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ENZYME-MEDIATED SYNTHESIS OF THE ANTIGENIC PORTION OF THE BLOOD GROUP O SUBSTANCE WITH SPECIFIC CARBON-13 ENRICHMENT

By

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ABSTRACT

ENZYME-MEDIATED SYNTHESIS OF THE ANTIGENIC PORTION OF THE BLOOD GROUP O SUBSTANCE WITH SPECIFIC CARBON-13 ENRICHMENT.

By

Paul Richard Rosevear

The use of partially purified glycosyltranferases and chemically synthesized sugar nucleotides provides an alternative method for synthesis of complex carbohydrates. Enzymatic synthesis of the glycosidic linkage has been shown to proceed with anomeric specificity, and high yield. This approach was used in the synthesis of mmole quantities of the anitgenic portion of the blood group O substance with specific carbon-13 enrichment.

Partially purified bovine N-acetylglucosaminide β (1-4) galactosyltransferase and porcine β galactoside α(1-2) fucosyltransferase were used with chemically synthesized sugar nucleotides in the synthesis of the antigenic trisaccharide Fuc α(1-2)Galβ(1-4)GlcNAc-β-hexanolamine.

The β-galactoside α(1-2) fucosyltransferase was partially purified from porcine submaxillary glands by a procedure similar to T.A. Beyer and R.L. Hill (unpublished results). The purification entailed; solubilization with Triton X-100, chromatography on the affinity adsorbant GDP-Sepharose, Sephadex G50 chromatography and concentration on GDP-Sepharose. The enzyme was purified 11,000 fold and was used in

the synthesis of mmole quantities of several fucosylated di and trisaccharides.

The enzyme-mediated synthesis of fucosylated oligosaccharides was performed by adding equimolar ratios of GDP-fucose and acceptor to the enzyme in sodium cacodylate buffer, pH 6.0 in the presence of manganese chloride at 33°. After 12 hours of incubation, yields of the fucosylated compounds were typically 80%. The remaining GDP-fucose was hydrolyzed to fucose during the incubation. Purification of the product di or trisaccharide was rapidly achieved by deproteinization, ion exchange chromatography and gel filtration. Enzymatic synthesis with specifically enriched UDP-[1-^{13}C]-D-Galactose, GDP-[1-^{13}C]-L-fucose or ^{13}C-enriched acceptors allowed preparation of singly or doubly enriched di and trisaccharides.

Specific ^{13}C-enrichment and comparison with ^{13}C-enriched model compounds allowed unambiguous resonance assignments of the carbons in the trisaccharide using $^{1}J_{C-C}$, $^{2}J_{C-C}$ and $^{3}J_{C-C}$ coupling constants. Derivatization of a carbon with another aldopyranosyl ring resulted in a 5-8 ppm downfield shift of that resonance. Carbons contiguous to the derivatized carbon also underwent small chemical shift changes. The β-galactoside α(1-2) fucosyltransferase was unambiguously shown to transfer fucose from GDP-L-fucose to C2 galactose when [1-^{13}C]Galβ(1-4)GlcNAc-β-hexanolamine was used as the acceptor. The C2 galactose was easily identified by a $'J_{C1'C2'}$ coupling of 46 Hz.

Inter-residue $^{3}J_{C-C}$, $^{3}J_{C-H}$ and $^{2}J_{C-C}$ coupling constants in the singly and doubly enriched fucosylated oligosaccharides allowed estimation of the most abundant conformer for the ψ and $\emptyset$ torsion angles in the Galβ(1-4)GlcNAc and Fucα(1-2)Gal glycosidic linkages.

DEDICATION

The author would like to dedicate this work to his wife

Cathryn Carson Rosevear

for the joy that she has given me, her love, and her continual support throughout the days of research and preparation of this work, and to his mother

Dorothy B. Rosevear

for her love, encouragment and help throughout the years.

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ABBREVEATIONS

2,5-diphenyloxazole, PPO; 1,4 bis [2(-4-methyl-5-phenyloxazolyl)]-benzene, POPOP; parts per million, ppm; guanosine monophoshate, GMP; guanosine diphosphate, GDP; guanosine triphosphate, GTP; uridine monophosphate, UMP; uridine diphosphate, UDP; uridine triphosphate, UTP; hydrochloric acid, HCl; lithium chloride, LiCl; sodium hydroxide, NaOH; N-acetylglucosamine, GlcNAc; L-fucose, Fuc; galactose, Gal; glucose , Glc; (6-amino-1-hexyl)-2-acetamido-2-deoxy-β-D-glucopyranoside, GlcNAc-β-hexanolamine; magnesium perchlorate, $Mg(ClO_4)_2$; mercuric cyanide, $Hg(CN)_2$; tetramethylsilane, TMS; lactose, Galβ(1-4)Glc; N-acetyllactosamine, Galβ(1-4)GlcNAc; (6-amino-1-hexyl)-4-O (β-D-galactopyranosyl)-2-acetamido-2-deoxy-D-glucose; Galβ(1-4)GlcNAc-β-hexanolamine; (6-amino-1-hexyl)-β-D-galactopyranoside, Gal-β-hexanolamine; ethyl-β-D-galactopyranoside, ethyl-Gal; methyl-α-L-fucopyranoside, methyl-Fuc; (6-amino-1-hexyl)-4-O-(fucopyranosylα(1-2) β-D-galactopyranosyl)-2-acetamido-2-deoxy-β-D-glucopyranoside, Fuc α(1-2)Galβ(1-4) GlcNAc-β-hexanolamine; (6-amino-1-hexyl)-2-O(α-L-fucopyranosyl)-galactopyranoside, Fuc α(1-2)Gal-β-hexanolamine; 2-O(α-L-fucopyranosyl)-D-galactopyranose, Fuc α(1-2)Gal; ^{13}C nuclear magnetic resonance spectroscopy, ^{13}C-nmr; 'H nuclear magnetic resonance spectroscopy, 'H-nmr; Sulfopropyl-Sephadex, SP-Sephadex, high pressure liquid chromatography, HPLC.

INTRODUCTION

Carbohydrates present on glycoproteins and glycolipids have recently become an active area of interest due to their probable role in cell-cell adhesion, density dependent growth inhibition, molecular recognition, hormonal control, blood clotting, immunological protection, structural support, and surface protection (1,2). Glycoproteins are ubiquitously distributed in nature, occuring in vertebrates, invertebrates, plants, bacteria and viruses.

One important class of glycoproteins and glycolipids are the ABO blood group substances. Aside from the recognized importance of the ABO antigens in blood typing and transfusion, they have been implicated in carcinoma, ulcers, pernicious anemia, hemolytic disease of the newborn, fetal loss and infertility (1,3).

A versatile chemical synthesis of these kinds of complex, biologically active, oligosaccharides has not been accomplished. Lemieux and coworkers, have succeeded in the chemical synthesis of the Lewis and B blood-group antigenic determinants (4,5). However, their method was extremely laborious, involving complex blocking groups, difficult α-glycosidations, purifications, and limited in the ability to vary the aglycon at the reducing end of the carbohydrate moiety.

A more versatile approach to the synthesis of biologically active oligosaccharides is needed due to the difficulty of isolation from natural sources, marked heterogenity when isolated, and limited availability. The necessity for a general method of oligosaccharide synthesis becomes increasingly important with the advent of ^{13}C nmr spectroscopy in the elucidation of structures and conformations of

oligosaccharides, antigenic determinants, glycoside-protein complexes, and micelles containing gangliosides (6,7,8,9,10,11,12). Carbon-13 nmr spectroscopy, with the use of appropriately labeled ^{13}C compounds, provides an excellent nondestructive tool for probing the conformations and biological interactions of this important class of compounds.

One approach to this problem is the use of a combination of chemical and enzymatic methods for the synthesis of complex oligosaccharides. This approach involves the chemical synthesis of enzyme substrates, with appropriate labels when necessary, and the use of these substrates with specific enzymes for the synthesis of oligosaccharide linkages. Enzymatic synthesis of the glycosidic linkage proceeds with specificity, high yield, and relative ease as compared to the chemical methods for synthesis of these linkages (4,5,13). One example of this type of synthesis is the use of the N-acetylglucosaminide β(1-4) galactosyltransferase in the synthesis of N-acetyllactosamine, glycosidic derivatives of N-acetyllactosamine and specifically labeled [^{13}C]-N-acetyllactosamine (13,14,15). This type of enzymatic approach to synthesis of carbohydrates has been used as early as 1955 by Bean and Hassid for the synthesis of several disaccharides (16). However, this approach has not been used for the synthesis of branched chain complex oligosaccharides found on cell surfaces that are responsible for antigenic activity. The combination of chemical and enzymatic syntheses requires that quantities of substrate and at least partially pure enzyme preparations be available.

The ABO blood group substances provide an excellent class of complex oligosaccharides to attempt a combination of chemical and enzymatic syntheses. Each carbohydrate is known to be transferred from

a sugar nucleotide donor to the nascent chain by action of a specific glycosyltransferase (17).

This thesis describes the solubilization and partial purification of a β-galactoside α(1-2) fucosyltransferase from porcine submaxillary glands. This enzyme is responsible for the transfer of L-fucose from GDP-fucose to oligosaccharides containing a terminal β-galactoside residue. Partial purification was achieved using the affinity adsorbant GDP-hexanolamine Sepharose. The use of this enzyme in conjunction with a bovine β-N-acetylglucosaminide β(1-4) galactosyltransferase and chemically synthesized sugar nucleotides, GDP-fucose and UDP-galactose, enabled the synthesis of quantities of the terminal trisaccharide portion of the blood group O antigenic substance sufficient for ^{13}C-nmr evaluation. Enzymatic synthesis of fucosylated disaccharides and trisaccharides containing specific ^{13}C enrichment was accomplished using chemically synthesized UDP-[1-^{13}C]-galactose and GDP-[1-^{13}C]-fucose. Specific enrichment with ^{13}C to the 90% level allowed vicinal carbon-carbon and carbon-hydrogen coupling constants to be evaluated in terms of the conformation of the oligosaccharide.

REVIEW OF THE LITERATURE

I. Current Concepts in Glycoprotein Biosynthesis

The discovery of glycogen synthetase by Leloir and Cardini (18) established the role of sugar nucleotides in complex carbohydrate synthesis as shown below;

$$\text{UDP-Glucose} + \text{Glycogen}_n \rightleftharpoons \text{Glycogen}_{n+1} + \text{UDP}$$

Recently attention has turned to the carbohydrate moiety which is covalently attached to either protein or lipid in glycoproteins and glycolipids, respectively. This interest has largely been due to the implication of carbohydrates in many important biological processes (1,2). Complex carbohydrates, in this context, are the oligosaccharide structure found in glycoproteins and glycolipids.

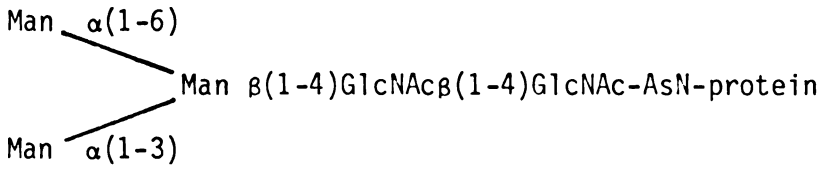
The general process for synthesis of complex carbohydrates consists of a transglycosylation reaction as shown below:

$$\text{XDP-O-R}_1 + \text{R}_2\text{-OH} \rightleftharpoons \text{R}_2\text{-O-R}_1 = \text{XDP}$$

where XDP-O-R_1 is any number of sugar nucleotides and $\text{R}_2\text{-OH}$ is the alcohol acceptor, usually a monosaccharide or oligosaccharide. The common sugar nucleotides used in the synthesis of complex carbohydrates are UDP-α-D-glucose, UDP-α-D-galactose, UDP-α-D-N-acetylglucosamine, UDP-α-N-acetylgalactosamine, GDP-α-D-mannose, GDP-β-L-fucose and CMP-α-sialic acid. The common anomeric linkages found for each of these sugars incorporated into complex carbohydrates are; α and β for galactose, N-acetylgalactosamine and mannose; β for N-acetylglucosamine, and α for fucose and sialic acid (1,2,19).

Two types of carbohydrate-protein linkages exist. Carbohydrates linked through asparagine are classified as N-glycosidically linked and those linked through serine or threonine as O-glycosidically linked carbohydrates. The N-glycosidically linked carbohydrates are found in plasma proteins, hormones, immunoglobulins and enzymes including bovine ribonuclease and α_1-acid glycoprotein (2). The O-glycosidically linked glycoproteins include those from mucous secretions, immunoglobulins, fetuin, plasma membranes and the Antarctic fish freezing-point depression glycoprotein (2).

The biosynthesis of N-glycosidically linked carbohydrates has recently been described by Schachter and Roseman (19). These structures share a common core containing mannose and N-acetylglucosamine. The assembly of this core occurs through a polyisoprenoid phospholipid acceptor, dolichol phosphate (20). Initiation of the oligosaccharide core occurs by the transfer of N-acetylglucosamine from UDP-GlcNAc to the lipid acceptor (20). The chain is then extended by the sequential transfer of N-acetylglucosamine and mannose from UDP-GlcNAc and GDP-Man to form Manβ(1-4)GlcNAcβ(1-4)GlcNAc-P-P-Dol. Further extension occurs with branching by the subsequent addition of mannose and glucose to form mannosylphosphoryl dolichol (19). The entire oligosaccharide is then transferred from dolichol phosphate to an asparagine residue on the nascent glycoprotein. This transfer of the oligosaccharide from dolicohol to the protein occurs in the rough endoplasmic reticulum (19). Further elongation occurs in the Golgi apparatus of the cell. Before the immature glycoprotein reaches the Golgi complex the oligosaccharide is processed to remove all glucose residues and some mannose residues. The processed nascent glycoprotein strucure is shown below:



Once the nascent glycoprotein reaches the Golgi area final elongation occurs by the sequential action of specific glycosyl transferases (19). The structures of the mature glycoproteins are dictated by the strict substrate specificities of the glycosyltransferases (21). The action of a number of these glycosyltransferases are mutally exclusive (21,22). Vacuoles then bud off from the Golgi apparatus and the mature glycoprotein is transferred to the exterior of the cell.

Biosynthesis of O-glycosidically linked oligosaccharides does not occur by the same mechanism as N-glycosidically linked oligosaccharides (19,23,24). Initiation does not involve pre-assembly of the oligosaccharide as a lipid intermediate (24). The Ser(Thr)-GalNAc linkage is synthesized by a UDP-GalNAc: polypeptide N-acetylgalactosaminyltransferase (25). Immediately after the incorporation of GalNAc there is a branch point in the synthetic pathway of the oligosaccharide (25). The incorporation of sialic acid α(2-6) to the GalNAc prevents further carbohydrate incorporation and the predominant form of ovine submaxillary mucin results. Incorporation of galactose β(1-3) to the GalNAc allows further incorporation of carbohydrates to form the common porcine submaxillary mucins. The relative proportions of these two enzymes control this important branch point (26). Additions of carbohydrates to the Galβ(1-3)GalNAc-Ser can

then proceed to form the ABO blood group megalosaccharides as shown in Figure (1) (27).

Figure 1. Structures Of The O-Glycosidically Linked Oligosaccharides. I, The predominant form of ovine submaxillary mucin. Incorporation of sialic acid α(2-6) to the GalNAc prevents further carbohydrate incorporation. II, the predominant form of porcine submaxillary mucin. Incorporation of GalNAc allows further carbohydrate incorporation. III, The complete carbohydrate structure of the ABO blood group megalosaccharides.

I Sialic acid α(2-6)GalNAc-O-(Thr)Ser

II Galβ(1-3)GalNAc-O-(Thr)Ser

III (A) GalNAc α(1-3)

or Gal β(1-4)GlcNAcβ(1-6)Galβ(1-3)GlcNAcβ(1-3)Galβ(1-3)GalNAc-O-(Thr)Ser

α(1-2)

(B) Gal α(1-3)

Fuc

(H)

Figure 1

II. The Biochemical Structure and Synthesis of the Blood Group Subtances

The blood group antigens are one important class of cell surface oligosaccharides. They are gene-dependent structures expressing the "individuality" of cell surfaces, body fluids, and secretions. Blood group antigens are classified into the following groups depending on their specificities: ABO, Lewis, MN, P,I,Y, and Rh antigens. There is no O-antigen, group O erythrocytes contain the H antigen, but the Group O designation has been retained for historical reasons. Pure ABO antigens were isolated in water-soluble form from pepsin, saliva, gastric juice, gastric mucin, ovarian cyst fluid and urine (28). Membrane bound ABO blood group antigens have recently been isolated from erythrocytes using a combination of organic solvents and detergents (29).

The scheme of synthesis of the blood group antigens, their participating genes and their primary products, glycosyltransferases, is schematically represented in Figure 2. Biologically, these substances are synthesized by glycosyltransferases through the sequential addition of monosaccharide units, derived from sugar nucleotides, to carbohydrate acceptors. These glycosyltransferases have strict specificity toward the structure of the sugar nucleotide and somewhat less specificity toward the structure of the precursor carbohydrate chain (acceptor).

The serologic specificity of these oligosaccharides is determined by the structure and linkage of the monosaccharides at the nonreducing ends of the carbohydrate chains. Two types of nonreducing ends, Type 1

Figure 2. The scheme of synthesis of type two blood group antigens, their participating genes and their primary products, glycosyltransferases.

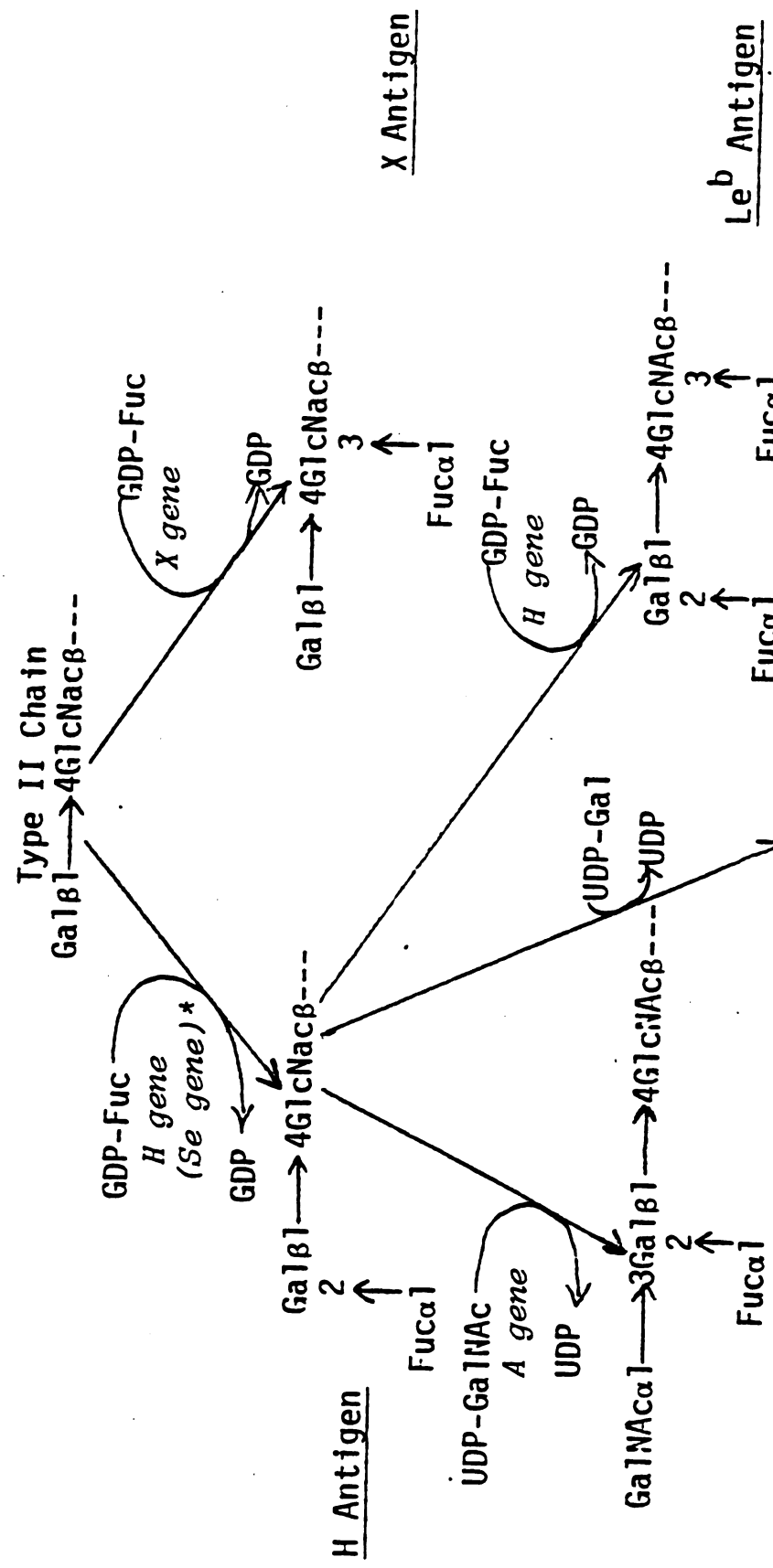

Type II Chain
Galβ1→4GlcNacβ---
GDP-Fuc
H gene
(Se gene)*
GDP
Galβ1→4GlcNacβ---
2
↑
Fucα1
H Antigen
GDP-Fuc
X gene
GDP
Galβ1→4GlcNacβ---
3
↑
Fucα1
X Antigen
UDP-GalNAc
A gene
UDP
GalNAcα1→3Galβ1→4GlcNacβ---
2
↑
Fucα1
A Antigen
UDP-Gal
UDP
Galα1→3Galβ1→4GlcNAcβ---
2
↑
Fucα1
B Antigen
GDP-Fuc
H gene
GDP
Galβ1→4GlcNAcβ---
2
↑
Fucα1
3
↑
Fucα1
Leb Antigen

Figure 2

and Type 2, are commonly found in the ABO and Lewis systems. Type 1 contains a Galβ(1-3)GlcNAc sequence while Type 2 contains a Galβ(1-4)GlcNAc sequence, commonly called N-acetyllactosamine. The Type 2 sequence can be synthesized by the galactosyltransferase isolated from milk (15). The A,B, and H determinants can be synthesized from either Type 1 or Type 2 chains. The most important carbohydrate in each chain is known as the immunodominant sugar and for H specificity this carbohydrate is L-fucose. A fucosyltransferase, the product of the H gene and designated β-galactoside α(1-2) fucosyltransferase, is responsible for the synthesis of the H-active substance through addition of L-fucose from GDP-fucose, to either Type 1 or Type 2 chain acceptors.

The Lewis system is characterized by the addition of fucose to the N-acetylglucosamine of Type 1 chains. This is an α(1-4) linkage and is catalyzed by β-galactoside α(1-4) fucosyltransferase which is a Le gene product. The fucose linked α(1-4) to the N-acetylglucosamine is the immunodominant structure of the Le^a antigen. However, the Le^b antigen contains not only fucose linked α(1-4) to N-acetylglucosamine but also a fucose α(1-2) linked to galactose to form it's immunodominant structure. The fucosyltransferase responsible for the α(1-2) linkage is the H gene product.

The immunodominant sugar in the blood group A substance is N-acetylgalactose linked α(1-3) to the galactose of the H-active substance. The N-acetylgalactosaminyltransferase designated [fucosyl α(1-2)] galactoside α(1-3)N-acetylgalactosaminyltransferase, responsible for this activity has very strict substrate specificity for Fucα(1-2)Galβ(-) and uses UDP-N-acetylgalactosamine as the sugar

nucleotide donor (30,31). This enzyme, the product of the A gene, has recently been purified from porcine submaxillary glands and characterized (30,31).

Finally, the immunodominant sugar in blood group B antigenic substance is galactose. The enzyme that catalyzes the transfer of galactose from UDP-galactose to Fucα(1-2)Galβ(-) is a galactosyltransferase designated [fucosylα(1-2)] galactoside α(1-3) galactosyltransferase, and is the B gene product.

III. Review on the Purification and Properties of Lactose Synthetase

Lactose Synthetase (UDP-galactose:glucose-β(1-4)galactosyltransferase; EC 2.4.1.22) catalyzes the synthesis of a galactose β(1-4) glycosidic linkage between N-acetylglucosamine or glucose. In the presence of a specific protein, α-lactalbumin, the enzyme will only transfer galactose to glucose to form lactose (32). However, in the absence of α-lactalbumin the enzyme shows a high acceptor specificity for N-acetylglucosamine (14).

Lactose synthetase activity and the levels of α-lactalbumin have been measured in the mammary glands from pregnant and lactating animals and shown to be approximately 20% higher than in non-lactating animals (33,34). Increased synthesis of lactose synthetase and α-lactalbumin were observed by treatment of mammary explants from virgin mice with insulin, hydrocortisone, and prolactin (35). The presence of progesterone was found to decrease the synthesis of α-lactalbumin but the synthesis of lactose synthetase remained unaltered (36). The transferase accumulates in Golgi membranes during the last half of pregnancy and once lactation commences the rate of α-lactalbumin synthesis increases tremendously. The greatly increased α-lactalbumin

synthesis is due to the drop in progesterone levels at parturition. Lactose after it's synthesis, together with α-lactalbumin, casein, and other milk proteins is enclosed in membranous vacuoles, derived from the Golgi, and secreted by exocytosis (37). This mechanism accounts for the release of proteins and lactose into the milk but also explains the presence of galactosyltransferase in the milk. The inner surface of the secretory vacuole corresponds to the intracisternal membrane of the Golgi apparatus to which the galactosyltransferase is embedded. Once the vacuole has fused with the cell surface, the transferase could either dissociate from the membrane into the milk or be attacked by proteolytic enzymes in the milk which cleave it from the membrane (39).

In tissues other than the mammary gland the galactosyltransferase functions in the catalysis of plasma glycoproteins (40). Under physiological conditions, competition of glucose with glycoproteins containing terminal N-acetylglucosamine residues is not possible since the Michaelis constant for glucose is 2.5 M in the absence of α-lactalbumin. The galactosyltransferase is secreted from the Golgi apparatus in vaccuoles and released into the extracellular fluid.

The enzymatic activity of lactose synthetase was first demonstrated by Watkins and Hassid (41). Brodbeck and Ebner (42) showed that the enzyme system is composed of two components, A and B, and Brew et al. (14) clearly identified the A component as UDP-galactose: N-acetylglucosamine β(1-4) galactosyltransferase. Babad and Hassid (43) were the first to report that the bovine galactosyltransferase was a particulate enzyme and to partially purify the soluble form from milk. The purification yielded a 70-fold enriched enzyme using ammonium sulfate precipitation, heat denaturation, and hydroxylappatite

chromatography (43). The most common assay system for the galactosyltransferase was also developed by Babad and Hassid (43) and consisted of using a low molecular weight acceptor, glucose or N-acetylglucosamine, and radioactive galactose in UDP-galactose. After the appropriate incubation period, the reaction was diluted with ice water and passed over a pipet column of Dowex 1 chloride resin. The unreacted UDP-galactose remains bound to the column and the radioactive galactose transferred to the acceptor passes through the column directly into a scintillation vial and is counted.

Pure preparations of galactosyltransferase were first obtained from bovine milk by chromatography on DEAE-Sepharose, cellulose phosphate and α-lactalbumin attached covalently to Sepharose (44). α-Lactalbumin was coupled to Sepharose by the cyanogen bromide activation method of Porath, Axen, and Ernback (45). The partially purified galactosyltransferase was applied to the α-lactalbumin Sepharose column in the presence of manganese and glucose. After all the inert protein had been eluted, the galactosyltransferase was specifically eluted by removal of the glucose in the buffer (44). The galactosyltransferase purified from the α-lactalbumin Sepharose was found to be pure by gel electrophoresis (44). Later, Barker, et al. (15) introduced the use of argarose derivatives of uridine diphosphate and N-acetylglucosamine for the purification of the bovine galactosyltransferase. The galactosyltransferase could be bound to the UDP-hexanolamine Sepharose affinity resin in the presence of manganese ions and specifically eluted by removal of the manganese ions and addition of EDTA. The N-acetylglucosamine Sepharose affinity column was also found to bind the galactosyltransferase in the presence of UMP or UDP. The enzyme could

be specifically eluted with the addition of glucose and removal of the UMP or UDP.

SDS-polyacrylamide gel electrophoresis showed three main bands with molecular weights corresponding to 54,000, 48,000 and 42,000. All three polypeptides have been shown to be active by gel filtration studies (38). Treatment of the three components with trypsin was found to give a progressive decrease in the high molecular weight form and increasing amounts of the lower molecular weight forms (39). The galactosyltransferase is a glycoprotein with a considerable amount of carbohydrate (44).

Bell et al. (46) have shown that the galactosyltransferase follows a random equilibrium mechanism. In the absence of α-lactalbumin, the enzyme first binds manganese and then can either bind UDP-galactose or acceptor (46). When α-lactalbumin is added to the assay system it has been shown to bind to either the E-Mn-UDP-Gal or the E-Mn-acceptor complex (46).

IV Review of the Literature on the Fucosyltransferase

Fucosyltransferases are known which transfer L-fucose, from GDP-L-fucose, onto various acceptor molecules in an α(1-2), α(1-3), α(1-4) and α(1-6) linkage (47,48,49,50,51,52,53).

The enzyme initially was reported and the products partially characterized in human milk and serum (48,52,53,54). Later, the enzyme was reported in human submaxillary glands, human stomach tissue, human bond marrow, rat small intestinal mucosa, porcine liver, porcine submaxillary glands, ovine submaxillary glands, testis, bovine spleen, human neuroblastoma cells, rat lymphocytes, Hela cells, and monkey

kidney cells (47,55,56,57,58,59,60). Enzymatic activity was measured either directly from the soluble source, after ammonium sulfate precipitation or after isolation of a Golgi or membrane fraction from the various tissues. The major problem with many of the studies except those done on the bovine spleen and porcine liver was that only radioactive GDP-fucose, containing no measureable mass, was used as the sugar nucleotide donor. One example of this is the use of 0.2 nmoles of GDP-L-[^{14}C]-fucose containing 135,000 cpm as the nucleotide donor and N-acetylactosamine as the acceptor in an enzyme assay. After 20 hours only 4% of the radioactivity migrated in an area corresponding to a trisaccharide following paper chromatography (55). The inherent low activity of the enzyme and lack of substrate saturating amounts of GDP-fucose lead to incubation times ranging from one hour to three days (54,58). Isolation of the product usually entailed descending paper chromatography, a double chromatographic method, or high voltage electrophoresis followed by descending paper chromatography (54,57,58). Thus, in addition to long incubation times, isolation of the product for scintillation counting required, at least, an additional 24 hours.

The possibility of various positional isomers of fucose in oligosaccharides led to the development of procedures for determination of the linkage position. A common procedure used to show fucose α(1-2) linked to galactose when low molecular weight acceptors were used took advantage of the Lobry de Bruyn-Alberda van Ekenstein transformation. Under alkaline conditions, at elevated temperatures, 2' fucosyllactose yields 2' fucosylgalactose, a small amount of 2' fucosyltalose and degradation products of glucose. The 2' fucosylgalactose can be identified by descending paper chromatography and compared against

standard 2' fucosylgalactose isolated from milk (47,48). Alternatively, an α(1-2) fucosidase, which is specific for the 2' fucosylgalactose linkage, can be isolated from Colstridium perfringens and used to determine if the fucose linkage is α(1-2) (58,61). Two fractions can be isolated from Trichomonas foetus that contain fucosidase activity (62). One is specific for the α(1-2) linkage to galactose and the second fraction specific for fucose linked either α(1-3) to N-acetylglucosamine (or glucose) or α(1-4) to N-acetylglucosamine (62,63).

Serological activity is often used for information on the linkage position of fucose. For example, fucose linked α(1-2) to galactose of N-acetyllactosamine, H-active trisaccharide, should inhibit the agglutination action on O cells of anti H sera. This type of inhibition of hemagglutination has been used to characterize the various fucosyl linkages in oligosaccharides (64,65). For determination of H-active products the lectins Ulex europeus and Lotus tetragonalobus were used with blood group O cells. Chester et al. (63) synthesized various methyl, phenyl, and nitrophenyl glycosides for use as artificial acceptors for the fucosyltransferase. One acceptor, β-D-phenylgalactopyranoside was said to be specific for accepting fucose in an α(1-2) linkage to galactose (63).

Most of the procedures described above make use of the following various artificial acceptors; lactose, N-acetyllactosamine and β-D-phenylgalactoside. Studies have also been done with macromolecular acceptors isolated from various tissue sources. Some of the macromolecular acceptors used are: asialofetuin, asialo-α_1-glycoprotein, and porcine submaxillary mucin. Fucosyltransferase activity is assayed by precipitation of the macromolecular acceptor with

trichloracetic acid and either the radioactivity determined directly or the protein subjected to chromatography (56). Glycolipids have also been used for acceptors in the fucosyltransferase reaction. The reaction product glycolipids are usually extracted with organic solvents and subjected to chromatographic analysis (58).

Jabbal and Schachter (57) published one of the first papers in which substrate saturating amounts of GDP-fucose were used to assay the enzyme. The enzyme was isolated from porcine liver by preparation of a 48,000 X g membrane pellet. Both low molecular weight and macromolecular acceptors were used. These were assayed for radioactive fucose transfer by high voltage electrophoresis followed by descending paper chromatography (57). Their enzyme was found to be unstable at 4°C and -20°C for over 2 to 4 days, respectively (57). The fucosyltransferase activity had a broad pH optimum between 6.5 and 8.5 and both magnesium and manganese were equally effective in stimulating enzyme activity. Triton X-100 (0.2%) and GTP (.5mM) had a stimulating effect. The enzyme was inhibited 65% by 50mM EDTA. It was shown that the enzyme obeyed Michaelis-Menten kinetics with a Km calculated to be 7.8×10^{-5} M for the nucleotide sugar. Basu et al. (58) using substrate saturating concentrations of GDP-fucose, investigated a fucosyltransferase in bovine spleen. They isolated a fraction rich in Golgi, endoplasmic reticulum and mitochondria enriched 24 to 46 fold over the crude homogenate (58). The membrane bound enzyme transferred fucose from GDP-fucose to various glycosphingolipids. Treatment of the enzyme with EDTA resulted in a 95% loss of activity. In contrast to the enzyme reported by Jabbal and Schachter (57), GTP (0.5 mM) was found to inhibit the enzyme 30 percent. Maximal activity was found with the

cationic detergent G3634A (58). GDP-Fucose was found to obey Michaelis-Menten kinetics with a K_m of 3.6×10^{-4}M (58). The calculated K_m for the acceptor lactosylceramide was found to be 6×10^{-4}M. Products were characterized by microimmunodiffusion using Ulex europeus and hemagglutination inhibition techniques (58).

The fucosyltransferase reported by Basu et al. (58) had a wider range of specificity for the acceptor substrate than the porcine liver enzyme reported by Jabbal and Schachter (57). These conclusions were based on enzymatic assay of crude tissue preparations containing membrane bound fucosyltransferase. It is important to note that the substrate specificities using membrane fragments can be assigned only tentatively since the influence of the membrane fragment is not known. Likewise, only apparent kinetic values and physical properties can be established. Further characterization of the fucosyltransferase with respect to the number of isoenzymes present, substrate specificities, (various glycoproteins verses glycolipids), kinetic properties and physical properties will have to await solubilization, purification and characterization of the enzyme from various tissue sources. Purification of these isoenzymes will be extremely difficult due to the inherant low activity (pmoles.hr^{-1}.mg^{-1} protein), difficulty in solubilization, separation of the isoenzymes, and characterization of their substrate specificities and products.

Several investigators attempted to overcome the solubilization difficulties by isolation of the enzyme from soluble sources such as serum (54,66). However, the major problem with soluble sources of the fucosyltransferase are the inherant low activities. A soluble

β-galactoside α(1-2)fucosyltransferase has been reported in all ABO secretors. All sera examined were also found to contain a β-galactoside α(1-3) fucosyltransferase of unknown function. Schenkel-Brunner et al. (67) reported an activity for both fucosyltransferases of 7.5 pmoles of product formed/25ml serum/72 hours. Such a low activity makes purification and characterization from soluble sources almost impossible.

Recently, the β-galactoside α(1-2) fucosyltransferase has been purified to homogenity from porcine submaxillary glands using solubilization with Triton X-100 and GDP-hexanolamine Sepharose affinity chromatography. (T.A. Beyer and R.L. Hill, personal communication).

V Review of ^{13}C-NMR Spectroscopy and ^{13}C-^{13}C Spin-Spin Coupling Constants.

Nuclear magnetic resonance spectroscopy was initially devoted almost entirely to the proton. The proton is ubiquitous in organic molecules has a high natural abundance of the 'H isotope (99.98%), a spin of 1/2, and a high magnetic susceptibility which produces strong resonance signals.

With the advent of fast Fourier transform techniques and pulsed excitation of nmr signals the observation of ^{13}C-nmr spectra and measurement of ^{13}C-X spin-spin coupling constants have become possible.

The common isotope of carbon, ^{12}C, has a nuclear spin of 0 and is nmr inactive. The isotope of carbon which is nmr active, ^{13}C, exists in nature to the extent of 1.1%. The second inherent problem with the ^{13}C nucleus is the low magnetic susceptibiltity, only 0.016 that of 'H. However, ^{13}C nmr spectroscopy does offer several

advantages over conventional 'H nmr. Carbon-13 nuclei resonate over 200 ppm of the applied field where 'H nuclei only resonate over 12 ppm of the applied field. ^{13}C NMR spectra, normally obtained with broad band 'H decoupling to remove spin-spin couplings between ^{13}C and 'H, are usually much less complex than corresponding 'H spectra allowing the interpretation of more complex molecules. Proton decoupling also enhances the carbon signals due to the nuclear Overhauser effect.

Carbohydrates provide excellent examples of the usefullness of ^{13}C nmr spectroscopy. Chemical shifts and coupling constants are very sensitive to substitution, configuration and conformation. This sensitivity often makes assignment of resonances quite difficult but once assignments are made, the same sensitivity yields information about structure that would not other wise be obtainable.

Assignments of chemical shifts in ^{13}C nmr spectra of carbohydrates have been established by selective [^{13}C] enrichment (68,69), deuterium isotope shifts (70,71), effects of derivatization on chemical shift (72) and the use of one bond ^{13}C-'H coupling constants (68,73).

Since organic compounds containing carbon also have other nuclei possessing spin in close proximity to the carbon nucleus, these spins may be coupled so that various combinations of spin states may differ in energy and fine structure in the resonance bands produced.

The first spin-spin couplings involving the carbon nucleus to be observed were one bond carbon-hydrogen couplings ($^1J_{CH}$). Due to the higher sensitivity of the 'H, these couplings were usually extracted from the 'H spectra. When specifically ^{13}C-enriched compounds are

available, however, the $^1J_{CH}$ coupling can be obtained more directly from the ^{13}C spectra.

Bock et al. (73), Bock and Pedersen (74) and Walker et al. (68) have measured ^{13}C-^1H coupling constants ($^1J_{CH}$) in hexoses, pentoses and their derivatives. Their data show that in pyranosyl derivatives $^1J_{C1-H1}$ is found to be approximately 10Hz smaller (160 Hz) when the H-1 is axial than when the H-1 is equatorial (170 Hz).

The magnitude and sign of two bond C-H couplings ($^2J_{CCH}$) in carbohydrates have been studied by Perlin and his coworkers (75, 76) and Walker et al. (68). Perlin and his coworkers (75,76,77) demonstrated that the orientation and electronegativity of the substituents on the ^{13}C nucleus affected the magnitude of the two bond coupling between ^{13}C and ^1H.

The establishment of a Karplus-type (78) relationship between vicinal coupling of ^1H nuclei and the magnitude of the torsional angles led to investigations concerning the angular dependence of three-bonded (vicinal) carbon-proton couplings. Calculations for propane suggest a classical Karpus-type relationship for $^3J_{CH}$ with a minimum at a dihedral angle of 90°, a maximum at 0° and a larger maximum at 180° (79). Experimental studies on aliphatic $^3J_{CH}$ values and conformational studies on propionaldehyde strongly suggest this dependence of coupling constant on dihedral angle (80,81). Schwarcz and Perlin (76) have established a Karpus type relationship for $^3J_{C-H}$ coupling in carbohydrates. Coupling constants of 0,2 and 6Hz were found to correspond approximately to dihedral angles of 100, 60 and 180°,

respectively. They also observed that vicinal ^{13}C-H coupling was very sensitive in the 60° region of the dihedral angle (76).

Measurement of natural abundance ^{13}C-^{13}C coupling constants is exceedingly difficult since two atoms of ^{13}C are present in the appropriate relationship in 1 of every 10^4 molecules. However, one bond ($^1J_{CC}$) coupling constants have been observed on natural abundance compounds using Fourier transform instruments and concentrations of compound near 70% (82). The vast majority of J_{C-C} values have been determined by the use of singly or multiply ^{13}C-enriched compounds. Carbon-13 enrichment is necessary when large amounts of compounds are not available and when small coupling constants are to be observed. Karabatsos et al. (83) have predicted that ^{13}C-^{13}C coupling constants would be 0.16-0.49 times the corresponding ^1H-^1H coupling constants. The small magnitudes of the coupling constants are compensated for by the simplicity in which they can be observed. Carbon-carbon coupling constants have recently been the subject of three excellant reviews (84,85,86).

The value of one bond, $^1J_{C-C}$, carbon-carbon coupling constants have been shown to be a function of hybridization, substitution, ring size and substituent orientation (87). Substituent orientation effects, when observed, appear to be small in most cases. Walker et al. (68) observed one bond carbon-carbon coupling constants in specifically enriched [1-^{13}C]-aldopyranoses in the range of 46 Hz. The magnitude of the $^1J_{C-C}$ coupling constant did not appear to be sensitive to substituent orientation at C1 or C2.

Two-bond couplings between carbon nuclei have been known for some time to depend on the hybridization of the carbons involved, the

electronegativity of substituents on the carbons and the bond angles between the two coupled carbon nuclei (88). Values for $^2J_{C-C}$ coupling between two sp^3 hybridized carbons fall in the range of 0.5-4.4 Hz (85). Walker et al. (68) observed in carbohydrates that ^2J(C1-O-C5) was large in α-anomers but small and not resolvable in the β anomers. This was explained in terms of the "dihedral angle rule" adopted by Perlin (75,76) to explain $^2J_{C-H}$ coupling constants. A substituent trans to the carbon gives a positive contribution and a substituent in the gauche position gives a negative contribution to the coupling. Carbons in the carbohydrate ring were assumed to be equivalent to oxygen ring atoms in their contribution to the coupling constant (68). However, this approach does not explain the behavior of $^2J_{(C1-C3)}$ coupling in carbohydrates.

Three bond carbon-carbon couplings have been investigated in aliphatic carboxylic acids and alcohols by Marshall et al. (89) and Barfield (90,91). They obtained a "modified" Karplus type curve with a minimum and maximum displaced from 90° and 180° when compared to the ^1H Karplus relationship. The dependence on the dihedral angle, Ø, was similar to the curve found for ^3J(FF) (92). Dihedral angles of 0, 80 and 180° were found to correspond to coupling constants of 2, 0 and 4Hz, respectively (89). Recently, Barfield (91) demonstrated from theoretical calculations that nonbonded interactions are substantial for coupling to ^{13}C and may lead to deviations from the "Karplus type" relationship of dihedral angle and coupling constant. This deviation has been explained by the importance of interactions of the "rear lobes" of the carbon hybrid orbitals in the trans arrangement (91).

Vicinal carbon-carbon couplings of the magnitude of 3-4Hz were observed in [1-^{13}C]-enriched aldopyranoses between C1 and C6 (68). This coupling constant corresponds to a trans relationship between C1 and C6 and may therefore represent a near maximum for $^{3}J_{C-C}$ coupling in carbohydrates. Coupling between C1 and C4 (dihedral angle = 60°) in the [1-^{13}C]-enriched carbohydrates was not observed (68). The vicinal relationship between C1 and C4 in the pyranose ring is gauche and therefore only a small coupling constant would be predicted. However, coupling within cyclic systems is complicated by the fact that there are two pathways through which coupling can occur. In the case of C1-C4 coupling, the pathway through C2 and C3 is the mirror image of the pathway through O5 and C5. If the couplings have different signs there would be a cancelation of couplings decreasing the likelihood observing a coupling which would have a small value. Although a well defined relationship between dihedral angle and coupling constant in carbohydrates for $^{3}J_{C-C}$ coupling has not been established, values of $^{3}J_{C-C}$ observed in the pyranose ring can serve as estimates for the maximal and minimal values expected for $^{3}J_{C-C}$ coupling in carbohydrates.

METHODS

I. Materials

Bovine milk was obtained from the Michigan State University dairy. Porcine submaxillary glands were purchased from Bio-Resources Dallas, Texas. [6-^{3}H]-L-Fucose, [UL-^{14}C]-L-fucose, GDP[UL-^{14}C]-L-fucose, UDP[UL-^{14}C]-D-galactose, [UL-^{14}C]-D-galactose, D-lyxose, and K[^{14}C]CN were purchased from New England Nuclear. Potassium [^{13}C] cyanide (K^{13}CN) was supplied by the Los Alamos Scientific Laboratory, University of California, Los Alamos, N.M., with 99.64 percent purity and 90.7 atom percent ^{13}C enrichment. Galactose, N-acetylglucosamine, L-fucose, hexanolamine, UDP-galactose, UMP, GMP, GDP, Dowex 50-X8, Dowex 1-X8, Bio-Gel P2 (-400 mesh), Sulfopropyl-Sephadex, Sephadex G50, Sepharose 4B and Chelex-100 were purchased from Sigma Chemical Company. Silica Gel G, 250 or 500 microns, thin layer chromatography plates were purchased from Analtech.

II. Analytical Procedures

1. Thin Layer Chromatography

Thin layer chromatography was performed on Silica Gel G plates, 250 or 500 microns, using two solvent systems: (I) ethyl acetate: hexane (1:1v/v); (II) n-propanol:glacial acetic acid:water (85:22:3 v/v). Plates were developed with 2N H_2SO_4 followed by heating at 110° for 5-15 min. Radioactivity was detected on a Berthold radioscanner. Ascending chromatography was performed on Whatman #1 paper in solvent system (III), isopropanol: 1.3 M aqueous sodium acetate buffer, pH 5.0 (7:3 v/v).

2. High Voltage Paper Electrophoresis

High voltage paper electrophoresis was performed on Whatman #1 paper in 0.05 M triethylammonium bicarbonate buffer, pH 7.4, at 2100V and 32 d.c. amps for 1-2 hr. Compounds were identified by either molybdate phosphate spray (93), ninhydrin spray (94), UV absorbance or radioactivity.

3. High Pressure Liquid Chromatography

High pressure liquid chromatography was performed on a Whatman Partisil PXS 10/25 SAX column at 1000 psi using 0.2M potassium phosphate buffer, pH 3.4. Compounds were detected by monitoring the column eluate with a Altex dual wavelength UV detector.

4. Gas Chromatography

Gas chromatography was performed on a Varian Aerograph Series 2100 gas chromatograph, with a flame ionization detector. The column was either an OV-17(3%) on High Performance Chromosorb G or a Hi-eff 3CP (10%) on GAS-CHROM Q, 80-100 mesh, (both supplied by Applied Science Laboratories, INC) with helium as the carrier gas. Silyl derivatives were prepared by drying 1-10 mg of material in a derivatization vial and adding 150 µl of pyridine followed by 250 µl N, O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) with 1% trimethylsilylchloride obtained from Pierce. The derivative mixture was incubated at 60° for 30 min. Separations were achieved with a linear temperature program from 100 to 250° at 8° per min.

5. Radioactivity Measurements

Radioactivity measurements were made with a Beckman LS-100 or LS-7000 scintillation counter. Aqueous samples (0.2 to 1.0 ml) were

mixed with 2-5 ml of scintillation fluid (3g PPO, 0.2g dimethyl POPOP, 1 liter Triton X-100 and 2 liters toluene) and counted.

6. Carbon-13 NMR Spectroscopy

Carbon-13 nmr spectra were obtained with a Bruker WP-60 or 180, 15.08 or 45 MHz, Fourier-transform spectrometer equipped with quadrature detection. Spectra were obtained in the proton-decoupled mode with 4K spectral points at 36°. High field carbon-13 nmr spectra were obtained on a Nicolet 360, 90 MHz, Forrier transform spectrometer equipped with quadrature detection. Spectra were obtained using a two level decoupling program to prevent excessive sample heating due to the high decoupling power required. A spectral window of 1800 Hz was used when the entire spectrum of the compound was required and a spectral window of 1000 Hz used for doubly enriched compounds. All spectra were obtained with 16384 spectral points at 14°. Chemical shifts are given in ppm and referenced to the C1 resonance of β-D-glucopyranose in a water solution and are accurate to $\pm$ 0.01 ppm with respect to internal TMS (68).

7. Quantitation of Hexanolamine Compounds

Quantitation of hexanolamine derivatized compounds containing a primary amino group was performed using the fluorescamine assay (95). Hexanolamine, 0 to 80 μmoles was used as a standard. To a solution containing the primary amino group, 1.5 ml of 0.2M borate buffer, pH 9.25, is added and the solution mixed well. With constant vortexing, 0.5 ml of a 30% w/v solution of fluorescamine in dry p-dioxane is added. Complete mixing is essential since fluorescamine is decomposed by water. The fluorescence was determined by excitation at 390 nm and emission at 475 nm on an Aminco-Bowman spectrophotofluorometer using a Xenon lamp

power supply. The fluorescamine solution in dioxane, if kept dry at 4°, remains good for about 2 months.

8. Protein Determination

Protein concentrations were measured using the fluorescamine assay of Bohler et al. (95) with bovine serum albumin as a standard.

III. Synthetic Procedures

1. GMP-Morpholidate and UMP-Morpholidate

GMP-Morpholidate and UMP-morpholidate were synthesized by the method of Moffat (96). The sodium salts of GMP and UMP were converted to the free acids by passage over a 4 x 20 cm column of Dowex 50 (hydrogen form; 20-50 mesh) resin. The progress of the reaction was followed by high voltage paper electrophoresis and was determined to be complete when only the morpholidate derivative was detected by UV and phosphate analysis. Trituration with ether yielded a fluffy white powder in approximately 60% yield.

2. 6-Amino-1-Hexanol Phosphate (I)

6-Amino-1-hexanol phosphate was prepared by the method of Barker et al. (68).

3. N-Trifluoroacetyl 6-Amino-1-Hexanol Phosphate (II)

N-Trifluoroacetyl 6-amino-1-hexanol phosphate was synthesized from 6-amino-1-hexanol phosphate (I) by the method of Barker et al. (68).

4. P'-(6-Amino-1-Hexyl)-P-(5'-Guanosine)-Pyrophosphate (III)

N-Trifluoroacetyl-6-amino-1-hexanol-GDP (III) was synthesized from compound (II) and GMP-morpholidate by the procedure of Moffat (96). Solutions containing 1mmole of GMP-morpholidate or 1mmole compound (II) plus 1 mmole tri-N-octylamine in 100 ml pyridine, that had been

distilled over calcium hydride, were evaporated three times with pyridine. The compounds were dryed in vacuo over $Mg(ClO_4)_2$. In a glove box, under a stream of N_2 gas, the GMP-morpholidate was dissolved in compound (II) and tri-N-octylamine. This was evaporated to dryness at 40° and another 50 ml dry pyridine added to dissolve the gum. The flask was then flushed with N_2 gas, stoppered, and stored in a desiccator over P_2O_5 under a slight vacuum for 48 hours at room temperature. The pyridine was then removed and 100 ml H_2O added. This was extracted 3 times with 50 ml aliquots of ether. The aqueous phase was applied to a 1 x 25 cm Dowex-1 (chloride form; 200-400 mesh) column and washed with 125 ml H_2O. A linear gradient of 200 ml 0.01 N HCl as starting solvent and 200 ml 0.01 N HCl containing 0.6 M LiCl as the limit solvent was applied. Fractions were collected every 6 ml and absorbance monitored at 262 nm. Three UV absorbing peaks were found, pooled, and analyzed by high voltage paper electrophoresis, ascending paper chromatography in solvent III and by ^{13}C-nmr spectroscopy. The first peak contained unreacted GMP-morpholidate and GMP. Peak II contained unreacted compound (II) and P^1, P^2-diquanosine-5'pyrophosphate. Peak III contained the desired product N-trifluoroacetyl 6-amino-1-hexanol-GDP in 73% yield. N-Trifluoroacetyl-6-amino-1-hexanol-GDP was identified by ^{13}C nmr spectroscopy. Compound III, 6-amino-1-hexanol-GDP, was obtained by hydrolysis of the trifluoroacetyl derivative at pH 12 overnight at room temperature. Completion of hydrolysis was checked by high pressure liquid chromatography.

D. P'-(6-Amino-1-Hexyl)-P-(5'-Uridine)-Pyrophosphate (IV)

Compound IV, 6-amino-1-hexanol-UDP, was synthesized as described above for compound III.

E. GDP-Hexanolamine Sepharose

The procedure used is modified from that of Cuatrecasas (97). Sepharose 4B (55g;110 ml) was washed with 2 liters of water and suspended in 55 ml 5M phosphate buffer, pH 12.0, and 55 ml of H_2O. This was placed in an ice bath with efficient stirring. Cyanogen bromide (22g), dissolved in 25 ml of acetonitrile, was injected into the Sepharose solution with a syringe and the reaction allowed to proceed for 9 min maintaining pH 12 wih 8N NaOH. This was quickly filtered and washed with 2-3 liters of ice water and the gel added to 0.70 mmoles of compound III in 70 ml of H_2O. Total time for filtering was 3 minutes. The initial pH of compound III and the activated gel was 12.5. After 5 minutes the pH was brought to 10.2 with HCl and the mixture shaken at 4°C for 15 hours. The gel was then filtered and washed with 3 liters of H_2O. The UV absorbance in the filtrate was used to determine that 5 μmoles of GDP-hexanolamine per ml of Sepharose was bound.

Before use in the purification of the fucosyltransferase, the affinity resin was washed with 300 ml each of 0.1M boric acid-potassium hydroxide, pH 9.0, containing 0.5M NaCl; water; 0.1M sodium acetate, pH 5.0, containing 1M NaCl; and finally 2 liters of water.

7. UDP-Hexanolamine Sepharose

UDP-hexanolamine Sepharose was prepared as described for GDP-hexanolamine Sepharose. This affinity resin was used for the preparation of the galactosyltransferase.

8. UDP-β-D-Galactose

β-D-Galactopyranose pentaacetate (V) was prepared by the procedure of Bates et al. (98). The product was characterized by gas

chromatography on a Hi-eff 3CP column and ^{13}C nmr spectroscopy in deuterated chloroform. The α:β ratio of the pure pentaacetates was found to be 10:90, respectively, by ^{13}C nmr. The C_1 resonances for the α and β anomers in $CDCl_3$ were found to be 90.6 and 93.3 ppm, respectively. α-D-Galactopyranose-1-phosphate (VI) was prepared by melting compound (V) in crystalline phosphoric acid following the procedure of McDonald (99). After deacetylation and removal of the lithium phosphate, according to the above procedure, ^{13}C nmr showed approximately an 85:15 ratio of the α and β sugar phosphates, respectively. The C_1 resonance for the α-galactose-1-phosphate appears as a doublet at 95.7 and 95.4 ppm. The galactose-1-phosphate was passed over a Dowex 50 (hydrogen cycle; 50-100 mesh) column and the eluate collected in cyclohexylamine. The overall yield for the synthesis of galactose-1-phosphate, based on phosphate analysis, was 58%. The anomers of galactose-1-phosphate were separated by passing 15 mmoles of galactose-1-phosphate in the cyclohexylammonium salt through a 6 X 48 cm Dowex 1 (bicarbonate form; 200-400 mesh) column, washing with 1.2 liters of H_2O and eluting the anomers with a linear gradient of 3.5 liters of H_2O as starting solvent and 3.5 liters of 0.35 M triethylammonium bicarbonate buffer, pH 7.4, as the limit solvent. Fractions of 10 ml were collected and assayed for phosphate. Two phosphate containing peaks were eluted from the column. The first peak was shown to be α-D-galactose-1-phosphate and the second peak β-D-galactose-1-phosphate by ^{13}C nmr spectroscopy. The α-D-galactose-1-phosphate was pooled, concentrated and converted to the pyridinium salt by repeated evaporation from pyridine. UDP-galactose was synthesized by condensing 1 mmole each of α-D-galactose-1-phosphate

and tri-n-octylamine with 1 mmole of UMP-morpholidate following the modified procedure of Moffat (96) used to synthesize compound (III). The reaction was followed by high voltage electrophoresis and high pressure liquid chormatography. After 24 hours of reaction at room temperature the reaction was judged to be complete based on UV absorbance under the peaks corresponding to UMP-morpholidate and UDP-galactose plus P^1, P^2-diuridine-5' pyrophosphate. The reaction mixture was concentrated to dryness, extracted three times with 200 ml ether, and the aqueous layers combined and applied to a 2 X 55 cm Dowex-1 (chloride form, 200-400 mesh) column at a flow rate of 50 ml per hour. The column was washed with 0.5 liters of water and eluted with a linear gradient of 2.25 liters of 0.01M HCl as starting solvent and 2.25 liters of 0.01M HCl with 0.1M LiCl as limit solvent. Fractions of 8 ml were collected and assayed for UV absorbance at 260 nm. Three peaks were eluted from the column in the order of increasing salt concentration and determined by high pressure liquid chromatography to be UMP-morpholidate, UDP-galactose, and P^1P^2-diuridine-5'pyrophosphate, respectively. The fractions containing UDP-galactose were pooled, concentrated, and desalted by passage over a P_2 column (-400 mesh). UDP-galactose was characterized by ^{13}C nmr and the purity checked by high pressure liquid chromatography and high voltage electrophoresis. The overall yield of UDP-galactose from galactose-1-phosphate was 20%.

9. GDP-α-L-Fucose

GDP-α-L-Fucose was synthesized by the method of Nunez et al. (100).

10. 2-Acetamido-3,4,6-Tri-O-Acetyl-2-Deoxy-Glucopyranosyl Chloride VII

Compound VII was synthesized by the procedure of Horton (101) and the purity determined by gas-liquid chromatography, on a high efficiency 3 CP column.

11. N-Trifluoroacetyl-6-Amino-1-Hexyl-2-Acetamido-2-Deoxy-β-D-Glucopyranoside VIII. Compound VIII was synthesized from compound (VII) by the procedure of Barker et al. (68).

12. 6-Amino-1-Hexyl-2-Acetamido-2-Deoxy-β-D-Glucopyranoside (IX)

Compound VIII was incubated overnight at pH 12 in NaOH and de-N-acetylation was judged complete by quantitative fluorescamine. The product, compound IX, GlcNAc-β-hexanolamine was purified by binding to a 1 X 10cm Dowex-50 (hydrogen form; 100-200 mesh) column and eluted, after washing with water, with 1M pyridinium acetate. The pyridinium acetate was removed by repeated evaporation.

13. Acetobromogalactose (2,3,4,6-Tetraacetylgalactopyranosyl Bromide) (X)

Galactose pentaacetate, [1-^{13}C]-galactose pentaacetate and [2-^{13}C] galactose pentaacetate were synthesized by the method of Bates et al. (98). Acetobromogalactose, 2,3,4,6-tetraacetylgalactopyranosyl bromide, was synthesized by dissolving 1.7 mmoles of dry galactose pentaacetate in 4ml glacial acetic acid and adding 4ml of 30% hydrogen bromide in glacial acetic acid. The reaction was allowed to proceed for 30 min at room temperature and then poured onto ice in a separatory funnel. Dichloromethane, 100ml, was added and the aqueous layer removed and discarded. The dichloromethane layer, containing the acetobromogalactose, was extracted once with 200ml of cold water, 3 times with cold saturated sodium bicarbonate, 2 times with water and

finally dried over $MgSO_4$ for 30 min. The $MgSO_4$ was removed by filtration through a pad of celite and the dichloromethane solution concentrated to a gum by rotatory evaporation at 40°. The gum was further dryed over $Mg(ClO_4)_2$ under vacuum for 1-2 h.

14. 6-Amino-1-Hexyl-α,β-D-Galactopyranoside (XI)

Compound XI was synthesized from 2,3,4,6-tetraacetylgalactopyranosyl bromide and N-trifluoroacetyl-6-amino-1-hexanol. N- Trifluoroacetyl-6-amino-1-hexanol was prepared as described for N-trifluoroacetyl-6-amino-1-hexanol phosphate (II) and dryed overnight before use over $Mg(ClO_4)_2$ under vacuum. Mercuric cyanide was also dryed overnight over $Mg(ClO_4)_2$ under vacuum. Nitromethane:benzene (1:1v/v) used in the condensation reaction was dryed over 4 Å molecular sieves before use.

Dry 2,3,4,6-tetraacetylgalactopyranosyl bromide, 1.6 mmoles, was mixed with 1.8 mmoles of N-trifluoroacetyl-6-amino-1-hexanol, 1.7 mmoles $Hg(CN)_2$ in 50 ml nitromethane:benzene (1:1v/v) and 2g 4 Å molecular sieves. The solution was heated in an oil bath, with efficient stirring, at 56°. After 12h the reaction was complete, as determined by ^{13}C nmr spectroscopy. The reaction mixture was filtered, concentrated to a gum by rotatory evaporation at 40°, diluted in dichloromethane and extracted with water to remove traces of $Hg(CN)_2$. The dichloromethane layer was again concentrated to a gum and 20 ml of triethylamine:methanol:water (1:11:4 v/v) added and the solution stirred overnight for complete de-O-acetylation of the galactosides. After removal of the triethylamine:methanol:water by rotatory evaporation, the gum was dissolved in 20 ml water and the pH maintained for 4h at 11.0 for de-N-acetylation. Carbon-13 nmr revealed 4 major components;

6-amino-1-hexyl β-D-galactopyranoside, 6-amino-1-hexyl α-D-glactopyranoside, β-D-galactopyranose and α-D-galactopyranose in the ratio of 36:36:21:8, respectively. The α and β anomers of the 6-amino-1-hexyl galactopyranosides were separated by chromatography on a 3 x 70 cm Dowex 1-X2 (hydroxide form; 200-400 mesh) column by elution with water. The α-anomer was the first to elute followed by the β-anomer. Unreacted galactose was converted to acids which remain bound to the column.

15. Ethyl-β-D-Galactopyranoside (XII)

Ethyl-β-D-galactopyranoside was prepared by the method of Chiang et al. (102). Compound X, 2,3,4,6,-tetracetylgalactopyranosyl bromide, was prepared as previously described. Acetobromogalactose, 2.8 mmoles, in 24 ml of dichloromethane is placed in a foil covered reaction flask. With efficient stiring, the following additions are made: 0.23 ml (4 mmoles) of ethanol which had been dryed over 3 Å molecular sieves, 0.55g silver carbonate that had been dryed at least 12h, 0.04g iodine and 1g 4 Å molecular sieves. The condensation was shown to be complete within 2h by tlc in solvent system (I). Ethyl-β-D galactose tetraacetate had a R_f of 0.43 and acetobromogalactose had a R_f of 0.54 in solvent system (I). The reaction mixture was filtered, concentrated to a gum and 20 ml triethylamine:methanol:water (1:11:4w/v) added for de-O-acetylation overnight. The triethylamine:methanol:water was removed by rotatory evaporation and the compound purified over a 3 X 20 cm Dowex 1-X2 (hydroxide form: 200-400 mesh) column by elution with water. Yield, based on weight was 92%. Carbon-13 nmr revealed the presence of only 1 anomer, β, at 104.2 ppm downfield from TMS.

16. 5-Deoxy-L-Lyxose (XIII)

Compound XIII was prepared by the oxidative decarboxylation of L-fuconic acid.

L-Fuconic acid was prepared by bromine oxidation of L-fucose, by the method of Isbell (103). Passage of the reaction mixture through a 4 X 30 cm Dowex 50-X8 (hydrogen form; 20-50 mesh) column and rotatory evaporation at 45° yielded the lactone as a syrup. The lactone was shown to be pure by glc of it's trimethylsilyl derivative and by ^{13}C-nmr spectroscopy. Yields of L-fuconolactone based on weight of the dryed syrup were 98%. Hydrolysis of the lactone to the sodium salt was accomplished by maintaining a pH of 9.4 with 5M sodium hydroxide for 1 hour.

The oxidative decarboxylation of the sodium salt of L-fuconic acid was carried out using the Ruff degradation (104). The sodium salt (60 mmoles) of [6-^{3}H]-L-fuconate at pH 9.5 was dissolved in 200 ml H_2O and 1.25 g barium acetate and 0.62 g ferric sulfate added. The mixture was boiled for 3 minutes and allowed to cool to 65°. Hydrogen peroxide (30%), 8 ml, was added and the mixture stirred with maintenance of the temperature between 65°-75°. When the temperature cooled to 65°, another 8 ml of 30% hydrogen peroxide was added and the temperature again maintained between 65-75°. This was repeated once more with an additional 8 ml of 30% hydrogen peroxide. After the reaction mixture had cooled to room temperature, 5 g decolorizing charcoal was added and the reaction mixture filtered through Celite. The solution was immediately placed on a 4 X 50 cm Dowex 50-X8 (hydrogen form; 20-50 mesh) column connected to a 2 X 50 cm Duolite A6 (hydroxide form; 20-50 mesh) column at 4°. The only species eluting from the columns was [5-^{3}H]-5-deoxy-L-lyxose. The 5-deoxy sugar was shown to be pure by

glc and C-13 nmr. The resonances for C1 through C5 in [5-^{3}H]-5-deoxy-β-L-lyxose are 102.3, 78.2, 79.9 and 15.3, respectively. The yield of [5-^{3}H]-5-deoxy-L-lyxose by radioactivity and weight as a dry syrup was routinely 40%.

17. [1-^{13}C, 6-^{3}H]-L-Fucose and [1-^{13}C, 6-^{3}H]-6-Deoxy-L-Talose (XIV)

Potassium [^{13}C] cyanide, 13.2 mmoles, was dissolved in 20 ml H_2O at 18° and the pH adjusted to 7.2 with 1M acetic acid. [5-^{3}H]-5-Deoxy-L-lyxose, 12 mmoles, was dissolved in 6 ml H_2O and added to the cyanide solution. The pH of the reaction was maintained at 7.2 for 30 minutes. After 20 minutes, glc showed two main TMS-derivatives appearing from the OV-17 3% column at 172 and 175°, corresponding to fucono and 6-deoxy-L-talononitriles, respectively. A small amount of unreacted 5-deoxy-L-lyxose was also present, emerging at 142°. The solution was adjusted to pH 4.2 and airated for 15 minutes to remove any excess cyanide. The nitriles were reduced catalytically with palladium-barium sulfate (5%, 60 mg/mmole nitrile) at 50 psi of hydrogen for 1.5 hours.

1-Amino-1-deoxyalditols and aldonic acids, produced as side products from the hydrogenation reaction were removed with Dowex 50-X8 (hydrogen form; 20-50 mesh) and Dowex 1-X8 (acetate form; 100-200 mesh), respectively, as previously described (105). Separation of the epimeric [1-^{13}C, 6-^{3}H]-L-fucose and [1^{13}C, 6-^{3}H]-L-talose was achieved using a 4 X 90 cm Dowex 50-X8 (barium form; 200-400 mesh) column and elution with water. [1-^{13}C, 6-^{3}H]-L-Fucose was found to elute near the void volume of the column and [1-^{13}C, 6-^{3}H]-L-talose eluted approximately 150 ml later. The

overall yield for the synthesis of [1-^{13}C, 6-^{3}H]-L-fucose and [1-^{13}C, 6-^{3}H]-6-deoxy-L-talose was 70% based on starting [5-^{3}H]-5-deoxy-L-lyxose.

18. [1-^{13}C]-D-Galactose (XX)

Compound XX was prepared by the general method of Serianni et al. (105) from D-lyoxse.

19. [2-^{13}C]-D-galactose (XXI)

Compound XXI was prepared by the sequential addition of cyanide to threose by the method of Serianni et al. (105).

20. Purification of Bovine UDP-Galactose:Glucose-β(1-4) Galactosyltransferase

The galactosyltransferase was purified by the method of Barker et al. (68). The purified enzyme, 0.05 mg/ml, was stored at 4° in 25 mM sodium cacodylate, pH 7.4, 25 mM $MnCl_2$ and 1 mM β-mercaptoethanol. Under these conditions no loss in enzymatic activity was observed in 2 years. Dilute solutions of the galactosyltransferase were concentrated in the presence of 0.01% Triton X-100. The nonionic detergent allowed concentrations greater than 1 mg protein/ml and stabilized the enzyme at these concentrations for up to a week at 4°. The rate of synthesis of N-acetylactosamine, lactose or (6-amino-1hexyl)-4-O-(β-D-galactopyranosyl)- 2-acetamido-2-deoxy-β-glucopyranoside was followed by measuring the rate of transfer of ^{14}C from UDP [UL-^{14}C] galactose into disaccharide as described previously (46). All assays were initiated with galactosyltransferase and incubated for 10 min at 37°. The enyzme concentration was adjusted so no more than 15% of the radioactivity was transferred. Standardization of enzyme solutions were preformed with 1.5mM UDP [UL-^{14}C]-galactose in the standard assay mixture.

21. Preparation of Galactosylated Disaccharides

Syntheses of the galactosylated compounds were carried out on a 140 μmole scale at 33° by a procedure similar to Nunez and Barker (13). The reaction mixture contained 16 ml of N-acetylglucosaminide β(1-4) galactosyltransferase (43 units/ml of enzyme solution) in 25 mM sodium cacodylate, pH 7.4, 25 mM $MnCl_2$ and 2 mM β-mercaptoethanol, 60μl 0.01 mCi/ml UDP [UL-^{14}C] galactose, 140 μmoles UDP-galactose, disodium salt, 150 μmoles acceptor, and 4 mg BSA in a total volume of 18 ml. After 6 h another 6 ml of N-acetylglucosaminide β(1-4) galactosyltransferase was added and the incubation continued for another 12 hr.

22. Purification of Galactosylated Disaccharides

Reaction mixtures were passed over a 1 X 4 cm column of Dowex 1-X8 (chloride form; 100-200 mesh) and concentrated. Protein was removed by repeated concentrations in a 10ml Amicon cell using an Amicon PM 10 membrane or by precipitation using the method of Somogyi (107). Alternatively, glycosides of hexanolamine could be purified by binding to a 1 X 5 cm column of Dowex 50-X8 (100-200 mesh) and elution with 1M pyridinium acetate. The pyridinium acetate could be removed by repeated evaporation in vaccu at 40°. Separation of the galactosylated disaccharide from the acceptor was achieved on a 4 X 90 cm column of Bio-Gel P2 (-400 mesh) equilibrated in 0.1M triethylammonium bicarbonate, pH 7.4. Triethylammonium bicarbonate could easily be removed from the final product by repeated evaporation in vaccu at 40°.

23. Assay of a Porcine β-Galactoside α(1-2) Fucosyltransferase

Fucosyltransferase activity was determined by incubating 100μl mixtures containing 0.5 μmoles lactose, N-acetyllactosamine, or Gal β(1-4)GlcNAc-β-hexanolamine, 5 μmoles sodium cacodylate, pH 6.0, 2

μmoles $MnCl_2$, 0.2 mg bovine serum albumin, 2 nmoles GDP-fucose containing approximately 5,000 cpm GDP [UL-^{14}C]-fucose and enzyme solution at 33° for 15-60 min. Appropriate controls were included to correct for the amount of nonspecific hydrolysis of GDP-fucose. Standardizations of enzyme solutions were performed, as above, with 0.08 μmoles GDP-fucose containing approximately 15,000 cpm of GDP [UL-^{14}C]-fucose. Activity units are defined as μmoles of fucose transferred from GDP-fucose to acceptor per minute. Reactions were stopped by placing the tubes on ice and adding 0.5 ml water. Two methods were then used in the work-up of the reaction mixtures. The first method was used only with crude enzyme preparations and involved the use of the acceptor Galβ(1-4)GlcNAc-β-hexanolamine. The reaction mixture was applied to a Pasteur pipet column of Dowex 50-X8 (hydrogen form; 100-200 mesh) and washed under gravity with 9.5 ml of water. The product was then eluted into a scintillation vial with 4 ml of 1 M pyridinium acetate. The pyridinium acetate was removed on a hot sand bath, scintillation fluid added and the vial counted for radioactivity. Reaction blanks were approximately 60 cpm per assay. Standard deviations of replicate analyses were 5 to 15 cpm. Alternatively, high voltage electrophoresis can be used to isolate and quantitate the product.

The second method was routinely used for assaying enzymatic activity in the purification of the fucosyltransferase. The reaction mixture is applied directly to a Pasteur pipet column of Dowex 1-X8 (chloride form; 100-200 mesh) and washed with 1.5 ml water. The eluate is collected directly into a scintillation vial, 14 ml scintillation

fluid added and the vial is counted for radioactivity. Standard deviations of replicate analysis were 2 to 8.

24. Purification of a Porcine β-Galactoside α(1-2) Fucosyltransferase.

The porcine β-galactoside α(1-2) fucosyltransferase was partially purified with modifications from a procedure developed by T.A. Beyer and R.L. Hill (personal communication).

The buffers employed are identified by letter as follows: Buffer A, 25 mM sodium cacodylate, pH 6.0; Buffer B, 25 mM sodium cacodylate, pH 6.0, 0.3M NaCl; Buffer C, 25 mM sodium cacodylate, pH 6.0, 0.15 M NaCl; Buffer D, 25 mM sodium cacodylate, pH 6.0, 0.5 M NaCl; Buffer E, 25 mM sodium cacodylate, pH 6.5; Buffer F, 25 mM sodium cacodylate, pH 6.0, 2 M NaCl.

The procedure used for large scale solubilization of the fucosyltransferase was modified from the procedure used to solubilize a porcine CMP-N-acetylneuraminate:β-D-galactoside α(2-3) sialyltransferase (106). All procedures were done at 4°.

Step 1: Removal of Mucin from the Porcine Submaxillary Glands

Porcine submaxillary glands, 2 kg, obtained frozen from Bio-Resources, were quickly thawed under cold running water and placed on ice. The glands were trimed of fat and connective tissue and ground in a meat grinder at 4°. Portions of the ground glands, 500g, were added to 2 liters of water and homogenized in a Waring blender on high for three 30 sec bursts with one min rest inbetween bursts. The homogenate was centrifuged at 7000 X g for 45 min. The fat and mucin layer at the top of the centrifuge tube was removed by aspiration. Pellets from 1kg of material were resuspended in 2 liters of Buffer A by homogenization in a Waring blender with three 30 sec bursts on medium

and 30 sec intervals inbetween bursts. The slurry was brought to 20 mM $MnCl_2$ by dropivise addition of 60 ml 1M $MnCl_2$ and stirred for 15 min. This slurry was centrifuged for 30 min as above and the supernatant removed by aspiration. The pellet was resuspended and the procedure repeated again. The washed pellets from 1 kg of tissue were resuspended in 1.5 liters of Buffer A by homogenization in a Waring blender with three 10 sec bursts on low and 15 sec intervals inbetween bursts. This procedure was repeated with the second 1kg batch.

Step 2 Triton X-100 Extraction

The resuspended pellets from 1 kg of starting material were brought to 1% Triton X-100 and 20 mM $MnCl_2$, over a period of 15 min by the addition of 100 ml 20% Triton X-100 and 40 ml 1M $MnCl_2$, and stirred for 45 min. After stirring for 45 min, the solution was centrifuged for 30 min as above. This was repeated with the second kg batch (II). The supernatants (I and II) were removed and combined. Pellets (I and II) were resuspended by homogenization on low in 1.5 liters of Buffer A and brought to 1% Triton X-100 and 20 mM $MnCl_2$, over a period of 15 min, and stirred for 45 min. This was again centrifuged, as above, for 30 min. The supernatant (III) was removed and the pellets (III) were discarded.

Step 3 Sulfopropyl-Sephadex Chromatography

The combined supernatants I and II were immediately treated batchwise with 150 g SP-Sephadex, previously equilibrated with Buffer A, and stirred for 30 min. Supernatant III was treated similarly with 50g SP-Sephadex. The gel, containing the bound fucosyltransferase, was poured into a 5 X 20 cm column, washed with 500 ml of Buffer A and eluted rapidly with Buffer B. Fractions, 20 ml, were collected and

tubes containing the fucosyltransferase pooled and made 50% (v/v) with ice cold glycerol. The gel, from supernatant (III) was similarly poured into a column and eluted with Buffer B.

Step 4 Sodium Chloride Elution of the Enzyme From GDP-Sepharose

The SP-Sephadex pooled enzyme from 4kg of tissue, stored in 50% glycerol, was diluted 50% with Buffer C and applied to a 5 X 15 cm column of GDP-Sepharose, equilibrated in Buffer C, at a flow rate of 100 ml/hr. After application of the enzyme solution, the column was washed with 250 ml of Buffer C followed by 250 ml Buffer D. A 500 ml linear gradient of 0.5 M NaCl in Buffer A as the starting solvent and 2.0 M NaCl in Buffer A as the limit solvent was applied to the column followed by 200 ml of Buffer F. Fractions of 20 ml were collected and assayed for fucosyltransferase activity. The enzyme was found to elute with 2 M NaCl. Fractions, 400 ml, containing the fucosyltransferase were pooled and stored at 4°. The enzyme was stable at 4° for at least 3 months.

Step 5 Sephadex G-50 Chromatography

Pooled fucosyltransferase from the GDP-Sepharose column was desalted in batches of 200 ml, by application to a 5 X 110 cm column of Sephadex G-50 (fine) equilibrated in Buffer A. Fractions of 20 ml were collected at a flow rate of 200 ml/hr. Fucosyltransferase activity was pooled and quickly applied to a second GDP-Sepharose column.

Step 6 Second GDP-Sepharose Column

The desalted fucosyltransferase, from Step 5, was applied at a flow rate of 50 ml/hr to a 1 ml GDP-Sepharose column equilibrated in Buffer A in a plastic syringe. The column was then washed with 20 ml Buffer A and the fucosyltransferase eluted with Buffer F. Fractions of 1 ml were collected and assayed for fucosyltranferase activity. The enzyme was

found to elute in a volume of 5-8 ml and could be stored with no loss of activity for at least 2 months at 4°. Longer periods of storage required dilution of the enzyme with 50% glycerol and storage at -20°.

25. Preparation of Fucosylated Oligosaccharides

All enzymatic syntheses were carried out at 33° and the extent of reaction followed by radioactivity or high pressure liquid chromatography. Incorporation of radioactivity from GDP[UL-^{14}C]-fucose into oligosaccharide was measured by removing a 20µl aliquot from the reaction and diluting it with 0.5 ml water. The sample was transferred to a Pasteur pipet column containing Dowex 1-X8 (chloride form; 100-200 mesh) and collected in a scintillation vial. The column was rinsed with 1.5 ml water and the eluate, collected in a scintillation vial, counted for radioactivity incorporated into neutral oligosaccharides. Nonspecific hydrolysis of GDP-fucose in the reaction mixture could be measured when glycosidic derivatives of hexanolamine were used as acceptors. A 20 µl aliquot was removed from the reaction mixture and diluted with 0.5 ml water. This sample was applied to a Pasteur pipet column containing equal proportions of Dowex 1-X8 (chloride cycle; 100-200 mesh) and Dowex 50-X8 (hydrogen cycle; 100-200 mesh). The column was washed with 1.5 ml of water and the eluate, collected in a scintillation vial, counted for radioactivity. Only radioactive fucose which had been hydrolyzed from GDP-fucose would elute from the column.

High pressure liquid chromatography on a Partisil PXS 10/25 SAX column could be used to estimate the proportions of GMP, GDP and GDP-fucose in the reaction mixture. Retention times for GMP, GDP-fucose and GDP, were 7, 12 and 24 min, respectively.

Synthesis of compounds were routinely performed in 50 μmole batches. The reaction mixture contained 4 ml of β-galactoside α(1-2) fucosyltransferase (0.62 units/ml enzyme solution), 30 μl 0.01 mCi/ml GDP [UL-^{14}C] fucose, 0.04 mmoles sodium cacodylate, pH 6.0, 0.15 mmoles $MnCl_2$, 7.5 mg BSA, 50-75 μmoles of acceptor oligosaccharide and 50 μmoles GDP-fucose in a total volume of 7.4 ml.
Reactions were incubated for 6 h and an additional 2 ml of β-galactoside α(1-2) fucosyltransferase added and incubation continued for 12 h.

26. Purification of Enzymatically Synthesized Oligosaccharides

Reaction mixtures were first placed in a 10 ml Amicon cell containing an Amicon PM10 membrane. The mixture was concentrated to 0.5 ml and diluted to 10 ml with water and reconcentrated. This was repeated 2 more times. The protein free filtrate was then applied to a small column containing 2 ml Dowex 1-X8 (chloride form; 100-200 mesh) and 0.5 ml Chelex-100 (sodium form) and eluted with water.
The mixture was concentrated and applied to a 4 X 90 cm Bio-Gel P2 (-400 mesh) column equilibrated in 0.1M triethylammonium bicarbonate, pH 7.4 and pumped at a flow rate of 5 ml/h. The fucosylated product was separated from the acceptor and concentrated. Occasionally, some salt eluted with the fucosylated product and was removed by rechromatography on a 1 X 40 cm Bio-Gel P2 (-400 mesh) column equilibrated in water at a flow rate of 5 ml/h. Nonreducing compounds were treated with 0.5 ml Chelex-100 (sodium form) and reducing compounds treated with 0.5 ml Chelex-100 (hydrogen form) before nmr analysis. Compounds containing glycosidically linked hexanolamine were completely eluted from the Chelex-100 (sodium form) with 1 M pyridinium acetate which was removed by rotatory evaporation.

RESULTS

I. Synthesis of Galactosylated Disaccharides

The N-acetylglucosaminide β (1-4) galactosyltransferase can be stored for 2 years with small losses in enzymatic activity in 25 mM sodium cacodylate, pH 7.4, 25 mM $MnCl_2$, 2 mM β-mercaptoethanol and 50 mM N-acetylglucosamine. The enzyme is stabilized by N-acetylglucosamine (68).

Before synthesis of Galβ(1-4)GlcNAc-β-hexanolamine the stablizer, N-acetylglucosamine, was removed by dialysis. The dialyzed enzyme was found to lose 30% of it's enzymatic activity during 5 days at 4°.

Yields for the synthesis of disaccharides varied depending on acceptor. When N-acetylglucosamine was used as an acceptor the yield was routinely 85%. Yields using GlcNAc-β-hexanolamine were always lower and varied between 60 and 75%.

II. Enzymatic Assay of the β-Galactoside α(1-2) Fucosyltransferase

During initial steps, the purification of this enzyme is complicated by the presence of large amounts of endogenous acceptors and hydrolysis of the sugar nucleotide, GDP-fucose. A small amount of enzymatic activity could be measured in the crude homogenate using the substrate Galβ(1-4)GlcNAc-β-hexanolamine. This acceptor, containing a primary amino group, allowed the fucosylated product, Fuc α(1-2) Galβ(1-4)GlcNAc-β-hexanolamine, to be bound to a cation exchange resin and eluted with pyridinium acetate after unreacted GDP-fucose, hydrolysis products, and fucosylated endogenous glycoproteins were washed through the column.

The routine assay of the β-galactoside α(1-2) fucosyltransferase, following removal of the mucin, utilized an anion exchange resin to bind unreacted GDP-fucose. The fucosylated product and free fucose are eluted into a scintillation vial for counting. Controls were used to account for free fucose released by the hydrolysis of GDP-fucose.

Using GDP-fucose, (0.01 mM) the concentration of enzyme was adjusted so that <10% of the GDP-fucose was utilized during the reaction. Saturating amounts of GDP-fucose were not used in routine assays during the enzyme purification because the substrate is difficult to prepare. The Michaelis constant (K_m) for GDP-fucose determined with GDP-Sepharose purified enzyme was 0.07 mM at pH 6.0. The maximal velocity with the same preparation is 0.10 μmoles/min/mg protein. Standardizations of fucosyltransferase activities were carried out using 0.8 mM GDP-fucose.

Low molecular weight acceptors were used in the assay of fucosyltransferase activity. An approximate K_m of 0.5 mM for the disaccharides, lactose and N-acetyllactosamine was determined. The monosaccharide, Gal-β-hexanolamine, was determined to have an approximate K_m of 6 mM.

The macromolecular acceptor asialo-α_1-glycoprotein, containing an oligosaccharide core with a terminal Galβ(1-4)GlcNAc-β-sequence is an excellent acceptor for the fucosyltransferase.

III. Purification of the Porcine β-Galactoside α(1-2) Fucosyltransferase

The 10,000 fold purification of the β galactoside α(1-2) fucosyltransferase, by a modification of the procedure developed by T.A. Beyer and R.L. Hill (personal communication), from 4 kg of porcine

submaxillary glands is summarized in Table I. Due to the small amount of enzymatic activity present it was necessary to process a large quantity of starting material. The procedure utilized is designed to obtain the maximal amount of enzyme sufficiently pure for the synthesis of fucosylated compounds. The partial purification of the β-galactoside α(1-2) fucosyltransferase can be accomplished in less than 10 days.

Fucosyltransferase has been shown to be membrane bound except in secretory individuals where the enzyme is also present in a soluble form in the secretions of several glands (17).

Two of the initial steps in preparing enyzme in large quantities entailed removal of the mucin and solubilization of the enzyme from the membrane. Much of the mucin could be removed by homogenization of the crude submaxillary glands with water and centrifugation. Isolation of a membrane fraction can be accomplished by high speed ultracentrifugation or aggregation of the membrane fraction with manganese chloride. Induced aggregation of the membrane fraction permits a large volume of homogenate to be centrifuged at low speed in a Lourdes Beta centrifuge (106,108). The membrane fraction is obtained as a pellet from which the supernatant containing soluble proteins can be removed by aspiration. The membranes are washed a second time and reaggregated by the addition of manganese chloride.

Several non-ionic detergents are capable of solubilizing the fucosyltransferase from the membrane. In the presence of 0.5M sodium chloride; Triton X-100, Brij 99 and octyl-β-D-glucopyranoside are capable of solubilizing practically all of the enzyme from the membrane. However, the ionic strength necessary to solubilize all of the enzyme from the membrane prohibited adsorption to SP-Sephadex. All of the

Table I

Purification of the Porcine Submaxillary β-Galactoside α(1-2) Fucosyltransferase

Results are shown for the preparation from 4 kg of submaxillary glands.

Step	Volume	Total Protein	Total activity	Specific Activity	Yield	Step purification	Total purification
	ml	mg	units[a]	units/mg	%		
Homogenate minus mucin	7930	198,250	16.6	0.00008	100	1	1
Membrane Fraction	5349	56,164	9.6	0.00017	60	2	2
Triton Extract	5100	39,780	9.6	0.00024	100	1.4	3
SP-Sephadex	1872	6,365	7.5	0.0012	45	5	15
GDP Sepharose I	326	46	6.0	0.132	36	110	1650
Sephadex G50	414	12	5.0	0.417	30	3.2	5212
GDP Sepharose II	60	4.2	3.7	0.881	22	2.1	11012

[a] One unit corresponds to 1 μmol of product formed per min. at saturating concentrations of GDP-fucose and lactose at 33°.

fucosyltransferase could not be extracted with Triton X-100 in low ionic strength buffer. Complete solubilization of the fucosyltransferase from the membrane is achieved by repeated extraction with the detergent in low ionic strength buffer. The enzyme in this extract is completely adsorpted on SP-Sephadex. Batch adsorption of the enzyme onto SP-Sephadex was used since the enzyme is inactivated in Triton X-100 and unstable on cation exchange resins (T.A. Beyer and R.L. Hill, personal communication). The fucosyltransferase is easily eluted from the SP-Sephadex resin with 0.3 M sodium chloride. Dilution of the enyzme at this stage in purification with 50% glycerol allows the enzyme to be stored for at least 6 months at -20° with little loss of activity.

The major purification is achieved on the affinity resin GDP-Sepharose. This resin was synthesized for use in preparation of the fucosyltransferase based on the success of similar resins in the purification of N-acetylglucosaminide β(1-4) galactosyltransferase, CMP-N-acetylneuraminate: α-N-acetylgalactosaminide α(2-6) sialyltransferase and [fucosyl α(1-2)] galactoside α(1-3) N-acetylgalactosaminyltransferase (30,31,68,106). The SP-Sephadex pooled fucosyltransferase was diluted with buffer for faster loading on the GDP-Sepharose column. No activity could be detected in the elute from the affinity resin. A linear sodium chloride gradient from 0.5 to 2.0 M eluted many proteins adsorbed through ionic interactions. Fucosyltranferase activity was eluted with 2.0 M sodium chloride, as shown in Figure 3, giving a 1600 fold purified enzyme preparation. The GDP-Sepharose I purified enzyme can be stored for at least 3 months at 4° without loss of enzymatic activity. The enzyme can be stored up to 6

Figure 3. Purification of The Porcine Submaxillary Gland β-Galactoside α(1-2) Fucosyltransferase By NaCl Elution From GDP-Sepharose. Enzyme was applied to a 5 X 15 cm GDP-Sepharose I column and eluted at the arrows (↓); A, with 25 mM sodium cacodylate, pH 6.0, 0.15 M NaCl; B, 25 mM sodium cacodylate, pH 6.0, 0.5 M NaCl; C, a 500 ml linear gradient of 25 mM sodium cacodylate, pH 6.0, 0.5 M NaCl as starting solvent and 25 mM sodium cacodylate, pH 6.0, 2.0 M NaCl as the limit solvent; D, 25 mM sodium cacodylate, pH 6.0, 2.0 M NaCl.

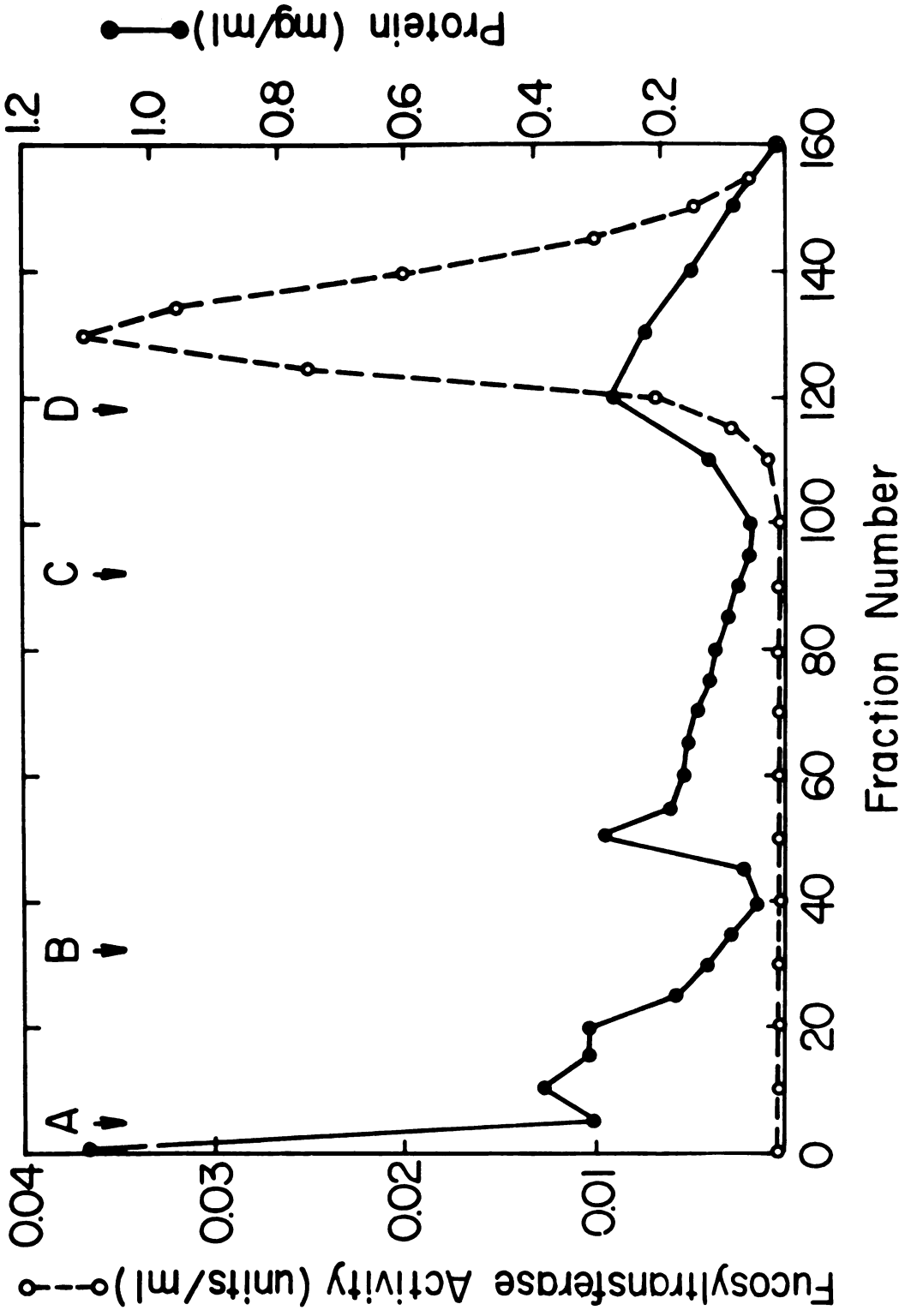

Fucosyltransferase Activity (units/ml)
Protein (mg/ml)
Fraction Number
0.04
0.03
0.02
0.01
1.2
1.0
0.8
0.6
0.4
0.2
0
20
40
60
80
100
120
140
160
A
B
C
D

Figure 3

months by dilution with 50% glycerol and storage at -20°.

The fucosyltransferase is not stable in low ionic strength buffers and desalting before application to another affinity column or a cation exchange column must be accomplished quickly. Dialysis of large volumes is difficult to accomplish technically and results in loss of enzymatic activity. Desalting was accomplished in 200 ml batches on a 5 X100 cm Sephadex G50 column at a flow rate of 150 ml/hr. Fractions of 20ml were collected. The Sephadex G50 chromatography step completely desalted the fucosyltransferase and resulted in a 3 fold purification, as shown in Figure 4. The enzyme was found to be extremely unstable in low ionic strength buffers, loosing 90% of it's activity in a 24 h period. Concentration of the Sephadex G50 pooled enzyme was achieved by adsorption to a 1 ml column of GDP-Sepharose, in a plastic syringe, at a flow rate of 50 ml/h. The column was washed with buffer and the enzyme eluted with 2 M sodium chloride as shown in Figure 5. Fractions of 1.0 ml were collected and those containing the fucosyltransferase pooled. The pooled enzyme is stable at 4° for at least 2 months. Storage for longer periods of time was best accomplished in 50% glycerol and storage at -20°.

IV Synthesis and Purification of Fucosylated Oligosaccharides

Fucosylation of galactosylated derivatives was performed at 33° because of the greater stability at this temperature of the GDP-Sepharose II enzyme.

Reactions were initially performed with a 2-fold excess of acceptor over the GDP-fucose concentration as described under "Methods". Using the acceptor, Galβ(1-4)GlcNAc-β-hexanolamine, it is possible to follow

Figure 4. Purification and Desalting of The β-Galactoside α(1-2) Fucosyltransferase. The β-galactoside α(1-2) fucosyltransferase was desalted on a 5 X 100 cm Sephadex G50 (fine) column equilibrated in 25 mM sodium cacodylate, pH 6.0. Fractions of 20 ml were collected.

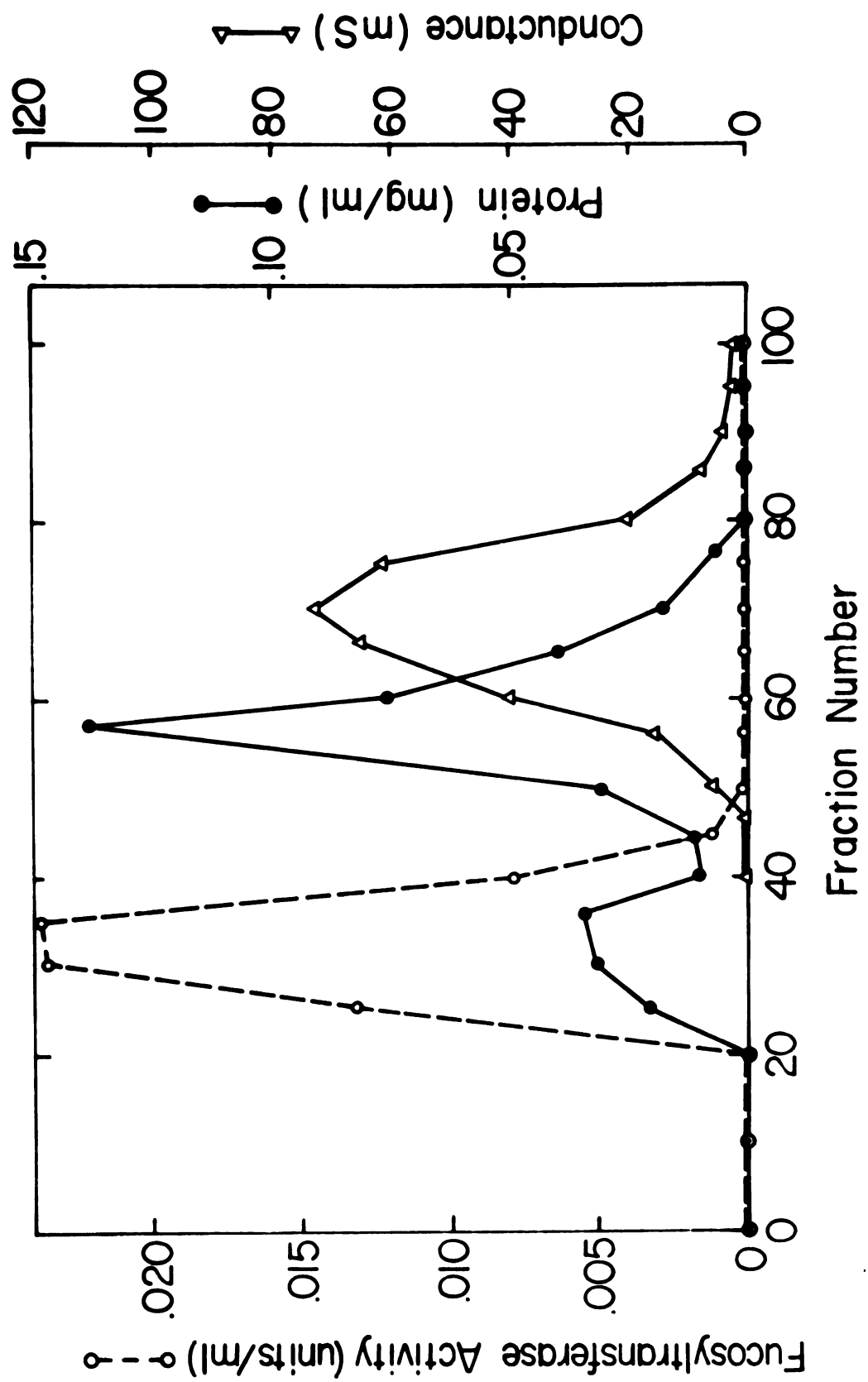

Fucosyltransferase Activity (units/ml) o - - o
.020
.015
.010
.005
0
.15
.10
.05
Protein (mg/ml) •——•
120
100
80
60
40
20
0
Conductance (mS) ▲——▲
0
20
40
60
80
100
Fraction Number

Figure 4

Figure 5. Concentration of The Porcine β Galactoside α(1-2) Fucosyltransferase By GDP-Sepharose II Chromatography. The desalted enzyme was adsorbed to a 1 ml GDP-Sepharose column and washed at the arrows (↓); A, with 25 mM sodium cacodylate, pH 6.0; B, 25 mM sodium cacodylate, pH 6.0, 2.0 M NaCl; C, 4 M NaCl and 4 M urea.

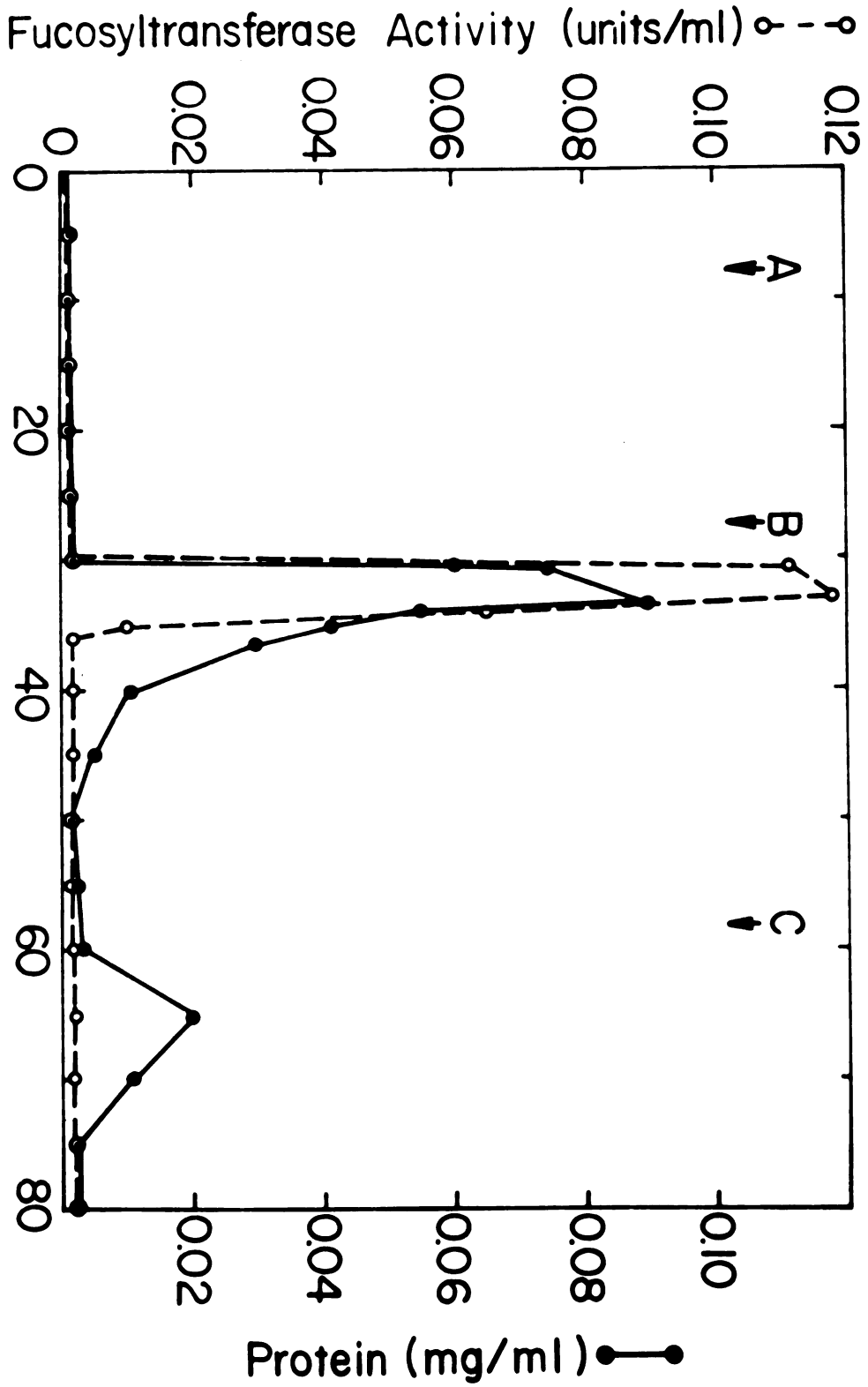

Fucosyltransferase Activity (units/ml)
0
0.02
0.04
0.06
0.08
0.10
0.12
Fraction Number
0
20
40
60
80
A
B
C
Protein (mg/ml)

Figure 5

the kinetics of the reaction and estimate GDP-fucose hydrolysis, as described under "Methods". Under these conditions, 94% utilization of GDP-fucose could be achieved with 72% incorporated into product and 22% hydrolysis to fucose, as shown in Figure 6.

Large scale reactions with ^{13}C-enriched acceptors were performed using a 1:1.2 ratio of GDP-fucose to acceptor. The reaction profile, for the synthesis of Fucα(1-2)[1-^{13}C]Galβ(1-4)GlcNAc followed by high pressure liquid chromatography, as described in "Methods", is shown in Figure 7. No unreacted GDP-fucose is detected in the reaction mixture after 20 h, Figure 7C, the only products observable are GMP and GDP. The yield of Fucα(1-2)[1-^{13}C]Galβ(1-4)GlcNAc was 83% based on starting GDP-fucose.

Hydrolysis of GDP-fucose to fucose was approximately 20% in all reactions, independent of acceptor concentration. The factors influencing hydrolysis of GDP-fucose were further investigated by determining the kinetics of hydrolysis in the absence of acceptor and in the absence of enzyme, as shown in figure 8. The hydrolysis of GDP-fucose by the enyzme preparation in the absence of acceptor indicates that the fucosyltransferase might act as a phosphohydrolase.

Purification of the large scale fucosylation reactions is easily achieved by deproteinization, deionization and gel filtration. Several methods for removal of proteins were investigated. Deproteinization with barium hydroxide and zinc sulfate by the method of Somogyi (107) gave a white precipitate of barium sulfate and protein which was difficult to filter and gave in large volumes of filtrate containing the product. Alternatively, products containing a primary amino group could be adsorbed to a small Dowex 50 (hydrogen) column and eluted with 1 M

Figure 6. The Kinetics Of Fucosylation Of Galβ(1-4)GlcNAc-β-Hexanolamine With The Porcine β Galactoside α(1-2) Fucosyltransferase. The rate of formation of product was monitored by assaying reactions at varying times of incubation. At different times, two aliquots were removed and terminated by dilution with water. One aliquot was applied to a Pasteur pipet column of Dowex 1 (chloride) and the second aliquot applied to a Pasteur pipet column containing both Dowex 50 (hydrogen) and Dowex 1 (chloride) resin. Both columns were eluted as described under "Methods" and counted for radioactivity. Radioactivity eluting from the Dowex 1 column represents the amount of fucose transferred to Galβ(1-4) GlcNAc-β-hexanolamine and the amount of GDP-fucose hydrolyzed. Radioactivity eluting from the column containing both Dowex 50 and Dowex 1 resin represents the amount of GDP-fucose hydrolyzed.

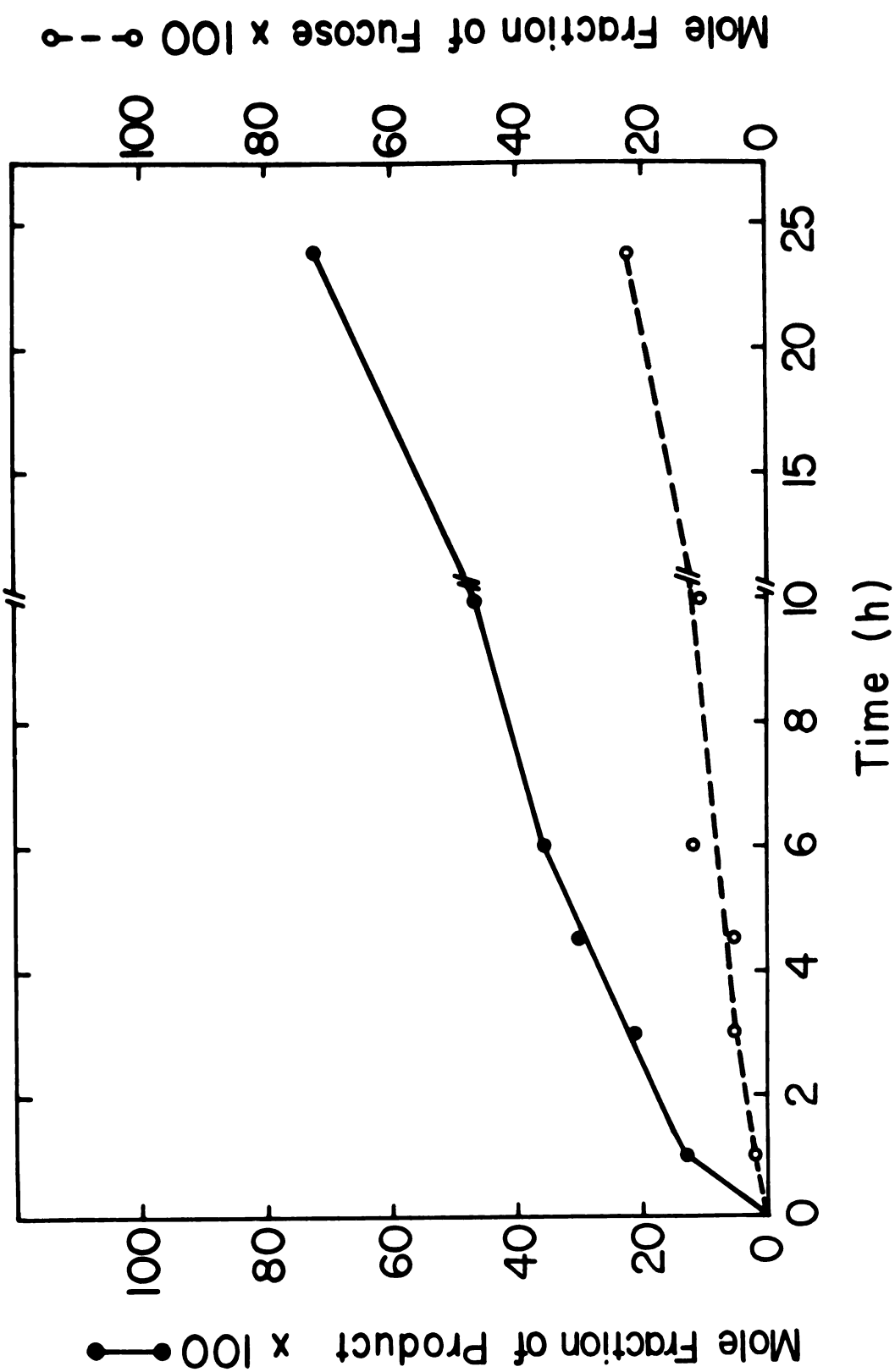

Mole Fraction of Product x 100 ●—●
Mole Fraction of Fucose x 100 o- - -o
Time (h)
0
20
40
60
80
100
0
2
4
6
8
10
15
20
25

Figure 6

Figure 7. Time Course Of Fucosylation Of [1-^{13}C]Galβ(1-4)GlcNAc Followed By High Pressure Liquid Chromatography. Aliquots were assayed by removal of 1.5μl from the incubation mixture, dilution to 40 μl with 0.2 M potassium phosphate buffer , pH 3.4, and 20μl injected into a Partisil PXS 10/25 SAX column. The three time profiles shown; A, B, and C, correspond to 0,1 and 20h of incubation, respectively. Retention times for GMP, GDP-fucose and GDP were 7,12 and 24 min, respectively.

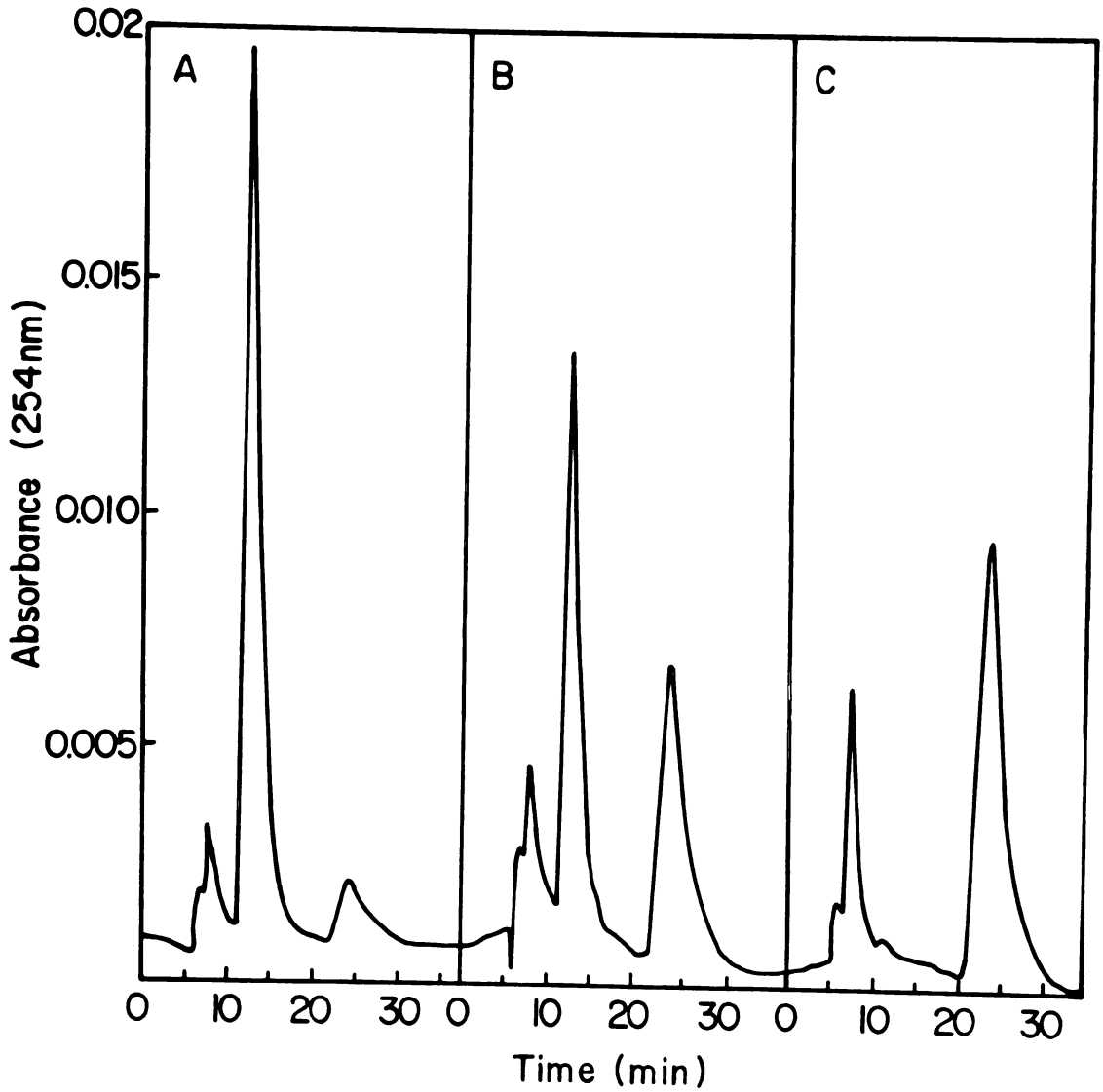

0.02
0.015
0.010
0.005
Absorbance (254nm)
A
B
C
0
10
20
30
0
10
20
30
0
10
20
30
Time (min)

Figure 7

Figure 8. Kinetics Of Hydrolysis Of GDP-Fucose By The β-Galactoside α(1-2) Fucosyltransferase In The Absence Of An Acceptor Substrate. Rates of GDP-fucose hydrolysis, in the absence of acceptor (●—●), by the fucosyltransferase were measured by removing aliquots from the incubation mixture, previously described in "Methods", and application to a Dowex 1 (chloride) column. The eluate was counted for radioactivity and was used as a measure of GDP-fucose hydrolysis. GDP-Fucose hydrolysis in the absence of enzyme in the incubation assay (O---O) was also measured using a Dowex 1 (chloride) column.

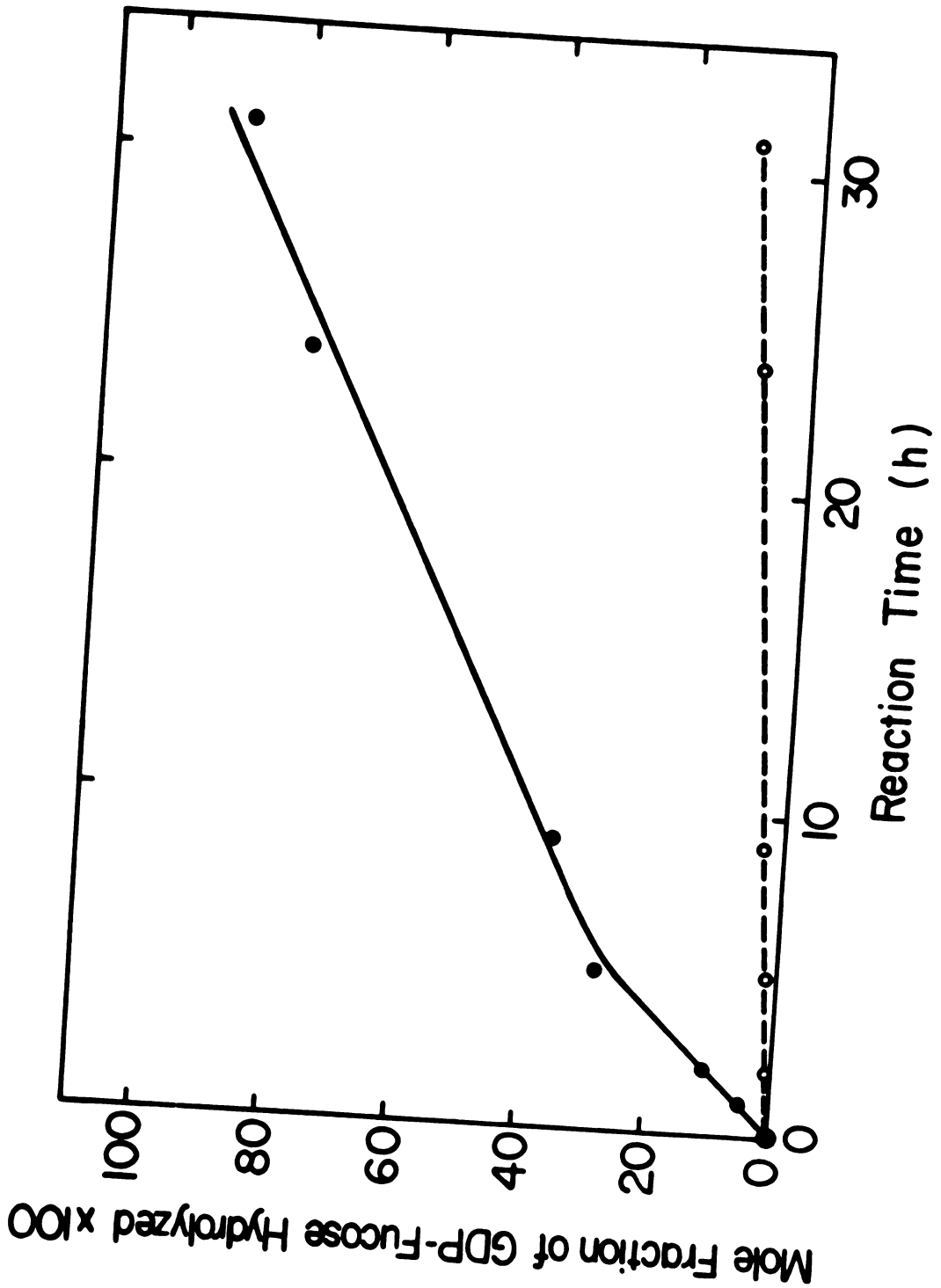

Mole Fraction of GDP-Fucose Hydrolyzed x100
0
20
40
60
80
100
0
10
20
30
Reaction Time (h)

Figure 8

pyridinium acetate. Removal of the pyridinium acetate by rotatory evaporation at 30° resulted in slightly acidic conditions that led to some fucose hydrolysis. However, purification of the disaccharide, Gal β(1-4)GlcNAc-β-hexanolamine from the galactosyltransferase reaction, with Dowex 50 (hydrogen) chromatography did not result in any detectable hydrolysis of the product.

The best method for removal of proteins from the fucosylation reaction was concentration by Amicon filtration. After washing the concentration cell several times, the product was recovered quantitatively in the filtrate free of proteins. Deionization and removal of GMP, GDP and GDP-fucose was achieved by passing the filtrate through a 1 X 4 cm column containing Dowex 1 (chloride) and Chelex 100 (sodium). The solution was then concentrated to 2 ml and applied to a 4 X 90 cm gel filtration column containing Bio-Gel P2 (-400 mesh) equilibrated in 0.1 M triethylammonium bicarbonate pH 7.5. A typical separation profile, for the fucosylated and unreacted acceptor is shown, in Figure 9, for Fucα(1-2)Gal-β-hexanolamine. A two fold excess of acceptor was used in the reaction incubation. The fucose containing disaccharide eluted first containing [UL-^{14}C]-L-fucose glycosidically linked to the galactose residue. The primary amino group of the hexanolamine, present in both Fucα(1-2)Gal-β-hexanolamine and Gal-β-hexanolamine, was quantitatively measured using a fluorescent assay for primary amines (95). Removal of the triethylammonium bicarbonate buffer by rotatory evaporation yielded the fucosylated product ready for nmr analysis. Fucosylated compounds synthesized with the β galactoside α(1-2)fucosyltransferase were; Fuc α(1-2) Galβ(1-4)GlcNAc-β-hexanolamine, Fucα(1-2)Gal-β-hexanolamine, Fucα(1-2)

Figure 9. Separation Of Fucα(1-2)Gal-β-Hexanolamine and Gal-β-Hexanolamine By Gel Filtration. Gel filtration was performed on a Bio-Gel P2 (-400 mesh) column equilibrated in 0.1 M triethylammonium bicarbonate, pH 7.5. The primary amino group in the hexanolamine aglycon was measured using a fluorescent assay, as described in "Methods". The [UL-^{14}C]-L-fucose present in the disaccharide, Fuc α(1-2) Gal-β-hexanolamine was transferred from GDP-[UL-^{14}C] fucose by the β-galactoside α(1-2) fucosyltransferase.

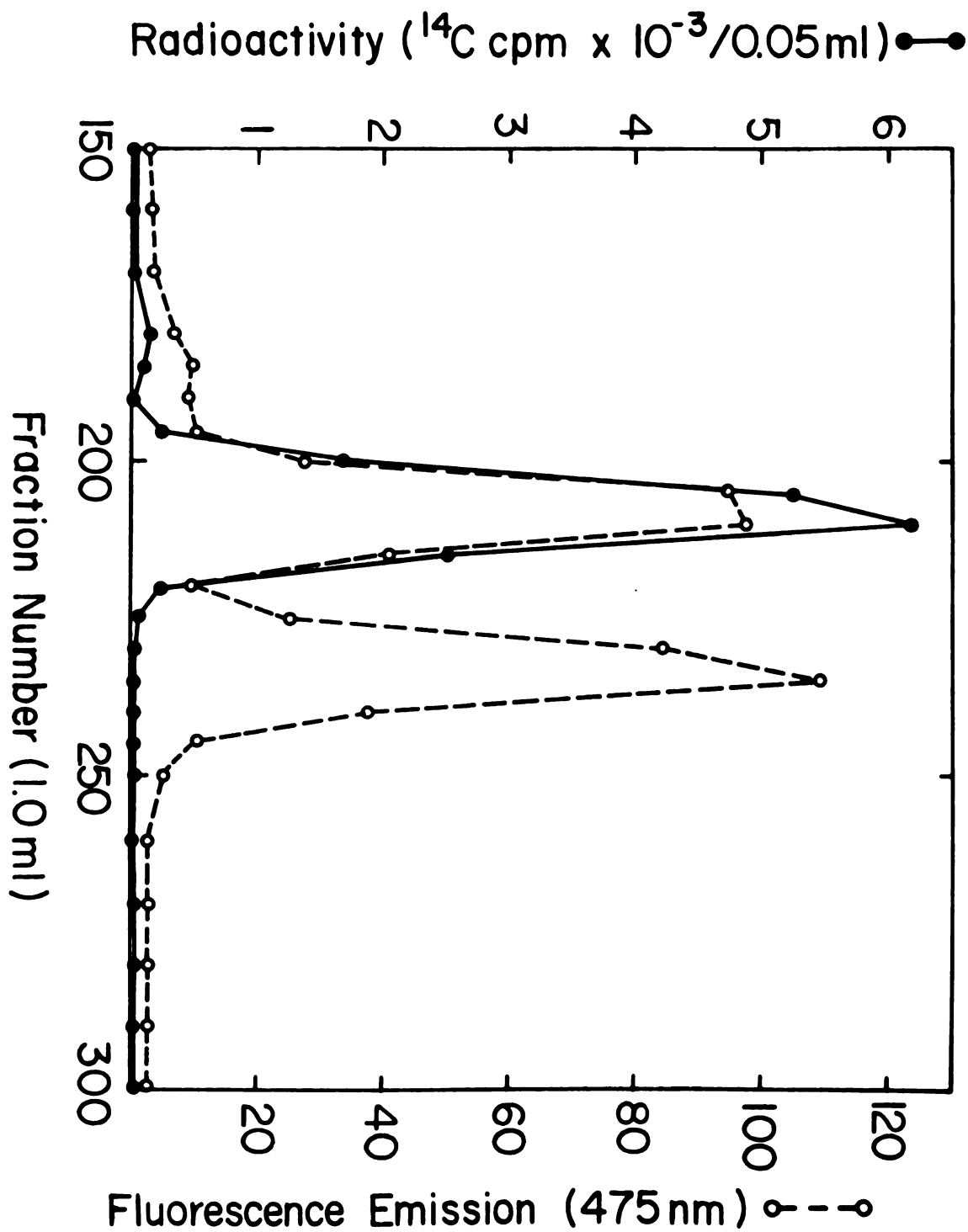

Radioactivity (^{14}C cpm x 10^{-3}/0.05ml)
1
2
3
4
5
6
150
200
250
300
Fraction Number (1.0 ml)
20
40
60
80
100
120
Fluorescence Emission (475nm)

Figure 9

[1-^{13}C]Galβ(1-4)GlcNAc-β-hexanolamine, [1-^{13}C]Fucα(1-2)[1-^{13}C]Galβ(1-4)GlcNAc-β-hexanolamine, Fucα(1-2)[1^{13}C]Galβ(1-4)GlcNAc, [1-^{13}C]Fucα(1-2)[1-^{13}C]Galβ(1-4)GlcNAc, [1-^{13}C]Fucα(1-2)[1-^{13}C]Galβ(1-4)Glc, [1-^{13}C]Fucα(1-2)[1-^{13}C] Gal-β-hexanolamine, [1-^{13}C]Fucα(1-2)[1-^{13}C]Gal-β-ethyl, Fucα(1-2)[1-^{13}C]Gal-β-ethyl, [1-^{13}C]Fucα(1-2)Gal-β-ethyl, and Fucα(1-2)[2-^{13}C]Galβ-ethyl.

V Carbon-13 NMR Analysis of Fucosylated Oligosaccharides

The enzymatic synthesis of Galβ(1-4)GlcNAc-β-hexanolamine was carried out using the bovine N-acetylglucosaminide β(1-4) galactosyltransferase. The 15MHz, proton decoupled, ^{13}C-nmr of this acceptor substrate is shown in Figure 10A. The C1 of GlcNAc, β glycosidically linked to hexanolamine, resonates at 102.4 ppm. Figure 10B, shows the 15 MHz, proton decoupled, ^{13}C nmr of the disaccharide Galβ(1-4)GlcNAc-β-hexanolamine synthesized by the enzymatic transfer of galactose to C4' of GlcNAc-β-hexanolamine. The C1 of Gal β glycosidically linked to C4' of GlcNc is found to resonant at 104.3 ppm. Derivatization of the C4' of GlcNAc results in a downfield shift from 71.6 to 79.9 ppm as discussed by Nunez and Barker (13). Glycosidation at C4'results in small chemical shifts in C3' (75.5 to 73.9 ppm) and C5' (77.4 to 76.5 ppm). Unequivocal assignment of carbon resonances in the dissaccharide were made using [1-^{13}C]Galβ(1-4)GlcNAc-β-hexanolamine and are given in Table II (13).

Synthesis of quantities of fucosylated compounds sufficient for ^{13}C-nmr analysis was attempted first with the acceptor Gal-β-hexanolamine. The 15.08 MHz, proton decoupled, ^{13}C-nmr spectrum

Figure 10. The 15.08 MHz, Proton Decoupled, ^{13}C-NMR Of; A. The Galactosyltransferase Acceptor Substrate GlcNAc-β-Hexanolamine, B. The Galactosylated Product, Galβ(1-4)GlcNAc-β-Hexanolamine. The Cl Gal is found to resonante at 104.3 ppm downfield of tetramethylsilane. Derivatization of GlcNAc-β-hexanolamine with Gal at C4' results in a downfield shift of C4'GlcNAc (71.6 to 79.9 ppm). The Cl'GlcNAc resonantes at 102.4 ppm. The disaccharide is an acceptor in the fucosyltransferase reaction.

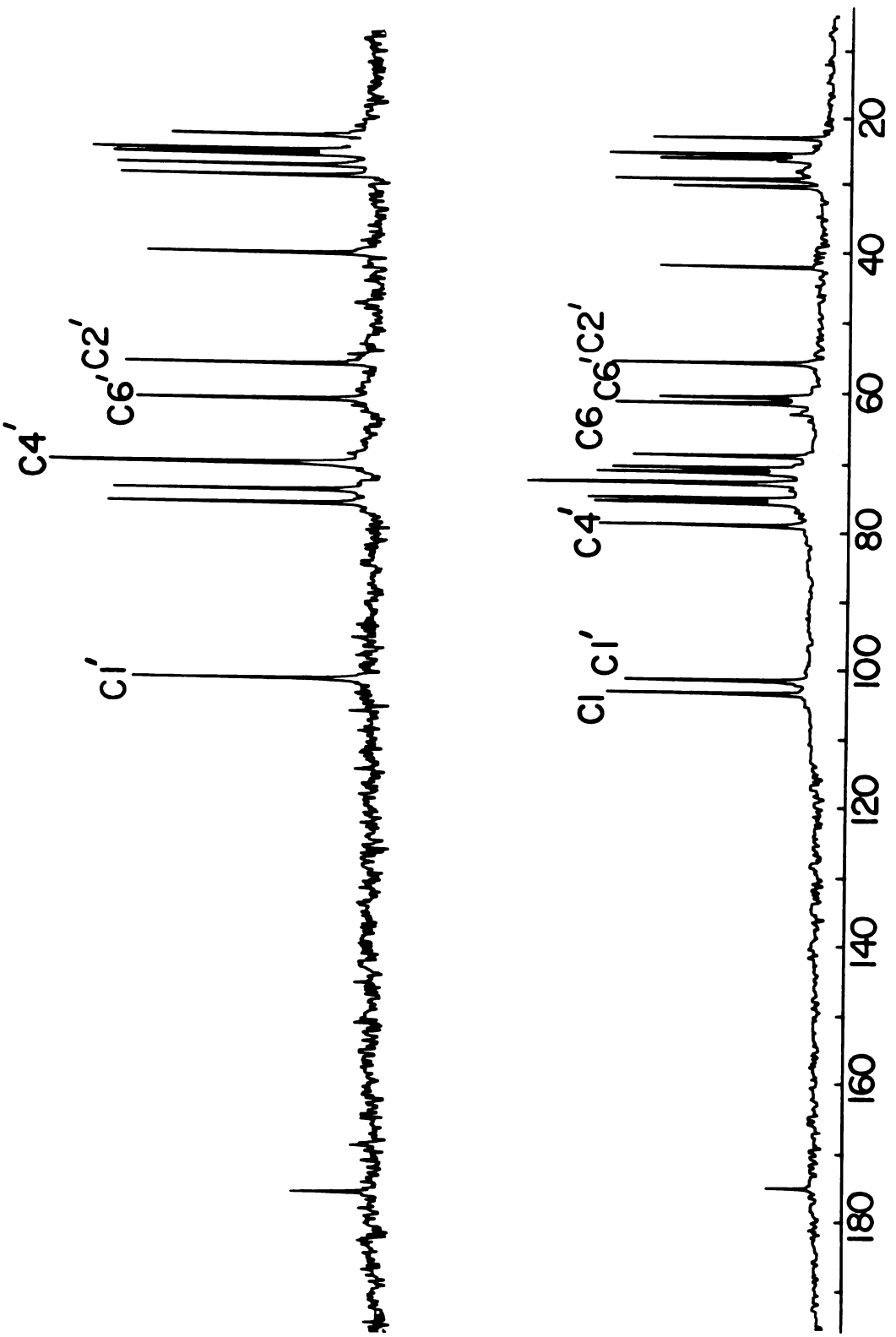


Figure 10

Table II
Carbon-13 Chemical Shift Assignments[a]

	R[b] α	R[b] β	Ethyl Gal	Methyl Fuc	GlcNAc[c] arm	GalGlcNAc[c] arm	FucGal GlcNAc arm	Gal arm	FucGal β-arm	GalGlcNAc[c]		FucGal GlcNAc α	FucGal GlcNAc β
C1													
Glc	93.6	97.4											
GlcNAc	92.2	96.4			102.7	102.4	102.7			91.6	96.2	92.1	96.5
Gal	93.8	98.0	104.0			104.3	101.7	104.3	103.5	104.1		102.3	
Fuc	93.8	97.8		101.2			100.9		101.3			101.4	
C2													
Glc	73.2	75.9											
GlcNAc	55.5	58.1			57.2	56.5	56.8			55.1	57.6	55.5	57.9
Gal	70.0	73.6	72.3			72.3	77.9	72.4	78.4	72.5		77.9	
Fuc	69.8	73.4		69.6			69.6		70.2			69.8	
C3													
Glc	74.5	77.5											
GlcNAc	72.1	75.3			75.5	73.9	73.8			70.6	74.9	75.1	76.8
Gal	70.8	74.4	74.4			74.0	75.0	74.6	75.8	73.9		72.0	
Fuc	71.0	74.6		71.3			71.0		71.5			71.2	
C4													
Glc	71.4	71.3											
GlcNAc	71.5	71.2			71.6	79.9	77.5			80.1	79.7	77.5	78.1
Gal	70.9	70.4	70.2			70.1	70.6	70.4	70.9	69.9		70.8	
Fuc	73.5	73.1		73.5			73.1		73.9			73.3	

	R[b] α	R[b] β	Ethyl Gal	Methyl Fuc	GlcNAc[c] arm	GalGlcNAc[c] arm	FucGal GlcNAc arm	Gal arm	FucGal β-arm	GalGlcNAc[c]		FucGal GlcNAc α	FucGal GlcNAc β
C5													
Glc	73.0	77.4											
GlcNAc	73.0	77.3			77.4	76.5	76.8			71.5	76.1	70.8	76.7
Gal	72.0	76.6	76.6			76.9	76.9	76.7	77.0		76.6	76.8	
Fuc	67.8	72.3		67.9			68.4		68.7			68.5	
C6													
Glc	62.3	62.5											
GlcNAc	62.0	62.2			62.4	61.5	61.5			61.3	61.3	61.6	61.6
Gal	62.8	62.6	62.5			62.5	61.6	62.6	62.9	62.3		62.7	
Fuc	17.2	17.2		16.9			16.3		17.4			16.8	
carboxyl of N-acetyl	175.9	176.2			175.9	175.7				176		176	
methyl of N-acetyl	23.3	23.5		56.6	23.8	23.6				23.3	23.5		
C1 of Hexanolamine						71.6	71.7	72.0	71.8	72.4			

a. Chemical shifts are given in ppm with reference to a chemical shift value of 97.4 ppm for C1 of the β-glucopyranose anomer in a water solution at 25° (68).

b. R specifies the chemical shift of the reducing moiety taken from Walker et al. (68).

c. Chemical shifts taken from Nunez and Barker (13).

Figure 11. The 15.08 MHz, Proton Decoupled, ^{13}C-NMR Of Gal-β-Hexanolamine. The downfield resonance at 104.3 ppm is C1 Gal. Carbon 2 of Gal was assigned to 72.4 ppm by comparison to [1-^{13}C]Gal-β-hexanolamine.

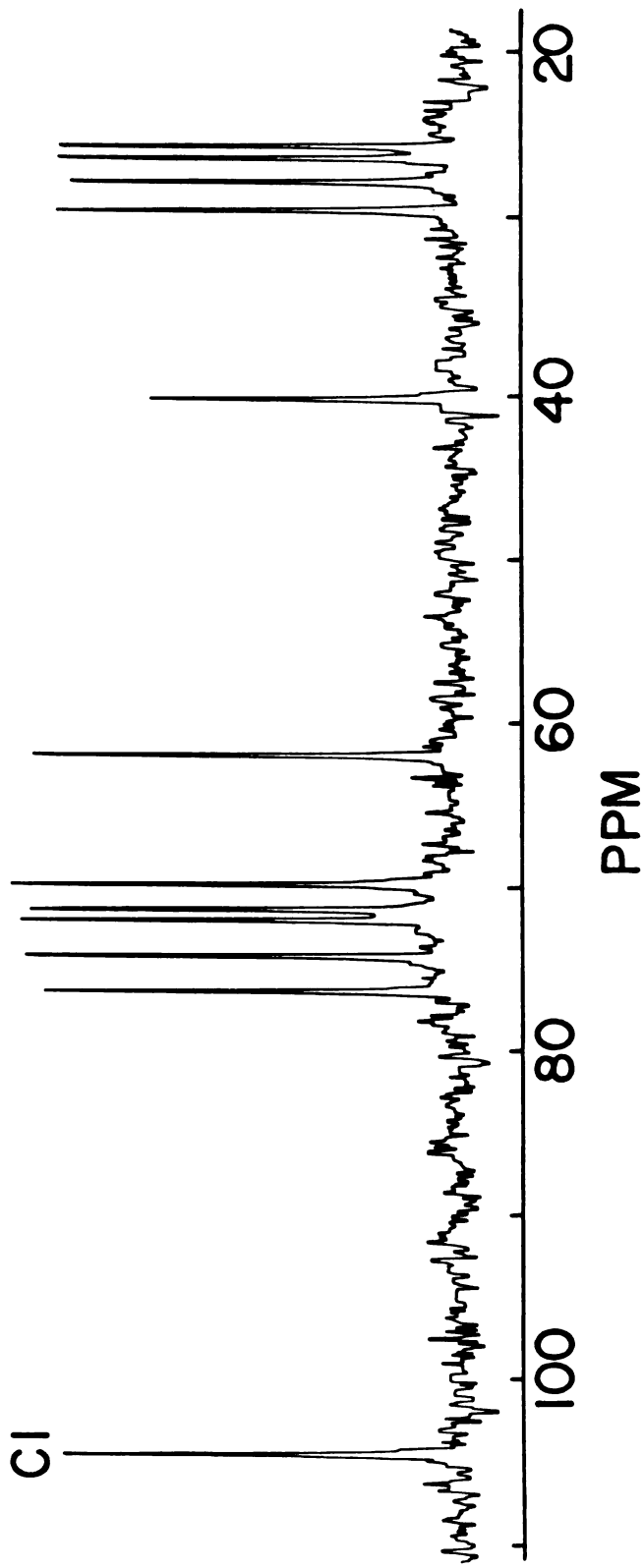


Figure 11

of Gal-β-hexanolamine is shown in Figure 11. The downfield resonance at 104.3 ppm was assigned to C1 of galactose β-glycosidically linked to hexanolamine. Carbon 2 of galactose was assigned to 72.4 ppm by comparison to hexanolamine β-D-[1-^{13}C] galactopyranoside. The 90 MHz, proton decoupled, ^{13}C-nmr of the fucosylated product, Fucα(1-2)Gal-β-hexanolamine is shown in Figure 12. The two resonances found at 103.5 and 101.3 ppm correspond to C1 of Gal and Fuc, respectively. The C1' Gal is shifted upfield as a result of derivatization at C2 with fucose. Fucose was shown to be α-glycosidically linked to galactose by comparison with standard methyl-α-L-[1-^{13}C] fucopyranoside and by enzymatic removal of the fucose by an α-L-fucosidase from bovine kidney. Derivatization at C2 with fucose resulted in a characteristic downfield shift (from 72.4 to 78.4 ppm) of the C2 galactose resonance. Carbon 3 was found to be shifted upfield as a result of derivatization at C2 with fucose (from 74.6 to 75.8 ppm). Carbon-1 was also shifted upfield (104.3 to 103.5 ppm). Complete assignment of the chemical shifts are given in Table II.

The antigenic portion of the blood group O substance was synthesized by enzymatic fucosylation of the disaccharide Galβ(1-4) GlcNAc-β-hexanolamine. The 90 MHz, proton decoupled, natural abundance ^{13}C-nmr spectrum of the antigenic trisaccharide, Fucα(1-2)Galβ(1-4) GlcNAc-β-hexanolamine is shown in Figure (13). Resonances marked, X, are due to the disaccharide acceptor Galβ(1-4)GlcNAc-β-hexanolamine, an impurity in the sample. The spectrum can be divided into three regions: a glycosidic region from 105 to 95 ppm containing C1 of the carbohydrate; a second region from 85 to 55 ppm containing C2 through C6 of the carbohydrates and C1 of the hexanolamine; and a third region from

Figure 12. The 90 MHz, Proton Decoupled, ^{13}C-NMR Of Fuc α(1-2)Galβ(1-4)GlcNAc-β-Hexanolamine. The two resonances found at 103.5 and 101.3 ppm correspond to C1 of Gal and Fuc, respectively. The C2'Gal is shifted downfield (from 72.4 to 78.4 ppm) as a result of derivatization with Fuc. The resonance X is an impurity.

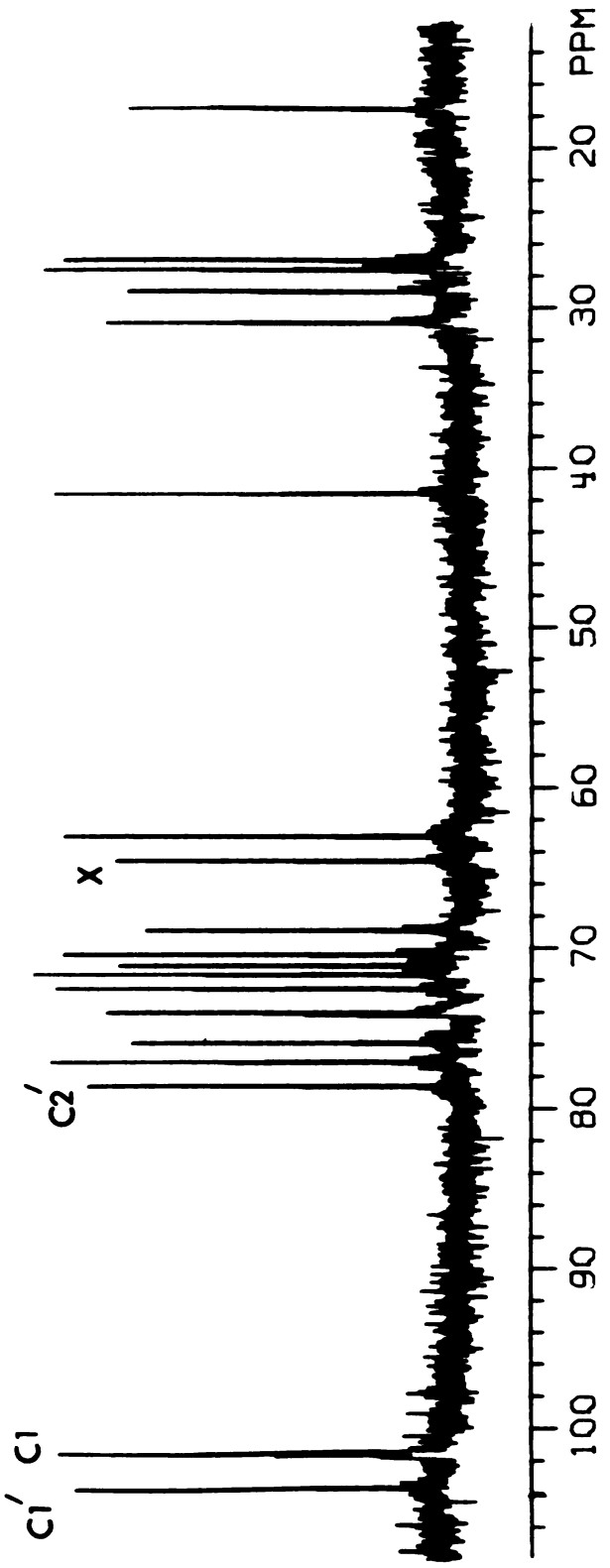


Figure 12

Figure 13. The 90 MHz, Proton Decoupled, Natural Abundance ^{13}C-NMR Spectrum Of The Antigenic Trisaccharide, Fucα(1-2)Galβ(1-4)GlcNAc-β-Hexanolamine. Resonances marked, X, are due to the disaccharide acceptor Galβ(1-4)GlcNAc-β-hexanolamine, an impurity in the sample. The spectrum can be divided into three regions: a glycosidic region from 105 to 95 ppm containing C1 of the carbohydrates; a second region from 85 to 55 ppm containing C2 through C6 of the carbohydrates and C1 of the hexanolamine; and a third region from 50 to 15 ppm containing C2 through C6 of the hexanolamine, C6 of fucose and the methyl carbon in the N-acetyl group of GlcNAc. Complete assignment of the resonances are given in Table II.

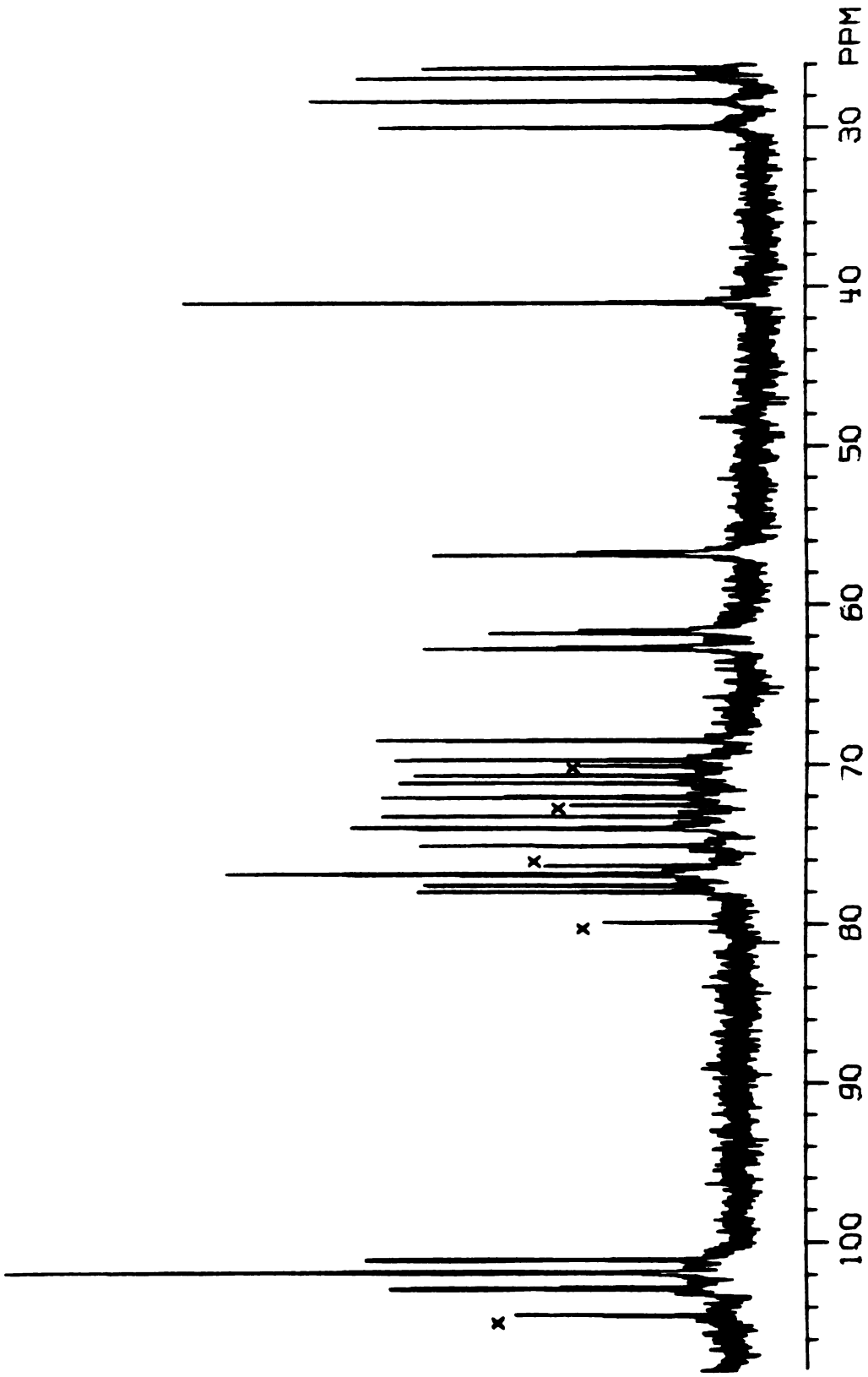


Figure 13

50 to 15 ppm containing C2 through C6 of the hexanolamine, C6 of fucose and the methyl carbon in the N-acetyl group of GlcNAc. Complete assignment of the resonances are given in Table II. Unequivocal resonance assignments were made using methyl-α-L-[1-^{13}C] fucopyranoside, ethyl-β-D-[1-^{13}C] galactopyranoside and Fuc α(1-2) [1-^{13}C]Galβ(1-4)GlcNAc-β-hexanolamine. An expanded 90 MHz, proton decoupled, ^{13}C-nmr spectrum (Figure 14) of the anomeric region of Fuc α(1-2)Galβ(1-4)GlcNAc-β-hexanolamine reveals chemical shift differences in the anomeric carbons as a result of derivatization with Fuc at C2. Resonances are labeled in accordance with IUPAC nomenclature with Fuc, Gal and GlcNAc being represented by C, C' and C'', respectively. Carbons Cld and Cl'd correspond to the contaminating disaccharide Galβ(1-4)GlcNAc-β-hexanolamine. Derivatization of C2' with Fuc results in a 2.6 Hz upfield chemical shift of Cl' (from 104.3 to 101.1 ppm). The Cl'' of GlcNAc is found to shift downfield by 0.11 ppm as a result of fucosylation. Carbon-1 of the α-L-fucosopyranosyl residue resonates at 100.9 ppm.

Th 45 MHz, proton decoupled ^{13}C-nmr of the trisaccharide Fuc α(1-2)[1-^{13}C]Galβ(1-4)GlcNAc-β-hexanolamine is shown in Figure 15. The carbon 1 of Gal is enriched with ^{13}C to the 90% level allowing unequivocal assignment of the Gal resonances in the trisaccharide. The anomeric region shows two carbon-13 enriched resonances. The enriched resonance at 104.3 ppm is the Cl' of Gal in the contaminating disaccharide [1-^{13}C]Galβ(1-4)GlcNAc-β-hexanolamine. Natural abundance carbons of the disaccharide are not observable. The carbon-13 enriched resonance at 101.7 ppm is the Cl' of Gal in the trisaccharide.

Figure 14. An Expanded 90 MHz, Proton Decoupled, ^{13}C-NMR Spectrum Of The Anomeric Region Of Fucα(1-2)Galβ(1-4)GlcNAc-β-Hexanolamine. Resonances labeled C, C' and C'' represent Fuc, Gal and GlcNAc, respectively. Carbons C1d and C1'd correspond to the disaccharide Galβ(1-4) GlcNAc-β-hexanolamine.

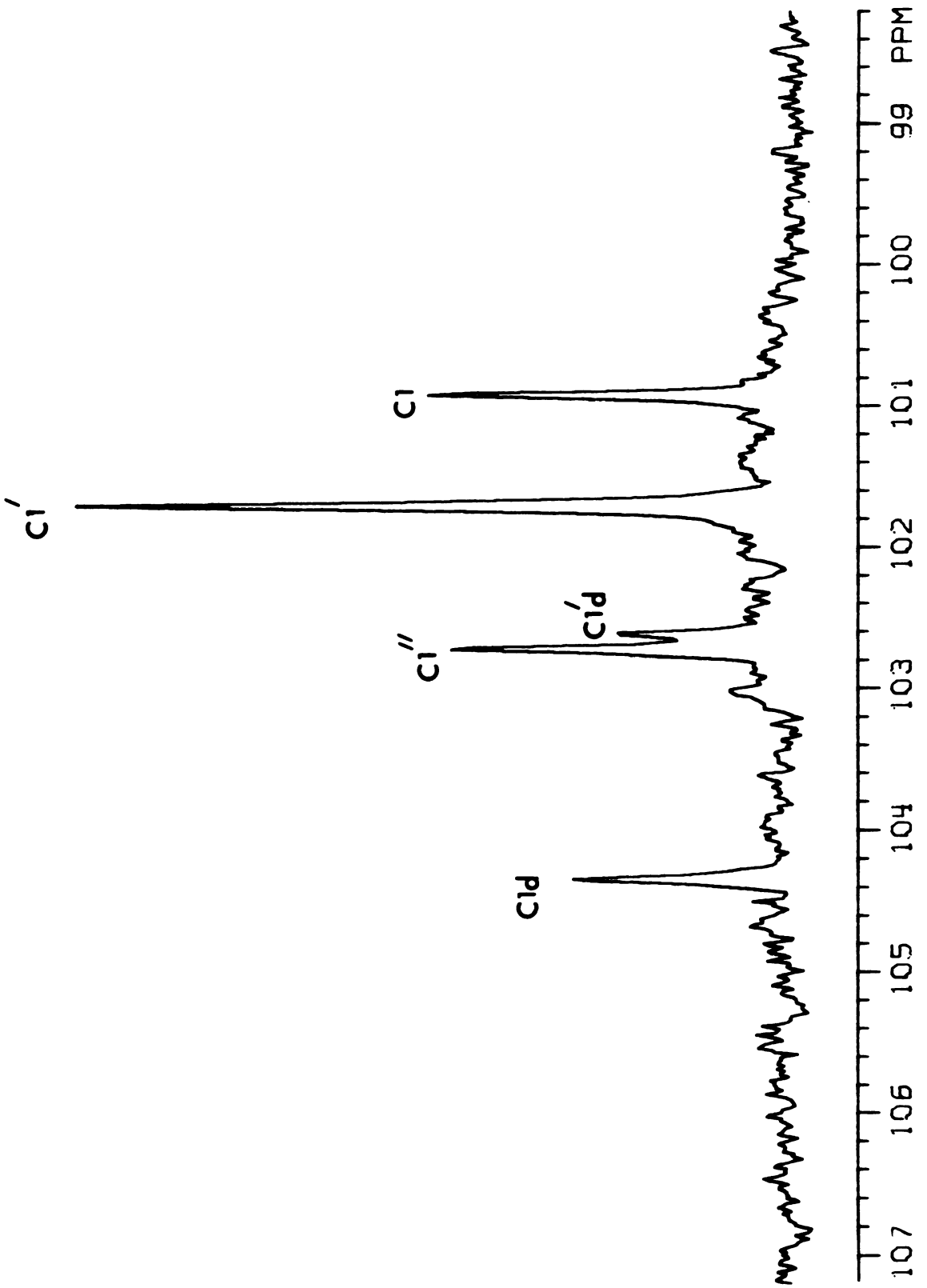


Figure 14

Figure 15. The 45 MHz, Proton Decoupled, ^{13}C-NMR Of The Trisaccharide Fucα(1-2) [1-^{13}C] Galβ(1-4)GlcNAc-β-Hexanolamine. The Cl Gal is enriched to the 90% level with ^{13}C. The enriched resonance at 104.3 ppm is the Cl' of Gal in the contaminating disaccharide [1-^{13}C] Gal β(1-4)GlcNAc-β-hexanolamine. Natural abundance carbons of the disaccharide are not observable. The C2 Gal is unequivocally assigned from the large one-bond coupling (46 Hz) from [1-^{13}C] Gal to C2 Gal.

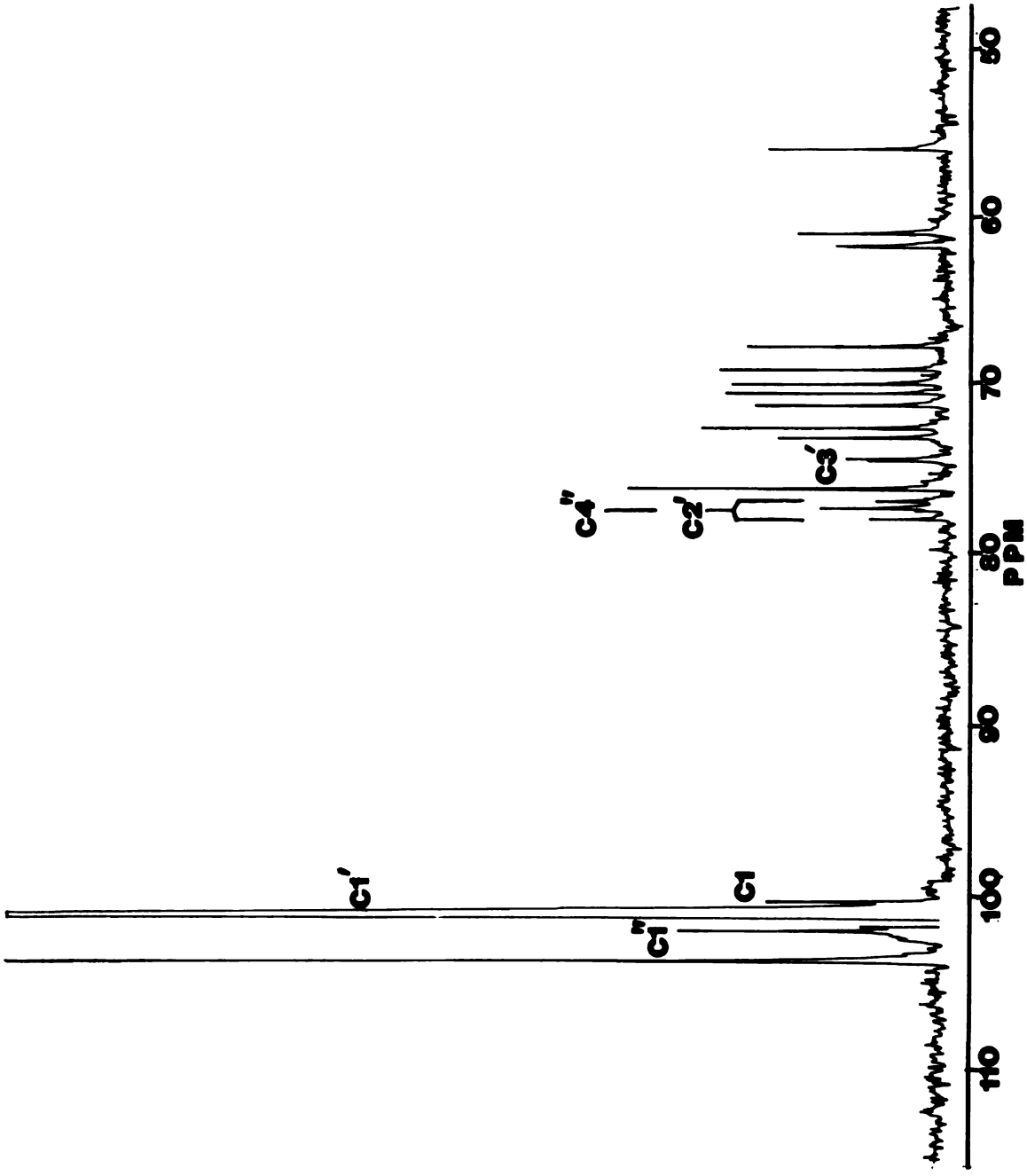


Figure 15

An expanded region from C2 to C6 of Fuc [1-^{13}C]Galβ(1-4) GlcNAc-β-hexanolamine is shown in Figure 16. Derivatization of Gal at C2 with fucose results in a downfield shift of C2' (from 72.3 to 77.9 ppm). The large one-bond coupling (46 Hz) from [1-^{13}C] Gal to C2 makes assignment of the C2 resonance unequivocal. The C4'' of GlcNAc derivatized with Gal is clearly observed at 70.6 ppm. Carbon-3 of Gal is easily assigned at 75.0 ppm by a $^{2}J_{C1-C3}$ coupling of 3.5 Hz, shown in Figure 16. Carbon 2 of GlcNAc is found to resonate upfield at 56.8 ppm as a result of derivatization with an N-acetyl group. Carbon 6 of Gal was assigned to 76.9 ppm as a result of a broadening due to a small $^{3}J_{C1-C6}$ interaction. The C6'' of GlcNAc was assigned to 61.5 ppm. The 6-deoxy carbon of fucose is found to resonate upfield at 16.3 ppm.

The reducing trisaccharide Fucα(1-2)[1-^{13}C]Galβ(1-4)GlcNAc was enzymatically synthesized by the sequential addition of [1-^{13}C] Gal and Fuc, from UDP-[1-^{13}C]-Gal and GDP-Fuc, by the galactosyltransferase and fucosyltransferase, respectively. Chemical shift assignments are reported in Table II and were determined as described for Fucα(1-2)[1-^{13}C]Galβ(1-4)GlcNAc-β-hexanolamine. The ratio of α:β GlcNAc in the trisaccharide was found to be 2:1. The chemical shifts of the [1-^{13}C] enriched galactose in Fucα(1-2) [1-^{13}C] Galβ(1-4)GlcNAc were found to differ (102.32 to 102.28) depending on the anomeric configuration at C1 of GlcNAc, α or β. Fuc α(1-2)[1-^{13}C]Galβ(1-4)Glc was also found to have an α:β ratio of 2:1 and differing chemical shifts of the [1-^{13}C] Gal depending on the anomeric configuration of Glc.

VI Conformational Analysis of the Fucosylated Oligosaccharides

Figure 16. An Expanded Region From C2 to C6 Of The 90 MHz, Proton Decoupled, ^{13}C-NMR Of Fucα(1-2) [1-^{13}C] Galβ(1-4)GlcNAc-β-Hexanolamine.

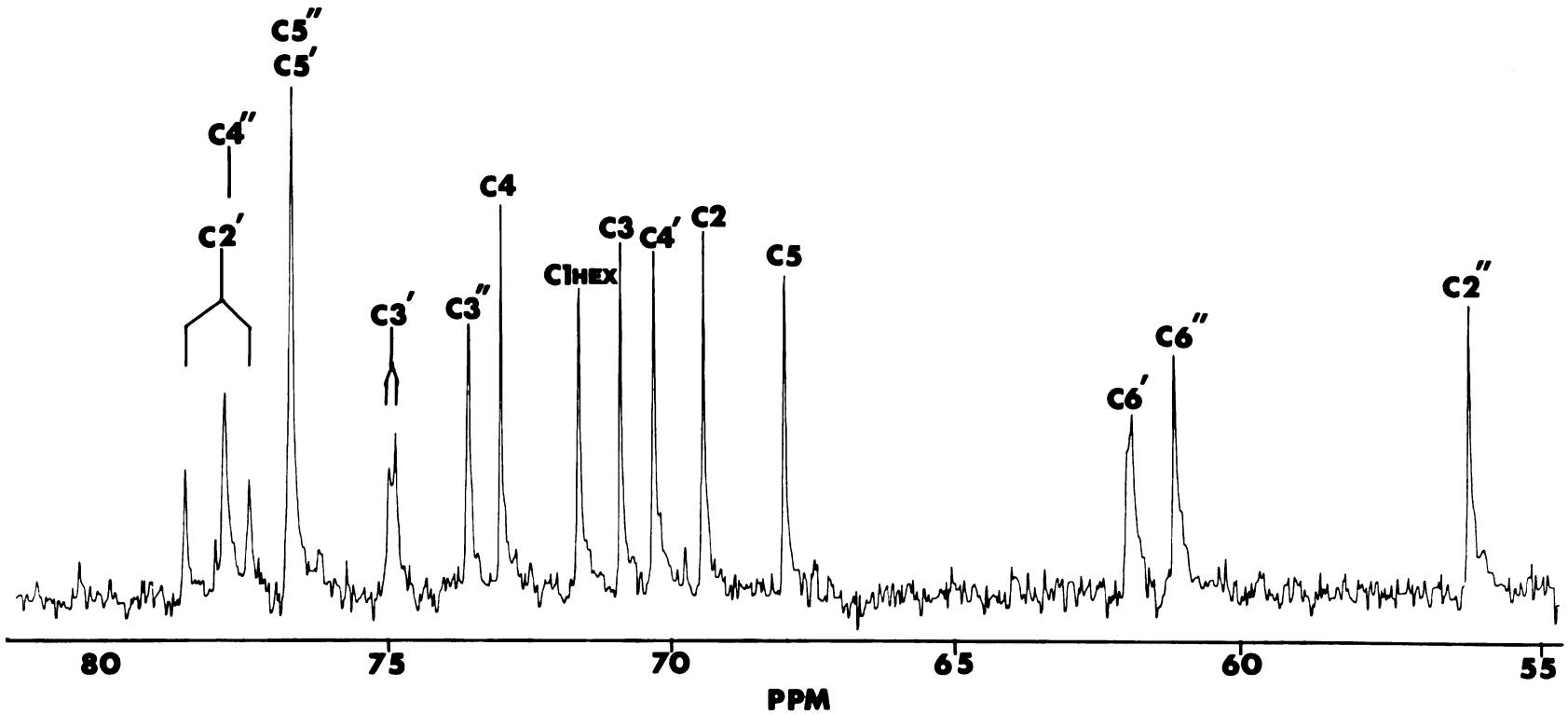


Figure 16

Specifically enriched carbon-13 carbohydrates can be used to evaluate the solution conformation of oligosaccharides by measurement of interresidue spin-spin coupling (^3J) between the 90% enriched ^{13}C nucleus of one residue and an appropriatively positioned ^{13}C or ^1H nucleus of the adjacent residue. Conformation about the glycosidic bond can be defined by two torison (dihedral) angles, ψ (C1-O-C2'-C1') and $\emptyset$ (C2'-O-C1-H1) as shown in Figure 17. The dihedral angles can be evaluated by measurement of the inter-residue coupling between carbohydrates.

The trisaccharide Fuc [1-^{13}C] Galβ(1-4)GlcNAc-β-hexanolamine was initially examined for interresidue spin-spin coupling between [1-^{13}C]C1 Gal and C1 Fuc. A partial spectrum of the trisaccharide Fucα(1-2)[1-^{13}C]Galβ(1-4)GlcNAc-β-hexanolamine is shown in Figure 18. Inter-residue $^3J_{C-C}$ coupling between [1-^{13}C]C1 Gal and C1 Fuc is small or absent since a coupling constant could not be observed at C1 Fuc, as shown in Figure 18. The linewidth of C1Fuc is broad in comparison with C1 of GlcNAc or any single resonance in the spectrum. Repeated attempts to resolve the broadening failed. The broadening is estimated to reflect a coupling of no more than 2Hz. To increase the sensitivity in the measurement of the small $^3J_{CC}$ coupling between [1-^{13}C] C1 Gal and C1 Fuc the trisaccharide was synthesized with 90% enriched ^{13}C at both C1Gal and C1Fuc. Again, no observable splitting pattern could be detected, however, inter-residue interactions could be detected by increased linebroadening in the two ^{13}C enriched resonances. Linebroadening was measured by comparison of the ^{13}C enriched linewidths in [1-^{13}C] Fucα(1-2)[1-^{13}C]Galβ-(1-4)GlcNAc-β-hexanolamine to Fucα(1-2)[1-^{13}C] Galβ(1-4)GlcNAc-β-

Figure 17. The Conformation About The Glycosidic Bond in Fucα(1-2)Gal Can Be Defined By Two Torison (dihedral) Angles, Ψ (C1-O-C2'-C1') and Ø (H1-C1-O-C2').

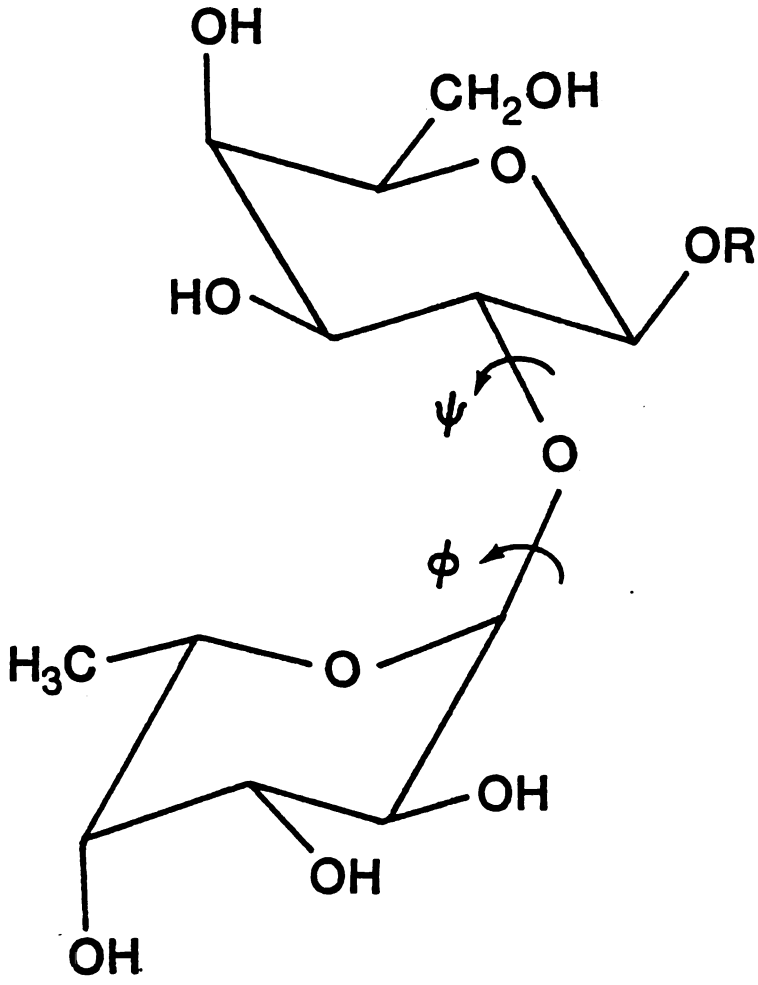


Figure 17

Figure 18. The 90 MHz, Proton Decoupled, ^{13}C-NMR Spectrum Of The Anomeric Region Of The Trisaccharide Fucα(1-2)[1-^{13}C]Galβ(1-4)GlcNAc-β-Hexanolamine. Carbons 1 of Fuc, Gal and GlcNAc are labeled C1, C1' and C1'', respectively.

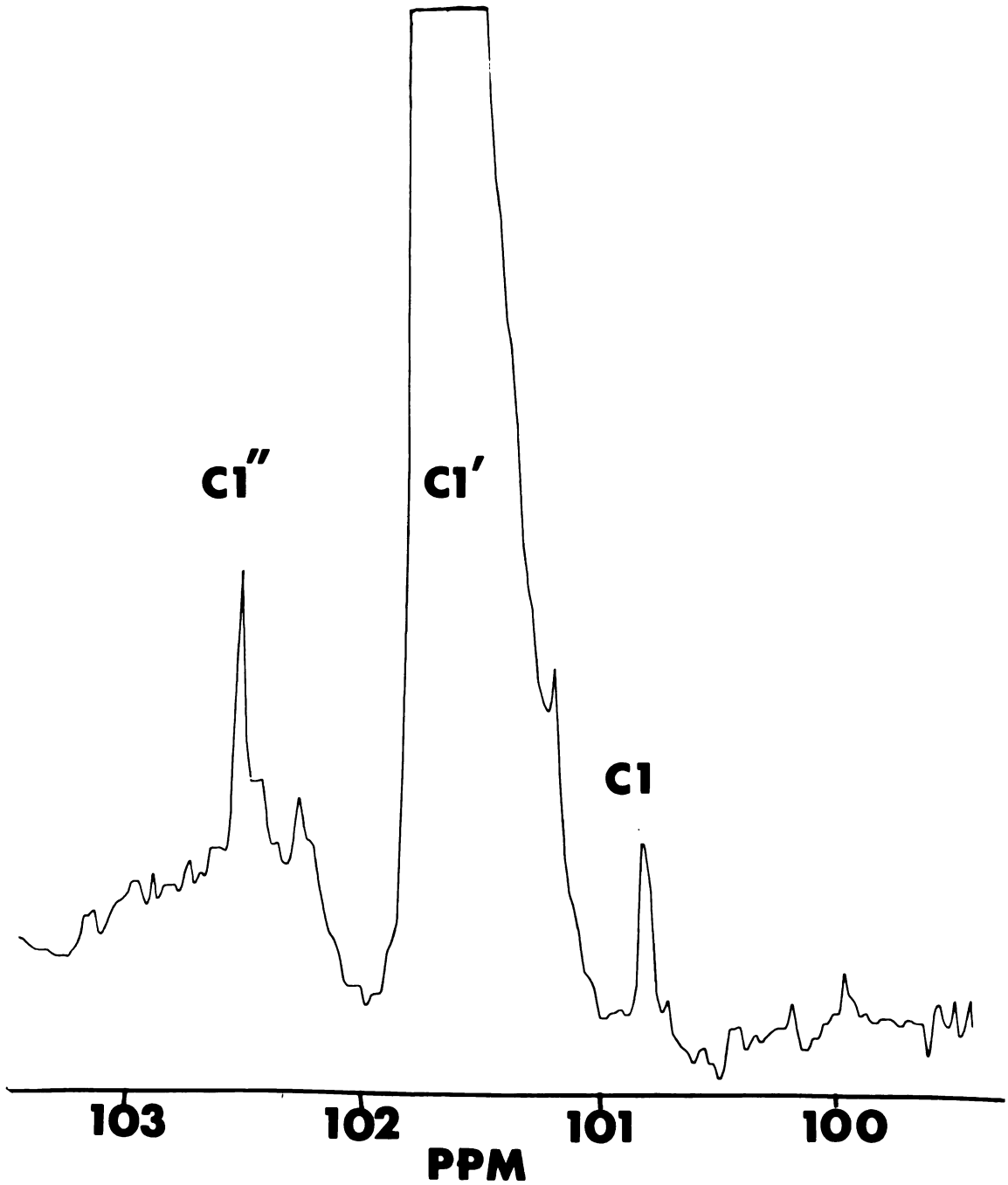


Figure 18

hexanolamine and [1-^{13}C]Galβ(1-4)GlcNAc-β-hexanolamine using dioxane as an internal standard. Linewidths of the ^{13}C enriched resonances in [1-^{13}C]Fucα(1-2)[1-^{13}C]Galβ(1-4)GlcNAc-β-hexanolamine were approximately 3 times greater than the linewidth of the ^{13}C enriched resonance in [1-^{13}C]Galβ(1-4)GlcNAc-β-hexanolamine as shown in Figure 19. Although a coupling constant ($^{3}J_{C1\text{-}O\text{-}C2'\text{-}C1'}$) cannot be measured for the torsion angle, a small unresolved coupling of 1 to 1.5 Hz must be occuring to account for the observable increased linebroadening. Several doubly enriched ^{13}C di and trisaccharides, [1-^{13}C]Fucα (1-2) [1-^{13}C] Gal-β-ethyl, [1-^{13}C]Fucα(1-2) [1-^{13}C] Galβ(1-4)GlcNAc, [1-^{13}C] Fucα(1-2)[1-^{13}C]Galβ(1-4)Glc and [1-^{13}C] Fuc α(1-2) [1-^{13}C] Gal-β-hexanolamine were prepared for evaluation of the ψ torsion angle by $^{3}J_{13C\text{-}13C}$ coupling constants. In no case was appreciable inter-residue coupling observed, but increased linebroadening of approximately 3 Hz was observed for all doubly enriched oligosaccharides when compared to the singly enriched analog.

Evaluation of the Ø torsion angle was attempted using proton nmr by inter-residue $^{3}J_{13C\text{-}'H}$ coupling. Inter-residue coupling involving sp^3 carbons contained in the sequence ^{13}C-O-C-'H have been shown to obey a "Karplus type" of relationship (125). The model compound Fucα(1-2)[2-^{13}C]Gal-β-ethyl was used to evaluate the Ø torsion angle about the Fucα(1-2)Gal glycosidic bond.

Assignment of resonances in the proton nmr of compounds as complex as disaccharides can be extremely difficult since coupling is not always

Figure 19. Carbon-13 NMR Linewidth Measurements Of The ^{13}C-Enriched Resonances in Fuc α(1-2)[1-^{13}C]Galβ(1-4)GlcNAc-β-Hexanolamine and [1-^{13}C]Fucα(1-2)[1-^{13}C]Galβ(1-4)GlcNAc-β-Hexanolamine. A, The ^{13}C-enriched resonance in Fucα(1-2)[1-^{13}C]Galβ(1-4)GlcNAc-β-hexanolamine; B, The ^{13}C-enriched resonances in [1-^{13}C]Fucα(1-2)[1-^{13}C]Galβ(1-4)GlcNAc-β-hexanolamine; and C, Dioxane.

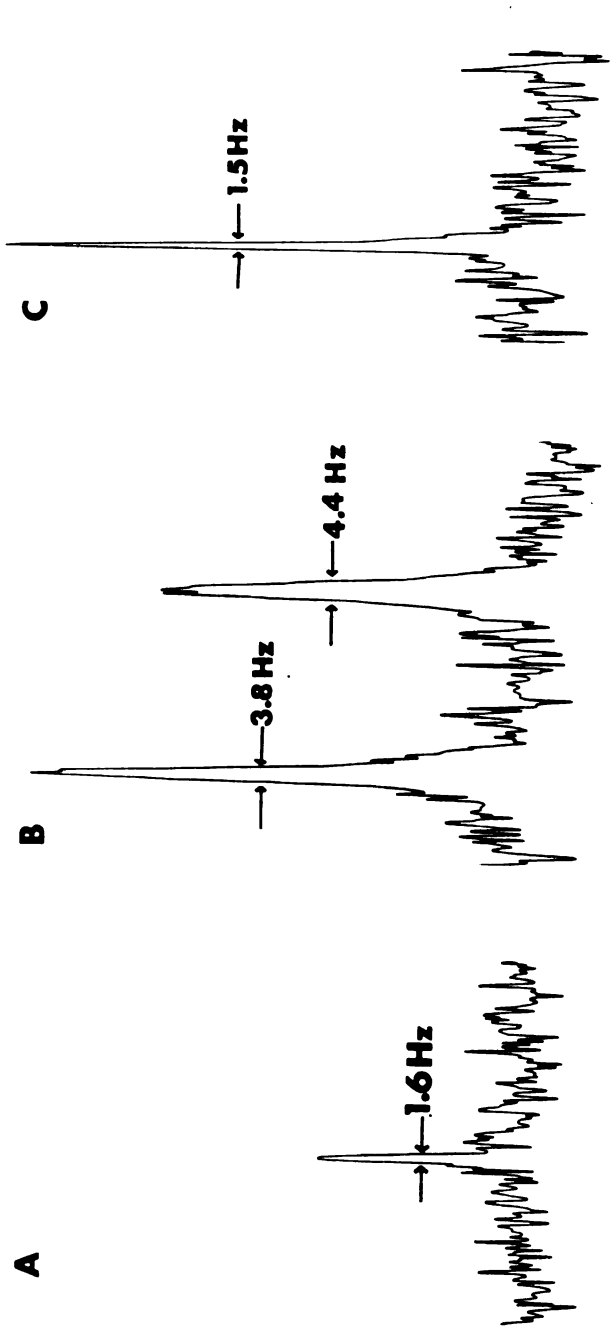


Figure 19

first order. One exception are the anomeric protons on the carbohydrates which resonate 1 to 2 ppm downfield from H2 through H6. Anomeric protons are usually observable as doublets as a result of a $^{3}J_{H1-H2}$ coupling. The H1Fuc resonance in Fucα(1-2)Gal-β-ethyl was found to resonate as a doublet at 5.04 ppm downfield of tetramethylsilane. A $^{3}J_{H1-H2}$ coupling of 3.4 Hz was measured for Fucα(1-2)Gal-β-ethyl, shown in Figure 20A. The Ø torsion angle was estimated using $^{3}J_{H1-^{13}C2}$ coupling in Fucα(1-2) [2-^{13}C]Gal-β-ethyl. Figure 21B shows the H1 resonance of Fuc in Fucα(1-2) [2-^{13}C]Gal-β-ethyl. The multiplet was interpreted as an ABX splitting pattern with a $^{3}J_{H1-H2}$ of 3.4 Hz and a $^{3}J_{H1-^{13}C2'}$ of approximately 3.2 Hz. Computer simulation would be needed to further refine the coupling constants. The $^{3}J_{H1-^{13}C2}$ coupling of approximately 3.2 Hz can be used to evaluate the Ø torsion angle (the position of C2'Gal with respect to the Fuc ring) in the Fucα(1-2)Gal glycosidic linkage.

Figure 20. The 180 MHz Proton NMR Showing The H1 Fuc Resonance In; A. Fuc α-(1-2)Gal-β-Ethyl, and B. Fuc α(1-2)[2-^{13}C]Gal-β-Ethyl.

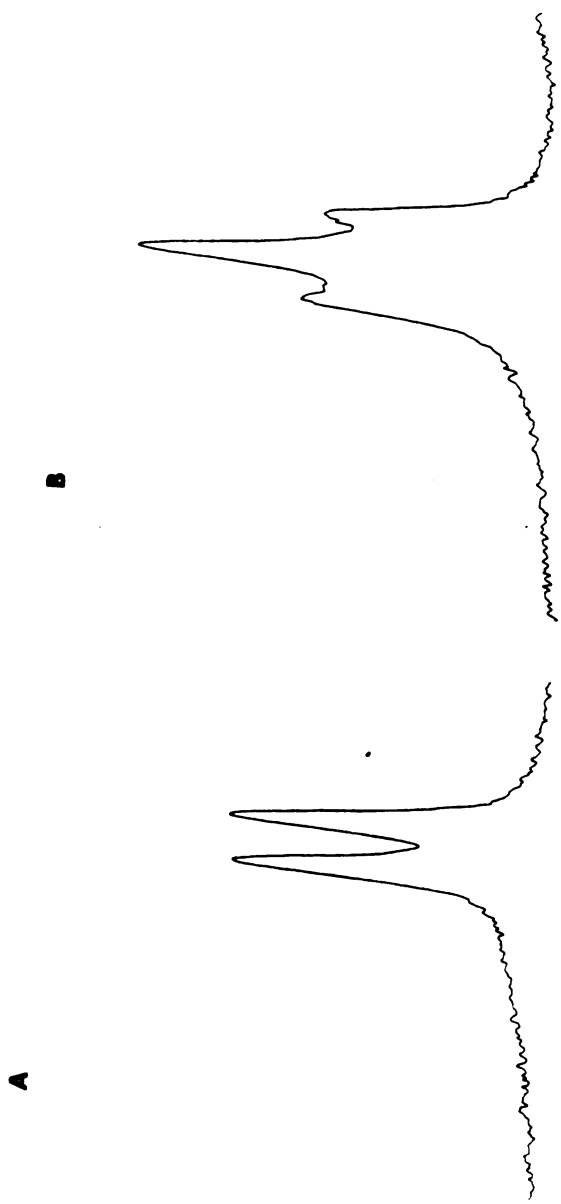


Figure 20

DISCUSSSION

I. Preparation of β-Galactoside α(1-2) Fucosyltransferase

Enzyme mediated synthesis of branched chain complex oligosaccharides proceeds with high yield and anomeric specificity in contrast to chemical methods for the synthesis of glycosidic linkages.

The chemical preparation of β-linked glycosides are usually carried out using the classical Koenigs-Knorr reaction (109) or a modification of this method. Such methods involve reaction of a blocked halogenated sugar with the agylcon (bearing a hydroxyl group) in the presence of silver or mercury salts to produce a β-linked glycosides.

Recently, much effort has been devoted to the stereoselective preparation of α-linked disaccharides in good yields (110). Much of this interest was due to the finding that many of the immunodominant sugars in glycoproteins and glycolipids are α-linked in these structures. The major breakthrough in this area was the so-called α-halide ion catalyzed glycosidation reaction developed by Lemieux and co-workers (4,5,111). Although this method does allow synthesis of α-fucosides, some limitations exist. Yields are low, complex blocking strategies are required, and extensive purification of the product is necessary before and after removal of the blocking groups. The number of readily obtainable derivatives is limited, most importantly those for blocking the reducing moiety of the di or trisaccharide. A second method for α-glycosidations has recently been described which involves the synthesis of sugar imidates and their alcoholysis in the presence of p-toluenesulfonic acid to obtain α-linked disaccharides (112). This

method also suffers from some of the same limitations as mentioned above.

The advent of carbon-13 nmr spectroscopy in the elucidation of structures and conformations of oligosaccharides, antigenic determinants and glycoside-protein complexes and the recent development of a general method for synthesis of carbon-13 enriched carbohydrates prompted investigation into a versatile synthetic method for synthesis of complex biologically active oligosaccharides. Enzymatic synthesis has been proven to be a useful method to obtain specificity and high yield. The enzyme mediated synthesis of the blood group O antigenic substance was performed using a N-acetylglucosaminide β(1-4) galactosyltransferase and a β-galactoside α(1-2) fucosyltransferase. The N-acetylglucosaminide β(1-4) galactosyltransferase has been purified to homogenity by Barker et al. (15) and has recently been used to synthesize carbon-13 enriched derivatives of Galβ(1-4)GlcNAc by Nunez and Barker (13). The product disaccharide, Galβ(1-4)GlcNAc, is the building block on which the antigenic sugars of the ABO blood group system are transferred by specific glycosyltransferases.

Synthesis of the terminal antigenic portion of the blood group O substance required the purification of a β-galactoside α(1-2) fucosyltransferase capable of transferring fucose from GDP-Fuc in an α(1-2) glycosidic linkage to a terminal galactose residue.

Several tissue sources were examined for fucosyltransferase activity. Porcine submaxillary glands were found to have good enzymatic activity, were easily obtainable and therefore were chosen as the tissue from which to purify the enzyme.

The two major criteria of the purification procedure were; to obtain a partially purified enzyme preparation that easily allowed synthesis of 0.1 mmole quantities of fucosylated compounds and to obtain the enzyme preparation in the highest yield since concentrations of the enzyme in the tissue were extremely low.

The β-galactoside α(1-2) fucosyltransferase had been previously reported in several tissues, shown to be membrane bound and purified 70 fold (57,58). Recently, the enzyme has been purified to homogenity from porcine submaxillary glands (T.A. Beyer and R.L. Hill, personal communication). A purification procedure was developed that involved removal of mucin from the glands, manganese chloride aggregation of the membranes, solubilization of the enzyme with 1% Triton X-100, adsorption to SP-Sephadex, purification on a GDP-Sepharose affinity adsorbant, desalting on Sephadex G50 and finally concentration on GDP-Sepharose. This approach allowed partial purification of the enzyme on a large scale. Aggregation of the membranes with manganese chloride was chosen to alleviate the necessity of high speed centrifugation of large volumes. Extraction with 1% Triton X-100 in 0.5 M sodium chloride quantitatively solubilized the enzyme from the membrane. However, the ionic strength prohibited adsorbing the enzyme to SP-Sephadex. The enzyme could be adsorbed batchwise to GDP-Sepharose and eluted with 2.0 M sodium chloride but the purification achieved was small and the high ionic strength needed to elute the enzyme presented problems in further purification. Therefore, the enzyme was extracted twice from the precipitated membranes with 1% Triton X-100 in low ionic strength buffer, adsorbed batch-wise to SP-Sephadex and eluted with 0.3 M sodium chloride. The enzyme, at this stage could be stored in 50% glycerol

at -20° for long periods of time. The enzyme was eluted from the SP-Sephadex resin in the absence of Triton X-100 since the enzyme was found to rapidly lose enzymatic activity in the presence of this detergent (T. Beyer and R.L. Hill, personal communication).

Large scale purification of the enzyme was achieved on the affinity adsorbant GDP-Sepharose. After adsorption of the enzyme to the column, the column was washed with a 0.5 M to 2.0 M sodium chloride gradient. The enzyme was eluted with 2.0 M sodium chloride giving a 1600 fold purification. The next step was to concentrate the enzyme so that it could readily be used in large scale synthesis of fucosylated oligosaccharides. To achieve this the fucosyltransferase was desalted on a Sephadex G50 column, adsorbed on a 1 ml GDP-Sepharose column and eluted with a samll volume of 2.0 M sodium chloride. The β-galactoside α(1-2) fucosyltransferase at this stage had been purified 11,000 fold, had a specific activity of 0.88 units/mg protein, and was used in all syntheses of fucosylated compounds reported here.

Increased purification could be achieved by specific elution of the second GDP-Sepharose column with 5 mM GMP and adsorption onto a Gal β(1-4)GlcNAc-Sepharose affinity column. The enzyme was eluted from the disaccharide affinity adsorbant by removal of the GMP. Enzyme prepared in this manner was over a 100,000 fold purified but the overall yield was only 5%.

A similar procedure was utilized by T.A. Beyer and R.L. Hill (personal communication) in the initial steps in the purification to homogenity of the β-galactoside α(1-2) fucosyltransferase.

II. Preparation of Fucosylated Oligosaccharides

The use of partially purified enzyme preparations of the β-galactoside α(1-2) fucosyltransferase for the synthesis of fucosylated oligosaccharides proceeds with anomeric specificity, high yields, and ease of purification, as shown in "Results".

The preparation of fucosylated di and trisaccharides was monitored by radioactivity and HPLC. Yields of the fucosylated products were typically 70-85% based on GDP-fucose. Hydrolysis of GDP-fucose was always 10-20%. The complete enzymatic hydrolysis of GDP-fucose in the absence of acceptor indicates that the β-galactoside α(1-2) fucosyltransferase may also act as a phosphohydrolase by transfering fucose from GDP-fucose to water. Recently, this observation was confirmed by T. Beyer and R. Hill (personal communication) who have now succeeded in purifying this enzyme to homogenity.

Purification of the fucosylated products was rapidly achieved by ultrafiltration, passage over a small Dowex 1 (chloride) and Chelex-100 (sodium) column and gel filtration. Yields from the purification procedure were routinely greater than 90%.

III. Carbon-13 NMR of Fucosylated Oligosaccharides

The assignment of resonances in the carbon-13 nmr spectra of Fucα(1-2)Gal-β-hexanolamine, Fucα(1-2)Galβ(1-4)GlcNAc-β-hexanolamine, Fucα(1-2)[1-^{13}C]Galβ(1-4) GlcNAc-β-hexanolamine, Fucα(1-2)[1-^{13}C]Galβ(1-4)GlcNAc and Fucα(1-2)Gal have been accomplished using selective ^{13}C-enrichment and ^{13}C-enriched model compounds (Table II).

Roberts and his co-workers (114) have shown that methylation of a hydroxyl group results in an 8-11 ppm downfield chemical shift in the α carbon atom and β-carbon chemical shifts of less than 1 ppm. Chemical

shift changes in resonances more remote are usually less than 0.3 ppm (114). The substituent effects were interpreted in terms of a steric hindrance or a proximity effect as was observed in inositols (113). Chemical shift differences can arise from neighbor-anisotropy effects, currents induced in regions of a molecule that are relatively remote from the carbon atom of interest, electrogenativity effects, and steric hinderance or proximity effects (12). The structure of several disaccharides and trisaccharides have been determined by ^{13}C-nmr using substituent effects and confirmed by biological degradation experiments (13,87,115,116).

In the synthesis of the disaccharide Galβ(1-4)GlcNAc-β-hexanolamine it was observed that glycosidation of galactose to C4' of GlcNAc resulted in a downfield shift of C4' (8.3 ppm). Substituent effects β to C4' resulted in upfield chemical shifts of 1.6 ppm for C3' and 0.9 ppm for C5' as predicted by Dorman and Roberts (114). Other nuclei were shifted upfield also (C1', 0.3ppm; C2', 0.7 ppm and C6', 0.9 ppm).

Fucosylation of the disaccharide, gave the antigenic trisaccharide Fucα(1-2)Galβ(1-4)GlcNAc-β-hexanolamine. Derivatization of C2' Gal with fucose was unequivocally shown when [1-^{13}C]Galβ(1-4)GlcNAc-β-hexanolamine was used as an acceptor. The large $^{1}J_{C1-C2}$ coupling of 46 Hz permitted easy assignment of this resonance. The C2' Gal was shifted downfield, deshielded, by 5.6 ppm (from 72.3 to 77.9 ppm). Substituent effects to the β-carbons, C1' and C3' of Gal, were also readily observed. The C3' Gal could be unambiguously assigned by a $^{3}J_{C1-C3}$ coupling of 3.5 Hz. The C3' Gal was shifted downfield as a result of derivatization at C2. The C1' Gal was shifted

downfield as a result of derivatization at C2. The C1' Gal was shifted upfield by 2.6 ppm (from 104.3 to 101.7 ppm).

Derivatization of C2' with fucose also resulted in a 0.11 ppm shift in C'' GlcNAc, as shown in Figure 15. This small chemical shift could result from steric effects or a small conformational change in the disaccharide as a result of derivatization with fucose.

Analysis of the reducing trisaccharide, Fucα(1-2)[1-^{13}C]Gal β(1-4)GlcNAc, was complicated by the presence of both α and β anomers of GlcNAc. Complete assignment of the spectrum was again aided by selective ^{13}C-enrichment and comparison to the disaccharide [1-^{13}C] Galβ(1-4)GlcNAc. The C1' Gal was found to differ (102.32 to 102.28 ppm) depending on the anomeric configuration at C1 of GlcNAc, α or β. The proportion of α:β anomers in the reducing trisaccharide was found to be 2:1.

Enzyme mediated synthesis of the antigenic portion of the blood group O substance allowed quantities of natural abundance and ^{13}C-enriched compounds to be obtained for nmr evaluation. Carbon-13 enrichment, in many cases, allowed unambiguous assignment of resonances in these compounds. Correct chemical shift assignments are imparative before the complex carbohydrate can be used for structural analysis or dynamic studies involving molecular interactions. At least one study on the interaction of a lectin with various glycosides has already been reported based on incorrect chemical shift assignments (11).

IV Conformation of the Fucosylated Oligosaccharides

The use of selectively enriched ^{13}C di and trisaccharides allow $^{3}J_{C-C}$ and $^{3}J_{C-H}$ coupling constants about the glycosidic bond to be evaluated. The abundance of glycosidic structures in nature and the

increasing importance of these structures in glycoproteins and glycolipids make a detailed study of O-glycosidic linkages of great importance.

The conformation about a glycosidic bond with an aglycon more complex than a methyl group requires the specification of two torsional linkages, ψ and ϕ, as shown in Figures 17 and 21 for the Fucα(1-2)Gal glycosidic linkage. Several studies have already attempted to define the conformation about the glycosidic linkage using primarily $^{3}J_{C\text{-}O\text{-}C\text{-}H}$ coupling, and $^{3}J_{C\text{-}O\text{-}C\text{-}C}$ coupling (13, 117, 188, 199).

Lemieux and Koto (119) developed the concept of exo-anomeric effect for the particular stability of certain conformations of the ϕ torsion angle in glycosidic linkages. The exo-anomeric effect is proposed to be due to the extra stability provided when the lone pair electrons on the ring oxygen are anti-periplanar to the glycosidic C-O bond (119). Two conformations about the ϕ torsion angle allow for an anti-periplanar orientation of the p-orbital of O1 with the O5-C1 bond. One conformation has the aglycon in a syn-clinal orientation to H1 and O5 and is expected to be more energetically favorable than the orientation in which the aglycon is syn-clinal to O5 and C2. Using a series of compounds ^{13}C-enriched at the aglyconic position and a modified ^{13}C to ^{1}H Karplus relationship a ϕ torsional angle was found in agreement with the exo-anomeric effect (119). X-Ray crystallographic data, where available, confirmed the torsional angles obtained by nmr.

The ψ torsion angle in peracetylated disaccharides labeled with ^{13}C at the C1 position having α(1-3), β(1-3), α(1-4) and β(1-4) glycosidic linkages were evaluated using $^{3}J_{^{13}C\text{-}O\text{-}Cn\text{-}Hn}$

Figure 21. Newman Projections Describing The Two Torsional (dihedral) Angles, Ψ And $\emptyset$, About The Glycosidic Bond In Fucα(1-2)Gal. The Ψ and $\emptyset$ angles describe the position of C1 Fuc with respect to the Gal ring and the position of C2 Gal with respect to the Fuc ring, respectively.

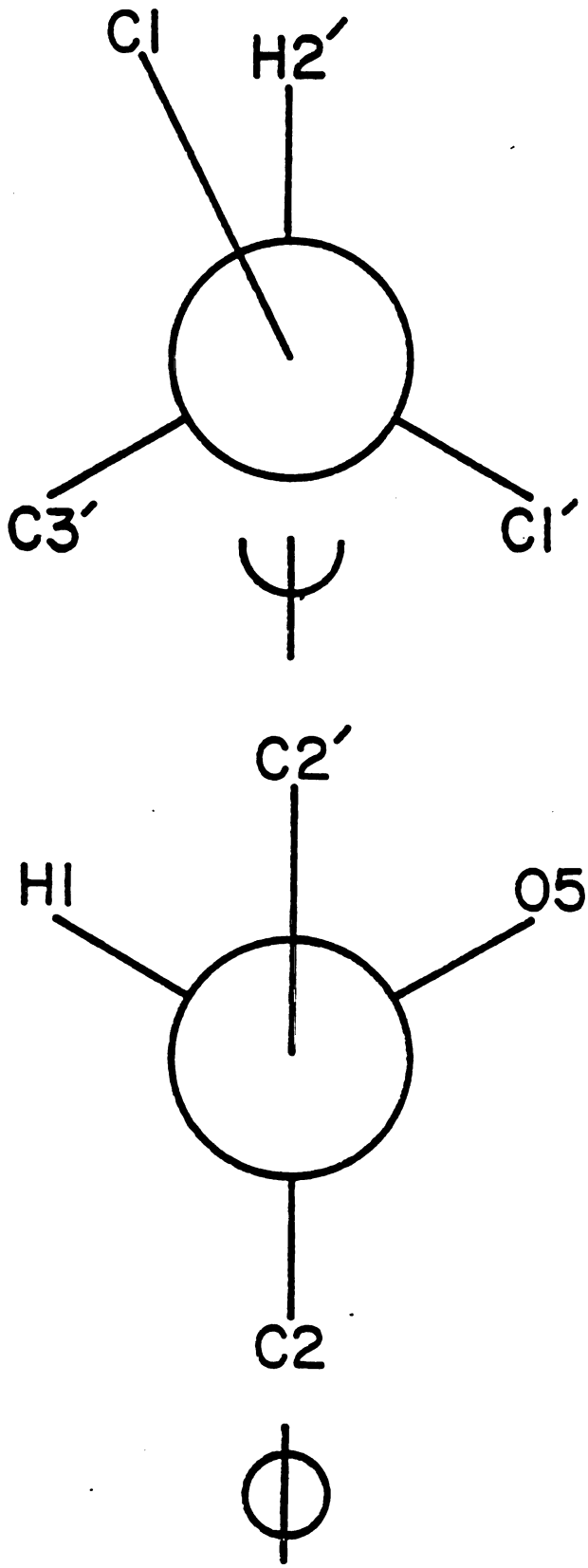


Figure 21

coupling (117). Coupling constants in the range of 3.7 to 5.5 Hz were observed in all cases (117). The 5.5 Hz coupling constant in β-cellulose octaacetate was related to the 15.7° angle determined by X-ray crystallography and the conclusion drawn that the ψ conformation was the same in solution as in the crystalline state (117). The value of the J^3 coupling constant was noted to be remarkabely constant regardless of the (1-3) or (1-4) linkage in these disaccharides (117).

Nunez and Barker (13) were the first to use $^3J_{C-C}$ inter-residue coupling to evaluate the ψ torsion angle in the glycosidic linkage, Gal β(1-4)GlcNAc-β-hexanolamine. Carbon-13 enrichment to the 90% level at C1 allowed evaluation of ^3J coupling from C1 to C3' and C1 to C5'. A coupling constant could not be observed to either C3' or C5' of the GlcNAc residue (13). However, C5' GlcNAc was shown to be broadened by > 1 Hz with respect to other singlets in the spectrum (13). This was interpreted as indicating a $^3J_{C1-C5} \leq 1.5$ Hz and a $^3J_{C1-C3'} = 0$ Hz (13). Since a series of model compounds with defined ψ torsion angles for an observed coupling constant are not available the value of the coupling constant was compared to intra ring $^3J_{C-C}$ coupling constants and dihedral angles in monosaccharides. Walker et al. (68) observed a $^3J_{C1-C6}$ intra ring coupling of 3.7 Hz in hexopyranosides in which C1 and C6 are trans. A $^3J_{C1-C4}$ coupling, corresponding to a dihedral angle in which C1 and C4 are gauche, was not observed in any of the aldopyranoses (68). A $^3J_{C1-C6}$ of 0 to 2.6 Hz (110 to 140°) for pentofuranoses, pentofuranosides and 1,4 aldonolactones has also been observed by Nunez, (personal communication). Using this data to establish limits for

$^{3}J_{C-C}$ coupling in carbohydrates a conformer which favored C1 Gal at an angle slightly greater than 120° with respect to C5' GlcNAc and slightly less than 120° with respect to C3' GlcNAc was postulated (13). This conformation, shown in Figure 22, was also proposed by X-ray crystallography (121) and theoretical calculations (122) for lactose and cellobiose which have the same β(1-4) glycosidic linkage as Galβ(1-4)GlcNAc-β-hexanolamine (13).

To obtain an approximation of the solution value for the Ø torsion angle, Nunez and Barker (13), relied on $^{2}J_{C1-O-C4'}$ coupling constants through the glycosidic linkage. Geminal, $^{2}J_{C-O-C}$, coupling through the ring oxygen was found to be influenced by the configuration of the C, O and H substituents on the carbon involved in the coupling (123). Both a ring oxygen and a ring carbon are considered to contribute equally to the coupling. An oxygen or carbon gauche to the coupled carbon is considered to make a negative contribution to the coupling whereas an oxygen or carbon anti to the coupled carbon makes a positive contribution to the coupling (68). Coupling, $^{2}J\ ^{13}C_{1-C4'}$, from [1-^{13}C] Gal to C4' GlcNAc was not observed in the disaccharide Galβ(1-4)GlcNAc-β-hexanolamine (13). However, a linebroadening of approximately 1 Hz was observed indicating an interaction between ^{13}C1 Gal and C4' GlcNAc (13). Two possible Ø torision angles would result in a small $^{2}J_{C1-C4'}$ coupling constant. The rotamer that was favored had C4' bisecting O5 and H1Gal again in agreement with the exo-anomeric effect (119) and X-ray data on lactose and cellobiose (121).

Fucosylation of [1-^{13}C] Galβ(1-4)GlcNAc-β-hexanolamine allowed the conformations about the angles ψ and Ø in the Galβ(1-4)GlcNAc

Figure 22. The Proposed ψ Torsion Angle For The Glycosidic Linkage In Galβ(1-4)GlcNAc-β-Hexanolamine (13).

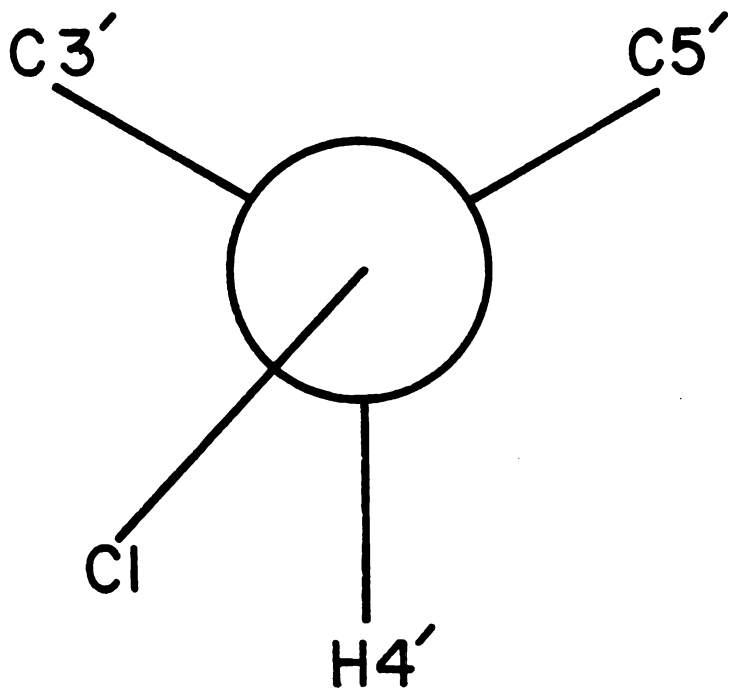


Figure 22

glycosidic linkage and the ψ torsion angle in the Fucα(1-2)Gal glycosidic linkage to be evaluated. The 90 MHz, proton decoupled, ^{13}C-nmr completely resolved all resonances except C5'' GlcNAc and C5' Gal as shown in Figure 16. The C3'' GlcNAc was not found to be broadened by or coupled to the ^{13}C1Gal. Broadening of the C5''GlcNAc could not be determined since it was not completely resolved from C5'Gal. However, the lack of broadening of the C3''GlcNAc is in agreement with the conformation of the ψ torsion angle postulated in Galβ(1-4)GlcNAc-β-hexanolamine since an appreciable change in coupling to C5'' GlcNAc would simultaneous cause an observable change in coupling to C3'' GlcNAc. Thus, it appears likely that fucosylation of the disaccharide does not appreciably affect the ψ torsion angle in the Galβ(1-4)GlcNAc glycosidic linkage. An alternative conformation could exist in which C1'Gal is gauche to both C3'' and C5'' GlcNAc since linebroadening from C1'Gal to C5''GlcNAc can not be resolved. This conformation is expected to be particularly unstable since H1Gal would sterically interact with H3'' and H5'' of GlcNAc. Also, hydrogen bonding between OH3'' of GlcNAc and O5'Gal, as predicted in the disaccharides Galβ(1-4)GlcNAc and lactose, would be loss in this alternative conformation (13,127).

Intramolecular interactions between contiguous residues have been shown to cause wide variations in the ψ angle around the glycosidic linkage (121). Since fucosylation does not appear to affect the torsion angle in the Galβ(1-4)GlcNAc glycosidic linkage, appreciable interaction of the fucopyranosyl ring with the Gal and GlcNAc rings in the trisaccharide may not occur.

The Ø torsion angle in the Galβ(1-4)GlcNAc glycosidic linkage in the trisaccharide was evaluated using $^{2}J\ ^{13}C_{1'-C4''}$ coupling. An angular dependence of ^{13}C substituents on the magnitude of the geminal coupling has been shown (68,76). However, this relationship between the conformation and the magnitude of geminal coupling is not well understood (68). The C4'' GlcNAc in the trisaccharide Fucα(1-2)[1-^{13}C]Galβ(1-4)GlcNAc- β-hexanolamine was observed to be broadened with respect to C2'', C4', C4 or any single resonance in the spectrum, shown in Figure 16. Although the line-broadening (1.0-1.5 Hz) can not be quantitatively interpreted with respect to the Ø torsional angle of the Galβ(1-4)GlcNAc glycosidic linkage, studies with [1-^{13}C] and [2-^{13}C] enriched aldopyranosides indicated that the aglycon carbon equivalent to C4'' GlcNAc is <u>trans</u> to C2. Ethyl-β-D-[2-^{13}C] galactopyranoside was found to have a $^{3}J\ ^{13}C_{2-C1'}$ coupling of 3.5 Hz indicating a <u>trans</u> relationship between C2 and C1'. Two bond, $^{2}J\ ^{13}C_{1-C1'}$, coupling in these simple glycosides resulted in increased linebroadening (1 Hz) of the C1' aglycon. The 1 to 1.5 Hz linebroadening in the C4'' GlcNAc resonance, equivalent to the C1' aglycon in the [1-^{13}C] and [2-^{13}C] enriched aldopyranosides indicated that C4'' GlcNAc is <u>trans</u> to C2' Gal and between a <u>syn</u>-clinal and elipsed orientation to H1' and O5' Gal. Derivatization at C2 of Gal with Fuc may affect the contribution of C2 to the coupling constant since C4' trans to C2 with OH as a substituent may differ signficantly from the same geometry with <u>O</u>-Fuc at C2. However, a rotamer with C4'' GlcNAc <u>syn</u>-clinal to H1' and O5 Gal not only accounts for the observed linebroadening but is in agreement with the exoanomeric effect, model

studies, theoretical calculations and X-ray crystallography (13, 121, 122, 124). This conformation also permits hydrogen bonding between OH3'' GlcNAc and O5' Gal as predicted for lactose by X-ray crystallography and proposed in the disaccharide Galβ(1-4)GlcNAc (13, 127).

Comparison of the intra-residue galactose couplings in the trisaccharide; $^{1}J_{C1-C2}$ = 46 Hz, $^{2}J_{C1-C3}$ = 3.5 Hz and a broadening of C6' as a result of $^{3}J_{C1-C6}$ coupling, with galactose indicate that the galactopyranosyl ring is not distorted in the trisaccharide.

The ψ torsion angle in the Fucα(1-2)Gal glycosidic linkage in the trisaccharide Fucα(1-2)[1-^{13}C]Galβ(1-4)GlcNAc-β-hexanolamine was evaluated by $^{3}J_{C-C}$ coupling. Carbon-carbon coupling from ^{13}Cl Gal to Cl Fuc was used in hope of obtaining a coupling constant which could be related to the ψ dihedral angle. The spectrum of the anomeric region of Fucα(1-2)[1-^{13}C]Galβ(1-4)GlcNAc-β-hexanolamine, shown in Figure 18, reveals no coupling from ^{13}Cl Gal to Cl Fuc although a broadening of the Cl Fuc resoanance of approximately 3 Hz is observed. Due to the length of time required to obtain natural abundance data on 100 μmoles of material it was thought that better resolution could be obtained with doubly ^{13}C-enriched compounds. The sugar nucleotide GDP[1-^{13}C] Fuc was synthesized and the β-galactoside α(1-2) fucosyltransferase used to prepare [1-^{13}C]Fucα(1-2)[1-^{13}C]Galβ (1-4)GlcNAc-β-hexanolamine. Again, no coupling was observable between Cl'Gal and ClFuc. Comparison of linewidths of the ^{13}C-enriched resonances in [1-^{13}C]Fucα(1-2)[1-^{13}C]Galβ(1-4)GlcNAc-β-hexanola-

mine with Fucα(1-2)[1-^{13}C]Galβ(1-4)GlcNAc-β-hexanolamine and [1-^{13}C]Galβ(1-4)GlcNAc-β-hexanolamine unambiguously shows a linebroadening of approximately 3 Hz , shown in Figure 19, in the doubly enriched trisaccharide. A detailed analysis and comparison of these lines indicate that a $^{3}J_{C1-C1'}$ coupling of approximately 1.5 Hz can account for the observed linebroadening. Resolution of the coupling constant could not be obtained between the temperatures of 45 to -40°. Linewidths at -40° in 60% methanol water for the ^{13}C enriched resonances in [1-^{13}C]Fucα(1-2)[1-^{13}C]Galβ(1-4)GlcNAc-β-hexanolamine were 16 Hz with an internal dioxane linewidth of 2 Hz. One possible explanation of the 16 Hz linewidths at -40° would be the existance of two stable populations of the Fucα(1-2)Gal glycosidic linkage. Further experiments are necessary to explain the observed linewidths at -40°. Several other doubly ^{13}C-enriched compounds, [1-^{13}C]Fucα(1-2)[1-^{13}C]Gal-β(1-4)GlcNAc, [1-^{13}C]Fucα(1-2)[1-^{13}C]Galβ(1-4)Glc and [1-^{13}C]Fucα(1-2)[1-^{13}C]Gal-β-ethyl were analyzed and compared against their respective singly ^{13}C-enriched compound. In all cases a $^{3}J_{C1-C1'}$ coupling could not be resolved but an interaction of 1.5 Hz could be estimated from the 3 Hz linebroadening. Using this data and assuming a Karplus type relationship for $^{3}J_{C-C}$ coupling the most stable rotamers about the Fucα(1-2)Gal glycosidic linkage depicting the ψ torsion angle are shown in Figure 23. Rotamers with C1 Fuc eclipsed or near eclipsed to either C1 or C3 Gal are omitted since these are highly unfavorable due to nonbonded interactions. Rotamer B with C1 <u>trans</u> to C1' can also be omitted since an observable

Figure 23. Possible Ψ Torsional Angles About The Fucα(1-2)Gal Glycosidic Linkages.

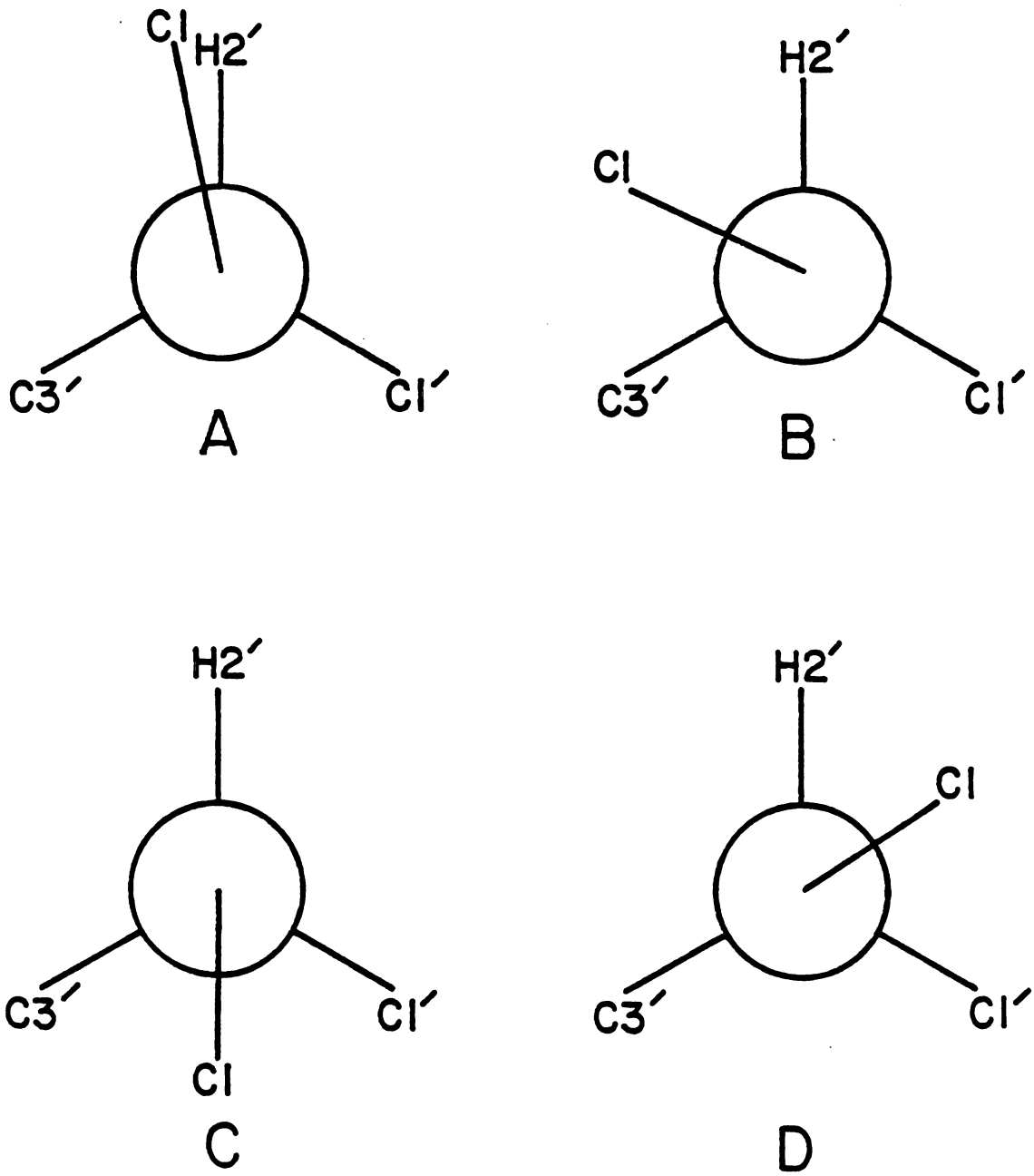
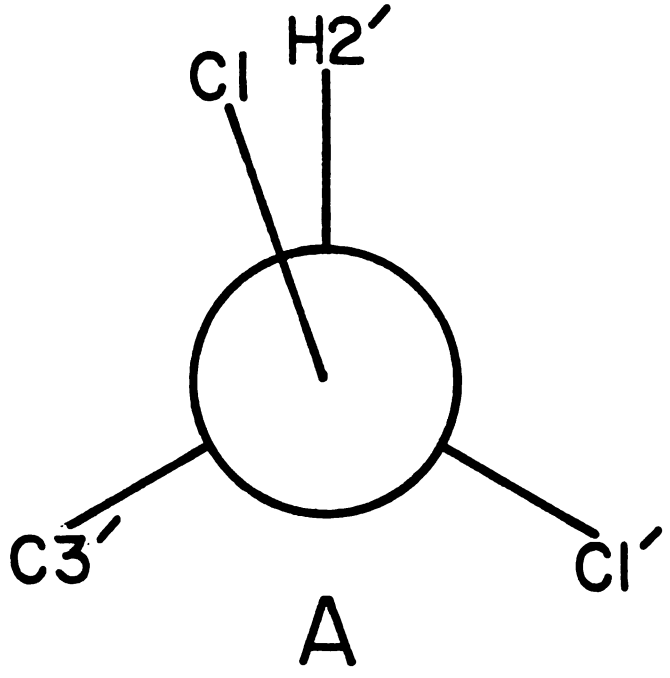


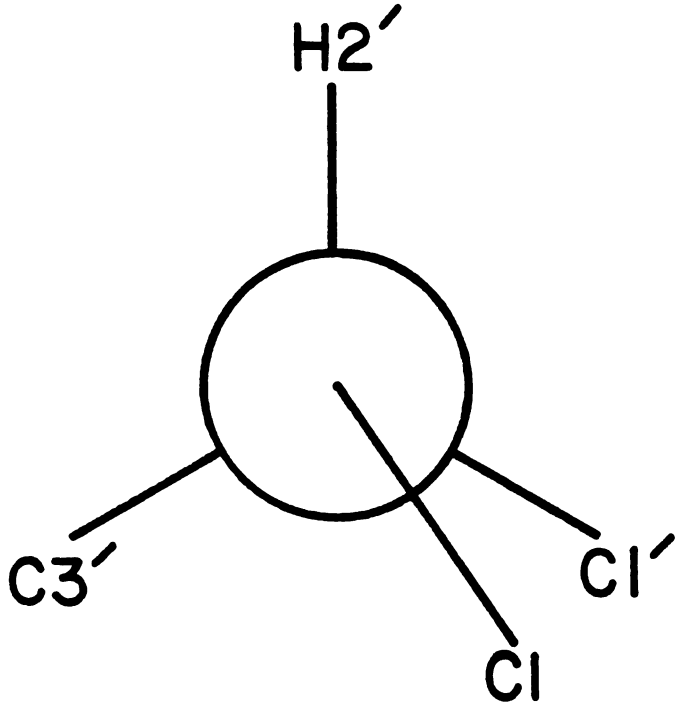
Figure 23

coupling constant of approximately 3.5 Hz would be expected for this arrangement. A trans relationship between C2 and the C1' methylene carbon in the ethyl group of ethyl-β-D-[2-^{13}C] galactopyranoside gives an obervable $^{3}J_{C2-C1'}$ coupling of 3.5 Hz. Similarly, $^{3}J_{C1-C6}$, through the ring oxygen, gives a 3.7 Hz coupling constant for a trans arrangement. Unfortunately, an appropriate model for $^{3}J_{C-C}$ coupling, through a glycosidic linkage, for an angle of 120° is not available. Values for the $^{3}J_{C-C}$ coupling in rotamers C and D would be expected to lie in the range of 0 to > 1.0 Hz for gauche situated carbons since $^{3}J_{C1-C4}$ in aldopyranoses, C1 gauche to C4, is not observable (68). A conformation with C1 Fuc less than 120° to C1 Gal would not be expected to result in a 3 Hz linebroadening since C3'GlcNAc is less than 120° to C1'Gal in the disaccharide Galβ(1-4)GlcNAc β-hexanolamine and no increased linebroadening was observed (13). The observed $^{3}J_{C1-C1'}$ interaction of a 3 Hz linebroadening, approximately a 1.5 Hz coupling, favors a ψ torsion angle with C1 at an angle slightly greater than eclipsed with H2' Gal (Figure 23 Rotomer A). NMR can not distinguish between a rotamer with C1 Fuc at an angle slightly greater than 120° (Figure 24 Rotomer A) from C1' Gal and a conformer with C1 turned through 180° as shown in Figure 24; Rotomer B. Model building studies do not completely resolve this ambiguity. Steric interactions appear greater in rotamer B where the fucopyranosyl ring interferes with the N-acetylglucosamine ring and the C1 Fuc is nearly eclipsed with C1 Gal. Results obtained from model building of the O antigenic trisaccharide and conclusions from theoretical calculations (122) X-ray crystallography (121) and nmr (13,117,118) on the ψ angle in other

Figure 24. Torsional Angles About The Fucα(1-2)Gal Glycosidic Linkage Expected To Give Approximately A 1.5 Hz Vicinal Coupling Between Cl Gal And Cl Fuc.



A



B

Figure 24

disaccharides favor the rotomer (A) in which Cl Fuc is at an angle slightly greater than 120° with respect to Cl Gal.

The Ø torsion angle about the Fucα(1-2)Gal glycosidic linkage was evaluated using the disaccharide Fucα(1-2)Gal-β-ethyl. Ethyl β-D-[2-^{13}C]-galactopyranoside was chemically synthesized and fucose added to it enzymatically with the use of GDP-fucose and the β-galactoside α(1-2) fucosyltransferase. The carbon-13 enriched and natural abundance Fucα(1-2)Gal-β-ethyl were used as a model for the blood group O trisaccharide. Evaluation of the Ø torsion angle could be obtained by $^{3}J\ ^{13}C_{2'}\text{-}H1$ coupling. There is ample experimental and theoretical evidence that a "Karplus-type" of relationship exists between $^{3}J_{C-H}$ coupling and the torsional angle (56). Homer et al. (125) have established a "Karplus-type" of relationship involving inter-residue sp^3 carbons contained in the specific sequence, ^{13}C-O-C-'H. The region of 0 to 60° was difficult to define precisely due to the lack of appropriate model compounds (125). The 180 MHz 'H nmr of Fucα(1-2)Gal-β-ethyl was obtained and the H1 Fuc examined (shown in Figure 20A). The anomeric proton of fucose resonates as a doublet at 5.04 ppm as a result of a 3.4 Hz $^{3}J_{H1-H2}$ coupling. No long range coupling was observed. The H1 Fuc resonance was confirmed by comparison with the 'H nmr of [1-^{13}C]Fucα(1-2)Gal-β-ethyl. The H1 of Fuc in Fucα(1-2)[2-^{13}C]Gal-β-ethyl is a multiplet (shown in Figure 20B) resulting from $^{3}J_{H1-H2}$ and $^{3}J_{H1-^{13}C2'}$ coupling. The multiplet is comprised of a $^{3}J_{H1-H2}$ splitting of 3.4 Hz and a ^{3}J H1-^{13}C2' splitting of approximately

3.2 Hz. The inter-residue coupling of 3.2 Hz corresponds to a torsional angle in which C2'Gal is syn-clinal to O5 and H1. NMR can not distinguish between this conformer and one turned through a 180°. However, a conformer turned through 180°, having C2' syn-clinal to C2 and O5 is sterically unfavorable as discussed previously (124). Interestingly, the former angle corresponds closely to the Ø torsion angle in gentabiose (glucoseβ(1-6)glucose) octaacetate ($^3J\ ^{13}C6\text{-}H1$ of 3.8 Hz) (118). Two bond, $J_{^{13}C1\text{-}C4'}$, in Galβ(1-4)GlcNAc also indicated a Ø torsion angle with C4' near syn-clinal with H1 and O5 as was observed in the crystalline structure of stereo-similar lactose (13). Lemieux and co-workers (129) estimated Ø for α and β alkyl-D-glycopyranosides and found $^3J_{C\text{-}H}$ values of approximately 3.8 and 4.2 Hz for the α and β anomers, respectively. The exo-anomeric effect appears to be the predominant force in stabilizing the conformation about the Ø torsion angle in the absence of extenuating circumstances. Small variations observed in the Ø angle may be the result of steric interactions, intramolecular hydrogen bonding and α verses β glycosidic linkage effects.

Enzyme mediated synthesis of complex oligosaccharides, using partially purified glycosyltransferases and chemical synthesized sugar nucleotides, has been shown to proceed with high yield, anomeric purity and ease of product purification. The galactosyltransferase and the fucosyltransferase have been shown to utilize acceptor substances containing a number of different aglycons. Partial purification of these enzymes using affinity adsorbants allows quantities of enzyme to be easily prepared in less than two weeks.

Sugar nucleotides may be synthesized from specifically ^{13}C-enriched carbohydrates and used with the glycosyltransferases to synthesize specifically ^{13}C-enriched complex oligosaccharides. High resolution ^{13}C-nmr allows product characterization and unambiguous assignment of many resonances with the use of specific ^{13}C-enrichment. Accurate resonance assignment is the first priority before using the synthesized oligosaccharide in biological studies and for future assignment of more complex biological oligosaccharides. Specifically ^{13}C-enriched compounds allow the measurement of inter-residue $^{3}J_{C-C}$, $^{2}J_{C-C}$ and $^{3}J_{C-H}$ coupling constants which can then be used to evaluate the solution conformation of the molecule. The specifically ^{13}C-enriched fucosylated compounds can now be used in biological studies to evaluate glycoside-protein interactions, in particular antigen-antibody interactions. Glycoproteins and glycolipids could also be used as acceptors for the glycosyltransferases allowing specific ^{13}C-enrichment of the carbohydrate moiety in the glycoprotein or glycolipid. Examination of these specifically ^{13}C-enriched glycoproteins and glycolipids may provide much information as to the role of carbohydrates in these molecules.

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APPENDIX

List of Publications

Rosevear, P., VanAken, T., Baxter, J., and Ferguson-Miller, S: Alkyl Glycoside Detergents: A Simpler Synthesis and Their Effects on the Kinetic and Physical Properties of Cytochrome c Oxidase (in press Biochemistry).

Nunez, H.A., O'Connor, J.V., Rosevear, P.R., and Barker, R.: The Chemical Sythesis and Characterization of α- and β-L-Fucopyranosyl Phosphates and GDP-Fucose (submitted to Can. J. Biochem.)

Rosevear, P.R., Murphy, K., and Barker, R.: Evaluation of the Mechanism of the Alkaline Degradation of Glucose Using Isotopically Enriched Substrates:The Retention of Hydrogen from C2 of Glucose (submitted to J. Am. Chem. Soc.)

Rosevear, P.R. and Barker, R.: Synthesis and ^{13}C NMR Analysis of 1-^{13}C Enriched L-Fucose and L-Rhamnose (in preparation for Carbhydr. Res.)