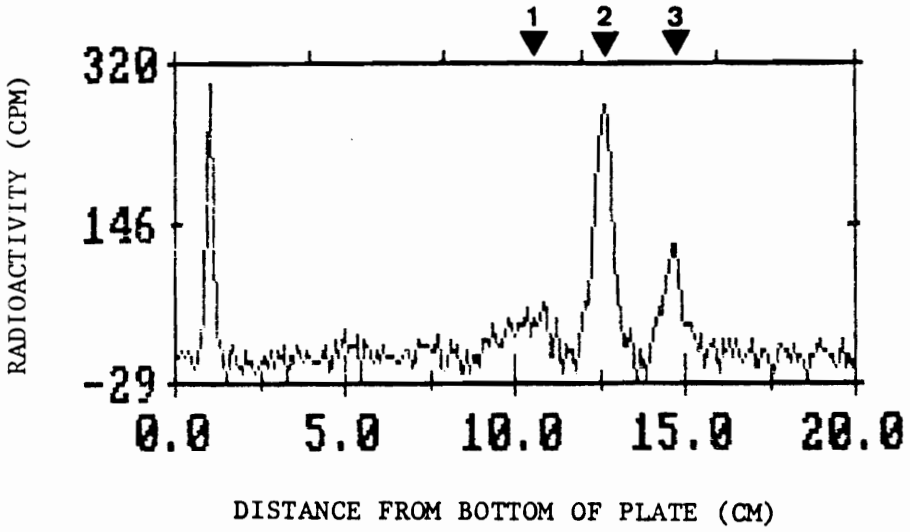


Figure 14.- Methylation analysis of ^{3}H-Gal-CDH-2-II.- ^{3}H-Gal-CDH-2-II was reduced, methylated and hydrolyzed according to the procedures described in chapter III. The partially methylated derivatives were analyzed by thin layer chromatography. Samples were spotted on aluminum backed plates (5 x 20 cm), and developed for 70 minutes in the solvent system acetone/water/ammonium hydroxide (250/3/1.5). Partially methylated standards are indicated, 1, 2,3,6-trimethylgalactose; 2, 2,3,4,6-tetramethylgalactose; and 3, 1,2,3,5,6-pentamethylglucitol.

We explained the presence of the 2,3,6-trimethylgalactose, by assuming that a small fragment of the ceramide remained attached to the anomeric hydroxyl group of the reducing galactose, in 13%-17% of the 2-II molecules after ozonolysis and alkaline beta elimination, which prevented the anomeric carbon, C-1 from being reduced.

Treatment of fraction 2-II with coffee bean α-galactosidase (0.05-0.1 U/ml, 5 hours at 37° C), totally converted this fraction into a monosaccharide (fig. 12A) as tested by applying the α-galactosidase digestion products to HPLC, using program #1. No resolution on the HPLC between the non-reducing terminal galactose and the reducing galactose, a proportion of which may contain an additional fragment attached to the anomeric hydroxyl group, was observed. This fraction also was subjected to jack bean β-galactosidase digestion (high concentration, 0.8 U/ml, for 24 hours at 37° C) and it was found to be resistant to this enzyme (see fig. 12B). The results from methylation of the unreduced fraction 2-II, compositional analysis, and coffee bean α-galactosidase digestion, indicate that the structure present in this fraction corresponds to galabiose, Galα1-4Gal. The results from methylation of the reduced fraction 2-II suggest that some of the galabiose in this fraction, contains a small fragment of the ceramide portion attached to the anomeric hydroxyl group of the reducing galactose, that prevents the reduction of this carbon.

It is interesting to notice that the RCA-I column used (0.7 x 50 cm) was able to retard galabiose to the same extent as it retards authentic human milk lacto-N-tetraose. This finding indicates, that the RCA-I lectin reacts weakly with non-reducing terminal galactose in an α1-4 linkage. To our knowledge this specificity has not been described before for the RCA-I lectin, however, to test

this, other experiments using other oligosaccharide standards containing terminal α1-4 galactose such as globotriose will be necessary.

Galabiosylceramide has been found to be present in normal human kidney [2], in human meconium [224], in gastric cancer tissue [165], and in some human colonic cell lines [225], but it has been found to be absent in normal colonic mucosa [225]. While these findings suggest that galabiose may be a colorectal carcinoma antigen, Siddiqui et al.[226], examined numerous colonic tumors that did not contain any galabiosylceramide, indicating that this glycolipid may not be a true marker for gastrointestinal cancer.

Fraction 2-I.- The ^{3}H-galactose-2-I fraction was hydrolyzed with 2N HCL and then applied to paper chromatography as described in chapter III. The hydrolyzed sample did not move from the origin, indicating either that this fraction was resistant to acid hydrolysis or that the hydrolysis products were other than galactose and glucose, which with the chromatography conditions used they stayed at the origin. No further experiments were done with this fraction. In addition, the ^{3}H-Gal-labelled peak 3 (fig.8), when subjected to strong acid hydrolysis, followed by paper chromatography, yielded products that did not move from the origin of the paper chromatogram (results not shown), similar to what was observed with fraction 2-I. These two fractions are probably by-products of the labelling that copurified with the neutral glycolipids but are not composed of galactose or glucose.

4.3.2 The Monosaccharide Fraction (Peak 1)

The ^{3}H-galactose-labelled monosaccharide fraction (peak 1, fig. 8A) was applied to paper chromatography (see fig. 10C) in a solvent system (ethyl

acetate/pyridine/water, 12/5/4) that separates glucose, galactose, mannose and fucose. In this fraction, 30% of the radioactivity corresponded to glucose and 70% to galactose (ratio of 2.33 Gal/1 Glc). The ceramide monohexoside fraction, separated by preparative thin layer chromatography from neutral-^{3}H-Gal-glycolipids, was found to contain similar proportions of glucose (32%) and galactose (68%), after it was subjected to strong acid hydrolysis and paper chromatography (results not shown). These results clearly indicate that SW1116 cells express glucosylceramide and galactosylceramide. It was not expected that 70% of the ceramide monohexoside fraction would be galactosylceramide, since this is a glycolipid mainly found in neural tissues [2]. However, several studies have reported the presence of galactosylceramide in normal small intestine [147] and in gastric cancer [165]. The expression of twice as much galactosylceramide as compared to glucosylceramide may be explained by reasoning that glucosylceramide is a precursor for most of the glycolipids, and that galactosylceramide only is used by mammalian cells to make galabiosylceramide [2] and ganglioside GD4 (NeuAcα2-3Gal-Cer). In addition, the proportion of glucose in this fraction may appear to be smaller than it was because the specific activity of the ^{3}H-glucose was lower than that of^{3}H-galactose. This was demonstrated by the ratio of galactose to glucose in ^{3}H-Gal-metabolically-labelled lactose from SW1116 cells (see section 4.3.1-Fraction 2-III), which corresponded to 1.22 Gal/1 Glc, instead of 1 Gal/1 Glc. Therefore, the actual proportions of Gal and Glc in the monosaccharide fraction, when calculated based on the ratio 1.22 Gal/1 Glc, should be 65% galactose and 35% glucose.

4.3.3 The Tetrasaccharide Peak (Peak 4)

The tetrasaccharide peaks labelled with ^{3}H-Gal and ^{3}H-GlcN were further purified further on the RCA-I column (0.7 x 50 cm). The ^{3}H-Gal-tetrasaccharide peak separated into three fractions (fig. 15A), 4-I which eluted in the void volume and corresponded to 32% of the tetrasaccharides; 4-II which cochromatrographed with authentic human milk lacto-N-tetraose, and was 60% of the tetrasaccharides; and, 4-III which eluted in the same position as human milk lacto-N-neotetraose and comprised only 8% of the tetrasaccharides. The ^{3}H-GlcN-tetrasaccharide peak was also resolved into three peaks (fig. 15B) that each eluted in the same positions as those labelled with ^{3}H-galactose, however, the percentages were different. The percentage of ^{3}H-GlcN-4-I was 44%, higher than that of ^{3}H-Gal-4-I; the percentage of ^{3}H-GlcN-4-II was 48%, lower than that of the corresponding ^{3}H-Gal-4-II; and the percentage of the ^{3}H-GlcN-4-III was 7%, slightly lower than that of the ^{3}H-Gal-4-III. The higher percentage of ^{3}H-GlcN-4-I as compared to that of ^{3}H-Gal-4-I may suggest that this fraction is composed of some oligosaccharide structures that contain a higher proportion of N-acetylglucosamine and/or N-acetylgalactosamine than that of galactose and/or glucose. The ratio of ^{3}H-Gal-4-II/^{3}H-Gal-4-III and that of ^{3}H-GlcN-4-II/^{3}H-GlcN-4-III were very similar, 7.5 and 7.0 respectively.

Fraction 4-II.- Compositional analysis of ^{3}H-Gal-4-II (see fig. 10D) demonstrated that 69% of the radioactivity in this fraction corresponded to galactose and 31% to glucose (ratio[3] of 1.83 Gal/1 Glc). Compositional analysis

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[3] The ratio was calculated based on the ratio of Gal/Glc obtained from lactose, 1.22 Gal/1 Glc. Ratio=%Gal/%Glc/1.22/1.

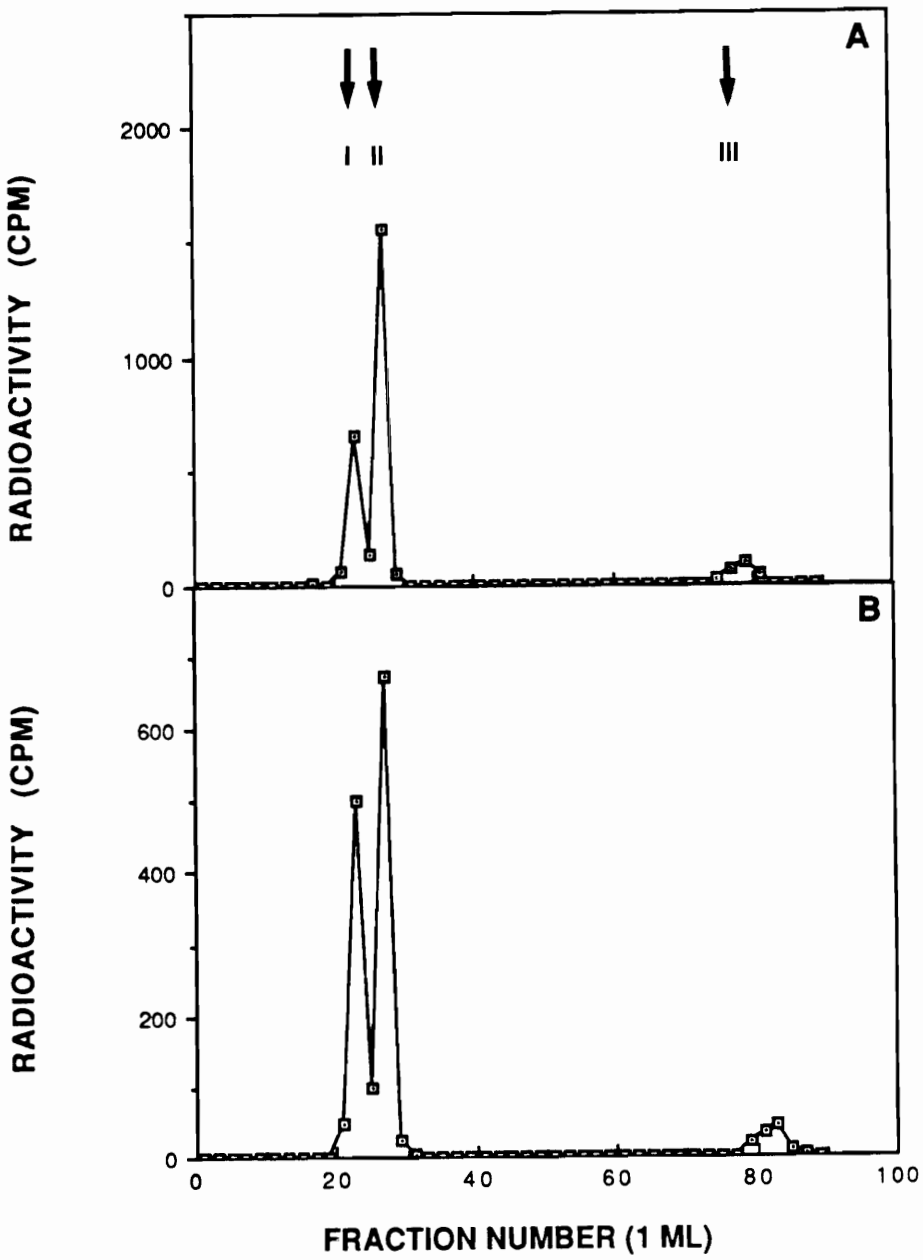


Figure 15.- Purification of the neutral tetrasaccharide fraction (peak 4) by affinity chromatography on RCA-I-agarose.- ^{3}H-Gal-peak 4 and ^{3}H-GlcN-peak 4 were separately applied to a RCA-I column (0.7 x 50 cm), equilibrated in phosphate buffered saline at room temperature. Samples were incubated for 15 minutes before eluting them in the starting buffer at a flow rate of 8 ml/hr. Aliquots of 1ml fractions were assayed for radioactivity. The void volume, I; the elution position of authentic human milk ^{3}H-LNTol, II; and ^{3}H-LNnTol, III, are indicated. Panel A, ^{3}H-Gal-peak 4, panel B, ^{3}H-GlcN-peak 4.

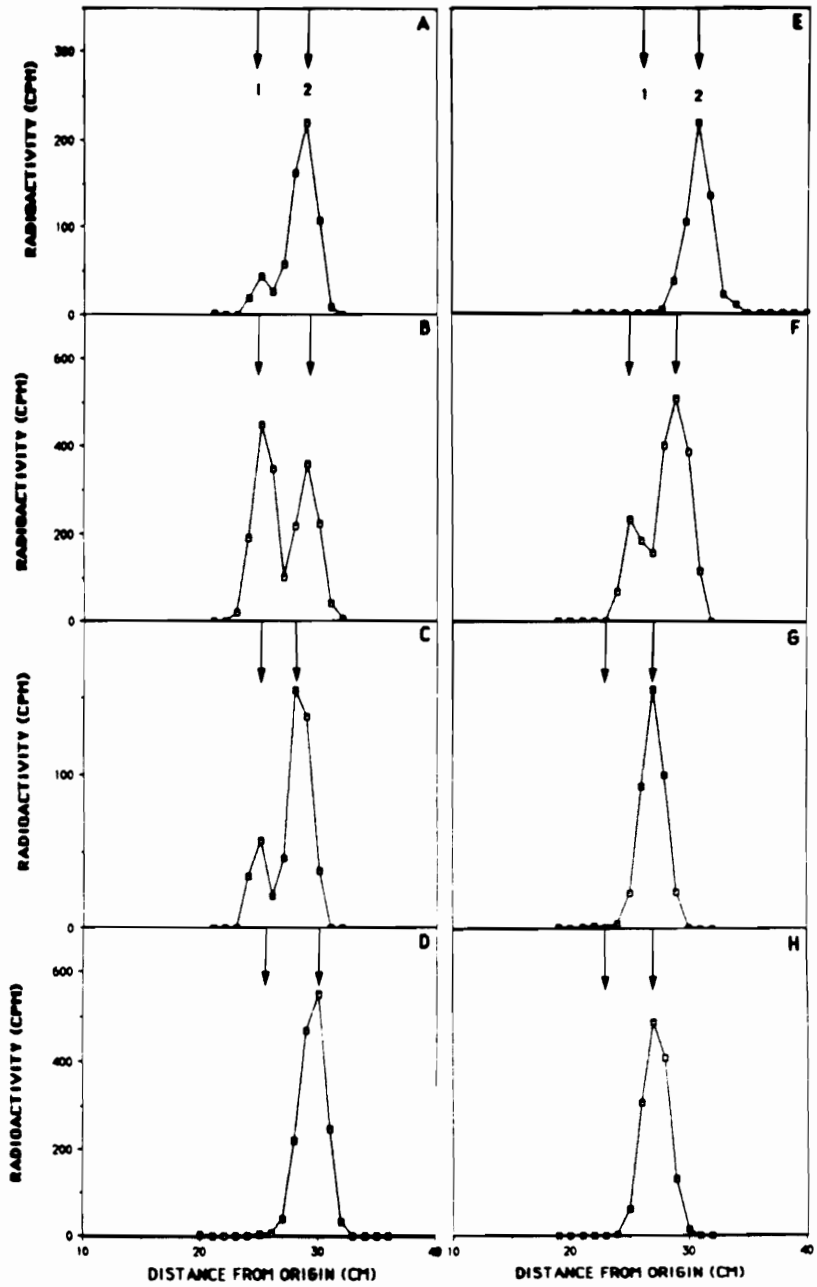


Figure 16.- Monosaccharide composition of ^{3}H-GlcN-4-I-a, ^{3}H-GlcN-4-I-b, ^{3}H-GlcN-4-I-c, ^{3}H-GlcN-4-II, ^{3}H-GlcN-4-III, ^{3}H-GlcN-5-4-I, ^{3}H-GlcN-5-4-II, ^{3}H-GlcN-5-4-III.- Samples subjected to 2N HCL hydrolysis for 24 h at 100°, were applied to descending paper chromatography and developed in the solvent system ethyl acetate/pyridine/water (12/5/4) for 48 h. Radioactive monosaccharides were localized by cutting the paper chromatogram into 1cm segments. Paper segments were placed in 7 ml beta-vials, to which 0.5 ml of water and 4 ml of scintillation cocktail were added, and then counted in a liquid scintillation counter for 5 min. Panel A, ^{3}H-GlcN-4-I-a; panel B, ^{3}H-GlcN-4-I-b; panel C, ^{3}H-GlcN-4-I-c; panel D, ^{3}H-GlcN-4-II; panel E, ^{3}H-GlcN-4-III; panel F, ^{3}H-GlcN-5-4-I; panel G, ^{3}H-GlcN-5-4-II; and panel H, ^{3}H-GlcN-5-4-III;. The positions of unlabelled galactosamine, 1; and glucosamine, 2, are indicated by the arrows.

of ^{3}H-GlcN-4-II showed that it only contained N-acetylglucosamine (see fig. 16D). Fraction 4-II was found to be lacto-N-tetraose (LNT), Galβ1-3GlcNAcβ1-3Galβ1-4Glc, based upon retardation on the RCA-I column, the monosaccharide composition, methylation analysis, and jack bean β-galactosidase digestion. Methylation analysis of reduced 4-II (fig. 17A) demonstrated the presence of a terminal non-reducing galactose (2,3,4,6-tetramethylgalactose, peak 2 in fig. 17A) (35%), a 3-substituted galactose (2,4,6-trimethylgalactose, peak 1 in fig. 17A) (46%), and a reducing-end glucose (1,2,3,5,6-pentamethylglucitol, peak 3 in fig. 17A) (19%). Using assay conditions (0.8-1.6 U/ml of enzyme) in which jack bean β-galactosidase will completely digest the Galβ1-4 linkage, and only 10-18% of the Galβ1-3 linkage, just 7% of ^{3}H-Gal-4-II was digested (fig. 18A). A second incubation of ^{3}H-Gal-4-II with jack bean β-galactosidase produced similar results (not shown). Also, fraction ^{3}H-Gal-4-II was resistant to digestion with coffee bean α-galactosidase (fig. 18B).

Fraction 4-III.- This fraction corresponded to lacto-N-neotetraose (LNnT), Galβ1-4GlcNAcβ1-3Galβ1-4Glc, by its retardation on the RCA-I column which was the same as authentic LNnT from human milk. Methylation analysis of ^{3}H-Gal-4-III (fig. 17B) showed a terminal non-reducing galactose (2,3,4,6-tetramethylgalactose, peak 2 in fig. 17B) (37%), a 3-substituted galactose (2,4,6-trimethylgalactose, peak 1 in fig. 17B) (39%), and a reducing-end glucose (1,2,3,5,6-pentamethylglucitol, peak 3 in fig. 17B) (24%). As expected, the results of methylation analysis of 4-III were exactly the same as those of 4-II. Compositional analysis of ^{3}H-Gal-4-III (see fig. 10E) revealed that it was composed of 73% galactose, and 27% glucose (ratio 2.22 Gal/1 Glc).

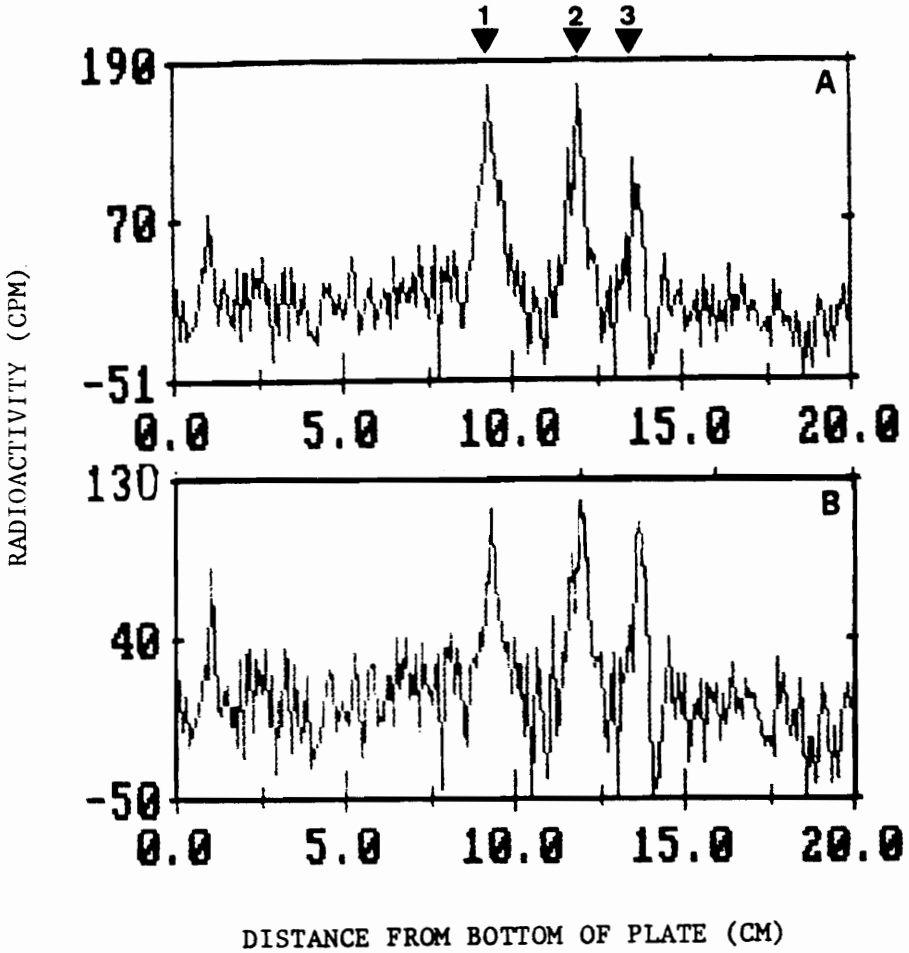


Figure 17.- Methylation analysis of ^{3}H-Gal-4-II and ^{3}H-Gal-4-III.- ^{3}H-Gal-4-II and -4-III were reduced, methylated and hydrolyzed according to the procedures described in chapter III. The partially methylated derivatives were analyzed by thin layer chromatography. Samples were spotted on aluminum backed plates (5 x 20 cm), and developed for 70 minutes in the solvent system acetone/water/ammonium hydroxide (250/3/1.5). Partially methylated standards are indicated, 1, 2,4,6-trimethylgalactose; 2, 2,3,4,6-tetramethylgalactose; and 3, 1,2,3,5,6-pentamethylglucitol. Panel A, ^{3}H-Gal-4-II; and panel B, ^{3}H-Gal-4-III.

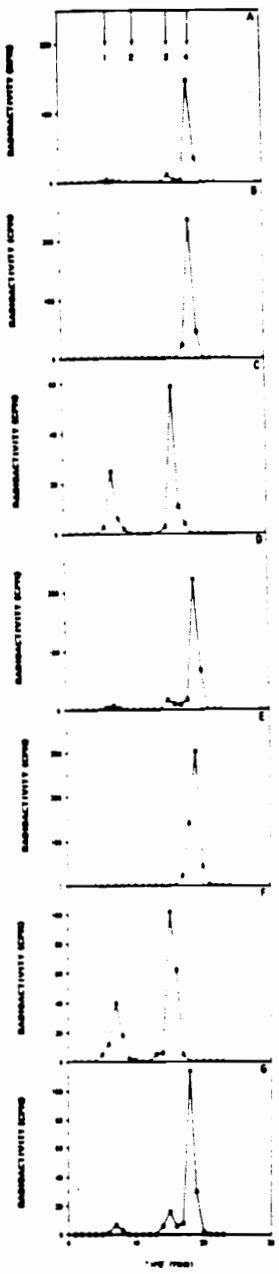


Figure 18.- HPLC of the jack bean β-galactosidase and/or coffee bean α-galactosidase digests of ^{3}H-Gal-4-II, ^{3}H-Gal-4-III, ^{3}H-Gal-5-4-II, ^{3}H-Gal-5-4-III, and ^{3}H-Gal-6-4-II.- Jack bean β-galactosidase and/or coffee bean α-galactosidase digestions from each sample were analyzed by HPLC as explained in figure 12. Elution times of authentic 1, ^{3}H-glucitol; 2, ^{3}H-lactitol; 3, ^{3}H-lacto-N-triaitol; 4, ^{3}H-LNTol, are indicated. Panel A, jack bean β-galactosidase digests of ^{3}H-Gal-4-II; panel B, coffee bean α-galactosidase digests of ^{3}H-Gal-4-II; panel C, jack bean β-galactosidase digests of ^{3}H-Gal-4-III; panel D, jack bean β-galactosidase digests of ^{3}H-Gal-5-4-II; panel E, coffee bean α-galactosidase digests of ^{3}H-Gal-5-4-II; panel F, jack bean β-galactosidase digests of ^{3}H-Gal-5-4-III; and panel G, jack bean β-galactosidase digests of ^{3}H-Gal-6-4-II.

Compositional analysis of ^{3}H-GlcN-4-III showed that it contained only N-acetylglucosamine (see fig. 16B).

Treatment with jack bean β-galactosidase, under assay conditions that will digest 100% of the Galβ1-4 linkage and only a 10-18% of the Galβ1-3 linkage (0.8-1.6 U/ml of enzyme), completely converted ^{3}H-Gal-4-III into a trisaccharide (fig. 18C).

Fraction 4-I.- Compositional analysis of ^{3}H-Gal-4-I (fig. 10F), showed that this fraction was composed of 77% galactose and 23% glucose (ratio of 2.74 Gal/1 Glc). Compositional analysis of ^{3}H-GlcN-4-I, demonstrated that it contained 34% of N-acetylgalactosamine and 66% of N-acetylglucosamine (fig. 16E). These results indicated that 4-I was most likely composed of more than one structure. Therefore, fractions ^{3}H-Gal-4-I and ^{3}H-GlcN-4-I were individually applied to HPLC using program #3 to further purify them.

Fractions 4-I from each labelling were separated into three components, 4-I-a, 4-I-b and 4-I-c according to their elution order (fig. 19). The components, ^{3}H-Gal-4-I-a, ^{3}H-Gal-4-I-b, and ^{3}H-Gal-4-I-c, constituted a 30%, 68% and a 2% of the total ^{3}H-Gal-4-I fraction. Likewise, ^{3}H-GlcN-4-I-a, ^{3}H-GlcN-4-I-b, and ^{3}H-GlcN-4-I-c were 21%, 64%, and 15% of the complete ^{3}H-GlcN-4-I fraction. Compositional analysis of ^{3}H-GlcN-4-I-a, -b, -c fractions (fig. 14A, B and C) showed that ^{3}H-GlcN-4-I-a, was constituted by 14% of N-acetylgalactosamine, and 86% of N-acetylglucosamine; ^{3}H-GlcN-4-I-b was composed of 57% of N-acetylgalactosamine, and 43% of N-acetylglucosamine; ^{3}H-GlcN-4-I-c, contained 18% of N-acetylgalactosamine, and 82% of N-acetylglucosamine (fig. 16D, E and F). A methylation was done only on the reduced ^{3}H-Gal-4-I-b fraction because there was insufficient material in other fractions.

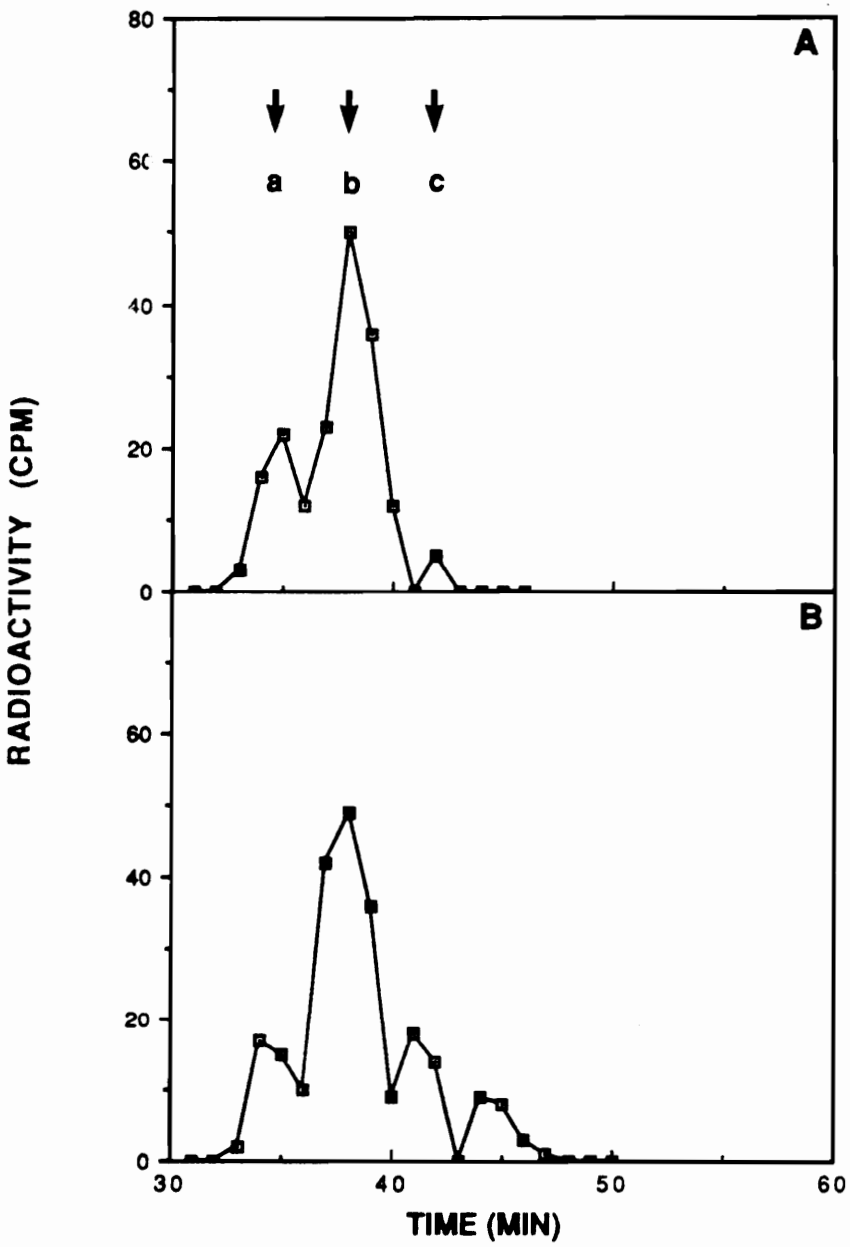


Figure 19.- HPLC of fraction 4-I.- ^{3}H-Gal- and ^{3}H-GlcN-4-I were individually applied to HPLC on an Alltech carbohydrate NH_2 column, using program # 3, described in chapter III. Aliquots of 1 ml fractions (0.5 min) were assayed for radioactivity. Position of elution of the different fractions obtained are indicated by, a, b, and c. Panel A, ^{3}H-Gal-4-I; and panel B, ^{3}H-GlcN-4-I.

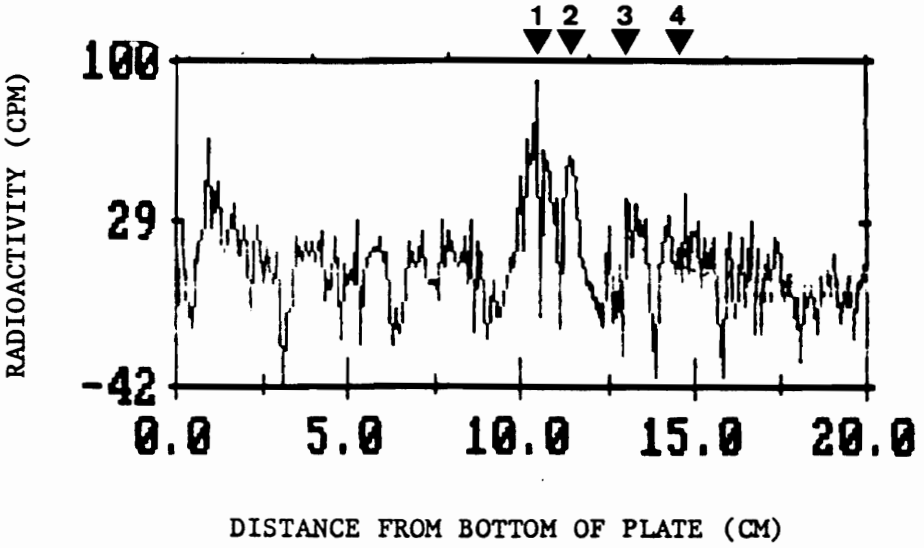


Figure 20.- Methylation analysis of ^{3}H-Gal-4-I.- ^{3}H-Gal-4-I was reduced, methylated and hydrolyzed according to the procedures described in chapter III. The partially methylated derivatives were analyzed by thin layer chromatography. Samples were spotted on aluminum backed plates (5 x 20 cm), and developed for 70 minutes in the solvent system acetone/water/ammonium hydroxide (250/3/1.5). Partially methylated standards are indicated, 1, 2,4,6-trimethylgalactose; 2, 2,3,6-trimethylgalactose; 3, 2,3,4,6-tetramethylgalactose; and 4, 1,2,3,5,6-pentamethylglucitol.

The results of methylation analysis showed a 3-substituted galactose (2,4,6-trimethylgalactose, peak 1 in fig. 20) (37%), a 4-substituted galactose (2,3,6-trimethylgalactose, peak 2 in fig. 20) (33%), a non-reducing terminal galactose (2,3,4,6-tetramethylgalactose, peak 4 in fig. 20) (16%), and a reducing glucose/galactose (1,2,3,5,6-pentamethylhexaitol, peak 4 in fig. 20) (14%). The data from the methylation analysis of ^{3}H-Gal-4-I-b, in conjunction with the compositional analysis of the corresponding ^{3}H-GlcN-4-I-b fraction, clearly indicate that 4-I-b is not composed by a single structure but by several tetrasaccharide species. Further analysis of 4-I-a. -b, -c will be necessary to correctly determine the tetrasaccharide structures present in these fractions.

4.3.4 The Pentasaccharide Peak (Peak 5)

The pentasaccharide peaks (peak 5, fig. 8, panels A and B) labelled with ^{3}H-Gal and ^{3}H-GlcN were subjected separately, to beef kidney α-fucosidase digestion, which converted approximately 95% of both samples into a tetrasaccharide (fig. 21A and B). These results showed that peak 5 was mainly composed of fucosylated tetrasaccharides. The tetrasaccharide fraction obtained, which was called, 5-4, was applied to the RCA-I-agarose column (0.7 x 50 cm). Fraction 5-4 from each labelling, was separated into 3 fractions in an equal pattern as peak 4 (section above) (fig. 22 panels A and B). The fractions were named 5-4-I, 5-4-II, and 5-4-III. The percentages of ^{3}H-Gal-5-4-I, -5-4-II, and -5-4-III were 3%, 93%, and 4% respectively. Likewise, the proportions of ^{3}H-GlcN-5-4-I, -5-4-II, and -5-4-III were 1%, 93% and 6%.

Fraction 5-4-I.- Fraction ^{3}H-Gal-5-4-I was subjected to acid hydrolysis and paper chromatography, showing that it contained 73% Gal and 27% Glc (ratio

of 2.21 Gal/1 Glc) (fig. 23A). Acid hydrolysis and paper chromatography of ^{3}H-GlcN-5-4-I demonstrated that this fraction was composed of 32% GalNAc and 68% GlcNAc (fig. 16F). The results from the compositional analysis suggest that 5-4-I probably was composed of multiple structures, and that those structures may be the same as those found in fraction 4-I.

Fraction 5-4-II.- Fraction 5-4-II, as fraction 4-II, eluted in the position of LNT after affinity chromatography on the RCA-I column. Methylation analysis of the ^{3}H-Gal-5-4-II (fig.24A) produced the same results as those of fraction 4-II, a terminal non-reducing galactose (2,3,4,6-tetramethylgalactose, peak 2 in fig. 24A) (36%), a 3-substituted galactose (2,4,6-trimethylgalactose, peak 1 in fig. 24A) (40%), and a reducing-end glucose (1,2,3,5,6-pentamethylglucitol, peak 3 in fig. 24A) (24%).

Jack bean β-galactosidase treatment using conditions that will cleave all the Galβ1-4 linkage but only 10-18% of the Galβ1-3 linkage (0.8-1.6 U/ml), digested only 11% of the sample (fig. 18D). A second digestion with jack bean β-galactosidase produced the same result. Fraction 5-4-II was also treated with coffee bean α-galactosidase, and it was found to be resistant to the action of this enzyme (fig. 18E).

Compositional analysis of ^{3}H-Gal-5-4-II showed (fig. 10H) that it was composed of 73% Gal and 27% Glc (ratio of 2.22 Gal/1 Glc); compositional analysis of the corresponding ^{3}H-GlcN-5-4-II fraction demonstrated that it contained only GlcNAc (fig. 16H). All this data confirms that 5-4-II is LNT.

Fraction 5-4-III.- This fraction, as did 4-III, corresponded to LNnT based on its elution from the RCA-I column.

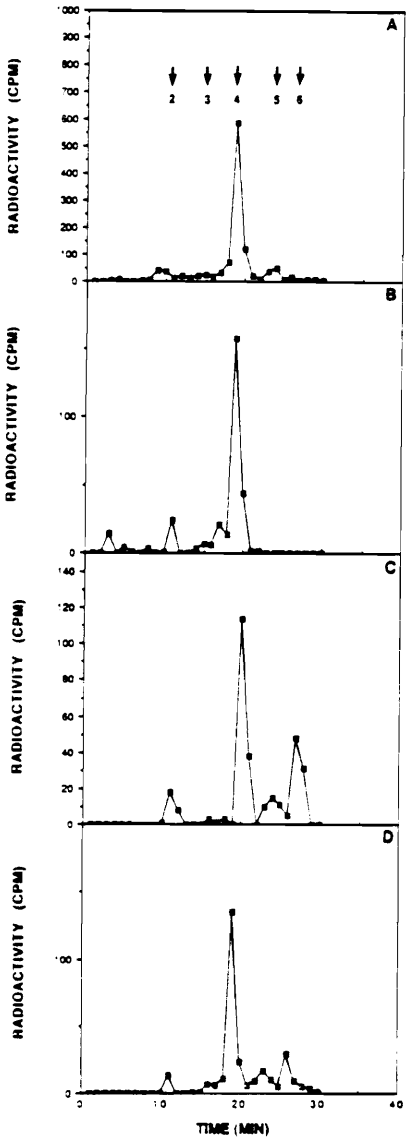


Figure 21.- HPLC of the beef kidney α-fucosidase digests of peak 5, and peak 6.- Beef kidney α-fucosidase digestions were carried as explained in chapter III and they were analyzed by HPLC as explained in figure 12. Elution times of authentic, 2, ^{3}H-lactitol; 3, ^{3}H-lacto-N-triaitol; 4, ^{3}H-LNTol; 5, ^{3}H-LNFol-I; 6, ^{3}H-lacto-N-neohexaitol, are indicated. Panel A, α-fucosidase digests of ^{3}H-Gal-peak 5; panel B, α-fucosidase digests of ^{3}H-GlcN-peak 5; panel C, α-fucosidase digests of ^{3}H-Gal-peak 6; panel D, α-fucosidase digests of ^{3}H-GlcN-peak 6.

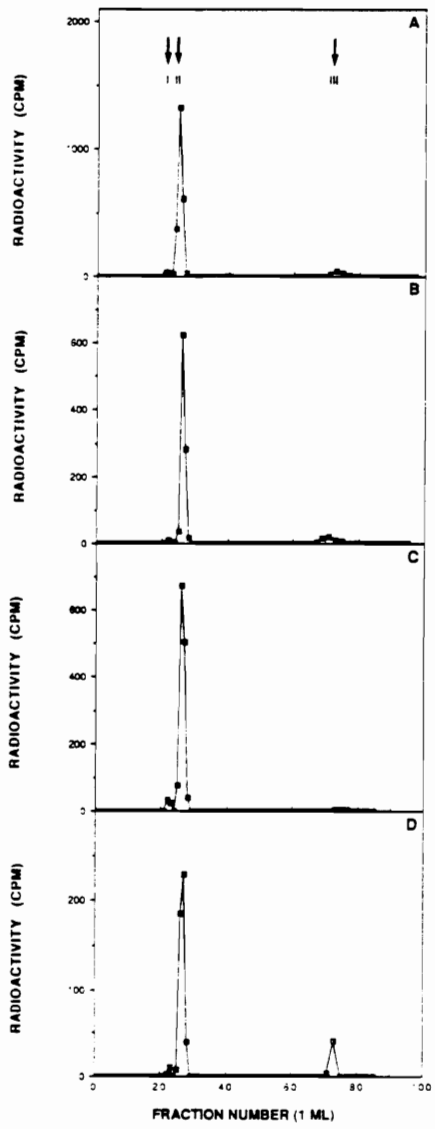


Figure 22.- Purification of the defucosylated peak 5 and peak 6 by affinity chromatography on RCA-I-agarose.- Defucosylated ^{3}H-Gal-peak 5 and ^{3}H-GlcN-peak 5; and defucosylated ^{3}H-Gal-peak 6 and ^{3}H-GlcN-peak 6 were separately applied to an RCA-I column (0.7 x 50 cm), equilibrated in phosphate buffered saline at room temperature. Samples were incubated for 15 minutes before eluting them in the starting buffer at a flow rate of 8 ml/hr. Aliquots of 1ml fractions were assayed for radioactivity. The void volume, I; the elution position of authentic human milk ^{3}H-LNTol, II; and ^{3}H-LNnTol, III, are indicated. Panel A, defucosylated ^{3}H-Gal-peak 5, panel B, defucosylated ^{3}H-GlcN-peak 5; panel C, defucosylated ^{3}H-Gal-peak 6, panel D, defucosylated ^{3}H-GlcN-peak 6.

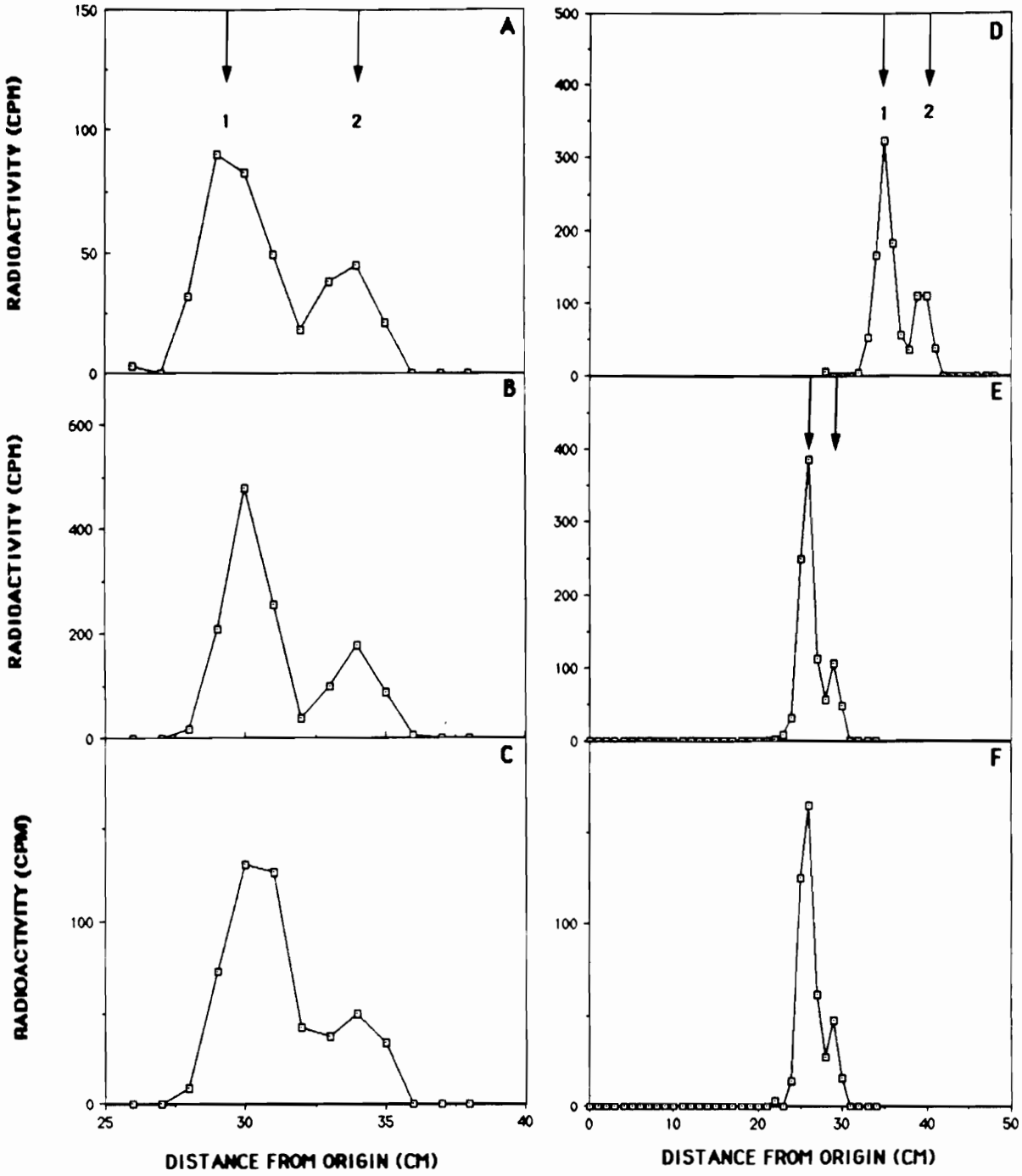


Figure 23.- Monosaccharide composition of ^{3}H-Gal-5-4-I, ^{3}H-Gal-5-4-II, ^{3}H-Gal-5-4-III, ^{3}H-Gal-6-4-II, ^{3}H-Gal-7b and ^{3}H-Gal-7c.- Samples subjected to 2N HCL hydrolysis for 24 h at 100°, were applied to descending paper chromatography and developed in the solvent system ethyl acetate/pyridine/water (12/5/4) for 24 h. Radioactive monosaccharides were localized by cutting the paper chromatogram into 1cm segments. Paper segments were placed in 7 ml beta-vials, to which 0.5 ml of water and 4 ml of scintillation cocktail were added, and then counted in a liquid scintillation counter for 5 min. Panel A, ^{3}H-Gal-5-4-I; panel B, ^{3}H-Gal-5-4-II; panel C, ^{3}H-Gal-5-4-III; panel D, ^{3}H-Gal-6-4-II; panel E, ^{3}H-Gal-7-b; and panel F, ^{3}H-Gal-7-c. The positions of unlabelled galactose, 1, and glucose, 2, are indicated by the arrows.

Methylation analysis of ^{3}H-Gal-5-4-III (fig. 24B) resulted in the same profile as that of 4-II, 4-III, and 5-4-II, a terminal non-reducing galactose (2,3,4,6-tetramethylgalactose, peak 2 in fig. 24B) (38%), a 3-substituted galactose (2,4,6-trimethylgalactose, peak 1 in fig. 24B) (44%), and a reducing-end glucose (1,2,3,5,6-pentamethylglucitol, peak 3 in fig. 24B) (18%). Digestion with jack bean β-galactosidase (0.8-1.6 U/ml) totally converted this tetrasaccharide into a trisaccharide (fig. 18F). Compositional analysis of ^{3}H-Gal-5-4-III (fig. 23C) indicated that it was composed of 72% galactose and 28% glucose (ratio of 2.1 Gal/1 Glc); the ^{3}H-GlcN-5-4-III fraction only contained GlcNAc (fig. 16H). All these data are consistent with 5-4-III being LNnT.

The results obtained from methylation indicated that peak 5 was composed mainly of fucosylated derivatives of LNT (93%), fucosylated derivatives of LNnT (4%) and fucosylated derivatives of unknown structures under fraction 5-4-I (3%). Two of the core structures were already identified, nevertheless, the position of the fucose in the tetrasaccharide cores was unknown. Since we did not have any method to separate peak 5 into the three components that it contained without removing the fucose, we proceeded to methylate a sample of the intact peak 5. We felt confident doing the methylation of peak 5 directly, since 93% of the fraction was composed by fucosyl-5-4-II, or fucosyl-LNT. Methylation of the intact ^{3}H-Gal-peak 5 (fig. 24C), produced the same profile as that of the tetrasaccharides 5-4-II and 5-4-III. The methylation analysis showed a non-reducing terminal galactose (27%), a 3-substituted galactose (55%), and the reducing glucose (18%), however, the proportions of the terminal galactose and the 3-substituted galactose were not the same as the ones observed in the methylation of the tetrasaccharides derived from this

fraction. The explanation for this may be that peak 5 is mainly composed of the Lewis^a pentasaccharide (Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc) and some of the "H1" pentasaccharide (Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc). The methylation of the ^{3}H-Gal-Lewis^a -pentasaccharide will give the same profile as that of the defucosylated structure, since the fucose is positioned in the GlcNAc. However, methylation of the ^{3}H-Gal-"H"-pentasaccharide should give a 2-substituted galactose, a 3-substituted galactose, the reducing glucose, and no non-reducing terminal galactose. However, the 3,4,6-trimethyl-galactose (2-substituted galactose) and the 2,4,6-trimethyl-galactose (3-substituted) do not separate very well by thin layer chromatography under the conditions used [240]. Therefore, in the thin layer chromatogram these two methylated derivatives will migrate nearly in the same position. The results obtained from methylation of ^{3}H-Gal-peak 5 fit this explanation. Comparison of the methylation of the tetrasaccharide 5-4-II and that of peak 5 showed the non-reducing terminal galactose was decreased in the methylation profile of peak 5, indicating that the difference (28%) most likely represents the proportion of terminal non-reducing galactose that was substituted by fucose at the 2-position ("H1" structure). The presence of glycolipids containing the H1-pentasaccharide and the Le^a pentasaccharide has been reported in SW1116 cells [198, 228, 229]. In these studies the carbohydrate structure of these glycolipids has not been directly confirmed, although both types of glycolipids have been detected by immunostaining of SW1116 glycolipids on thin layer chromatograms using monoclonal anti-H1 and anti-Le^a serum. In these studies the synthesis of the two glycolipid antigens has been assessed by incubation of SW1116 cell extracts with radiolabeled UDP-monosaccharide

precursors, and exogenously added acceptor glycolipids. Analysis of the the H1 and Le^a glycolipids synthesized in this way by SW1116 was done by cochromatography with authentic glycolipid standards and immunostaining on thin layer chromatograms.

The fact that one of the core saccharides present in peak 5 was LNnT, indicated that fucose may be attached α1-2 to the terminal galactose forming the H-type 2 structure or H2 (Fucα1-2Galβ1-4GlcNAcβ1-3Galβ1-4Glc). Alternatively, fucose may have been linked α1-3 to the GlcNAc forming the Le^x antigen (Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4Glc), which is the positional isomer of the Le^a pentasaccharide.

4.3.5 The Hexasaccharide Peak (Peak 6)

The hexasaccharide peaks (peak 6, fig. 8, panels A and B) labelled with ^{3}H-Gal and ^{3}H-GlcN were individually subjected to beef kidney α-fucosidase digestion (fig. 21C and D). Most of peak 6 (85%) was converted to a tetrasaccharide, which was called 6-4. This result suggested that peak 6 was primarily composed of difucosylated tetrasaccharides. 6-4 was applied to RCA-I chromatography (fig. 22C and D) and it was separated in a similar fashion as that of peak 4 and peak-5-derived tetrasaccharides. The proportions of ^{3}H-Gal-6-4-I, -II, and -III were 4%, 94% and 2% respectively. The proportions of the ^{3}H-GlcN-6-4-I, -6-4-II, and 6-4-III corresponded to 2%, 93%, and 5% respectively.

Fraction 6-4-II.- This fraction eluted in the position of LNT from the RCA-I column. Compositional analysis of ^{3}H-Gal-6-4-II (see fig. 23D), indicated that it was composed of 73% galactose and 27% glucose (ratio of 2.21 Gal/1 Glc);

compositional analysis of ^{3}H-GlcN-6-4-II showed that it contained only GlcNAc (fig. 25A). Treatment with jack bean β-galactosidase, using the same conditions as in the experiments indicated above, digested 15% of the sample (fig. 18G). Repetition of the β-galactosidase treatment, gave a comparable result. Methylation of ^{3}H-Gal-6-4-II resulted (fig.24D) in the same pattern as that of fraction 4-II, confirming that 6-4-II was actually LNT.

Fraction 6-4-III.- The amount of ^{3}H-Gal-6-4-III available only permitted compositional analysis of this sample, which showed that this sample contained 73% galactose and 27% glucose (ratio of 2.21 Gal/1 Glc). Compositional analysis of ^{3}H-GlcN-6-4-III showed that it contained GlcNAc.

Methylation analysis of intact ^{3}H-Gal-peak 6 (fig. 24E) yielded a 2,4,6-trimethylgalactose (3-substituted) or 3,4,6-trimethylgalactose (2-substituted) (both migrate under the same peak) that constituted 50% of the partially methylated derivatives, and the reducing glucose peak, 1,2,3,5,6-pentamethylglucitol (22%). The methylation profile also showed a peak that comigrated with dimethylgalactose and that amounted to 28% of the partially methylated derivatives. The results of methylation peak 6 and that of the defucosylated derivative 6-4-II indicated that this fraction contains, at least, the Lewis^{b} hexasaccharide (Fucα1-2Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc).

However, the fact that 28% of the radioactivity was recovered as a component that cochromatographed with dimethylgalactose in the thin layer chromatogram suggested the presence of another component in this fraction. This component may be a difucosylated derivative of LNT, since 93% of peak 6 is composed by this core saccharide. There are two possible difucosylated LNT structures that will yield a dimethylgalactose and 2/3,4,6-trimethylgalactose,

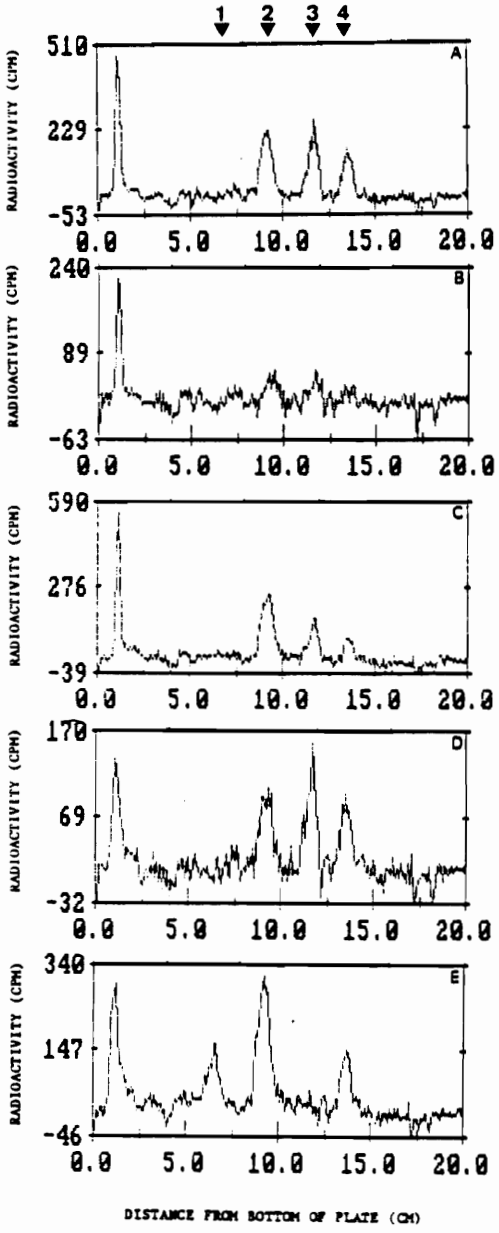


Figure 24.- Methylation analysis of ^{3}H-Gal-5-4-II and ^{3}H-Gal-5-4-III, ^{3}H-Gal-peak 5, ^{3}H-Gal-6-4-II, and ^{3}H-Gal-peak 6.- Samples were reduced, methylated and hydrolyzed according to the procedures described in chapter III. The partially methylated derivatives were analyzed by thin layer chromatography. Samples were spotted on aluminum backed plates (5 x 20 cm), and developed for 70 minutes in the solvent system acetone/water/ammonium hydroxide (250/3/1.5). Partially methylated standards are indicated, 1, dimethylgalactose; 2, 2,4,6-trimethylgalactose; 3, 2,3,4,6-tetramethylgalactose; and 4, 1,2,3,5,6-pentamethylglucitol. Panel A, ^{3}H-Gal-5-4-II; panel B, ^{3}H-Gal-5-4-III; panel C, ^{3}H-Gal-peak 5; panel D, ^{3}H-Gal-6-4-II; and panel E, ^{3}H-Gal-peak 6.

but no 2,3,4,6-tetramethylgalactose, upon methylation. One of them is Fucα1-2Galβ1-3GlcNAcβ1-3[Fucα1-2/4/6]Galβ1-4Glc and Fucα1-2[Fucα1-3/4/6]Galβ1-3GlcNAcβ1-3Galβ1-4Glc. The last structure, which contains two fucoses attached to the terminal non-reducing galactose, has never been observed in known glycoconjugates. The first structure proposed has never been found in glycolipids either, but the trisaccharide, Fucα1-2Galβ1-4Glc, or 2'fucosyllactose, which forms part of the reducing end trisaccharide sequence of the proposed hexasaccharide, has been reported as a human milk oligosaccharide. To prove the existence of either of these two structures further analyses are necessary, probably using fucosidases that are specific for a type of fucose linkage.

The glycolipid containing the Lewis^b hexasaccharide structure has been reported to be present in SW1116 cells [[180, 198, 229]. Liepkans et al. [198] studied the synthesis of this antigen in SW1116 cells by incubation of cell extracts with radiolabeled UDP-monosaccharide precursors, and exogenously added acceptor glycolipids. Detection of the Le^b glycolipid synthesized by SW1116 was done by cochromatography with authentic glycolipid standards and immunostaining on thin layer chromatograms.

4.3.6 The Heptasaccharide Peak (Peak 7)

The heptasaccharide fractions (peak 7, fig. 3 panels A and B), ^{3}H-Gal- and ^{3}H-GlcN-labelled, were further purified by applying them to HPLC using program #3. Peak 7 was separated into 3 fractions, that were named 7a, 7b and 7c (fig. 26). The proportions of ^{3}H-Gal-7a, -7b, and -7c were 17%, 63%, and 20% respectively. The percentages of ^{3}H-GlcN-7a, -7b, and -7c, were 14%, 65%, and 21% respectively.

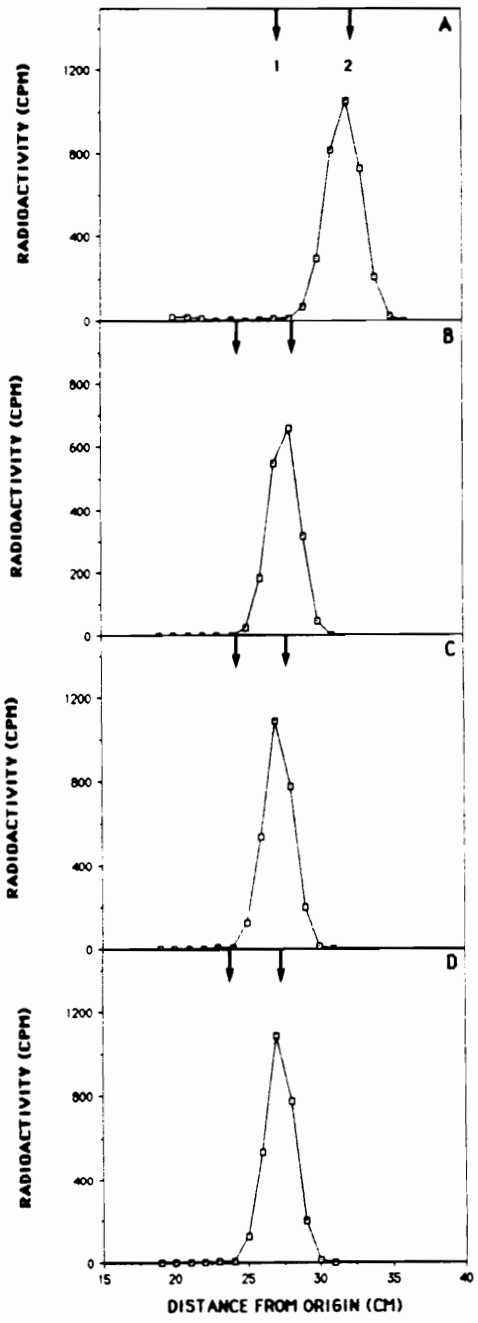


Figure 25.- Monosaccharide composition of ^{3}H-GlcN-6-4-II, ^{3}H-GlcN-7-a, ^{3}H-GlcN-7-b, ^{3}H-GlcN-7-c.- Samples subjected to 2N HCL hydrolysis for 24 h at 100º, were applied to descending paper chromatography and developed in the solvent system ethyl acetate/pyridine/water (12/5/4) for 48 h. Radioactive monosaccharides were localized by cutting the paper chromatogram into 1cm segments. Paper segments were placed in 7 ml beta-vials, to which 0.5 ml of water and 4 ml of scintillation cocktail were added, and then counted in a liquid scintillation counter for 5 min. Panel A, ^{3}H-GlcN-6-4-II; panel B, ^{3}H-GlcN-7-a; panel C, ^{3}H-GlcN-7-b; panel D, ^{3}H-GlcN-7-c. The positions of unlabelled galactosamine, 1; and glucosamine, 2, are indicated by the arrows.

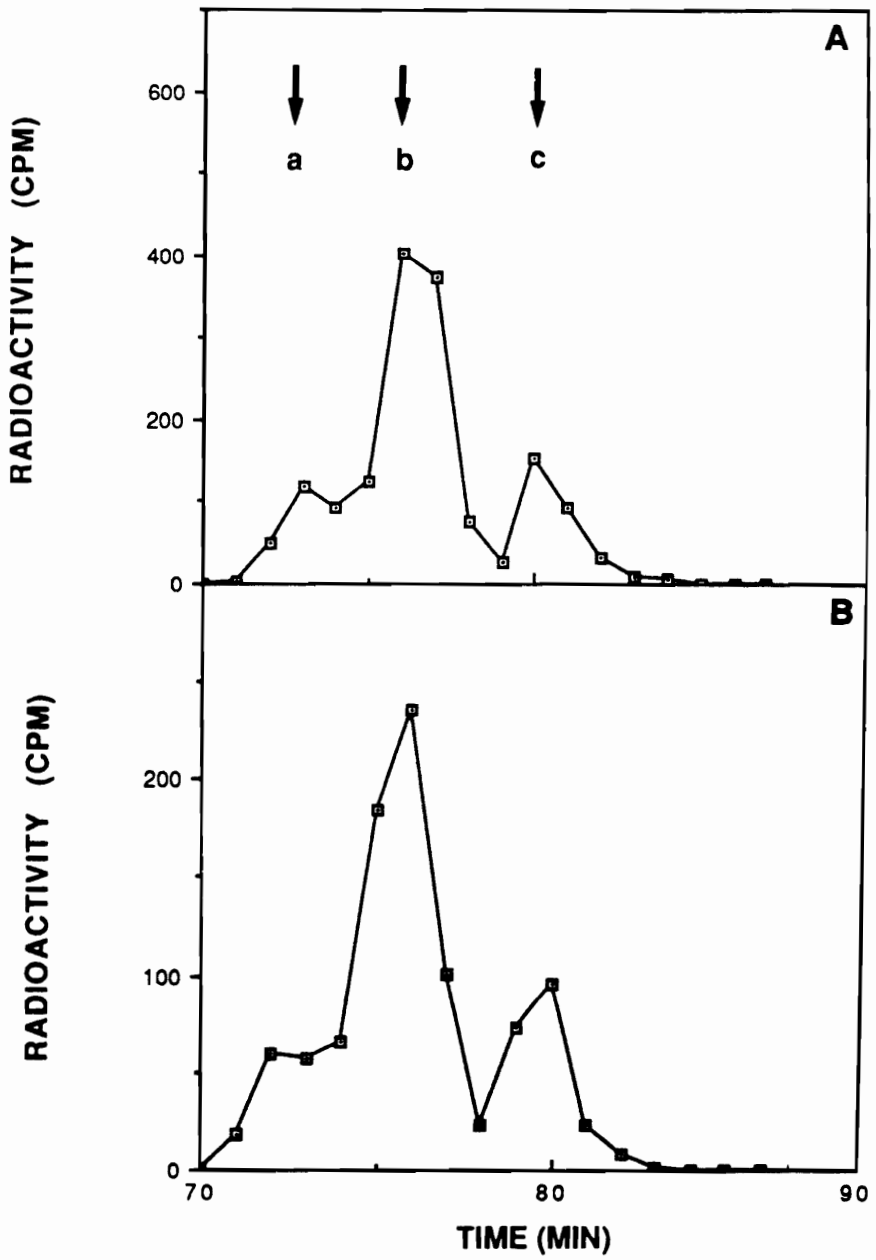


Figure 26.- HPLC of peak 7.- ^{3}H-Gal-peak 7 and ^{3}H-GlcN-peak 7 were individually applied to HPLC on an Alltech carbohydrate NH_2 column, using program # 3. Aliquots of 1 ml fractions (0.5 min) were assayed for radioactivity. Position of elution of the different fractions obtained are indicated by, a, b, and c. Panel A, ^{3}H-Gal-peak 7; and panel B, ^{3}H-GlcN-peak 7.

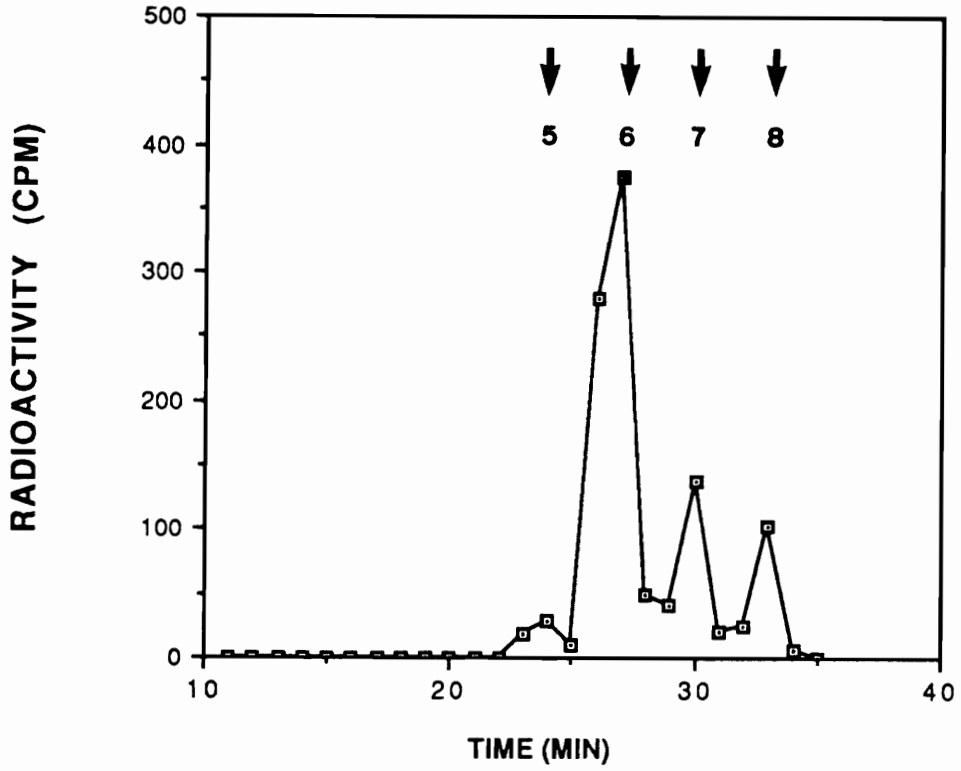


Figure 27.- HPLC of the beef kidney α-fucosidase digest of ^{3}H-Gal-7-b.- Beef kidney α-fucosidase digestion of H-Gal-7-b was carried as explained in chapter III and it was analyzed by HPLC as explained in figure 12. Elution times of authentic, 5, human milk ^{3}H-LNFol-I; 6, human milk ^{3}H-lacto-N-neohexaitol; 7, rabbit erythrocyte ^{3}H-heptaitol; and 8, ^{3}H-Gal-SW1116 octasaccharide, are indicated.

Compositional analysis of ^{3}H-GlcN-7a demonstrated that it contained only GlcNAc (fig. 25B). Compositional analysis ^{3}H-Gal-7c (see fig. 23F) showed that it was composed of 80% galactose and 20% glucose (ratio of 3.23 Gal/1 Glc), and that of ^{3}H-GlcN-7c demonstrated that it contained only GlcNAc (see fig. 25D). The presence of only GlcNAc, and no GalNAc in fractions 7a and 7c, suggested that the components of these fractions were derivatives of lacto or lactoneo type chains.

Fraction 7b.- ^{3}H-Gal-7b was treated with beef kidney α-fucosidase, and the digestion products were analyzed by HPLC, showing that they were composed of 5% of a pentasaccharide, 65% of a hexasaccharide, 18% of heptasaccharide, and 12% of contaminating octasaccharide (fig. 27). Since the hexasaccharide was the major product, just this hexasaccharide was subjected to further analysis. The ^{3}H-Gal-7b-hexasaccharide was subjected to α-fucosidase digestion for a second time, but no further digestion occurred. The hexasaccharide was applied to the RCA-I column and it was eluted in the position of LNT (fig. 28), indicating that it contained a non-reducing terminal galactose forming a β1-3 linkage. Methylation analysis of ^{3}H-Gal-7b-hexasaccharide (fig. 29) yielded 2,3,4,6-tetramethylgalactose (non-reducing terminal galactose) (29%), 2,4,6-trimethylgalactose (3-substituted) (58%), and 1,2,3,5,6-pentamethylglucitol (reducing glucose) (13%). Methylation analysis of the intact fraction ^{3}H-Gal-7b (fig. 30) demonstrated the presence of a 3-substituted galactose (90%) and a reducing glucose (10%). Compositional analysis of ^{3}H-Gal-7b (fig. fig. 23E) showed that 79% of the radioactivity corresponded to galactose and that 21% to glucose (ratio of 3.08 Gal/Glc). ^{3}H-GlcN-7b only contained GlcNAc.

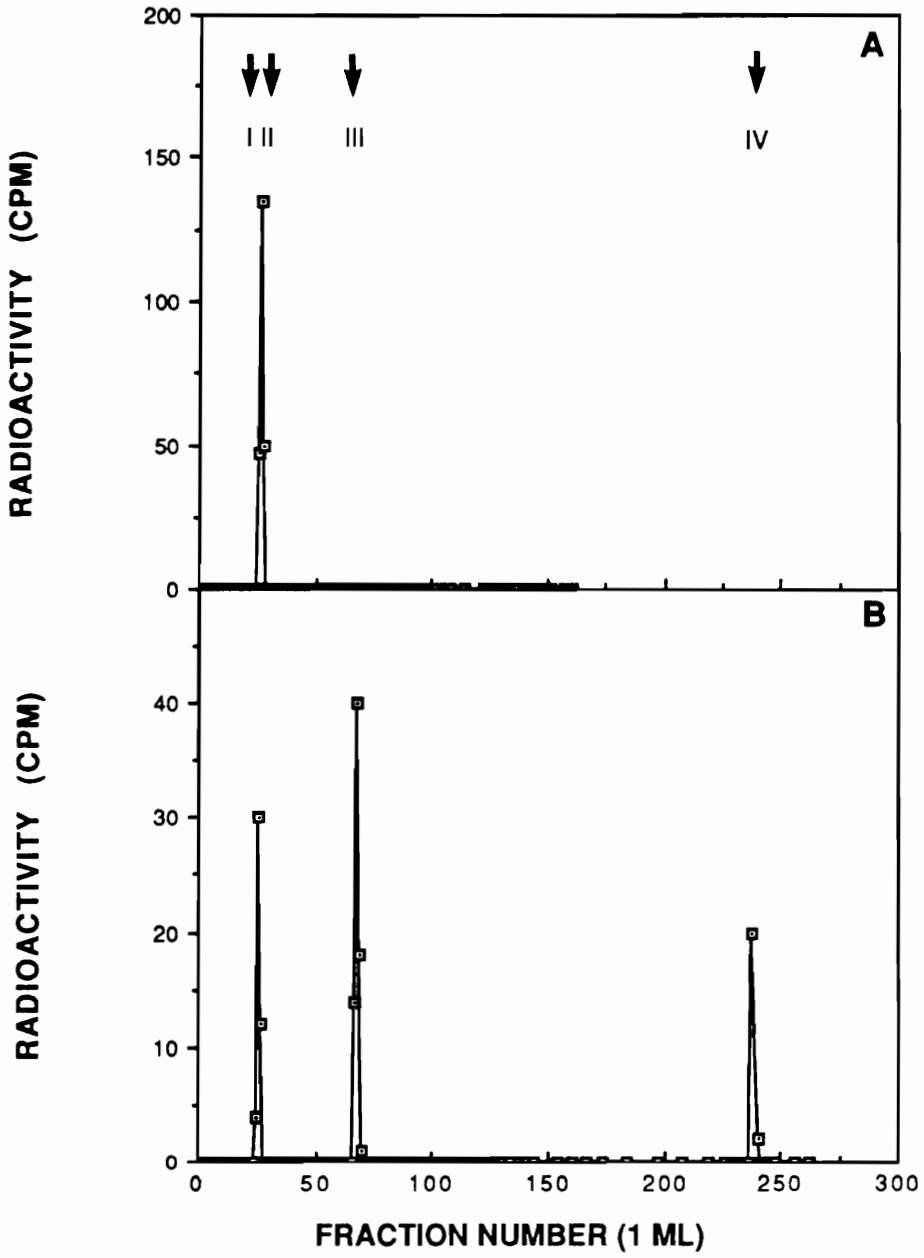


Figure 28.- Purification of the defucosylated 7-b-hexasaccharide and defucosylated asialo MSP VI-hexasaccharide by affinity chromatography on RCA-I-agarose.- Defucosylated ^{3}H-Gal-7-b-hexasaccharide; and defucosylated ^{3}H-Gal-asialo MSP VI-hexasaccharide from figure 40 were applied to an RCA-I column (0.7 x 50 cm), equilibrated in phosphate buffered saline at room temperature. Samples were incubated for 15 minutes before eluting them in the starting buffer at a flow rate of 8 ml/hr. Aliquots of 1ml fractions were assayed for radioactivity. The void volume, I; the elution position of authentic human milk ^{3}H-LNTol, II; ^{3}H-LNnTol, III; and ^{3}H-lacto-N-neohexaitol, IV, are indicated. Panel A, defucosylated ^{3}H-Gal-7-b-hexasaccharide, panel B, defucosylated ^{3}H-Gal-asialo-MSP VI-hexasaccharide.

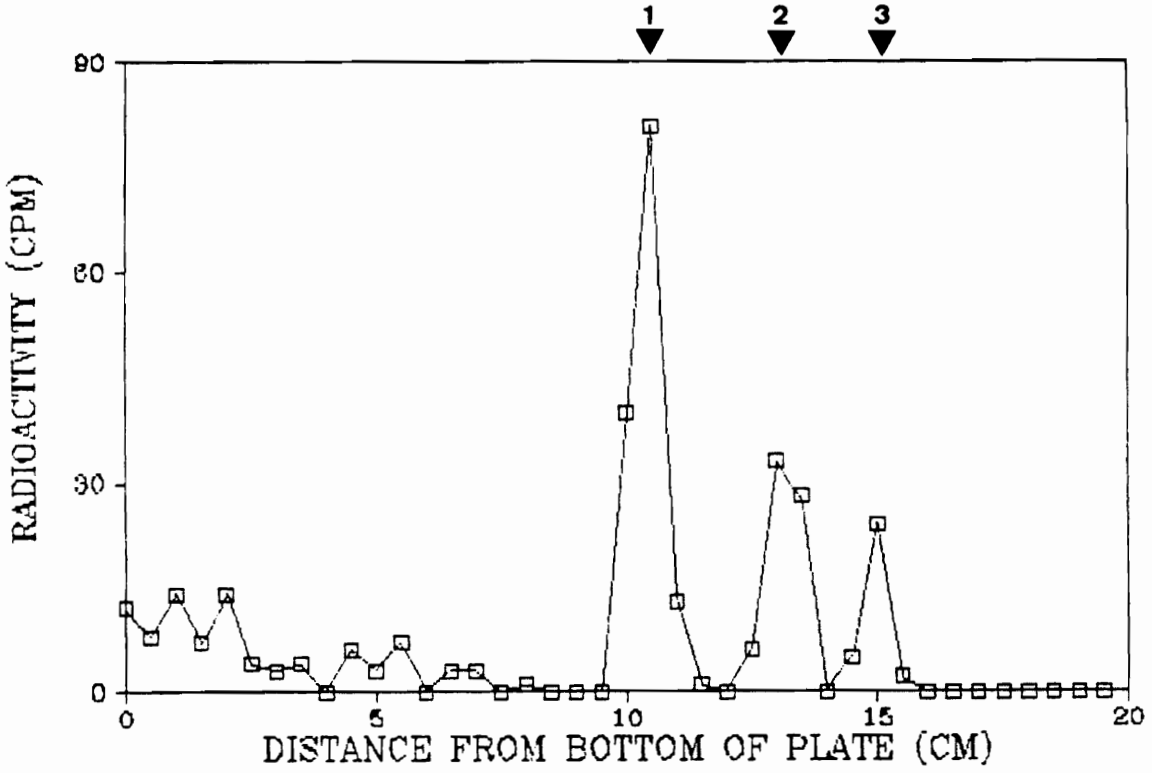


Figure 29.- Methylation analysis of defucosylated ^{3}H-Gal-7-b-hexasaccharide.- Defucosylated ^{3}H-Gal-7-b-hexasaccharide was reduced, methylated and hydrolyzed according to the procedures described in chapter III. The partially methylated derivatives were analyzed by thin layer chromatography. Samples were spotted on aluminum backed plates (5 x 20 cm), and developed for 70 minutes in the solvent system acetone/water/ammonium hydroxide (250/3/1.5). The dry chromatogram was divided into 0.5 cm segments from bottom to top. The plate was sprayed lightly, with distilled water, the silica was scraped from each segment into a 7 ml scintillation vial, 0.5 ml of water were added and then 4 ml of scintillation fluid. Each vial was mixed vigorously in a vortex mixer for 30 seconds, and counted for 5 minutes in a liquid scintillation counter. Partially methylated standards are indicated, 1, 2,4,6-trimethylgalactose; 2, 2,3,4,6-tetramethylgalactose; and 3, 1,2,3,5,6-pentamethylglucitol.

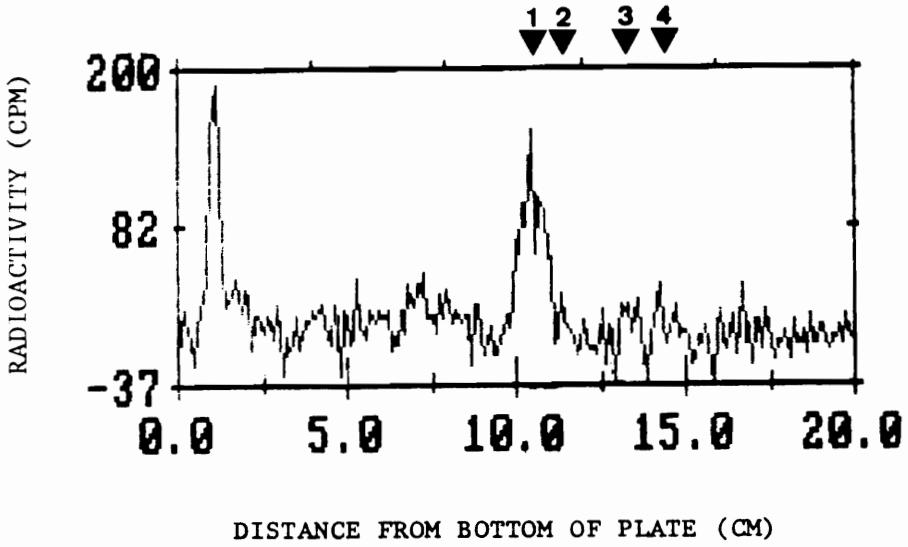


Figure 30.- Methylation analysis of ^{3}H-Gal-7-b.- ^{3}H-Gal-7-b was reduced, methylated and hydrolyzed according to the procedures described in chapter III. The partially methylated derivatives were analyzed by thin layer chromatography. Samples were spotted on aluminum backed plates (5 x 20 cm), and developed for 70 minutes in the solvent system acetone/water/ammonium hydroxide (250/3/1.5). Partially methylated standards are indicated, 1, 2,4,6-trimethylgalactose; 2, 2,3,6-trimethylgalactose; 3, 2,3,4,6-tetramethylgalactose; and 4, 1,2,3,5,6-pentamethylglucitol.

All these results suggested that the heptasaccharide of fraction 7b had the following structure, Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-3/4GlcNAcβ1-3Galβ1-4Glc.

4.4 SEPARATION AND STRUCTURAL ANALYSIS OF ACIDIC OLIGOSACCHARIDES

^{3}H-Gal- and ^{3}H-GlcN-labelled oligosaccharides released from the acidic glycolipids by ozonolysis/alkali fragmentation were individually subjected to DE-53-cellulose chromatography. Acidic oligosaccharides were separated into peaks 1-7 (fig. 31). Peaks 1-4, and 7 were identified based on their elution on the DE-53-cellulose column and cochromatography with authentic human milk acidic oligosaccharides, as neutrals (peak 1, fig. 31), monosialyl high molecular weight oligosaccharides (peak 2, fig. 31), monosialylpentasaccharides and monosialyltetrasaccharides (peak 3, fig. 31), monosialyllactose (peak 4, fig. 31), and disialyl- and trisialyloligosaccharides (peak 7, fig. 31). Peaks 5, and 6 did not cochromatograph with known standards, and their characterization was not acomplished in this work. Also, the diasialyl and trisialyl oligosaccharides, peak 7, were out of the scope of this study. The proportions of each of these fractions are given in table 5. The ^{3}H-Gal- and the ^{3}H-GlcN-monosialyltetrasaccharide and pentasaccharide fraction (peak 3, fig. 31) represented the major component of the total monosialyloligosaccharides. The disialyloligosaccharide fraction from both labellings also constituted a considerable percentage of the total acidic oligosaccharides.

In preliminary studies the monosialyltetra- and pentasaccharide fracion (peak 3) and the monosialyllactose fraction (peak 4) were subjected to affinity chromatography, using lectins that bind specific types sialic acid linkages. One of the affinity columns used was *Maackia amurensis* (MAL)-agarose which binds terminal non-reducing sialic acic linked α2-3 to lactoneo cores (type 2 chain) [245], for instance, it binds the sialyltetraose derived from sialylparagloboside (NeuAcα2-3Galβ1-4GlcNAβ1-3Galβ1-4Glc). MAL-agarose does not bind any structure containing sialic acid linked α2-6. The other affinity column employed was *Sambucus nigris* (SNA)-agarose which binds acidic oligosaccharides containing sialic acid linked α2-6 to galactose or N-acetylgalactosamine [245]. For example, it binds authentic STc (NeuAcα2-6Galβ1-4GlcNAβ1-3Galβ1-4Glc) from human milk. The monosialyloligosaccharide fractions (peaks 3 and 4) from SW1116 acidic glycolipids failed to bind either of these lectin affinity columns, indicating that oligosaccharides fulfilling the structural requirements for binding to MAL or SNA were not present in those fractions.

4.4.1 The Monosialyllactose Fraction (MSL, Peak 4)

The ^{3}H-Gal- and ^{3}H-GlcN-MSL fractions, when applied separately to HPLC using program #2, eluted as a single component in the position of authentic human milk 3'sialyllactose (NeuAcα2-3Galβ1-4Glc) (fig. 32). Treatment with *Arthrobacter ureafaciens* neuraminidase completely converted ^{3}H-Gal-MSL into a disaccharide (fig. 35). This disaccharide was applied to the RCA-I column, and it eluted in the position of lactose (fig. 36A). Methylation analysis of ^{3}H-Gal-MSL (fig. 37A), showed a 3-substituted galactose (2,4,6-

trimethylgalactose) (66%), some terminal non-reducing galactose (2,3,4,6-tetramethylgalactose) (10%), maybe present because of some desialylation of the sample, and the reducing glucose (1,2,3,5,6-pentamethylglucitol) (24%). Methylation after neuraminidase digestion of ^{3}H-Gal-MSL (fig. 37B) only showed a terminal non-reducing galactose (56%), and the reducing glucose (44%), demonstrating that the sialic acid was attached to the third hydroxyl group of the galactose in an α-linkage. These results confirm that MSL is composed of 3'sialyllactose. Compositional analysis of ^{3}H-Gal-MSL (fig. 33 A), indicated that it contained 60% galactose and 40% glucose, which corresponded to a ratio of 1.5 Gal/1Glc[4]. The ^{3}H-GlcN labelled MSL was digested with neuraminidase. After removal of sialic acid, the neutral and the acidic fractions were separated on a DE-52-cellulose column (0.4 x 3 cm); all the radioactivity was recovered in the acidic fraction. The fraction ^{3}H-GlcN-MSL, should contain all the radiolabel as sialic acid, since the glucosamine incorporated by the cells is used only to make N-acetylmanosamine, sialic acid, N-acetylglucosamine and N-acetylgalactosamine.

4.4.2 The Monosialyltetrasaccharide and Pentasaccharide Fraction (MSP, Peak 3)

The ^{3}H-Gal-, and ^{3}H-GlcN-monosialyltetrasaccharide and pentasaccharide fractions (peak 3, fig. 31, panels A and B) were individually applied to HPLC (program #2) and they were separated into 5 fractions (fig. 38 A and B), that were called MSP I, MSP II, MSP III, MSP IV, and MSP VI. The major component

[4]The ratio of Gal/Glc from MSL (1.5 Gal/Glc) was used to calculate the ratio of Gal/Glc for the rest of the acidic glycolipids, since they were isolated from a different batch of ^{3}H-Gal-metabolically labelled SW1116 cells.

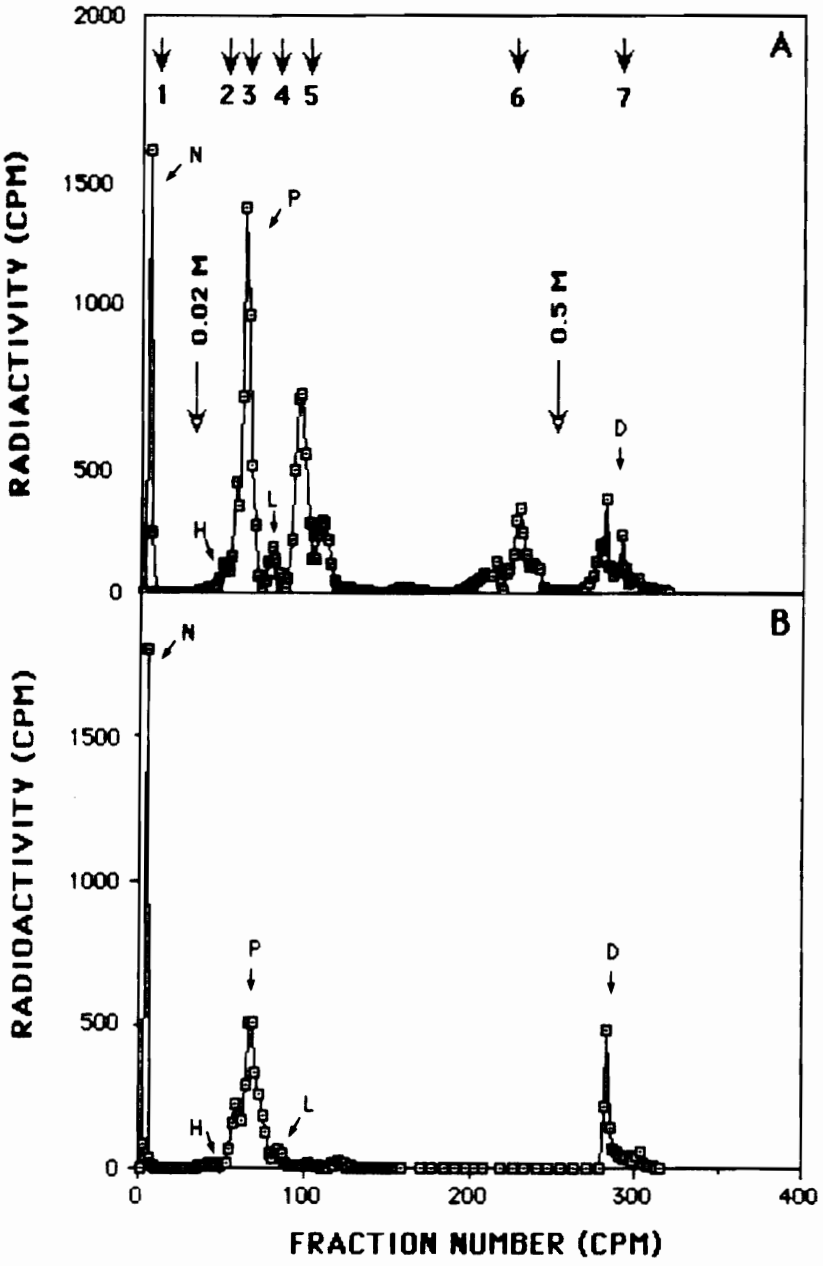


Figure 31.- DEAE-53 chromatography of oligosaccharides derived from acidic glycolipids.- ^{3}H-Gal- and ^{3}H-GlcN-oligosaccharides derived from acidic glycolipids were applied to a DEAE-53-cellulose column (0.7 x 20 cm) previously equilibrated in 0.002 M pyridine acetate buffer. The column was eluted with 25 ml of the starting buffer, 200 ml of 0.02 M pyridine acetate, and 100 ml of 0.5 M pyridine acetate. The elution positions of neutral oligosaccharides, 1; human milk high molecular weight monosialyloligosaccharides, 2; human milk monosialylpenta- and tetrasaccharides, 3; human milk monosialyllactoses, 4; and human milk di-and trisialyloligosaccharides, 7, are indicated. Peaks 5 and 6 did not comigrate with known standards. Aliquots of 1 ml fractions were assayed for radioactivity. Panel A, ^{3}H-Gal-acidic oligosaccharides; panel B, ^{3}H-GlcN-acidic oligosaccharides.

TABLE 6

Proportions of the Fractions Obtained from Figure 31

Fraction from fig. 31	^{3}H-Galactose (%)	^{3}H-Glucosamine (%)
N (peak 1)	10.4	28.0
SHMW (peak 2)	0.1	0.3
MSP (peak 3)	30.0	46.0
MSL (peak 4)	3.0	3.0
Peak 5	25.0	2.7
Peak 6	13.5	0.0
Di- & Trisialyls (peak 7)	18.0	20.0

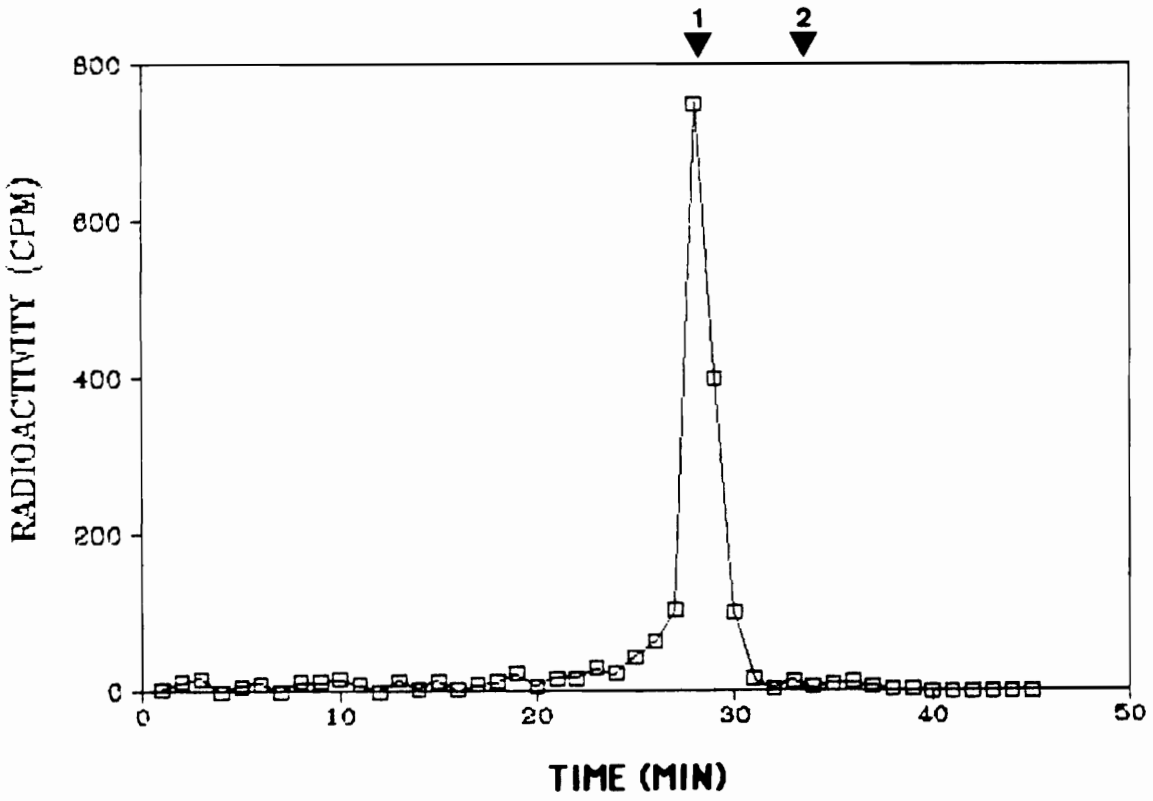


Figure 32.- HPLC of the ^{3}H-Gal-MSL fraction.- The ^{3}H-Gal-monosialyllactose fraction (MSL) was applied to HPLC using program # 2. Aliquots of 1 ml fractions (0.5 min) were assayed for radioactivity. The elution times of authentic human milk 3'monosialyllactose, 1; and 6'monosialyllactose, 2, are indicated.

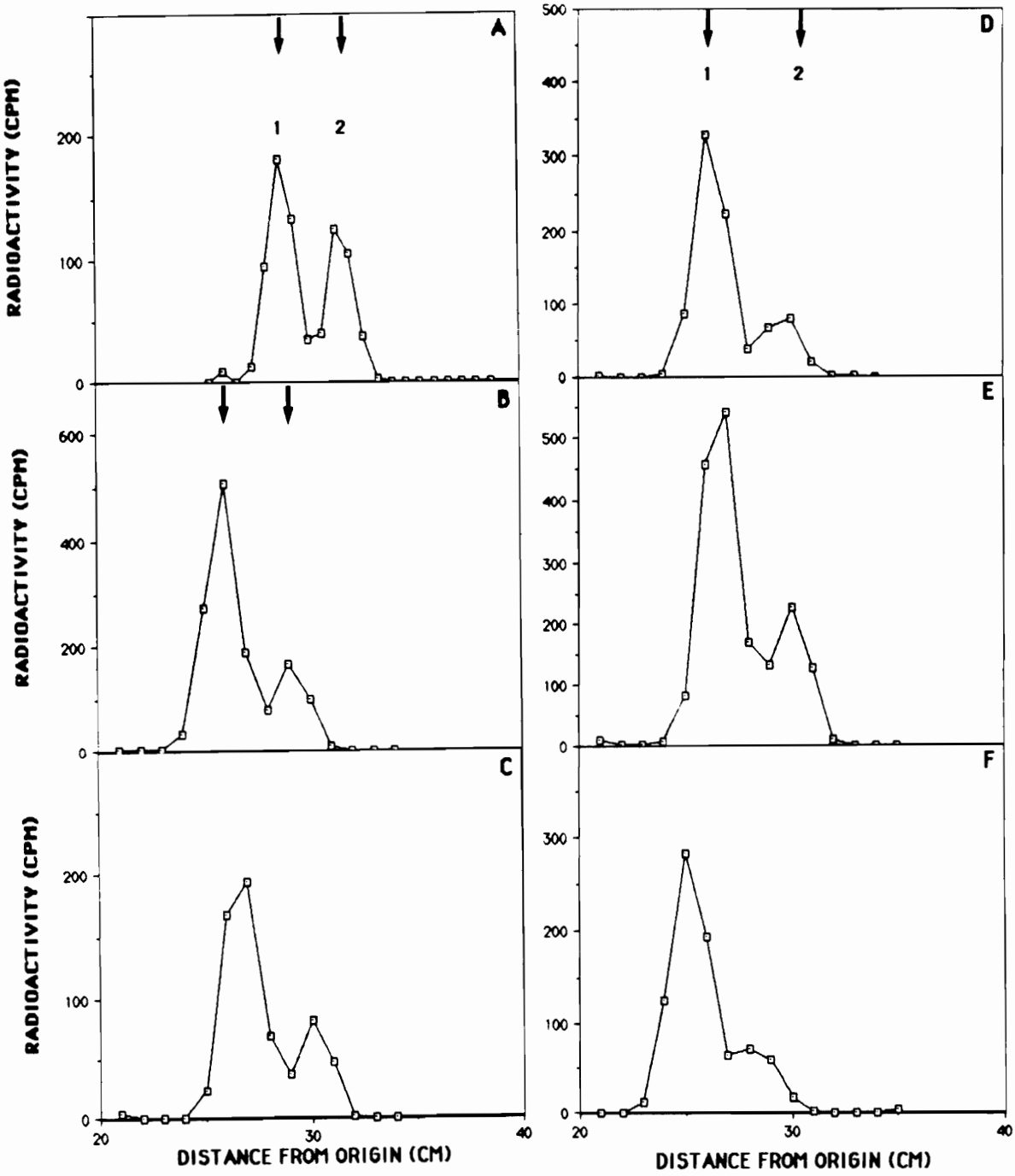


Figure 33.- Monosaccharide composition of ^{3}H-Gal-MSL, ^{3}H-Gal-MSP I, ^{3}H-Gal-MSP II, ^{3}H-Gal-MSP III, ^{3}H-Gal-MSP IV and ^{3}H-Gal-MSP VI.- Samples subjected to 2N HCL hydrolysis for 24 h at 100°, were applied to descending paper chromatography and developed in the solvent system ethyl acetate/pyridine/water (12/5/4) for 24 h. Radioactive monosaccharides were localized by cutting the paper chromatogram into 1cm segments. Paper segments were placed in 7 ml beta-vials, to which 0.5 ml of water and 4 ml of scintillation cocktail were added, and then counted in a liquid scintillation counter for 5 min. Panel A, ^{3}H-Gal-MSL; panel B, ^{3}H-Gal-MSP I; panel C, ^{3}H-Gal-MSP II; panel D, ^{3}H-Gal-MSP III; panel E, ^{3}H-Gal-MSP IV; and panel F, ^{3}H-Gal-MSP VI. The positions of unlabelled galactose, 1, and glucose, 2, are indicated by the arrows.

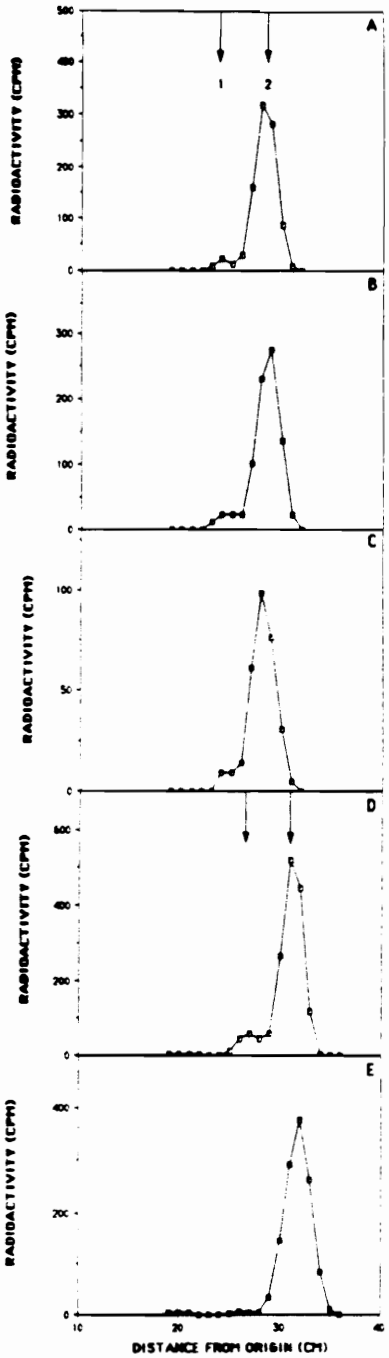


Figure 34.- Monosaccharide composition of ^{3}H-GlcN-MSP I, ^{3}H-GlcN-MSP II, ^{3}H-GlcN-MSP III, ^{3}H-GlcN-MSP IV, ^{3}H-GlcN-MSP VI.- Samples subjected to 2N HCL hydrolysis for 24 h at 100°, were applied to descending paper chromatography and developed in the solvent system ethyl acetate/pyridine/water (12/5/4) for 48 h. Radioactive monosaccharides were localized by cutting the paper chromatogram into 1cm segments. Paper segments were placed in 7 ml beta-vials, to which 0.5 ml of water and 4 ml of scintillation cocktail were added, and then counted in a liquid scintillation counter for 5 min. Panel A, ^{3}H-GlcN-MSP I; panel B, ^{3}H-GlcN-MSP II; panel C, ^{3}H-GlcN-MSP III; panel D, ^{3}H-GlcN-MSP IV; panel E, ^{3}H-GlcN-MSP VI. The positions of unlabelled galactosamine, 1; and glucosamine, 2, are indicated by the arrows.

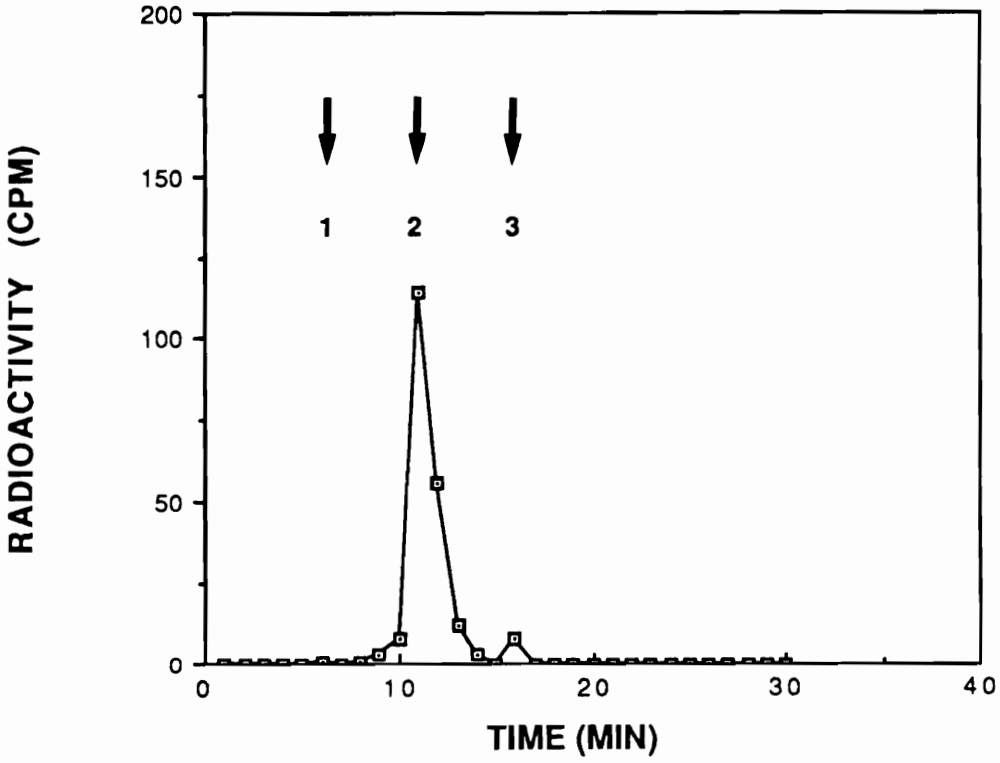


Figure 35.- **HPLC of the *Arthrobacter ureafaciens* neuraminidase digest of ^{3}H-Gal-MSL.-** Neuraminidase digestion of H-Gal-MSL was carried as explained in chapter III and the asialo-derivative was analyzed by HPLC as explained in figure 12. Elution times of ^{3}H-galactose, 1; of ^{3}H-lactitol, 2; and ^{3}H-lacto-N-triaitol, 3, are indicated.

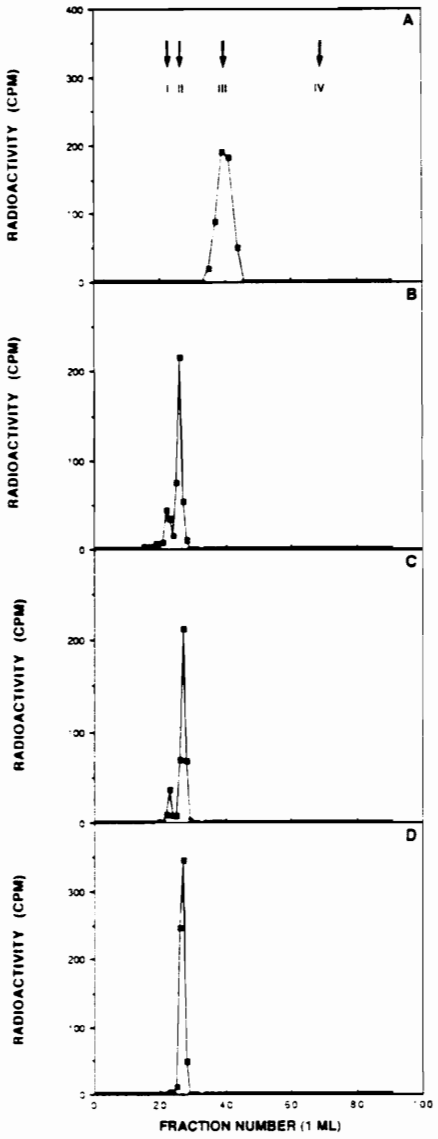


Figure 36.- Purification of the desialylated fractions MSL, MSP I, MSP II, and defucosylated-MSP IV by affinity chromatography on RCA-I-agarose.- Desialylated ^{3}H-Gal-MSL, -MSP I, MSP II, and desialylated and defucosylated ^{3}H-Gal-MSP IV were separately applied to an RCA-I column (0.7 x 50 cm), equilibrated in phosphate buffered saline at room temperature. Samples were incubated for 15 minutes before eluting them in the starting buffer at a flow rate of 8 ml/hr. Aliquots of 1ml fractions were assayed for radioactivity. The void volume, I; the elution position of authentic human milk ^{3}H-LNTol, II; ^{3}H-Gal-SW1116-lactose, III and human milk^{3}H-LNnTol, IV, are indicated. Panel A, desialylated ^{3}H-Gal-MSL, panel B, desialylated ^{3}H-Gal-MSP I; panel C, desialylated ^{3}H-Gal-MSP II; panel D, desialylated and defucosylated ^{3}H-Gal-MSP IV.

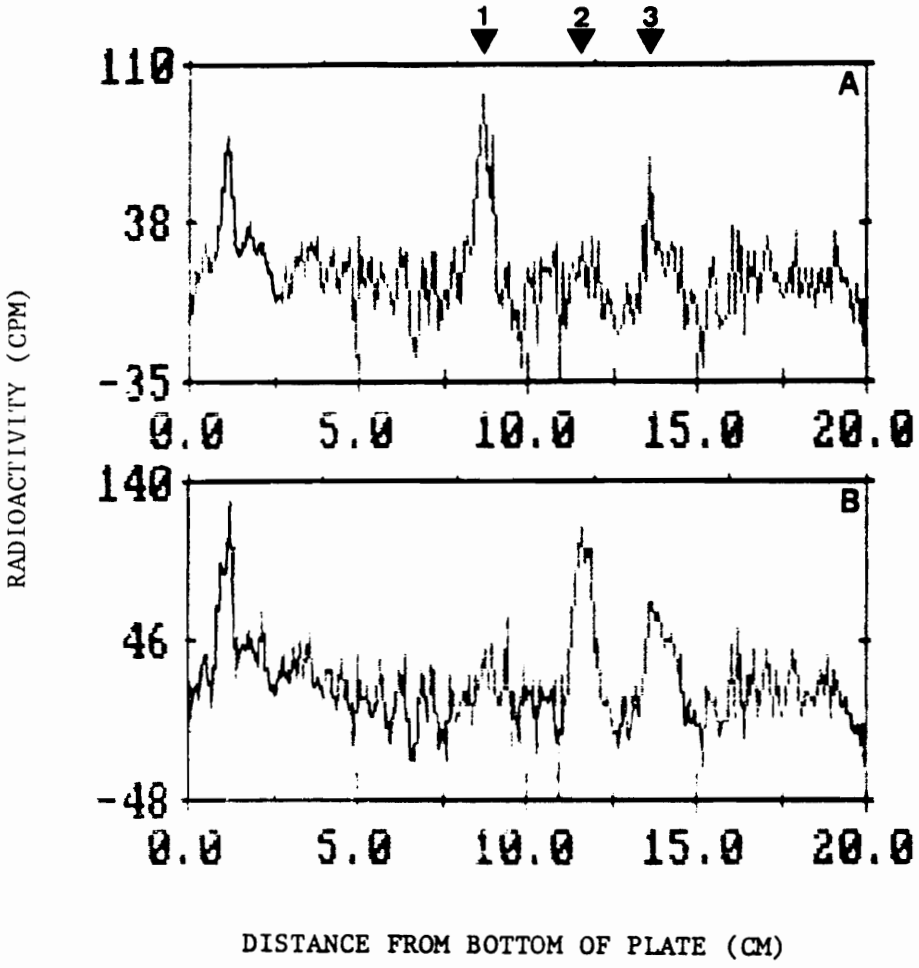


Figure 37.- Methylation analysis of ^{3}H-MSL before and after neuraminidase treatment.- ^{3}H-Gal-MSL and desialylated ^{3}H-Gal-MSL were reduced, methylated and hydrolyzed according to the procedures described in chapter III for sialylated samples. The partially methylated derivatives were analyzed by thin layer chromatography. Samples were spotted on aluminum backed plates (5 x 20 cm), and developed for 70 minutes in the solvent system acetone/water/ammonium hydroxide (250/3/1.5). Partially methylated standards are indicated, 1, 2,4,6-trimethylgalactose; 2, 2,3,4,6-tetramethylgalactose; and 3, 1,2,3,5,6-pentamethylglucitol. Panel A, ^{3}H-Gal-MSL; panel B, desialylated ^{3}H-Gal-MSL.

of this fraction was MSP IV, which contained the sialyl Le^a pentasaccharide. The proportions of each of these components are given in table 6.

MSP I.- On HPLC separation, this fraction eluted with authentic human milk sialyltetrasaccharide a, STa (NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc). Neuraminidase treatment converted this sample into a tetrasaccharide (fig. 39A), most of which, when applied to the RCA-I column, eluted in the position of LNT (fig. 36B). Treatment of the tetrasaccharide with 0.8-1.6 U/ml of jack bean β-galactosidase, only digested 18% of the sample (fig. 39 B). A second digestion gave a similar result. Compositional analysis showed that total radioactivity in ^{3}H-Gal-MSP-I was recovered as 74% galactose and 26% glucose (ratio of 1.90 Gal/1 Glc) (fig. 33B). ^{3}H-GlcN-MSP-I was composed of sialic acid (30%), GlcNAc (67%) and GalNAc (3%) (fig. 34A) Methylation analysis of ^{3}H-Gal-MSP-I, showed a 3-substituted galactose (2,4,6-trimethylgalactose) (81%), some 2,3,4,6-tetramethylgalactose (7%), probably due to some desialylation of MSP-I, and 1,2,3,5,6-pentamethylglucitol (12%) (fig. 40A). Methylation after removal of sialic acid demonstrated the presence of a terminal non-reducing galactose (2,3,4,6-tetramethylgalactose) (43%), indicating that the sialic acid was linked α2-3 to the terminal galactose, a 3-substituted galactose (2,4,6-trimethylgalactose) (35%), and the reducing glucose (1,2,3,5,6-pentamethylglucitol) (22%) (fig. 40B). These results confirmed that the major component of MSP-I was STa. Some GM1(Galβ1-3GalNAcβ1-3[NeuAcα2-3]Galβ1-4Glc) may be present in this fraction, since it contained GalNAc.

MSP II.- This fraction comigrated on HPLC with authentic human milk sialyltetrasaccharide b, STb (Galβ1-3[NeuAcα2-6]GlcNAcβ1-3Galβ1-4Glc).

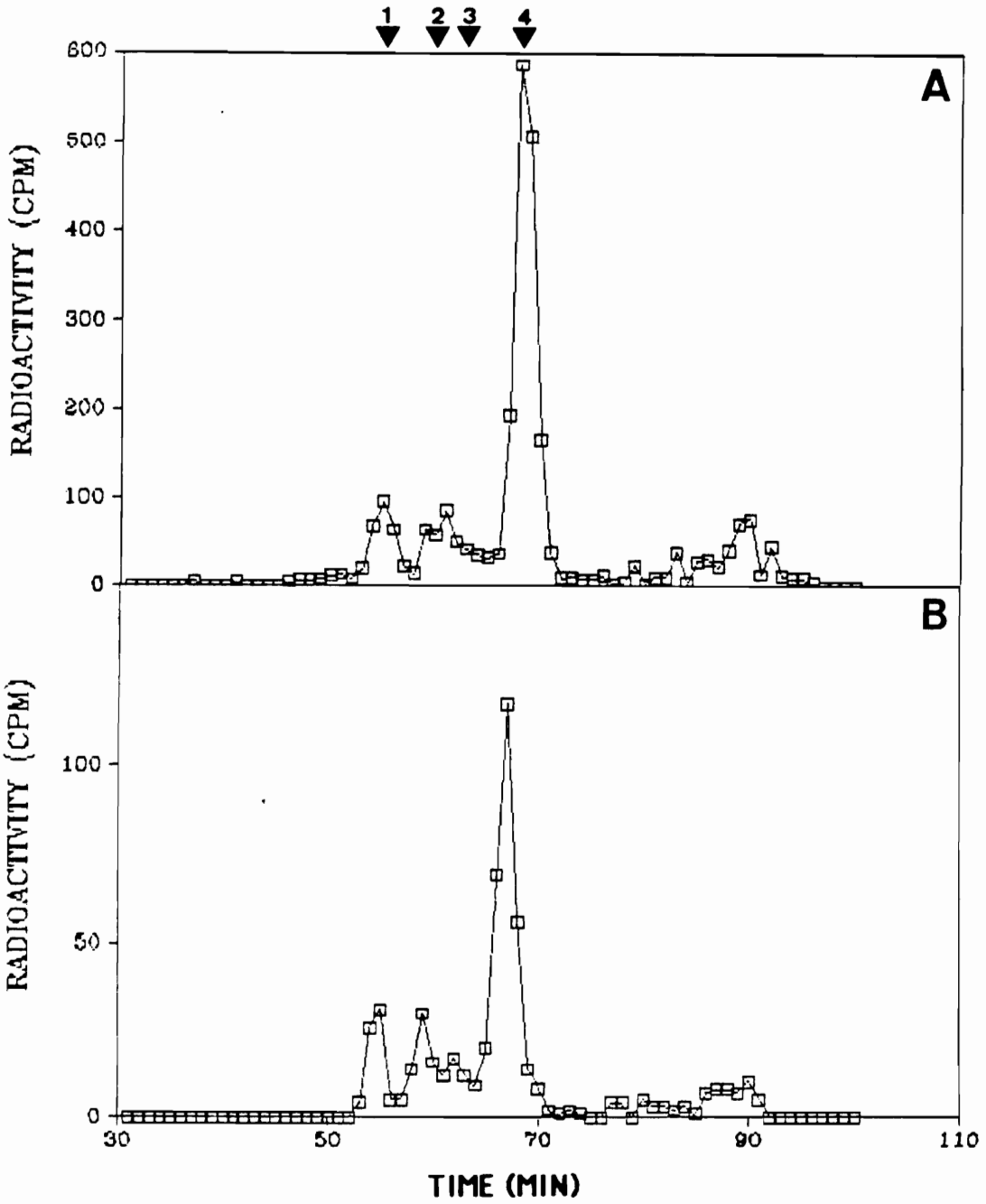


Figure 38.- HPLC of the ^{3}H-Gal-MSP and ^{3}H-GlcN-MSP fractions.- The ^{3}H-Gal and ^{3}H-GlcN-monosialylpenta-and -tetrasaccharide fractions (MSP) were separately applied to HPLC using the program # 2, described in chapter III. Aliquots of 1 ml fractions (0.5 min) were assayed for radioactivity. The elution times of authentic human milk sialyltetrasaccharide a, 1; sialyltetrasaccharide b, 2; sialyltetrasaccharide c, 3; and sialylpentasaccharides, 4 are indicated. Panel A, ^{3}H-Gal-MSP and Panel B, ^{3}H-GlcN-MSP.

TABLE 7

Proportions of Monosialylpenta- and tetrasaccharide Peaks Obtained from Figure 38

Peaks from fig. 38	^{3}H-Galactose (%)	^{3}H-Glucosamine (%)
MSP I	11	9
MSP II	5	11
MSP III	9	5
MSP IV	58	42
MSP VI	11	20

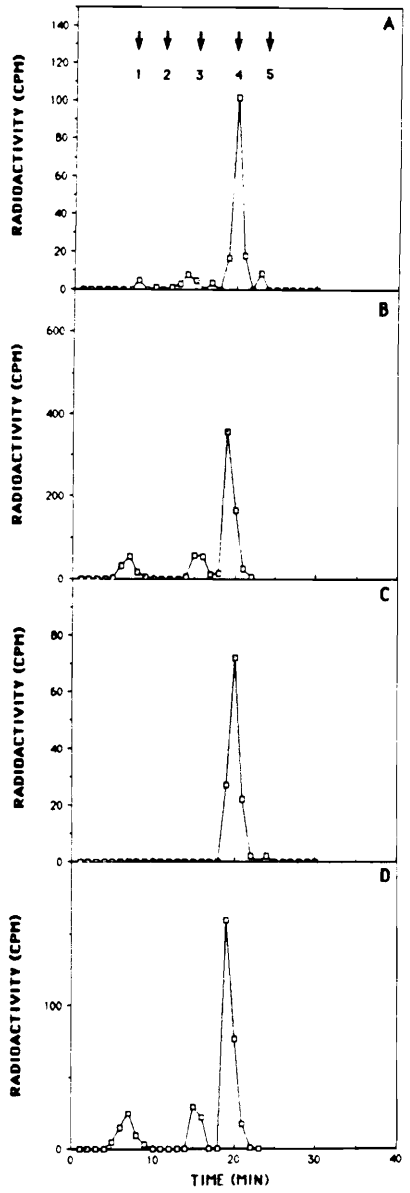


Figure 39.- HPLC of the *Arthrobacter ureafaciens* neuraminidase and jack bean β-galactosidase digests of ^{3}H-Gal-MSP I and -MSP II.- Neuraminidase digestion of ^{3}H-Gal-MSP I and -MSP II were carried as explained in chapter III and the asialo-derivatives were analyzed by HPLC as explained in figure 12. Subsequently, the desialylated fractions ^{3}H-Gal-MSP I and -MSP II were subjected to jack bean β-galactosidase digestion as explained in chapter III. The corresponding β-galactosidase digestion products were analyzed by HPLC as explained in figure 12. Elution times of ^{3}H-galactose, 1; of ^{3}H-lactitol, 2; ^{3}H-lacto-N-triaitol, 3; ^{3}H-lacto-N-tetraitol, 4; and ^{3}H-lacto-N-fucopentaitol, 5, are indicated. Panel A, neuraminidase digestion products of ^{3}H-Gal-MSP I; panel B, β-galactosidase digestion products of asialo ^{3}H-Gal-MSP I; panel C, neuraminidase digestion products of ^{3}H-Gal-MSP II; and, panel D, β-galactosidase digestion products of asialo ^{3}H-Gal-MSP II.

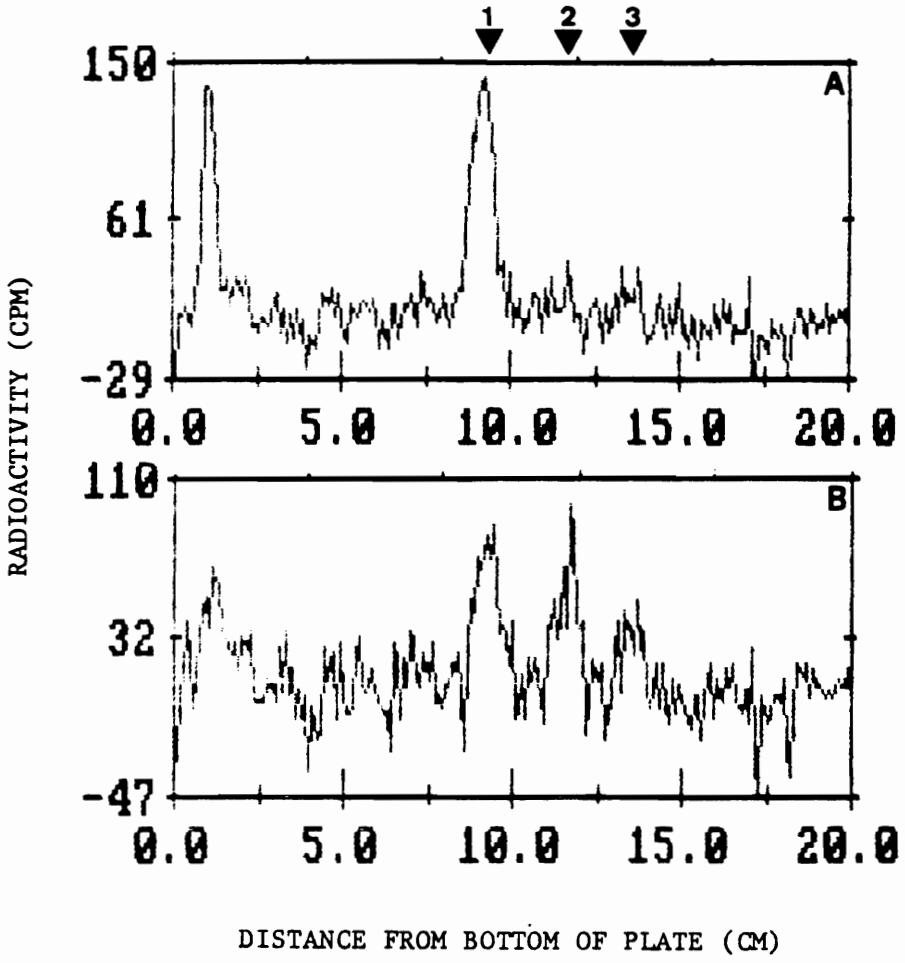


Figure 40.- Methylation analysis of ^{3}H-MSP I before and after neuraminidase treatment.- ^{3}H-Gal-MSP I and desialylated ^{3}H-Gal-MSP I were reduced, methylated and hydrolyzed according to the procedures described in chapter III for sialylated samples. The partially methylated derivatives were analyzed by thin layer chromatography. Samples were spotted on aluminum backed plates (5 x 20 cm), and developed for 70 minutes in the solvent system acetone/water/ammonium hydroxide (250/3/1.5). Partially methylated standards are indicated, 1, 2,4,6-trimethylgalactose; 2, 2,3,4,6-tetramethylgalactose; and 3, 1,2,3,5,6-pentamethylglucitol. Panel A, ^{3}H-Gal-MSP I; panel B, desialylated ^{3}H-Gal-MSP I.

Neuraminidase treatment totally converted ^{3}H-Gal-MSP II into a tetrasaccharide (fig. 39C). When this tetrasaccharide was applied to the RCA-I column, it yielded the same elution profile as did asialo MSP-I (fig. 36C). Jack bean-β-galactosidase digestion resulted in conversion of 17% of asialo ^{3}H-Gal-MSP-II into a trisaccharide (fig. 39D). When this experiment was repeated, a comparable result was obtained. ^{3}H-Gal-MSP-II was composed of 73% Gal and 27 % Glc (ratio of 1.8 Gal/1 Glc) (fig. 33C). ^{3}H-GlcN-MSP-II contained 48% of sialic acid, 48% of GlcNAc and 4% of GalNAc (fig. 34B). Methylation analysis of ^{3}H-Gal-MSP-II before and after neuraminidase digestion (fig. 41A and B) presented the same profile, a terminal non-reducing galactose (2,3,4,6-tetramethylgalactose) (43%), a 3-substituted galactose (2,4,6-trimethylgalactose) (42%), and a reducing glucose (1,2,3,5,6-pentamethylglucitol) (15%). These results indicated that the major component of this fraction was a sialylated derivative of LNT, with the sialic acid linked to the GlcNAc, since the methylation of the intact compound and that of the asialo derivative presented the same pattern. This structure most probably corresponds to STb. But some contamination with another component may exist since this fraction, also contained some GalNAc.

MSP III.- MSP III comigrated on HPLC with authentic human milk sialyltetrasaccharide c, STc (NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc). However, when MSP III was subjected to neuraminidase digestion and the digestion product were analyzed by HPLC, it was observed that the desialylated fraction was composed of about equal amounts of a tetrasaccharide and a pentasaccharide. The presence of the pentasaccharide can be ascribed to contamination from fraction MSP IV which eluted immediately after MSP III

from the HPLC. The tetrasaccharide was pooled and applied to the RCA-I agarose column. Contrary to what was expected (all the counts being retarded in the position of LNnT), the tetrasaccharide separated on the RCA-I-agarose column into 3 fractions similar to the ones obtained with the the neutral tetrasaccharide fraction (peak 4). From this three fractions, the one that comigrated with LNnT was a minor component (results not shown). In addition, MSP-III was subjected to affinity chromatography on *Sambucus nigris* (SNA)-agarose and no retardation of the sample was observed (results not shown). SNA binds sialic acid linked α2-6 to terminal galactose, and SNA-agarose retards authentic human milk STc. There was not enough material to analyze the 3 fractions separately, therefore, only compositional analysis was performed on the complete MSP III. The ^{3}H-Gal-MSP-III contained 79% galactose and 21% glucose (ratio of 2.5 Gal/1 Glc) (fig. 33D). Asialo ^{3}H-GlcN-MSP-III (fig. 34C) was composed of 94% GlcNAc and 6% GalNAc (the amount of sialic acid was not determined).

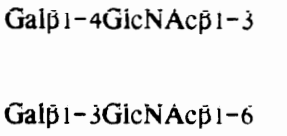
MSP-IV.- MSP IV eluted from HPLC in the position of a human milk sialylpentasaccharide. MSP IV was the major component of the sialyltetra- and pentasaccharide fraction. Since the main ganglioside made by SW1116 cells is the sialyl Lewis^{a} [183] (NeuAcα2-3Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc), fraction MSP-IV was most probably this structure. Neuraminidase treatment of ^{3}H-Gal-MSP-IV resulted in total conversion of this fraction into a pentasaccharide (fig. 42A). Fucosidase digestion of this pentasaccharide completely converted it into a tetrasaccharide (fig. 42B). When this tetrasaccharide was applied to RCA-I, most of it comigrated with LNT (fig. 35D). Jack bean β-galactosidase treatment (0.8-1.6 U/ml) of the tetrasaccharide, only

digested 4% of the sample (fig. 42C). When the sample was subjected to a second digestion with jack bean β-galactosidase, it gave a comparable result. The tetrasaccharide, when subjected to coffee bean α-galactosidase digestion, proved to be resistant to this enzyme (fig. 40D). ^{3}H-Gal-MSP-IV contained 72% Gal and 28% Glc (ratio of 1.71 Gal/1 Glc) (see fig. 33E). ^{3}H-GlcN-MSP-IV contained 34% sialic acid, 60% GlcNAc and 6% GalNAc (fig. 34D).

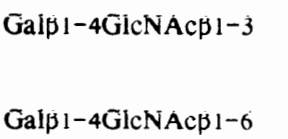
Methylation of ^{3}H-Gal-MSP-IV, before and after sialic acid removal (fig. 43 and 44), presented the same profiles as those obtained for intact and desialylated ^{3}H-Gal-MSP-I respectively. This indicated that sialic acid was attached to the terminal galactose in an α2-3 linkage. Methylation analysis of ^{3}H-Gal-MSP-IV after neuraminidase and fucosidase digestion (fig. 45) showed the same pattern as that of the desialylated derivative, indicating that the fucose was attached to the GlcNAc residue and, confirming that MSP-IV was mostly composed of sialyl Lewis^{a}, NeuAcα2-3Galβ1-3[Fucα1-4] GlcNAcβ1-3Galβ1-4Glc. A minor component/s was also present in this fraction since it contained GalNAc and some 4-substituted galactose, as seen in the methylation profile.

MSP VI.- This fraction eluted from HPLC, long after the elution time of the sialylpentasaccharides, and after the elution time of sialylhexasaccharides, indicating that MSP VI was composed of sialylheptasccharides or octasaccharides. ^{3}H-Gal-MSP-VI was treated with neuraminidase, and the digestion products were analyzed by HPLC (fig. 46A), which demonstrated that the asialo derivatives were a heptasaccharide (84%) and an octasaccharide (16%). Only the heptasaccharide was further analyzed, since the octasccharide represented very little material. The heptasaccharide was treated with α-fucosidase, which converted 90% of it into a hexasaccharide

(fig. 46B). The hexasaccharide was then applied to the RCA-I column. The profile of the hexasaccharide on the RCA-I column (fig. 28B), showed that it was composed of a fraction (II) that comigrated with LNT (27%), indicating that the hexasaccharide/s under this peak contained a terminal Galβ1-3 linkage; another fraction (III) that eluted in the position of LNnT (61%), which suggested the presence of a Galβ1-4 linkage under this peak; and a strongly retarded fraction (IV) (12%), that probably corresponded to a branched hexasaccharide with two terminal Galβ1-4 units. The ^{3}H-Gal-MSP-VI-hexasaccharide was found to be composed of 82% galactose, and 18% glucose (ratio of 3.04 Gal/1 Glc) (fig. 33F). The ^{3}H-GlcN-MSP-VI-hexasaccharide only contained GlcNAc (fig. 34E). The amount of sialic acid in the intact ^{3}H-GlcN-MSP-VI fraction was not measured. Due to the limited amounts of this fraction, methylation analysis was not possible. The information obtained about the MSP-VI fraction, suggested that it was composed of sialylated and fucosylated hexasaccharides with the following possible structures, 1, Galβ1-3GlcNAcβ1-3Galβ1-3/4GlcNAcβ1-3Galβ1-4Glc (fraction II, RCA-I); 2, Galβ1-4GlcNAcβ1-3Galβ1-3/4GlcNAcβ1-3Galβ1-4Glc (fraction III, RCA-I), or,



and 3 (fraction IV from RCA-I chromatography),



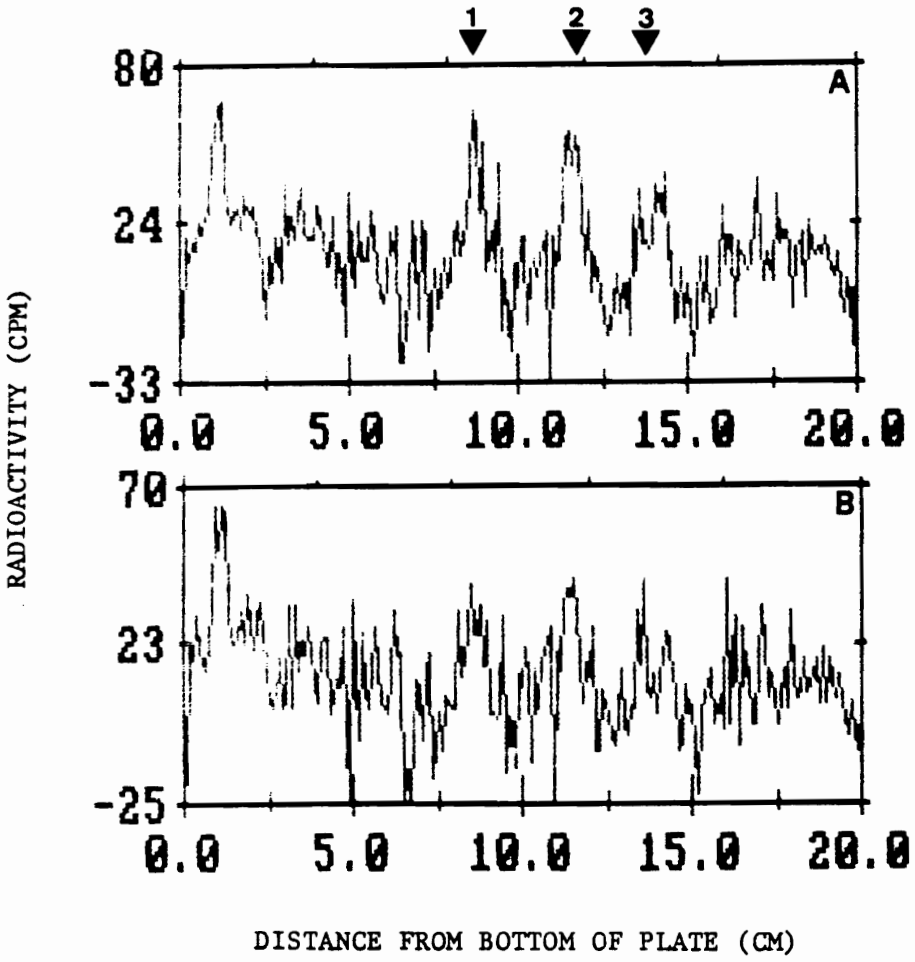


Figure 41.- Methylation analysis of ^{3}H-MSP II before and after neuraminidase treatment.- ^{3}H-Gal-MSP II and desialylated ^{3}H-Gal-MSP II were reduced, methylated and hydrolyzed according to the procedures described in chapter III for sialylated samples. The partially methylated derivatives were analyzed by thin layer chromatography. Samples were spotted on aluminum backed plates (5 x 20 cm), and developed for 70 minutes in the solvent system acetone/water/ammonium hydroxide (250/3/1.5). Partially methylated standards are indicated, 1, 2,4,6-trimethylgalactose; 2, 2,3,4,6-tetramethylgalactose; and 3, 1,2,3,5,6-pentamethylglucitol. Panel A, ^{3}H-Gal-MSP II; panel B, desialylated ^{3}H-Gal-MSP II.

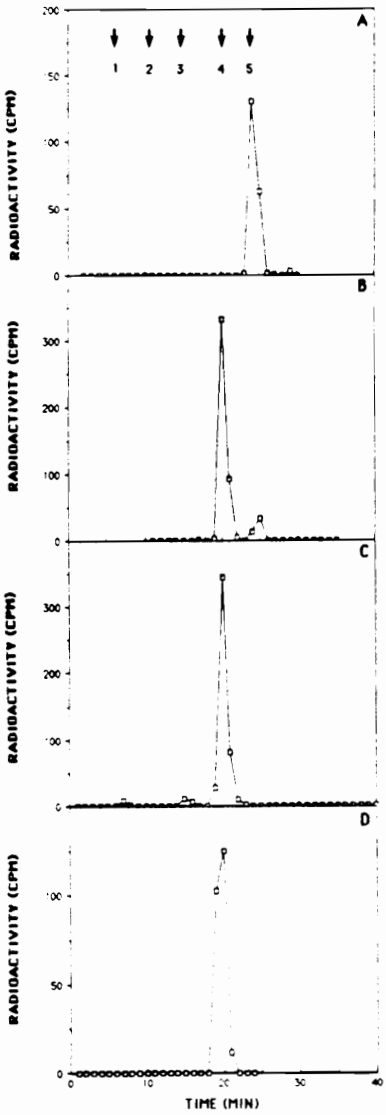


Figure 42.- HPLC of the *Arthrobacter ureafaciens* neuraminidase, beef kidney α-fucosidase, jack bean β-galactosidase, and coffee bean α-galactosidase digests of ^{3}H-Gal-MSP IV.- Neuraminidase digestion of ^{3}H-Gal-MSP IV was carried as explained in chapter III and the asialo-derivative was analyzed by HPLC as explained in figure 12. The asialo-derivative of ^{3}H-Gal-MSP IV was in turn, treated with beef kidney α-fucosidase, and the digestion products were analyzed by HPLC as described in figure 12. Subsequently, the desialylated and defucosylated fraction ^{3}H-Gal-MSP IV was subjected to jack bean β-galactosidase and coffee bean α-galactosidase digestion as explained in chapter III. The corresponding β-galactosidase and α-galactosidase digestion products were analyzed by HPLC as explained in figure 12. Elution times of ^{3}H-galactose, 1; of ^{3}H-lactitol, 2; ^{3}H-lacto-N-triaitol, 3; ^{3}H-lacto-N-tetraitol, 4; and ^{3}H-lacto-N-fucopentaitol I, 5 are indicated. Panel A, neuraminidase digestion products of ^{3}H-Gal-MSP IV; panel B, fucosidase digestion products of desialylated ^{3}H-Gal-MSP IV; panel C, β-galactosidase digestion products of desialylated and defucosylated ^{3}H-Gal-MSP IV; and, panel D, α-galactosidase digestion products of desialylated and defucosylated ^{3}H-Gal-MSP IV.

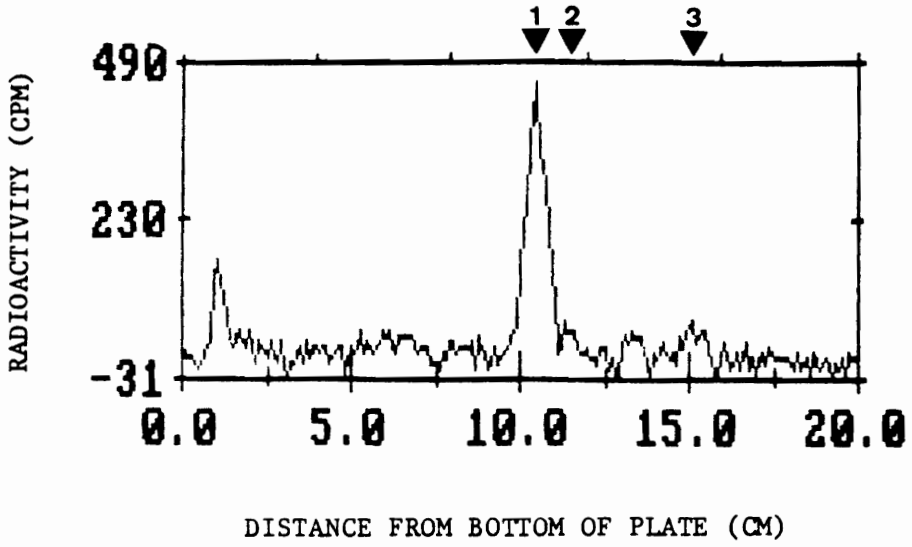


Figure 43.- Methylation analysis of ^{3}H-MSP IV .- ^{3}H-Gal-MSP IV was reduced, methylated and hydrolyzed according to the procedures described in chapter III for sialylated samples. The partially methylated derivatives were analyzed by thin layer chromatography. Samples were spotted on aluminum backed plates (5 x 20 cm), and developed for 70 minutes in the solvent system acetone/water/ammonium hydroxide (250/3/1.5). Partially methylated standards are indicated, 1, 2,4,6-trimethylgalactose; 2, 2,3,6-trimethylgalactose; 3, 2,3,4,6-tetramethylgalactose; and 4, 1,2,3,5,6-pentamethylglucitol.

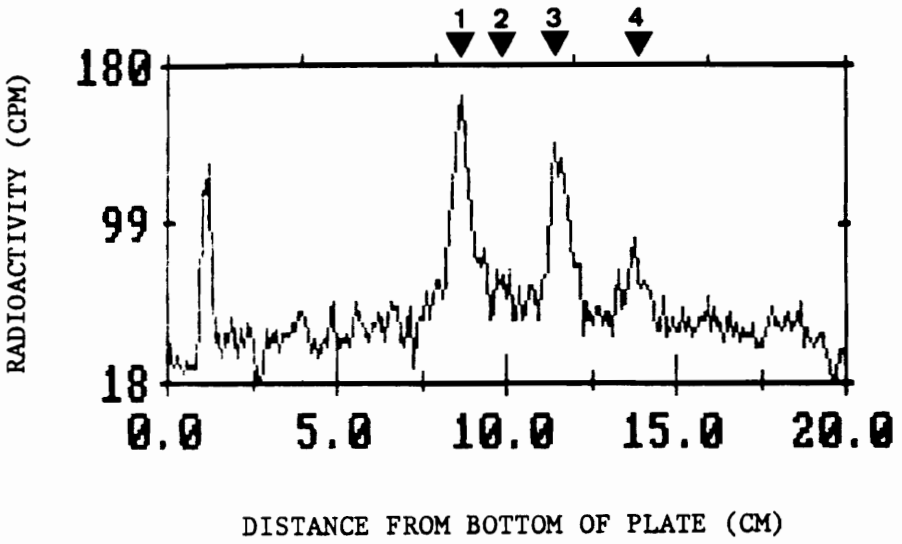


Figure 44.- Methylation analysis of desialylated ^{3}H-MSP IV .- Desialylated ^{3}H-Gal-MSP IV was reduced, methylated and hydrolyzed according to the procedures described in chapter III for sialylated samples. The partially methylated derivatives were analyzed by thin layer chromatography. Samples were spotted on aluminum backed plates (5 x 20 cm), and developed for 70 minutes in the solvent system acetone/water/ammonium hydroxide (250/3/1.5). Partially methylated standards are indicated, 1, 2,4,6-trimethylgalactose; 2, 2,3,6-trimethylgalactose; 3, 2,3,4,6-tetramethylgalactose; and 4, 1,2,3,5,6-pentamethylglucitol.

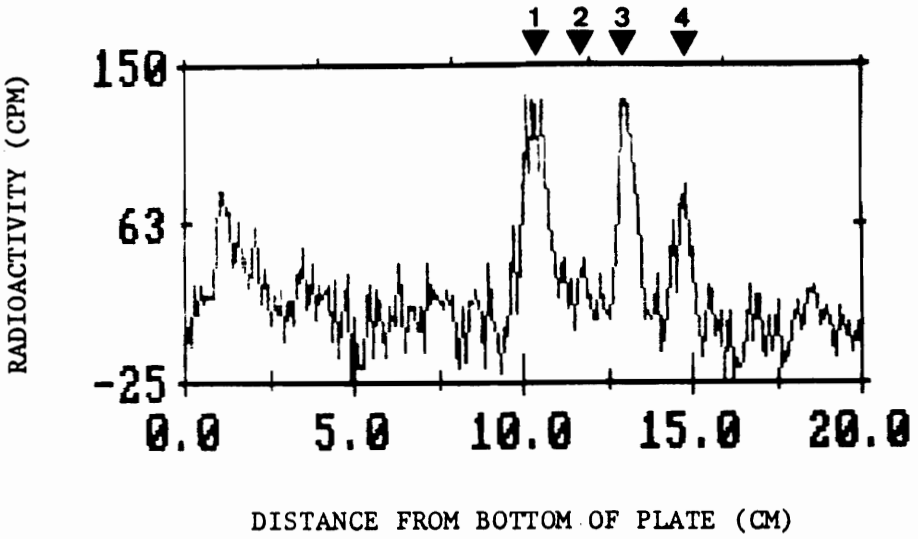


Figure 45.- Methylation analysis of desialylated and defucosylated ^{3}H-MSP IV .- Desialylated and defucosylated ^{3}H-Gal-MSP IV was reduced, methylated and hydrolyzed according to the procedures described in chapter III. The partially methylated derivatives were analyzed by thin layer chromatography. Samples were spotted on aluminum backed plates (5 x 20 cm), and developed for 70 minutes in the solvent system acetone/water/ammonium hydroxide (250/3/1.5). Partially methylated standards are indicated, 1, 2,4,6-trimethylgalactose; 2, 2,3,6-trimethylgalactose; 3, 2,3,4,6-tetramethylgalactose; and 4, 1,2,3,5,6-pentamethylglucitol.

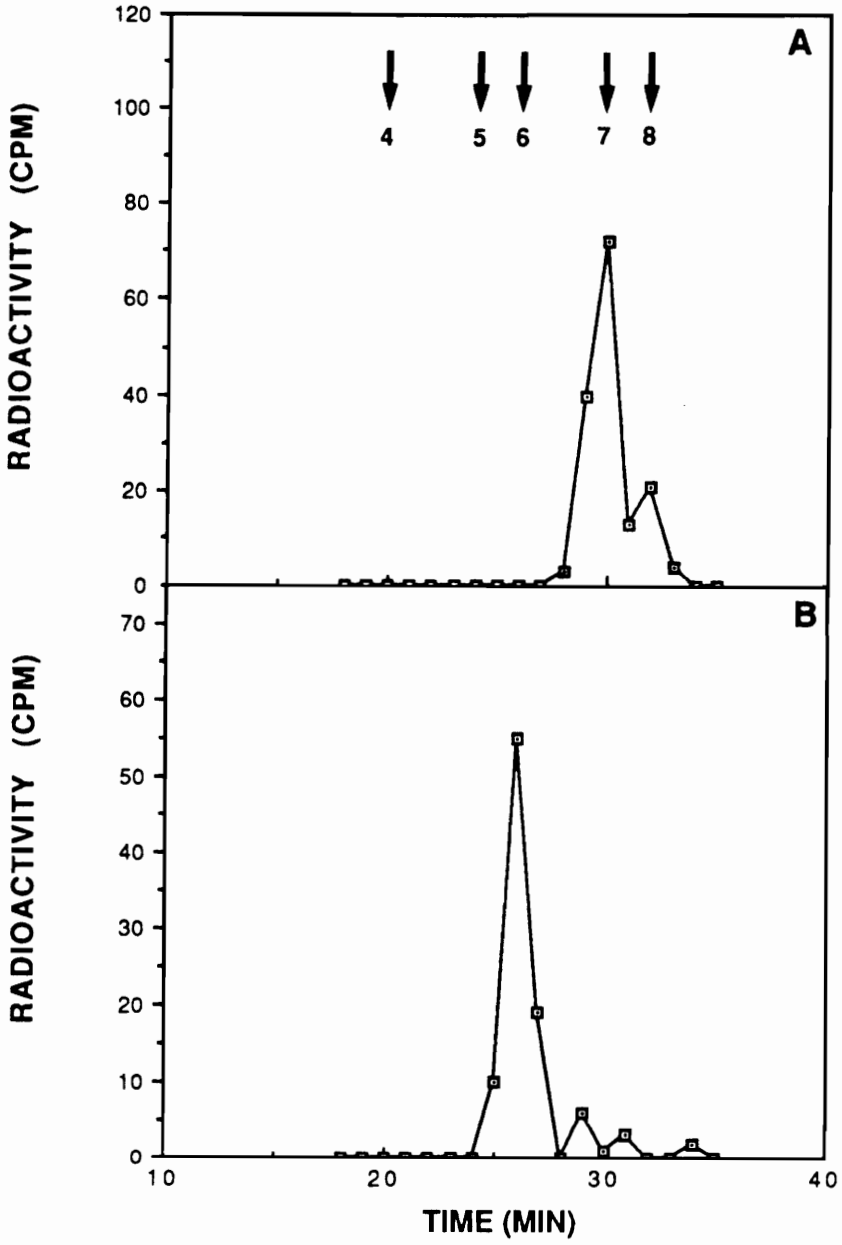


Figure 46.- HPLC of the *Arthrobacter ureafaciens* neuraminidase and beef kidney α-fucosidase digests of ^{3}H-Gal-MSP VI .- Neuraminidase digestion of ^{3}H-Gal-MSP VI was carried as explained in chapter III and the asialo-derivatives were analyzed by HPLC as explained in figure 12. The heptasaccharide peak was pooled and it was subsequently subjected to beef kidney α-fucosidase digestion as explained in chapter III. The corresponding α-fucosidase digestion products were analyzed by HPLC as explained in figure 12. Elution times of ^{3}H-lacto-N-tetraitol, 4; ^{3}H-lacto-N--fucopentaitol I, 5; ^{3}H-lacto-N-neohexaitol, 6; rabbit erithrocyte ^{3}H-heptaitol, 7; and SW1116 ^{3}H-Gal-octaose, 8, are indicated. Panel A, neuraminidase digestion products of ^{3}H-Gal-MSP VI; panel B, α-fucosidase digestion products of asialo ^{3}H-Gal-MSP VI-heptasaccharide.

V

CONCLUSIONS

SW1116 cells were metabolically labelled with ^{3}H-galactose and ^{3}H-glucosamine, and their glycolipids were structurally analyzed. The results from these analyses showed that 91% of the total radioactivity incorporated into SW1116 glycolipids corresponded to neutral glycolipids and that the remaining 9% corresponded to acidic glycolipids. The low amounts of acidic glycolipids may reflect a slow turnover and biosynthesis of these glycolipids, but to confirm this possibility pulse-chase experiments should be performed. Alternatively, acidic glycolipids may be shed from the plasma membrane into the culture media, as has been observed with other tumors and cancer cell lines. Nevertheless, in normal intestinal epithelial cells the content of acidic glycolipids is very low (21 ng/mg protein) representing only 0.54 % of the weight of total glycolipids [149]. Therefore, comparing the proportion of acidic glycolipids in normal intestinal mucosa with that of SW1116 cells, the amounts of acidic glycolipids in SW1116 cells is increased 17-fold. This increase may be associated with malignant transformation of this intestinal epithelium-derived cell line.

The glycolipids from SW1116 cells were mainly composed of type 1 lacto-series glycolipids, and to a much lesser extent of type 2 lactoneo-series glycolipids (tables 8, 9, 10). The glycolipid composition of SW1116 cells resembled that of normal intestinal mucosa, which preferentially expresses type 1 lacto-series glycolipids over type 2 lactoneo-series glycolipids. Other

colon cancer cell lines, such as Colo 205 [73], also synthesize glycolipids that are mostly composed of type 1 lacto-series glycolipids, and trace amounts of glycolipids derived from type 2 lactoneo structures.

Blood group active glycolipids were present in the neutral glycolipid fraction from SW1116 cells. One of the most abundant was the Le^a pentaglycosylceramide (Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc-Cer). The H-type 1 pentaglycosylceramide (Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc-Cer) was part of the neutral glycolipid fraction as well as was the Le^b hexaglycosylceramide (Fucα1-2Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc-Cer). Another glycolipid with H1 antigenicity was the heptaglycocylceramide present in peak 7 (Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-3/4GlcNAcβ1-3Galβ1-4Glc-Cer). The H1, the Le^a, and the Le^b glycolipids have been detected in SW1116 cells by immunostaining with specific monoclonal antibodies on thin layer plates [198, 228-229]. The H1 pentaglycosylceramide, the H1 heptaglycosylceramide and the Le^b are considered tumor-associated antigens when they are expressed by colonic tumors from non-secretor individuals, since they are incompatible with the phenotype of the individual. In the same way these antigens, when expressed by colonic tumors from the distal segments of the colon, are regarded as tumor-associated antigens, because normal distal colon mucosa synthesizes only trace amounts of blood group antigens (see section 2.2). SW1116 cells were established from a colonic tumor from an individual that was O-blood type, however, the secretor status of that individual was not recorded. Therefore, we cannot determine if the presence of H1 and Le^b glycolipids in SW1116 accounts for incompatible expression of blood group glycolipids by the tumor cells (see section 2.2). Likewise, since

the portion of the colon from which the tumor (proximal or distal) was removed was not reported, it is not possible to establish if the H1 and Le^b glycolipids represent reexpression of blood group antigens in the distal part of the colon (see section 2.2). In other words, with the available information about the colonic tumor and its donor, it is impossible to determine if H1 and Le^b glycolipids were originally expressed by the colonic mucosa of the donor (since we do not know the secretor status of the donor and the location of the tumor within the colon) or if they are tumor-associated glycolipid antigens that were only expressed by the original colonic tumor and its derived cell line, SW1116.

In addition to the Le^b structure, another oligosaccharide was detected in the neutral hexasaccharide fraction. This structure was one of the most abundant glycolipids in SW1116. Its structure was not fully identified due to technical limitations. Based on the information gathered, we proposed two possible structures, Fucα1-2Galβ1-3GlcNAcβ1-3[Fucα1-2/4/6]Galβ1-4Glc and Fucα1-2[Fucα1-3/4/6]Galβ1-3GlcNAcβ1-3Galβ1-4Glc. Both of these structures have H antigenicity since they have a fucose linked α1-2 to the terminal galactose, and either of them may represent novel blood group antigens, or perhaps novel tumor-associated antigens if they do not occur in normal colonic tissue. Further studies to identify the structure/s present in this fraction may be worthwhile.

The Le^x pentaglycosylceramide and the Le^y hexaglycosylceramide may be present in SW1116 cells at very low concentration since their core saccharide, the type 2 LNnT structure, was detected in minimal amounts (see table 8).

Glycolipids with A or B antigenicity were not detected in SW1116 cells, since oligosaccharides derived from neutral glycolipids failed to bind to the HP-

agarose column (specific for A antigens) and the GSI-agarose column (specific for B antigens). This is in disagreement with other studies in which SW1116 cells were positively immunostained with monoclonal antibodies directed to A or B antigens [61, 163]. Since we did not find any glycolipids with A or B antigenicity, we presumed that glycoproteins carrying those antigens were responsible for the positive immunostaining results. We applied SW1116 glycopeptides metabolically labelled with ^{3}H-galactose to an HP-agarose column (results not shown) but no binding of the glycopeptides occurred. Although SW1116 cells were immunostained with the A and B antibodies, the activities of the A and B enzymes were not detectable in SW1116 cell homogenates [163], indicating that those antibodies may be crossreacting with similar carbohydrate structures carried by SW1116 glycoconjugates.

Other neutral glycolipids that did not belong to the lacto or lacto-neo series glycolipids, identified in SW1116 cells, were the monhexosylceramides, glucosylceramide and galactosylceramide (table 10), with galactosylceramide accounting for 65% of this fraction (table 8). This is consistent with other studies that have reported galactosylceramide in normal colon and in gastric cancer [147, 165]. The dihexosylceramides were composed mainly of lactosylceramide and galabiosylceramide. Galabiosylceramide constituted 80% of this fraction. Since lactosylceramide is the precursor for the synthesis of all glycolipids, except for galactosylceramide and galabiose, its steady-state concentration in SW1116 cells was low. Galabiosylceramide has been reported in colon cancer cell lines [225] and in gastric cancer tissue [165].

The gangliosides accounted for only 9% of the total glycolipids. The major component of the acidic glycolipids was the sialyl Le^{a} antigen, which is in

agreement with results from previous studies [183]. STa ceramide and STb ceramide were present in very low concentrations (table 9). STc ceramide was not detected, inconsistent with results reported by Law and Smith [74], who detected STc by immunostaining of the gangliosides on thin layer chromatograms with a polyclonal antibody directed to this structure. Since the antibody used was polyclonal, crossreactivity with other gangliosides might account for the positive reaction of SW1116 gangliosides with this antibody. Sialylated heptasaccharides also were detected in the ganglioside fraction (MSP VI). MSP VI was composed of sialyl derivatives of fucosylated hexasaccharides. These hexasaccharides were a mixture of linear and branched lacto/lactoneo cores. The gangliosides, as were the neutral glycolipids, were composed mainly of type 1, lacto-series glycolipids and some type 2, lactoneo-series glycolipids (table 10). GM3 was the only ganglioside not belonging to the lacto/lactoneo-series, but rather to the ganglio-series, that was detected in the monsialyloligosaccharide fraction. The concentration of GM3 in SW1116 cells was low (table 9).

From the glycolipid-derived oligosaccharide structures from SW1116 cells, several potential tumor-associated carbohydrate markers, that do not carry blood-group antigenicity could be proposed. One example is galabiosylceramide, however, this glycolipid has a short oligosaccharide chain, and can be easily masked by other glycoconjugates at the cell surface. Other examples are lacto-N-tetraosylceramide, lacto-N-neotetraosylceramide, which constitute, 5.46% and 0.73% of the total glycolipids of SW1116 cells. From these two glycolipids, probably lacto-N-tetraosylceramide will be more antigenic at the cell surface due to its higher concentration. The amount of

lacto-N-tetraosylceramide is 2.25 fold as compared to that of the sialyl Lewis^a glycolipid antigen in SW1116 cells, indicating that lacto-N-tetraosylceramide could represent a suitable tumor marker. Although, the sialyl Lewis^a glycolipid is in less concentration than lacto-N-tetraosylceramide, its capacity for being antigenic may be endowed by its greater structural complexity (it has a longer chain, and a fucose and sialic acid residue). Sialyltetraosylceramide a and and sialyltetraosylceramide b could be proposed as possible tumor antigens, too. Their concentration in SW1116 cells was found to be very low, which may not be sufficient to induce a good immune response, however, the quantities of these two gangliosides may be greater in other tumor cells. The capability of any of the glycolipids mentioned, for being tumor antigens has to be tested by determining their uniqueness to cancer cells and their absence in the corresponding normal cells. In addition, the usefulness of these structures as diagnostic tools would depend on their expression as secreted mucin type glycoproteins in blood, and/or their shedding from the tumor plasma membrane into the blood.

In this dissertation I have described the structural analysis of the carbohydrate portion of glycolipids by metabolically labelling cells in culture with radiolabelled monosaccharide precursors. SW1116 cells (1 x 10^6 cells) metabolically labelled with 222 μCi of either ^{3}H-galactose or ^{3}H-glucosamine for 30 hours, incorporated 1%-3% of the radioactivity into their glycoconjugates. Approximately 63% of the radiolabelled glycoconjugates were glycolipids when labelling was with ^{3}H-galactose and about 12% when labelling was with ^{3}H-glucosamine. When glycolipids were labelled with ^{3}H-galactose, most of the radioactivity was recovered as ^{3}H-galactose and ^{3}H-

glucose. And, when they were labelled with ^{3}H-glucosamine, the radioactivity was recovered as N-acetylglucosamine, N-acetylgalactosamine and as N-acetylneuraminic acid. Using this approach we have been able to determine the structures of minor components such as STa ceramide which constitutes 0.46% of the total glycolipids, STb ceramide which is only 0.21% of the total glycolipids, and GM3 that is 0.42% of the total glycolipids, and have done this with relatively small number of cells (6 x 10^{6} cells). Other fractions such as 4-I, 5-4-I and 6-4-I, were not characterized since they were in very low concentration and composed of several structures that could not be separated into the different components with the methods that we employed.

The use of the metabolic labelling approach can be extended to other areas of glycolipid research. For example, using this method one can determine changes in glycolipids associated with differentiation and malignant transformation that could not be detected using immunochemical techniques or physico-chemical methods such as NMR, MS and FAB-MS. Smith et al. [241] determined the presence of the Forssman glycolipid in F9 teratocarcinoma cells and the disappearance of this glycolipid after retinoic acid differentiation of F9 cells. Likewise, using this technique, the changes in glycolipid expression from CHO cells transfected with glycosyltransferases genes have been determined [242]. In a recent publication, Weis and Davis [243], reported analysis of the ganglioside composition of mutant CHO cells (clone *ldl*), deficient in the enzyme 4'-epimerase, when grown in the presence and absence of galactose in the culture media, by metabolically labelling the cells with N-[^{3}H]-acetylmannosamine. The enzyme 4'-epimerase catalyzes the interconversion of UDP-glucose into UDP-galactose, therefore cells grown in

media that only contains glucose fail to synthesize gangliosides since all gangliosides contain galactose (except for GM4). Using the metabolic labelling approach, they were able to see qualitative and quantitative changes in ganglioside expression by the *ldl* clone induced by the addition of galactose to the culture medium, in a fast and direct way [243]. Metabolic radiolabelling of glycolipids may also be applied to the identification of the glycolipid biosynthetic pathways, their site of synthesis, intracellular sorting, recycling, and turnover. Metabolically labelled glycolipids are the endogenous biosynthetic products of the cells, therefore, by this method, glycolipids synthesized *de novo* by the cells could be differentiated from exogenous glycolipids taken up by cells from the culture media.

The metabolic labelling of glycolipids may be regarded as a technique that is as sensitive as the immunostaining of glycolipids on thin layer plates. Nevertheless, the metabolic labelling of glycolipids requires lower numbers of cells, than does the immunostaining method. In addition, the metabolic labelling procedure provides unequivocal definition of the glycolipid structures while the immunostaining techniques usually require an independent method to confirm the complete glycolipid structure. Also, the immunostaining technique is not very useful for determining subtle changes in glycolipid expression induced by differentiation or transformation. The immunostaining technique may be preferrable over the metabolic labelling technique when the presence of an specific glycolipid needs to be determined. For instance, if we want to know if globoside is present in a certain cell line, it will be faster to immunostain a thin layer chromatogram of the total glycolipid extract with a monoclonal antibody, than it will be to go through the

procedure of metabolic labelling the cells, isolation of the glycolipids, release of the oligosaccharides from the ceramide, purification on HPLC and then lectin affinity chromatography. On the other hand, if we want to determine all the glycolipid structures in this same cell line, the use of the metabolic labelling technique would be the preferred aproach. Trying to determine all the glycolipid structures using the immunostaining method would require monoclonal antibodies against all possible structures, a procedure that may be impractical.

Although, NMR, MS and FAB-MS provide unambiguous structural assignment of the glycolipid structures, these techniques require the availability of enormous amounts of tissue or cells in culture, in order to obtain quantities of pure glycolipids that are within levels of detection of these methods. Glycolipids for identification by these methods, must be purified by thin layer chromatography and silicic acid chromatography using organic solvents. These two methods principally separate glycolipids based on their size, as a consequence a single purified peak from silicic acid chromatography, or a band from thin layer chromatography may contain different species of glycolipids with the same size. The fractionation of glycolipids into pure components is a very tedious procedure requiring many chromatographic steps on silicic acid columns. Using the metabolic labelling approach, the radiolabelled carbohydrate portion can be released from the ceramide by chemical (ozonolysis/alkali fragmentation) or enzymatic methods (endoglycoceramidase), and the free oligosaccharides can be efficiently separated by a combination of conventional chromatographic techniques and lectin or antibody affinity chromatography. However, in the metabolic

labelling approach the ceramide portion of the glycolipid would not be analyzed, unless the carbohydrates and the ceramide are double labelled with different isotopes, and the oligosaccharide is released by the enzymatic method since ozonolysis/alkali fragmentation would destroy the ceramide moiety.

Metabolically labelling of glycolipids may be limited to established cells in culture, nevertheless, procedures may be developed to extend the application of this technique to organ-tissue culture, and to the *in vivo* metabolic labelling of small animals.

TABLE 8

Distribution of the Radioactivity Incorporated into Oligosaccharides Derived from 6-[^{3}H]-Galactose-Metabolically-Labelled Neutral Glycolipids

Fraction	% [a]	Component	% [b]
Monosaccharide	9.11	Glc	5.92
		Gal	3.19
Disaccharide[c]	7.28	Lactose	1.46
		Galabiose	5.82
Tetrasaccharide	9.10	4-I	2.91
		LNT	5.46
		LNnT	0.73
Pentasaccharide	13.00	5-4-I	0.38
		Le^a	8.0
		H1	4.0
		Fuc-LNnT	0.55
Hexasaccharide	12.00	6-4-I	0.47
		Le^b	3.11
		Difuc-LNT	8.0
		Difuc-LNnT	0.24
Heptasaccharide	8.17	7a	1.39
		7b	5.14
		7c	1.64

[a]The numbers represent the percent of radioactivity incorporated in each fraction obtained from the HPLC of neutral oligosaccharides.

[b]The numbers represent the percent of radioactivity incorporated into each neutral component from the radioactivity incorporated into total glycolipids, neutrals and acidics.

[c]The disaccharide peak obtained from HPLC constituted 18% of the radioactivity incorporated into neutral oligosaccharides. However, we report 8% because we deduced from the 18%, the proportion or radioactivity corresponding to fraction 2-I. This fraction did not contain Gal or Glc, therefore we considered it as a non-glycolipid, radiolabelled contaminant.

TABLE 9

Distribution of the Radioactivity Incorporated into Oligosaccharides Derived from 6-[^{3}H]-Galactose-Metabolically-Labelled Acidic Glycolipids

Fraction	% [a]	Component	% [b]
MSL	0.42	3'sialyllactose	0.42
MSP	4.20	STa	0.46
		STb	0.21
		MSP III	0.38
		Sialyl Le^a	2.43
		MSP VI	0.46

[a]The numbers represent the percent of radioactivity from the acidic glycolipids incorporated into the fractions obtained from the DE-53-cellulose column. Percents were recalculated by substracting percents of the neutral fraction (peak 1) and peak 5, from the total. Peak 5 labelled with ^{3}H-galactose, when subjected to acid hydrolysis, yielded products that were not galactose or glucose (results not shown).

[b]The numbers represent the percent of radioactivity incorporated into each component from the radioactivity incorporated into total glycolipids (neutrals, and acidics).

TABLE 10

Glycolipids Detected in SW1116 Cells

Structure	Trivial Name
Gala Series	
Gal-Cer	Galactosylceramide
Galα1-4Gal-Cer	Galabiosylceramide
Hemato Series	
Glc-Cer	Glucosylceramide
Galβ1-4Glc-Cer	Lactosylceramide
Ganglio Series	
NeuAcα1-3Galβ1-4Glc-Cer	GM3
Lacto Series	
Galβ1-3GlcNAcβ1-3Galβ1-4Glc-Cer	Lacto-N-tetraosylceramide
Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer	Lacto-N-neotetraosylceramide
Galβ1-3GlcNAcβ1-3Galβ1-4Glc-Cer 4 Fucα1	Le^a pentaglycosylceramide
Galβ1-3GlcNAcβ1-3Galβ1-4Glc-Cer 2 Fucα1	H1 pentaglycosylceramide

TABLE 10 (CONT.)

Glycolipids Detected in SW1116 Cells

Structure	Trivial Name
Lacto Series (cont.)	
Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer 3 Fucα1	Le^x pentaglycosylceramide
Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer 2 Fucα1	H2 pentaglycosylceramide
Galβ1-3GlcNAcβ1-3Galβ1-4Glc-Cer 2 4 Fucα1 Fucα1	Le^b hexaglycosylceramide
Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer 2 3 Fucα1 Fucα1	Le^y hexaglycosylceramide
Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1- 3/4GlcNAcβ1-3Galβ1-4Glc-Cer	Fucosyllacto-N-norhexaosylceramide
NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1- 4Glc-Cer	Sialyltetraosylceramide a
Galβ1-3GlcNAcβ1-3Galβ1-4Glc-Cer 6 NeuAcα2	Sialyltetraosylceramide b
NeuAcα2-3Galβ1-3[Fucα1-4]GlcNAcβ1- 3Galβ1-4Glc-Cer	Sialyl Le^a pentaglycosylceramide

V I

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VII

VITA

Maria Teresa Tarrago Medina was born in Santo Domingo, Dominican Republic, the third of August of 1960. She finished her high school studies at Instituto Veritas, Santo Domingo, in 1978. In August of the same year she entered Universidad Nacional Pedro Henriquez Urena, in Santo Domingo, where she finished her course work in December 1982. She completed and defended a thesis as a requirement for graduation in February 1984. She graduated Summa Cum Laude as Licenciate in Chemistry, and was awarded a silver medal for holding the best point average in the College of Science, in April 1984. In September of 1984 she was accepted in the Department of Biochemistry and Nutrition at Virginia Polytechnic Institute and State University, Blacksburg, Virginia, as a candidate for a Master's degree. During the course of the Master's program, she had an scholarship from the Agency for International Development (AID) and the Latin American Scholarship Program for American Universities (LASPAU). She completed the Master's degree in December 1986. She entered the Ph.D. program in the Biochemistry and Nutrition Department, at Virginia Polytechnic Institute and State University, Blacksburg, Virginia, January 1987, and completed her research work in September 1989. Maria Teresa Tarrago Medina is married to Dr. Antonio A. Trani, who is currently an assistant professor at the Department of Engineering, Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

María T. Tarrago-Trani