PARTIAL CHARACTERIZATION OF THE GLUCOSAMINE-CONTAINING O-LINKED OLIGOSACCHARIDES OF A MAJOR SIALOGLYCOPROTEIN OF AN ASCITES RAT MAMMARY ADENOCARCINOMA

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NOMENCLATURE

ABH	-	alkaline borohydride
ASGP-1	-	ascites sialoglycoprotein-1
cm	-	centimeter
cpm	-	counts per minute
DEAE	-	diethylaminoethyl
dpm	-	disintegrations per minute
fuc	-	fucose
galNacOH	-	N-acetylgalactosaminitol
gal	-	galactose
GC-MS	-	gas chromatography-mass spectrometry
GLC	-	gas liquid chromatography
gln	-	glucosamine
glcNac	-	N-acetylglucosamine
gm	-	gram(s)
g	-	acceleration due to gravity
Gdn-HCl	-	guanidine hydrochloride
HPLC	-	high performance liquid chromatography
hr	-	hour(s)
in	-	inch(es)
MAT	-	mammary ascites tumor
MAT-B	-	a morphologically distinct cell line derived from a benzanthracene induced solid rat mammary adenocarcinoma

MAT-B1	-	a non-xenotransplantable subline derived from MAT-B cells
MAT-C	-	a cell line morphologically distinct from MAT-B
MAT-C1	-	a xenotransplantable subline derived from MAT-C cells
MAT-C2	-	a non-xenotransplantable variant subline derived from MAT-C1 cells
MAT-C3	-	a non-xenotransplantable variant subline derived from MAT-C2 cells
MAT cMR6-s	-	a xenotransplantable variant derived from MAT-B1 cells
m/z	-	mass to charge ratio
ml	-	milliliter
mM	-	millimolar
min	-	minute(s)
NANA	-	N-acetylneuraminic acid
N	-	normal
PMAA	-	partially methylated alditol acetate
PAGE	-	polyacrylamide gel electrophoresis
rpm	-	revolutions per minute
SA	-	sialic acid
SDS	-	sodium dodecyl sulfate
TA3-Ha	-	an allotransplantable mouse mammary adenocarcinoma
TA3-St	-	a non-allotransplantable mouse mammary adenocarcinoma
st	-	weight
µCi	-	microcurie
µl	-	microliter
µ	-	micron

CHAPTER I

INTRODUCTION

Sialoglycoproteins and the Tumor Cell Surface

Sialoglycoproteins have been implicated in social interactions between cells such as contact inhibition of cell growth (Knecht and Lipkin, 1977), cell recognition and adhesion (Edwards, 1978), and cell aggregation (Kang et al., 1974). Since neoplastic cells are considered to exhibit abnormal social interactions (Stoddart, 1979) it is not surprising that many reports of qualitative and quantitative alterations in cell surface sialoglycoproteins resulting from malignant transformation of a normal cell have appeared recently (Buck et al., 1970; Shin et al., 1975; Burridge, 1976; Glick, 1976; Startling et al., 1978).

Some reports have indicated that these alterations may be changes in the carbohydrate. Many laboratories have noted an increased agglutination by lectins of several tumorigenic cells compared to agglutination of their normal counterparts (Aub et al., 1965; Nicolson, 1974; Becker, 1974; and Walborg et al., 1975), alterations in the sizes of the oligosaccharides of cellular glycoproteins of tumorigenic cells when compared to their normal counterparts (Warren et al., 1973), changes in glycosyl transferases in some tumors compared to normal tissues (Patt et al., 1977; Bauer et al., 1977; Bauer et al., 1978), and loss of the glycocalyx of highly metastatic tumor cells (Kim et al.,

1975). The glycoproteins in some of these studies were characterized and found to have oligosaccharides N-linked to asparagine residues. Recently, sialomucins, sialoglycoproteins containing alkali-labile oligosaccharides O-linked to serine or threonine residues, have been found on the cell surface of many tumor cells. Tumor cell systems in which they have been found include ascites mammary adenocarcinomas (Codington et al., 1978); Huggins et al., 1980), melanomas (Bhavanandan et al., 1977; and Bhavanandan et al., 1981), cultured malignant human breast cells (Chandrasekaran et al., 1979), spontaneously and chemically induced rat hepatomas and human colon adenocarcinoma (Price and Stoddart, 1976). The Thomsen-Friedenreich antigen (T-antigen), which is the disaccharide precursor for mucin sialooligosaccharides, has been implicated in some studies in malignant breast lesions (Springer et al., 1975 and Springer et al., 1977). The best characterized membrane sialomucin is epiglycanin. This cell surface molecule has been found in the TA3-Ha mouse mammary adenocarcinoma and has been postulated to be responsible for its allotransplantability (Sanford et al., 1973). Its structural and functional properties will serve as a model system by which other membrane sialomucins can be compared.

Epiglycanin

Scanning electron microscopy of whole cells, transmission electron microscopy of whole cells treated with polycationic ferritin (Miller et al., 1977), together with electron microscopy of metal-contrasted replicas of tryptic fragments of cells (Slayter et al., 1973) suggested that epiglycanin is a highly extended rod-shaped sialoglycoprotein found in TA3-Ha mouse mammary adenocarcinoma. The fibrillar rods

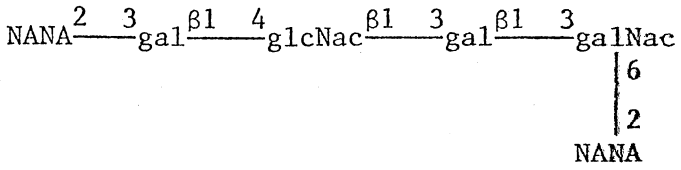
extend 200-300 nm into the extracellular fluid surrounding a cell.

Epiglycanin, isolated from whole cells by tryptic digestion and gel filtration (Codington et al., 1972), has been analyzed for its carbohydrate composition and amino acid composition (Codington et al., 1978). The major monosaccharides found were galactose, N-acetylglucosamine, N-acetylgalactosamine, and N-acetyl neuraminic acid. The most prominent amino acids found were serine and threonine. These compositional data indicate that epiglycanin is a mucin-type sialoglycoprotein. Physicochemical studies have shown that more than 500 carbohydrate chains are attached to a single polypeptide chain of about 1300 amino acid residues in a molecule of about 500,000 molecular weight.

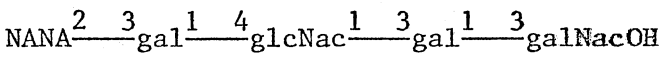
Partial characterization of the O-linked oligosaccharides of epiglycanin has been achieved (Van den Eijnden et al., 1979). Two acidic or sialylated and three neutral oligosaccharides were isolated. The major neutral oligosaccharide is the Thomsen-Friedenreich disaccharide, gal$\overset{\beta 1\quad 3}{\text{———}}$galNacOH, comprising 60% of the carbohydrate chains. The two sialylated saccharides accounted for only 18% of the chains. The major sialylated chain, 13% of the chains, has the following structure:

$$\text{NANA}\overset{2\quad 3}{\text{———}}\text{gal}\overset{\beta 1\quad 3}{\text{———}}\text{galNacOH}$$

the same trisaccharide found in fetuin (Spiro and Bhooyroo, 1974) and glycophorin (Thomas and Winzler, 1969). The second sialosaccharide is proposed to have the structure:



Codington et al. (Van den Eijnden et al., 1979) considered this to be the most complex sialosaccharide yet found on mammalian membrane sialomucin. However, oligosaccharides isolated from canine submaxillary mucin (Lombart et al., 1974), ovarian cyst fluid glycoproteins (Kabat et al., 1976), and porcine gastric glycoproteins (Derevitskaya et al., 1978) have quite similar structures. Chandrasekaran and Davidson (Chandrasekaran et al., 1979) have reported the presence of a monosialylated pentasaccharide with the structure:



in a surface molecule of human B16 melanoma.

Epiglycanin has been implicated in the transplantability of the TA3-Ha adenocarcinoma via a masking phenomena on the surface of the cell (Codington et al., 1973 and Sanford et al., 1973). The physical characteristics of epiglycanin are ideally suited for such a masking phenomena. Structural features of epiglycanin responsible for transplantability have not yet been identified but since another subline which is not transplantable does not contain cell surface epiglycanin they suggest that the presence of this sialomucin is at least partly responsible for its transplantability properties.

ASGP-1

Recently Sherblom et al. (1980a) have reported the presence of a mucin type ascites sialoglycoprotein, ASCP-1, on the surface of a transplantable rat mammary adenocarcinoma. Unlike the mouse mammary adenocarcinoma studied by Codington, the glycoprotein is also present on a non-transplantable cell line. This suggests that more subtle differences in glycoprotein structure may be important in the transplantation phenomena exhibited.

The 13762 rat mammary adenocarcinoma is a dimethyl benzanthrene induced solid tumor. The tumor was adapted for ascites growth at Mason Research Institute. Three sublines: MAT-A, MAT-B, and MAT-C were isolated (Segaloff, 1966). In addition, a cell culture line, MR, was derived from the MAT-B ascites line by growth in soft agar. After a few months of passages in Dr. Carraway's laboratory, the MAT-B and MAT-C ascites lines became the more stable MAT-B1 and MAT-C1 lines.

By scanning electron microscopy MAT-C1 cells exhibit a highly irregular surface, covered with microvilli extending as branched structures from the cell body. MAT-B1 cells have a more normal appearance, with unbranched microvilli, ruffles, ridges and blebs associated closely with the cell body (Carraway et al., 1978). The MAT-C1 subline has been shown to be xenotransplantable into mice while the MAT-B1 subline is not xenotransplantable. Both sublines contain a major sialoglycoprotein (ASGP-1) which exhibits low electrophoretic mobility in SDS polyacrylamide gels. ASGP-1 has been purified from membrane vesicles by extraction with 4 M guanidine

hydrochloride (Sherblom et al., 1980a). The composition of ASGP-1 from each subline is high in serine, threonine, galactosamine, glucosamine, galactose, sialic acid, and fucose with a carbohydrate content of 67% and 73% for MAT-B1 and MAT-C1, respectively. Amino acid compositions of ASGP-1 from the two sublines are essentially the same. The major differences between them is a three-fold greater content of sialic acid per unit protein for MAT-C1 ASGP-1 than MAT-B1 ASGP-1 and the presence of sulfated carbohydrate in MAT-B1 ASGP-1 but not in MAT-C1 ASGP-1. Molecular weights of 570,000 (MAT-B1) and 690,000 (MAT-C1) were estimated by sedimentation velocity analysis and gel filtration in 4 M guanidine hydrochloride. Fractionation of oligosaccharides from alkaline borohydride treated ASGP-1 on Bio-gel P4 gives patterns that are quantitatively different for MAT-B1 and MAT-C1. This suggests that the differences in oligosaccharide composition may be a factor involved in the transplantation phenomena exhibited by these cells.

The expression of ASGP-1 appears to be modulated according to the environment of growth conditions of the tumor as evidenced by the observation that ASGP-1 is present in the ascites cells but virtually absent in the solid tumor (Huggins et al., 1980). Furthermore the cells, when adapted for cell culture, lose ASGP-1 but upon re-passaging *in vivo* the ASGP-1 abruptly reappears at about the 16th passage (Howard et al., 1980). ASGP-1 appears to be shed from the cells by a proteolytic cleavage mechanism into the ascites fluid of the tumor bearing animal. The rates of shedding *in vitro* appear to be the same for the MAT-B1 and MAT-C1 sublines. However the amount of ASGP-1 in ascites fluid is 80% greater for MAT-C1 cells than for MAT-B1 cells. This

suggests that other processes beside shedding are involved in determining the amount of soluble ASGP-1 in ascites fluid for the MAT-C1 cells (Howard et al., 1981). Another glycoprotein, ASGP-1, has been found to be associated with ASGP-1 in detergent extracts of both cell types (Sherblom et al., 1980b). Lectin induced redistribution studies indicate that ASGP-1 and ASGP-2 are associated at the cell surface (Helm et al., 1981). ASGP-2 is believed to play an important role in the mechanism by which ASGP-1 is both attached to and shed from the ascites cell surface.

The function of ASGP-1 is still obscure. It is possible that it plays some role in the transplantability properties of these cells. The role of ASGP-1 in transplantability may partly involve the carbohydrate moiety as well as other factors such as modulation of ASGP-1, shedding of ASGP-1, and association of ASGP-2 with ASGP-1. The purpose of the studies herein was to investigate structural details of the alkali labile oligosaccharides not only to better understand the biological function of this major cell surface sialomucin but also to gain insight into cell surface sialomucin biosynthesis and its control.

CHAPTER II

PARTIAL CHARACTERIZATION OF THE GLUCOSAMINE-CONTAINING O-LINKED OLIGOSACCHARIDES OF ASGP-1

Introduction

The 13762 rat mammary adenocarcinoma contains on its surface a major sialoglycoprotein, ASGP-1 (Sherblom et al., 1980a). The expression of ASGP-1 on the cell surface has been shown to be modulated in response to environmental stresses (Howard et al., 1980). Evidence has been presented to suggest that ASGP-1 may be necessary for the existence of the ascites form of the mammary adenocarcinoma. With increasing evidence in other systems (Warren et al., 1978) for the role of sialoglycoproteins in tumorigenesis it was of interest to characterize the oligosaccharide structure of ASGP-1. Preliminary data indicated ASGP-1 is a heavily glycosylated (>70% by weight) mucin type glycoprotein with sialic acid a prominent component. The presence of sialic acid on tumor cell surfaces has been known for some time (Jeanloz et al., 1976). However the isolation and structural characterization of sialoglycoproteins of tumor cells has been reported for only one glycoprotein, epiglycanin (Van den Eijnden et al., 1979). Once structural features have been defined it may be possible to elucidate oligosaccharide biosynthetic principles which may aid in the understanding of the cell surface phenomena attributed to glycoproteins.

Materials

D-(6-^{3}H) glucosamine (2-6 Ci/mmol), D-(1-^{14}C) glucosamine (56 mCi/mmol), (^{3}H) $NaBH_4$ (300 mCi/mmol) were from Amersham; Instagel was from Packard; CsCl (99.9%) was a product of Varlacoid; guanidine hydrochloride (Grade 1) was from Sigma. Carbohydrate standards included inositol (Supelco), N-acetylglucosamine, N-acetylgalactosamine, arabinose, galactose, N-acetylneuraminic acid, and fucose (Sigma). N-acetylgalactosaminitol was prepared by reduction of N-acetylgalactosamine (Laine et al., 1972). α-L-fucosidase from bovine epididymis, α-galactosidase from Aspergillus Niger, and β-galactosidase (Grade V) from Aspergillus Niger were from Sigma and not purified further.

Methods

Cells

MAT-B and MAT-C ascites sublines of the 13762 rat mammary tumor were obtained from Mason Research Laboratories and were maintained as described previously (Carraway et al., 1976). MAT-B1 and MAT-C1 designate the sublines which arose from the MAT-B and MAT-C lines by routine weekly passage for one year in Carraway's laboratory (Carraway et al., 1978). The distinguishing features of these sublines have been stable for over two years since the original observations. Cells are passaged by weekly transfers into 2-3 month old female Fischer 344 rats and maintained as frozen stocks. Cells used for these studies were washed three times with cold phosphate buffered saline after recovery from the peritoneal cavity.

MAT cMR6-s is a variant subline originally obtained from the MAT-B1 subline. MAT-B1 cells were adapted to cell culture, cloned, and after the sixth passage in cell culture re-injected into rats and passaged in vivo (Howard et al., 1980). During passage in cell culture the cells lost ASGP-1. After the sixteenth passage in vivo there was a sudden reappearance of ASGP-1. The Bio-gel P4 chromatographic profile of released oligosaccharitols was similar to the profile of MAT-B1 ASGP-1 oligosaccharitols (Figure 1).

During the passage of MAT-C1 cells the host, Fischer 344 female rats, contracted a bacterial infection which quantitatively affected the oligosaccharitol content of ASGP-1. These variant cells also exhibited a loss of xenotransplantability. This new subline derived from MAT-C1 cells was designated MAT-C2. Passage of MAT-C2 cells in healthy rats gave rise to another variant cell line after approximately 25 passages. This variant subline, designated MAT-C3, regained the original oligosaccharitol content of MAT-C1 ASGO-1 but did not regain the ability to transplant across species barriers.

Cell Labeling

Radioactive monosaccharides (25 μCi ^{14}C-gln; 100 μCi ^{3}H-gln; or 100 μCi ^{3}H-fuc) in 0.2 ml of 0.9% saline were injected into the peritoneal cavity of a tumor-bearing rat 16 hr prior to sacrifice and recovery of cells.

Isolation of ASGP-1 from Membrane Vesicles

Washed cells were suspended in 10 volumes of 10mM Tris-HCl pH 8.0, and allowed to stand on ice for 2 min prior to centrifugation at 600g

for 2 min. The swollen pellet was suspended in 10 volumes of the same buffer and homogenized by four to five strokes of a Dounce homogenizer with a tight pestle. Immediately after homogenization the suspension was brought to a concentration of 3mM in Mg^{2+} and 1mM in Na^{+} by addition of 30mM $MgCl_2$ and 10mM NaCl. The homogenate was centrifuged at 1000g for 1 min and the supernatant was centrifuged at 10,000g for 10 min. Membrane vesicles were collected by centrifugation of the supernatant at 100,000g for 90 min. Preformed CsCl gradients were prepared by gently layering under each other 2 ml each of 4M Gdn-HCl and 10mM Tris-HCl, pH 7.4, containing 1.58, 2.0, 2.37, 2.79, and 3.15M CsCl in 5/8 x 3 in centrifuge tubes. Membrane vesicles were suspended in 4M Gdn-HCl in 10mM Tris-HCl, pH 8.0 with the aid of a hand homogenizer. 1 ml (∿5 mg of membrane protein) was layered onto the CsCl gradient and the gradients were centrifuged in a Beckman 75 Ti rotor at $4^{o}C$ for 16-24 hr at 55,000 rpm. After centrifugation the bottom of the tube was punctured using an ISCO fractionator and 10-20 drop fractions were collected. The density of selected fractions was determined by the difference in the dry and filled weight of a 25 µl glass constriction pipet. Fractions containing radioactivity and eluting at a density of 1.38-1.42 g/ml were pooled and CsCl and Gdn-HCl were removed by dialysis/concentration with a colloidion bag apparatus. The final dialyzate concentrate contained glycoprotein and if not used immediately it was lyophilized and stored in a screw cap test tube at $-20^{o}C$.

Aklaline Borohydride Degradation of ASGP-1

The glycoprotein in 1.0 ml of distilled water (∿5% by wt. aqueous solution) was mixed with 0.5 ml of a standard alkaline borohydride

solution prepared by dissolving 0.18 gm. of $NaBH_4$ in 1.3 ml of distilled water to which 0.26 ml of 1.0N NaOH was added. The mixture was incubated at 45°C for 16 hrs. After incubation the reaction was terminated by the dropwise addition of glacial acetic acid until bubbling subsided. The ABH digest was then mixed with 1.5 ml of a suspension of Dowex 50(H^+). The suspension was centrifuged at low speed and the supernatant saved. The resin was washed three times with distilled water. The supernatant and washings were combined, lyophilized, and stored at -20°C until fractionated on gel filtration media. Oligosaccharides released from the peptide and reduced in this manner are referred to as oligosaccharitols.

Gel Filtration of Oligosaccharitols

The supernatants and washings from Dowex 50(H^+) treatment usually in 1.0-1.5 ml of distilled water were layered gently onto a 0.9 x 100 cm column of Bio-gel P4 (200-400 mesh). The column was eluted with 0.1M pyridine acetate pH 6.0 and 45 drop fractions were collected using a Gilson microfractionator. Appropriate aliquots of selected fractions were mixed with two to four ml of Insta-gel and analyzed for radioactivity using an LKB 1212 Rack Beta scintillation counter. Fractions containing radioactivity were pooled, frozen, lyophilized, and stored at -20°C until further analyzed.

DEAE-Sephadex Ion Exchange Chromatography of Oligosaccharitols

Oligosaccharitol fractions from Bio-gel P4 gel filtration chromatography in 1.0-3.0 ml of distilled water were applied to a 2.0 x 30 cm

column of DEAE-Sephadex A-25 (Sigma). The column was first eluted with 0.05M pyridine acetate pH 5.0 and 120 drop fractions were collected. After ten to fifteen fractions the column was then eluted with a linear gradient of 0.05 to 0.65M pyridine acetate pH 5.0 formed by gradual mixing of 120 ml of each pyridine acetate solution. 120 drop fractions were collected and appropriate aliquots of selected fractions were assayed for radioactivity. Pyridine acetate concentrations of pooled fractions were estimated by comparison with the conductivity of standard pyridine acetate solutions. Pooled fractions were lyophilized and stored at -20°C.

Carbohydrate Analysis

Oligosaccharitol compositions were determined by GLC of trimethylsilyl derivatives or by GLC of alditol acetates. GLC of trimethylsilyl derivatives was done essentially as described by Reinhold (Reinhold, 1972). Aliquots of oligosaccharitols were lyophilized in borosilicate ampoules and hydrolyzed with 0.5 ml of 0.5M methanolic HCl for 16 hr at 65°C under N_2. Re-acetylation with acetic anhydride, de-O-acetylation with methanolic HCl, trimethylsilyl derivatization, extraction with hexane, and gas liquid chromatography were performed with inositol or perseitol as the internal standard.

GLC of the alditol acetate derivatives was done essentially as described by Griggs (Griggs et al., 1971). Aliquots of oligosaccharitols in borosilicate ampoules were hydrolyzed with aqueous 4N HCl for four hrs at 100°C. After reduction with $NaBH_4$ and acetylation with acetic anhydride the alditol acetates were analyzed by GLC. Gas chromatography was on 3% ECNSS on Gas Chrom-Q in 6 ft glass columns

using a temperature program of 140-200°C at 4°C/min.

Mild Acid Hydrolysis of Sialic Acid-Containing Oligosaccharitols

Oligosaccharitol fractions purified by sequential Bio-gel P4 chromatography and DEAE-Sephadex chromatography and dissolved in distilled water were mixed with an equal volume of 0.1N H_2SO_4 and incubated at 80°C for 1 hr. After incubation the reactants were cooled to room temperature and neutralized with dropwise addition of 1.0N NaOH. The mild acid hydrolyzate was percolated over a Dowex-1 (formate) resin of 1 cm height in a pasteur pipette. The column was washed with five volumes of water. The eluant and washings contain the desialylated oligosaccharitol. The column was then eluted with five column volumes of either 0.3N formic acid or 1.0M pyridine acetate pH 6.0 to recover the released sialic acid. The desialylated neutral fraction was rechromatographed over Bio-gel P4 to desalt the solution as well as monitor the integrity of the acid treatment.

GC-MS Linkage Analysis

Partially methylated alditol acetates were prepared from oligosaccharitols by sequential methylation, hydrolysis, reduction with sodium borodeuteride, and acetylation as described by Bjorndal (Bjorndal et al., 1970) and Stellner (Stellner et al., 1973) except that chemical ionization and methane were used with mass fragmentography (Hancock et al., 1976; Laine et al., 1977; and McNeil et al., 1977) of the (MH-60) ion to generate a profile of the partially methylated alditol acetates. Gas chromatography was on Supelcoport 100/120, with

helium carrier gas at 35 ml/min, using a programmed temperature gradient from 155° to 240°C at 4°C/min with injectors and transfer lines at 250°C. The instrument used was a Finnigan 3300-6110, with 1 Torr methane pressure in the ion source. Ionizing electron energy was 150 eV and source temperature was 80°C. Spectra were scanned from m/z 100 to 450 at a rate of 2 s/scan.

β-Elimination of ASGP-1 in the Presence of ^{3}H-$NaBH_4$

The procedure used for β-elimination was essentially that described by Aminoff (Aminoff et al., 1981). The lyophilized glycoprotein was suspended in 40 µl of 0.05N NaOH in a 0.3 ml Reacti-Vial (Pierce). 10 µl of a standard solution of ^{3}H-$NaBH_4$ was added and 40 µl more of 0.05N NaOH was added. A standard solution of ^{3}H-$NaBH_4$ was prepared by dissolving ^{3}H-$NaBH_4$ (300 mCi/mmol) in 0.05N NaOH to a concentration of 100 mCi/ml and stored dessicated at -20°C. Unlabeled $NaBH_4$ was added to bring the concentration of borohydride to 1.0M. The reaction mixture was incubated at 45°C for 16 hr. The reaction was stopped by dilution with an equal volume of water and a few drops of glacial acetic acid. Before further analysis the reduced digest was lyophilized at least 3 times with water to remove $^{3}H_2O$.

Periodate Oxidation of Sialic Acid-Containing Oligosaccharitols

The procedure for periodate oxidation was essentially that described by Gathmann (Gathmann et al., 1981). Oligosaccharitols were treated with 0.1M sodium periodate at 4°C using 0.1M sodium

acetate buffer, pH 4.5 for 18 hr. After destruction of excess periodate by the addition of glycerol the oxidized oligosaccharitols were separated into neutral and anionic components by passage through a small column (1 ml) of AG1 (Cl^-) followed by elution with 0.5M pyridine acetate pH 5.0 to elute the anionic components from the resin.

HPLC of Oligosaccharitols

Oligosaccharitols were dissolved in 50-200 µl of distilled water. 10-50 µl of sample containing at least 3,000 dpm was injected onto a column (0.4 x 25 cm) of 5 µ silica gel modified with 1,4 diaminobutane and equilibrated with 70% CH_3CN in 0.05% w/v 1,4 diaminobutane. The system used was a Waters M6000 pump with an automatic sample injector WISP 710 B, data module, and system controller. The column was eluted with the equilibration buffer at a flow rate of 1.0 ml/min. 1.0 ml fractions were collected. The column was routinely calibrated with a maltodextrin mixture.

Exoglycosidase Treatments

α-Fucosidase digestions were done in 50mM sodium citrate pH 5.0. α(β)-Galactosidase digestions were done in 50mM sodium citrate pH 4.5. 0.01-0.1 unit of the enzymes were added to oligosaccharitol in 100 µl of the appropriate buffer and incubated for 1 hr to 3 days at 37°C under a toluene atmosphere. The digestion was terminated by heating for 3 min at 100°C. The digest was desalted by passage through an ion exchange column (pasteur pipette) consisting of 0.15 ml of Dowex-1 (formate) on the bottom and 0.15 ml of Dowex 50(H^+) on the top. The

column was then eluted successively with 0.5 ml H_2O and 0.5 ml absolute methanol. The eluant and washings were evaporated in a vacuum dessicator and the residue brought up in an appropriate volume in preparation for HPLC analysis.

Analytical Methods

Protein content was analyzed by the method of Lowry (Lowry et al., 1951). Hexose was measured by the anthrone assay (Kabat and Mayers, 1961). Sialic acid was measured colorimetrically by the TBA method as described by Warren (Warren, 1959). TCA precipitable radioactivity of biological samples was measured in the following way. 10 µl aliquots of sample were spotted on Whatman filter paper (3MM), dried in a 60°C oven, washed with ice cold 10% w/v trichloroacetic acid for 15 minutes, 5% trichloroacetic acid for 15 minutes, 1:1 v/v ethanol/water for one hour, and 1:1 ethanol/ether v/v for 30 minutes. The filter paper was then mixed with two to four ml of Insta-gel and analyzed for radioactivity.

Results

Isolation of Purified ASGP-1

Tumor bearing rats, when injected with ^{3}H or ^{14}C-glucosamine, incorporates greater than 70% of the radioactivity into a component which exhibits low electrophoretic mobility in SDS-PAGE (Sherblom et al., 1980a). This component has been designated ASGP-1, a major ascites sialoglycoprotein on the surface of 13762 rat mammary adenocarcinoma. ASGP-1 can be purified from ascites cells by extraction from membrane vesicles with 4M Gdn-HCl followed by isopycnic

centrifugation in CsCl. ASGP-1 migrates to a density of 1.4 g/ml (data not shown) in the CsCl gradient. SDS-PAGE of this fraction results in only one radiolabeled component that exhibits low electrophoretic mobility. Isolated ASGP-1 represents 0.1% of the total cellular protein (Table I) and 0.7% of the total cellular hexose. There is a 135-fold purification of ASGP-1 with respect to total cellular protein and a 16-fold purification of ASGP-1 with respect to total cellular hexose. From 5×10^9 cells 1.3 mg of ASGP-1 which contains 146 µg of hexose can be isolated. The hexose measurement is a low estimate of the carbohydrate content since it does not account for any of the sialic acid or amino sugars present on ASGP-1.

O-Linked Oligosaccharitols of ASGP-1

Release of the O-linked oligosaccharitols of glucosamine labeled ASGP-1 was accomplished by alkaline borohydride (ABH) β-elimination under conditions which prevent degradation of the oligosaccharide (Carlson et al., 1970). Bio-gel P4 chromatography of the ABH digest of MAT-B1 ASGP-1 results in the separation of three major glucosamine-containing oligosaccharitol fractions designated II, III, and IV (Figure 1). Fraction I co-elutes with the void volume and most probably represents undigested glycoprotein. The fractions II, III, and IV represent 18%, 36% and 36% respectively of the glucosamine label. Since glucosamine is metabolized to n-acetyl neuraminic acid this quantitation may be misleading. A more accurate quantitation was obtained by elimination of the oligosaccharides in the presence of ^3H-$NaBH_4$. This quantitatively labels the reducing end sugar of

TABLE I

YIELDS AND SPECIFIC ACTIVITIES OF THE SUBCELLULAR FRACTIONS° OBTAINED DURING ISOLATION OF ASGP-1 FROM 13762 RAT MAMMARY ASCITES TUMOR CELLS

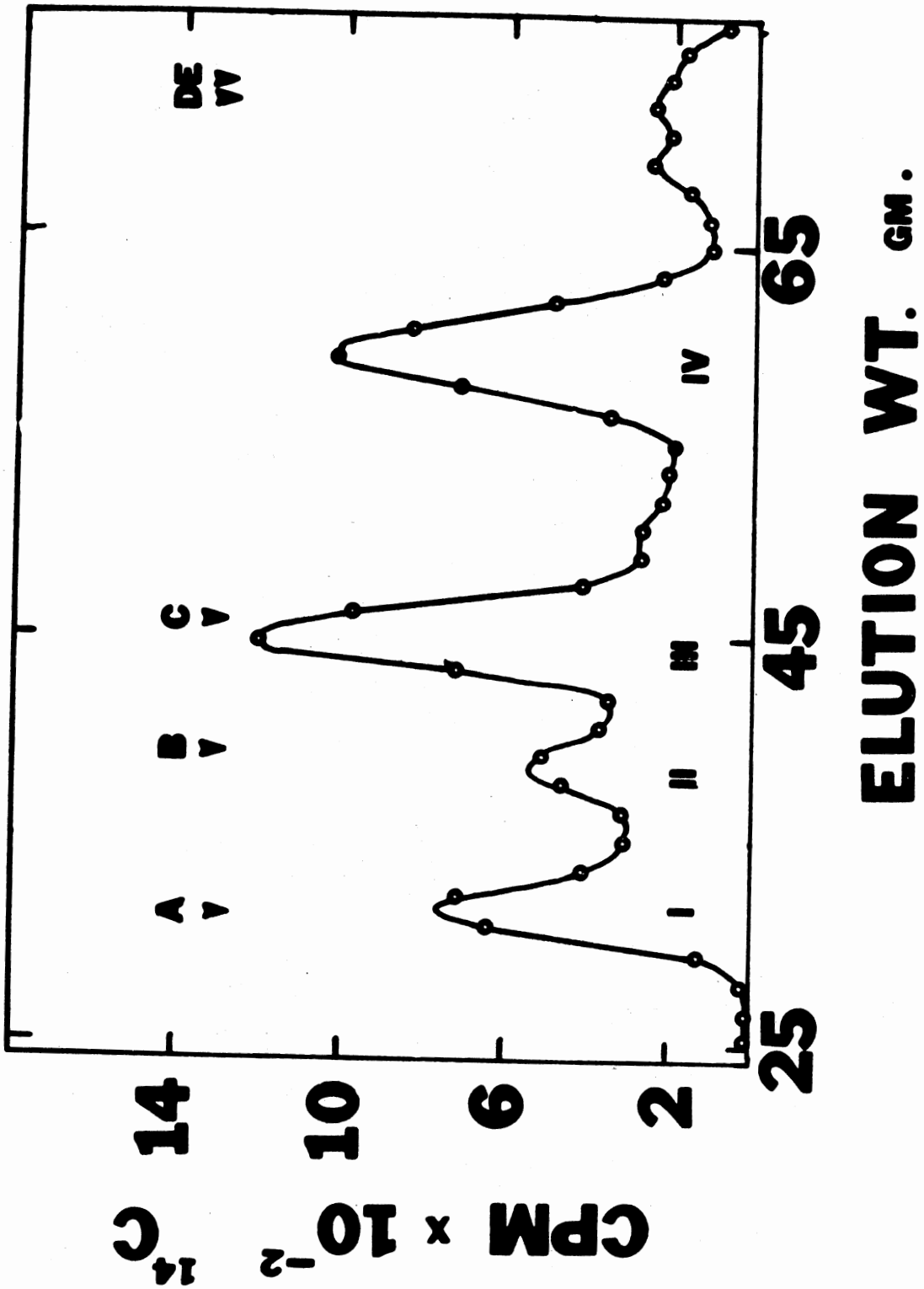
Fraction	Total Yield*		Specific Radioactivity	
	Protein (g)	Hexose (mg)	Protein basis† (dpm/μg)	Hexose basis† (dpm/μg)
Cells	1.64	21.2	2.54	196
Membranes	0.076	5.0	18.9	288
ASGP-1	0.0013	0.146	343	3060

*From 5×10^9 tumor cells.

†25 μCi/rat of ^{14}C-glucosamine.

°ASGP-1 was isolated from tumor cells as described in Methods. After isolation of each indicated fraction appropriate aliquots were analyzed for protein, hexose, and acid-precipitable radioactivity as described in Methods.

Figure 1. Bio-gel P4 Chromatographic Profile of the ABH Digest of ^{14}C-Glucosamine Labeled MAT-B1 ASGP-1. ^{14}C-gln labeled MAT-B1 ASGP-1 was treated with alkaline borohydride, desalted on Dowex 50(H^+), and chromatographed on Bio-gel P4. The column was standardized with A) undegraded ASGP-1; B) $NANA_2gal_1galNacOH_1$; C) $NANA_1gal_1galNacOH_1$; D) $gal_1galNacOH_1$, and E) galNacOH.


CPM x 10⁻² ¹⁴C
14
10
6
2
25
45
65
ELUTION WT. GM.
A
B
C
DE
I
II
III
IV
V

each oligosaccharide chain as it is cleaved from the polypeptide. Bio-gel P4 chromatography of ^{14}C-gln ASGP-1 digested with alkali in the presence of ^{3}H-$NaBH_4$ is shown in Figure 2. The distribution of radioactivity (Table II) indicates that fractions II, III, and IV comprise 9%, 27%, and 45%, respectively of the galactosamine. Further separation of the gel filtration fractions according to their sialic acid content was achieved by anion exchange chromatography. DEAE-Sephadex chromatography (Figure 3) indicated that MAT-B1-II consisted primarily of a highly anionic fraction designated MAT-B1-II-3; MAT-B1-III primarily contained an intermediate anionic component designated MAT-B1-III-2; and MAT-B1-IV was comprised solely of a neutral fraction designated MAT-B1-IV-1. From the recovery of ^{3}H-label in each of these fractions after β-elimination with ^{3}H-$NaBH_4$ almost half of the glucosamine-containing oligosaccharitols released from MAT-B1 ASGP-1 are the neutral fraction IV-1 whereas the sialylated saccharitols represent 35% of the chains released from ASGP-1 by ABH treatment.

Carbohydrate Composition of II-3, III-2, and IV-1

The carbohydrate composition of the oligosaccharitol fractions II-3, III-2, and IV-1 (Table III) indicated that II-3 and III-2 are di-sialylated and mono-sialylated oligosaccharitol fractions. Oligosaccharitol fractions III-2 and IV-1 contained small but significant amounts of fucose. Since the homogeneity of these fractions is suspect definite structural assignments cannot be made but the data indicated that each fraction contained at least the tetrasaccharitol sequence, $gal_2glcNac_1galNacOH_1$.

Figure 2. Bio-gel P4 Chromatography Profile of ^{3}H-$NaBH_4$ β-Eliminated MAT-B1 ASGP-1. ^{14}C-gln (o—o) labeled MAT-B1 ASGP-1 was treated with alkaline borohydride using ^{3}H-$NaBH_4$ (●--●). The ABH digest was processed according to the detailed procedure given in methods and chromatographed on Bio-gel P4.

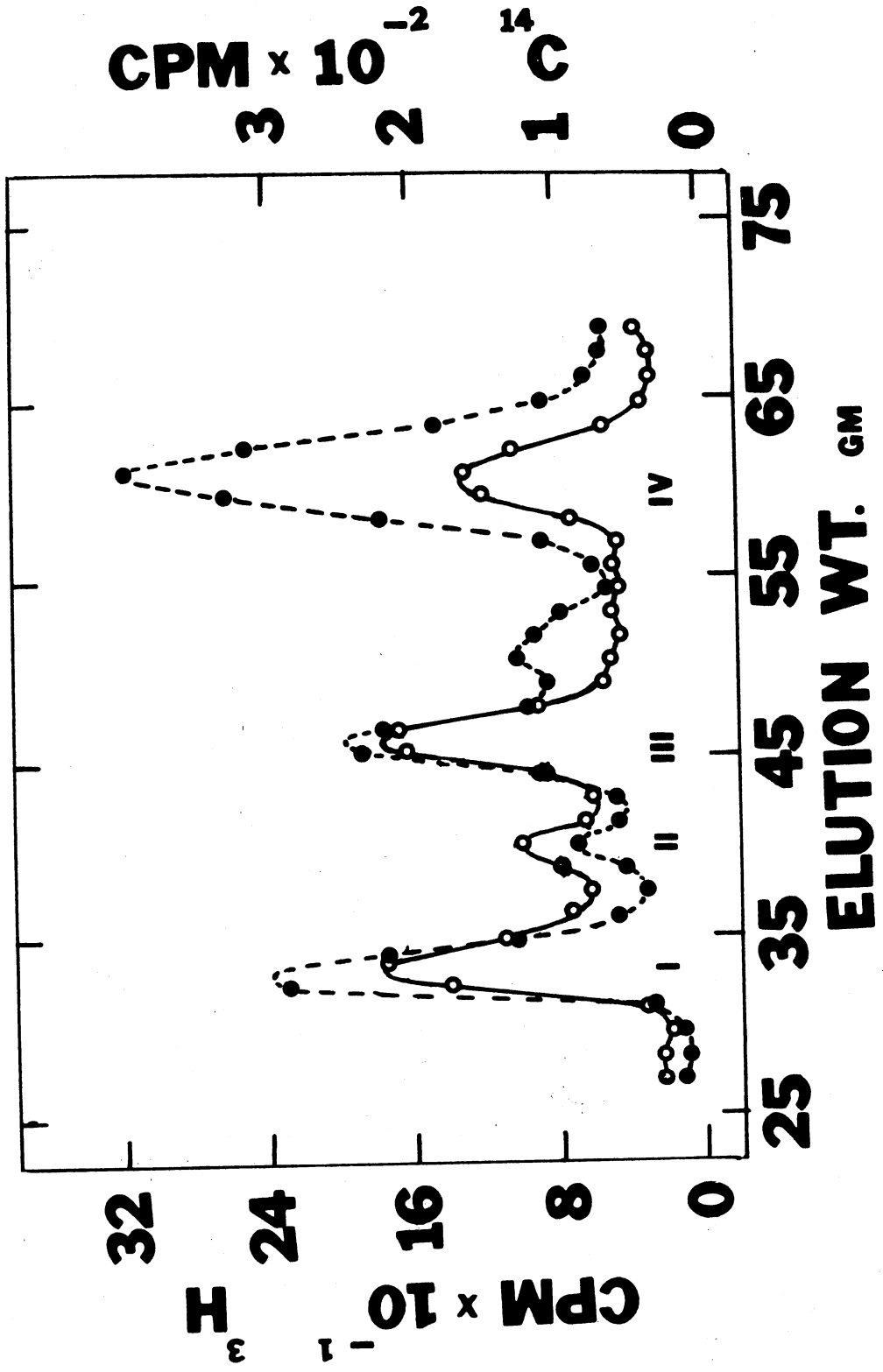

CPM x 10^-2 14C
3
2
1
0
75
65
55
45
35
25
ELUTION WT. GM
IV
III
II
I
32
24
16
8
0
CPM x 10^-1 3H

TABLE II

RELATIVE QUANTITATION° OF ABH RELEASED OLIGOSACCHARIDES OF MAT-B1 ASGP-1

Fraction	% Total Recovery		Relative Number of Oligosaccharide Chains	
	GalNacOH	GlcNac	GalNacOH	GlcNac
II	9	18	1	1
III	27	36	3	2
IV	45	36	5	2

°The radioactivity recovered in the Bio-gel P4 fractions II-IV from profile of Figure 2 was quantitated and the percentage of label recovered in each fraction calculated by comparison to the total label recovered in fractions I-IV. The relative number of chains was calculated by setting the fraction II to 1.0.

Figure 3. DEAE-Sephadex Chromatographic Profiles of the O-Linked Oligosaccharitols of MAT-B1 ASGP-1. ^{14}C-gln labeled oligosaccharitol fractions II-IV as isolated on Bio-gel P4 were chromatographed on DEAE-Sephadex as described in Methods.

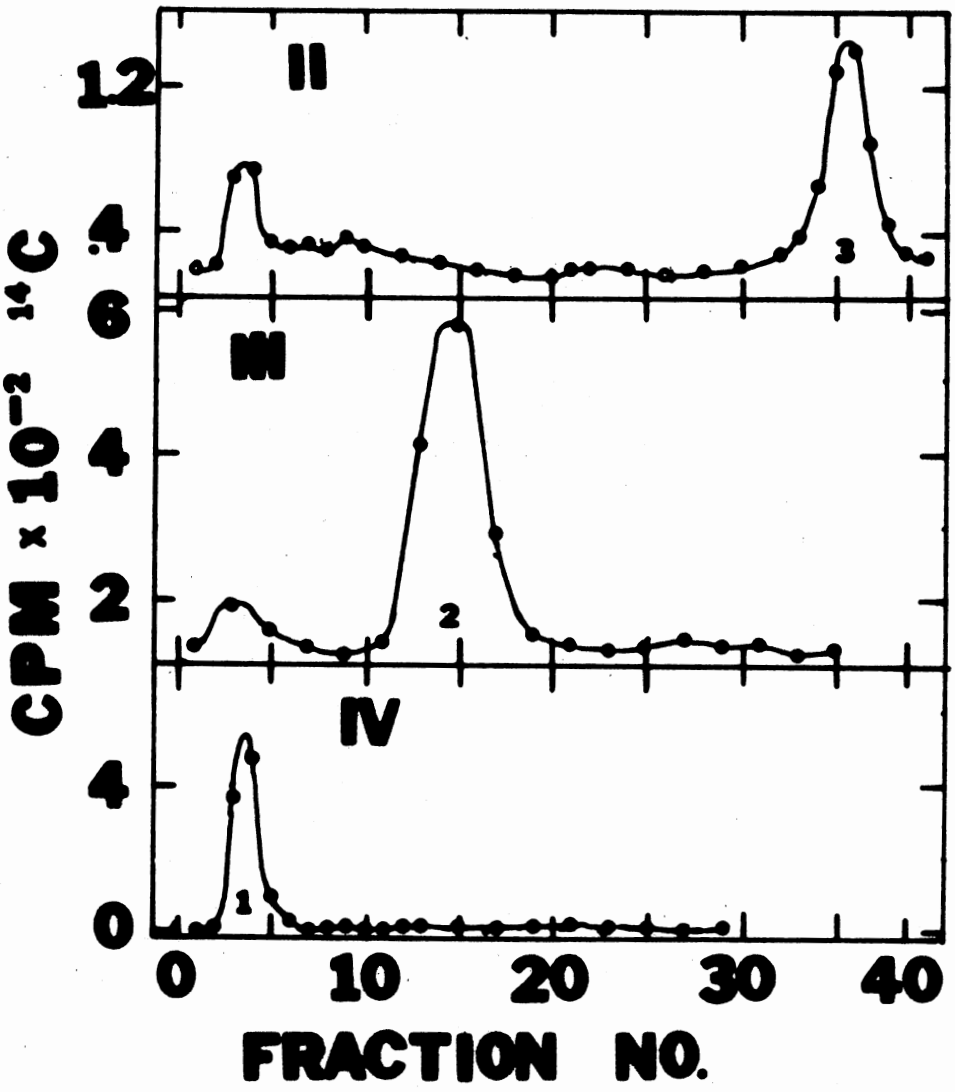

II
III
IV
1
2
3
12
4
6
4
2
4
0
CPM x 10⁻³ ¹⁴C
0
10
20
30
40
FRACTION NO.

TABLE III

CARBOHYDRATE COMPOSITION[a] OF THE OLIGOSACCHARITOL FRACTIONS II-3, III-2, AND IV-1

Fraction	mole/mole galNacOH			
	glcNac	gal	fuc	SA
II-3[b]	0.78	1.79	-	2.15[e]
III-2[c]	0.93	2.0	0.33	0.90
IV-1[d]	1.16	2.41	0.41	-

[a]Determined as their trimethylsilyl derivatives as described in Methods.

[b]Average of four determinations; ranges are glcNac: 0.67-0.92; gal: 1.5-2.4; SA: 1.82-2.60.

[c]Average of two determinations; ranges are glcNac: 0.76-1.10; gal: 1.99-2.01; fuc: 0.30-0.37; SA: 0.71-1.10.

[d]Average of two determinations; ranges are glcNac: 1.16-1.17; gal: 1.99-2.8; and fuc: 0.28-0.52.

[e]Also determined colorimetrically (Warren, 1959), average of two determinations = 2.2, range: 2.0-2.4.

The Core Oligosaccharitol(s) of the Oligosaccharitol Fractions II-3 and III-2

The possibility that the three fractions II-3, III-2, and IV-1 have a common core tetrasaccharitol was initially investigated by comparing the de-sialylated core oligosaccharitols II-3D and III-2D with the neutral oligosaccharitol fraction IV-1 on Bio-gel P4. ^{3}H-gln labeled II-3D co-chromatographed with ^{14}C-gln labeled IV-1 (Figure 4A) indicated that II-3D eluted at the low molecular weight region of IV-1. ^{3}H-gln labeled III-2D co-chromatographed with ^{14}C-gln labeled IV-1 (Figure 4B) indicated that III-2D co-eluted with IV-1. This observation prompted an investigation into the further fractionation of the oligosaccharitol fractions II-3D, III-2D, and IV-1 by HPLC.

Apparent separation to homogeneity was achieved by elution of the aforementioned oligosaccharitol fractions on 5μ silica gel modified with 1,4-diaminobutane (Turco, 1981). Comparative HPLC co-elution profiles of the fractions are shown in Figure 5. ^{3}H-gln labeled II-3D co-chromatographed with ^{14}C-gln labeled IV-1 (Figure 5A) indicated that II-3D consisted of one component oligosaccharitol which co-eluted with the smaller of the two components found in IV-1. ^{3}H-gln labeled III-2D co-chromatographed with ^{14}C-gln labeled IV-1 indicated that III-2D contained two component oligosaccharitols which co-eluted with the two oligosaccharitols of IV-1.

Carbohydrate Composition of IV-1a and IV-1c

Comparative HPLC co-elution profiles (Figure 5) indicated that

Figure 4. Bio-gel P4 Co-elution Profiles of the Core Oligosaccharitol Fractions of II-3 and III-2 with the Neutral Oligosaccharitol Fraction IV$_{14}$-1. (A) Co-elution of ^{3}H-gln labeled II-3D (o—o) and ^{14}C-gln labeled IV-1 (o--o), ^{3}H-gln labeled II-3 (o—o) was also eluted as an internal standard. (B) Co-elution of ^{3}H-gln labeled III-2D (o—o) and ^{14}C-gln labeled IV-1 (o--o), III-2 labeled with ^{3}H-gln (o—o) was also eluted as an internal standard.

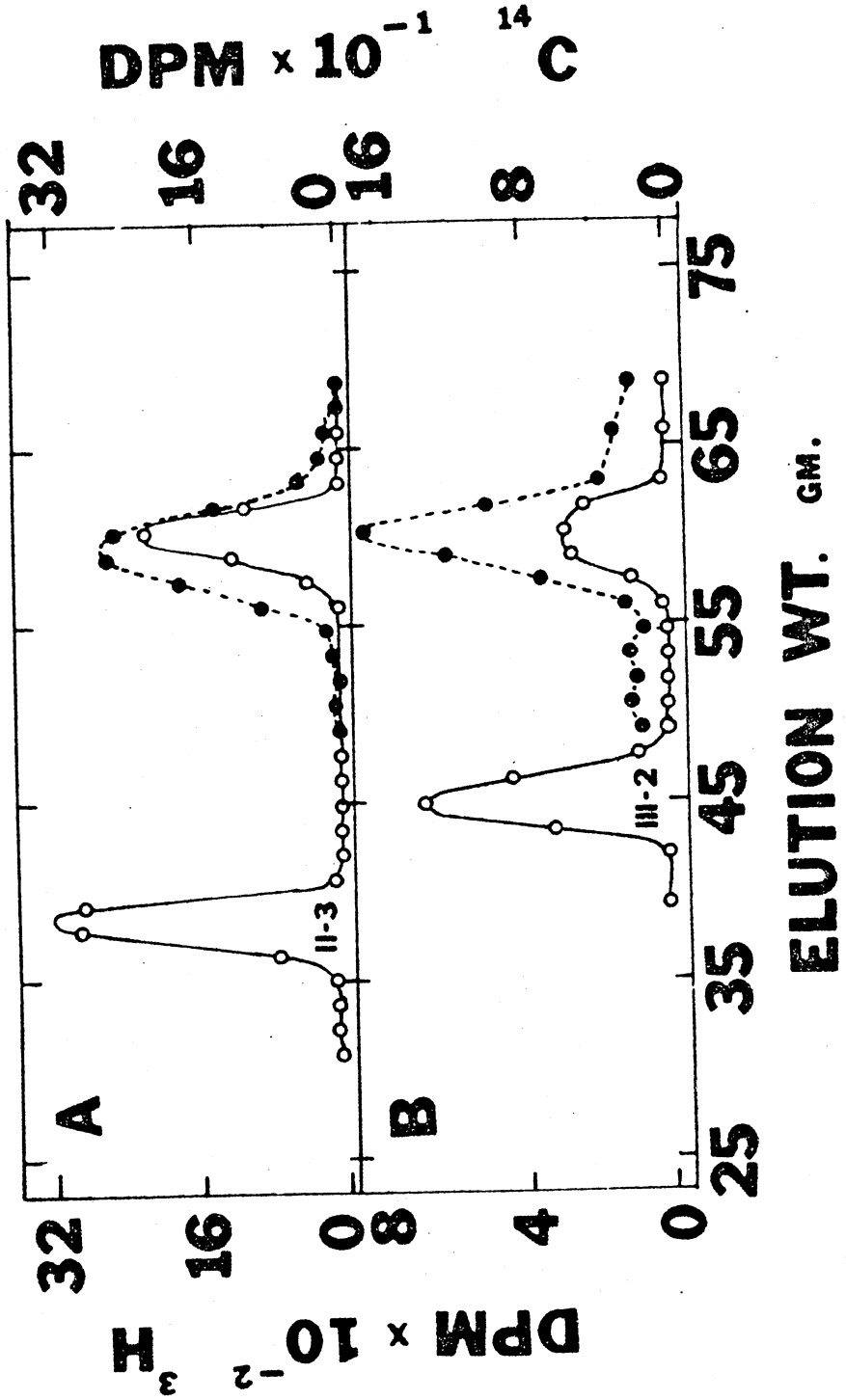
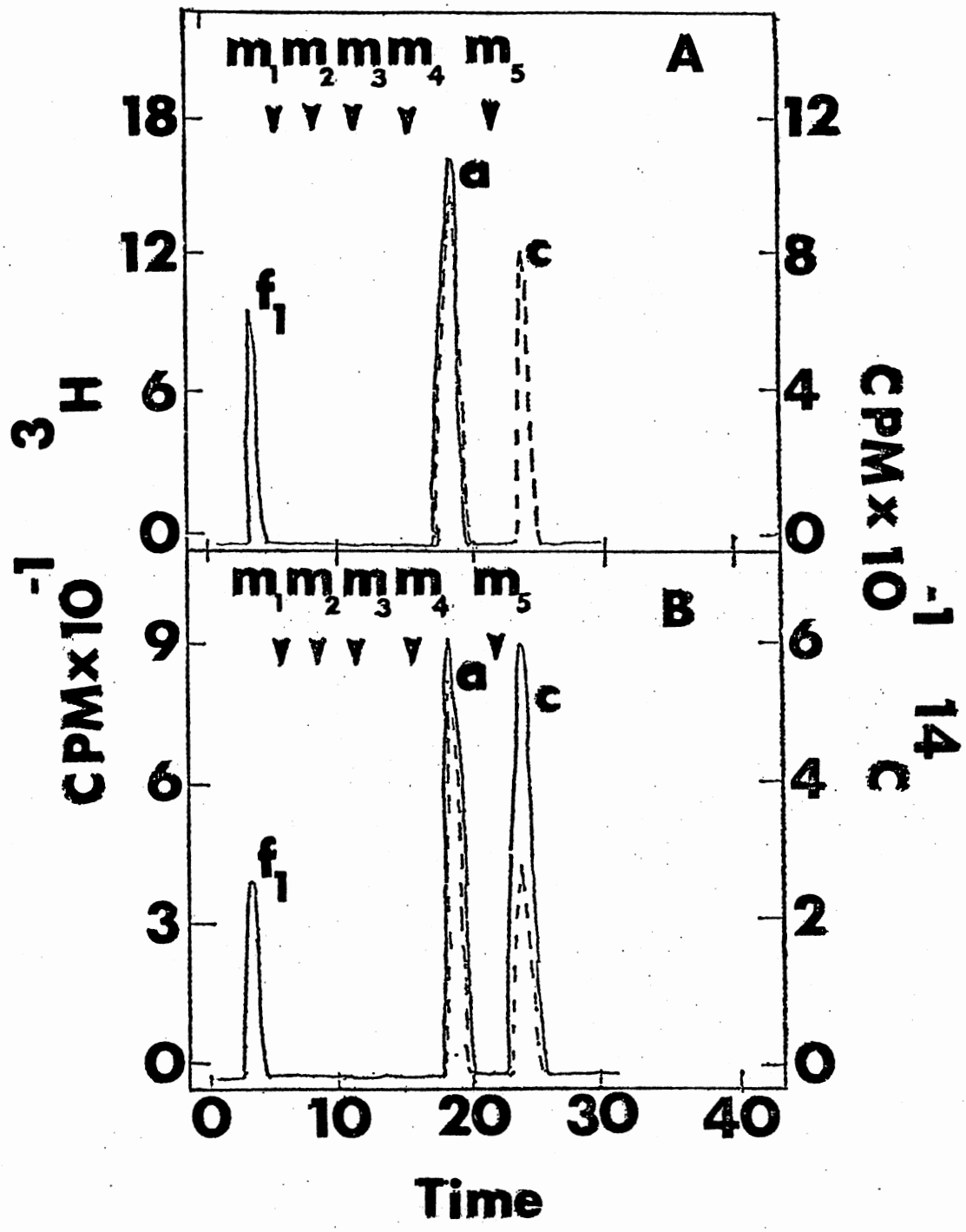

DPM x 10⁻¹ ¹⁴C
32
16
0
16
8
0
75
65
55
45
35
25
ELUTION WT. GM.
A
B
II-3
III-2
32
16
0
8
4
0
DPM x 10⁻² ³H

Figure 5. HPLC Co-elution Profiles of the Core Oligosaccharitol Fractions of II-3 and III-2 with the Neutral Oligosaccharitol Fraction IV-1. (A) ^{3}H-gln labeled II-3D (——) was co-eluted with ^{14}C-gln labeled IV-1 (---) on 5μ silica gel modified with 1,4 di-aminobutane as described in Methods. (B) ^{3}H-gln labeled III-2D (——) was co-eluted with ^{14}C-gln labeled IV-1 (---) as in (A). The silica gel column was calibrated with a maltodextrin mixture. M_1 = glucose, M_2 = maltose, M_3 = maltotriose, M_4 = maltotetrose, M_5 = maltopentose. f_1 is some unknown ^{3}H-labeled contaminant.


m1 m2 m3 m4 m5
A
18
12
6
0
a
c
f1
3H
CPM x 10-1
12
8
4
0
m1 m2 m3 m4 m5
B
9
6
3
0
a
c
f1
CPM x 10-1
14C
6
4
2
0
0
10
20
30
40
Time

II-3D, III-2D, and IV-1 contain a common oligosaccharitol (II-3Da, III-2Da, and IV-1a) which apparently elutes as a tetrasaccharitol and III-2D and IV-1 contain a second common oligosaccharitol (III-2Dc and IV-1c) which apparently elutes as a pentasaccharitol.

Since oligosaccharitol fraction IV is more convenient to work with the carbohydrate composition of the two components IV-1a and IV-1c isolated by HPLC was analyzed (Table IV). The data indicated that IV-1a is the tetrasaccharitol, $gal_2glcNac_1$ $galNacOH_1$ and IV-1c is the pentasaccharitol, $gal_3glcNac_1$ $galNacOH_1$.

Structure of II-3

HPLC analysis indicated that II-3D was homogeneous (Figure 5A). Carbohydrate compositional analysis indicated that the de-sialylated core of II-3 was the tetrasaccharitol $gal_2glcNac_1$ $galNacOH_1$ (Table IV) and that II-3 results from the transfer of two sialic acid residues to this tetrasaccharitol (Table III). The possible structures of a core tetrasaccharitol of the proposed composition are:

I. Gal—GlcNac—Gal—GalNacOH

II.
```
Gal—GlcNac—GalNacOH
                   |
                  Gal
```

III. Gal—Gal—GlcNac—GalNacOH

IV.
```
GlcNac—Gal—GalNacOH
                   |
                  Gal
```

V. GlcNac—Gal—Gal—GalNacOH

VI.
```
Gal—Gal—GalNacOH
                |
             GlcNac
```

TABLE IV

CARBOHYDRATE COMPOSITION[a] OF OLIGOSACCHARITOL FRACTIONS IV-1a AND IV-1c

Fraction	Relative Molar Ratio		
	gal	glcNac	galNacOH
IV-1a[b]	1.98	1.0	1.06
IV-1c[c]	2.82	1.0	1.12

glcNac taken as 1.0.

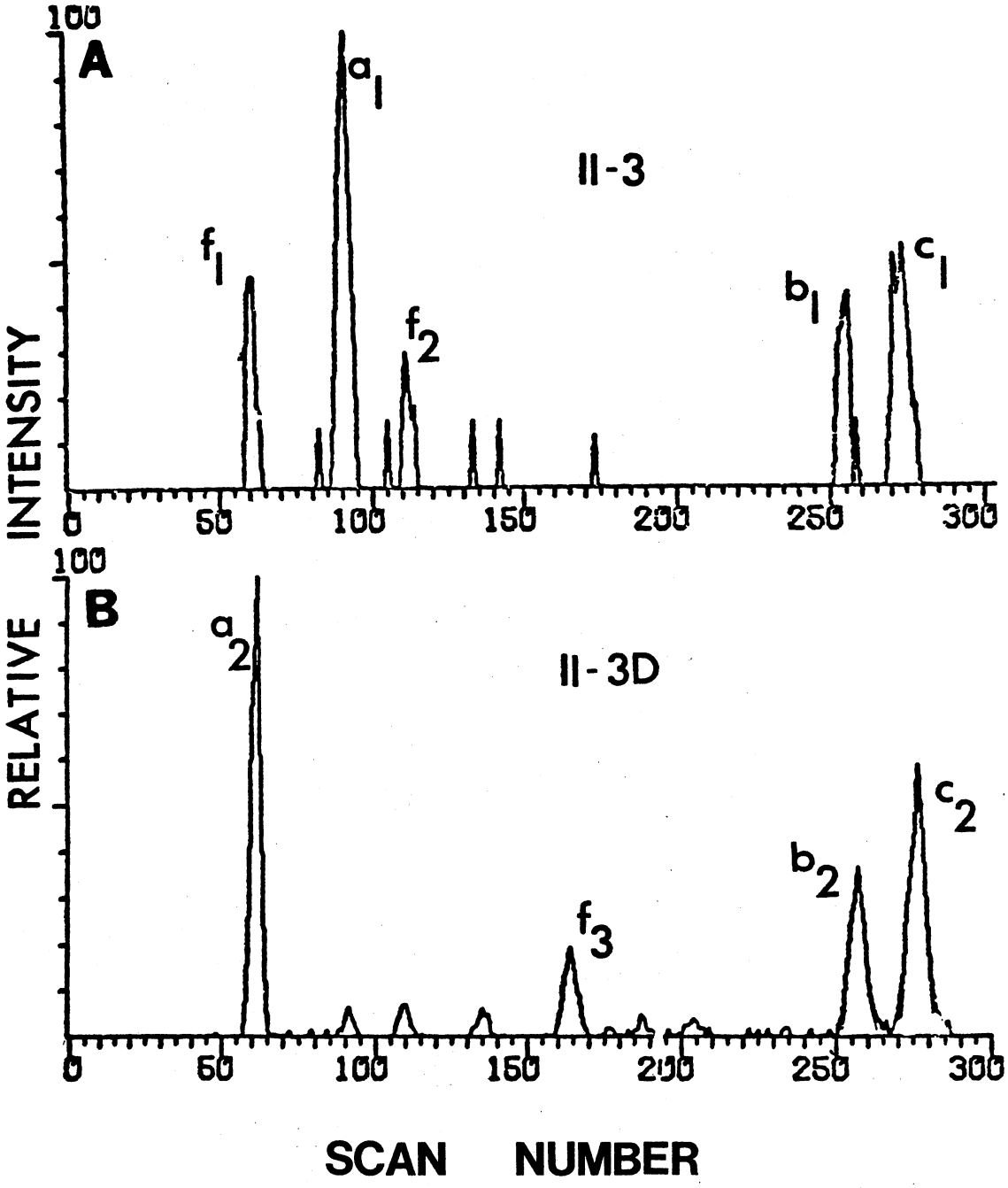
[a]Determined as their alditol acetates as described in Methods.

[b]Average of two determinations, range: gal: 1.76–2.21; galNacOH: 1.03–1.08.

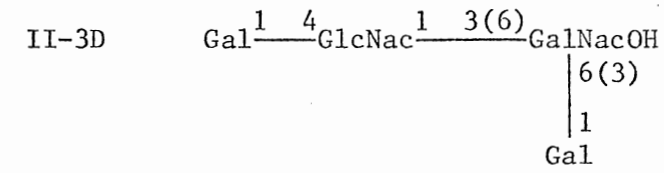
[c]Average of two determinations, range: gal: 2.69–2.95; galNacOH: 1.10–1.14.

To determine which of these structures is most likely the partially methylated alditol acetates of the intact and de-sialylated II-3 core were analyzed by GC-MS. Data for II-3 was obtained from MAT-C1 ASGP-1. Sufficient material for analysis was not obtainable from MAT-B1 ASGP-1. II-3 from MAT-C1 ASGP-1 has identical carbohydrate composition to and co-elutes with MAT-B1 II-3 on Bio-gel P4. II-3D from both sublines co-elute on 5μ silica gel activated with 1,4-diaminobutane. The GC profiles of II-3 (Figure 6A) and II-3D (Figure 6B) indicate that both the intact and the de-sialylated oligosaccharitol contain three major components. The identification of these components was achieved by comparison of retention times with standards and the chemical ionization mass fragmentogram which generates the characteristic MH, MH-32, and MH-60 mass fragments of the various partially methylated alditol acetates (Laine, 1981). The data indicated that II-3 contained a_1: 3-linked galactose, b_1: 3,6-linked galNacOH, and c_1: 4-linked glcNac. II-3D contained a_2: terminal galactose, b_2: 3,6-linked galNacOH, and c_2: 4-linked glcNac. Comparison of peak areas of the amino sugars suggested that they are present in approximately equimolar amounts which is in agreement with the carbohydrate composition data. The fact that 3,6-linked galNacOH is present in both II-3 and II-3D suggested that a sialic acid moiety is not directly linked to galNacOH. II-3 contained only 3-linked galactose whereas II-3D contained only terminal galactose. Thus the two sialic acid residues of II-3 are linked to galactose via a 1→3 linkage. Only one structure of those proposed is consistent with the GC-MS data for the core II-3. It's most likely structure is:

Figure 6. Gas Chromatographic Elution Profiles of the Partially Methylated Alditol Acetates of II-3 and II-3D. The multiple mass chromatogram of the partially methylated alditol acetates of (A) II-3 and (B) II-3D. Data were recorded on a Finnigan Model 3300-6110 instrument. Conditions are described in Methods. The ions traced in the mass chromatogram are at M/Z 264, 292, 304, and 393. The abscissa records the scan number at 2s/scan and the ordinate is relative intensity. The identification of the peaks are: a_1, 3-linked gal; a_2, terminal gal; b_1 and b_2, 3,6-linked galNacOH; c_1 and c_2, 4-linked glcNac; f_1, unknown contaminant, mass spectrum shows no significant hexose fragments; f_2, possible mixture of mono-substituted and di-substituted glucose probably introduced into sample during dialysis steps and Sephadex chromatography employed during isolation procedures; f_3, a possible side product of acetolysis.

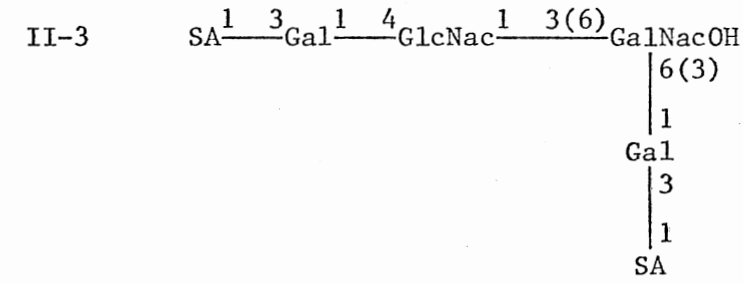

100
A
a_1
II-3
f_1
f_2
b_1
c_1
0
50
100
150
200
250
300
RELATIVE INTENSITY
100
B
a_2
II-3D
f_3
b_2
c_2
0
50
100
150
200
250
300
SCAN NUMBER

II-3D

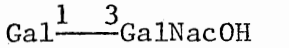


It follows that the most likely structure for the intact oligosaccharitol II-3 is:

II-3



The question remains as to which positions of GalNacOH the galactose and n-acetylglucosamine residues are attached. A tentative assignment has been made on the basis of the lectin agglutinability of ASGP-1. ASGP-1 has been previously shown to be a peanut agglutinin receptor (Huggins et al., 1980). This lectin recognizes the disaccharitol:



suggesting that this is an oligosaccharitol present in ASGP-1. Sherblom (Sherblom et al., 1980b) found this disaccharitol to be present in ASGP-1. It is not detected readily in these studies because the tracer used is glucosamine. This disaccharitol has been implicated as a biosynthetic precursor in mucin biosynthesis (Springer et al., 1976). It seems logical that II-3 and II-3D contain this disaccharitol. Thus the linkages to galNacOH are deduced to be:

II-3 —GlcNac$\overset{1\quad 6}{\text{——}}$GalNacOH
|3
|1
Gal
|

Heterogeneity of III-2

HPLC co-elution profiles (Figure 5B) indicated that III-2D consisted of at least two component oligosaccharitols which are chromatographically identical to the two components of the neutral oligosaccharitol fraction IV-1. Comparison of the HPLC profiles of II-3D and III-2D (Figures 5A and 5B) indicate that the smaller of the two components of III-2D, III-2Da, is the same tetrasaccharitol found in II-3, II-3Da. Carbohydrate compositional analysis (Table III) indicated that III-2 contained small significant amounts of fucose. HPLC analysis (Figure 7) of metabolically dual labeled ^{3}H-fucose and ^{14}C-glucosamine III-2D indeed reveals that a fucosylated oligosaccharitol, III-2Db, which does not co-elute with either of the components, III-2Da and III-2Dc, is present.

GC-MS Linkage Analysis of III-2 and III-2D

Although the oligosaccharitol fractions III-2 and its de-sialylated core III-2D consist of at least three oligosaccharitol chains the entire fractions as isolated by sequential gel filtration and anion exchange chromatography were analyzed by GC-MS in order to insure enough material was present. The results are summarized in Table V.

Consistent with the compositional data III-2 and III-2D contain equimolar amounts of the amino sugars, 3,6-linked galNacOH and 4-linked

Figure 7. HPLC Elution Profile of ^{3}H-Fucose and ^{14}C-Glucosamine Dual Labeled III-2D. ^{3}H-fucose (●--●) and ^{14}C-gln (o—o) III-2D was analyzed on 5μ silica gel as described in Methods. Column was calibrated with a maltodextrin mixture.

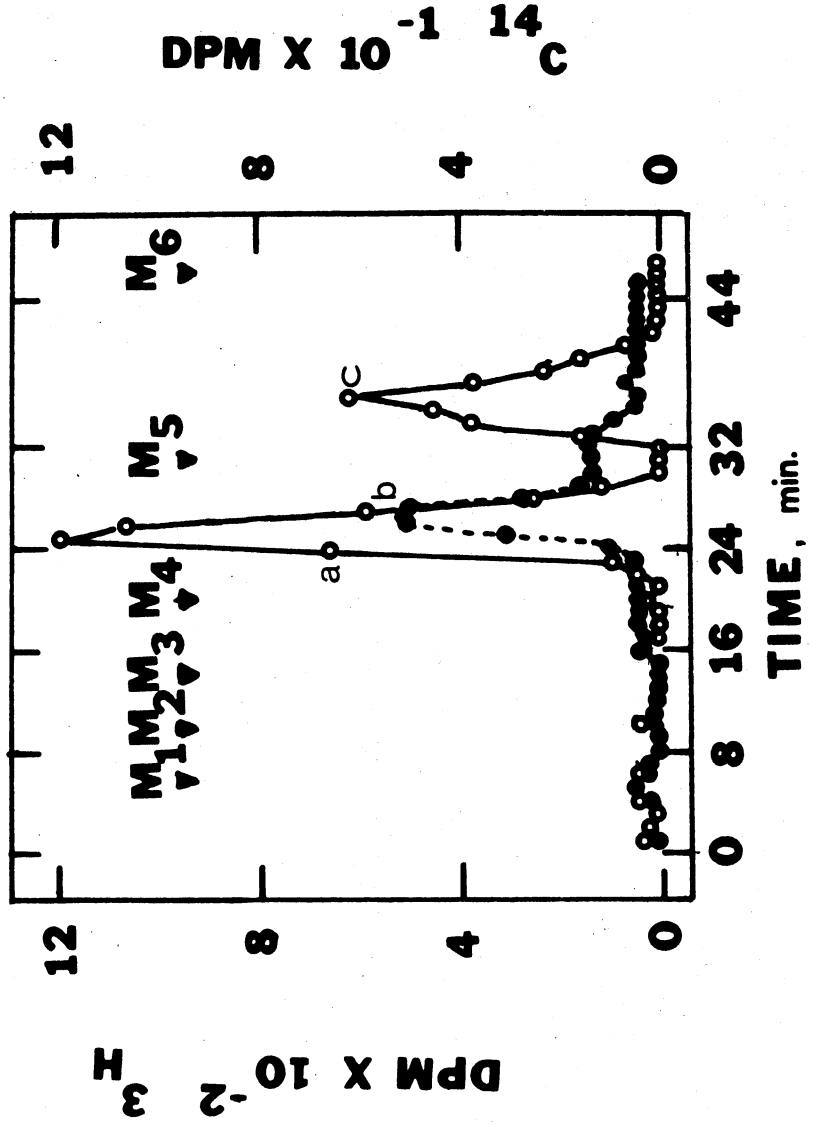

DPM X 10^-1 14C
12
8
4
0
M6
M5
M4
M3
M2
M1
a
b
c
0
8
16
24
32
44
TIME, min.
DPM X 10^-2 3H

TABLE V

RELATIVE SUGAR CONTENTS OF THE PARTIALLY METHYLATED ALDITOL ACETATES OF III-2 AND III-2D°

Component	Relative Amount	
	III-2	III-2D
Terminal fucose	0.03	0.14
Terminal galactose	0.97	1.7
2-linked galactose	+*	+*
3-linked galactose	2.2	0.9
3,6-linked galNacOH	1.0	1.0
4-linked glcNac	1.26	0.87

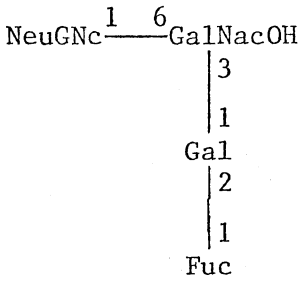
*Its presence is suspected, arguments presented in text. 3,6-linked galNacOH taken as 1.0.

°The PMAA derivatives of III-2 and III-2D were prepared and analyzed by GC-MS analysis as described in Methods. Relative ratios of each derivative were estimated by comparing the intensities of m/z 234 (t-fucose), m/z 264 (t-hexose), m/z 292 (3 linked galactose), m/z 364 (3,6-linked galNacOH), and m/z 393 (4 linked glcNac). Fetuin tetrasaccharitol was run as a standard for the identification of 3,6-linked galNacOH and an estimation of the relative response of 3 linked gal and 3,6-linked galNacOH. No response factor was used for terminal fucose and terminal galactose.

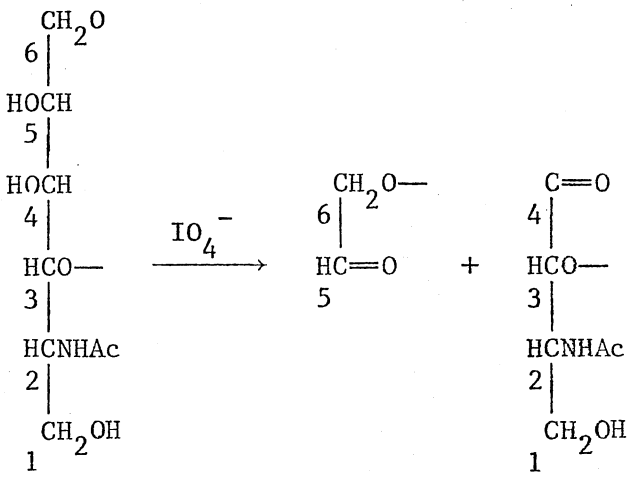
glcNac. The presence of di-substituted galNacOH in both fractions indicated that sialic acid is not linked to this sugar. Comparison of the 3-linked galactose:3,6-linked galNacOH ratio in III-2 and III-2D shows a loss of approximately one 3-linked galactose/3,6-linked galNacOH after de-sialylation. A similar comparison of the terminal galactose:3,6-linked galNacOH shows a concomitant increase of one terminal galactose/3,6-linked galNacOH upon de-sialylation. This result indicated that the one sialic acid residue of III-2 is 3-linked to galactose. Interestingly III-2D still contains internal 3-linked galactose. If this fraction consisted only of the core tetrasaccharitol substituted with one sialic acid residue this result would be unexpected. This observation may be indicative of a galactose residue substituted in the C-3 position present in one or both of the oligosaccharitol components III-2Db and/or III-2Dc (Figure 7).

Periodate Oxidation of III-2

Insight into the site of attachment of the one sialic acid residue to each of its three oligosaccharitol core components can be gained by periodate oxidation with subsequent separation of the fragments by anion exchange chromatography. Recently, Gathmann and associates (Gathmann et al., 1981) have used this methodology to verify the position of sialic acid attachment to the core of the porcine submaxillary blood group oligosaccharitol:

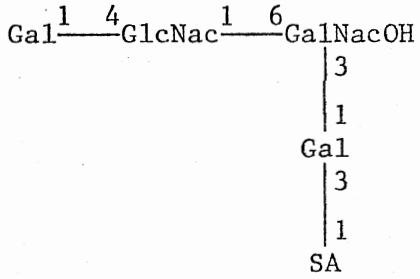


Periodate oxidation of 3,6-linked oligosaccharitols will result in the cleavage of the galNacOH between the C4 and C5 bond:



to give two fragments, one which contains the 3-linked branch and the other which contains the 6-linked branch. Separation of these fragments according to their sialic acid content can be effected by anion exchange chromatography. The distribution of ^{3}H label from periodate treated ^{3}H gln labeled III-2 between the unadsorbed and adsorbed fraction after anion exchange chromatography should reveal the position of sialic acid in relation to glcNac and thus reveal the galactose residue to which it is linked. Two control experiments were simultaneously performed with periodate treatment. One portion of ^{3}H-gln III-2 was incubated in buffer for 18 hr at $0^{o}C$. This control is necessary since it is possible that during the incubation

time the acidic environment (pH 4.5 buffer) could cause the cleavage of sialic acid. Another control was the treatment of III-2 with mild acid. This treatment will de-sialylate the oligosaccharitol and separation of the sialic acid from the core will give an estimate as to the amount of the glucosamine label that has metabolized into sialic acid. The analysis (Table VI) of the control indicated that a minimal amount (15%) of the oligosaccharitol was de-sialylated during the incubation in 0.1M sodium acetate, pH 4.5 under the conditions for periodate oxidation. Analysis of the mild acid treated sample indicated that 20-25% of the label was metabolized into sialic acid. Analysis of the periodate treated sample gave an elution profile identical to the mild acid treated sample indicating that the glucosamine label can be separated from the sialic acid. This result established that all of the mono-sialylated oligosaccharitols of III-2 have the glucosamine and sialic acid in different branches of the core and the sialic acid is linked to the core tetrasaccharitol as shown below:



Periodate treatment with subsequent anion exchange chromatography of the fragments from dual labeled ^{3}H-fucose and ^{14}C-glucosamine III-2 resulted in the co-elution of ^{3}H and ^{14}C in the unadsorbed fraction suggesting that fucose and n-acetylglucosamine are in the same branch

TABLE VI

BEHAVIOR OF ^{3}H RADIOACTIVITY AFTER PERIODATE OXIDATION OF ^{3}H-GLN III-2°

Treatment	% Total Gln Recovered	
	Unadsorbed	Adsorbed
Control	16	84
Mild acid	76	24
Periodate	78	22

Control: 0.1M sodium acetate, pH 4.5, 0°C, 18 hr.

Mild acid: 0.05N H_2SO_4, 80°C, 2 hr.

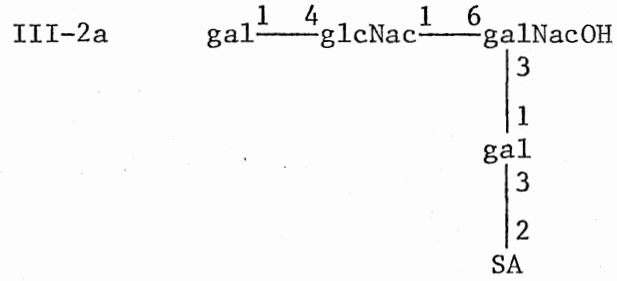
Periodate: 0.1M $NaIO_4$ in 0.01M sodium acetate, pH 4.5, 0°C, 18 hr.

°^{3}H-gln labeled III-2 was treated under the three indicated conditions. After each treatment the samples were then analyzed by anion exchange chromatography and the unadsorbed and adsorbed label was quantitated.

of the core tetrasaccharitol.

Structure of III-2a

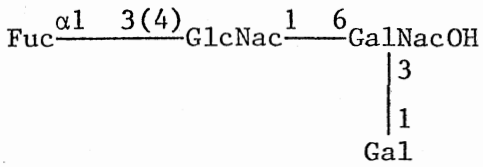
HPLC analysis (Figure 5A and 5B) indicated that III-2Da is identical to II-3Da. GC-MS linkage analysis indicated that every chain in III-2 contained 3,6-linked galNacOH and 4-linked glcNac. Carbohydrate compositional analysis (Table IV) indicated that III-2Da was the tetrasaccharitol $gal_2glcNac_1$ $galNacOH_1$. Periodate oxidation of III-2 (Table VI) indicated that the one sialic acid residue was linked to the galactose which was 3-linked to galNacOH. The structure for III-2a which is consistent with these data is:



Structure of III-2b

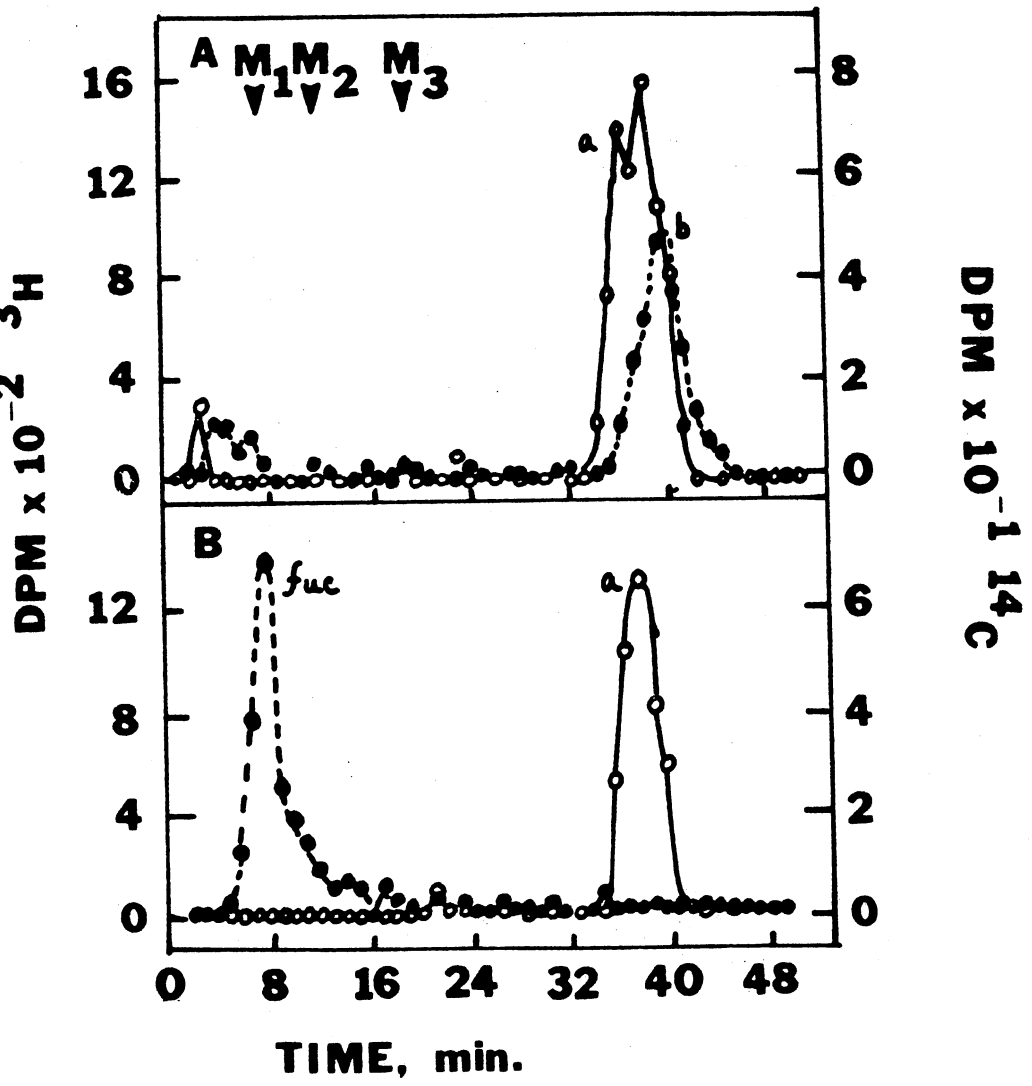
From the glucosamine label that co-elutes with the fucose label on 5μ silica gel (Figure 7) the fucosylated oligosaccharitol III-2b is 12% of the total glucosamine recovered in III-2. The limited amount of this fucosylated component and its poor separation by HPLC made analysis of this oligosaccharitol difficult. Carbohydrate analysis (Table III) had shown that there were at least two galactose and one N-acetylglucosamine residues/N-acetylgalactosaminitol. This suggests that the fucosylated component of III-2 is the product of fucosylation of the core tetrasaccharitol. Thus far the only fucose

to galactose linkages reported for mucous glycoproteins is α1→2. The evidence for a 2-linked galactose from GC-MS linkage (Table V) analysis is weak but there may be two good explanations. First, the fucosylated component is only minor and such a hexose is present in such small amounts that it is not easily detectable. Secondly, a small peak elutes in the region where 2-linked galactose is expected to elute. The positive identification of this peak was hampered not only by its low recovery but also by the presence of hexose contamination possibly introduced into the sample by dialysis and column chromatography steps during isolation procedures. Evidence to support the contention that III-2Db is the fucosylated derivative of the core tetrasaccharitol was obtained from α-fucosidase digestion of this component. The experiments described were done on the identical component present in the more abundant neutral fraction IV-1. The digestion (Figure 8) resulted in the cleavage of 100% of the fucose as evidenced by the label eluting in the monosaccharide region. After removal of fucose the core tetrasaccharitol, a, was retained and the generation of any smaller oligosaccharitols was not evident. This result rules out the possibility that the fucosylated component could be a structure like:

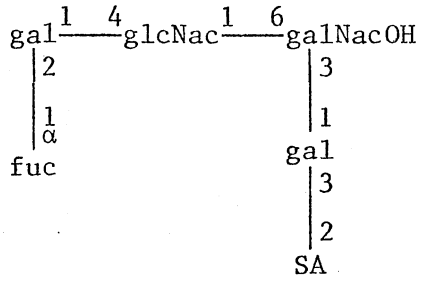


The results of the α-fucosidase digestion are consistent with the structure:

Figure 8. Separation of α-Fucosidase Digestion Products of IV-1a(b) on HPLC. ^{3}H-fucose (•--•) and ^{14}C-gln (o—o) IV-1a(b) was treated with (A) 50mM sodium citrate, pH 5.0 for 48 hr at 37°C under a toluene atmosphere and (B) 0.1 unit of α-fucosidase in 50mM sodium citrate, pH 5.0 for 48 hr at 37°C under a toluene atmosphere. The digestion products were then separated on 5μ silica gel. The peaks are defined as: a. core tetrasaccharitol, $gal_2glcNac_1$ $galNacOH_1$; b. fucosylated oligosaccharitol of unknown composition; fuc: free fucose.


A
M1 M2 M3
a
b
B
fuc
a
16
12
8
4
0
8
6
4
2
0
0
8
16
24
32
40
48
TIME, min.
DPM x 10-2 3H
DPM x 10-1 14C

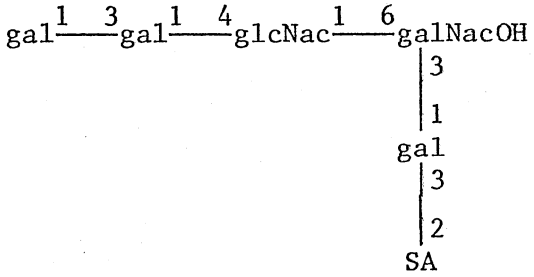
III-2b



However the possibility that fucose is linked α1→3 to galactose cannot be ruled out.

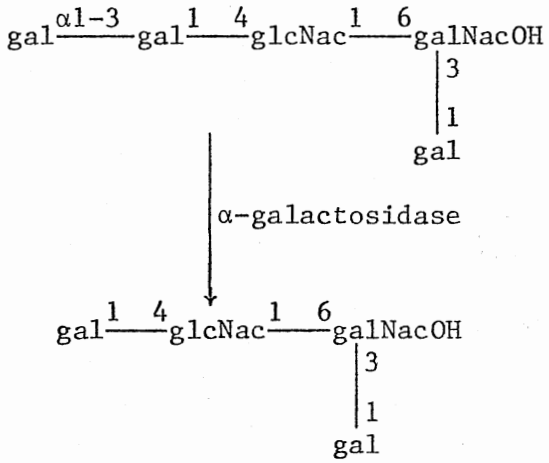
Structure of III-2c

From the glucosamine label recovered in III-2Dc (Figure 7) this oligosaccharitol is ∿40% of the total glucosamine label recovered in the oligosaccharitol fraction III-2D. From carbohydrate analysis (Table IV) this component is the pentasaccharitol, $gal_3glcNac_1 galNacOH_1$. GC-MS linkage analysis of III-2 (Table V) indicated that every chain of III-2 contained at least the trisaccharitol, glcNac 1-6(gal 1-3)galNacOH. Linkage analysis of III-2D (Table V) indicated that after de-sialylation there was still 3-linked galactose present. From this data and the periodate data (Table VI) the most likely structure for III-2c is:



This structure has been found in blood group substances, most recently in human cervical mucin (Yurewicz et al., 1981). In this oligo-

saccharitol the gal→gal linkage is α1→3 whereas the other galactose linkages are β1→3(4). Confirmatory evidence of this structure and the structure of the tetrasaccharitol, III-2Da (II-3Da), was obtained from exoglycosidase digestions. Because of its ease of isolation the exoglycosidase digestions were performed on the fraction IV-1 which has been shown (Figure 5A and 5B) to have the identical components found in II-3D and III-2D. ^{3}H-glucosamine labeled IV-1 was treated with α-galactosidase (Figure 9) and the digestion products analyzed by HPLC on 5μ silica gel. This treatment resulted in the disappearance of IV-1c (III-2Dc) and the retention of IV-1a (II-3Da, III-2Da). This is consistent with the cleavage of an α-linked galactose residue of the pentasaccharitol (below) to produce the core tetrasaccharitol:



These results further suggest that the remainder of the galactose linkages are β-linkages. ^{3}H-glucosamine labeled IV-1 was then treated with β-galactosidase and α-galactosidase simultaneously and the digestion products separated on 5μ silica gel (Figure 10). Conditions were such that complete digestion was not expected.

Figure 9. HPLC Elution Profiles of α-Galactosidase Digestion Products of IV-1. ^{3}H-gln (o—o) IV-1 was treated with (A) 50mM sodium citrate, pH 4.5 for 1 hr at 37°C under a toluene atmosphere, and (B) 0.1 unit of α-galactosidase in 50mM sodium citrate, pH 4.5 for 1 hr at 37°C under a toluene atmosphere. The digestion products were analyzed on 5 μ silica gel.

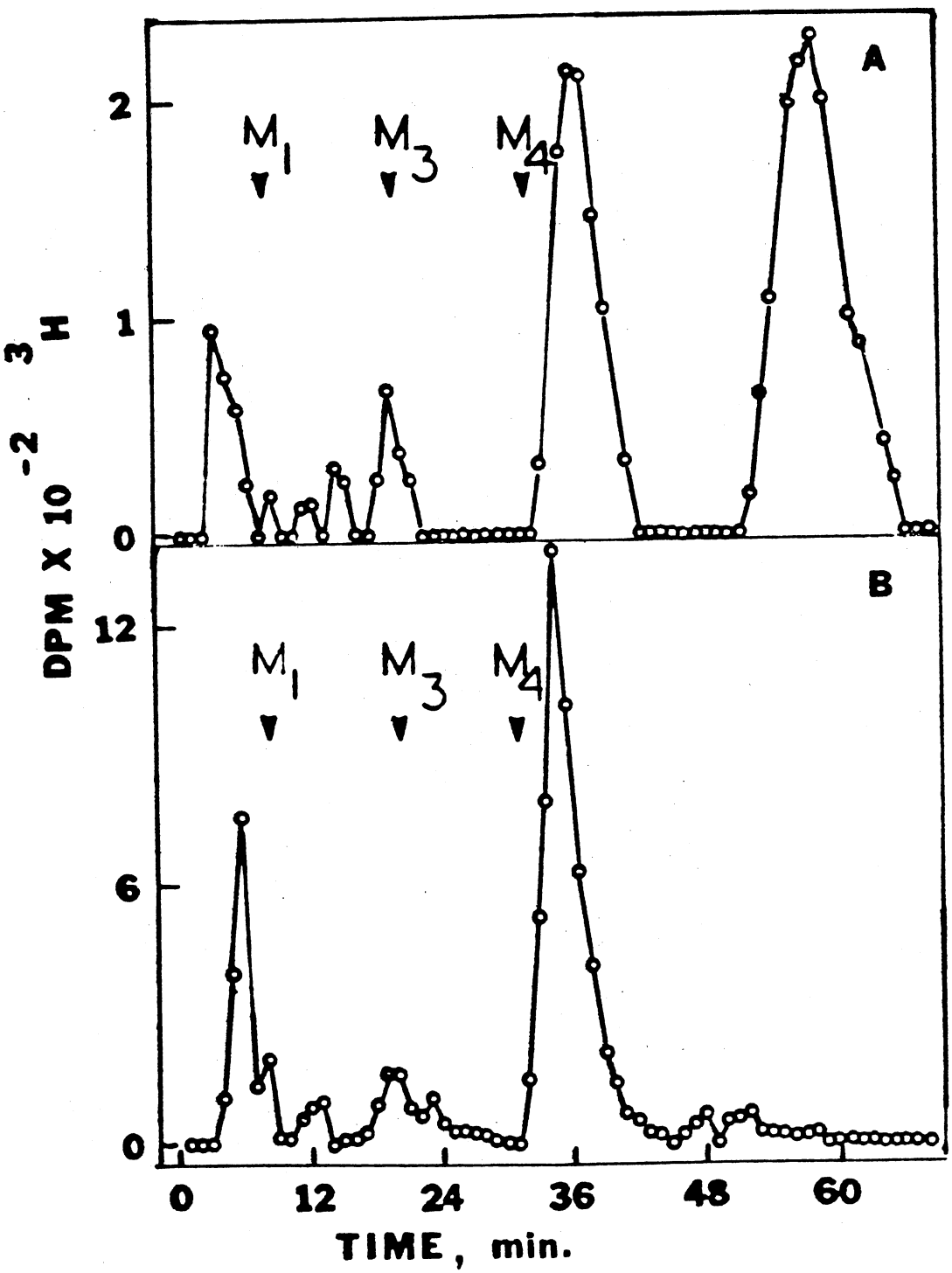
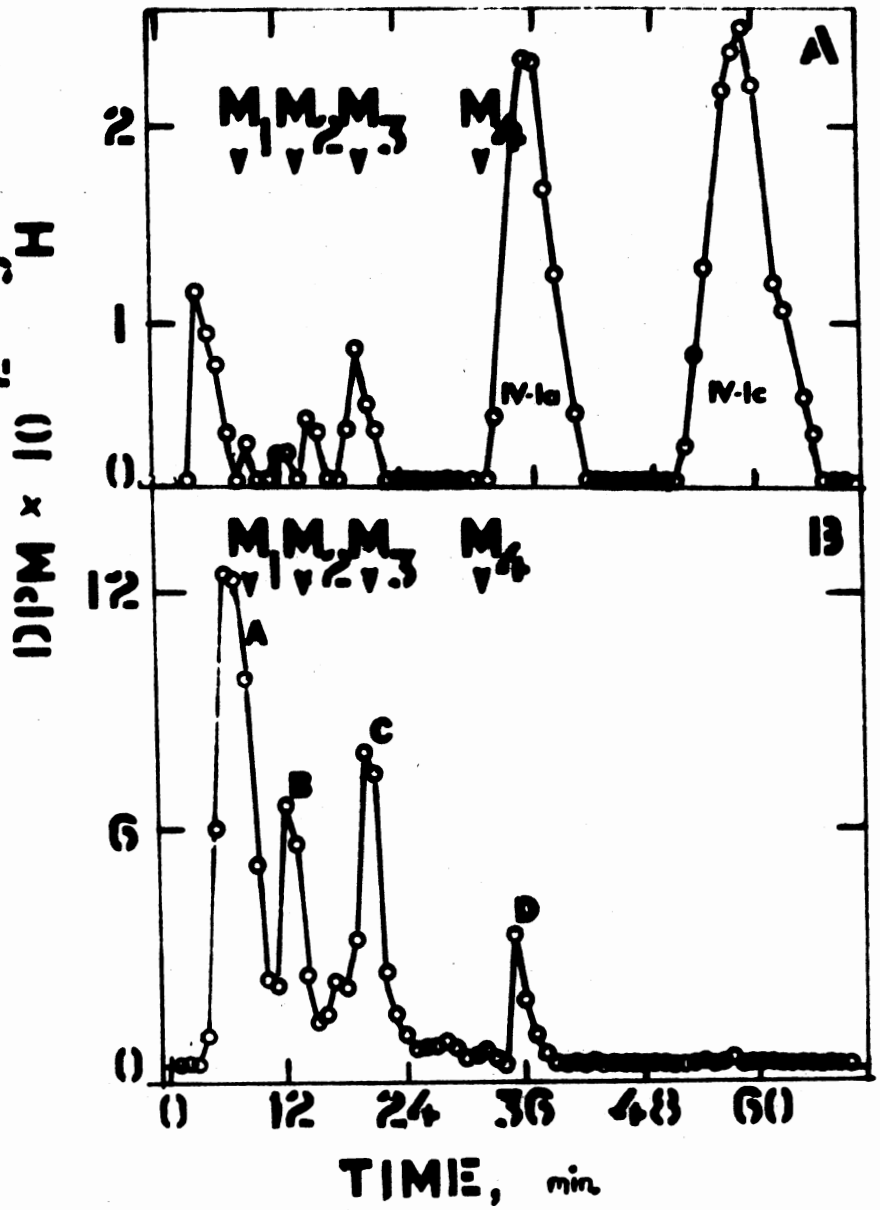

A
B
M1
M3
M4
DPM X 10-2 3H
2
1
0
12
6
0
0
12
24
36
48
60
TIME, min.

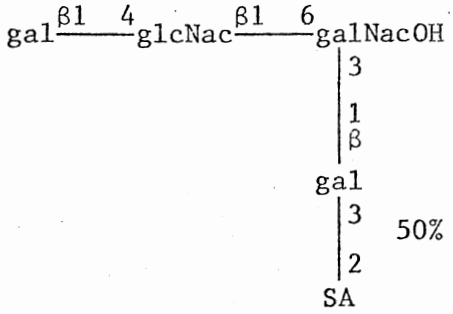
Figure 10. HPLC Elution Profiles of α-Galactosidase and β-Galactosidase Digestion Products of IV-1. ^{3}H-gln (o—o) IV-1 was treated with (A) 50mM sodium citrate, pH 4.5 for 1 hr at 37°C under a toluene atmosphere and (B) 0.1 unit of β-galactosidase + 0.015 unit of α-galactosidase in 50mM sodium citrate, pH 4.5 for 1 hr at 37°C under a toluene atmosphere. The digestion products were analyzed on 5 μ silica gel.


A
M1 M2 M3 M4
IV-1a
IV-1c
B
M1 M2 M3 M4
A
B
C
D
DPM x 10^-2 ³H
0
1
2
0
6
12
0
12
24
36
48
60
TIME, min.

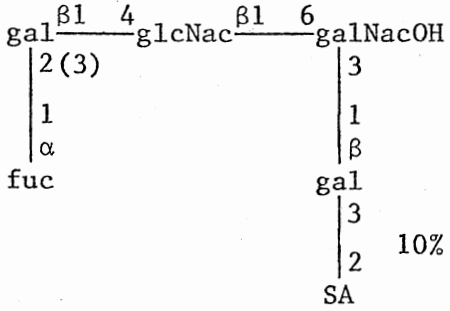
These conditions were employed to minimize the action of a contaminating β-hexosaminidase (<2.0%) in β-galactosidase and to generate all the possible digestion products. 5%, 24%, 19%, and 52% of the glucosamine label eluted as a tetrasaccharitol, trisaccharitol, disaccharitol, and monosaccharide(itol), respectively. The tetrasaccharitol, D, is presumably the core structure, IV-1a (II-3Da, III-2Da) resulting from the cleavage of the α-linked galactose residue of the pentasaccharitol, IV-1c (III-2Dc). The trisaccharitol, C, is presumably $gal_1glcNac_1$ $galNacOH_1$ which would result from the loss of one galactose residue from the tetrasaccharitol, IV-1a (II-3Da, III-2Da). The disaccharitol, B, is $gal_1galNacOH_1$ resulting from the loss of glcNac and/or $glcNac_1$ $galNacOH_1$ resulting from the loss of two galactose residues from the core tetrasaccharitol. Either is likely because from analysis of a glucosamine labeled ASGP-1 hydrolyzate on an amino acid analyzer the radioactive label was essentially evenly distributed between glucosamine and galactosamine. The label eluting in the monosaccharide(itol) region is presumably glcNac resulting from the action of the contaminating β-hexosaminidase and/or galNacOH resulting from the complete digestion of the fraction IV-1. The results of these digestion studies indicated that IV-1c (III-2Dc) is the α-galactosylated product of the core tetrasaccharitol, IV-1a (III-2Da) and that the linkages of the core tetrasaccharitol are of the β-conformation. From gel filtration data, carbohydrate composition, GC-MS linkage analysis, HPLC analysis, periodate oxidation studies, and exoglycosidase studies the fraction III-2 was deduced to be a mixture of three oligosaccharitols, III-2a, III-2b, and III-2c which represent

50%, 10%, and 40% of the total glucosamine recovered.

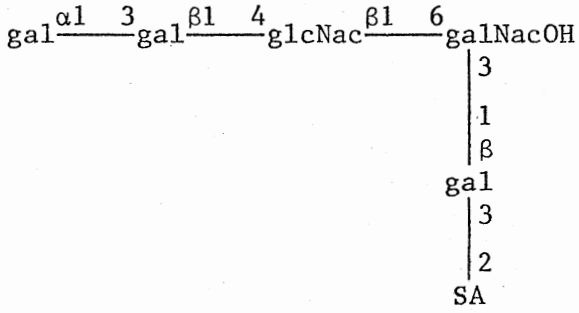
III-2a:



III-2b:



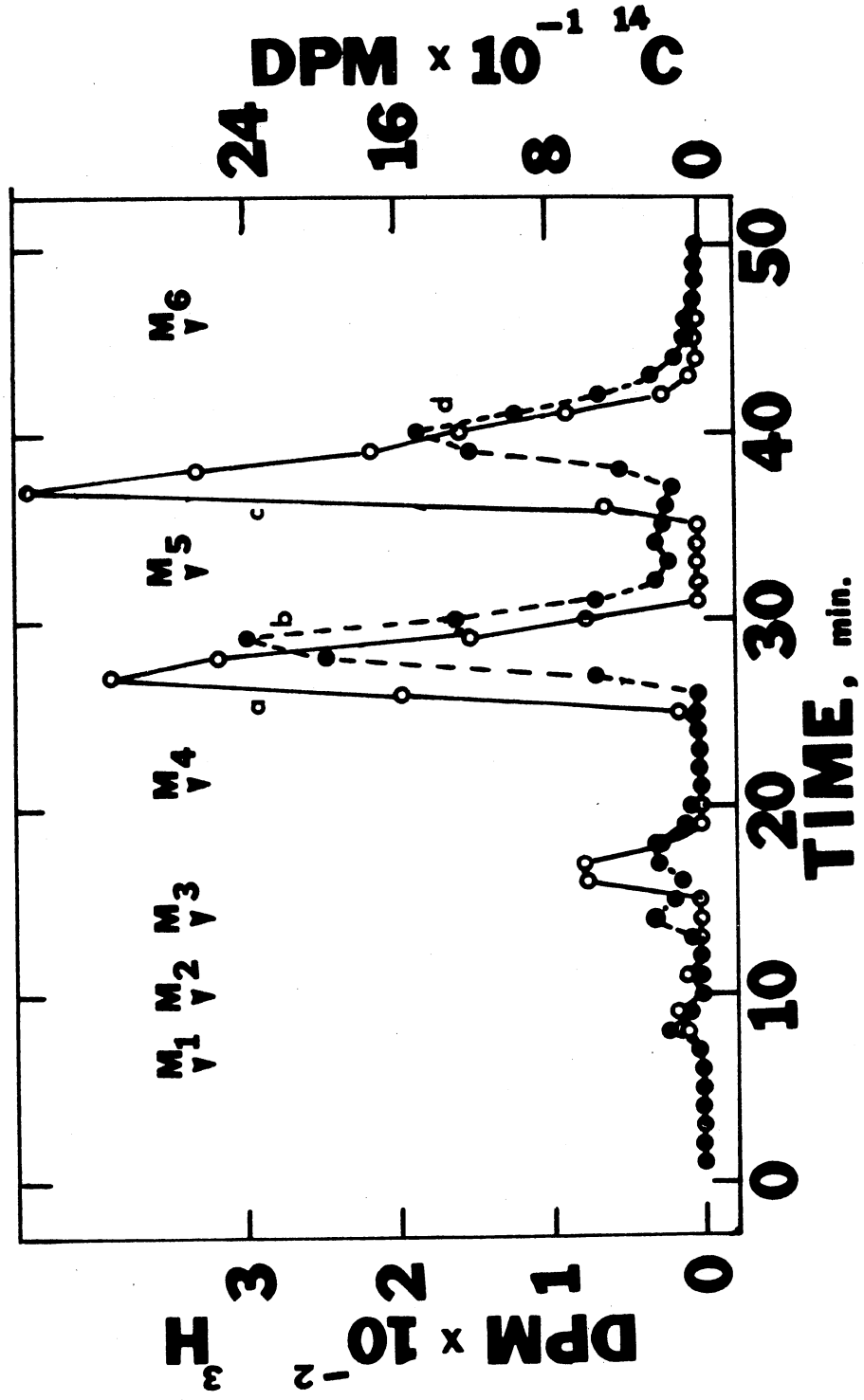
III-2c:



Heterogeneity of IV-1

The heterogeneity of IV-1 was studied by HPLC analysis (Turco, 1981; White et al., 1980). ^{3}H-fucose and ^{14}C-glucosamine dual labeled IV-1 HPLC elution profile (Figure 11) revealed at least four oligosaccharitol chains, two non-fucosylated (IV-1a and IV-1c) and two fucosylated (IV-1b and IV-1d). An attempt to better quantitate the components of IV-1 was made by dividing the Bio-gel P4 fraction into three components and analyzing each separately by HPLC. The

Figure 11. HPLC Elution Profile of IV-1. Dual labeled ^{3}H-fucose (•--•) and ^{14}C-gln (o—o) IV-1 was analyzed on 5 μ silica gel modified with 1,4-diaminobutane as described in Methods.


DPM x 10⁻¹ ¹⁴C
24
16
8
0
M6
M5
M4
M3
M2
M1
a
b
c
d
e
50
40
30
20
10
0
TIME, min.
DPM x 10⁻² ³H
3
2
1
0

three fractions analyzed were IVa (Figure 12A), IV (Figure 12B), and IVb (Figure 12C), the high, median, and low molecular weight fractions, respectively, of IV-1. Quantitation of the four oligosaccharitols was estimated by recovery of glucosamine label and is summarized in Table VII. From this experiment it appeared that the nonfucosylated oligosaccharitols made up almost 70% of the oligosaccharitols of IV-1.

GC-MS Linkage Analysis of IV-1

To obtain linkage information on IV-1, GC-MS analysis of the partially methylated alditol acetates was performed. The entire fraction from Bio-gel P4 was analyzed to insure that enough material was available for analysis. The results are summarized in Table VIII. IV-1 contained approximately equimolar amounts of 3,6-linked galNacOH and 4-linked glcNac. This fraction contained 2-3 times more terminal galactose than internal 3-linked galactose. Terminal fucose was detected in very small amounts. 2-linked galactose was possibly present in small quantities however its precise identification was hampered by its low recovery. The low recovery of fucose could be indicative of a small amount of fucosylated oligosaccharitols in IV-1. Thus all indications are that IV-1 is a heterogeneous fraction in which all chains contain at least the trisaccharitol glcNac1-6(gal 1-3)-galNacOH to which galactose and fucose are variously substituted.

Structure of IV-1a

Comparative HPLC profiles (Figure 5) indicated that IV-1a and II-3D are chromatographically identical. Carbohydrate composition (Table IV) indicated that IV-1a is the tetrasaccharitol, $gal_2glcNac_1$-

Figure 12. HPLC Elution Profiles of the Sub-fractions of IV-1. ^{3}H-fucose (●--●) and ^{14}C-gln (o—o) IV-1 was sub-fractionated into (A) IVa, the high molecular weight sub-fraction of IV-1 from Bio-gel P4, (B) IV, the medium molecular weight fraction of IV-1 from Bio-gel P4, and (C) IVb, the low molecular weight fraction of IV-1 from Bio-gel P4 and subsequently analyzed by HPLC on 5 μ silica gel modified with 1,4-diaminobutane as described in Methods.

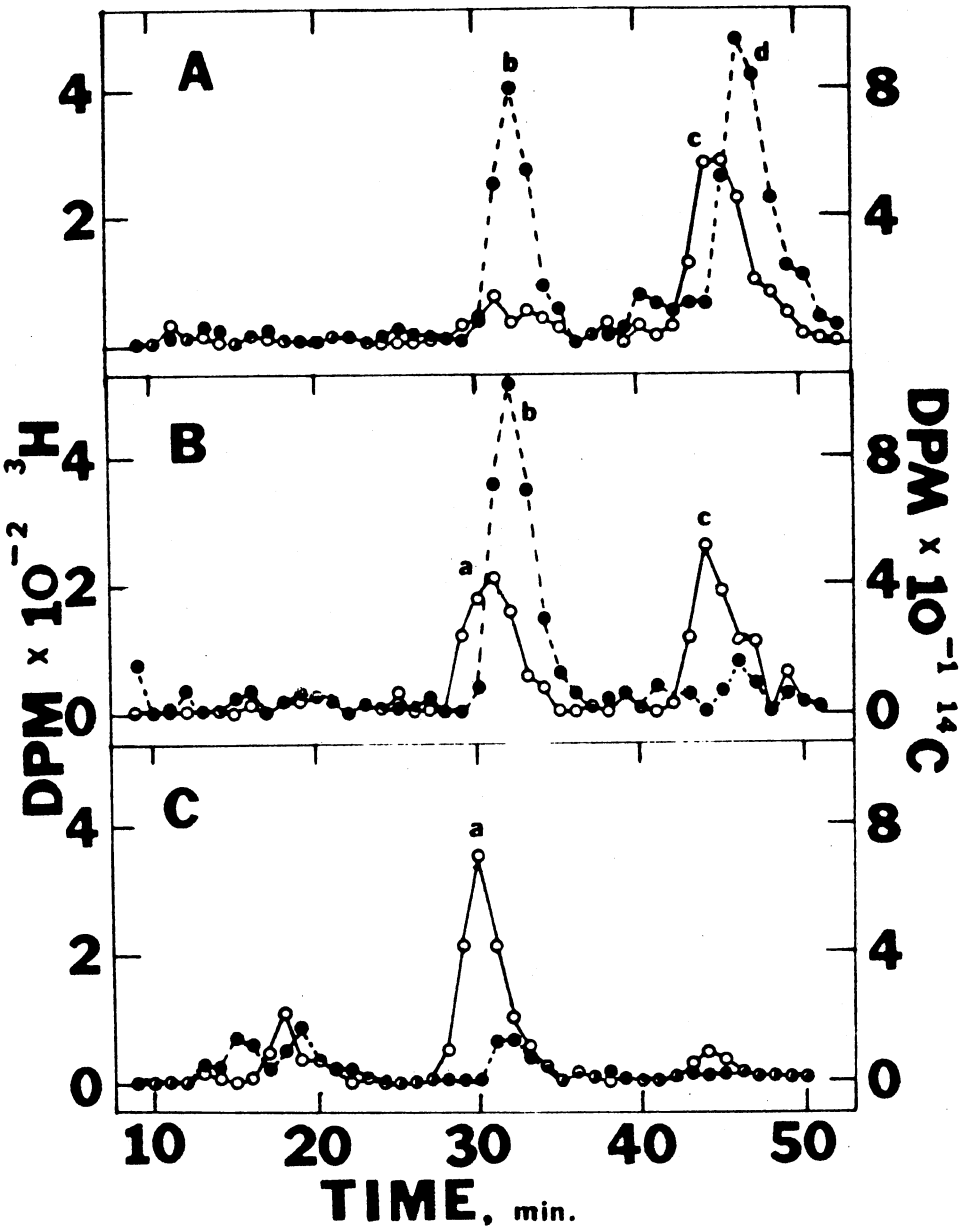

A
B
C
a
b
c
d
DPM x 10^-2 ³H
DPM x 10^-1 ¹⁴C
TIME, min.
10
20
30
40
50
0
2
4
8

TABLE VII

RECOVERY AND RELATIVE AMOUNTS OF THE FOUR OLIGOSACCHARITOLS OF IV-1

Fraction	% GlcNac Recovered°	Relative Ratio
IV-1a	35	2.7
IV-1b	13	1
IV-1c	33	2.5
IV-1d	18	1.4

°Radioactive counts in each fraction were quantitated and compared to the total (all fractions) recovered.

TABLE VIII

THE PARTIALLY METHYLATED ALDITOL ACETATES OF IV-1°

Component	Relative Amount
Terminal fucose	0.17
Terminal galactose	1.48
3-linked galactose	0.53
2-linked galactose	+*
3,6-linked galNacOH	1.0
4-linked glcNac	0.87

*Its presence is suspected, arguments presented in text. 3,6-galNacOH taken as 1.0.

°Analysis by GC-MS and comparison to standards was done as described in legend to Table V.

galNacOH$_1$. GC-MS data (Table VIII) indicated that every chain in the fraction IV-1 contained 3,6-linked galNacOH and 4-linked glcNac. These data are consistent with the contention that IV-1a has the identical structure proposed earlier for II-3D and III-2Da.

Structure of IV-1c

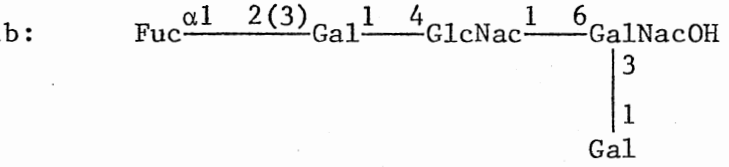
Carbohydrate compositional analysis (Table IV) indicated that IV-1c was the pentasaccharitol, gal$_3$glcNac$_1$galNacOH$_1$. Digestion of the fraction IV-1 with α-galactosidase (Figure 9) indicated that the third galactose residue of IV-1c is α-linked to the tetrasaccharitol IV-1a. HPLC co-elution (Figure 5) indicated that IV-1c is chromatographically identical to III-2Dc. From these arguments it follows that IV-1c has the structure identical to III-2Dc.

Structure of the Fucosylated Oligosaccharitols, IV-1b and IV-1d

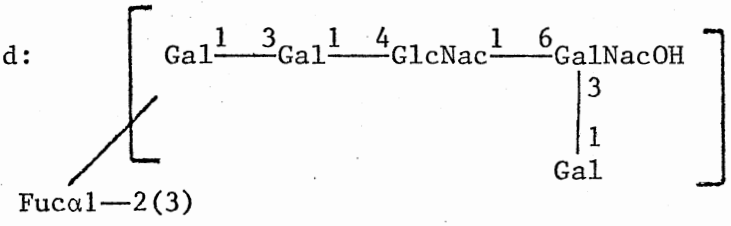
The fucosylated oligosaccharitols were available only in limited amounts for structural analysis. For this reason structural information has been deduced from experiments with the complete fraction IV-1 rather than the individual components separated on silica gel. α-Fucosidase treatment of ^{3}H-fucose and ^{14}C-gln dual labeled IV-1 resulted in a 95% release of the fucose as monitored by HPLC analysis (data not shown). This suggested that the fucose linkage to the saccharide is an α-linkage. Comparison of the de-sialylated core oligosaccharitol fraction III-2 with IV-1 by HPLC on 5 μ silica gel (compare Figure 7 with Figure 11) suggested that IV-1b is identical to III-2Db. Thus it appears that IV-1b is simply the core tetra-

saccharitol fucosylated at one of the galactose residues. On the basis of periodate oxidation studies of III-2 described previously the galactose linked 1→4 to glcNac is fucosylated. It follows from these arguments for IV-1b that IV-1d is the fucosylated product of IV-1c. Further evidence that these fractions are the fucosylated products of IV-1a and IV-1c was obtained from degradation studies. IV-1 treated with 0.05N H_2SO_4 at 100°C for two hours, conditions shown by other workers (Kobata et al., 1978) to preferentially remove fucose, resulted in the retention of the non-fucosylated components IV-1a and IV-1c without any evidence for the generation of any smaller oligosaccharitols (Figure 13). Treatment with α-fucosidase gave similar results. From these experiments a tentative assignment of structure for the fucosylated oligosaccharitols of IV-1 are:

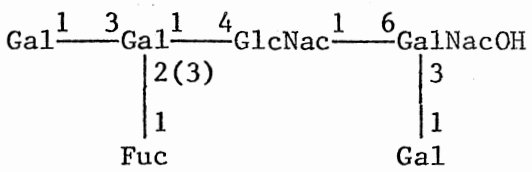
IV-1b:



IV-1d:

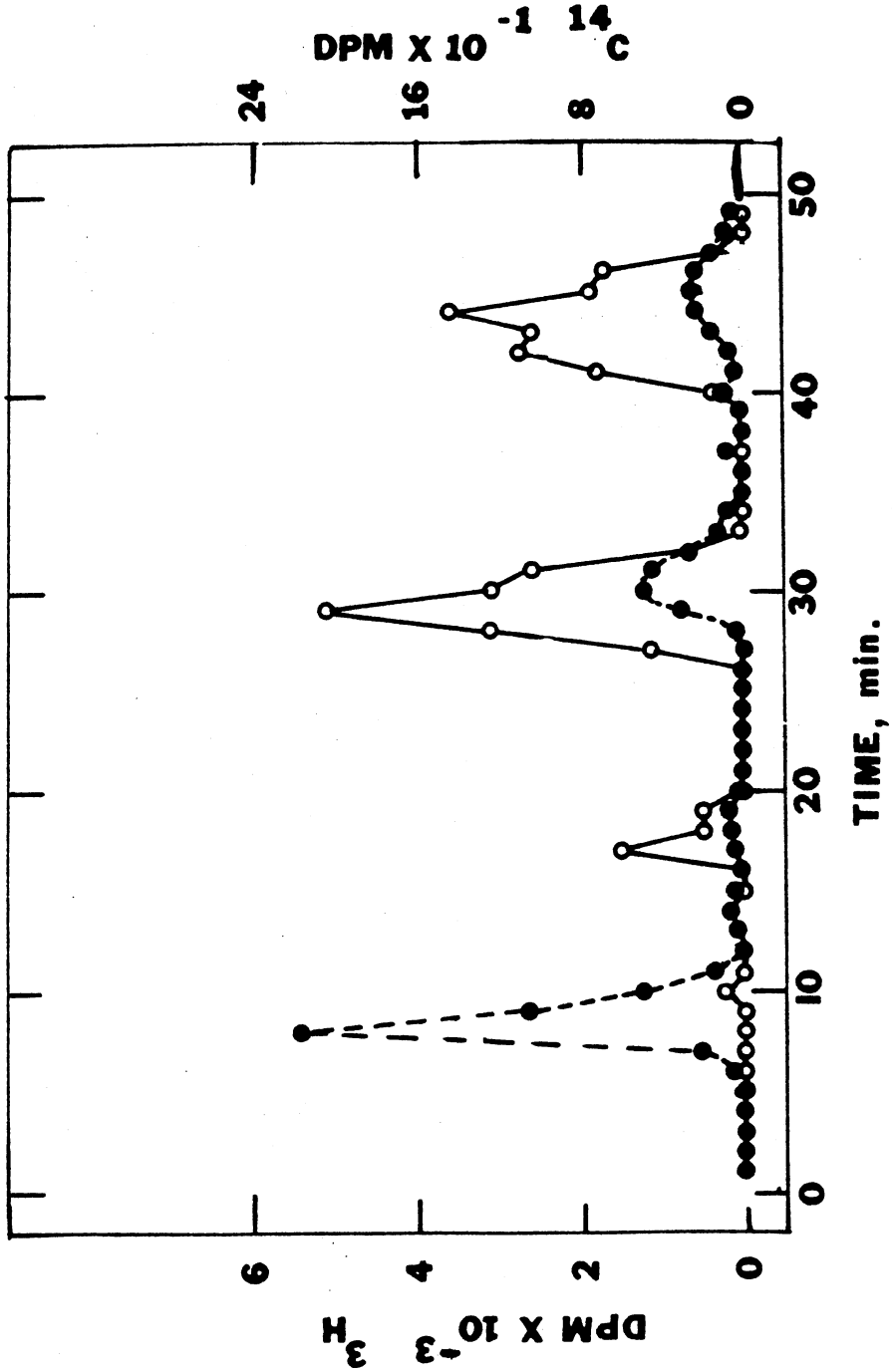


The most likely structure for IV-1d is:

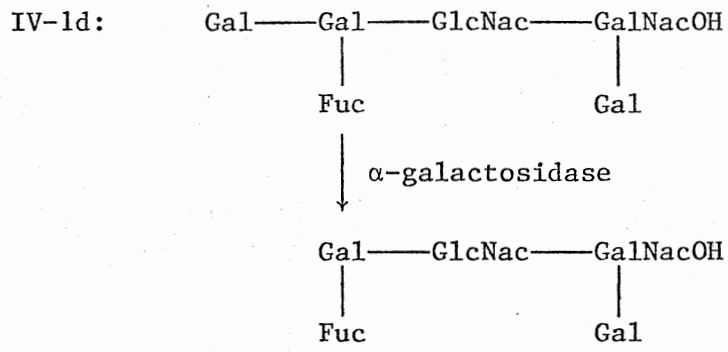


α-Galactosidase treatment of glucosamine labeled IV-1 (Figure 9)

Figure 13. HPLC Elution Profile of IV-1 Treated with Mild Acid Under Conditions Which Preferentially Cleave Fucose Residues. ^{3}H-Fucose (●--●) and ^{14}C-gln (o—o) dual labeled IV-1 was treated with mild acid (0.05N H_2SO_4, 100°C, 2 hr), desalted as described in Methods and analyzed on 5 μ silica gel.


DPM X 10 -1 14 C
24
16
8
0
50
40
30
20
10
0
TIME, min.
6
4
2
0
DPM X 10 -3 3 H

resulted in the complete disappearance of the IV-1c and IV-1d with the retention of IV-1a and IV-1b. This would be consistent with the proposed structure of IV-1d since loss of one galactose residue would convert IV-1d into IV-1b:

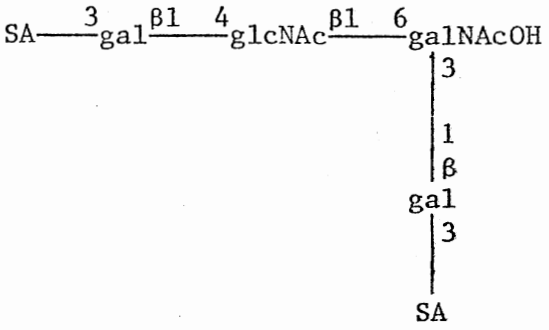


Discussion

From the data accumulated, ASGP-1 contains eight glucosamine-containing oligosaccharitols, four acidic (Figure 14) and four neutral (Figure 15). It appears that the sialylated oligosaccharitols of ASGP-1, II-3, III-2a, III-2b, and III-2c, have a common biosynthetic precursor core tetrasaccharitol galβ1-4glcNacβ1-6 (galβ1-3)galNacOH. This biosynthetic precursor has been previously isolated from human ovarian cyst blood group substance (Rovis et al., 1973), human gastric mucin (Oates et al., 1974), desialylated ovine colostrum casein (Soulier et al., 1980), human cervical mucin (Yurewicz et al., 1981), and the ascites hepatoma, AH 66 (Funakoshi et al., 1982). II-3 and the major fraction of III-2 were established to be the di-sialo and mono-sialo adducts, respectively of the core tetrasaccharitol. This is an interesting finding in that very few oligosaccharitols with the glcNac 1-6(galβ1-3) galNacOH sequence have been found to be sialylated. Williams and associates (Williams et al., 1980) speculated that this sequence

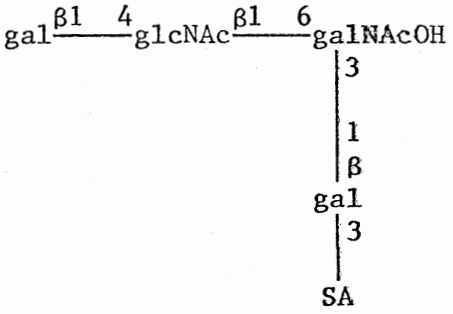
Figure 14. Proposed Structures of the Sialic Acid Containing Oligosaccharitols of ASGP-1.

II-3:



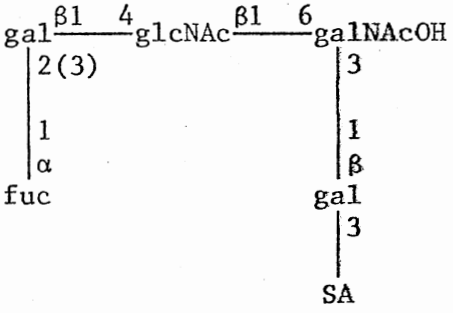
9%

III-2a:



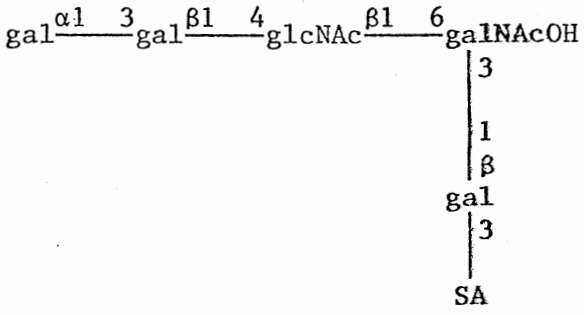
13%

III-2b:



3%

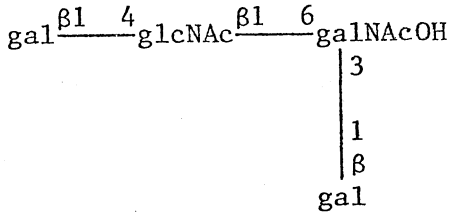
III-2c:



10%

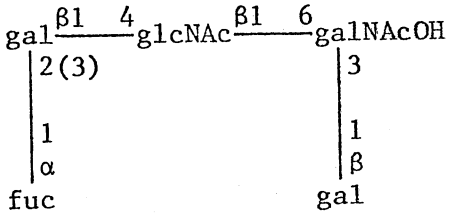
Figure 15. Proposed Structures of the Neutral Oligosaccharitols of ASGP-1.

IV-1a:



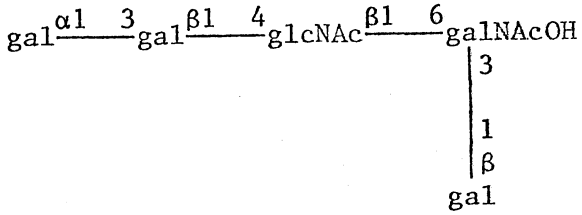
17%

IV-1b:



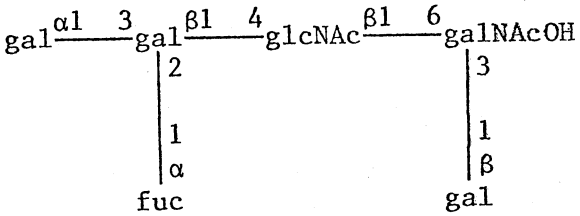
6%

IV-1c:



17%

IV-1d:



10%

signaled the mucin biosynthetic machinery to synthesize a neutral oligosaccharitol. The only other mucins to my knowledge that contain such sialic acid containing oligosaccharitols are surface glycopeptides of the ascites hepatoma, AH 66, characterized by Funakoshi (Funakoshi et al., 1982) and human cervical mucin recently characterized by Yurewicz and colleagues (Yurewicz et al., 1981). Yurewicz reported the presence of the mono-sialylated derivative of the core tetrasaccharitol. The disialohexasaccharitol, II-3, and the monosialopentasaccharitol, III-2a, are found in glycopeptides isolated from the ascites hepatoma, AH 66. It appears then that the theory put forth by Williams is not applicable to the assembly of all mucous-like glycoproteins. An alternative explanation is that the core tetrasaccharitol is sialylated in some early step soon after the assembly of the neutral tetrasaccharitol. At a later stage during oligosaccharitol assembly some regulatory mechanism, possibly in the form of a neuraminidase activity, is turned on or off depending on a number of factors including substrate availability, intracellular pool concentration, or pH. In the case of ASGP-1 the regulatory mechanism may not be as effective as in other mucins and thus the sialic acid would not be removed from ASGP-1 as readily as from other mucous glycoproteins.

The amount of fucosylated oligosaccharitols of ASGP-1 are relatively minimal. From HPLC analysis of ASGP-1 metabolically labeled with fucose at least 10% of the oligosaccharitols of III-2 are fucosylated. Thus ASGP-1 contains some oligosaccharitols with both fucose and sialic acid. Such an oligosaccharitol has been reported only in a few systems, one being porcine submaxillary

mucin (Carlson, 1968). Dische (Dische, 1963) first observed a reciprocal relation between the sialic acid content and fucose content of mammalian glycoproteins. An enzymatic basis for this observation was described by Beyer (Beyer et al., 1979). Beyer found that only the fucα1→2galβ1→3(NeuAcα1-6)galNac structure could be synthesized. The oligosaccharitol III-2b is similar to this structure in one respect. The fucose and sialic acid are found in separate branches of the structure. It seems tempting to speculate that the proximity of a sialic acid to the site of fucose transfer may provide an additional means of control. From periodate studies it was concluded that fucose and sialic acid are in different branches of the core tetrasaccharitol. GC-MS linkage analysis and α-fucosidase digestions suggested that fucose is linked α1→2 to the galactose which is linked 1→4 to glcNac. The absence of any fucosylated oligosaccharitol in which the galactose linked 1→3 to galNacOH is fucosylated suggests that fucosylation is not a random reaction but instead strictly controlled. One control mechanism could be manifested in the sialylation of the core tetrasaccharitol. Before fucosylation can occur the core must first be sialylated to give II-3, de-sialylated to give the mono-sialylated core which is then a substrate for a fucosyltransferase. III-2c is a unique sialylated oligosaccharitol and has not been reported to my knowledge in the literature. It can easily be generated in a similar fashion as proposed for III-2b. Instead of the transfer of fucose to galactose the transfer of galactose is effected by a galactosyl transferase. A galactosyl transferase which catalyzes this transfer has been isolated from human plasma (Nagai et al., 1978) and Ehrlich

ascites tumor cells (Blake et al., 1981).

The neutral fraction IV-1 consists of at least four oligosaccharitols. Three of the chains, IV-1a, IV-1b, and IV-1c, are identical to the structures of de-sialylated II-3, III-2a, III-2b, and III-2c. IV-1d is a fucosylated component not found in any of the other fractions. On the basis of mild acid hydrolysis, α-fucosidase digestion, α-galactosidase digestion, and its elution behavior on 5 μ silica gel this oligosaccharitol appears to have a structure identical to the B blood group determinant found in the blood group substances. This structure was also recently found in cervical mucin (Yurewicz et al., 1982).

Thus it appears that the oligosaccharitols of ASGP-1 are all assembled from the core tetrasaccharitol, IV-1a. The peripheral sugars sialic acid, fucose, and galactose are apparently transferred to the core tetrasaccharitol via a complex process that appears to be partly controlled since only a few of the possible structures are present in detectable amounts. The nature of this complex process is the subject of present research efforts.

CHAPTER III

COMPARISON OF THE OLIGOSACCHARITOLS OF ASGP-1 FROM MAT-B1 AND MAT-C1 ASCITES RAT MAMMARY ADENOCARCINOMA CELLS

Introduction

MAT-B1 and MAT-C1 ascites tumor cells, variants obtained from the 13762 rat mammary adenocarcinoma, have been shown to exhibit distinct differences in certain cell surface properties which are summarized in Table VIII. It is interesting that the two sublines exhibit a difference in transplantability but both contain the major cell surface sialoglycoprotein, ASGP-1. Cell surface sialoglycoproteins have been implicated in transplantation phenomena. The allotransplantable mouse mammary adenocarcinoma, TA3-Ha, contains a major sialoglycoprotein, epiglycanin, which is not present on the non-allotransplantable cell, TA3-St. Codington and his colleagues (Codington et al., 1973) have postulated that epiglycanin masks the histocompatability antigens on the cell surface. The structural detail of such a mechanism has not been elucidated. The presence of sialic acid could possibly play a role by creating a negative charge on the cell surface that could prevent the approach of any circulating antibodies.

The rat mammary system may provide a system in which structural requirements of xenotransplantability can be studied. MAT-B1 and

TABLE IX

CELL SURFACE PROPERTIES OF TWO RAT MAMMARY ASCITES TUMORS

Property	B1	C1
Xenotransplantability	-	+
Receptor mobility	+	-
Branched microvilli	-	+
ASGP-1	+	+

MAT-C1 both contain ASGP-1. The amino acid composition of ASGP-1 is similar for both sublines. MAT-C1 ASGP-1 contains at least 2 times more sialic acid than MAT-B1 ASGP-1. The oligosaccharitol structure of ASGP-1 has been partially characterized (Chapter II). It is of interest to compare the oligosaccharitols of MAT-B1 and MAT-C1 ASGP-1 to determine if any differences in structure can be correlated with xenotransplantability. Any differences in structure may provide clues to the structure-function relationships of sialoglycoproteins involved in transplantation phenomena of tumor cells. In addition, differences in structure may provide valuable insights into the biosynthetic mechanisms by which mucous membrane glycoproteins are glycosylated.

Materials

D-(6-^{3}H) glucosamine (2-6Ci/mmole), D-(1-^{14}C) glucosamine (56mCi/mmole), and D-(1-^{3}H) fucose (25mCi/mmole) were from Amersham; Instagel was from Packard. Silica gel and acetonitrile were from Waters Associates.

Methods

Xenotransplantation

For each tumor line tested, ten C57B1/6J female mice were injected with 0.25 ml of 0.9% NaCl, pH 7.4. Controls were injected with 0.25 ml saline. Mice were observed for at least 90 days. All other methods are as described in Chapter II.

Results

Comparison of the O-linked Oligosaccharitols of MAT-B1 and MAT-C1 ASGP-1

ASGP-1 from MAT-C1 cells metabolically labeled with ^{3}H-glucosamine and ASGP-1 from MAT-B1 cells metabolically labeled with ^{14}C-glucosamine were mixed together, treated with alkaline borohydride to release O-linked oligosaccharides, and separated by gel filtration on Bio-gel P4 (Figure 16). MAT-B1 ASGP-1 and MAT-C1 ASGP-1 contain three major glucosamine-containing oligosaccharitol fractions II, III, and IV. MAT-B1-II, MAT-B1-III, and MAT-B1-IV represent 15, 40, and 35%, respectively of the glucosamine label. MAT-C1-II, MAT-C1-III, and MAT-C1-IV represent 39, 34, and 10% of the glucosamine label (Table IX).

Sialylated Oligosaccharitols of MAT-B1 and MAT-C1 ASGP-1

Oligosaccharitol fractions II and III of ASGP-1 have been shown by anion exchange chromatography to be sialic acid containing components. DEAE-sephadex co-chromatography of MAT-B1 and MAT-C1-II (Figure 17A) suggested that II from both sublines was disialylated and co-chromatography of MAT-B1 and MAT-C1-III (Figure 17B) suggested that III from both sublines is monosialylated. In agreement with previous findings (Buck et al., 1980) the gel filtration profile (Figure 16) indicated that there was at least two times as much disialosaccharitol II in MAT-C1 ASGP-1 as in MAT-B1 ASGP-1. Therefore the possibility exists that increased sialic acid content of ASGP-1 can be correlated with xenotransplantability.

Figure 16. Bio-gel P4 Elution Profile of the O-linked Oligosaccharitols of MAT-B1 ASGP-1 Chromatographed with MAT-C1 ASGP-1 O-linked Oligosaccharitols. ASGP-1 from MAT-C1 cells and MAT-B1 cells metabolically labeled with ^{3}H-gln (•--•) and ^{14}C-gln (o—o) respectively was isolated, mixed together, treated with alkaline borohydride, and eluted on Bio-gel P4 as described in Methods.

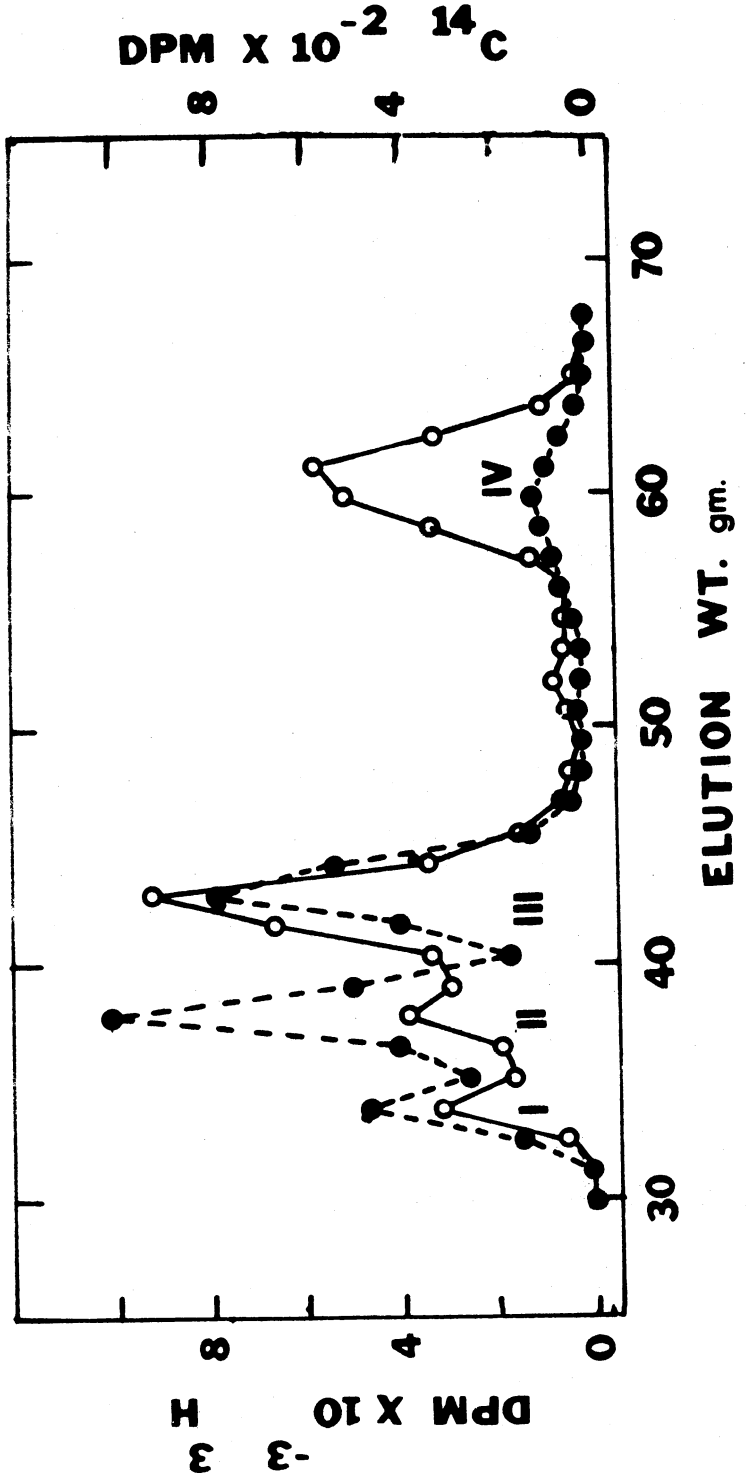

DPM X 10^{-2} ^{14}C
8
4
0
70
60
50
40
30
ELUTION WT. gm.
IV
III
II
I
8
4
0
DPM X 10^{-3} ^{3}H

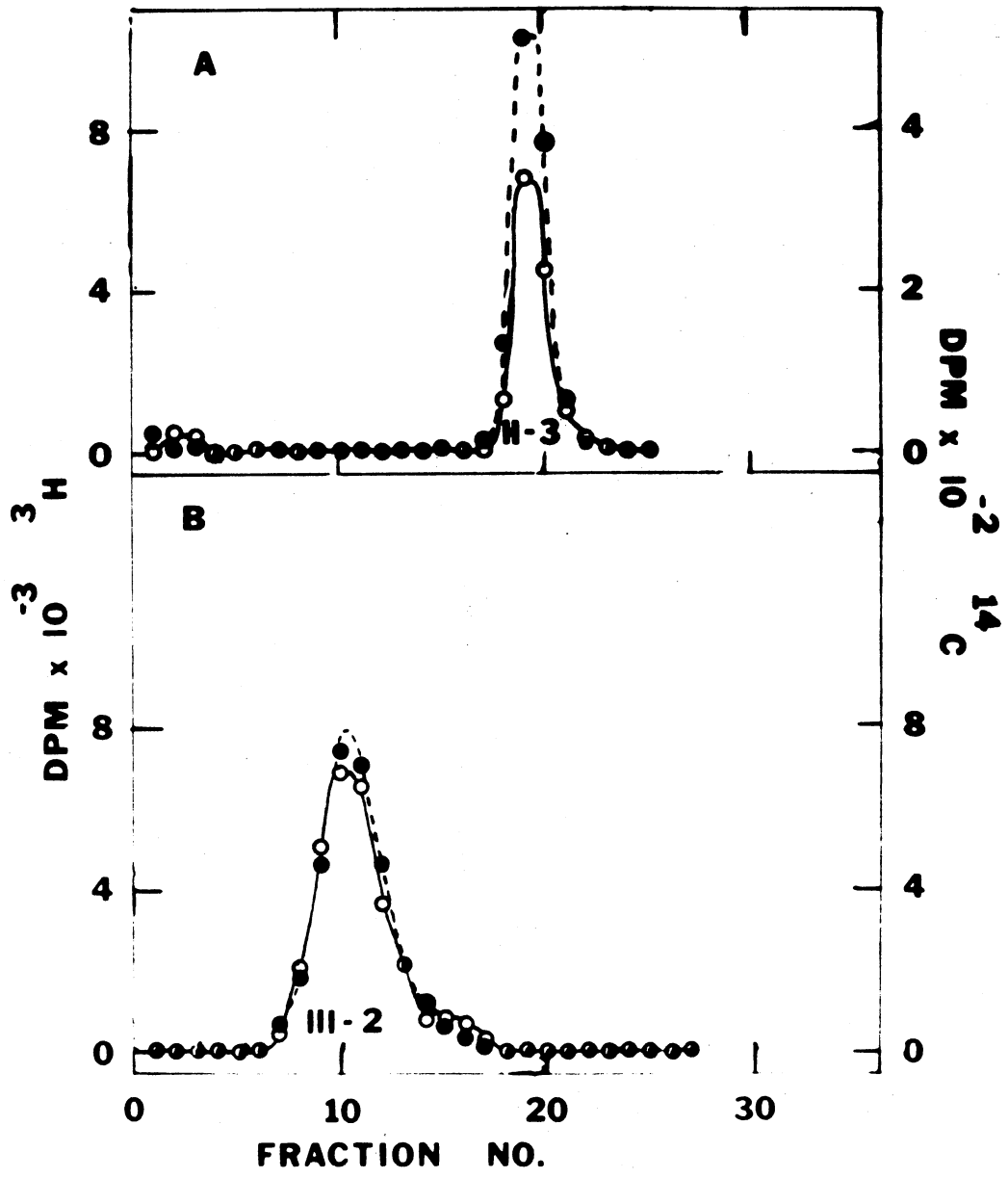
TABLE X

RECOVERY OF GLUCOSAMINE LABEL IN THE OLIGOSACCHARITOL FRACTIONS OF MAT-B1 AND MAT-C1 ASGP-1

Fraction	% Glucosamine Recovered°	
	MAT-B1	MAT-C1
I	8.7	16.3
II	15.6	39.3
III	40.5	34.5
IV	35.0	9.8

°Radioactive counts were quantitated for each fraction and compared to the total (all fractions) recovered.

Figure 17. DEAE-Sephadex Elution Profiles of the Sialylated Oligosaccharitols of MAT-B1 ASGP-1 Chromatographed Together with the Sialylated Oligosaccharitols of MAT-C1 ASGP-1. (A) ^{3}H-gln (●--●) MAT-C1-II and ^{14}C-gln (o—o) MAT-B1-II. (B) ^{3}H-gln (●--●) MAT-C1-III and ^{14}C-gln (o—o) MAT-B1-III.

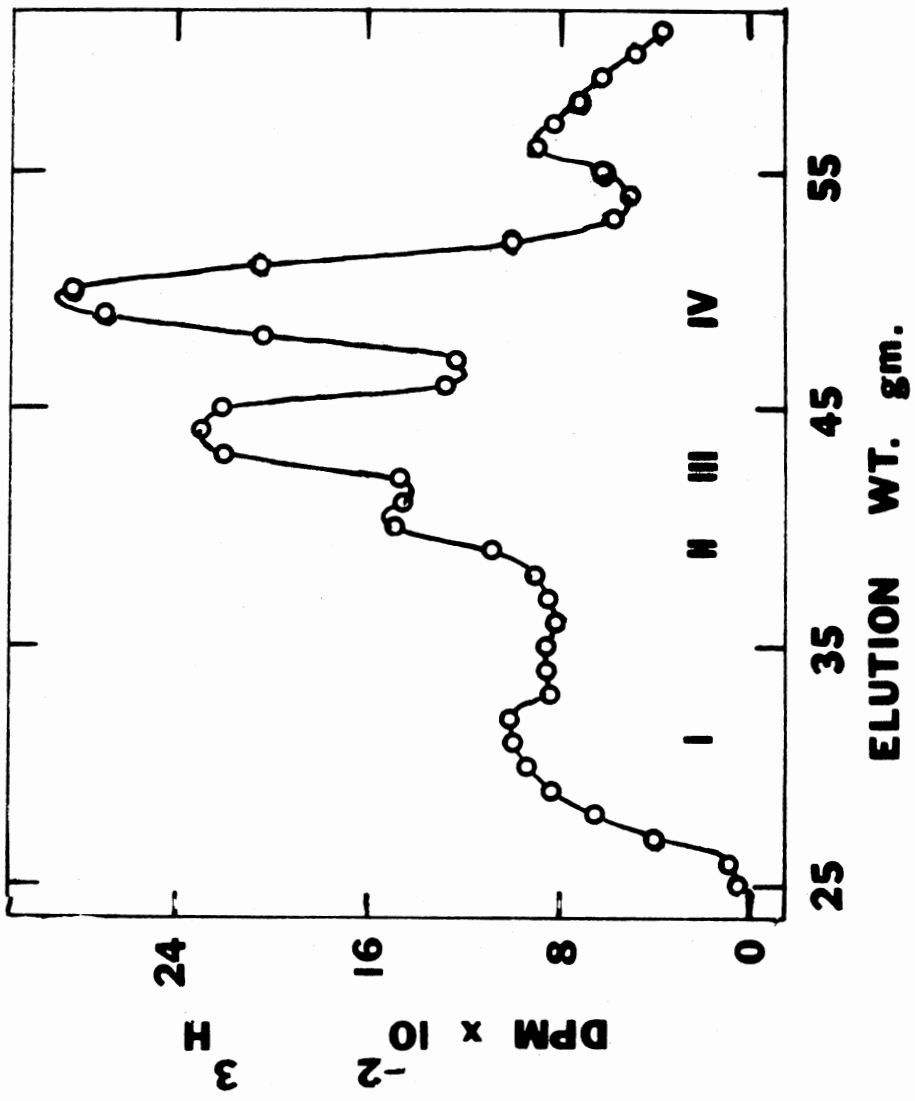

A
B
III-3
III-2
DPM x 10^-3 ^3H
DPM x 10^-2 ^14C
0
4
8
2
10
20
30
FRACTION NO.

Sialylated Oligosaccharitols of MAT-cMR6-s,
a Variant of MAT-B1

MAT-cMR6-s is a variant subline originally obtained from the MAT-B1 subline. MAT-B1 cells were adapted to cell culture, cloned, and after the sixth passage in cell culture re-injected into rats and passaged *in vivo* (Howard et al., 1980). During passage in cell culture the cells lost ASGP-1. After the sixteenth passage *in vivo* there was a sudden reappearance of ASGP-1. The oligosaccharitols of ASGP-1 from MAT cMR6-s as separated on Bio-gel P4 (Figure 18) are similar to MAT-B1 ASGP-1. Oligosaccharitol fractions II and III have apparently shifted to a lower molecular weight (compare Figure 16 and Figure 18). This effect arose because of differences in protocol. After β-elimination of the MAT-B1 and MAT-C1 ASGP-1 the digest was first desalted with Dowex 50(H^+) before elution on Bio-gel P4. The desalting step was not used in the analysis of the MAT-cMR6-s digest. Without desalting it is possible that the sodium ions present mask the negative charge of the sialic acid and thus the sialic acid-containing oligosaccharitols elute on the column according to their size. Desalting the digest removes sodium ions thus exposing the negatively charged sialic acid groups. Such negatively charged oligosaccharitols have been shown to behave like molecules larger than their true size. This effect is termed the charge-exclusion effect (Etchison et al., 1977).

Unlike the MAT-B1 cells, the MAT-cMR6-s cells were xenotransplantable. The ASGP-1 of MAT-cMR6-s has only 15% of the glucosamine label associated with the disialosaccharitol fraction II. This

Figure 18. The Bio-gel P4 Elution Profile of the O-linked Oligosaccharitols Released from MAT-cMR6-s ASGP-1 by ABH Treatment. ASGP-1 from MAT-cMR6-s cells was isolated, treated with alkaline borohydride, and eluted on Bio-gel P4 as described in Methods.


DPM x 10^-2 ³H
0
8
16
24
25
35
45
55
ELUTION WT. gm.
I
II
III
IV

indicated that the increased amount of this oligosaccharitol cannot account for the xenotransplantability of the MAT-C1 tumor cells.

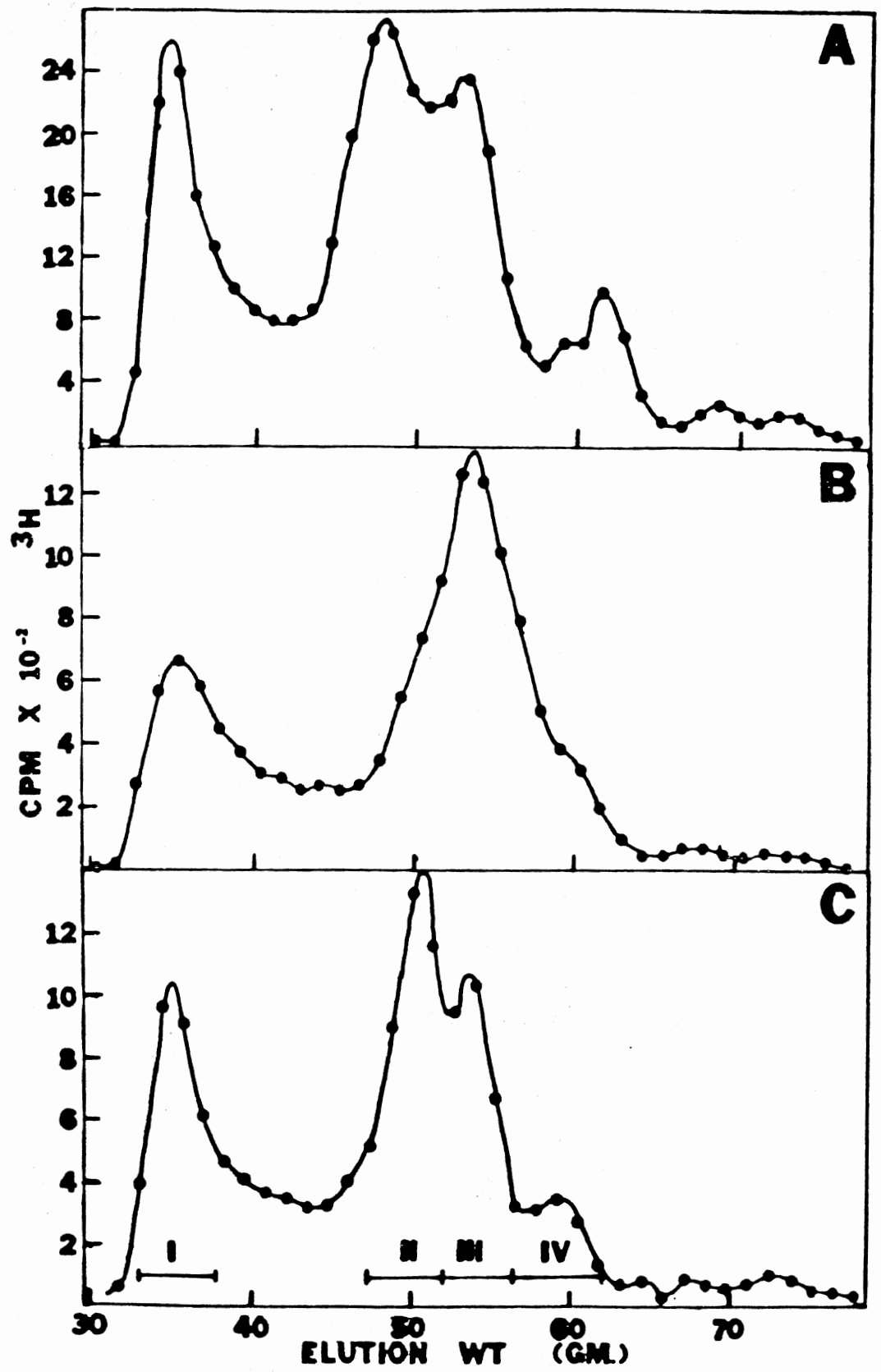
Sialylated Oligosaccharitols of MAT-C1 Variant Sublines

During the passage of MAT-C1 cells the host, Fischer 344 female rats, contracted a bacterial infection which affected the pattern of oligosaccharitols (Figures 19A, 19B, and 19C) on Bio-gel P4. The change from MAT-C1 (Figure 19A) to MAT-C2 (Figure 19B) was accompanied by a decreased amount of the oligosaccharitol fraction II. Interestingly the change was also accompanied by a loss of xenotransplantability suggesting a correlation with degree of sialylation of oligosaccharitols. The rats were quarantined and treated with antibiotics. Passage of the MAT-C2 cells were continued in healthy animals. Over about 25 passages the ASGP-1 reverted back to an oligosaccharitol profile on Bio-gel P4 resembling that of MAT-C1 (Figure 19C). The reverted cell line, however, was still found to be nonxenotransplantable. Since it exhibited this difference the new subline was designated MAT-C3. MAT-C3 ASGP-1 thus regained the level of sialylation found in MAT-C1 ASGP-1 without regaining xenotransplantability. This observation supports the contention that the increased sialylation of ASGP-1 cannot account for the xenotransplantability of the MAT-C1 cells.

Comparison of the Structures of the Desialylated Oligosaccharitols of MAT-B1 and MAT-C1 ASGP-1

Since the degree of sialylation of oligosaccharitols of ASGP-1

Figure 19. Bio-gel P4 Elution Profiles of the O-linked Oligosaccharitols of MAT-C1, MAT-C2, and MAT-C3 ASGP-1. ASGP-1 from (A) MAT-C1 cells, (B) MAT-C2 cells, and (C) MAT-C3 cells metabolically labeled with ^{3}H-gln (•—•) was treated with alkaline borohydride and the resultant digest eluted on Bio-gel P4 as described in Methods.


A
B
C
CPM X 10⁻³ ³H
24
20
16
12
8
4
12
10
8
6
4
2
I
II
III
IV
30
40
50
60
70
ELUTION WT (GM.)

did not correlate with xenotransplantability it was of interest to compare the desialylated core oligosaccharitols of the two ASGP-1 molecules to determine a difference, if any, in core structure which may be correlated with xenotransplantability. Carbohydrate compositional analysis suggested that the desialylated core is a tetrasaccharitol with the composition, $gal_2glcNac_1galNacOH_1$. Bio-gel P4 co-chromatography of desialylated II-3 of MAT-B1 and MAT-C1 ASGP-1 (Figure 20) indicated that the core oligosaccharitols, II-3D, from both sublines are similar in size. The possibility existed that the co-eluting peaks may represent oligosaccharitols with identical composition but isomeric with respect to spatial arrangement. Further analysis of the core oligosaccharitols was performed by HPLC. The analysis (Figure 21) indicated that the core oligosaccharitols co-elute. Since this chromatographic system is very sensitive to branching of oligosaccharitols (Turco, 1981), MAT-B1 and MAT-C1 II-3D are identical.

Bio-gel P4 co-chromatography of MAT-B1 and MAT-C1 III-2 desialylated core (Figure 22) shows that the two core structures, III-2D, are similar in size. The broadness of the peak suggested that III-2D is heterogeneous. Previous analysis indicated that MAT-B1 III-2D consisted of at least three components, two pentasaccharitols and a tetrasaccharitol. HPLC analysis of MAT-B1 and MAT-C1 III-2D (Figure 23) shows differences in the elution behavior of the two oligosaccharitol fractions. MAT-C1 III-2D did not contain appreciable amounts of III-2Dc. This component is the pentasaccharitol, $gal_3glcNac_1galNacOH_1$, which results from transfer of one galactose to the core tetrasaccharitol.

Figure 20. Comparison of the Core Oligosaccharitols of ^{3}H-gln (●--●) Labeled MAT-C1 II-3 and ^{14}C-gln (o—o) Labeled MAT-B1 II-3 on Bio-gel P4. II-3 from both sublines was eluted to serve as an internal standard.

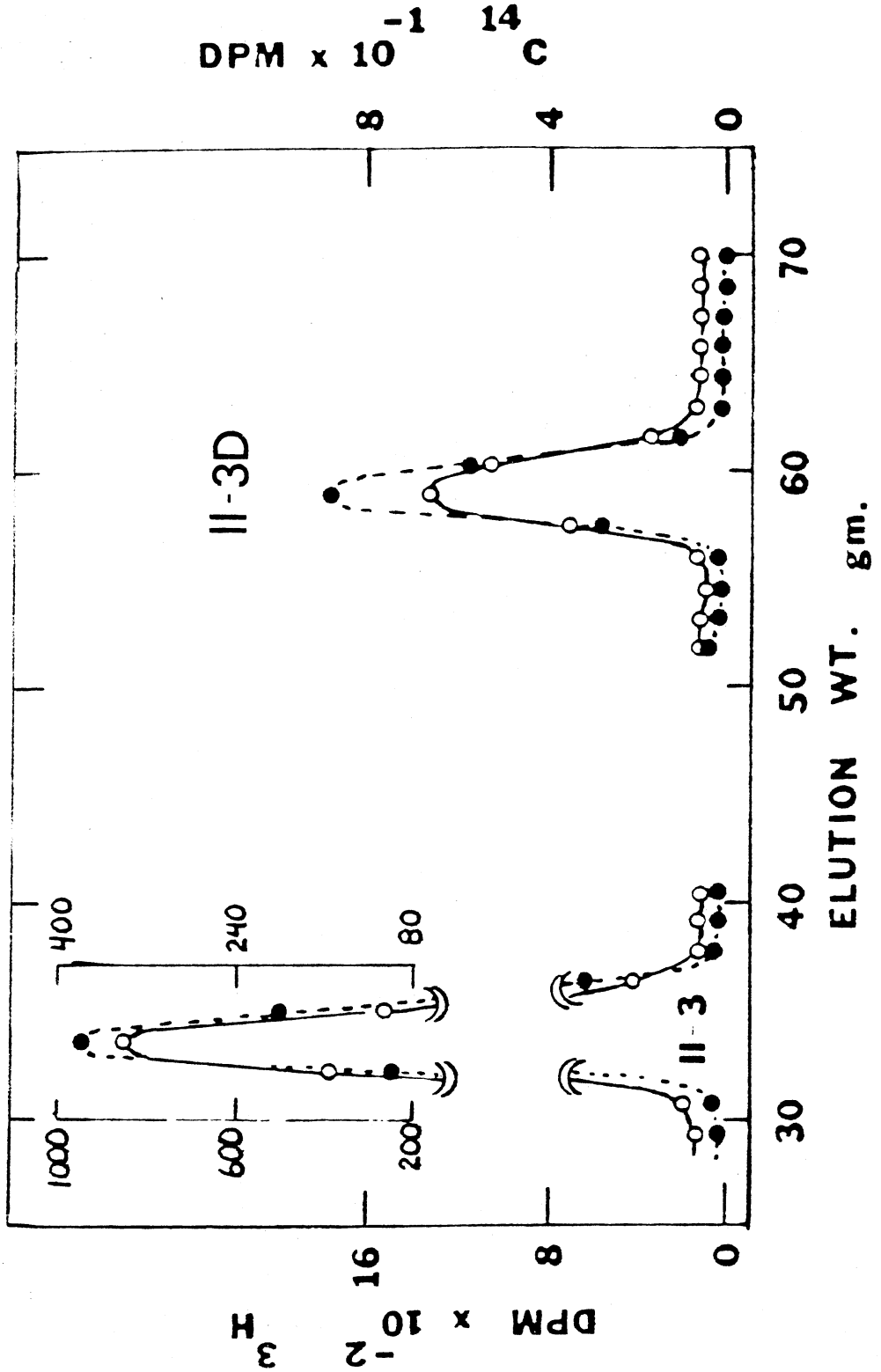

DPM x 10^-1 14C
8
4
0
II-3D
II-3
400
240
80
1000
600
200
16
8
0
DPM x 10^-2 3H
70
60
50
40
30
ELUTION WT. gm.

Figure 21. HPLC Elution Profile of the Core Oligosaccharitol of MAT-C1 II-3 Chromatographed Together with the Core Oligosaccharitol of MAT-B1 II-3. The core oligosaccharitols of ^{3}H-gln (●--●) labeled MAT-C1 II-3 and ^{14}C-gln (o—o) labeled MAT-B1 II-3 were prepared and analyzed by HPLC on 5 μ silica gel as described in Methods.

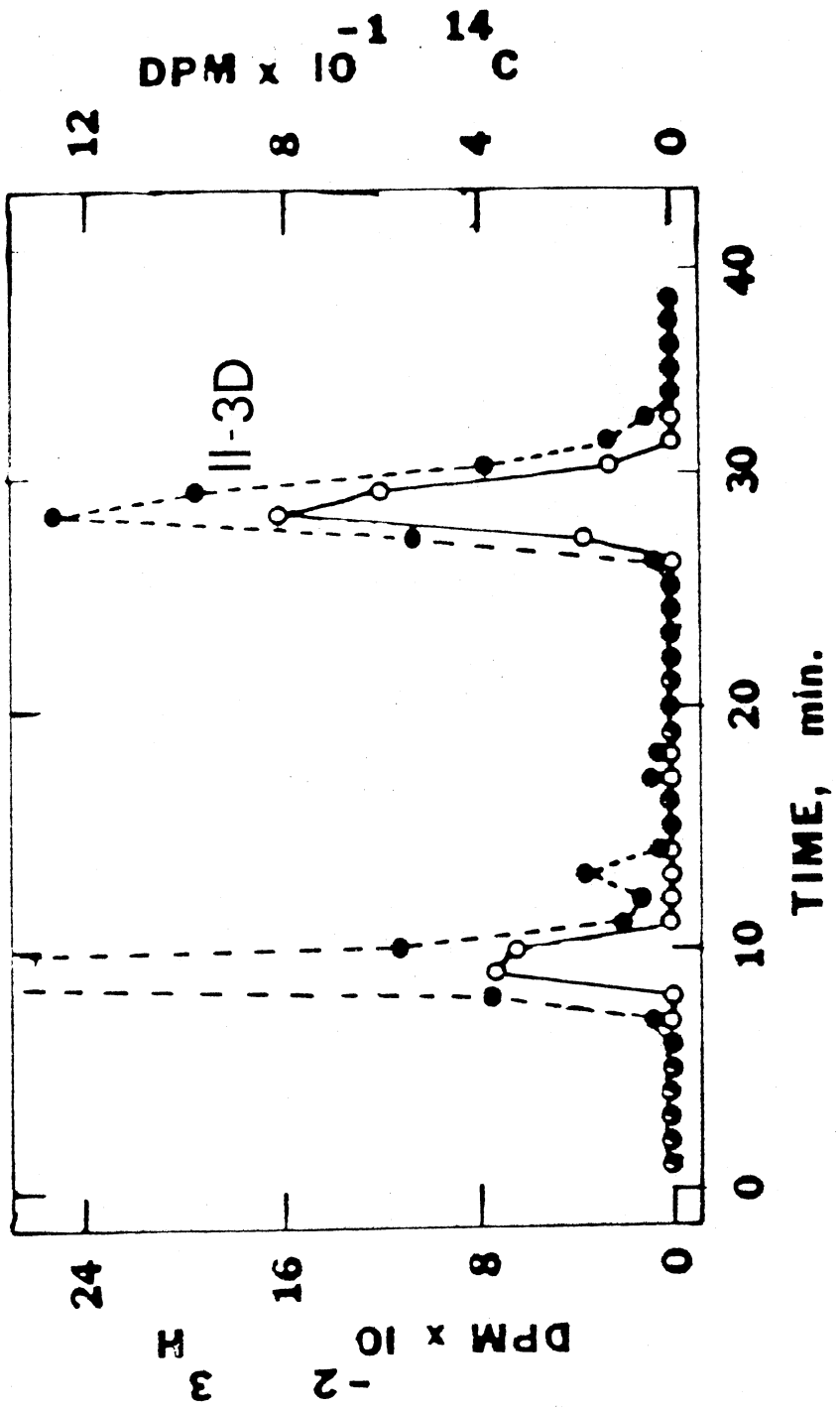

DPM x 10^-1 14C
12
8
4
0
II-3D
40
30
20
10
0
TIME, min.
24
16
8
0
DPM x 10^-2 3H

Figure 22. Bio-gel P4 Elution Profile of the Core Oligosaccharitol Fraction of MAT-C1 III-2 Chromatographed Together with the Core Oligosaccharitol Fraction of MAT-B1 III-2. The core oligosaccharitol fractions of ^{3}H-gln labeled (●--●) MAT-C1 and ^{14}C-gln labeled (o--o) MAT-B1 III-2 were prepared, mixed together, and eluted on Bio-gel P4 as described in Methods. III-2 from MAT-B1 and MAT-C1 ASGP-1 (first peak) was eluted as an internal standard.

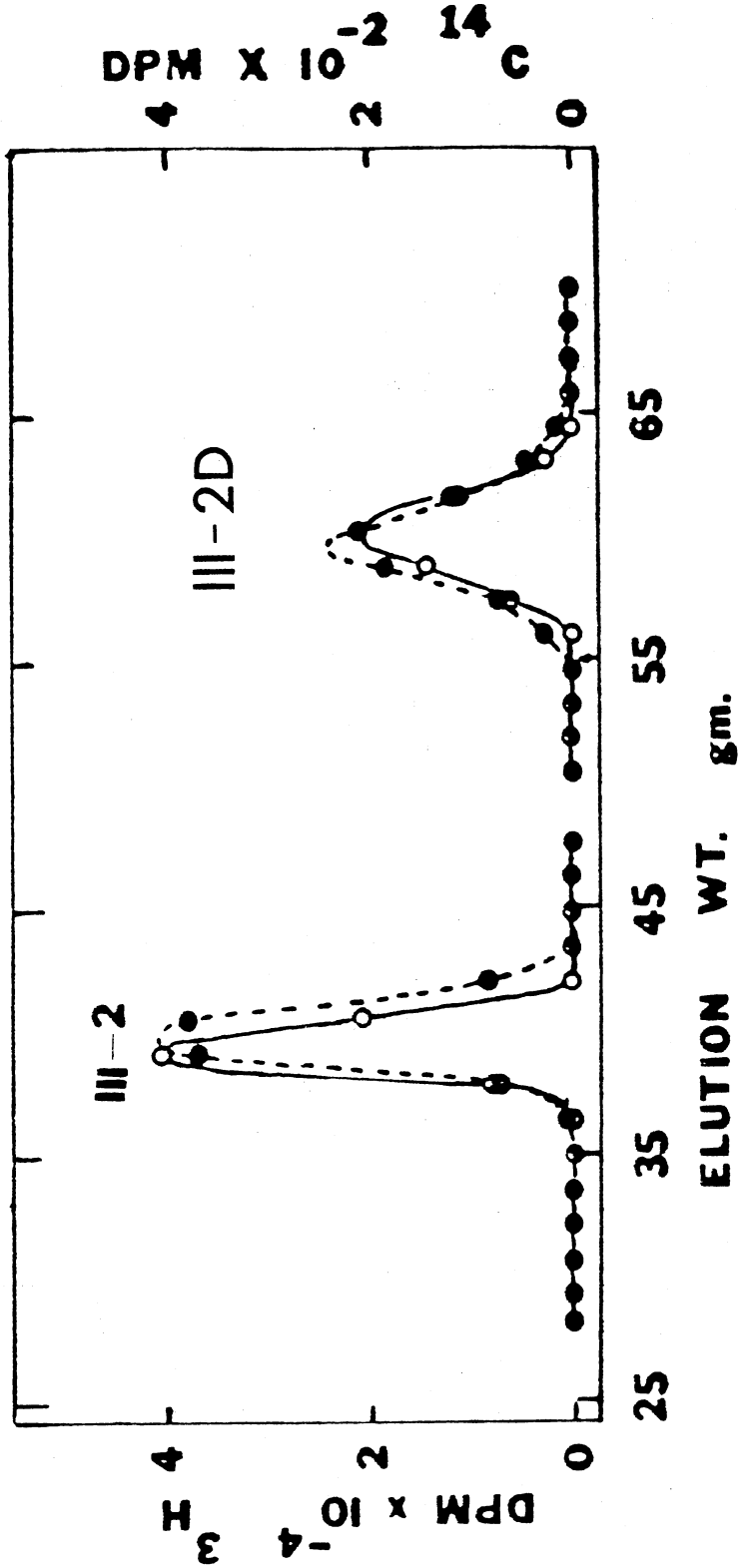
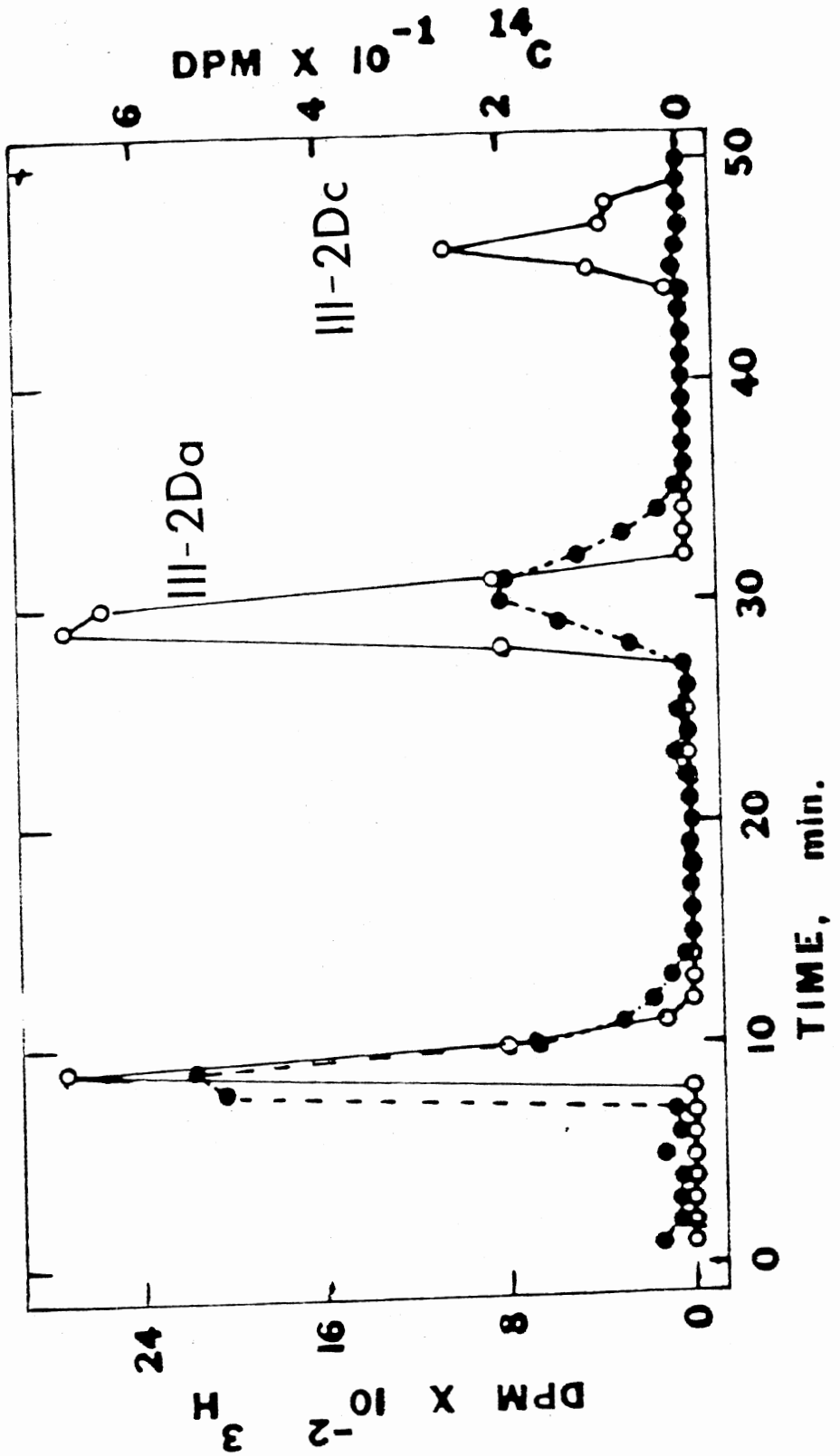

DPM X 10^-2 14 C
4
2
0
III-2D
III-2
65
55
45
35
25
ELUTION WT. gm.
DPM x 10^-4 3H
4
2
0

Figure 23. HPLC Elution Profile of the Core Oligosaccharitol Fraction of MAT-C1 III-2 Chromatographed Together with the Core Oligosaccharitol Fraction of MAT-B1 III-2. The oligosaccharitol fraction of ^{3}H-gln labeled (•--•) MAT-C1 III-2 and ^{14}C-gln labeled (o—o) MAT-B1 III-2 was prepared, mixed together, and analyzed by HPLC on 5 μ silica gel.

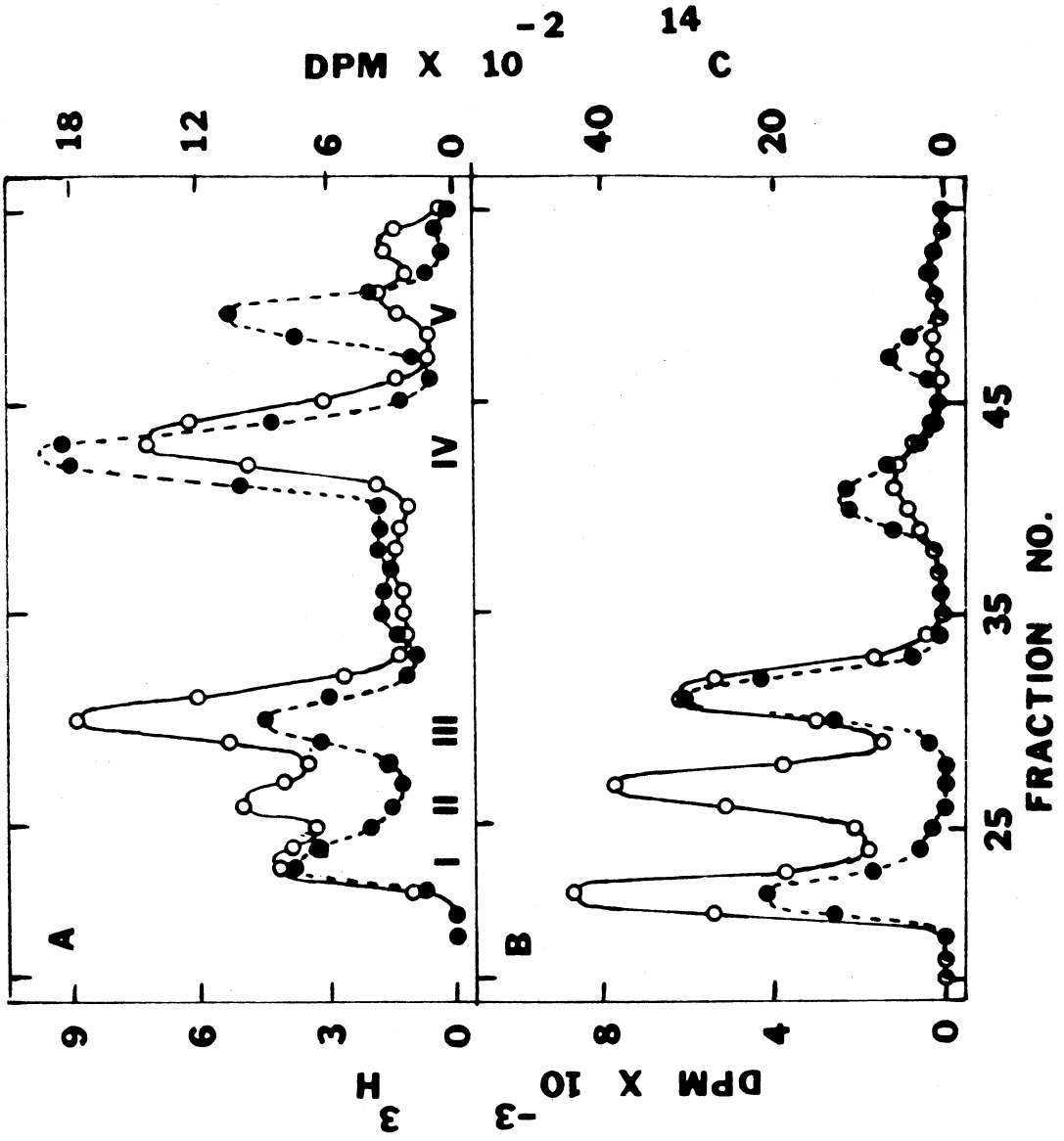

DPM X 10^{-1} ^{14}C
6
4
2
0
III-2Dc
III-2Da
50
40
30
20
10
0
TIME, min.
24
16
8
0
DPM X 10^{-2} ^{3}H

Thus MAT-C1 III-2 was more homogeneous than MAT-B1 III-2 with respect to component oligosaccharitols. There was also a difference in III-2Da. This peak represents the core tetrasaccharitol and its fucosylated derivative. MAT-C1 III-2Da appears to be more heterogeneous with respect to component MAT-B1 III-2Da. This may represent a difference in the amounts of the fucosylated component of III-2 between the two sublines. This possibility prompted an investigation into the fucosylation of MAT-B1 and MAT-C1 ASGP-1.

Fucosylated Oligosaccharitols of MAT-B1 and MAT-C1 ASGP-1

Fucosylated oligosaccharitols of the two glycoproteins were fractionated by gel filtration and anion exchange chromatography of ASGP-1 metabolically labeled with ^{3}H-fucose and ^{14}C-glucosamine. The separation of oligosaccharitols on Bio-gel P4 is shown for MAT-B1 and MAT-C1 ASGP-1 in Figure 24A and 24B, respectively. Each glycoprotein contains three major fucose-containing fractions III, IV, and V. The fucose label elutes at the high molecular weight side of IV and V indicating a possible mixture of oligosaccharitols with respect to fucosylation. It appears that the major fucosylated oligosaccharitol fraction of MAT-B1 ASGP-1 is IV and III is the major fucosylated fraction of MAT-C1 ASGP-1. Characterization of these two fractions by anion exchange chromatography and HPLC analysis was undertaken to determine the nature of the differences in fucosylation of MAT-B1 and MAT-C1 III and IV.

Figure 24. Bio-gel P4 Elution Profiles of the O-linked Fucosylated Oligosaccharitols of MAT-B1 ASGP-1 and MAT-C1 ASGP-1. (A) ^{3}H-fucose (•--•) and ^{14}C-gln (o—o) dual labeled MAT-B1 ASGP-1 was digested with ABH and the released oligosaccharitols were separated on Bio-gel P4 as described in Methods. (B) ^{3}H-fucose (•--•) and ^{14}C-gln (o—o) dual labeled MAT-C1 ASGP-1 was treated in similar fashion as MAT-B1 ASGP-1.


DPM X 10^-2 14 C
DPM X 10^-3 3 H
FRACTION NO.
A
B
I
II
III
IV
V
0
6
12
18
20
40
3
6
9
4
8
25
35
45

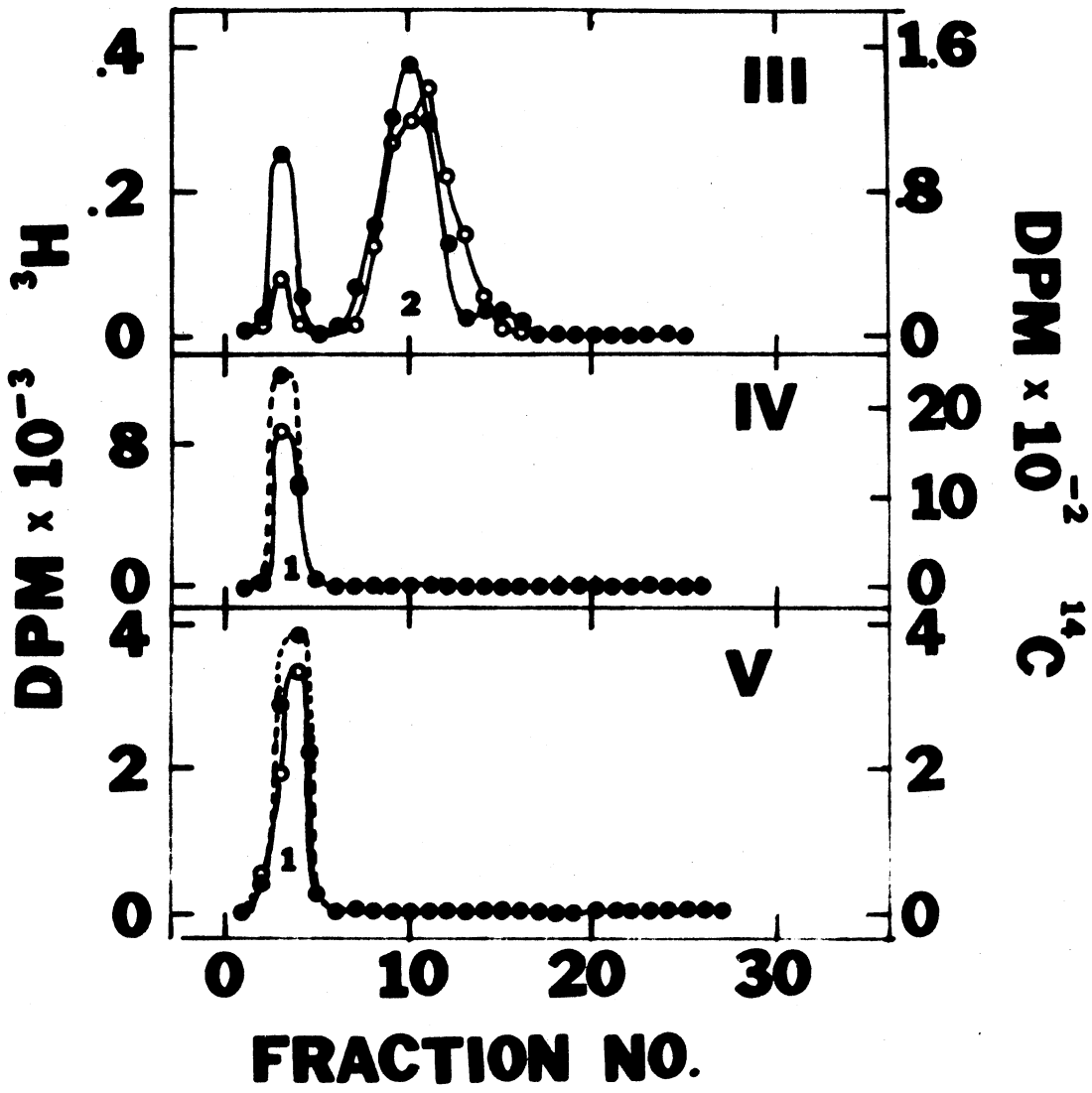
Fucosylation of MAT-B1 and MAT-C1 Oligosaccharitol Fraction III

DEAE-Sephadex anion exchange chromatography of the dual labeled MAT-B1 (Figure 25) and dual labeled MAT-C1 (Figure 26) oligosaccharitols indicated that III-2 from both sublines contains fucose. Gel filtration comparative profiles indicated that III from MAT-B1 ASGP-1 contains 18% (Table XI) of the recovered fucose label whereas III from MAT-C1 ASGP-1 contains 49% of the recovered fucose label. From this data there is apparently a 2-3 fold greater amount of the fucosylated III-2 fraction in MAT-C1 ASGP-1 as compared to MAT-B1 ASGP-1. HPLC comparative profiles of ^{3}H-fucose and ^{14}C-glucosamine dual labeled III-2D from MAT-B1 and MAT-C1 ASGP-1 (Figure 27) indicated that both MAT-B1 III-2 and MAT-C1 III-2 contain the same fucosylated component, III-2b. Therefore the difference in fucosylated III-2 of MAT-B1 and MAT-C1 ASGP-1 is quantitative not qualitative. A possible correlation of increased amount of fucosylated III-2 with xenotransplantability is suggested from these results. It would be interesting to investigate this by studying the fucosylation of MAT-C3 ASGP-1. Unfortunately this variant subline is no longer available.

Fucosylation of MAT-B1 and MAT-C1 Oligosaccharitol Fraction IV

From gel filtration analysis oligosaccharitol fraction IV is the major fucosylated fraction of MAT-B1 ASGP-1 and the least abundant fucosylated fraction of MAT-C1 ASGP-1. The comparison of this fraction from the two sublines was made by HPLC on 5μ silica gel

Figure 25. DEAE-Sephadex Chromatography of ^{3}H-Fucose (●--●) and ^{14}C-gln (o—o) Dual Labeled MAT-B1 ASGP-1 Oligosaccharitol Fractions III, IV, and V. Oligosaccharitol fractions III, IV, and V from Bio-gel P4 chromatography were eluted on DEAE-sephadex as described in Methods.


III
IV
V
1
2
DPM x 10⁻³ ³H
DPM x 10⁻² ¹⁴C
.4
.2
0
8
0
4
2
0
1.6
.8
0
20
10
0
4
2
0
0
10
20
30
FRACTION NO.

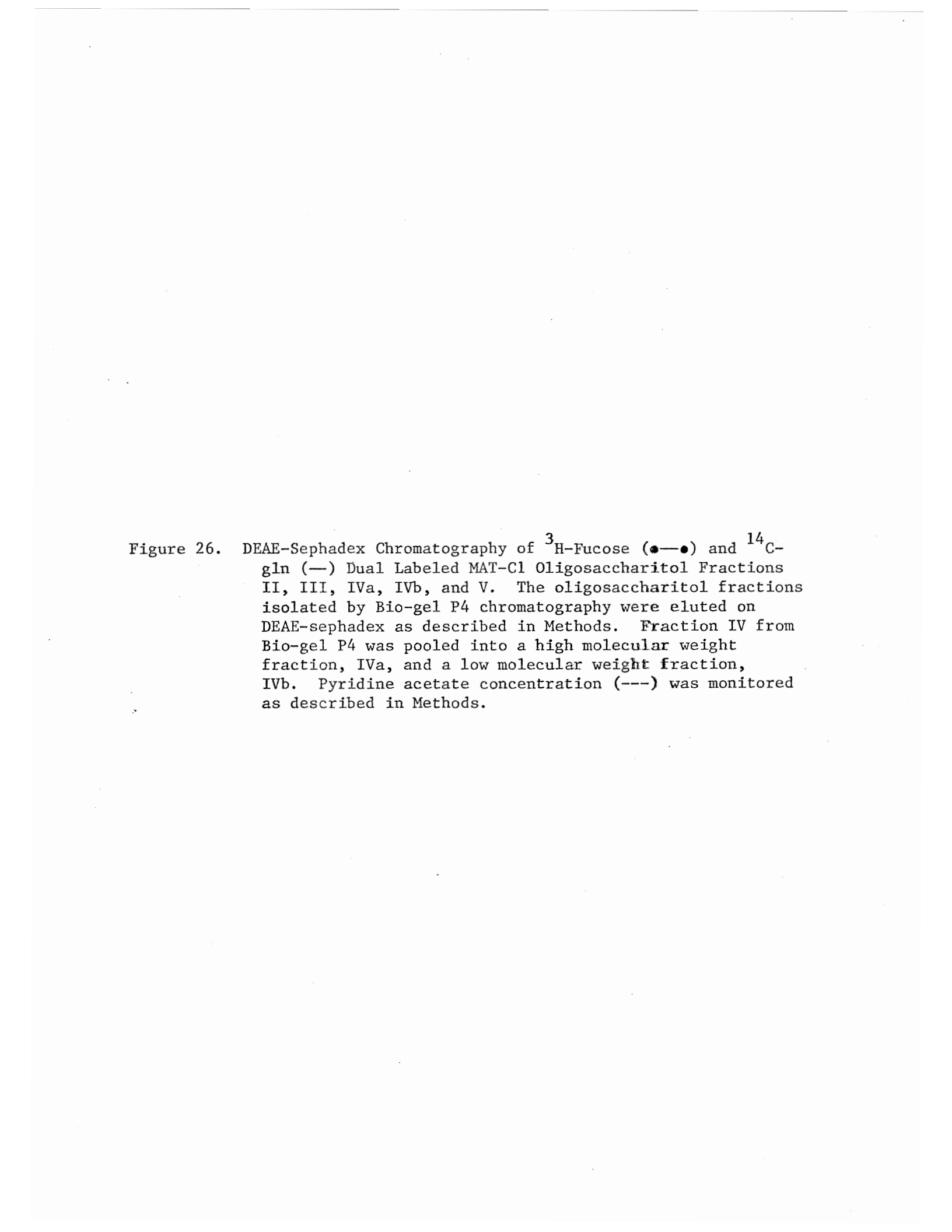


Figure 26. DEAE-Sephadex Chromatography of ^{3}H-Fucose (●—●) and ^{14}C-gln (—) Dual Labeled MAT-Cl Oligosaccharitol Fractions II, III, IVa, IVb, and V. The oligosaccharitol fractions isolated by Bio-gel P4 chromatography were eluted on DEAE-sephadex as described in Methods. Fraction IV from Bio-gel P4 was pooled into a high molecular weight fraction, IVa, and a low molecular weight fraction, IVb. Pyridine acetate concentration (---) was monitored as described in Methods.

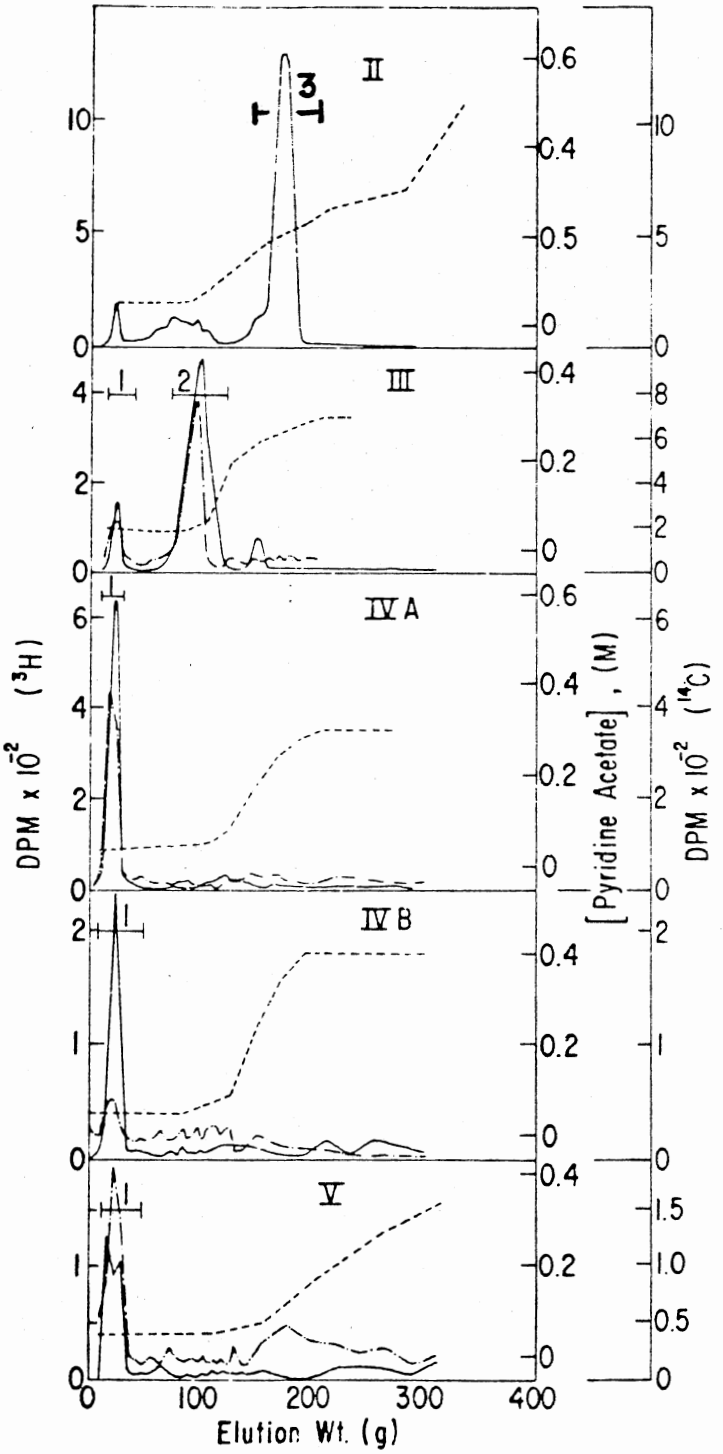

II
T3
T4
III
1
2
IV A
IV B
V
DPM x 10⁻² (³H)
[Pyridine Acetate], (M)
DPM x 10⁻² (¹⁴C)
Elution Wt. (g)
0
100
200
300
400

TABLE XI

RECOVERY OF FUCOSE RADIOACTIVE LABEL IN THE OLIGOSACCHARITOL FRACTIONS I, III, IV, AND V OF MAT-B1 AND MAT-C1 ASGP-1

MAT-B1

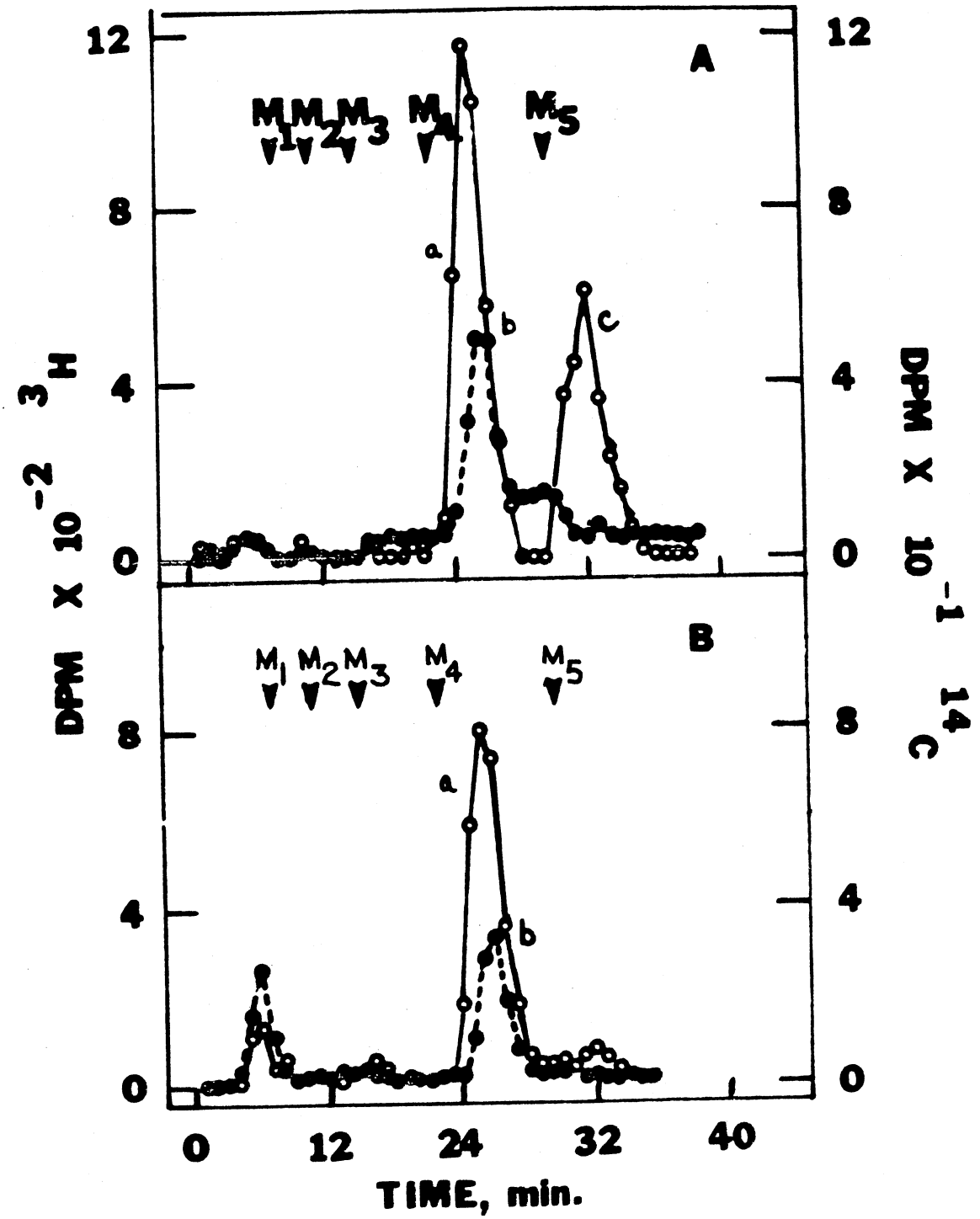
Fraction	% Fucose Recovered°
I	15.3
III	17.9
IV	48.1
V	18.7

MAT-C1

Fraction	% Fucose Recovered°
I	28.1
III	43.8
IV	21.9
V	6.2

°Radioactive counts in each fraction were quantitated and compared to the total (all fractions) counts recovered.

Figure 27. HPLC Elution Profiles of ^{3}H-Fucose (●--●) and ^{14}C-Glucosamine (o—o) Dual Labeled MAT-B1 III-2D and MAT-C1 III-2D. (A) MAT-B1 III-2D and (B) MAT-C1 III-2D were prepared from the oligosaccharitol fraction III-2 and analyzed by HPLC on 5μ silica gel as described in Methods.


A
B
M1 M2 M3 M4 M5
a
b
c
DPM X 10-2 3H
DPM X 10-1 14C
TIME, min.
0
12
24
32
40
4
8
12

(Figure 28).

From recovery of glucosamine label, MAT-C1 IV-1 contains the core tetrasaccharitol, IV-1a, and fucosylated core, IV-1b, in a 40:60 ratio whereas these oligosaccharitols are present in an 80:20 ratio in MAT-C1 IV-1. MAT-C1 IV-1 does not contain an appreciable amount of IV-1c, instead it has a small amount of another fucosylated component IV-1e. It appears that MAT-C1 IV-1 and MAT-B1 IV-1 contain the fucosylated component, IV-1d.

Discussion

MAT-C1 ascites cells are xenotransplantable into mice but MAT-B1 ascites cells are not. The possible role of ASGP-1 carbohydrate structure was investigated by comparison of their oligosaccharitol structure and are summarized in Table XII. Gel filtration comparative profiles (Figure 16) indicated an increased amount of the disialohexasaccharitol II-3, on the xenotransplantable subline, MAT-C1. This finding was of particular interest since many authors have suspected a role for sialic acid in the masking of cell surface antigens. This had been ascertained by the enhanced antigenicity created by SA removal from the cell surface. Many authors have reported the reduced transplantability of neuraminidase-treated tumor cells, both in allogeneic (Sanford, 1967; Currie, 1967; Currie and Bagshawe, 1968a,b) and syngeneic (Simmons et al., 1973; Sethi and Brandis, 1973; Bekesi et al., 1971) systems. The mechanism of the role of sialic acid is not well understood. Possible explanations are: (1) the sialic acid might serve to protect penultimate galactose residues that could serve as receptor sites for cytotoxic cells much like they serve as

Figure 28. HPLC Elution Profiles of ^{3}H-Fucose (●--●) and ^{14}C-Glucosamine (o—o) Dual Labeled MAT-B1 IV-1 and MAT-C1 IV-1. (A) MAT-B1 IV-1 and (B) MAT-C1 IV-1 were analyzed by HPLC on 5μ silica gel as described in Methods.

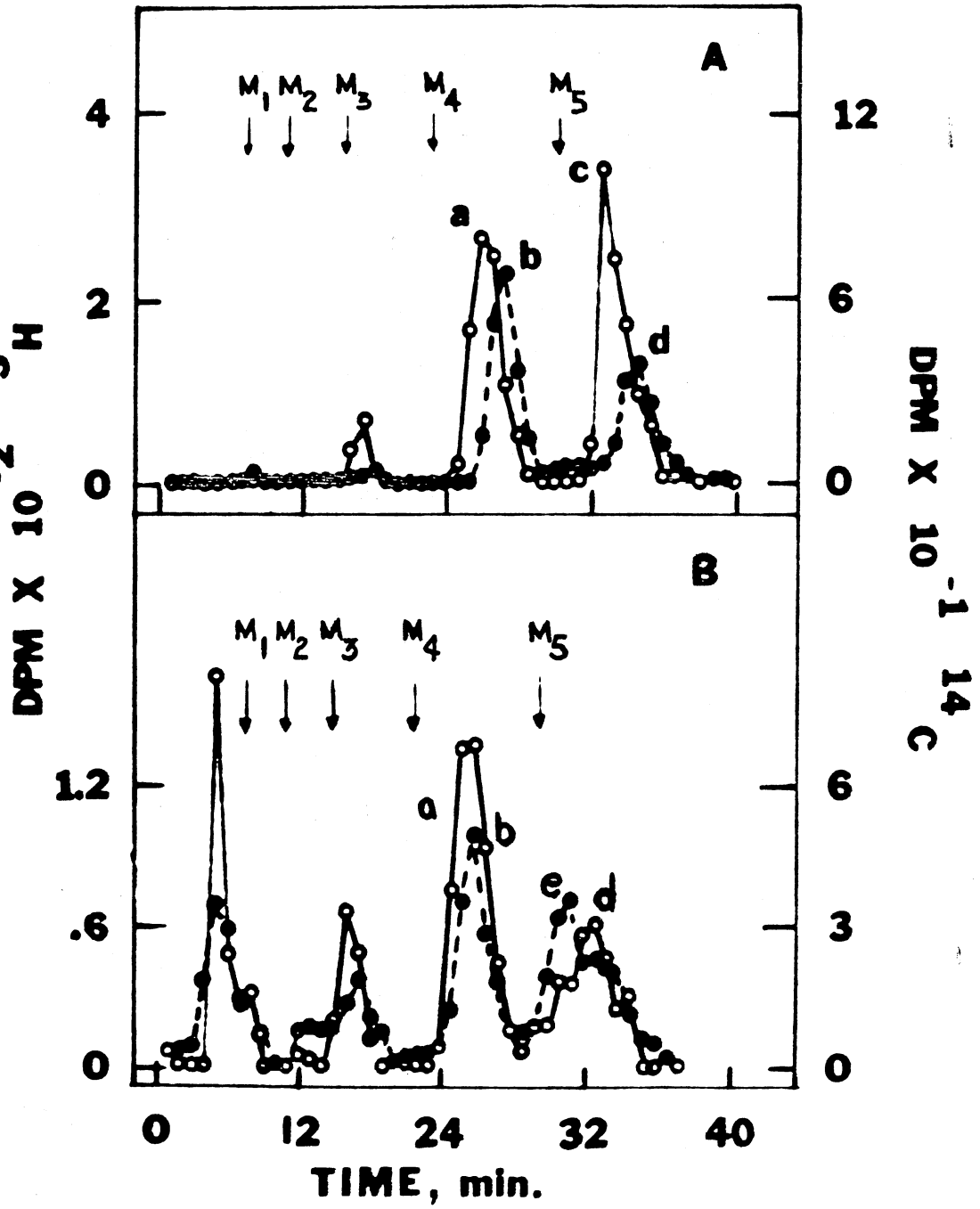

A
B
M1 M2 M3 M4 M5
a
b
c
d
e
4
2
0
12
6
0
1.2
.6
0
3
0
12
24
32
40
TIME, min.
DPM X 10-2 3H
DPM X 10-1 14C

TABLE XII

SUMMARY OF STRUCTURAL COMPARISON OF MAT-B1 and MAT-C1 ASGP-1

Property	MAT-B1 ASGP-1	MAT-C1 ASGP-1
Amount of II-3	+	++++
Core tetrasaccharide	+	+
Amount of III-2Da	+	+++
Amount of III-2Dc	+	-
Amount of IV-1	++++	+
Amount of Terminal galactose	+++++	+

receptors for the binding of serum glycoproteins to the liver and other organs (Pricer and Ashwell, 1971; Ashwell and Morell, 1974) and/or (2) the loss of sialic acid could lower the negative charge at the cell surface, thus increasing the deformability of the cell (Weiss, 1965) and enhancing its susceptibility to phagocytosis (Lee, 1968). If we could correlate the presence of sialic acid with xenotransplantability of MAT cells we might possibly have a system in which we could study possible mechanisms for the participation of sialic acid in transplantability. However, based on two observations, namely that (1) II-3 is present in limited amounts on both a non-xenotransplantable subline, MAT-B1, and a xenotransplantable subline, MAT cMR6-s and (2) II-3 is present as the major sialylated oligosaccharitol on a xenotransplantable subline, MAT-C1 and non-xenotransplantable subline, MAT-C3, we conclude that this disialohexasaccharitol is not responsible for the xenotransplantability of the ascites rat mammary adenocarcinoma. This conclusion agrees with the observations of Sanford and Codington (Sanford and Codington, 1971; and Sanford et al., 1973). They concluded that the sialic acid of epiglycanin, 12% of the carbohydrate content, did not appear to play any direct role in the masking process since its removal did not significantly alter the adsorption of H-2 antisera.

Glick and associates (Glick et al., 1978) have noted the peculiar properties of fucose and its presence on the surface of tumor cells. It has been shown that cancer patients contain high amounts of fucose in their serum glycoproteins (Waalkes et al., 1978) probably due to the elevated levels of fucosyl transferases (Bauer et al., 1978). Evidence has been presented to indicate that the presence of fucose

at the cell surface (Trefts et al., 1976), more specifically the disaccharide sequence fucα1-2gal- (Parish et al., 1978) may be important for the binding of cytotoxic cells such as B lymphocytes. Thus it appears that fucose may play some role in transplantation. In the MAT system the non-xenotransplantable and xenotransplantable sublines both contain fucosylated oligosaccharitols. The xenotransplantable subline contained approximately two times as much of the acidic fucosylated oligosaccharitol, III-2b, as the non-xenotransplantable cell line. The majority of the fucosylated oligosaccharitols of the non-xenotransplantable subline are neutral. These observations suggest that the proximity of sialic acid to fucα1-2gal linkages may prevent the approach and/or binding of circulating lymphocytes. However, the fucosylated oligosaccharitols make up at the most 19% of the O-linked oligosaccharitols of ASGP-1. It thus seems unlikely that the difference in fucosylation of ASGP-1 could be correlated with xenotransplantability. It would be interesting to study the fucosylation of cell surface glycoproteins in other systems to explore further the possible synergistic role of sialic acid and fucose in transplantability.

Since the presence of terminal galactose has been correlated with the increased binding of glycoproteins to liver cells (Ashwell and Morell, 1974) it seems possible that terminal galactose may be involved in the complex binding processes of transplantation. In these studies it is evident that MAT-B1 ASGP-1 had more terminally exposed galactose than MAT-C1 ASGP-1. From gel filtration comparisons MAT-B1 IV-1 comprised at least 40% of the O-linked glucosamine-containing oligosaccharitols of MAT-B1 ASGP-1 compared to only 10% of

the chains of MAT-C1 ASGP-1. This observation suggested that in the MAT system the presence of terminal galactose may be necessary for a cell to be recognized by attacking cytotoxic cells. This possibility is unlikely since MAT cMR6-s, a variant subline of MAT-B1, contained a similarly high proportion of IV-1 but was found to be xenotransplantable.

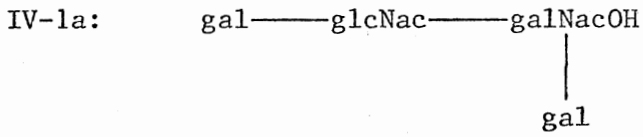
The fact that ASGP-1 is found on both the xeno- and non-xenotransplantable cell lines coupled with the finding that oligosaccharitol structure appears not to be correlated with xenotransplantability suggest that ASGP-1 is not directly involved in xenotransplantability as epiglycanin is in the TA3 mouse mammary adenocarcinoma. The only correlation that has been consistently correlated with transplantability in the rat mammary adenocarcinoma is a branched cell surface morphology (Howard et al., 1982). This does not rule out the possibility that ASGP-1 may be involved in concert with other factors to bring about xenotransplantation. The differences in glycoprotein structure suggest differences in biosynthetic mechanisms in the two cells. These two cell lines may provide a tool for the study of mucous glycoprotein synthesis and its control. Future studies will be directed towards isolation and characterization of biosynthetic precursors of ASGP-1, glycosyl transferases, and glycosyl hydrolases.

CHAPTER IV

SUMMARY

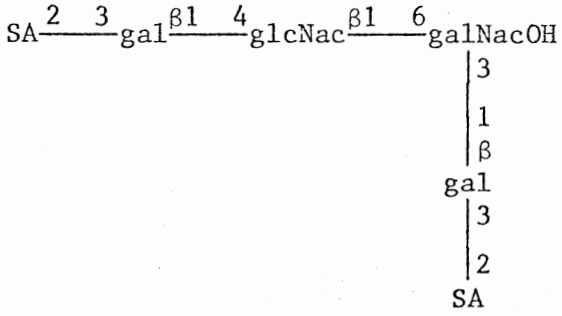
Oligosaccharitols of a major cell surface sialoglycoprotein, ASGP-1, have been isolated by specific alkaline β-elimination from the polypeptide backbone, separation by gel permeation chromatography, anion exchange chromatography, and high performance liquid chromatography. The structural characterization of these oligosaccharitols has been accomplished by carbohydrate compositional analysis, GC-MS linkage analysis, periodate oxidation studies, and specific degradation with exoglycosidases. The glucosamine-containing oligosaccharitols of ASGP-1 appear to be assembled from a core tetrasaccharitol, IV-1a:

IV-1a:



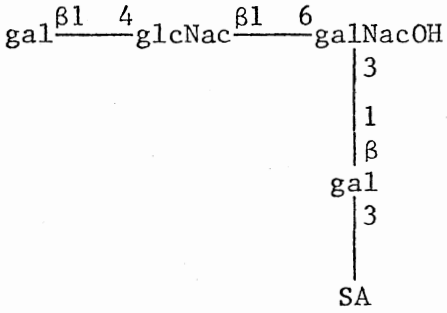
This core tetrasaccharitol is variously substituted with sialic acid, fucose, and galactose. In MAT-B1 ASGP-1 9% of the glucosamine-containing oligosaccharitols result from the addition of two sialic acid residues to the core tetrasaccharitol to give the disialohexasaccharitol, II-3:

II-3:



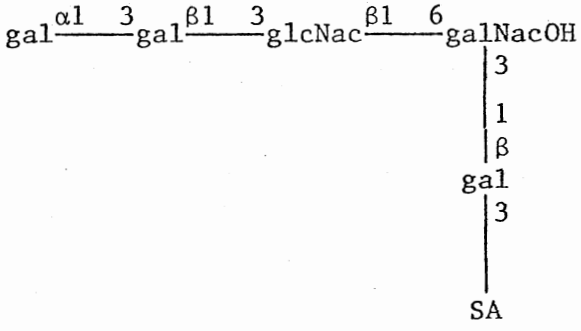
This oligosaccharitol is the major carbohydrate chain released from MAT-C1 ASGP-1 as at least 30% of the O-linked chains are disialylated. Monosialylated oligosaccharitols comprise 26% of the chains released from MAT-B1 ASGP-1. The sialic acid moiety is only found on the galactose residue linked 1→3 to GalNacOH. One half of the mono-sialylated oligosaccharitols result from the sialylation of the core tetrasaccharitol to give, III-2a:

III-2a:



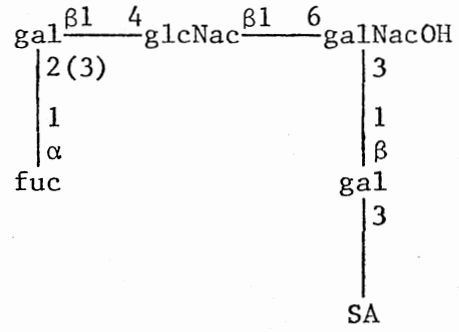
40% of the mono-sialylated chains also contain another galactose α-linked to the remaining galactose residue to give III-2c:

III-2c:



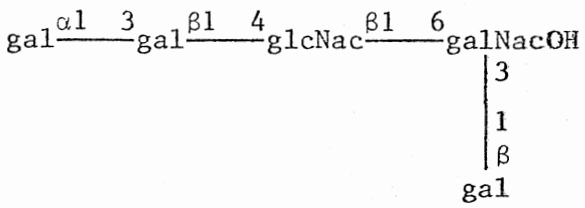
The remainder of the mono-sialylated oligosaccharitols of MAT-B1 ASGP-1, only 3% of the total, also contain fucose α-linked to the remaining galactose residue to give III-2b:

III-2b:



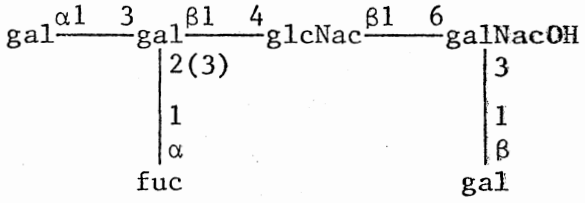
MAT-C1 ASGP-1 and MAT-B1 ASGP-1 have approximately identical amounts of mono-sialylated oligosaccharitols, however, the two glycoprotein molecules differ in the amount of III-2b and III-2c present. MAT-C1 ASGP-1 does not have detectable amounts of III-2c and instead has more of the fucosylated component, III-2b. It appears from HPLC analysis that 60% of the mono-sialylated oligosaccharitols are non-fucosylated III-2a and 40%, only 10% of the total carbohydrate chains of MAT-C1 ASGP-1, are the fucosylated component, III-2b. The neutral (non-sialylated) oligosaccharitols of MAT-B1 ASGP-1 represent almost 50% of the O-linked oligosaccharitols. Although the neutral oligosaccharitols are isolated in one broad peak on gel filtration media, four distinct oligosaccharitol fractions are isolated by high performance adsorption chromatography on 5μ silica gel modified with 1,4-diaminobutane. 17% of the oligosaccharitols of MAT-B1 ASGP-1 result from the addition of galactose to the core tetrasaccharitol to give IV-1c:

IV-1c:



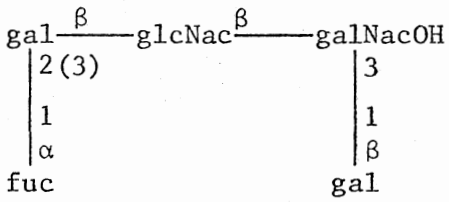
From exoglycosidase digestions it has been established that this galactose is α-linked. 10% of the chains of MAT-B1 ASGP-1 are products of the addition of galactose and fucose to the core tetrasaccharitol to give IV-1d:

IV-1d:



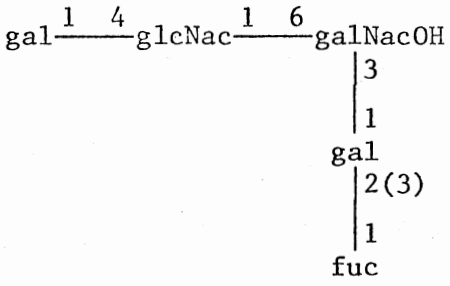
6% of the chains result from the fucosylation of the core tetrasaccharitol to give IV-1b:

IV-1b:



The remainder of the chains, 17% of the total, are the core tetrasaccharitol, IV-1a. MAT-C1 ASGP-1 differs from MAT-B1 ASGP-1 in that the neutral fraction IV as isolated from gel filtration represents only 9% of the carbohydrate chains. From HPLC analysis this neutral fraction also appears to contain four components, three of which co-chromatograph and are identical to IV-1a, IV-1b, and IV-1d of MAT-B1 ASGP-1. One fucosylated component of MAT-C1 IV is not found

in MAT-B1 ASGP-1. Its low recovery during isolation made its further characterization impossible, however it seems reasonable that it is an isomeric oligosaccharitol of IV-1b, possibly:



These results suggest that there are only quantitative differences in oligosaccharitol content of MAT-B1 and MAT-C1 ASGP-1. It appears that none of these differences are directly related to xenotransplantability. It is apparent that ASGP-1 of the xenotransplantable MAT-C1 cells contains at least two times as much sialylated oligosaccharitol II-3 as MAT-B1 ASGP-1. Its presence however is not responsible for transplantation since a variant subline, MAT-cMR6s, contains only minimal amounts of II-3 and yet is xenotransplantable. Furthermore, another cell line, MAT-C3, was found to be non-xenotransplantable and its major oligosaccharitol is II-3. MAT-C1 ASGP-1 contains at least two times as much of the fucosylated mono-sialylated oligosaccharitol, III-2b, as MAT-B1 ASGP-1. It is possible that the close proximity of fucose and sialic acid may play a role in transplantation. This is unlikely though since this fucosylated oligosaccharitol is only a minor fraction, 10% of the oligosaccharitols of MAT-C1 ASGP-1. MAT-B1 ASGP-1 appears to contain at least three times as much terminal galactose as MAT-C1 ASGP-1. With the known binding capacity of terminal galactose (Morell and Ashwell, 1974) it

seems possible that such a structural feature could be responsible for the decreased transplantability of MAT-B1 cells. This is also unlikely since the xenotransplantable cell variant, MAT cMR6-s has ASGP-1 with similar amounts of terminal galactose.

All indications are that the carbohydrate structure of ASGP-1 does not play any consistent role in xenotransplantation. The MAT rat mammary system however may provide an interesting means of studying the biosynthetic principles of glycosylation of mucous like membrane glycoproteins. This study raises such interesting questions as: Why does MAT-C1 ASGP-1 contain more sialylated oligosaccharitols than MAT-B1 ASGP-1? Is the addition of sialic acid the final step in the assembly of the oligosaccharide or is it an early step after which sialidase activities are determining factors in the extent of galactosylation and fucosylation of the core tetrasaccharitol? These and many more questions can be explored by studying the enzyme levels, both glycosyl transferases and glycosyl hydrolases, in the various sublines of the 13762 rat mammary adenocarcinoma.

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