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ELISA Assay, Substrate Specificity and Inhibitors of *N*-Acetylglucosaminyltransferase V

BY

Suzanne Christine Crawley

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

IN

Food Chemistry
Department of Food Science and Nutrition

EDMONTON, ALBERTA
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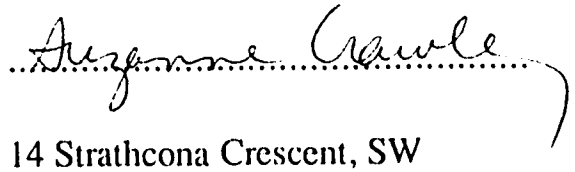

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ELISA Assay, Substrate Specificity and Inhibitors of *N*-Acetylglucosaminyltransferase V

submitted by: **Suzanne Christine Crawley**
in partial fulfillment of the requirements for the
degree of **Doctor of Philosophy**
in **Food Science and Nutrition**

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Date: ..June 13, 1993.....

Abstract

UDP-*N*-acetylglucosamine: α-mannoside β(1-6)*N*-acetylglucosaminyltransferase V (GlcNAcT-V) (EC 2.4.1.155) is a membrane-bound, Golgi enzyme which transfers GlcNAc to the α(1-6)mannose residue of asparagine-linked oligosaccharides. Increased levels of GlcNAcT-V activity and concommitant βGlcNAc(1-6)αMan(1-6) branching of *N*-linked carbohydrates are associated with malignant transformation; thus this enzyme offers an attractive target for development of mechanism-based inhibitors. βGlcNAc(1-2)αMan(1-6)-βGlc-O-octyl was previously determined to be a good acceptor for this enzyme.

An ELISA was developed for GlcNAcT-V. Immobilized [βGlcNAc(1-2)αMan(1-6)βMan-O-$(CH_2)_8$CO-]$_n$-BSA served as the acceptor, and tetrasaccharide product formed by action of GlcNAcT-V was recognized by affinity-refined polyclonal antibodies derived from immunization with synthetic tetrasaccharide-BSA conjugate. Anti-rabbit IgG-alkaline phosphatase conjugates were used as secondary antibodies, and conditions were established for a linear relationship between formation of *p*-nitrophenol and both GlcNAcT-V concentration and time. Radiolabelling showed that 50-300 fmol of product were formed per microtiter well. GlcNAcT-V activity was measured in hamster kidney extract and, for the first time, in human serum.

Analogs of βGlcNAc(1-2)αMan(1-6)βGlc-O-octyl (K_m=26μM) permitted kinetic evaluation of the enzyme's acceptor binding site. H, F, and OMe substitutions of the reactive 6'-OH were all tolerated by GlcNAcT-V, yielding competitive inhibitors with K_i's from 24 to 71 μM. Replacement of the neighboring 4'-OH with an OMe surprisingly abolished all transfer activity, affording instead a good competitive inhibitor (K_i=14 μM). Tri-O-methyl and tri-O-benzyl-βGlc analogs were good substrates, demonstrating that none of these hydroxyl groups formed important hydrogen-bonding interactions with the enzyme active site. Substitutions at the 3-, 4- and 6- positions of the βGlcNAc, however, seriously impaired GlcNAcT-V recognition of the resulting trisaccharides.

Two bisubstrate analog inhibitors differing by a single oxygen atom were kinetically evaluated. The phosphate analog had the lower K_i (48 μM) but behaved more as a UDP analog. The phosphonate analog behaved as a true bisubstrate inhibitor (K_i=73 μM), supporting results from previous kinetics which indicated a random mechanism for this enzyme.

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Thanks to Dr. Monica Palcic, for her commitment to research, which has given me the opportunity to do research in a stimulating and productive environment. I must express deepest appreciation for her patience, and for sharing the joy and power of enzymes and enzyme kinetics.

Thanks also to Professor Ole Hindsgaul, for generously sharing his knowledge, ideas, time and enthusiasm, and whose synthetic carbohydrate program permitted much interesting study of GlcNAcT-V.

Thanks, also, to Dr. Hindsgaul's talented post-doctoral fellows who synthesized---and entrusted me with--- a whole box-full of "toys" for GlcNAcT-V; specifically: Dr. Osamu Kanie, who synthesized GlcNAc-modified trisaccharide analogs and provided many stimulating discussions; Dr. Shaheer Khan, who synthesized mannose-modified analogs; Dr. Ingvar Lindh, who synthesized bisubstrate analogs and other interesting compounds; Dr. Torsten Linker, who synthesized glucose-modified analogs; Drs. Kanwal Kaur and Om Srivastava, whose substrates have contributed in important ways to the current knowledge of GlcNAcT-V.

Personal thanks to Gordon Alton, for instruction, assistance, NMR-spectra, BSA-conjugates, affinity supports, moral support, and many valuable discussions over the years. Thanks also to my talented colleagues who have been my friends and family for the past few years, especially SG, CS, AAC, YK, TLL, CAC, LDH, RO, LK, MA, JS, HG, and IN.

Finally, to my best friend and colleague, Ken Wlasichuk:

Thank-you, for your support.

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List of Abbreviations

AU	absorbance units
Asn	asparagine
β(1→6) branch	βGlcNAc(1→6)αMan(1→6)βMan(1→) branch of *N*-linked oligosaccharides
BHK	baby hamster kidney
BSA	bovine serum albumin
CHO	Chinese hamster ovary
Dol	dolichol
dpm	disintegrations per minute
E. C.	Enzyme Commission
EDTA	ethylenediaminetetraacetic acid
Fuc	L-fucose (6-deoxy-L-galactopyranose)
Gal	D-galactose
GalNAc	2-acetamido-2-deoxy-D-galactopyranoside or *N*-acetylgalactosamine
GDP	guanosine 5'-diphosphate
GlcNAc	2-acetamido-2-deoxy-D-glucopyranoside or *N*-acetylglucosamine
k_{cat}	catalytic rate constant
K_m	Michaelis constant
$K_{m,app}$	apparent Michaelis constant
L-PHA	leukoagglutinating phytohaemagglutinin
LAMP	lysosome-associated membrane glycoprotein
MES	2-[*N*-morpholino]ethanesulfonic acid
mU	milliunits of enzyme activity, nmol/min
NMR	nuclear magnetic resonance
nU	nanounits of enzyme activity, pmol/min
PBS	phosphate buffered saline
ppm	parts per million
PY-BHK	polyoma virus transformed BHK
RS-BHK	Rous sarcoma virus transformed BHK
Tris	tris(hydroxymethyl)aminomethane or 2-amino-2(hydroxymethyl)-1,3-propanediol
UDP	uridine-5'-diphosphate
UDP-GlcNAc	uridine-5'-diphospho-*N*-acetylglucosamine
υ	reaction velocity
V_{max}	maximum velocity of enzyme-catalyzed reaction

Chapter 1
Introduction

Significance and Roles of Asparagine-linked Oligosaccharides

A wide variety of complex carbohydrates are found in nature; these different classes of molecules occupy different niches within cells and tissues, serving different structural, recognition and regulatory functions. Some representative examples of complex carbohydrates are shown in Figure 1.1, and include glycosphingolipids (A) (1), *N*-linked (B) (2-7) and *O*-linked glycoproteins (C) (8), proteoglycans (D) (9), and lipopolysaccharides (E) (10). The carbohydrate moieties of glycoconjugates have been implicated in key biological roles, often serving as ligands in molecular recognition phenomena or serving as modulators of the function of the glycoconjugates to which they are attached (2-6, 11, 12). A broad spectrum of carbohydrate structures serve as ligands in cell-cell and protein-receptor interactions (13-15); some specific, diverse examples are illustrated in Figure 1-2: Selectins are expressed on the surfaces of leukocytes and endothelial cells and involved in such processes as lymphocyte homing and neutrophil adhesion to the vascular endothelium; they are thought to recognize the sialyl Lewis^x structure (**A**) (13, 15); some uropathogenic *E. coli* adhere to host cells *via* internal or terminal Galα1$\rightarrow$4Gal glycolipid sequences (**B**) (16; reviewed in Reference 17); and influenza virus hemagglutinin attaches to host cells via sialic acid receptors (C) (18, 19). *N*-linked glycoprotein hormones are recognized and cleared from circulation by hepatic receptors specific for the 4-SO_4^--βGalNAc(1$\rightarrow$4)βGlcNAc(1$\rightarrow$2)αMan(1$\rightarrow$)R terminal sequence (**D**) (20; reviewed in 21); cholera toxin binds to host cells via G_{M1} glycolipid receptors (**E**) (22); polysialic acid structures have been implicated in regulation of neural cell adhesion (**F**) (23). Protein glycosylation has been found to play an important role in glycoprotein function, affecting such properties as stability, folding and association of subunits, targeting to organs and organelles, immunogenicity and clearance (3, 4, 6, 11). Not surprisingly, then, changes in sugar structures have also been found to correlate with diverse physiological changes and diseases (11, 12), with some of the most consistent observations of altered glycosylation accompanying both normal and abnormal cellular differentiation (24-26).

Evidence that cell surface carbohydrates play an important biological role has accumulated during the last several decades,

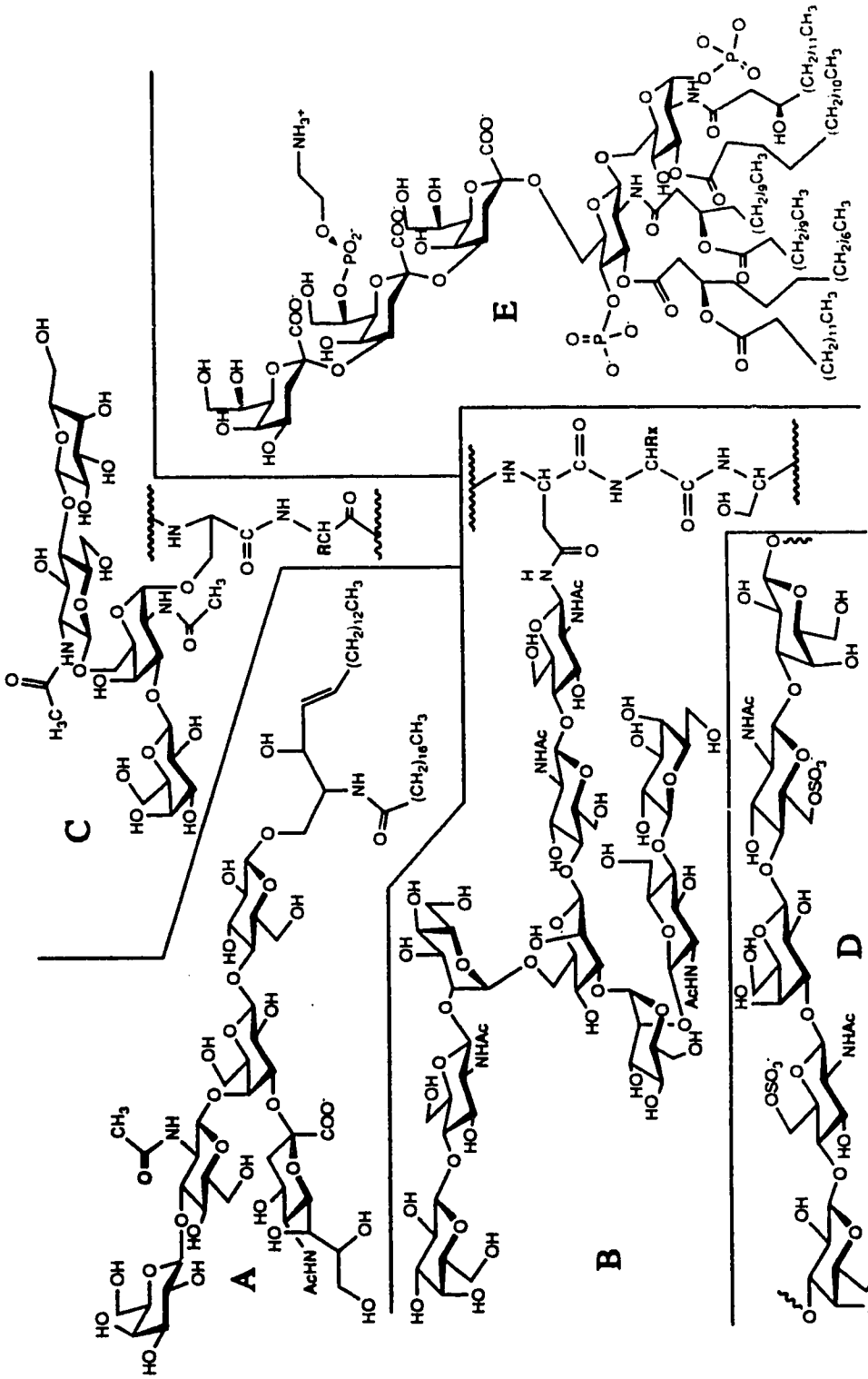


Figure 1.1: Examples of glycoconjugates: (A) glycosphingolipid (1); (B) *N*-linked glycoprotein (2-7); (C) *O*-linked glycoprotein (8); (D) proteoglycan (9); and (E) lipopolysaccharides (10).

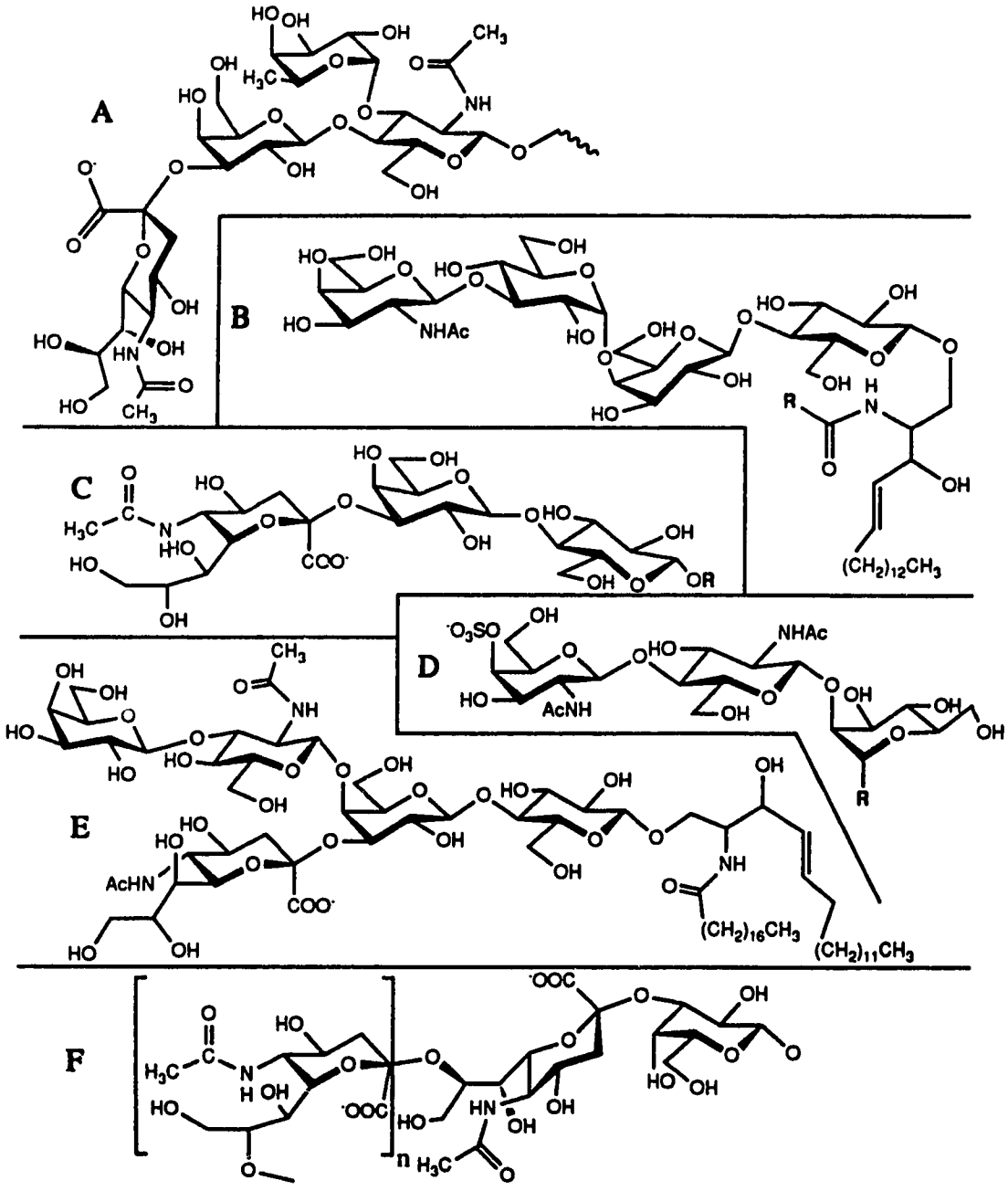


Figure 1.2: Oligosaccharides involved in recognition phenomena: (**A**) Sialyl Le^x (13-15); (**B**) αGal(1→4)βGal; (**C**) sialyllactose (18,19); (**D**) 4-SO_4^--βGalNAc (1→4)βGlcNAc(1→2)αMan(1→)R terminal sequence of glycoprotein hormones (20); (**E**) G_{M1} glycolipid (22); (**F**) polysialic acid (23). See text for details of carbohydrate functions.

but although chromatographic, analytical and synthetic methods for studying complex carbohydrates have expanded enormously; and although many tools in the form of cell lines, enzymes, lectins, synthetic carbohydrates and cDNA clones of glycoproteins and glycosyltransferases have recently become available, very few recurring motifs in carbohydrate structure and function have emerged (11, 12). Detailed cause-and-effect relationships remain to be established, and our limited understanding of these phenomena has yet to manifest itself as a really practical application. To achieve this, we look to gain a better understanding, and perhaps some control, over the factors -- particularly glycosyltransferases -- which ultimately govern glycosylation. Specifically, development of inhibitors which can perturb the actions of glycosyltransferases within the cell should provide tools for investigating those cellular phenomena which are accompanied by changes in carbohydrates. These same inhibitors may also provide a basic design for drugs capable of affecting those phenomema which constitute disease.

Early Steps of Asparagine-linked Oligosaccharide Biosynthesis

A defined repertoire of glycosyl-transferring and hydrolyzing enzymes controls the synthesis and re-structuring of the carbohydrate moieties of asparagine-linked oligosaccharides by the step-wise addition and removal of individual monosaccharide residues. A general scheme of the machinery involved in this biosynthesis is illustrated in Figures 1.3 and 1.4 (27, 7). An oligosaccharide comprised of three linear glucose units and nine nonlinear mannose residues attached via a chitobiose disaccharide to dolicholpyrophosphate (i.e. Glc_3-Man_9-$(GlcNAc)_2$-*P*-*P*-Dol) is synthesized in a stepwise fashion as a heptasaccharide in the cytosol. This lipid-linked oligosaccharide is translocated into the endoplasmic reticulum (ER) where conversion to the 14-mer is completed employing hexose-*P*-*P*-dolichol activated sugar donors (Figure 1.3). Dolichyldiphosphoryl oligosaccharide: polypeptide oligosaccharyltransferase transfers this large sugar to the asparagine residue within the consensus sequence Asn-X-Ser/Thr (X is any amino acid except proline) of a nascent polypeptide as it is synthesized on a ribosome of the ER. During or after synthesis of the polypeptide, glucosidases I and II remove the three terminal glucose residues and an $\alpha(1\rightarrow2)$mannosidase hydrolyzes one or more of the $\alpha Man(1\rightarrow2)$ linkages (7).

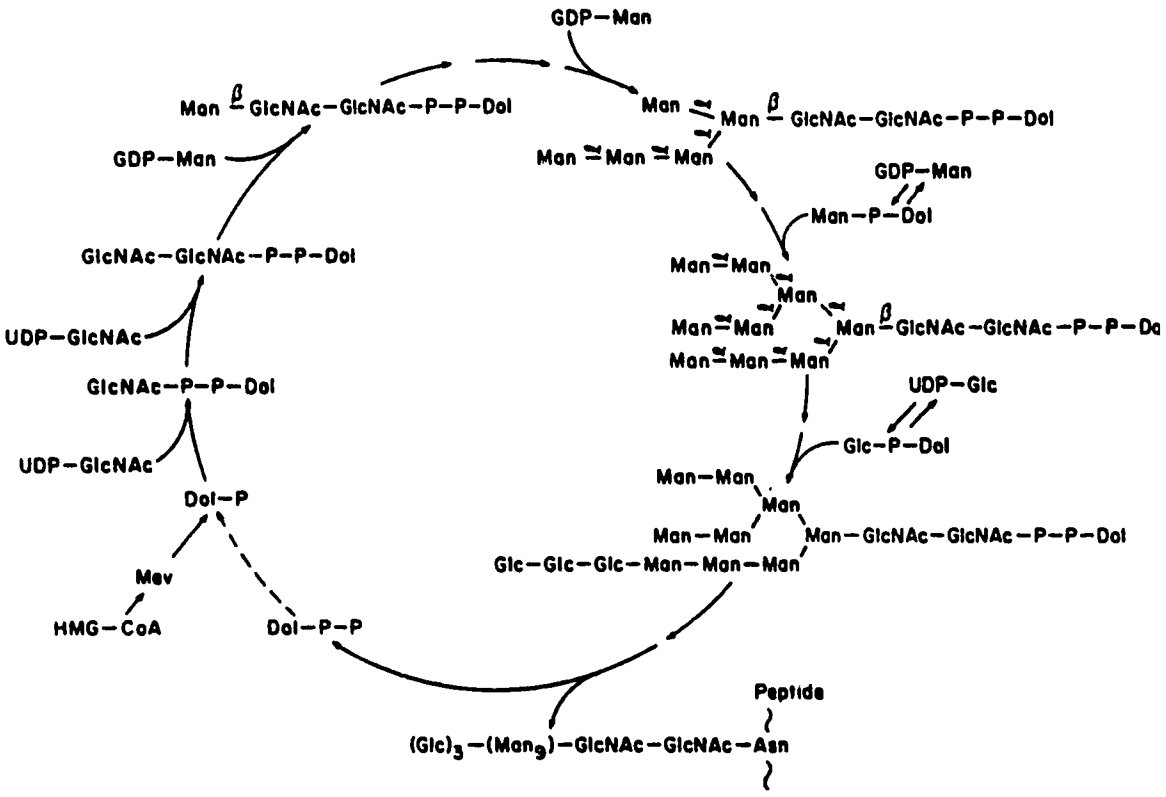


Figure 1.3: Dolichol pathway for synthesis of $Glc_3Man_9(GlcNAc)_2$-PP-dolichol in the cytosol and endoplasmic reticulum.

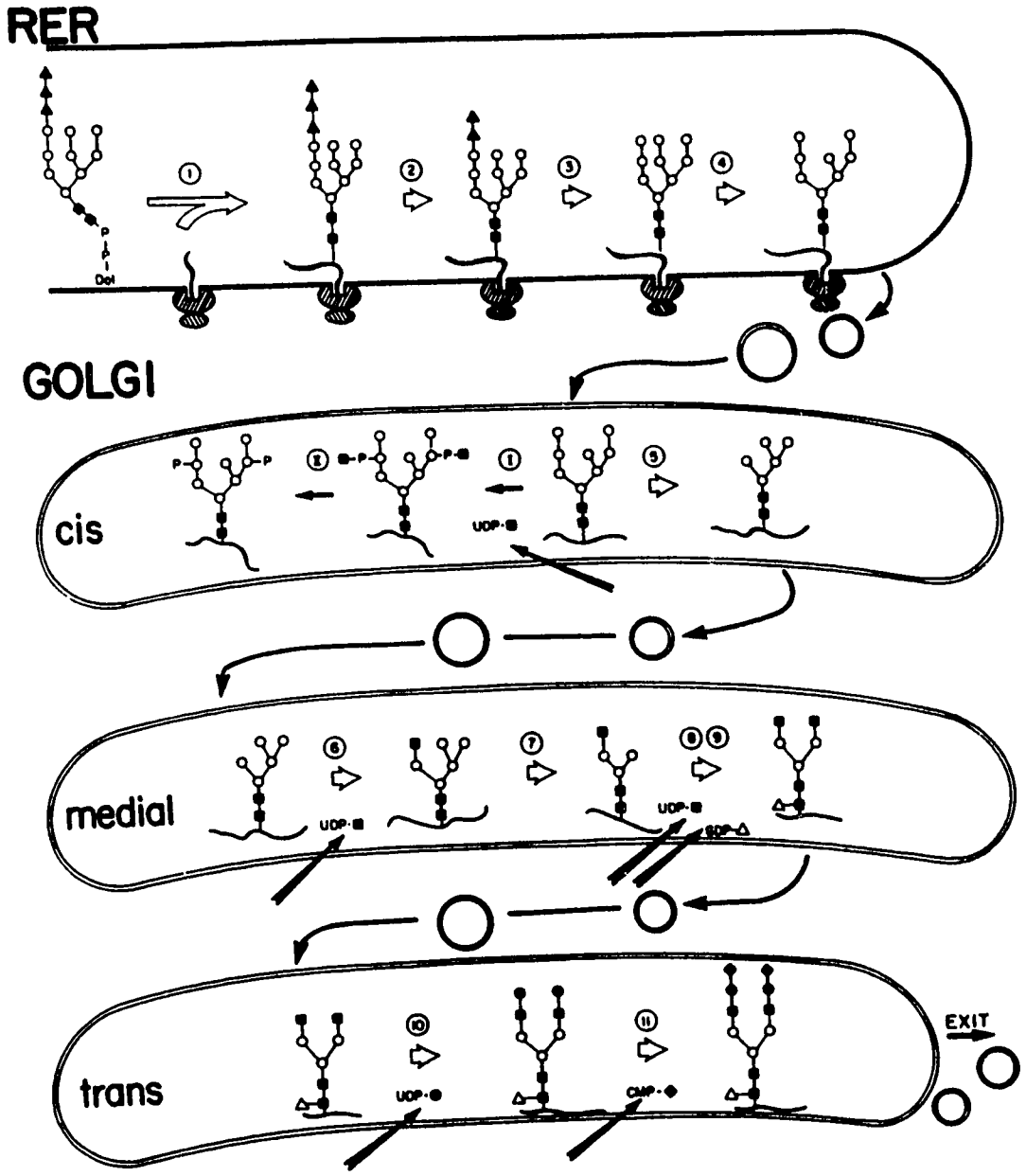


Figure 1.4: Pathway of oligosaccharide processing of *N*-linked glycoproteins. Reactions are catalyzed by: 1) oligosaccharyltransferase; 2) α-glucosidase I; 3) α-glucosidase II; 4) ER α(1→2)mannosidase; I) GlcNAc-1-phosphate transferase; II) GlcNAc-1-phosphodiester-α-*N*-acetylglucosaminidase; 5) Golgi α-mannosidase I; 6) GlcNAcT-I; 7) Golgi α-mannosidase II; 8) GlcNAcT-II; 9) fucosyltransferase; 10) galactosyltransferase; 11) sialyltransferase. Symbols used are: ■-GlcNAc; o-mannose; ▲-glucose; Δ-fucose; ●-galactose; ♦-sialic acid. From Kornfeld and Kornfeld (1985) *Annu Rev Biochem* **54**: 631.

After the polypeptide is synthesized it is translocated with the cell's normal vesicular traffic to the *cis*-Golgi, where α-mannosidase I removes the remaining α(1→2)mannose residues, giving rise to an *N*-linked oligosaccharide substrate for *N*-acetylglucosaminyltransferase I (GlcNAcT-I), which is thought to act in the next (medial) Golgi compartment (7). This GlcNAc transfer from UDP-GlcNAc to the αMan(1→3) of the core trimannoside, forming a β1→2 linkage, represents the first committed step in the conversion of the high mannose structure(s) to hybrid and then to complex type structures (Figures1.4 and 1.5) (28, 29), just as transfer of a phosphorylated version of *N*-acetylglucosamine to the penultimate mannose residues in the *cis*-Golgi is the first step in the conversion of the glycoprotein to the mannose-6-phosphate class which is targeted specifically to lysosomes. Subsequent removal of two terminal mannose residues and GlcNAcT-II-catalyzed addition of a GlcNAc residue to the αMan(1→6) both occur in the medial Golgi, and these reactions may be followed by GlcNAc transferase III-, IV-, V- and VI-mediated transfers to the three mannose residues, giving rise to different types and degrees of branching. These reactions are illustrated using biochemical abbreviations in Figure 1.5 (3, 28, 29).

The transfer of GlcNAc in a β1→4 linkage to the internal mannose reside is catalyzed by GlcNAcT-III, giving rise to a "bisected"-type structure. This enzyme can transfer to the GlcNAcT-I product before the removal of the two non-reducing terminal mannose residues, preventing subsequent mannosidase reactions and giving rise to hybrid-type structures comprised largely of mannose sugar residues. Alternatively, GlcNAcT-III can act after the GlcNAcT-II-catalyzed transfer of GlcNAc to the αMan(1→6) branch, giving rise to a bisected-type structure which is poorly recognized by other branching and elongating glycosyltransferases acting later in the biosynthesis (3, 29-31). This is one of several examples in which final carbohydrate structures are the outcome of competition among different glycosyltransferases with different activities, occurring in different compartments of the cell, and with very different--broad and narrow--substrate specficities.

Branching of *N*-linked Oligosaccharides is Determined by the Action of GlcNAc Transferases

The biantennary structure formed by GlcNAc transferase II is a substrate for GlcNAc transferases III, IV, and V, whereas GlcNAc transferase VI (to date only demonstrated in avian tissues) requires the

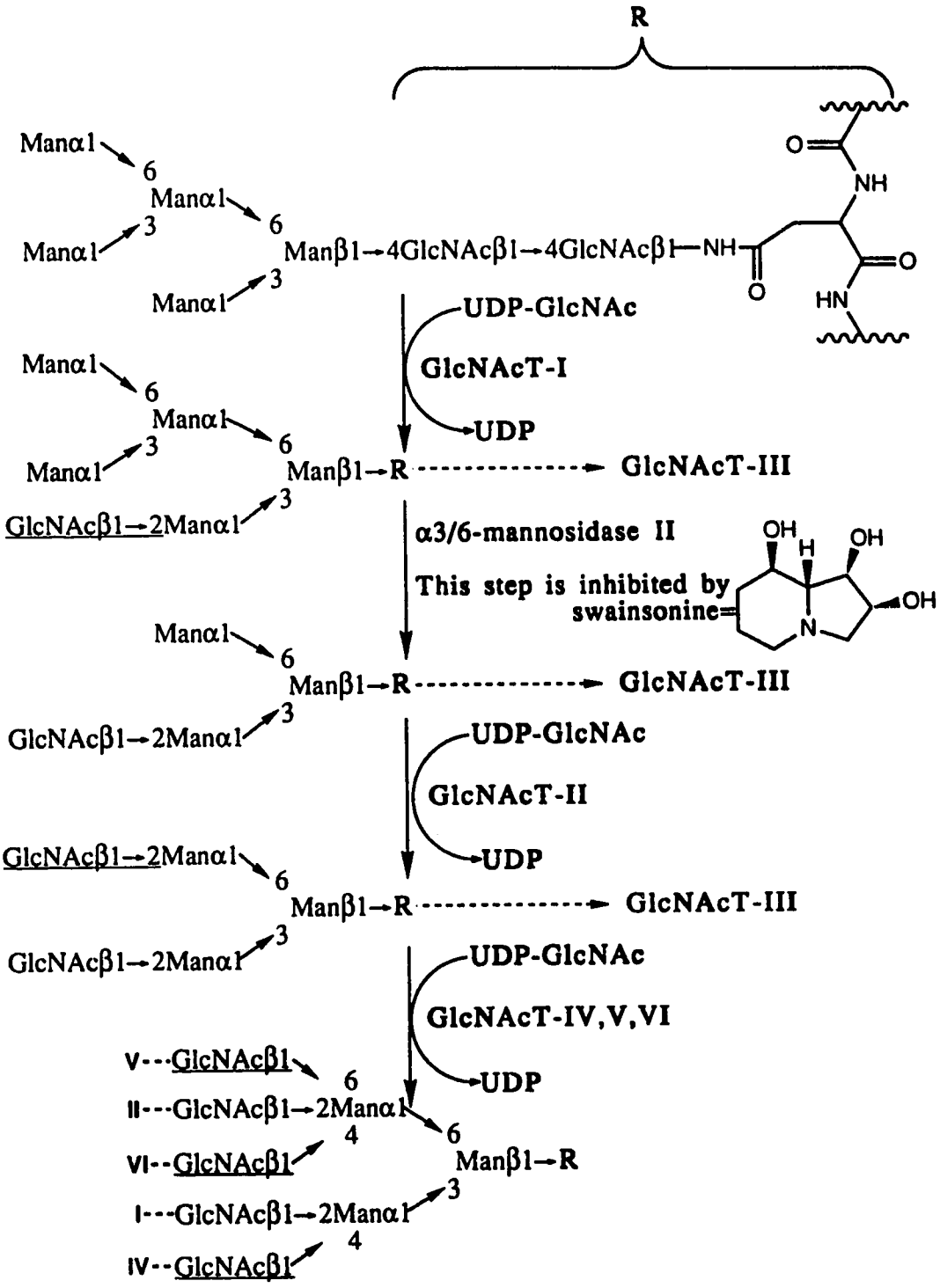


Figure 1.5: Early processing steps of *N*-linked oligosaccharides in the *cis*-Golgi (Adapted from A. Kobata (1992) *Eur J Biochem* 209: 483)

prior action of GlcNAc transferase V (29,30,32). Linkages formed by these enzymes are represented by the carbohydrate structure at the bottom of Figure 1.5. Not all of the prerequisite enzymes are present in all tissues, and a set of rules has been derived from high performance liquid chromatographic studies of the substrate specificities of hen oviduct enzymes involved in this *N*-linked oligosaccharide branching (29-31). Whereas the action of GlcNAcT-III in transferring a GlcNAc residue in a β(1→4) linkage to the βMan(1→4) has been found to prevent subsequent transfers by GlcNAcT's IV and V, it does not prevent transfer by GlcNAcT-VI. Addition of a GlcNAc β(1→4) to αMan(1→6) prevents GlcNAc transfer by GlcNAcT-V. The effects of the different substrate specificities of these enzymes on the course of *N*-linked oligosaccharide branching is illustrated in Figure 1.6 (adapted from Reference 32).

The rigid specificities and consequent ordered action of these GlcNAc transferases, presumably in the medial Golgi, has suggested that increased levels or the absence of a particular enzyme will have a significant effect on final GlcNAc branching of *N*-linked oligosaccharides. Therefore, the number, position, type and density of branching of oligosaccharides is largely controlled at this stage of biosynthesis, and it can be seen that an alteration in the level of a GlcNAc transferase enzyme activity may have an impact on the final *N*-linked structures formed. All of the non-reducing terminal GlcNAc residues are substrates for β(1→3) and β(1→4) galactosyltransferases of the trans Golgi, which act to form βGal(1→3)βGlcNAc(1→) and βGal(1→4)βGlcNAc(1→) (Type I and Type II) terminal disaccharides. These disaccharide sequences are, in turn, acceptors for an array of "elongating" and "terminating" glycosyltransferases, including other GlcNAc transferases, fucosyltransferases, sialyltransferases, galactosyltransferases, and *N*-acetylgalactosaminyltransferases, which also act in an ordered way dictated by their respective substrate specificities (Figure 1.7) (33). Some of the elongating and terminating glycosyltransferases exhibit a degree of preference for particular branches of *N*-linked oligosaccharides, as well (34). Thus, competing action of these glycosyltransferases gives rise to mature, complex-type oligosaccharides, which may be further modified by addition of sulfate, *O*-acetyl, phosphate and other functional groups (7).

The occurrence of very different types of *N*- and *O*-linked sugars at different sites on a given glycoprotein, as well as the occurrence at these particular sites of a characteristic distribution of related but different structures ("microheterogeneity"), is evidence for and a consequence of the dynamic interplay of glycosylating enzymes and

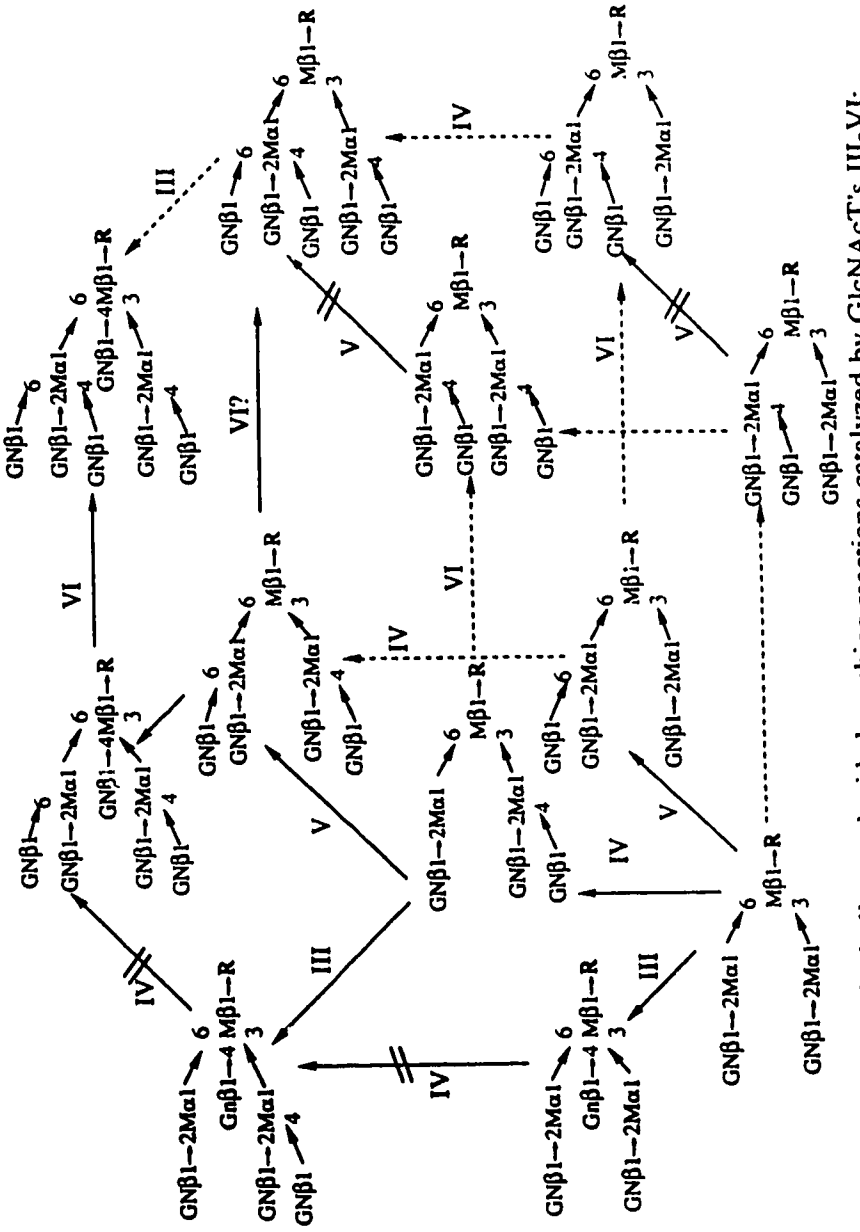


Figure 1.6: *N*-linked oligosaccharide branching reactions catalyzed by GlcNAcT's III-VI; dotted lines indicate probable reactions which have not been demonstrated conclusively *in vitro*. GN=GlcNAc; M=Man; R=4GlcNAc(1→4)βGlcNAc(1→Asn). (adapted from Brockhausen, I. *et al* (1989) *J Biol Chem* **264**: 11211)

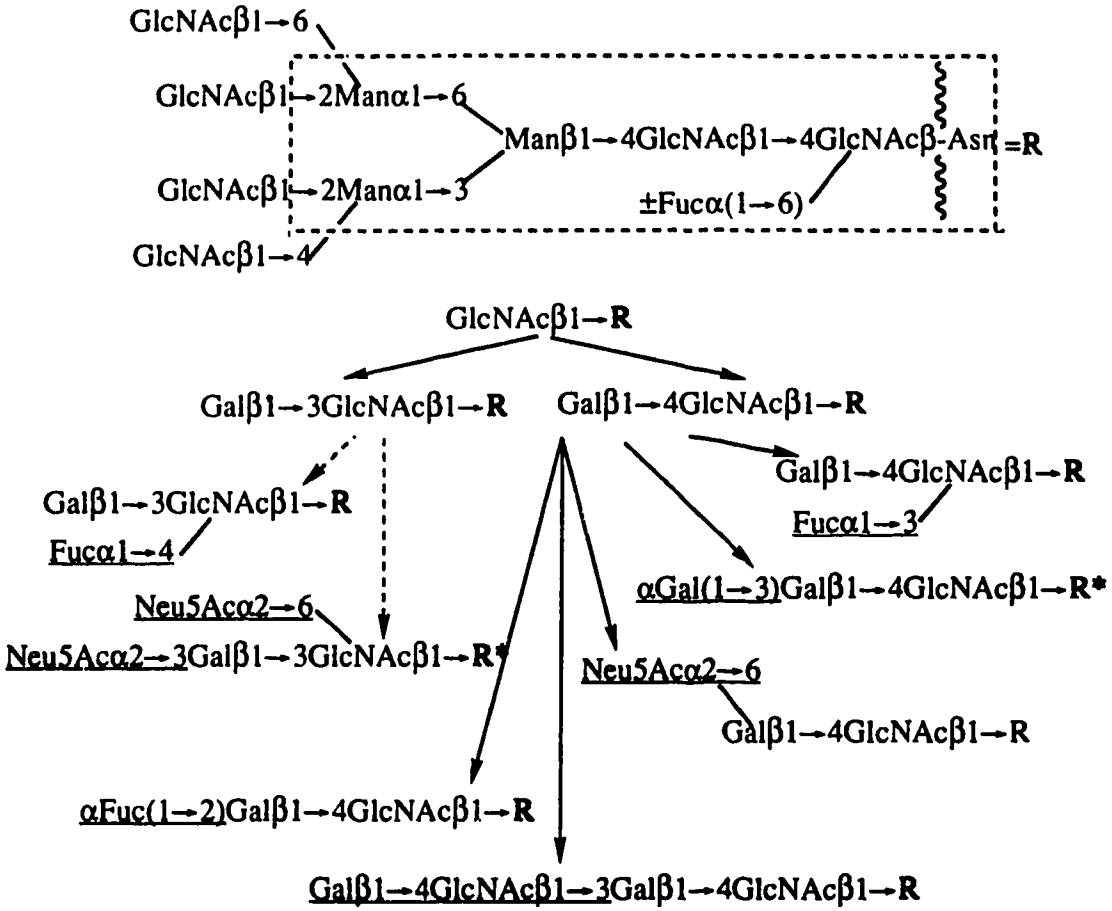


Figure 1.7: Glycosyltransferase-catalyzed "maturation" reactions forming some common and uncommon (*) outer chains of *N*-linked carbohydrates (Adapted from Kornfeld and Kornfeld (1985) *Annu Rev Biochem* **54**: 634).

protein structure i.e. polypeptide conformation and folding, *N*-glycosylation site accessibility, and amino acid sequence (3, 6, 7). Because of the ordered mode of action of the enzymes involved in this synthetic process, dictated by their compartmentalization but also by their respective substrate specificities, different levels or the lack of a particular enzyme activity can have dramatic effects on the types and abundance of different structures that can be formed by a particular cell. This gives rise to cell-line specific, tissue- and organ- and species-specific glycosylation of glycoproteins. This phenomemon is particularly well-demonstrated in the differential "maturation" of *N*-linked oligosaccharides of the same glycoproteins expressed in different tissues, species and cell lines; γ-glutamyltranspeptidases, interferon-β1, erythropoetin, granulocyte/macrophage colony stimulating factor and chorionic gonadotropin are a few of the particularly well-studied examples of this phenomenon (3, 4, 11). This kind of diversity is a serious consideration in the production of recombinant glycoprotein therapeutics in cases where the asparagine-linked oligosaccharides contributes significantly to *in vivo* and *in vitro* activity of the protein, as well as to factors such as biological half-life and immunogenicity (4).

Some other factors which are thought to affect the final carbohydrate structures of glycoproteins include the spatial -- and therefore temporal -- compartmentalization of specific classes of transferases relative to their respective glycoprotein acceptor substrates, transit time, availability of cofactors (eg Mn^{2+}) and availability of co-substrates, including activated donors of monosaccharide residues, sulfates, methyl and acetyl groups *et cetera*.

The synthesis of *O*-linked glycoproteins is not as well-understood, but is known to be initiated post-translationally by transfer of GalNAc from UDP-GalNAc to a serine or threonine hydroxyl group of a polypeptide chain, forming an α linkage. Elongation and branching of *O*-linked oligosaccharides is carried out by glycosyltransferases with substrate specificities quite distinct from those involved in *N*-linked sugar biosynthesis, but terminal glycosylation steps are likely carried out by many of the same enzymes (8).

Some of the properties of GlcNAc transferases I-VI are described in Table 1.1 (35-40). GlcNAc transferases I, II, III and V have been purified to near-homogeneity using detergent extraction from microsomes or acetone powder, followed by multiple conventional chromatographic steps, and affinity chromatography, in which sugar-nucleotide donor analogs and acceptor analogs are attached to a solid support (35-37). Typically, the enzyme levels are low, with GlcNAcT-V occurring at a level of 3 milliunits per gram of rat kidney

homogenate protein and GlcNAcT-I present as 300 milliunits per gram of rat liver homogenate protein. Overall yields obtained from enzyme purifications are typically low also (less than 3% for all GlcNAcT's which have been purified to date, except GlcNAcT-V which required only a two-column isolation procedure (37)). GlcNAcT-I, -III and -V have been cloned (36, 38-40) and analysis of their amino acid sequences have shown them to be type II transmembrane proteins, as are all glycosyltransferases cloned to date, with short cytoplasmic amino terminal sequences followed by a hydrophobic signal/anchor transmembrane sequence, a proteolytically-sensitive "neck" region and then a carboxy-terminal catalytic domain (41). No sequence homology with other glycosyltransferases was reported but greater than 95% homology was found between rat and mouse GlcNAcT-V at the nucleotide level (40), and 92% homology was found between human and rabbit GlcNAcT-I (39). This type of relationship between the same enzymes from different sources has been observed for other glycosyltransferases (41, 42). Another manganese-independent βGlcNAc(1→6) transferase, the core 2 enzyme, has been recently cloned and found to be a 50,000 Dalton type II transmembrane protein with two potential *N*-glycosylation sites and no sequence homology with other glycosyltransferases cloned to date (43). The cloning of these and other glycosyltransferases represents one important strategy for investigating and eventually influencing glycosylation phenomena (44).

Elucidation of the specific interactions which dictate the substrate specificities of these and other glycosyltransferases also offers a strategy for perturbing complex carbohydrate synthesis, *via* the development of specific enzyme inhibitors. Oligosaccharide-binding sites can be investigated using synthetic oligosaccharides to test roles of individual hydroxyl groups in the enzyme-carbohydrate interactions (44). This approach is particularly applicable to glycosyltransferases when synthetic acceptor analogues are more readily available in significant amounts than are the enzymes. This approach has been employed by a number of the groups engaged in studying these enzymes, and results of this kind of study on the substrate specificty of hamster kidney GlcNAcT-V are presented in Chapters 3 and 4.

Levels of GlcNAcT-V and βGlcNAc(1→6)αMan(1→6) Branching are Associated with Transformation and Metastasis

Since the early 1970's qualitative changes in cell-surface carbohydrates in differentiation and tumor progression have been observed and documented (27, 45). In some cases observed changes

GlcNAcT (source)	Characteristics of purified or cloned enzyme	$[Mn^{2+}]$(mM) and pH optima	Selected substrates and their K_m values (mM)
I (rat liver)	45,000 (54,000 and 50,000 minor bands)[a]; 52,000[b]	20-100 mM pH 5.6[a]	Manα(1,6)[Manα(1,3)]Manα(1,6)[Manα(1,3)] Manβ(1,4)βGlcNAc(1,4)βGlcNAc=0.25[a] UDP-GlcNAc=0.078[a]
II (rat liver)	48,000 (43,000)[a]	10-15 mM pH6.0-6.5[a]	βGlcNAc(1,2)αMan(1,6)[βGlcNAc(1,2)αMan (1,3)]βMan(1,4)βGlcNAc(1,4)βGlcNAc=0.19[a] UDP-GlcNAc=0.96[a]
III (rat kidney)	62,000(52,000) 3 putative *N*-glycosylation sites[c]	12 mM pH 6-7[a]	βGlcNAc(1,2)αMan(1,6)[βGlcNAc(1,2)αMan (1,3)]βMan(1,4)βGlcNAc(1,4)βGlcNAc=0.23[a] UDP-GlcNAc=1.1 [a]
IV (hen oviduct)	not purified	12 mM pH 7.0[a]	βGlcNAc(1,2)αMan(1,6)[βGlcNAc(1,2)αMan (1,3)]βMan(1,4)βGlcNAc(1,4)βGlcNAc[a]
V (rat kidney)	69,000; 75,000[d] 95,000[e]; 6 putative *N*-glycosylation sites[e]	no Mn2+ requirement pH6.6[d]	βGlcNAc(1,2)αMan(1,6)βMan-O$(CH_2)_8COOCH_3$=0.09 UDP-GlcNAc=11[d]
VI (hen oviduct)	not purified[a]	75-100 mM pH7.0-8.4[a]	βGlcNAc(1,2)[βGlcNAc(1,6)]αMan(1,6)βMan-O$(CH_2)_8COOCH_3$[a]

Table 1.1: Properties of branching *N*-acetylglucosaminyltransferases.

a. Schachter, H, *et al* (1989) *Methods in Enz* 179:351.
b. Kumar, R, *et al* (1990) *Proc Natl Acad Sci USA* 87:9948.
c. Nishikawa, A, *et al* (1992) *J Biol Chem* 267:18199.
d. Shoreibah, MG, *et al* (1992) *J Biol Chem* 267:2920.
e. Shoreibah, M, *et al* (1993) *J Biol Chem* (in press).

have involved an increase in the average size of *N*-linked oligosaccharides, which has been attributed to increased sialylation (46, 47) and increased GlcNAc branching (48, 49). In particular, βGlcNAc(1→6)αMan(1→6) branching of the oligosaccharides isolated from membrane glycoproteins of baby hamster kidney (BHK) cells was increased when cells were transformed either by polyoma virus (50) or by Rous sarcoma virus (51). These studies both noted an increase in poly-*N*-acetyllactosamine sequences. Similarly, membrane glycoproteins isolated from NIH 3T3 cells transformed with DNA from human carcinoma cell lines containing T24H-*ras* oncogenes showed enhanced binding affinity for leukoagglutinating phytohemagglutinin (L-PHA); this lectin specifically recognizes the βGlcNAc(1→6)αMan(1→6) branch of *N*-linked oligosaccharides(52). The biosynthesis of this βGlcNAc(1→6)αMan(1→6) branch was shown earlier to be catalyzed by manganese-independent GlcNAcT-V, assayed using radiolabelled glycopeptides and leukophytohemagglutinin lectin chromatography (53). Yamashita *et al* established that a doubling of the activity of GlcNAcT-V (measured using oligosaccharides isolated from urine of patients with exoglucosidase deficiencies) coincided with the increased βGlcNAc(1→6) branching upon polyoma transformation of baby hamster kidney cells, while other GlcNAc transferase activities remained unchanged (54). The same result was obtained by Arango and Pierce, who assayed the enzyme in Rous sarcoma-transformed BHK cells using a synthetic trisaccharide acceptor (55). In this latter study, it was also suggested that GlcNAcT-V activities from transformed and untransformed BHK cells were essentially the same enzyme, as defined by their kinetic parameters and pH optima. The reaction catalyzed by GlcNAcT-V *in vivo* is shown in Figure 1.8.

A direct association between increased βGlcNAc(1→6)αMan(1→6)-branching of *N*-linked glycoproteins and metastatic phenotype was demonstrated by Dennis *et al*, who found that L-PHA lectin selection of a highly metastatic murine lymphoma cell line gave rise to two mutants, both of which were non-metastatic and also showed 80% reduction in GlcNAcT-V activity (56). Glycosylation of one plasma membrane protein, gp130,was particularly affected as indicated by intense ^{125}I-L-PHA binding to this protein band in SDS gels, and enhanced L-PHA binding to gp130 was directly correlated with metastatic propensity of tumor cells. It was suggested that increased βGlcNAc(1→6) branching conferred a metastatic advantage on particular tumor cells when they were injected subcutaneously, as evidenced by the 100% occurrence of metastases in mice injected with

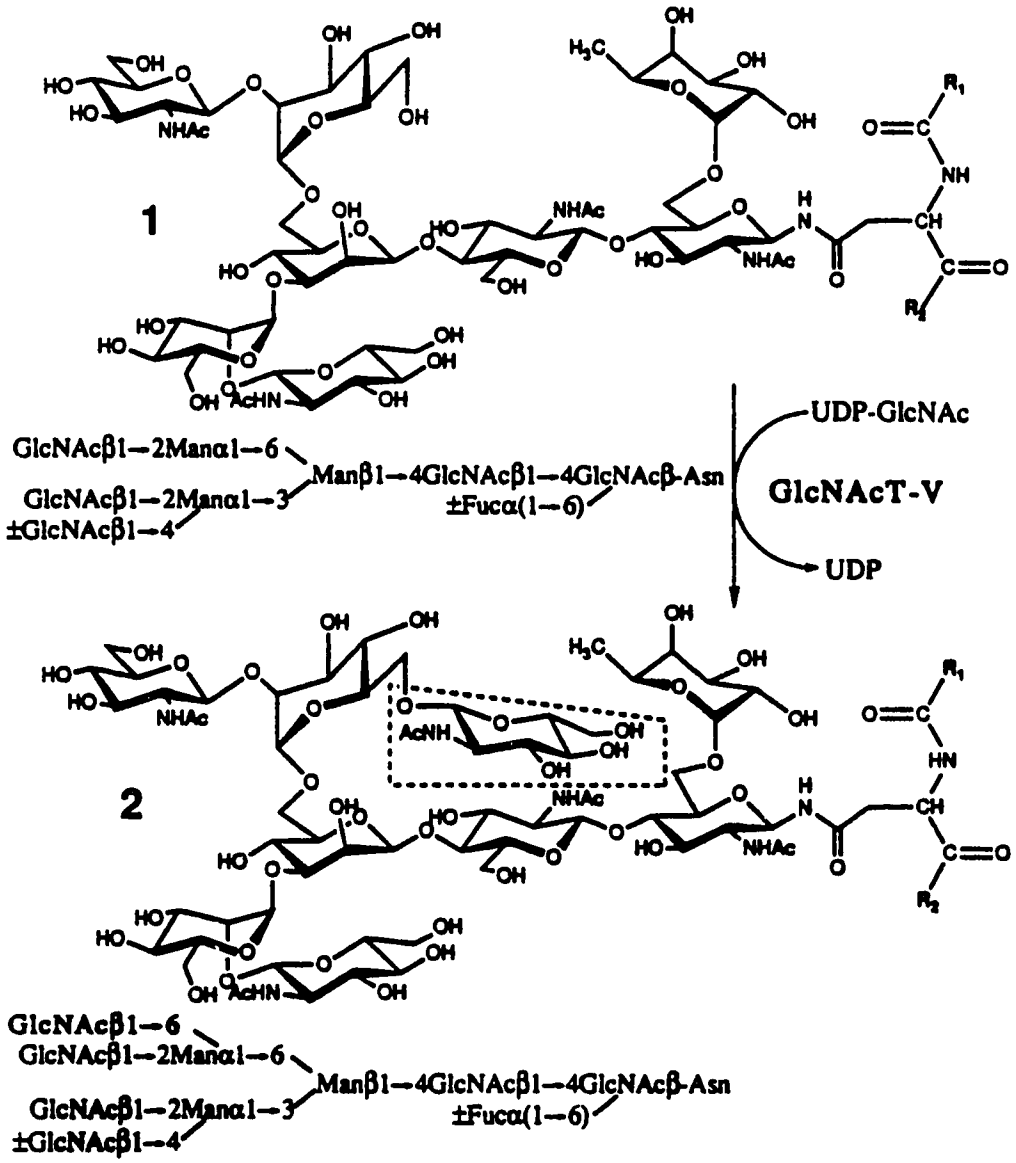


Figure 1.8: Reaction catalyzed by GlcNAcT-V *in vivo*, with GlcNAc transfer to acceptor **1** to generate product **2**.

pooled pSV_2 *neo*-transfected SP1 cells. In later work, gp130 was identified as LAMP-1 (lysosome associated membrane glycoprotein), which had an unusual localization to the cell surface rather than to the lysosomes, and it was suggested that the heavy glycosylation might provide accessible carbohydrate ligands for interaction with receptors in other tissues (ie. providing a mechanism for arrest of tumour cells) and/or might sterically impair normal cell-matrix and cell-cell interactions, permitting intravasation and migration (57). Because of other, less pronounced, changes in GlcNAcT-I and IV activities, the authors suggested that expression of a number of glycosyltransferases might be under the control of a single regulatory element that was affected by the mutation(s) responsible for the metastatic phenotype (56).

Further investigation into a detailed mechanism(s) of GlcNAcT-V-mediated effects on tumor cell growth metastasis have involved comparison of adhesion assay and invasiveness assay results obtained for metastatic/L-PHA-reactive cells lines (SP1*neo5* and SP1T24*ras*1) versus non-metastatic/non-reactive SP1 cells. The former cell lines were significantly less adhesive but 3-4 times more invasive in assays using amnionic basement membranes. This behaviour suggested that the type of glycosylation particular to these cells may have somehow reduced adhesion to extracellular matrix proteins at the primary tumour site and also enhanced invasion (extravasation) at a new site (58). MDAY-D2 tumor cells with glycosylation machinery impaired by mutations or swainsonine treatment showed slower growth in the presence of sub-optimal concentrations of growth factors, and this slowed growth was correlated with growth rates of tumour cells injected subcutaneously (59). (Swainsonine is an α3/6-mannosidase II inhibitor which essentially prevents the conversion of hybrid-type *N*-linked oligosaccharides to complex-type oligosaccharides, by preventing conversion of GlcNAcT-I product to GlcNAcT-II substrate, as indicated in Fig 1.5) The parent cell line showed comparatively relaxed growth requirements, suggesting that these cells produced their own growth factors or that the function of a receptor and/or transporter was affected by the altered glycosylation. Transcription of tissue inhibitor of metalloproteinases (TIMP) is enhanced in tumor cells where *N*-linked glycosylation is inhibited either by swainsonine or somatic mutation, suggesting that specific types of glycosylation might actually affect expression of specific genes (60). Also, histological L-PHA staining for βGlcNAc(1→6) branched carbohydrate structures in human esophageal carcinomas has shown that these sugars can predominate on cells that

are located at the outer surfaces of the tumor masses, adjacent to the surrounding tissue (61). Whereas these authors suggested that such a distribution of the β(1→6)GlcNAc structures was indicative of a role for these carbohydrates in the process of invasion, such inhomogeneous expression of these specific carbohydrates also suggests that the synthesis of these structures may be stimulated by external factors. Possibly related to these two sets of observations, a recent study has found that GlcNAcT-V activity is 3.2-fold higher in subconfluent versus confluent HepG2 cell cultures; the authors suggested that enzyme levels and concommitant expression of βGlcNAc(1→6)αMan(1→6) branching of (transferrin) oligosaccharides may be under control of regulators which modulate the liver-specific phenotype of the cells (62).

Interestingly, it has been found in one system that the alterations in carbohydrate structures of glycoproteins arising after activation of the *ras* proto-oncogene in NIH 3T3 cells occurred at almost the same time as expression of the $p21^{ras}$ (at approximately 20 hours after induction) whereas other phenotypic changes (eg invasiveness) occurred only after 2 days (63). The authors suggest that this temporal relationship is evidence that the $p21^{ras}$-mediated malignant phenotype may in part be derived from its effect on *N*-linked glycosylation (63).

Tumorigenic potential of adenovirus transformants of rat 3Y1 cells was found to correlate with βGlcNAc(1→6) branching of asparagine-linked sugars, such that transformation with either one (E1) or with two different (E1 plus E4) regions of the adenovirus genome gave rise to poorly or highly tumorigenic cells, respectively, which also expressed 60% and 150% higher molar ratios of βGlcNAc(1→6) branched *N*-linked sugar structures (determined by hydrazinolysis, methylation analysis and lectin chromatography) (64). The E4 genes (for the T antigen) as well as the E1 genes, known to be involved in initiation of transformation and expression/maintenance of the transformed phenotype, were required for full tumorgenicity, and both were required to give the maximum increase in βGlcNAc(1→6) branching which was observed.

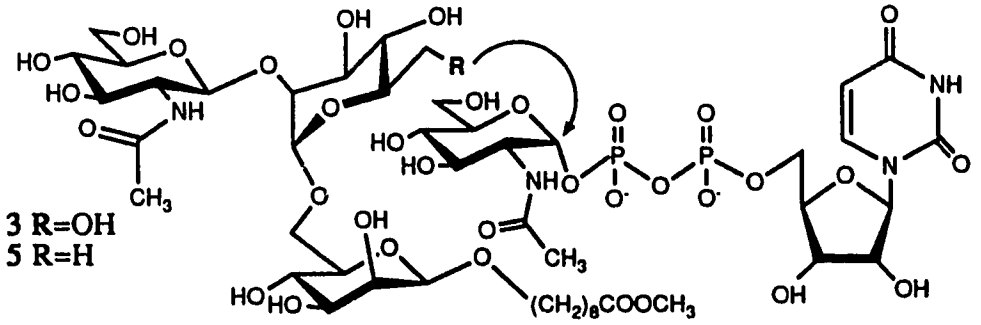
Levels of GlcNAcT-V have also been shown to be substantially elevated in malignant versus benign human breast cancer biopsies (65); and several oncogenes (v-*fps* and H-*ras*) which induce a metastatic phenotype when transfected into rat2 fibroblasts, also produced an increase in β(1→6)branching (66). Related work by this and other groups has been reviewed (67).

The earlier observation that increased βGlcNAc(1→6) branching coincided with increased poly-*N*-acetyllactosamine structures was expanded by the work of van den Eijnden *et al* in a very thorough study

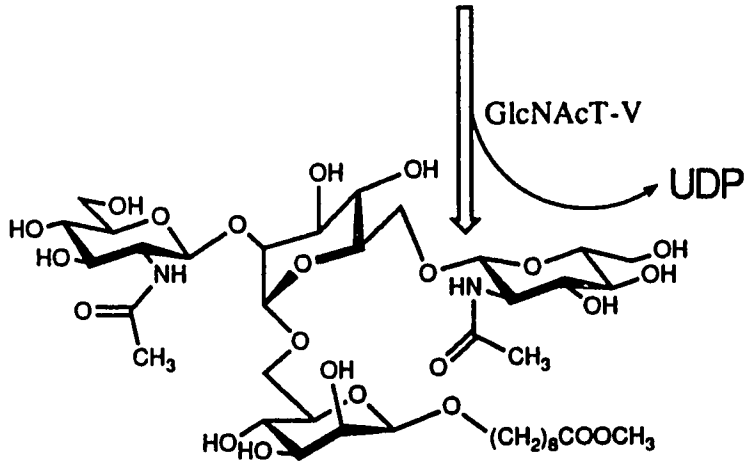
of the *N*-acetyllactosaminide-GlcNAcβ(1→3) ("i") transferase from Novikoff tumor cell ascites fluid. This glycosyltransferase was found to display greatest catalytic efficiency in transferring to *N*-linked oligosaccharide acceptors with the βGal(1→4)βGlcNAc(1→6) αMan(1→6) branch (34), and this lead the authors to suggest that, because of the branch preferrence displayed by this enzyme, the formation of GlcNAcT-V product must favor initiation of polylactosaminoglycan chains and disfavor transfer by enzymes with opposite branch specificities. It was also suggested that this might be an important mechanism whereby alteration in the activity of a single glycosyltransferase could "yield a totally different spectrum of cell surface oligosaccharide structures" (34).

A specific synthetic trisaccharide acceptor substrate for GlcNAcT-V, comprised of a truncated version of the natural *N*-linked oligosaccharide, was developed. Figure 1.9 shows synthetic acceptor **3** and product **4** (68). Synthetic substrate **3** and a series of analogs of this acceptor permitted testing of the earlier proposal by Arango and Pierce (55) that GlcNAcT-V enzymes from transformed and untransformed BHK cells were kinetically identical (69). Enzymes from BHK cells, Rous sarcoma-transformed BHK cells, and an L-PHA lectin-resistant cell line LP3.3 (selected for reduced cell-surface expression of the GlcNAcT-V product) were kinetically evaluated using sugar-nucleotide donor, synthetic trisaccharide acceptor (**3**), and modified versions of this acceptor. As well, an acceptor analog which was deoxygenated at the reactive 6-hydroxyl position of the αMan(1→6) to give a non-substrate trisaccharide **5** (Figure 1.9), was tested and found to be a competitive inhibitor. K_m and K_i values were the same for all substrates and for inhibitor, respectively, with enzymes from all three sources; on the other hand, V_{max} was approximately doubled for virally transformed cells, and dramatically reduced for LP3.3. This work lent further support to the idea that apparent increases in enzyme activity upon viral transformation are attributable to increases in levels of expression of GlcNAcT-V, rather than to some phenomenon which enhances the enzyme's catalytic efficiency.

Work involving three murine tumor cells lines and their respective malignant or metastatic counterparts showed 70 and 320% increase in core 2 GlcNAc transferase and GlcNAcT-V enzyme levels, respectively, for T24H-*ras*-mediated transformation of rat2 cells, and 70 and 1050% increases upon T24H-*ras* or 12-*O*-tetradecanoylphorbol-13-acetate-mediated transformation of nonmalignant SP1 tumor cells into metastatic cell lines (70). Corresponding levels of *N*- and *O*-linked



3 GlcNAcβ(1→2)Manα(1→6)Manβ-$(CH_2)_8COOCH_3$ UDPGlcNAc



4 GlcNAcβ(1→2)[GlcNAcβ(1→6)]Manα(1→6)Manβ-$(CH_2)_8COOCH_3$

Figure 1.9: Reaction catalyzed by GlcNAcT-V with synthetic trisaccharide acceptor **3**, to generate product **4**. Deoxygenated trisaccharide **5** is a competitive inhibitor.

poly-*N*-acetyllactosaminoglycans in cell membranes were evaluated and found to be increased in these two cases. Levels of these structures were reduced only on the non-metastatic KBL-1 revertants which had also exhibited reduced GlcNAcT-V levels, suggesting that the level of GlcNAcT-V as well as βGlcNAc(1→3) transferase level controls synthesis of *N*-linked poly-*N*-acetyllactosaminoglycan structures. A model was proposed in which β(1→6)GlcNAc branching of *N*-linked and *O*-linked glycoproteins is controlled by levels of their respective synthetic enzymes; and this branching, in turn, controls levels of poly-*N*-acetyllactosamine chains if the "i" GlcNAcβ(1→3) transferase is not limiting. The poly-*N*-acetyllactosaminoglycan chains formed are substrates for the subsequent action of fucosyl- and sialyltransferases. Additionally, retinoic-acid induced differentiation of mouse F9-teratocarcinoma cells, from embryonic to endoderm-like cells, is accompanied by particularly dramatic increases in GlcNAcT-V activity, giving rise to a corresponding increase in expression of L-PHA-reactive oligosaccharides and poly-*N*-acetyllactosamine structures on membrane glycoproteins (including LAMP-1) (71). Activity of another βGlcNAc(1→6) branching enzyme, core 2 GlcNAc transferase (6), was also elevated under these circumstances, supporting the idea that this enzyme may be regulated in a fashion similar to GlcNAcT-V and may have a similar, important role in cell growth and social behavior.

Easton *et al* have recently synthesized their results into a similarly unifying "enzymatic amplification" theory, which also implicated the β(1→3)GlcNAc "i" transferase in a key role (72). Induction of the N-*ras* proto-oncogene in a stable NIH 3T3 cell line (t15) gave rise to an invasive phenotype accompanied by changes in cell-surface glycoproteins. These changes were accompanied by increased GlcNAc transferase III, V, "i", increased β(1→4) galactosyltransferase and sialyltransferase activities, slightly decreased α(1→3)galactosyltransferase activity, and unaltered GlcNAcT-I, -II and -IV activities. The authors suggested that these specific changes in apparent enzyme levels were responsible for an altered course of glycosylation, giving rise to cell surface carbohydrates with increased βGlcNAc(1→6)αMan(1→6) branching, elongated poly-*N*-acetyllactosamine structures and an increased tendency for terminal sialylation (72). Their observation of altered activity in a number of glycosyltransferases again suggests that the genes coding for these enzymes are coordinately regulated. The same group has also compared activities of GlcNAc transferases I, II, III, IV and V in single metastasizing and non-metastasizing variants of rat prostate tumor cell lines (R3327-MatLyLu and -H); they found that the former had higher

GlcNAcT-V and -III activities, giving ratios of 2.6:1 and 6.5:1 for the two enzymes, respectively (73).

Saitoh *et al*, who examined glycosylation of LAMP 1 and 2 glycoproteins of highly metastatic versus poorly metastatic cell lines, found no difference in GlcNAcT-V or βGlcNAc(1→3) transferase activities; but they did observe slightly higher poly-*N*-acetyllactosamines (74). It was therefore suggested that increased poly-*N*-acetyllactosamine formation accompanied by increased sialyltransferase and/or decreased fucosyltransferase activities gives rise to more terminal sialyl Lewis^x structures which could interact with selectins of endothelial cells and platelets. This type of theory provides one functional connection (*via* poly-*N*-acetyllactosamine structures) between observation of increased βGlcNAc(1→6) branching and increased tumorigenicity or metastatic capacity of tumor cell lines (74).

In consideration of the profound effect that this βGlcNAc(1→6)αMan(1→6) branching seems to have on the phenotype of the tumor cell lines discussed above, and in light of the recent discoveries of carbohydrates as ligands of cell adhesion molecules (13-15), it will be surprising if other correlations between activities of glycosyltransferases, such as GlcNAcT-V, and cell growth and differentiation are **not** found. Because of GlcNAcT-V's potentially key role in cell-surface glycosylation and in tumour cell metastasis, this enzyme offers a very attractive target for development of mechanism-based inhibitors. Observations that changing cell surface glycosylation reduces the metastatic potential of tumor cell lines (56, 67) lends credence to the idea that tampering with the glycosylation machinery of tumor cells may be a reasonable approach to altering their malignant phenotype, and work by Dennis *et al*, using swainsonine to inhibit growth of human melanoma tumor xenografts in mice, has demonstrated the feasibility of developing inhibitors of glycosyation for therapeutic as well as investigative applications (25, 67, and references therein).

An ELISA-based assay has been developed for this enzyme and is described in Chapter 2 (75). Immobilized [βGlcNAc(1→2)αMan(1→6) βMan-O-$(CH_2)_8$CO-]$_n$-BSA served as acceptor, and tetrasaccharide product formed by action of GlcNAcT-V was recognized by affinity-refined polyclonal antibodies derived from immunization with synthetic tetrasaccharide-BSA conjugate. Anti-rabbit IgG-alkaline phosphatase conjugates were used as secondary antibodies, and conditions were established for a linear relationship between formation of *p*-nitrophenol and both GlcNAcT-V concentration and time. Radiolabelling showed

that 50-300 fmol of product were formed per microtiter well. GlcNAcT-V activity was measured in hamster kidney extract and, for the first time, in human serum. This is a non-radiochemical method that will permit multiple enzyme assays with product identification as well as sensitive detection. Although originally conceived as a diagnostic tool for assay of GlcNAcT-V in human serum (No disease state has been correlated with altered serum enzyme levels.), this method also lends itself well to assays of column fractions and cell lines and also can be utilized for screening of natural products for GlcNAcT-V inhibiting compounds. The assay is 50 times more sensitive than the radiochemical assay and is the only assay available which is sensitive enough to detect and therefore study this enzyme in human serum.

A series of substrate analogs of GlcNAcT-V acceptor **3** has been synthesized by Drs. O. Kanie, I. Lindh, K. Kaur, O. Srivastava, S. Khan and T. Linker in the group of Professor O. Hindsgaul, Department of Chemistry, University of Alberta. Kinetic evaluation of these analogs as acceptors and inhibitors of GlcNAcT-V (Chapter 3) permitted characterization of the enzyme's active site in terms of the key interactions formed with acceptor trisaccharides. H, F, and OMe substitutions of the reactive 6'-OH were all tolerated by GlcNAcT-V, yielding competitive inhibitors with K_i's from 24 to 71 μM. Replacement of the neighboring 4'-OH with an OMe surprisingly abolished all transfer activity, affording instead a good competitive inhibitor (K_i=14 μM). Tri-O-methyl and tri-O-benzyl-βGlc analogs were good substrates for the enzyme, demonstrating that none of these hydroxyl groups forms important hydrogen-bonding interactions with the enzyme active site. On the other hand, substitutions at the 3-, 4- and 6- positions of the βGlcNAc seriously impaired GlcNAcT-V recognition of the resulting trisaccharides.

Two bisubstrate analogs inhibitors, a phosphonate analog and a phosphate analog differing by a single oxygen atom (synthesized by Dr. Ingvar Lindh); these have been kinetically evaluated (Chapter 4). The phosphate analog had the lower K_i (48 μM) but behaved more as a UDP analog. The phosphonate analog behaved as a true bisubstrate inhibitor (K_i=73 μM), supporting results from previous kinetics which indicated a random mechanism for this enzyme.

Thus, several competitive inhibitors for this enzyme have been discovered using different strategies, and a characterization of the substrate combining sites has been achieved which should greatly facilitate development of more potent inhibitors with properties better suited for cell uptake and enhanced residence time.

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Chapter 2
An Enzyme-linked Immunosorbent Assay for *N*-Acetylglucosaminyltransferase V[1]

Introduction

UDP-GlcNAc:α-Mannoside β(1→6)*N*-acetylglucosaminyltransferase (*N*-acetylglucosaminyltransferase-V, GlcNAcT-V, E.C. 2.4.1.155) is a key enzyme involved in the branching of asparagine-linked oligosaccharides (1, 2). Interest in the development of assays for GlcNAcT-V activity stems from several observations that transformation of cells by tumor viruses (3, 4) or oncogenes (5) results in increased GlcNAcT-V activity and concomitantly altered cell-surface glycosylation. Increases in intracellular GlcNAcT-V activity have also been shown to correlate with the metastatic potential of both rodent and human tumor cells (6-8).

Biosynthetically, GlcNAcT-V transfers a GlcNAc residue from UDP-GlcNAc to acceptors having the minimum oligosaccharide structure **1**, converting it to structure **2** which bears the additional branch at the 6-position of the Manα(1→6) arm (1, 2). Early assays for GlcNAcT-V involved quantitation of the transfer of radiolabelled GlcNAc to **1** or its derivatives isolated from natural sources, to produce labelled **2** (1, 3, 4). After separation of UDP-GlcNAc and its degradation products, **2** was then structurally characterized by its retention volume on Bio-Gel P-4 and/or reactivity towards glycosidases or lectins. Later assays involved HPLC techniques for separation of products (9, 10). Advantage was also taken in these assays of the observation that GlcNAcT-V was the only *N*-acetylglucosaminyltransferase which did not have an absolute requirement for Mn^{+2}, and was therefore active in the presence of EDTA. Subsequently, the synthetic trisaccharide **3** was prepared and shown to be an acceptor specific for GlcNAcT-V, which converted it to the tetrasaccharide **4** (11-13). In radioactive assays, labelled product **4**

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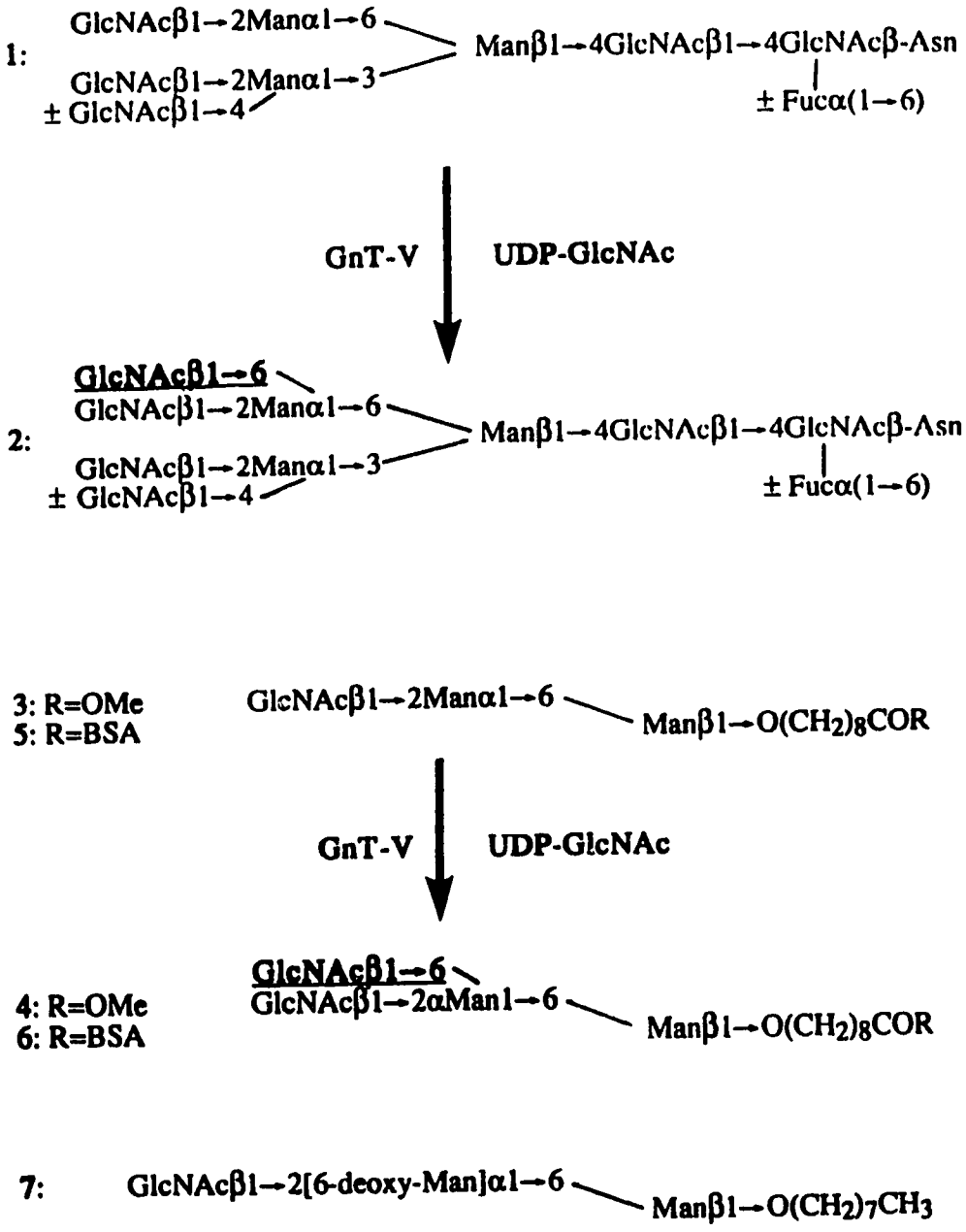
[1]. This chapter has been published as Reference 24. O. Hindsgaul provided affinity-refined polyclonal antiserum, trisaccharide acceptor **3** and inhibitor **7**. G. Alton synthesized BSA conjugates for immunizations and enzyme assays. M. Pierce provided GlcNAcT-V enzyme extracts from BHK and RS-BHK cells, and from hamster kidney. M. Palcic determined original assay design and conditions.


1:
GlcNAcβ1→2Manα1→6
GlcNAcβ1→2Manα1→3
± GlcNAcβ1→4
Manβ1→4GlcNAcβ1→4GlcNAcβ-Asn
± Fucα(1→6)
GnT-V
UDP-GlcNAc
2:
GlcNAcβ1→6
GlcNAcβ1→2Manα1→6
GlcNAcβ1→2Manα1→3
± GlcNAcβ1→4
Manβ1→4GlcNAcβ1→4GlcNAcβ-Asn
± Fucα(1→6)
3: R=OMe
5: R=BSA
GlcNAcβ1→2Manα1→6
Manβ1→O(CH2)8COR
GnT-V
UDP-GlcNAc
4: R=OMe
6: R=BSA
GlcNAcβ1→6
GlcNAcβ1→2αMan1→6
Manβ1→O(CH2)8COR
7:
GlcNAcβ1→2[6-deoxy-Man]α1→6
Manβ1→O(CH2)7CH3

Scheme 2.1

could be readily quantitated after adsorption onto C-18 sample-preparation cartridges by virtue of the hydrophobic nature imparted to it by its lipid-like 8-methoxycarbonyloctyl aglycone (14).

We report here the development of an enzyme-linked immunosorbent assay (ELISA) for GlcNAcT-V which utilizes a polyclonal rabbit antiserum for detection and quantitation of enzymatically produced **4** when it is covalently attached to BSA. The advantages of this ELISA over the previously reported radiochemical assays lie in the simultaneous structural identification of the product, its high sensitivity, and its amenability to extensive automation allowing the rapid measurement of GlcNAcT-V activity in a large number of samples.

Experimental

Materials: Hamster kidney acetone powder extract was prepared as described for *N*-acetylglucosaminyltransferase I (15), except that 2[*N*-morpholino]-ethanesulfonic acid (MES, 50 mM, pH 6.5) was used for the extraction steps instead of cacodylate buffer; and protease inhibitors (0.05 mg/mL of each of soybean trypsin inhibitor, aprotinin; and 0.1 mM phenylmethylsulfonyl fluoride) were incorporated during homogenization and in subsequent steps. One unit of enzyme activity is defined as the quantity of enzyme producing 1 μmol/minute of product in radiochemical assays containing 1.1 mM ^{3}H-UDP-GlcNAc (40,000 dpm/nmol) and 2 mM concentration of acceptor **3**, in 50 mM MES pH 6.5 with 1 % Triton X-100, at 37°C. Human serum used as a source of GlcNAcT-V was prepared by allowing blood to clot at room temperature for 2 h, refrigerating overnight at 4°C and centrifuging to remove red blood cells. Serum samples were made 0.01% (w/v) in NaN_3 and stored frozen at -20°C. Microsomal extracts of BHK and RS-BHK cells were prepared and assayed previously described (12, 13). Alkaline phosphatase conjugate of goat anti-rabbit IgG (whole molecule, adsorbed with human serum proteins) and alkaline phosphatase substrate tablets containing 5 mg *p*-nitrophenyl phosphate were from Sigma. UDP-[6-^{3}H]*N*-Acetylglucosamine (26.8 Ci/mmol) was from New England Nuclear and ACS scintillation cocktail was from Amersham. Removable flat bottomed wells of Immulon 2 were from Dynatech. Chromosorb P was from Johns Manville. The

following buffers were used: PBS: 7.8 mM Na_2HPO_4, 2.2 mM KH_2PO_4, 0.9% NaCl and 15 mM NaN_3 , pH 7.4 and PBST: PBS with 0.05% Tween 20.

*Preparation of BSA-Conjugates **5** and **6**:* Compounds **3** and **4** (11, 12) were coupled to BSA via their acyl azides as described by Pinto and Bundle (16). The carbohydrate content of **5** and **6** were determined using the phenol-sulfuric acid assay (17) with **3** and **4**, respectively, as reference standards. Incorporations of 11-13 oligosaccharides per BSA molecule were achieved.

Rabbit Immunizations and Refining of Antisera: Three rabbits were immunized with **6** following the protocol described by Lemieux *et al* (18). The acyl azide of **3** was covalently attached to silylaminated Chromosorb P beads as previously described (19) to provide an affinity matrix (20) with an incorporation of 0.35 μmol/gm. The rabbit antisera (1.0 mL) were added to 1.5 mL microfuge tubes containing 0.2 gm of the affinity support derived from **3**, and rotated end over end at 4°C for 4 h. After centrifugation, the supernatants were removed and used directly in the ELISA assays. One of the adsorbed antisera showed background binding to wells coated with **5** (A_{405} = 0.082 after 1 h development) and was used in all subsequent GlcNAcT-V ELISAs.

Plate Coating: Microtiter plates were coated as previously described (21) by incubation with 100 μL of synthetic BSA-glycoconjugate **5** or **6** (20 μg/mlL) in 50 mM sodium phosphate buffer pH 7.5 containing 5 mM $MnCl_2$ and 15 mM NaN_3 for 16 h at ambient temperature. This solution was then removed by aspiration and replaced with 5% BSA in PBS (200 μL). After 4 h, this solution was removed and wells were washed three times with PBS (200 μL) and once with H_2O (200 μL), air dried for 1 h and stored at 4°C. Plates were washed again with H_2O (200 μL) immediately before use.

ELISA For GlcNAcT-V: GlcNAcT-V assays were performed by adding buffer and enzyme directly to microtiter plates coated with **5** and initiating the reaction by addition of UDP-GlcNAc or, alternatively, by combining buffer, enzyme and UDP-GlcNAc (final volume 100 μL) and adding this mixture to the coated wells to initiate

carried out in triplicate with the buffer composition, concentrations of enzyme and nucleotide donor noted in the Figure legends for the different enzyme sources. The microtiter plate was incubated at 37°C for 60 or 90 min. The reaction mixture was removed by aspiration; wells were washed (2 x 200 µL H_2O and 1 x 200 µL PBST) and incubated for 2 h at room temperature with the refined rabbit antiserum (100 µL of 1/8000 dilution in 1% BSA/PBST). Wells were aspirated, washed with 5 x 200 µL of PBST and then incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (100 µL of 1/1000 dilution in 1% BSA/PBST) for 2 h. This solution was aspirated and wells were washed (3 x 200 µL PBST, 1 x 200 µL H_2O and 1 x 300 µL H_2O) before adding *p*-nitrophenyl phosphate substrate solution (1.0 mg/ml in 1 M diethanolamine-HCl buffer pH 9.8 containing 1% BSA and 500 µM $MgCl_2$). Increase in absorbance at 405 nm was monitored over time using a Bio-Tek EL-309 or EL-311 EIA plate reader. Data was acquired at 10 min intervals and readings reported are for 60 min, except where noted. Both ELISA plate readers exhibited linear response to an absorbance of 2.8. The experiments for the quantitation of radiolabelled GlcNAc transfer onto wells were carried out at reduced nucleotide donor concentrations and included 0.8 µCi UDP-[^{3}H]GlcNAc in the incubation mixtures. After color development, wells were counted in 10 mL ACS cocktail with a Beckman LS1801 scintillation counter. The average background transferred onto the microtiter wells for the radiochemical experiments was 49 ± 5 dpm, while enzyme incubations gave 45-204 dpm above background.

Results and Discussion

Acceptor **3** and product **4** were covalently attached to BSA to provide the synthetic glycoconjugates **5** and **6** having 11-13 moles of carbohydrate per mole of BSA. Three rabbits were immunized with **6**, resulting in the production of antisera containing antibodies which detected both **5** and **6** immobilized on microtiter plates. For one of these antisera, the cross-reacting antibodies binding acceptor structure **5** could be completely removed by adsorption with an affinity matrix prepared by immobilization of trisaccharide **3** on Chromosorb P, a calcined diatomaceous earth. The refined, mono-specific antiserum thus obtained was used at 1/8000 dilution in all subsequent work. Sufficient antiserum was obtained from this one rabbit to perform 4 x

10^6 ELISAs for GlcNAcT-V.

Microtiter plates were coated with acceptor conjugate **5** in admixture with 0.1-0.8% of product **6** in order to simulate the plate that would result from the action of GlcNAcT-V on immobilized **5**. In Figure. 2.1 (A), product detection by the affinity purified antiserum, amplified by alkaline phosphatase-conjugated goat anti-rabbit IgG, is seen to be linearly proportional to the amount of coated product up to about 0.5%. The slope of this standard curve varied over the range 2.0 ± 0.4 (A_{405}/ % product **6**) when new batches of plates were coated and new solutions of ELISA reagents were prepared. ELISA response, however, always remained linear up to 0.5% product. The saturation at concentrations greater than 0.5% is not due to non-linear microplate reader response since saturation was also observed for readings taken at 40 and 50 min with lower absolute values. Figure 2.1 (B) shows that, as expected, antibody detection of **6** is inhibited by the soluble hapten **4**. The antiserum was therefore concluded to specifically recognize the product tetrasaccharide structure present in **6**.

A Triton X-100 extract of hamster kidney acetone powder was used as a known source (14) of GlcNAcT-V in the experiments summarized in Figures 2.2 and 2.3. Addition of this GlcNAcT-V-containing extract and unlabelled UDP-GlcNAc to microtiter plates coated with **5** resulted in the production of immobilized **6** as detected by the antiserum. The amount of enzymatically-formed product is seen to be proportional to enzyme concentration in Figure 2.2. In Figure 2.3, the formation of product is shown to be linear with time up to about 2 h. In order to obtain an estimate of the absolute amount of product being generated by GlcNAcT-V, and detected in ELISA by the antiserum, the experiments summarized in Figure 2.3 were performed using labelled UDP-[^{3}H]GlcNAc. After the absorbances of the plate were measured using the ELISA reader, the radioactivity of individual wells was then quantitated by liquid scintillation counting. The amount of radioactivity incorporated onto each well is seen in Figure 2.3 to parallel the product formation detected spectrophotometrically by ELISA. The ELISA response was found to be linear with the enzymatically generated product in the range of 50-300 fmol/well, corresponding to an absorbance of 0.2-1.2 at 405 nm after a 1 h color development. The linear range is dependent on the amount and source of enzyme, as well as the concentration of UDP-GlcNAc used and must

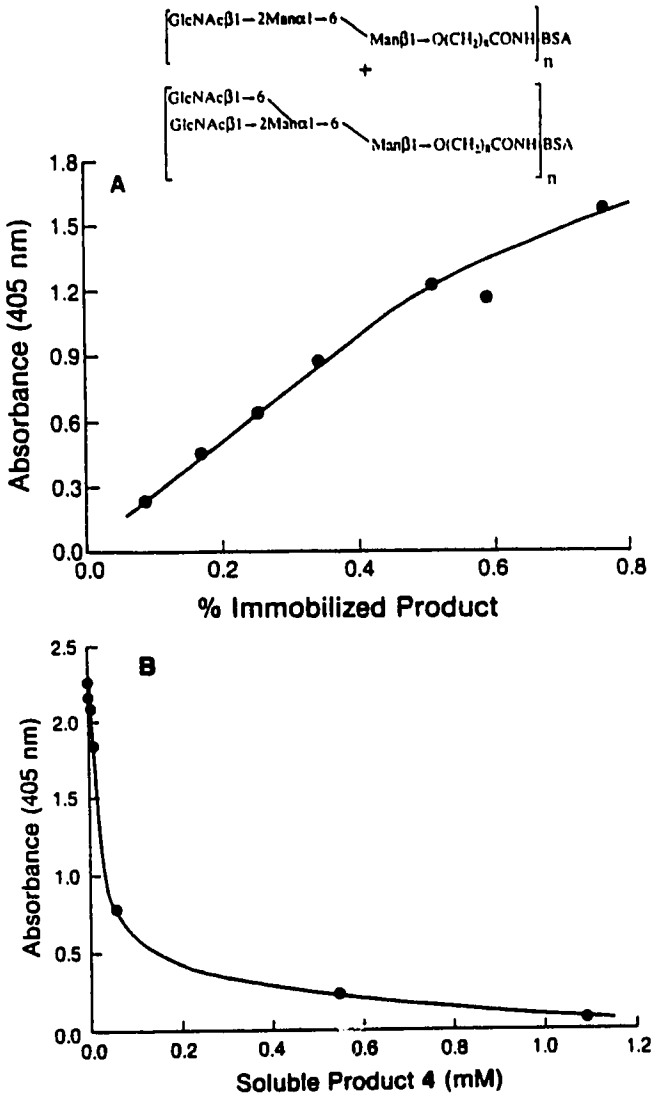


Figure 2.1: (A) Standard curve for ELISA response of wells coated with increasing ratios of product 6 and substrate 5. Absorbance was measured after 60 min incubation with *p*-nitrophenyl phosphate after incubating with refined antiserum at 1/8000 dilution, then alkaline phosphatase-conjugated IgG antibodies as described in **Materials and Methods**. (B) Competition of soluble product 4 with the bound BSA product conjugate 6 for antibody in the antibody-binding step of the ELISA. Assays were performed as described in **Materials and Methods** except that wells coated with 100% 6 were used, and in the primary antibody-binding step, 100 µl of 1/8000 dilution of affinity-purified antiserum was replaced with 50 µl of 1.1 to 109 nmol of 4 in 1% BSA/PBST plus 50 µl of 1/4000 dilution of antibody.

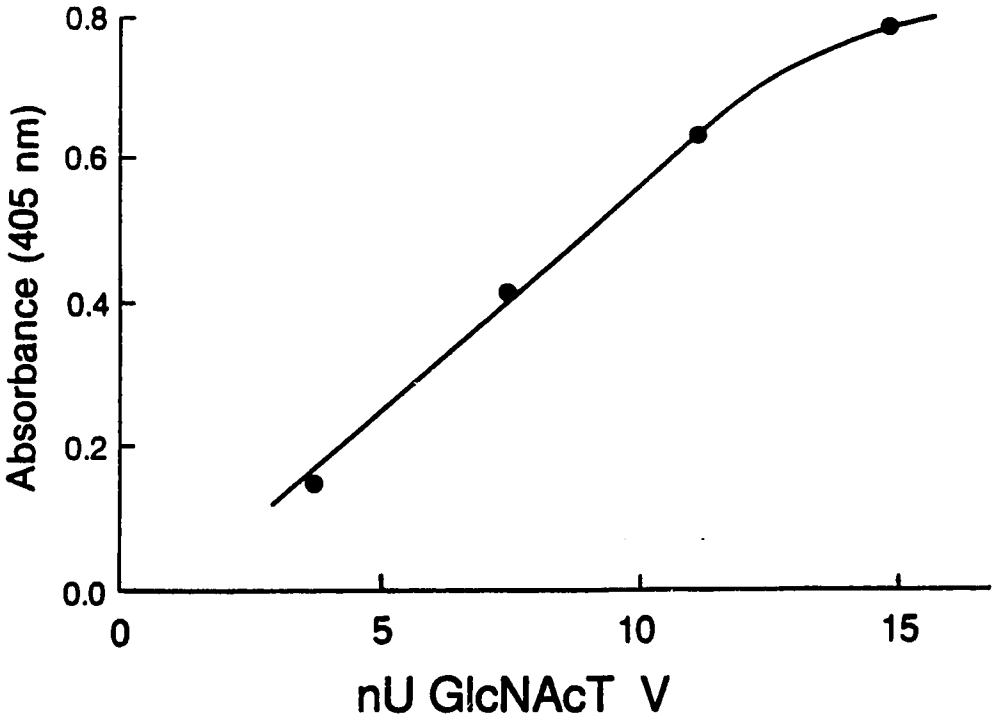


Figure 2.2: Effect of enzyme concentration on product formation. Enzyme incubations contained 4 to 15 nU of GlcNAcT-V enzyme from hamster kidney acetone powder extract (0.17 to 0.68 μg protein) and 563 nmol UDP GlcNAc in 30 mM MES buffer pH 6.5 with 1% Triton X-100. Enzyme incubations were carried out at 37°C for 60 min. After aspiration, ELISA was carried out as described in **Methods and Materials**, with color development measured at 70 min.

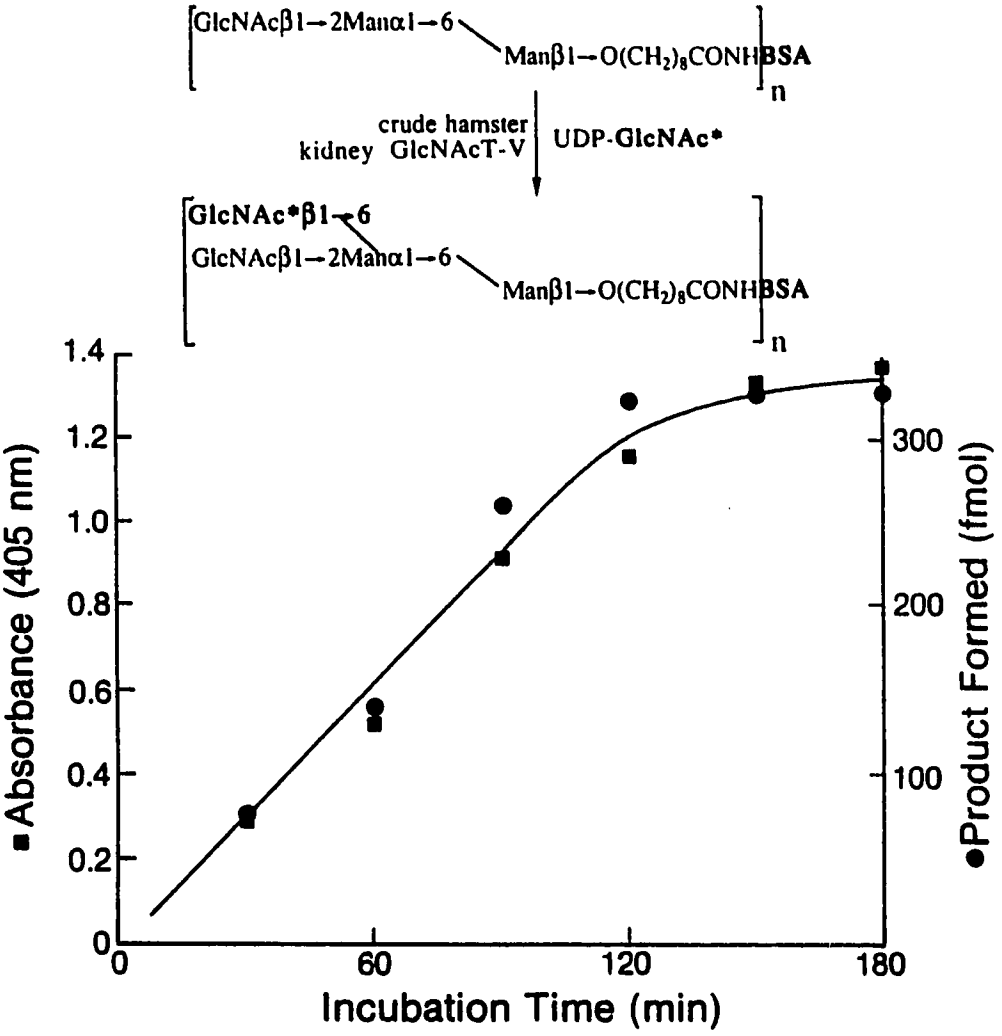


Figure 2.3: Dependence of the ELISA response on time of GlcNAcT-V reaction (squares). Wells coated with 5 were incubated with 0.3 nU of hamster kidney acetone powder GlcNAcT-V extract, 2.8 nmol UDP-GlcNAc and 0.8 μCi ^{3}H-UDP-GlcNAc, for the indicated times at 37 oC. Absorbance was measured after 70 min incubation with *p*-nitrophenyl phosphate (circles). After absorbance measurements, the amount of ^{3}H-GlcNAc transferred to the wells was quantitated by liquid scintillation counting.

be operationally defined for a given sample.

The ELISA also detected GlcNAcT-V activity in human serum (Figure 2.4 (A)) where product formation was proportional to enzyme in the 0.2-0.7 AU response range for a 1 h ELISA development. Additional evidence that serum GlcNAcT-V was indeed responsible for the production of antibody-detected **6** was obtained by performing the incubation in the presence of the deoxy-trisaccharide **7**, an inhibitor specific for GlcNAcT-V (22). The amount of product formed using serum as the enzyme source decreased with increasing inhibitor concentration (Figure 2.4(B)), as would be expected for the GlcNAcT-V catalyzed reaction. The ELISA-detected serum GlcNAcT-V activity was also not inhibited by up to at least 20 mM EDTA, a characteristic property of the enzyme (1). The K_m of the serum enzyme for UDP-GlcNAc was estimated, by ELISA, to be 0.3 mM (data not shown). To our knowledge this is the first report of GlcNAcT-V activity in human serum.

Finally, Rous sarcoma virus-transformation of BHK cells has been shown (4) to result in a two-fold increase in the specific activity of GlcNAcT-V, detected using radiochemical assays. Figure 2.5 shows that this increase was also detected by the ELISA using BHK and RS-BHK microsomes as the sources of GlcNAcT-V. The specific activity of GlcNAcT-V in the RS-BHK cells was estimated by ELISA to be two-fold higher than the activity in BHK cells using absorbance readings in the linear range of approximately 0.2-0.8 AU.

In conclusion, GlcNAcT-V activity can be specifically detected by ELISA in crude biological samples and the sensitivity of this assay exceeds that of conventional radiochemical methods. GlcNAcT-V activity measured in this manner is proportional to enzyme concentration and incubation time over appropriate experimentally-determined absorbance ranges. This ELISA is currently in routine use in our laboratories both for monitoring column fractions for GlcNAcT-V activity in the course of purification of the enzyme and in screening clinical serum samples for alterations in GlcNAcT-V activity. The approach described here using synthetic glycoconjugates as acceptor-product pairs for the development of an ELISA for GlcNAcT-V, coupled with earlier reports (21, 23) of similar assays for both a galactosyltransferase and a fucosyltransferase, suggest the general applicability of ELISAs for the detection and quantitation of glycosyltransferase activities. A major impediment to the development of ELISA assays for glycosyltransferases, as described above, is the

requirement of acceptor-product pairs of oligosaccharides which must generally be prepared by multi-step chemical synthesis. The increasing commercial availabilty of synthetic oligosaccharides should, however, allow the development of such assays outside synthetic chemical laboratories.

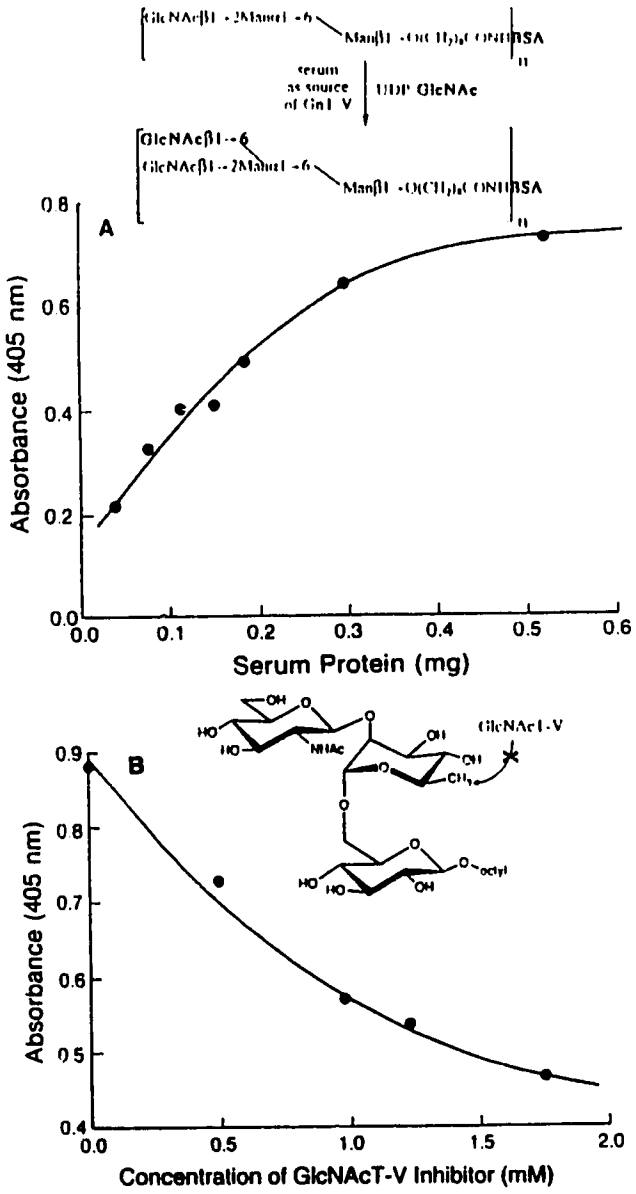


Figure 2.4: (A).Effect of serum concentration on GlcNAcT-V activity. Enzyme incubations contained 0.5 to 7 µl of serum (37 to 520 µg protein) and 11 nmol UDP-GlcNAc and were carried out in 75 mM sodium cacodylate buffer with 3.5 mM $MnCl_2$, pH 7.2 for 60 min at 37 °C. (B) Effect of the specific GlcNAcT-V inhibitor, 7, on apparent enzyme activity. Incubations contained 4 µl serum (298 µg protein), 352 nmol UDP-GlcNAc and increasing concentrations of 7, in a total volume of 100 µl in 40 mM sodium cacodylate buffer pH 7.2. After reaction at 37°C for 90 min, solutions were aspirated and ELISA carried out as described in **Materials and Methods.**

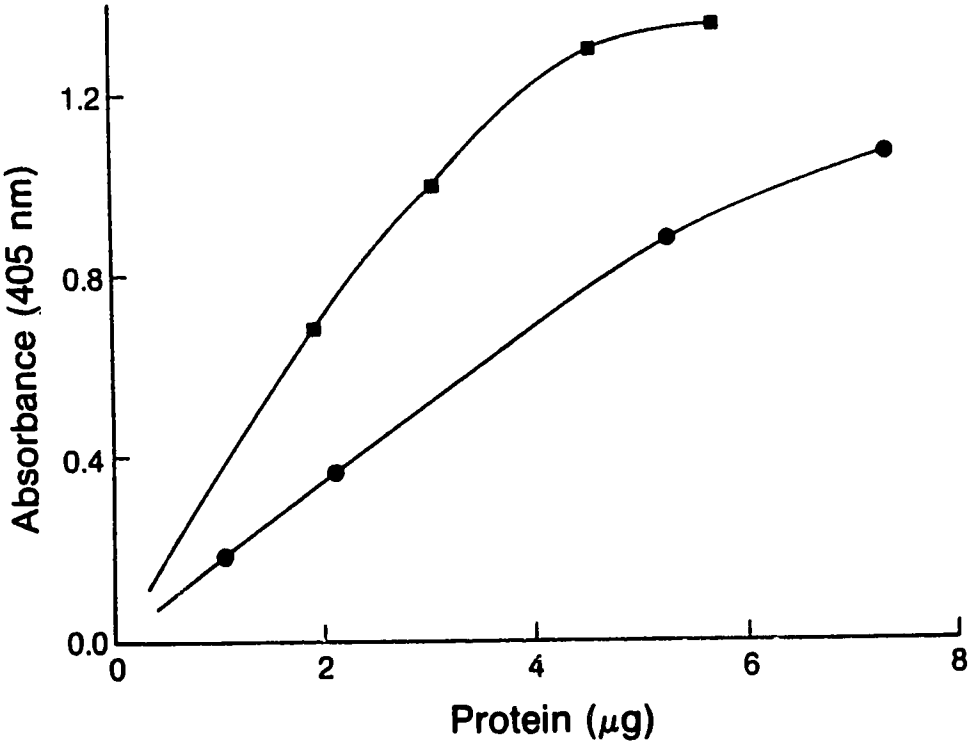


Figure 2.5: Comparison of GlcNAcT-V activities of Rous sarcoma virus transformed (squares) and non-transformed parental BHK cells (circles) using microsomal extracts. Enzyme incubations contained 1.9 to 5.7 μg protein and 1.0 to 7.4 μg protein for RS-BHK and BHK extracts, respectively, and 11 nmol UDP-GlcNAc in 38 mM MES buffer, pH 6.5 with 0.8% Triton X-100. Incubations were carried out for 60 min at 37 °C, solutions removed by aspiration and ELISA carried out as described in **Materials and Methods**, with absorbance readings taken at 65 min.

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Chapter 3
Definition of Acceptor Specificity of *N*-Acetylglucosaminyltransferase V using Synthetic Trisaccharide Substrate Analogs[1]

Introduction

Cell surface carbohydrates of glycoproteins and glycolipids act as recognition markers to direct cell-cell, cell-virus, cell-bacterium, cell-protein and even carbohydrate-carbohydrate interactions (4-8). One particularly interesting class of *N*-linked oligosaccharide structures is synthesized by UDP-GlcNAc: α-D-mannoside β(1→6) *N*-acetylglucosaminyltransferase V (GlcNAcT-V; E.C. 2.4.1.155), which catalyzes transfer of a GlcNAc sugar residue to asparagine-linked oligosaccharides as shown in Figure 3.1 (9), forming βGlcNAc(1→6)αMan(1→6) branched structures. Interest in this enzyme has been stimulated by observations that glycopeptides isolated from membrane glycoproteins of cells transfected by virus or DNA express higher levels of the structures formed by this enzyme (10-12). Yamashita *et al* established that a doubling of the activity of GlcNAcT-V coincided with the increased βGlcNAc(1→6) branching upon polyoma transformation of baby hamster kidney cells, while other GlcNAc transferase activities remained unchanged (13). The same result was obtained by Pierce and Arango, who assayed the enzyme in Rous sarcoma-transformed BHK cells using a synthetic trisaccharide acceptor (11). Also, a direct association between increased βGlcNAc(1→6)αMan(1→6)-branching of *N*-linked glycoproteins and metastatic phenotype was demonstrated by Dennis *et al*, who found that L-PHA lectin selection of a highly metastatic murine lymphoma cell line gave rise to two mutants, both of which were non-metastatic and also showed reduced GlcNAcT-V activity (15). Levels of GlcNAcT-V have also been shown to be substantially elevated in malignant versus benign human breast cancer biopsies (16); and several oncogenes (v-*fps* and H-*ras*), which induce a metastatic phenotype when transfected into rat2 fibroblasts, also produced an increase in β(1→6)branching (17). Related work by this and other groups has been reviewed (18).

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1. Parts of this chapter have been published as References 1-3. Acceptor analogs were chemically synthesized by the group of O. Hindsgaul. Specifically: K. Kaur synthesized **3** and **11**; O. Kanie synthesized GlcNAc-modified trisaccharides **13-23**; S. Khan synthesized Man-modified analogs **7-10**, and **12**; and T. Linker synthesized acceptors **5**, **6** and **24**.

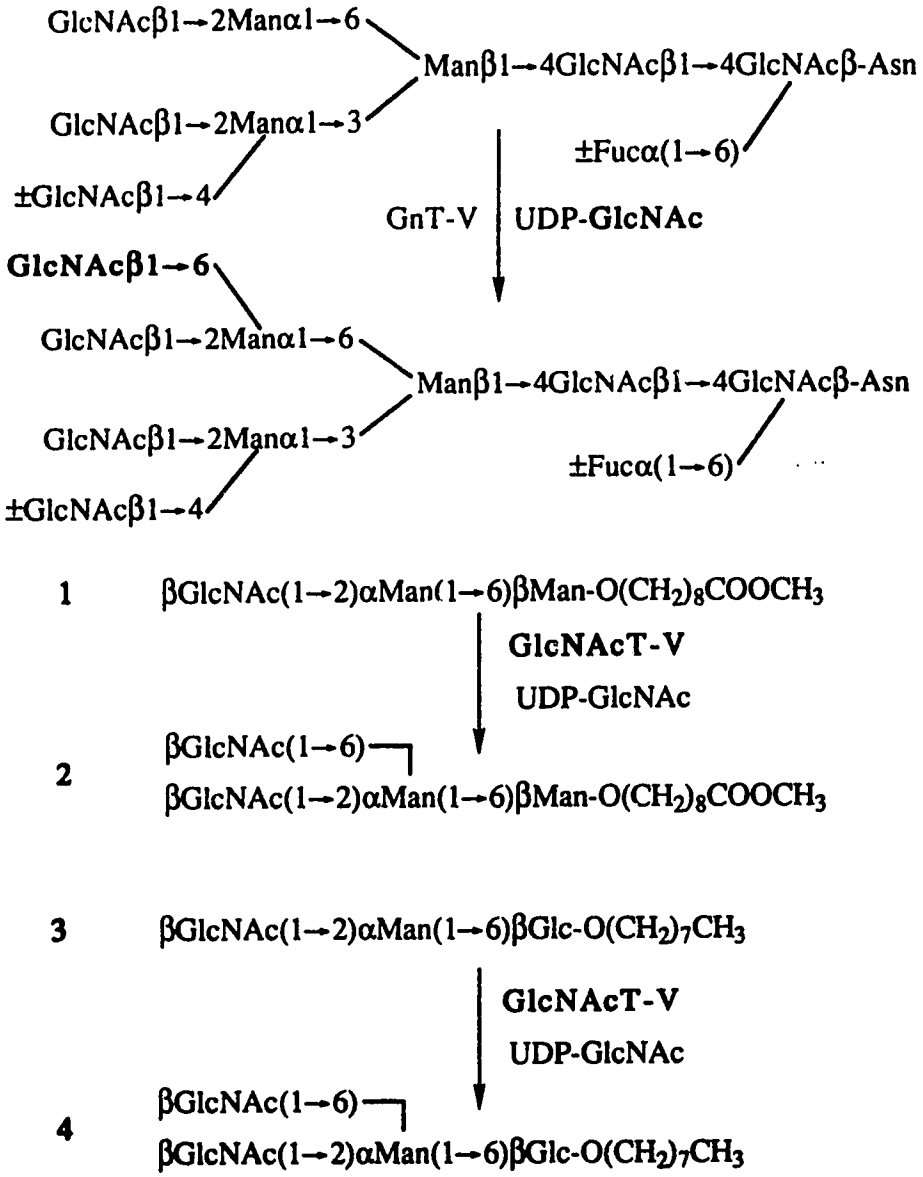

Figure 3.1: Natural (top) and synthetic substrates (1 and 3) and products (2 and 4) of the GlcNAcT-V transfer reaction.

Activities of GlcNAcT-I, -II, -III, -IV and -V have also been measured in a metastasizing and a non-metastasizing variant of rat prostate tumor cell lines (R3327-MatLyLu and -H) and it was found that the former had higher GlcNAcT-V and -III activities giving ratios of 2.6:1 and 6.5:1 for the two enzymes, respectively(19). Similarly, Heffernan *et al* observed that retinoic acid-induced differentiation of mouse F9-teratocarcinoma cells from embryonic to endoderm-like cells is accompanied by particularly dramatic increases in GlcNAcT-V activity, giving rise to a corresponding increase in expression of L-PHA-reactive oligosaccharides and poly-*N*-acetyllactosamine structures on membrane glycoproteins including LAMP-1 (20). The tumorigenic potential of adenovirus transformants of rat 3Y1 cells was found to correlate with βGlcNAc(1→6) branching of *N*-linked sugar chains, and expression of two particular genes of the adenovirus genome (where one is known as the T antigen) were found to be involved in this branching (21). Models have been proposed in which βGlcNAc(1→6) branching of *N*-linked glycoproteins is controlled by levels GlcNAcT-V and this branching, in turn, affects directly the levels poly-*N*-acetyllactosamine structures (22, 23).

Specific inhibitors for this and other glycosyltransferases would allow very specific alterations to be made to the course of protein glycosylation within a malignant cell type, permitting study of the resultant phenotype, and, ideally, reversion to a more normal state. Toward this end of developing a GlcNAcT-V inhibitor, the synthetic trisaccharide **1** was determined to be the minimum structure required for acceptor substrate recognition by this glycosyltransferase (24), and six analogs of acceptor trisaccharide **1** were synthesized in order to probe the enzyme's acceptor binding site (25). Among other modifications it was found that conversion of the reducing-end sugar from the *manno* to the *gluco* configuration gave an acceptor (**3**) with almost identical reactivity, and this modification has been retained in many subsequent trisaccharide analogs in order to facilitate their chemical syntheses. A rapid, sensitive radiochemical assay was developed for this and other glycosyltransferases, taking advantage of the hydrophobic aglycone in order to separate radiolabelled tetrasaccharide product **4** from radiolabelled UDP-GlcNAc donor (26). The first inhibitor developed for GlcNAcT-V was designed by replacement of the reactive 6'-OH group with a hydrogen, and was employed, with several other acceptor analogs, in a comparative kinetic study of this enzyme in normal, Rous sarcoma-transformed and lectin-resistant BHK cell lines (27). Since all of the enzymes had the same

K_m's and K_i's, but different V_{max}'s, it was determined that altered enzyme activities probably arose from differential levels of expression rather than from expression of catalytically different forms of the enzyme.

A kinetic study of hamster kidney GlcNAcT-V has been undertaken, employing trisaccharide acceptor analogs in which individual reactive hydroxyl groups have been replaced by one or more functional groups. The result is a picture of an acceptor recognition site which is dominated by polar interactions with three key polar groups of the GlcNAc residue, an essentially hydrophobic pocket for the reducing-end Man residue, and a recognition site for the αMan(1→6) which does not rely heavily on polar interactions, but which must ensure very specific orientation of the reactive 6'-OH, particularly relative to its neighboring 4'-OH group.

Experimental

General Materials and Methods: EDTA, MES, Triton X-100, UDP and UDP-GlcNAc were obtained from Sigma. Liquid scintillation cocktail was from ICN (Ecolite(+)). Reverse-phase C18 SepPak cartridges from Waters Associates were pre-equilibrated with 20 mL methanol and 30 mL water before use. UDP-[6-^{3}H(N)]GlcNAc was obtained from American Radiolabelled Chemicals Inc.; in order to reduce background values obtained in radioassays, this material was lyophilized, passed through a C18 SepPak cartridge pre-equilibrated with water, and then re-lyophilized and dissolved in ethanol:water (70:30) for later use. Hamster kidneys were obtained from Pel Freez Biologicals, Rogers, Arkansas. Synthetic trisaccharides **3** and **11** were obtained from previous work (25, 27). Synthesis of **5**, **6** and **24** are described in Reference 3; synthesis of **8** and **9** have been reported (2) and synthesis of **16-21** are reported in Reference 1. Synthesis of **7**, **10** and **12** will be reported in a separate communication (S Khan and O Hindsgaul) as will synthesis of compounds **13-15**, **22** and **23** (O Kanie and O Hindsgaul). Other materials were of reagent grade.

Enzyme Preparation: *N*-acetylglucosaminyltransferase-V was partially purified from hamster kidney acetone powder using a procedure similar to that of Shoreibah *et al* (29), originally derived from the GlcNAcT-I purification by Oppenheimer and Hill (30). Dialyzed extract from 24 g of hamster kidney was applied to a 3.4 mL bed-volume UDP-hexanolamine Sepharose column (7 μmol/mL). This

column was washed with 50 mL buffer (50 mM sodium cacodylate, pH 6.5, containing 10 mM EDTA, 0.25% Triton X-100 and 20% glycerol), and then with the same buffer containing 0.1 M NaCl. GlcNAcT-V was eluted with 20 mL each of buffer containing 0.1 M NaCl and 5 mM UDP, and then with buffer containing 0.25 M NaCl and 5 mM UDP. Eluates were concentrated to less than 1.5 mL by ultrafiltration and dialyzed into 50 mM sodium cacodylate buffer, pH 6.5, containing 10 mM EDTA, 20% glycerol and 0.1% Triton X-100 (Buffer A). Precipitate formed during ultrafiltration and dialysis was removed by centrifugation. A typical preparation yielded 6 mU of GlcNAcT-V, with 1 mU/mg protein, where 1 mU is defined as the amount of enzyme catalyzing the transfer of 1 nmol GlcNAc per minute, using 1.1 mM UDP-GlcNAc and 400 μM synthetic trisaccharide acceptor **3** in Buffer A at 37°C.

General GlcNAcT-V Enzyme Kinetic Methods: GlcNAcT-V was assayed radiochemically using reverse-phase C18 SepPak cartridges to separate the labelled hydrophobic product tetrasaccharide **4** from unreacted radiolabelled sugar-nucleotide donor UDP-GlcNAc as described (26). Unless otherwise stated, kinetic studies with acceptor analogs contained 11 nmol UDP-GlcNAc (30,000 dpm/nmol); substrates were lyophilized in 600 μL plastic microfuge tubes, and enzyme and buffer were added to give a final volume of 10 μL. The tube containing substrates and enzyme was vortexed, microfuged briefly and incubated at 37°C for up to 2 hours. The reaction was quenched using 0.4 mL water and the reaction mixture transferred with water onto a pre-equilibrated C18 SepPak cartridge. Unreacted radiolabelled donor was removed by washing with 100 mL water, and labelled product was eluted slowly with 2 X 3 mL MeOH and collected for liquid scintillation counting. Kinetic constants (± standard errors) were obtained by fitting rate data to the appropriate equations (31) using unweighted nonlinear regression (SigmaPlot 4.1, MacIntosh version). Apparent Michaelis constants ($K_{m,app}$) were obtained by fitting rate data to Equation 1. Equation 2 was used to determine competitive inhibition constants (K_i) from measurements of reaction rates over a range of acceptor (A) concentrations at two or three different inhibitor (I) concentrations.

$$\upsilon = \{V_{max,app}[A]\}/\{K_{m,app} + [A]\} \qquad (1)$$

$$\upsilon = \{V_{max,app}[A]/K_{m,app}\}/\{1 + [A]/K_{m,app} + [I]/K_i\} \quad (2)$$

Specific Protocols Used for Different Analogs:

A. 1.2 μU GlcNAcT-V (specific activity of 2.3 mU/mg) was used in radioassays. Incubations were carried out for 32 to 60 minutes. Acceptors were evaluated as acceptors using 150 to 4000 μM of **24** and 6 to 400 μM of **3** and **5**.

B. Due to solubility problems, acceptor analog **6** was dissolved in 1:4 DMSO/Buffer A and added as a solution (0.12-8 nmol) to 22 nmol of lyophilized UDP-[^{3}H]-GlcNAc. DMSO/Buffer A was added to bring the volume to 10 μL, and then 1.4 μU enzyme in 10 μL Buffer A was added, so that the final reaction mixtures contained 10% DMSO in a final volume of 20 μL. For comparison, reaction rates were measured using the regular trisaccharide acceptor **3** as substrate under the same conditions, adding first 10 μL 1:4 DMSO/Buffer A and then 10 μL enzyme in Buffer A to lyophilized acceptor **3** (0.12-8 nmol) and donor (22 nmol of UDP-[^{3}H]-GlcNAc).

C. Assays of **7** and **13** contained 11 nmol ^{3}H-UDP-GlcNAc (60,000 dpm/nmol). Enzyme in buffer (0.09 μU/μL; 2.1 mU/mg protein) was added to lyophilized donor to give 1.1 mM final concentration, and 10 μL aliquots of this donor/enzyme mixture were transferred to microfuge tubes containing 0.04 to 10 nmol of lyophilized acceptor (**3**, **7**, or **13**), and reactions were incubated 28 to 51 minutes.

Assays of **14**, **15**, **19** and **20** used 4.5 μU of hamster kidney enzyme and 0.04 to 10 nmol lyophilized acceptor. Long-term incubations of these acceptors were carried out as a precaution to ensure that greater than 10% turnover could be achieved and to estimate the fraction of **3** present. Only the 6"-substituted compounds were found to contain a significant amount of **3**, which was estimated to be between 3 and 6.5% by GlcNAcT-V and bovine milk β(1→4)galactosyltransferase labelling experiments, respectively; as expected, both 6'-substituted analogs contained the same relative amount of **3.D.** Radioassays contained 2 μU of GlcNAcT-V and 0.06-5.6 nmol acceptor **3** or **9**. K_m values for UDP-GlcNAc and acceptors were determined by measuring reaction rates while varying concentrations of both substrates (5-560 μM acceptor **3** or **9** and 0.1 to 6 mM UDP-GlcNAc). Rate data were fit to Equation 3 for a rapid-

equilibrium random bireactant mechanism because independent studies using a bisubstrate analog have shown the order of addition of substrates to be random[2]. (A and B refer to acceptor **3** and UDP-GlcNAc substrates; K_A and K_B refer to their respective Michaelis constants.)

$$\upsilon = V_{max}[A][B]/\{\alpha K_A K_B + \alpha K_A[B] + \alpha K_B[A] + [A][B]\} \quad (3)$$

The rates of transfer of radiolabel from ^{3}H-UDP-GlcNAc to equimolar mixtures of trisaccharide acceptors **3** and **9** were measured and the values obtained were compared with those predicted for the two cases where either two different enzymes are catalyzing transfer to the two different acceptors (Equation 4), or one enzyme is catalyzing transfer to both acceptors (Equation 5). The results are presented in Table 3.2, and bold subscripts in equations refer to compound numbers from Table 3.2.

$$\upsilon = V_{max3}[S_3]/\{K_{m3}+[S_3]\}+V_{max9}[S_9]/\{K_{m9}+[S_9]\} \quad (4)$$

$$\upsilon = \{V_{max3}[S_3]/K_{m3}+V_{max9}[S_9]/K_{m9}\}/\{1+[S_3]/K_{m3}+[S_9]/K_{m9}\} \quad (5)$$

[Compound **3**] (μM)	[Compound **9**] (μM)	measured rate (pmol/hr)	predicted rate -2 enzymes	predicted rate -1 enzyme
30	30	67	124	77
60	60	93	181	95
30	0	27	40	40
0	30	85	84	84
60	0	43	51	51
0	60	136	130	130

Table 3.1: Substrate competition experiment to determine whether GlcNAc transfer to acceptors **3** and **9** is catalyzed by a single enzyme. Rates were measured as described in **Protocol D**. Assays contained 0.6 μU of enzyme and were incubated for 30 minutes.

Additional evidence that **3** and **9** are acting as acceptors for the same enzyme is provided by the observation that the 6'-deoxy inhibitor

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2 See Chapter 4.

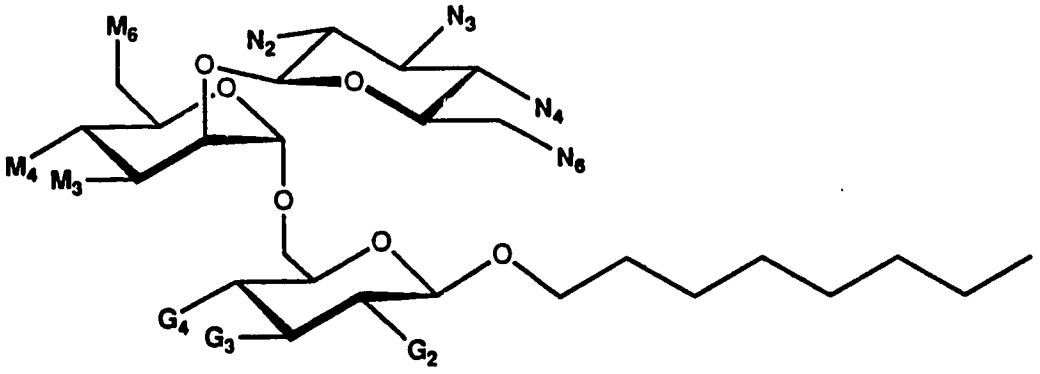
11 inhibited transfer to both acceptors. At 30 μM acceptor concentrations, 80 μM **11** gave a reduction in rate from 85 to 10 pmol/hr using **9** as acceptor. This may be compared with a reduction in rate from 27 to 12 pmol/hr when using **3** as acceptor.

Compound **8** was tested as a competitive inhibitor by evaluating acceptor kinetics at 0, 33 and 67 μM **8**. Primary data obtained from this evaluation is presented in the form of an inverse plot in Figure 3.3**A**. In order to determine whether **8** affected kinetic parameters of UDP-GlcNAc, the $K_{m,app}$ for UDP-GlcNAc was measured with and without **8** (0.080 mM). Results of this experiment are presented in Figure 3.3**B**.

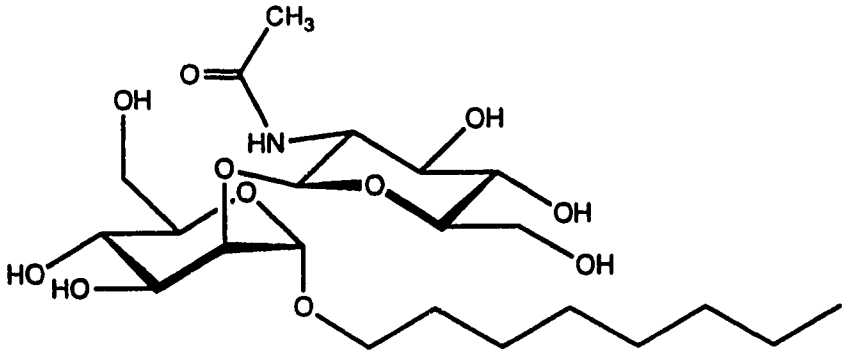
E. Acceptor analog inhibitors **10**, **11** and **12** were lyophilized and GlcNAcT-V in Buffer A (0.15 μU/μL; 0.7 mU/mg) added to give 0, 33 and 67 μM final concentrations. 10 μL aliquots of this mixture were transferred to microfuge tubes containing lyophilized acceptor **1** (0.06 to 8 nmol) and donor, and assays were incubated 61.5 minutes (no inhibitor added), 92 minutes (**12** and **10**) or 98 minutes (**11**).

F. 3.6 nmol of 4"-substituted acceptor analogs **16-21** were incubated for 26 hours with GlcNAcT-V (9 μU) and UDP-GlcNAc (35.2 nmol; 38,300 dpm/nmol) in Buffer A, and reactions were quenched and radiolabelled product quantitated as described above. Apparent conversions of substrate to product ranged between 0.3 and 5.7% (0.5% **16**, 5.7% **17**, 5.3% **18**, 5.3% **19**, 2.5% **20** and 0.3% **21**).

The ability of β(1→4)galactosyltransferase to transfer galactose from UDP-galactose to the 4"-OH position of the terminal GlcNAc residue of acceptor **3** was exploited to determine if there were small amounts of **3** contaminating the trisaccharides **16-21**. 80 nmol of 4"-substituted acceptors were lyophilized with 7.2 nmol UDP-Gal (22,000 dpm/nmol) in microfuge tubes. As a control, trace amounts of normal acceptor **3** with a free 4"-OH group were also galactosylated by pre-incubation (at 37°C for 10.6 hours) with 18 mU bovine milk β(1→4)galactosyl-transferase (from Sigma) in 50 mM sodium cacodylate buffer, pH 7.2, with 1 mg/mL BSA, 20% glycerol and 2 mM $MnCl_2$ in a final volume of 10 μL. Preliminary experiments had shown that these conditions were sufficient to convert at least 1.6 nmol of trisaccharide **3** to 4"-galactosylated product which was no longer an acceptor for GlcNAcT-V. Fractions of the potential substrates which were converted to β(1→4)galactosyltransferase product (as determined by radiolabel incorporation) varied over the range 0.2 to 2.6% (**16**,



compounds **1-23**



compound **24**

Figure 3.2: Structures of compounds tested in Table 3.2.

ana-log	G2	G3	G4	M3	M4	M6	N2	N3	N4	N6	K_m (mM)	$\%V_{max,rel}$*	(V/K) rel*	K_i (μM)
3[a]	OH	OH	OH	OH	OH	OH	NHAc	OH	OH	OH	.026±.002	100±2[a]	100	--
5[a]	OMe	OMe	OMe	OH	OH	OH	NHAc	OH	OH	OH	.033±.002	145±3[a]	116	--
3[b]	OH	OH	OH	OH	OH	OH	NHAc	OH	OH	OH	.037±.001	100±5[b]	100	--
6[b]	OBn	OBn	OBn	OH	OH	OH	NHAc	OH	OH	OH	.058±.005	17±1[b]	10.6	--
7[c]	OH	OH	OH	OMe	OH	OH	NHAc	OH	OH	OH	.06±.02	47±3	18	--
8[d]	OH	OH	OH	OH	OMe	OH	NHAc	OH	OH	OH	--	0	0	14±2
9[d]	OH	OH	OH	OH	H	OH	NHAc	OH	OH	OH	.074±.006	460±10	143	--
10[e]	OH	OH	OH	OH	OH	OMe	NHAc	OH	OH	OH	--	--	--	70±10
11[e]	OH	OH	OH	OH	OH	H	NHAc	OH	OH	OH	--	--	--	30±5
12[e]	OH	OH	OH	OH	OH	F	NHAc	OH	OH	OH	--	--	--	24±3
13[c]	OH	OH	OH	OH	OH	OH	OH	OH	OH	OH	.068 ± .006	178 ± 4	68	--
14[c]	OH	OH	OH	OH	OH	OH	NHAc	NH_2	OH	OH	1.10 ± .06	34 ± 1	0.80	--
15[c]	OH	OH	OH	OH	OH	OH	NHAc	NHAc	OH	OH	1.94 ± .01	21.7±0.5	0.29	--
16[f]	OH	OH	OH	OH	OH	OH	NHAc	OH	OMe	OH	4±1	1.9±0.2	0.02	--
17[f]	OH	OH	OH	OH	OH	OH	NHAc	OH	OH-epi	OH	4.4±.4	10.8±0.5	0.11	--
18[f]	OH	OH	OH	OH	OH	OH	NHAc	OH	F	OH	3.9±.5	11.3±.9	0.12	--
19[f]	OH	OH	OH	OH	OH	OH	NHAc	OH	NH2	OH	6±2	13±2	0.09	--
20[f]	OH	OH	OH	OH	OH	OH	NHAc	OH	NHAc	OH	13.09±.04	13.8±0.2	0.045	--
21[f]	OH	OH	OH	OH	OH	OH	NHAc	OH	H	OH	1.4±.2	0.95±0.05	0.03	--
22[c]	OH	OH	OH	OH	OH	OH	NHAc	OH	OH	NH_2	not substrate	--	0	--
23[c]	OH	OH	OH	OH	OH	OH	NHAc	OH	OH	NH-acetyl	not substrate	--	0	--
24[a]	--	--	--	OH	OH	OH	NHAc	OH	OH	OH	1.8±.4	137±9[a]	2.0	--

Table 3.2: Kinetic parameters obtained for synthetic acceptor analogs tested with GlcNAcT-V.
*$\%V_{max,rel}$ and $(V/K)_{rel}$ were determined relative to compound 3 under the same experimental conditions. a, b, c, d, e, and f refer to GlcNAc transferase V assay protocols A, B, C, D, E, and F, respectively.

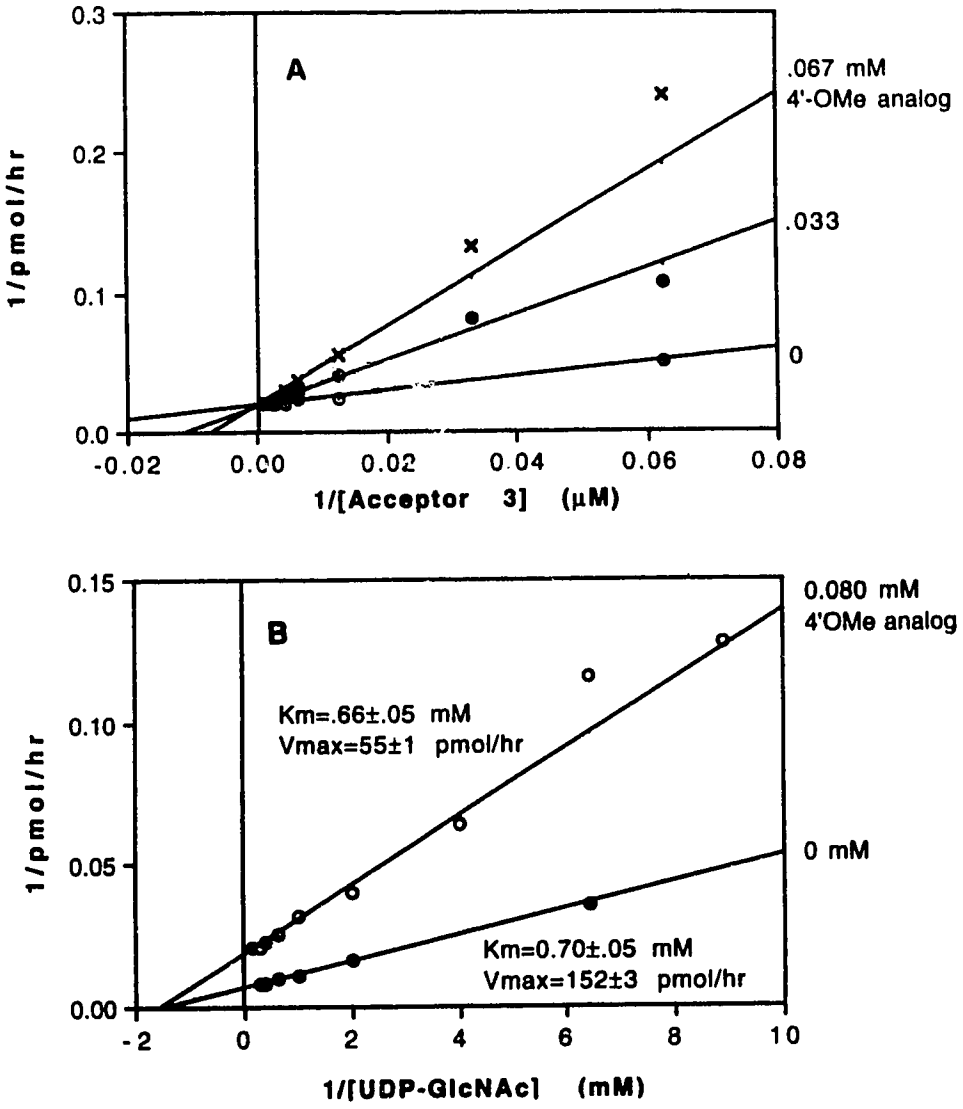


Figure 3.3: Inverse plot of rate data obtained from inhibitor kinetic evaluation of 4'-OMe acceptor analog **8**. (A) Reaction velocities were measured over a range of concentrations of **3** (6 to 560 μM), with **8** added in amounts indicated on figure. Assays contained 1 microunit of enzyme and were incubated for up to 54 minutes. (**B**) Non-competitive inhibition by **8** versus UDP-GlcNAc. Reaction rates were measured at a fixed concentration of acceptor **3** (80 μM) with and without 80 μM 4'-OMe acceptor analog **8** as indicated on the figure. UDP-GlcNAc concentration was varied from 0.11 to 6.0 mM. Assays contained 1 microunit of enzyme and were incubated for 35 or 66 minutes.

17, **18**, **19**, **20**, and **21** were 2.3, 2.6, 2.0, 0.2, 1.0, and 0.4% radiolabelled,respectively). Finally, compounds **16-21** were tested as substrates for the GlcNAcT-V reaction after ensuring galactosyltransferase-mediated elimination of the small fraction of **3** present in the acceptor preparations by performing pre-incubations as described above for radiolabelling experiments, using 10, 20 and 80 nmol of potential acceptors and unlabelled UDP-Gal. After these pre-incubations, GlcNAcT-V reactions were initiated by addition of GlcNAcT-V as well as 22 nmol [^{3}H]-UDP-GlcNAc (25,400 or 31,800 dpm/nmol) as described for 26-hour incubations above. For comparison, the GlcNAcT-V reaction rate with the normal acceptor **3** was measured under similar conditions, except that galactosyltransferase was replaced with buffer only in the pre-incubation step and reactions were only allowed to proceed for 7.5-10 minutes instead of 2 hours. Reactions were quenched and radiolabelled product isolated and quantitated as described above.

Results and Discussion

Analogs **5-24** of trisaccharide acceptor **3** have been tested as substrates or, where appropriate, inhibitors of hamster kidney GlcNAcT-V. Kinetic parameters are presented in Table 3.2; V_{max} and V_{max}/K_m values are given relative to the parent compound **3** tested under the same conditions. All acceptor analogs which were found to be inhibitors showed a competitive mode of inhibition versus acceptor **3**. A diagramatic representation of the enzyme's substrate specificity as deduced from these studies is provided in Figure 3.4. Transfer of radiolabelled GlcNAc to acceptor analogs was quantitated by measuring incorporation of label into product isolated on reverse-phase cartridges as described. The new glycosidic linkage formed in each case was assumed to be $\beta(1\rightarrow6)$ to the αMan as deduced for the original trisaccharide acceptor, where the enzymatic reaction product was isolated and its ^{1}H-NMR spectrum compared with that of synthetic tetrasaccharide **2** for structural confirmation (24). 10 mM EDTA was included in all assays to eliminate interference by manganese-dependent GlcNAc transferases, and no other divalent-cation independent glycosyltransferases are known which can transfer GlcNAc to the types of structures employed in these studies.

Effects of substitutions at the Reducing-terminal Mannose Residue: The important role of the reducing-end sugar residue in acceptor

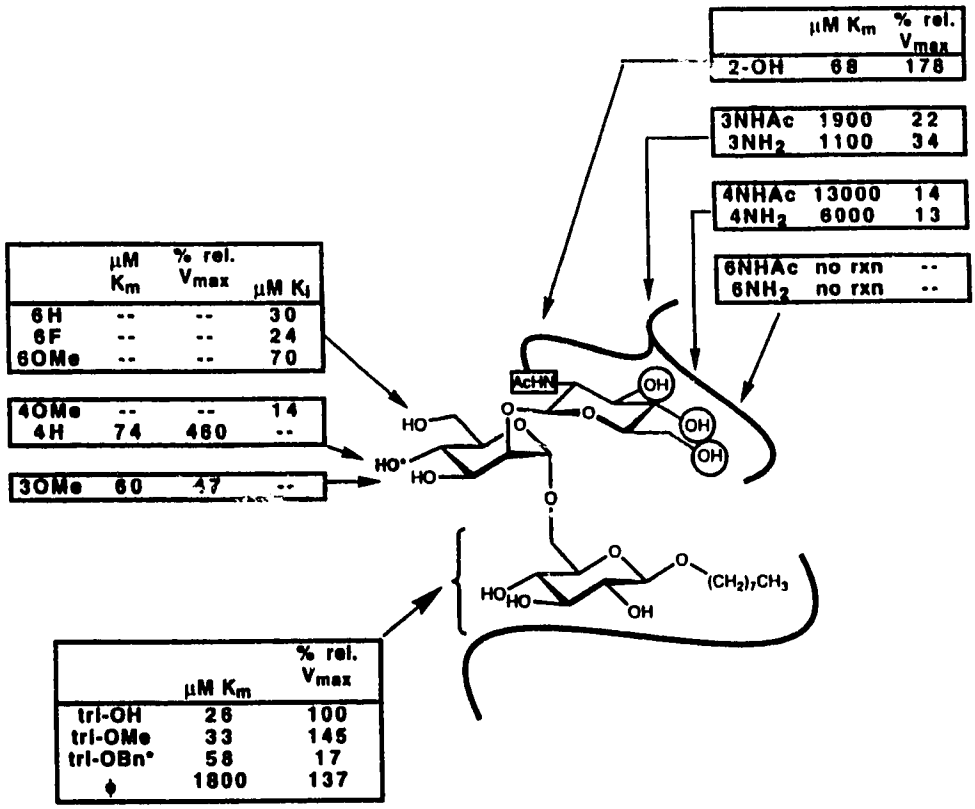


Figure 3.4: Summary of substrate specificity of hamster kidney GlcNAcT-V for synthetic trisaccharide acceptors. Circled groups could not be replaced by other functional groups. Rectangular boxes designate groups which could be replaced only by specific functional groups. Substitutions of remaining hydroxyl groups were well tolerated.

recognition is demonstrated by the 71-fold increase in K_m shown for **24** (1.84 mM) versus **3** (0.026 mM) (Table 3.2). However, 2, 3, 4-substituted analogs **5** and **6** had K_m values comparable to those of the parent compound **3** assayed under the same condition, suggesting that none of these three hydroxyl groups form critical hydrogen-bond donating interactions with the enzyme active site. The tri-O-methyl trisaccharide **5** was found to be an excellent substrate, with kinetic parameters almost indistinguishable from those of the parent **3**, and the tri-O-benzyl compound **6** (assayed in 10% DMSO to overcome solubility problems) had a K_m 60% higher than the parent compound evaluated under the same conditions. The V_{max} for the tri-O-benzylated compound **6** was considerably lower than the parent compound. Interestingly, the transfer reaction proceeded faster in the presence of 10% DMSO as reflected in a 100% increase in V_{max} (data not shown).

Formation of the enzyme-substrate complex is therefore seen to be only marginally affected by what amounts to very large perturbations of the Glc residue. These perturbations block all of the hydroxyl groups and dramatically increase the size and potential steric interactions of the acceptor analogs with the enzyme combining site. The possibility that the hydroxyl oxygen atoms participate in important polar (hydrogen-bond accepting) interactions could not be eliminated by this work, but the earlier study by Srivastava *et al* had demonstrated that deoxygenation at C4 did not affect the enzyme reaction rate at 1 mM concentration of acceptor; and epimerization at C2 was also shown to not affect the reactivity of the acceptor (25). Participation by ring and glycosidic oxygens in protein-carbohydrate interactions is also not eliminated by this study, but the results do suggest a hydrophobic recognition of the Glc residue and its aglycone by GlcNAcT-V.

Putting these results into a biological context, it is known that in the natural substrate shown in Figure 3.1, transfer of GlcNAc by GlcNAcT-III to the 4 position of this reducing-end βGlcNAc renders the *N*-linked oligosaccharide a non-substrate for GlcNAcT-V and several other glycosyltransferases. Earlier results by Srivastava *et al* (25) and results obtained here for compounds **5** and **6** suggest that recognition may be prevented by the steric bulk of the added βGlcNAc(1→4) residue rather than by disruption of some specific interaction with the 4-OH group, and that this same type of steric interference is not provided by the benzyl groups of **6**.

Effects of substitutions at the αMan(1→6) Residue: Deoxygenation of the reactive 6'-OH group of the GlcNAcT-V acceptor was previously shown to be an effective strategy for providing a competitive inhibitor for this enzyme, with K_i one-third of K_m for enzyme from BHK cells (27), and K_i almost twice K_m for enzyme extracted from hamster kidneys (28). This strategy was also found to be successful for four glycosyltransferases out of the eight glycosyltransferases for which it was tested (28). The implication is that, for GlcNAcT-V and several other glycosyltransferases, there is no essential hydrogen-bonding interaction between the enzyme active site and the reactive hydroxyl group. Substitution of the 6' reactive hydroxyl with either a fluorine or a hydrogen gave inhibitors **11** and **12** with essentially the same K_i's (30 and 24 μM, respectively), whereas O-methylation gave a slightly less potent inhibitor (**10**) with a K_i of 71 μM (Table 3.2). A useful consequence of this study is that any compound recognized as an acceptor by this enzyme can probably be re-synthesized as an inhibitor, with a K_i comparable to the K_m, by removing the reactive hydroxyl group.

The 4'-O-methyl-trisaccharide **8**, which still contains the target 6'-OH group to which GlcNAcT-V transfers, was initially tested as an acceptor and found to be completely inactive. The assays were performed under conditions that would have detected less than 1% of the activity of the standard non-methylated acceptor **3**. The simplest explanation was that the 4'-O-methyl group simply prevented trisaccharide **8** from binding to the enzyme, but testing of this compound as an inhibitor of GlcNAcT-V showed it to be a competitive inhibitor of the binding of **3** with a K_i of 14 ± 2 μM (Figure 3.3**A**; Table 3.2).

It was possible that the binding of UDP-GlcNAc was somehow affected by the presence of the 4'-O-methyl group immediately adjacent to the reactive 6'-OH group in **8**, so enzyme kinetics were evaluated in the presence and absence of **8** at a fixed, sub-saturating concentration of acceptor **3** over a range of UDP-GlcNAc concentrations (Figure 3.3**B**). If **8** had any effect on the randomly-ordered binding of UDP-GlcNAc to the enzyme active site, it should have manifested itself as an alteration (i.e. an increase) in the $K_{m,app}$ for UDP-GlcNAc in the presence of the 4'-O-methyl analog. However, this value remained unchanged (700 ± 50 μM compared with 660 ± 50 μM) in the presence of **8** (80 μM), whereas the V_{max} decreased from 152 to 55 pmol/hr. This is the behaviour expected if **8** were an inhibitor which was non-

competitive with the donor UDP-GlcNAc.

That **8** was inactive as an acceptor, but still bound tightly to the enzyme, could also have been explained by implicating the free 4'-OH group of **3** directly in the catalytic mechanism. Alternatively, this OH group might be an essential recognition element somehow interacting (e.g. hydrogen-bonding) with the enzyme and allowing the reorganization of the active site towards the transition-state for glycosyl transfer. These possibilities were tested by synthesizing the 4'-deoxy analog **9** where the 4'-OH group had been removed. Deoxy-trisaccharide **9** was an excellent acceptor for GlcNAcT-V with a K_m 3-fold higher than the parent hydroxylated compound **3** and almost five-fold higher V_{max} (Table 3.2). The 4'-OH group in **3** is therefore not required for the transfer reaction to occur. In fact, the transfer reaction is five times faster without this group present.

The possibility that deoxygenation of the 4'-OH group affected(i.e. enhanced) the enzyme's ability to bind UDP-GlcNAc was eliminated by measuring GlcNAcT-V reaction rates over a range of concentrations of both donor and acceptor **9**. Data obtained were fit to the velocity equation for a rapid-equilibrium random bireactant mechanism in order to obtain a K_m for UDP-GlcNAc which could be compared with the value determined using the unsubstituted trisaccharide acceptor **3**. The two constants were not significantly different: $K_m = 1.1 \pm 0.2$ mM with normal acceptor **3** and 1.4 ± 0.2 mM for UDP-GlcNAc with the 4'-deoxy acceptor **9** (data not shown). The increased rate of transfer of GlcNAc to **9** therefore appears to result from a facilitated transfer process of the bound reactants.

The kinetic data reported for **9** (Table 3.2) were obtained from experiments that quantitated the formation of a radioactive product which was not structurally characterized. The GlcNAcT-V preparation used was only partly purified and it was therefore possible that **9** might have been a substrate for some other divalent cation-independent GlcNAc-transferase. If **3** and **9** were indeed substrates for different enzymes, then incubations containing mixtures of these acceptors should result in increased rates of radioactive-product formation. Reaction velocities should be calculable from their kinetic constants in Table 3.2, using Equation 4 (presumes two transferases acting independently) and Equation 5 (for the case of a single enzyme acting on two acceptors). Incubation of equimolar subsaturating concentrations of **3** and **9** (30 and 60 μM) with the GlcNAcT-V preparation yielded reaction rates compatible only with the presence of a single enzyme (Table 3.1).

In addition, the 6-deoxy-trisaccharide **11** inhibited the transfer to both **3** and **9**. Using acceptor concentrations of 30 μM, in the presence of **3** (80 μM), transfer to **3** and **9** were inhibited by 88 and 55%, respectively. Taken together, these data indicate that both **3** and **9** are substrates for a single enzyme.

The minimum energy solution conformation of **3**, and the conformation that appears to be acted upon by GlcNAcT-V, have been deduced through an NMR study of a series of conformationally-restricted oligosaccharides (32). The NMR data for compounds **8** and **9** confirm that substitutions at C-4', whether O-methylation or deoxygenation, do not cause any serious alterations compared with the conformation deduced for the parent trisaccharide **3**. Since the deoxy-trisaccharide **9** remains a good substrate for GlcNAcT-V, OH-4' cannot be involved in catalytically-important recognition by this enzyme. The 4'-methoxy analog **8** still binds to the enzyme, as evidenced by its low K_i; yet it does not interfere with the binding of UDP-GlcNAc. The ternary complex [GlcNAcT-V]-[UDP-GlcNAc]-[**8**] forms but does not react because of the presence of the added methyl group. We suggest that the steric bulk of this added methyl group precludes the transfer reaction either by preventing a necessary re-orientation of substrates within the active site, such as movement of the two bound substrates towards each other, or by preventing a protein conformational change required for catalysis. Such a conformational change might be essential either for activation of the pyrophosphate leaving group or for deprotonation of the reacting 6'-OH group, or for both. The fact that a fairly conservative substitution such as addition of a methyl group can absolutely block transfer to the free 6'-OH suggests that the orientation or re-orientation of reacting groups is very precise and rigid relative to the neighboring 4'-OH group.

The dramatic effects obtained in the study of compounds **8** and **9** suggests that this might be a useful approach for development of inhibitors for other glycosyltransferases, particularly where the deoxygenation approach has failed (28). These results also suggest that effects of substitutions at positions in some proximity to the reactive hydroxyl group of an acceptor should be evaluated carefully, particularly if there is an effect on reaction rate and particularly if different substitutions at a single position give very different results. For example, 3'-OMe analog **7** has a higher K_m but half the V_{max} of **3**, and so the question arises as to whether this group also has an effect, albeit slight, on the actual transfer reaction to the 6'-OH.

Effects of substitutions at the GlcNAc Residue: Finally, effects of some substitutions on the GlcNAc residue have been evaluated, and it is at this non-reducing part of the acceptor that the only unequivocally critical hydroxyl groups are located (33). Replacement of the NHAc of the GlcNAc residue with a hydroxyl group produced a surprisingly good acceptor **13** (Table 3.2). The 4 position of this sugar was expected to be important because it was known from the original work with this enzyme that galactosylation at this position blocked transfer by GlcNAcT-V (9). 4"-substituted compounds **16-21** were found to be only weakly active as substrates, and it was difficult to achieve either saturation of the enzyme or more than 1% turnover. The rate of transfer to these compounds was also decreased after 2 hours. These observations suggested the presence of small amounts of contaminating acceptor **3**, potentially present since all of compounds **16-21** were derived from the same synthetic precursor where the "normal" 4-OH group was present. In order to detect and quantitate **3** in **16-21**, these compounds were reacted with commercially-available bovine milk β(1→4)galactosyltransferase and UDP-^{3}H-galactose. Less than 2.6% of each acceptor **16-21** was radiolabelled in each case (This is lower than the limit detectable by ^{1}H-NMR), with negligible amounts present in HPLC-purified compounds **19** and **20**, as expected.

In order to evaluate compounds **16-21** as GlcNAcT-V substrates, then, each was pre-treated similarly with galactosyltransferase in the presence of unlabelled UDP-galactose in order to galactosylate small amounts of contaminating **3**, and then the resulting products were evaluated as acceptors for GlcNAcT-V by subsequent addition of this enzyme with radiolabelled donor as usual. Decreases in activity of between 55-95% were indeed observed after the galactosylation reactions, but the activities were not completely abolished. If these decreases in activity were in fact due to the presence of contaminating **3**, then the level of contamination could be estimated from the kinetic parameters for **3** and those for analogs **16-21** (Table 3.2) to be under 1%. Had contaminating **3** been the sole acceptor substrate in **16-21**, the V_{max} values for all the compounds should have approached the same value, assuming no inhibition by any of the acceptors or products formed. Since the V_{max} values differ over 100 fold for galactosyltransferase-treated acceptors (Table 3.2), the presence of **3** cannot be solely responsible for the observed very weak activities.

The kinetic parameters eventually obtained for **16-21** show all six 4"- analogs to be extremely poor acceptors with K_m values between

30-300 fold higher than **3** and V_{max} values between 7-105 fold slower. V_{max}/K_m values for these analogs, indicators of enzyme efficiency, were between 0.12 and 0.02% of the value obtained for **3**. The 4-OH group of the GlcNAc residue in **3** is therefore a critical recognition element for this enzyme active site (33). Galactosylation of **1** (and **3**) therefore destroys the acceptor activity of these compounds not by introducing the steric bulk of an additional sugar residue but by disrupting a critical H-bonding interaction (or network) between the 4-OH group and the enzyme combining site. This 4-OH group of the terminal GlcNAc residue, according to the classification of Lemieux (33), can therefore be designated as a key polar group in the recognition of substrates by GlcNAcT-V.

3"-substituted analogs **14** and **15** were similarly found to be poor acceptors for GlcNAcT-V (Table 3.2). 6"-substituted analogs were found by GlcNAcT-V and bovine galactosyltransferase labelling experiments to contain between 3 and 6.5% **3**, as expected from the synthetic methods used. No evidence of transfer by GlcNAcT-V to these 6"-substituted analogs could be obtained. Although the possibility remains that other functional groups than the NH_2 and NHAc may be tolerated at the 3"- and 6"-positions, the similar results obtained for the analogous 4"-substituted acceptors **19-20** suggest that the 3"- and 6"-hydroxyls may also be designated as key polar groups.

In summary, every hydroxyl of the GlcNAcT-V acceptor **3** has been replaced with at least one different functional group to give a "map" of the acceptor binding site shown in Figure 3.4. Recognition of the two reducing-end residues seems to rely mainly on hydrophobic interactions since no substitutions of specific hydroxyl groups on these mannose residues seriously impaired the enyzme's ability to recognize the resulting trisaccharide, whereas removal of the reducing-end β-mannose residue gave an acceptor with dramatically-increased K_m. Replacement of hydroxyl groups of the GlcNAc residue were found to not be tolerated, and, in particular, none of the functional groups H, F, NH_2, NHAc, OMe or $OH_{(axial)}$ could replace the 4"-OH group. 6"-substituted analogs were not substrates at all, suggesting that the enzyme's inability to tolerate substitutions at nearby 3"- and 4"-positions might arise partly from disruption of one or more intramolecular interactions between two or three of these groups. As observed previously, substitutions at the reactive 6'-OH group were well-tolerated by GlcNAcT-V, giving rise to enzyme inhibitors which were competitive versus acceptor **3**. It was demonstrated by different substitutions of the 4'-OH that the transfer reaction could be

dramatically affected by nearby functional groups. Specifically, recognition of **8** by GlcNAcT-V was almost identical (judging by K_i) to that of **3**, but GlcNAc transfer was completely prevented by the presence of the O-Me group at the 4'-position even though the reactive 6'-OH was free. None of the modifications tested in this study showed promise of any profound improvements in enzyme-substrate recognition of the sort required for tight-binding, but in terms of inhibitor and drug design, there are apparently many positions at which different types of substitutions can be made to alter the properties of a potential GlcNAcT-V inhibitor.

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Chapter 4.
Bisubstrate Analog Inhibitors of *N*-Acetylglucosaminyltransferase V

Introduction

Glycosyltransferases catalyze the transfer of a monosaccharide from an activated sugar nucleotide donor to the hydroxyl group of a sugar, shown as a general reaction in Figure 4.1. In the glycosylation of mammalian *N*-linked and *O*-linked glycoproteins, individual transferases are specific for a particular sugar-nucleotide donor, for the type and the position of the linkage formed, and for certain elements of the acceptor which usually encompass at least one sugar residue (1). A cell's repertoire of glycosyltransferase activities determines, to a large extent, the carbohydrate structures present on its surface glycoproteins (2,3); and variations in glycosylation can have very profound effects on the biological activity and stability of some proteins (4-6). Because many glycoproteins occur at the cell surface, glycosyltransferase activities can affect the external phenotype of the cell, as demonstrated by the use of lectins to select transferase-deficient mutants (7), and by the use of lectins and carbohydrate-specific antibodies to isolate cells expressing products of certain glycosyltransferases (8). Similarly, introduction of a single glycosyltransferase gene has been shown to permit expression of a specific type of carbohydrate, the ELAM-1 ligand, at the surfaces of cells which previously lacked this structure (9, 10). Also, it has been found that drugs such as swainsonine can affect expression of cell-surface carbohydrates by interrupting the normal processing of *N*-linked oligosaccharides, making certain substrates unavailable to glycosyltransferases which act later in the overall biosynthetic scheme (Reviewed in 11, 12 and 13). Glycosyltransferases have also been used as reagents to alter carbohydrates on the surfaces of cells *in vitro* (14).

N-acetylglucosaminyltransferase V (GlcNAcT-V; E. C. 2.4.1.155) catalyzes the transfer of GlcNAc from UDP-GlcNAc to the α(1→6)mannose of *N*-linked oligosaccharides (1), forming a β(1→6) linkage as illustrated in Figure 4.2 (15). *In vivo*, this transfer reaction follows those of GlcNAc transferases I and II which act in the medial Golgi to form the first two out of a possible five GlcNAc branches on the trimannoside core of asparagine-linked glycoproteins (16). Synthetic trisaccharide acceptor **3** (Figure 4.3) was designed as a minimum tructure required by the enzyme for recognition, and has

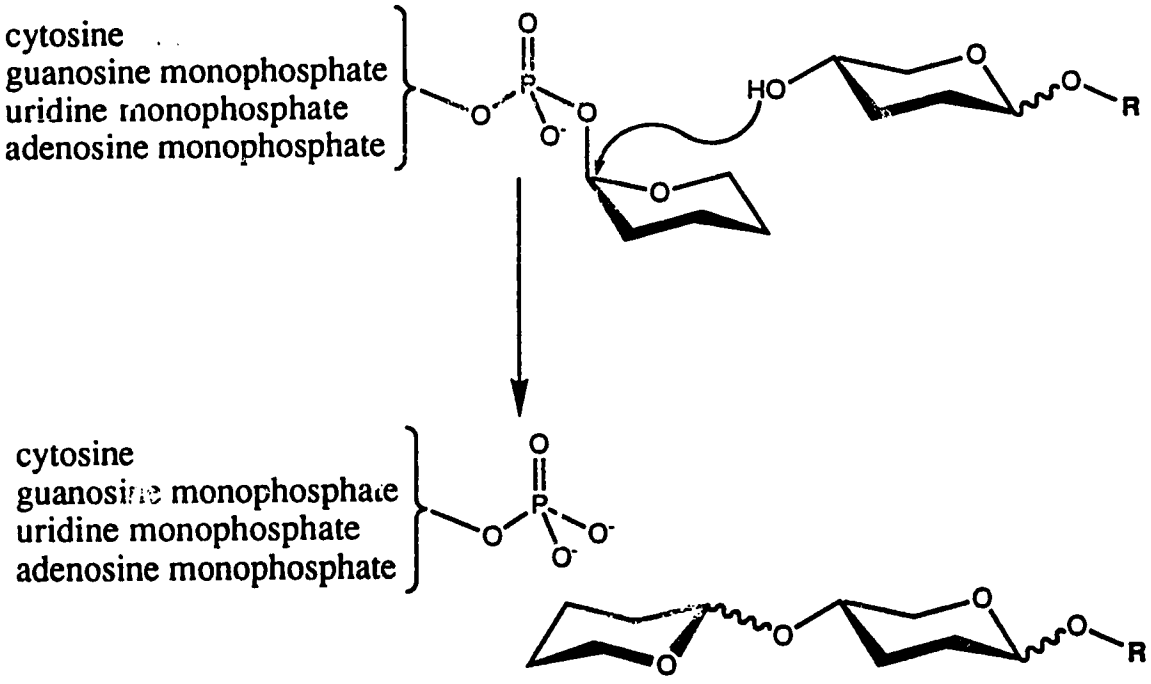


Figure 4.1: Generalized glycosyltransferase-catalyzed reaction between an activated sugar-nucleotide donor and an unspecified carbohydrate acceptor.

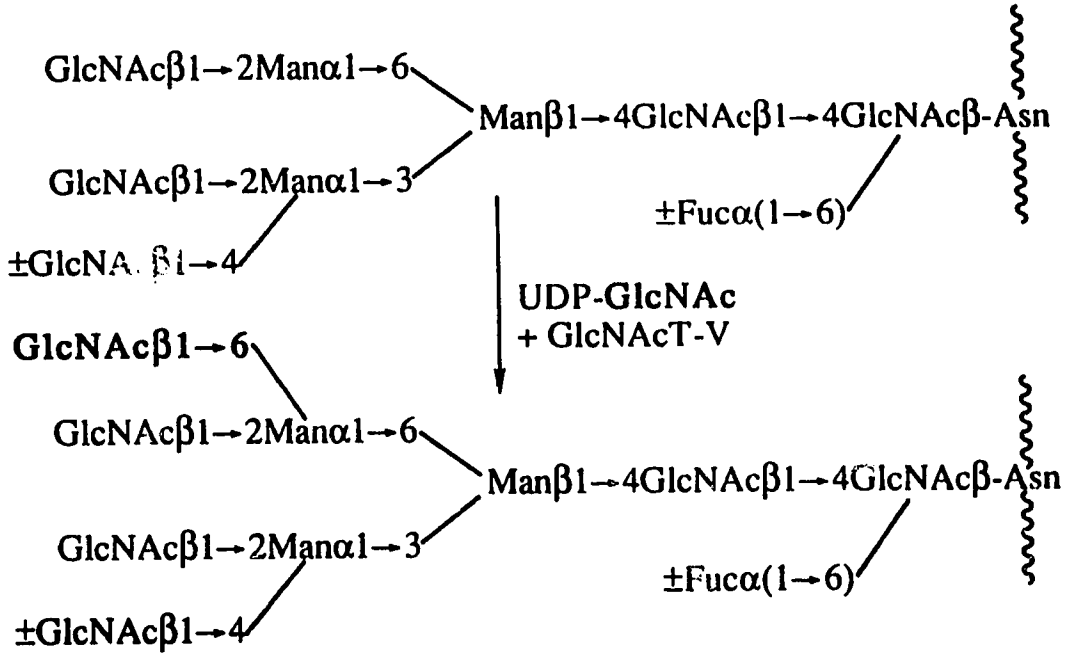
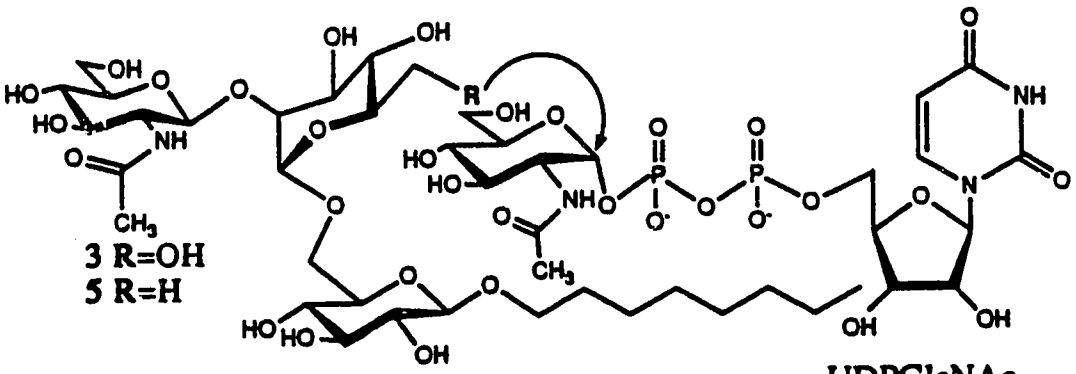


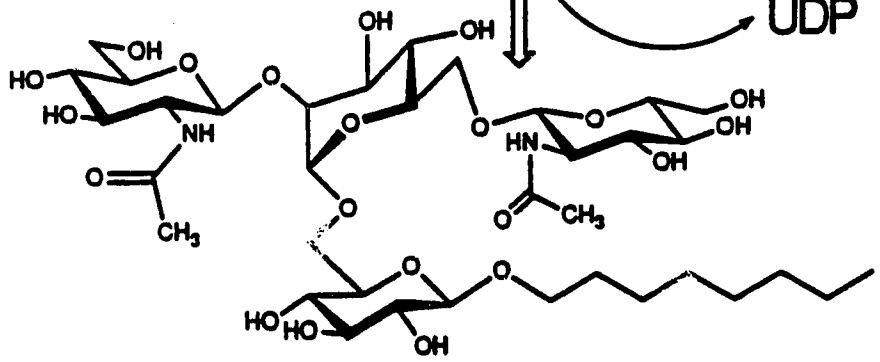
Figure 4.2: *In vivo* transfer of GlcNAc from UDP-GlcNAc to the αMan(1→6) of a biantennary (minus βGlcNAc(1→4)) or triantennary (plus βGlcNAc(1→4)) asparagine-linked oligosaccharide.



3 GlcNAcβ(1→2)Manα(1→6)Glcβ-$(CH_2)_7CH_3$

GlcNAcT-V

UDP



4 GlcNAcβ(1→2)[GlcNAcβ(1→6)]Manα(1→6)Glcβ-$(CH_2)_7CH_3$

Figure 4.3: Reaction catalyzed by GlcNAcT-V with synthetic trisaccharide acceptor **3**, to generate product **4**. Deoxygenated trisaccharide **5** is a competitive inhibitor for this enzyme.

been used to develop a radioassay, taking advantage of the hydrophobic character of the aglycone for adsorption of labelled tetrasaccharide product **4** on reverse-phase cartridges (Figure 4.3) (17-19).

Interest in controlling GlcNAcT-V activity has arisen from investigations which have shown positive correlations between the malignant transformation of cells and levels of both GlcNAcT-V activity and its βGlcNAc(1→6)αMan(1→6) branch product, but the underlying mechanism(s) of action of this and other transformation-associated glycosyltransferases is unknown (20-22; reviewed in 23-25). However, because of the βGlcNAc(1→6) branch preference exhibited by the apparently-limiting βGlcNAc(1→3) "i" transferase involved in their initiation and extension, GlcNAcT-V is postulated to play a critical role in the elaboration of poly-*N*-acetyllactosamine linear repeating carbohydrate structures (26-28).

A number of different strategies have been tested for specific inhibition of glycosyltransferases in general and GlcNAcT-V in particular, based on modifications to acceptor and donor molecules. The design and synthesis of potential inhibitors of glycosyltransferases has been recently reviewed in Reference 13. Deoxygenation of the reactive 6'-hydroxyl group of synthetic trisaccharide **3** provided the first acceptor-based inhibitor **5** for GlcNAcT-V (Figure 4.3) (29); and this also proved to be a useful general strategy for certain other glycosyltransferases (30). A very effective refinement of this deoxygenated inhibitor was achieved by conformational restraint of the flexible αMan(1→6) linkage of **5**[1], suggested by previous work of Lindh and Hindsgaul (31), and steric blocking of the transfer reaction has been achieved by addition of a single methoxy substituent at the 4'-position neighboring the reactive 6'-OH of **3** (32).

Some other strategies for acceptor-based design of glycosyltransferase inhibitors which have appeared in the recent literature have involved incorporation of photo-activated functional groups to form covalent bonds within the glycosyltransferase active site (33-35), and replacement of normal sugar residues of glycosyltransferase acceptors with aza sugar analogs (36,37). Kajihara *et al* recently reported that the substitution of a sulfhydryl group at a position neighboring the reactive hydroxyl group of an acceptor produced a slow substrate which was also a good competitive inhibitor for bovine β(1→4)galactosyltransferase (38).

A bisubstrate analog inhibitor of α(1→2)fucosyltransferase with

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[1] I. Lindh, S. Crawley, M. Palcic, and O. Hindsgaul, unpublished results.

combined elements of acceptor and sugar-nucleotide co-substrate has also been synthesized and tested (39). This inhibitor was comprised of an acceptor moiety (phenyl-galactose) joined by an ethylene bridge from the reactive hydroxyl group to the β-phosphoryl oxygen of sugar-nucleotide analog GDP. It was found to be a competitive inhibitor versus both of the transferase's substrates, with a K_i the same order of magnitude as that of GDP (39). These results implied that the enzyme reaction involved random-order binding of substrates (40). The fact that there was no dramatic enhancement of the affinity of the bisubstrate analog for the enzyme suggested that any entropic advantage derived from having elements of the enzyme's two substrates joined into one molecule were apparently off-set by sub-optimal distances, orientations or individual interactions. However, this work also demonstrated that the attachment of a nucleotide to an acceptor with lower apparent affinity for the enzyme active site could enhance the latter's binding to the enzyme active site; thus, a bisubstrate analog can potentially lend specificity to otherwise non-specific nucleotide-based inhibitors.

The multisubstrate analogue approach to inhibitor design has been successful for a number of other bisubstrate enzymes, for example adenylate kinase (41,42), where P^1, P^4-di(adenosine-5')tetraphosphate and P^1, P^5-di(adenosine-5')pentaphosphate and their derivatives showed K_i<10μM (versus 0.3 mM K_m for ATP) and were used to demonstrate random versus ordered binding of substrates. Similar bisubstrate adducts are also inhibitors of deoxynucleoside kinases (43), displaying similarly low K_i values which may be compared with 85 μM K_m for deoxycytidine. β-thioglycinamide ribonucleotide 5,8-dideazafolate has been designed as a mimic of the glycinamide ribonucleotide/ tetrahydrofolate reaction intermediate of glycinamide ribonucleotide transformylase. It has a dissociation constant of 250 picomolar and displays slow, tight-binding inhibition (44). Other enzymes for which sucessful multisubstrate analogue inhibitors have been designed include L-aspartate transcarbamoylase (inhibited by phosphonacetyl-L-aspartate), methionine adenosyltransferase (inhibited by a $(PO^-_3)_3$-NH-S-adenosylmethionine adduct), thymidylate synthase (inhibited by 5-deazafolate derivatives of 2'-deoxyuridine 5'-monophosphate), and dopamine β-hydroxylase, which is inhibited by an adduct of a 3, 4-difluoro-3-hydroxy-*N*-benzyl moiety (tyrosine mimic) and imidazolethione (oxygen mimic). These are reviewed in Reference 45.

Based on the simple model of a single-displacement transfer reaction, two bisubstrate analogs of the GlcNAcT-V reaction

intermediate have been designed and synthesized (Figure 4.4)[2]. Each analog is comprised of a trisaccharide moiety identical to **3** joined to UDP by an ethylene bridge extending from the 6'-oxygen to the β-phosphate, forming either a phosphonate linkage to the phosphorous (**5**), or an phosphoester linkage to oxygen (**6**) (Figure 4.4). We report here the kinetic evaluation of these two bisubstrate analogs as inhibitors of the GlcNAcT-V-catalyzed reaction.

Experimental and Results

Materials and Methods: Triton X-100, UDP, bovine serum albumin, and UDP-GlcNAc were obtained from Sigma Chemical Co. UDP-6-^{3}H(N)-GlcNAc (6 or 25 mCi/mmol) was purchased from NEN DuPont or American Radiolabeled Chemicals Inc. Background adsorption of radiolabel to C18 Sep-Pak Plus cartidges (Waters) was reduced by raising the pH of the MilliQ water used for washing steps by addition of approximately 5 ppm NH_3, and/or by passing radiolabeled UDP-GlcNAc in water through a pre-equilibrated C18 Sep-Pak cartridge prior to use in assays. Ecolite (+) scintillation cocktail was obtained from ICN. GlcNAcT-V was partially purified from frozen hamster kidneys (Keystone Biologicals, Cleveland, Oklahoma) essentially as described previously (32). The crude Triton X-100 detergent extract of a kidney acetone powder was chromatographed on a UDP-hexanolamine Sepharose column (7 μmol/mL ligand; 4.5 mL bed volume) which was washed with 40 mM sodium cacodylate buffer (pH 6.5) containing 8 mM EDTA, 20% glycerol (v/v), and 0.1% Triton X-100. GlcNAcT-V used in the present studies was eluted with 45 mL of the same buffer containing 0.1 M NaCl and 4 mM UDP; this eluate was ultrafiltered to 1 mL final volume using a YM30 membrane (Amicon) and dialyzed versus 50 mM sodium cacodylate, pH 6.5, containing 20% glycerol, 1 mM EDTA, 0.1 M NaCl and 0.1% Triton X-100. This extract had a specific activity of 0.2 mU/mg protein and was used for all experiments unless otherwise stated. Protein was measured using a bicinchoninic assay kit from Sigma with bovine serum albumin as the protein standard. GlcNAcT-V activity was assayed at 1 mM UDP-GlcNAc and 264 μM **3**.

Compounds **5** and **6** had very similar but distinct ^{1}H-NMR spectra which included the following signals for **5** (D_2O, 360 mHz): δ 4.872 (d, J≈1.5 Hz, H-1'), 4.562 (d, J=8.5 Hz, H-1"), 4.437 (d, J=8.0

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2 I. Lindh and O. Hindsgaul (to be published elsewhere).

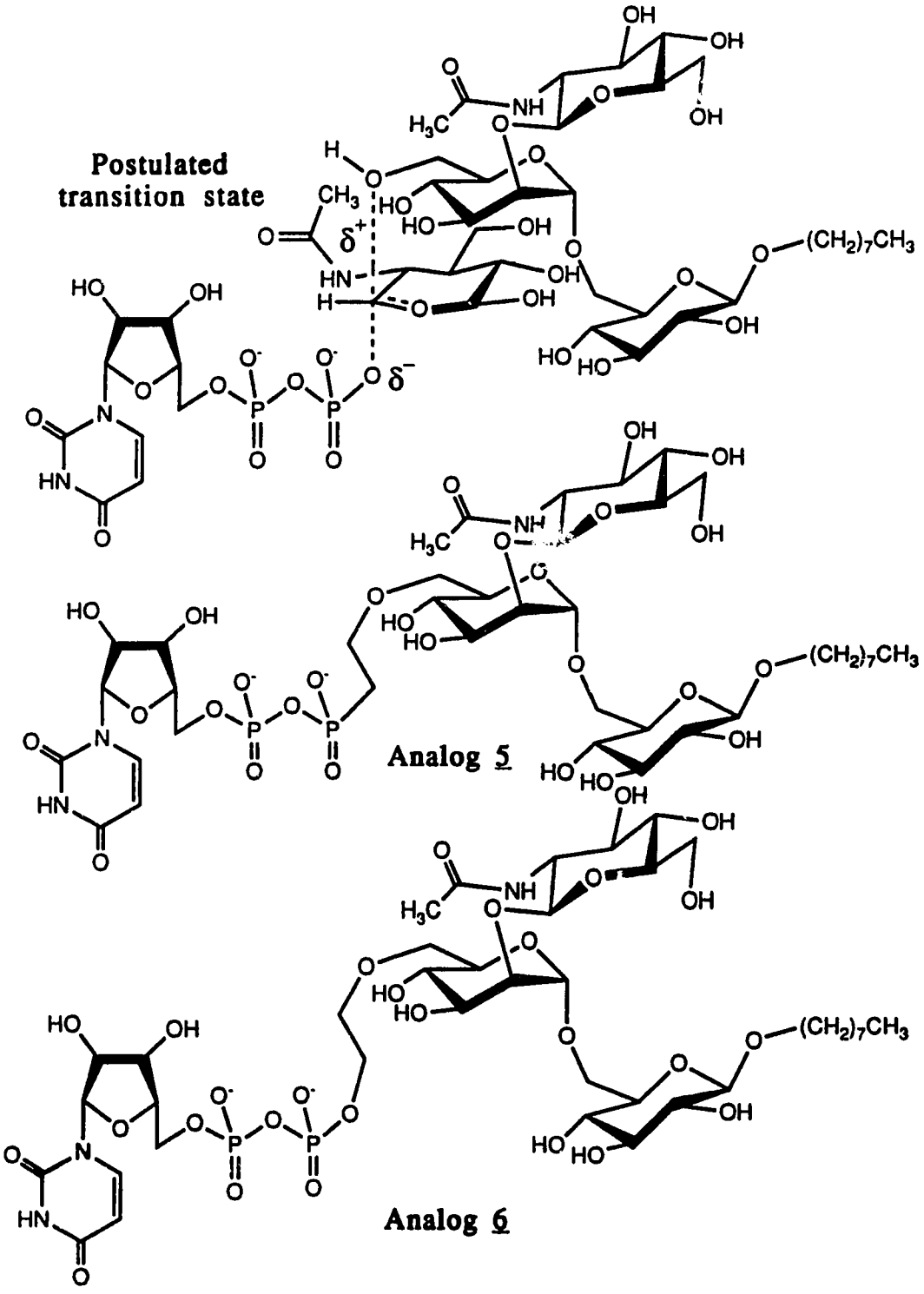


Figure 4.4: Structures of bisubstrate analog inhibitors **5** and **6**, with a possible transition-state intermediate included for comparison (top).

Hz, H-1). For **6**, signals included (D_2O, 360 mHz): δ 4.868 (d, J≈1.5 Hz, H-1'), 4.565 (d, J=8.5 Hz, H-1''), 4.440 (d, J=8.0 Hz, H-1). The ^{1}H-NMR spectrum of **5** was readily differentiated by signals for the CH_2-P methylene protons which resonated as a complex ^{31}P-coupled multiplet at 2.20-2.05 ppm.

Kinetic Evaluations:

A. *Enzyme Substrate Kinetics*: The dependence of reaction velocity on the concentration of both substrates was evaluated over a range of concentrations of both acceptor **3** (6 to 264 μM) and UDP-GlcNAc (0.125 to 6 mM; 60,000 dpm/nmol). Acceptor and donor substrates were lyophilized together in 500 uL plastic microfuge tubes, 1.1 μU of GlcNAcT-V (diluted 10-fold in 50 mM sodium cacodylate buffer, pH 6.5, containing 20% glycerol, 10 mM EDTA, 0.1% Triton X-100 detergent and 1 mg/mL bovine serum albumin,) was added to give a final volume of 10 μL. Tubes were vortexed and centrifuged briefly before incubating at 37°C for 30 minutes. Reactions were quenched by addition 0.5 mL of 32 mM EDTA-4Na$^+$ and kept below 0°C until product isolation. Radiolabelled product **4** was separated from radiolabeled UDP-GlcNAc using C18 Sep-Pak cartridges by transferring each reaction mixture (with 5 ppm NH_4OH in water) onto a Sep-Pak cartridge pre-equilibrated with methanol, water and dilute NH_4OH; the cartridge was washed with same to remove unreacted radiolabel (60 mL), then with 10 mL water, and finally unreacted **3** and labeled product **4** were eluted slowly with 2 X 3 mL methanol (19). Reaction velocities were calculated from the number of disintegrations per minute (dpms) in this methanol elution, subtracting blank values obtained from incubations without acceptor. Despite precautions, blank values increased linearly with increasing amounts of added UDP-GlcNAc, so a standard curve of background dpm versus amount of added UDP-GlcNAc was constructed from individual blanks and this was used to calculate blank values for different donor concentrations. Unweighted values for enzyme reaction rates were fit to Equation 1 for a rapid-equilibrium random bireactant mechanism (43) using a nonlinear regression analysis microcomputer program (SigmaPlot^R, MacIntosh version, obtained from Jandel Scientific). (A = acceptor **3**; B = UDP-GlcNAc; K_a and K_b are Michaelis constants (K_m's) for A and B, respectively; α is the factor by which binding of A affects the dissociation constant for B and *vice versa* (43)).

$$\upsilon = \frac{V[A][B]/\alpha K_a K_b}{1 + [A]/K_a + [B]/K_b + [A][B]/\alpha K_a K_b} \quad (1)$$

B. *Enzyme Inhibitor Kinetics with Both Substrate Concentrations Varied*: The effects of inhibitors **5** and **6** on GlcNAcT-V recognition of acceptor **3** were investigated by evaluating substrate kinetics as described above, in the presence of 0.106 and 0.317 mM **5**, or in the presence of 0.106 and 0.315 mM **6**. A mixture of inhibitor and GlcNAcT-V was added as a 10 uL aliquot to each microfuge tube containing lyophilized donor and acceptor; tubes were incubated 45 to 110 minutes, and radiolabelled product isolation and quantitation carried out as described above. All rate data obtained from these assays were fit to Equations 2, 3, and 4, in order to determine the mode(s) of inhibition and kinetic parameters (43, 44) (I = inhibitor; K_i = inhibition constant; β = factor by which binding of acceptor to enzyme affects the dissociation constant of the inhibitor and *vice versa*; γ = factor by which binding of donor to enzyme affects the dissociation constant of the inhibitor and *vice versa).* Parameters obtained from these analyses are given in Table 4.1.

$$\upsilon = \frac{V[A][B]/\alpha K_a K_b}{1 + [A]/K_a + [B]/K_b + [I]/K_i + [A][I]/\beta K_a K_i + [B][I]/\gamma K_b K_i + [A][B]/\alpha K_a K_b} \quad (2)$$

$$\upsilon = \frac{V[A][B]/\alpha K_a K_b}{1 + [A]/K_a + [B]/K_b + [I]/K_i + [A][I]/\beta K_a K_i + [A][B]/\alpha K_a K_b} \quad (3)$$

$$\upsilon = \frac{V[A][B]/\alpha K_a K_b}{1 + [A]/K_a + [B]/K_b + [I]/K_i + [A][B]/\alpha K_a K_b} \quad (4)$$

Lineweaver-Burk plots of rate data as a function of substrate concentrations are provided in Figures 4.5 (compound **5**) and 4.6 (compound **6**), where a separate plot is shown for each substrate and each inhibitor concentration. The same data is replotted in Figures 4.7 and 4.8 for **5** and **6**, respectively, where separate lines correspond to different inhibitor concentrations, and separate plots correspond to different concentrations of fixed substrate. These plots demonstrate, in

Eqn.	Kinetic Parameter	Phosphonate Bisubstrate 5[a]	Phosphate Bisubstrate 6[a]	Phosphonate Bisubstrate 5[b]	Phosphate Bisubstrate 6[b]
2	β		10 ± 3		6 ± 2
	γ		23 ± 17		11 ± 6
	K_i		0.051 ±.006		0.07 ± .02
	V		166 ± 3		130 ± 2
	α		0.46 ± .06		0.6 ± .2
	K_b		1.7 ± .2		1.0 ± .2
	K_a		0.043 ± .004		0.039 ± .007
	norm		14.2		54.5
3	β	38 ± 50	13 ± 4	28 ± 20	10 ± 4
	K_i	0.11 ± .01	.045 ± .004	0.09 ± .01	0.048 ± .009
	V	166 ± 2	165 ± 2	134± 2	130 ± 2
	α	0.49 ± .07	0.47 ± .06	0.8 ± .1	0.8 ± .2
	K_b	1.6 ± .2	1.6 ±.2	0.8 ± .1	0.8 ± .2
	K_a	0.042 ±.005	0.043 ± .004	0.033 ± .004	0.035 ± .007
	norm	17.7	14.5	33.0	55.92
4	K_i	0.10 ± .01		.073 ± .002	
	V	166 ± 2		134.2± .4	
	α	0.50 ±.07		1.01 ± .04	
	K_b	1.6 ± .2		0.75 ± .02	
	K_a	0.041 ± .004		0.0283 ± .0008	
	norm	17.8		8.0	

Table 4.1: Kinetic parameters obtained from fit of rate data to Equations 2-4.
a. Rates were measured over a range of concentrations of both substrates at 0, 0.1 and 0.3 mM 5 or 6.
b. Rate data were obtained from experiments in which non-varied substrate was subsaturating and inhibitor concentrations ranged from .1 to .4 mM.

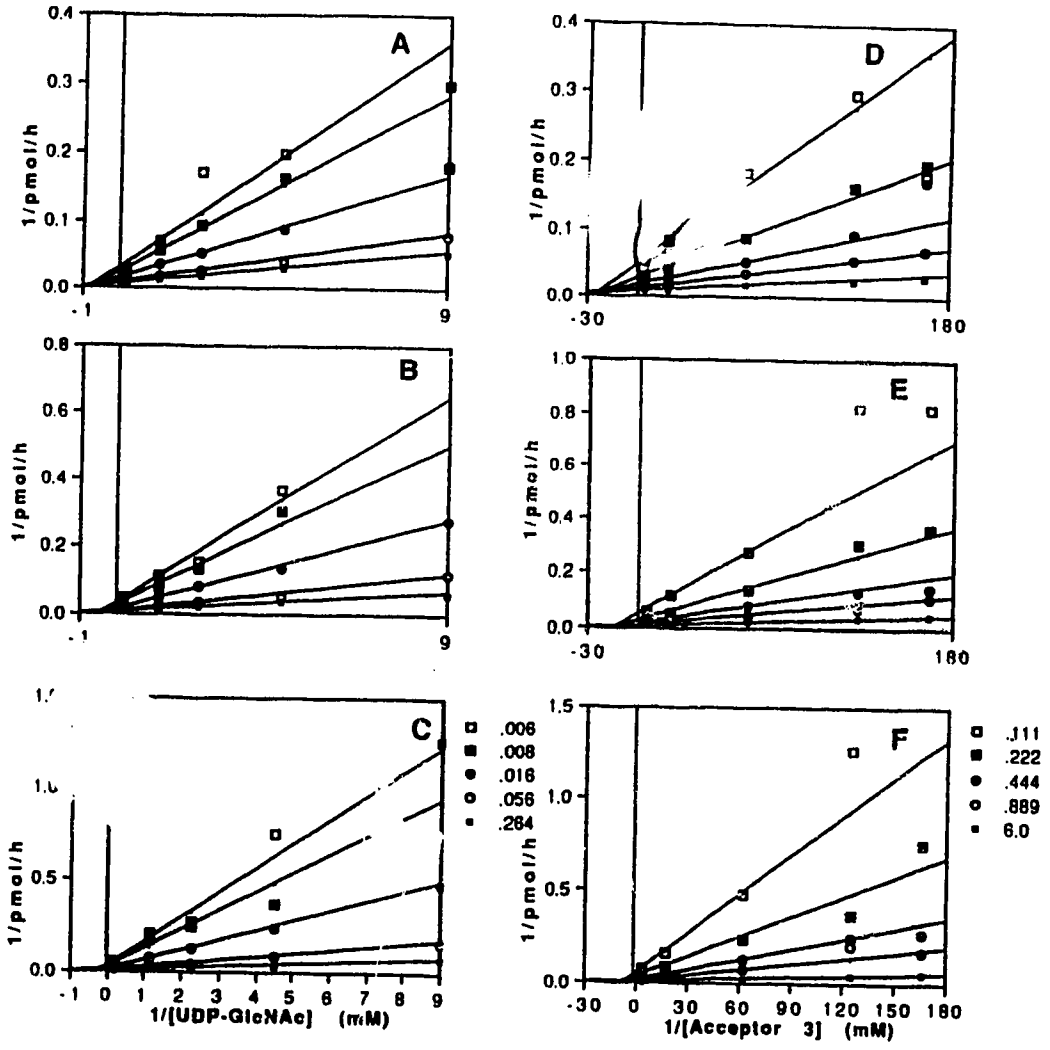


Figure 4.5: Inverse plots of rate data obtained from evaluation of inhibitor **5**, with concentrations of both UDP-GlcNAc (**A-C**) and acceptor trisaccharide **3** (**D-F**) varied as described in **Experimental** section **B**. Individual lines represent different concentrations of the fixed substrate, with millimolar concentrations indicated in a legend for each set of 3 plots. Lines were generated using theoretical values for velocity generated by the SigmaPlot^R program from curve fitting to Equation 4. Inhibitor concentrations were 0 (**A** and **D**), 0.106 (**B** and **E**), and 0.317 (**C** and **F**) mM.

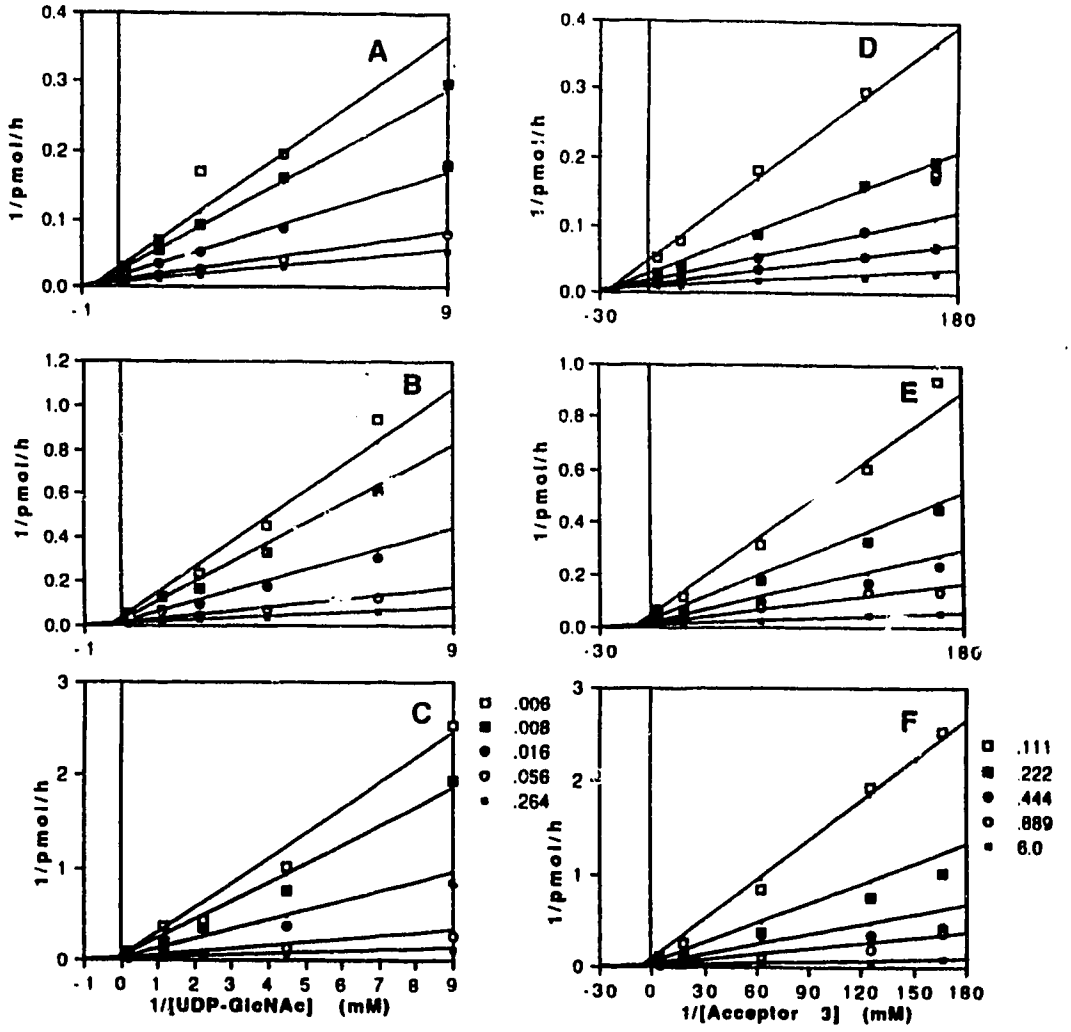


Figure 4.6: Inverse plots of rate data obtained from evaluation of inhibitor **6**, with concentrations of both UDP-GlcNAc (**A-C**) and acceptor trisaccharide **3** (**D-F**) varied as described in **Experimental** section **B**. Individual lines represent different concentrations of the fixed substrate, with millimolar concentrations indicated in a legend for each set of 3 plots. Lines were generated using theoretical values for velocity generated by the SigmaPlot^{R} program from curve fitting to Equation 3. Inhibitor concentrations were 0 (**A** and **D**), 0.106 (**B** and **E**), and 0.315 (**C** and **F**) mM.

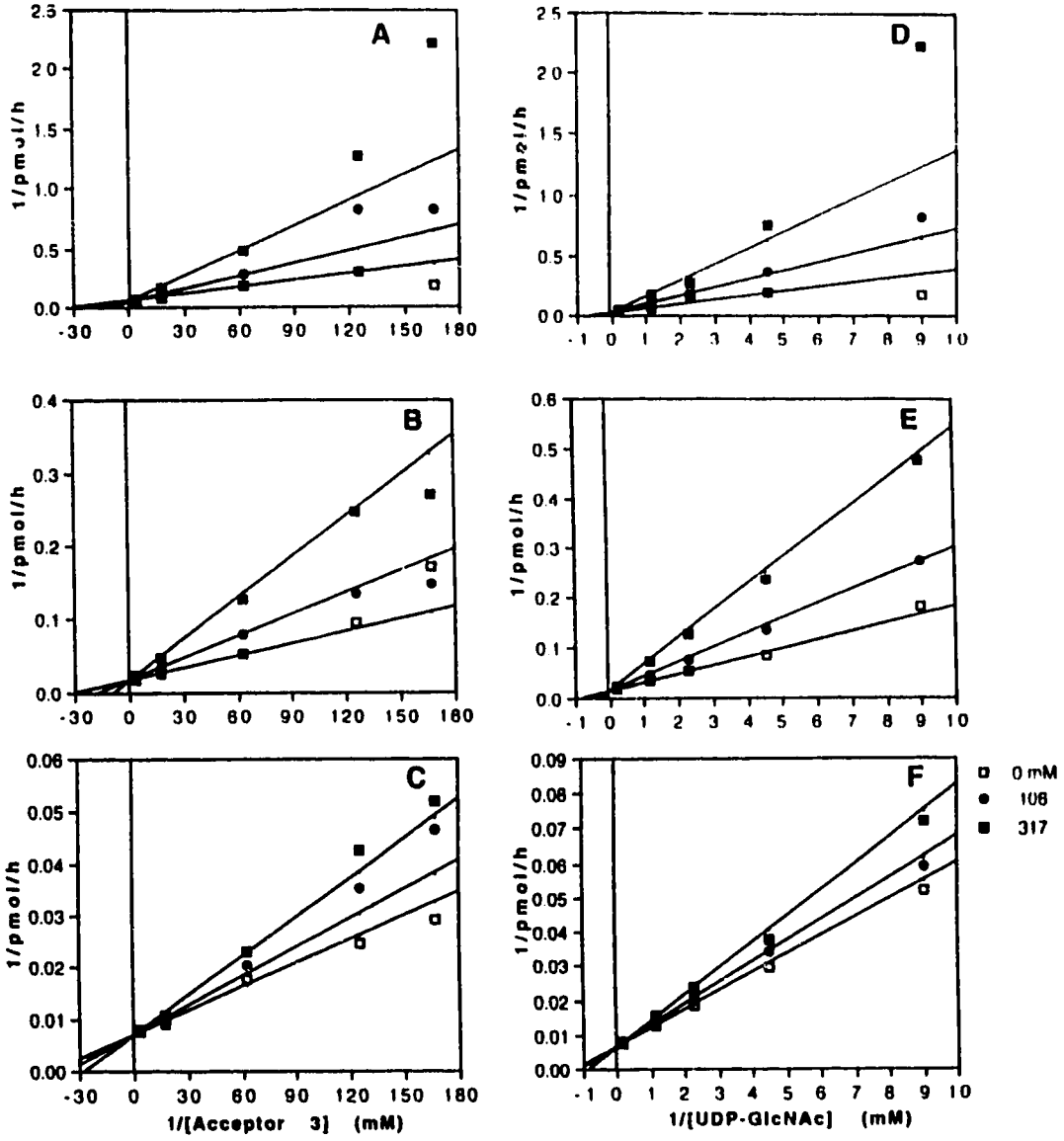


Figure 4.7: Selected inverse plots of rate data obtained from evaluation of inhibitor 5 (Experimental section B), where individual lines represent different concentrations of inhibitor 5 (millimolar concentrations indicated in the legend). Data from plots A, B, and C were obtained at [UDP-GlcNAc] = 0.111, 0.444, and 6.0 mM, respectively. Rates plotted in D, E, and F were obtained at [acceptor 3]=0.006, 0.016, and 0.264 mM. Lines were generated using theoretical values for velocity generated by the SigmaPlot^R program from curve fitting of experimental rate data to Equation 4.

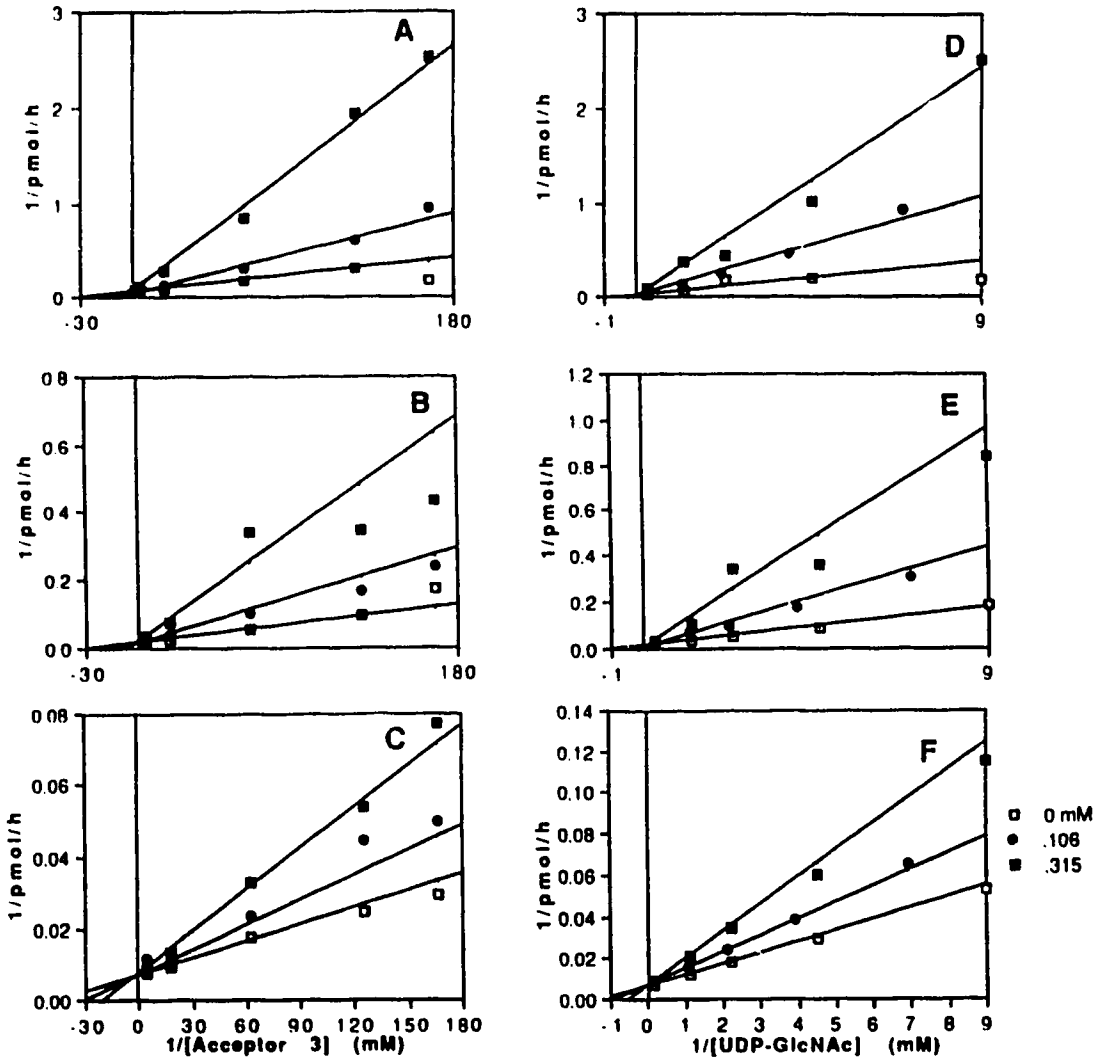


Figure 4.8: Selected inverse plots of rate data obtained from evaluation of inhibitor **6** (**Experimental** section **B**), where individual lines represent different concentrations of inhibitor **6** (millimolar concentrations indicated in the legend). Data from plots **A**, **B**, and **C** were obtained at [UDP-GlcNAc] = 0.111, 0.444, and 6.0 mM, respectively. Rates plotted in **D**, **E**, and **F** were obtained at [acceptor **3**]=0.006, 0.016, and 0.264 mM, respectively. Lines were generated by using theoretical values for velocity generated by the SigmaPlot^{R} program from curve fitting of experimental rate data to Equation 3.

a qualitative way, effects of increasing inhibitor concentration on y-axis intercepts ($1/V_{max,app}$) and x-axis intercepts ($-1/K_{m,app}$).

Data was further evaluted by fitting individual 5-point data sets to the Michaelis-Menten Equation 5, where the concentration of one substrate (S) is variable while inhibitor and alternate substrate concentrations are fixed at some value.

$$\upsilon = \frac{V_{max,app}[S]}{[S] + K_{m,app}} \qquad (5)$$

This provided experimental values for apparent K_m and V_{max} ($K_{m,app}$ and $V_{max,app}$), which are replotted (versus inhibitor concentration) as $K_{m,app}$, apparent (K_m/V_{max}) and $1/V_{max,app}$ in Figures 4.9 and 4.10 for inhibitor **5**, and in Figures 4.11 and 4.12 for inhibitor **6**. Standard errors (SE) of values for $K_{m,app}$ and $V_{max,\ app}$, which were obtained from curve fitting to Equation 5, were used to calculate errors (which are shown as error bars in Figures 4.9 to 4.12) by using values for SE to set upper and lower limits on the values of the various parameters. Thus, the error for apparent $1/V_{max}$ was simply estimated as

$$error_{(1/Vmax)} = 0.5*\{(V_{max}-SE_{Vmax})^{-1} - (V_{max}+SE_{Vmax})^{-1}\}$$

Similarly, error for apparent (K/V) was estimated as

$$error_{(K/V)} = 0.5*(K_{m,app}+SE_{Km})/(V_{max,app}-SE_{Vmax}) - (K_{m,app}-SE_{Km}/V_{max,app}+SE_{Vmax})$$

The values for these apparent kinetic parameters versus inhibitor concentration were also calculated from values for V_{max}, K_a, K_b, α, β, and γ (Table 4.1) using Equations 6 and 7 (44), and such values are represented as lines in Figures 4.8 to 4.12 to allow further, qualitative comparison with experimentally determined values (shown as data points). Separate figures have been provided for each variable substrate, and experimental values for kinetic parameters are compared with theoretical values (represented as lines) generated from both of the equations applied to data for each inhibitor (ie. either Equations 3 and 4 for inhibitor **5**; or Equations 2 and 3 for inhibitor **6**). The significance of the equations which were used, and significance of the parameters which were obtained from curve fitting, are described further under **Discussion**.

$$K_{m,app} = \frac{\alpha K_a(1 + [I]/\gamma K_i) + \frac{\alpha K_a K_b}{[B]}(1 + [I]/K_i)}{1 + (\alpha K_b/[B])(1 + [I]/\beta K_i)} \quad (6)$$

for A as the variable substrate and [B] constant

$$K_{m,app} = \frac{\alpha K_b(1 + [I]/\beta K_i) + \frac{\alpha K_a K_b}{[A]}(1 + [I]/K_i)}{1 + (\alpha K_a/[A])(1 + [I]/\gamma K_i)}$$

for B as the variable substrate and [A] constant

$$V_{max,app} = 1 + (\alpha K_b/[B])(1 + [I]/\beta K_i) \quad (7)$$

for A as variable substrate

$$V_{max,app} = 1 + (\alpha K_a/[A])(1 + [I]/\gamma K_i)$$

for B as variable substrate

C. *Enzyme Inhibitor Kinetics with one Substrate Concentration Constant and Subsaturating:* Effects of inhibitors on substrate kinetics were evaluated over a range of acceptor concentrations (6 to 560 μM **3**) in the presence of subsaturating UDP-GlcNAc (0.6 mM), to obtain apparent K_m values for **3** (ie. $K_{a,app}$) in the absence of inhibitors and in the presence of 0.11, 0.21, 0.32, and 0.42 mM concentrations of **5**, and 0.10, 0.20, 0.29, and 0.39 mM **6**. In all cases, the non-varied substrate was lyophilized and combined with enzyme and buffer to give a 10-fold dilution; aliquots of this mixture were transferred to tubes containing different amounts of lyophilized donor to give the indicated concentrations, and 10 μL aliquots of these mixtures, containing fixed substrate, inhibitor and GlcNAcT-V, were added to tubes containing different amounts of varied substrate (lyophilized). Assay mixtures were incubated for 50 to 110 minutes and then quenched and analyzed as described above. Rate data is presented as Lineweaver-Burk plots in Figure 4.13 (inhibitor **5**) and Figure 4.14 (inhibitor **6**).

Reaction velocities obtained over a range of substrate concentrations for individual inhibitor concentrations were also fit to the Michaelis-Menten equation (Equation 5) in order to obtain apparent K_m, V_{max} and K_m/V_{max} values, which are plotted versus inhibitor

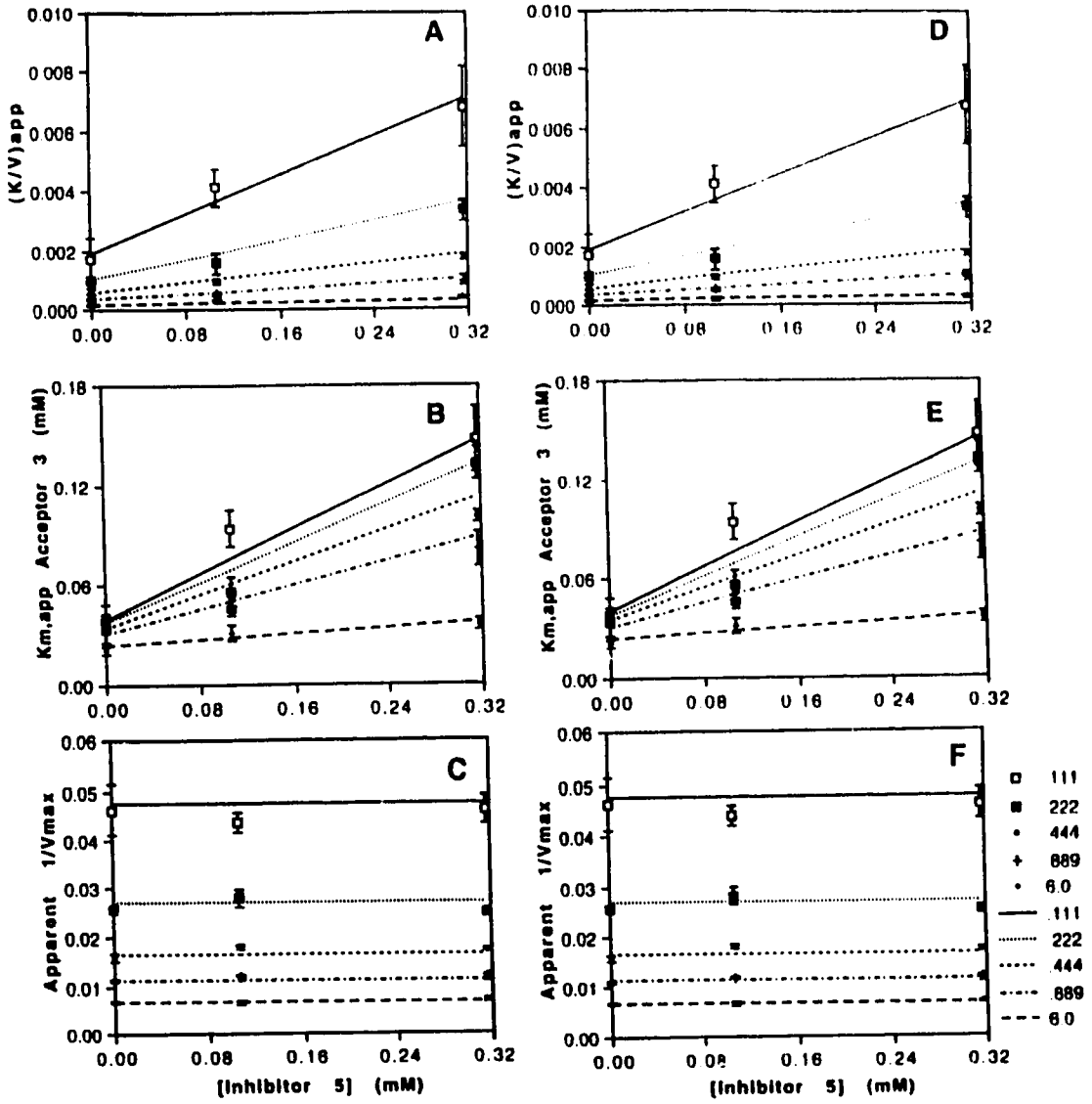


Figure 4.9: Effect of inhibitor 5 on behavior of apparent kinetic parameters for acceptor 3 as the variable substrate (Experimental section B). Experimental points were deduced from fits of 5-point data sets to the Michaelis-Menten equation (Equation 5), where each data set corresponds to a specific inhibitor concentration and a fixed UDP-GlcNAc concentration (mM) as indicated in the figure legend. Lines were drawn using theoretical values of (K/V)app, Km,app and apparent 1/Vmax which were calculated from Equations 6 and 7 by substituting parameters generated from curve fitting to Equation 3 (A-C) or Equation 4 (D-F) (Table 4.1).

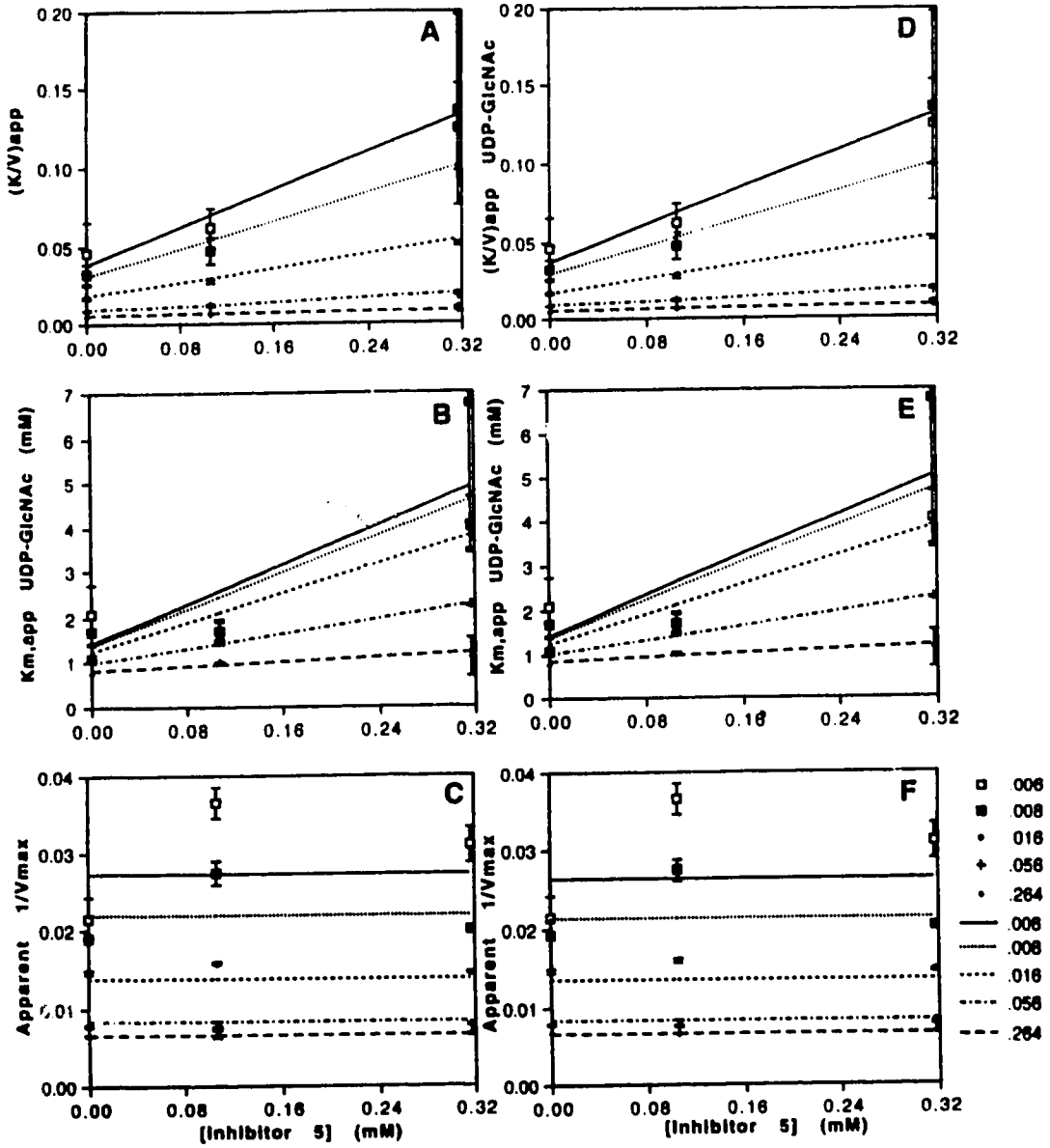


Figure 4.10: Effect of inhibitor 5 on behavior of apparent kinetic parameters for UDP-GlcNAc as the variable substrate (**Experimental** section **B**). Experimental points were deduced from fits of 5-point data sets to the Michaelis-Menten equation (Equation 5), where each data set corresponds to a specific inhibitor concentration and a fixed concentration of acceptor 3 as indicated in the figure legend (mM). Lines were drawn using theoretical values of (K/V)app, Km,app and apparent 1/Vmax which were calculated from Equations 6 and 7 by substituting parameters generated from curve fitting to Equation 3 (**A-C**) or Equation 4 (**D-F**) (Table 4.1).

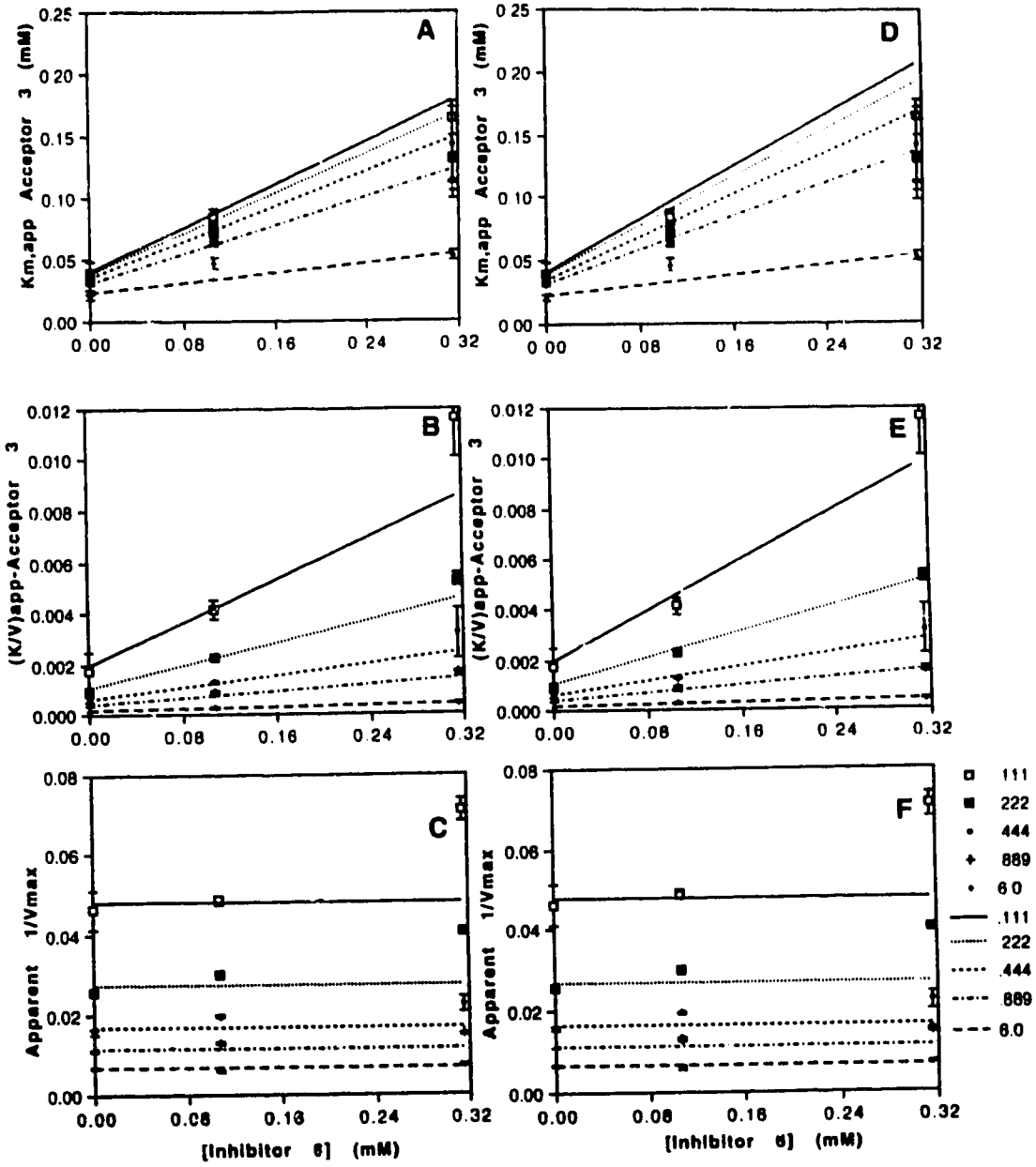


Figure 4.11: Effect of inhibitor 6 on behavior of apparent kinetic parameters for acceptor 3 as the variable substrate (**Experimental** section **B**). Experimental points were deduced from fits of 5-point data sets to the Michaelis-Menten equation (Equation 5), where each data set corresponds to a specific inhibitor concentration and a fixed UDP-GlcNAc concentration (mM) as indicated in the figure legend. Lines were drawn using theoretical values of (K/V)app, Km,app and apparent 1/Vmax calculated from Equations 6 and 7 by substituting parameters generated from curve fitting to Equation 2 (A-C) or Equation 3 (D-F) (Table 4.1).

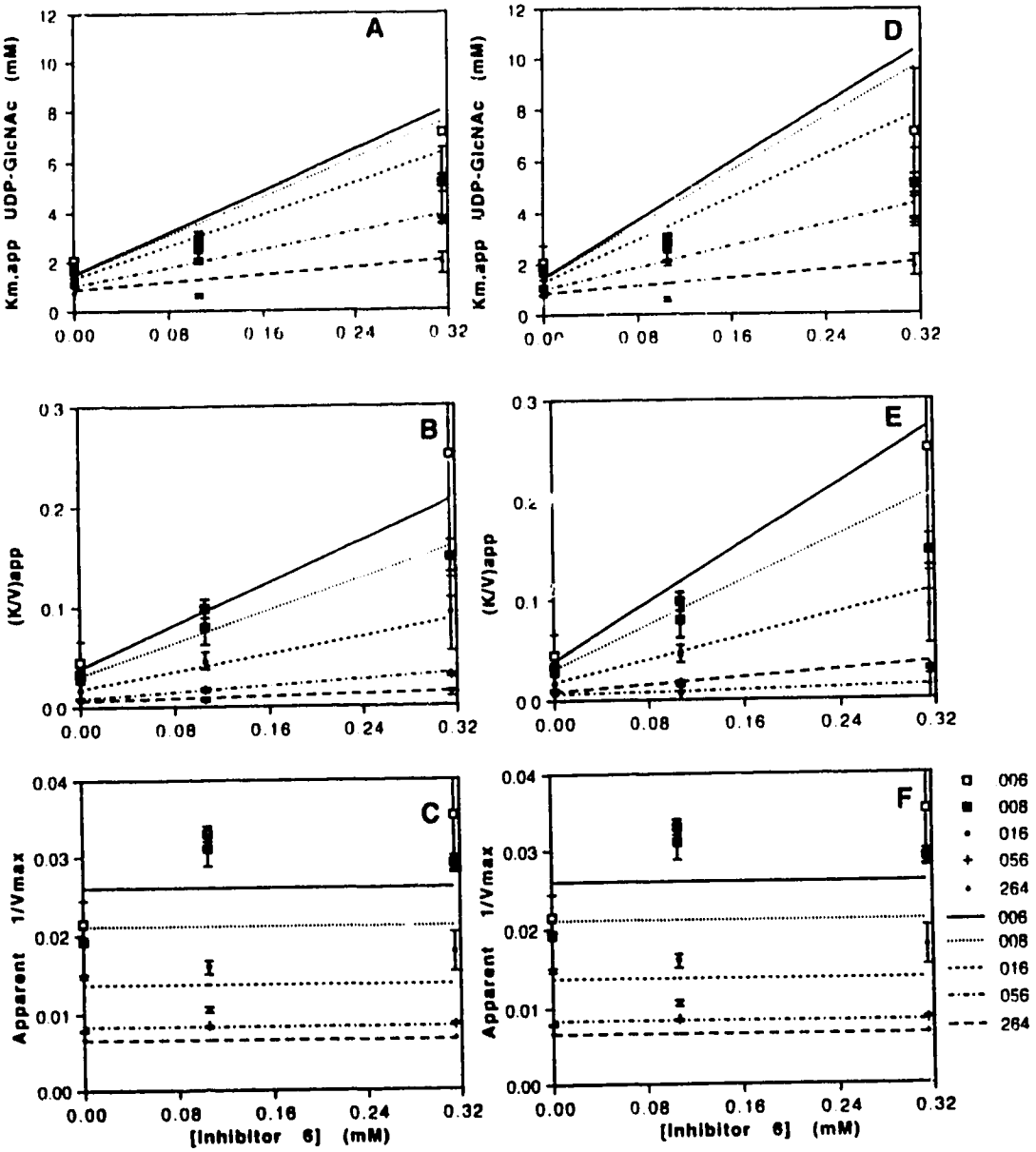


Figure 4.12: Effect of inhibitor **6** on behavior of apparent kinetic parameters for UDP-GlcNAc as the variable substrate (**Experimental** section **B**). Experimental points were deduced from fits of 5-point data sets to the Michaelis-Menten equation (Equation 5), where each data set corresponds to a specific inhibitor concentration and a fixed concentration of acceptor 3 as indicated in the figure legend (mM). Lines were drawn using theoretical values of (K/V)app, Km,app and apparent 1/Vmax calculated from Equations 6 and 7 by substituting parameters generated from curve fitting to Equation 2 (**A-C**) or Equation 3 (**D-F**) (Table 4.1).

concentration in Figures 4.15 (compound **5**) and 4.16 (compound **6**). Estimated errors in these apparent kinetic parameters were calculated as described in **Experimental** section **B** and are represented in figures as error bars.

Inhibitor kinetic data was also combined with substrate kinetic data obtained from earlier, independent experiments (section **A** above) and fit to Equations 2, 3, and 4 to obtain values for kinetic parameters. Further kinetic experiments were performed at nearly-saturating concentrations of fixed substrate to see if data obtained under such conditions might reduce the size of errors on β and γ values and thus permit distinction among the possible kinetic mechanisms. Specifically, acceptor kinetics of **3** (0.006 to 0.560 mM) were evaluated in the presence 6.0 mM UDP-GlcNAc, in the absence of inhibitors and then in the presence of either 0.211 mM **5** or 0.196 mM **6**. Similarly, reaction rates were measured over a range of donor concentrations (0.125 to 10 mM) in the presence and absence of each inhibitor at 0.56 mM fixed concentration of acceptor trisaccharide **3**. All assays contained the same amount of enzyme as previous kinetic studies and incubations were carried for 15 minutes. Data from these three types of experiments (i.e. substrate kinetics of section **A**, and inhibitor kinetics obtained at bothsubsaturating and saturating concentrations of fixed substrate (described in this section **C**) were combined and fit to Equations 2, 3 and 4. The kinetic parameters obtained from this evaluation are provided in Table 4.1; values were essentially the same as those obtained for previous experiments (section **B**).

D. *UDP Inhibition Kinetics*: For purposes of comparison, GlcNAcT-V reaction rates were measured with and without 0.1 mM UDP added, over a range of UDP-GlcNAc concentrations (0.1 to 10 mM; 30,000 dpm/nmol) with acceptor **3** concentration fixed at 0.080 mM. Assays were performed under slightly different conditions and using a different enzyme extract. Reactions contained 1.3 μM GlcNAcT-V (specific activity 0.6 mU/mg protein), 50 mM sodium cacodylate pH 6.5 with 20 % glycerol, 10 mM EDTA and 0.1% Triton X-100. Incubations were carried out for 32 to 90 minutes. Rate data were fit to Equation 4 in order to obtain an estimate of the value for the K_i of UDP; K_a, K_b, and α values (0.0283 mM, 0.75 mM, and 1.01 respectively) were taken from Table 4.1. The opposite experiment (i.e. with acceptor **3** as the variable substrate) was also performed at 0 and 0.1 mM UDP. UDP-GlcNAc concentration was 1.1 mM and acceptor concentration was varied over the range 0.004 to 1.6 mM. Reactions were allowed to

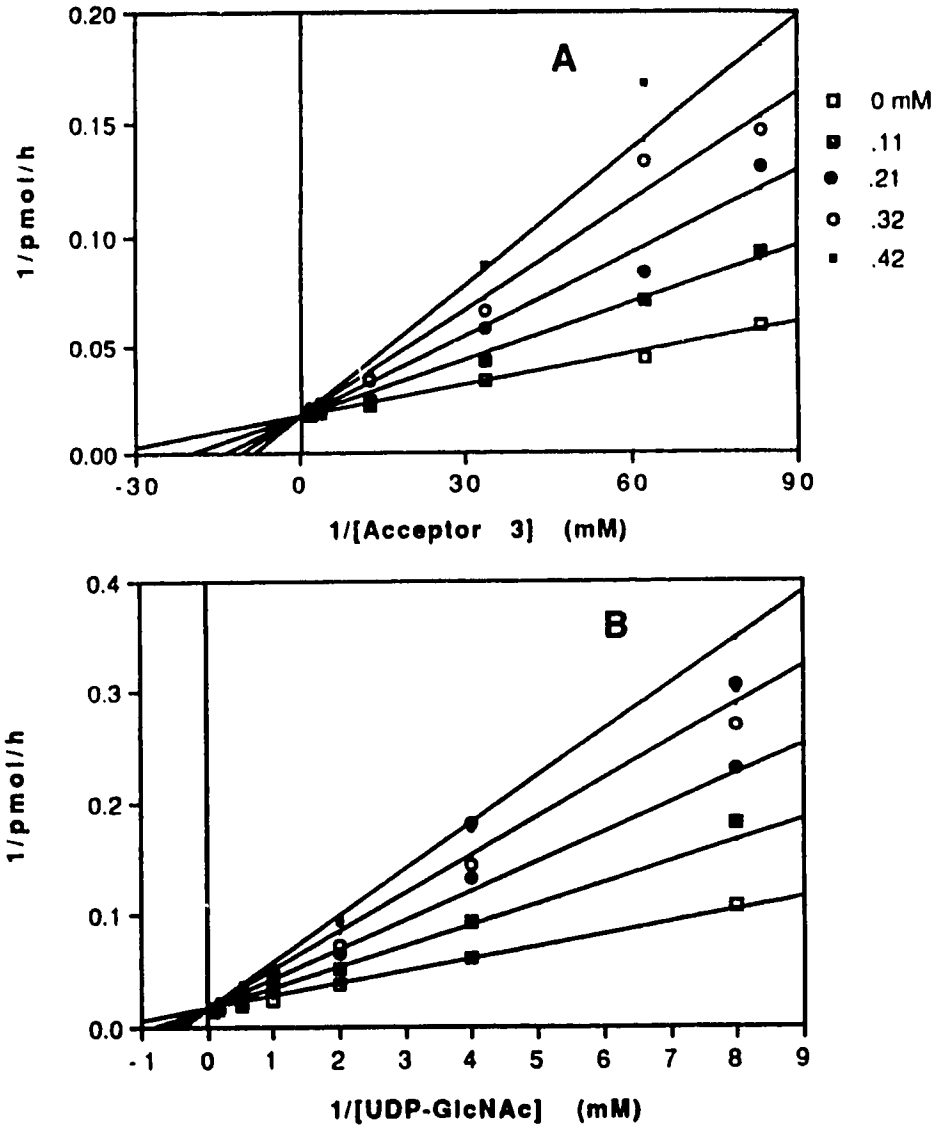


Figure 4.13: Inverse plots of rate data for inhibitor 5, with either acceptor 3 (A) or UDP-GlcNAc (B) as variable substrate, as described in **Experimental** section C. Individual lines corresponding to different inhibitor concentrations (millimolar concentrations indicated in legend) were drawn using theoretical values for velocities generated by curve fitting procedure and Equation 4.

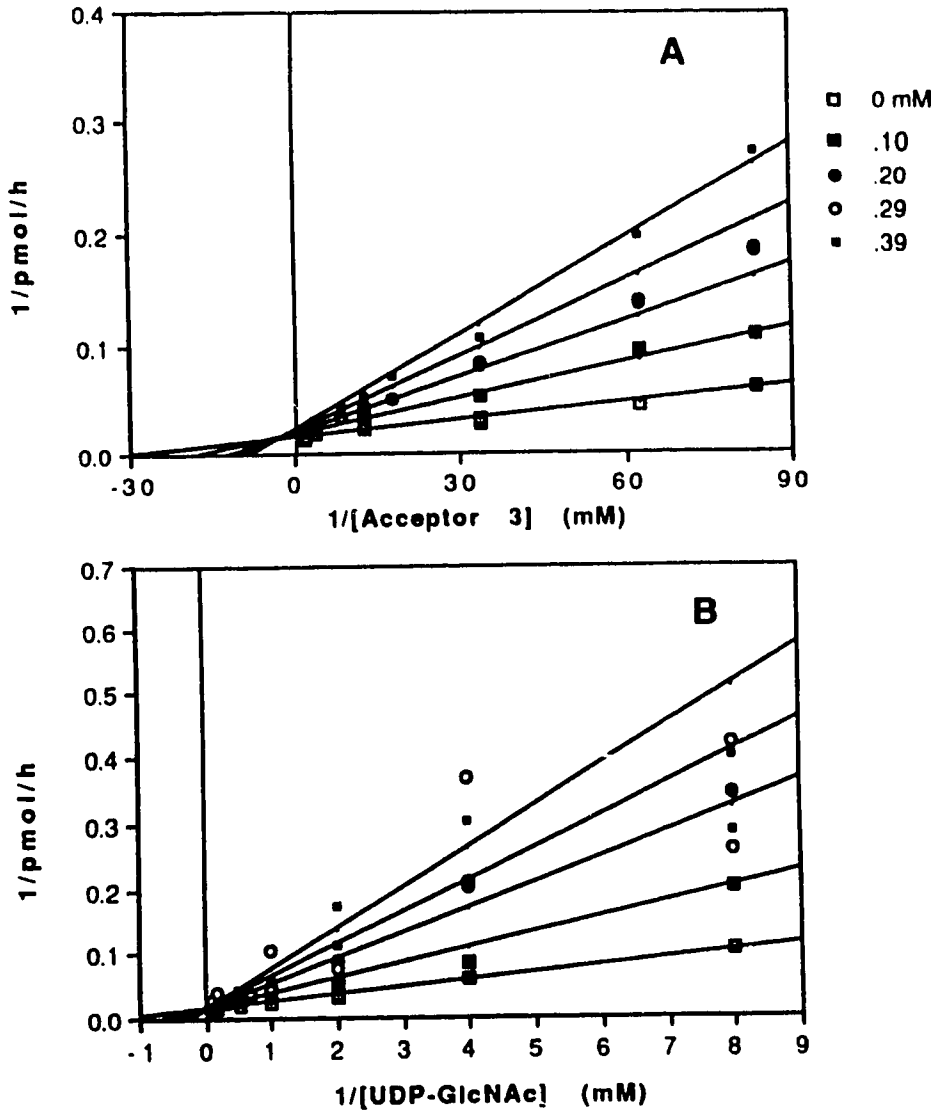


Figure 4.14: Inverse plots of rate data for inhibitor 6, with either acceptor 3 (A) or UDP-GlcNAc (B) as variable substrate, as described in **Experimental** section **C**. Individual lines corresponding to different inhibitor concentrations (millimolar concentrations indicated in legend) were drawn using theoretical values for velocities generated by curve fitting procedure and Equation 3.

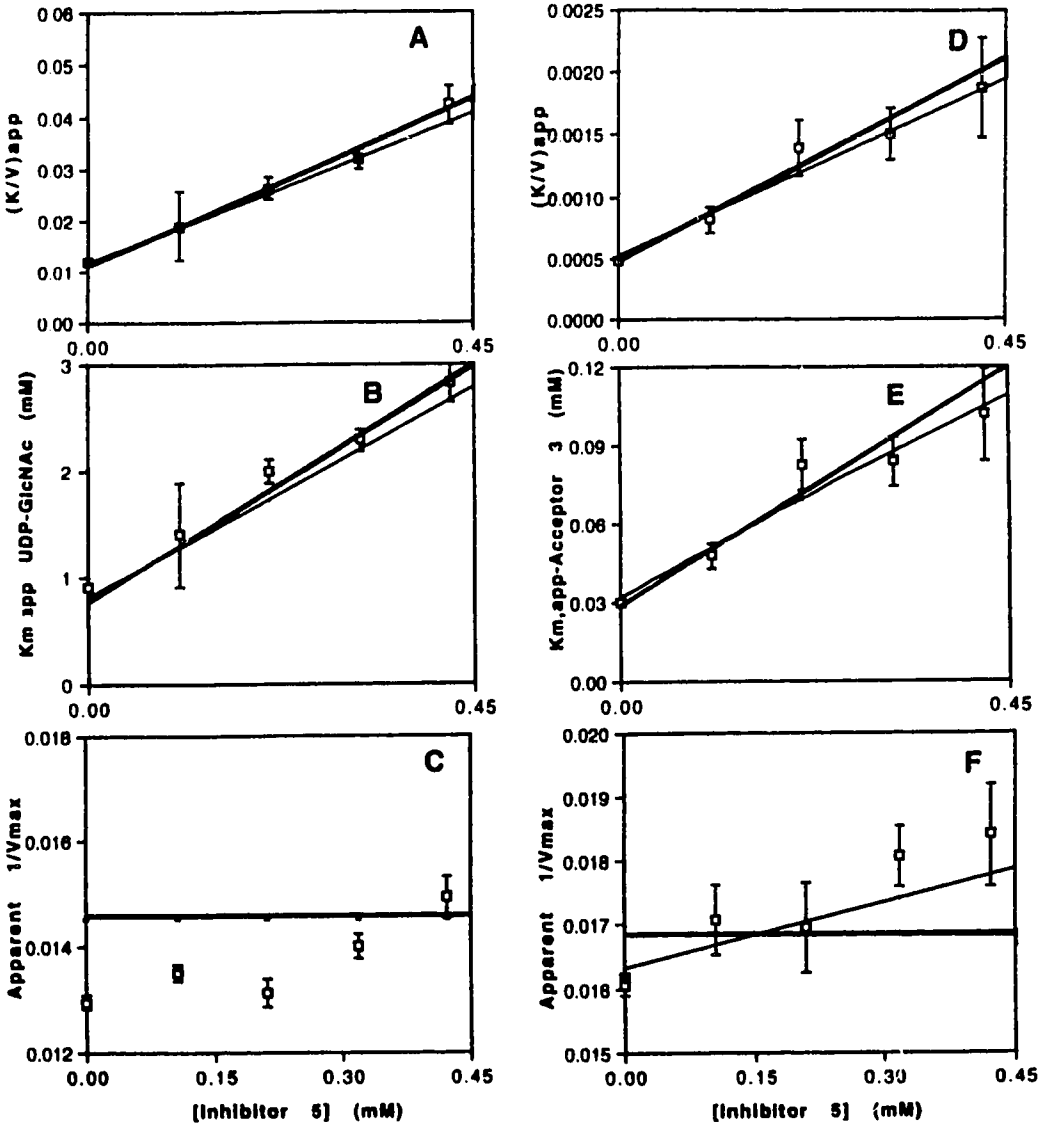


Figure 4.15: Effect of inhibitor **5** on behavior of apparent kinetic parameters for either UDP-GlcNAc (**A-C**) or acceptor **3** (**D-F**) as the variable substrate (**Experimental** section **C**). Experimental points were deduced from fits of substrate kinetic data to the Michaelis-Menten equation (Equation 5), where each data set corresponds to a specific inhibitor concentration. Lines drawn through values of $(K/V)_{app}$, $K_{m,app}$ and apparent $1/V_{max}$ were calculated from slopes ($K_{m,app}/V_{max}$) and y-axis intercepts of inverse plots of the theoretical velocities obtained from the curve fitting procedure using Equation 3 (line) or Equation 4 (**bold line**).

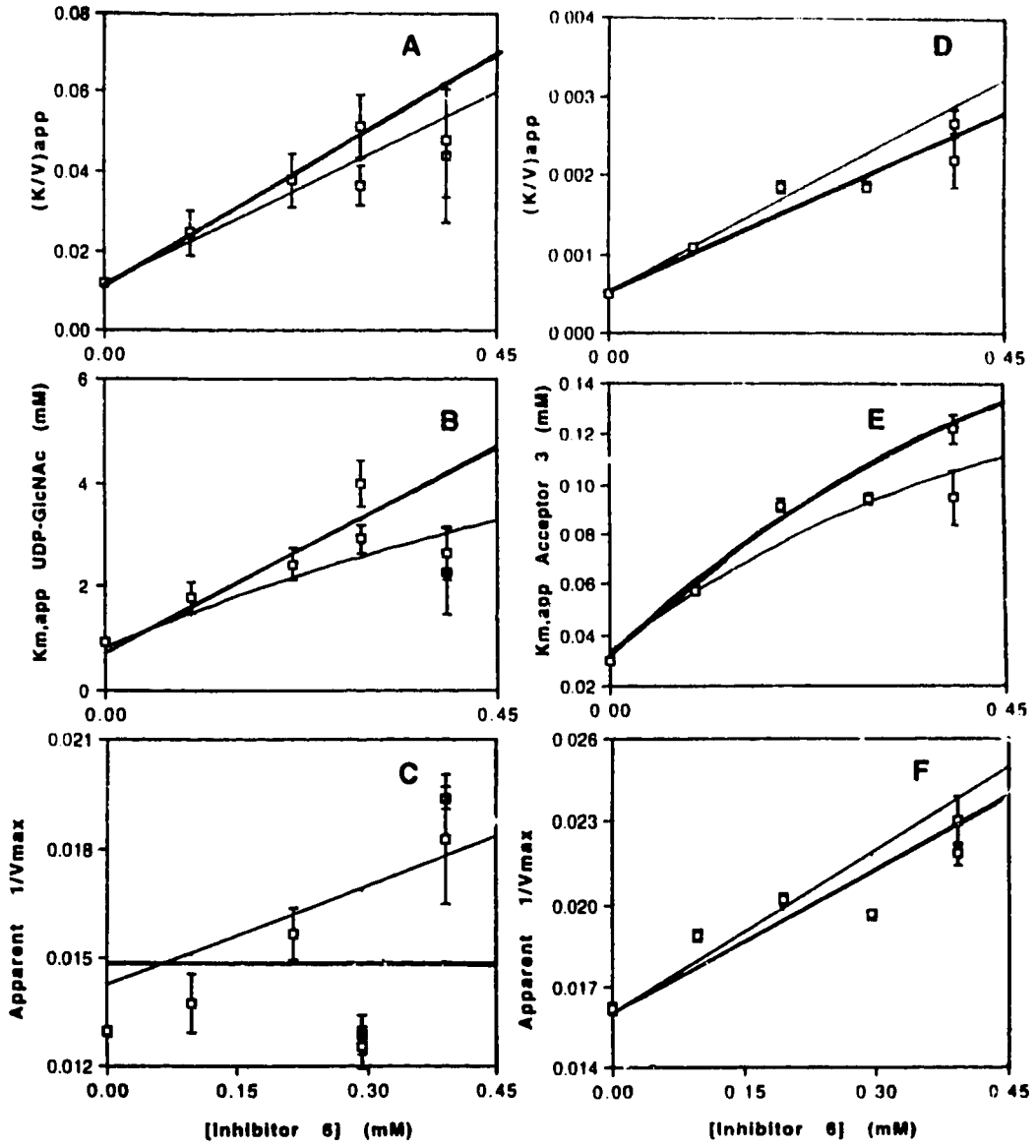


Figure 4.16: Effect of inhibitor 6 on behavior of apparent kinetic parameters for either UDP-GlcNAc (A-C) or acceptor 3 (D-F) as the variable substrate (Experimental section C). Experimental points were deduced from fits of substrate kinetic data to the Michaelis-Menten equation (Equation 5), where each data set corresponds to a specific inhibitor concentration. Lines drawn through values of $(K/V)_{app}$, $K_{m,app}$ and apparent $1/V_{max}$ were calculated from slopes ($K_{m,app}/V_{max}$) and y-axis intercepts of inverse plots of the theoretical velocities obtained from the curve fitting procedure using Equation 2 (line) or Equation 3 (bold line).

proceed for 20.5 or 73 minutes. Inverse plots of rate data from both of these experiments are presented in Figure 4.17.

Discussion

Substrate Kinetics and Enzyme Mechanism: Enzyme-catalyzed sequential reactions can involve either random or ordered binding of substrates to enzyme to form a ternary enzyme-substrates complex (represented **E*A*B**). An enzyme's reaction mechanism can have important implications in substrate and inhibitor design and in enzyme purification. Rapid-equilibrium random versus ordered bisubstrate mechanisms can often be distinguished using initial-rate kinetics, by analyzing dependence of reaction rates on substrate concentrations and by investigating modes of inhibition by substrate analog inhibitors and products. The rapid-equilibrium assumption that break-down of the **E*A*B** complex to form products is the rate-limiting step of the reaction is used to simplify equations describing the kinetic behavior of bireactant enzymes.

In a general way, substrate kinetics examine if and how varyingthe concentration of one substrate affects apparent K_m and V_{max} values for the other substrate. Although values of kinetic parameters can be analyzed simply and directly by non-linear regression analysis of experimentally-determined reaction rates as functions of the concentrations of ligands (eg substrates, inhibitors, or activators) using rate equations such as Equation 1, graphical analysis is traditionally used for qualitative and quantitative presentation and analysis of rate data. Very importantly, graphical representation of experimental *versus* theoretical rate data can also be used to determine whether deviations from predicted behavior (i.e. the distributions of residuals) are random or not.

Substrate kinetics for GlcNAcT-V have been evaluated (**Experimental,** section **A**) by measuring reaction rates over a range of concentrations of acceptor **3** at a number of concentrations of the alternate substrate UDP-GlcNAc. This data can be presented in two types of inverse plots, with either the sugar-nucleotide donor as the variable substrate ($1/[\mathbf{B}]$ *versus* $1/v$, giving separate lines for different acceptor concentrations as in Figure 4.5**A**) or with acceptor **3** as the variable substrate ($1/[\mathbf{A}]$ versus $1/v$, with separate lines for donor concentrations as in Figure 4.5**B**). If a reaction mechanism involves ordered addition of substrates to the enzyme, the two inverse plots for the two variable substrates are different: The lines of the inverse plot

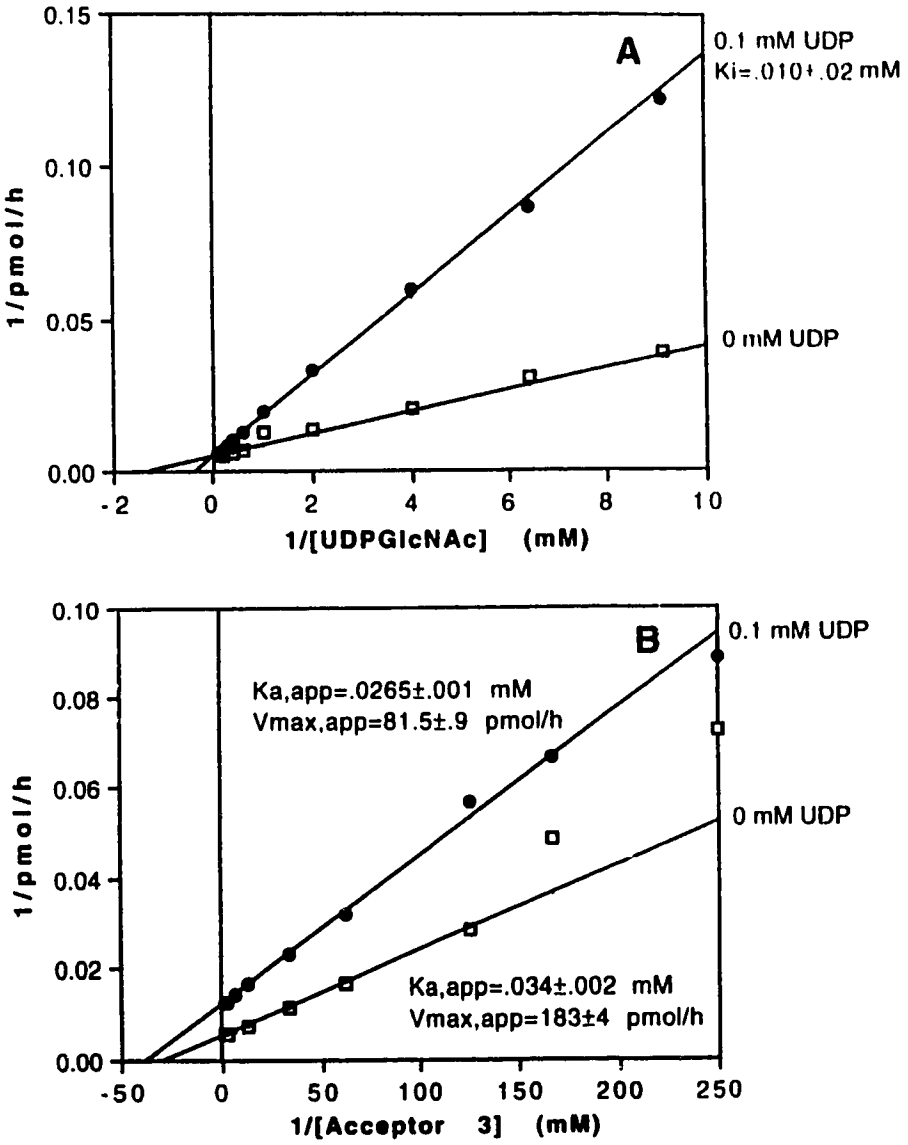


Figure 4.17 Effect of the inhibitor UDP on donor (**A**) and acceptor (**B**) kinetics (**Experimental** section D). Inhibitor concentrations are indicated on plots along with a K_i (*versus* UDP-GlcNAc) in **A**, and apparent (acceptor) kinetic parameters in **B**.

for the second substrate **B** typically intersect on the y-axis. This arises because the 1/y-axis intercept is equal to $V_{max,app} = k_{cat}[\mathbf{E^*A^*B}]$ (k_{cat} is the catalytic constant or turnover number) and **[E*A*B]** is, in turn, dependent on **[B]** which has essentially been extrapolated to infinity at 1/**[B]** = 0. On the other hand, the value of $K_{b,app}$ (-1/x-axis intercept) is dependent on the concentration of the first substrate **A**, approaching a minimum value (ie K_b) as **[A]** approaches saturation. The lines of the inverse plot for the first substrate (**A**) typically intersect to the left of the y-axis, giving a decreasing $K_{a,app}$ and increasing $V_{max,app}$ with increasing **[B]**.

If free enzyme is capable of forming a complex with either of the two substrates, then the mechanism is considered to be random and lines of inverse plots for both substrates intersect to the left of the y-axis as demonstrated for GlcNAcT-V in Figures 4.5**A** and 4.5**B**. Substrate kinetic data obtained as described in **Experimental** section **A** were fit to Equation 1, which gave values of $\alpha = 1.6 \pm 0.4$; $K_b = 0.50 \pm 0.1$ mM; $K_a = 0.021 \pm 0.004$ mM; $V_{max} = 128 \pm 3$ pmol/h (standard error = 8.0). These results were also fit to the general equation for an ordered mechanism (same as Equation 1 but without α constants and without the $[\mathbf{B}]/K_b$ term accounting for the **E*B** complex which does not form in an ordered mechanism) (46), but the standard error was larger (39.5) and the distribution of residuals was obviously non-random, suggesting that the enzyme did not bind substrates in an ordered fashion (results not shown).

Other evidence that the GlcNAcT-V-catalyzed reaction follows random order of substrate binding is offered by the results of substrate analog inhibitor kinetics. Previous work has shown that a 4'-methoxy derivative of acceptor **3** is a competitive inhibitor *versus* acceptor **3**, with $K_i = 0.014$ mM (32), increasing the apparent K_m of acceptor but not affecting V_{max} in inhibitor kinetic studies. V_{max} is unaffected because **[E*A*B]** is not altered by the presence of inhibitor when **[A]** and **[B]** are saturating. On the other hand, a higher concentration of **A** is required to achieve this saturation because inhibitor competes for the same site on the enzyme. Hence, there is an increase in $K_{m,app}$. (Note that in the case of inhibitor studies, "apparent" kinetic constants are kinetic constants which are somehow affected by the presence of inhibitor). The same 4'-OMe trisaccharide inhibitor is non-competitve versus UDP-GlcNAc; that is, it does not affect the donor's K_m but produces a substantially-reduced $V_{max,app}$. Thus, whereas inverse plots for a competitive inhibitor may be represented as a family of lines, with

each line corresponding to a different inhibitor concentration, which intersect on the y-axis (i.e. $1/V_{max}$ is not affected), non-competitive inhibition is often characterized by intersection of lines on the x-axis (i.e. $K_{m,app}$ is not affected). The inverse plots of rate data from inhibition studies of the 4'OMe derivative of acceptor **3** as an inhibitor of GlcNAcT-V which is competitive *versus* acceptor **3** and noncompetitive versus sugar-nucleotide donor are shown in Chapter 3, Figure 3.3. Similarly, UDP has been partially evaluated as an inhibitor of GlcNAcT-V in **Experimental** section **D**. It was found to be a competitive inhibitor *versus* UDP-GlcNAc, with $K_i = 0.010 \pm 0.002$ mM (Figure 4.17**A**). UDP was a non-competitve inhibitor versus acceptor **3** (Figure 4.17**B**). These two types of inhibitors provide support for the idea that the reaction catalyzed by GlcNAcT-V proceeds via a random mechanism because inhibitors which compete with the second substrate of an ordered mechanism will usually produce non-competitive-type kinetics (46).

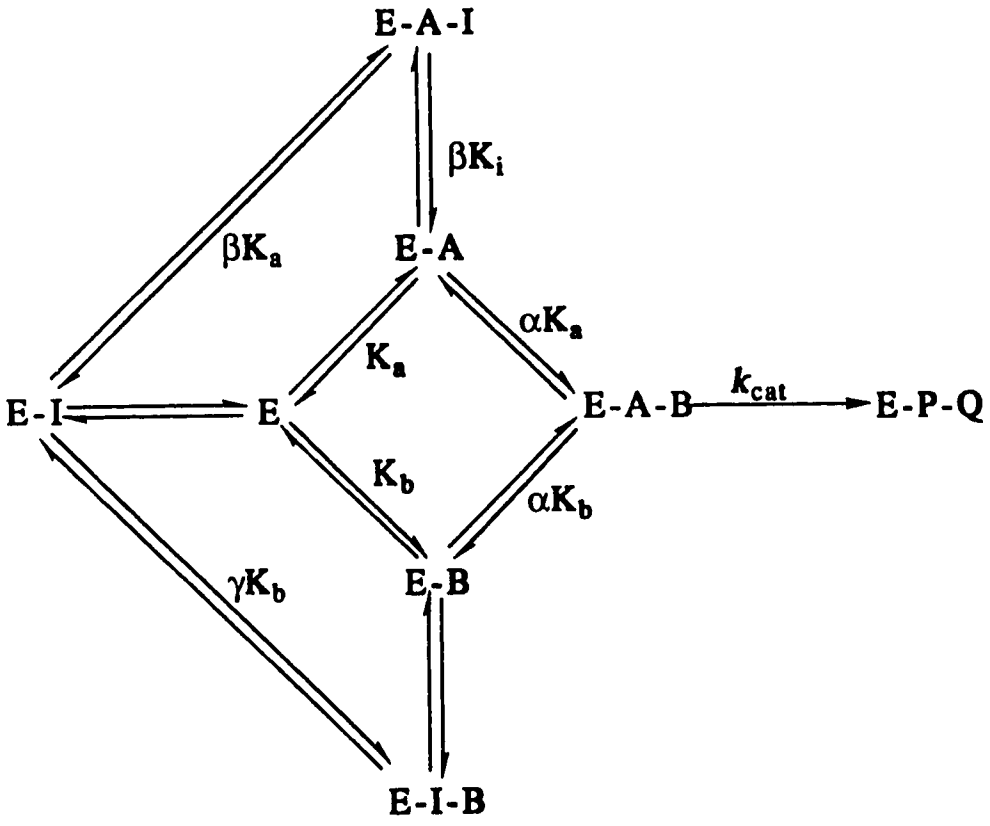
The Theorell-Chance mechanism is a special type of ordered mechanism in which the enzyme forms abortive complexes with both the second substrate bound and the first product released, and this mechanism is indistinguishable from a random mechanism on the basis of the usual substrate, inhibitor and product inhibition experiments (48). Fromm has suggested that a Theorell-Chance ordered mechanism may be distinguished from a random order of substrate binding by the fact that it is only for the latter case that a bisubstrate analog will bind to free enzyme producing inhibition that is purely competitive versus both substrates of the bireactant enzyme (40).

Bisubstrate analog inhibitor kinetics: Bisubstrate analogs **5** and **6** were tested as inhibitors of hamster kidney GlcNAcT-V in two different types of experiments. Whereas simple inhibition kinetics are usually measured at close to saturating concentration of the non-varied substrate, so that apparent K_m and V_{max} values are as close as possible to their true values in the absence of inhibitor, a bisubstrate inhibitor will not give any measurable inhibition under these conditions unless it is tight-binding. Evaluation of bisubstrate analogs **5** and **6** was therefore undertaken using a 5 X 5 matrix of donor and acceptor concentrations in order to obtain complete substrate kinetics at several inhibitor concentrations (**Experimental**, section **B**). Primary rate data for this experiment is shown in Figures 4.5 and 4.6, and replotted as inhibitor kinetics in Figures 4.8 and 4.7. A second experiment was also performed by measuring reaction rates at sub-saturating concentrations of fixed

substrate (**Experimental**, section **C**), with primary rate data shown in Figures 4.13 and 4.14). Since α, K_a and K_b values could not be deduced from this latter experiment **C**, more complete substrate kinetics were measured independently (**Experimental**, section **A**) and rate data included in the curve fitting procedure. Alternatively, these kinetic parameters could have been determined separately and entered as constants in the curve-fitting routine. The kinetic parameters obtained from these two different types of experiments (**B** and **C**), with each set of data fit to two different equations, are provided in Table 4.1.

Some of the possible modes of interaction between a putative bisubstrate analog and two substrate binding sites are represented in Scheme 4.1. Ideally, a bisubstrate analog inhibitor (**I**) should bind only to free enzyme (**E**), forming an **E*I** complex (40), but other types of competitive interactions are available to inhibitors which resemble one or both substrates of a bireactant enzyme (42). Equation 2 represents the dependence of GlcNAcT-V reaction velocity (υ) on the concentrations of acceptor 3 ([**A**]) and of UDP-GlcNAc ([**B**]), as well as on the concentration of inhibitor **5** or **6** ([**I**]) for the case in which the inhibitor is capable of binding to both sub-sites, in the presence or absence of the alternate substrate. Equations 3 applies to the case where inhibitor does not bind to enzyme while UDP-GlcNAc is bound; that is, the species **E*I*B** does not contribute significantly to the over-all equilibrium. When neither **E*A*I** nor **E*I*B** species are formed to an appreciable extent and **E*I** is the major species, and the inhibitor is a true bisubstrate analog inhibitor, Equation 4 is applicable. K_i is the dissociation constant for **E*I** complex formation, and β and γ terms account for the effect on K_i of the binding of one molecule of acceptor or donor, respectively. Thus, βK_i and γK_i are (maximum) values for dissociation constants representing the equilibrium between inhibitor **I** and **E*A** or **E*B** at saturating concentration of **A** or **B**, respectively; on the other hand, K_i is the dissociation constant for an inhibitor with free enzyme. Rate data were fit to these three equations in order to discriminate the predominant mode of interaction of the inhibitor with the enzyme, and to obtain values for the various kinetic parameters (Table 4.1).

An equation for the case in which the **E*I*B** complex -- but not the **E*A*I** complex -- forms readily, was not included because it did not apply to either of the two inhibitors studied, as deduced from the tendency for γ to always be larger than β. Binding of inhibitors to an "active" site versus individual substrate-binding sites was not distinguished in these equations. Also, a term has not been included to



Scheme 4.1: General model for possible modes of interaction of a non-exclusive bisubstrate analog inhibitor with GlcNAcT-V in a rapid-equilibrium random bireactant mechanism.

account for the possible binding of two inhibitor molecules to a single generated from theoretical values of velocities or kinetic parameters, enzyme molecule, forming an **E*I*I** complex.

As described in the figure legends, lines through kinetic data were both generated from the curve-fitting procedure, using the simplest equation applied in each case (ie. Equation 4 for inhibitor **5**, and Equation 3 for inhibitor **6**). It can be seen that deviations of experimental values from theoretical values are not systematic in any way. Lines generated by the more complicated alternate Equations 3 and 2 for inhibitors **5** and **6**, respectively, did not differ signficantly from those shown. The kinetic parameters obtained from these two different types of experiments (**B** and **C**) (Table 3.1) are quite similar not only in terms of absolute values and implications for the modes of inhibition displayed by each bisubstrate analog; but results obtained from the two types of experiments are also similar in terms of the uncertainty in the values of the kinetic parameters which were calculated. Both **5** and **6** are moderately inhibitory in an essentially competitive way *versus* both acceptor and donor; this is demonstrated in a qualitative way by the tendency for lines of inverse plots to intersect at the y-axis. As well, large β and γ values suggest that both bisubstrate analogs have little or no capacity to bind to either **E*A** or **E*B** complexes. Kinetic data was further evaluated in order to determine whether the subtle differences in the effects of the two inhibitors (Table 4.1) were significant.

Apparent kinetic parameters generated by these two types of experiments were determined as described in their respective experimental sections and plotted versus inhibitor concentration in order to determine whether experimental data deviated significantly from theoretical values generated by either of the two kinetic models obtained for each inhibitor (Figures 4.9-4.12 and Figures 4.15 and 4.16). It can be seen that such secondary plots did not provide evidence to eliminate any of the possible models.

K_i values 105 ± 11 and 46 ± 4 μM for **5** and **6**, respectively, were of the same order of magnitude as those of the available single-substrate analogs, including the 6'-deoxygenated acceptor analog ($K_i = 30$ μM) and UDP ($K_i = 10$ μM). This indicated that no net advantage was obtained from combining elements of the two GlcNAcT-V substrates into the same molecule. From the values of K_i, β and γ, it can be seen that the two compounds, although similar in structure, behave slightly differently in the enzyme active site. Phosphonate bisubstrate **5**, which had the higher K_i of the two compounds, was almost purely competitive

versus both substrates, as indicated by its large values for β (39 $\pm$ 50) and γ (>10^5) obtained from the best fits of the data for this compound (Table 4.1). Compound **6**, on the other hand, behaved as a mixed competitive inhibitor versus both substrates. Although UDP-GlcNAc has a higher K_m (K_b) (1.7 $\pm$ 2 mM) than acceptor **3** (K_a = 40 $\pm$ 7 μM), binding to the donor-binding site appears to dominate interations of inhibitor with GlcNAcT-V's active site. Unfortunately, the data was not of sufficient quality and inhibitor concentrations used were too low to eliminate all but one possible mode of inhibition and determine precise values for β and γ.

Whereas the kinetic evidence has suggested that the reaction catalyzed by GlcNAcT-V proceeds with random order of binding of substrates, the behavior of the enzyme with the phosphonate bisubstrate analog **5** eliminates the possibility of a Theorell-Chance ordered type of mechanism, which is expected to display noncompetitive and competitive modes of bisubstrate inhibition versus first and second substrates, respectively (40).

The simplest interpretation of the kinetic data obtained for the two bisubstrates, then, is that the phosphonate **5** bisubstrate analog interacts mainly with free enzyme, spanning both substrate binding sites, whereas **6**, possibly due to its better fit into the donor binding site, behaves mostly as an inhibitor which is competitive with this substrate while still impairing, acceptor binding. It may also be that the subtle differences between the dimensions (eg. number of bond lengths), orientations and flexibility of the two bisubstrate analogs are responsible for their different modes of interaction with enzyme.

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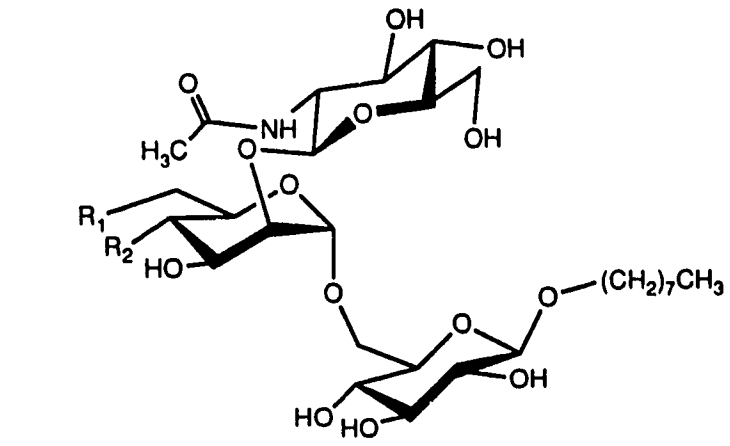
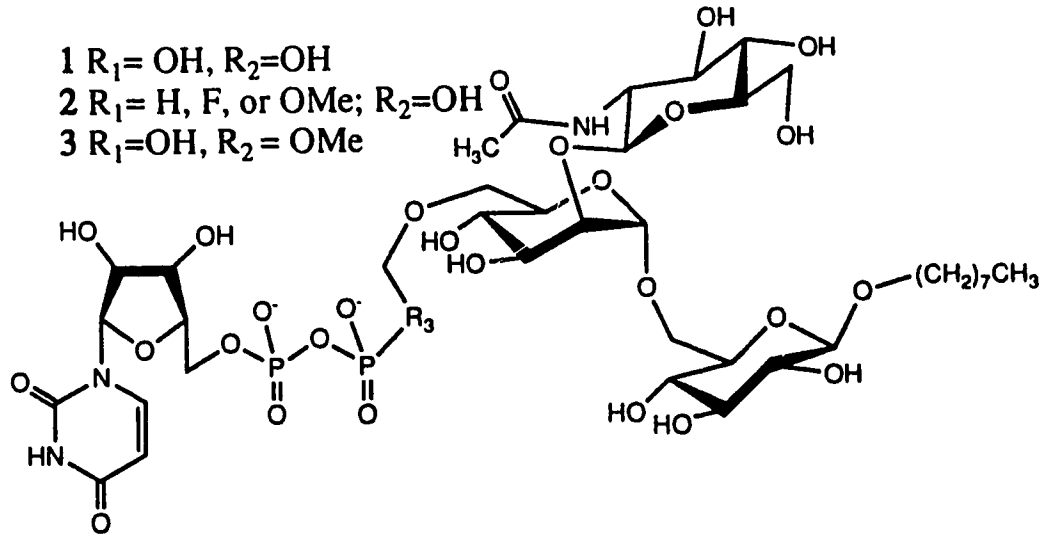
Chapter 5
Concluding Remarks

Introduction

N-acetylglucosaminyltransferase V (GlcNAcT-V) in cell and tissue homogenates has been measured and studied by a number of groups intent on discerning the relationship between levels of this glycosyltransferase, and the glycosylation of proteins of transformed cells (1-8). GlcNAcT-V can be detergent-extracted from rat and hamster kidney acetone powders (9,10); it has been purified to homogeneity from rat kidney acetone powder using affinity chromatography (10) and cloned (11). Hamster kidney GlcNAcT-V is an unusual GlcNAc transferase in several respects: It has a low K_m (0.026 mM) for its acceptor compound **1** (Figure 5.1) compared with most other GlcNAc transferase (see Table 1.1 and references therein). This K_m is also low compared to synthetic acceptor K_m values obtained for most other glycosyltransferases in general. This may not be physiologically relevant but due to some particularly favorable property of the synthetic trisaccharide **1**, or due to the absence of a requirement for neighboring sugars and/or protein to induce an optimum conformation for acceptor recognition. The acceptor K_m for the hamster kidney enzyme is slightly lower than that of the BHK enzyme and rat enzyme which are 0.213 (4) and 0.087 mM (8), respectively. Values for the K_m of UDP-GlcNAc appear to vary among enzymes derived from different sources, ranging from 0.75 and 1.2 mM for hamster kidney and baby hamster kidney (4; this study) to 11 mM for the enzyme purfied from rat kidney (8). Substrate and inhibitor kinetics (Chapter 4) indicate a random order of binding of substrates; this is in contrast to similarly-characterized GlcNAc transferases I, II, and mucin $\beta(1\rightarrow6)$, which display an ordered sequential mechanism where UDP-GlcNAc binds first and UDP departs from the active site last (12-14). This enzyme has no divalent cation requirement, a property common to other $\beta(1\rightarrow6)$GlcNAc transferases (15, 16), as well as to the sialyltransferases (17). Coordination of the nucleotide phosphates is the role usually postulated for the metal ion, but it appears that the manganese-independent enzymes are



1 R_1= OH, R_2=OH
2 R_1= H, F, or OMe; R_2=OH
3 R_1=OH, R_2 = OMe



4 R_3= CH_2
5 R_3= OCH_2

Figure 5.1: Structures of acceptor 1 and inhibitors 2-5 which have been designed for GlcNAcT-V (References30-32; Chapters 3 and 4).

mechanistically different from most other glycosyltransferases in this respect. The possibility that these enzymes possess tightly-bound metal cofactors cannot be discounted either. No sequence similarities have been found between the two β(1→6)GlcNAc transferases which have been cloned, namely the rat liver GlcNAcT-V (9) and UDP-GlcNAc: Galβ(1→3)GalNAc-R (GlcNAc to GalNAc) β(1→6)GlcNAc transferase (16).

An ELISA for GlcNAcT-V has been Developed

Since the original publication of a GlcNAcT-V ELISA (18; Chapter 2), the number of available ELISA-based glycosyltransferase assays has gone from two (19, 20) to ten. They include A and B blood group Gal(NAc) transferases (21, 22), the Lewis α(1→4)fucosyltransferase (20), β(1→4)galactosyltransferase assays (23-26), hormone-specific GalNAc transferase (27) and α(2→6)sialyltransferase.(28). These advances in this area, coming from a number of laboratories with different interests in such assays, have demonstrated the need for sensitive, rapid and specific assays for these enzymes because of their low abundance and their expensive substrates. This GlcNAcT-V assay has proven useful for measurement of the enzyme in crude human serum where it is present at levels which are lower than the limit of detection for the available radioassay; hence, this is the only assay permitting study of the soluble human enzyme. This ELISA has also been used for monitoring column fractions during enzyme purification, and to identify and quantitate GlcNAcT-V in cells transfected with a vector containing the putative GlcNAcT-V (9).

The affinity of GlcNAcT-V for its immobilized BSA-conjugated acceptor is demonstrated by the readiness with which the enzyme transfers GlcNAc to the small amount of acceptor trisaccharide (25 picomoles) which is coated onto each well. (This would yield a final "concentration" of 0.25 μM in a typical 100 μL enzyme reaction mixture.) The observation that almost 2 mM concentration of soluble deoxygenated acceptor **2** (K_i=0.03 mM) is required to produce 50 % inhibition in the ELISA assay suggest that this inhibitor is not as effective with the human serum GlcNAcT-V. However, it is more likely (since a similar behavior was observed with hamster kidney enzyme), that the enzyme has a higher affinity for the immobilized acceptor-BSA conjugate of acceptor versus the soluble form. This has practical implications if the GlcNAcT-V ELISA is to be applied to the task of randomly screening compounds to find enzyme

inhibitors.

Synthetic Substrate Analogs have Permitted Evaluation of the GlcNAcT-V Active Site and Mechanism

The efforts of five post-doctoral fellows, comprising approximately eleven post-doc years of carbohydrate synthesis and hundreds of chemical steps, have provided a number of completely different inhibitors for GlcNAcT-V (Figure 5.1) and detailed information, from substrate kinetics, about the enzyme's active site. This information should aid in the development of more potent, related inhibitors, as well as in the design of new inhibitors. An illustration of the trisaccharide acceptor **1** without non-essential functional groups, as deduced from the present studies, is shown in Figure 5.2.

A few interesting observations have arisen from studies in Chapter 3:

1) Even though specific (but limited) substitutions have been tried at every hydroxyl position (4, 29-32), only one critical hydroxyl group (4"-OH) has been unequivocally identified on trisaccharide acceptor **1** (30); the 3"- and 6"-hydroxyl groups are very strong possibilities as well (Table 3.2). All critical hydroxyl groups are located at the non-reducing end of a single non-reducing terminal sugar residue.

2) Despite the apparent lack of any single, strong interactions between the enzyme and the β-mannose, this residue is important for high-affinity binding of acceptor to GlcNAcT-V. The conformation about the α(1→6) linkage has also been demonstrated to be important although results with conformationally *restrained* versus conformationally *biased* (C-methyl) trisaccharide analogs appear to be contradictory (29, 33). The absence of key polar interactions between enzyme and ring hydroxyl groups of the αMan(1→6)βMan-OR disaccharide suggests that van der Waals interactions between complementary surfaces of the enzyme and the sugar must predominate, although polar interactions between the enzyme active site and ring and glycosidic oxygens are also possible. Although the requirement for a trisaccharide structure poses serious synthetic challenges to practical inhibitor design, the hydrophobic character of the acceptor

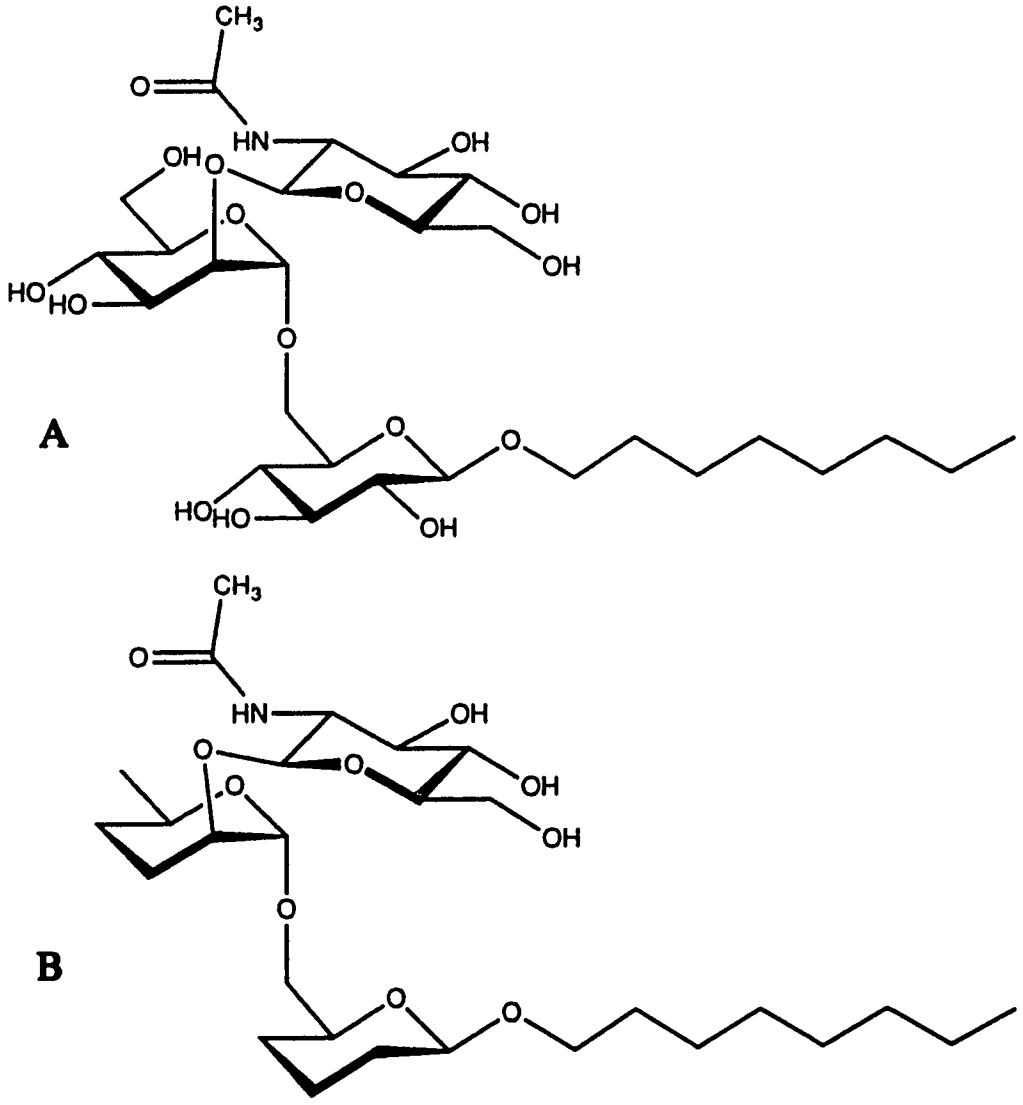


Figure 5.2: Structure of GlcNAcT-V synthetic trisaccharide acceptor (**A**) and representation of structure with known non-essential functional groups removed (**B**).

binding site, and especially the tolerance for bulky substitutions on the reducing-end mannose also offers some flexibility in design of inhibitors, particularly for enhancing the membrane permeability of a potential inhibitor(32).

3) The surprising ability for an O-methyl group at the 4'-position to completely block the transfer reaction without impairing enzyme recognition of the trisaccharide **3** (Figure 5.1), suggests that the reactive 6'-OH is very precisely oriented relative to the 4'-OH and relative to the sugar-nucleotide donor in the enzyme active site (31). This result has offered a new strategy for inhibition of these and other glycosyltransferases, but it also indicates that effects of hydroxyl group substitutions must be interpreted with caution. Results obtained with the 4'-OMe analog **3** also suggest that the orientation of the acceptor portion of bisubstrate analogs **4** and **5**, relative to the UDP portion of these compounds, is not optimal for binding to the enzyme active site.

Future prospects

The enzymological studies described here for GlcNAcT-V have illustrated the usefulness of enzyme kinetics in elucidation of the mechanism and active site properties of an enzyme which has only recently been purified to homogeneity and for which overall yields from tissue (eg 9.5 mU from 300 g rat kidneys, Reference 8) will never be high enough for most other methods of biochemical characterization. Since the enzyme has recently been cloned (9), perhaps larger quantities will soon be available, and the factors regulating this enzyme and its expression can be explored in order to discover its role in transformation and development of cells. Meanwhile, the essential role of carbohydrate chemistry has also been demonstrated: Synthetic substrates and affinity matrices have been indispensible to advancing the current understanding of this and almost all other glycosyltransferases currently being studied. Although the enzymatic synthesis of complex carbohydrates is slowly becoming practical as cloned enzymes slowly become available, development of inhibitors for carbohydrate-binding proteins, as demonstrated here, is being most effectively achieved by synthetic chemistry.

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