THE ROLE OF THE CARBOHYDRATE CHAINS OF Gal β1-4 GlcNAc α2-6 SIALYLTRANSFERASE FOR ENZYME ACTIVITY

by

Darren Glenn Fast

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

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I would like to thank J. C. Jamieson, G. McCaffrey, G. Harder, R. Belo, G. Molgat, D. Pererria, L. Donald, and H.W. Duckworth for technical help, thought provoking discussions, moral support, and their different perspectives on the values of independence, industry, cooperation, acumen and the value and realization of goals. I am indebted to the University of Manitoba, the Department of Chemistry, University of Manitoba and the Chemistry Graduate Students for financial assistance in the form of scholarships and stipends.

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ABSTRACT

Gal β1-4 GlcNAc α2-6 Sialyltransferase (α2,6ST) is a rat liver Golgi enzyme responsible for the addition of terminal sialic acid residues to carbohydrate chains of glycoproteins. The enzyme is synthesized in the rough endoplasmic reticulum and passes to the Golgi complex where it expresses its catalytic activity. The enzyme contains three potential sites for glycosylation (Weinstein *et al.* 1987, J. Biol Chem. **262**: 17735-17743) and it has been shown to be a glycoprotein. In this study the importance of the carbohydrate chains for catalytic activity was studied. Treatment of native α2,6ST with N-glycanase™, which cleaves N-linked oligosaccharide chains, resulted in the loss of about 80% of enzyme activity after 6 hours of incubation; controls, incubated in the absence of N-glycanase, showed little loss of activity under the same conditions. It was found that the presence of up to 10% methanol or ethanol was essential for efficient removal of carbohydrate chains from native α2,6ST BY N-Glycanase.

Immunoblot analysis of N-glycanase treated α2,6ST showed three bands. One corresponded to the native enzyme with M_r 42,000, a second corresponded to a completely deglycosylated form of the enzyme with M_r 38,000 and a third band was intermediate between the two. The completely deglycosylated α2,6ST was the main form of the enzyme found after treatment of the native or denatured enzyme with N-glycanase for 18 hours. The results show that catalytic activity of α2,6ST is dependent on the presence of the carbohydrate chains which presumably influence the conformation of

the enzyme. The work also suggests that the presence of carbohydrate chains of the correct structure on α2,6ST may influence the expression of the catalytic activity of the enzyme in the Golgi complex.

ABBREVIATIONS

Asn	aspargine
BSA	bovine serum albumin
CMP-NeuAc	cytidine-5′-monophosphate-N-Acetylneuraminic acid
Dol	dolichol
Dol-P	dolichol-phosphate
Dol-P-P	dolichol pyrophosphate
dpm	disintegrations per minute
EDTA	ethylene diamine tetraacetate
endo F	endo-β-N-acetylglucosaminidase F (E.C. 3.2.1.96)
endo H	endo-β-N-acetylglucosaminidase H (E.C. 3.2.1.96)
ER	endoplasmic reticulum
EtOH	ethanol
g	gram
Glc	glucose
GlcNAc	N-Acetylglucosamine
hr.	hour
HSA	human serum albumin
Man	mannose
MeOH	methanol
min.	minute
Mn^{2+}	manganese
M_r	relative molecular mass
NeuAc	N-acetylneuraminic acid

N-Gly	N-glycanase™ (peptide-N^4-(N-acetyl-β-glucosylaminyl)-asparagine amidase F) (E.C. 3.5.1.52)
SDS	sodium dodecyl sulfate
SDS PAGE	SDS polyacrylamide gel electrophoresis
Ser	serine
α2,6ST	galactose β1,4-N-acetylglucosamine α2,6-sialyltransferase (EC 2.4.99.1)
TEMED	N,N,N′,N′-tetramethylethylenediamine
Thr	threonine
Tris	2-amino-2-hydroxymethylpropane-1,3-diol
WGA	wheat germ agglutinin

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

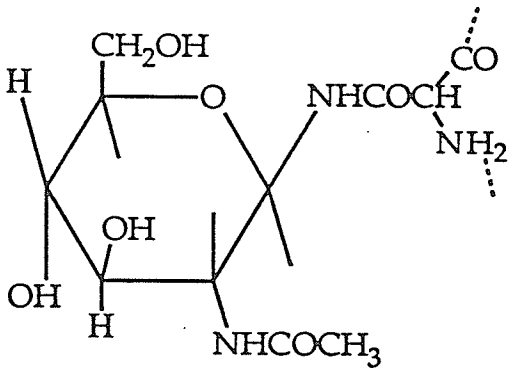
INTRODUCTION:

History

Glycoproteins are found widely distributed in nature with the possible exception of procaryotes. Halobacteria are the only procaryotes that have been shown to contain glycoproteins (Sharon and Lis, 1982). They occur in both soluble and membrane bound forms, in both the inter and intracellular matrices, as well as in extracellular fluids. Types of proteins which may be glycoproteins include enzymes, immunoglobulins, hormones, toxins, lectins and structural proteins. Glycoproteins may be secreted, soluble, membrane bound or structural in nature (Sharon and Lis, 1982). The carbohydrate content of glycoproteins varies from 1 to over 85% of the total weight. Their existence has been known for over 100 years with the first paper published in 1865 (Maley *et al.*, 1989). However, glycoprotein research did not become widespread until the early 1960's, when a linkage between the anomeric carbon of N-Acetylglucosamine and the amide nitrogen of asparagine was defined (Fig. 1). There are several other linkages which have been defined to date. The carbohydrate portion of glycoproteins is made up of heterosaccharide chains consisting of a number of glycosidically linked monosaccharides. Of the greater than 150 monosaccharides found in nature only 11 are commonly found in glycoproteins (Sharon and Lis, 1982). The number of carbohydrate residues on a glycoprotein can vary widely from a mono- or disaccharide (collagen) to branched oligosaccharide containing about 20 monosaccharide units (plasma glycoproteins); to linear polysaccharides of more than 100 residues (proteoglycans). The number of carbohydrate units can range from 1 to more than 100 and are usually distributed unevenly along the polypeptide backbone. More than one type of carbohydrate-peptide linkage

Figure 1. Bond between N-acetylglucosamine and asparagine.

Bond between N-acetylglucosamine and asparagine. This structure is the link for all N-linked type oligosaccharide chains. The asparagine is always part of a sequon consisting of Ser(Thr)-X-Asn. Where X is any amino acid except proline.



may occur in the same protein (Sharon and Lis, 1982). The role of the protein moiety has been defined for most glycoproteins, but the contribution of the carbohydrate portion remains undefined for many proteins. Since monosaccharides are able to combine with each other at different linkage positions (ie. 1-2, 2-6, or 1-4, etc.) and can form branched structures, and since the glycosidic linkages may be either α or β, a very small number of monosaccharides can be combined to give many different polysaccharide structures. For example, three molecules of glucose can form 176 different trisaccharides and, if three different hexose sugars were used, up to 1056 different trisaccharides could be formed, whereas three different amino acids could only form 6 tripeptides (Sharon and Lis, 1982). Therefore, we can see that carbohydrates are more difficult to analyze than amino acid chains of the same size. Often there is microheterogeneity of the carbohydrate chains (this is the observation that different molecules of a glycoprotein may differ in size and composition of their carbohydrate chains) which adds to the difficulty in determining the exact structure of the carbohydrate on any given glycoprotein.

Acute Phase Response

The acute phase response has been known since ancient times when the Greeks recognized that red cells from the blood of an ill person sedimented rapidly in contrast to those of a healthy person (Kushner, 1988). The acute phase response occurs as a result of the body being challenged by inflammatory stimuli. These stimuli may include bacterial infection, trauma, bone fractures, burns, tissue infarction, immunological responses or even severe exertion (Kushner, 1988). In experimental animals, the acute phase response is often induced by infection, trauma, burns or by injection of turpentine or other substances that result in a local inflammatory response. Within hours of the stimulus, changes in

metabolic, endocrine, and physiological functions begin to occur. The most obvious of these in humans is fever. However, major physiological changes occur in the liver, where concentrations of large numbers of plasma proteins are changed. The acute phase reactants are mainly liver synthesized secretable glycoproteins. Accompanying these changes are other alterations such as increases in hepatic glycosyltransferases and in liver pools of nucleotide sugars. There are decreases in lysosomal enzyme activities, changes in liver ultrastructure, dilation of the ER, and proliferation of the Golgi (Turchen *et al.*, 1977). Decreases in serum iron and zinc levels, increases in copper levels, altered nitrogen and lipid metabolism (Beisel, 1980; Langstaff *et al.*, 1980; Milanino *et al.*, 1988), and increases in the levels of a variety of hormones (Jamieson *et al.*, 1987) are also seen. Increased concentrations of acute phase proteins can be assumed to be helpful to the organism in permitting survival, recovery and healing following injury or infection (Kushner, 1988), as many of the acute phase proteins are clotting factors. The acute phase response is thought to be beneficial to the organism by restoring homeostasis disturbed by injury. Proteins whose concentrations increase at least 25% upon acute phase induction are called positive acute phase reactants. Those whose concentrations decrease are called negative acute phase reactants. Table 1 shows the best studied human acute phase proteins. The known acute phase proteins vary greatly in physiological role and function. Of the more than 20 acute phase proteins identified to date, most can be classified into functional classes of plasma proteins such as; proteinase inhibitors, complement components, immunological modulators, and transport proteins. Most of the acute phase reactants are glycoproteins, such as α_1-acid glycoprotein, fibrinogen, and haptoglobin. Mediators of the acute phase response are called cytokines and it is often necessary to have a complicated network of cytokine responses to fully stimulate the response with several

Table 1. Acute phase proteins.

Acute phase proteins are increased in the serum upon trauma or injury. The response can be as great as an increase of 1000 fold over normal levels.

Table 1. Acute Phase Proteins

Protein	Species
Concentration Increases Several Hundred Fold	
C-reactive protein	man, rat
Serum amyloid A protein	man, mouse
Cysteine proteinase inhibitor	rat
$\alpha_2$macroglobulin	rat
Concentration increases two to five fold	
α_1 acid glycoprotein	man, rat, mouse
α_1 antitrypsin	man
α_1 antichymotrypsin	man
Fibrinogen	man, rat, mouse
Haptoglobin	man, rat, mouse
Complement	mouse
Ceruloplasmin	mouse

Adapted from Gauldie, 1989 and Fey and Gauldie, 1989

cytokines acting in harmony or sequence (Koj *et al.*, 1988). Changes in the pattern of glycosylation of a number of glycoproteins are seen upon induction of the acute phase response, and this is thought to be due to the differences in the amount and activity of various glycosylating enzymes (see later section) and the increase in mRNA for the acute phase proteins (Pos *et al.*, 1988). The major site of synthesis of acute phase reactants has been shown to be the liver with multiple acute phase proteins being produced by individual hepatocytes (Kushner, 1988; Harder *et al.*, 1990).

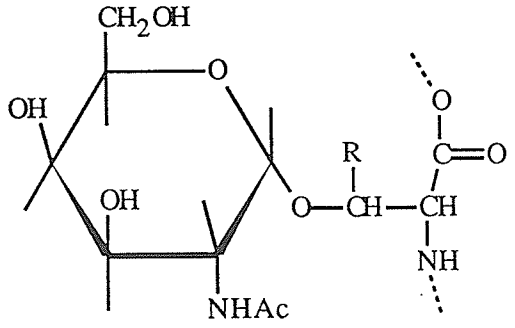
In this thesis the acute phase response was used as a means to elevate the levels of glycoproteins, in particular sialyltransferase, so that studies could be undertaken on the regulation and structure of this enzyme (Jamieson *et al.*, 1987).

Biosynthesis of Carbohydrate Chains on Glycoproteins

There are two main groups of glycosylated proteins; the N-linked type with the carbohydrate attached to asparagine residues or the O-linked type with the carbohydrate linked to serine or threonine (Baenziger, 1984) as shown in Fig. 2. Only the N-linked type of glycoproteins will be dealt with in this thesis. All N-linked glycoproteins have a common core oligosaccharide, called a chitobiose unit with the structure shown in Fig. 3. The high mannose type has an additional 2-6 mannose residues arranged in a branched structure as shown in Fig. 4. Complex type chains can have two, three, or four additional groups of sugars attached to the mannose residues of the core oligosaccharide giving bi-, tri- or tetra-antennary structures (Fig. 5). These structures can also be bisected or intersected by other sugar groups or be of a hybrid type with a high mannose chain on one arm and a complex type chain on the other, as shown in Fig. 6. Carbohydrate groups can contain phosphate, fucose or sulfate. Sulfate groups are sometimes attached to GlcNAc residues of complex type carbohydrates at

Figure 2. O-linked Carbohydrate.

Bond of O-linked carbohydrate type. This is commonly found linked to serine or threonine residues on the protein. This type of structure is commonly found in glycoproteins from animal sources. There are other types of O-linked carbohydrate bonds not mentioned in this thesis.



R= H SERINE

or

CH_3 THREONINE

Figure 3. Core chitobiose Structure.

Core chitobiose structure common to all N-linked carbohydrate structures. This is universal among all N-linked oligosaccharides.

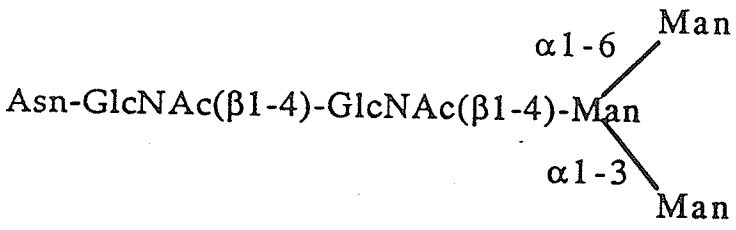
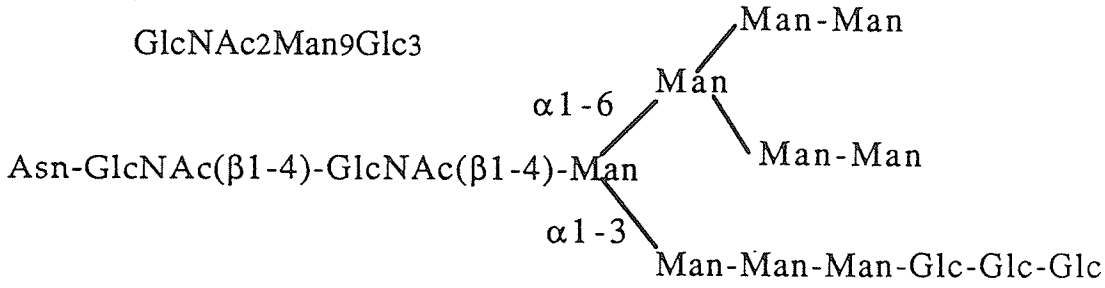


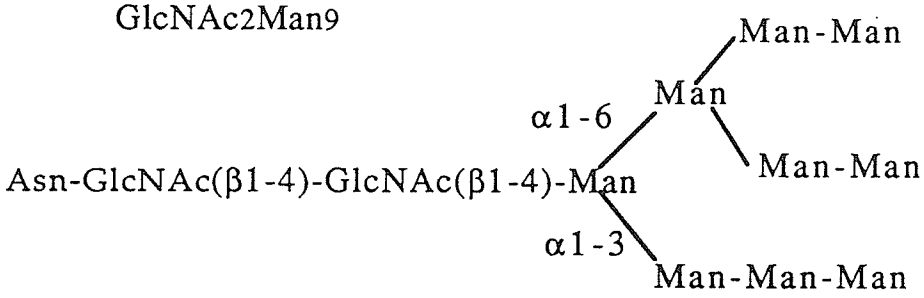
Figure 4. High mannose oligosaccharide structures.

Examples of high mannose oligosaccharide structures. The $GlcNAc_2Man_9Glc_3$ structure shown is the initial structure of all N-linked oligosaccharides prior to any processing reactions. The $GlcNAc_2Man_9$ structure is a commonly found on many glycoproteins. The $GlcNAc_2Man_5$ structure represents a processed form of the high mannose oligosaccharides. A GlcNAc intersected structure is a novel high mannose oligosaccharide.

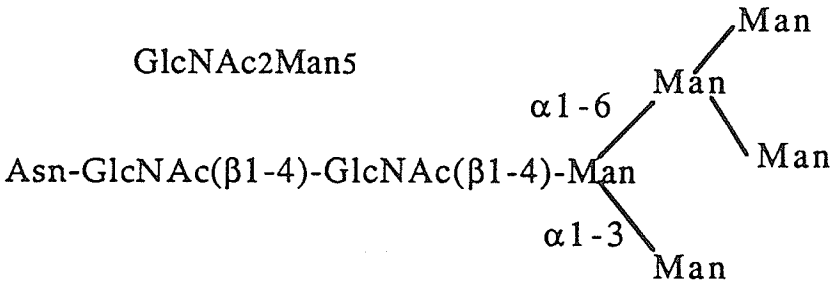
GlcNAc2Man9Glc3



GlcNAc2Man9



GlcNAc2Man5



Intersected GlcNAc2Man9

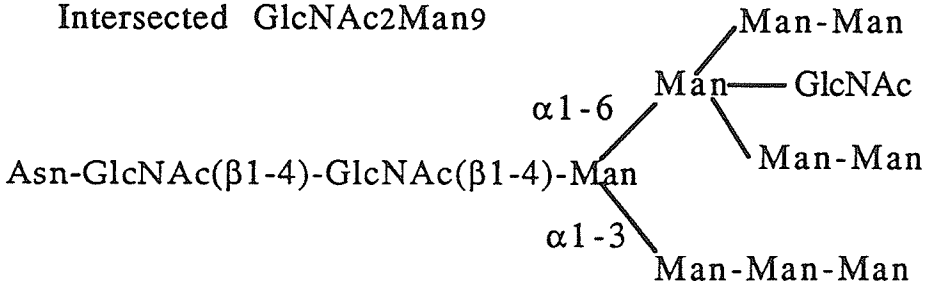
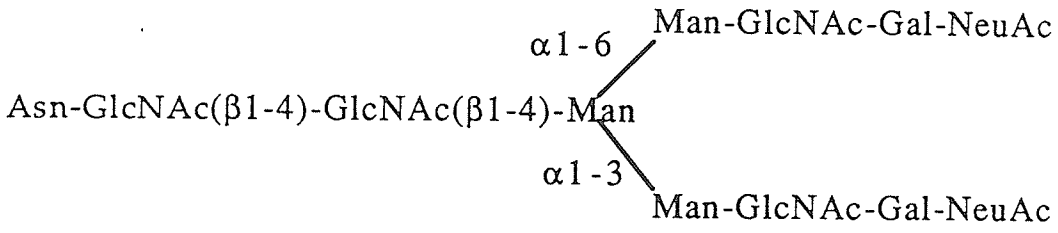


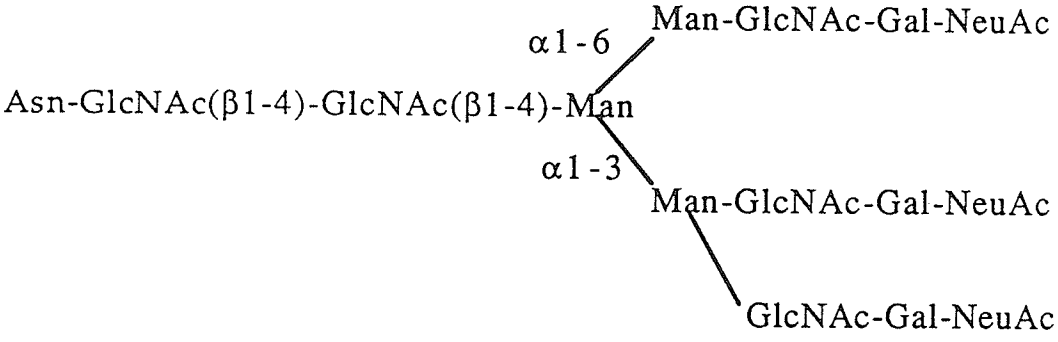
Figure 5. Complex type oligosaccharide structures.

Three common complex type oligosaccharides are bianntennary, triantennary, and tetrantennary. Branching at the core residues allows for the attachment of up to three terminal triplet sequences per mannose residue. The bisected biantennary oligosaccharide, with a GlcNAc in a β1-4 linkage to the interior mannose of the core, represents one of the several possible bisected complex structures. In addition to the structures shown here, it is also possible to add fucose or other sugars at a variety of sites on the oligosaccharide chain.

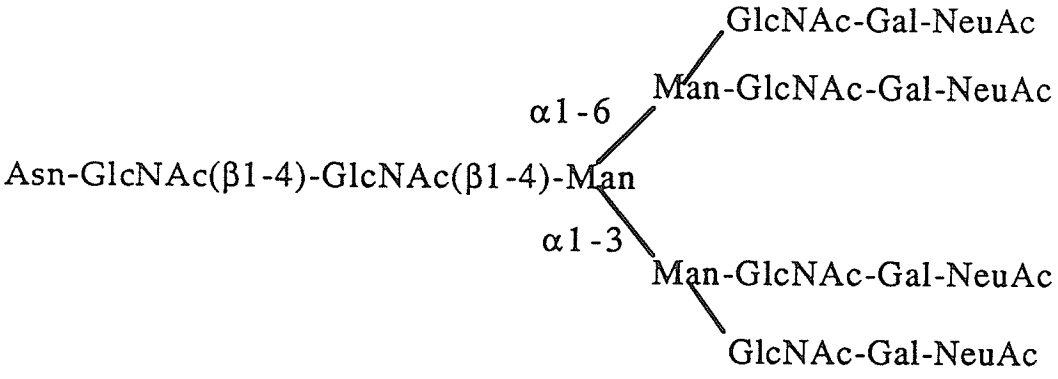
Biantennary



Triantennary



Tetraantennary



Bisected
biantennary

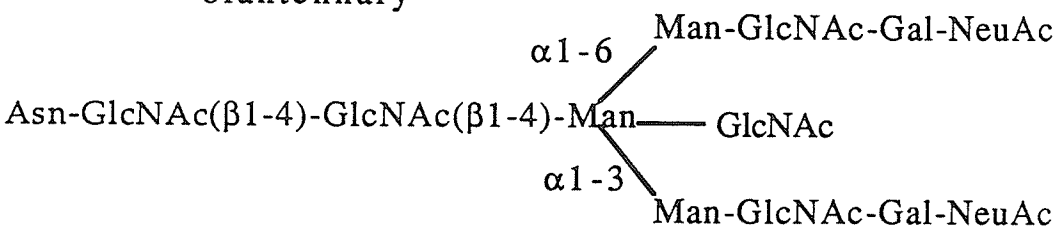
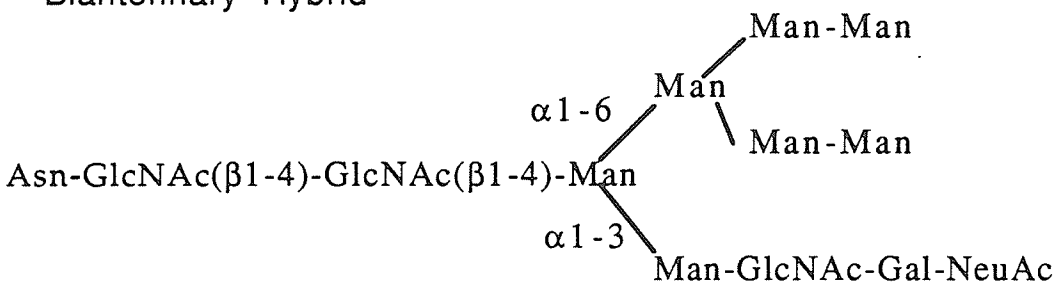


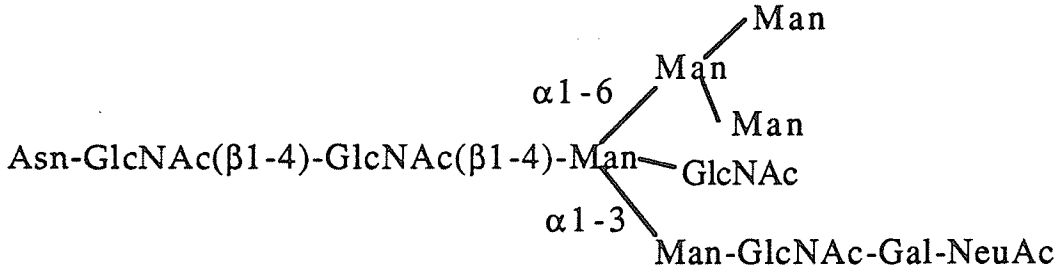
Figure 6. Hybrid oligosaccharide structures.

Hybrid oligosaccharides contain one arm of partially processed high mannose oligosaccharides with a second branch containing a complex terminal triplet sequence. Hybrid oligosaccharides may be bisected or non-bisected.

Biantennary Hybrid



Bisected Biantennary Hybrid



position 6 (Roux *et al.*, 1988) and are most often found where the terminal linkage is an α2-6 linked sialic acid group. Sulfate groups can also be attached to galactose residues at position 3 in place of sialic acids as a terminal addition to the chains (Spiro and Bhoyroo, 1988). The Gal-3-SO_4 groups occur on both biantennary and more highly branched chains whereas the GlcNAc-6-SO_4 occurs only on the latter (Spiro and Bhoyroo, 1988). Sulfate groups have also been shown to be attached to GalNAc structures in the pituitary (Yokoyama *et al.*, 1988). Addition of sulfate to carbohydrate chains is widespread and it is believed to act as a determinant of functional specificity (Freeze and Varki, 1986). As can be seen there exists great variability in the type and or size of the carbohydrate chains and to generate these a large number of enzymes are required.

Dolichol Cycle

The synthesis of N-linked glycoproteins is complex and starts with a common oligosaccharide precursor synthesized *via* the dolichol cycle. The dolichol cycle is shown in Fig. 7. Dolichol is a linear polyisoprene of 16-23 repeating units, with the most common eukaryotic dolichol having 19 of these units (see Fig. 8) (Hirschberg and Snider, 1987). The first seven sugar residues added to dolichol-phosphate come from nucleotide sugars (UDP-GlcNAc and GDP-Man) and the last seven from lipid linked sugars (Man-P-Dol and Glc-P-Dol). The lipid linked sugars are made from Dol-P and the corresponding nucleotide sugars (Hirschberg and Snider, 1987). The synthesis of this oligosaccharide lipid complex takes place within the lumen of the ER after the precursor sugar residues have been transported across the membrane from the cytoplasm where they are made. The transport mechanisms involve nucleotide transporters which transfer the nucleotides across the membrane to the lumenal side of the organelles, with the exception of GDP-Man which does not have a

Figure 7. The dolichol cycle.

The Dolichol cycle is the sequence of sugar additions to the lipid carrier molecule resulting in the ultimate formation of the $GlcNAc_2Man_9Glc_3$ oligosaccharide, which is transferred to the asparagine residue of the sequon. In the alternate pathway, a truncated oligosaccharide containing only five mannose residues may also be transferred to the protein. (Jamieson, 1983).

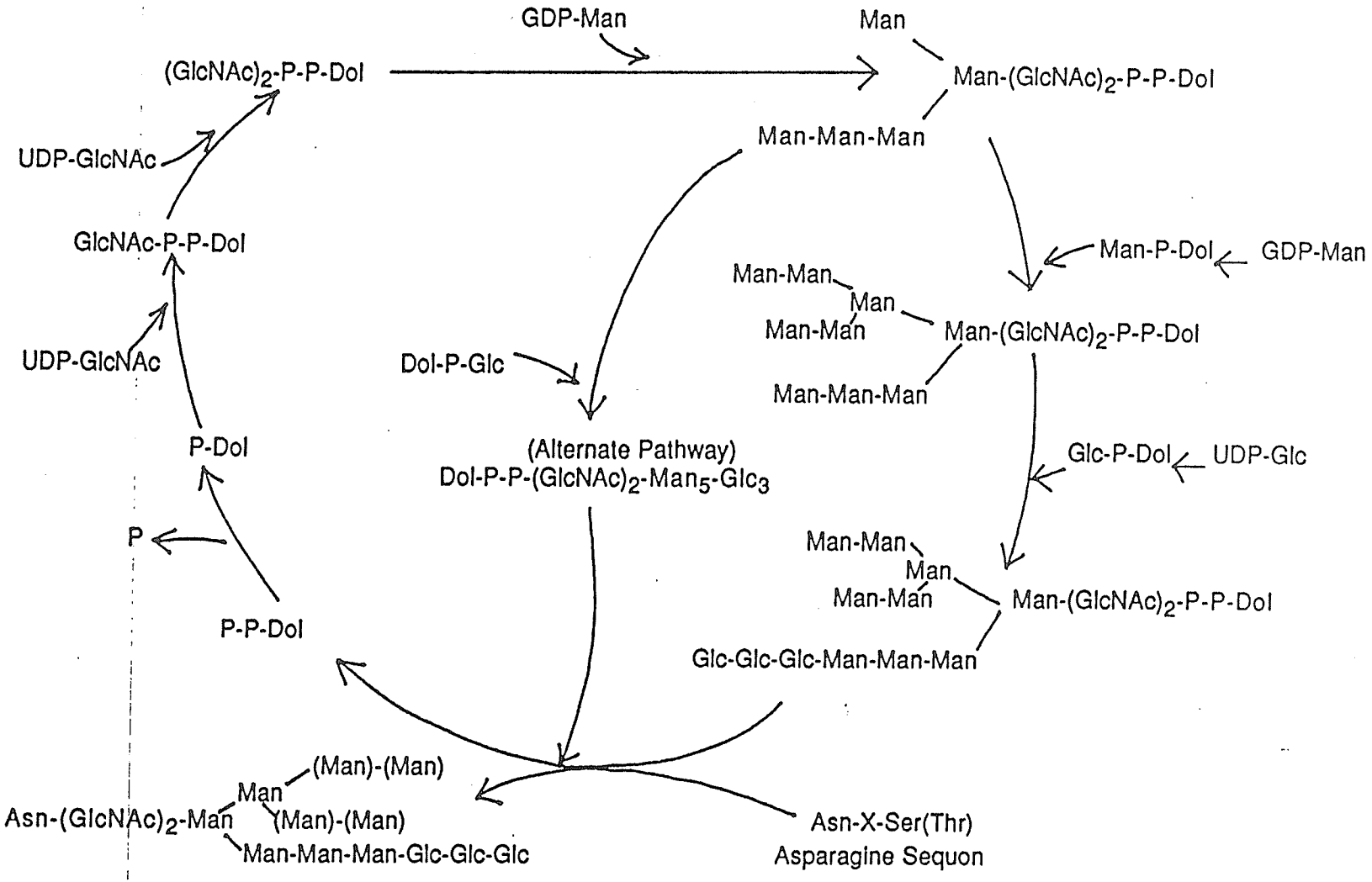
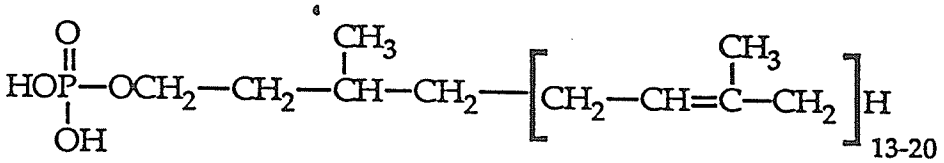

GDP-Man
Man
(GlcNAc)2-P-P-Dol
Man-(GlcNAc)2-P-P-Dol
Man-Man-Man
UDP-GlcNAc
GlcNAc-P-P-Dol
Man-P-Dol
GDP-Man
Man-Man
Man
Man-Man
Man-(GlcNAc)2-P-P-Dol
Man-Man-Man
UDP-GlcNAc
Dol-P-Glc
P-Dol
(Alternate Pathway)
Dol-P-P-(GlcNAc)2-Man5-Glc3
Glc-P-Dol
UDP-Glc
P
Man-Man
Man
Man-Man
Man-(GlcNAc)2-P-P-Dol
P-P-Dol
Glc-Glc-Glc-Man-Man-Man
(Man)-(Man)
Man
Asn-(GlcNAc)2-Man
(Man)-(Man)
Man-Man-Man-Glc-Glc-Glc
Asn-X-Ser(Thr)
Asparagine Sequon

Figure 8. Structure of dolichol.

Structure of dolichol. Dolichol may contain from 13-20 repeating units, with 19 being most common. This is utilized in the dolichol cycle as shown in Fig. 7.



DOLICHOL MONOPHOSPHATE

transporter. The enzymes which transfer the activated sugar residues, usually from a nucleotide sugar, onto a growing carbohydrate chain are called glycosyltransferases. There may be as many as 100 different glycosyltransferases necessary to generate all the terminal carbohydrate arrangements possible (Paulson and Colley, 1989). The end product of this cycle (see Fig. 4) is a lipid linked oligosaccharide which contains three glucose, nine mannose and two GlcNAc residues (Hirschberg and Snider, 1987). There is also an alternate pathway in which a $Glc_3Man_5GlcNAc_2$ structure may be transferred to a protein. The steps involved in building up the oligosaccharides *via* the dolichol cycle are as follows.

Step 1. Synthesis of $GlcNAc_2$-PP-Dol, this may occur on either the lumenal or cytoplasmic face of the ER. 2: Synthesis of $Man_5GlcNAc_2$-PP-Dol, the first five mannose residues are added from GDP-Man on the cytoplasmic face of the ER. This is then flipped to the lumenal face of the ER by a mechanism which is not yet understood. 3: Synthesis of Dol-P-Man occurs on the cytoplasmic face and is then flipped to the lumenal face of the ER. The Dol-P mannosyltransferase may catalyze the flip as suggested by its preference for membranes which contain phospholipids that prefer non-bilayer arrangements (Jensen and Schutzbach, 1985, 1986, 1988). 4: Synthesis of $Man_9GlcNAc_2$-PP-Dol, the last four mannose residues are added from Man-P-Dol on the lumenal face of the ER. 5: Synthesis of Dol-P-Glc, this may be occurring on the cytoplasmic side and then flipped to the lumenal face or may be occurring in the lumen after UDP-Glc is translocated into the lumen. 6: Transfer of Glc residues to $Man_9GlcNAc_2$-PP-Dol occurs on the lumenal face of the ER. 7: Transfer of $Glc_3Man_9GlcNAc_2$-PP-Dol to the peptide acceptor, the carbohydrate portion of the precursor is then transferred *en bloc* to the polypeptide by oligosaccharide transferase, forming an N-glycosylamine bond between GlcNAc and asparagine residues (see Fig. 1), the

Dol-PP is released on the lumenal face of the ER and recycled after being converted to Dol-P.

The site of attachment on the protein is known as the asparagine sequon and is found at the site of attachment of all N-linked oligosaccharides. The amino acid sequence at the sequon is Asn-X-Ser(Thr), where X is any amino acid other than proline and the third amino acid on the carboxyl side is either serine or threonine. The secondary structure of this region is thought to be in β turn. Only a third of potential glycosylation sites are actually used thus suggesting that the surrounding amino acids must contribute to the short range interactions and thus affect the rate and extent of glycosylation. Protein glycosylation requires vitamin A (Chan and Wolf, 1987) as vitamin A deficiency causes accumulation of Dol-P-P-$GlcNAc_2Man_5$. The vitamin A is required at the transfer of oligosaccharide from the lipid carrier to the protein acceptor. Vitamin A may act to destabilize the membrane thus allowing transmembrane movement of substrates. The transfer from the lipid linked oligosaccharide to the protein occurs on the inner face of the lumen of the ER (Kaplan *et al.*, 1987); however, the timing of this event does not appear to be the same for all proteins. For most proteins initial glycosylation occurs while they are still being translated (Lingappa *et al.*, 1978), however, there are some that are glycosylated after being completely translated (Kaplan *et al.*, 1987). For glycosylation which is occurring cotranslationally there may only be a short period of time when the asparagine sequon is exposed in the correct conformation for glycosylation, since after the protein is folded the sites may no longer be accessible to the oligosaccharide transferase; this may explain why some proteins contain unglycosylated sequons. The extent of glycosylation may also be dependent on the amount of the precursor available as it has been shown that increasing the amount of Dol-P increases the glycosylation of secreted RNase from 12 to 90% in bovine pancreas

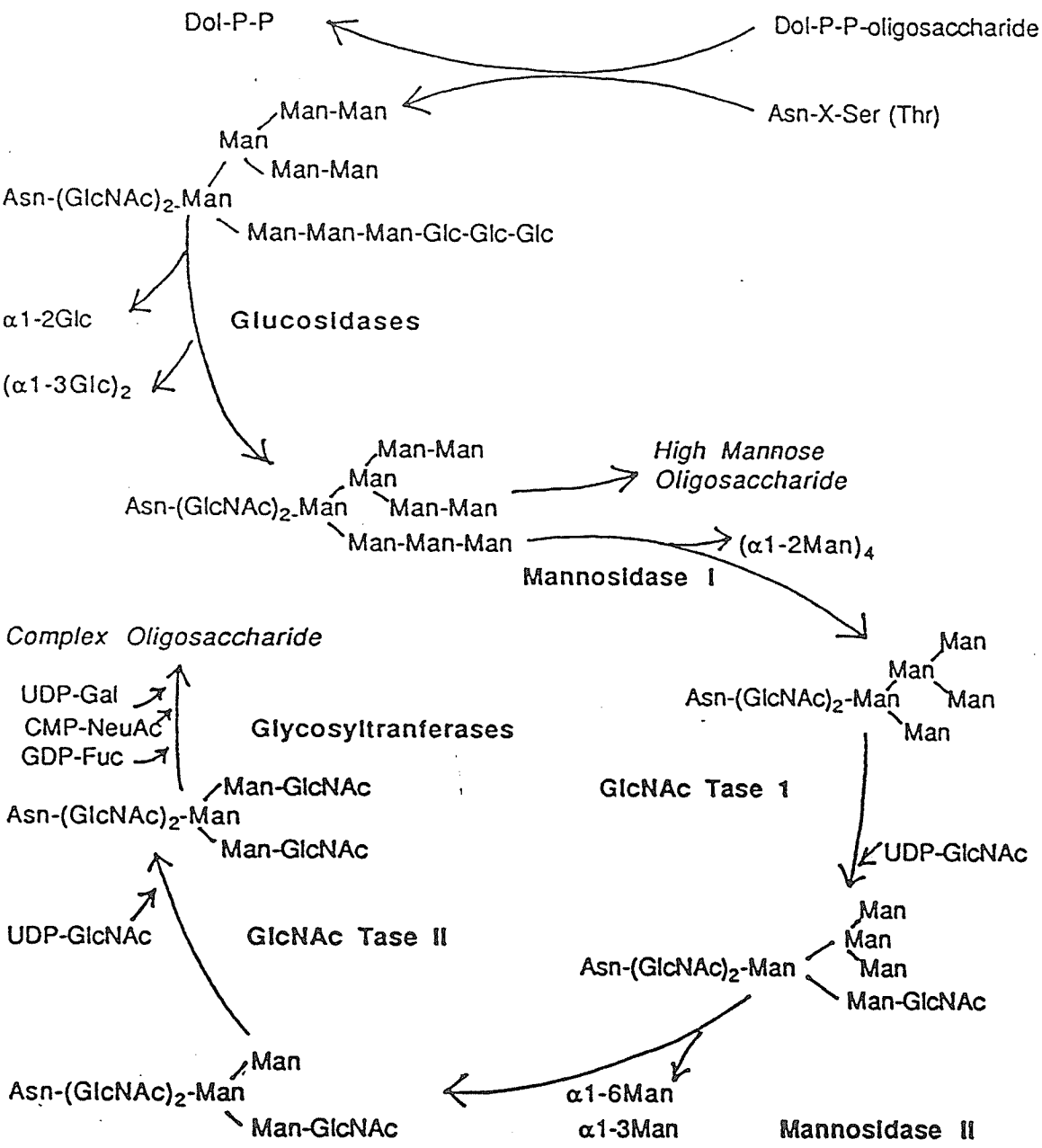
(Kornfeld and Kornfeld, 1985). It has been shown that there must be a spacer of at least 32 amino acid residues between the sequon and the ribosome for glycosylation to occur. The acute phase response causes a 3-4 fold increase in the amount of these intermediates to be made (Silanovich and Jamieson, 1987). The functional significance of these differences in timing is not clear.

Processing

After the transfer of oligosaccharide, some of the sugars are removed from the precursor glycoprotein by oligosaccharide processing enzymes (Roth, 1987). This process is complex and is summarized in Fig. 9. The first sugars to be removed are the glucose residues and this occurs in the ER immediately following glycosylation. There are at least two enzymes which are responsible for the removal of the glucose residues called glucosidases I and II, both are found in the ER membrane. Glucosidase I removes the terminal α1,2 linked glucose and glucosidase II removes the inner two α1,3 linked glucose residues. Next, an α1,2 linked mannose is removed in the ER or Golgi by an α1,2-mannosidase of which there are several (Lubas and Spiro, 1988). The steps mentioned thus far can occur cotranslationally for some proteins (Roth, 1987). It is interesting to note the ER α-mannosidase is not inhibited by the mannose analog deoxymannojirmycin, whereas other mannose processing enzymes are sensitive to the presence of this inhibitor. In some resident ER proteins, the glucose residues are added back onto the carbohydrate chain and it is thought that this may act to help protect the oligosaccharide from degradation (Kornfeld and Kornfeld, 1985). The next step in oligosaccharide processing is to transport the non-resident ER proteins to the *cis* Golgi. The proteins travel in vesicles which bud from the ER and fuse to the Golgi membrane. It has been shown that 1-deoxynojirimycin, a specific inhibitor of both glucosidases I and II, delays exit

Figure 9. Oligosaccharide processing reactions.

The processing reactions remove the three glucose residues resulting in the formation of the high mannose oligosaccharide ($GlcNAc_2Man_9$). The oligosaccharide may remain as a high mannose oligosaccharide or be further modified by mannosidases I and II, and the transferase enzymes which add the sugar residues found in the hybride and complex oligosaccharides (Jamieson, 1983).


Dol-P-P
Dol-P-P-oligosaccharide
Asn-X-Ser (Thr)
Man-Man
Man
Man-Man
Asn-(GlcNAc)2-Man
Man-Man-Man-Glc-Glc-Glc
α1-2Glc
(α1-3Glc)2
Glucosidases
Man-Man
Man
Asn-(GlcNAc)2-Man
Man-Man
Man-Man-Man
High Mannose
Oligosaccharide
(α1-2Man)4
Mannosidase I
Complex Oligosaccharide
UDP-Gal
CMP-NeuAc
GDP-Fuc
Glycosyltranferases
Man-GlcNAc
Asn-(GlcNAc)2-Man
Man-GlcNAc
UDP-GlcNAc
GlcNAc Tase II
Man
Man
Asn-(GlcNAc)2-Man
Man
Man-GlcNAc
GlcNAc Tase 1
UDP-GlcNAc
Man
Man
Asn-(GlcNAc)2-Man
Man
Man-GlcNAc
α1-6Man
α1-3Man
Mannosidase II

of some, but not all glycoproteins from the ER. Also, it has been postulated that glucose trimming may be necessary for efficient movement from the ER to the Golgi by forming a recognition site for a transport receptor, or that the presence of glucose influences the folding of proteins in such a way that transport is affected (Lodish and Kong, 1984).

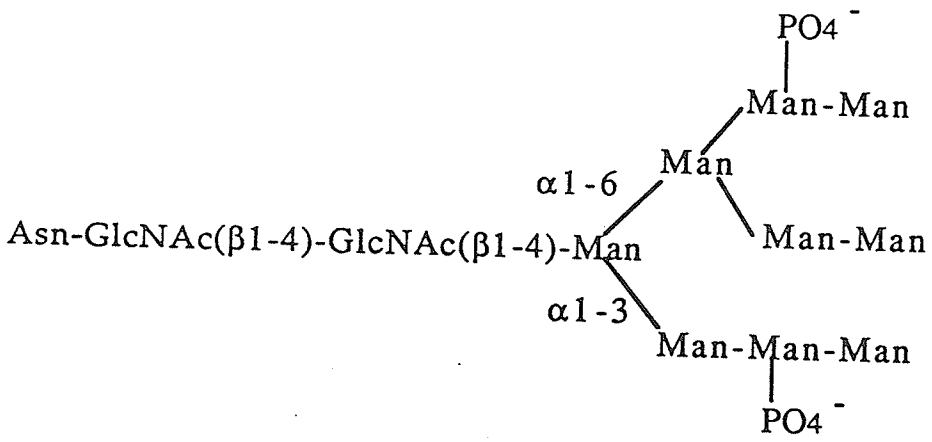
Once the glycoprotein is in the Golgi it is transported from the *cis* through the *medial* and to the *trans* cisternae by non-clathrin coated vesicular transport. If the glycoprotein is a lysosomal enzyme, it is acted on by N-acetylglucosaminyl-phosphotransferase and N-acetylglucosamine-1-phosphodiester α-N-Acetylglucosaminidase in a sequential manner to produce a high mannose chain with a mannose 6-phosphate signal (see Fig. 10). This is believed to occur in the *cis* or *medial* Golgi cisternae (Kornfeld, 1990).

The high mannose oligosaccharides on non-lysosomal enzymes are trimmed by Golgi α1,2 mannosidases (Golgi mannosidases IA and IB) to yield a $Man_5GlcNAc_2$ structure. If these residues are not removed then a high mannose type glycoprotein is produced; removal of these mannose residues is a necessary first step towards becoming a complex type glycoprotein. This occurs in the *cis* or *medial* Golgi. Those oligosaccharides destined to become complex type structures are then processed by addition of a GlcNAc by N-acetylglucosaminyltransferase I to the terminal α1,3 mannose residue. This is followed by the removal of two mannose (α1,3 and α1,6) residues by Golgi α-mannosidase II which gives the GlcNAc-$Man_3GlcNAc_2$ structure. This step is dependent on the action of the GlcNAc transferase I reaction.

Further glycosylation reactions can take place by addition of more GlcNAc residues to mannose residues or the addition of fucose to the innermost GlcNAc. The assembly of biantennary complex chains is initiated by GlcNAc transferase II. If addition of a second GlcNAc to the inner α1,3 mannose by GlcNAc

Figure 10. Mannose 6-phosphate signal.

The structure of the mannose 6-phosphate signal, this is used to direct lysosomal enzymes to the lysosomes. It is detected by a mannose 6-phosphate receptor which is located in the Golgi.



transferase IV occurs, the pathway for triantennary complex chains is initiated. A second GlcNAc can also be added to the β linked mannose residue of the core by GlcNAc transferase III. This GlcNAc is linked between the two α-linked mannoses and is called the "bisecting" GlcNAc (see Fig. 5). Addition of this residue alters the conformation of the inner core and prevents Golgi α-mannosidase II, GlcNAc transferase II and fucosyltransferase from acting and thus prevents further processing of hybrid structures (Roth, 1987). Another mechanism used to control the order of glycosylation is the compartmentalization of the various glycosyltransferases into ER, *cis*, *medial* or *trans* Golgi. It has been shown that the glycosyltransferases involved in the terminal glycosylation steps are distributed across the *cis* to *trans* gradient of the Golgi, roughly in the order that they act (Kornfeld and Kornfeld, 1985). The final oligosaccharide structure of the proteini s determined by which enzyme(s) are present and their specificity (Yet *et al.*, 1988). Most processing enzymes act in a sequential order using the product of the previous reaction as a substrate (Roth, 1987). After this complex carbohydrates are built up by the action of N-Acetylglucosamine-, galactosyl-, sialyl-, sulfo-, and fucosyl-transferases (Kornfeld and Kornfeld, 1985). These late acting glycosyltransferases have been detected in the *trans* Golgi by density gradient centrifugation and by immunocytochemical labeling (Roth, 1987). It is known that the terminal glycosylation steps are the most important in determining the structure of the oligosaccharide chains. Control of this process is thought to derive from the protein structure which determines the accessibility of the glycosyltransferases, the specificity of the glycosyltransferases and the level of expression of the glycosyltransferases. Glycosyltransferase expression is regulated at the level of transcription (Paulson and Colley, 1989).

It was found that the protein structure influences the rate of synthesis and processing of carbohydrate side chains as well as the sugar composition (Shao and Wold, 1988). Shao and Wold studied three Golgi located processing enzymes and suggested that the protein matrix affected the catalytic efficiency rather than the substrate affinity of the processing enzymes. N-Linked oligosaccharides at different glycosylation sites on a single polypeptide can vary widely in structure possessing different degrees of processing and terminal glycosylation. (Hubbard, 1988; Paulson, 1989). Variations such as these reflect the effect of the polypeptide structure in determining the oligosaccharide composition by sterically preventing access of one or more processing glycosidases or terminal glycosyltransferases.

Role of Carbohydrate Chains on Glycoproteins

The role of carbohydrate chains on proteins has only recently begun to be understood. Many glycoproteins require their carbohydrate chains for activity *in vivo*. The oligosaccharide chains may influence the final structure of the protein, by directing the folding of the protein into the correct conformation (Paulson, 1989). This is reasonable since the oligosaccharides are often added cotranslationally. However this is not the case for all glycoproteins since some are unaffected by inhibitors of glycosylation such as tunicamycin which prevents N-glycosylation. Oligosaccharide chains of glycoproteins are often found on the surface of cells and thus may function as receptors or mediators for cell-cell interactions (Yet *et al.*, 1988; Paulson, 1989). For example; myoblast cells with defective carbohydrate chains do not fuse properly (Spearman *et al.*, 1987). Glycosylation has also been shown to be important as a signal for protein targeting, with the best known example of this being the mannose 6-phosphate sequence of lysosomal proteins which are responsible for targeting these proteins

to the lysosomes (described above). Another example of the importance of carbohydrate chain structure on transport is seen for α_1-antitrypsin and α_1-antichymotrypsin where glucose removal from the $Glc_{1-3}Man_9GlcNAc_2$ structure is required prior to these proteins leaving the ER (Lodish and Kong, 1984). Glycosylation has been shown to be important for the biosynthesis, secretion and function of erythropoietin made in kidney cells. This protein has 3 N-linked and one O-linked chains. If the chains are removed, there is rapid degradation of the protein (Dube *et al.*, 1988). Oligosaccharide chains have also been shown to be important for biological activity, for example, the Na^+/H^+ antiporter from rat renal brush border membranes must have intact N-linked oligosaccharides of the biantennary complex type for transport of Na^+/H^+, removal of the chains by endoglycosidases also decreased the rate of Na^+/H^+ transport (Yusufi *et al.*, 1988). The location of the carbohydrate on the protein is important (Yet *et al.*, 1988) as it may confer some partial structural stability (Gibson *et al.*, 1980). For example, inhibiting glycosylation of the low density lipoprotein receptor with tunicamycin causes the receptor to be smaller in size, have a lower binding capacity, both due to a less stable structure (Filipovic, 1989). For some proteins the presence or absence of carbohydrate chains can help to determine its biological activity. A good example of this is prolactin, the unglycosylated form was shown to have lower activity and to be more selective in its action, but this change of activity is species dependent (Markoff *et al.*, 1988). This is also true for the human transferrin receptor which has a much lower affinity for its substrate if it is unglycosylated (Hunt *et al.*, 1989). Glycosylation has also been shown to be important for the subunit assembly and folding of a number of hormones (Ronin *et al.*, 1987). For example, the subunits of thyrotropin combine early in their biosynthesis in the ER, and assembly is completed as the chains are trimmed and elongated to complex structures, allowing the hormone to be active. This

probably involves discrete conformational changes dependent on the dimeric hormone complex (Ronin *et al.*, 1987). The degree of processing may also have an effect on the activity of some glycoproteins, with incompletely processed chains. The human fibronectin receptor can be assembled and inserted into the plasma membrane, but is not active unless the chains are fully processed (Akiyama *et al.*, 1989).

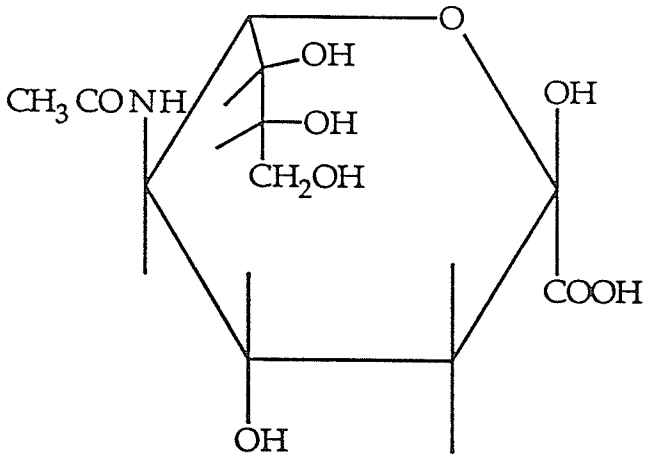
When hormones have been modified to remove or prevent glycosylation from occurring it has been shown that the glycosylation site nearest the amino terminus of the hormone is most important for hormone signal transduction (Sairam, 1989).

Sialyltransferase

β-galactoside α-2,6-sialyltransferase (EC 2.4.99.1) is the enzyme that is responsible for attaching a sialic acid group (see Fig. 11) in an α2,6 linkage to the terminal galactose residue of serum N-linked glycoproteins. Recently, it has been shown that the enzyme may not be as specific as once thought, and that it may add NeuAc to mannose residues which are linked to GlcNAc although with a lower affinity for the mannose substrate (van Pelt *et al.*, 1989). This is only one of up to twelve sialyltransferases known to date (Weinstein *et al.*, 1987). The α2,6ST responsible for attachment of NeuAc to secretable serum glycoproteins is located on the lumenal face of the *trans* Golgi and the *trans* Golgi network of liver (Roth *et al.*, 1985; Carey and Hirschberg, 1981;Weinstein *et al.*, 1987). In intestinal absorbtive cells, a different sialyltransferase is localized throughout the *cis, medial* and *trans* Golgi regions (Roth *et al.*, 1986) although it has widespread tissue distribution. The α2,6ST is translated from a mRNA that includes a 1209 nucleotide open reading frame and an extensive 3'-untranslated region (Weinstein *et al.*, 1987). There appears to be tissue specific expression of α2,6ST

Figure 11. Neuraminic Acid.

The structure of neuraminic acid, one of the most common sialic acids.



with multiple sizes of mRNA generated by what is thought to be alternate splicing of mRNA in various tissues (O'Hanlon *et al.*, 1989; Paulson *et al.*, 1989). This also causes differences in the amounts of mRNA expressed in each tissue. For the rat the highest levels were seen in the liver, with intermediate levels seen in ovary, kidney, spleen and lung, and the lowest levels seen in brain and heart (Paulson *et al.*, 1989). The nucleic acid sequence has been determined, for both rat and human α2,6ST (Weinstein *et al.*, 1987 and Grundmann *et al.*, 1990) and both predict 46.7 kDa polypeptides with a short amino-terminal cytosolic region, a single transmembrane domain, and a large carboxy-terminal domain which contains the catalytic region. The human and rat forms of α2,6ST show 87.6% homology in protein sequence as shown in Figs. 12 and 13 (Grundmann *et al.*, 1990). The human α2,6ST has two potential N-glycosylation sites whereas the rat α2,6ST has three potential sites. There is also a soluble form of the enzyme which can be released into the serum, this is derived by cleaving the catalytic domain from the membrane anchor by a cathepsin D-like proteinase activity (most likely the lysosomal enzyme cathepsin D) which has a preference for acidic pH (Lammers and Jamieson, 1988, 1989, 1990). The signal for retaining α2,6ST in the Golgi is to be found in the 57 amino acid region at the N-terminus since removal of these causes the α2,6ST to be secreted. Thus, the catalytic domain of the enzyme cannot be responsible for retaining the protein in the Golgi (Colley et al., 1989). The soluble form of the enzyme has M_r of about 40,000 (Lammers and Jamieson, 1988; Weinstein *et al.*, 1982), thus suggesting that the purified α2,6ST is a catalytically active fragment of the membrane bound form.

α2,6ST has been shown to be an acute phase reactant (Kaplan *et al.*,1983; Lammers and Jamieson, 1986; Woloski *et al.*, 1986; Lammers *et al.*, 1986) which increases up to 5 fold in the serum of rats. The mechanism of induction of the α2,6ST response in the acute phase state is unknown, but is preceded by an

Figure 12. The cDNA and amino acid sequence of rat α2,6ST.

The cDNA and amino acid sequence of rat galactose β1,4 N-acetylglucosamine α2,6 sialyltransferase (Weinstein *et al.*, 1987). Note the site of cleavage by cathepsin D at amino acid 63. The potential N-glycosylation sites are boxed (residues 146, 159, and 285).

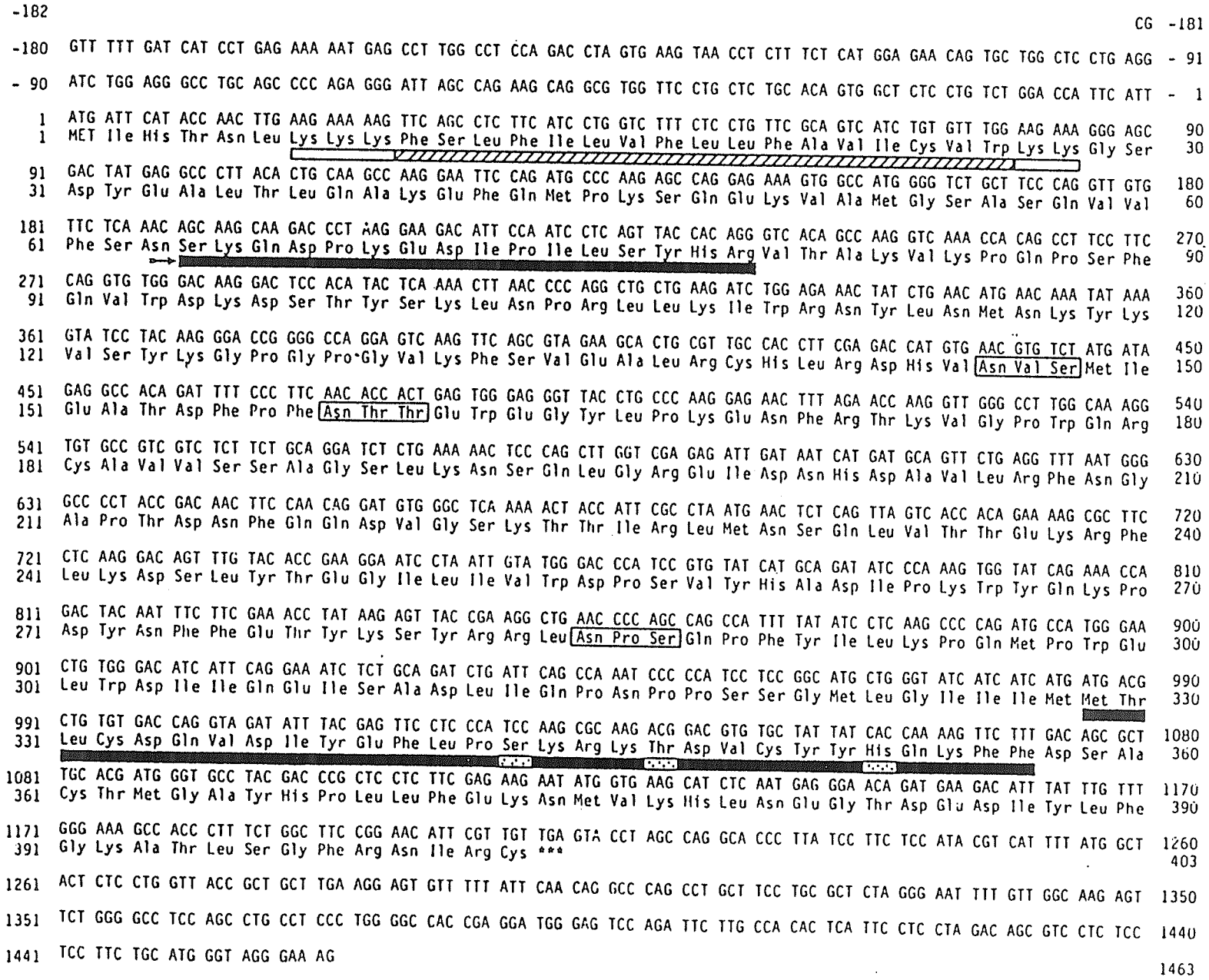
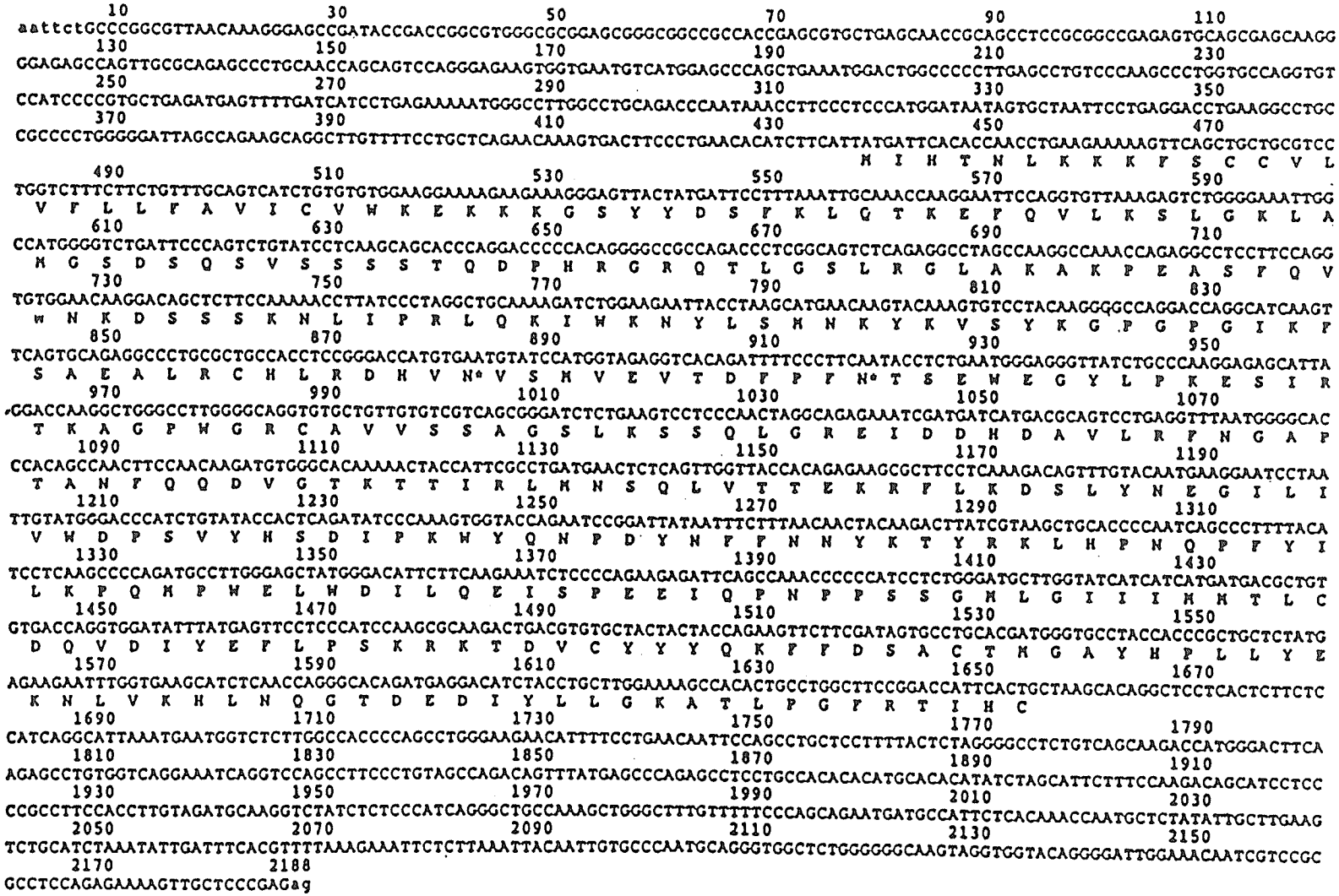

-182
CG -181
-180 GTT TTT GAT CAT CCT GAG AAA AAT GAG CCT TGG CCT CCA GAC CTA GTG AAG TAA CCT CTT TCT CAT GGA GAA CAG TGC TGG CTC CTG AGG - 91
- 90 ATC TGG AGG GCC TGC AGC CCC AGA GGG ATT AGC CAG AAG CAG GCG TGG TTC CTG CTC TGC ACA GTG GCT CTC CTG TCT GGA CCA TTC ATT - 1
1 ATG ATT CAT ACC AAC TTG AAG AAA AAG TTC AGC CTC TTC ATC CTG GTC TTT CTC CTG TTC GCA GTC ATC TGT GTT TGG AAG AAA GGG AGC 90
1 MET Ile His Thr Asn Leu Lys Lys Lys Phe Ser Leu Phe Ile Leu Val Phe Leu Leu Phe Ala Val Ile Cys Val Trp Lys Lys Gly Ser 30
91 GAC TAT GAG GCC CTT ACA CTG CAA GCC AAG GAA TTC CAG ATG CCC AAG AGC CAG GAG AAA GTG GCC ATG GGG TCT GCT TCC CAG GTT GTG 180
31 Asp Tyr Glu Ala Leu Thr Leu Gln Ala Lys Glu Phe Gln Met Pro Lys Ser Gln Glu Lys Val Ala Met Gly Ser Ala Ser Gln Val Val 60
181 TTC TCA AAC AGC AAG CAA GAC CCT AAG GAA GAC ATT CCA ATC CTC AGT TAC CAC AGG GTC ACA GCC AAG GTC AAA CCA CAG CCT TCC TTC 270
61 Phe Ser Asn Ser Lys Gln Asp Pro Lys Glu Asp Ile Pro Ile Leu Ser Tyr His Arg Val Thr Ala Lys Val Lys Pro Gln Pro Ser Phe 90
271 CAG GTG TGG GAC AAG GAC TCC ACA TAC TCA AAA CTT AAC CCC AGG CTG CTG AAG ATC TGG AGA AAC TAT CTG AAC ATG AAC AAA TAT AAA 360
91 Gln Val Trp Asp Lys Asp Ser Thr Tyr Ser Lys Leu Asn Pro Arg Leu Leu Lys Ile Trp Arg Asn Tyr Leu Asn Met Asn Lys Tyr Lys 120
361 GTA TCC TAC AAG GGA CCG GGG CCA GGA GTC AAG TTC AGC GTA GAA GCA CTG CGT TGC CAC CTT CGA GAC CAT GTG AAC GTG TCT ATG ATA 450
121 Val Ser Tyr Lys Gly Pro Gly Pro Gly Val Lys Phe Ser Val Glu Ala Leu Arg Cys His Leu Arg Asp His Val Asn Val Ser Met Ile 150
451 GAG GCC ACA GAT TTT CCC TTC AAC ACC ACT GAG TGG GAG GGT TAC CTG CCC AAG GAG AAC TTT AGA ACC AAG GTT GGG CCT TGG CAA AGG 540
151 Glu Ala Thr Asp Phe Pro Phe Asn Thr Thr Glu Trp Glu Gly Tyr Leu Pro Lys Glu Asn Phe Arg Thr Lys Val Gly Pro Trp Gln Arg 180
541 TGT GCC GTC GTC TCT TCT GCA GGA TCT CTG AAA AAC TCC CAG CTT GGT CGA GAG ATT GAT AAT CAT GAT GCA GTT CTG AGG TTT AAT GGG 630
181 Cys Ala Val Val Ser Ser Ala Gly Ser Leu Lys Asn Ser Gln Leu Gly Arg Glu Ile Asp Asn His Asp Ala Val Leu Arg Phe Asn Gly 210
631 GCC CCT ACC GAC AAC TTC CAA CAG GAT GTG GGC TCA AAA ACT ACC ATT CGC CTA ATG AAC TCT CAG TTA GTC ACC ACA GAA AAG CGC TTC 720
211 Ala Pro Thr Asp Asn Phe Gln Gln Asp Val Gly Ser Lys Thr Thr Ile Arg Leu Met Asn Ser Gln Leu Val Thr Thr Glu Lys Arg Phe 240
721 CTC AAG GAC AGT TTG TAC ACC GAA GGA ATC CTA ATT GTA TGG GAC CCA TCC GTG TAT CAT GCA GAT ATC CCA AAG TGG TAT CAG AAA CCA 810
241 Leu Lys Asp Ser Leu Tyr Thr Glu Gly Ile Leu Ile Val Trp Asp Pro Ser Val Tyr His Ala Asp Ile Pro Lys Trp Tyr Gln Lys Pro 270
811 GAC TAC AAT TTC TTC GAA ACC TAT AAG AGT TAC CGA AGG CTG AAC CCC AGC CAG CCA TTT TAT ATC CTC AAG CCC CAG ATG CCA TGG GAA 900
271 Asp Tyr Asn Phe Phe Glu Thr Tyr Lys Ser Tyr Arg Arg Leu Asn Pro Ser Gln Pro Phe Tyr Ile Leu Lys Pro Gln Met Pro Trp Glu 300
901 CTG TGG GAC ATC ATT CAG GAA ATC TCT GCA GAT CTG ATT CAG CCA AAT CCC CCA TCC TCC GGC ATG CTG GGT ATC ATC ATC ATG ATG ACG 990
301 Leu Trp Asp Ile Ile Gln Glu Ile Ser Ala Asp Leu Ile Gln Pro Asn Pro Pro Ser Ser Gly Met Leu Gly Ile Ile Ile Met Met Thr 330
991 CTG TGT GAC CAG GTA GAT ATT TAC GAG TTC CTC CCA TCC AAG CGC AAG ACG GAC GTG TGC TAT TAT CAC CAA AAG TTC TTT GAC AGC GCT 1080
331 Leu Cys Asp Gln Val Asp Ile Tyr Glu Phe Leu Pro Ser Lys Arg Lys Thr Asp Val Cys Tyr Tyr His Gln Lys Phe Phe Asp Ser Ala 360
1081 TGC ACG ATG GGT GCC TAC GAC CCG CTC CTC TTC GAG AAG AAT ATG GTG AAG CAT CTC AAT GAG GGA ACA GAT GAA GAC ATT TAT TTG TTT 1170
361 Cys Thr Met Gly Ala Tyr His Pro Leu Leu Phe Glu Lys Asn Met Val Lys His Leu Asn Glu Gly Thr Asp Glu Asp Ile Tyr Leu Phe 390
1171 GGG AAA GCC ACC CTT TCT GGC TTC CGG AAC ATT CGT TGT TGA GTA CCT AGC CAG GCA CCC TTA TCC TTC TCC ATA CGT CAT TTT ATG GCT 1260
391 Gly Lys Ala Thr Leu Ser Gly Phe Arg Asn Ile Arg Cys *** 403
1261 ACT CTC CTG GTT ACC GCT GCT TGA AGG AGT GTT TTT ATT CAA CAG GCC CAG CCT GCT TCC TGC GCT CTA GGG AAT TTT GTT GGC AAG AGT 1350
1351 TCT GGG GCC TCC AGC CTG CCT CCC TGG GGC CAC CGA GGA TGG GAG TCC AGA TTC TTG CCA CAC TCA TTC CTC CTA GAC AGC GTC CTC TCC 1440
1441 TCC TTC TGC ATG GGT AGG GAA AG 1463

Figure 13. The cDNA sequence of human α2,6ST.

The cDNA sequence of human galactose β1,4-N-acetylglucosamine α2,6-sialyltransferase (Grundmann *et al.*, 1990). The two potential N-linked glycosylation sites are at amino acids 149 and 161. The protein sequence shows 87.6% similarity to the rat protein sequence.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M I H T N L K K K F S C C V L
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610 630 650 670 690 710
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970 990 1010 1030 1050 1070
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1810 1830 1850 1870 1890 1910
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1930 1950 1970 1990 2010 2030
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2050 2070 2090 2110 2130 2150
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2170 2188
GCCTCCAGAGAAAAGTTGCTCCCGAGag

increase in the amount of mRNA present (Wang *et al.*, 1989). During the acute phase response α2,6ST is released into the serum as the soluble form of the enzyme. The cleavage site on the protein is shown in Fig. 12 (Weinstein *et al.*, 1987). Increased amounts of α2,6ST have also been found in the serum of patients suffering from cancer and other pathological conditions and therefore may be important physiologically as a marker of disease (Jamieson *et al.*, 1987).

Introduction to the Current Work

The aims of the studies which are presented in this thesis were to identify the importance of the carbohydrate chains for the activity of α2,6ST. This was done with a combination of oligosaccharide processing inhibitors and endoglycosidases as described below. Therefore, by looking at loss of activity and/or change in molecular weight by either modifying or removing carbohydrate chains, I studied the relationship between structure and function of the oligosaccharide group for catalytic activity of α2,6ST.

The α2,6ST catalyses the final step in the synthesis of complex type chains and it is a glycoprotein with three potential glycosylation sites. As we have seen above, α2,6ST reaches the Golgi complex after protein synthesis and glycosylation in the ER and then undergoes oligosaccharide processing as it passes from the ER to the Golgi. However, the enzyme only expresses activity when it reaches the Golgi complex. One explanation for this is that α2,6ST is catalytically inactive in the ER because it contains unprocessed oligosaccharide chains instead of the fully processed chains found on the enzyme in the Golgi. It has been suggested that the α2,6ST may contain both high mannose and complex type chains in its fully processed form (Paulson, personal communication). Two approaches were used to examine the importance of oligosaccharide chains for catalytic activity of α2,6ST. The first involved removing the carbohydrate chains

from native α2,6ST with N-Gly and it has been shown that the activity of the enzyme is greatly decreased and the molecular weight is changed showing that deglycosylation, not proteolysis is occurring. The effect of endo H on the molecular weight and catalytic activity were also examined. The second approach involved the use of monensin which blocks transfer of α2,6ST from the ER to the Golgi and should freeze the oligosaccharide chains on α2,6ST in their high mannose form (this was also done with the glycosylation inhibitors; 1-deoxymannojirimycin, castanospermine). This form of the enzyme has been shown to have reduced activity and was not secreted from the cell (Fast and Jamieson, 1989). The acute phase response was used as previously (Jamieson *et al.*, 1987) to increase the levels of α2,6ST.

The various inhibitors used in this study and their mechanism of action is described below together with information on the specificities of the glycosidases used for cleaving oligosaccharide chains.

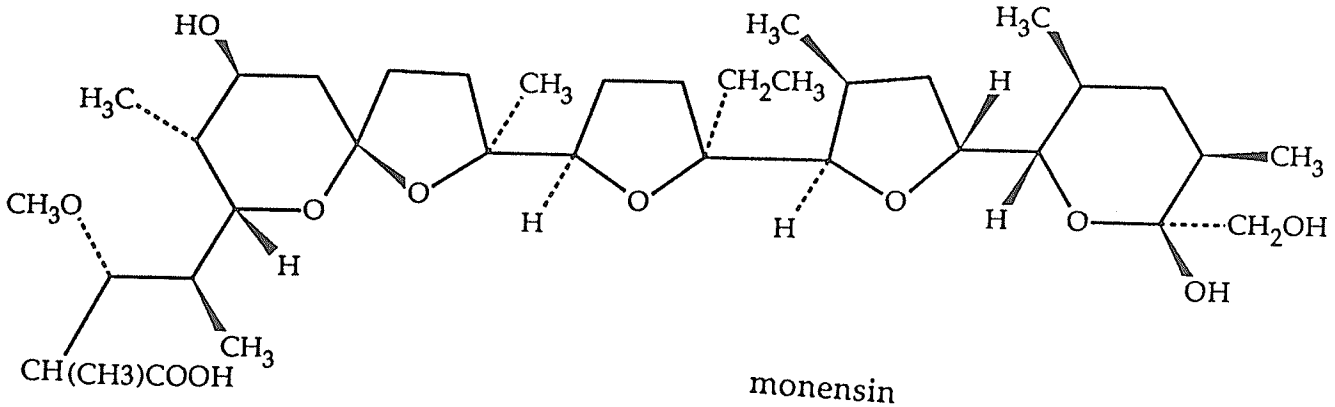
Inhibitors

Monensin

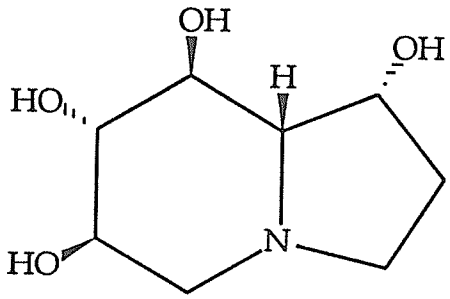
Monensin (2-[5-ethyltetrahydro-5-[tetrahydro-3-methyl-5-[tetrahydro-6-hydroxy-6-(hydroxymethyl)-3,5-dimethyl-2H-pyran-2-yl]-2-furyl]-2-furyl]-9-hydroxy-β-methoxy-α, γ, 2, 8,-tetramethyl-1,6-dioxaspiro[4,5]decane-7-butyric acid) is a monovalent cationic ionophore from *Streptomyces cinnamonensis* which is able to insert into the membranes of the Golgi (see Fig. 14). Monensin disrupts sodium and proton gradients across biological membranes. Monensin interferes with cellular transport of glycoproteins from the ER to the Golgi in rat liver cells (Machamer and Cresswell, 1984) and from the Golgi to the cell membrane (Sai and Tanzer, 1984), by allowing exchange of sodium and potassium ions across

Figure 14. Structures of processing inhibitors.

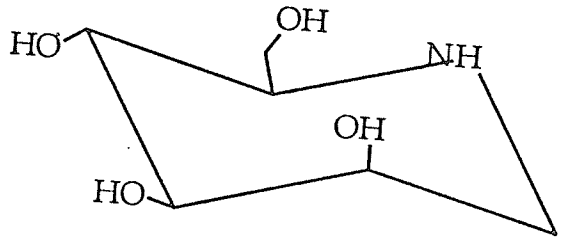
Structures of processing inhibitors. Monensin, castanospermine and deoxymannojirimycin are shown. Monensin is an inhibitor of transport between the ER and Golgi. Castanospermine and deoxymannojirimycin are inhibitors of glucosidases and mannosidases, respectively. See text for mechanism of action.



monensin



castanospermine



deoxymannojirimycin

membranes. Monensin causes the Golgi to become swollen (Tartakoff and Vassalli, 1978), and this blocks the release of secretory vesicles from the Golgi. As a result of this there is a dose dependent reduction in the intracellular transport of newly synthesized macromolecules. Studies have shown that non-secreted proteins accumulate in the Golgi and on long incubations can "pile up" in the ER (Ledger and Tanzer, 1984). The effects of monensin are reversible. Due to the blockage of secretory traffic, monensin acts mainly to block processing beyond the high mannose stage (Sai and Tanzer, 1984), however in some cell lines this high mannose containing material is able to be secreted from the cells. Monensin does not interfere with *de novo* protein synthesis at concentrations up to 50μM (Oda and Ikehara, 1982; Rustan *et al.*, 1985; Tartakoff, 1983; Elbein, 1987). Glycoproteins in monensin treated cells get trapped in the ER and never get to the Golgi where they are normally processed into complex type chains (Griffiths *et al.*, 1983) or are phosphorylated to produce a mannose 6-phosphate group which acts as a signal for transport to lysosomes. Thus most of the carbohydrate chains of monensin treated cells should have a high mannose conformation (Schmaljohn *et al.*, 1986). The effects of monensin on perfused rat liver have been examined and it was shown that monensin treated livers are still viable, as oxygen consumption and release of potassium were not significantly altered (Kloppel *et al.*, 1986). Monensin also delays the secretion of newly synthesized plasma proteins from rat liver, and inhibits internalization and degradation of the asialoglycoprotein receptor (Kloppel *et al.*,1986).

Castanospermine

Castanospermine (8aβ-indlizidine-1α,6β,7α,8β-tetrol or 1,6,7,8-tetra-hydroxy–octahydroindolizine; Fig. 14) inhibits the processing of the oligo-saccharide chains of glycoproteins by acting as a competitive inhibitor of

glucosidases I and II. It is a plant alkaloid isolated from the nuts of an Australian tree, *Castanospermum australe* (Elbein, 1987). The processing glucosidases are fooled into accepting the castanospermine into their active site and thus cannot process the chains past the $GlcNAc_2Man_{7-9}Glc_{1-3}$ structure. The glucosidases remove terminal glucose residues from the $GlcNAc_2Man_9Glc_3$ structure (Elbein, 1987). Castanospermine is not thought to inhibit transport of newly synthesized proteins and this would allow a less active form of the α2,6ST to be released into the medium. Castanospermine (at concentrations up to 530μM) is not cytotoxic and does not inhibit the growth of mammalian cells (Boehringer Mannheim data sheet).

Deoxymannojirimycin

Deoxymannojirimycin (1,5-dideoxy-1,5-imino-D-mannitol), a chemically synthesized mannose analog (Fig. 14) inhibits the removal of mannose residues from the $GlcNAc_2Man_{7-9}$ structure by Golgi α-mannosidase I thus causing a build up of $GlcNAc_2Man_8$ and $GlcNAc_2Man_9$ structures (Elbein, 1987). Deoxymannojirmycin does not inhibit any mannosidase found in the ER or the Golgi α-mannosidase II. The mechanism is thought to involve binding to the active site of the enzyme (Boehringer Mannheim data sheet). Deoxymannojirimycin only affects the processing of complex type chains and will have no effect on the processing of high mannose type chains.

Endoglycosidases

Endoglycosidases are defined as any enzyme that releases a disaccharide or larger unit during its course of action. Endoglycosidases have been widely used to determine structure function relationships, clarifying the biosynthesis of complex oligosaccharides, to study the role of carbohydrate in transport and

targeting of glycoproteins, hormone function, receptor action, and the pathogenicity of viruses (Maley *et al.*, 1989). There are several types of endo-glycosidases each with their own specificity. Not all carbohydrate chains on a protein are equally susceptible to these enzymes as shown for human thyrotropin treated with N-glycosidase F (N-gly) and Endo F (Ronin *et al.*, 1987) where some chains were more easily removed than others.

Endo H

Endo β-N-acetylglucosamide H was first isolated from *Streptomyces plicatus* and has since been cloned into *E. coli*. Endo H is specific for high mannose type chains (Fig. 4). The minimum required structure for Endo H to work is a chitobiose unit with a mannose linked to the α1-6 mannose arm as shown in Fig. 15 (Maley *et al.*, 1989). Endo H has a M_r 29,000-33,000. The pH optimum is between 5 and 6 and the enzyme is very stable, even at 37°C (Maley *et al.*, 1989). Endo H is also active in the presence of detergents such as SDS although it may be inactivated by high concentrations (Trimble and Maley, 1984). Substitutions or additions to the high mannose chain structure such as addition of fucose to the branched trimannosyl core may affect the ability of Endo H to digest high mannose type chains (Tarentino *et al.*, 1989). The site of endo H cleavage is shown in Fig. 16.

N-Glycanase™

N-Glycanase (peptide-N^4-(N-acetyl-β-glucosylaminyl)-asparagine amidase F) is purified from cultures of *Flavobacterium meningosepticum* and hydrolyzes N-linked oligosaccharides from glycoproteins and glycopeptides giving a free oligosaccharide with an intact di-N-acetylchitobiose, ammonia and a protein/peptide containing aspartic acid at the glycosylation site (instead of asparagine originally present there) as shown in Fig. 16. Therefore this is not an

Figure 15. Minimum structure required for endo H cleavage.

Minimum structure required for endo H cleavage of oligosaccharide chains. The implications of this are that it is possible for endo H to cleave hybrid type chains, although it does this with lower efficiency.

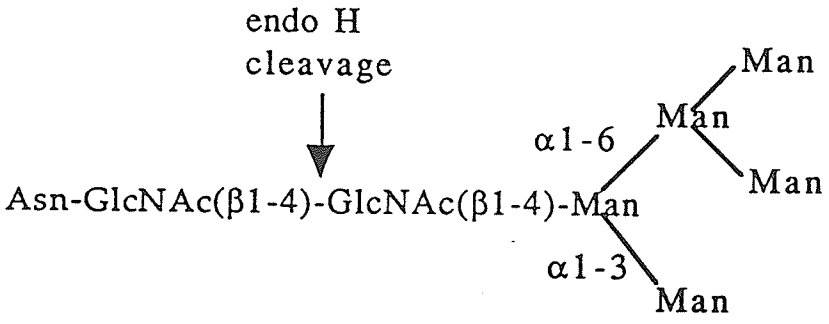
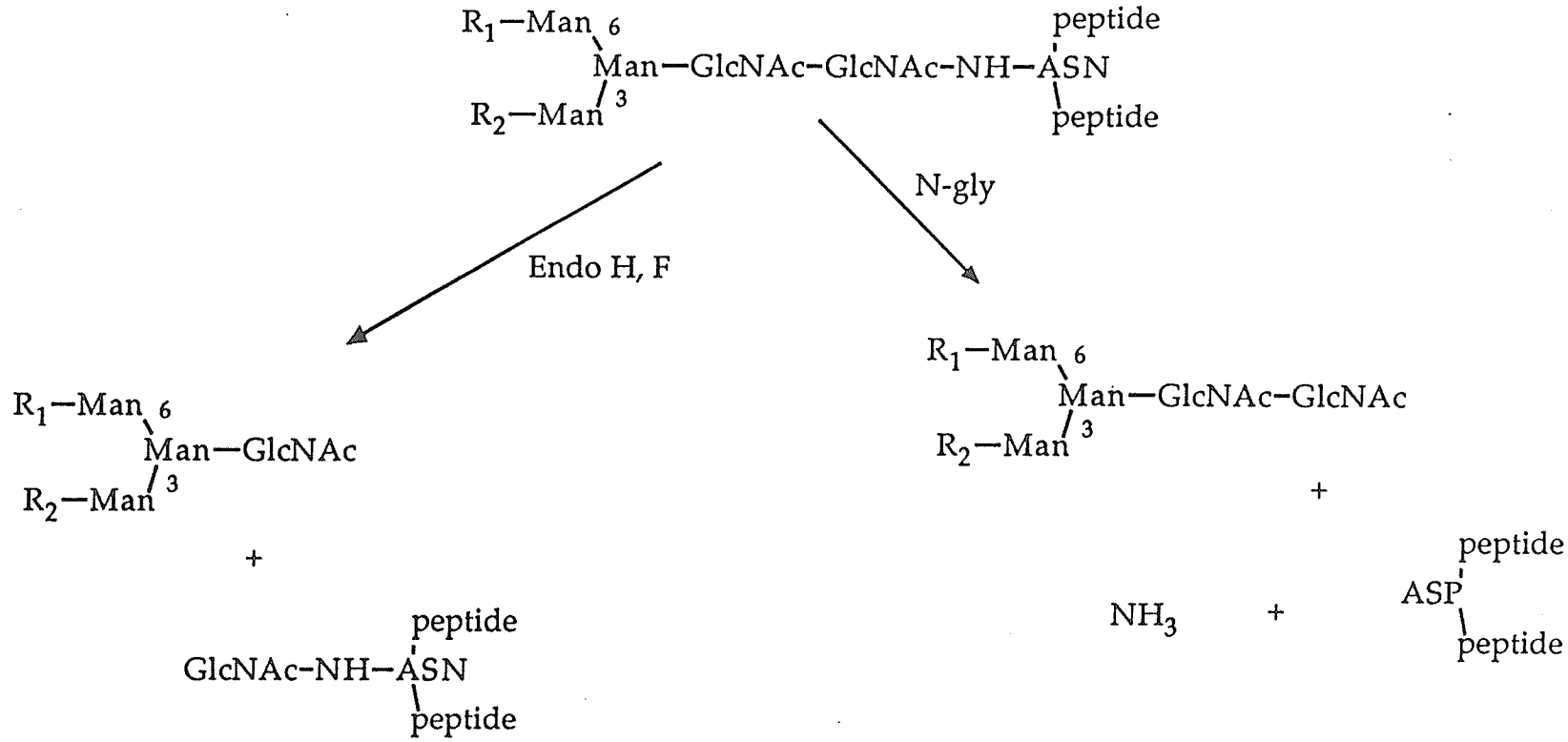


Figure 16. Cleavage of oligosaccharide chains by endoglycosidases.

Cleavage of oligosaccharide chains by endoglycosidases. N-gly is not a true glycosidase, but rather an amidase. The cleavage of oligosaccharides by N-gly leaves an aspartic acid residue where there was initially an asparagine linked to a GlcNAc. N-gly cleaves off all types of N-linked oligosaccharides. Endo H is specific for high mannose type chains.


R1—Man 6
Man—GlcNAc—GlcNAc—NH—ASN
R2—Man 3
peptide
peptide
N-gly
Endo H, F
R1—Man 6
Man—GlcNAc
R2—Man 3
+
peptide
GlcNAc—NH—ASN
peptide
R1—Man 6
Man—GlcNAc—GlcNAc
R2—Man 3
+
NH3
+
peptide
ASP
peptide

N-glycosidase but rather an amidase. N-Glycanase has M_r 35,500, and a pH optimum of 8.6. However, the enzyme retains 60% of its activity at pH 6.5 and 9.5. It is very stable in glycerol and is not affected by high concentrations of EDTA or 2.5M urea. In 5M urea it still retains 40% of its activity (Maley *et al.*, 1989). The enzyme prefers SDS denatured glycoproteins/glycopeptides but it is effective against native glycoproteins/glycopeptides at higher concentrations. Deglycosylation of native fetuin, human transferrin and ribonuclease B has been shown (Tarentino *et al.*, 1985). N-glycanase has been shown to cleave all types of N-linked oligosaccharides, including bi-, tri- and tetra-antennary complex, fucose-substituted, sulfated, hybrid and high mannose chains (Tarentino *et al.*, 1989). The oligosaccharide type has little effect on hydrolysis except for small differences in the rate of digestion, with triantennary > biantennary > high-mannose > tetraantennary. The enzyme does not act on oligosaccharide chains found on the N- or C-terminus of proteins. N-glycanase is completely inhibited by citrate buffer, an observation made by Genzyme and reported in its technical bulletin. The mechanism of action or concentration of citrate required to inhibit N-gly is unknown (Genzyme technical bulletin, personal communication). Recently, the enzyme has been sequenced (Tarentino *et al.*, 1990) and cloned into *E. coli.* (Barsomian *et al.*, 1990) and is available commercially from Genzyme.

MATERIALS AND METHODS

Materials

Radioactive Compounds

Cytidine-5'-Monophosphate-[4,5,6,7,8,9-^{14}C]-N-Acetylneuraminic Acid (247 μCi/mmol) was from New England Nuclear Corp., Lachine, Que.; [^{125}I] - protein A was from Amersham, Oakville, Ont.

Enzymes and Proteins

N-Glycanase™, endo-β-N-acetylglucosaminidase H, and sialyltransferase were obtained from Genzyme Co., Boston, Mass. BSA was from Sigma Chemical Co., St. Louis, MO.; Soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, BSA, and phosphorylase b were used as M_r standards and were obtained from Bio-Rad laboratories, Richmond, CA. Human serum albumin (purity 99%) was obtained as a gift from Dr. Rick Janzen of the Winnipeg. Rh Institute. Antibodies to α2,6ST were obtained as a gift from Dr. J.C. Paulson, Department of Biological Chemistry, School of Medicine, University of California. Asialo α_1 acid glycoprotein was prepared from rat serum as described by Kaplan *et al.*, (1983).

Chemicals and Chromatographic Media

Bio-Gel P4, Affi-Gel Blue, Acrylimide, N'N'-Bis-methylene-acrylamide, SDS, Glycine, Ammonium persulfate, TEMED and Bio-Rad Protein Reagent were from Bio-Rad laboratories, Richmond, CA; Cytidine-5'-Monophosphate-N-Acetylneuraminic Acid, McCoy's medium, HEPES buffer, Triton CF-54, Triton X-100, β-mercaptoethanol, bromophenol blue, Tris, WGA-Sepharose and Con A sepharose were obtained from Sigma Chemical Co., St. Louis, MO.; Nitrocellulose membranes were obtained from Fisher Scientific and Turpentine

Oil was obtained from Sargeant Welch. ACS liquid scintillation fluid was from Amersham Corp., Oakville, Ont; Other chemicals were of analytical grade obtained from local suppliers.

Inhibitors

Monensin was from Sigma Chemical Co., St. Louis, Mo.; Deoxymannojirimycin was from Boehringer Mannheim Biochemicals, Dorval, P.Q. and Castanospermine was from Calbiochem, La Jolla, Ca.; 1,10 ortho Phenanthroline was from Genzyme.

Methods

Physical and Chemical Methods

Absorbance was measured in a Phillips PU8620 UV/Vis/NIR spectrophotometer, radioactivity was determined with a LKB model 1215 RackBeta II liquid scintillation Counter using 10 ml of ACS scintillation fluid. Conversions to dpm were performed using an automatic external standard and a preprogrammed calculation routine as shown in appendix B. Determination of pH was done with a Fisher Accumet model 915 pH meter. Fractions were collected on either an LKB Ultrorac fraction collector or with a Spectrum Spectra/Chrom fraction collector. Distilled deionized water was obtained from a NanoPure II water purifying system (Barnstead) with a final 0.22μM filter. Centrifugations were carried out in a Sorval model RCB-2 centrifuge with a SS-34 head.

Treatment of Animals

Male Long Evans Hooded rats of 200-250 g body weight were purchased locally and were maintained under constant light conditions on a diet of Purina

Rat Chow and water *ad libitum*. Rats which received injections were anesthetized under ether. Experimental inflammation was induced by subcutaneous injection of 0.5ml turpentine oil per 100 g body weight into the dorsolumbar region (Ashton *et al.*, 1970), controls received injections of 0.15M sodium chloride. Animals were sacrificed 24 hours after injection by severing the jugular veins as described by Jamieson *et al.*, 1970. Rats were starved for 16 hrs. prior to sacrifice. Livers were perfused *in situ via* the portal vein with ice cold 0.15M NaCl, rapidly excised and transferred to ice cold 0.15 M NaCl. The blood was collected for use in preparing the asialo α_1 acid glycoprotein acceptor.

Slice Preparation

Slices were prepared as previously described (Janzen *et al.*, 1987) using perfused livers from either control or 24 hr turpentine inflamed rats. Slices were cut on a pre-cooled aluminum template with grooves 7mm wide and 0.36mm deep. The template was covered during slicing with ice cold 0.15M NaCl. Slices were washed with 0.15M NaCl and samples of 300 ± 10 mg (wet weight) slices were transferred onto 60 x 15mm tissue culture dishes (Corning) and 2.0ml of modified McCoy's medium added to the slices along with 10µl stock solutions of monensin, castanospermine, deoxymannojirimycin, 50% ethanol or water (see Results section for details). The modified McCoy's medium consisted of McCoy's medium and 20mM HEPES, adjusted to a final pH of 7.35 with HCl. The role of the HEPES was to provide additional buffering capacity. Prior to use the medium was saturated with a 95:5 mixture of O_2:CO_2 for 30 min. The slices were then placed in an atmosphere of 95% O_2: 5% CO_2 and swirled gently for up to 20 hours. The normal incubation time was 12 hours. Liver was separated from medium by aspirating the medium with a Pasteur pipette and the liver

slices were then washed with 10ml of 0.9% saline and centrifuged for 20 min. at 2000rpm in the Sorvall centrifuge at 4°C using the SS-34 rotor (g_{av}= 478).

Sialyltransferase Assay

Sialyltransferase was assayed as described previously (Kaplan *et al.*, 1983; Lammers and Jamieson, 1986). Briefly, a sample of homogenate, medium, or a fraction from a column (see Results section for details) was incubated in the presence of 250µg of rat asialo α_1-acid glycoprotein, 0.05M imidazole buffer pH 7.00, and 20nCi/5nmoles of ^{14}CMP-NeuAc, in a final volume of 150µl. The incubations were done at 37°C in a shaking water bath for 1-3 hrs. The assays were stopped by rapidly cooling the samples to 0°C. 120µl were transferred to two 2.5cm diameter Whatman No. 1 filter disks (60µl each) and allowed to dry. These disks were then washed in ice cold 10% TCA for 10 min, followed by two 5 min washes in ice cold 5% TCA. The disks were then washed for approximately 8 min in a 2:1 ethanol:ether mixture and finally dried in ether for 5 min. The disks were then transferred to scintillation vials along with 10ml of ACS scintillation fluid and counted in a LKB rack Beta counter for 5 min. One unit of α2,6ST activity is defined as equal to the transfer of 1 pmol of NeuAc from CMP-NeuAc to asialo α_1 acid glycoprotein per min. The conversion from dpm to picomoles of NeuAc transferred per min. per mg protein (or ml of volume) is shown in appendix B.

Protein Assay

Protein was determined using the Bio-Rad method (Bradford, 1976) using bovine serum albumin (0-160µg) as a standard. If samples contained any detergent, it was included at the same concentration in the standards.

Columns

Affi-Gel Blue Sepharose

The column (20cm x 1.0.cm) was first washed with a minimum of 800ml of buffer A (see appendix A) as a modification of the method of Sticher *et al.*, 1988. Homogenates of liver slices were prepared for running down the column by homogenizing in buffer A using 7.5ml per g (wet weight) of liver slices, then centrifuged at 2000rpm for 20 min. (Sorval) and the pellet rehomogenized in one half the original volume used and recentrifuged. The supernatants were then combined and loaded onto the column. Medium samples were first centrifuged as above to remove any particulate material, and then the supernatant loaded directly onto the column. 100 drop fractions were collected and 150ml of buffer A eluted through the column to wash off any unbound material. The buffer was then changed to include a linear salt gradient of 0.15M to 2.5M NaCl (300ml total volume) in buffer A. The fractions were then assayed for sialyltransferase and protein. In some experiments, the samples containing enzyme activity were pooled and vacuum dialysed against buffer A to remove the excess salt as well as to concentrate the protein. These samples were used for determination of K_m and V_{max}, as well as for further column work.

Bio Gel P4

These columns were used to separate the glycerol from the sialyltransferase obtained from the Affi-Gel Blue column. The glycerol interfered with the lectin columns and therefore had to be removed. This was accomplished by eluting the sample through the column with a 50mM Tris buffer pH 7.0 and collecting 50 drop fractions. Each fraction was assayed for both

protein and α2,6ST activity. Fractions containing activity were then immediately run down a WGA-sepharose column.

WGA sepharose

The WGA sepharose columns (1.0 x 10cm or 0.5 x 10cm) were washed prior to use with approximately 20 volumes of 50mM Tris (pH 8.00), 1% Triton CF-54. Samples of up to 750µl were placed on the column after being collected and pooled from the Bio-Gel P4 column as above and left to soak for 15 min.; the column was then washed with the above buffer and after approximately 40 fractions were collected, the buffer was then changed to include 0.1M GlcNAc to elute any glycoproteins containing complex carbohydrate chains. The fractions were collected into glycerol so that the final glycerol concentration was 25% and then vortexed and stored on ice. The glycerol was present to stabilize the α2,6ST (Sticher *et al.*, 1988). The volume of the fractions varied between experiments but was usually between 20 and 50 drops. Fractions were assayed for sialyltransferase and protein. For WGA sepharose columns using partially purified sialyltransferase (from the Bio-Gel P4 columns) the buffer used to wash and run the column consisted of 50mM Tris (pH 8.00), 0.1% Triton CF-54 and fractions collected as before. WGA sepharose columns were run at room temperature and the fractions stored on ice.

Endoglycosidase Experiments

N-glycanase digestions of native α2,6ST were carried out according to the information provided by Genzyme Corp. For native proteins the incubation mixture consisted of; 0.2M Sodium Phosphate (pH 8.6), up to 10% methanol (methanol was found to enhance cleavage of oligosaccharide chains from α2,6ST, see Results section for more details), 1.2µl N-Gly, 10µl α2,6ST (approximately 0.188 µg), and 0.375 µg HSA in a final volume of 30µl (multiples of these volumes

were used for some experiments). Control samples had an equal volume of 50% glycerol/2.5mM EDTA solution in place of the N-gly. The assay was incubated for up to 20 hours at 37°C and stopped by placing the tubes on ice; 10µl aliquots of this assay were tested for α2,6ST activity or M_r was determined on SDS PAGE. Denatured proteins were treated the same way except that the incubation mixture also included 7.5% NP-40. For complete digestion of native α2,6ST with N-gly, an additional amount of N-gly (1.2µl) was added after 10 hours and the incubation allowed to proceed for 24 hours. Control experiments were done to examine the exact methanol concentration required to maximize digestion of the oligosaccharide chains of α2,6ST without causing too much disruption of the enzyme. Controls were also done to determine the concentration of HSA that could be used to inhibit proteases without affected α2,6ST activity.

Endo H experiments were done on either native or denatured α2,6ST. The α2,6ST was denatured by boiling in a 1.2 fold weight excess of SDS for 3-4 min. Then 50mM Sodium Citrate buffer pH 5.5 was added to the sample so that the final SDS concentration was less than 0.2mg/ml as concentrations higher than this adversely affected endo H activity (Trimble and Maley, 1984). Endo H was then added and the incubation was allowed to proceed for up to 20 hours at 37°C. The incubation was stopped by placing the samples on ice. Aliquots of the assay were used for determination of the M_r of proteins on SDS PAGE. For digestions of native α2,6ST with endo H the assay did not include any SDS and was not boiled, but some samples contained methanol (5%) to promote accessibility of the α2,6ST oligosaccharide chains to digestion. Digestions with native α2,6ST were for up to 12 hours at 37°C, with 10µl aliquots removed at various time intervals.

Immunoblotting of Sialyltransferase

SDS polyacrylamide-gel electrophoresis was performed by the method of Laemmli, 1970. A Bio-Rad Mini-Protean II electrophoresis system was used. Samples were prepared for electrophoresis by heating in a boiling water bath for 4 min. in 0.125M Tris/HCl (pH 6.8) containing 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol and 0.02% (w/v) Bromophenol Blue. Samples were then centrifuged for 10 min. at 7000 rpm in a microfuge, and 2-10 µl of each sample were applied to individual tracks of 10% (w/v) acrylamide gels. Electrophoresis was for 1 hr. at 200 V using a tank buffer of 0.025 M Tris, 0.192M Glycine (pH 8.3), 0.1% SDS. Electroblotting was performed using the Bio-Rad Mini Trans-blot cell. Protein transfer to 0.45µm pore size nitrocellulose was for 1 hr. at 100V with a 0.025M Tris, 0.192M glycine (pH 8.3), 20% methanol blotting buffer. Protein M_r standards were soybean trypsin inhibitor (M_r=21,600), carbonic anhydrase (M_r=31,000), ovalbumin (M_r=42,699), BSA (M_r=66,200), and phosphorylase b (M_r=97,400). Immunodetection of α2,6ST was as described by Haas and Bright (1985) using anti-sialyltransferase (1:200) and affinity purified ^{125}I-Protein A and autoradiographed on Hyperfilm-βmax film for 3-7 days.

RESULTS

Effect of Inhibitors of Oligosaccharide Processing on Sialyltransferase in Liver Slices

Effect of Monensin Concentration

Fig. 17 shows the effect of monensin concentration on the release of α2,6ST into the medium in experiments with liver slices from 24 hour inflamed rats. For liver slices from 24 hour inflamed rats the maximum inhibition of release occurred at about 1μM and no further inhibition was seen up to 20μM for a 12 hr incubation at 37°C. Fig. 18 shows the effect of monensin concentration on release of α2,6ST into the medium for liver slices from control rats. Maximum inhibition of release occurred with 0.5μM monensin and enzyme activity is not further decreased with monensin concentrations up to 20μM. For both control and inflamed liver slices the controls consisted of slices treated with 0.25% ethanol because the monensin was dissolved in 50% ethanol.

The amount of α2,6ST released into the medium by liver slices after 12 hours of incubation at 37°C differs significantly between samples from control and inflamed animals, a factor of 2 fold at 24 hours after inflammation; that is 10.7 units for 24 hour inflamed animals and 5.2 units for control animals. This is similar to the increase in α2,6ST released into the serum of inflamed and control rats which have values of 20 and 5 units respectively (Kaplan *et al.*, 1983).

Effect of Monensin on Time of Release of α2,6ST from Liver Slices

Fig. 19 shows the effect of 2μM monensin on the release of α2,6ST from liver slices from 24 hour inflamed rats, inhibition of release was evident as early as three hours after the start of incubation. The inhibitory effect of monensin was

Figure 17. Effect of monensin concentration on the release of α2,6ST from liver slices from inflamed rats.

Samples (300mg) of liver slices from 24 hour inflamed rats were incubated with 2ml of McCoy's medium for 12 hours at 37°C in the presence of up to 20μM monensin (final concentration). The amount of sialyltranferase activity released from slices in the absence of monensin (0.25% ethanol, see Materials and Methods) was taken as 100%. This value represents 10.2 units of α2,6ST activity. Each point represents the mean from at least three experiments; reproducibility was within ±15%.

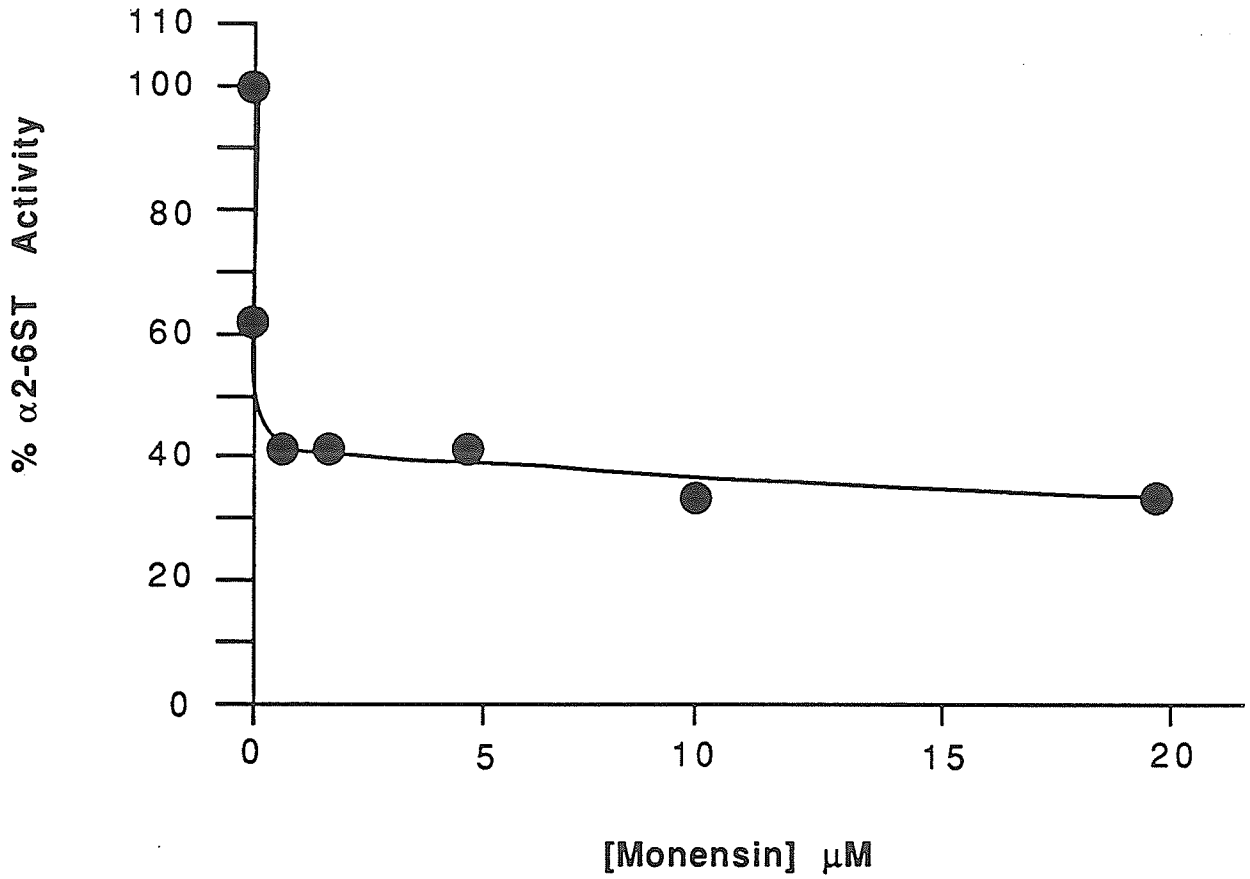

110
100
80
60
40
20
0
% α2-6ST Activity
0
5
10
15
20
[Monensin] μM

Figure 18. Effect of monensin concentration on the release of α2,6ST from control liver slices.

Samples (300mg) of liver slices from control rats were incubated with 2ml of McCoy's medium for 12 hours at 37°C in the presence of up to 20μM monensin (final concentration). The amount of α2,6ST activity released from slices in the absence of monensin (0.25% ethanol) was taken as 100%. This value represents 5.1 units of α2,6ST activity. Each point represents the mean from at least three experiments; reproducibility was within ±15%.

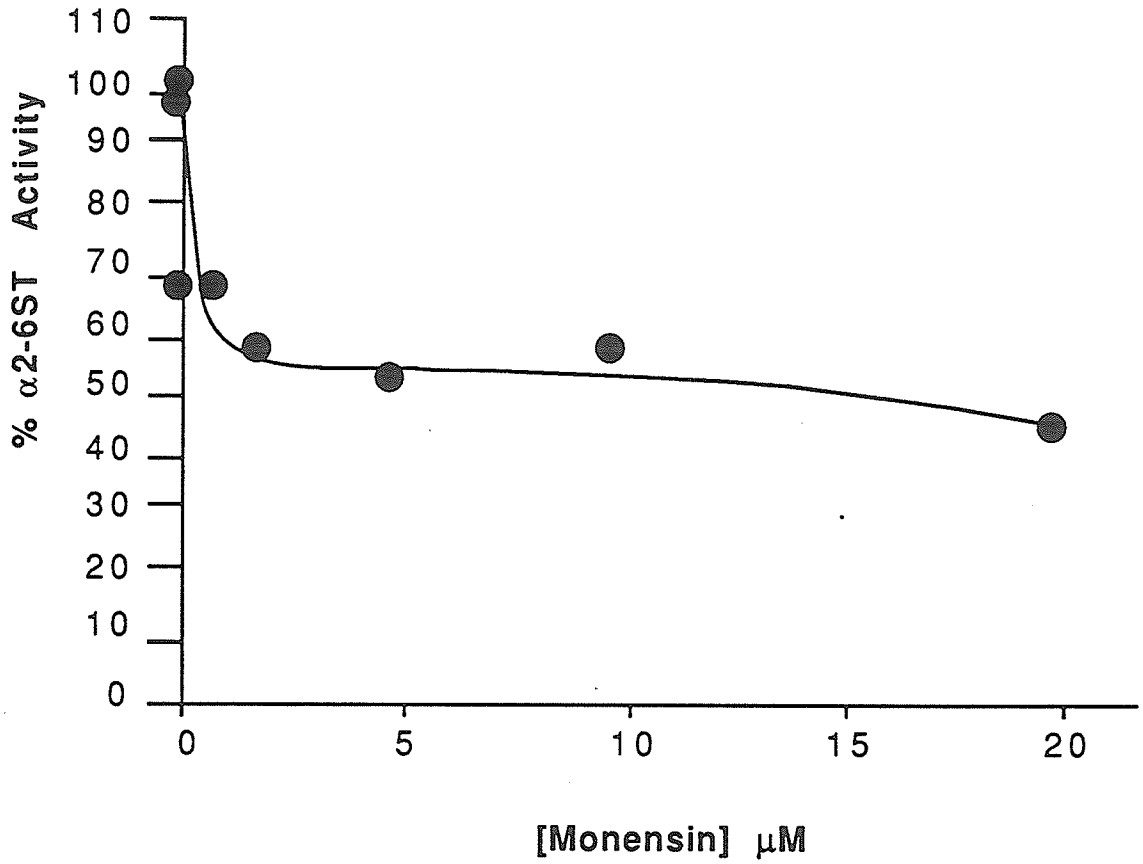
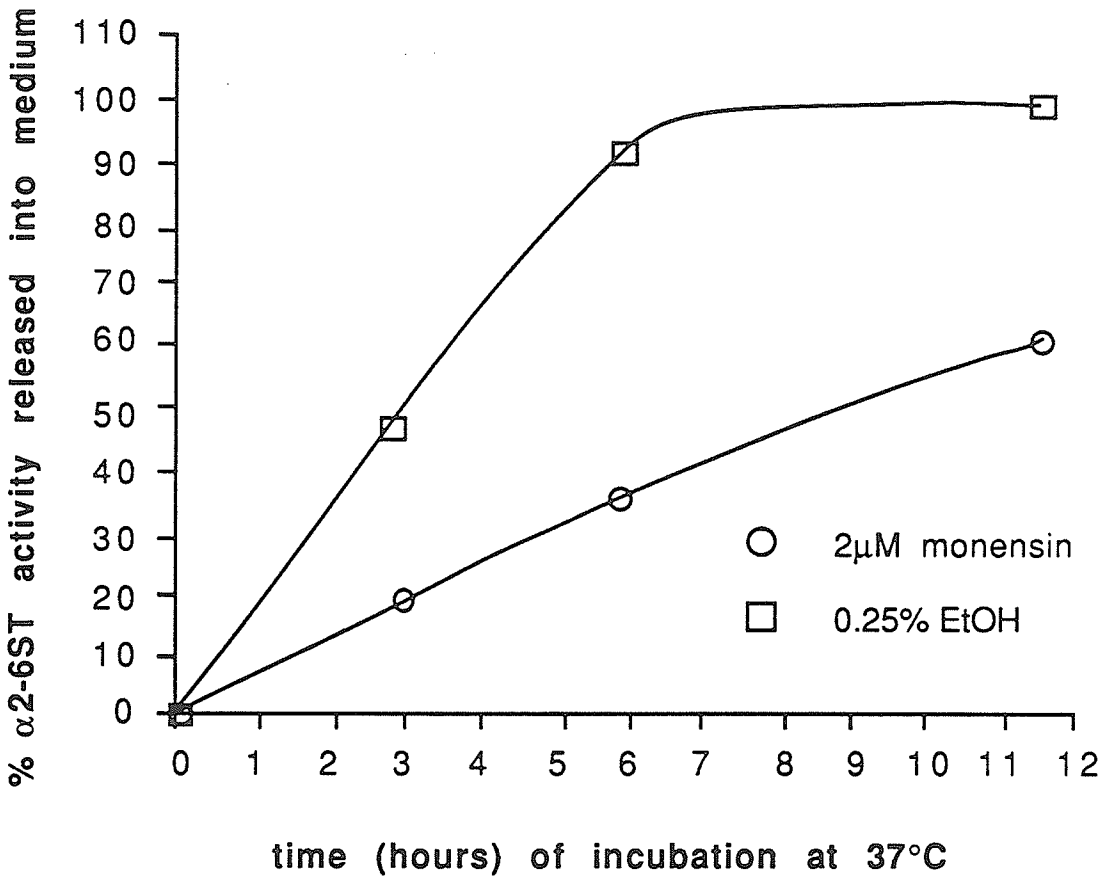

% α2-6ST Activity
110
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
[Monensin] μM

Figure 19. Effect of incubation time on release of α2,6ST from liver slices obtained from 24 hour inflamed rats, treated with 2μM monensin.

Slices were incubated as described in the legend to Fig. 17, in the presence of either 2μM monensin ○ or 0.25% ethanol □, and the release of α2,6ST into the medium was monitored as a function of time of incubation. The 100% values were those described in the legend to Fig. 17. Each point represents the mean of three experiments; reproducibility was within ±15%.


% α2-6ST activity released into medium
110
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12
time (hours) of incubation at 37°C
2μM monensin
0.25% EtOH

more pronounced with slices from inflamed rats. Fig. 20 shows the effect of 10μM monensin on the release of α2,6ST from 24 hour inflamed slices as a function of time. When the two concentrations of monensin are compared it can be seen that there is not much difference in the release of α2,6ST, although for the 10μM monensin treated slices, the release of α2,6ST leveled off after 6 hours of incubation at 37°C. Fig. 21 shows the time course for the release of α2,6ST from liver slices from control rats using 10μM monensin; compared with the inflamed rat the amount of α2,6ST released was much lower. At low concentrations of monensin (1μM) variability was observed, therefore higher concentrations of monensin were used throughout. In view of the known mechanism of action of monensin these results suggest that hepatic α2,6ST is a glycoprotein with at least one complex type carbohydrate chain in its fully mature form. The acute phase response also seems to be important for this process as it appears to require less monensin to inhibit transport then in the control slices.

Effect of Castanospermine Concentration

Fig. 22 shows the effect of castanospermine concentration on the release of α2,6ST from 24 hour inflamed rat liver slices after 12 hours of incubation at 37°C. There was a reduction of α2,6ST activity in the medium of only 35%. The release of α2,6ST activity from the slices was inhibited maximally at 5μM and no further reduction in the amount of α2,6ST activity released is seen up to 175μM castanospermine. These results were disappointing since castanospermine is a potent inhibitor of glucosidases I and II. This suggests that the enzyme may still be able to get out of the liver even though it has oligosaccharides with a high mannose type structure and that the enzyme will still be active although possibly at a reduced level. The exact nature of the carbohydrate chains on the ST which were released into the medium is unknown as there may be be a pool of enzyme

Figure 20. Effect of incubation time on release of α2,6ST from liver slices of 24 hour inflamed rats treated with 10μM monensin.

Slices were incubated as described in the legend to Fig. 17, in the presence of either 10μM monensin ○ or 0.25% ethanol ❑, and the release of α2,6ST into the medium was monitored as a function of time of incubation. The 100% values were those described in the legend to Fig. 17. Each point represents the mean of three experiments; reproducibility was within ±15%.

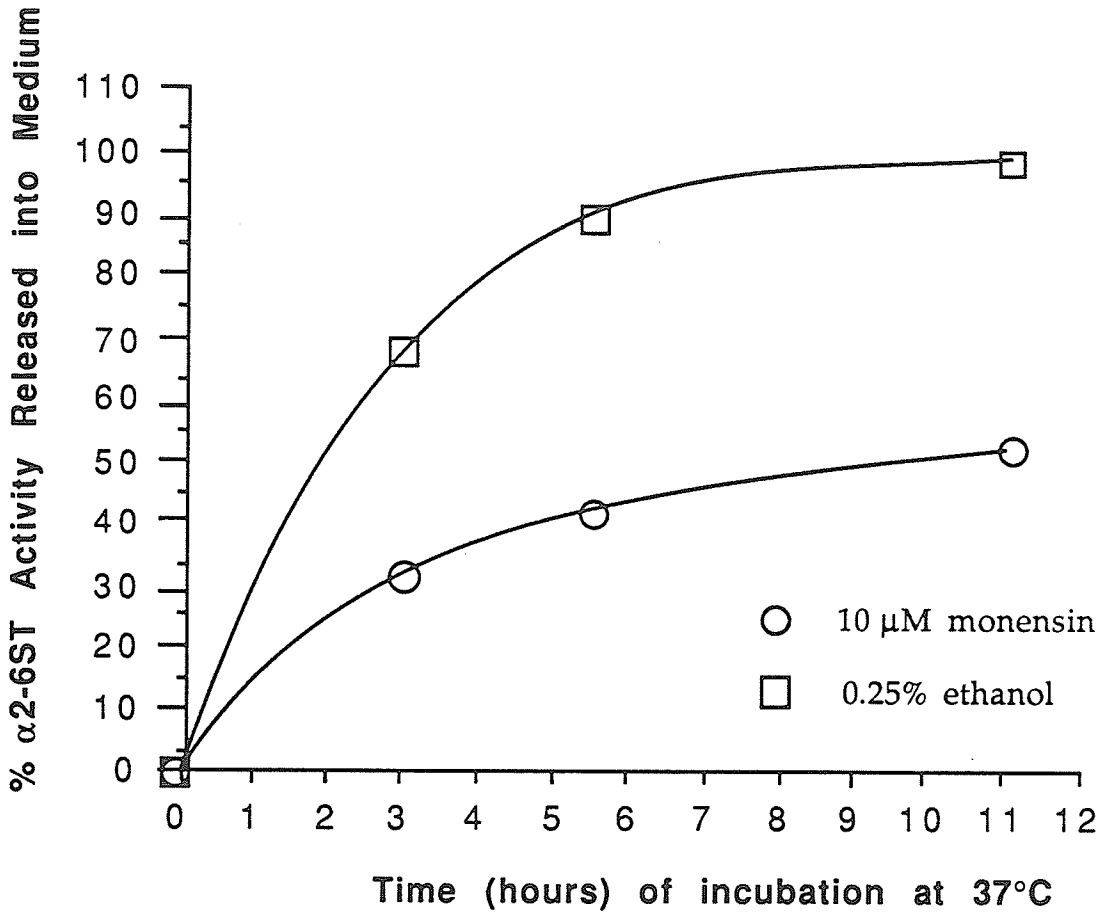

% α2-6ST Activity Released into Medium
110
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (hours) of incubation at 37°C
10 μM monensin
0.25% ethanol

Figure 21. Effect of incubation time on release of α2,6ST from liver slices of control rats treated with 10μM monensin.

Slices were incubated as described in the legend to Fig. 18, in the presence of either 2μM monensin ○ or 0.25% ethanol ❑, and the release of α2,6ST into the medium was monitored as a function of time of incubation. The 100% values were those described in the legend to Fig. 18. Each point represents the mean of three experiments; reproducibility was within ±15%.

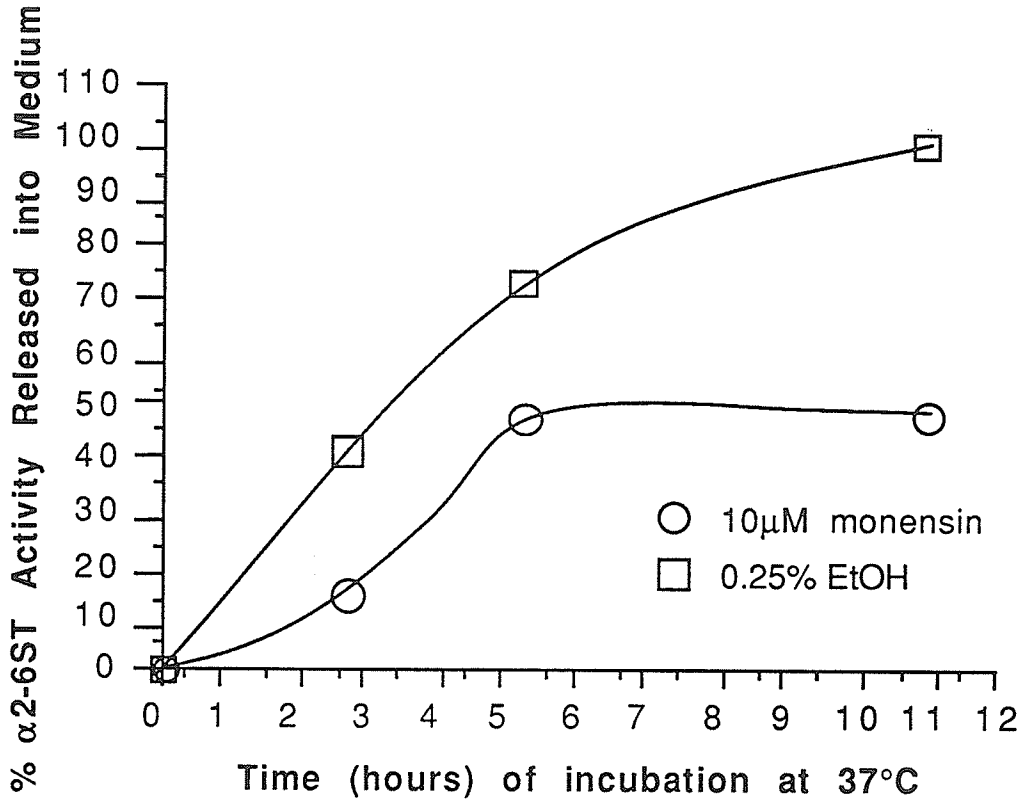
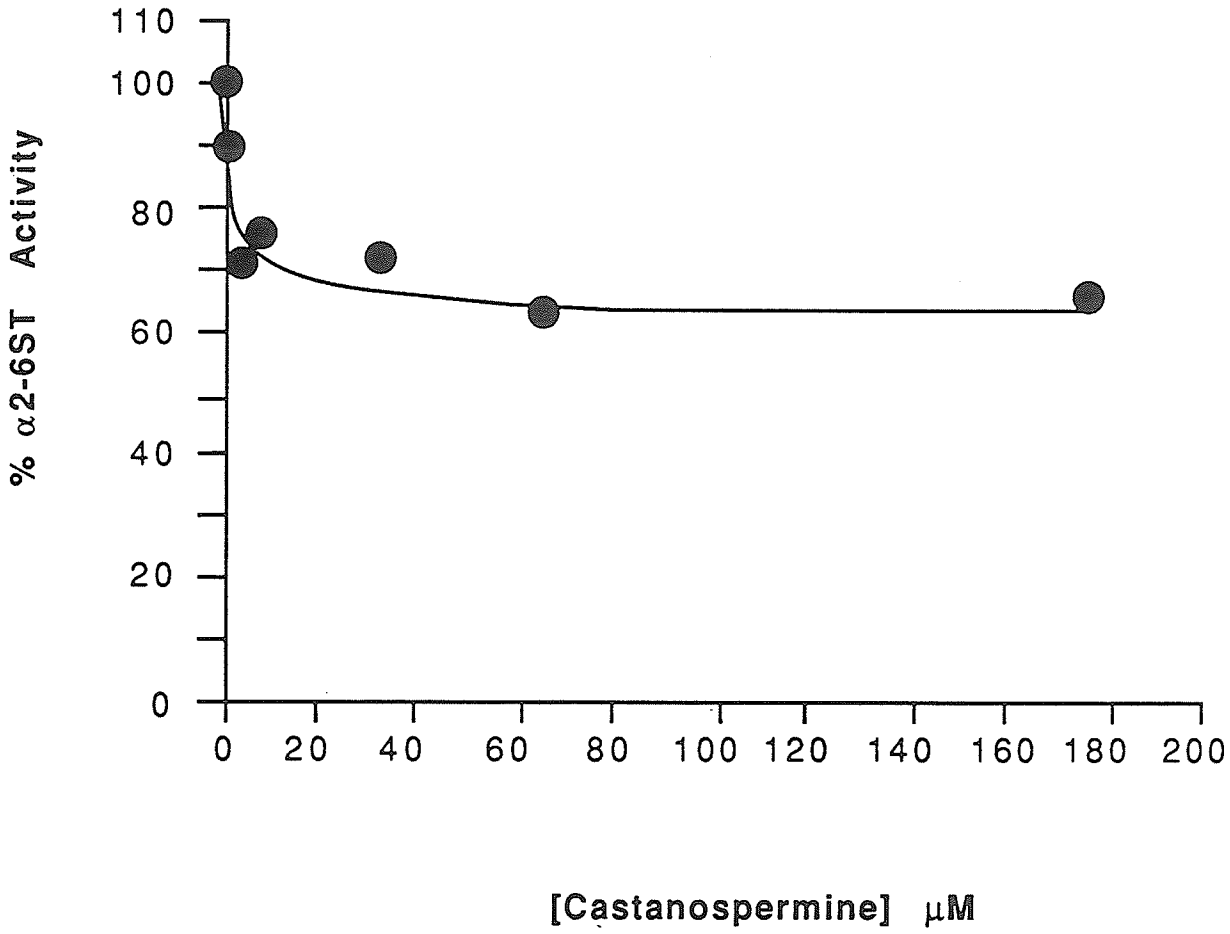

% α2-6ST Activity Released into Medium
110
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (hours) of incubation at 37°C
10μM monensin
0.25% EtOH

Figure 22. Effect of castanospermine concentration on the release of α2,6ST from liver slices of 24 hour inflamed rats.

Slices were incubated as described in the legend to Fig. 17, in the presence of up to 180μM castanospermine and the release of α2,6ST into the medium was monitored after 12 hours of incubation at 37°C. The 100% values were those described in the legend to Fig. 17. Each point represents the mean of three experiments; reproducibility was within ±15%.


110
100
80
60
40
20
0
% α2-6ST Activity
0
20
40
60
80
100
120
140
160
180
200
[Castanospermine] μM

which is released or the enzyme "leaks out" and is processed even in the presence of castanospermine.

Activity of Sialyltransferase in Liver Homogenates

Kinetic data

The kinetic parameters of the α2,6ST remaining in the slices after treatment with 10μM monensin, 50μM castanospermine, 1mM deoxymanno-jirimycin or 0.25% ethanol were examined after partially purifying the α2,6ST on an Affi-Gel Blue column and are shown in Table 2. From this it can be seen that the values for K_m and V_{max} have changed as a result of inhibitor addition to the slices. For all inhibitors the V_{max} and K_m values are decreased. The values for K_m were decreased approximately one order of magnitude as were the V_{max} values. This suggests that the α2,6ST with high mannose chains bind the asialo α_1 acid glycoprotein tighter than does the α2,6ST from control slices, although it does not utilize the substrate to its maximum potential (see Discussion). Concentration and time course studies were not done using deoxymannojirimycin as it was too costly and has basically the same effect on the structure as does castanospermine.

Activity of Sialyltransferase in the Medium

The asialo α_1 acid glycoprotein K_m and V_{max} values for α2,6ST released into the medium after incubation with both 2μM monensin and 0.25% EtOH are shown in Table 3. It can be seen that the secreted α2,6ST was not greatly affected by treatment of the slices with inhibitors and this suggests that the enzyme which does get out of the cells is fully processed. This is possible since there is a preexisting pool of fully processed α2,6ST in liver slices at the start of experiments which may be released after the start of incubation.

Table 2. Kinetic parameters of α2,6ST found in liver after inhibitor treatment

Kinetics of α2,6ST partially purified from liver slices treated with various inhibitors. Samples were prepared as stated in materials and methods. All protein concentrations were adjusted to 1.5mg/ml prior to start of kinetic studies. Values are the means of three experiments and are within ±15%.

Table 2 Effect of various inhibitors on the Kinetic parameters of partially purified ST from liver slices.

	Control	10μM Monensin	50μM Castano-spermine	1mM Deoxy-mannojirmycin
V_{max} (pmol/min/mg)	1823.2	269.9	474.2	111
K_m (μM)	74.5	17.5	28.73	15

Table 3. Kinetic parameters of α2,6ST released into medium in the presence of monensin.

Kinetics of α2,6ST released into the medium, partially purified on an Affi-Gel blue column. Samples were prepared as stated in materials and methods. All protein concentrations were adjusted to 1.5mg/ml prior to start of kinetic studies. Values are the means of three experiments and are within ±15%.

Table 3 Kinetic parameters for secreted ST from 24 hr. inflamed rat liver slices treated with monensin.

Sample	K_m	V_{max}
2μM Monensin	112μM	2.54 pmol/min/ml
0.25% EtOH	88μM	3.32 pmol/min/ml

Partially Purified α2,6ST from Slice Experiments

The M_r of samples treated with monensin, ethanol, castanospermine and deoxymannojirimycin could not be determined due to the extremely low levels of α2,6ST present. The samples used were obtained after partial purification on an Affi-Gel Blue column as described in the Materials and Methods section. Fig. 23 shows a typical activity/protein profile for an Affi-Gel Blue column. The latter peak (typically fractions 90 to 125) was pooled and concentrated to a total protein concentration of 1.5mg/ml. Samples at this stage were either studied using SDS PAGE or WGA sepharose columns (after removal of glycerol on a Bio-Gel P4 column).

WGA Columns

Concentrated fractions from the Affi-Gel Blue column were then run down a Bio-Gel P-4 column, the fractions containing protein were pooled and then immediately run down a WGA sepharose column. The purpose of the Bio-Gel P4 column was to remove the glycerol stabilizing the α2,6ST, because glycerol interfered with the lectin column. Fig. 24 shows a typical Bio-Gel P4 column profile with a single protein and α2,6ST activity peak. This sample was from 0.25% ethanol (control) treated liver slices obtained from 24 hour inflamed rats. This type of profile was seen for both control and inhibitor treated α2,6ST preparations. A difficulty with the inhibitor treated slice preparations is that the α2,6ST produced is not as active and may not have been as stable. Samples from monensin or castanospermine treated slices gave very similar results. The WGA columns did not produce acceptable results when used with monensin treated slice preparations since the specific activity of the α2,6ST was lower. Fig. 25 shows a typical elution profile of control α2,6ST passed over a WGA sepharose

Figure 23. Affi-Gel Blue column profile.

Rat liver slices were homogenized in 7.5ml buffer A per g (wet weight) liver slices, homogenized and centrifuged for 20 min at 2000 rpm in a Sorval SS34 rotor. The pellet was rehomogenized in 50% of the initial volume of buffer A and recentrifuged. The two supernatants were combined and applied to the Affi-Gel Blue column and 100 drop fractions were collected. After approximately 150ml of buffer A was passed through the column to wash off any unbound material; the buffer was then changed to include a linear salt gradient of 0.15M to 2.5M NaCl (300ml total volume) in buffer A. The fractions were then assayed for α2,6ST and protein. Fractions from the second peak were vacuum dialyzed against buffer A and adjusted to a final protein concentration of 1.5mg/ml. Samples were then run down a BioGel P4 column or used for kinetic studies.

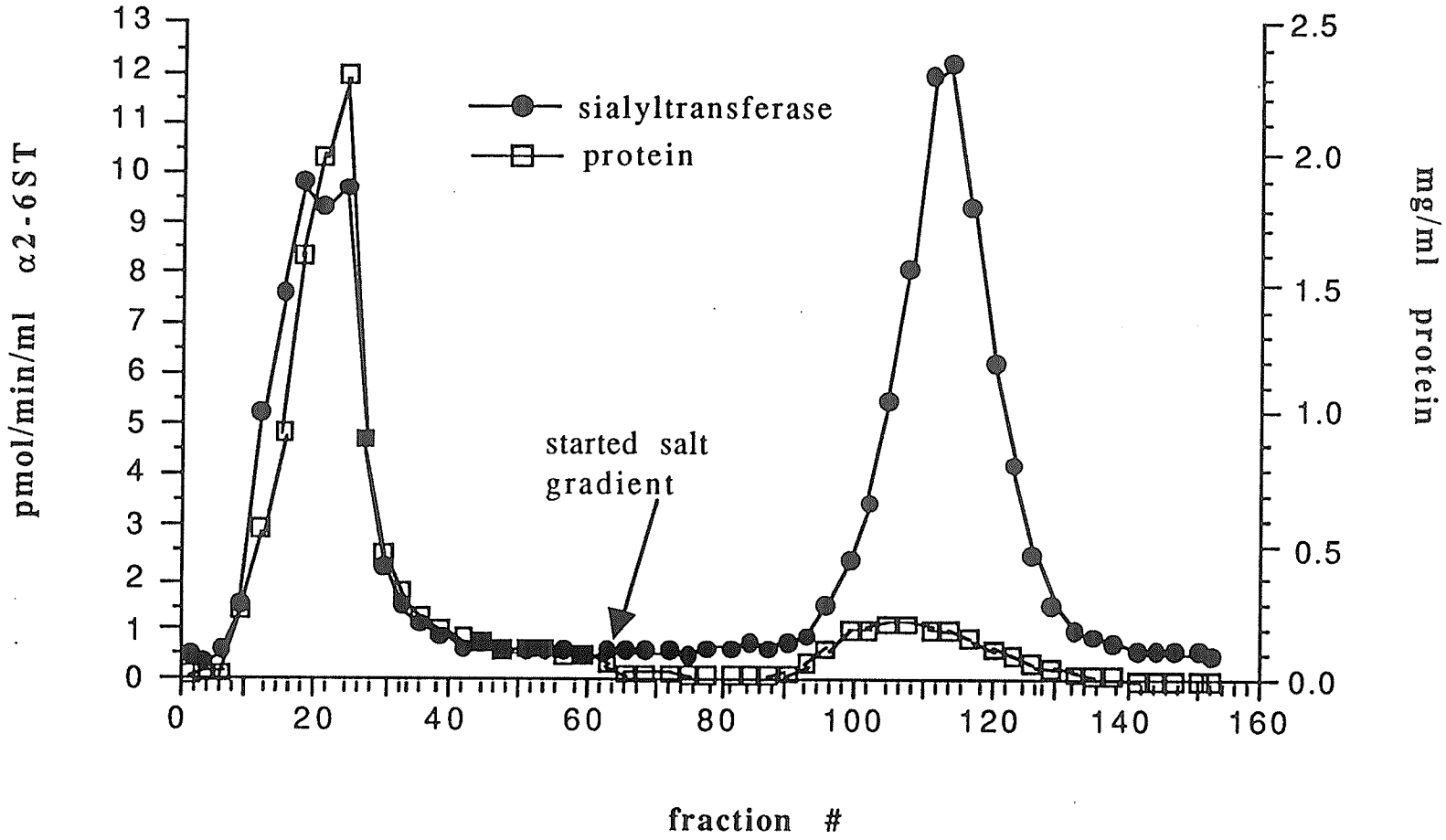

pmol/min/ml α2-6ST
mg/ml protein
sialyltransferase
protein
started salt
gradient
fraction #
0
1
2
3
4
5
6
7
8
9
10
11
12
13
0.0
0.5
1.0
1.5
2.0
2.5
0
20
40
60
80
100
120
140
160

Figure 24. Bio Gel P4 column profile.

Samples (500-750µl) obtained from the Affi-Gel Blue column were applied to the column and allowed to soak in for approximately 15 minutes. Then the column was eluted with 50mM Tris (pH 7.0) into 50 drop fractions. The fractions were kept on ice and assayed for protein and α2,6ST. Fractions containing α2,6ST activity were immediately pooled and run down a WGA sepharose column. The purpose of the BioGel P4 column was to remove any glycerol present in the samples from the Affi-Gel Blue column.

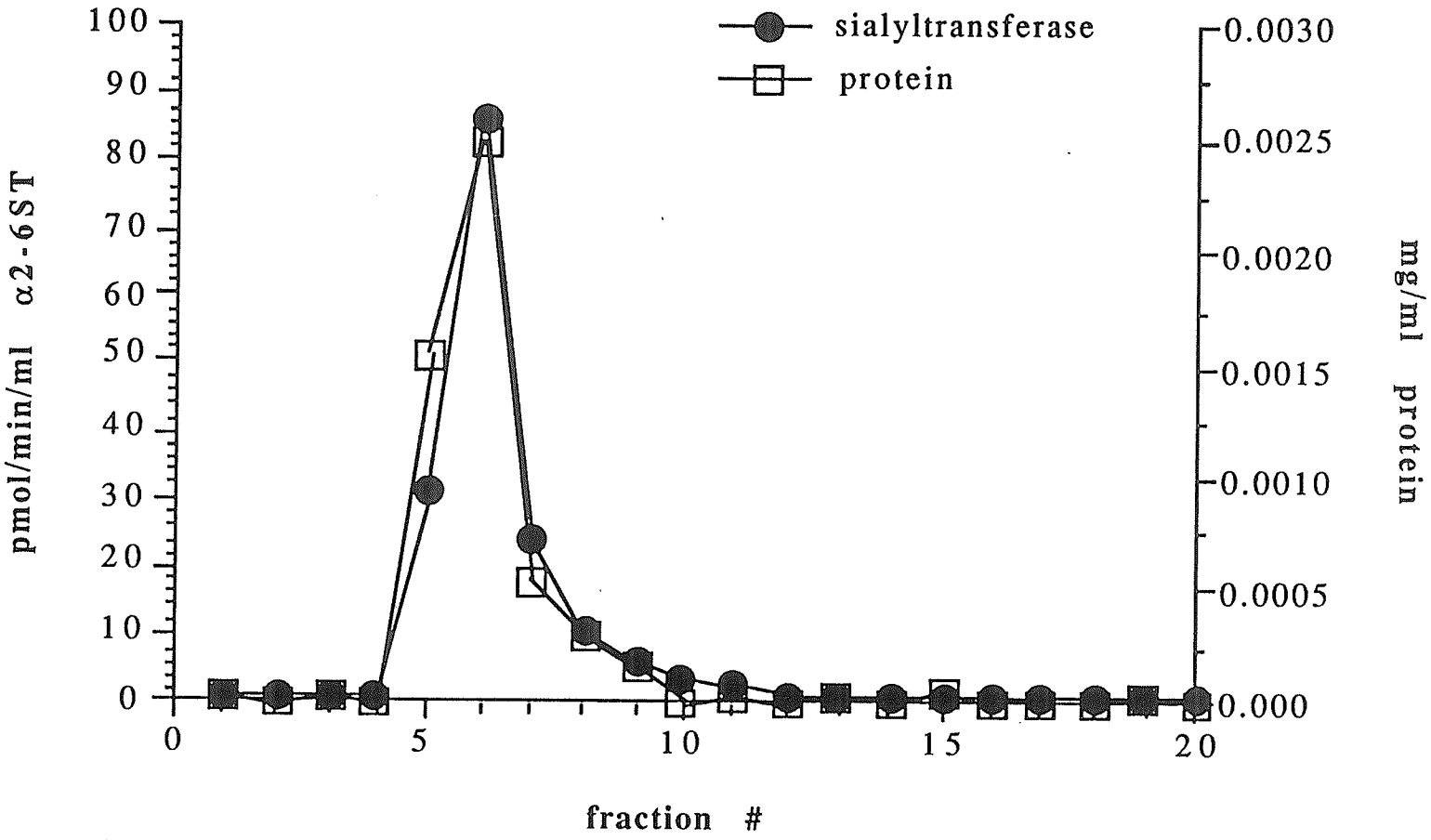
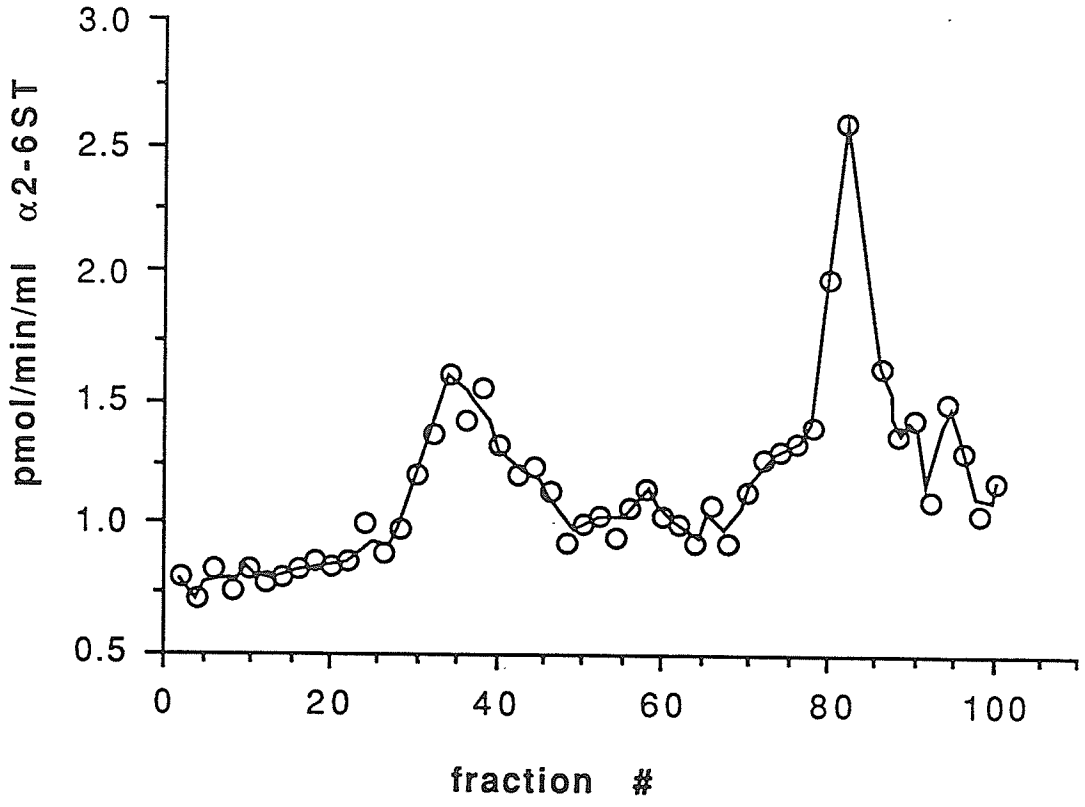

sialyltransferase
protein
pmol/min/ml α2-6ST
mg/ml protein
fraction #
100
90
80
70
60
50
40
30
20
10
0
0.0030
0.0025
0.0020
0.0015
0.0010
0.0005
0.000
0
5
10
15
20

Figure 25. WGA sepharose column profile.

This sample is from 0.25% ethanol treated slices obtained from a 24 hour inflamed rat. The samples were applied to the column at room temperature and allowed to soak in for 15 minutes. Then 50 drop samples were eluted into 500 µl of 25% glycerol and stored on ice. After 50 fractions the buffer was changed to include 0.1M GlcNAc to elute any α2,6ST containing complex type carbohydrate chains. All samples were assayed for protein and α2,6ST. Protein was below the limit of detection.


3.0
2.5
2.0
1.5
1.0
0.5
pmol/min/ml α2-6ST
0
20
40
60
80
100
fraction #

column. Protein concentration could not be measured as it was below the sensitivity of our assay system.

Digestion of Sialyltransferase with Endoglycosidases

Although the presence of monensin and other processing inhibitors caused a reduction in the release of α2,6ST from liver slices into medium which correlated well with the apparent accumulation of high mannose type oligosaccharides in liver; the results were not definitive. Therefore, an alternative approach was used to obtain information on the importance of oligosaccharide chains for α2,6ST activity. This involved the removal of oligosaccharide chains from purified α2,6ST with endoglycosidases.

N-glycanase

N-glycanase Time Course

Samples of α2,6ST supplied by Genzyme, were incubated in the presence of N-gly for up to 20 hours and the effect of the digestion on the activity of the α2,6ST was measured. Fig. 26 shows that digestions in the presence of 5% methanol cause approximately 75% of the α2,6ST activity to be lost after 6 hours of incubation when compared to controls in the absence of N-gly. Fig. 27 shows the loss of activity in the presence of 10% methanol over time. The activity loss was similar with 10% methanol present; 75% loss of activity occurred after approximately 6 hours of incubation at 37°C. 5% methanol was therefore used in further experiments to minimize any denaturation that may be caused by higher concentrations of methanol. All the time course experiments were done in the presence of a 2 fold excess (weight/weight) of albumin to eliminate any proteolytic activity which could be present in either the N-gly or α2,6ST preparations and controls contained EDTA and Mn^{2+}.

Figure 26. Activity of α2,6ST upon N-gly digestion over time in the presence of 5% methanol.

Samples consisted of 30µl pure α2,6ST, 0.2M NaPO4 (pH 8.6), 5% methanol, HSA and 3.6µl of N-gly (●) in a total volume of 90µl. Controls were set up in parallel and contained 3.6µl of 50% glycerol/2.5mM EDTA (❑) in place of N-gly. The samples were incubated for up to 15 hours at 37°C and 10µl aliquots removed at the times indicated. Samples were assayed for α2,6ST activity for 1 hour as described in the Materials and Methods section. Results are the means of three experiments and are within ±15%.

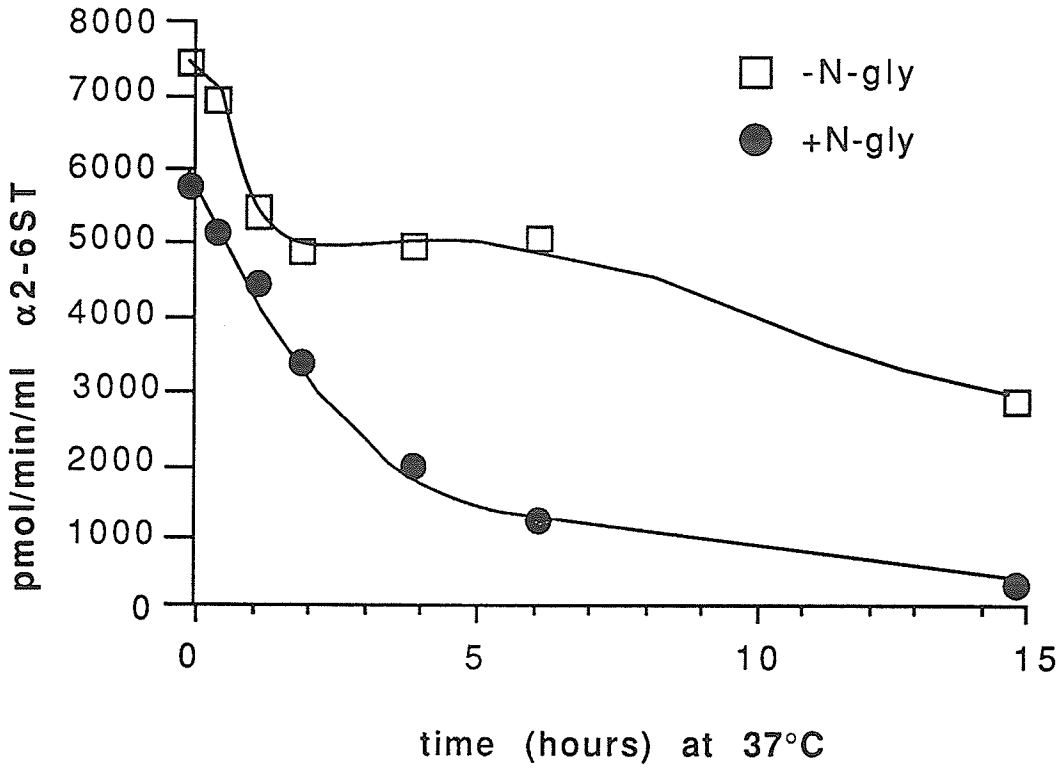
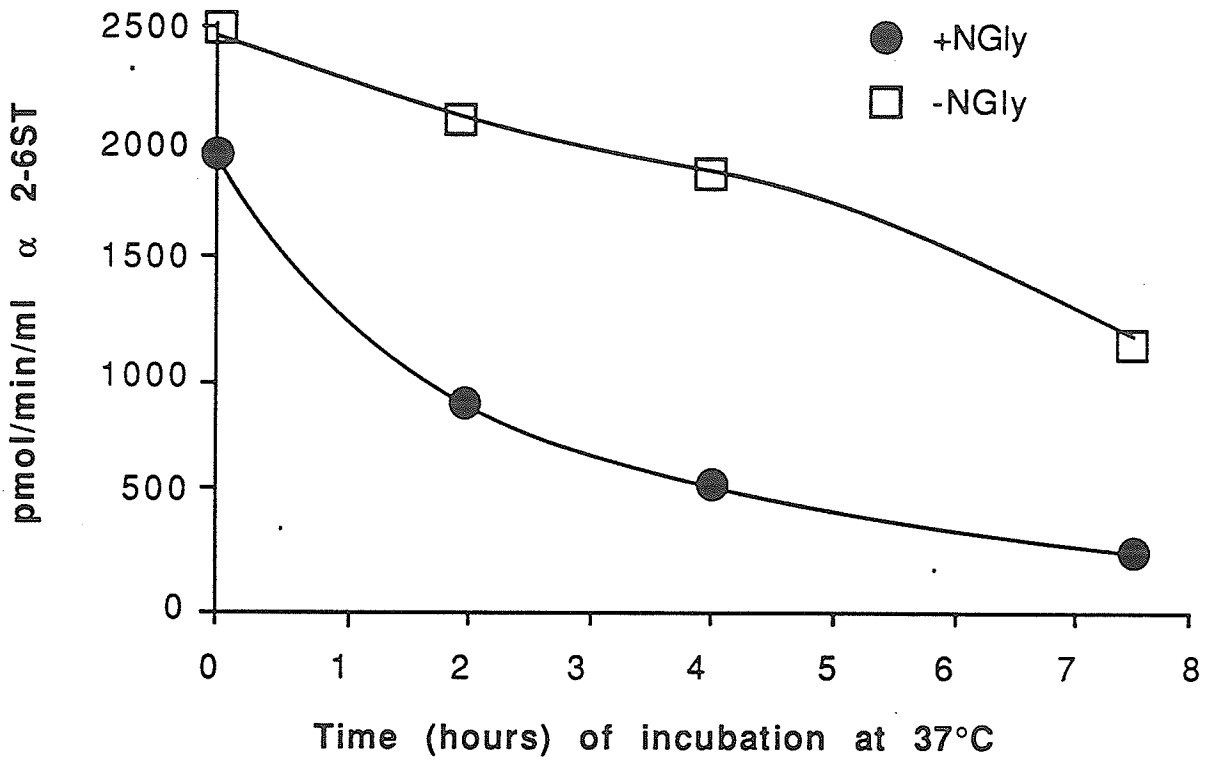

8000
7000
6000
5000
4000
3000
2000
1000
0
pmol/min/ml α2-6ST
0
5
10
15
time (hours) at 37°C
-N-gly
+N-gly

Figure 27. Activity of α2,6ST as a result of N-gly digestion over time in the presence of 10% methanol.

Samples were prepared and assayed as in Fig. 26 with the exception that the methanol concentration was 10%. Incubation was for up to 8 hours. Results are the means of three experiments and are within ±15%.


2500
2000
1500
1000
500
0
pmol/min/ml α 2-6ST
+NGly
-NGly
0
1
2
3
4
5
6
7
8
Time (hours) of incubation at 37°C

It was important to carry out control experiments to determine the conditions for digestion of α2,6ST with N-gly as experiments done according to the Genzyme technical bulletin did not provide enough information. Methanol was initially used to dissolve a protease inhibitor 1,10 orthophenathroline as recommended by Genzyme, but the 1,10 phenanthroline inhibited the α2,6ST activity. Therefore, HSA was used as a protease inhibitor. Tests were carried out to determine what concentration of methanol best promoted the deglycosylation of α2,6ST by N-gly. Ethanol, closely related to methanol was also examined as a potential enhancer of N-gly action on α2,6ST.

Molecular weight studies of N-gly treated sialyltransferase

SDS PAGE of native α2,6ST showed that there was a molecular weight change on digestion with N-gly. Fig. 28 shows three bands, one of which corresponded to the native enzyme (M_r = 42,000). The other bands had M_r = 40,000 and 38,000 and would correspond to forms of α2,6ST which had lost one and two carbohydrate chains, respectively. This corresponds well with the loss of activity, suggesting that the carbohydrate may be important for activity. After a 24 hour digestion of native α2,6ST with N-gly, there is only one band seen at M_r = 38,000 (as shown in Fig. 28) which corresponds to the band seen when denatured α2,6ST was treated with N-gly. Denatured α2,6ST treated with N-gly showed a M_r change with only the lower, M_r = 38,000 weight band being seen after 6 hours of incubation at 37°C, suggesting that the protein was not subject to proteolytic degradation on digestion with N-gly.

Effect of Methanol

In view of the results described above, in which methanol was found to improve removal of oligosaccharide chains with N-gly, a more comprehensive study was undertaken. Fig. 29 shows the effect of methanol concentration on

Figure 28. Molecular weight studies of α2,6ST after treatment with N-gly.

Gels showing the change in molecular weight of α2,6ST after treatment of the native enzyme with N-gly for 6 hours, 18 hours, and also the denatured enzyme for 6 hours. A. Pure α2,6ST is shown in lane 1 and has M_r 42,000. Lane 2 shows native α2,6ST treated for 6 hours with N-gly. Three bands can be seen at M_r 42,000, 40,000 and 38,000. Lane 3 shows the control sample with a single band at M_r 42,000. B. Pure α2,6ST is shown in lane 1, native α2,6ST treated for 18 hours in the presence of N-gly is shown in lane two with a single band at M_r 38,000. Lane 3 shows the control sample also incubated for 18 hours at 37°C. C. Digestion of denatured α2,6ST by N-gly for 6 hours is shown in lane 1, with M_r 38,000 and the control sample is shown in lane 2 with M_r 42,000.

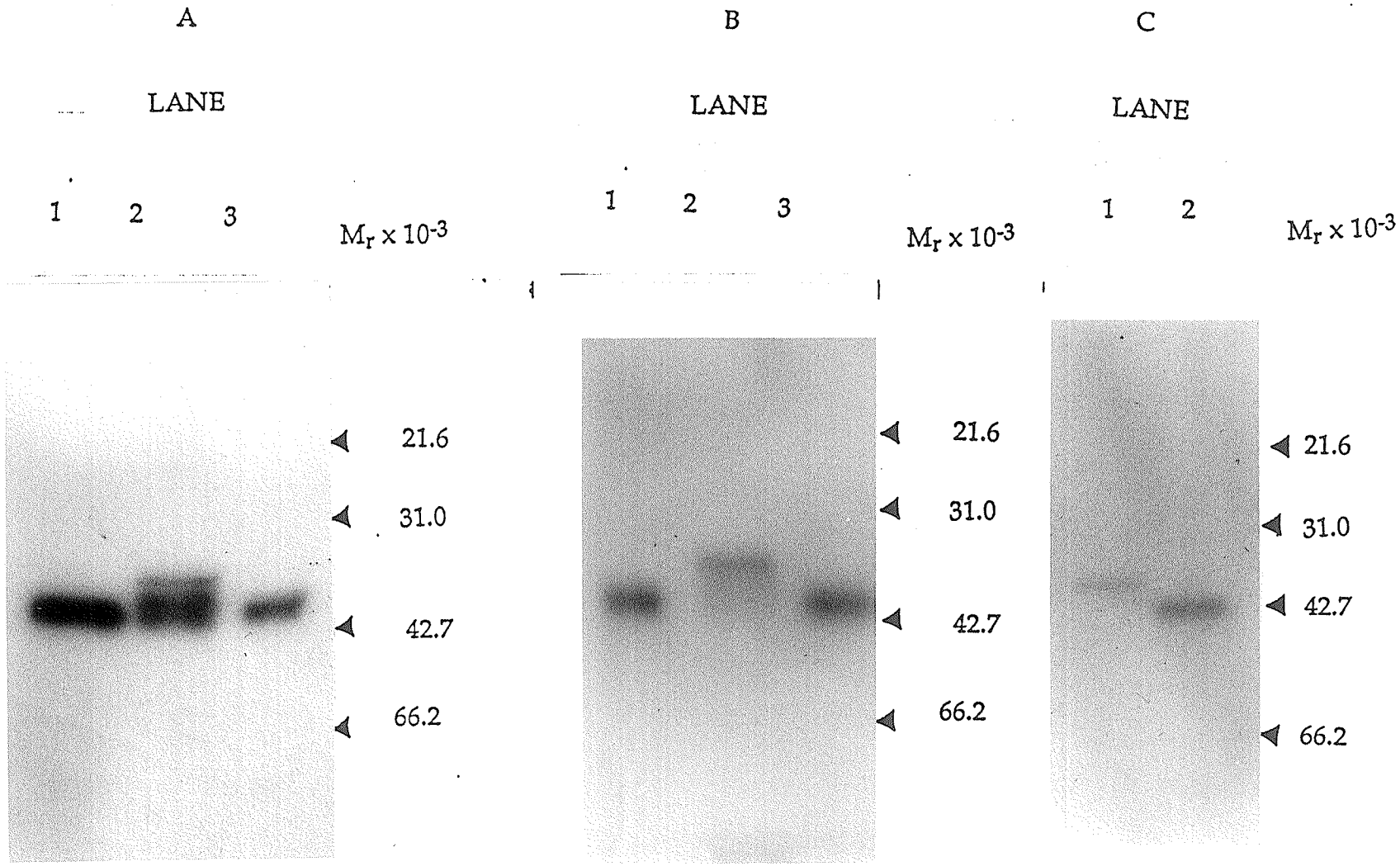
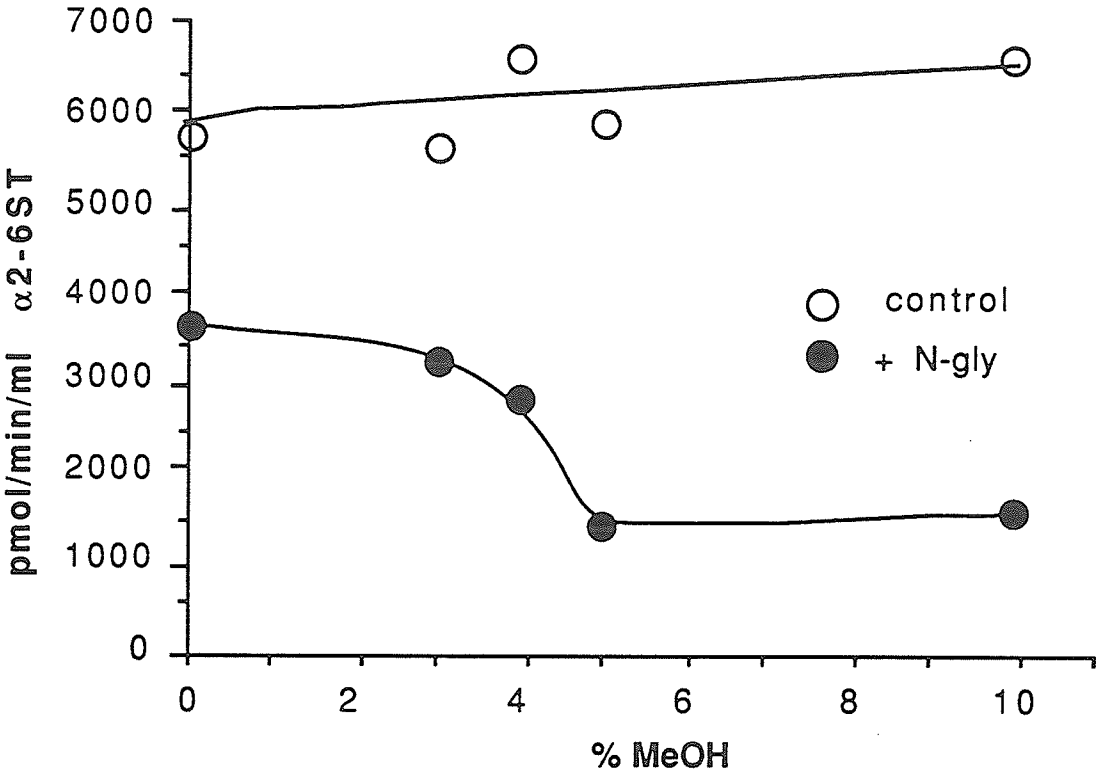

A
LANE
1
2
3
$M_r \times 10^{-3}$
21.6
31.0
42.7
66.2
B
LANE
1
2
3
$M_r \times 10^{-3}$
21.6
31.0
42.7
66.2
C
LANE
1
2
$M_r \times 10^{-3}$
21.6
31.0
42.7
66.2

Figure 29. α2,6ST activity in the presence of methanol after 6 hours at 37°C.

Activity of α2,6ST in the presence of various methanol concentrations at 0 hours of incubation at 37°C. Conditions are as for Fig. 26 and the α2,6ST assay was incubated for 1 hour. Results are the means of three experiments and are within ±15%.


7000
6000
5000
4000
3000
2000
1000
0
pmol/min/ml α2-6ST
0
2
4
6
8
10
% MeOH
control
+ N-gly

α2,6ST activity after 6 hours at 37°C. Fig. 30 shows the effect of methanol concentration on α2,6ST activity at 0 hours of incubation at 37°C. From this we can see that the methanol is not affecting the α2,6ST activity even after 6 hours of incubation at 37°C.

Effect of other Alcohols

The ability of ethanol to promote N-gly digestion of α2,6ST is shown in Fig. 31. From this we can see that both ethanol and methanol do not work equally well in promoting the digestion of α2,6ST. However, ethanol does allow some carbohydrate to be removed from α2,6ST. The effect of ethanol on the ability of N-gly to promote loss of α2,6ST activity is not as pronounced as that of methanol. Reasons for differences in ability to promote deglycosylation are outlined in the discussion.

Effect of Manganese and EDTA on sialyltransferase

Table 4 shows the effects of Mn^{2+} and EDTA on α2,6ST. Samples were tested after 4 hours of incubation at 37°C. There was no significant difference between those samples with EDTA and those without EDTA. Adding Mn^{2+} to the samples with EDTA did not significantly affect the activity. Therefore the EDTA present in the commercial N-gly preparation was not responsible for the difference in activity of α2,6ST after digestion with N-gly. EDTA was present in all control samples for N-gly digestion at the concentration equal to that found in the N-gly preparation.

Effect of Albumin

Human serum albumin (99.9%) was added to α2,6ST to inhibit any proteases which may be present in either the α2,6ST or the N-gly preparations. Fig. 32 shows concentrations up to 5 fold weight excess albumin (compared to

Figure 30. α2,6ST activity in the presence of methanol prior to incubation. Activity of α2,6ST in the presence of various methanol concentrations at 0 hours of incubation at 37°C. Conditions are as for Fig. 26 and the α2,6ST assay was incubated for 1 hour. Results are the means of three experiments and are within ±15%.

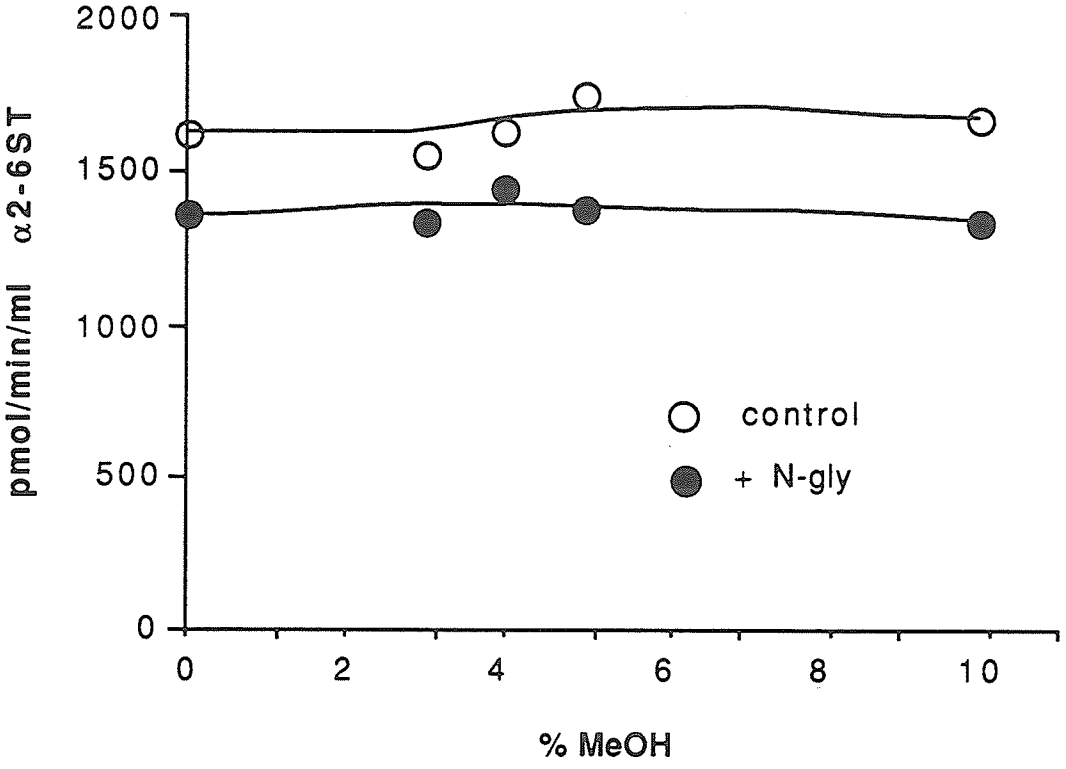

2000
1500
1000
500
0
pmol/min/ml α2-6ST
0
2
4
6
8
10
% MeOH
control
+ N-gly

Table 4. Effect of Mn^{2+} and EDTA on α2,6ST activity

Effect of Manganese and EDTA on the Activity of α2,6ST. Samples were prepared as stated in materials and methods. Manganese and EDTA were added to ensure compounds in the N-gly preparation were not interferring with the results. Values are the means of three experiments and are within ±15%.

Table 4. Activity of Sialyltransferase upon addition of EDTA and/or manganese

all values in pmol/min/ml.

+EDTA/Glycerol		-EDTA/Glycerol
$+Mn^{2+}$	$-Mn^{2+}$	$-Mn^{2+}$
1200	1095	1261.6

Figure 31. Activity of α2,6ST as a result of N-gly digestion over time in the presence of 5% ethanol.

Samples were prepared and assayed as in Fig. 26 with the exception that the 5% ethanol was used in place of methanol. Incubation was for up to 12 hours at 37°C. Results are the means of three experiments and are within ±15%.

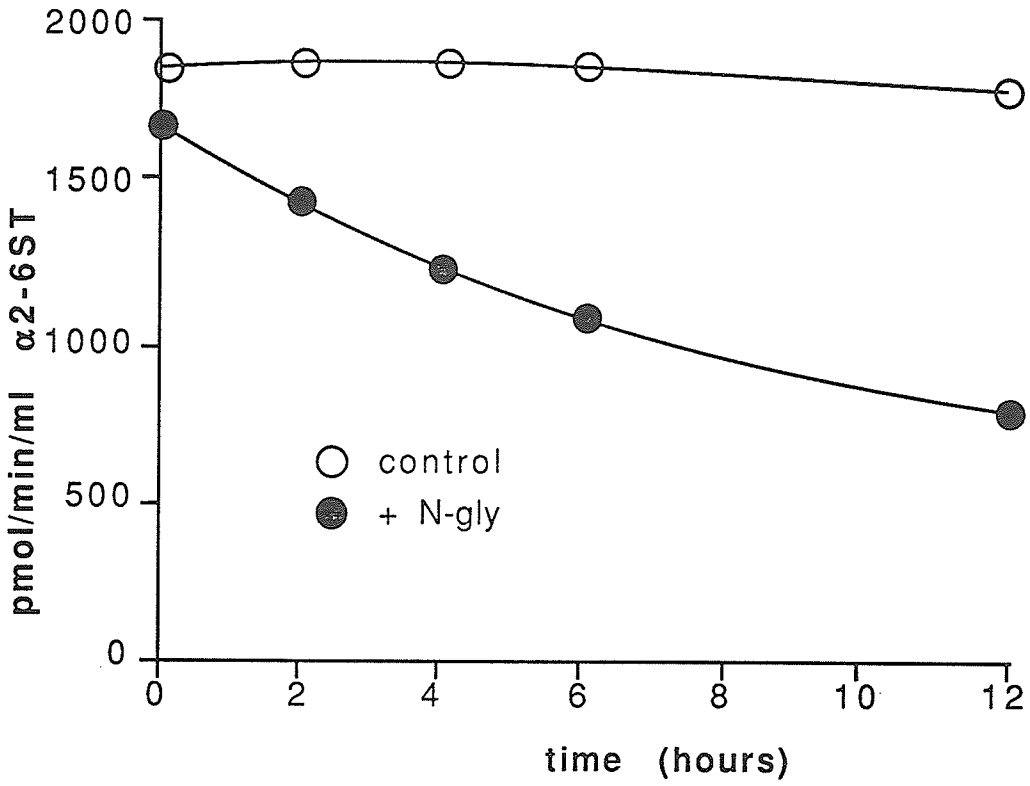
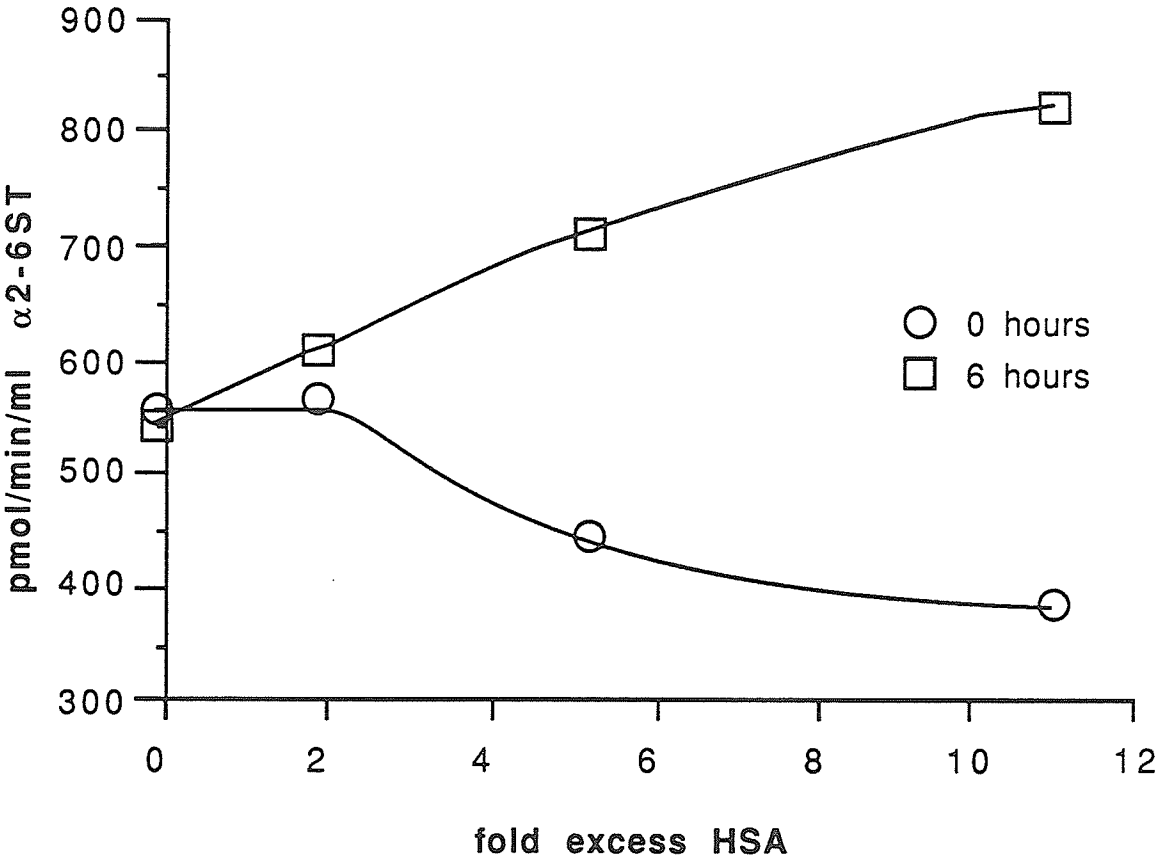

2000
1500
1000
500
0
pmol/min/ml α2-6ST
control
+ N-gly
0
2
4
6
8
10
12
time (hours)

Figure 32. Activity of α2,6ST in the presence of HSA.

Activity is shown at 0 and 6 hours of incubation at 37°C in the presence of up to 5 fold weight excess of HSA over α2,6ST. HSA was used as a protease inhibitor. Results are the means of three experiments and are within ±15%.


900
800
700
600
500
400
300
pmol/min/ml α2-6ST
0
2
4
6
8
10
12
fold excess HSA
0 hours
6 hours

α2,6ST) were used. α2,6ST activity was measured after 0 and 6 hour incubations at 37°C and it was found that a 2 fold excess of albumin did not greatly affect activity. At higher concentrations the albumin appeared to increase the activity of the α2,6ST, but only after incubation at 37°C for 6 hours. Therefore a 2 fold excess of albumin was added to all further experiments to inhibit proteases.

Endo H treatment of α2,6ST

Table 5 shows the inability of endo H to affect the activity of native α2,6ST even after 12 hours of incubation at 37°C. The presence of 5% methanol did not promote or reduce the ability of endo H to change the α2,6ST activity. Fig. 33 shows the effect of Endo H on the M_r of denatured α2,6ST after a 6 hour incubation. From this we can see that there are two bands, one at approximately M_r = 42,000 and the other at M_r = 40,000. Since no activity loss is seen, but the molecular weight changes by approximately one carbohydrate chain on digestion with endo H, we can assume that even if there are high mannose type chains on the α2,6ST, they are not important for activity.

Table 5. Effect of endo H on α2,6ST activity

Effect of endo H digestion of naitve α2,6ST on activity. Samples were prepared as stated in materials and methods. Some of the samples contained 5% methanol as did the N-gly experiments. Samples were incubated for 6 hours in the presence of endo H and the α2,6ST assay was for 1 hour. Values are the means of three experiments and are within ±15%.

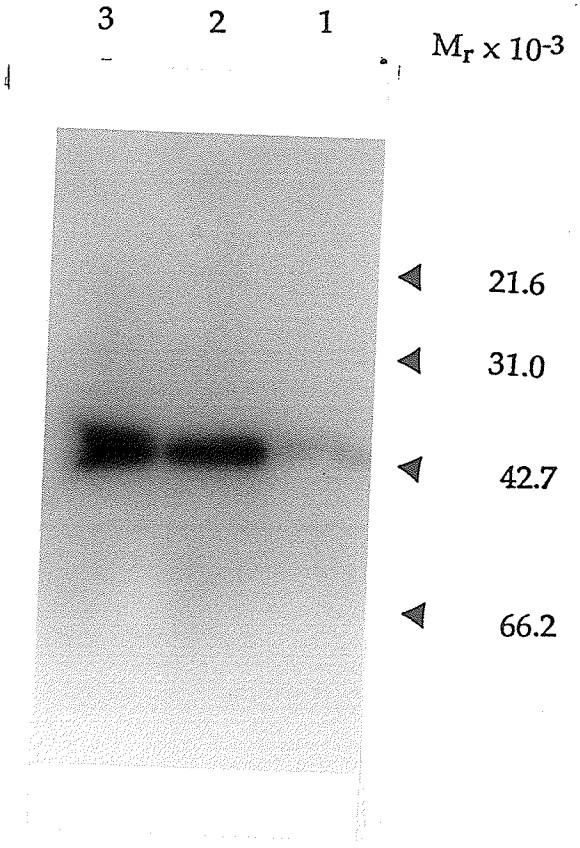
Table 5. Endo H digestion of α2,6ST.

All values are in pmol/min/ml

time (hours)	+ 5% methanol		without methanol	
	+ endo H	- endo H	- endo H	+ endo H
0	1542	1687	2218	2289
1	1573	1660		
2	1607	1742		
4	1628	1731	2245	2377
6	1690	1719		
11	1302	1497	1861	2213

Figure 33. Change in Molecular weight as a result of endo H digestion.

Samples were prepared and run as stated in Materials and Methods. Lane 1 shows pure α2,6ST (Mr 42,000), Lane 2 shows the control sample (Mr 42,000) and Lane 3 shows the endo H treated sample with two bands, at Mr 42,000 and 40,000. The α2,6ST was denatured by first boiling in SDS for 3 min. and then treated with endo H for 6 hours at 37°C.


LANE
3
2
1
$M_r \times 10^{-3}$
21.6
31.0
42.7
66.2

DISCUSSION

Liver Slices

Liver slices, while different from intact liver, maintain most of the biochemical properties, including synthesis and secretion of plasma proteins. Liver slices maintain a large degree of organization and therefore are advantageous over cultured or isolated cells where cells are removed from their natural environment. Liver slices represent a complete and heterogeneous system, and the results obtained reflect integration of the various processes occurring within the tissue (Fraga and Tappel, 1988). Thus, liver slices can be considered very close to an *in vivo* system. Liver slices from inflamed rats exhibit elevated rates of synthesis of acute phase reactants as does intact liver (Janzen *et al.*, 1987; Woloski, 1983). Although some cell damage does occur, liver slices can be incubated for up to 48 hours and are viable as shown by the incorporation of [^{3}H] leucine into total liver and medium proteins (Janzen *et al.*, 1987); and can express the acute phase response over these long incubation times. Liver slices have been used previously in the presence of glycosylation processing inhibitors (Jamieson, 1988). Deoxynojirimycin, a processing inhibitor which prevents oligosaccharide from being modified beyond the $Glc_{1-3}Man_9GlcNAc_2$ stage, was used to prevent the release of α2,6ST from slices from both control and inflamed rats.

Glycosylation Inhibitor Studies

As stated in the results, a series of glycosylation processing inhibitors were used to modify the carbohydrate chains of α2,6ST. All affected the kinetic parameters of K_m and V_{max}. Studies of this nature have been done previously with the nicotinic acetylcholine receptor, where it was shown that inhibitors of

oligosaccharide processing altered the kinetics of the receptor (Covarrubias *et al.*, 1989).

As mentioned earlier, monensin is a monovalent ionophore which interferes with intracellular transport by disrupting the Na^+/H^+ gradient across ER and Golgi membranes. As a result of this, glycoprotein processing beyond the high mannose stage is inhibited. Thus, any glycoproteins produced in the presence of monensin should have high mannose type chains (Ledger *et al.*, 1983). Therefore a buildup of α2,6ST with high mannose type oligosaccharides should be observed in the ER and Golgi of the liver slices treated with monensin. The α2,6ST isolated from 10μM monensin treated slices had different kinetic properties than that of control (0.25% ethanol) treated slices. Treatment with monensin caused α2,6ST to be produced with different types of carbohydrate chains than were normally found. As a result of this the K_m is lowered and the enzyme binds the asialo α_1 acid glycoprotein substrate tighter than normal, thus preventing the enzyme from utilizing it efficiently. The V_{max} of the α2,6ST is also affected with it being decreased to approximately 15% of its control value, suggesting that it cannot utilize the substrate properly. The α2,6ST obtained from the medium of 2μM monensin treated liver slices did not have significantly different kinetic parameters than that from ethanol treated slices. This suggests that the blockage of transport and processing by monensin is incomplete and some α2,6ST is processed and transported out of the cell. Another possibility is that there is an intracellular pool of α2,6ST with fully processed complex type carbohydrate chains, which is released from the Golgi by a cathepsin D like proteinase activity even in the presence of inhibitor. This source of α2,6ST should have fully processed chains and therefore the kinetic parameters should not be different. Also, it is not likely that the α2,6ST being secreted by the monensin treated slices contains only high mannose type chains, because

monensin does not block transport completely and it is believed that a small amount of transport still occurs and there is no evidence to suggest that monensin directly affects any of the glycosyltransferases along the secretory pathway. More transport could probably be inhibited by using higher concentrations of monensin, but this would cause inhibition of protein synthesis and other cellular functions as well, thus making the system less than ideal.

The results show that α2,6ST release from rat liver slices into the medium is inhibited by treatment with monensin versus controls treated with ethanol. This occurred with liver slices from both control and 24 hour inflamed rats. For slices from inflamed rats the maximum inhibition of release occurred at about 1μM and no further inhibition was seen up to 20μM for a 12 hour incubation at 37°C. For control slices, maximum inhibition occurred with 0.5μM monensin and enzyme activity was not further decreased with monensin concentrations up to 20μM. The amount of α2,6ST activity released into the medium in the presence of monensin was reduced to approximately 40% of the controls for slices from inflamed rats and only to approximately 50% for slices from control rats. The effect of monensin as an inhibitor was more pronounced when liver slices from inflamed rats were used, as glycoprotein biosynthesis is enhanced, therefore requiring more inhibitor to stop transport of enzymes from the ER to the Golgi. This has also been seen when deoxynojirmycin was used as an inhibitor of glycoprotein processing (Jamieson, 1988).

Although kinetic parameters of α2,6ST from control liver slices were not examined, it is possible that the acute phase response is responsible for changes in the pattern of glycosylation, thereby leading to differences in kinetics. This is also a possible explanation for the decreased sensitivity to monensin observed in slices from inflamed rats. The acute phase response is known to cause some changes in the pattern of glycosylation for some proteins (Pos *et al.*, 1988), as

observed by enhanced affinity of some glycoproteins to the lectin concanavalin A which typically binds high mannose type oligosaccharide chains.

The glycoprotein processing inhibitor castanospermine also affected the activity of α2,6ST made by rat liver slices. This inhibitor should produce chains with a $GlcNAc_2Man_9Glc_3$ structure as shown in Fig. 4. From the results we can see that castanospermine inhibits the release of α2,6ST into the medium or changes its kinetic parameters. The maximum inhibition of release occurred with 5μM castanospermine and no further release was seen up to 175μM castanospermine. The α2,6ST found in the liver slices treated with 50μM castanospermine does not have the same kinetics as α2,6ST isolated from control slices. The K_m was decreased by a factor of 4 fold and the V_{max} was approximately 25% of the α2,6ST obtained from control treated slices, suggesting that the carbohydrate chain structure plays an important role in the activity of the enzyme as well as in specificity and catalytic efficiency.

Deoxymannojirimycin, a processing inhibitor of Golgi located α–mannosidases was also found to change the kinetic parameters of α2,6ST isolated from liver slices treated with 1mM deoxymannojirimycin. Oligosaccharides produced in the presence of deoxymannojirimycin should all have $GlcNAc_2Man_9$ type structures The V_{max} was only 6% of the control value and the K_m was 20% of the control. These values are similar to those obtained for α2,6ST from monensin and castanospermine treated slices. This makes sense since all the inhibitors claim to stop processing at almost the same stage. Thus the α2,6ST with high mannose type oligosaccharide chains must be able to bind the asialo α_1 acid glycoprotein substrate well, but be unable to use it efficiently. It is possible that the carbohydrate chains play a role in maintaining the tertiary structure in a correct conformation and that altering the type of oligosaccharide chains changes the conformation at or near the active site, causing the α2,6ST to bind the CMP-

NeuAc substrate tighter than normal, thus preventing it from being cleaved and a NeuAc added to a growing carbohydrate chain. A possible role of oligosaccharides in enzyme activity is that the oligosaccharides may prevent the correct folding of the enzyme as it passes through the Golgi and is processed by the processing enzymes. It is also possible that the processing enzymes require a certain protein conformation, which is caused as a result of correct oligosaccharide chain structure, to act on the oligosaccharide.

For all kinetic studies the α2,6ST was partially purified by running either liver homogenate or medium from liver slice experiments down an Affi-Gel blue column. The purpose of this was to remove a large number of impurities and proteases from the enzyme solution. Profiles of this column with either control or monensin treated samples usually gave two peaks of protein content. The first peak was always quite large with approximately 90-95% of the protein in this peak. The first peak also contained about 50% of the α2,6ST activity. The second peak typically had very little protein and a large amount of α2,6ST activity. The basis of separation when using an Affi-Gel blue column is that hydrophobic proteins stick to the column and are then eluted using a linear salt gradient (Sticher *et al.*, 1988). The partially purified α2,6ST from the control slices which was run down the WGA sepharose column showed that the enzyme had at least some complex type chains as there was a peak after addition of GlcNAc which is typical of proteins containing complex type oligosaccharides. α2,6ST from monensin and castanospermine treated slices were also run down the WGA sepharose column, but no enzyme could be detected by either the protein assay or the sialyltransferase assay as the levels of the α2,6ST was too low. A reason for this very low level of α2,6ST is that the enzyme is very unstable once the glycerol is removed and the column needed to be run at room temperature for it to work, thus promoting the denaturation of the enzyme. Therefore the α2,6ST

probably was denatured before it could be recovered from the lectin column. Other possible reasons that detection of the α2,6ST on the lectin columns was difficult is that the enzyme is unstable as a result of the modified carbohydrate chains and is therefore quickly degraded before detection can occur. Stability may be a role of the oligosaccharide chains of α2,6ST.

These results suggest that hepatic α2,6ST is a glycoprotein with at least one complex type carbohydrate chain in its fully mature form. The main role of the acute phase response in this study was to provide a larger amount of enzyme to be studied. This has been done previously in order to obtain larger amounts of rare proteins (Silanovich and Jamieson, 1989).

Endoglycosidase Studies

As seen in the results section, digestion of native α2,6ST with N-gly under appropriate conditions resulted in the loss of approximately 85% of the catalytic activity of α2,6ST after a 6 hour incubation. This time dependent loss of activity suggested that as the carbohydrate chains were removed from the α2,6ST it lost catalytic activity. From the molecular weight studies of the native and denatured α2,6ST treated with N-gly we can postulate that the α2,6ST has two carbohydrate chains. This is seen in the number of bands on the immunoblot of the native α2,6ST treated with N-gly for 6 hours where there are three bands, the first corresponding to the pure α2,6ST (M_r =42,000), the second corresponding to the loss of one oligosaccharide chain (M_r = 40,000) and the third corresponding to the loss of two oligosaccharide chains (M_r = 38,000). This is consistent with two N-linked oligosaccharides being removed, each changing the mass by approximately 2,000 daltons. This last band correlates well with the 24 hour digestions of native α2,6ST and digestions of denatured α2,6ST for six hours where only one band was seen corresponding to the removal of two carbohydrate chains (M_r = 38,000).

N-gly removes all types of N-linked oligosaccharide chains as reviewed in the introduction and therefore we cannot determine what type of oligosaccharide chains are being released by N-gly digestion of α2,6ST. It is possible that one or both of the chains may be a high mannose type, complex type, hybrid type, or a branched type structure.

The removal of oligosaccharide chains with N-gly causes an aspartic acid residue to be formed where there was originally an asparagine-GlcNAc bond. This aspartic acid residue has a negative charge and could possibly be the cause of decreased activity of α2,6ST seen after digestion with N-gly. A negative charge could disrupt folding of the protein, thus leading to reduced activity. This possibility has not been explored further as the tertiary structure at or near the glycosylation sites is unknown. This could probably be studied using molecular biology techniques, in which the DNA could be changed so that an aspartic acid residue would be in place when the α2,6ST was synthesized. This would determine if it was the introduction of a negative charge at the glycosylation sites which is causing the loss of activity.

Treatment of native α2,6ST with endo H did not result in a reduction of activity over controls, suggesting that even if there are high mannose type chains, they are either not important for activity or are inaccessible to endo H under the conditions used. Treatment of denatured α2,6ST with endo H did result in a M_r change of approximately 2000 Da, two bands were seen at Mr = 42,000 and 40,000. These probably correspond to the fully glycosylated α2,6ST and a species with one high mannose type chain removed. This suggests that only one of the carbohydrate chains is probably a high mannose type, although it is not important for activity of α2,6ST. The other possibility is that heterogeneity of the oligosaccharide chains exists and that only some of the α2,6ST has two complex type chains and some have both complex and high mannose and some have only

high mannose type chains. It is also possible that some of the bands seen were proteolytic fragments of the α2,6ST as there may be trace proteases in either the endo H or α2,6ST preparations, although this is unlikely since other proteolytic fragments would have been detected as well, and care was taken to guard against proteolysis by incubating all samples in the presence of HSA.

Experiments designed to minimize the degradation of α2,6ST by proteases present in the enzyme preparations also provided some interesting results. Addition of HSA at up to 5 fold excess over α2,6ST caused an increase in α2,6ST activity after 6 hours of incubation at 37°C. The reason for this is unknown, but it can be speculated that, like the methanol, the albumin somehow disrupted the micelles surrounding the enzyme and allowed the substrates easier access, thus leading to higher values for the samples containing 5 fold weight excess of albumin. Therefore only a two fold weight excess of HSA was added as this did not seem to affect α2,6ST activity. Ideally, one would have liked to add a protease inhibitor such as 1,10 phenanthroline to inhibit proteases, but it was found that this inhibitor interfered with the α2,6ST, causing it to be inactive. It is believed that the α2,6ST requires trace amounts of Mn^{2+} to be active (Sticher *et al.*, 1988) and that the 1,10 phenanthroline chelates divalent cations, thereby preventing the α2,6ST from being active in its presence. Other protease inhibitors were not tested.

Role of Organic solvents in digestion by N-gly

The loss of α2,6ST activity on treatment with N-gly was seen in the presence of both methanol and ethanol, although less of an effect was observed in the latter. Methanol was shown to be important for the removal of carbohydrate from α2,6ST by N-gly. In the absence of methanol, experiments with N-gly were inconclusive and α2,6ST activity was not lost at a rate greater

than the control in which N-gly was absent. At high concentrations of methanol the α2,6ST was probably subject to denaturation and activity is lost rapidly after a few hours. At up to 5% methanol alone, only about 15% of the α2,6ST activity is lost compared to the control (0% methanol) even after 6 hours of incubation at 37°C. From this it was determined that 5% methanol should be used in all further experiments since this allowed maximal α2,6ST activity after 6 hours of incubation for controls and a large loss of activity on treatment with N-gly. The effect of ethanol on the ability of N-gly to digest α2,6ST was also examined and it was found that ethanol also promoted digestion although to a lesser extent than did methanol. After 6 hours of incubation with N-gly the α2,6ST only lost approximately 45% of its activity in the presence of 5% ethanol compared to 85% in the presence of 5% methanol. The role of organic solvents in allowing the N-gly to work on native α2,6ST is puzzling. One explanation for this effect is that methanol and ethanol may disrupt micelles surrounding the α2,6ST or N-gly and allow the N-gly easier access to the oligosaccharide chains of α2,6ST. This is quite possible since the α2,6ST is prepared from rat liver Golgi membranes and probably has a high lipid content. Other possible effects of methanol and/or ethanol are that the structure of either the N-gly or the α2,6ST are changed enough to allow the N-gly to cleave the carbohydrate as methanol and/or ethanol could create unfavorable interactions with charged residues on the surface of either protein (Nakano and Fink, 1990). It is also possible that methanol and ethanol are activators of N-gly although no support has been found in the literature regarding this hypothesis. In the presence of organic solvents the structure of the α2,6ST may be changed so as to make the oligosaccharide chains more accessible to the N-gly, possibly by disrupting the tertiary structure, although this would probably adversely affect the catalytic activity of α2,6ST. The reason that methanol is more effective than ethanol at

promoting N-gly digestion of α2,6ST is that methanol creates a more hydrophobic environment than ethanol (Nakano and Fink, 1990) and therefore is more disruptive to the micelles or proteins, thus allowing easier access to the oligosaccharide chains. Ethanol has been known to cause a number of disruptions to membranes and lipids. Concentrations of ethanol of greater than 200mM (approximately 1.3%) have been shown to disorder membranes (Taraschi and Rubin, 1985) and it is possible that disruption of lipid packing is a factor in the ability of methanol and ethanol to attenuate N-gly activity on the carbohydrate chains of α2,6ST.

It was also observed that in all N-gly digestions of α2,6ST, the zero time point value was approximately 10% lower for samples containing N-gly than for controls. A possible explanation for this is that there may be some component in the N-gly preparation which interferes with the α2,6ST activity. It is also possible that a small amount of N-gly activity could be active when the α2,6ST is being assayed.

Role of carbohydrate chains

Much work has been done looking into the role of carbohydrate chains on glycoproteins. The simplest explanation for oligosaccharide chains is that they are important for recognition, that is they represent specific chemical messages that are recognized and read by receptors within cells and then translated into specific interactions between molecules and cells with their assorted consequences (Yet *et al.*, 1988). The processing enzymes along the transport pathway and the protein structure determine the exact oligosaccharide composition as the glycoprotein is synthesized. The number and kind of oligosaccharides on the protein may also help determine the final oligosaccharide structure (Yet *et al.*, 1988). For human interferon β1 it has been suggested that the carbohydrate

chains of the protein affect stability, solubility, and metabolic destination; this is very important in light of the fact that recombinant products therefore must have the same type of carbohydrate chains for the interferon to function properly (Kagawa *et al.*, 1988). For human chorionic gonadotropin β subunit it has been shown that both of its oligosaccharide chains must be present for efficient secretion and assembly with the α subunit of its secreted dimer form. The chains are also likely to be important for proper folding of the β subunit of the hormone (Matzuk and Boime, 1988). It has been shown that glycosylation and some oligosaccharide processing are both necessary for the epidermal growth factor receptor to bind substrates properly (Gamou and Shimizu, 1988). Oligosaccharides may also play a role in molecular recognition. It has been shown that for myoblast cells in culture, changing the type of oligosaccharide produced with glycosylation processing inhibitors (castanospermine and deoxynojirimycin) to $Glc_{1\text{-}3}Man_9GlcNAc_2$ caused inhibition of fusion reactions which were reversible when the inhibitors were removed (Spearman *et al.*, 1987). It was also noted that changing the carbohydrate chains to a hybrid type caused interference with the receptors which normally recognized high mannose type oligosaccharides. It has been suggested that oligosaccharide chains may influence the activity of other proteins (endogenous lectins); and that other proteins may exhibit control over various glycoproteins by influencing their carbohydrate chains (Feizi, 1988). This could possibly be a mechanism used to control the glycoprotein processing enzymes. Using a variety of glycoprotein processing inhibitors, it has been shown that for thyrotropin, the oligosaccharide structure determines stability and half life of the protein. Thyrotropins with glucosylated high mannose structures have been shown to be degraded more rapidly than those with high mannose or complex type chains (Stannard *et al.*, 1989). It has been suggested that the oligosaccharide chains may be useful in

maintaining the structure for stability or activity; transport, targeting, signalling, etc. Therefore we can see that the role of the carbohydrate on the glycoprotein is protein specific. There is no universal code for the function of oligosaccharide chains on proteins and therefore they must be studied on an individual basis.

Role of carbohydrate chains for the activity and structure of α2,6ST

As the α2,6ST is being translated from its mRNA into a protein it is glycosylated at a minimum of two sites by the enzyme oligosaccharyl transferase in the ER (see introduction) and then the carbohydrate is processed from glucose containing high mannose type chains to high mannose type chains. At this stage, while in the ER /*cis* Golgi, the enzyme does not have any catalytic activity since the carbohydrate chains are not in the correct conformation. The protein may not have achieved its final tertiary structure, a process that may be controlled by the structure of the oligosaccharide chains. Regardless of the explanation, the α2,6ST is not catalytically active in its early structural forms. As the enzyme moves through the Golgi at least one of the chains is processed to a complex type conformation, thus allowing the enzyme to fold properly and assume an active conformation. The role of the carbohydrate may be to allow the α2,6ST to be transported through the Golgi as a result of it maintaining a certain conformation. As is already known, the α2,6ST is not released from the Golgi until it is cleaved by a cathepsin D like protease (Lammers and Jamieson, 1988). However for α2,6ST the carbohydrate appears to be important for activity as α2,6ST without carbohydrate or with modified carbohydrate has distinctly different activity than normal α2,6ST. Removing the carbohydrate chain(s) with N-gly causes the α2,6ST to lose approximately 85% of its activity after 6 hours of incubation, whereas modifying the chains with 10μM monensin causes the V_{max} to be lowered approximately 85% as well suggesting that the carbohydrate chains

must be fully processed for the α2,6ST to function properly. The V_{max} and K_m of the N-gly treated α2,6ST were not examined due to the extremely small amounts of enzyme present. The carbohydrate chains may also be important for stability of α2,6ST as shown by the inability to detect the enzyme on the lectin columns. From this information it is probable that the α2,6ST contains two N-linked oligosaccharide chains. One is definitely of the complex type and the other may be complex or high mannose type. The finer structure of the complex type chain could not be determined from the tests done. Clearly, the carbohydrate chains are required for activity and the proper conformation of these chains is important for efficient activity of this enzyme.

Thus there are four main stages of α2,6ST existence in liver. In the first stage the α2,6ST is synthesized with two high mannose type oligosaccharides attached. It then may be cleaved off the membrane and then move through the ER to Golgi pathway while at least one of the carbohydrate chains is being processed to a complex type. Once in the *trans* Golgi, the fully active α2,6ST is believed to bind to a receptor which anchors the enzyme in the correct position, and with the correct orientation to sialylate any glycoproteins in transit through the Golgi. In the acute phase state, due to a defect in trafficking of lysosomal cathepsin D, the catalytic unit of α2,6ST is subject to cleavage by this enzyme which results in the secretion of α2,6ST activity. The details of this mechanism are currently under study by others in the laboratory.

Suggestions for Future Study

The work presented here suggests possible further study related to this work that was beyond the scope of this thesis.

It may be possible to try to add the carbohydrate chains back onto α2,6ST after removing them with N-gly or other endoglycosidases using an *in vitro*

glycosylation system. This would provide insight into the role of carbohydrate chains. Processing inhibitors such as castanospermine, and swainsonine could be used to block processing at various stages in order to look at the minimum structure required for catalytic activity of α2,6ST. It would also be possible to look at the role of carbohydrate chains in activity using sequential exoglycosidases such as neuraminidase and galactosidase to remove carbohydrate and then look at activity to find the minimum chain structure required for the α2,6ST to function properly. It would be necessary to use a combination of neuraminidase and galactosidase since if just neuraminidase was used the α2,6ST would self sialylate, defeating the purpose of the exoglycosidase treatment. Another study would be aimed at studying the kinetics of the N-gly and endo H treated α2,6ST to see if values for K_m are changed. The structures of the oligosaccharides released by treatment with N-gly or endo H could be studied using HPLC or some other method. Another set of experiments would be to look at the parameters for N-gly digestion of α2,6ST using the recombinant enzyme (Barsomian *et al.*, 1990) which is commercially available from Genzyme. This would reduce the need to add protease inhibitors to the assay system and thus clarify the results.

APPENDICES

A. Composition of Buffer A

10mM Sodium Cacodylate (pH 6.5)

0.15M NaCl

25% (v/v) Glycerol

0.1% (v/v) Triton CF-54

B. DPM-pmol conversion

The parameters required for conversion of dpm values to pmol/min/mg(ml) are as follows

Parameter	Abbreviation
dpm from counter	dpm
specific activity of [14C] CMPNeuAc (dpm/pmol)	sp. act.
total assay volume (µl)	TAV
spotted volume (counted) (µl)	SV
aliquot volume (µl)	AV
time of incubation at 37°C (minutes)	time
concentration of protein in sample (mg/ml)	conc.

The calculation is as follows

dpm / sp. act * TAV/SV * 1000µl/ml/ AV /time / conc.

This gives a value of pmol/min/mg protein.

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