Studies on glycosyltransferases in the acute phase response to injury: activity changes and mechanism of retention in the Golgi apparatus.

By

Kevin Brian Richardson

A Thesis Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

Department of Chemistry
University of Manitoba
Winnipeg, Manitoba
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FACULTY OF GRADUATE STUDIES
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Studies on Glycosyltransferases in the Acute Phase Response to Injury: Activity Changes and Mechanism of Retention in the Golgi Apparatus

BY

Kevin Brian Richardson

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree

of

DOCTOR OF PHILOSOPHY

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To my parents,
who did not possess college degrees,
but had the wisdom and generosity to guarantee mine.

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Abbreviations

Materials and Methods

MES	(2-[*N*-Morpholino]ethanesulfonic acid)
AMP	Adenosine monophosphate
DTT	Dithiothreitol
rev	revolution
min	minute
g_{av}	average gravitational force
W	watt
DMSO	dimethylsulfoxide
s	second
Ci	Curie
TIMDPS	Triton X-100; Imidazole; Manganese chloride; DTT; sucrose
LIMDPS	Lubrol W; Imidazole; Manganese chloride; DTT; sucrose
wt	weight
vol	volume

General Text

CMP	Cytidine 5'-monophosphate
CMP-NeuAc	Cytidine 5'-monophospho-*N*-acetylneuraminic acid
DNA	Deoxyribonucleic Acid
Cer	Ceramide
G_{D2}	Ganglioside; D= Disialic acid; 2= Glc-Cer
G_{D3}	Ganglioside; D= Disialic acid; 3= Gal-Glc-Cer
G_{M3}	Ganglioside; M= Monosialic acid; 3= Gal-Glc-Cer

G_{T3}	Ganglioside; T= Trisialic acid; 3= Gal-Glc-Cer
RNA	Ribonucleic acid
UDP	Uridine diphosphate
UDP-GlcNAc	Uridine diphosphate *N*-acetyl-D-glucosamine
TGN	*Trans* Golgi Network
CGN	*Cis* Golgi Network
AGP	α_1-acid glycoprotein
TX-100	Triton X-100
M_r	relative molecular mass

Carbohydrate Subunits

Glc	D-glucose
Man	D-mannose
Gal	D-galactose
GlcNAc	D-*N*-acetylglucosamine
GalNAc	*N*-acetylgalactosamine
Fuc	L-fucose
Xyl	D-xylose
NeuAc	*N*-acetyl-D-neuraminic acid (sialic acid)
GlcA	D-glucuronic acid
IdoA	D-Iduronic acid
Eth	ethanolamine

Glycosyltransferases

General

GT	glycosyltransferase
GluT	glucosyltransferase
GalT	galactosyltransferase

ST	sialyltransferase
GlcNAcT	*N*-acetylglucosaminyltransferase
GalNAcT	*N*-acetylgalactosaminyltransferase

*Specific**

ST6Gal I	β-D-Gal-(1→4)-α-D-GlcNAc-α-(2→6) ST
GlcNAcα2,6ST	NeuAc-α-(2→3)Gal-β-(1→3)GlcNAc-β-R α-(2→6) ST
ST3Gal III	β-D-Gal-(1→3(4))-α-D-GlcNAc-α-(2→3) ST
SAT-I	β-D-Gal-(1→4)-α-D-Glc-β-1-O-ceramide α-(2→3) ST
GlcNAcT-I	*N*-acetylglucosaminyltransferase I
GlcNAcT-III	*N*-acetylglucosaminyltransferase III
GlcNAcT-V	*N*-acetylglucosaminyltransferase V

* The nomenclature for the sialyltransferases has often been criticized as being too ambiguous since more than one name has often been used for the same enzyme. Recently, a systematic method has been applied to naming these enzymes (Tsuji, S. *et al.*, 1996). The four elements that make up the system are: ST,x,y,z, where ST represents the sialyltransferase gene family, x is the sialic acid on the acceptor sugar to which the sialic acid is attached, y is the name of the acceptor sugar and z is a Roman numeral assigned to each new distinct gene as it is discovered. *It must be stressed that the authors recommended that this system only be applied to cloned sialyltransferases for which the cDNA sequence is known and the acceptor specificity has been determined.* In accordance with this recommendation, of the four sialyltransferases analyzed in this thesis only ST6Gal I and ST3Gal III apply to these rules, as the enzymes for SAT-I and GlcNAcα2,6ST have not been cloned and sequenced. Therefore, the original abbreviation for the ganglioside enzyme, SAT-I, will be used throughout this thesis. A more difficult problem presents itself in the case of GlcNAcα2,6ST. Very little has been reported on this enzyme and a standard abbreviated form has yet to be published. I have therefore modeled my own nomenclature for this enzyme. Due to the rarity of GlcNAc acting as an acceptor for the sialyltransferases and the fact that this particular enzyme transfers the sialic acid in a α2,6 linkage pattern (see figure 2 for explanation), GlcNAcα2,6ST should be an acceptable abbreviation in that it gives a basic description of the reaction and most importantly will not be confused with other known sialyltransferases that exist.

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Abstract

Glycosyltransferases are the key enzymes involved in the cells' ability to synthesize glycoconjugate macromolecules. Since these structures of glycoconjugates often change during the onset of many diseases, a primary focus of this thesis was to study how the behavior of these enzymes may change during the acute phase response to injury. The liver is the primary source of acute phase proteins. Therefore, the subcellular Golgi organelle (the key source of glycosyltransferase activity) was isolated *via* sucrose density gradients from liver cells. The Golgi organelles isolated in this manner were effectively used to assay a total of seven glycosyltransferases by monitoring the amount of radioactive donor transferred to the specific enzymes' acceptor. Results from these studies indicated that while some enzymes increase in activity during acute inflammation, others decrease, explaining why there may be a change in the glycoconjugate structure of some molecules within a host during its response to acute inflammation. In addition, while one of these enzymes (ST6Gal I; 2.4.99.1) was noted as significantly increasing in activity in the serum during inflammation, due to being cleaved from it's membrane anchor, others did not demonstrate significant levels of activity in the serum. This sets the stage for an interesting problem since these enzymes were shown to be proteolytically cleaved in much the same manner. Furthermore, this cleavage was prevented from occurring during the presence of pepstatin A, a strong inhibitor of the lysosomal enzyme cathepsin D, indicating that this proteinase was responsible for the removal of the enzymes from their membrane anchor.

Previous reports have demonstrated the existence of a protein matrix located between the Golgi cisternae which is capable of maintaining the structural integrity of the Golgi. By solubilizing the Golgi membranes with detergent, then extracting with salt, the glycosyltransferases were isolated. Removing the salt *via* dialysis resulted in reassociation of the enzymes with the matrix. Therefore, the purpose of the matrix may be to bind the glycosyltransferases *via* their cytoplasmic tail, as sialyltransferases cleaved from their membrane anchor were not able to reassociate with the matrix. The reason for this interaction is proposed to help orientate the enzymes to the appropriate Golgi membrane, that is, *cis*, *medial*, or *trans* Golgi vesicles.

Chapter 1: Introduction to Glycobiology

I. Glycobiology: A New Frontier

The existence of life is dependent on its ability to remedy any disturbance of its homeostasis due to tissue injury, infection, neoplastic growth, or any one of a multitude of immunological disorders. Even subtle changes in pH, temperature or ionic strength in the surrounding environment are challenges that are continually being met by the smallest organisms. If homeostasis is not restored, then severe consequences will be met with death the most likely. For this reason mankind continually strives to understand what methods could be used for improving the quality of life. It is this foundation that directs us into which areas of the biological sciences we enter and how much effort we feel is justified once we are there. Of course, the direction that is taken is often impeded by the fact that technology normally does not advance in a particular niche of science unless a clear need for it is seen. However, this need may never be fully appreciated unless the appropriate technology is present, hence allowing the ideas to be brought to full capacity. This has resulted in serendipity being the driving force behind many breakthroughs.

Since the advent of biochemistry the study of nucleic acids and proteins has held center stage. Although their importance cannot be overstated, it has perhaps caused the scientific community to underestimate other facets of biochemical research. Carbohydrates have long been known to serve as a source

of energy or as the structural or protective materials for cells, but with quantum leaps in the aforementioned areas being made by Watson and Crick (structure of DNA; 1953), Fredrick Sanger (structure of the first protein; 1953), and the like, biochemists had little interest in pursuing the possibility that carbohydrates may be involved in any sort of recognition phenomena. In fact, the idea that these compounds contained any sort of biological specificity was almost completely neglected. As a result, even those carbohydrates that were in covalent association with proteins did not attract much attention until the 1960's. It was at this time that their role in biological recognition, such as in host-pathogen, cell-cell, and cell-molecule interactions, was beginning to be revealed, and thus began the age of glycobiology. As more structures of these cell surface carbohydrates were elucidated and characterized it soon became apparent that they were a major component of the outer surface of mammalian cells and that they very often characterized the cell types. Throughout much of the 1970's and 80's most of the attention was focused towards understanding how the cell assembled these structures and characterizing the enzymes that did so. The one fundamental problem which had always perplexed scientists was that there was little understanding as to what function these structures performed. Today however, we are beginning to obtain a wealth of information regarding their participation in cellular activity. For instance, carbohydrate structures change dramatically during mammalian development. Specific sets of carbohydrates are expressed at different stages of differentiation and in many instances these carbohydrates are recognized by specific lectins, thus providing differentiation antigens. In mature organisms,

expression of distinct carbohydrates is eventually restricted to specific cell types, providing cell-type-specific carbohydrates. Aberrations in these cell surface carbohydrates are associated with various pathological conditions, including malignant transformation (Taylor-Papadimitriou, J. *et al.*, 1994; Rademacher, T.W., 1992; Rademacher, T.W., 1998; Alper, J., 1993).

Oligosaccharides are unique in the complexity of their structure. In contrast to DNA and proteins, which are constructed from nucleosides and amino acids respectively, which in turn bond together in a consistent linkage pattern, carbohydrates are composed of monosaccharides that have the ability to bind together in a variety of combinations. In other words, DNA and proteins derive their 'biological information' from the *order* of their subunits, while oligosaccharides obtain theirs through not only the order, but also the *position* in which their subunits link together. For instance, one monosaccharide contains three or four different hydroxyl groups which each have the potential to bind additional monosaccharides. Second, the linkage between the two monosaccharide residues can be one of two isomers, referred to as α- and β-linkages. Third, oligosaccharides are able to branch out, due to multiple linkage sites on a single monosaccharide. This particular characteristic separates carbohydrates from the rest of the biological macromolecules, which contain almost exclusively linear structures at the primary level. Because of this complexity, carbohydrates can provide almost unlimited variations in structure. Thus, carbohydrates are excellent candidates for providing multiple recognition signals that serve as ligands for recognition by other molecules.

With such an overwhelming potential for new and exciting discoveries,

glycobiology has opened many doors in the areas of molecular biology, cell biology, immunology and many other biological sciences. Many pharmaceutical companies now regard glycobiology as a 'sleeping giant' that is soon and certain to awaken. Article titles such as "Carbohydrates surge through clinical trials" (Alper, J., 1993) and "Potential glycotherapeutics may outnumber protein-based compounds" (Wrotnowski, C., 1996) are becoming more common and demonstrate the widespread popularity that these macromolecules are achieving. Of particular interest is the change in carbohydrate structures that occurs on the surface of various cell types, which may be either the cause or result of any one of a number of autoimmune diseases, cancers, or developmental abnormalities.

An obvious question that presents itself is *how* do these carbohydrates differentiate during the aforementioned conditions. For instance, what causes the carbohydrate chains on the surface of many cells to become more branched when they become cancerous (Crocker, P.R. *et al.*, 1996) or specific antibodies to alter their carbohydrate structures upon the onset of autoimmune disorders such as rheumatoid arthritis or systemic lupus erythematosis (Delves, P.J., 1998). The answer to these and other related questions lies within understanding the enzymes that synthesize the carbohydrate chains. Collectively known as *glycosyltransferases* these enzymes are an integral feature found mainly in the ER and Golgi apparatus of all mammalian cells. Specific cell types contain distinct classes of these enzymes and in addition to this vary in the active concentration of each enzyme. It is these two features that are often modified during detrimental circumstances such as stress, illness, or other external factors. When these factors affect the level

and activity of all or even just one enzyme in the Golgi of a particular cell type it essentially changes the carbohydrate building machinery, therefore altering the final structure or structures that are normally constructed by that cell. For this reason, many investigators are currently involved in trying to gain a deeper understanding of the behavior of the glycosyltransferases and how various external, as well as internal stimuli affect their behavior.

II. Carbohydrates: Information in Formation

Chemically, carbohydrates are aldehyde or ketone compounds with multiple hydroxyl groups. But their ability to link together in numerous fashions, accommodate multiple substitution groups, and in turn attach to various molecules in differing linkage patterns allow them to make up most of the organic matter on earth because of their multiple roles in all life forms (Stryer, L., 1988). For instance, carbohydrates serve as energy stores, fuels, and metabolic intermediates. The sugars ribose and deoxyribose form part of the structural framework of DNA and RNA while polysaccharides are structural elements in the cell walls of bacteria and plants. In addition, their ability to bind proteins and lipids has prompted investigators to study their ability to contribute to cell-cell recognition, since many of these conjugated structures are often found on cell surfaces. As further information has been gathered regarding the location, function, and chemical composition of carbohydrates, investigators have found that trying to neatly classify these structures into specific categories had led to overlap, with some structures

falling under more than one category.

In order to communicate in any discipline it is essential to have a command and familiarity with the appropriate terminology. The field of carbohydrate biochemistry is characterized by the application of a large set of rules of nomenclature. Various types of compounds consisting of carbohydrates covalently linked with other types of chemical constituents are classified under the general name of glycoconjugates. The major groups of glycoconjugates are the glycophospholipids, glycosaminoglycans, glycoproteins, and ceramide-linked oligosaccharides (fig. 1). The structures depicted in this figure represent the core unit of each of the particular classes of glycoconjugates and become increasingly complex during transport through the Golgi apparatus. *N*-linked glycans are appropriately named due to the nitrogen atom in the amino acid asparagine binding to the GlcNAc residue in the pentasaccharide core. *O*-linked glycans involve the oxygen atom of either a serine or threonine residue binding to the monosaccharide GalNAc. Although the early stages of synthesis differ greatly from *N*-linked glycans, the final steps of chain elongation within the Golgi are quite similar. Ceramide-linked glycans are commonly located by virtue of the two hydrophobic tails that anchor into the plasma membrane. The various classes of these structures are defined by the monosaccharides of which they are composed. As with the *O*-linked glycans, the early stages of synthesis are unique to these compounds but many of the later enzymes involved in oligosaccharide synthesis are probably shared with *N*- and *O*-glycans. Oligosaccharides attached to xylose *via* a *O*-linkage to serine residues on a variety of 'core protein' polypeptides that have arrived from the ER.

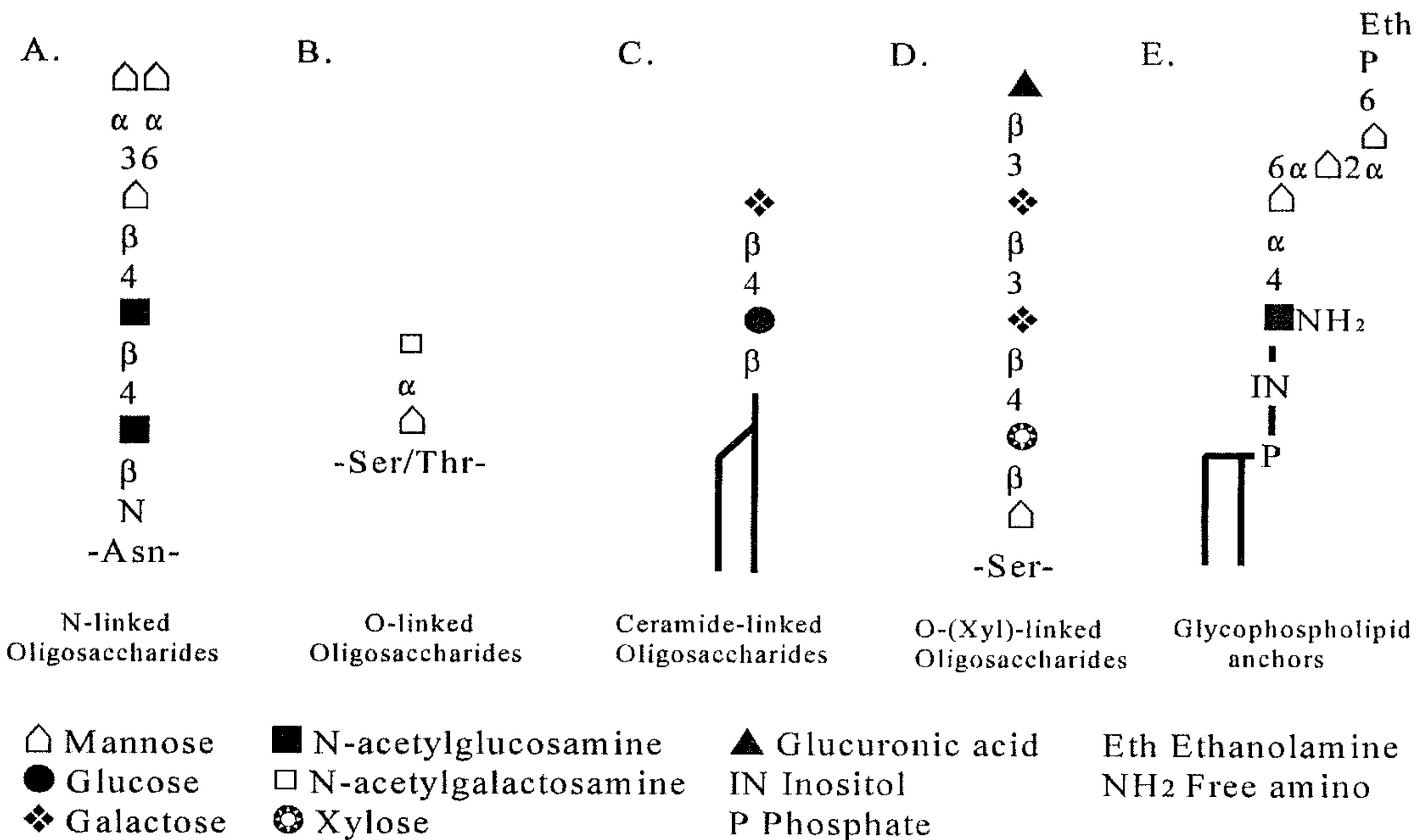


Figure 1. The major classes of glycoconjugates.(a) N-linked glycans. The numbers represent the carbon atom in the monosaccharide which is involved in the linking, while the configuration of the anomeric carbon of the monosaccharide in question is indicated with either an α or β. (b) O-linked glycans involve the oxygen atom of either a serine or threonine residue binding to the monosaccharide GalNAc. (c) Ceramide-linked glycans. (d) Xylose-linked oligosaccharides. (e) GPI anchors. (Diagram adapted and modified from Varki, A., 1998).

These structures are then extended by alternating disaccharide units such as GlcA-GlcNAc, generating heparan sulphate (which has been implicated in the control of differentiation and cell growth) or GlcA-GalNAc, generating dermatan sulphate (which is involved in the construction of connective tissue such as skin, blood vessels, and heart valves). Glycophospholipid inositol (GPI) anchors are preformed in the ER and then used to replace the C-terminal membrane-spanning regions of certain proteins- i.e. providing an alternative means to anchoring proteins to the cell surfaces. The common core structure is then elongated and modified in several ways in the Golgi apparatus. Each of these described compounds are divided into multiple subclasses, many of which can overlap in classification themselves. This undoubtedly can lead to confusion when one is reading literature on the subject. In fact, the nomenclature of the subclasses are often refashioned due to the constant accumulation of structural data that is obtained from glycosylated molecules (Hayes, P.A., 1998). Therefore, for reasons of clarity and brevity, much of the following discussion on the structure and location of glycoconjugates will be those that are most relevant to this thesis, the glycoproteins and a specific class of ceramide-linked oligosaccharides, the gangliosides.

A. Glycoproteins

A glycoprotein is a protein containing carbohydrate covalently linked to protein. The carbohydrate may be in the form of monosaccharide(s), disaccharide(s), oligosaccharide(s), or their derivatives (e.g., sulfo- or phospho-

substituted). Although the number of naturally occurring monosaccharides is over 200, there are essentially six main monosaccharides involved in the creation of glycoproteins (fig.2). The manner in which these molecules link together, combined with further modifications by substituents such as phosphate, sulphate, acetate, or methyl groups add further complexity to an already complex system. All of the monosaccharides represented in figure 2 are composed of a 6-carbon ring and differ in the position of the hydroxyl groups and presence or absence of acetyl groups. Of all the monosaccharides known, sialic acid is perhaps one of the most complex due to the addition of the 3-carbon chain at position 6. The hydroxyl groups on these carbons are prone to substitution with phosphate, sulphate, or acetate groups. Glycoproteins can generally be divided into two families; the so called '*O*-linked', referring to the fact that the carbohydrate chain is attached to the oxygen atom of a serine or threonine residue, or the '*N*-linked', relating to the fact that the carbohydrate is attached to the nitrogen atom of an asparagine residue. While both macromolecules are initially synthesized differently (*O*-linked glycopeptides have the first sugar (GalNAc) incorporated onto the protein primarily in the Golgi apparatus, while *N*-linked synthesis initiates itself with the creation of a Asn-GlcNAc linkage in the endoplasmic reticulum) they do share the same common feature in that they are transported through the Golgi apparatus in order to obtain a significant portion of their carbohydrate chains. Aside from this similarity, there are numerous differences between the two classes of glycoproteins, however the immediate focus here is directed towards enzymes which are involved in the creation of *N*-linked glycoproteins, although some overlap with the former

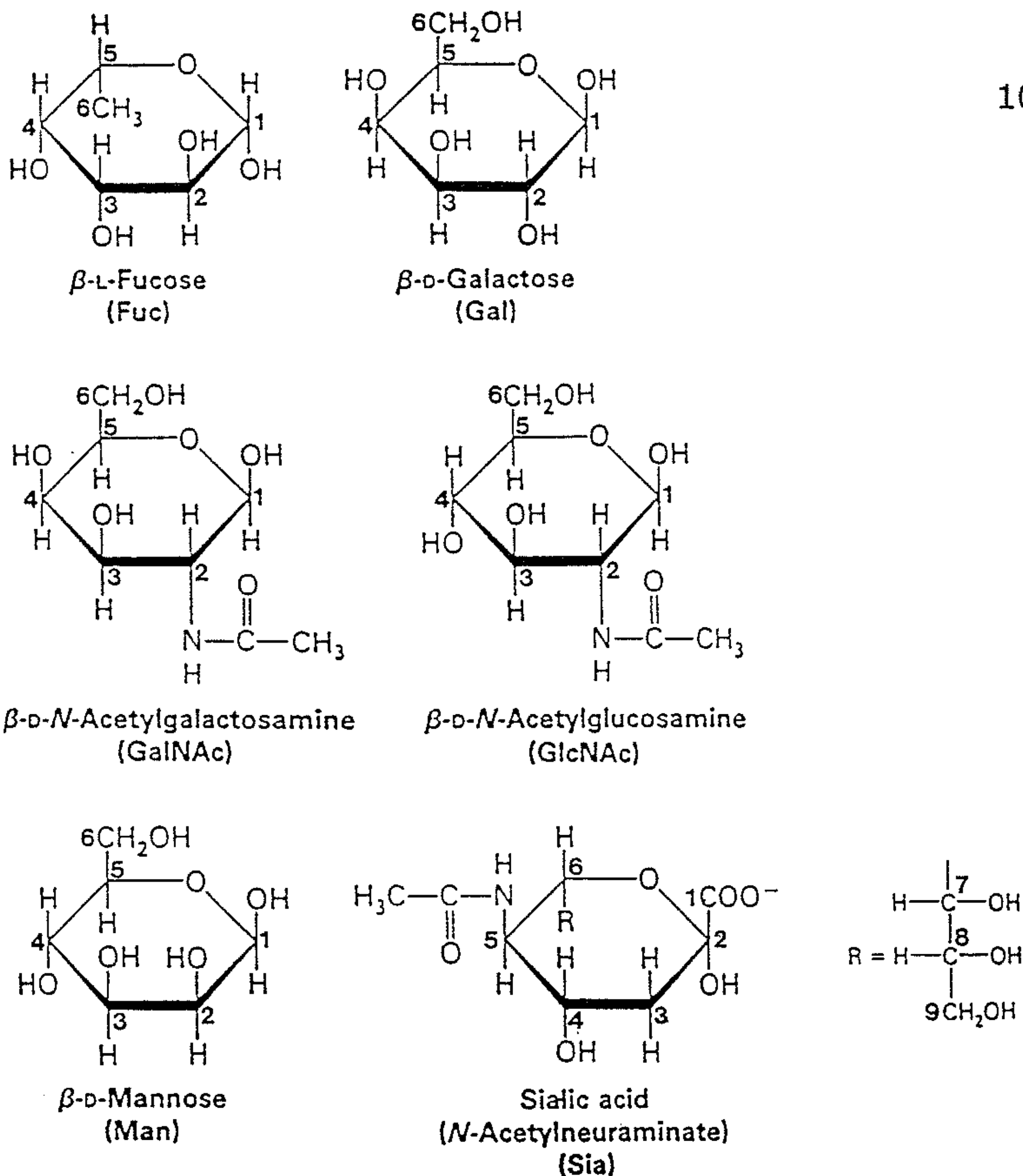
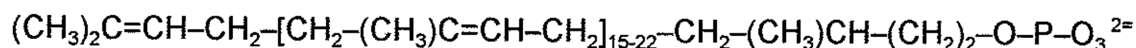


Figure 2. The common monosaccharides which are used in the synthesis of glycoproteins. The carbon at position 1 (position 2 for sialic acid) is known as the 'anomeric carbon'. If the hydroxyl group is above the plane of the ring, it is regarded as β, and α when below the plane. Fucose is an exception to this rule, as it is classified as an 'L' sugar, while the remaining ones are 'D', referring to the absolute configuration of the asymmetric carbon furthest from the aldehyde or keto group. Therefore all of the monosaccharides are regarded as β, except for sialic acid, which is α. It should be noted that in solution monosaccharides exist in both the α and β form at equilibrium, with a ratio of 2:1 in favor of the β form. However, the glycosyltransferases generally recognize the structures shown above, so the remainder of this thesis will focus on these particular forms of the monosaccharides presented. This nomenclature is used to describe how these monosaccharides are linked to one another in glycoconjugates. For example, if the hydroxyl group at C-1 of GlcNAc were to bind to the hydroxyl group of mannose at C-4, via a dehydration reaction, it would be termed a 'β1→4' linkage pattern. Similarly, if the C-2 hydroxyl group of sialic acid were to bind to the C-6 hydroxyl group of galactose, through the same dehydration reaction, this would be referred to as an 'α2→6' linkage pattern. (Diagram taken and modified from Stryer, L., 1988).

class of glycoprotein does occur.

The biosynthesis of Asn-linked oligosaccharides is separated into discrete enzymatic steps, which are generally categorized as early-stage processing, middle-stage processing, and late-stage processing. These stages involve the endoplasmic reticulum (ER), and various compartments of the Golgi apparatus. In the early stages of development an oligosaccharide precursor, $Glc_3Man_9GlcNAc_2$, is synthesized by a complex yet well orchestrated cascade of events now known as the "dolichol phosphate cycle" (Sharon *et al.*, 1982) which is depicted in figure 3. Dolichol phosphate is a polyisoprenoid that contains an α-saturated isoprene residue and which usually has 16-23 isoprene units with eukaryotic cells typically having 19 units (Schutzbach, J.S., 1997):



$$(CH_3)_2C{=}CH{-}CH_2{-}[CH_2{-}(CH_3)C{=}CH{-}CH_2]_{15\text{-}22}{-}CH_2{-}(CH_3)CH{-}(CH_2)_2{-}O{-}P{-}O_3^{\,2-}$$

These dolichol phosphate molecules situate themselves in the ER by embedding the highly hydrophobic polyisoprene segment within the ER membrane itself, thus allowing for the hydrophilic phosphate portion to expose itself away from the outer surface of the lipid bilayer. The ability of dolichol phosphate to translocate itself across the ER membrane is a crucial step in this cycle, as it permits the growing oligosaccharide to come into contact with its monosaccharide substrates at the various stages of development. Upon completion of the $Glc_3Man_9GlcNAc_2$ precursor, it is then transferred from the dolichol phosphate molecule onto a nascent polypeptide chain. This transfer process is catalyzed by the enzyme

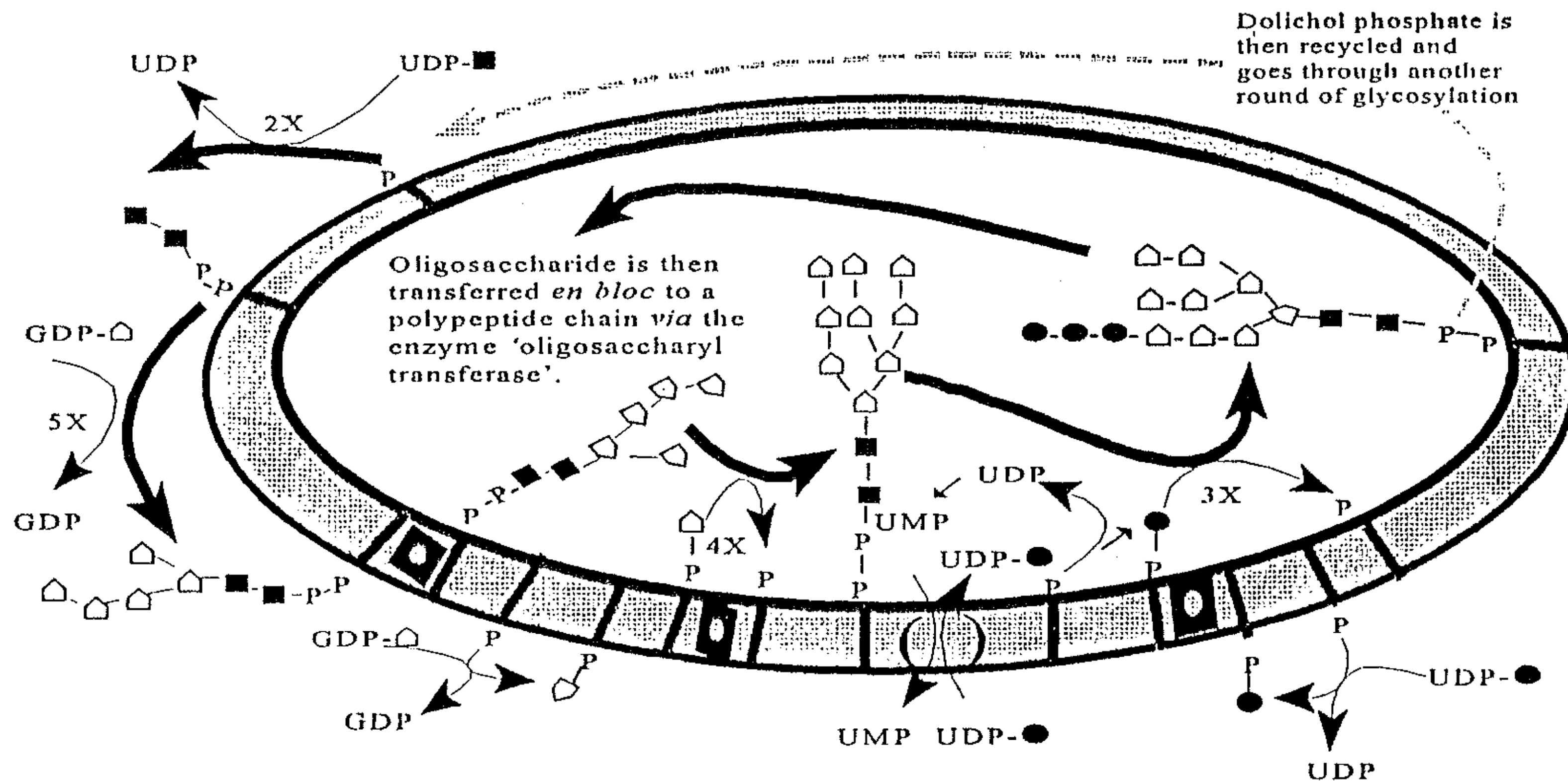


Figure 3. Dolichol phosphate cycle. The long hydrophobic dolichol tail (|) is buried in the membrane of the ER and is 'flipped', along with the carbohydrate portion *via* a yet undefined mechanism (◘). The glucose containing structure is then attached to the asparagine residue in a protein (see fig.1a) and through a variety of quality control mechanisms, the properly folded N-linked glycoprotein is transported to the Golgi complex where is undergoes further carbohydrate modifications. Abbreviations are as follows; ■: GlcNAc; ⌂: Man; ●: Glc; P: phosphate. Diagram is based on data presented by Cacan, R. *et al.*, 1998.

oligosaccharyltransferase, which recognizes a specific sequence of amino acids known as a 'sequon'. The sequon is generally a Asn-X-Ser(Thr) tripeptide, where X can be any amino acid except proline (Moremen, K.W. *et al.*, 1994). However, there are cases of Asn-X-Cys being glycosylated (Vance, B.A. *et al.*, 1997). The carbohydrate itself it linked to the nitrogen atom in the asparagine residue, hence the term '*N*-linked', but on average only about one third of the sequons within the protein population are actually glycosylated. This is most likely due to the fact that other parameters, such as the rate at which the protein folds, preventing access to the asparagine residue, contribute to the rate of glycosylation. The oligosaccharide is then clipped from the dolichol phosphate and eventually transferred to lysosomes whereupon it is degraded (Cacan, R. *et al.*, 1992). Once linked to a protein however, this glycosylation process is immediately followed by sequential deglycosylation steps leading to the release of the three glucoses and up to six mannose residues (figure 4). De-glycosylating, or 'trimming' the carbohydrate permits two important processes to occur. First, it promotes an ingenious method for the quality control of proper glycoprotein folding within the ER. Second, it creates additional substrates which will aid in predetermining the final overall structure of the carbohydrate as it travels through the Golgi apparatus.

Obviously, proteins that have not folded into their proper orientation could produce detrimental effects if they were to be released from the ER, since their intended function may be eliminated. Two enzymes, glucosidase I and II remove the terminal glucose and two internal glucoses respectively, upon which it is delivered to the Golgi complex for further modifications. However, if the protein has

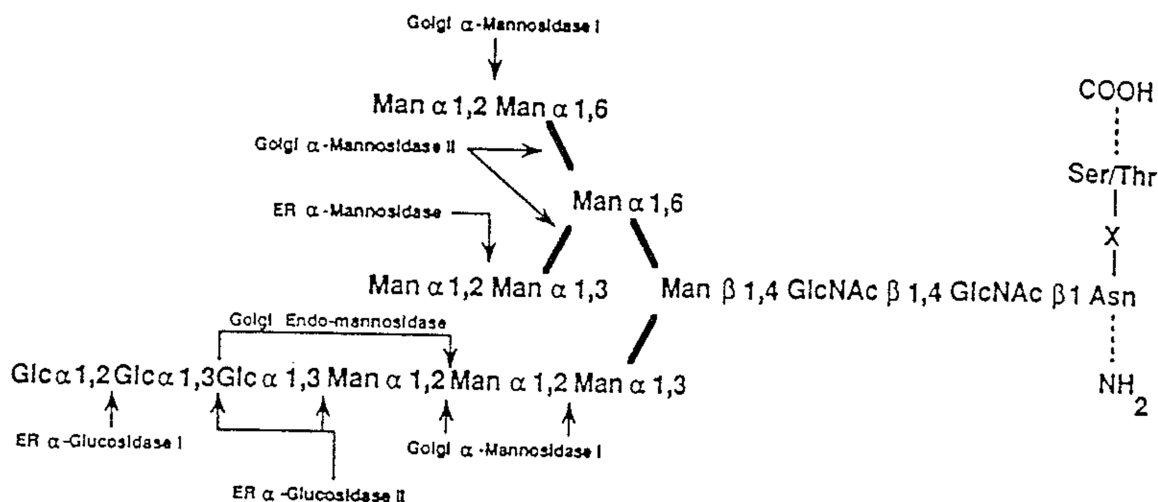


Figure 4. Glycosidases involved in the initial synthesis of N-linked glycoproteins. The detailed structure of the final $Glc_3Man_9GlcNAc_2$ oligosaccharide produced by the dolichol phosphate cycle. The specific action of the mannosidase enzymes are crucial in directing the final structure of the carbohydrate chain, determining if it will be classified as 'high mannose', 'hybrid', 'complex' or 'bisected' as described in figure 7. Removal of the glucose residues are involved in controlling the release of the glycoprotein from the ER into the Golgi. (Diagram taken from Cummings, R.D., 1992).

not folded properly it is re-glucosylated by a specific glucosyltransferase (GluT) which attaches a glucose onto the same Manα1,2Manα1,2Manα1,3 arm that the three glucose residues were previously located (figure 4). This glucose acts as a key recognition marker for one of two homologous molecular chaperones of the ER, calnexin and calreticulin. In fact, "substrate specificity studies have identified the single terminal glucose residue as a critical determinant recognized by both chaperones since oligosaccharides containing 0, 2, or 3 glucose residues fail to bind" (Spiro, R.G. *et al.*, 1996) However, the possibility that some of the internal mannose residues are recognized as well has not been excluded (Cacan, R. *et al.*, 1998). It has therefore been suggested that when a glycoprotein has not folded properly, GluT reattaches a glucose residue, which in turn is recognized by one, or both of the chaperones which prevent it from exiting the ER. The chaperones then interact with nascent glycoproteins in cycles of binding and release regulated by glucosidase II, which removes the single glucose residue and by GluT which reattaches it, if it has not yet completely folded. Both chaperones have been studied extensively, and both are believed to act in much the same manner with the same specificity. It is unclear why the ER of most eukaryotic species contain two homologous chaperones with similar properties, but "it may reflect the redundancy in the ER quality control and protein folding systems or the two chaperones could conceivably function in a coordinated fashion" (Vassilakos, A. *et al.*, 1998). The fundamental question then is, how does GluT know that the protein has not folded properly, and thus must reattach the glucose residue? Although this has yet to be answered with certainty, several hypotheses may be advanced concerning the

protein domains interacting with GluT. They may be formed by a) specific amino acid sequences common to all glycoproteins, b) certain specific amino acids that are separated in the primary sequence but become close in the denatured conformations, and c) nonspecific amino acids with common three-dimensional structures shared by all denatured glycoproteins but generated by totally different amino acid sequences (Parodi, A.J., 1998). Therefore, the GluT involved in this "glyco-deglyco" process behaves as a sensor for the varying degrees of folded protein conformations. The two chaperones, in turn, recognize the monoglucosylated oligosaccharide by recognizing the single glucose molecule and thus are able to retain the glycoproteins in the ER as long as the protein is not properly folded.

Once a glycoprotein has been examined by the ER quality control system and deemed to be properly folded, it is transported to the Golgi apparatus. The difficulty here lies within the fact that the highly hydrophilic carbohydrate portion has to first be translocated across the hydrophobic lipid membrane of the ER. Although the exact mechanism of this phenomena has yet to be unveiled (Krag, S.S ., 1998) it has been proposed that a few specialized proteins are involved in the process (Rush, J.S. *et al.*, 1995). However, once transported to the *cis* region of the Golgi complex, it is further glycosylated by a network of enzymes which are segregated in a highly organized fashion throughout the entire Golgi apparatus. Termed glycosyltransferases (GT), they attach monosaccharides one at a time in a sequential manner until a final structure has been created (figure 5). It is apparent that many thousands of different oligosaccharide structures could be

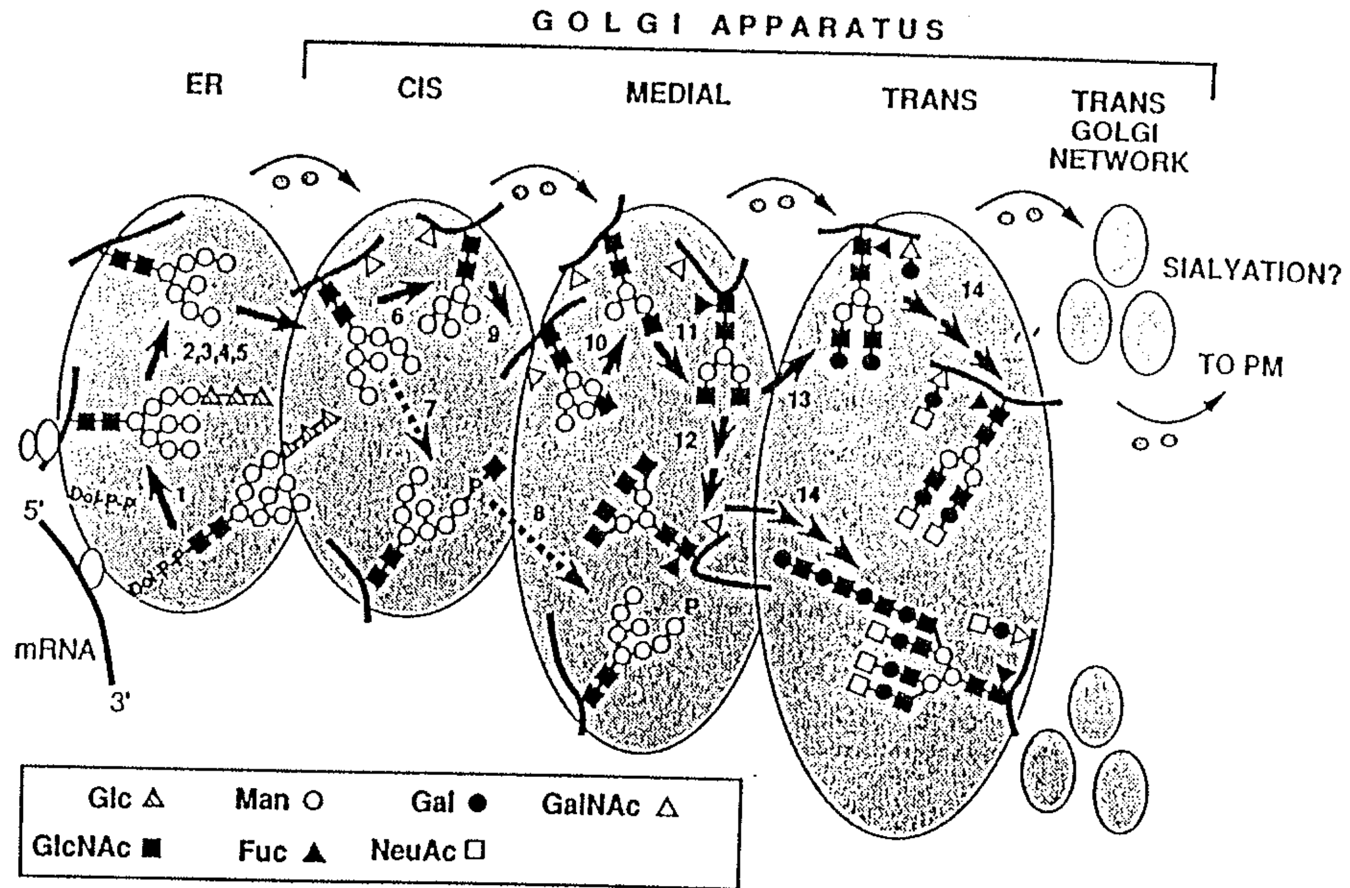
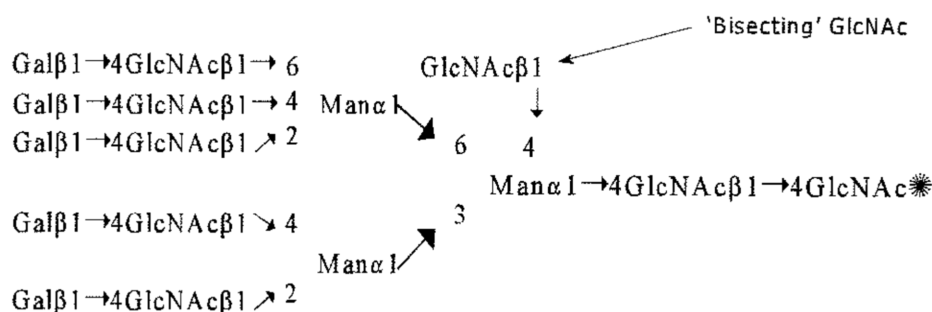


Figure 5. Involvement of the Golgi apparatus in the synthesis of glycoconjugates. 1. The oligosaccharyltransferase transfers oligosaccharide from dolichol-linked oligosaccharide to nascent polypeptide; 2,3,4,5 and 6 are glycosidases involved in trimming the carbohydrate chains; 7 and 8 are responsible for phosphorylating specific mannose residues in order to direct these glycoproteins into lysosomes if they are lysosomal enzymes (see chapter 3, figure 2); 9. GlcNAc transferase I; 10. mannosidase II; 11. GlcNAc transferase II; 12. GlcNAc transferase IV and V; 13. galactosyltransferase; 14. Terminal glycosylation reactions involving sialyltransferases, GlcNAc transferases and galactosyltransferases which attach the monosaccharides sialic acid, GlcNAc and galactose, respectively. (Diagram taken from Cummings, R.D., 1992).

assembled from the combined action of these processing enzymes. Yet in fact the synthesis of only a limited number of structures is observed which is due to the rigid substrate specificity of these enzymes. For instance, the first committed step in complex oligosaccharide synthesis is catalyzed by Golgi α-mannosidase II which removes the terminal α1,3 and α1,6 linked mannose residues from $GlcNAcMan_5GlcNAc_2$-Asn (figure 4). This enzyme has a very strict substrate specificity and cannot remove the mannose residues from structures that contain a 'bisecting' GlcNAc which has been attached to the core β-mannose (figure 6). This reaction is catalyzed by GlcNAcT-III and represents a committed step to hybrid oligosaccharide synthesis. Therefore the relative abundance of α-mannosidase II and GlcNAcT-III in a given tissue could determine whether it synthesizes complex or hybrid oligosaccharides. Furthermore, if GlcNAcT-III acts on the product to attach this 'bisecting' GlcNAc residue then the oligosaccharide cannot be further branched. It is easy to see then, that as a glycosidase or glycosyltransferase creates a new structure, it in turn becomes a new substrate that is specific for the next group of competing enzymes. The availability of these enzymes, which can change due to physical trauma (Khansari, D.N., *et al.*, 1990) can therefore explain why there are structural changes in the glycoconjugates synthesized within cells during periods of stress on the system. The fact that inhibitors of the various enzymes may be factored into the equation greatly complicates an already complex phenomenon (Kleineidam, R.G. *et al.*, 1997). In addition, naturally occurring substituents like acetates, sulfates and phosphates can make the possible arrangement of structures quite substantial.

1.



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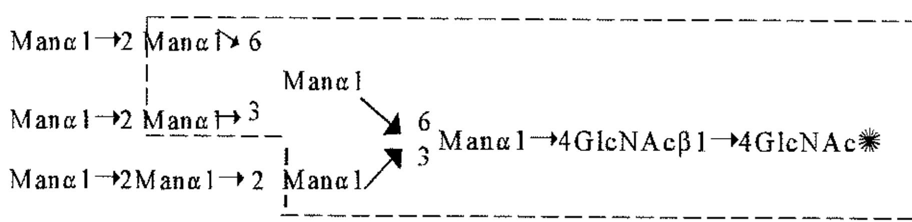

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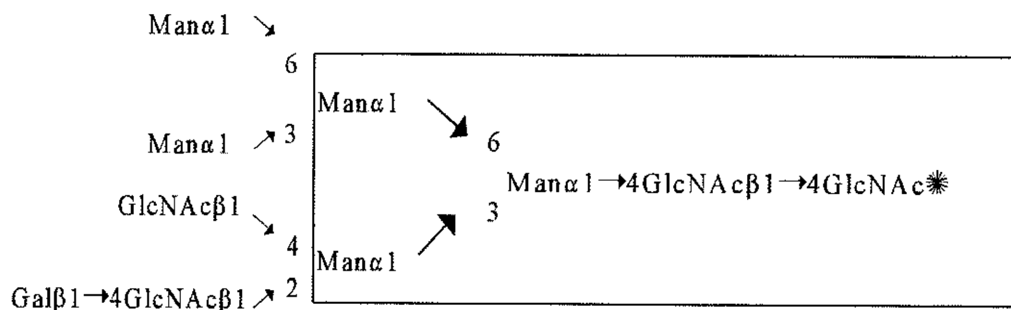
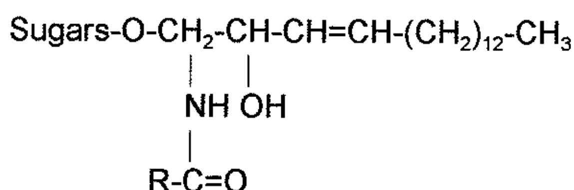


Figure 6. The subgroups of N-linked sugar chains. (1) Complex-type. (2) High-mannose-type. (3) Hybrid-type. The structure enclosed with the dotted line (depicted in 2) is the common heptasaccharide found in all high-mannose-type sugar chains. The structure within the solid line (depicted in 3) is the pentasaccharide structure common to *all* N-linked sugar chains. All of these carbohydrate chains are attached to an asparagine residue which is located within the sequon Asn-X-Ser(Thr) of the protein which is represented by the ✱. The number of branching chains extending from the common pentasaccharide core is used to define a generic nomenclature for the various structures. For example, structure (1) would be considered 'tetraantennary' as it contains five chains. Structure (2) is noted as a 'triantennary' as it contains three chains. Structure (2) is noted as a 'triantennary' structure by virtue of its three chains, 'biantennary' would have two chains, and so on.

B. Gangliosides

Glycolipids are ubiquitous membrane components of mammalian cells. The majority of these lipids are believed to be localized at the outer leaflet of the plasma membrane, with their hydrophilic sugar chain extending towards the external surface. Because of the presence of glycolipids at the cell surface and their unique structural diversity in different tissues and cell types of normal and diseased states, there has been an outburst of interest among many scientists to examine the possible function of these molecules. Although glycolipids show heterogeneity in both the oligosaccharide and ceramide portions, as depicted below, they are characterized and identified on the basis of their carbohydrate structures.



Structure of ceramide. The R group represents a fatty acid which varies in chain length and degree of unsaturation. The amount of sugars found in gangliosides range from 4 to 18-22, (Zdaebske, E. *et al.*, 1978)

The major types of sugars that are found located within the brain and nervous system (Svennerholm,L. *et al.*, 1989) are sialic acids, glucose, galactose, fucose, GalNAc and GlcNAc, with other sugars being identified in rare instances. Glycolipids containing one or more sialic acids attached to the neutral sugars by an α-ketosidic bond are known as gangliosides and are primarily found in most other

tissues in much smaller concentrations where they are thought to perform specific functions.

Sialic acids are a defining feature of the gangliosides. Their proposed pathway for biosynthesis is depicted in figure 7. It is, however, unclear how many different sialyltransferases are involved in this synthesis. Furthermore, the identity of some of them are in doubt (Lloyd, K.O. *et al.*, 1998). Another important question being studied is which of the enzymes are glycolipid specific and which are active on both glycolipid and glycoprotein acceptors. It is therefore easy to see that much of the research involving the synthesis and function of these unique molecules can be confusing at times. Because, in certain instances, a ganglioside can be an acceptor for more than one glycosyltransferase, the levels and efficiency of key enzymes are important. Competition between SAT-I / β1-4GalNAc-T; SAT-II / β1-4GalNAc-T and SAT-III / β1-4GalNAc-T plays an important role, controlling entry into the A, B, and C pathways, respectively. Some advance in understanding how gangliosides are synthesized and how a cell regulates and controls the final desired structure is now being accomplished by the cloning of the genes for some of the transferases involved. By transfecting cDNA of a particular enzyme into a cell, observations on the ganglioside structures produced by the cells are giving some insight into how a cell is meeting its requirements for the necessary structures. For example, transfection of SAT-II cDNA into 3T3 fibroblast cells converted a cell line expressing only 'A' pathway gangliosides into one expressing 'B' series gangliosides (Ruan, S. *et al.*, 1997). There is also evidence that subtle differences in the ratios of the various glycosyltransferases within a given pathway can

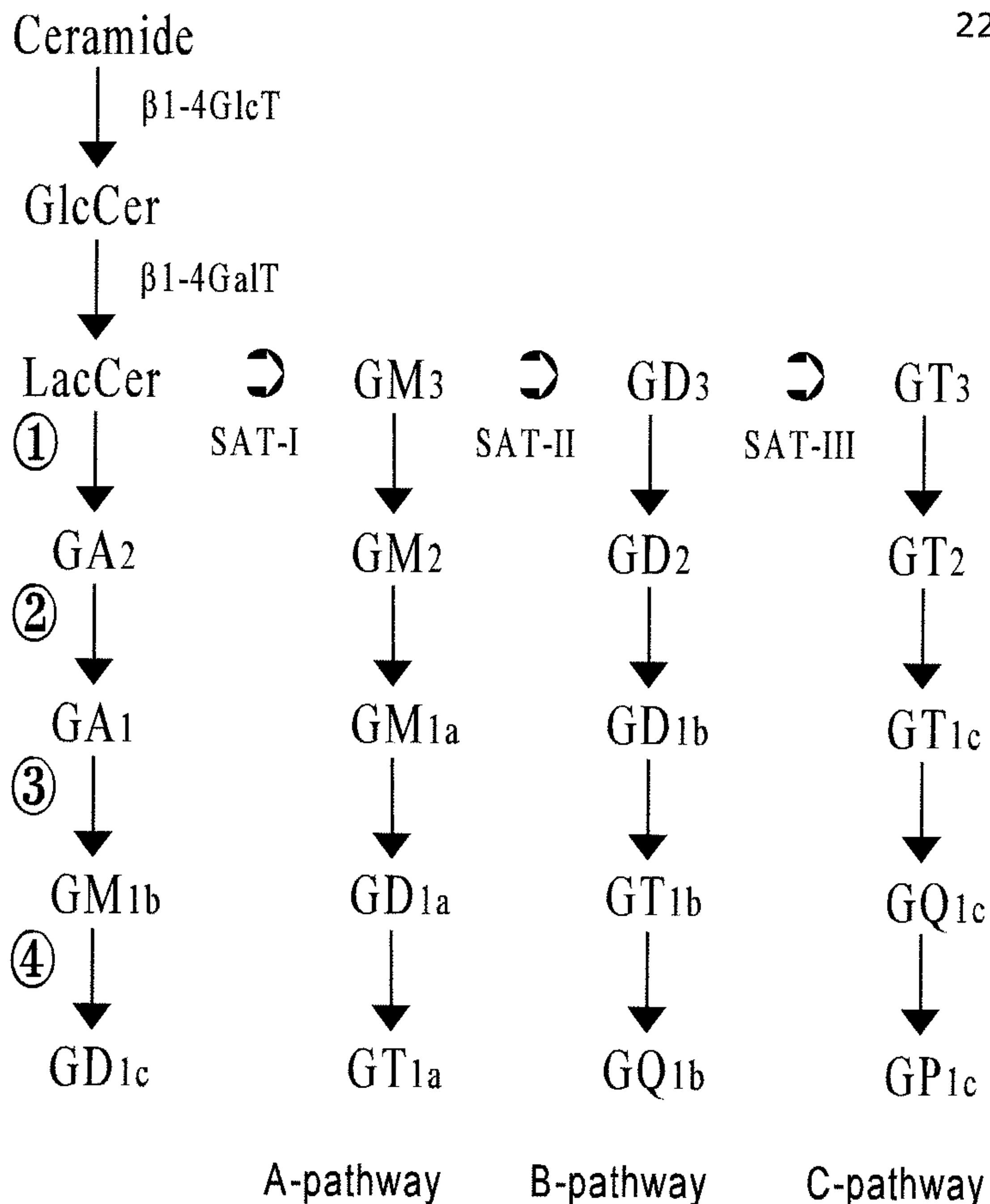


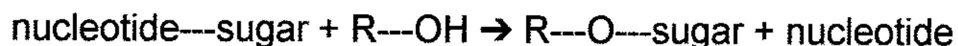
Figure 7. Biosynthetic pathway of gangliosides. The activity levels of SAT-I (α2,3 sialyltransferase), SAT-II (α2,8 sialyltransferase) and SAT-III (α2,8 sialyltransferase) are paramount in determining which pathway, A, B or C is followed. These enzymes are indicated by the circled numbers and are as follows; ① β1-4GalNAcT; ② β1-3GalT; ③ SAT- IV (α2,3 sialyltransferase); ④ SAT-V (α2,8 sialyltransferase). The nomenclature is used to indicate the degree of sialylation on the ganglioside; i.e. those with an 'A' are 'asialo'; 'M' are 'mono sialo'; 'D' are 'disialo'; 'T' are 'trisialo'; 'Q' are 'quadsialo' and 'P' are 'pentasialo'. (Diagram modified from Lloyd, K.O. *et al.*, 1998).

influence the ganglioside profile of cells. For instance, the high levels of G_{D2} characteristic of neuroblastoma cells are determined not only by high SAT-II levels but also by low levels of the following enzyme in the B pathway, β1-3GalT, which would convert the G_{D2} into GD_{1b} (Ruan, S. *et al.*, 1992). Unfortunately, no particular role can be assigned to an individual ganglioside, but the general concept has emerged that gangliosides contribute to cell-cell recognition interactions through carbohydrate - carbohydrate interactions (Jessel, T.M. *et al.*, 1990), that are important in development and tissue organization.

Chapter 2: Glycosyltransferases

I. Glycosyltransferases: The Tools of Glycosylation

The glycosyltransferases (GTs) catalyze the synthesis of the carbohydrate portions of glycoproteins, glycolipids, and other such glycoconjugates. Most transfer one sugar in one linkage and are encoded by a unique gene. Thus, synthesis of a branched carbohydrate may require expression of at least 30 GT genes (Stanley, P. *et al.*, 1995). Their primary function is to transfer a sugar residue from an activated donor, usually a nucleotide sugar, to a growing carbohydrate group. The specificity of the enzymes for their donor and acceptor substrates constitute the primary basis for determining the structures of the sugar chains produced by a cell. It is estimated that 100 or more GTs are required for the synthesis of known carbohydrate structures on glycoproteins and glycolipids, and most of these are involved in elaborating the highly diverse terminal sequences (Paulson, J.C. *et al.*, 1989). The enzymes are named according to the monosaccharide that they transfer. For instance, sialic acid (NeuAc) is transferred by sialyltransferase (ST), N-acetylglucosamine (GlcNAc) is transferred by N-acetylglucosaminyltransferase (GlcNAc-T), galactose (Gal) is transferred by galactosyltransferase (GalT) and so on. The most common reaction catalyzed by these enzymes is:



nucleotide---sugar + R---OH → R---O---sugar + nucleotide

where R is a free monosaccharide, or a monosaccharide linked to other saccharide units, a protein or a lipid. As depicted in figure 8, the Golgi glycosyltransferases have a short NH_2-terminal cytoplasmic tail, a 16-20 amino acid signal-anchor domain, and an extended stem region which is followed by the large COOH-terminal catalytic domain. The signal-anchor domain acts as a transmembrane binding region which spans the lipid bilayer of the Golgi and locates itself in a type II membrane orientation, meaning that the C-terminal region of the protein is in the lumen while the N-terminal region is in the cytoplasm. This places the catalytic domain within the lumen of the Golgi, where the nucleotide sugars are located and thus allows for optimal activity of the enzymes (Kawakita, M. *et al.*, 1998). In addition, the stem region acts as a flexible tether, allowing the catalytic domain to glycosylate carbohydrate groups of membrane bound and soluble proteins of the secretory pathway as transport occurs through the Golgi apparatus. In addition to the Golgi, soluble forms of GTs have been demonstrated and purified from milk, serum, and other body fluids (Sadler, J.E., 1984), and increased serum levels have been noted in disease states (Delves, P.J., 1998) and inflammation (Lammers, G. *et al.*, 1989). The origin of these enzymes has long been thought to result from proteolytic release from the membrane bound form of the enzymes (Paulson, J.C. *et al.*, 1987; Strous, G.J.A.M. *et al.*, 1982). It is believed that the soluble forms of these enzymes could result from the release of membrane-bound enzymes by endogenous proteases, presumably by cleavage between the catalytic domain and the transmembrane domain (Lammers, G. *et al.*, 1989).

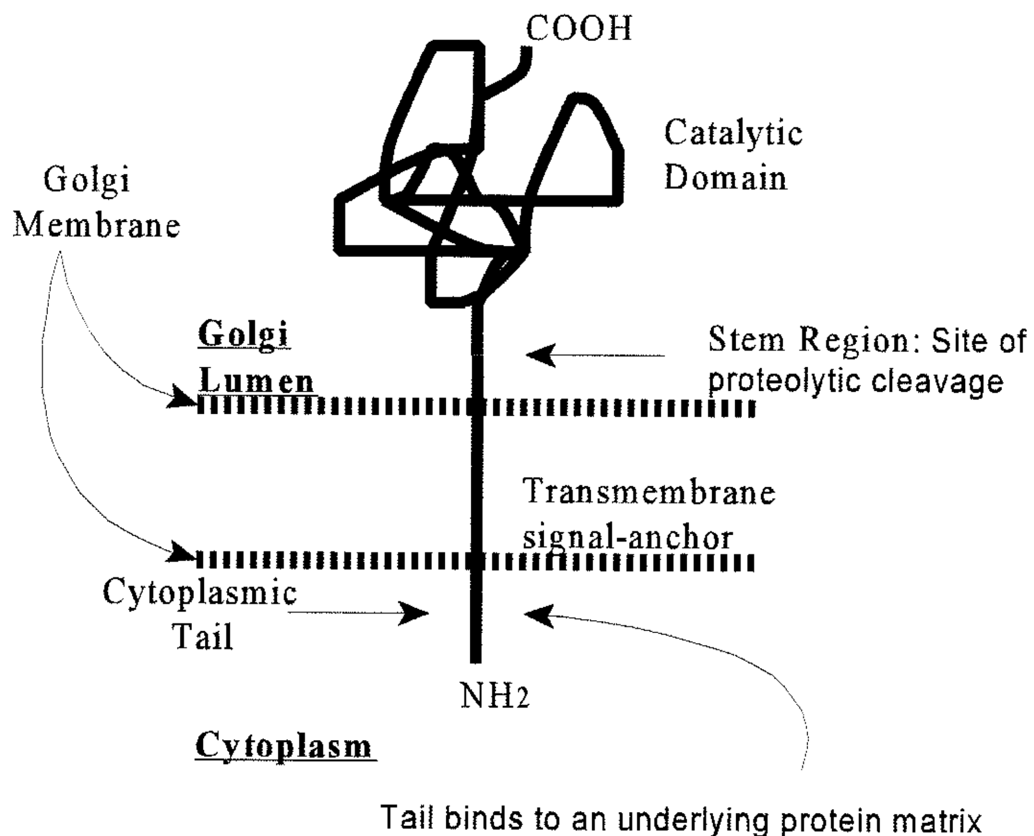


Figure 8. Common Topology of Glycosyltransferases found within the Golgi apparatus. The cytoplasmic tail is quite short, with an average length of 5-17 amino acids, while the signal-anchor domain is anywhere from 11-29 amino acids, depending on the enzyme. The exact length of the stem region is not known (Schachter, H., 1994) since it is unclear how many amino acid residues can be removed from the catalytic region before there is a compromise in the enzyme's activity.

There is abundant evidence that terminal glycosylation sequences are differentially expressed in cells and are subject to change during development, differentiation, and oncogenic transformation (Muthing, J. *et al.*, 1994; Ladisch, S. *et al.*, 1995; Osanai, T. *et al.*, 1997; Yao, M. *et al.*, 1998). The concept that the cellular glycosylation machinery largely determines the structures of glycoprotein sugar chains stems from observed differences in the carbohydrate structures elaborated on viral glycoproteins produced in various cultured cell lines (Jenkins, N. *et al.*, 1994) and from the sugar structures of glycoproteins naturally expressed in different tissues (Rademacher, T.W. *et al.*, 1988). Although the structure of the protein or lipid to which the carbohydrate is attached places secondary constraints of accessibility on the glycosylation machinery (Baenziger, J.U., 1994), it should be realized that the terminal glycosylation sequences produced by a cell are presumed to reflect the expression of the corresponding GTs which synthesize them. Therefore, whether the enzymes are removed from their proper Golgi compartments *via* proteolytic cleavage or have their overall activity affected at the level of transcription, it will result in a change in structure of the oligosaccharide itself. Support for this comes from examples of altering the cellular glycosylation machinery by transfection of cells with DNA fragments of expression vectors containing cDNAs coding for GTs which synthesize terminal glycosylation sequences. For example, although Chinese hamster ovary cells produce N-linked carbohydrate groups with the NeuAcα2,3Gal linkage, it was demonstrated that when these cells were stably transfected with the Gal α2,6-ST gene, they were

able produce both the NeuAcα2,6Gal and NeuAcα2,3Gal linkages (Lee, E.U. *et al.*, 1989). Furthermore, GT expression is most likely regulated at the level of transcription as has been demonstrated by the observation that the level of Gal α2,6-ST mRNA varies 50-100 fold in various rat tissues, correlating with the activity of the enzyme (Paulson, J.C. *et al.*, 1989). It is then conceivable that by knowing the enzymes which are present, their relative concentrations, the specifics of their kinetics, as well as the substrate residence times in the reaction compartments, that one could predict the final structure of many of the glycoconjugates produced by a cell. In fact, quite recently two mathematical models have been proposed that were able to predict the N-linked glycoforms synthesized by a cell (Umana, P. *et al.*, 1997) and specifically, the level and type of sialylation within a cell (Monica, T.J. *et al.*, 1997) by setting up the aforementioned parameters. Finally, one of the more interesting features of GTs is exemplified in their primary structure. Although the enzymes, as a whole, differ greatly in amino acid composition (see below) from one another the same enzymes are quite similar when compared from different species, showing that their structures have been conserved readily throughout evolution. However, the greatest variability occurs in the 'stem' region, where there is only about 30-40% amino acid similarity whereas the sequence similarity is ~80-90% in regions where functional domains have been located, i.e. the transmembrane domain and C-terminal catalytic domains (Schachter, H., 1994).

In order to gain a thorough appreciation of the data exhibited in the following report, a brief review of the GTs, specifically the STs and GlcNAcTs, which are

most relevant to these studies will be presented.

A. Sialyltransferases

These enzymes are a subset of the GT family that use CMP-NeuAc as the activated sugar donor to catalyze the transfer of sialic acid residues, usually to terminal non-reducing positions of oligosaccharide chains of glycoproteins and glycolipids. There is very little similarity among them at the primary sequence level, except for the two sialyl motifs, L (for large) which is about 50 amino acids in length and S (for small) which is 23 amino acids long (Tsuji, S., 1996). This compares to the overall length of STs which ranges in size from ~350-400 amino acids (Schachter, H., 1994). The results of site-directed mutagenesis have indicated that the L motif is involved in the binding of the donor substrate, CMP-NeuAc (Datta, A.K. *et al.*, 1995), while the S motif is involved in recognizing both the donor and acceptor substrates (Datta, A.K. *et al.*, 1998). The subcellular localization of these enzymes has been extensively studied. They have been shown to be restricted to the *trans* cisternae of the Golgi apparatus and to the *trans* Golgi network (Mellman, I. *et al.*, 1992). However, some studies have suggested that these enzymes may have a more diffuse distribution throughout the Golgi apparatus in some cells (Roth, J., 1987). Overall, in spite of the fundamental role played by STs in the biosynthesis of specific sialylated sequences, there is limited information available on their secondary protein structure, mechanism of enzyme action and on the cellular mechanisms involved in the regulation of their transcriptional expression.

<u>**(i) Galβ1-4GlcNAc α2,6-sialyltransferase (EC 2.4.99.1; ST6Gal I)**</u>

The ST6Gal I enzyme is the only sialyltransferase able to transfer sialic acid to the 6-hydroxyl group of the galactose residue at the terminal position of the disaccharide Galβ1-4GlcNAc, where the acceptor may be a free disaccharide or may represent a non-reducing terminal N-acetyllactosamine unit on an N– or O-linked oligosaccharide (Harduin-Lepers, A. *et al.*, 1995). The enzyme is highly specific for type 2 oligosaccharide chains (Galβ1-4GlcNAc) and is unable to transfer sialic acid onto type 1 oligosaccharide chains (Galβ1-3GlcNAc) or onto the T-antigen (Galβ1-4GalNAc; Weinstein, J. *et al.*, 1982). However, in *in vitro* assays, this enzyme transfers sialic acid onto other oligosaccharide structures, such as lactose (Galβ1-4Glc); Paulson, J.C. *et al.*, 1977), GalNAcβ1-4GlcNAc (Nemansky, M. *et al.*, 1992) or Manβ1-4GlcNAc (Van Pelt, J. *et al.*, 1989). In fact, a precise determination of the acceptor specificity using synthetic modified Type 2 acceptors indicates the ST6Gal I requires the 6-hydroxyl group of the β-Gal and the 2-acetamido group of the GlcNAc, and that most of the other hydroxyl groups accept modifications (Wlasichuk, K.R. *et al.*, 1993) Type 2 sequences occur mainly in complex N-glycan chains. Furthermore, ST6Gal I preferentially sialylates the Gal residue at the Galβ1-4GlcNAcβ1-2Man<u>α1-3</u> branch rather than the Galβ1-4GlcNAcβ1-2Man<u>α1-6</u> branch in a biantennary structure, and the presence of an additional Galβ1-4GlcNAcβ1-6Man<u>α1-6</u> branch in tri- and tetra-antennary glycans diminishes the rate of transfer into both branches (Joziasse, D.H. *et al.*, 1987).

ST6Gal I is widely expressed in a number of tissues and cells, especially in the liver where it participates in the sialylation of serum glycoproteins. A soluble form of the enzyme has also been purified from bovine colostrum and was shown to arise from the membrane-bound enzyme by the proteolytic cleavage of the non-catalytic N-terminal part of the polypeptide that contains the membrane signal anchor (Hesford, F.J. *et al.*, 1984). A soluble form of ST6Gal I is also present in serum (Kaplan, H.A. *et al.*, 1983; Lammers, G. *et al.*, 1989a) and its level is modulated according to physiological or pathological conditions (cirrhosis, pregnancy, cancer, etc.). It is increased during the acute-phase response of the liver to tissue injury. The release of ST6Gal I from liver to serum involves a cathepsin D-like protease which is induced during the inflammatory response (Lammers, G. *et al.*, 1989b) and can be considered an acute-phase protein (Jamieson, J.C. *et al.*, 1993) as described in chapter 4.

(ii) NeuAcα2-3Galβ1-3GlcNAcβ-R α2,6-sialyltransferase (EC2.4.99.7; GlcNAcα2-6ST)

This enzyme catalyses the following reaction:

CMP-NeuAc + NeuAcα2-3Galβ1-3**GlcNAc**β-R →
NeuAcα2-3Galβ1-3[NeuAcα2-6]**GlcNAc**β-R + CMP

where R is a complex-type N-linked glycan. Very little is known about this enzyme, as only one publication to date has focused on it (Paulson, J.C. *et al.*, 1984), with minor reference to its existence occurring in three others (De Heij, H.T. *et al.*, 1986; Richardson, K. *et al.*, 1994; Harduin-Lepers, A. *et al.*, 1995). It is involved in the

biosynthesis of the terminal NeuAcα2-3Galβ1-3[NeuAcα2-6]-GlcNacβ1-R group occurring in human milk oligosaccharides and in the glycan chains of several N-glycoproteins. This enzyme generally recognizes NeuAcα2-3Galβ1-3GlcNAcβ1-R as an acceptor and shows very low or no activity towards Galβ1-3GlcNAcβ1-R or GlcNAcβ1-R acceptor structures, respectively. Finally, the biosynthesis of the disialylated sequences first requires the action of the Galβ1-3/4GlcNAc α2,3-ST (see below), which attaches the terminal NeuAcα2,3 to the structure, before α2,6 sialylation of GlcNAc residues.

(iii) Galβ1-3(4)GlcNAc α2,3-sialyltransferase (EC 2.4.99.6; ST3Gal III)

Although this enzyme prefers Type 1 chain (Galβ1-3GlcNAc) acceptors, it also transfers sialic acid into Type 2 acceptors (Galβ1-4GlcNAc), but with a slightly lower (~10%) efficiency (Weinstein, J. *et al.*, 1982b). The exact acceptor specificity was determined using synthetic modified type 1 and type 2 disaccharide acceptors, and demonstrates that rat liver ST3Gal III requires the 3-, 4- and 6- hydroxyls of the terminal β-Gal, and some assistance from the subterminal sugar, which would shed light on the dual-reactivity of this enzyme for the type 1 and type 2 acceptors (Wlasichuck, K.B. *et al.*, 1993). ST3Gal III acts weakly on the Galβ1-3[NeuAcα2-6]GlcNAcβ sequence (Chandrasekaran, E.V. *et al.*, 1995). As discussed above, this indicates that the main pathway leading to the disialyl tetrasaccharide sequence NeuAcα2-3Galβ1-3[NeuAcα2-6]GlcNAcβ first involves ST3Gal III before the α2-6 sialylation of GlcNAc. Like ST6Gal I, the enzyme is also cleaved from its

membrane anchor by a cathepsin D-like proteinase (Richardson, K. *et al.*, 1994), yet there is no evidence that it appears in the serum in a catalytically active form during the acute-phase response.

(iv) Galβ1-4Glcβ1-O-Ceramide α2,3-sialyltransferase (EC 2.4.99.9; SAT-1)

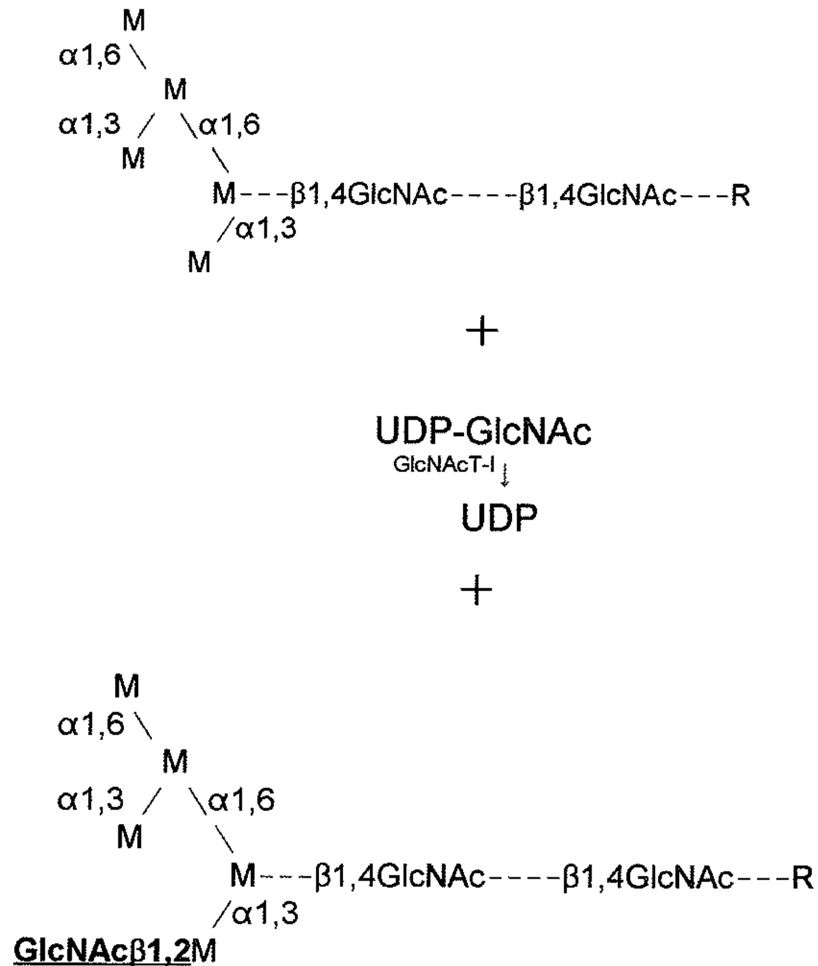
Although this enzyme is effectively able to transfer sialic acid onto the 3-hydroxyl group of the terminal Gal residue of lactosylceramide (**Gal**β1-4Glcβ1-O-ceramide), SAT-1 has also been noted to recognize glucosylceramide (Glcβ1-O-ceramide) and galactosylceramide (Galβ1-O-ceramide). The ganglioside asialo-G_{M1a} (Galβ1-3GalNacβ1-4**Gal**β1-4Glcβ1-O-ceramide) has also been shown to be an acceptor for SAT-1, but to a much lesser extent (Melkerson-Watson, L.J. *et al.*, 1991).

B. *N-Acetylglucosaminyltransferases*

GlcNAc-Ts are a subset of the GT family that use UDP-GlcNAc as the activated sugar donor to catalyze the transfer of GlcNAc residues which are located within internal positions of oligosaccharide chains of glycoproteins. As is the case with STs, there is very little sequence homology among these enzymes. On average they are larger than the STs as they range in size from ~450-500 amino acids.

(i) β-1,2-N-Acetylglucosaminyltransferase I (EC 2.4.1.101; GlcNAcT-I)

GlcNAcT-I plays a key role in controlling the conversion of high mannose N-glycans to hybrid and complex structures by catalyzing the following reaction:

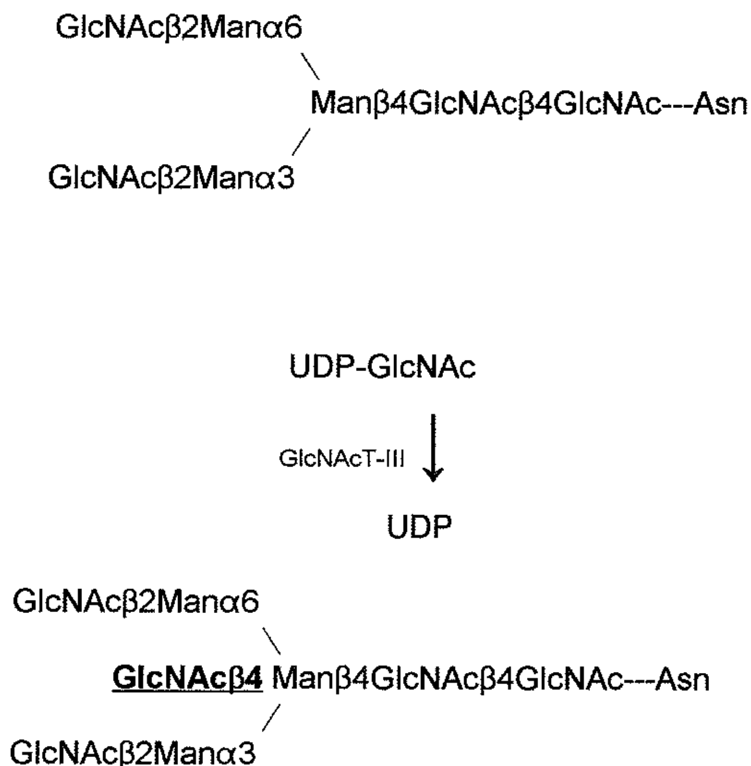


where R can be any aglycon group, but is usually N-linked to an asparagine residue. Chinese hamster ovary mutant cell lines lacking GlcNAcT-I are unable to make hybrid and complex N-glycans but are nevertheless viable and have normal growth characteristics (Stanley, P., 1989). This is somewhat surprising since none

of the other GlcNAc-Ts involved in glycoconjugate biosynthesis can act until this one has acted (Schachter, H., 1986). The subcellular localization of this enzyme has been determined and it is found to occur in almost equal amounts in the *medial* and *trans* regions of the Golgi apparatus (Rabouille, C. *et al.*, 1995). Purification of the enzyme from rabbit (Nishikawa, Y. *et al.*, 1988) revealed two molecular weight species, the larger a membrane bound form, and a smaller soluble form. The smaller form was sequenced and it was found to be about 215 base pairs long, leading to the conclusion that it was cleaved in the stem region, a likely result of proteolysis of the high molecular weight form.

(ii) β-1,4-N-Acetylglucosaminyltransferase III (EC 2.4.1.144; GlcNAcT-III)

The activity of GlcNAcT-III adds a 'bisecting' GlcNAc in β1,4 linkage to the β-linked mannose of the core of Asn-linked oligosaccharides as shown:

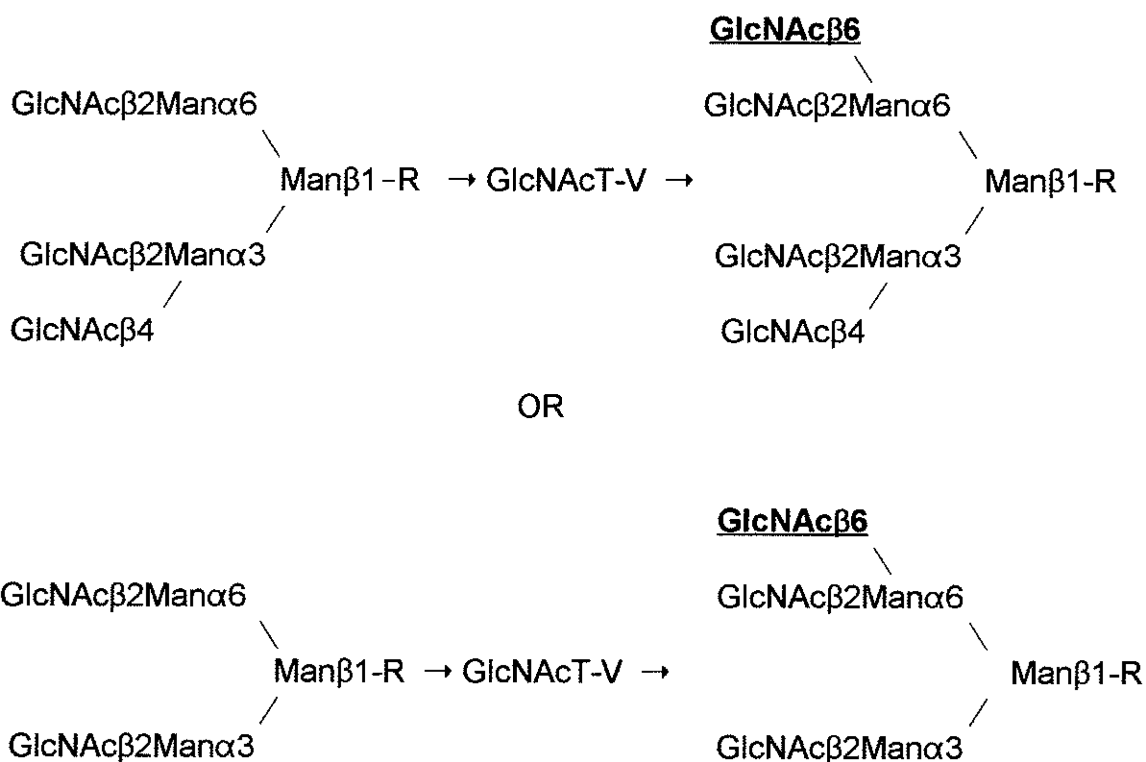


The carbohydrate structures of normal liver glycoproteins do not have bisecting GlcNAc residues in their Asn-linked oligosaccharides (Kobata, A., 1984). But conflicting reports on the presence or absence of bisecting GlcNAc in liver cancer have appeared. For instance, it was shown that 50% of the oligosaccharide chains of carcinoembryonic antigen purified from liver metastases of colon cancer patients had bisecting GlcNAc (Yamashita, K. *et al.*, 1987), yet bisecting GlcNAc residues in the oligosaccharide chains of α_1 acid glycoprotein isolated from liver

metastases of colon cancer patients were not detected (Chandrasekaran, E.V. *et al.*, 1983). Overall, bisecting GlcNAc residues appear to be restricted to glycoproteins from tissues such as hematopoietic cells, kidney, oviduct, malignant tissues and abnormal skin fibroblasts (Narasimhan, S. *et al.*, 1988). GlcNAcT-III itself has been purified from rat kidney (Nishikawa, A. *et al.*, 1992), however, its subcellular localization has not been specifically analyzed. Although the bisecting linkage pattern created by the enzyme is only found on N-linked proteins, the presence of the Asn residue and protein backbone is not an absolute requirement for its action, as the GlcNAcβ4GlcNAcβ4Man-Asn-protein has been replaced with the hydrophobic group, $-O(CH_2)_8-COO-CH_3$ (Khan, S.H. *et al.*, 1994).

(iii) β-1,6-N-acetylglucosaminyltransferase V (EC 2.4.1.155; GlcNAcT-V)

The activity of GlcNAcT-V transfers a GlcNAc from UDP-GlcNAc to the C-6 position of the β1,6-linked mannosyl residue in the trimannosyl core of complex-type N-linked oligosaccharides which can be biantennary or triantennary:



where R follows the same classifications as for GlcNAcT-III. Because of the above newly created linkage pattern, it is commonly referred to as the '2,6-branched mannose'. This enzyme is well recognized as initiating β1-6 branching in oligosaccharides, which in turn are thought to contribute directly to the malignant or metastatic phenotypes of tumor cells (Yao, M. *et al.*, 1998). In addition, the

activity of GlcNAcT-V is elevated in cells following DNA or RNA tumor viruses, resulting in increased 2,6-mannose branching of complex-type N-linked oligosaccharides (Dennis, J.W. *et al.*, 1989). Increases in 2,6-branching and elevation in GlcNAcT-V activity are also associated with activation of human T4 and T8 cell populations (Lemaire, S. *et al.*, 1994). GlcNAcT-V has been purified from rat kidney and cDNA encoding this enzyme has been cloned (Shoreibah, M.G. *et al.*, 1993). The cDNA predicts that the mature membrane-bound enzyme may be 95 kDa, making GlcNAcT-V one of the largest GTs cloned to date. Although its subcellular location has not been specifically determined, it would most likely be primarily located within the medial region of the Golgi apparatus since this enzyme does not act on oligosaccharides with outer galactosyl and sialyl residues (Do, K-Y. *et al.*, 1994), which are transferred within the *trans* and TGN regions of the Golgi (see chapter 1). Studying the substrate specificity for GlcNAcT-V to act on a glycoprotein has shown that two important criteria must be met (Do, K-Y. *et al.*, 1994). First, glycoprotein acceptors for GlcNAcT-V must have the correct oligosaccharide structure, i.e. bi- or triantennary oligosaccharides lacking galactosyl residues and terminating in GlcNAc residues. Second, the oligosaccharides on acceptor glycoproteins must be accessible to GlcNAcT-V. Thus, for many glycoproteins the lack of 2,6-branched mannose on their bi / triantennary N-linked oligosaccharides is due primarily to the inaccessibility of their oligosaccharides to action by GlcNAcT-V.

Chapter 3: Controlling Glycosylation

I. The Golgi Apparatus: A Hundred Years of Dispute

Although it has been 100 years since Camillo Golgi discovered the uniquely shaped organelle which is now named after him (Golgi, C., 1898) , researchers are still mystified by even some of the basic operations of the Golgi complex. Not surprisingly, this organelle, which over the years has offered an array of puzzling questions, began its inception into cell biology with frequent debates over its actual existence. The primary reason behind this was that the Golgi could not be visualized in living cells and when visualization could be achieved, it was performed with a staining method which was difficult to reproduce and in turn stained additional structures. However, with the dawn of electron microscopy (EM) the Golgi was finally shown to be a bona fide organelle in 1954, 57 years after its initial discovery (Dalton *et al.*, 1954). In 1969, two findings were uncovered which were the key to implicating the Golgi complex in glycoprotein synthesis. First, preparation of Golgi fractions showed high levels of galactosyltransferase activity compared to other regions of the cell (Fleischer, B. *et al.*, 1969) and secondly it was demonstrated that the sugar galactose was directed into the Golgi (Whur, P. *et al.*, 1969). Another major development in this same era was the realization that the Golgi apparatus consists of distinct subcompartments. Furthermore, it was noted that the enzymes in this organelle were not distributed uniformly across the stacks

of the these various subcompartments (Farquhar, M.G. *et al.*, 1981). With the knowledge that the Golgi was involved in the production of carbohydrates, much of the 1970's and 80's were spent examining the detail with which the Golgi was able to create *N*-linked and *O*-linked glycoproteins, as well as other glycoconjugates such as proteoglycans. From these studies, it soon became clear that differences did exist among different cell types in the distribution of marker enzymes within the Golgi. However, as more information was compiled on the structural, chemical and physiological properties of the Golgi, it appeared as though more questions were being asked than were being answered. For instance, how is the Golgi able to maintain its unique architecture with such a high degree of cellular trafficking? It has been estimated that 1100-3400 vesicles of 100 nm diameter reach the Golgi complex from the ER per second (Weiland, F.T. *et al.*, 1987). This has investigators wondering if transport of cellular material through the Golgi itself is guided by vesicles, tubules, or a combination of the two (Presley, J.F. *et al.*, 1998). In general, glycosylation enzymes are localized within the Golgi cisternae in the same sequence in which they act to modify oligosaccharide substrates (Colley, K.J.,1997). The possibility that this enzyme subcompartmentalization may control the types of oligosaccharides expressed by a cell has led to an interest in the signals and mechanisms directing enzyme localization within the Golgi. However, their lack of sequence homology suggests that these proteins' Golgi retention signals are not linear amino acid sequences, but most likely involve general characteristics or conformations of larger protein

domains. Although there have been many contradictory results and several unexplained discoveries, a general model of the Golgi has been developed that has gained general acceptance throughout the cell biology community (fig. 9). It is generally accepted that the Golgi is organized into three functionally distinct regions; the Golgi stack (consisting of *cis*, *medial*, and *trans* cisternae), and two tubulo-vesicular networks, namely the *cis*-Golgi network (CGN) and the *trans*-Golgi network (TGN). Although depicted as a distinct compartment, it has been suggested that the CGN is actually a specialized region of the ER. The TGN is functionally distinct from the *trans*-cisternae and is the sorting station of the Golgi where proteins are packaged into vesicles and shipped to various destinations, including the cell surface, lysosomes, and secretory storage granules. Flanked by the CGN and TGN are the *cis*, *medial*, and *trans* cisternae, which represent functionally, biochemically and morphologically distinct sub-compartments. These compartments differ from one another in the density of their membranes, pH, and most importantly in the populations of resident proteins which they contain. However, detailed biochemical characterization of the individual cisternae is lacking as the current methods are inappropriate to allow their purification.

II. Mannose 6-phosphate Receptors: Dangerous Cargo

It is clear that the Golgi apparatus has two main roles: the first of these is the modification of newly synthesized proteins and lipids as they pass through the

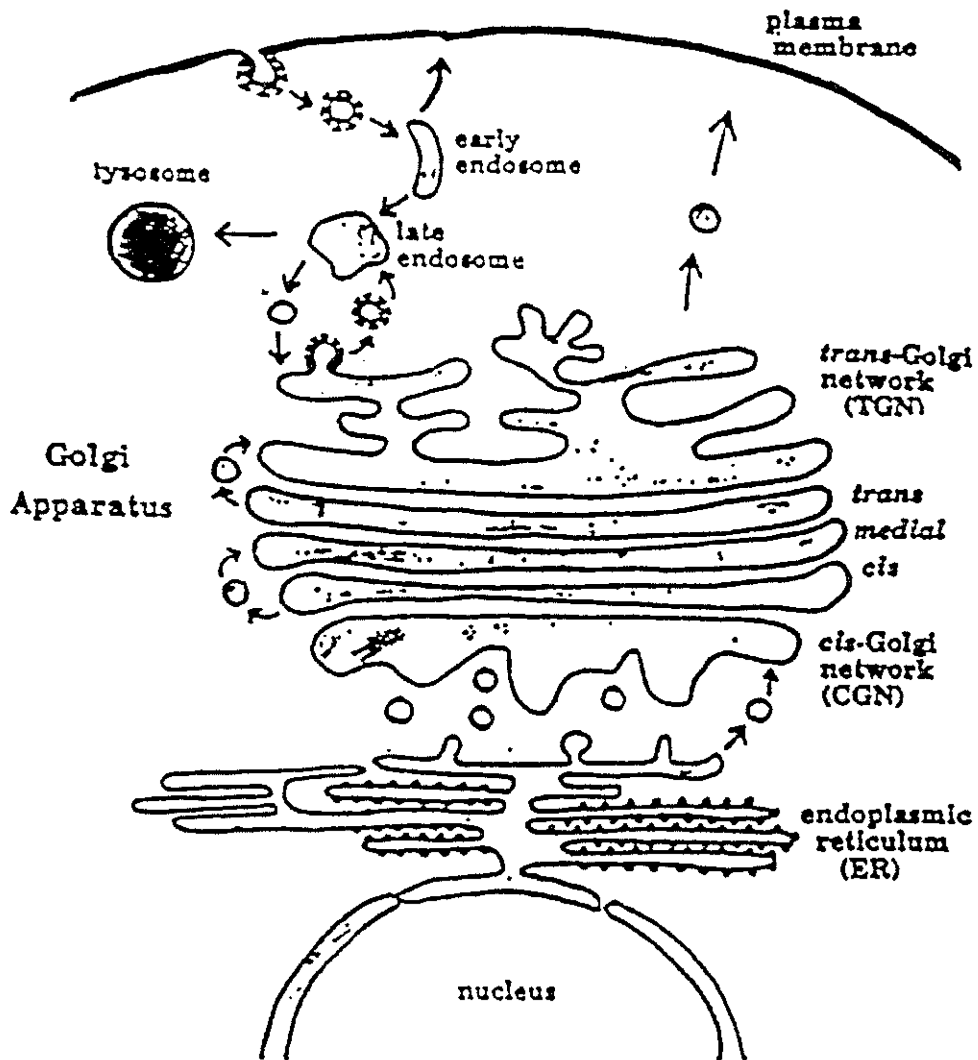
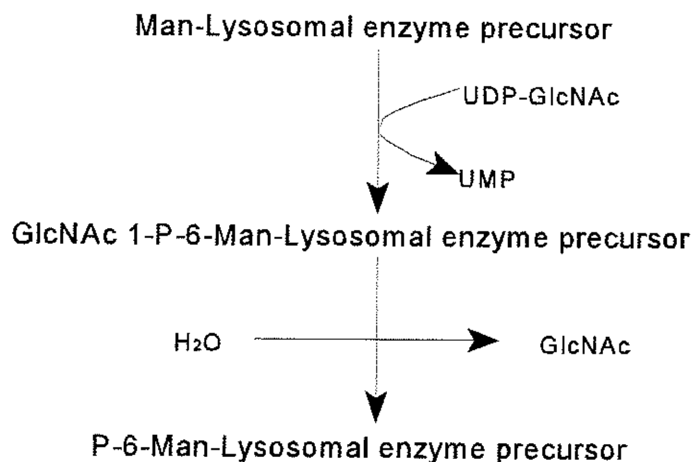


Figure 9. Diagram of the Golgi apparatus and it surrounding environment.
(Diagram taken from Baenziger, J.U., 1994).

organelle. As discussed in Chapter 1, the modification enzymes of the Golgi include the glycosidases and glycosyltransferases which are responsible for synthesizing the huge diversity of oligosaccharides that are attached to glycoproteins and glycolipids. Other Golgi enzymes catalyze the sulphur atom of tyrosines and sialic acids, promote the attachment palmitoyl groups, and catalyze proteolytic cleavage of specific proteins (Munro, S., 1998) The second role of the Golgi is to serve as a major sorting point in the secretory pathway, with proteins and lipids being selectively targeted to several different organelles. Easily the most familiar of these mechanisms is the mannose 6-phosphate (M6P) sorting signal which is involved in directing specific proteolytic enzymes to the lysosome. Lysosomes are organelles involved in intracellular digestion and contain the enzymes needed to digest all types of macromolecules. Many of these are digestive enzymes which aid in the accumulation of nutrients, or are involved in the immune response by degrading foreign invaders or degrading blood clots that were originally designed to localize the infection. A cell can also survive a period of starvation by selectively digesting portions of itself to remain alive. One of the most remarkable aspects about lysosomes is that they accomplish all these tasks without releasing their digestive enzymes into the cytoplasmic matrix, a catastrophe that would destroy the cell, which is evident by the fact that this is a process carried out during cell death. When lysosomal enzymes are synthesized in the rough endoplasmic reticulum, delivering them to their final destination requires a multi-step process involving a series of interactions between cellular components and

recognition signals present on the enzymes.

Lysosomal enzymes usually contain two or more oligosaccharides. The signal that permits the enzyme to eventually be directed into the lysosomes is a phosphate residue, which is attached to the C-6 position of a mannose on the N-linked oligosaccharide chain(s). The phosphate is attached in the *cis* region of the Golgi and the glycoprotein is then transported as usual through the remainder of the Golgi complex (fig. 10). The enzyme catalyzing the first reaction in the phosphate transfer is UDP-N-acetylglucosamine:lysosomal enzyme *N*-Acetlyglucosaminyl-1-phosphotransferase (referred to as the phosphotransferase) attaches GlcNAc-P to the mannose residue *via* the phosphate that was originally in the nucleotide sugar UDP-GlcNAc. As shown below, the second step is a hydrolysis reaction which removes the GlcNAc sugar, and thus exposes the phosphate:



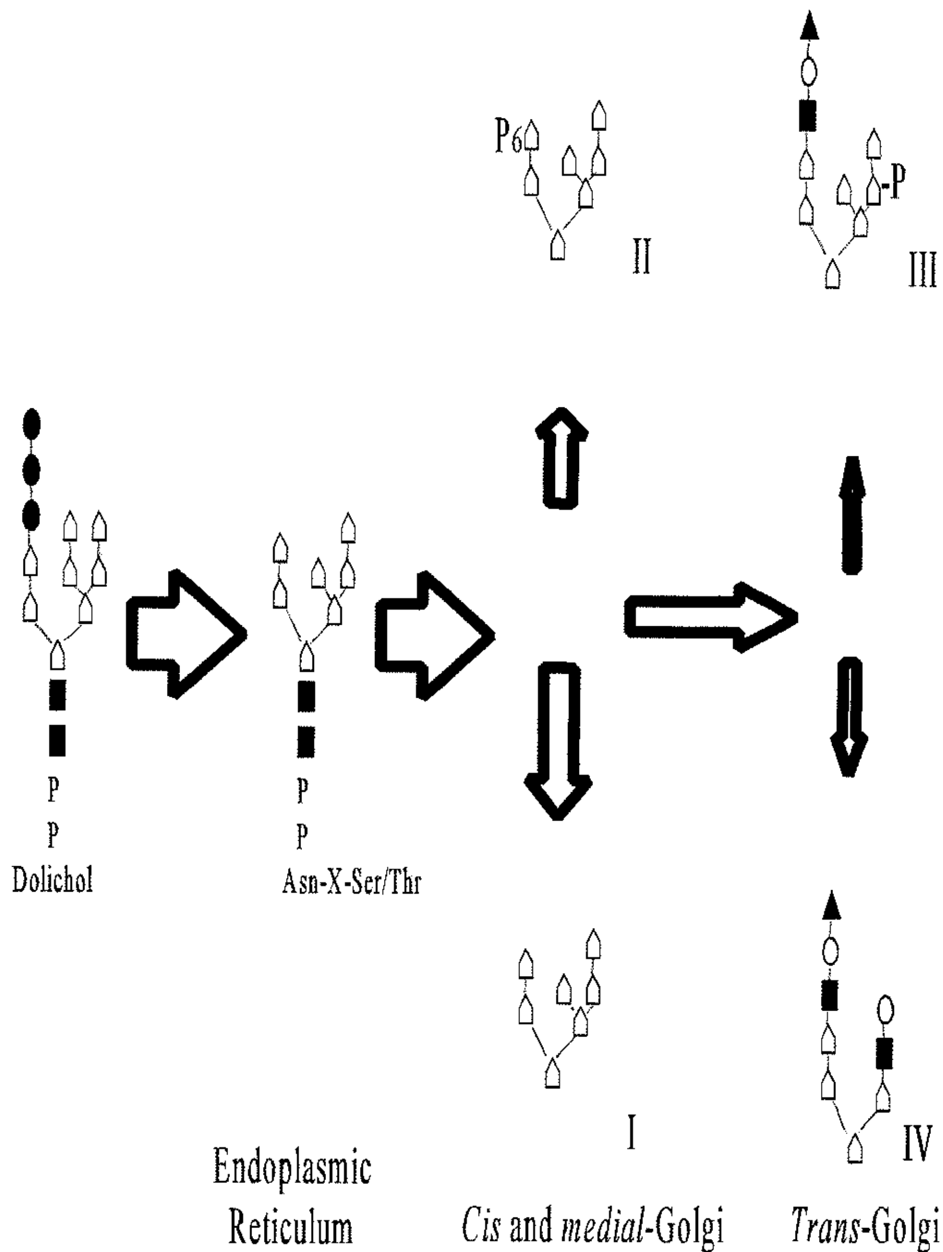


Figure 10. Main stages in the processing of oligosaccharides in lysosomal enzymes. The four most common structures that have been found in lysosomal enzymes as formed in different parts of the Golgi are shown: I = high-mannose, II = phosphorylated high-mannose, III = phosphorylated hybrid, IV = complex oligosaccharide. The arrows indicate the relative abundance of the four oligosaccharide types. Abbreviations: ■, GlcNAc; ⌂, Mannose; ●, Glucose; ○, Galactose; ▲, NeuAc; P, phosphate. (Diagram modified from Figura, K.V. *et al.*, 1986).

This phosphorylation of high-mannose oligosaccharides is not detectable in many non-lysosomal glycoproteins, although a few exist (Dahms, N. M. *et al.*, 1996) and isolated oligosaccharides from the lysosomal enzymes are not phosphorylated very efficiently (Reitman, M.L. *et al.*, 1981). This is because lysosomal enzymes contain a unique structure within the protein itself that is distinct from the acceptor oligosaccharide, and is recognized by the phosphotransferase. Primary structures of several lysosomal enzymes have been determined and no homologies have been found in the primary sequences in the vicinity of the glycosylation sites of these enzymes (Shewale, J.G. *et al.*, 1984). However, because the signal is sensitive to treatment with heat or trypsin, it is probably dependent on the tertiary rather than primary structure (Lang, L. *et al.*, 1984). A very common and well documented lysosomal enzyme, cathepsin D, was examined in relation to what regions were important for attaching the mannose 6-phosphate. Mutagenesis studies identified a phosphotransferase recognition region consisting of Lys-203 and residues 265-292 (Baldwin, E.T. *et al.*, 1993). The carbohydrate chain which was phosphorylated was attached to Asn-70, which was 32Å distant from the aforementioned region. It was subsequently shown that the high degree of flexibility of the carbohydrate chain itself allowed for the terminal mannose residues to be brought within close contact of the recognition region. Convincingly, this same study was able to superimpose the 3-D structure of residues 265-292 in cathepsin D onto related sequences in other lysosomal enzymes, demonstrating

that a tertiary structure of the protein involving ~30 amino acids plays a significant role in tagging lysosomal enzymes with mannose 6-phosphate. Therefore, it seems as though the phosphotransferase first recognizes the 3-D motif within the protein backbone of the enzyme, then transfers the UDP-GlcNAc onto the mannose of the enzyme's carbohydrate chain, provided that it is in reach. At the time of phosphorylation, the bulk of oligosaccharides contain six to nine mannose residues, however, those with only five residues have been demonstrated. Furthermore, phosphorylation of glucosylated high-mannose oligosaccharides is possible in branches that do not contain glucose (Gabel, C.A. *et al.*, 1982). In all studies to date, oligosaccharides with one phosphate group were found to be two to five times more frequent than oligosaccharides with two phosphate groups. However, which mannose and how many have the GlcNAc-1-P attached to it, solely depends on the phosphotransferase's ability to recognize the mannose in question while bound to the initial 3-D recognition marker (Takeshima, H. *et al.*, 1995).

Once the lysosomal enzyme is tagged with its phosphate residue in the *cis* region, it travels through the remainder of the Golgi complex, allowing the oligosaccharide chains to be modified further. Once in the TGN, the lysosomal enzymes are packaged into the lysosomes *via* a receptor which recognizes the mannose-6-phosphate residue (Baranski, T.J. *et al.*, 1991). Specifically, there are two distinct mannose-6-phosphate receptors. One is the larger 300-kDa mannose-6-phosphate / insulin-like growth factor 2 receptor (M6P/IGF2R) which is cation-independent (Chen, H. J. *et al.*, 1997) while the smaller 46-kDa form is cation-

dependent and binds only mannose-6-phosphate (Roberts, D. L. *et al.*, 1998). Although the larger receptor is involved in most of the trafficking, it isn't totally clear as to why there are two distinct receptors and why the larger of the two has additional binding capabilities. In fact M6P/IGF2R has been shown to have its trafficking altered by binding retinoic acid (Kang, J. X. *et al.*, 1998) and has been shown to play a crucial role in the control of cell growth in fetal development (Ludwig, T. *et al.*, 1996) and carcinogenesis (Piao, Z. *et al.*, 1997; Mills, J. J., 1998). Both receptors are continually recycled, therefore a preventative mechanism must insure that they do not enter the lysosomes or they will be degraded. There does appear to be a distinct difference in the manner in which each receptor is able to do this. For instance, the smaller receptor has been shown to contain at least four distinct signals within its 67 amino acid cytoplasmic tail which have not been located in its larger counterpart (Schweizer, A. *et al.*, 1997). There is additional evidence that the enzymes themselves can be targeted to the lysosomes either through the biosynthetic or endocytic pathway (fig. 11). The biosynthetic pathway is simply transfer of the enzymes through the Golgi as described above while the endocytic pathway is the uptake of extracellular enzymes, as a small portion of them are typically secreted by cells (Kornfeld, S., 1987). If these enzymes are not properly transferred into the lysosomes, damaging consequences can be the result. Over 30 inherited lysosomal storage disorders are known to occur in man with I-cell disease being the most common. Patients with this disease have severe psychomotor retardation and skeletal deformities. Their lysosomes contain large

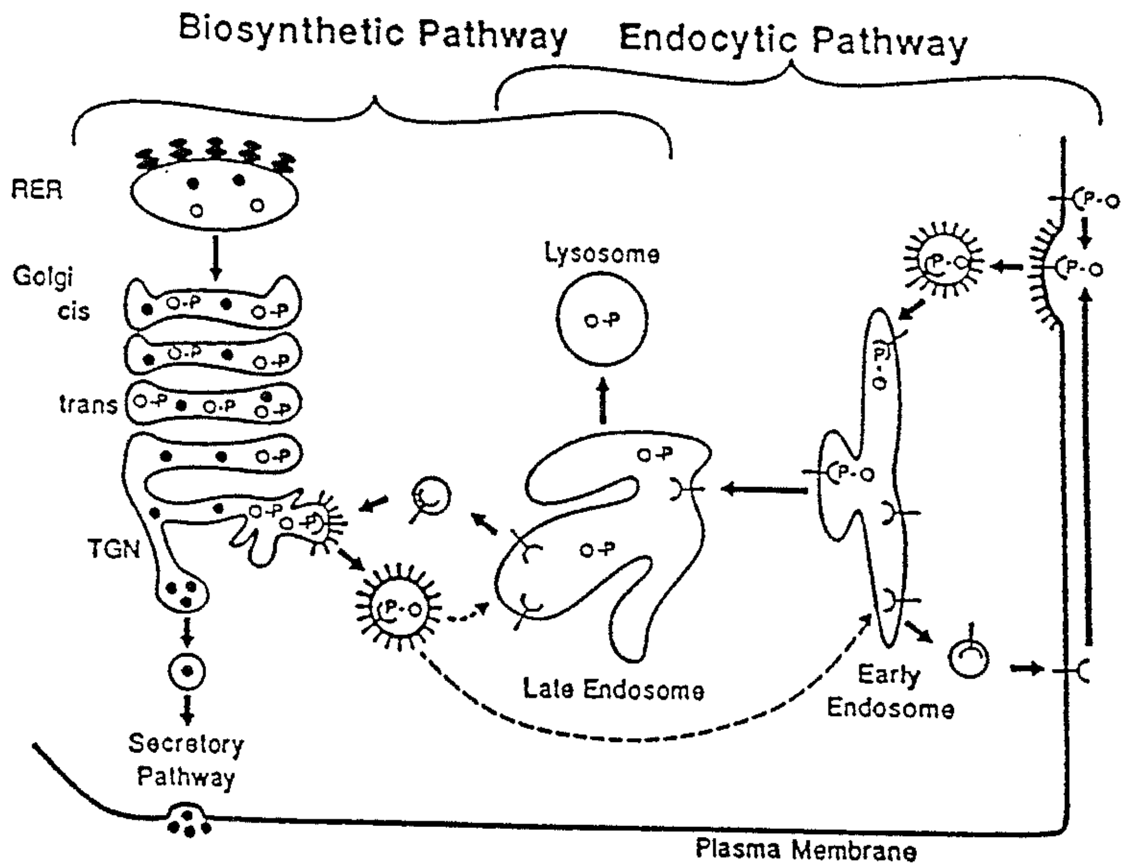


Figure 11. Model for lysosomal enzyme targeting to lysosomes. Lysosomal enzymes (O) and secretory proteins (●) are synthesized in the rough endoplasmic reticulum (RER) and transported to the Golgi where the lysosomal enzymes acquire phosphomannosyl residues (O-P). Most of the lysosomal enzymes bind to M6P receptors (Y) in the trans Golgi network (TGN) and are translocated to late and/or early endosomes where they are released from the receptor due to the intraorganelle acidification. The enzymes are then packaged into lysosomes while the receptors cycle back to the Golgi or to the plasma membrane. (Diagram taken from Dahms, N.M. *et al.*, 1989).

inclusions of undigested glycoconjugates, a result of missing at least eight enzymes required for their degradation (Stryer, L., 1988). The reason for the lack of enzymes is that they are not tagged with the necessary phosphate groups.

III. Golgi Retention: Locating the Proper Compartment

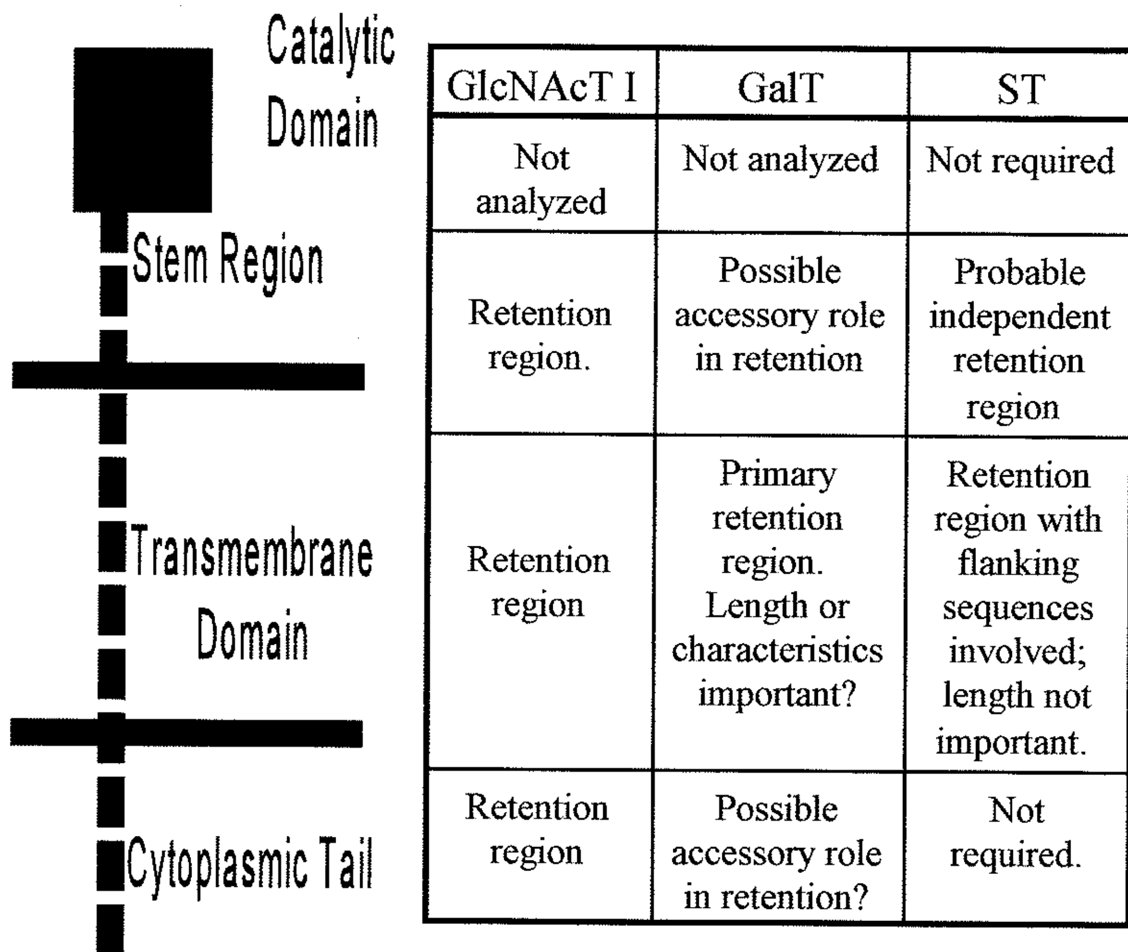
As briefly discussed above, a major issue that is presently being debated is how the glycosyltransferases within the Golgi membrane are able to localize themselves within the correct cisternal compartments. For instance, sialyltransferases are generally found within the *trans* and TGN Golgi compartments (Rabouille, C. *et al.*, 1995), NAGT I overlaps in the *medial* and *trans* sections (Burke, J. *et al.*, 1995), and GalT in typically found in the *trans* cisternae (Russo, R.N. *et al.*, 1992). The examination of the primary sequences of these enzymes did not reveal in an obvious membrane binding signal, such as the carboxy terminal sequence KDEL, which is used by soluble ER proteins to keep them located in the ER (Pelham, H.R., 1990) or the dileucine-containing peptide motif which has been shown to direct the transport of Man-6-phosphate receptors from the TGN to the late endosomes (Johnson, K.F. *et al.*, 1992). In fact, the overall localization signals of non-Golgi proteins can be categorized as hydrophilic motifs located on either the cytoplasmic or luminal domains of the protein (Gleeson, P.A. *et al.*, 1994). However, the localization signals of the glycosyltransferases has proven to be quite elusive.

Since the early part of this decade several groups have attempted to identify the targeting signal responsible for the localization of glycosyltransferases. A common strategy has been employed by most groups to identify a putative Golgi retention signal(s) by analyzing the localization, in transfected mammalian cells, of hybrid molecules containing limited sequences derived from Golgi glycosyltransferases. In all known cases, the membrane spanning domains of the Golgi glycosyltransferases have been shown to direct at least partial localization of hybrid molecules to the Golgi apparatus. In fact, it was demonstrated that the transmembrane domain of GalT and GlcNAcT-I can specifically localize hybrid proteins to the *trans* and *medial* cisternae respectively (Nilsson, T. *et al.*, 1991; Burke, J. *et al.*, 1994). However, there have been numerous studies that have shown that sequences flanking the transmembrane domain also play auxiliary roles in mediating Golgi localization. For example, additional sequences from the stem region and the cytoplasmic tail increase the efficiency of Golgi localization of ST6Gal I (Munro, S., 1991) and GlcNAcT-I (Tang, B.L. *et al.*, 1992), although the tail and / or stem of either of these enzymes alone is not capable of retaining a reporter molecule in the Golgi. At the same time, however, there have been contradictory results which showed that removal of the stem region from wild type ST6Gal I (Colley, K.J. *et al.*, 1992) or GalT (Aoki, D. *et al.*, 1992) does not disrupt Golgi localization. As will be discussed shortly, these flanking sequences contain charges that may be crucial in the enzymes ability to properly orientate itself within the Golgi membrane.

Since the transmembrane domain is the only part of the glycosyltransferase to actually make significant contact with the cisternal membrane it is still surprising that it plays a central role in the targeting and localization of resident Golgi glycosyltransferases, as localization signals of many other proteins are hydrophilic regions of the cytoplasmic or luminal domains. The involvement of a hydrophobic stretch of amino acids in targeting seemed to indicate a unique mechanism for the localization of these resident Golgi proteins. Unfortunately, there is considerable variability in the results obtained between groups, even when comparing the same glycosyltransferase. A classic example of this was observed when it was reported that the transmembrane domain of ST6Gal I resulted in very efficient Golgi localization of a hybrid molecule (Wong, S.H. *et al.*, 1992), whereas a different hybrid construct containing the equivalent ST6Gal I domain, resulted in leakage to the cell surface (Munro, S., 1991). Site-directed mutagenesis of residues of the transmembrane domain of GalT suggested that uncharged polar residues are critical for the ability of these hydrophobic domains to mediate Golgi retention (Aoki, D. *et al.*, 1992). Yet there have been numerous studies which have indicated that considerable alterations can be made to the transmembrane domain of glycosyltransferases without abolishing Golgi retention. First, it was demonstrated that the transmembrane domain of ST6Gal I could be totally replaced by a poly-leucine sequence of similar length, without affecting the enzymes localization (Munro, S., 1991). This same study also reported that the length of the poly-leucine segment seemed to be important in maintaining efficient Golgi localization

since a transmembrane domain of 23 leucine residues showed leakage to the cell surface. Contrasting this length requirement, when the 17 amino acid transmembrane domain of ST6Gal I was replaced with the 29 amino acid long transmembrane domain from influenza neuraminidase there did not appear to be a disruption of the retention signal (Dahdal, R.Y. *et al.*, 1993). A study which further demonstrated just how contradictory these results could become was highlighted when it was shown that swapping the transmembrane domains of two cell surface proteins resulted in hybrid molecules which either accumulated in the Golgi or were retarded in transport through the Golgi apparatus (Low, S.H. *et al.*, 1994). From all of these results it is quite evident that the hydrophobic transmembrane domain *in relation to its charged flanking sequences* is important in transport to the Golgi apparatus, and most likely the specific compartments within it. As discussed above, additional sequences from the stem and cytoplasmic regions of these enzymes have been implicated in the localization of these enzymes, although the potential role of the cytoplasmic domain has been overlooked in most studies. Figure 12 gives a breakdown of three glycosyltransferases, the four regions of which they are composed, and how each of these regions may be involved in the Golgi localization of these enzymes. As cautioned by Colley, the Golgi retention requirements for the enzymes listed in this table are purely general and it should be emphasized that these may vary in different cell types.

There may be a few reasons for the apparent lack of agreement between these Golgi localization studies, as pointed out by Gleeson. In the majority of



GlcNAcT I	GalT	ST
Not analyzed	Not analyzed	Not required
Retention region.	Possible accessory role in retention	Probable independent retention region
Retention region	Primary retention region. Length or characteristics important?	Retention region with flanking sequences involved; length not important.
Retention region	Possible accessory role in retention?	Not required.

Figure 12. Glycosyltransferase domain structure and general Golgi retention requirements. It should be noted that the requirements for the enzymes listed in the table are purely general and they may vary in different cell types. (Diagram taken and modified from Colley, K.J., 1997).

studies, sequences involved in Golgi localization have been identified by their ability to transport a reporter protein. Since different reporter molecules have been used by each of the groups it is possible that "these molecules may affect the conformation of the putative retention motif" (Gleeson, P.A. *et al.*, 1994) Secondly, the exact defining region of the transmembrane domain has varied among research groups. Usually, charged residues signify the boundaries of this domain, yet in a few cases these charged amino acids have been included as part of the transmembrane domain, which in turn would change amino acids which defined the stem region and cytoplasmic tail. In fact, three separate groups had independently cloned and sequenced the same sialyltransferase enzyme, but each had reported the transmembrane domain to occur in a different region of the protein (Sasaki, K. *et al.*, 1994; Nara, K. *et al.*, 1994; Haraguchi, M. *et al.*, 1994). Another obvious, yet equally critical factor to consider is that there really isn't any direct evidence that the enzymes localized within the various Golgi subcompartments are retained by identical mechanisms. There may be subtle differences between the way in which the *cis*, *medial*, *trans* and TGN recognize and retain the enzymes specific for their respective compartments.

Another issue that has drawn attention to the Golgi localization quandary is determining the mechanism which is used by these enzymes to locate themselves into the proper compartment. For instance, even if it were shown that the transmembrane domain itself was responsible for locating all the Golgi enzymes in their proper cisternal membrane, it still wouldn't answer *how* these enzymes

organize themselves in this manner. It is unlikely that localization of glycosyltransferases involves a simple receptor-ligand interaction where the receptor is fixed in the Golgi cisternae, since it has been widely shown that over-expression of wild-type transferases does not result in saturation of the retention mechanism (Teasdale, R.D. *et al.*, 1992; Aoki, D. *et al.*, 1992; and Nilsson, T. *et al.*, 1991). Furthermore, a retrieval system which brings proteins back to their proper compartments, as is the case for ER proteins, could be conceived, except studies have shown that enzymes which escape the compartments in which they are designed to locate themselves do leak to the cell surface and are not retrieved back to the Golgi apparatus (Wong, S.H. *et al.*, 1992 and Teasdale, R. *et al.*, 1994). Therefore, two potential Golgi retention mechanisms have been proposed, the oligomerization or 'kin-recognition' model and the lipid-sorting, or 'bilayer-thickness' model. The kin-recognition model proposes that the enzymes in a particular cisterna interact to form structures too large to enter transport vesicles (fig. 13). This model is based on three assumptions. First, that the Golgi enzymes are homodimers because their lumenal domains, which comprise of the catalytic and stem region, are bound together. The second assumption is that the spanning domains of Golgi enzymes can specifically bind to those of their 'kin'. In other words, *medial* enzymes should bind to each other, but not to *trans* enzymes. This is demonstrated in the figure by having sialyltransferase (ST) and galactosyltransferase (GalT) bind together while GlcNAcT-I and mannosidase II (Mann II) link to each other in the same manner. Although the latter two enzymes

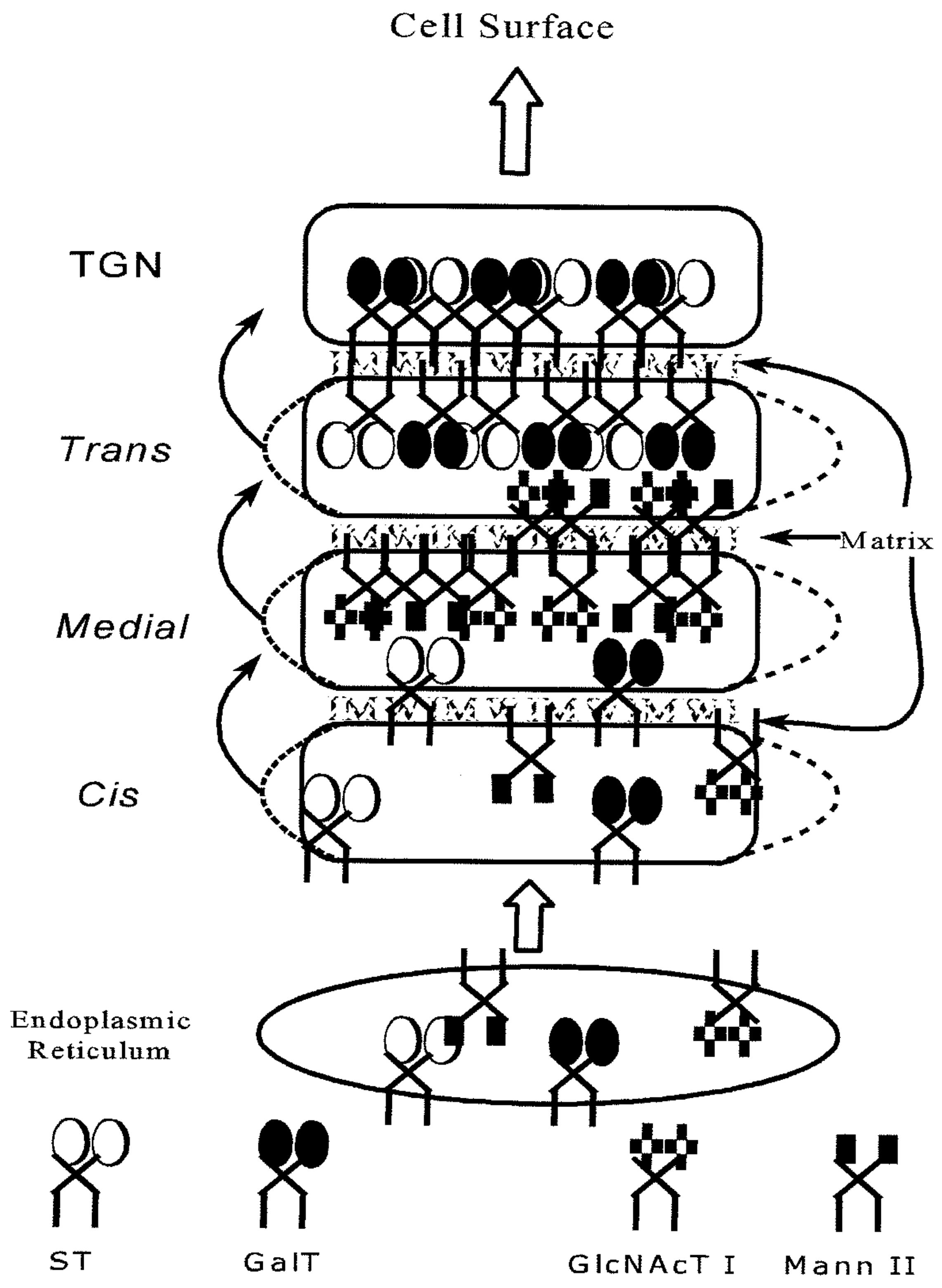


Figure 13. Model for kin-recognition.

are considered *medial* enzymes, they have been found in the *trans* region of the Golgi in many cell types. The last assumption is that the hetero-oligomers are anchored to the cisternal membrane, acting almost as a long chain. By forming such an interaction, the size of the hetero-oligomer would not be able to fit into transport vesicles budding from the cisternal rims, as indicated by the dashed lines. Therefore, there would have to be an intercisternal environmental factor which precipitates the formation of these oligomers in the proper compartment. The bilayer-thickness model envisages that the bilayer of the Golgi cisternae is not homogeneous, but contains distinct lipid domains in which the enzymes locate themselves depending on the length of their transmembrane domain. In fact, it has been demonstrated that the Golgi apparatus contains substantial amounts of cholesterol, and there is some evidence that it forms a gradient which in turn affects the thickness of the bilayer (Bretscher, M.S., 1993). Simply put, a shorter transmembrane domain within the enzyme in question would make it energetically unfavorable for a protein to enter the domain where the bilayer is thicker. It should be noted that the two mechanisms may not be exclusive and that in a variety of circumstances the two mechanisms may be employed at the same time.

Chapter 4: The Acute Phase Response

I. Inflammation: The Double Edged Sword

In the aftermath of injury, trauma or infection of a tissue, a complex series of reactions are executed by the host in an effort to prevent ongoing tissue damage, to isolate and destroy the infective organism and to activate the repair process. However, a key and essential feature of these immune reactions is the ability to return to the same state as it was before it was challenged. In fact, an excessive production of inflammatory mediators may have a negative or even lethal effect, as in the case of bacterial septic shock. This may develop within a few hours following infection by certain Gram-negative bacteria including *E. coli.* and the symptoms, which often are fatal, include a drop in blood pressure and widespread blood clotting in various organs. It is therefore essential that the early and unspecific but highly complex reactions which control the immune reaction be under strict control at all times. This process is known as inflammation, and the early and immediate set of reactions that are induced are known as the acute phase response (APR). Specifically, the APR is a predetermined and well-orchestrated sequence of processes which are initiated at the site of infection or trauma, leading to the release of soluble mediators that mobilize the metabolic response of the whole organism.

As shown schematically in Fig. 14, the APR can be considered as two separate, yet co-dependent systems, the local and systemic reactions. If there is

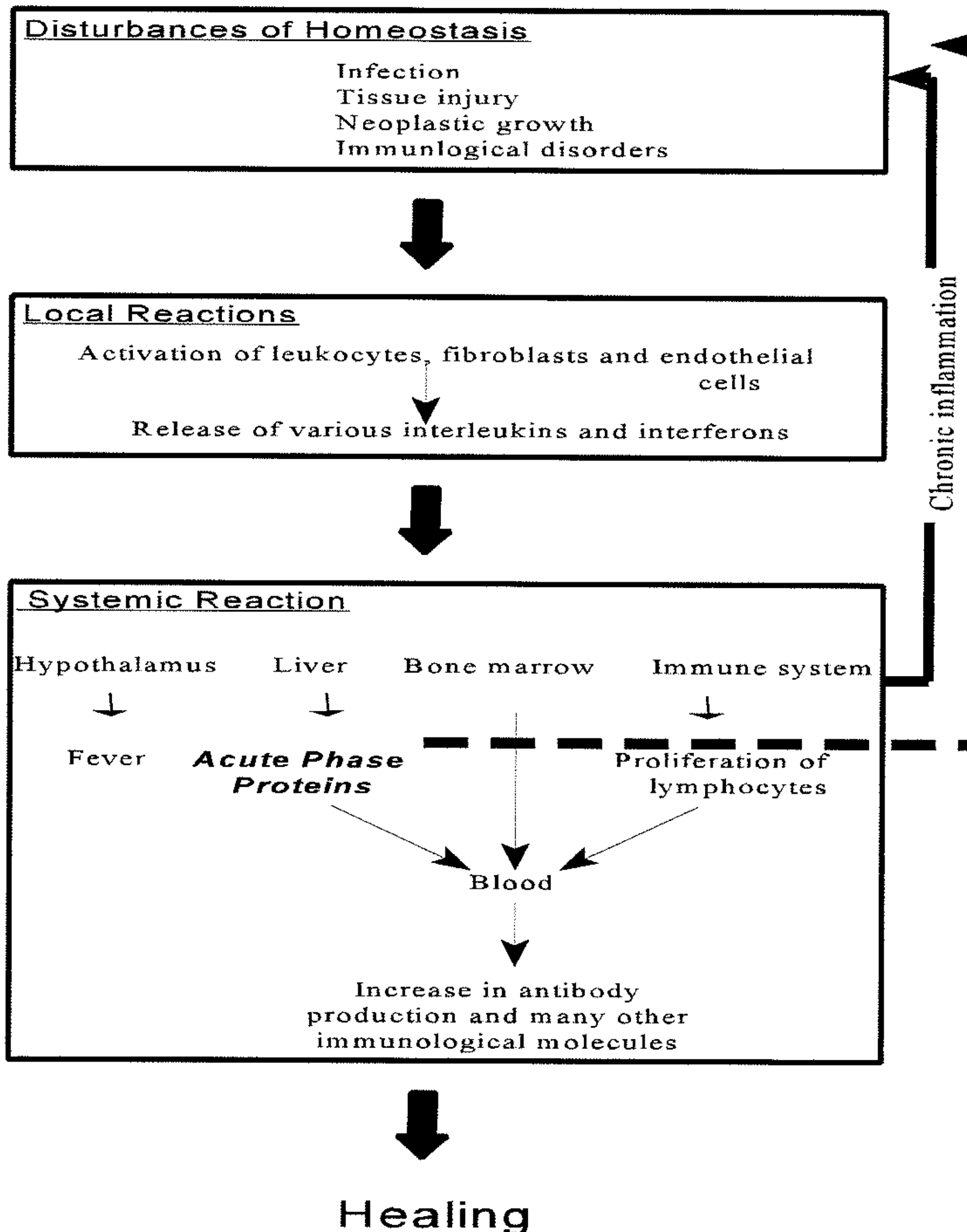


Figure 14. Schematic representation of the acute phase response. The multitude of systemic reactions, like fever, proliferation of white blood cells and the release of acute phase proteins from the liver (dashed line), all contribute to the healing of the wound by preventing the spread of infection and localizing the site of the tissue damage. If this cascade of events is not regulated properly, but continues to act on the system long after it is needed, then chronic flare ups (indicated by the solid line) could result which may last a lifetime.(Diagram modified from Heinrich, P.C. *et al.*, 1990).

a disturbance in this process, the immediate response is to localize the cause of the disturbance and prevent it from spreading to other regions. The two main features of this localization are the formation of blood clotting factors, which help to create a physical barrier around the inflamed tissue and dilation which causes blood vessels to leak and permits the accumulation of immune cells, such as leukocytes. The increased permeability of the surrounding blood vessels also allow for the release of 'messenger molecules' like the interleukins and interferons. These mediators act on specific target cells in a wide variety of places, such as bone marrow and the hypothalamus, which in turn cause the systemic reactions. The APR typically lasts from 2 - 4 days, however, as illustrated by the arrows on the right side of figure 14 these systemic reactions can lead to chronic inflammatory disorders, such as arthritis or colitis, if not properly regulated. In fact, it is not known which control step is crucial to the conversion from natural APR to chronic inflammation and an understanding of this change will benefit the possibility of therapeutic intervention to reverse the damaging aspects of this event.

A primary characterization of the systemic reactions is the dramatic change in the concentration of some plasma proteins, commonly referred to as acute phase reactants. Upon stimulation from mediators released from the local reaction, primarily interleukin-6, (Koj, A., 1996) the liver becomes the major organ for the synthesis of acute phase reactants (Miller, L. L., 1951). In fact, the response to local inflammation is quite often referred to as the hepatic acute phase response, because of the primary role that the liver plays. As shown in figure 15, these acute

phase reactants have a wide range of functions, which vary from directly neutralizing inflammatory agents to participating in tissue repair and regeneration. In addition, the acute phase reactant patterns vary from one species to another and in the level to which they increase or decrease in the blood as shown in table 1 (Stadnyk, *et al.*, 1991). Acute phase proteins are commonly defined as those whose plasma concentration rises by at least 25% (Stadnyk, A.W. *et al.*, 1991). However, any change in plasma level as the result of an inflammatory response may constitute a protein as an acute phase protein, even negative changes. Major increases are defined as 10 - 100 fold while medium are 2 - 10 fold. Those which decrease in activity during the acute phase response do so to allow for the increase in the capacity of the liver to synthesize the induced proteins. Two of the major acute phase proteins are the C-reactive and serum amyloid A proteins. Ironically, out of all the proteins the activities of these two are the least well known. Numerous aspects of the acute phase response have been studied, from examining the effect of diet (Jennings, G. *et al.*, 1996) to how circadian variation and the time of day play a role in the responses of various local and systemic reactions (Perdiz, P. *et al.*, 1996). However, it still remains that the true functions of most acute phase reactants have evaded many investigators (Koj, A., 1997).

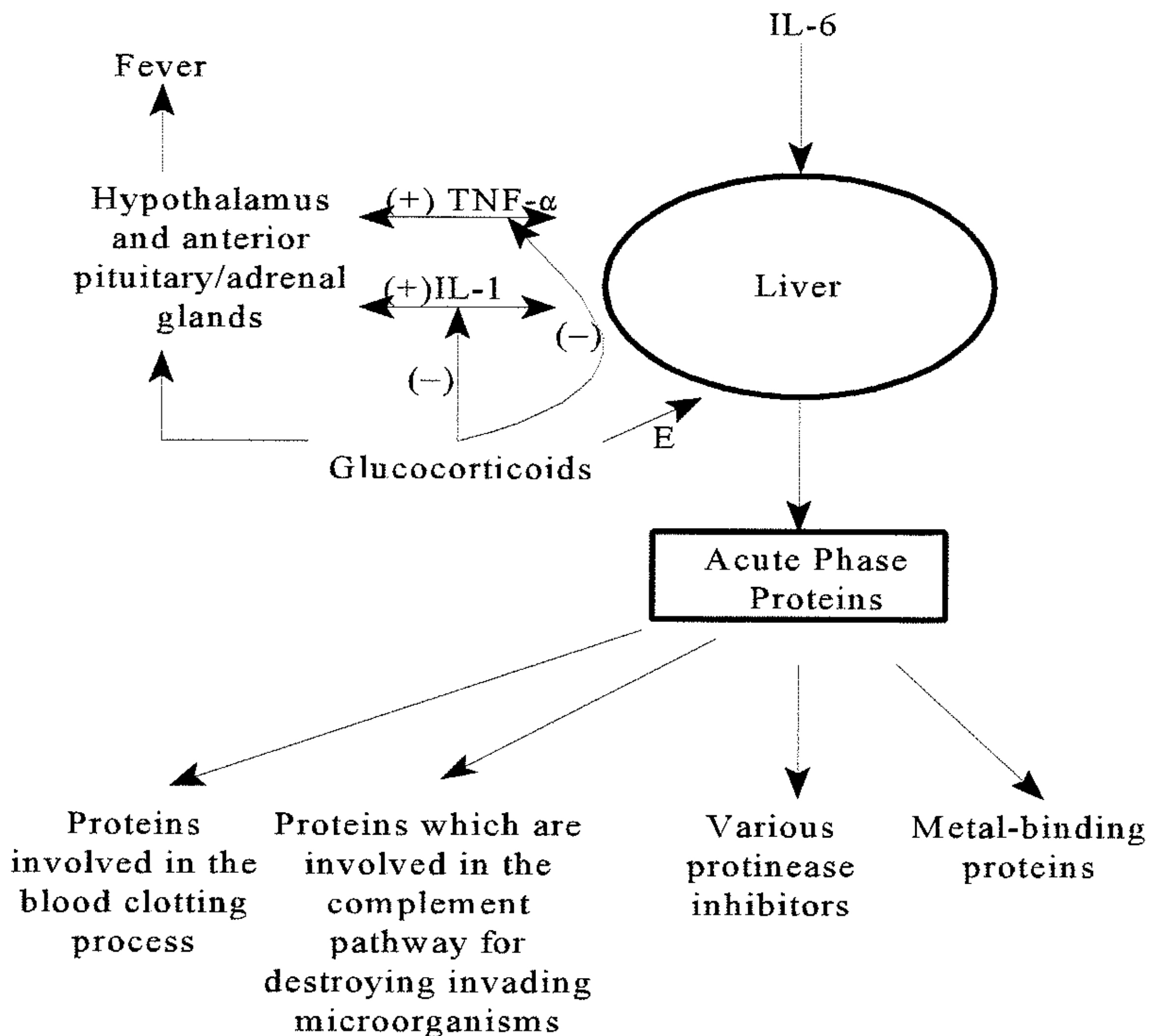


Figure 15. Inflammatory mediators that modulate hepatic APR. The liver is the primary source of acute phase proteins, which it synthesizes when acted upon by various cytokines that are released from the site of inflammation. The most potent of these is IL-6 and it alone has been shown to induce the liver into creating acute phase proteins when the recombinant human form was administered in rats (Geiger, T. *et al.*, 1988). Interestingly, many of the genes of acute phase proteins have the same consensus sequence, CTGGGAA, in the promoter region. IL-6, along with appropriate transcription factors, have been shown to be directly involved with this consensus sequence and the subsequent increases in the mRNA levels (Tsuchiya, Y. *et al.*, 1987). Abbreviations: (+), stimulation of activity; (-), inhibition of activity; (E), enhancement of activity. (Diagram modified from Baumann, H. *et al.*, 1994).

Protein	Species	Biological role
Major concentration increases		
C-reactive protein	Human, rabbit, rat	Immunomodulating
α1Acid glycoprotein	Most species	Transport protein
α2 Macroglobulin	Rat	Antiprotease/transport protein
Serum amyloid A protein	Human, mice	Assists in macrophage binding
Medium concentration increases		
ST6Gal I	Human, rat, mouse	Localize tissue damage (?)
Haptoglobin	Most species	Binds hemoglobin
Angiotensinogen	Human, Rat	Blood pressure
Fibrinogen	Most species	Coagulation
Ceruloplasmin	Most species	Oxygen scavenger
Negative changes in concentration		
Albumin	Most species	Transport protein
Transferrin	Most species	Transport protein

Table 1. The acute phase proteins and their biological roles.

II. Glycosylation: A role in the APR?

It has been well documented that the acute phase reactants vary in concentration during the inflammatory response, not only in the various tissues which produce them, but in the serum. However, a growing curiosity in the glycoconjugates and glycosyltransferases is taking place within the biochemical community (Kornfeld, S., 1998). This interest is driven by the fact that the degree of branching on the glycan chains of many glycoconjugates either increase or decrease in complexity. For instance, α_1-acid glycoprotein (AGP) is a normal plasma glycoprotein which increases by two- to five-fold in the host response to tissue injury. Additionally, forty-five per cent of its molecular mass is composed of carbohydrate units (Van Dijk, W. *et al*, 1995). AGP in humans (Schmid, K. *et al.*, 1977) possess five complex-type N-linked glycans and has been thoroughly investigated because this is one of the few serum glycoproteins that contains tetraantennary as well as di- and triantennary, N-linked glycans. Rat (Yoshima, H. *et al* 1981) and mouse (Baumann, H. *et al.*, 1984) AGP contain 4-6 N-linked complex-type glycans which are also heavily glycosylated. In fact, at least 12 glycoforms of AGP can be detected in normal human serum (Van der Linden, E.C.M. *et al.*, 1994), which differ in the degree of branching as well as in the degree of fucosylation and sialylation. Studies from numerous laboratories have shown that this microheterogenity of AGP does not represent a non-specific process but is strongly dependent on the physiological conditions (Van der Linden,

E.C.M. *et al.*, 1998). The basic reason behind the differentiation in structure is the change in the availability and/or organization of the glycosylation machinery of the cell. Several mechanisms can be used to explain how carbohydrate structures change on glycoconjugates:

1. Glycosyltransferase changes in concentration due to a decrease in synthesis of the particular enzyme.
2. Alterations in intracellular transport and organization of molecules.
3. Recycling, shedding, and degradation of glycoconjugates may be affected.
4. Alterations of nucleotide sugar synthesis and transport.
5. Abnormal degradation by glycosidases.
6. Change in the level of inhibitors or co-factors, such as metal ions that contribute to the activity of glycosyltransferases.

In many cases, more than one of these factors may play role in the final outcome of the glycosylated product. A primary factor that has been determined as a source of change in glycosylation patterns during disease is the role of the glycosyltransferases. Carbohydrate abnormalities that are detected in disease are usually linked to an abnormality in these enzymes (table 2). If research shows that a particular disease is primarily the result of a specific change in the structure of a carbohydrate, then it is not unreasonable to assume that treating the ailment could be accomplished by controlling the biosynthesis of the glycoconjugate. In other words, the ability to control the activity of a specific glycosyltransferase at the intracellular level, and thus in turn control the final structure created, could pave

Disease	Carbohydrate abnormality	Glycosyltransferase abnormality
Bladder cancer	Less sulfation More branching More O-acetyl sialylation	Incr. Gal-T Incr. GlcNAcT-V
Choriocarcinoma	Unusual N-glycans More branching More α6 Fucosylation Less sialylation	Decr. ST Incr. GlcNAcT activity
Lung Cancer	More branching	Incr. GlcNAcT activity Incr. Serum α3 FT Incr. β4GalT Incr. ST Incr. α2 FT
Cystic Fibrosis	More O-glycosylation More mucin sulfation More α6 Fucosylation in fibroblasts	Incr. GlcNAcT activity
Rheumatoid arthritis	Less Gal on IgG	Decr. GalT
Leukemia	More sialylation Less α3 Fucosylation	Incr. ST
AIDS	More branching Incr. Bisected structures	Incr. ST Decr. GlcNAcT-III (?) Incr. FT

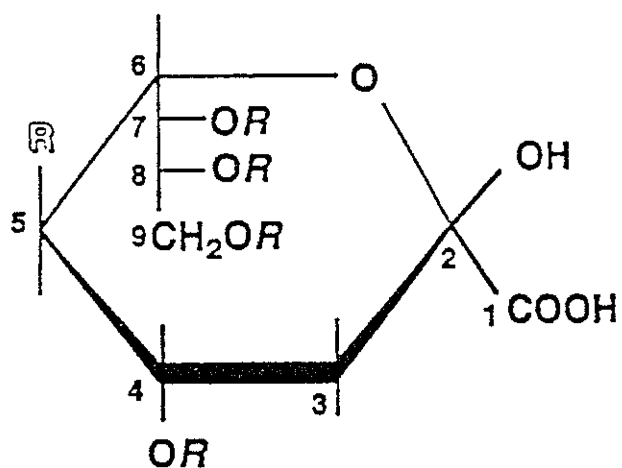
Table 2. Glycosylation changes in human disease. Data taken from Brockhausen, I.,1993

a new direction in the treatment of certain specified diseases. For example, the mechanisms of tumor cell detachment from the primary tumor, survival in the blood, resistence to immune attack and growth may all be influenced by cell surface carbohydrates (Nicolson, G. L. *et al.*, 1984). Viruses often contain membrane lipids and glycoproteins on their surfaces. The role of these carbohydrates may be to "shield the virion from immune attack or from degradation or to interact with components of the host cell or other viruses" (Andersson, A.M. *et al.*, 1997). If the specific glycosyltransferases from cancer cells could be inhibited, or effectively altered in such a way so as to prevent the proper carbohydrate chain from being created, then it is conceivable that an attack on the host's system could be kept at a minimum. Such an agent, might, for instance, be one of the hormones or cytokines which have been shown to influence glycosylation (Mackiewicz, A. *et al.*, 1989). If the defect has been pinpointed to a specific gene of a glycosyltransferase, then gene therapy may be possible, either to "counteract the defective gene product or to increase or decrease an aberrant level of expression" (Delves, P.J., 1998)

III. Sialylation: The Terminal Fingerprint

Carbohydrates may influence immune recognition in at least two ways: firstly, they influence the conformation of glycoproteins and sometimes modulate their biological activity (Sharon, N., 1984) and secondly, they may serve as recognition

determinants because they contain such a high potential for structural variation (Cook, G.M.W., 1986). Sialic acids contribute greatly to both of these effects. They constitute a family of 9-carbon nonulosonic acids usually found at the non-reducing termini of oligosaccharides. In addition to the commonest sialic acid, N-acetylneuraminic acid, over 40 natural sialic acids, varying in terms of the nature of the *N*-substituent of neuraminic acid and in the number, position or combination of *O*-substituents have been identified so far (Klein, A. *et al.*, 1998). The substituent on the carbon 5 can be one of an amino, an acetamido, a glycolylamido, or a hydroxyl group and defines the four major types of sialic acid: neuraminic acid (Neu), *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid and ketodeoxy nonulosonic acid (KDN) (fig. 16). Most of the other members derive from substitutions of the hydroxyl groups present on carbons 4, 7, 8 and 9 by a methyl, an acetyl, a lactyl, a sulphate, or a phosphate group (Varki, A., 1992). No other natural sugar demonstrates such a degree of structural diversity, although every species expresses only a limited number of modified sialic acids (Schauer, R., 1985). The most important function of sialic acids, with respect to immune recognition, is probably their ability to act as "biological masks via steric hindrance and/or electrostatic repulsion". This is due to the highly electronegative and hydrophilic properties of the molecule (Pilatte, Y., 1993). With the ability to act as a recognition molecule, and a strong ability to affect the overall structure of the carbohydrate chain itself, understanding *how* and *why* carbohydrate chains are



Ⓡ	*R*	sialic acid	Abbreviation
CH_3CONH	H	*N*-Acetylneuraminic acid	Neu5Ac
$HOCH_2CONH$	H	*N*-Glycoloylneuraminic acid	Neu5Gc
OH	H	Ketodeoxynonulosonic acid	KDN
	CH_3CO-	*O*-acetylated sialic acid	Sia Ac

Figure 16. The nature, position of the substituent, and name of the sialic acids.

sialylated during various physiological conditions is crucial. Obviously sialyltransferases are of primary importance in that they determine the type of glycosidic bond between sialic acids and the penultimate sugars. However, there is evidence that the degree of sialylation of specific glycoconjugates on the surface of immune cells does not correlate with the levels of sialyltransferase activity within the cells. For example, specific cell surface molecules such as MHC class II molecules are more heavily sialylated in B-cells than in T lymphocytes, whereas these differences are not correlated with the levels of sialyltransferase activity (Merlu, B., *et al.*, 1989). Likewise, the overall level of sialyltransferase activity has been shown to be either increased or unaffected in T lymphocytes following mitogenic stimulation where the degree of sialylation in some of the key glycosylated molecules actually decreased (Landolfi, N.F. *et al.*, 1985). These contradictions may be attributed to the fact that phenomena involving both the organization and localization of sialyltransferases within cells, as discussed in chapter 3, have yet to be discovered. Examples of such possible phenomena may include a change in the intracellular trafficking of specified cells upon stimulation of the immune response where the glycoconjugates in question are not brought into contact with the proper sialyltransferases. It may also be that the sialyltransferases themselves are transported to other cellular locations due to proteolytic cleavage from their membrane anchor (Richardson, K. *et al.*, 1994). Along with this finding, it has recently been demonstrated that soluble glycosyltransferases glycosylate less efficiently than their membrane bound counterparts (Zhu, G. *et al.*, 1998).

It is clear that a more defined link between sialylation and the immune response is needed. The aim of the following thesis is two-fold: first, to study how the acute inflammatory response affects the overall levels of sialyltransferase activity throughout the host and in particular, how the organization of these enzymes may be affected at the intracellular level. Secondly, a study which examines how the sialyltransferases are able to orient themselves properly in the Golgi membrane for optimum catalytic efficiency is undertaken. Other enzymes, such as the *N*-acetylglucosaminyltransferases are also investigated for comparative purposes.

Introduction to Current Work

The acute phase response to injury is standard protocol in the immune system's constant challenge to localize tissue damage and infection. A major source of proteins and enzymes involved in the immune response are derived from the liver. Earlier work in this laboratory had demonstrated that ST6Gal I increased in activity 2-fold in the liver during inflammation, while serum levels had increased 5-fold over the same time period. The ST6Gal I in the serum is thought to be due to the proteolytic release of the membrane bound form of the enzyme, a result of the lysosomal enzyme cathepsin D cleaving the enzyme somewhere within its 'stem' region. This was proven by using liver slices, a method which has been employed in this laboratory for 25 years (Jamieson, J.C. *et al.*, 1975). The incubation of thin slices of liver in appropriate medium mimics the natural tissue system of the hepatocytes. Evidence that cathepsin D was the proteolytic enzyme responsible for the release of ST6Gal I was achieved in two ways. Firstly, pepstatin A is a well known inhibitor of cathepsin D (Lammers, G. *et al.*, 1988). By supplementing both liver slices and sonicated Golgi membrane samples with this inhibitor, the amount of enzyme released was significantly reduced. Secondly, pure cathepsin D isolated from bovine spleen was administered to sonicated Golgi membranes at reduced pH, which resulted in more enzyme being released from the membranes than when there wasn't any supplementation with cathepsin D.

A primary goal of this thesis was to examine other glycosyltransferases from the liver and to see if they also were released into the serum during the acute phase response. Three additional sialyltransferases (ST3Gal I, SAT-I and GlcNAcα2-6ST) and three *N*-acetylglucosaminyltransferases (GlcNAcT-I, GlcNAcT-III and GlcNAcT-V) were assayed from the liver and serum of animals before the onset of inflammation. These values were compared to samples taken at different time intervals over a 72 hour period following a subcutaneous injection of turpentine so that the maximum change in the level of activity in the liver and serum could be identified. As discussed, cathepsin D was shown to be responsible for the release of ST6Gal I from its membrane anchor in the Golgi complex. Using the same procedures, Golgi membranes isolated from hepatocytes were opened with sonication or detergent at reduced pH. All four enzymes studied in this set of experiments, ST3Gal III, SAT-1, GlcNAcα2-6ST and GlcNAcT-I were released in a soluble form at the same optimal pH, 5.6, as determined for ST6Gal I. Addition of exogenous cathepsin D to disrupted Golgi membranes at pH 5.6 caused an increased amount of these enzymes to be released. This suggested that a similar mechanism involving cathepsin D was responsible for the release of these glycosyltransferases from their Golgi membrane anchor. Although an attempt is made in this thesis to find where in the stem region the cleavage site occurs, it is difficult to locate since the target sequence requirements for proteolytic cleavage promoted by cathepsin D has remained elusive (Van Noort, J. M. *et al.*, 1989).

The fact that ST6Gal I increases in the serum during the acute phase response prompted an investigation to see if other glycosyltransferases behaved similarly. Of the four enzymes that were released from their membrane anchor at reduced pH in the presence of cathepsin D, only GlcNAcT-I and GlcNAcα2-6ST were found to appear in serum in a catalytically active form. Liver slice experiments were used to demonstrate a correlation between the level of activity in the liver and serum. GlcNAcT-I increases in activity in the liver and in the liver slice medium while GlcNAcα2-6ST decreases in both. These results demonstrate that as there is a change in the activity levels of the glycosyltransferases within the liver, serum levels are directly affected.

As mentioned, the liver is the primary source of acute phase reactants. However, an interest in examining other tissues as donors of serum glycosyltransferases was sparked by evidence that an acute phase response occurs in human intestinal epithelial cells (Molmenti, E.P. *et al.*, 1993). Thus, the kidney and spleen were selected for examination. Although the investigation of these two organs and their involvement in the acute phase response was not as thorough as that of the liver, their levels of glycosyltransferase activity, in some instances, did appear to be affected by the onset of acute inflammation.

The original Golgi isolation procedure resulted in two visibly identifiable fractions, the first one primarily enriched with Golgi membranes and one directly

beneath it enriched in smooth endoplasmic reticulum (Leelavathi, D. D. *et al.*, 1970). In the past, the work in our laboratory had understandably discarded the latter fraction and instead collected only the Golgi fraction to measure enzyme activity. However, the inflammatory response would very likely influence the density of the Golgi apparatus due to the increased amount of cellular trafficking that would be occurring. Therefore both fractions were assayed for the same glycosyltransferases before and during inflammation. Evidence from these studies suggested that there was an increase in Golgi membrane concentration in the fraction which was originally enriched in smooth endoplasmic reticulum, when isolated under control conditions. Expanding on these findings, the two fractions were pooled together and then separated on a continuous sucrose gradient *via* ultracentrifugation. Individual fractions from this gradient were then assayed for glycosyltransferase activity. The ultimate purpose for performing this experiment was to demonstrate the possibility that during inflammation these enzymes not only change in level of activity but may also re-route in subcellular location within the Golgi.

It is unclear how the Golgi apparatus is able to maintain its unique architecture, given the huge flow of cellular traffic that it endures. Some of the most convincing evidence of how this is achieved was presented when structures, shown to be composed of proteins, were identified between the individual cisternal membranes of the Golgi *via* electron microscopy (Cluett, E.B. *et al.*, 1992). When these structures were digested with proteinases, the Golgi membranes began to

unstack. This was followed by the report of a protein matrix isolated from the rat liver Golgi membranes which was capable of binding only *medial* enzymes (Slusarewicz, P. *et al.*, 1995). This was achieved by solubilizing the Golgi membrane with detergent so that the underlying matrix would be exposed while solubilizing as little of the *medial* enzyme as possible. These investigators then suggested that the cytoplasmic tail portion of the enzyme was responsible for the association to the matrix. Support for this idea derived from the finding that salt could disassociate the enzyme-matrix union, while subsequent removal of the salt by dialysis resulted in the enzymes ability to rebind to the matrix. Research in this laboratory is primarily interested in the sialyltransferases, which are *trans* Golgi membrane based enzymes (Harduin-Lepers, A. *et al.*, 1995). Therefore, there was an interest in identifying an intercisternal matrix from the Golgi that would be capable of binding these enzymes. Using similar methods as the above study, a matrix was identified from the Golgi apparatus which was able to associate with the sialyltransferases in the same salt dependent manner. In an effort to understand how and why this association occurs, a model, suggesting the importance of the positive charge of the cytoplasmic tail, is presented. The positive charge on the N-terminal region of proteins is thought to play an integral role in determining their topology by binding to a 'negatively charged partner' (Parks, G. D. *et al.*, 1991; Nilsson, I. *et al.*, 1990). The topology of the sialyltransferases, and most glycosyltransferases, are orientated in the Golgi so that the catalytic region is situated within the lumen where the nucleotide sugar donors and glycoconjugate

acceptors are located. It is conceivable that this matrix is indeed the negatively charged partner to which the positively charged N-terminal cytoplasmic tail associates with.

The goal of this thesis is two-fold: first, to present a study on the effects of acute inflammation on the activity levels of the glycosyltransferases and how the lysosomal enzyme cathepsin D may be involved. Secondly, to identify a matrix in the *trans*-region of the Golgi complex which would be capable of associating with the sialyltransferases. This work will contribute towards a deeper understanding of how the complex array of reactions which constitutes glycoconjugate biosynthesis orchestrates itself within the Golgi during normal homeostatic conditions as well as diseased states.

Experimental

Materials

CMP-[4,5,6,7,8,9-^{14}C] NeuAc (247mCi/mmol) was obtained from New England Nuclear Corp., (Bedford, MA). UDP-[Acetyl-1-^{14}C]-N-acetyl-D-glucosamine (25mCi/mmol) was from ICN Pharmaceuticals, Inc., (Costa Mesa, CA). Sep-Pak cartridges were from Waters Chromatography Division (Milford, MA). Whatman 3MM filter paper and 2.5 cm diameter Whatman No. 1 filter paper discs were from Fisher Scientific, Edmonton, AB, Canada. Dowex 1-X8 (Cl^- form, 200-400 mesh) was from Bio-Rad, Mississauga, ON, Canada. Sephadex (G-50 fine) was purchased from Pharmacia Biotech Inc., Baie d'Urfe, PQ, Canada. Rat asialo α_1 AGP and asialoagalacto α_1 AGP were prepared as previously described (Kaplan *et. al*, 1983). LS-Tetrasaccharide-a (LSTa) was purchased from Oxford GlycoSciences, Inc., (Rosedale, NY). Lactosyl ceramide was from Sigma Chemical Co., St. Louis, MO. Acceptors for the N-acetylglucosaminyltransferase I, III, and V enzymes were a generous gift from Dr. Ole Hindsgaul (Department of Chemistry, University of Alberta, Edmonton, AB, Canada). Cacodylate, MES, AMP, GlcNAc, and CMP-NeuAc were obtained from Sigma Chemical Co., St. Louis, Mo. β-galactosidase from both bovine testes and *Diplococci pneumoniae* was purchased from Sigma. Turpentine oil was purchased from Sargent-Welch Scientific Co., Skokie, Ill. Aqueous Counting Scintillant (ACS) was obtained from Amersham

Corp., Oakville, Ont. Pepstatin A, bovine spleen cathepsin D, penicillin G, Proteinase K and streptomycin sulphate were obtained from Sigma. Manual fraction collector was generously supplied by Dr. Herb Lejohn (Department of Microbiology, University of Manitoba, Winnipeg, MB, Canada). Imidazole and TX-100 were purchased from Sigma. Lubrol W was a gift from Imperial Chemical Industries, U.K. Dialysis membrane tubing was from Spectrum, Gardena, CA.

Methods

1. Preparation of serum and tissue samples

A. Source of mammalian samples

Male Long-Evans hooded rats (150-200 g) were purchased from Charles River Inc., St. Constance, Quebec and maintained on a diet of Purina laboratory chow and purified water. Inflammation was induced according to Ashton *et al.* (1970). The rats were anesthetized with ether, and then given a subcutaneous injection into the dorsolumbar region of 0.5 ml oil of turpentine per 100 g body weight. Control animals received injections of sterile 0.15 M NaCl.

B. Preparation of serum and liver homogenate samples

Serum was prepared as described by Jamieson *et al.* (1972). Rats were anesthetized with ether and sacrificed by severing the jugular veins. Blood was collected into a beaker and allowed to clot for an additional hour while kept on ice. The serum was obtained by centrifuging the clotted blood at 2500 g_{av} for 10 min at 4°C. Livers were perfused *in situ* by severing the portal artery and inserting an 18 gauge needle into the hepatic vein and flushing the livers with ice-cold 0.15 M NaCl until the organ appeared clear of any internal blood. The liver was then excised and rinsed twice by gentle agitation in 200 ml of ice-cold 0.15 M NaCl. Liver homogenates were prepared according to Kaplan *et al.* (1983). Perfused livers were weighed, minced into several small pieces with a surgical blade on an ice-cold

aluminum block and homogenized in 7.5 volumes of ice-cold 0.25 M sucrose per 1 g of liver tissue using 10 up and down strokes of a Potter-Elvehjem homogenizer at 2000 rev / min.

C. Preparation of liver slices

Liver slices were prepared according to Jamieson *et al.* (1975). Liver tissue from livers that had been perfused with 0.15 M NaCl was placed on an ice-cold aluminum template containing a groove 7 mm wide and 0.36 mm deep (Hultin *et al.,* 1960; Simkin *et al.,* 1967), and bathed in ice-cold 0.15 M NaCl. Slices were prepared manually using a blade supplied for the Stadie-Riggs tissue slicer, rinsed twice by gentle agitation in 200 ml of ice-cold 0.15 M NaCl, and maintained on ice in 0.15 M NaCl until use.

D. Preparation of liver slice medium samples

Incubation of liver slices and preparation of medium samples was according to the method of Jamieson *et al.* (1975) as modified by Kaplan *et al.* (1983). Freshly-prepared liver slices were transferred from 0.15 M NaCl and washed by gentle agitation in 200 ml of ice-cold incubation medium that had previously been purged with 95% O_2: 5% CO_2 for 30 min. Liver slices were blotted on filter paper, weighed, transferred to 25 ml incubation flasks that contained fresh medium and incubated at 37°C in an atmosphere of 95% O_2 : 5% CO_2 in a gently shaking water bath. Incubations were terminated by transferring the flasks to ice. Medium was

aspirated by hand using a Pasteur pipet, centrifuged for 15 min in a Micro-Centrifuge, model 235A (Fisher Scientific) to remove particulate material. Incubation medium contained KCl (77 mM), NaCl (39 mM), $NaHCO_3$ (32.5 mM), KH_2PO_4 (0.6 mM), $MgSO_4$ (3.1 mM), $CaCl_2$ (1.3 mM), glucose (25 mM), penicillin G (25 mg/L) and streptomycin sulfate (25 mg/L) (Marsh and Drabkin, 1958). Each 5 ml of incubation medium was supplemented with 0.1 ml of a stock solution of L-amino acids that contained alanine (48 mM), arginine (22 mM), asparagine (3.8 mM), aspartic acid (3.8 mM), cysteine (3.8 mM), glutamic acid (19 mM), glutamine (19 mM), glycine (43 mM), histidine (9 mM), isoleucine (9 mM), leucine (17 mM), lysine (48 mM), methionine (7 mM), phenylalanine (8 mM), proline (24 mM), serine (29 mM), threonine (29 mM), tryptophan (6.9 mM), tyrosine (9 mM) and valine (20 mM); this produced an amino acid concentration in the medium that was approximately twice that normally present in serum (Clemens *et al.*, 1970; Woloski *et al.*, 1983). Experiments designed to determine the effect of turpentine-induced inflammation on the release of sialyltransferase and N-acetylglucosaminyl transferase used 1 g of slices per 5 ml medium. The slices were incubated in the nutrient medium for 3 hours at 37°C in an atmosphere of 95% O_2 : 5% CO_2 in sterile tissue-culture plates that had been placed on a rotary shaker inside a Isotemp Incubator, model 655D (Fisher Scientific), centrifuged at 175,000 g_{av} for 30 min and the supernatant then assayed for enzyme activity. Experiments conducted to determine the effect of pepstatin A on the release of sialyltransferase and N-acetylglucosaminyltransferase from liver slices used 250 mg slices per 2.0 ml

medium. Liver slices were incubated at 37°C for 18 hours as described above.

E. Preparation of liver Golgi membranes

Golgi membranes free of lysosomal material were isolated according to the method of Leelavathi *et al.* (1970) as modified by Moremen and Touster (1986). All sucrose solutions were prepared using a 0.1 M potassium phosphate buffer, pH 6.5, containing 5 mM $MgCl_2$. Excised livers were first minced by hand on an ice-cold aluminum chopping block, and then gently homogenized on ice for 30 s in 4 volumes of 0.25 M sucrose using a Potter-Elvehjem homogenizer at 1000 rev / min. Large granule material was removed by centrifugation for 10 min at 600 g_{av} in a Ti60 rotor. Supernatants were layered on top of 20 ml of 1.3 M sucrose solution and centrifuged for 60 min at 75,000 g_{av} in a Ti60 rotor. Clear supernatant solutions were aspirated by hand using a Pasteur pipet and discarded. The crude Golgi membrane fractions appearing immediately above the 1.3 M sucrose layer were aspirated, combined as appropriate, and adjusted by weight to 1.1 M sucrose with sucrose. Ten ml aliquots of this initial Golgi membrane fraction were then layered on top of 20 ml of 1.25 M sucrose, covered with 5 ml of 0.5 M sucrose and centrifuged for 90 min at 80,000 g_{av} in a SW28 rotor. The dense white Golgi bands appearing between the 0.5 M and the 1.1 M sucrose layers were carefully collected by aspiration, and after adjustment to 1.1 M sucrose were subjected to a second identical discontinuous sucrose density gradient centrifugation. The resultant Golgi membrane bands were then carefully aspirated, pooled, diluted four-fold with 0.25

M sucrose, and then sedimented by centrifugation for 45 min at 175,000 g $_{av}$ in a Ti60 rotor. Golgi membranes were gently resuspended by homogenization by hand in ice-cold 0.25 M sucrose, adjusted with 0.25 M sucrose to a protein concentration of 10 mg / ml, and stored refrigerated on ice until required for use. Protein content was assayed according to the method of Lowry *et al.* (1951), as modified by Miller (1959), using crystalline bovine serum albumin as the standard. The final protein concentration was brought to 2.0 µg / µl, unless stated otherwise. The specific activity of the sialyltransferases and *N*-acetylglucosaminyltransferases present in each purified Golgi membrane preparation was assayed immediately prior to use, and the enzymes were typically found to be at least 40-fold and 30-fold greater in activity, respectively, than that of the liver homogenate from which it was prepared.

F. Detection of proteolytic cleavage of enzymes in the liver Golgi membranes

Golgi membranes resuspended at a concentration of 10 mg Golgi membrane protein / ml in ice-cold 0.25 M sucrose, containing 0.1 M potassium phosphate buffer, pH 6.5 / 5 mM $MgCl_2$, were ultrasonicated in 2.5 ml portions at 50 W using an Artek dismembrator fitted with a 1 mm-diameter titanium probe. Since sonication was found to inactivate the SAT-I enzyme, Golgi membranes were solubilized with the same 0.1 M potassium phosphate buffer, supplemented with 0.1% TX-100, for 30 min. By using this concentration of detergent, it ensured that the membranes were disrupted enough to release any cleaved enzymes, yet did not solubilize the

SAT-I enzyme from the membranes to any great extent (Richardson *et al.*, 1994). Since GlcNAcT-I is known to be resistant to TX-100 extraction (Slusarewicz *et al.*, 1994) membranes solubilized with 2% of the detergent were used to examine the release of this enzyme due to proteolytic cleavage. The release of GlcNAcT-I was also studied from sonicated Golgi membranes as described above. Golgi membranes disrupted by detergent solubilization were then centrifuged for 45 min at 175,000 g_{av} in a Ti60 rotor followed by careful resuspension in the buffered detergent. Samples of intact and permealbilized Golgi vesicle suspensions were adjusted to various pH values using equivalent amounts of 50 mM McIlvaine sodium phosphate-citric acid buffers (Elving *et al.*, 1956), placed in 2.5 ml Eppendorf tubes and incubated in a block heater at 37 °C either alone, with bovine speen cathepsin D, or with pepstatin A. Protein concentration was kept at 1 mg of Golgi membrane protein / ml. At the end of incubation, tubes were transferred to ice, pH was adjusted to 7.0 with 0.1 M imidazole-HCl buffer, pH 9.0, and incubation mixtures were adjusted to equal volumes with deionized water. Incubation mixtures were centrifuged for 45 min at 175,000 g_{av} in a Ti70 rotor. Supernatants were carefully removed using a Pasteur pipet, and pellets were gently resuspended in an equal volume of ice-cold 0.25 M sucrose containing 0.1 M potassium phosphate buffer, pH 6.5 / 5 mM $MgCl_2$ using a 1 ml-capacity Potter-Elvehjem homogenizer at 2000 rev / min. Supernatant and pellet samples were assayed immediately for sialyltransferase and *N*-acetylglucosaminyltransferase activities, and determination of protein content.

Stock solutions of pepstatin A dissolved in DMSO were added as appropriate to produce final concentrations of either 10^{-4} or 10^{-9} M with each incubation mixture containing equivalent amounts of DMSO; control incubation mixtures contained DMSO alone. Bovine spleen cathepsin D was present in incubation mixtures at a concentration of 1 µg per mg Golgi membrane protein.

G. Detection of cleavage site within stem region of sialyltransferases

The exact site of cleavage within the stem region of the enzymes was analyzed based on an earlier model (van Noort *et al.*, 1989). Cathepsin D was predicted to recognize a seven amino acid residues with the following consensus sequence: P_2 (hydrophobic) - P_1 (hydrophobic; leucine / aromatic) - P_1' (hydrophobic) - P_2' (charged; basic) - P_3' (x) - P_4' (x) - P_5' (charged; basic), with the cleavage site occurring between the P_1 and P_1' residues. It is difficult to pinpoint where the stem region ends because it is not known at which point the loss of a particular amino acid from the N-terminal region of the catalytic domain results in the inactivity of a particular glycosyltransferase (Paulson *et al.*, 1989a). In this study attention was focused on the first 100 amino acids in the N-terminal region. This number was more than adequate for the investigation as many soluble forms of glycosyltransferases have been produced during their purification, most likely due to the action of a variety of proteinases to which they had been exposed during the isolation procedure (Schachter, H., 1994). According to molecular mass determination, these soluble forms typically accounted for 85% of the membrane

bound enzyme. Sialyltransferases range in size from 340 - 410 amino acids (Tsuji, S., 1996). Thus, the cleavage site of the enzyme should be contained in the first 100 amino acids from the N-terminal, with the stem region being the main focus.

H. Preparation of kidney and spleen samples for enzyme assays

Kidneys were removed from rats as described in 1B. The fatty tissue was trimmed from the kidneys followed by two washes in 0.15 M NaCl. They were then weighed and minced with surgical scissors on a ice-cold aluminum block and homogenized on ice for 30 s in 4 volumes of 0.5 M sucrose in the same manner as the liver. Granular debris such as red blood cells, fat, and nuclei were removed by centrifugation for 10 min at 600 g_{av} in a Ti 60 rotor. The pellet was washed and resuspended in 0.25 M sucrose and adjusted to a protein concentration of 1 µg / µl then stored at 4°C. The supernatant fraction was layered onto of 20 ml of 1.3 M sucrose solution and centrifuged for 90 min at 75,000 g_{av} in a Ti60 rotor. This resulted in the appearance of three bands, a major band appearing immediately above the 1.3 M sucrose layer and two minor bands appearing in succession directly beneath this layer. Each of the three bands were aspirated with a Pasteur pipet and pooled then was diluted with 4 volumes of 0.25 M sucrose and centrifuged for 45 min at 175,000 g_{av} in a Ti70 rotor. The pellets were washed and resuspended in 0.25 M sucrose then adjusted to a protein concentration of 2 µg / µl.

The spleen was removed by carefully trimming away the connective tissue then washing it twice in 0.15 M NaCl. It was then minced, homogenized and centrifuged as stated above. The supernatant fraction was layered on top of 20 ml of 1.3 M sucrose solution and centrifuged for 90 min at 75,000 g_{av} in a Ti60 rotor. This resulted in the formation of a band on top of the 1.3 M layer; no other bands were visible. This fraction was aspirated with a Pasteur pipet, diluted with 4 volumes of 0.25 M sucrose and centrifuged for 45 min at 175,000 g_{av} in a Ti70 rotor. The pellet was washed and resuspended in 0.25 M sucrose and protein concentration adjusted to 2 µg / µl.

2. Measuring glycosyltransferase activity

A. Preparation of Substrates

Asialo human α_1 acid glycoprotein was used as the substrate acceptor for the ST6Gal I. The sialic acid residues of α_1-acid glycoprotein were removed by hydrolysis with 0.05 N H_2SO_4 at 80°C for 1 h to produce human asialo-α_1-acid glycoprotein as previously described by (Kaplan *et al.*, 1983). The sulfuric acid and liberated sialic acids were removed by dialysis with frequent changes of deionized water followed by lyophilization to remove the remaining water. The freeze-dried sample was resuspended in deionized water and brought to a final concentration of 1 µg / µl. Asialo rat α_1-acid glycoprotein was employed for the assay of the ST3Gal III. The sialic acids were hydrolyzed and removed as described above. The carbohydrate chains, which contain both Galβ1-3 and Galβ1-4 residues, then

had the Galβ1-4 selectively removed by the enzyme β-galactosidase from *Diplococcus pneumoniae* as described by Weinstein *et al.*, 1982. Acceptors for other two sialyltransferases, SAT-I and GlcNAcα2-6ST, were purchased and used directly as recommended by the supplier. Lactosyl ceramide and LS-tetrasaccharide-a were used as acceptors in assaying both SAT-1 and GlcNAcα2-6ST activity.

The acceptors for the three GlcNAc transferases, GlcNAcT-I, -III and -V were all kindly supplied by Dr. Ole Hindsgaul. The interesting feature of these acceptors is that they all contain a synthetic hydrophobic 'grease-arm' with the structure $(CH_2)_7CH_3$, which is exploited for the simple isolation of the product. The specific acceptors for GlcNAcT-I and -V were both synthesized as previously described (Kaur *et al*, 1991; and Lindh *et al*, 1991, respectively). The acceptor for the GlcNAcT-III assay was prepared by synthesizing a di-O-methylated pentasaccharide (Khan *et al.*, 1994). This acceptor was prepared by a block synthesis approach where the two hydroxyl groups which would normally act as acceptors for GlcNAcT-IV and -V are replaced with methyl groups, preventing the attachment of the respective GlcNAc sugars. Therefore, the detection of any radioactive GlcNAc to the acceptor could be attributed to the action of GlcNAcT-III. Furthermore, hydrophobic aglycon groups were incorporated into the acceptor in order to facilitate product isolation.

B. Enzyme Assays

GlcNAcα2-6ST was assayed based on a system previously developed by Paulson *et al*, 1984. The standard reaction mixtures (50 μl) contained 50 mM sodium cacodylate pH 6.0, 20 mM $MnCl_2$, 0.1% Triton CF-54, 0.15 mM LS-tetrasaccharide and 65 μg of Golgi membrane protein. In the case of kidney and spleen assays 100 μg of protein was used because these samples were not as pure as the liver Golgi membranes. A stock solution of the nucleotide sugar donor CMP-NeuAc was prepared by combining appropriate amounts of CMP-[^{14}C]NeuAc (20 nCi / nmol), unlabeled CMP-NeuAc and deionized water such that a 10 μl aliquot provided each incubation mixture with 20 nCi of radioactivity (80 nCi for spleen and kidney assays) and 15 nmol of nucleotide sugar. Reactions were carried out for 30 min (or 60 min for the case of spleen and kidney assays) at 37°C then stopped by placing on ice and quenching the mixture with 1 ml of cold 10 mM sodium phosphate, pH 6.8. The product, ^{14}C-labeled disialyllacto-N-tetraose, was quantified by separating it from the remaining CMP-[^{14}C]NeuAc on 3.5 inch Pasteur pipet columns of Dowex 1-phosphate (100-200 mesh). Essentially what this entailed was applying the quenched product onto the column, collecting the sample which flowed through into a scintillation vial, rinsing the assay tube with another 1 ml of cold 10 mM sodium phosphate and applying this to the same Pasteur pipet in order to ensure that all of the product was collected. The amount of disialated product recovered was corrected by a factor of 1.25 since only 80% of the formed

product was eluted (Paulson *et al*, 1984).

Human asialo α_1-acid glycoprotein and rat asialo α_1-acid glycoprotein, containing terminal Galβ1-3 linkages, were used as the acceptors for the ST6Gal I and ST3Gal III, respectively, as described in 2A. The assay mix contained 10 µg (15 µg for spleen and kidney assays) human asialo α_1 acid glycoprotein (for ST6Gal I) or 40 µg (60 µg for spleen and kidney) rat asialoagalacto α1 acid glycoprotein (for ST3Gal III), 50 µg bovine serum albumin, 50 mM cacodylate buffer, pH 6.0, 0.5% Triton CF-54, 9 nmol CMP[^{14}C] NeuAc with 20 nCi of radioactivity (80 nCi in the case of spleen and kidney) and Golgi membranes containing 50 µg protein, 75 µg protein for kidney and spleen, in a total volume of 60 µl. The assay was carried out for 30 min, or 60 min for spleen and kidney samples. Samples were then immediately transferred to ice and 50 µl samples were spotted on 2.5 cm diameter circles of Whatman No. 1 filter paper and washed as described before (Lammers *et al*, 1988).

SAT-I was assayed according to Melkerson-Watson *et al* (1991). The reaction mixture contained 50 µg of Golgi protein, 0.2 µM lactosyl ceramide, 9 nmol CMP[^{14}C] NeuAc (20 nCi of radioactivity , 50 mM cacodylate buffer, pH 6.5, 10 mM $MnCl_2$ and 1% Triton X-100 in a final volume of 100 µl. Samples were incubated for 60 min and the product of the reaction was isolated by reversed phase chromatography on Sep-pak C18 cartridges as described by Melkerson-Watson *et al* (1991).

The assay for GlcNAcT-I was performed as described by Schachter *et al* (1989). Samples were incubated at a final volume of 50 µl and contained 15 µg (20 µg for spleen and kidney) of the synthetic acceptor 8-methoxycarbonyloctyl 3,6-di-O-(α-D-mannopyranosyl)-β-D-mannopyranoside; 100 mM of MES, pH 6.0; 20 mM $MnCl_2$; 50 nmol UDP[1-^{14}C]GlcNAc , 20nCi of radioactivity (80 nCi in the case of spleen and kidney); 0.2% TX-100; 0.2% bovine serum albumin; 5 mM AMP, 100 mM GlcNAc and 50 µg of Golgi membranes (75 µg in the case of spleen and kidney). The mixtures were then incubated at 37°C for 30 min, or 60 min for spleen and kidney samples. The reaction was stopped by adding 0.4 ml of 20 mM sodium tetraborate-1 mM disodium EDTA . The product was then collected into scintillation counting vials by passing the sample through a Sep-pak C18 cartridge as described by Palcic *et al* (1988). The assay of GlcNAcT-V was carried out and the product collected as described above with the following exceptions. 10 µg (15 µg for spleen and kidney assays) of the synthetic trisaccharide βGlcNAc(1-2)αMan(1-6)βGlc-$O(CH_2)_7CH_3$ was used as the acceptor for the enzyme and 100 mM of EDTA since this is the only known branching GlcNAc-transferase in the asparagine-linked oligosaccharide pathway which retains activity in the absence of manganese.

The assay of GlcNAcT-III was performed as described by Nishikawa *et al*, (1988). The assays contained 100 µg of substrate; 125 mM MES, pH 6.00; 10 mM $MnCl_2$; 50 nmol UDP[1-^{14}C]GlcNAc ,20 nCi or 80 nCi in the case of spleen and kidney samples; 0.5% TX-100;0.2% bovine serum albumin; 5 mM AMP, 100 mM

GlcNAc and 50 μg of Golgi protein or 75 μg of protein when assaying spleen and kidney samples. Samples were incubated for 30 min, or 60 min in the case of spleen and kidney samples, at 37°C. At the end of the incubation the reaction was stopped with 0.2 ml ice-cold 0.15 M NaCl - 5 mM NaN_3 and transferred to ice; the solution was then applied to a Sep-pak C18 cartridge and the product was collected as previously described.

In all enzyme assays, conditions were established such that the product formation was linear with the amount of enzyme protein used. Endogenous activities were determined using controls where exogenous acceptor was omitted; these values were subtracted from total values. All samples were collected into vials and counted using ACS cocktail in a liquid scintillation counter. One unit of sialyltransferase activity is defined as the transfer of 1 pmol of NeuAc from CMP-NeuAc to acceptor per min. One unit of N-acetylglucosaminyltransferase activity is defined as the transfer of 1 pmol of GlcNAc from UDP-GlcNAc to acceptor per min. Protein was determined according to Lowry *et al* (1951) as modified by Miller (1959).

3. Density gradient formation of Golgi homogenates

A. Discontinuous gradient formation

Liver homogenate was prepared as described under methods and poured onto a layer of 1.3 M sucrose as described in figure 17. It was then centrifuged for 60 min at 75,000 g_{av} in a Ti60 rotor. The crude Golgi membrane fraction appearing

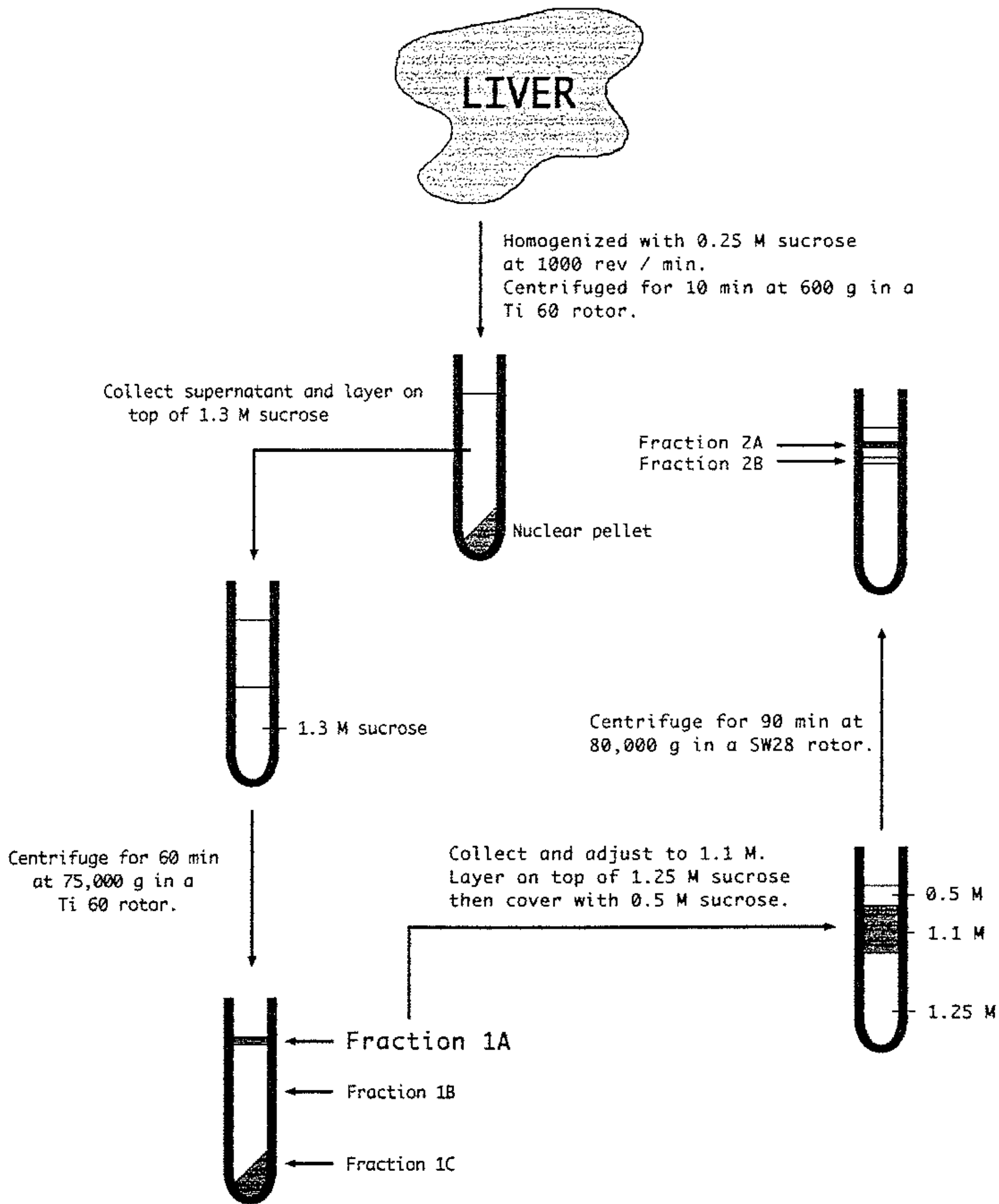


Figure 17. Subfractionation of rat liver homogenates.

immediately above the 1.3 M sucrose layer was aspirated, combined as appropriate and kept at 4°C. A small aliquot of this fraction was adjusted to a final protein concentration of 2.0 µg / µl to be used for enzyme assay purposes. This was designated as fraction 1A. Beneath this layer was a turbid 1.3 M sucrose medium (fraction 1B) which was removed and adjusted to a protein concentration of 2.0 µg / µl. A sediment which appeared at the bottom of the tube was resuspended in the original phosphate buffer, homogenized and adjusted to a final protein concentration of 2.0 µg / µl (fraction 1C). The remaining portion of fraction 1A was layered on 20 ml of 1.25 M sucrose and covered with 5 ml of 0.5 M sucrose then centrifuged for 90 min at 80,000 g_{av} at 4°C. This resulted in two main membrane fractions, one at the interface between 1.1 M sucrose and the homogenizing medium and the other at the interface between 1.25 M and 1.1 M sucrose. The two fractions were collected seperately and designated fractions 2A and 2B, respectively. Both fractions were resuspended in 4 volumes of 0.25 M sucrose and centrifuged for 45 min at 175,000 g_{av} in a Ti60 rotor. The pellets were washed twice with 0.25 M sucrose, resuspended, and homogenized in the same 0.25 M sucrose solution. The final protein concentration of each suspension was then brought to 2 µg / µl.

B. Continuous density gradient formation

A continuous density gradient ranging in concentration from 0.5 M to 2.0 M sucrose was prepared by the standard method of a density gradient formation

device. Essentially the gradient was created by adding 15 ml of 0.5 M sucrose to one well and 2.0 M sucrose to the other and clamping each of the wells to prevent the flow of each of the solutions (fig. 18). Both solutions were diverted into the same small plastic tube which in turn emptied into a SW28 tube which was continually being stirred at a slow rate. The 2.0 M solution was first unclamped and its contents allowed to empty into the SW28 tube. Immediately following this, the 0.5 M solution was unclamped, permitting it to flow into the plastic tube where the 2.0 M sucrose was. This enabled the solution, which initially contained 2.0 M sucrose, to become increasingly diluted with the 0.5 M sucrose. This process, coupled with the slow stirring of the effluent, allowed for the creation of a continuous gradient which started at 0.5 M sucrose and reached a final concentration of 2.0 M sucrose at the bottom of the SW28 tube. The gradient was then stored on ice. Five ml of fraction 1A was then incubated with 250 µg of proteinase K for 30 min at 20°C to unstack the Golgi membranes. This suspension was then adjusted to 0.25 M sucrose, cooled to 4°C, layered onto the continuous density gradient and centrifuged at 90,000 g_{av} for 2 h in a SW28 rotor. The plastic SW28 tube was then removed and placed in a manual fraction collector where the sample was stabilized and a small needle attached to a thin tube carefully inserted into the bottom of the tube so as not to disturb the gradient. The first 15 ml were then collected and discarded. The remaining solution was collected 1 ml at a time so that 20 × 1 ml samples were obtained. These samples were measured for their various glycosyltransferase activities by assaying 35 µl of each fraction. The

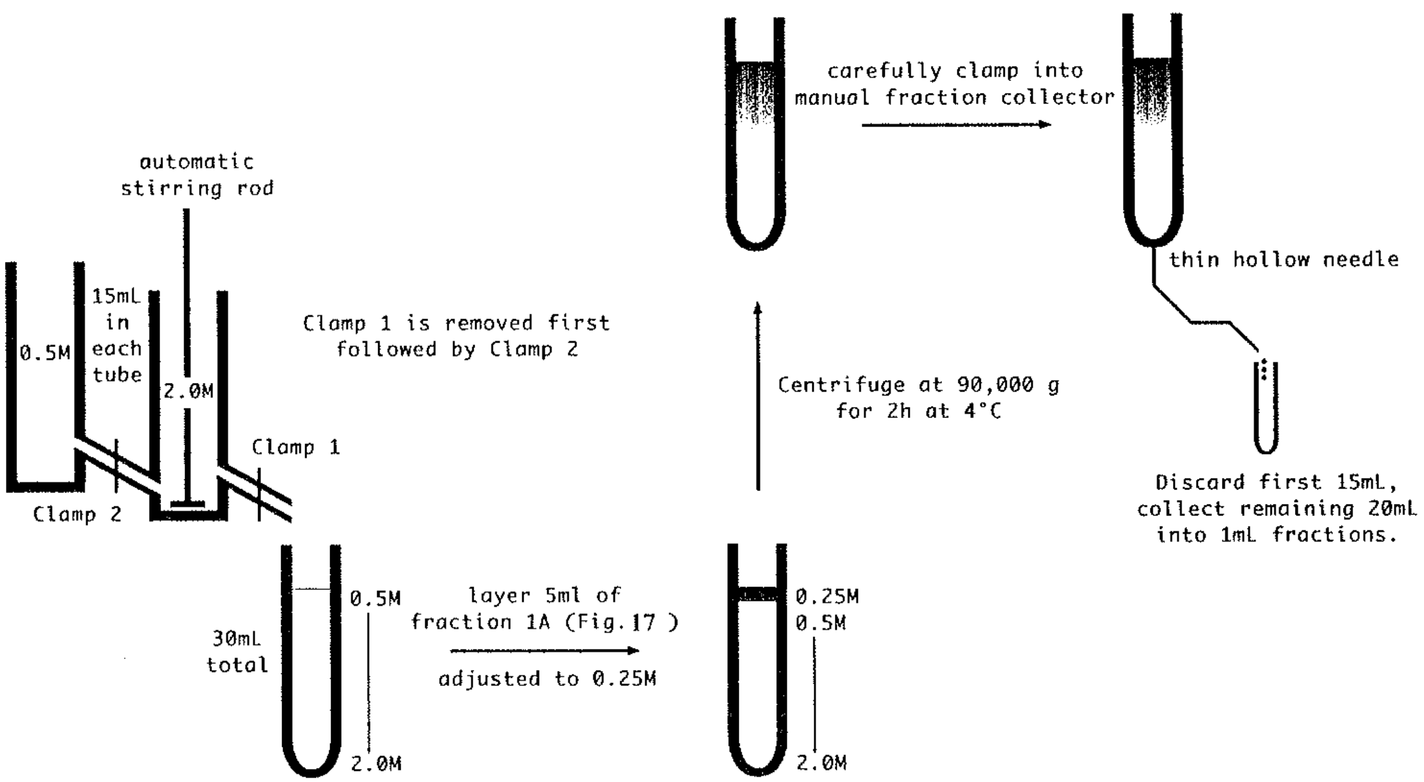


Figure 18. Creation of a continuous gradient.

protein concentration of each fraction was determined so that the final specific activity of the enzymes could be calculated.

4. Detection of intercisternal Golgi matrix

A. Preparation of Golgi membranes from liver

Purified Golgi membranes were isolated as described in section 1E. However, after the final centrifugation step, the pellets were gently resuspended in IMDPS buffer (75 mM imidazole/HCl, pH 7.0; 0.2 mM $MnCl_2$; 1 mM DTT; 0.1 mM pepstatin A, and 25 mM sucrose), and adjusted to a final protein concentration of 2 µg / µl, as outlined in figure 19.

B. Extraction of Golgi membranes with detergent

Golgi membranes in IMDPS buffer were resuspended in an equal volume of this same buffer supplemented with 0,1, 2, 4, or 6% (wt / vol) detergent, either Triton X-100 (TIMDPS) or Lubrol W (LIMDPS), followed by gentle homogenization in a Potter-Elvehjem homogenizer and its volume adjusted to give a final concentration of 0, 0.5, 1, 2, or 3% detergent with 1 µg protein / µl. The suspension was incubated on ice for 30 min then centrifuged at 90,000 g_{av} for 30 min at 4°C. A small aliquot of this sample was set aside for 100% activity determination. The supernatant was carefully removed, kept on ice and set aside for further analysis. This was labeled as '% solubilized'. The pellet was washed gently with IMDPS

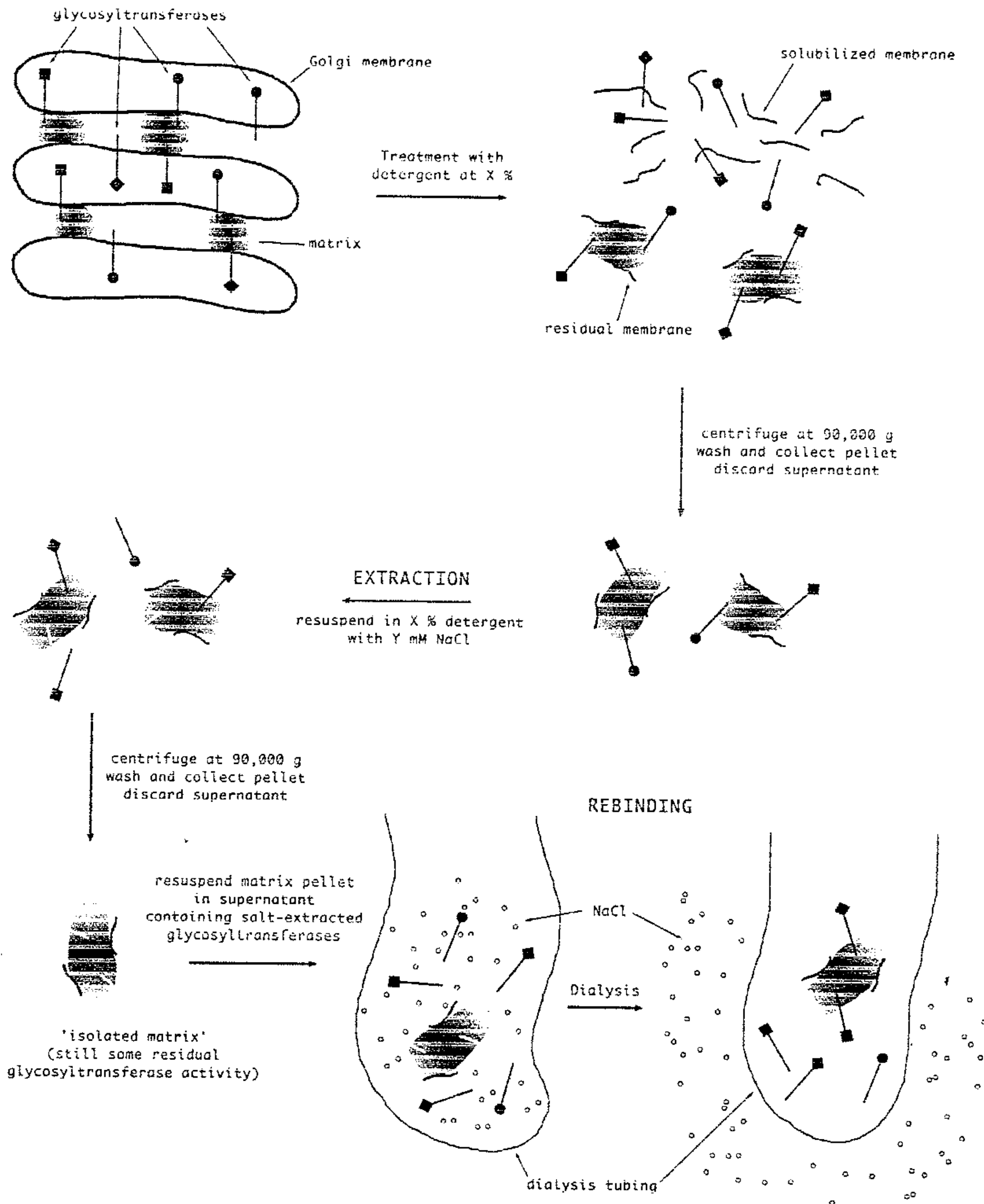


Figure 19. General principle behind isolation of matrix.

containing the same concentration of the respective detergent, homogenized as above, followed by resuspension in the original volume of buffered detergent. This activity was labeled as '% pellet'.

C. Salt extraction of glycosyltransferases from matrix

After solubilization and centrifugation of the Golgi membranes, the pellets were then resuspended and gently homogenized with the same 0, 1, 2, or 3% Lubrol buffer supplemented with 0 mM, 50 mM, 100 mM, 150 mM or 200 mM NaCl. This was stored on ice for 30 min and 50 µl set aside for 100% activity determination. The remaining solution was centrifuged at 90,000 g_{av} for 30 min at 4°C. The supernatant was aspirated with a Pasteur pipet and stored on ice for enzyme activity analysis. The pellet was resuspended and homogenized in a Potter-Elvehjem homogenizer with the same volume and concentration of buffered detergent minus the 50 µl taken for the 100% activity assay. This was stored on ice for enzyme activity analysis. Both the pellet and supernatant were assayed and compared to the 100% activity value. In order to study the effect of NaCl on the activity of the glycosyltransferases, Golgi membranes were resuspended in a 2% lubrol in IMDPS buffer supplemented with either 0, 50, 100, 150, or 200 mM NaCl and adjusted to a final concentration of 1.0 µg / µl. The protein mixture was then assayed directly and the relative enzyme activity values compared.

D. Reconstitution of glycosyltransferases into Golgi matrix

All enzymes to be reconstituted with the Golgi matrix were extracted with 2.0 ml of LIMDPS (2% Lubrol), containing either 70 mM NaCl or 150 mM NaCl from Golgi membranes solubilized with 2% Lubrol. After the extraction process, the samples were centrifuged as described above and the 2.0 ml of supernatant was assayed for the appropriate enzyme activity. Before the reconstitution process, each of the matrices was assayed for each of the respective endogenous enzyme activities. Due to the fact that a matrix still contains glycosyltransferase activity after the salt extraction process, these values were accounted for so that the *net increase* in enzyme activity of the matrix would indicate how much rebinding had actually occurred. In addition, the amount of activity which remained in the supernatant after the newly reconstituted matrix was centrifuged was calculated. This was to ensure that, if there is a net increase in activity of the matrix due to enzyme rebinding, that there should likewise be a *decrease* in the original activity of salt extracted enzyme.

Golgi matrices were prepared as follows: solubilizing Golgi membranes with just 2% Lubrol; 2% Lubrol / 70 mM NaCl; 2% Lubrol / 150 mM NaCl; 2% TX-100; 2% TX-100 / 70 mM NaCl and 2% TX-100 / 150 mM NaCl. As mentioned, these matrices each have various levels of enzyme activity of each of the four glycosyltransferase activities. The endogenous activities in each prepared matrix was predetermined and this value subtracted from the activity measured after rebinding of the salt extracted enzymes. This was performed by preparing 2.0 ml

of Golgi membrane in IMDPS buffer at a protein concentration of 1.0 μg / μl, followed by centrifugation at 90,000 g_{av} for 30 min at 4°C. This pellet was then resuspended in 2.0 ml of each of the six aforementioned detergent and salt combinations, kept on ice for 30 min then centrifuged at 90,000 g_{av} for 30 min at 4°C. Each of these pellets were then resuspended with 2.0 ml of either 2% Lubrol (LIMDPS) with 70 mM NaCl or 2% Lubrol (LIMDPS) with 150 mM NaCl, then gently homogenized as previously described; this is due to the fact that the salt extracted forms of the enzymes are present in 2.0 ml of both of these solutions. The sample is then dialyzed as described below, centrifuged at 90,000 g_{av} for 30 min at 4°C, the pellet (matrix) then resuspended in 2.0 ml of the same buffer and salt solution in which it was resuspended in, and assayed for enzyme activity. This step was carried out just as the reconstitution step itself, except that there was no salt extracted enzyme present, thus allowing for a background value to be measured.

The reconstitution procedure itself was performed by adding 2.0 ml of either 70 mM or 150 mM NaCl from Golgi membranes solubilized with 2% lubrol as described above. Therefore, two extracted forms of each enzyme was to be tested for rebinding; one extracted with 70 mM NaCl and the other with 150 mM NaCl. The 2.0 ml of salt extracted enzyme, which contained a known measure of activity, was then added to a matrix pellet, also of known enzyme activity, and gently resuspended with a Potter-Elvehjem homogenizer. The combined sample was then placed in dialysis tubing with a 12 - 14 kD molecular weight cut-off . Samples

were dialyzed for 18 h at 4°C against 3 liters of IMDPS buffer, which was changed every 2 - 4 h. After dialysis the contents were carefully aspirated with a needle and centrifuged at 90,000 g_{av} for 30 min at 4°C. Supernatants were removed with a Pasteur pipet and assayed directly for glycosyltransferase activity. The matrix pellets were then washed gently, resuspended in 2.0 ml of the original 2% lubrol / 70 or 150 mM NaCl buffer which was used to extract the enzyme, then gently resuspended and homogenized. The samples were then kept on ice until assayed for enzyme activity. Control samples were run by omitting dialysis and incubating the mixture for 18 h at 4°C with a gentle shaking. In all enzyme assays, 25 µl was removed from the respective 2.0 ml sample and then assayed as described under section 2B. However, there does exist the possibility that protein was lost during the dialysis procedure, which would in turn affect the activity values as they have been measured up to this point. Therefore, in the particular case of the matrix studies, the enzyme activity was measured in units / ml of homogenate, where a unit is defined as 1 pmol NeuAc or GlcNAc transferred per minute to the acceptor, but was then divided by 0.025 ml to account for the sample removed from the homogenate.

E. Reconstitution of salt extracted glycosyltransferases into a 2% lubrol / 500 mM NaCl Golgi matrix

A Golgi matrix was prepared using 2% Lubrol and 500 mM NaCl. ST6Gal I, ST3Gal III, GlcNAcα2-6ST, and GlcNAcT-I were extracted from Golgi membranes

with 70 mM NaCl as described. The salt-extracted forms were separated by centrifugation as described in 4C and then reconstituted into the 500 mM NaCl matrix as described in 4D. All enzymes activities were measured in units / ml homogenate as explained in 4D.

F. Reconstitution of clipped forms of sialyltransferase into the Golgi matrix

Three sialyltransferases; ST3Gal III, ST6Gal I, and GlcNAc 2-6ST were clipped from their membrane anchor and released as described in 1F. After centrifugation, the supernatant, which contained the clipped form of the enzymes, was supplemented with NaCl so as to bring the final concentration to 70 mM NaCl. This was then assayed and the activity measured in 'units / ml homogenate' as described in 4D. Although this enzyme was not extracted by salt, adding the NaCl placed the enzymes in the same environment as those that were salt extracted. An attempt was made to reconstitute the clipped form of the enzymes, which had a known amount of the enzymes activities, *via* dialysis. The Golgi matrix in this case was prepared as described in 4D by solubilizing the Golgi membranes with 2% Lubrol and 70 mM NaCl. The enzyme activity was compared in the matrix before and after the reconstitution procedure (as well as to the activity that was in the supernatant containing the clipped version of the enzymes) so that the percent that bound to the matrix could be calculated.

G. Determination of charge on sialyltransferase cytoplasmic tails

To determine the charge of the cytoplasmic tails, the amino acids constituting the transmembrane domain were determined by employing the MacVector 4.0 program which uses the Kyte-Doolittle hydropathy analysis with a window size of 7. Starting with the middle of the putative domain, the first charged residue in either direction was located. These amino acids were taken to be the boundaries of the membrane - spanning segment. Starting from these boundaries the charge of the cytoplasmic tail was calculated using the following parameters: arginine, lysine, and the N-terminal NH_2 group were given a value of +1; histidine, +0.5; and aspartate and glutamate, -1. The charge of the corresponding lumenal segment was calculated in an identical fashion by using the same number of amino acids that were determined in the cytoplasmic tail of the enzymes.

Results

A. *The Effects of the Acute Phase Response on Glycosyltransferase Activities from Liver, Kidney, and Spleen*

To effectively reproduce conditions in which the acute phase response could be studied, rats were injected with 0.5 ml of oil of turpentine per 100 g of body weight as described previously (Kaplan, H. A. *et al.*, 1983). After allowing the animals to endure the inflammatory response for various lengths of time, the liver, kidneys and spleen were removed and prepared for analysis.

Golgi liver membranes were purified and resuspended in a phosphate buffer to a constant protein concentration (see Methods). From these preparations four sialyltransferases and three *N*-acetylglucosaminyltransferases were assayed. As seen in figure 20, all the sialyltransferases decreased in specific activity by 50%, 24-48 hours post inflammation, with the exception of ST6Gal I, which increased by over 100% during this same period. By three days post inflammation, all enzyme activities had returned to normal. In the case of the GlcNAc transferases, all three showed an increase in activity within the same time period as the sialyltransferases (fig. 21). GlcNAcT-I displayed similar behavior as ST6Gal I increasing in activity 2-fold. The levels of GlcNAcT-III and -V were barely detectable in the liver before the initiation of the inflammatory response. In the case of the kidneys, the entire organ was homogenized followed by ultracentrifugation to remove debris such as

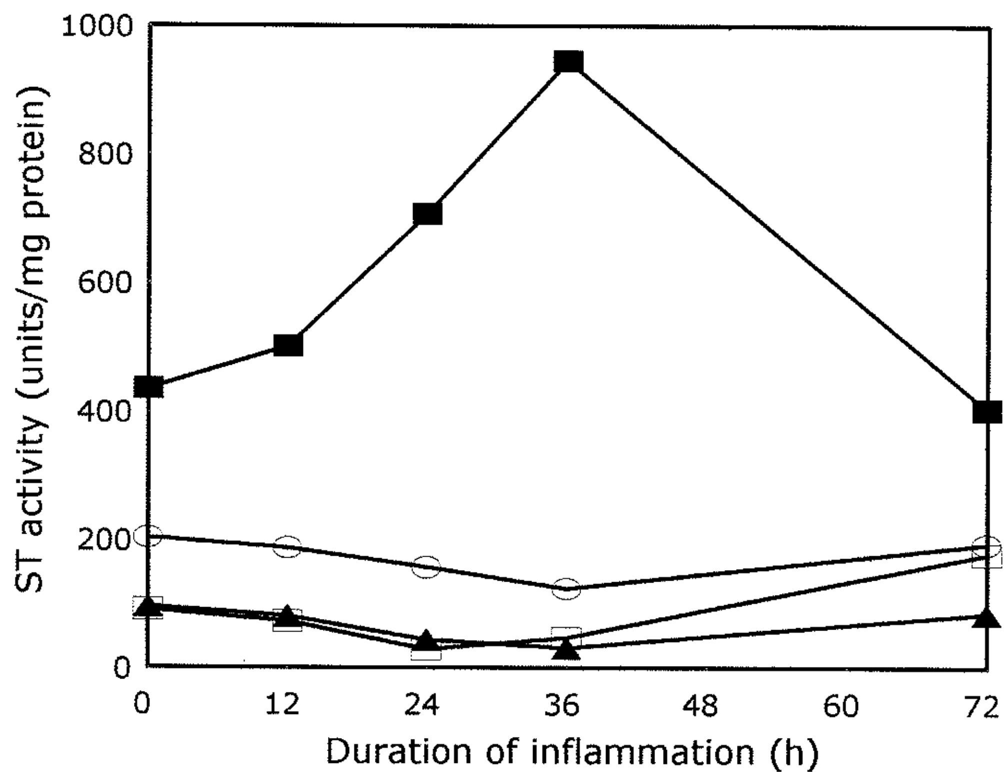


Figure 20. The effect of turpentine-induced inflammation on the activity of sialyltransferases from rat liver Golgi membranes. The sialyltransferases ST6Gal I (■),ST3Gal III (O), GlcNAc α2-6ST (□) and SAT-1 (▲) were each assayed from the various liver samples. Each experiment was a combination of two livers and each data point represents the average of three experiments. Results were reproducible within ±10%.

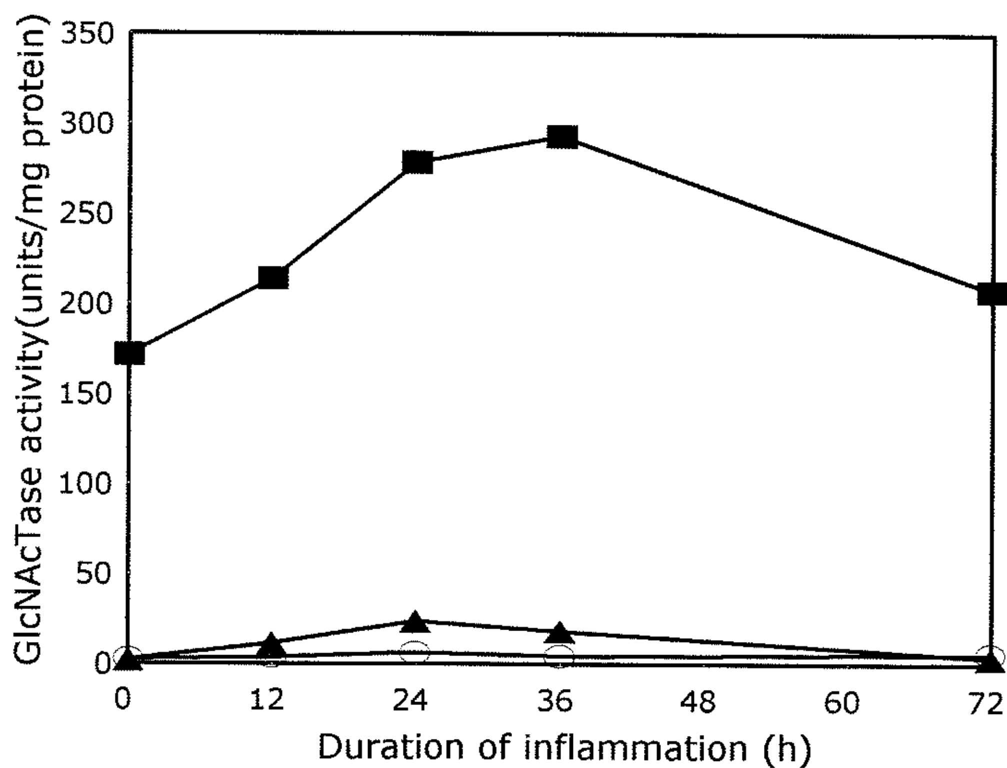


Figure 21. The effect of turpentine-induced inflammation on the activity of N-acetylglucosaminyl transferases from rat liver Golgi membranes. GlcNAcT-I (■), GlcNAcT-III (▲) and GlcNAcT-V (○) were each assayed from the various liver samples. Each experiment was a combination of two livers and each data point represents the average of three experiments. Results were reproducible within ±10%.

fatty tissue, red blood cells, nuclei and unbroken cells. The supernatant was then layered on a sucrose density gradient as described under methods and the band which formed was removed by pipette and assayed for the same seven glycosyltransferases as above. All three sialyltransferases behaved quite similarly by increasing slightly (~15%) and at the same time frame of 24-36 hours (fig. 22). GlcNAcT-III and -V increased slightly, however GlcNAcT-I showed about a 20% drop in activity before returning to normal levels of activity (fig. 23).

The source of enzyme from the spleen was prepared similarly to the kidney. The supernatant was then assayed for three sialyltransferases (fig. 24) and the three *N*-acetylglucosaminyl transferases (fig. 25). A notable observation here is seen in figure 25 where the enzyme GlcNAcT-V responds relatively soon upon the onset of inflammation by increasing its activity by a factor of 25% within the first 12 hours, which is close to its maximum level. Furthermore, GlcNAcT-III increases in activity by about 2-fold after 36 hours, which is quite substantial when compared to the moderate increases of this enzyme in the liver and kidney.

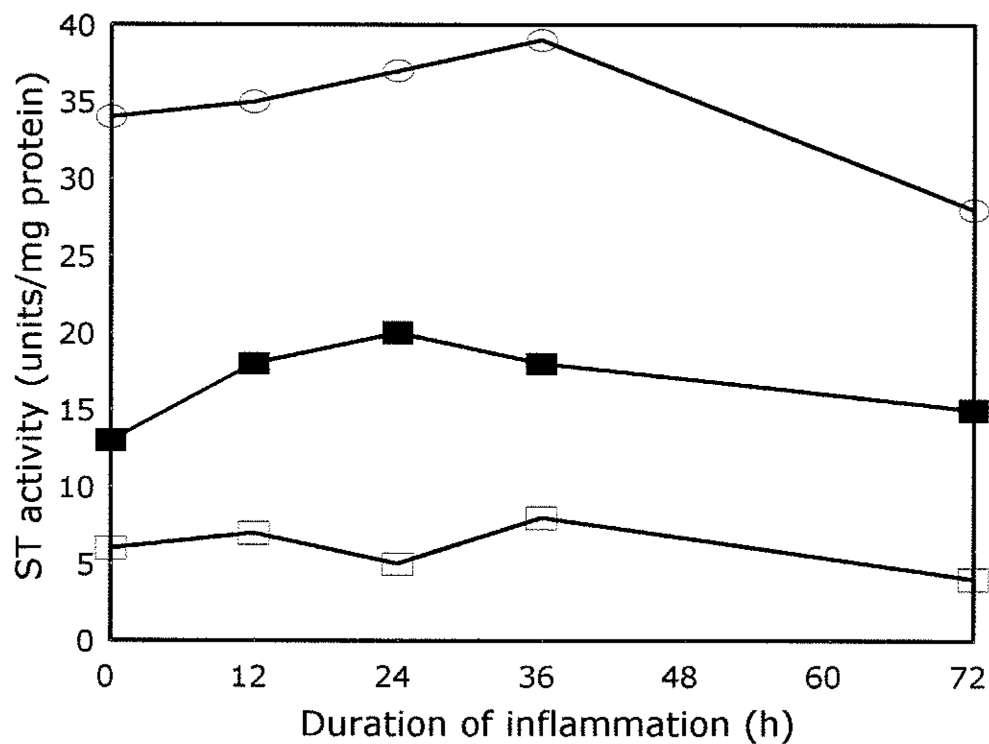


Figure 22. The effect of turpentine- induced inflammation on the activity of sialyltransferases from kidney. The three sialyltransferases analyzed, ST6Gal I (■), ST3Gal III (○), and GlcNAc α2-6 (□) did show variable levels of activity from the supernatant. Each experiment represents a combination of four kidneys from two rats. A total of three experiments were performed and results were reproducible within ±10%.

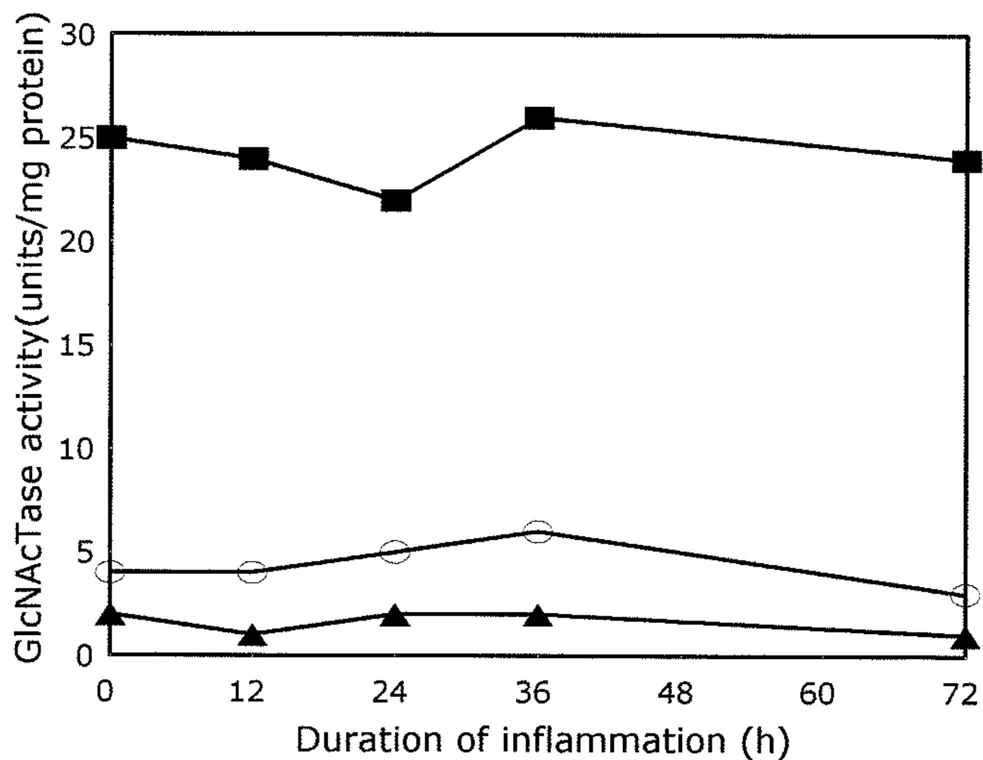


Figure 23. The effect of turpentine-induced inflammation on the activity of N-acetyl glucosaminyl transferase from the kidney. Analysis of the three acetylglucosaminyltransferase enzymes, GlcNAcT-I (■), GlcNAcT-III (O), and GlcNAcT-V (▲), were derived from the supernatant as described under methods. Each experiment represents a combination of four kidneys from two rats. A total of three experiments were performed and results were reproducible within ±10%.

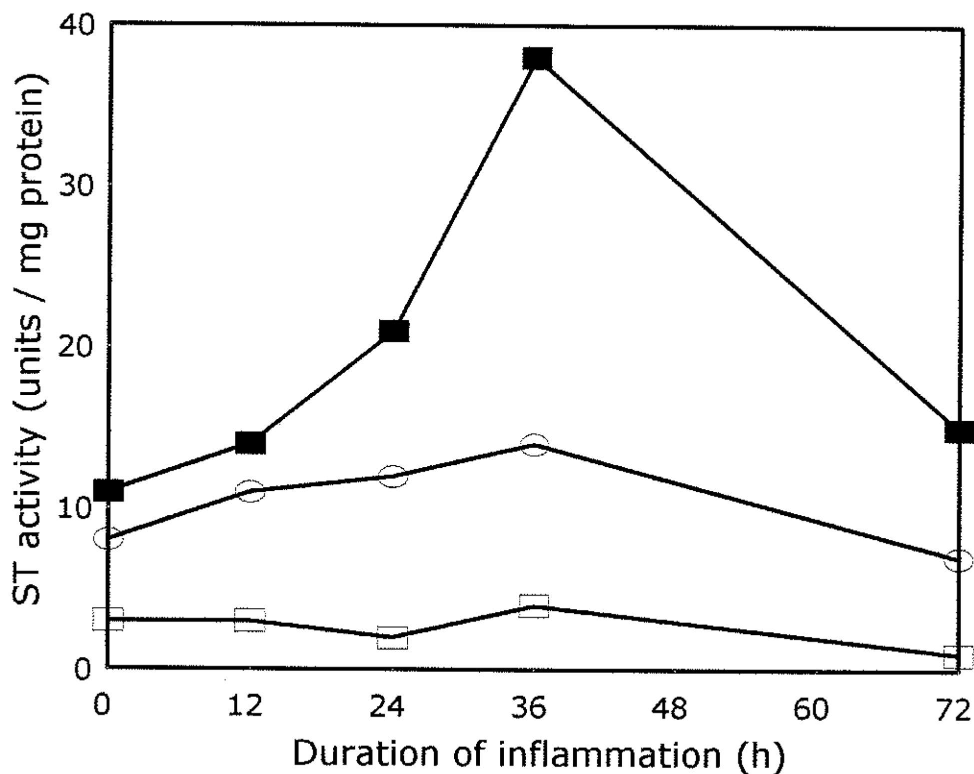


Figure 24. The effect of turpentine- induced inflammation on the activity of sialyltransferases from spleen. Analysis of the three enzymes ST6Gal I (■), ST3Gal III (○), and GlcNAcα2-6ST (□) were performed as described under methods. Each experiment represents a combination of two spleens. A total of three experiments were performed and results were reproducible with ±10%.

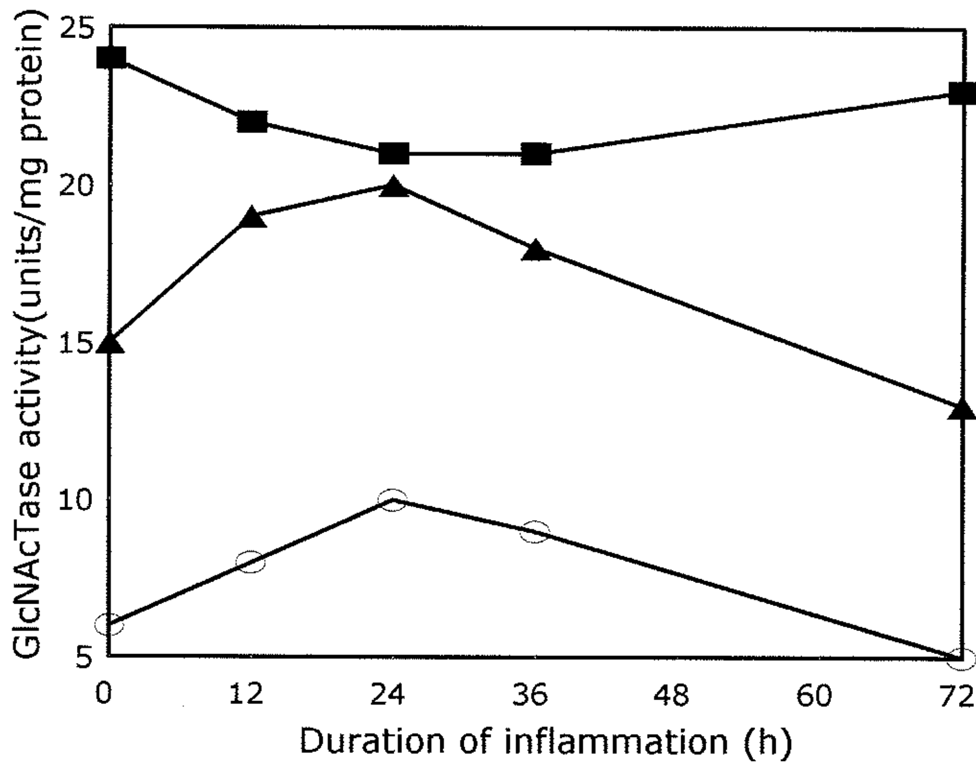


Figure 25. The effect of turpentine-induced inflammation on the activity of N-acetylglucosaminyl transferases from spleen. Fractions for enzyme analysis were prepared as described under methods and assayed for GlcNAcT-I (■), GlcNAcT-III (O), and GlcNAcT-V (▲). Each experiment represents a combination of two spleens. A total of three experiments were performed and results were reproducible with ±10%.

B. A Study on the Release of Glycosyltransferases from Disrupted Hepatic Golgi Vesicles

Whole liver tissue was used as a source to isolate Golgi vesicles (Leelavathi *et al.*, 1970) from both control and inflamed animals. The membranes were subsequently disrupted to expose the lumenal portion of the organelle to its surrounding environment. In order to effectively study the release of glycosyltransferases from their Golgi membrane anchors, a method had to be developed, which would disrupt the integrity of the vesicles, yet still retain membrane bound, catalytically active glycosyltransferase. Ultrasonic vibration was used to disrupt the Golgi membranes when studying the proteolytic release of the sialyltransferases. The effect of reduced pH on sonicated Golgi vesicles demonstrated that all three *N*-linked processing enzymes, ST6Gal I, ST3Gal III and GlcNAcα2-6ST, were released from the membrane at the same pH of 5.6, as seen in figures 26, 27 and 28 respectively. Furthermore, about 60-70% of the enzymes were released from liver Golgi membranes prepared from both control and inflamed rats. The enzyme SAT-I was studied by disrupting the Golgi membranes with a final concentration of 0.1% TX-100; sonication is known to destroy SAT-I activity (Trinchera, M. *et al.*, 1991). As seen in figure 29, the same optimal pH of 5.6 released SAT-I from its membrane anchor, although only 16-18% of the total enzyme activity was cleaved from both control and inflamed Golgi membranes. Because GlcNAcT-I is able to resist extraction with TX-100 and is not susceptible

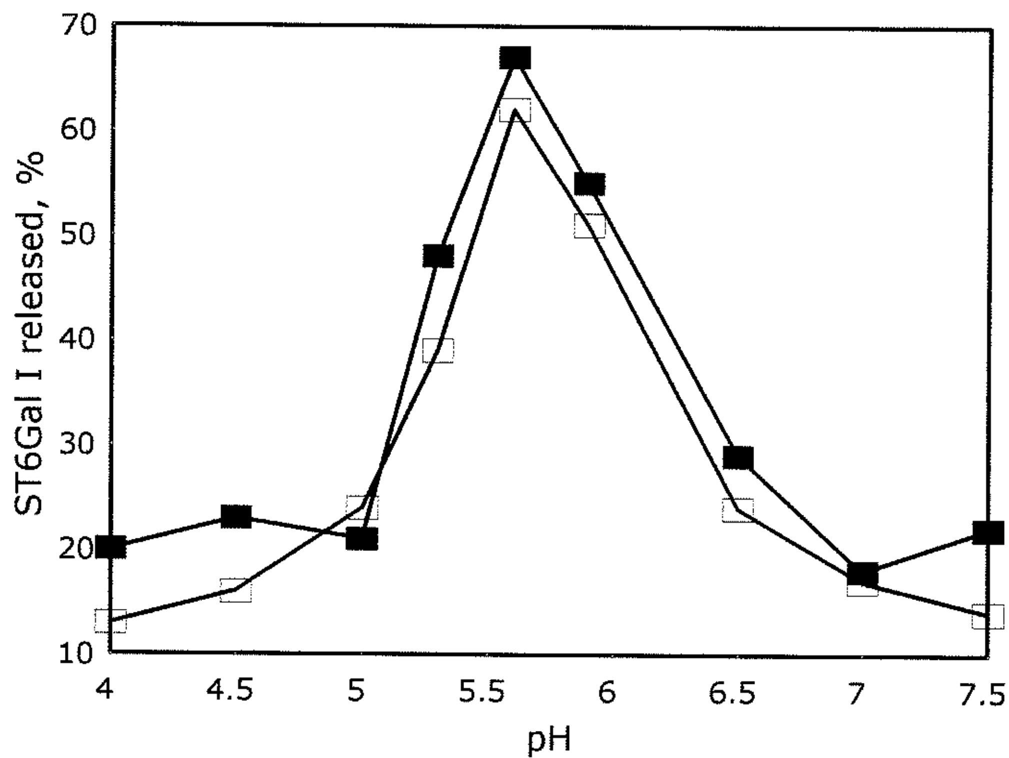


Figure 26. Effect of pH on the release of ST6Gal I sialyltransferase from sonicated Golgi membranes. Results are expressed as % ST6Gal I activity released from the membrane. The 100% values for control (□) and 36 h inflamed (■) ST6Gal I activity were 365.5 and 836.9 units / mg Golgi protein, respectively. Each result was performed with a combination of two rat livers and experiments repeated three times with a reproducibility of ±10%.

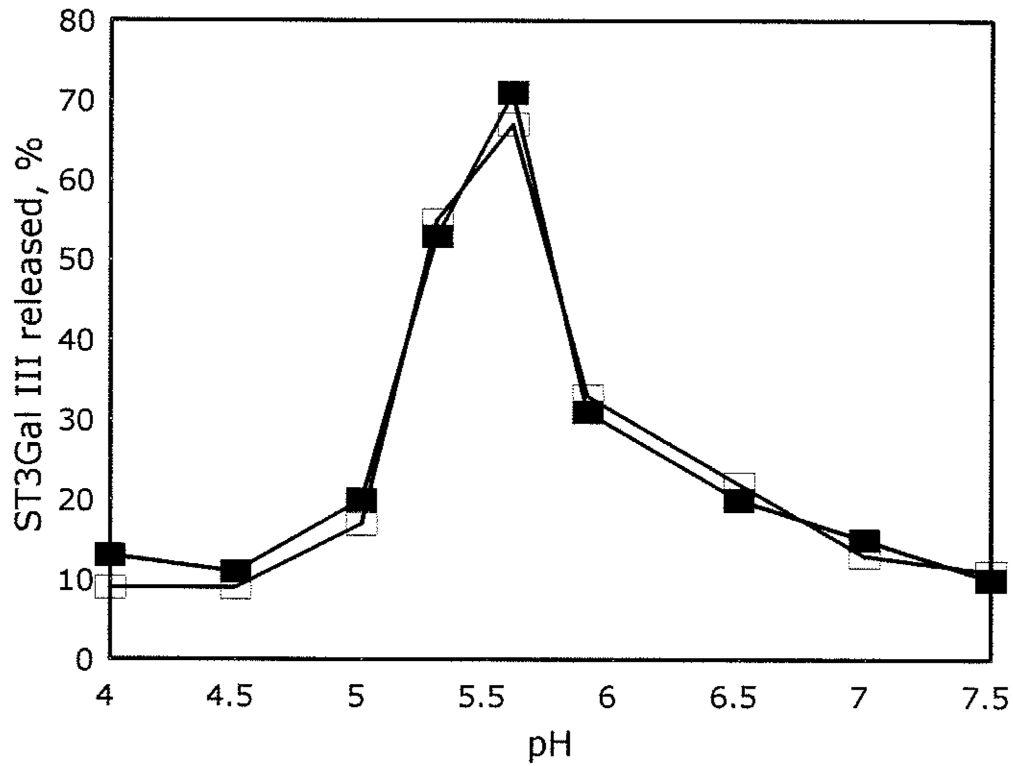


Figure 27. Effect of pH on the release of ST3Gal III sialyltransferase from sonicated Golgi membranes. Results are expressed as % ST3Gal III activity released from the membrane. The 100% values for control (□) and 36 h inflamed (■) ST3Gal III activity were 262.5 and 173.4 units / mg Golgi protein, respectively. Each result was performed with a combination of two rat livers and experiments repeated three times with a reproducibility of ±10%.

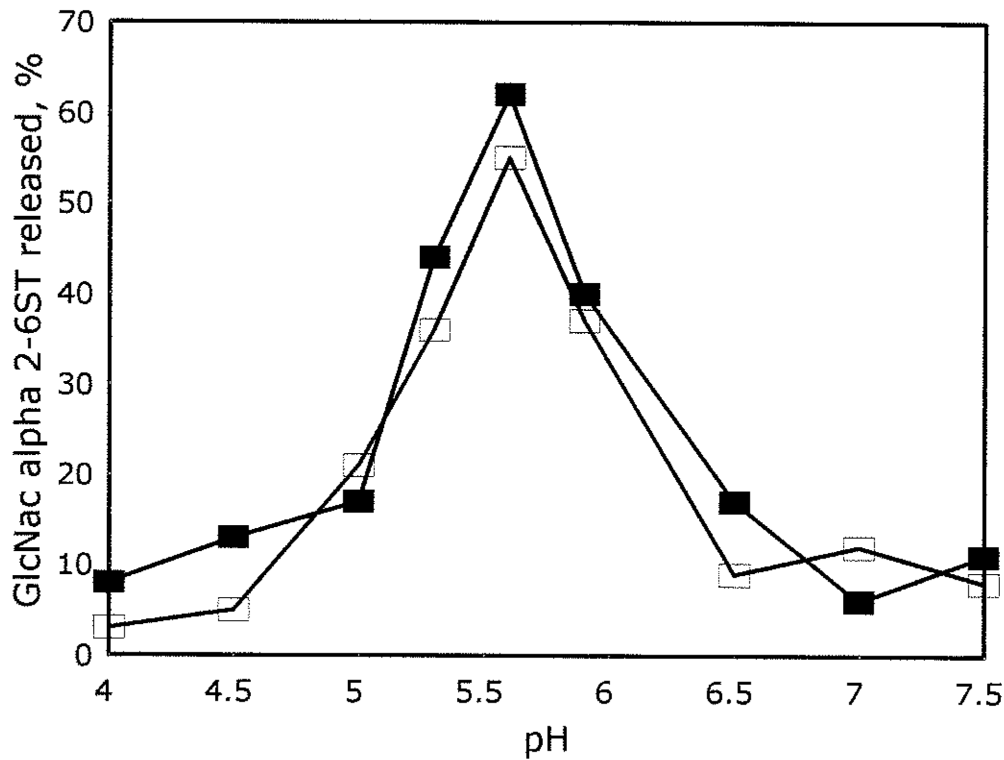


Figure 28. Effect of pH on the release of GlcNAcα2-6 sialyltransferase from sonicated Golgi membranes. Results are expressed as % GlcNAcα2-6 ST activity released from the membrane. The 100% values for control (□) and 36 h inflamed (■) GlcNAcα2-6 ST activity were 92.7 and 76.1 units / mg Golgi protein, respectively. Each result was performed with a combination of two rat livers and experiments repeated three times with a reproducibility of ±10%.

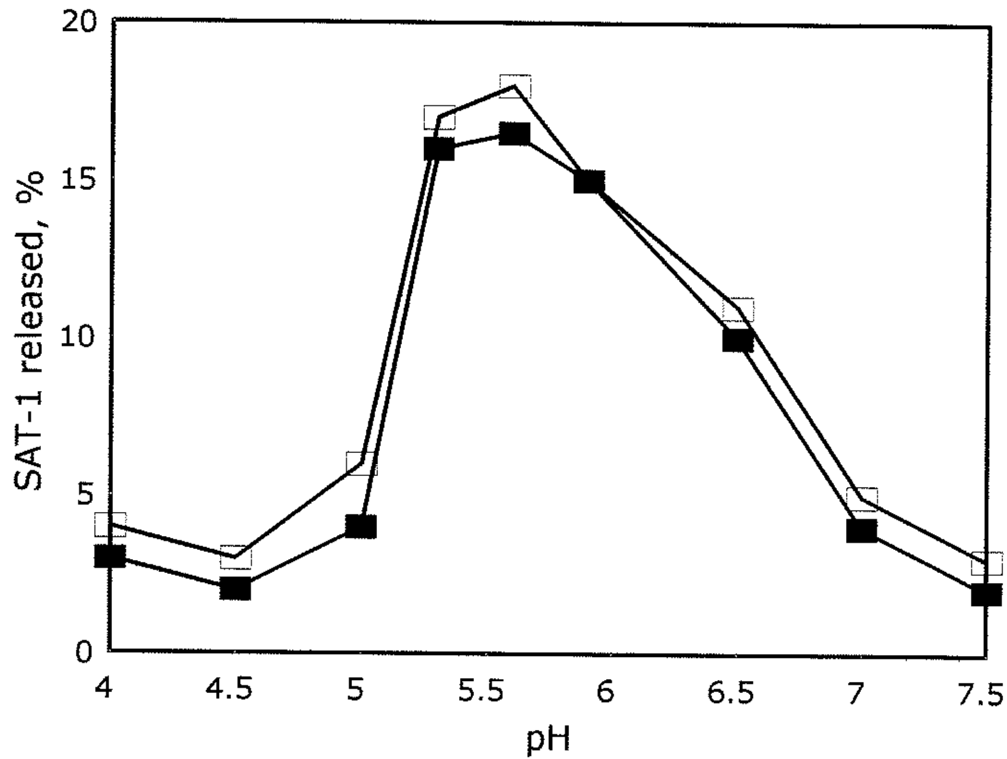


Figure 29. Effect of pH on the release of SAT-I from Golgi membranes disrupted with 0.1% Triton X -100. Results are expressed as % SAT-I activity released from the membrane. The 100% values for control (□) and 36 h inflamed (■) SAT-I activity were 96.3 and 44.9 units / mg Golgi protein, respectively. Each result was performed with a combination of two rat livers and experiments repeated three times with a reproducibility of ±10%.

to inactivation by ultrasonic vibration (Slusarewicz, P. *et al.* 1994), both techniques were employed to disrupt the Golgi membranes, with the exception that TX-100 was adjusted to a final concentration of 2.0%. As shown in figure 30, a pH of 5.6 was required for proteolytic release from the membrane anchor. In addition, only 10-15% of the enzyme was cleaved and released from the membrane, regardless of which method was used to disrupt the Golgi vesicles. As with the other enzymes, Golgi membranes from both control and inflamed liver tissue demonstrated similar properties in the optimal pH required to obtain efficient release of enzyme activity. Demonstrated in both figure 31 and 32 are the effects of incubation time on the release of the glycosyltransferases from disrupted Golgi vesicles at pH 5.6. Both ST6Gal I and GlcNAcT-I show a greater amount of enzyme being released over time from inflamed samples as compared to control conditions. ST3Gal III, GlcNAcα2-6ST, and SAT-I all showed the same gradual release of enzyme over the same period of time, however, the control samples registered a larger amount of released activity as compared to the Golgi membranes isolated from inflamed liver.

Previously, it had been identified that the aspartic proteinase cathepsin D was responsible for cleaving ST6Gal I from its Golgi membrane anchor (Lammers, G. *et al.* 1989). Therefore, experiments were performed to determine if cathepsin D was responsible for the release of the other glycosyltransferases. The first such set of experiments involved incubating disrupted Golgi membranes at pH 5.6 with commercially available bovine spleen cathepsin D. For reasons of comparative

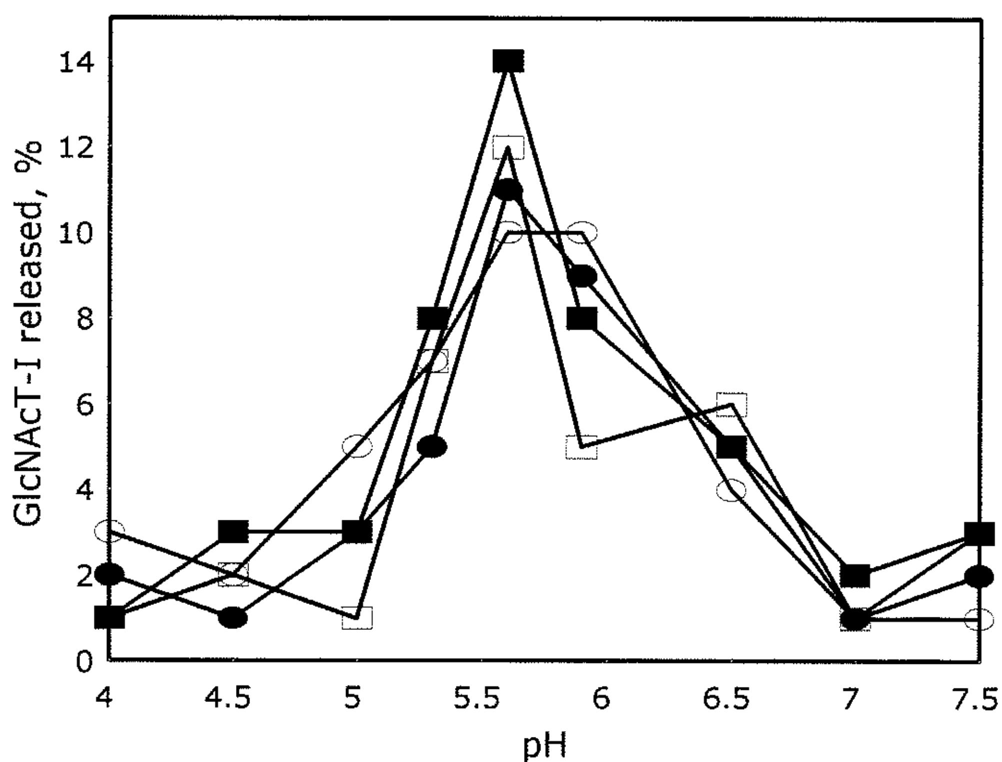


Figure 30. Effect of pH on the release of GlcNAcT-I from Golgi membranes disrupted by treatment with either 2.0% Triton X-100 or ultrasonic vibration. Results are expressed as % GlcNAcT-I activity released from the membranes. For membranes disrupted with detergent, the 100% values for control (□) and 36 h inflamed (■) GlcNAcT-I activity were 465.6 and 887.8 units / mg Golgi protein, respectively. For membranes which were disrupted by ultrasonic vibration, the 100% values for control (○) and 36 h inflamed (●) were 217.5 and 402.1 units / mg Golgi protein, respectively. Each result was performed with a combination of two rat livers and experiments repeated three times with a reproducibility of ±10%.

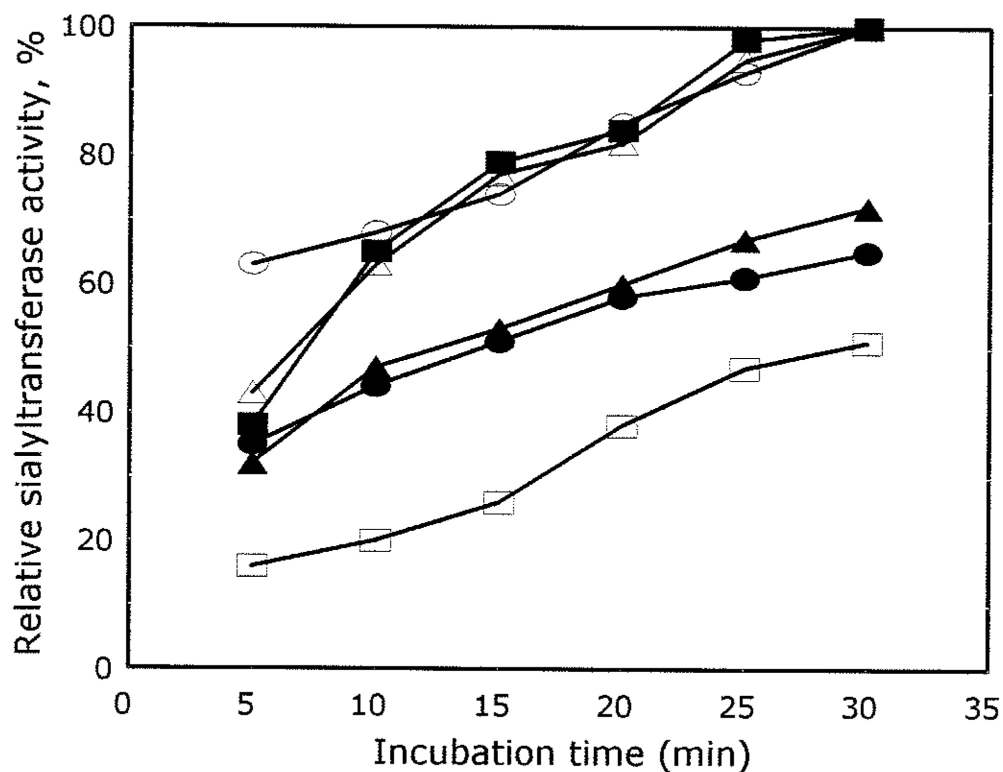


Figure 31. Effect of incubation time on the release of sialyltransferases from sonicated Golgi membranes at pH 5.6. Golgi membranes from control rats (open symbols) and those which had been inflamed for 36 hours (closed symbols) were exposed to ultrasonic vibration and the pH adjusted to 5.6. The samples were then incubated at 37°C for 5 to 30 min, centrifuged, and the supernatants assayed for ST6Gal I (□,■); ST3Gal III (O,●) and GlcNAcα2-6ST (△,▲) activity. The 100% value was taken as the highest amount of activity released for each of the three enzymes, ST6Gal I (593.6 units / mg Golgi protein), ST3Gal III (174.3 units / mg protein) and GlcNAcα2-6ST (63.2 units / mg protein). For ST6Gal I this was from the inflamed Golgi while the 100% values for ST3Gal III and GlcNAcα2-6ST were obtained from control samples, since these enzymes were shown to decrease slightly in activity with the onset of inflammation. The Golgi membranes were from a total of three rat livers and the experiments were repeated three times with a reproducibility of ±15%.

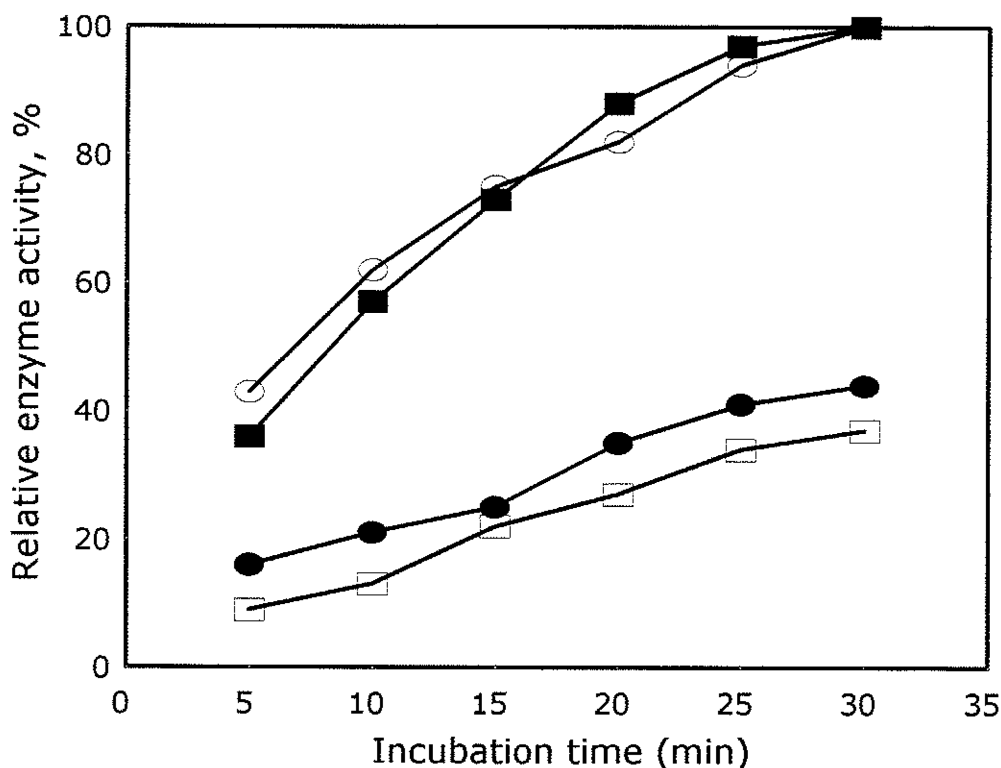


Figure 32. Effect of incubation time on the release of SAT-I and GlcNAcT-I from Golgi membranes disrupted Triton X -100. Golgi membranes from control rats (open symbols) and those which had been inflamed for 36 hours (closed symbols) were disrupted by treatment with either 0.1% Triton X-100 (for SAT-I release) and 2.0% Triton X-100 (for GlcNAcT-I release) for 30 min on ice followed by ultracentrifugation. The disrupted Golgi membrane pellet was washed and resuspended in the original buffer as described under methods. The solution was then adjusted to a pH of 5.6 and the samples incubated at 37°C for 5 to 30 min, readjusted to a neutral pH and centrifuged for 30 min. The supernatant was then assayed for SAT-I (O,●) and GlcNAcT-I (□,■) activity. The 100% value was taken as the highest amount of activity released for each of the two enzymes. For GlcNAcT-I activity this was from the inflamed Golgi (466.3 units / mg protein) while the 100% values for SAT-1 (83.1 units / mg protein) were obtained from the control samples, since this enzyme was shown to decrease in activity with the onset of inflammation. The Golgi membranes were from a total of three rats and the experiments were repeated three times with a reproducibility of ±15%.

analysis, the original experiment studying ST6Gal I was repeated (table 3). As the data indicate, the addition of exogenous cathepsin D resulted in a greater quantity of the enzyme being released as compared to control samples. Furthermore, a greater amount of the enzyme was released from inflamed samples, which correlates with the findings that there is an increase in activity of the enzyme in the liver during the acute inflammatory response. Similar results were obtained when ST3Gal III (table 4) and GlcNAcα2-6ST (table 5) were tested for release with exogenous cathepsin D. In both cases however, the Golgi membranes from control samples were able to release substantially more enzyme, which again correlates with the decrease in activity of the two enzymes observed from isolated Golgi membranes during inflammation. Tables 6 and 7 show the results of the studies with SAT-I and GlcNAcT-I, respectively, in which Golgi membranes were treated with bovine spleen cathepsin D. In both cases, Golgi membranes were disrupted with TX-100 to expose the lumenal side of the membranes to the cytoplasm. Similar results to those of the above studies were achieved with samples containing endogenous cathepsin D showing a greater amount of enzyme released. The same trend was observed for control and inflamed samples. Control membranes released a higher amount of SAT-I than the inflamed membranes, while contrasting results were observed with GlcNAcT-I. Again, these results support the findings that exogenous bovine spleen cathepsin D releases the greatest amount of enzyme from Golgi membranes isolated from either control or inflamed samples.

In order to gain further evidence that the action of cathepsin D is the primary

		ST6Gal I activity released (units/mg of Golgi membrane protein)			
		Control		Inflamed	
Incubation time (min)	CD	−	+	−	+
2		54	70	72	81
6		162	208	243	403
12		289	350	535	727

Table 3. Release of ST6Gal I sialyltransferase from sonicated Golgi membranes incubated with bovine spleen cathepsin D at pH 5.6. Sonicated Golgi membranes were incubated either with (+) or without (−) cathepsin D (CD) purified from bovine spleen at 37°C, for either 2, 6, or 12 min at a pH of 5.6. Experiments were repeated on three occasions, each time with Golgi membranes prepared from the combination of two rat livers. Results were obtained with a reproducibility of ±10%.

		ST3Gal III activity released (units/mg of Golgi membrane protein)			
		Control		Inflamed	
Incubation time (min)	CD	–	+	–	+
2		29	58	17	13
6		96	177	63	82
12		210	329	143	187

Table 4. Release of ST3Gal III sialyltransferase from sonicated Golgi membranes incubated with bovine spleen cathepsin D at pH 5.6. Sonicated Golgi membranes were incubated either with (+) or without (-) cathepsin D (CD) purified from bovine spleen at 37°C for either 2, 6, or 12 min at a pH of 5.6. Experiments were repeated on three occasions, each time with Golgi prepared from the combination of two rat livers. Results were obtained with a reproducibility of ±10%.

Incubation time (min)	CD	GlcNAc α2-6ST activity released (units/mg of Golgi membrane protein)			
		Control		Inflamed	
		–	+	–	+
2		10	16	12	19
6		28	43	25	39
12		64	79	41	58

Table 5. Release of GlcNAc α2-6 sialyltransferase from sonicated Golgi membranes incubated with bovine spleen cathepsin D at pH 5.6. Sonicated Golgi membranes were incubated either with (+) or without (-) cathepsin D (CD) purified from bovine spleen at 37°C for either 2, 6, or 12 min at a pH of 5.6. Experiments were repeated on three occasions, each time with Golgi prepared from the combination of two rat livers. Results were obtained with a reproducibility of ±10%.

		SAT-I activity released (units/mg of Golgi membrane protein)			
		Control		Inflamed	
Incubation time (min)	CD	–	+	–	+
2		1	2	—	2
6		8	13	2	5
12		11	16	3	8

Table 6. Release of SAT-I sialyltransferase from Golgi membranes disrupted with 0.1% Triton X-100 and incubated with bovine spleen cathepsin D at pH 5.6. Sonicated Golgi membranes were incubated either with (+) or without (-) cathepsin D (CD) purified from bovine spleen at 37°C, for either 2, 6, or 12 min at a pH of 5.6. Experiments were repeated on three occasions, each time with Golgi prepared from the combination of two rat livers. Results were obtained with a reproducibility of ±10%.

		GlcNAcT-I activity released (units/mg of Golgi membrane protein)			
		Control		Inflamed	
Incubation time (min)	CD	–	+	–	+
2		2	11	9	15
6		13	43	17	49
12		32	71	46	83

Table 7. Release of GlcNAcT-I from Golgi membranes disrupted with 2.0% Triton X-100 and incubated with bovine spleen cathepsin D at pH 5.6. Sonicated Golgi membranes were incubated either with (+) or without (-) cathepsin D (CD) purified from bovine spleen at 37°C for either 2, 6, or 12 min at a pH of 5.6. Experiments were repeated on three occasions, each time with Golgi prepared from the combination of two rat livers. Results were obtained with a reproducibility of ±10%.

reason for the release of these glycosyltransferases from their membrane anchor, pepstatin A, a potent inhibitor of cathepsin D, was used. When Golgi membranes were disrupted and incubated at the optimal pH of 5.6, a large decrease in the amount of released enzyme was observed with increasing concentrations of pepstatin A (table 8). Golgi membranes isolated from both inflamed and control animals demonstrated similar trends in the ability of pepstatin A to prevent proteolytic cleavage of the enzymes from their membrane anchors.

Glycosyltransferase activities were assayed in the sera from control and inflamed animals as a possible destination of the clipped form of the enzymes. GlcNAcT-III and -V, ST3Gal III, and SAT-I were not detected in the serum of control or experimentally inflamed animals (table 9). GlcNAcT-I and GlcNAcα2-6ST were detected in low levels, however, large increases in ST6Gal I serum levels were seen. The findings that ST6Gal I increases in activity in the liver during the acute phase response, is released by liver slices into the culture medium, and is elevated in activity in the serum during inflammation have been previously reported (Kaplan, H. A. *et al.*, 1983). Although the six aforementioned glycosyltransferases were assayed for the same biological properties, none of them appear to behave like ST6Gal I with regards to changes in catalytic activity in the Golgi and serum. The K_m values for these enzymes were not calculated, however earlier work had compared the kinetics of ST6Gal I from both control and inflamed sources. It was concluded that there was little change in the catalytic activity of this enzyme when it was released from the Golgi membranes and into the serum (Kaplan, H. A.,

Pepstatin A (M)	Glycosyltransferase activity released into supernatant, %									
	ST6Gal I		ST3Gal III		SAT-I		GlcNAcα2-6ST		GlcNAcT-I	
	C	I	C	I	C	I	C	I	C	I
none present	31	69	73	36	20	7	63	28	5	11
10^{-9}	12	30	19	14	11	3	26	12	1	3
10^{-4}	4	11	14	6	3	1	4	5	1	1

Table 8. Effect of pepstatin A on the release of glycosyltransferases from disrupted Golgi membranes at pH 5.6. Golgi membranes form control rats (C) and those suffering from acute inflammation for 36 hours (I) were disrupted either by ultrasonic vibration (ST6Gal I, ST3Gal III, and GlcNAc α2-6ST), 0.1% Triton X-100 (SAT-I), or 2.0% Triton X-100 (GlcNAcT-I), as described under methods. Experiments were repeated three times, each with the combination of two rat livers. Results were obtained with a reproducibility of ±10%.

Enzyme	Glycosyltransferase activity (pmol/min/ml serum)	
	Control	Inflamed
GlcNAcT-I	1.6	2.7
GlcNAcT-III	ND*	ND*
GlcNAcT-V	ND*	ND*
ST6Gal I	6.2	24.8
ST3Gal III	ND*	ND*
GlcNAc α2-6ST	1.4	0.9
SAT-1	ND*	ND*

ND*, not detected.

Table 9. The effect of turpentine induced inflammation on the serum levels of sialyl and N-acetylglucosaminyltransferases. Serum from control rats and those suffering from acute inflammation for 36 hours was collected and assayed as described under methods. Each experiment represents a combination of serum from two animals. A total of five experiments were performed and results were reproducible with ±15%.

1983).

In order to perform studies which would give a better indication of how these enzymes are behaving while in a whole cell environment, liver slice experiments were performed. The glycosyltransferases listed in table 10 had shown varying degrees of cleavage from their membrane anchor, only ST6 Gal I demonstrated the ability to be released from the liver slices and into the surrounding culture medium, a finding which was concluded earlier (Jamieson, J.C., 1988). While ST3Gal III, GlcNAcT-III nor GlcNAcT-V were shown to be secreted in liver slice experiments only extemely low levels of GlcNAcT-I and GlcNAcα2-6ST activities were secreted.

Further studies were conducted to observe the inhibitory effects of pepstatin A on glycosyltransferase secretion by liver slices. Because ST6Gal I, ST3Gal III, and GlcNAcα2-6ST are the only enzymes released from the liver slices, studies were restricted to these enzymes. Pepstatin A was previously reported to inhibit the release of ST6Gal I from liver slices, results which were confirmed in figure 33. Similar results were obtained for GlcNAcα2-6ST and GlcNAcT-I, which resulted in similar results as shown in figures 34 and 35 respectively. The only notable difference was seen with the release of GlcNAcα2-6ST, where slice samples from control animals were affected more than samples taken from inflamed animals.

Enzyme	Glycosyltransferase activity (pmol/min/ml of medium)	
	Control	Inflamed
GlcNAcT-I	1.6	2.3
ST6Gal I	5.5	19.7
GlcNAc α2-6ST	1.8	0.7

Table 10. The effect of turpentine induced inflammation on the release of glycosyltransferases from liver slices. Although ST3Gal III, SAT-I, GlcNAcT-III and GlcNAcT-V were each assayed, they did not demonstrate any significant levels of activity in the liver slice medium from control or inflamed animals. Each sample consisted of slices from two rats and experiments were repeated 4 times with a reproducibility of ±10%

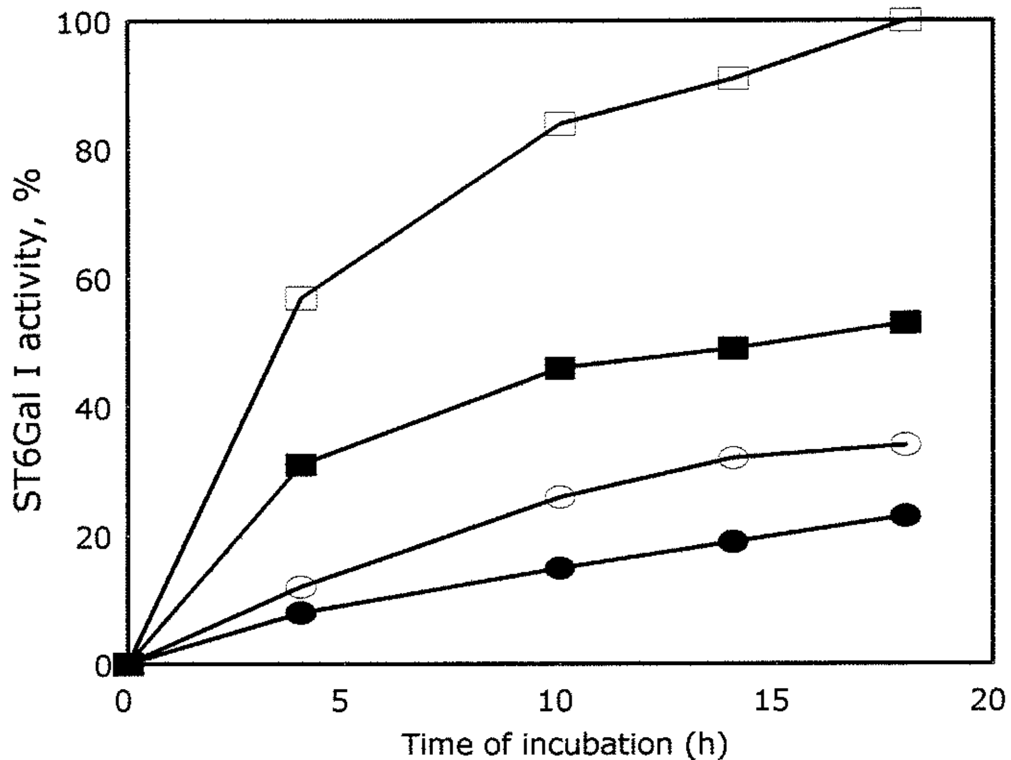


Figure 33. Effect of Pepstatin A on Release of ST6Gal I Sialyltransferase from Rat Liver Slices. Liver slices from control rats were incubated in a nutrient culture medium in which 10^{-4} M of pepstatin A was either present (●) or absent (○). Liver slices were also taken from rats suffering from acute inflammation for 36 hours and incubated in the same medium either with 10^{-4} M pepstatin A present (■) or absent (□). The highest level of enzyme activity released into the medium occurred from inflamed samples that did not contain pepstatin A, and was therefore determined as 100% activity. Experiments were repeated three times ans were reproducible within ±10%.

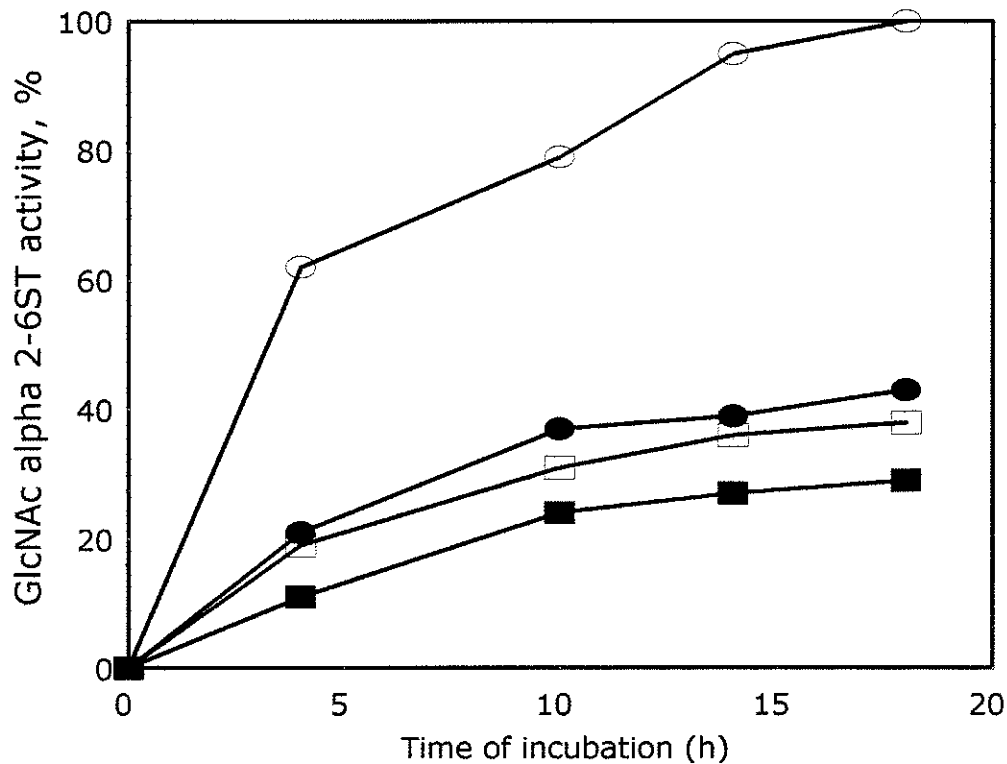


Figure 34. Effect of Pepstatin A on Release of GlcNAcα2-6 Sialyltransferase from Rat Liver Slices. Liver slices from control rats were incubated in a nutrient culture medium in which 10^{-4} M of pepstatin A was either present (●) or absent (○). Liver slices were also taken from rats suffering from acute inflammation for 36 hours and incubated in the same medium either with 10^{-4} M pepstatin A present (■) or absent (□). The highest level of enzyme activity released into the medium occurred from the control sample that did not contain pepstatin A, and was therefore determined as 100% activity. Experiments were repeated three times and were reproducible with ±10%.

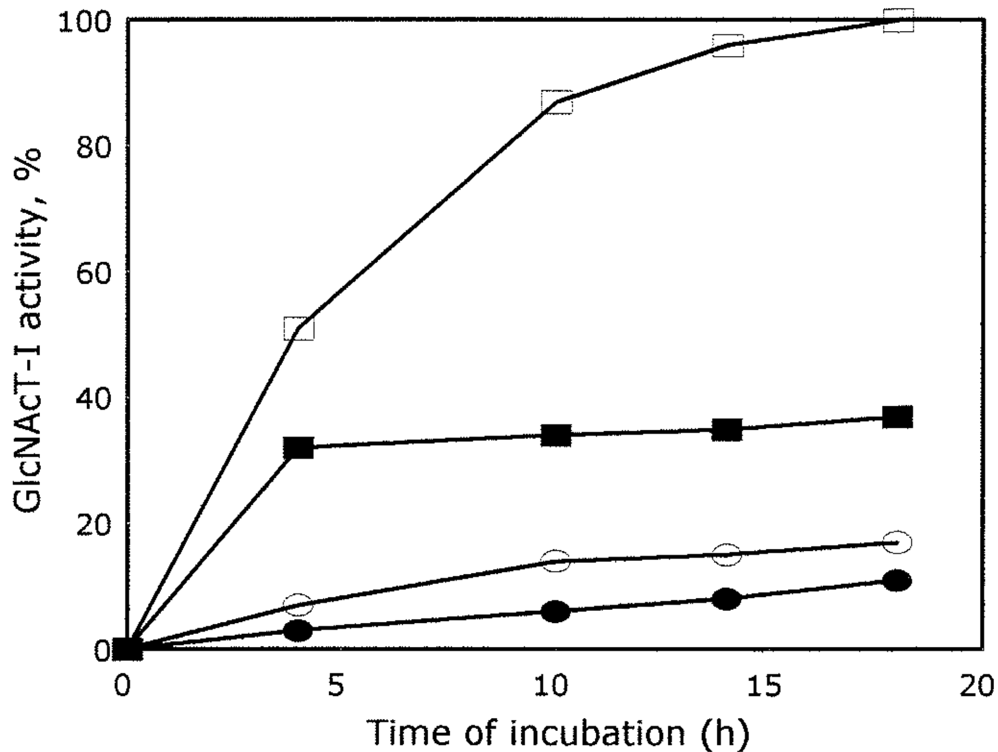
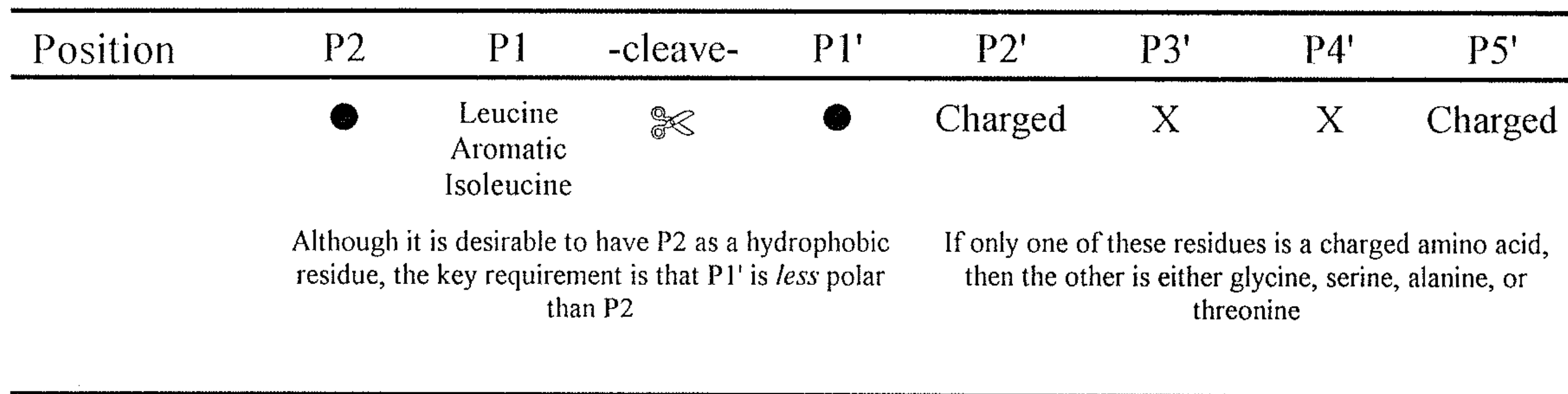


Figure 35. Effect of Pepstatin A on Release of GlcNAcT-I from Rat Liver Slices. Liver slices from control rats were incubated in a nutrient culture medium in which 10^{-4} M of pepstatin A was either present (●) or absent (○). Liver slices were also taken from rats suffering from acute inflammation for 36 hours and incubated in the same medium either with 10^{-4} M pepstatin A present (■) or absent (□). The highest level of enzyme activity released into the medium occurred from the inflamed sample that did not contain pepstatin A, and was therefore determined as 100% activity. Experiments were repeated three times and were reproducible with ±10%.

C. Identification of the proteolytic site recognized by cathepsin D within the stem region of glycosyltransferases

Cathepsin D is an aspartic proteinase that has been shown to have broad substrate specificity (Van Noort, J.M. *et al.*, 1989) However, for this study, a more precise restriction was placed on the amino acid sequences that cathepsin D is thought to recognize as shown in figure 36 and table 11. The putative site of proteolytic cleavage was determined by looking for such sequences within the stem region of the glycosyltransferases that had been shown to be cleaved by cathepsin D. The exact length of the stem region cannot be stated with accuracy because it is not known how much of the amino-terminal sequence can be removed without loss of catalytic activity. However, the molecular weight of many soluble forms of various glycosyltransferases, derived from the membrane anchored form, have been determined. The remaining membrane bound segments of the enzymes were always well less than 100 amino acids in length, indicating that a search for a proteolytic sequence beyond this point was not likely to contain a sequence that would result in cleavage from the membrane anchor. Both ST6Gal I (Weinstein, J. *et al.*, 1987) and ST3Gal III (Wen, D.X. *et al.*, 1992) from rat liver have been cloned and sequenced. As shown in figure 37, only one predicted cleavage site, at position 61-62, is found in the ST3Gal III sequence, Two possible sites were predicted in the ST6Gal I sequence, one at the 34-35 position and the other between amino acids 74 and 75. GlcNAcT-I has not been sequenced from rat liver, but it has been cloned and sequenced from human, mouse, and rabbit



Position	P2	P1	-cleave-	P1'	P2'	P3'	P4'	P5'
	●	Leucine Aromatic Isoleucine	✂	●	Charged	X	X	Charged
	Although it is desirable to have P2 as a hydrophobic residue, the key requirement is that P1' is *less* polar than P2				If only one of these residues is a charged amino acid, then the other is either glycine, serine, alanine, or threonine			

Figure 36. Preferred site of sequence recognized and cleaved by cathepsin D. Cathepsin D is believed to recognize a sequence spanning seven residues designated P2 → P5' with the cleavage site occurring between P1 and P1' (✂). P1 must be either a leucine, an aromatic, or isoleucine amino acid. P2 and P1' are hydrophobic residues (●), however P1' must be less polar than P2 while P2' and P5' are charged amino acids. P3' and P4' do not seem to be required in the recognition process and therefore their identity is not considered important.

Amino Acid	1 - letter code	Side chain	Polarity
Alanine	A	Non polar	1.6
Arginine	R	Charged polar (basic)	-12.3
Aspartic acid	D	Charged polar (acidic)	-9.2
Asparagine	N	Uncharged polar	-4.8
Cysteine	C	Uncharged polar	2.0
Glutamic acid	E	Charged polar (acidic)	-8.2
Glutamine	Q	Uncharged polar	-4.1
Glycine	G	Non polar	1.0
Histidine	H	Charged polar	-3.0
Isoleucine	I	Non polar	3.1
Leucine	L	Non polar	2.8
Lysine	K	Charged polar (basic)	-8.8
Methionine	M	Non polar	3.4
Phenylalanine	F	Non polar	3.7
Proline	P	Non polar	-0.2
Serine	S	Uncharged polar	0.6
Threonine	T	Uncharged polar	1.2
Tryptophan	W	Non polar	1.9
Tyrosine	Y	Uncharged polar	-0.7
Valine	V	Non polar	2.

Table 11. Abbreviation and chemical properties of the amino acids. The 'standard' amino acids are listed in alphabetical order along with their one letter code abbreviation. Side chain properties are those predominating at pH 7.0. The polarity values are expressed in kcal / mol and are the free energies for the transfer of an amino acid residue in a α helix from the membrane interior to water (Engelman, D.M. *et al.*, 1986).

ST3Gal III

1	M	G	L	L	V	F	V	R	N	L	L	L	A	L	C	15
16	L	F	L	V	L	G	F	L	Y	Y	S	A	W	K	L	30
31	H	L	L	Q	W	E	D	S	N	S	L	I	L	S	L	45
46	D	S	A	G	Q	T	L	G	T	E	Y	***D***	X	X	***G***	60
61	***F***	***L***	***L***	K	L	D	S	K	L	P	A	E	L	A	T	75
76	K	Y	A	N	F	S	E	G	A	C	K	G	P	Y	A	90
91	S	A	M	M	T	A	I	F	P	R						100

ST6Gal I

1	M	I	H	T	N	L	K	K	K	F	S	L	F	I	L	15
16	V	F	L	L	F	A	V	I	C	V	W	K	K	G	***S***	30
31	X	X	***E***	***A***	***L***	***T***	L	Q	A	K	E	F	Q	M	P	45
46	K	S	Q	E	K	V	A	M	G	S	A	S	Q	V	V	60
61	F	S	N	S	K	Q	E	P	K	G	D	I	***P***	***I***	***L***	75
76	***S***	X	X	***R***	V	T	A	K	V	K	P	Q	P	S	F	90
91	Q	V	W	D	K	D	S	T	Y	S						100

Figure 37. Primary sequences of ST3Gal III and ST6Gal I from rat liver. The amino acids at position P3' and P4' have been changed to an 'X' for purposes of clarity. The site of cleavage for ST6Gal I (Weinstein, J. *et al.*, 1987) and ST3Gal III (Wen, D.X. *et al.*, 1992) is underlined and the location of the putative transmembrane domain is identified by the underlying black bar. Although ST6Gal I demonstrates two possible sites of cleavage, the one nearest the transmembrane domain is the least likely for two reasons. First, being that close to the membrane may make it difficult for cathepsin D to bind to the sequence for sterical reasons. Secondly, the difference in polarity between P2 and P1' is not that significant, which would make the sequence less susceptible to proteolytic cleavage.

sources. Figure 38 shows the alignment of all three sequences. Interestingly, even with the variety of choices made possible by the multiple amino acid substitutions, only one site, between amino acids 71 and 72, appears to match the predictive recognition parameters.

Earlier results from our laboratory had shown that galactosyltransferase was not cleaved from its membrane anchor under the same conditions as ST6Gal I. A likely explanation for the lack of cleavage by cathepsin D may be due to the absence of a corresponding recognition sequence. Because the enzyme has not been sequenced from rat liver, sources from cow, human, and mouse were analyzed for cathepsin D cleavage sites (fig. 39). Again, even with the large array of possible recognition sites due to amino acid substitutions, there were no sites which resembled regions which could potentially be cleaved by cathepsin D.

An additional parameter which may play an important role in the structural features which are recognized by cathepsin D is the secondary structure of the proteins. Since the suggested heptapeptide sequence recognized by cathepsin D is most likely to occur within an amphiphilic α-helical structure (Van Noort, J. M. *et al.*, 1989), the primary sequences of ST3Gal III, ST6Gal I and GlcNAcT-I were investigated for potential α-helical structures. By using the MacVector© protein prediction program, which employs both the Chou-Fasman and Robson-Garnier methods for secondary structure prediction, a search for helices was performed within the first 100 amino acids of these glycosyltransferases. The Chou-Fasman

Human	1	M	L	K	K	Q	S	A	G	L	V	L	W	G	A	I	15
Mouse	1	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	15
Rabbit	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	15
Human	16	L	F	V	A	W	N	A	L	L	L	L	F	F	W	T	30
Mouse	16	I	-	-	G	-	-	-	-	-	-	-	-	-	-	-	30
Rabbit	16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	30
Human	31	R	P	A	P	G	R	P	P	S	V	S	A	L	D	G	45
Mouse	31	-	-	-	-	-	-	L	-	-	D	-	-	-	G	D	45
Rabbit	31	-	-	V	-	S	-	L	-	-	D	N	-	-	-	D	45
Human	46	D	P	A	S	L	T	R	E	V	I	R	L	A	Q	D	60
Mouse	46	-	-	-	-	-	-	-	-	-	-	H	-	-	E	-	60
Rabbit	46	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	60
Human	61	A	E	V	E	L	E	***R***	X	X	***G***	***L***	***L***	***Q***	Q	I	75
Mouse	61	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	75
Rabbit	61	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	75
Human	76	G	T	N	P	A	L	S	S	Q	R	G	R	V	P	T	90
Mouse	76	K	S	H	Y	-	-	W	R	-	-	W	-	-	-	-	90
Rabbit	76	R	S	H	H	-	-	W	-	-	-	W	K	-	-	-	90
Human	91	A	A	P	P	A	Q	P	R	V	P						100
Mouse	91	V	-	-	-	-	W	-	-	-	-						100
Rabbit	91	-	-	-	-	-	-	-	H	-	-						100

Figure 38. Primary sequence of GlcNAcT-I from human, mouse, and rabbit. GlcNAcT-I was cloned and sequenced from human (Schachter, H. *et al.*, 1991), mouse (Pownall, S. *et al.*, 1992), and rabbit (Sarkar, M. *et al.*, 1991) sources. The only sequence which would seem to be recognized by cathepsin D is indicated in bold italics and the site of cleavage underlined. The amino acids at position P3' and P4' have been changed to an 'X' for purposes of clarity. The putative transmembrane domain is identified by the underlying black bar.

Cow	1	M	K	F	R	E	P	L	L	G	G	S	A	A	M	P	15
Human	1	-	R	L	-	-	-	-	-	S	-	●	-	-	-	-	15
Mouse	1	-	R	-	-	-	Q	F	-	-	-	-	-	-	-	-	15
Cow	16	G	A	S	L	Q	R	A	C	R	L	L	V	A	V	C	30
Human	16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	30
Mouse	16	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	30
Cow	31	A	L	H	L	G	V	T	L	V	Y	Y	L	A	G	R	45
Human	31	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	45
Mouse	31	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-	45
Cow	46	D	L	R	R	L	P	Q	L	V	G	V	H	P	P	L	60
Human	46	-	-	S	-	-	-	-	-	-	-	-	S	T	-	-	60
Mouse	46	-	-	S	-	-	-	-	-	-	-	-	S	S	T	-	60
Cow	61	Q	G	S	S	H	G	A	A	A	I	G	Q	P	S	G	75
Human	61	-	-	G	-	N	S	-	-	-	-	-	-	S	-	-	75
Mouse	61	-	-	G	T	N	-	-	-	-	S	K	-	-	P	-	75
Cow	76	E	L	R	L	R	G	V	A	P	P	P	P	L	Q	N	90
Human	76	-	-	-	T	G	-	A	R	-	-	-	-	-	G	A	90
Mouse	76	-	Q	-	P	-	-	A	R	-	-	-	-	-	G	V	90
Cow	91	S	S	K	P	R	S	R	A	P	S						100
Human	91	-	-	Q	-	-	P	G	G	D	-						100
Mouse	91	-	P	-	-	-	P	G	L	D	-						100

Figure 39. Primary sequence of galactosyltransferase from cow, human, and mouse. Galactosyltransferase (EC 2.4.1.38) was cloned and sequenced from bovine (Narimatsu, H. *et al.*, 1986), human (Appert, H. E. *et al.*, 1986), and mouse (Shaper, N. L. *et al.*, 1988) sources. The human form of the enzyme does not have an amino acid at position 11, relative to the cow and mouse, which is represented by ●. The putative transmembrane domain is identified by the underlying black bar.

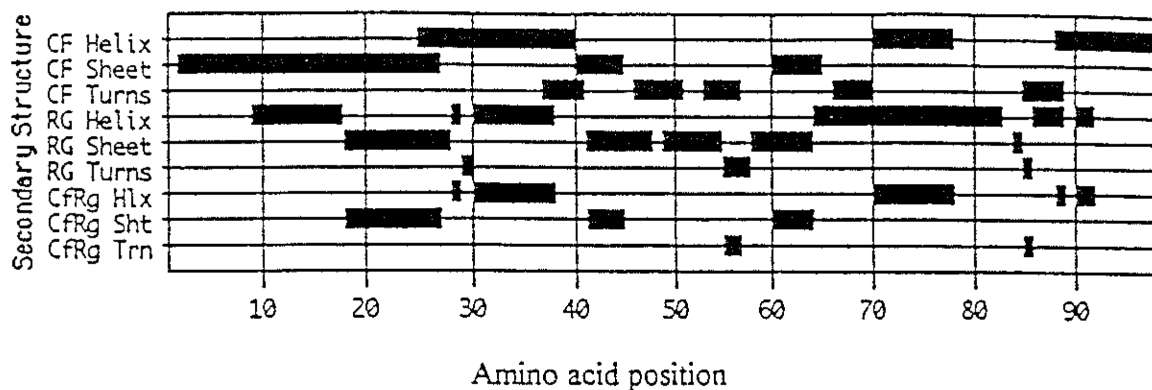
method was originally intended to be performed by hand, which leads to a major drawback when it is adapted for computers. The same region of the protein often appears to be equally likely contain a helical or sheet conformation and the rules do not resolve this conflict by indicating the relative weight to place on each possible conformation at a given region. By performing the method manually, the researcher resolves these conflicts by making subjective judgments (possible biased by foreknowledge). But when the method is computerized, the programer must explicitly assign the weights to be used to resolve such conflicts. Because each programmer may resolve them differently, one implementation of the Chou-Fasman method may yield different predictions from another implementation; MacVector deals with this problem by ignoring it. Thus a single region may be predicted to be in more than one conformational state. This form of presentation underscores the uncertainties of the method (or any current method for secondary structure prediction) and is a reminder that the predictions should not be over interpreted.

The Robson-Garnier method is based on information theory. Empirical studies show that an amino acid exerts a significant effect on the conformation of residues up to eight residues distant; therefore, the information for the conformation of residue N can be based on the information contributions of the 16 nearest neighbors of N. Using these information parameters, the likelihood of a given residue assuming each of the four possible conformations (alpha, beta, reverse

turn, or coil) is calculated, and the conformation with the largest likelihood is assigned to the residue.

By graphing the secondary predictions only where both the Chou-Fasman and Robson-Garnier methods agree, does one hope to locate those regions that are most likely to be in the predicted conformation. However, it is well to remember that at best, each method has about a 60 percent probability of being correct and that the consensus of two possibly wrong predictions does not give a correct prediction. The most likely cathepsin D cleavage site in ST3Gal III is between amino acids 61 and 62, however, neither method predicts an α-helix to occur within this region (fig. 40A) Two potential cleavage sites in ST6Gal I are between residues 34 and 35 and between 74 and 75, with only the former site having an α-helix predicted by both methods (fig. 40B). The best overall prediction of a cathepsin D cleavage site appeared within the GlcNAcT-I enzyme. Primary sequence analysis selected the site to occur between amino acids 71 and 72. When secondary structural analysis was performed, this region was indeed predicted to occur directly within the middle of a long α-helical structure (fig. 41).

A.



B.

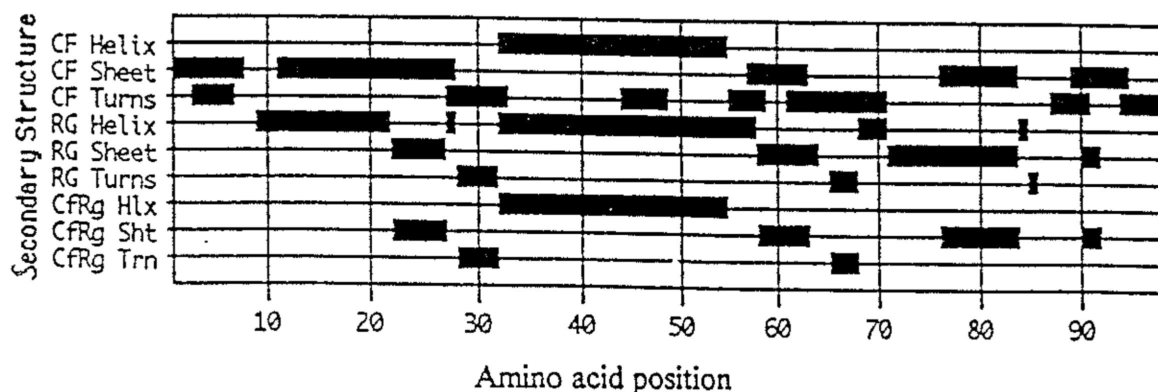


Figure 40. Secondary structure prediction of ST3Gal III and ST6Gal I from rat liver. Two standard methods were employed, the Chou-Fasman (CF; Chou *et al.*, 1974) and the Robson-Gaumier (RG; Robson *et al.*, 1986) prediction models. These models were used in combination with the aid of the MacVector© protein structural prediction program as described under methods. Both models combine to predict the location of α-helices (CfRg Hlx), β-sheets (CfRg Sht) and β-turns (CfRg Trn), although only the location of the α-helices are relevant to this study. According to these models, ST3Gal III (A) has a high probability of containing α-helices between amino acid residues 30-38 and 70-78, while ST6Gal I (B) is likely to contain one long α-helix between residues 32 and 55.

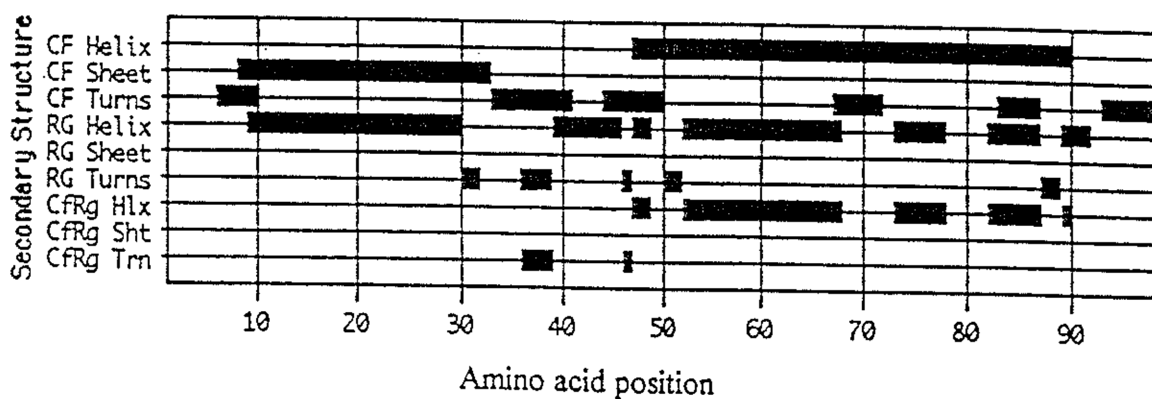


Figure 41. Secondary structure prediction of GlcNAcT-I from rabbit liver. The combined models predicted the location of an α-helix (CfRg Hlx) to occur from amino acid residues 52-86, with two small breaks occurring around position 70 and 80.

D. Determination of glycosyltransferase activity from various rat liver fractions before and during the acute phase response

The various fractions created during the Golgi isolation protocol were assayed to monitor how the distribution of glycosyltransferase activities were affected during the APR. ST6Gal I, ST3Gal III, GlcNAcα2-6ST and GlcNAcT-I were each assayed from the nuclear pellet, fraction 1A, 1B and 1C as described in figure 17 on page 96. Fraction 1A was collected and used for the final purification of the Golgi vesicles, as described in Methods and Materials. Table 12 displays the results of these assays while table 13 demonstrates the effectiveness of the purification procedures by illustrating that 70-80% of the respective glycosyltransferases were recovered in fraction 1A.

When fraction 1A was centrifuged on a discontinuous sucrose gradient as described in figure 17, two clearly visible fractions appeared. The first, appearing just above the 0.5 M / 1.1 M boundary (fraction 2A), typically considered the 'Golgi fraction' was collected and assayed for sialyltransferase activity from both control and inflamed sources. The fraction immediately beneath this one (fraction 2B) commonly referred to as the 'smooth endoplasmic reticulum fraction' was likewise assayed for the same enzymes. Interestingly, all three enzymes had varying degrees of increases in activity in fraction 2B during the acute phase response, whereas only ST6Gal I increased in activity in fraction 2A. Both ST3Gal III and

	Activity of glycosyltransferases (units / mg protein)							
	ST6Gal I		ST3Gal III		GlcNAcα2-6ST		GlcNAcT-I	
Fraction	I	C	I	C	I	C	I	C
1A	428	205	140	221	185	196	437	382
1B	43	19	26	7	34	31	32	17
1C	74	33	9	22	26	23	16	6
Nuclear pellet	4	7	5	12	11	3	4	10

Table 12. Determination of glycosyltransferase activity from fractions 1A, 1B, 1C, and the nuclear pellet of homogenized rat livers. Glycosyltransferase activity was assayed from both control (C) and 36 hour inflamed (I) rats. Fractionation procedure was performed as described in figure 17. Experiments were repeated 3-5 times with a reproducibility of ±10%.

	Activity of glycosyltransferases (units / mg protein)							
	ST6Gal I		ST3Gal III		GlcNAcα2-6ST		GlcNAcT-I	
Fraction	I	C	I	C	I	C	I	C
1A	428	205	140	221	185	196	437	382
1B	43	19	26	7	34	31	32	17
1C	74	33	9	22	26	23	16	6
Nuclear pellet	4	7	5	12	11	3	4	10

Table 12. Determination of glycosyltransferase activity from the fractions 1A, 1B, 1C, and the nuclear pellet of homogenized rat livers. Glycosyltransferase activity was assayed from both control (C) and 36 hour inflamed (I) rats. Fraction procedure was performed as described in figure 17. Experiments were repeated 3-5 times with a reproducibility of ±10%.

% of glycosyltransferase activity within fraction 1A							
ST6Gal I		ST3Gal III		GlcNAcα2-6ST		GlcNAcT-I	
C	I	C	I	C	I	C	I
80.0	77.7	77.8	84.4	72.3	77.5	89.4	92.0

Table 13. Percentage of glycosyltransferase activity within subfraction 1A from rat liver. Enzyme activity from fraction 1A was compared to the total activity recovered in the nuclear pellet, fraction 1C, fraction 1B and fraction 1A itself. Results were from both control (C) and 36 hour inflamed (I) rats. Experiments were repeated 3-5 times with a reproducibility of ±10%.

GlcNAcα2-6ST demonstrated a decrease in enzymatic activity from fraction 2B (table 14).

Since there seemed to be a change within activity levels of the sialyltransferases during inflammation between fraction 2A and 2B, a continuous sucrose gradient was formed with fraction 1A, which contains both 2A and 2B. When 1A was separated into 20 fractions by this method, using samples from both control and inflamed liver, all four enzymes displayed unique characteristics of density localization. Under control conditions ST6Gal I peaked in one specific region, fractions 12-15, while the inflamed samples resulted in two major peaks of activity (fig. 42). The larger one appeared from fractions 6-11, while the smaller of the two occurred from 14-18. In control samples ST3Gal III presented most of its activity in the early fractions, 3-6, and in the later fractions 14-16, which is where the predominant amount of ST6Gal I activity was found from control tissue (fig. 43). During inflammation the activity of ST3Gal III seemed to form three main peaks of activity, with the majority of the activity seeming to shift from fractions 3-6 and spreading throughout 5-11. There appeared to be little change in activity of this enzyme in the later fractions, as both control and inflamed samples produced similar levels of ST3Gal III activity in fractions 14-16. The results with GlcNAcα2-6ST activity proved to be quite interesting. Although both control and inflamed samples resulted in similar peaks of activity in fractions 15-17, a larger peak of the enzyme's activity, located in fractions 3-6 seems to disappear altogether (fig. 44).

Enzyme	Sialyltransferase activity (units/mg protein)			
	Fraction 2A		Fraction 2B	
	C	I	C	I
ST6Gal I	405	873	37	51
ST3Gal III	287	168	71	133
GlcNAcα2-6ST	96	60	16	103

Table 14. Sialyltransferase activity in the Golgi (fraction 2A) and smooth endoplasmic reticulum layer (fraction 2B) from control and inflamed rats. Both fraction 2A and 2B were aspirated from control rats (C) and those which had been inflamed for 36 hours (I). The fractions were then assayed for sialyltransferase activity. Each experiment was repeated three times with a combination of two rat livers. Results were reproducible within ±15%.

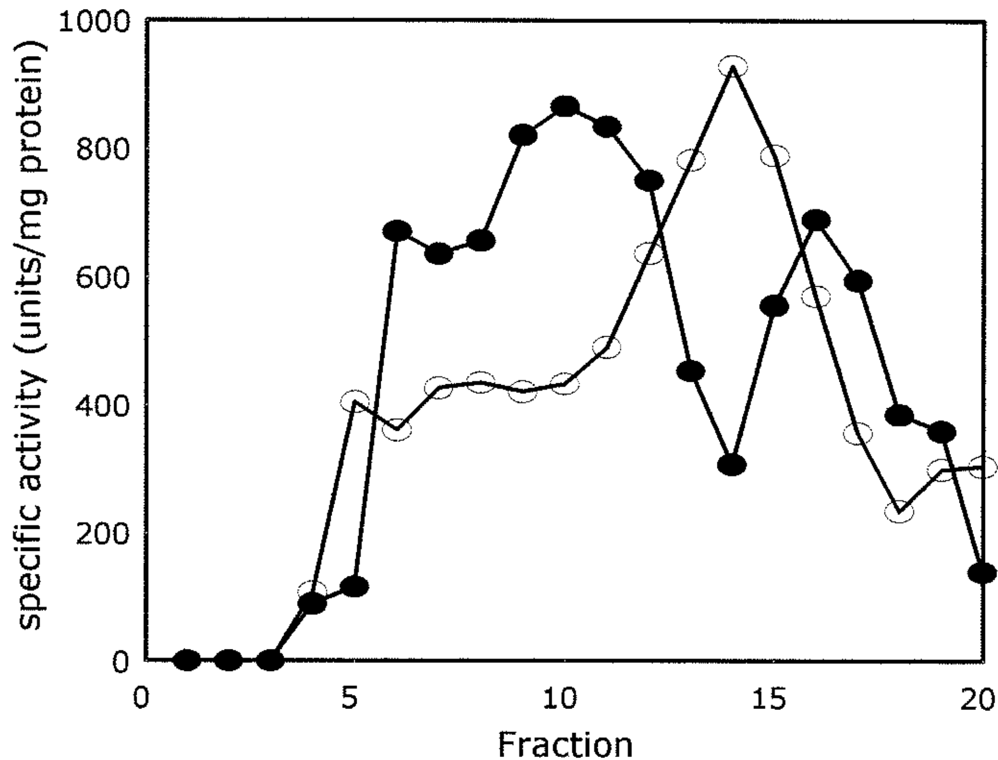


Figure 42. Determination of ST6Gal I activity from fraction 1A density layer following subfractionation on a continuous sucrose density gradient. Fraction 1A was collected as described in figure 17. It was then centrifuged on a continuous sucrose gradient and samples collected as described under Materials and Methods and figure 18. Each experiment was repeated three times with Golgi prepared from the liver of control rats (O) and those which have been inflamed for 36 hours (●). Results were reproducible within ±5%.

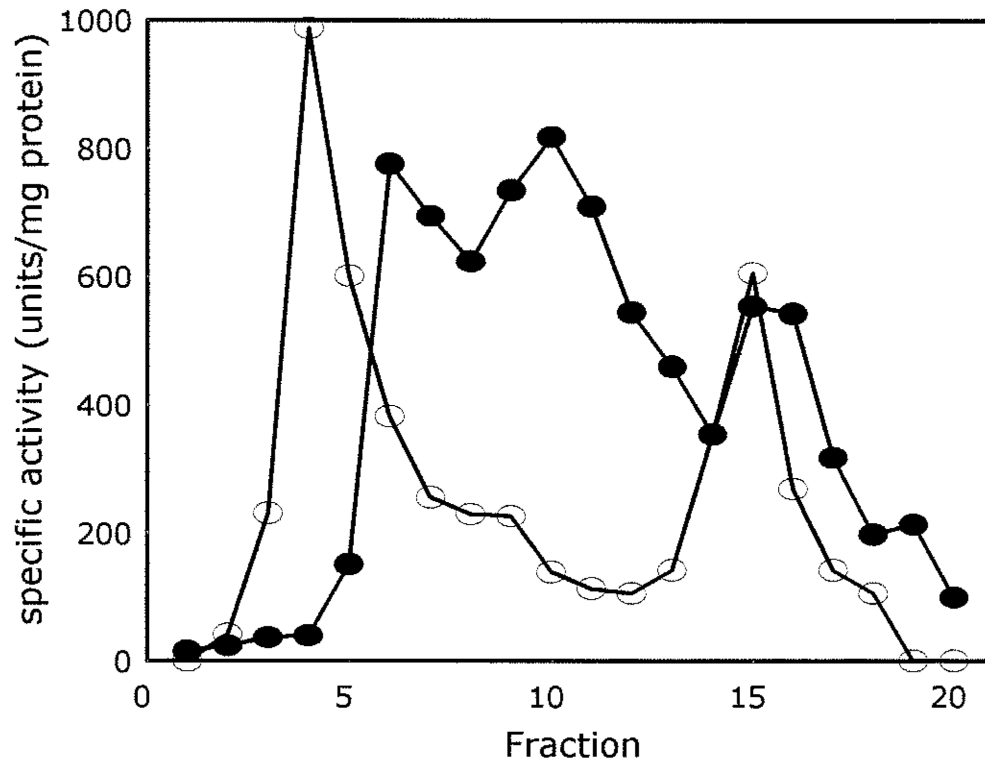


Figure 43. Determination of ST3Gal III activity from fraction 1A which was subfractionated on a continuous sucrose density gradient. Fraction 1A was collected as described in figure 17. It was then centrifuged on a continuous sucrose gradient and samples collected as described under Materials and Methods and figure 18. Each experiment was repeated three times with Golgi prepared from the liver of control rats (O) and those which have been inflamed for 36 hours (●). Results were reproducible within ±5%.

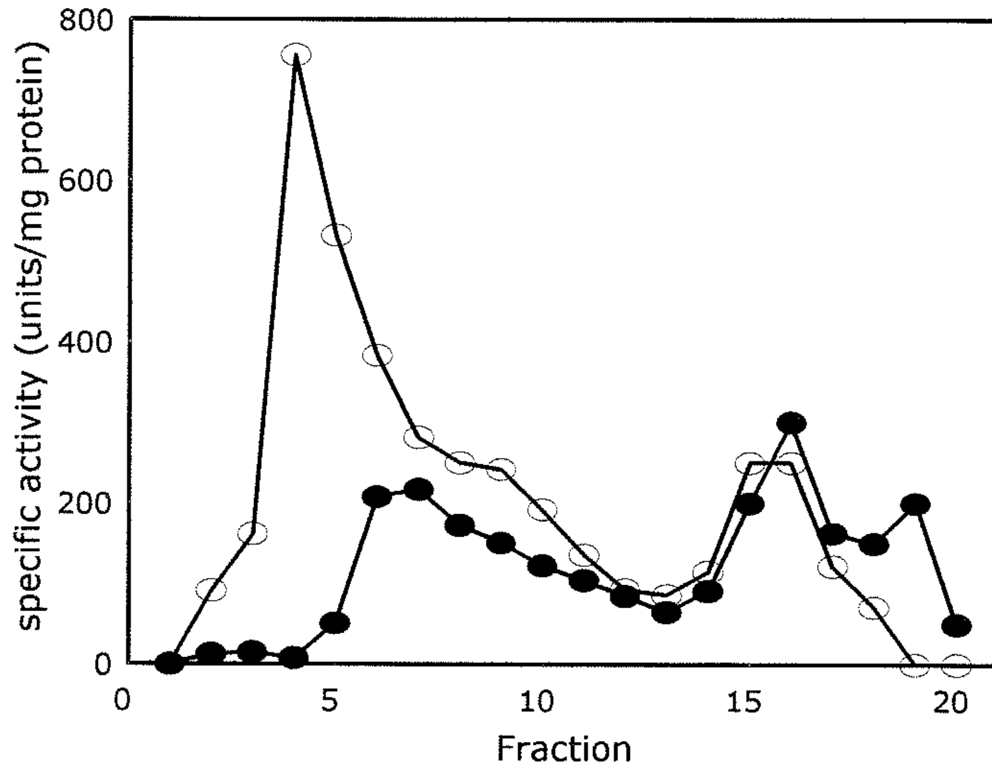


Figure 44. Determination of GlcNAcα2-6ST activity from fraction 1A which was subfractionated on a continuous sucrose density gradient. Fraction 1A was collected as described in figure 17. It was then centrifuged on a continuous sucrose gradient and samples collected as described under Materials and Methods and figure 18. Each experiment was repeated three times with Golgi prepared from the liver of control rats (O) and those which have been inflamed for 36 hours (●). Results were reproducible within ±5%.

The interesting feature of this result is that prior to subfractionation, both control and inflamed samples contained equal levels of activity of the GlcNAcα2-6ST enzyme, as presented in table 13. Although the availability of the acceptor limited the number of fractions that could be assayed for GlcNAcT-I activity, a clear trend in the differences between control and inflamed fractions could still be observed (fig. 45) From the subfractions taken from control samples two peaks of activity were evident. The smaller one appeared at about fraction 4 and the larger at fractions 15-17. Inflamed samples also demonstrated two peaks of activity, at fraction 3 and at fraction 12. However, unlike the control samples, these peaks were closer together and displayed the same levels of GlcNAcT-I activity.

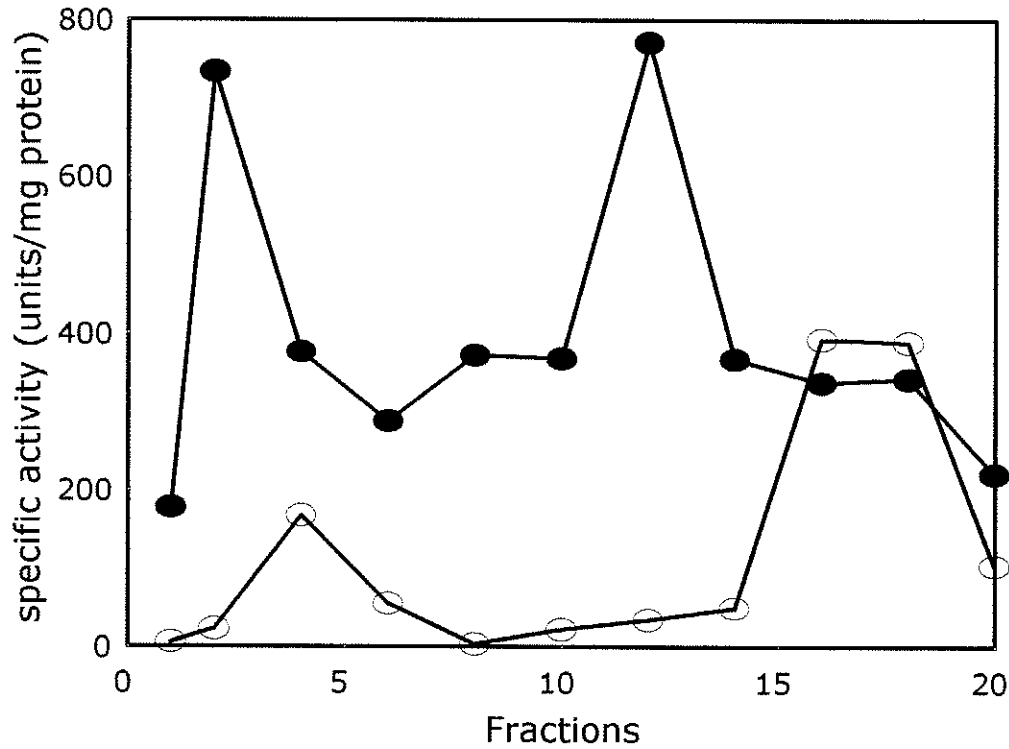


Figure 45. Determination of GlcNAcT-I activity from fraction 1A which was subfractionated on a continuous sucrose density gradient. Fraction 1A was collected as described in figure 17. It was then centrifuged on a continuous sucrose gradient and samples collected as described under Materials and Methods and figure 18. Each experiment was repeated three times with Golgi prepared from the liver of control rats (O) and those which have been inflamed for 36 hours (●). Results were reproducible within ±5%.

E. Detection of a Golgi matrix capable of binding sialyltransferases

In order to expose an underlying matrix situated between the Golgi cisternae a detergent was required which would solubilize the membrane lipids but not the sialyltransferases. Although TX-100 had been used in this manner to isolate a matrix from the *medial* region of purified Golgi membranes (Slusarewicz, P. *et al.*, 1994) this detergent proved to be unsatisfactory for this procedure as it solubilized the sialyltransferases, even at low concentrations. However, Lubrol was shown to be an effective detergent for these experiments. As seen in table 15, ST6Gal I was not readily solubilized by lubrol when compared to TX-100, even at a concentration of 3%. Although the amount of activity recovered was roughly 80% after the membranes were treated with Lubrol, most of this remained in the pellet. As expected, the ability of TX-100 to solubilize ST6Gal I was seen even at concentrations of 0.5% and by 2% most of the enzyme had been extracted. The ability of Lubrol and TX-100 to solubilize ST3Gal III (table 16) and GlcNAcα2-6ST (table 17) from purified Golgi membranes was similar to the results found for the ST6Gal I enzyme. GlcNAcT-I was unique in that it was able to resist extraction equally well by both detergents (table 18). This result was expected since the initial studies which isolated the *medial* matrix, did so by using GlcNAcT-I as a marker since it was not solubilized by TX-100.

Detergent	Activity of ST6Gal I in detergent (units/mg protein)			
	100% activity	% solubilized	% pellet	% recovered
Lubrol (%)				
0.0	262	1	81	82
0.5	271	7	72	79
1.0	295	11	63	74
2.0	318	14	68	82
3.0	493	22	59	81
TX-100 (%)				
0.0	262	1	93	94
0.5	362	61	58	119
1.0	431	83	21	104
2.0	518	89	8	97
3.0	647	96	3	99

Table 15. Ability of detergent to solubilize ST6Gal I from purified Golgi membranes. Experiments combined purified Golgi membranes from two rat livers and results were repeated three times with a reproducibility of ±10%.

Detergent	Activity of ST3Gal III in detergent (units/mg protein)			
	100% activity	% solubilized	% pellet	% recovered
Lubrol (%)				
0.0	166	1	92	93
0.5	180	4	87	91
1.0	248	10	84	94
2.0	338	14	73	87
3.0	374	19	68	87
TX-100 (%)				
0.0	166	1	97	98
0.5	219	34	84	118
1.0	282	52	61	113
2.0	377	83	29	112
3.0	436	95	12	107

Table 16. Ability of detergent to solubilize ST3Gal III from purified Golgi membranes. Experiments combined purified Golgi membranes from two rat livers and results were repeated three times with a reproducibility of ±10%.

Detergent	Activity of GlcNAcα2-6ST in detergent (units/mg protein)			
	100 % activity	% solubilized	% pellet	% recovered
Lubrol (%)				
0.0	76	1	94	95
0.5	93	12	74	86
1.0	98	15	75	90
2.0	96	21	70	91
3.0	103	29	56	85
TX-100 (%)				
0.0	76	1	94	95
0.5	91	29	63	92
1.0	113	58	31	89
2.0	147	72	29	101
3.0	153	87	22	109

Table 17. Ability of detergent to solubilize GlcNAcα2-6ST from purified Golgi membranes. Experiments combined purified Golgi membranes from two rat livers and results were repeated three times with a reproducibility of ±10%.

Detergent	Activity of GlcNAcT-I in detergent (units/mg protein)			
	100% activity	% solubilized	% pellet	% recovered
Lubrol (%)				
0.0	192	1	93	94
0.5	202	7	94	101
1.0	295	12	83	95
2.0	312	14	82	96
3.0	343	17	80	97
TX-100 (%)				
0.0	192	1	95	96
0.5	202	3	97	100
1.0	197	6	103	109
2.0	209	11	74	95
3.0	233	25	68	93

Table 18. Ability of detergent to solubilize GlcNAcT-I from purified Golgi membranes. Experiments combined purified Golgi membranes from two rat livers and results were repeated three times with a reproducibility of ±10%.

Before an examination of the ability of salt to extract the sialyltransferases could be tested, its effect on the enzyme activities was tested. Although there is very little activity lost for the enzymes studied up to 150 mM NaCl, the salt appears to inhibit GlcNAcT-I, ST6Gal I, and ST3Gal III when the salt concentration reaches 200 mM (table 19), while GlcNAcα2-6ST was not affected. Golgi membranes which were solubilized with 2% Lubrol buffer, but supplemented with NaCl ranging from 50 mM to 200 mM. The extraction process was also performed without salt, so as to act as a control. The other variable which was tested during the extraction was the concentration of Lubrol that was used to solubilize the Golgi membranes. As seen in figure 46, as more NaCl is used, there is an increase in the amount of ST6Gal I that is extracted, at all concentrations of Lubrol used to solubilize the Golgi membranes. However, NaCl was much more effective at extracting ST6Gal I from the matrix when higher concentrations of Lubrol were used. Without Lubrol treatment, NaCl only extracted low levels of the enzyme at very low levels, even at high concentrations. Solubilizing the Golgi membranes with 1% Lubrol facilitates NaCl extraction of higher levels of ST6Gal I, while solubilization with 2% and 3% Lubrol allowed for even greater levels of extraction by NaCl. Similar results were achieved when ST3Gal III (fig. 47) and GlcNAcα2-6ST (fig. 48) were extracted with salt. Overall, 150 mM - 200 mM NaCl was capable of extracting about 50% of the sialyltransferases which remained bound to the Golgi matrix after 2% or 3% Lubrol treatment of the Golgi lipid bilayer. Although GlcNAcT-I followed the same trend as the sialyltransferases, relatively higher levels of the enzyme were extracted

	Glycosyltransferase activity (units/mg protein)				
[NaCl] mM	0	50	100	150	200
GlcNAcT-I	312	316	293	280	287
GlcNAcα2-6ST	96	94	99	90	93
ST6Gal I	318	303	296	281	168
ST3Gal III	338	348	327	298	117

Table 19. Effect of NaCl on glycosyltransferase activity. Experiments were repeated 3 times with a reproducibility of ±10%.

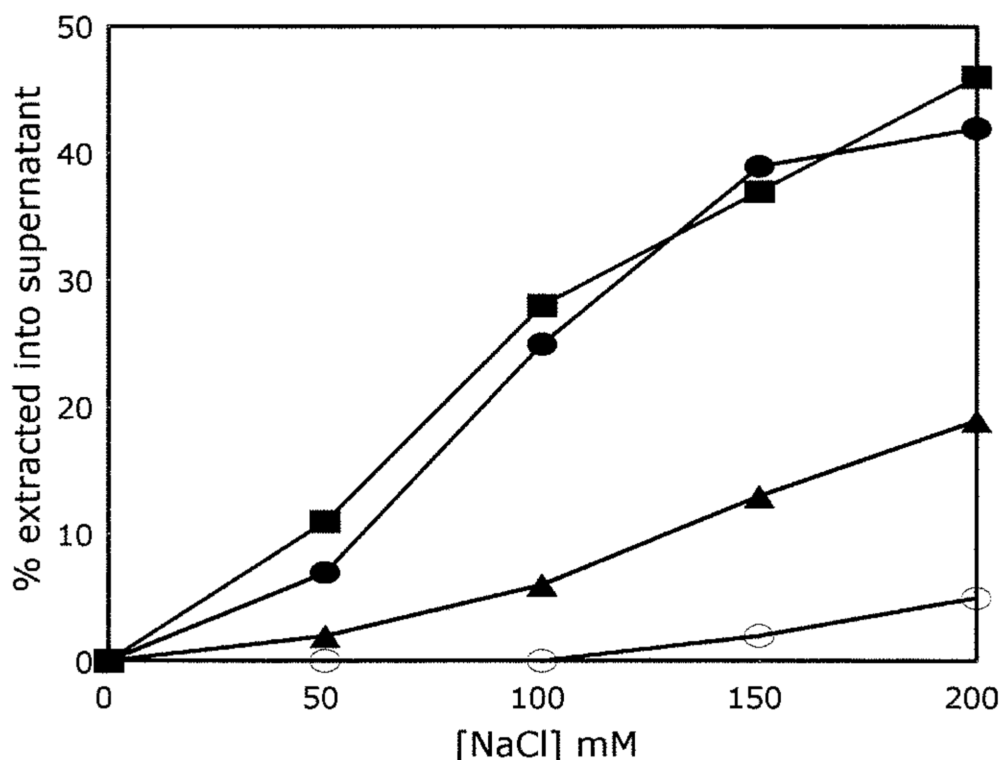


Figure 46. Extraction of ST6Gal I with NaCl from Golgi membranes solubilized with Lubrol. Purified Golgi membranes were solubilized with either 3% (■), 2% (●), 1% (▲), or no Lubrol (O) and then centrifuged as described under methods. The pellet was then gently resuspended in a buffer containing the same concentration of Lubrol supplemented with various concentrations of NaCl. This solution was then incubated on ice for 30 min, a small aliquot set aside for 100 % activity determination, then centrifuged so that the supernatant and pellet could be assayed. Recovery of ST6Gal I activity was 97 ±5 %, regardless of salt concentration. The value in the supernatant, compared to the 100% value, was taken to be the '% extracted into supernatant'. The 100 % values were calculated as pmol/min/mg protein and were determined for each Lubrol %. About 4 % of the enzyme was detected in the supernatant after treatment with 0 mM NaCl, which was subsequently subtracted from all other values where salt extraction had occurred. Therefore all values were equally readjusted to give a graphical illustration where the data represented the % extracted due totally to the presence of NaCl. Experiments were repeated three times with a reproducibility of ±15%.

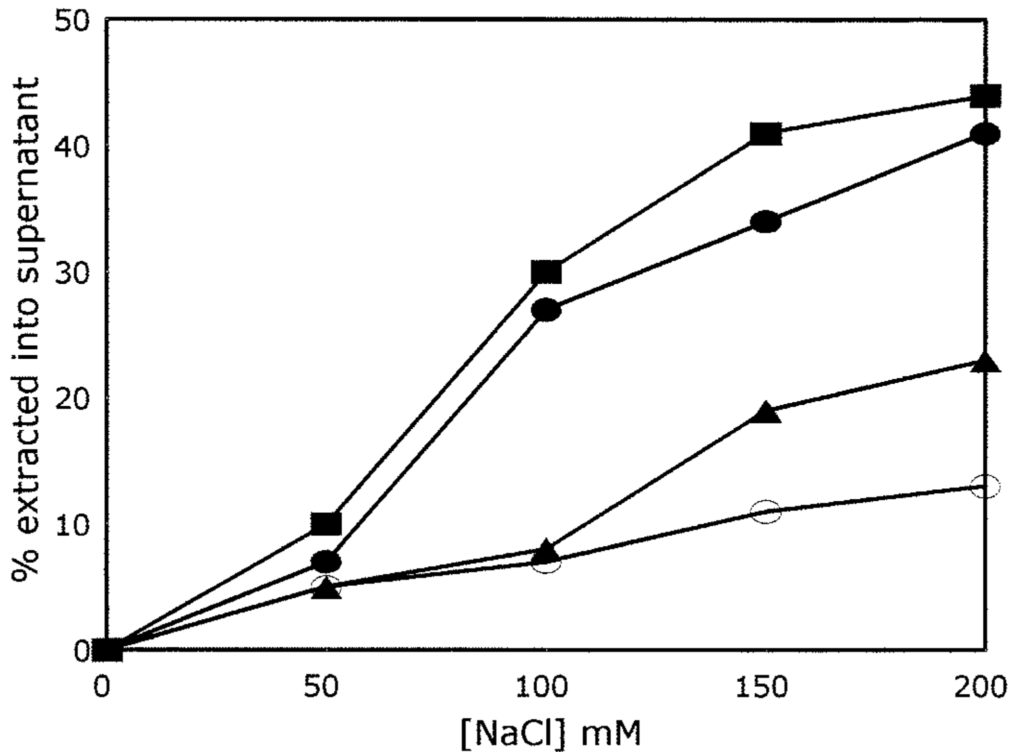


Figure 47. Extraction of ST3Gal III with NaCl from Golgi membranes solubilized with Lubrol. Purified Golgi membranes were solubilized with either 3% (■), 2% (●), 1% (▲), or no Lubrol (O) and then centrifuged as described under methods. The pellet was then gently resuspended in a buffer containing the same concentration of Lubrol supplemented with various concentrations of NaCl. This solution was then incubated on ice for 30 min, a small aliquot set aside for 100 % activity determination, then centrifuged so that the supernatant and pellet could be assayed. Recovery of ST3Gal III activity was 101 ±3 %, regardless of salt concentration. The value in the supernatant, compared to the 100% value, was taken to be the '% extracted into supernatant'. The 100 % values were calculated as pmol/min/mg protein and were determined for each Lubrol %. About 6 % of the enzyme was detected in the supernatant after treatment with 0 mM NaCl, which was subsequently subtracted from all other values where salt extraction had occurred. Therefore all values were equally readjusted to give a graphical illustration where the data represented the % extracted due totally to the presence of NaCl. Experiments were repeated three times with a reproducibility of ±15%.

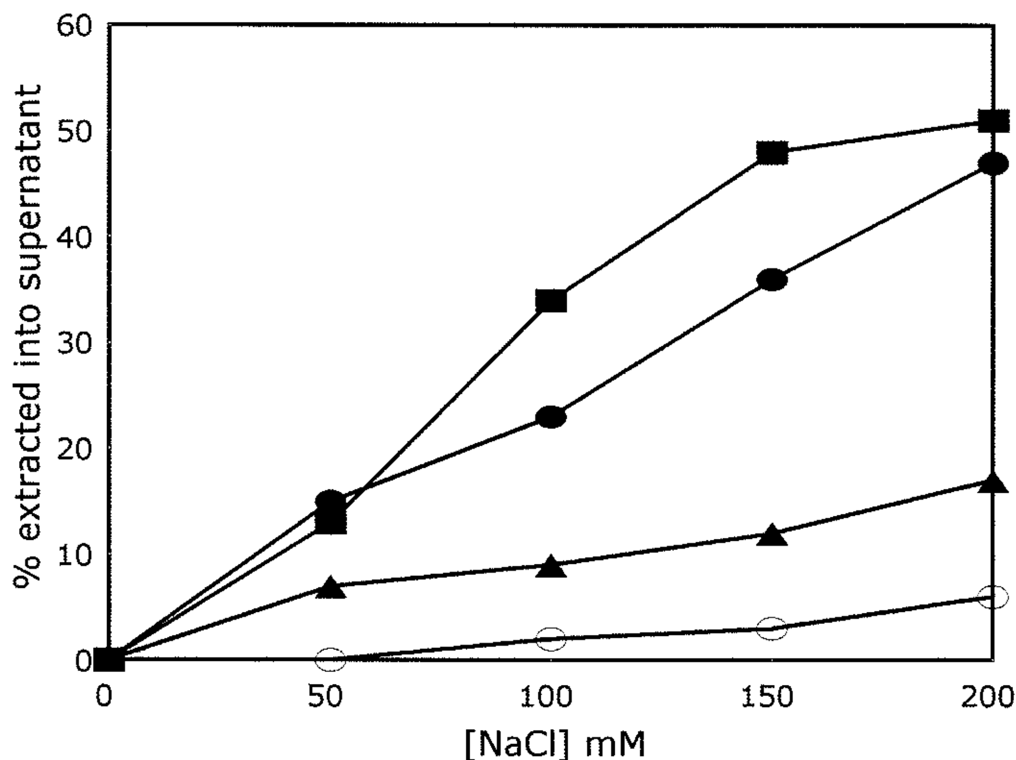


Figure 48. Extraction of GlcNAcα2-6ST with NaCl from Golgi membranes solubilized with Lubrol. Purified Golgi membranes were solubilized with either 3% (■), 2% (●), 1% (▲), or no Lubrol (O) and then centrifuged as described under methods. The pellet was then gently resuspended in a buffer containing the same concentration of Lubrol supplemented with various concentrations of NaCl. This solution was then incubated on ice for 30 min, a small aliquot set aside for 100 % activity determination, then centrifuged so that the supernatant and pellet could be assayed. Recovery of GlcNAcα2-6ST activity was 98 ±6 %, regardless of salt concentration. The value in the supernatant, compared to the 100% value, was taken to be the '% extracted into supernatant'. The 100 % values were calculated as pmol/min/mg protein and were determined for each Lubrol %. About 2 % of the enzyme was detected in the supernatant after treatment with 0 mM NaCl, which was subsequently subtracted from all other values where salt extraction had occurred. Therefore all values were equally readjusted to give a graphical illustration where the data represented the % extracted due totally to the presence of NaCl. Experiments were repeated three times with a reproducibility of ±15%.

under the same conditions. For instance, even when Lubrol concentrations of 1% were used, 150 mM - 200 mM NaCl was able to extract about 80% of the GlcNAcT-I, while 2% and 3% Lubrol allowed about 90% extraction (fig. 49).

In order to fully test the binding capabilities in the matrix, experiments were designed where glycosyltransferases that had been extracted with salt were able to be reconstituted with the matrix upon removal of the salt *via* dialysis. Furthermore, matrices were prepared by solubilizing the Golgi membranes with either Lubrol or TX-100 and varying concentrations of NaCl. In total, matrices were prepared by six different methods (table 20A) and the enzymes that were analyzed for rebinding capabilities were extracted from a matrix (prepared by solubilizing Golgi membranes with 2% Lubrol) with either 70 mM or 150mM NaCl. Although both salt extracted forms were able to rebind all of the six prepared matrices to varying degrees, matrices prepared by solubilizing the membranes with Lubrol clearly were more efficient at rebinding ST6Gal I than ones prepared with TX-100 (table 20B). Comparing matrices using Lubrol, ST6Gal I extracted with 70 mM NaCl was able to rebind the matrix with about a two-fold increase in binding efficiency compared to the enzyme extracted with 150 mM NaCl. For example, when a matrix was prepared *only* by Lubrol solubilization, 39 % of the 70 mM NaCl extracted ST6Gal I was able to rebind, while only 21% of the 150 mM NaCl extracted enzyme did so. Furthermore, using salt to prepare the matrix did seem to have an effect on the matrices' ability to rebind both extracted forms. For instance, when a matrix

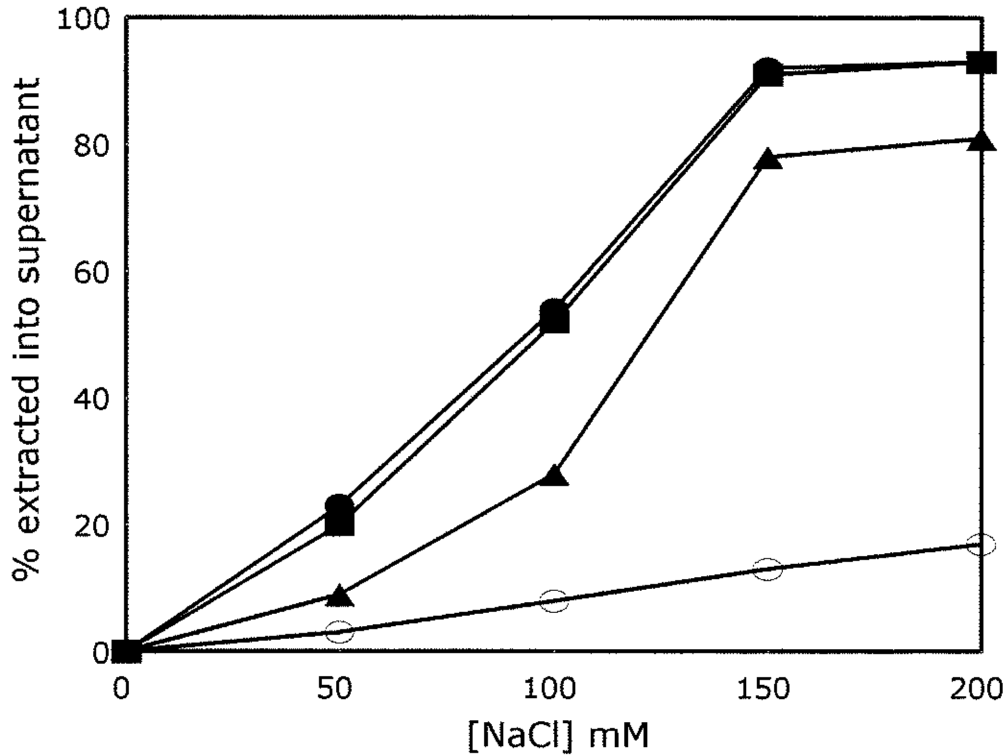


Figure 49. Extraction of GlcNAcT-I with NaCl from Golgi membranes solubilized with Lubrol. Purified Golgi membranes were solubilized with either 3% (■), 2% (●), 1% (▲), or no Lubrol (O) and then centrifuged as described under methods. The pellet was then gently resuspended in a buffer containing the same concentration of Lubrol supplemented with various concentrations of NaCl. This solution was then incubated on ice for 30 min, a small aliquot set aside for 100 % activity determination, then centrifuged so that the supernatant and pellet could be assayed. Recovery of GlcNAcT-I activity was 94 ±2 %, regardless of salt concentration. The value in the supernatant, compared to the 100% value, was taken to be the '% extracted into supernatant'. The 100 % values were calculated as pmol/min/mg protein and were determined for each Lubrol %. About 3% of the enzyme was detected in the supernatant after treatment with 0 mM NaCl, which was subsequently subtracted from all other values where salt extraction had occurred. Therefore all values were equally readjusted to give a graphical illustration where the data represented the % extracted due totally to the presence of NaCl. Experiments were repeated three times with a reproducibility of ±15%.

MATRIX	ST6Gal I activity in matrix (units / ml homogenate)	ST6Gal I activity in 70 mM NaCl extracted solution (units / ml homogenate)	ST6Gal I activity in matrix after reconstitution of 70mM NaCl extracted enzyme	Activity remaining in the 70 mM NaCl supernatant	Total activity of extracted 70 mM NaCl ST6Gal I recovered, (%)
A	220	33	233	18	31 (94)
B	185	33	194	20	29 (88)
C	122	33	129	23	30 (91)
D	41	33	43	30	32 (97)
E	30	33	33	29	32 (97)
F	22	33	26	28	32 (97)

MATRIX	ST6Gal I activity in matrix (units / ml homogenate)	ST6Gal I activity in 150 mM NaCl extracted solution (units / ml homogenate)	ST6Gal I activity in matrix after reconstitution of 150 mM NaCl extracted enzyme	Activity remaining in the 150 mM NaCl supernatant	Total activity of extracted 150 mM NaCl ST6Gal I recovered, (%)
A	220	87	238	67	85 (98)
B	185	87	199	72	86 (99)
C	122	87	133	77	88 (101)
D	41	87	49	79	87 (100)
E	30	87	42	74	86 (99)
F	22	87	32	75	85 (98)

Table 20A. Measurement of ST6Gal I activity reconstituted into matrices prepared in various salt and detergent concentrations. Matrices were prepared from purified Golgi membranes in six different manners as described under methods as follows: (A) 2% lubrol; (B) 2% lubrol / 70 mM NaCl; (C) 2% lubrol / 150 mM NaCl; (D) 2% TX-100; (E) 2% TX-100 / 70 mM NaCl; (F) 2% TX-100 / 150 mM NaCl. The amount of ST6Gal I activity which was extracted with the 70 mM and 150 mM NaCl solution was assayed and determined as 33 and 87 units / ml homogenate respectively. From this value, the amount of activity in the respective matrix could be compared, and therefore a % of ST6Gal I rebinding could be calculated (table 20B). Furthermore, the amount of ST6Gal I from both salt extracted solutions, which didn't rebind the matrix was determined as described under methods. This value was added to the amount which the matrix had increased by in ST6Gal I activity after reconstitution of the salt extracted. Therefore, *the sum of these two values represented the total activity of ST6Gal I recovered.* Comparing this total to the amount of activity originally extracted with either 70 mM or 150 mM NaCl indicated the amount of enzyme activity recovered.

MATRIX	The % of 70 or 150 mM NaCl extracted ST6Gal I that is reconstituted into Golgi matrices which have been prepared by a variety of techniques	
	70 mM NaCl extracted	150 mM NaCl extracted
2% Lubrol	39	21
2% Lubrol / 70 mM NaCl	27	16
2% Lubrol / 150 mM NaCl	21	13
2% TX100	6	10
2% TX100 / 70 mM NaCl	9	14
2% TX100 / 150 mM NaCl	11	12

Table 20B. Reconstitution of salt extracted ST6Gal I into Golgi matrices prepared by various detergents and salt concentrations. Although control samples were performed, as described under methods, they produced negligible results with typically less than 1% rebinding efficiency in the absence of dialysis. Experiments were repeated three times and the purified Golgi was derived from three to four rat livers. Results were reproducible within ±15%.

was prepared in the absence of salt, 39 % of the 70 mM NaCl extracted form was able to rebind, but when exposed to 70 mM NaCl, this dropped to 27 % rebinding, then to 21 % rebinding when the matrix was prepared with 150 mM NaCl. These same observations cannot be made for matrices prepared with TX-100. These same general results were obtained for the other two sialyltransferases ST3Gal III and GlcNAcα2,6ST (table 21A / B; table 22 A / B). The important feature of these results, as noted in tables 20 A, 21A and 22A, is that recovery of the enzymes was close to 100 %, accounting for the enzyme activity that did not rebind the respective matrices. As expected, the rebinding efficiency of GlcNAcT-I was significantly greater than the sialyltransferases, averaging at about 50% in all cases (table 23B). Furthermore, salt concentrations and detergent types did not seem to play a meaningful role in the rebinding. As with the other enzymes, there was close to 100% recovery of GlcNAcT-I (table 23A).

Because salt has been shown to have an adverse effect on the structural nature of the Golgi *medial*-matrix (Slusarewicz, P. *et al.*, 1994), and did affect rebinding of the sialyltransferases in these studies, the ability of the glycosyltransferases to rebind a matrix exposed to a high salt environment was examined. In this case, a matrix prepared by solubilizing Golgi membranes with 2% Lubrol / 500 mM NaCl was used. A separate sample of Golgi membranes solubilized with 2% Lubrol then had their glycosyltransferases extracted with 70 mM NaCl. The extracted forms of the sialyltransferases did not rebind the 2% Lubrol

MATRIX	ST3Gal III activity in matrix (units / ml homogenate)	ST3Gal III activity in 70 mM NaCl extracted solution (units / ml homogenate)	ST3Gal III activity in matrix after reconstitution of 70 mM NaCl extracted enzyme	Activity remaining in the 70 mM NaCl supernatant	Total activity of extracted 70 mM NaCl ST3Gal III recovered, (%)
A	243	37	256	26	39 (105)
B	203	37	213	25	35 (90)
C	147	37	156	27	36 (97)
D	94	37	96	34	36 (97)
E	61	37	67	29	35 (90)
F	38	37	43	30	35 (90)

MATRIX	ST3Gal III activity in matrix (units / ml homogenate)	ST3Gal III activity in 150 mM NaCl extracted solution (units / ml homogenate)	ST3Gal III activity in matrix after reconstitution of 150 mM NaCl extracted enzyme	Activity remaining in the 150 mM NaCl supernatant	Total activity of extracted 150 mM NaCl ST3Gal III recovered, (%)
A	243	100	256	84	97 (97)
B	203	100	224	81	102 (102)
C	147	100	164	80	97 (97)
D	94	100	101	91	98 (98)
E	61	100	74	86	99 (99)
F	38	100	49	90	101 (101)

Table 21A. Measurement of ST3Gal III activity reconstituted into matrices prepared by various salt and detergent concentrations. Matrices were prepared from purified Golgi membranes in six different manners as described under methods as followed: (A)2% Lubrol; (B)2% Lubrol / 70 mM NaCl; (C) 2% Lubrol / 150 mM NaCl; (D)2% TX-100; (E)2% TX-100 / 70 mM NaCl; (F)2% TX-100 / 150 mM NaCl. The amount of ST3Gal III activity which was extracted with the 70 mM and 150 mM NaCl solution was assayed and determined as 37 and 100 units / ml homogenate respectively. From this value, the amount of new activity in the respective matrix could be compared, and therefore a % of ST3Gal III rebinding could be calculated (table 21B). Furthermore, the amount of ST3Gal III from both salt extracted solutions, which didn't rebind the matrix was determined as described under methods. This value was added to the amount which the matrix had increased by in ST3Gal III activity after reconstitution of the salt extracted. Therefore, *the sum of these two values represented the total activity of ST3Gal III recovered.* Comparing this total to the amount of activity originally extracted with either 70 mM or 150 mM NaCl indicated the amount of enzyme activity recovered.

MATRIX	The % of 70 or 150 mM NaCl extracted ST3Gal III that is reconstituted into Golgi matrices which have been prepared by a variety of techniques	
	70 mM NaCl extracted	150 mM NaCl extracted
2% Lubrol	34	13
2% Lubrol / 70 mM NaCl	28	21
2% Lubrol / 150 mM NaCl	25	17
2% TX100	5	7
2% TX100 / 70 mM NaCl	16	13
2% TX100 / 150 mM NaCl	13	11

Table 21B. Reconstitution of salt extracted ST3Gal III into Golgi matrices prepared by various detergents and salt concentrations. Although control samples were performed, as described under methods, they produced negligible results with typically less than 2% rebinding efficiency in the absence of dialysis. Experiments were repeated three times and the purified Golgi was derived from three to four rat livers. Results were reproducible within ±15%.

MATRIX	GlcNAcα2-6ST activity in matrix (units / ml homogenate)	GlcNAcα2-6ST activity in 70 mM NaCl extracted solution (units / ml homogenate)	GlcNAcα2-6ST activity in matrix after reconstitution of 70 mM NaCl extracted enzyme	Activity remaining in the 70 mM NaCl supernatant	Total activity of extracted 70 mM NaCl GlcNAcα2-6ST recovered, (%)
A	72	11	76	7	11 (100)
B	57	11	61	5	9 (81)
C	45	11	48	10	13 (118)
D	43	11	44	9	10 (91)
E	34	11	36	6	8 (73)
F	19	11	21	7	9 (81)

MATRIX	GlcNAcα2-6ST activity in matrix (units / ml homogenate)	GlcNAcα2-6ST activity in 150 mM NaCl extracted solution (units / ml homogenate)	GlcNAcα2-6ST activity in matrix after reconstitution of 150 mM NaCl extracted enzyme	Activity remaining in the 150 mM NaCl supernatant	Total activity of extracted 150 mM NaCl GlcNAcα2-6ST recovered, (%)
A	72	27	76	21	25 (93)
B	57	27	62	20	25 (93)
C	45	27	47	24	26 (96)
D	43	27	44	24	25 (93)
E	34	27	36	25	27 (100)
F	19	27	20	22	23 (85)

Table 22A. Measurement of GlcNAcα2-6ST activity reconstituted into matrices prepared by various salt and detergent concentrations. Matrices were prepared from purified Golgi membranes in six different manners as described under methods as followed: (A)2% Lubrol; (B)2% Lubrol / 70 mM NaCl; (C) 2% Lubrol / 150 mM NaCl; (D)2% TX-100; (E)2% TX-100 / 70 mM NaCl; (F)2% TX-100 / 150 mM NaCl. The amount of GlcNAcα2-6ST activity which was extracted with the 70 mM and 150 mM NaCl solution was assayed and determined as 11 and 27 units / ml homogenate respectively. From this value, the amount of new activity in the respective matrix could be compared, and therefore a % of GlcNAcα2-6ST rebinding could be calculated (table 22B). Furthermore, the amount of GlcNAcα2-6ST from both salt extracted solutions, which didn't rebind the matrix was determined as described under methods. This value was added to the amount which the matrix had increased by in GlcNAcα2-6ST activity after reconstitution of the salt extracted. Therefore, *the sum of these two values represented the total activity of GlcNAcα2-6ST recovered.* Comparing this total to the amount of activity originally extracted with either 70 mM or 150 mM NaCl indicated the amount of enzyme activity recovered.

MATRIX	The % of 70 or 150 mM NaCl extracted GlcNAcα2-6ST that is reconstituted into Golgi matrices which have been prepared by a variety of techniques	
	70 mM NaCl extracted	150 mM NaCl extracted
2% Lubrol	35	14
2% Lubrol / 70 mM NaCl	32	15
2% Lubrol / 150 mM NaCl	26	19
2% TX100	7	4
2% TX100 / 70 mM NaCl	17	9
2% TX100 / 150 mM NaCl	15	8

Table 22B. Reconstitution of salt extracted GlcNAcα2-6ST into Golgi matrices prepared by various detergents and salt concentrations. Although control samples were performed, as described under methods, they produced negligible results with typically less than 1% rebinding efficiency in the absence of dialysis. Experiments were repeated three times and the purified Golgi was derived from three to four rat livers. Results were reproducible within ±15%.

MATRIX	The % of 70 or 150 mM NaCl extracted GlcNAcT-I that is reconstituted into Golgi matrices which have been prepared by a variety of techniques	
	70 mM NaCl extracted	150 mM NaCl extracted
2% Lubrol	64	48
2% Lubrol / 70 mM NaCl	57	44
2% Lubrol / 150 mM NaCl	53	41
2% TX100	43	45
2% TX100 / 70 mM NaCl	45	53
2% TX100 / 150 mM NaCl	55	47

Table 23B. Reconstitution of salt extracted GlcNAcT-I into Golgi matrices prepared by various detergents and salt concentrations. Although control samples were performed, as described under methods, they produced negligible results with typically less than 4% rebinding efficiency in the absence of dialysis. Experiments were repeated three times and the purified Golgi was derived from three to four rat livers. Results were reproducible within ±15%.

/ 500 mM NaCl matrix with much efficiency (table 24A / B). Interestingly, about 20% of the GlcNAcT-I was still able to rebind the matrix, but this compares with the 44% - 57% that was able to rebind a 2% Lubrol / 70 mM NaCl matrix or the 41% - 53% of the enzyme that was able to rebind a 2% Lubrol / 150 mM NaCl matrix.

In an effort to understand how these enzymes rebind the matrix, sialyltransferases which were proteolytically cleaved by cathepsin D from their membrane anchors were used. This form of the enzyme was devoid of its N-terminal region, which is thought to recognize the matrix. An effort to rebind the cleaved sialyltransferases into Golgi matrices by supplementing them with 70 mM NaCl, followed by dialysis to promote the rebinding, resulted in little re-association to the matrix (table 25). Typically, less than 3 % of the sialyltransferases which lacked a cytoplasmic tail were capable of associating with a Golgi matrix that was prepared by 2% Lubrol / 70 mM NaCl. This compares to 27% - 32% rebinding efficiency of the 70 mM NaCl extracted forms of the sialyltransferases with the aforementioned Golgi matrices prepared by treating Golgi membranes with 2% Lubrol / 70 mM NaCl.

A property of membrane spanning proteins is the ability to properly orient within the membrane. Although protein chemists are not certain of how this is achieved, a few models have been proposed. One of the more attractive theories stems from the fact that a difference in charges on either side of the first signal-

Enzyme	Activity in 500 mM NaCl matrix (units / ml homogenate)	Activity in 70 mM NaCl extraction	Activity in matrix after reconstitution	Activity remaining in 70 mM NaCl supernatant	Total activity of extracted 70 mM NaCl recovered, (%)
ST6Gal I	37	33	39	27	29 (88)
ST3Gal III	21	37	23	42	44 (122)
GlcNAcα2-6ST	8	11	8	8	8 (73)
GlcNAcT-I	13	102	31	93	111 (109)

Table 24A. Measurements of glycosyltransferase activities reconstituted into matrices prepared by solubilization of Golgi membranes with 2% Lubrol and 500 mM NaCl.

Enzyme	The % of 70 mM NaCl extracted enzyme reconstituted into a Golgi matrix exposed to 500 mM NaCl, compared to one which was only exposed to 70 mM NaCl.	
	500 mM NaCl Matrix	70 mM NaCl Matrix
GlcNAcα2-6ST	6	32
ST6Gal I	5	27
ST3Gal III	–	28
GlcNAcT-I	17	57

Table 24B. Reconstitution of salt extracted glycosyltransferases into a Golgi matrix exposed to treatment with 500 mM NaCl. Purified Golgi membranes were solubilized with a 2% Lubrol buffer supplemented with 500 mM NaCl as described under methods. Experiments were repeated three times and Golgi membranes were purified from the combination of three rat livers. Results were reproducible within ±15%.

MATRIX	The % of proteolytically cleaved sialyltransferases that are reconstituted into the Golgi matrix		
	GlcNAcα2-6ST	ST6Gal I	ST3Gal III
2% Lubrol / 70 mM NaCl	–	3	1

Table 25. Reconstitution of sialyltransferases which have been proteolytically cleaved in the stem region. Sialyltransferases were cleaved from their membrane anchor as described under Methods, resulting in a form of the enzyme which lacked a cytoplasmic tail, transmembrane domain, and a significant portion of its stem region. The activity was measured in 'units / ml homogenate' as described under Methods. Experiments were repeated three times and Golgi membranes were purified from the combination of three rat livers. Results were reproducible within ±15%.

anchor determines its orientation (Sakaguchi, M. *et al.*, 1992; Parks, P.D. *et al.*, 1991). Essentially, this model states that the more positive flanking sequence faces the cytoplasmic side of the membrane. In this case, it would mean that the N-terminal cytoplasmic tail of the sialyltransferases would have a charge more positive than the sequence of identical length flanking the lumenal side of the membrane. When the sequences of 31 sialyltransferases that have been cloned to date were analyzed in terms of this model, 30 of them were found to agree with its prediction (table 26). A possible explanation for this may be that the matrix has a negative charge which recognizes the greater positive on the N-terminal flanking region of the sialyltransferases. In this way, the C-terminal region is allowed to enter the lumenal side of the Golgi membrane, giving the enzymes their characteristic type-two (C_{in} / N_{out}) membrane orientation.

Enzyme	Source*	LTD	LCR	N	C	N-C
Galβ1-3GalNAc α2,3ST (I)	pig (1)	19	11	+5.0	+1.5	+4.0
	mouse (2)	29	7	+5.0	0.0	+5.0
	chick (3)	18	10	+5.0	+0.5	+4.5
	human (4)	19	10	+5.0	+0.5	+4.5
Galβ1-3GalNAc α2,3ST (II)	mouse (5)	21	6	+3.0	+1.0	+2.0
	human (6)	21	6	+3.0	+1.0	+2.0
	rat (5)	21	6	+3.0	+1.0	+2.0
Galβ1-3(4)GlcNAc α2,3ST (III)	rat (7)	20	8	+2.0	+0.5	+1.5
	human (8)	20	8	+2.0	+0.5	+1.5
	mouse (9)	20	8	+2.0	+0.5	+1.5
Galβ1-4(3)GlcNAc α2,3ST (IV)	human i (10)	18	8	+4.0	-1.0	+5.0
	human ii (11)	18	7	+2.0	-1.0	+3.0
	mouse (9)	18	8	+3.5	-1.0	+4.5
Galβ1-4GlcNAc α2,6ST	rat (12)	17	9	+4.5	0.0	+4.5
	human (13)	17	9	+4.5	+3.0	+1.0
	mouse (14)	17	9	+4.5	0.0	+4.5
	chick (15)	17	9	+3.5	+1.0	+2.5
GalNAc α2,6ST (I)	chick (16)	27	10	+4.0	+2.0	+2.0
Galβ1-3GalNAc GalNAc α2,6ST (II)	chick (17)	17	8	+4.0	-0.5	+4.5
	human (18)	27	5	+2.0	+1.0	+1.0
	mouse (19)	24	10	+4.0	0.0	+4.0
NeuAcα2,3Galβ1-3GalNAc α2,6ST(III)	rat (20)	17	8	+4.0	0.0	+4.0
GD3 synthase (ST8 Sia I)	human (21)	22	12	+4.0	0.0	+4.0
	rat (22)	22	12	+5.0	0.0	+5.0
	mouse (23)	25	8	+4.0	+1.0	+3.0
Polysialic acid Synthase (ST8 Sia II)	rat (24)	17	6	+2.0	-3.0	+5.0
NeuAcα2,3Galβ1-4GlcNAc α2,8ST (ST8 Sia III)	mouse (23)	25	8	+4.0	+1.0	+3.0
Polysialic acid Synthase (ST8 Sia IV)	hamster (25)	13	7	+5.0	+2.0	+3.0
	mouse (26)	13	7	+5.0	+2.0	+3.0
	human (27)	13	7	+5.0	+2.0	+3.0
α2,8ST (ST8 Sia V)	mouse (28)	22	16	+2.0	+3.0	-1.0

* See Table 27 for an abbreviated form of the listed references.

Table 26. Analysis of the charge differences on the sequences flanking the sialyltransferases. The location and subsequent length of the transmembrane domain (LTD) was identified as described in Materials and Methods. The number of amino acids on the N-terminal side of the membrane gives the length of the cytoplasmic tail. This same value was then used for the length of the lumenal segment flanking the LTD. The total charge of each of these two charged regions (LCR) was summed as described in Materials and Methods. The charge on the lumenal segment (C) was then subtracted from the charge of the cytoplasmic tail (N) to give a charge difference (N-C) between the two LCRs.

Reference Number	Reference
(1)	Gillespie, W., *et al* (1992)
(2)	Lee, Y.-C., *et al* (1993)
(3)	Kurosawa, N., *et al* (1995)
(4)	Chang, M.-L., *et al* (1995)
(5)	Lee, Y.-C., *et al* (1994)
(6)	Giordanengo, V., *et al* (1997)
(7)	Wen, D. X., *et al* (1992)
(8)	Kitagawa, H., *et al* (1993)
(9)	Kono, M., *et al* (1997)
(10)	Sasaki, K., *et al* (1993)
(11)	Kitagawa, H., *et al* (1994)
(12)	Weinstein, J., *et al* (1987)
(13)	Grundmann, U., *et al* (1990)
(14)	Hamamoto, T., *et al* (1993)
(15)	Kurosawa, N., *et al* (1994a)
(16)	Kurosawa, N., *et al* (1994b)
(17)	Kurosawa, N., *et al* (1994c)
(18)	Soutiropoulou, G., *et al* (1994)
(19)	Kurosawa, N., *et al* (1996)
(20)	Sjoberg, E.R., *et al* (1996)
(21)	Nara, K., *et al* (1994)
(22)	Watanabe, Y., *et al* (1996)
(23)	Yoshida, Y., *et al* (1995a)
(24)	Livingston, B.D., *et al* (1993)
(25)	Ekhard, M., *et al* (1995)
(26)	Yoshida, Y., *et al* (1995b)
(27)	Nakayama, J., *et al* (1995)
(28)	Kono, M., *et al* (1996)

Table 27. List of references used to compile data in table 26.

Discussion

Studies on the acute phase response on glycosyltransferase activities

Previous work in this laboratory was the first to demonstrate that ST6Gal I increases in activity by 2-fold in rat liver during acute inflammation, while GalT (EC 2.4.1.38) showed little, if any increase in activity (Kaplan, H.A., *et al.*, 1983; Lammers, G., *et al.*, 1988). Another observation was that ST6Gal I activity levels increased in the serum 5-fold during this same time period of inflammation (Kaplan, H. A. *et al.*, 1983). The source of this serum based enzyme activity was subsequently proven to be derived mainly from the liver by using a preparation of liver slices (McCaffrey, G., 1990). Furthermore, experiments which employed isolated Golgi membranes from rat, mouse and guinea pig liver demonstrated that about 70% of the ST6Gal I is released from its membrane anchor by proteolytic cleavage in the stem region *via* the lysosomal enzyme cathepsin D (Lammers, G. *et al.*, 1989; Lammers, G. *et al.*, 1990). In conjunction with these studies, additional sialyltransferases as well as *N*-acetylglucosaminyltransferases were studied in this thesis so that a comparison of their activity levels during the acute phase response could be made. As expected, liver activities of ST6Gal I increased 2-fold during the inflammatory response, while ST3Gal III, SAT-I and GlcNAcα2-6ST decreased by about 50% over the same time period (fig. 20). GlcNAcT-I behaved like ST6Gal I by increasing almost 2-fold in activity while GlcNAcT-III and GlcNAcT-V increased slightly (fig. 21). The reason(s) for these changes in

glycosyltransferase activities during the acute phase response to injury is not clear.. The respective enzyme activities could be seen as a reflection of the change in oligosaccharide structures required during the period of acute inflammation. It would not be unreasonable to suggest that some of these changes are the result of a series of chemical events, some damaging and some enhancing, within the cell when it is exposed to turpentine. This is an unfortunate limitation of these experiments. However, there is a substantial amount of research that supports the claims of this thesis (Mackiewicz, A. *et al.*, 1995 and references within), which is glycosyltransferase activities are altered due to the *response* of acute inflammation, no matter what the initial stimuli may be. It is well known that carbohydrate chains increase in branching complexity and that the level of glycoprotein synthesis increases when a stress is put on the immune system during episodes of inflammation, cancer, or any of a multitude of diseases (Mackiewicz, A. *et al.*, 1995).

It is conceivable that the structure which a glycosyltransferase synthesizes under control conditions could otherwise hinder the hosts immune response during episodes of the aforementioned conditions. An interesting example of this is demonstrated in pioneering results which revealed that gangliosides contained immunosuppresive activity (Ladisch, S. *et al.*, 1992). While the exact mechanism of how this occurs remains unclear, there is evidence that gangliosides may be secreted into the extracellular environment that have the ability to inhibit various

immunologically active cells, including T and B lymphocytes and macrophages (Ladisch, S. *et al.*, 1992). This same group later showed that even chemically synthesized gangliosides, which are not known to occur in nature, had the ability to suppress the immune system (Ladisch, S. *et al.*, 1995). In accordance with these findings, a plausible reason for the decrease in SAT-I activity could be that because it is the initial enzyme used in the biosynthesis of all gangliosides, suppressing its activity would be a desirable response. It would be interesting to administer a ganglioside, possibly GM_3, into the bloodstream of an animal which was suffering from acute inflammation. Comparing this to controls (animals which were inflamed *via* turpentine, but were not injected with GM_3) with regard to T and B cell levels, as well as macrophage concentrations, at various times of inflammation, would give more of an insight into the relationship between gangliosides and the acute phase response.

The increase in GlcNAcT-I activity in the liver can easily be explained by the fact that there is an overall net increase in the activity of the glycosylation machinery of the hepatocytes, as discussed in the introduction. Since this enzyme is a prerequisite for the synthesis of hybrid and complex *N*-linked glycoprotein, both of which increase in concentration during the immune response (Schachter, H.,1986), then an increase in its activity would almost be expected. GlcNAcT-III and V are generally only found in exceedingly small amounts in rat liver (Nishikawa, A. *et al.*, 1990). However, both of these enzymes have been shown to increase

substantially during various types of cancers (Easton, E.W. *et al.*, 1992; Yoshimura, M. *et al.*, 1995; Yao, M. *et al.*, 1998). Although there was a minor increase in both of these enzymes during the acute phase response (fig. 21), the change in the level of activity could not be considered substantial enough to warrant any further investigation in this study.

It is clear that the liver is the primary source of acute phase reactants, as discussed in the introduction. However, evidence that the acute phase response can be elicited in extrahepatic tissues and cell types, such as human intestinal epithelial cells has been presented (Molmenti, E.P. *et al.*, 1993). While sialyltransferase activities were undetectable in any intestinal homogenates produced in these studies, which agreed with earlier results (Paulson, J.C. *et al.*, 1989), they also did not seem to appear during the onset of inflammation. However, in an effort to see if other tissues responded with dramatic changes in their glycosyltransferase levels during the acute phase response, the kidney and spleen were assayed for various glycosyltransferase activities. The primary reason for choosing these two organs was that in the aforementioned work by Paulson, a thorough investigation of the levels of ST6Gal I and ST3Gal III was undertaken in the liver, brain, ovaries, kidneys, spleen, lungs and heart, as well as various regions of the intestine as discussed above. These results indicated that the kidney, spleen and ovaries had the highest amounts of ST6Gal I and ST3Gal III activities.

As presented in figures 22 and 23, the kidney did not respond with dramatic changes in the levels of glycosyltransferases assayed. It is difficult to say if these changes are significant. However, the spleen did have a more significant response (fig. 24). ST6Gal I increased in activity almost 4-fold, which is interesting because this is close to the increase in the level of activity found in the serum over the same time period of inflammation found in these and earlier studies (Kaplan, H. A. *et al.*, 1983). With this in mind, it is difficult to say for sure if the spleen is increasing its rate of ST6Gal I synthesis, or if this is merely enzyme from the serum itself. This is due to the fact that the spleen is a complex filter interposed in the blood stream. One of its primary biological functions is clearing the blood of particulate matter, so it may be that the spleen is in some way absorbing the enzyme from the serum for regulatory purposes associated with the immune response. For instance, the spleen contains a large amount of lymphoid tissue and possess a peculiar type of blood vessel that allows the circulating blood to come into contact with great numbers of macrophages (Kuby, J., 1992). Activated macrophages not only function as phagocytes but also specifically secrete an enormous variety of biological substances into surrounding tissues, therefore securing themselves as crucial factors in the host's immune response. However, the mechanism of *how* a macrophage is activated is not clearly understood (Stites, D.P. *et al.*, 1996). It is known that a macrophage continually samples its surrounding environment by pinocytosis and through an extensive array of receptors on its surface (Stites, D.P.

et al., 1996). Perhaps by recognizing an increase in ST6Gal I concentration, the macrophage is alerted to the fact that the immune system is being challenged.

While the slight change in GlcNAcT-I activity in the spleen cannot be considered significant , the increases in GlcNAcT-III and -V are interesting in that these enzymes are not found in the serum, in normal or inflammatory conditions (as explained later in table 9). An interesting feature of these two enzymes is that they are in direct competition with each other; that is, once one of the GlcNAc transferase has linked a GlcNAc onto an oligosaccharide in its specific linkage, the newly created structure is no longer a substrate for the other enzyme (Do, K-Y. *et al.*, 1994). It is possible that this is necessary to keep a balance between the different glycoconjugate structures, so that there is no need to separate the enzymes into different sub Golgi compartments. Overall, it is difficult to comment on these findings with any degree of certainty. It is clear that not only do various glycosyltransferases behave differently during the acute phase response within the same organ, but also among other organs. This would at first seem completely logical, since specific organs each have their own agenda with regards to the production of glycoconjugates. However, the assay of enzyme activity in these organs is complicated by the fact that different *regions* within the same organ could be controlling the level of a specific enzyme in a completely different manner. A clear example of this was presented by Coughlan, C.M. *et al.*, 1996. They were focused towards trying to understand the tissue specific regulation of

sialyltransferase activities in rats. As expected, ST6Gal I and ST3Gal III activity levels were found to differ in the liver, kidney and brain. But an interesting finding in this study was the observation that certain regions of the brain (partial cortex; hippocampus; brainstem and cerebellum) displayed different levels of sialyltransferase activity. In addition, they found that corticosteroids (steroid based compounds which have anti-inflammatory and immunosuppressive activities) were able to affect the activities of these enzymes, indicating that sialyltransferase activities in certain tissues is under the control of various inflammatory metabolites.

Future work in this area should be directed towards examing *why* these changes in glycosyltransferase activity occur. One possible suggestion would be to delete the gene of a specific glycosyltransferase, but only within the hepatocyte. Such an experiment would be possible using transgenic mice (Orban, P.C. *et al.*, 1992) Without ST6Gal I, which has long been known to be released into the serum during acute inflammation, it would be possible to evaluate how the acute phase response would be affected when this enzyme is missing.

Studies on the release of glycosyltransferases from disrupted Golgi vesicles

Previous work in this laboratory had shown that ST6Gal I was released from its membrane anchor when sonicated Golgi membranes were exposed to an acidic pH, with optimal release occurring at pH 5.6 (Lammers, G. *et al.*, 1988). In an effort to see if this phenomenon was a common property of the various sialyltransferases,

ST3Gal III, GlcNAcα2-6ST and SAT-I were analyzed under similar conditions. For comparative purposes, the effect of an acidic pH on the release of ST6Gal I was repeated and, as expected, it was released from its membrane anchor, with the greatest proficiency occurring at pH 5.6. Furthermore, about 70 % of the total enzyme activity was released from both the control and inflamed rat livers (fig. 26). ST3Gal III (fig. 27) and GlcNAcα2-6ST (fig.28) displayed similar results, with both enzymes being released from their membrane anchors at the optimum pH of 5.6, indicating that a similar mechanism of release was very likely. Disrupting the Golgi by means of ultrasonic vibration was not an effective method of disrupting vesicles to study the release of SAT-I because this enzyme is known to be rapidly inactivated by such treatment (Trinchera, M. *et al.*, 1991); Therefore, membranes were disrupted with a low concentration of Triton X-100. Although there was a small amount of enzyme solubilized along with the membrane, there was still a sufficient level of activity associated with the Golgi vesicle to study the effect of an acidic pH on the release of SAT-I. As shown in figure 29, SAT-I is indeed released from the membrane, with a pH optimum of 5.6, resulting in the greatest amount of release. Interestingly, only about 15% of the SAT-I activity is released, in comparison to 70% of the other three sialyltransferases. It is conceivable that 0.1% Triton X-100 solution was not adequate to disrupt the membranes. This would hinder the release of SAT-I that was cleaved from its membrane anchor. Advantage was taken of the fact that GlcNAcT-I is neither solubilized by Triton X-100 (Slusarewicz, P. *et al.*, 1994) nor affected by treatment with ultrasonic vibration

(Paulson, J.C. *et al.*, 1989). This allowed both methods of membrane disruption to be compared with regard to the ability of the enzyme to be released from its membrane anchor *via* proteolytic cleavage. Since sonication causes total disruption of the Golgi membranes, any enzyme cleaved from its membrane would be unhindered in its release into the supernatant. Although the greatest amount of GlcNAcT-I was released at the same optimal pH of 5.6 from membranes that were opened with ultrasonic vibration, thus accounted for only 14% of the enzyme to be released (fig. 30). However, membranes disrupted by with treatment with 0.1% Triton X-100 also demonstrated the same level of release at similar pH values. These results demonstrate that opening the Golgi membranes by treatment with detergent is satisfactory in allowing the maximum amount of cleaved enzyme to be released into the supernatant. Therefore, it can be concluded that ~70% of the activities of the three sialyltransferases involved in the glycosylation of *N*-linked proteins are cleaved from their membrane anchors while this value is only ~15% for SAT-I and GlcNAcT-I. It was noted, however, that although the reduction of the pH of the incubation medium to below 5.6 did cause significant amounts of ST6Gal I to be released from Golgi membranes, an increasing amount of solubilized enzyme was apparently being degraded under these conditions (McCaffrey, G. 1990). Thus a pH of 5.6 is considered optimum in that it releases the highest amount of catalytically active enzyme from the Golgi membranes. As for SAT-I though, there was very little activity remaining in the pellet after the solubilized membranes had been incubated at a pH of 5.6, indicating that possibly more than the reported 15%

had actually been cleaved from its membrane anchor. Indeed, SAT-I is known to be less stable than sialyltransferases involved in the glycosylation of *N*-linked glycoproteins, particularly when released from the Golgi membrane (Melkerson-Watson, L.J. *et al.*, 1991; Trinchera, M. *et al.*, 1991).

The question as to why only ~15% of the GlcNAcT-I activity is released is not quite as clear. As with the first three sialyltransferases, there is close to 100% recovery of the enzyme activity. There is little doubt regarding the stability of this enzyme, since the purified version of this enzyme is an active truncated form which was cleaved in the stem region and is removed from its natural Golgi membrane environment (Nishikawa, Y. *et al.*, 1988). Furthermore, it has recently been demonstrated that removal of the first 106 amino acids of this 447 residue long protein does not inactivate GlcNAcT-I (Sarkar, M. *et al.*, 1998). A possible explanation for GlcNAcT-I being released from its membrane anchor may be that only a small population of the enzyme is vulnerable to the mechanism which cleaves GlcNAcT-I. For instance, other resident proteins which are not susceptible to the same mechanism of cleavage may be in close enough contact to the stem region of GlcNAcT-I, where the enzyme is thought to be cleaved (see Results section C,). Although GlcNAcT-I is generally regarded as a *medial* resident enzyme, in actuality about 40% of the enzyme is still located in the *trans* region of the Golgi isolated from liver (Rabouille, C. *et al.*, 1995). Therefore the question remains as to why the sialyltransferases, which are located within this same Golgi

region, wouldn't be as immune to the effect of cleavage as GlcNAcT-I. After incubation at pH 5.6, much of the GlcNAcT-I was still active in the Golgi membrane, indicating that a proteolytic process which would release the enzyme from its membrane-bound anchor was not occurring.

However, there are still many fundamental issues which need to be addressed with regard to the organization of the glycosyltransferases within the individual cisternae in the Golgi complex. For instance, two forms of ST6Gal I have recently been found to populate the Golgi complex of the rat liver. The first is a monomer while the second is the disulfide-bonded dimer which comprise 70% and 30% of the ST6Gal I enzyme population, respectively (Ma, J. *et al.*, 1996). Furthermore, the dimer was found to have no catalytic activity, yet it could act as a lectin by binding galactose. There was also a distinct possibility that the sub-Golgi localization of these two forms did indeed differ. This group of researchers then identified two different monomeric populations of the ST6Gal I enzyme (Ma, J. *et al.*, 1997). Both were identical to each other except for a substitution at position 123, where one ST6Gal I possesses a tyrosine residue and the other a cysteine. Referred to as ST tyr and ST cys, both, as expected, were located within the *trans*-region of the Golgi. Interestingly though, ST tyr was identified as being able to move past this compartment and go to the cell surface. The final conclusion based on their data was that ST tyr and ST cys are localized within different regions of the cell and that only the ST tyr has access to any proteases that would cleave

it, permitting it to be cleaved and released in its soluble form.

In the case of ST6Gal I, significant impairment of the catalytic efficiency was not found to occur following release at reduced pH nor were the kinetics of the enzyme affected during the acute inflammation (Kaplan, H. A. *et al.*, 1983). This is not surprising since enzymes involved in the processing of *N*-linked glycosylation usually do not have their kinetics affected when the catalytic portion is removed from its membrane-anchor, as recently demonstrated (Cho, S.K. *et al.*, 1997). The glycosyltransferases involved in the biosynthesis of glycolipids are usually regarded as less stable when removed from their natural Golgi membrane environment, as exemplified by SAT-I in this report. Extremely interesting results were recently published which had demonstrated that two glycosyltransferases glycosylate less efficiently in the soluble form than their membrane bound counterparts (Zhu, G. *et al.*, 1998). The first enzyme involved in the biosynthesis of gangliosides was, not surprisingly, found to have undetectable levels of activity in the soluble form. However, the other enzyme studied was ST6Gal I and it was determined that cells expressing only the soluble form of the enzyme contained 2- to 4-fold less of the α2-6 linked sialic acid structure on the glycoproteins produced within the cell. The conclusion reached was that the soluble form of ST6Gal I was not as effective at glycosylating its structures as the membrane bound form. Interestingly, it was noted that the ST tyr form of ST6Gal I was employed in these studies, which raises the immediate question as to whether ST cys would behave in a similar manner.

Obviously there is a complex mixture of parameters which need to be accounted for when studying the mechanisms involved in the release of glycosyltransferases from the Golgi membrane. Why there is only about a 70% release of sialyltransferases involved in *N*-linked glycosylation, and not the total amount, is difficult to say. Figures 31 and 32 clearly demonstrate that 30 min at pH 5.6 was optimal for the release of the soluble form of the enzymes examined. Therefore, increasing this period of incubation would not result in further release of enzyme. Overall, it is difficult to say why complete solubilization of these sialyltransferases did not occur.

A major issue which had been a focus of this laboratory in earlier years was determining what mechanism exactly was at work in order to cleave the ST6Gal I enzyme from its membrane anchor. Evidence from these studies that indicated that cathepsin D is indeed the proteinase responsible for cleaving the ST6Gal I was three-fold. First, the addition of pepstatin A prevented the release of the enzyme. Pepstatin A is a microbial-derived pentapeptide that contains a novel hydroxyamino acid (4*S*-amino-3*S*-hydroxy-6-methyl-heptanoic acid; Umezawa, H. *et al.*, 1970). This residue is responsible for binding pepstatin A tightly to aspartic proteinases, such as cathepsin D, in a one-to-one ratio (Metcalf, P. *et al.*, 1993). Second, antiserum to cathepsin D was observed to prevent the release of the enzyme, while finally the addition of cathepsin D from bovine spleen was able to initiate the release of additional ST6Gal I.

In order to elucidate the ubiquitous action of cathepsin D towards sialyltransferases, the latter study was extended to ST3Gal III, GlcNAcα2-6ST and SAT-1. Furthermore, since GlcNAcT-I displayed a tendency to be released from its membrane anchor at a similar pH optimum, it too was examined in the same fashion. As demonstrated in previous studies, the addition of exodogenous cathepsin D does indeed increase the amount of ST6Gal I released from its membrane anchor (Table 3). Also noted is the release of additional enzyme from inflamed Golgi over control, a reflection of the fact that ST6Gal I increases in concentration during the inflammatory response. Tables 4, 5 and 6 certify that supplementing the samples with cathepsin D cause further removal of ST3Gal III, GlcNAcα2-6ST and SAT-1 respectively. However, the highest amounts of activity are released from the control Golgi, again a reflection of the findings that these three sialyltransferases seem to decrease in concentration during the acute inflammatory response. Even though endogenous cathepsin D appeared to abet the release of GlcNAcT-I (table 7), there seemed to be little difference between control and inflamed samples, although Golgi from inflamed liver did present a slightly higher level of enzyme activity, which is in accordance with the increase in activity found during inflammation. As discussed, pepstatin A is a powerful inhibitor of cathepsin D. It would then stand to reason that if this enzyme was indeed responsible for the proteolytic cleavage of these glycosyltransferases, then the addition of pepstatin A should prevent these enzymes from being released into their surrounding environment. Even at concentrations of 10^{-9} M, pepstatin A does

indeed have an effect on the release of all five glycosyltransferases, with a larger degree of inhibition occurring with increasing pepstatin A (table 8).

ST6Gal I has long been known to increase in activity in serum during the acute inflammatory response by nearly 5-fold (Kaplan, H.A. *et al.*, 1983). Of the seven glycosyltransferase examined in this study, only three were found to occur in an active state in the serum during control conditions (table 9). During the course of the inflammatory response the present study revealed that while the level of ST6Gal I activity did indeed increase appreciably, the levels of GlcNAcα2-6ST and GlcNAcT-I activities in the serum did not change in a substantial way. It is of interest, however, that while GlcNAcT-I and GlcNAcα2-6ST increased and decreased in activity in the liver, respectively, the activities of these enzymes were accompanied by the same change in the serum.

Studies with liver slices have already been used to demonstrate that the source of the ST6Gal I in the serum was due to the action of cathepsin D cleaving its membrane bound counterpart within the Golgi of the rat hepatocytes. This permitted the release of a catalytically active soluble form of the enzyme to circulate throughout the blood in increasing levels during the acute inflammatory response. Although there was little change in the serum levels of GlcNAcT-I and GlcNAcα2-6ST during acute inflammation, GlcNAcT-I did increase slightly, while GlcNAcα2-6ST decreased, both by about 50% respectively. This complemented the findings

that GlcNAcT-I increased in the liver during inflammation and GlcNAcα2-6ST decreased. The logical conclusion would be that the serum levels of all three of these enzymes are controlled by biochemical processes occurring within the liver. The use of liver slices has shown that the liver is indeed responsible for releasing ST6Gal I into the blood upon the onset of inflammation (Lammers, G. *et al.*, 1989). Using the same procedure, the release of GlcNAcT-I and GlcNAcα2-6ST from liver slices and into the surrounding medium was parallel to the data in table 9. As shown in table 10, levels of ST6Gal I activity increased as expected, while the liver slices isolated from rats enduring the acute phase response released a greater amount of GlcNAcT-I and a lesser amount of GlcNAcα2-6ST. This is likely due to the fact that the activity levels of these enzymes change in the liver during the course of inflammation. In other words, because there is more GlcNAcT-I activity in the liver during the acute phase response, there is a larger amount of it released by cathepsin D, while the same reasoning holds true for GlcNAcα2-6ST.

The release of the glycosyltransferases from hepatic Golgi membranes during the acute phase response is hypothesized to be the result of increased interaction between the membrane bound enzymes and incorrectly-targeted lysosomal proteinases. For this reason the Leelavathi method of Golgi membrane preparation, as modified by Moremen and Touster (See Methods) was chosen in order to minimize the amount of lysosomal contamination. However, it is entirely possible that some of the lysosomal enzymes were contaminated in the Golgi

membrane preparation. Concerned with this possibility, earlier studies affirmed that the cathepsin D was indeed an endogenous Golgi proteinase by performing the aforementioned liver slice experiments, but supplementing the medium with pepstatin A to inhibit the *in vitro* release of ST6Gal I (Lammers, G. *et al.*, 1989). These experiments were repeated in order to further validate that GlcNAcT-I and GlcNAcα2-6 were released into the surrounding medium of the liver slices and thus into the serum due to identical processes. As demonstrated in figures 33-35, the addition of pepstatin A does impede the release of the enzymes into the surrounding medium. Incubation times of up to 18 h were necessary due to the fact that pepstatin A has difficulty in permeating membrane bilayers (Dean, R.T., 1977). Pepstatin A was shown to inhibit the release of all three glycosyl transferases from both control and inflamed liver slices, with ST6Gal I and GlcNAcT-I from inflamed samples having the greatest influences. GlcNAcα2-6ST from liver slices from control animals was inhibited to a greater extent. Collectively, the results presented thus far indicate that cathepsin D is the lysosomal proteinase responsible for the proteolytic release of these enzymes from their membrane anchors. Just as important however, is the fact that the changes in the activities of the enzymes found within the serum during inflammation parallel the changes in their activities in the Golgi. This supports the conclusion that during inflammation, these enzymes are correctly targeted to their Golgi compartments and then abruptly cleaved by cathepsin D, thus permitting their release into the serum. It would be of interest to see if GlcNAcT-I has its distribution throughout the Golgi changed during

inflammation since it is considered a *medial* enzyme. For instance, it may be that the additional GlcNAcT-I synthesized by the cell during inflammation may be directly targeted further down the secretory pathway where cathepsin D is located, while the level of activity in the *medial* region would remain unchanged.

The biological significance of these soluble forms of the glycosyltransferases within the serum remains to be determined. Due to the extremely low levels of nucleotide sugar donors within the serum (Kaplan, H. A. *et al.*, 1983) the rates of any sugar transfer reactions promoted by these enzymes would be extremely low. However, one of the primary functions of blood is to act as a vehicle for transport of nutrients and metabolites. It is therefore conceivable that ST6Gal I, for instance, is being carried to the source of infection where it would perform a yet unknown function. Possibly, the soluble forms of the glycosyltransferases could act as ligands by binding to the surface of carbohydrate structures located on specific immune cells which may help to control their regulation. In fact, it has been suggested that, based on sequence homology, the secreted signaling molecules found in *Drosophila* might be glycosyltransferases (Yuan, Y. P. *et al.*, 1997).

Identification of the proteolytic site recognized by cathepsin D

Although there is little doubt that cathepsin D is the proteinase responsible for cleaving the glycosyltransferases to produce the soluble form of the enzyme, there is still a question of *where in the stem region the specific site of cleavage is*

taking place. When glycosyltransferases were first purified, it was often revealed that a large (membrane bound) and a small (soluble form) of the enzyme was present. This smaller form almost always had the same kinetic activity as its membrane bound counterpart and usually represented 85 - 90% of the Mr of the larger form. This indicated that the linker or 'stem' portion of these enzymes seemed to be quite susceptible to proteolytic cleavage (figure 8). Interestingly, it is the stem region of the glycosyltransferases that demonstrates the greatest sequence variability. These enzymes generally share a sequence similarity of over 90% in regions where the functional domains have been located (transmembrane anchor and catalytic domain; Schachter, H., 1994). For instance, whether a specific glycosyltransferase is isolated from a human, bovine or murine source, *most* of the primary sequence is identical, indicating that these enzymes have been conserved through much of evolution. The reasoning for the stem region having escaped these evolutionary constraints is unclear. It could very well be that each species has developed its own unique mechanism for release of the soluble form of the enzyme, and has thus preserved certain sequences which are recognized by specific proteases. The search for a cathepsin D cleavage site within the stem region of these glycosyltransferases was complicated by the fact that the exact location of the stem region, in these enzymes, is not known. However, the studies in this thesis limited the region to the first 100 amino acids based on current knowledge about the structure of the glycosyltransferases (see Methods).

Only two studies have seriously examined the preferential amino acid sequence which would be recognized by cathepsin D. The first study was not thought to produce accurate data since the conditions under which the proteins were subjected to proteolysis by cathepsin D were questionable (Imoto, T. *et al.*, 1987). The two primary problems with this study were that the pH was quite low (3.5) and the incubation period was at 37°C for prolonged periods of time. For instance, the low pH could decrease the stability of the enzyme, possibly exposing sites that would not normally be presented to cathepsin D. The long period of incubation would make it difficult to set apart cleavage sites initially cleaved by cathepsin D and those recognized only after exposure as a result of preceding cleavages. The second study cleaved proteins with cathepsin D under milder and more 'realistic' conditions in that the pH was close to that found in the Golgi apparatus and the incubation period was substantially shorter (Van Noort, J. M. *et al.*, 1989). Results demonstrated that cathepsin D is quite flexible with regard to what amino acids are required for its recognition with the one common denominator being that the sequence had to extend over seven amino acids in length (fig. 36 and table 11).

When examining the stem regions of the various glycosyltransferases for these parameters, ST3Gal III showed one possible recognition site, with the scissile bond at residues 61 - 62 (fig. 37). ST6Gal I had two possible sites, with the scissile bonds at 34 - 35 and 74 - 75, with the latter being the most likely (see legend of

figure 37). Unfortunately, the lack of availability of sequences for SAT-I and GlcNAcα2-6ST prevented their analysis. Although a sequence for GlcNAcT-I from rat liver was not available as well, sources from human, mouse and rabbit were analyzed (fig. 38). A cathepsin D recognition site was identified at the same location for all three, with the scissile bond at position 71 - 72. It is of interest to note that when this enzyme was purified from rabbit liver, a soluble form of the enzyme was purified that had been derived from the membrane bound precursor by proteolytic cleavage at "about base position 215 in the stem region" (Sarkar, M. *et al.*, 1991). This would place the scissile bond at position 71 - 72, indicating that cathepsin D may have been responsible for creating the soluble form of the enzyme.

Galactosyltransferase was previously observed not to be cleaved from its membrane anchor under the same conditions which had released the soluble form of ST6Gal I, demonstrating that this enzyme is resistant to the action of cathepsin D (Lammers, G. *et al.*, 1988). It could then be assumed that galactosyltransferase might lack a recognition sequence for cathepsin D in its stem region, which would account for the lack of proteolytic cleavage. To date this enzyme has not been cloned and sequenced from a rat source, however sources from human, cow and mouse have revealed interesting results (fig. 39). Taking into consideration that cathepsin D displays a wide range of freedom with respect to its recognition region, coupled to the fact that three enzymes with differing stem sequences were

searched for possible cathepsin D sites, it is impressive that none were found. This supports the earlier finding that galactosyltransferase was not cleaved from the Golgi membranes of rat livers, which may now be due to the lack of a region of amino acids which would be recognized by cathepsin D.

The seven amino acid sequence pattern recognized by cathepsin D has been envisioned to occur within an α-helical segment of the protein (Van Noort, J. M. *et al.*, 1989). However, a search of the secondary structure of these enzymes did not reveal any such constraints (fig. 40). As for ST3Gal III, an α-helical structure was not predicted to occur at position 61 - 62. Of the two sites in ST6Gal I which had fit the cathepsin D recognition parameters, one was predicted to occur within the constraints of an α-helical structure. However, this sequence, at position 34 - 35, is not as likely to be the site of cleavage as that which is found at 74 - 75 (see legend of fig. 37) based on this model. Results from our laboratory have indicated that the difference in molecular weight between the membrane bound and soluble forms of ST6Gal I was roughly 7,000 Da (Lammers, G. *et al.*, 1990). This would predict a cleavage site at about position 63 - 64. This discrepancy could be attributed to the limitations inherent in the SDS electrophoresis of glycosylated proteins. The site which is *predicted* to contain the cathepsin D recognition parameter for GlcNAcT-I, at position 71 - 72, does seem to fall within a region of the enzyme which is α-helical in nature. However, it must be taken into account that the program that predicts these secondary structures are at best 60 %

accurate (as suggested by the programers) , which is comparable to most other programs on the market today. Therefore, until a crystal structure of these enzymes has been obtained, it will remain uncertain as to what are the secondary structural requirements for cathepsin D recognition. However, if the site of cathepsin D cleavage is to be identified, future studies should be aimed at sequencing the N-terminal region of the soluble glycosyltransferases.

Determination of glycosyltransferase activity from various liver fractions

It has been well established that the structure of glycoconjugates are very often altered during the onset of many disorders, whether they be as insignificant as minor acute inflammation, or as serious as cancer. In order to achieve a deeper understanding as to what some of the contributions to these changes in structure are, a study was performed which observed a modification in the distribution of the glycosyltransferase activity within a density gradient. The original concept for developing these gradients was conceived when it was realized that there is an increase in the level of ST3Gal III and GlcNAcα2-6ST activity in fraction 2B over fraction 2A (fig. 17). Fraction 2A is the Golgi fraction used in assaying the various glycosyltransferases throughout much of this research, while 2B contained primarily smooth endoplasmic reticulum , and in the past has been discarded in the research carried out in our laboratory. However, this particular method of isolating the Golgi membranes was developed using rat livers from animals that did not suffer from any form of disease or inflammation. The large increase in protein trafficking which

occurs in the Golgi during such times would likely change its density, and perhaps the shape and organization of other organelles involved in protein synthesis. Even under the conditions which were originally constructed to isolate the Golgi membranes, it was noted that the smooth endoplasmic reticulum fraction (fraction 2B) contained "tubular profiles similar in appearance to those associated with the Golgi cisternae in the previous fraction is present in discrete amounts" (Leelavathi, D. D. *et al.*, 1970). Therefore it would be expected that an increase in density of the Golgi apparatus would result in more of its membrane components shifting to the 2B fraction.

Tables 12 and 13 confirm that fraction 1A (fig. 17) contains a relatively constant amount of sialyltransferase activity, whether isolated from control or inflamed samples. Therefore, this fraction, which contains the combined fractions of 2A and 2B, was collected from the gradient in figure 17 and used in the continuous gradient studies. Table 14 demonstrates that indeed there is a shift in some of the sialyltransferase activities from fraction 2A to 2B during inflammation. While ST6Gal I indicates a two-fold increase in activity in fraction 2A (as expected since this just the Golgi fraction), fraction 2B shows little activity. However, although earlier results had indicated that ST3Gal III and GlcNAcα2-6ST decrease slightly in activity within the Golgi membranes isolated, there does seem to be a corresponding increase in activity of both of these enzymes in fraction 2B. Therefore, attempting to separate fraction 1A on a continuous sucrose gradient was

a logical conclusion, as it would be interesting to see if the activity of these enzymes shifted along the gradient during inflammation.

After unstacking the Golgi apparatus with proteinase K, which has been shown to unstack the Golgi membranes without affecting the activity of the glycosyltransferases (Cluett, E.B. *et al.*, 1992; Slusarewicz, P. *et al.*, 1994), fraction 1A was applied to a continuous sucrose gradient as described in figure 18. As seen in figure 42, under control circumstances ST6Gal I has one major peak of activity at about fraction 14, while inflamed samples give two peaks, one at ~fraction 10 and the other ~fraction 16. This indicates that there is a shift in the organization of the glycosyltransferase activity which goes beyond a simple explanation that a change in the activity of the gradient is due to a change in the density of the individual Golgi compartments. If this was that case, then the one major peak at fraction 14 from control samples would simply shift to the left because it was more dense and thus came off the gradient sooner.

The activity gradient assayed for ST3Gal III activity poses a couple of interesting possibilities (fig. 43). First, ST3Gal III competes with ST6Gal I for identical substrates (Galβ1-4GlcNAc residues) so it would be expected to find the enzymes in the same fractions. This is indeed the case as exemplified by the fact that during control conditions ST3Gal III has two major peaks of activity, one of which is at ~fraction 14, the same as ST6Gal I. During inflamed situations, the

activity gradient displays a 'double hump' activity gradient with peak values at fraction 10 and 15, exactly the same as ST6Gal I. The major peak of activity for ST3Gal III in the control samples is at fraction 4. This is interesting because this is where the only major peak of activity is identified for GlcNAcα2-6ST (fig. 44). When this enzyme was first discovered, it was noted that the substrate could not be recognized by GlcNAcα2-6ST until it has been sialylated by ST3Gal III (Paulson, J. C. *et al.*, 1984).

Perhaps the most intriguing data are the fact that GlcNAcα2-6ST activity all but disappears from the gradient during inflammation. Table 13 indicates that GlcNAcα2-6ST activity was present when fraction 1A was applied to the gradient, so the loss is interesting. This may have occurred due to proteinase K sensitivity or perhaps the enzyme was damaged when exposed to high g-forces in centrifugation. The peculiar behavior of GlcNAcα2-6ST should be the subject of future investigations and the above possibilities ruled out. GlcNAcT-I demonstrates two peaks of activity, during both control and inflamed conditions, with a greater amount of activity occurring during inflammation (fig.45).

It is not likely that these different peaks of activity represent differing parts of the Golgi, such as the *medial* and *trans* regions, since sialyltransferase activity was also found in both of these peaks. Overall, it is very difficult to give any definite reasons as to why these glycosyltransferases display different levels of activity

along their gradient. Clearly, these experiments are in the embryonic stage, but do pose further questions about the role these enzymes have in the construction of glycoconjugates during both control and disease states. Future studies should be aimed at trying to identify what fractions are *cis*, *medial* and *trans* regions. Although a *complete* separation of these regions would not be expected, knowing which fractions predominate in these Golgi subcompartments would be very informative.

Detection of a Golgi matrix capable of binding sialyltransferases

The second main focus of the thesis was aimed at identifying a possible protein matrix which might be located between the Golgi cisternae and could be capable of binding three sialyltransferases and the GlcNAcT-I enzyme, all located within the *trans* region of the Golgi apparatus. Originally when the Golgi was purified from plant (Morre, D.J. *et al.*, 1964) and rat liver (Morre, D.J. *et al.*, 1970) the closely opposed cisternae were found to resist unstacking. This suggested that there are links between adjacent cisternae and that these links are plentiful because the width of the intercisternal space is relatively constant (Cluett, E.B. *et al.*, 1992). The likely prospects for forming such links would be the glycosyltransferases, which are relatively abundant proteins making up a few percent of each cisternal membrane. Furthermore, they are present throughout the entire Golgi stack with their exact position varying from enzyme to enzyme and from cell line to cell line (Roth, J., 1987). As discussed in the introduction, many of these enzymes have been cloned and sequenced. The cytoplasmic tails in the enzymes

vary from 5 to 24 amino acids, with no obvious consensus sequence being identified. Therefore, the longest tail could only span a distance of 36 Å as an α-helix, which is a third the width of the intercisternal space (100 Å; Cluett, E.B. *et al.*, 1992). Obviously then, additional proteins would be needed to link these cytoplasmic tails.

To date, very little work has been done regarding the possibility of a matrix system which may be involved in the stacking of the Golgi apparatus. Although publications on this subject are few and far between, components of an intercisternal matrix have been visualized by several workers, as early as the mid 1960's, in a variety of systems. They appear either as "inter-cisternal elements of electron-dense material running between and parallel to the cisternae" (Amos, W.B. *et al.*, 1968; Mollenhauer, H.H. *et al.*, 1973) or as "regularly spaced intercisternal cross-bridges" (Franke, W.W. *et al.*, 1972). The first report which was focused towards the existence of a potential intercisternal Golgi matrix was presented by Hilton Mollenhauer (Mollenhauer, H.H., 1965). He had reported that the cisternae of the Golgi apparatus may appear flat or curled in transverse sections but, in all cases, the cisternal elements remained separated from each other by a relatively constant minimal distance. Expanding on this, he stated that although there was only a limited number of Golgi samples that had been studied by electron microscopy, there was a "frequent visualization of intercisternal structures", suggesting that it was perhaps a universal feature of the Golgi apparatus. Not

surprisingly, such cross-bridging structures were subsequently found to connect membranes of several organelles such as the endoplasmic reticulum and the thylakoid membranes of chloroplasts (Franke, W. W. *et al.*, 1971). Although such structures have been seen in other Golgi samples since then, there has yet to be a full explanation as to what the exact function of these cross-bridges, or 'matrices' may have, although maintaining the structural integrity of the Golgi apparatus is a common suggestion.

Since little is understood regarding the nature, make-up, and overall properties of a matrix located between the Golgi membranes, attempting to find evidence for such a matrix was indeed a difficult task. Furthermore, the matrix was to be removed from the surrounding Golgi membranes, yet still contain the resident *trans* enzymes, the sialyltransferases. The reason for attempting to find a '*trans*-matrix' was based on the fact that a matrix was isolated from rat liver Golgi membranes which was capable of binding the *medial* enzymes mannosidase II and GlcNAcT-I , but *not* the *trans* enzyme galactosyltransferase (Slusarewicz, P. *et al.*, 1994). As an approach to the isolation of an intercisternal matrix, this group had used procedures similar to those used for the characterization of the nuclear lamina. The lamina is described as "a meshwork of intermediate filaments linking chromatin to proteins in the inner nuclear envelope membrane" (Worman, H.J. *et al.*, 1988) and was isolated as a rapidly sedimenting structure resistant to extraction with TX-100 and low salt (Dwyer, N. *et al.*, 1976). When 2%TX-100 was

used to solubilize purified Golgi membranes, they found that about 80% of the *medial* enzymes were still located in the pellet following centrifugation, while only 10-35% of enzymes from the *cis*, *trans* and TGN regions of the Golgi remained in the pellet, with those from the *trans* and TGN comprising the smallest amount. Furthermore, the pellet was identified as being composed primarily of protein. Since such a high percentage of detergent was used relative to the concentration of Golgi membrane (1μg / μl), there is undoubtedly a large amount of cisternal membrane removed from the Golgi. This would mean that the predominant amount of medial enzymes which remained behind would likely be attached not by their membrane anchors, but to an underlying matrix, composed largely of proteins, via the enzymes cytoplasmic tails.

Although the aforementioned studies have demonstrated evidence of an intercisternal matrix throughout the entire Golgi apparatus, there is additional proof that a *trans* matrix does indeed exist. Upon studying these protein matrices, Cluett had used Golgi membranes which were isolated from other cellular material by use of a sucrose gradient (Cluett, E.B. *et al.*, 1992), much in the same way the Golgi was isolated in these studies. In his studies he noted that "the second band from the top, representing the 0.9 / 1.0 M (sucrose) interface and containing the highest concentration of *intact* Golgi complexes was harvested and used for experiments.....and that this 0.9 / 1.0 M interface was highly enriched in galactosyltransferase activity......". Since galactosyltransferase is located within the

trans region of the Golgi (Berger, E.G. *et al.*, 1985), the data from Cluett's study supports the existence of a matrix located within the same region as the *trans* Golgi enzymes because all of the intercisternal spaces within the galactosyltransferase enriched Golgi stacks contained matrices. These structures were shown to be proteinaceous in nature since treatments with various proteases were capable of 'unstacking' the Golgi membranes.

Following the removal of the membrane lipids, the *medial*-matrix bound enzymes were extracted with NaCl, then when the salt was removed by dialysis the enzymes were able to rebind the matrix (Slusarewicz, P. *et al.*, 1994). In order to test similar properties of a matrix which was capable of binding the *trans* enzyme, sialyltransferase, the Golgi membranes had to first be solubilized while still leaving the sialyltransferases attached to the underlying matrix. Obviously Triton X-100 could not be used for this purpose as it was in the *medial* -matrix isolation, since this non-ionic detergent is highly efficient at solubilizing the sialyltransferases (Tsuji, S., 1996). In an effort to find a detergent that would fit these requirements, those which were non ionic were investigated, since resistance to extraction is a common feature of the cytoskeleton and its associated proteins when using these detergents (Brown, S. *et al.*, 1976). In addition, they are efficient in disrupting lipid-lipid and lipid-protein interactions in membranes, but are ineffective when it comes to disrupting interactions between proteins (Helenius, A. *et al.*, 1975).

When Lubrol, a non-ionic detergent, was used to solubilize the Golgi membranes, most of the sialyltransferase activity was still located within the pellet. This compared to TX-100 which solubilized most of the sialyltransferase activity along with the Golgi membranes. As seen in table 15, about 68% of the total ST6Gal I activity remained in the pellet when the Golgi membranes were solubilized with 2% Lubrol, which compares to about 8% when TX-100 was used. Furthermore, since the percent recovery was only ~80% when using Lubrol, as opposed to ~100% when using TX-100, the 68% of ST6Gal I which remained in the pellet could actually be considered to be even higher. This same trend in solubilization appeared for ST3Gal III (table 16) and GlcNAcα2-6ST (table 17). Not surprisingly, GlcNAcT-I was resistant to extraction by either detergent (table 18), since TX-100 had already been shown to be ineffective at solubilizing this enzyme from Golgi membranes at a 2% concentration (Slusarewicz, P. *et al.*, 1994).

Most of the ensuing matrix studies used detergent concentrations of 2% for three main reasons. First, higher concentrations would obviously extract a greater amount of enzyme. As seen in table 17, activity dropped from 70% in the pellet when 2% Lubrol was used to solubilize the membranes to 56% when the detergent concentration was raised to 3%. This would affect the accuracy of the ensuing data as higher overall activity levels allow an increase in the reproducibility of the experiments. Secondly, higher concentrations of detergent may affect the structural integrity of the matrix itself (Slusarewicz, P. *et al.*, 1994). Thirdly, lower

concentrations of detergent would not expose optimal amounts of the underlying matrix (see below).

Salt extraction of the enzymes from the matrix was performed by resuspending the Lubrol pellets in buffer supplemented with varying concentrations of NaCl. The effect of NaCl on enzyme activity did not really have a significant role in the subsequent studies. Salt extraction and reconstitution experiments were primarily concerned with samples which contained up to 150mM NaCl, which had little effect on the activity of the enzymes when compared to samples that did not contain any salt, although the overall activities of ST6Gal I and ST3Gal III were influenced by the addition of 200 mM NaCl (table 19). In fact, in the case of GlcNAcα2-6ST, optimal activity was observed with salt concentrations of 0.15 M or 20 mM $MnCl_2$, while "stimulation of activity of the two salts was not additive" (Paulson, J.C. *et al.*, 1984). Therefore, the $MnCl_2$ in the buffer would have negated any effect that the NaCl may have had.

When ST6Gal I was extracted from Lubrol pellets of varying concentrations with varying amounts of NaCl an interesting trend was observed. Whether the matrix was exposed to 0%, 1%, 2%, or 3% Lubrol, increasing amounts of NaCl extracted increasing amounts of enzyme (figure 46). In the case where Golgi membranes had not been exposed to Lubrol, salt was not very effective at extracting the enzyme. When Lubrol was increased to 1%, thus exposing some of

the underlying matrix, the salt was able to extract ST6Gal I, but only ~10% at most when 150-200 mM NaCl was used. There was little difference in the salt's ability to extract ST6Gal I from membranes that were solubilized with either 2% or 3% lubrol, although a greater amount of the enzyme (~40%) could be extracted with 150-200 mM NaCl.

Two conclusions can be drawn from these observations. First, because there is a correlation between the amount of Lubrol used to solubilize the Golgi membranes and the salt's ability to extract ST6Gal I, this would lend evidence towards the existence of an underlying matrix being exposed by the detergent. Second, solubilization with more than 2% Lubrol does not increase the salt's ability to extract more of the enzyme, indicating that at this concentration a maximum amount of the lipid bilayer has been removed under the conditions used. The same general trend was observed for ST3Gal III (figure 47), GlcNAcα2-6ST (figure 48) and GlcNAcT-I (figure 49). However, a primary difference was observed in the case of GlcNAcT-I, where ~90% of the enzyme was able to be extracted when 2% or 3% Lubrol solubilized membranes have been extracted with 150-200 mM NaCl. This compares well with the 100% extraction which was obtained when 2% TX-100 was used to solubilize the membranes and 150 mM NaCl used to extract the enzyme (Slusarewicz, P. *et al.*, 1994). This result is extremely important, as it indicates that both Lubrol and Triton X-100 solubilize equal amounts of lipid membrane under similar conditions.

A true measure of the matrices' abilities to bind the Golgi enzymes was obtained by removing the salt by dialysis. In this way, the same enzyme which was extracted by salt from the matrix, could also be tested for its proficiency at re-binding the same matrix. Furthermore, the extracted enzyme could be tested for its ability to bind matrices which were exposed by using various amounts and types of detergent as well as concentrations of NaCl, since both factors were thought to play a role in the structural integrity of the protein matrix itself. When attempting to rebind the enzymes, two preparations were used; a 70 mM NaCl and a 150 mM NaCl extracted form. These values were chosen because data from the above study observed that the matrix proteins were partially solubilized at 150 mM NaCl, therefore this group had used enzymes that were extracted with 70 mM NaCl, since the matrix itself was intact at this lower concentration. Although this study was focused towards the *medial* matrix, it is difficult to say for sure if the matrix in the *trans* region of the Golgi would behave in a similar fashion when exposed to these same salt concentrations. In other words, there is not a guarantee that the *trans*-matrix would be unaffected by an exposure of 70 mM NaCl as was its *medial* located counterpart, yet using a lower concentration of salt was not really an option since the amount of enzyme extracted at these lower values was too low to be of any use in this study. Besides testing both a 70 mM and 150 mM NaCl extracted preparation for rebinding, six matrices were prepared by using either 2% lubrol or 2% TX-100 either alone, or simultaneously with 70 mM or 150 mM NaCl.

In the case of the three sialyltransferases it is important to note that in all cases there was close to 100% recovery of the salt extracted enzymes (tables 20A; 21A and 22A). Therefore the enzyme which didn't bind the matrix would be accounted for in the supernatant. ST6Gal I clearly rebound a matrix prepared from lubrol solubilization compared to one prepared with TX-100 (table 20B). Furthermore, 70 mM extracted ST6Gal I rebound the lubrol matrices with an overall better proficiency than the 150 mM extracted form. Again, these trends were observed with the other subsequent *trans* Golgi located enzymes ST3Gal III (table 21B) and GlcNAcα2-6ST (table 22B).

A likely explanation for these phenomena would be that TX-100 is much more damaging to the *trans* Golgi matrix itself and therefore would prevent any extracted Golgi enzymes from reattaching to the structurally altered matrix. Slusarewicz *et al.*, had concluded that they isolated a *medial* matrix from the Golgi because the *medial* enzymes mannosidase II and GlcNAcT-I were able to rebind the matrix prepared from Golgi membranes treated with 2% TX-100 and 150 mM NaCl, but could not rebind the *trans* Golgi enzyme galactosyltransferase. With the addition of the data presented here, it is reasonable to assume that the TX-100 was responsible for disrupting the *trans* Golgi matrix, but not the *medial* Golgi matrix, and therefore would prevent any rebinding of *trans* Golgi located enzymes.

An explanation as to why the 70 mM NaCl extracted sialyltransferases rebind

the Lubrol matrices with a greater efficiency than their 150 mM NaCl counterpart (about 2-fold) may be due to the fact that at the higher salt concentration, some of the matrix proteins may have been partially solubilized themselves. If a few crucial ones involved in the actual binding process were small enough (below the 12-14 kDa threshold of the dialysis tubing), then their loss would have been a factor in the lower binding proficiency. An alternate explanation may be that the matrix proteins were still attached to the enzymes during extraction with 150 mM NaCl, whereas with 70 mM only the enzymes were removed and a fully intact matrix was left behind. Rebinding of enzymes that still had partial attachment of the matrix proteins during 150 mM NaCl extraction may somehow inhibit or slow the rebinding to the remaining matrix.

An explanation of the rebinding values for GlcNAcT-I is complicated by the fact that although it is traditionally considered a *medial* Golgi enzyme, ~40% of it is located within the *trans* region of the Golgi, with the exact value depending on the cell type (Nakamura, N. *et al.*, 1995). From the results shown in table 23B, neither the type of detergent or concentration of salt appeared to have any great affect on the rebinding abilities of GlcNAcT-I. Both of these parameters were a factor in the rebinding of all three sialyltransferases, which suggests that the *trans* Golgi matrix may be more susceptible to structural damage than the *medial* Golgi matrix. Therefore, although about 50% of the GlcNAcT-I activity is capable of rebinding all six of the matrices described in table 23B, a predominant amount of

this is likely occurring within the *medial* Golgi matrix. However, an interesting observation is noted in the case of the Lubrol matrices. GlcNAcT-I has its highest rebinding efficiency when the 70 mM extracted form is rebound into a matrix exposed with just 2% Lubrol, and this efficiency decreases slightly as more salt is introduced. As noted, the sialyltransferases can rebind a matrix when Lubrol is used, but not very well when TX-100 is used. GlcNAcT-I can rebind both, but does so better when Lubrol is used. Therefore Lubrol has less of an effect on the structural integrity of either the *trans* or *medial* Golgi matrix, accounting for the higher degree of GlcNAcT-I rebinding, which is found in both the *trans* and *medial* regions of the Golgi apparatus. The overall decrease in binding seen in the TX-100 matrices may be due to the possibility that TX-100 destroys some of the *trans* matrix, permitting rebinding to occur primarily within the *medial* Golgi region. As indicated in table 23A there was close to 100% recovery of the salt extracted forms.

Although the detergent had a definitive effect on the matrices, the effect of salt was not as clear. It was noted by electron microscopy that the proteins in the pellet were partially solubilized at 150 mM NaCl (Slusarewicz, P. *et al.*, 1994). However, there is no indication that these proteins themselves were directly involved in the rebinding properties of the matrix. In addition, when studying the intercisternal matrices of the Golgi, it was noted that "......addition of KCl or NaCl in concentrations ranging from 0.5 M to 1.0 M failed to disrupt the Golgi stacking, although increasing membrane damage was seen at higher salt concentrations.

Even at 2.0 M NaCl, when extensive membrane damage was observed, a significant number of Golgi cisternae remained stacked into a cohesive unit" (Cluett, E.B. *et al.*, 1992). Although salt does have an affect on the rebinding of the sialyltransferases. For instance, ST6Gal I rebinding of the 70 mM extracted form in the 2% Lubrol exposed matrix is 38%, while in the 2% Lubrol / 150 mM NaCl it is 20% (table 20B), the effect is not as dramatic with GlcNAcT-I. By comparison, rebinding of GlcNAcT-I only drops from 64% to 53% under the same conditions (table 23B).

In order to obtain a better understanding of the salt's effect on the matrices' abilities to rebind the enzymes, a matrix was exposed by solubilizing the Golgi membranes with 2% Lubrol and 500 mM NaCl. When the 70 mM NaCl extracted form was dialyzed in the presence of this matrix which was exposed to a higher salt concentration, there was a dramatic decrease in the enzymes' ability to rebind (table 24B). The rebinding efficiency of GlcNAcT-I decreased more than 3-fold (compared to the 70mM NaCl matrix), but the rebinding of the sialyltransferases was almost non existent. Table 24A demonstrates ~100% recovery of 70 mM NaCl extracted enzyme. This suggests that salt may have a greater effect on the *trans* matrices abilities to rebind enzymes as opposed to the *medial* matrix, even though, as stated by Cluett, E.B. *et al.*, 1992, there does not appear to be much damage to the matrix at high salt concentrations (at least when observed with an electron microscope). Therefore, the salt may be playing a more subtle role in preventing

the enzymes from rebinding. For instance, although the matrix may not have endured any substantial damage, there would be an increase in the ionic strength of the environment, which would promote the solubilization of the enzymes. This solubility is a result of : a.) polar interactions with aqueous solvent, b.) ionic interactions with the salts present and c.) to a smaller extent the repulsive electrostatic forces between like-charged molecules or small (soluble) aggregates of molecules (Scopes, R. K., 1987). A salt concentration of about 100 mM would not be unexpected within the confines of the Golgi lumen, since most of these glycosyltransferases require this concentration of salt (usually $MnCl_2$) for optimal catalytic efficiency (Harduin-Lepers, A. *et al.*, 1995). It is feasible that higher salt concentrations, such as 500 mM, although greater than physiological, could maintain the enzymes' solubilities, due to the aforementioned ionic interactions with the salts.

If such an intercisternal protein matrix within the *trans* region of the Golgi is capable of binding the glycosyltransferases, the obvious questions are *how* and *why* this binding is occurring. The obvious candidate for binding to the matrix is the cytoplasmic tail of the sialyltransferases, since the transmembrane domain is responsible for anchoring the enzymes to the cisternal membrane. Sialyltransferases which had been proteolytically cleaved within the stem region, and therefore lacked a cytoplasmic tail and transmembrane domain, were not capable of rebinding a matrix prepared by either treatment with 70 mM NaCl and

lubrol or TX-100. Matrices prepared with 70 mM NaCl and 2% Lubrol were able to rebind 28-32% of the 70 mM NaCl extracted sialyltransferase activity (tables 21B, 22B, 23B), whereas only 1-3% of the proteolytically cleaved sialyltransferase activity was able to rebind the same matrix (table 25). Although these same enzymes do not rebind to matrices prepared with 2% Lubrol / 150 mM NaCl as well (9-17%; tables 21B, 22B, 23B), they bind the same matrices with even less efficiency (3-5%) when the cytoplasmic tail and stem region have been proteolytically cleaved.

From the evidence provided thus far, it is possible that an intercisternal Golgi matrix within the *trans* region is capable of binding resident enzymes through, in some capacity, their cytoplasmic tail. The function of *trans* Golgi enzymes and *medial* Golgi enzymes binding to such a matrix is not quite clear. It is well understood that the retention of Golgi enzymes and proteins in the stack is mediated by the transmembrane domain (Machamer, C.E., 1993; Nilsson, T. *et al.*, 1993a; Sevier, C.S. *et al.*, 1998; Webb, R.J. *et al.*, 1998). Thus, the cytoplasmic tail would likely play a minor role in enzyme retention. A role in stacking the individual cisternae has been a common suggestion since proteolysis of the matrix with various chemical and enzymatic methods has consistently been shown to unstack the matrix (Cluett, E.B. *et al.*, 1992; Slusarewicz, P. *et al.*, 1994). Furthermore, GlcNAcT-I has been localized to both the *medial* and *trans* cisternae (Nilsson, T. *et al.* 1993b) so stacking could result if the GlcNAcT-I molecules in these cisternae

bound to the same matrix. Recently, a few of the proteins which are involved in the composition of these matrices have been discovered and examined. For instance, GRASP65 has been shown to be a protein involved in the stacking of Golgi membranes (Barr, F. A. *et al.*, 1997). It was determined that this in turn interacts with another protein, GM 130. Although located within the *medial* and *trans* regions of the Golgi, GM 130 was primarily located within the *cis* region (Nakamura, N. *et al.*, 1995), strengthening the notion that the intercisternal matrices may differ in their composition. Lending support to this idea is the observation that additional proteins, p230 and p200, were located on the cytoplasmic side of the *trans* membranes, but scarcely located in other regions of the Golgi (Gleeson, P.A. *et al.*, 1996), while β-spectrin was found exclusively in the TGN and was thought to play a role in the structural formation of this region of the Golgi (Beck, K. A. *et al.*, 1997).

The exact functions of these proteins have yet to be elucidated, and there is the possibility that they may indeed perform dual roles. For instance, even though p230 is thought to be involved in the stacking of the Golgi, it is associated with vesicles budding from the TGN and therefore may be involved in the trafficking of cellular cargo leaving the Golgi apparatus. How these proteins, and the many others which have yet to be discovered, stack the membranes of the Golgi could be viewed as "a specialized form of docking, that of one cisterna with another. This docking event, unlike that which occurs when a vesicle interacts with its target membrane, would not result in fusion of the two membranes but rather in a stable

interaction" (Beck, K. A. *et al.*, 1997).

Retention of these enzymes within the Golgi apparatus is well known to be mediated by the membrane spanning domain, so binding to the matrix could, at best, only enhance the binding process. As stated in the introduction, these enzymes are consistently situated in such a way so that the catalytic region is located within the lumen of the cisternae, while the short N-terminal 'tail' is situated on the cytoplasmic face of the membrane. Evidence given here illustrates that the sialyltransferases are capable of binding to the matrix, most likely *via* an interaction with their cytoplasmic tail. Therefore, besides acting as a type of scaffolding for the Golgi complex, the matrix may play a role in the orientation of the glycosyltransferases. It was recognized that in eukaryotic cells most transmembrane proteins acquire their final membrane orientation during or immediately after synthesis on the rough endoplasmic reticulum based on a specific set of amino acid sequences (Rapoport, T. A., 1986). Although exactly how this occurs remains unknown, a rule was developed which was used to predict how proteins would orientate themselves within a membrane based on the charges of the amino acids located on opposing sides of the membrane (Hartmann, E. *et al.*, 1989). This rule, as described under Methods, states that it is not the total charge on the region flanking the transmembrane domain that is important, but rather that there is a sufficient charge difference on either side of the transmembrane domain. For instance, proteins with the N-terminal region on the cytoplasmic face of the ER

membrane usually had a greater positive charge within this region, when compared to the sequence of equal length on the lumenal side of the membrane. The authors proposed a model which stated that "a competition between translocation competence of the segments at both sides of the signal anchor sequence was occurring (and that) the more positively charged segment would be more difficult to translocate across the membrane, perhaps because of binding to a negatively charged partner.....". In accordance with this model, I believe that such a phenomenon may be occurring within the Golgi apparatus and that the "negative partner" could be the matrix itself.

As seen in table 26, all known sequenced sialyltransferases were analyzed for their respective charges within the cytoplasmic tail and a lumenal segment of equal length (so that a fair comparison of charge differences could be performed). As predicted by the aforementioned model, the N-terminal region is more likely to have a greater positive charge than its lumenal counterpart, since 30 / 31 of the enzymes obeyed the charge difference rule. Interestingly, about 10 years ago, a transient transfection study on the protein TGN38 (named because it is 38 kDa and located specifically in the TGN) was performed which made reference to the charge on the cytoplasmic tail of the enzyme (Luzio, J.P. *et al.*, 1990). This protein is a type 1 membrane protein, meaning that it has a C-terminal cytoplasmic tail that loops around within the lumen, embedding the N-terminal region into the same membrane. They had concluded that the cytoplasmic tail of TGN38 contained

some level of important information for correct intracellular targeting and retention. Expanding on this, they noted that "the 33-amino acid cytoplasmic tail of TGN38 is very basic in character with a net positive charge of +9". As has been stated, the glycosyltransferases have all been regarded as type 2 membrane proteins, with their characteristic short C-terminal cytoplasmic tail and lumenal N-terminal region. However, recently a glycosyltransferase involved in glycosphingolipid biosynthesis has been shown to be a type 3 membrane protein. The GluT enzyme has a short N-terminal segment on the lumenal side and a long cytosolic tail, however it should be cautioned that the authors noted that "a detailed empirical study using cDNA is necessary to confirm the topology of the enzyme" (Ichikawa, S. *et al.*, 1996). Although an exception to the standard glycosyltransferase topology, I had observed that the short N-terminal region had an overall charge of -1 and the corresponding cytoplasmic region a charge of +3.5. Therefore, a change in enzyme orientation does not appear to have an effect on the aforementioned charge rule.

Perhaps the most conclusive and interesting findings to date involving the cytoplasmic tail of glycosyltransferases were reported when the cytoplasmic tail of human ST6Gal I was partially replaced with a negatively charged epitope (Yang, W. *et al.*, 1996). By constructing various chimera followed by locating their intracellular location by immunofluorescence, Yang's group had concluded that "positioning of negative charge, in particular, close to the membrane, typically produces a failure of type 2 Golgi glycosyltransferases to exit the ER / CGN,

presumably due to quality control mechanisms". Therefore, the role of a Golgi intercisternal matrix may be three-fold. First, to act as a scaffolding which helps keep the membrane stacks intact. Second, to help retain the residential enzymes by binding to their cytoplasmic tail in a charge related manner. Third in some way to be involved in the distribution of Golgi glycosyltransferase to the Golgi apparatus itself *via* recognition of the cytoplasmic tail of the enzymes.

Glycobiology is quickly becoming a complete and separate discipline among the sciences and not just relegated to a sub group in cell biology or biochemistry as it has been in the past. In the last few years new journals devoted entirely to them are being published, many research centers and small start-up pharmaceutical companies are investing all of their capital in glycobiology and larger companies such as Novopharm Biotech are increasing their financial commitment to carbohydrate analysis in certain medical disorders. While this is encouraging to glycobiologists, there are still many fundamental questions that need to be answered before any quantum leaps in this innovative field can be made. It is clear that we understand that glycoconjugates are extremely complex and diverse in structure. There isn't a great need at the moment to simply discover additional structures and present these data as novel findings, which is continually being done, since this will only reinforce what we already know: that they are complex! What we should be setting our sights on now is trying to find out *why* they are structured in a particular way, *what* this relevance is under the given set of

circumstances, whether it be involved in the natural development of an organism or due to a particular disease. Furthermore there should be a concentrated effort to determine *what* parameters are involved at the intracellular level in their construction.

The aim of this thesis has been to gain a deeper understanding of these matters by examining some key players in the synthesis of glycoconjugates, namely the glycosyltransferases, with specific attention to the sialyltransferases. Results from these studies have indicated that there is a carefully controlled orchestration of the these enzymes during inflammation, as indicated by the fact that the specific activities of each of the seven enzymes studied change in a consistent manner and return to normal over the same period of time. These changes in activity undoubtedly affect the structural formation of certain glycoconjugates. Therefore, future studies should be aimed at unmasking these newly formed carbohydrate molecules and what their specific functions are. By doing so, it will bring us closer to treating diseases if they are found to occur due to a defect in their glycosylation machinery, as is the case in a family of multisystemic congenital diseases known as carbohydrate-deficient glycoprotein syndromes (Charuk, J.H.M. *et al.*, 1995). By understanding how these enzymes localize themselves within the Golgi, it is conceivable that in the future a new class of 'Golgi-drugs' could be envisioned where we would be able to control a specific cell's ability to synthesize a certain structure. An ideal situation would be seen in

the case of cancer cells where their surface carbohydrates play a role in their ability to metastasize (Yarema, K.J. *et al.*, 1998; Taylor-Papadimitriou, J. *et al.*, 1994). Therefore, by regulating the structure of these carbohydrates *via* manipulation of the cell's glycosyltransferases, the chances of a tumor spreading could be lessened. Obviously there are many questions that will have to be answered regarding the organization of the glycosyltransferases, prior to and during a specific disease, before such interventions can be undertaken. However, as we head into the next millennium, there is indeed a great deal of anticipation regarding the large array of possibilities that the advancement of glycobiology could bring mankind.

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