THE UNIVERSITY OF MANITOBA

STUDIES ON THE EFFECTS OF ALTERED GLYCOPROTEIN GLYCOSYLATION ON DIFFERENTIATION AND METABOLISM OF RAT L6 MYOBLASTS

By

Maureen A. Spearman

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Submitted to the Faculty of Graduate Studies

In Partial Fulfilment of the Requirements for the Degree

Doctor of Philosophy

Department of Chemistry

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MAUREEN A. SPEARMAN

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

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To Dean, my family, and my grandmother, Helen.

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LIST OF ABBREVIATIONS

Asn	Asparagine
BHK	Baby hamster kidney
^{14}C	Carbon 14
cAMP	Cyclic adenosine 5'-monophosphate
CCCP	Carbonyl cyanide *m*-chlorophenylhydrazone
cDNA	Complimentary deoxyribonucleic acid
CHO	Chinese hamster ovary
Ci	Curie
Cl2V1	Clone 2 variant 1
Cl5V1	Clone 5 variant 1
CMP	Cytidine 5'-monophosphate
con A	Concanavalin A
CPK	Creatine phosphokinase
cpm	Counts per minute
C^R	Concanavalin A-resistant
Csn	Castanospermine
DMD	Duchenne muscular dystrophy
dN	1-Deoxynojirimycin
Dol-P	Dolichol monophosphate
Dol-P-P	Dolichol pyrophosphate
dpm	Disintegrations per minute
EDTA	Ethylene diamine tetraacetic acid
EM	Electron microscopy
endo H	Endo-ß-N-acetylglucosaminidase H
ER	Endoplasmic reticulum

FGF	Fibroblast growth factor
Gal	Galactose or galactosidase
GalNAc	N-acetylgalactosamine
g_{av}	Average gravitational force
GlcNAc	N-Acetylglucosamine
GDP	Guanosine 5'-diphosphate
Glc	Glucose or glucosidase
gp	Glycoprotein
^{3}H	Tritium
HA	Viral hemagglutinin
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
Hex	ß-Hexosaminidase
HLA	Human leucocyte antigen
h.p.l.c.	High pressure liquid chromatography
I	Iodine
IgD	Immunoglobulin D
IgM	Immunoglobulin M
K_m	Michaelis constant
Lec^R	Lectin resistant
LCA	*Lens culinaris* agglutinin
L-CAM	Liver cell adhesion molecule
Man	Mannose or mannosidase
α-MEM	α-Minimal essential medium
MES	2[N-Morpholino]ethanesulfonic acid
MHV	Mouse hepatitis virus
MdN	N-methyldeoxynojirimycin

mRNA	Messenger ribonucleic acid
mw	Molecular weight
NADPH	Nicotinamide-adenine dinucleotide phosphate
N-CAM	Neural cell adhesion molecule
NeuAc	N-Acetylneuraminic acid
NK	Natural killer
n.m.r.	Nuclear magnetic reasonance
P	Phosphate
PBS	Phosphate buffered saline
PHA	Phytohemagglutinin
RCA	*Ricinus communis* agglutinin
RER	Rough endoplasmic reticulum
RGD	Arginine-glycine-aspartic acid
RS	Rous sarcoma virus
S.D.	Standard deviation
Ser	Serine
SRP	Signal recognition particle
Sw	Swainsonine
Tase	Transferase
TGF-ß	Transforming growth factor-ß
Thr	Threonine
U	Units
UDP	Uridine 5'-diphosphate
V_{max}	Maximum velocity
VSV	Vesicular stomatitis virus
W	Watt
WGA	Wheat germ agglutinin

ABSTRACT

The effects of altered glycoprotein metabolism in rat L6 myoblasts were investigated through the use of oligosaccharide processing inhibitors, which block specific reactions in the formation of asparagine-linked oligosaccharides. Modification of cell surface oligosaccharides using the α-glucosidase inhibitors, 1–deoxynojirimycin, N-methyldeoxynojirimycin, and castanospermine, and the α–mannosidase II inhibitor, swainsonine, resulted in a substantial reduction in fusion capactiy of the wild type L6 myoblasts, which was reversible upon removal of the inhibitors. The mannosidase I inhibitor, 1-deoxymannojirmycin, did not affect the fusion capacity. Creatine phosphokinase, an enzyme which increases during muscle differentiation, was also significantly reduced in the L6 myoblasts treated with 1–deoxynojirimycin, N-methyldeoxynojrimycin, castanospermine, and swainsonine. Lectin binding studies with concanavalin A (con A) and wheat germ agglutinin (WGA) indicated that cell surface oligosaccharide content was altered in a manner consistent with the known oligosaccharide structures resulting from the treatment with oligosaccharide processing inhibitors. These results provide further evidence for the involvement of cell surface oligosaccharides, particularily of the $Man_{8\text{-}9}GlcNac_2$ type, in the fusion reaction required for the differentiation of myoblasts to myotubes.

The oligosaccharide processing inhibitors, swainsonine and deoxymannojirimycin also increased binding activity of the insulin receptor, another cell surface glycoprotein, whereas castanospermine slightly decreased insulin binding capacity in the myoblasts. Addition of the processing inhibitors to myoblast glycosidase assays indicated that oligosaccharide processing enzymes were sensitive to their respective inhibitors. The lysosomal enzymes, ß-galactosidase and ß-hexosaminidase, were also significantly reduced in myoblasts cultured in the presence of the glucosidase inhibitors.

The effects of altered glycoprotein metabolism in L6 myoblasts were also studied using a con A-resistant L6 myoblast line which does not fuse or undergo biochemical differentiation (Parfett *et al.*, 1981). The con A-resistant myoblasts have truncated high mannose oligosaccharides, which may have an effect on several aspects of glycoprotein metabolism within the cells. Lysosomal α-mannosidase activity was substantially reduced in the con A-resistant myoblasts, and extracellular ß-hexosaminidase was increased. Both sialyl transferase and galactosyl transferase activities were significantly altered in the con A-resistant myoblasts in comparison with the wild type myoblasts, when using the glycoprotein acceptor fetuin. However, no significant differences in activities and enzyme kinetics were observed using α_1-acid glycoprotein as the acceptor glycoprotein substrate. Altered populations of sialyl and galactosyl transferse enzymes may exist in the con A-resistant myoblasts.

Aspects of glycoprotein metabolism were also studied in fibroblasts taken from Duchenne muscular dystrophy patients and age-matched controls. No significant differences were observed in lysosomal α-mannosidase or ß-hexosaminidase activities between the two groups. Con A binding studies also showed that there were no significant differences in the cell surface carbohydrate content .

INTRODUCTION

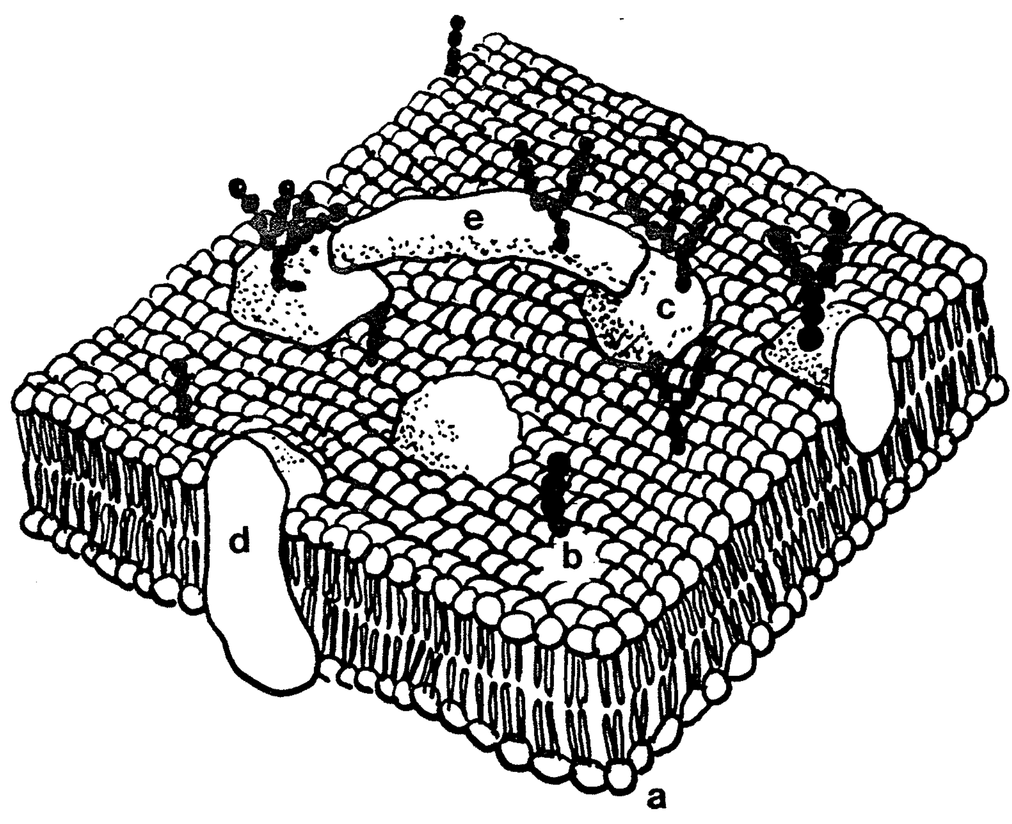
A. *Membrane Glycoproteins in Cell Recognition and Adhesion*

Plasma membranes were originally conceived as merely barriers to contain cellular organelles and the cytoplasm. Eventually, it became evident that to allow passage of some molecules through the membrane and restrict movement of other molecules, the membrane must contain highly specialized structures. Theories and models have developed leading to the presently accepted fluid mosaic lipid bilayer structure (Singer and Nicolson, 1972). This model accounts for such well known properties of membranes as selective permeability, fluidity and asymmetry. The basic structure is comprised of a lipid bilayer with intrinsic and extrinsic membrane proteins, glycoproteins and glycolipids (Figure 1). Membrane glycoproteins and glycolipids are externally orientated, and together with adsorbed glycoproteins and proteoglycans comprise the glycocalyx, the carbohydrate-rich layer located on the periphery of the plasma membrane. The membranes of active cells are constantly in a state of regeneration to replenish portions internalized by endocytosis, to replace modified or defective molecules, and to add new surface molecules for growth and differentiation.

The determination of the structure and function of membrane glycoproteins and their carbohydrate moieties is of prime interest in many fields of study involving the plasma membrane. Membrane glycoproteins are classified into several different groups based on their functional properties, such as hormone receptors, membrane pumps directing movement across the membrane, antigenic determinants, enzymes and intercellular attachment sites such as desmosomes. There are many more cellular processes in which membrane glycoproteins have been implicated, but their exact function remains unknown. Often associated with the question of glycoprotein function, is the unknown function of the carbohydrate moiety. Cell-cell and cell matrix adhesion or interaction are two closely associated biological events which occur in all types of eukaryotic cells. The significance of cell surface glycoconjugates

Figure 1.

A diagrammatic representation of the plasma membrane as suggested by the Singer-Nicholson model. The phospholipid bilayer (a) contains glycolipids (b) and glycoproteins (c) with carbohydrate extending into the extracellular space. Intrinsic membrane proteins (d) may be positioned on either the intracellular or extracellular face of the lipid bilayer or transverse the lipid bilayer. Adsorbed glycoproteins (e) may also be closely associated with other structural elements of the membrane.


e
c
d
b
a

and their carbohydrate in the recognition and adhesion process has become much more evident within the last ten to fifteen years.

1. Cell-cell Recognition and Adhesion

Cell-cell interactions are required in many cellular and physiological events in the development and maintenance of an organism. Plasma membrane glycoproteins have been the focus of several studies on cellular interactions such as slime mold aggregation, aggregation of sponge cells, contact inhibition, embryonic development, myoblast fusion, recognition of target cells by natural killer (NK) cells and macrophages, sperm-egg binding and lymphocyte homing. The basic concept of intercellular recognition in animal cells requires two components, the receptor and the complementary ligand. There are several examples of carbohydrate recognition which have served as model systems for studying the basis of cell-cell recognition and adhesion mediated through glycoproteins. A proposal by Roseman and associates suggested that adhesion between cells of the same tissue type was due to non-covalent linkages between a glycosyl transferase exhibiting lectin-like activity, and a carbohydrate moiety on the plasma membrane of an adjacent cell (Roth *et al.*, 1971a,b). Mammalian lectins which recognize specific sugar residues have been found on the surface of rat (Maynard and Baenzinger, 1981, 1982) and chicken hepatocytes (Drickamer and Mamon, 1982) and have also been proposed for myoblast adhesion (Gartner and Podleski, 1975). The receptor-mediated recognition and targetting of lysosomal enzymes containing mannose-6-phosphate residues (reviewed by Sly and Fischer, 1982; Jamieson, 1983; Kornfeld and Kornfeld, 1985; Kornfeld, 1986, 1987) and the hepatocyte cell surface receptor which recognizes and internalizes asialo glycoproteins (Ashwell and Morell, 1974; Hudgin *et al.*, 1974) are well-known model systems of specific receptor-carbohydrate interactions.

Intercellular adhesion is a complex process, generally thought to be initiated by receptor-ligand binding, which may culminate in fusion, phagocytosis, intracellular signalling and other secondary events. The identification of specific glycoproteins as contributing factors to the adhesion process has relied largely on the use of antisera and monoclonal antibody preparations to purify and characterize cell surface components that influence cell-cell adhesion.

A good model system for studying cell-cell interactions is the aggregation of the slime mold, *Dictyostelium discoideum* , in the formation of its multicellular structure. The glycoprotein composition of the plasma membrane is developmentally regulated (Henderson, 1984), and inhibitors of glycoprotein synthesis inhibited growth and differentiation (Lam and Siu, 1982) and reduced the production of a specific glycoprotein known as gp80. Gp80 is a membrane component of an area of adhesion, known as contact site A (Yamada *et al.*, 1982; Hirano *et al.*, 1982). Originally, the glycoprotein was thought to protect against proteolytic cleavage (Hirano *et al.*, 1983), but more recent evidence indicates that mannose-containing carbohydrates are directly involved in cell adhesion (Hirano *et al.*, 1985). However, monoclonal antibodies against gp80 failed to inhibit cell adhesion. Also, two mutants with altered gp80 expression retained their adhesive properties. This questions the theory of gp80 requirement for cell adhesion (Henderson, 1984), and therefore further studies are clearly required.

Discoidins I and II, cell surface lectins of *D. discoidieum* (Reitherman *et al.*, 1975; Rosen *et al.*, 1973), have also been implicated in cell adhesion during differentiation. Competitive inhibition studies with various sugars and glycoproteins indicated that oligosaccharides were the recognized ligands (Bartles and Frazier, 1980). However, there is no evidence for lectin-carbohydrate recognition between discoidins and the glycoproteins of contact site A (Olden *et al.*, 1982b).

Other plasma membrane glycoproteins involved in cell-cell adhesion interactions have been extensively reviewed (Damsky *et al.*, 1984; McClay and Ettensohn, 1987). Several cell adhesion glycoproteins, known as CAM's (cell adhesion molecule), have been identified through the use of broad spectrum antisera which block cell adhesion. N-CAM (nerve-cell adhesion molecule) was one of the first to be identified and has since been found in a variety of cells including chicken, rat, human and mouse neural tissue (Damsky *et al.*, 1984). Heterogeneity in the size of N-CAM in adult and embryonic forms is due to differences in sialic acid content, and this may be significant in embryonic neural develpment (Edelman, 1983). One possible function for N-CAM is the formation of myoneural junctions through muscle-nerve interactions (Rutishauser *et al.*, 1983); N-CAM in muscle tissue is closely associated with innervation of the muscle (Sanes *et al.*, 1986). N-CAM also has an affinity for heparan sulfate which may suggest how it elicits intercellular recognition and adhesion (Barnstable, 1986). Since the discovery of N-CAM, several glycoprotein adhesion molecules have been found in other types of tissues such as, L–CAM (liver adhesion molecule) (Neilsen *et al.*, 1981) and CAM-105 (Ocklind and Obrink, 1982; Ocklind *et al.*, 1983).

During embryonic development, intercellular adhesion and recognition events are obligatory. Also, dramatic changes in cell surface proteins and glycoproteins occur in the early stages of embryogenesis. Profiles of oligosaccharides from chick embryonic fibroblasts reveal a shift to more complex oligosaccharides during embryogenesis from day 8 to day 16 (Codogno *et al.*, 1983). A specific membrane glycoprotein involvment in embryonic develpment was demonstrated using antibody prepared against a glycoprotein shed from human mammary carcinoma. The antibody blocked compaction (and thus blastocyst formation) in mouse embryo cells (Damsky *et al.*, 1983).

Some studies implicating glycoproteins in embryogenesis have been of a more general nature. The requirement for asparagine-linked glycoprotein synthesis in gastrulation of sea urchin embryos is known (Lennarz, 1983, 1985). Glycosyl transferase enzymes appeared to be developmentally regulated in sea urchin embryos (Welply *et al.*, 1985) and are significantly elevated at the blastocyst stage in mouse embryos (Armant *et al.*, 1986). The increase in cell-cell interaction during the blastocyst stage of mouse embryonic development, coupled with the inhibition of blastocyst formation by tunicamycin, suggested a correlation between increased glycoprotein synthesis and embryonic cellular interactions (Surani, 1981). Examples of other studies which support a cell surface glycoprotein requirement in embryogenesis are: inhibition of aggregation of red blood cells by embryonal carcinoma cells by mannose-rich polymers (Grabel *et al.*, 1979); ß-galactosidase inhibition of aggregation of teratoma cells which express embryonic cell character (Oppenheimer, 1975); and the discover of a cell surface galactosyl transferase on teratoma cells, responsible for intercellular adherence (Shur, 1982, 1983). The importance of carbohydrate-containing glycoproteins on the embryonic cell surface is further emphasized by the expression of a special class of N-linked glycans called the polylactosamines (Muramatsu *et al.*, 1978; Ivatt, 1985).

Cell-cell and cell-ligand interactions of the immune system also require cell surface glycoproteins. Some examples are cell surface carbohydrate recognition by antibodies (reviewed by Feizi and Childs, 1987), activation and regulation of the complement system (Nose and Wigzell, 1983), binding of soluble mediators to cell surface carbohydrate (Reading, 1984), NK (natural killer) cell recognition of target cells (Ades *et al.*, 1981; Pohajdak *et al.*, 1984, 1986; Dennis and Laferte, 1985), macrophage recognition of target cell (Shepherd *et al.*, 1981; Largent *et al.*, 1984) and lymphocyte recognition and homing (Yednock *et al.*, 1987a,b). Contact inhibition of growth may also be associated with cell surface recognition of carbohydrate on

adjacent cells. Plasma membrane preparations from confluent cells inhibited DNA synthesis in human embryonal lung fibroblasts, however, membrane preparations from cells treated with tunicamycin or ß-galactosidase abolished this inhibitory effect (Weiser *et al.*, 1985).

2. Cell-Substratum Interactions

Membrane glycoproteins have also been recognized as important mediators of cell-substratum adhesion. One model suggested a transient, primary recognition followed by recruitment of receptors to intensify cell surface-extracellular matrix interactions (Damsky *et al.*, 1984). Many studies have identified proteins or glycoproteins of the plasma membrane which adhere to the extracellular matrix and its components, or to immobilized glycoconjugates (Schnaar, 1984). Knudsen and coworkers (1981) first report of a fibroblast membrane glycoprotein (140,000 mw) which adhered to the extracellular matrix, was soon followed by identification of cell membrane glycoproteins in other systems which had an affinity for collagen (Chiang and Kang, 1982; Mollenhauer and von der Mark, 1983; Neumeier *et al.*, 1984; Rubin *et al.* , 1986), fibronectin (Hansen and Clemmensen, 1982; Pytela *et al.*, 1985; Hasegawa *et al.*, 1985) and laminin (Malinoff and Wicha, 1983). Transmembrane glycoproteins, which bind to one or more components of the extracellular matrix, have been newly classed as integrin receptors (Hynes, 1987; Buck and Horwitz, 1987). Their existence in a wide variety of cell types is paralleled by their role in numerous cell adhesion processes such as fibroblast adhesion to the extracellular matrix, platelet adhesion, T lymphocyte and natural killer cell interactions, leukocyte adhesion to endothelia and monocyte and neutrophil adhesion (Hynes, 1987). Some integrin receptors recognize an RGD (Arg-Gly-Asp)-containing amino acid sequence within the matrix proteins. However, studies with peptides containing RGD sequences indicate the RGD sequence alone is not sufficient to promote attachment in some fibroblast cell lines

(Singer *et al.*, 1987) suggesting some other structure may be necessssary for recognition. The transmembrane orientation of the integrin receptors and their interaction with cytoskeletal elements may indicate a function as a transmembrane-linker between the extracellular matrix and intracellular structural elements (Hynes, 1987).

Plasma membrane glycoproteins are widely recognized as receptors in cell-matrix attachment, but the requirement for glycosylation of a receptor or the recognition of carbohydrate residues of extracellular components, has not been well documented. However, there are some studies which suggest cell surface carbohydrates may be involved in the adhesion process. Mouse fibroblasts which were defective in glycosylation due to a lack of production of N-acetylglucosamine, had a reduced adherence to substratum which could be reversed upon addition of N-acetylglucosamine (Pouyssegur *et al.*, 1977). *Ricinus communis* agglutinin-resistant cells, which are reduced in exposed ß-galactose residues, have poor adherence to fibronectin coated plates (Pena *et al.*, 1979). The attachment of macrophages to bone was inhibited by various sugar residues and the glycoprotein, fetuin, which implicated carbohydrate in these cell-matrix interactions (Bar-Shavit *et al.*, 1983). Also, monoclonal antibody preparations, specific for the fucosyl (poly)-N-acetyllactosamine structure of F9 embryonal carcinoma cells, inhibited cell substratum adhesion (Nomoto *et al.*, 1986). Although these studies have not assigned a precise structure-function role for carbohydrate in adhesion, they do emphasize the importance of glycosylation in cell-matrix interactions.

One field of study, in which cell-cell and cell-matrix adhesion is very important, is research in tumor growth and metastases. The relationship between cell surface and extracellular matrix glycoproteins and tumorigenicity is a very intriguing question and has been the focus of much research. Studies in the areas of neoplastic transformation and the metastatic process have correlated altered phenotypes with

dramatic changes in glycosylation and glycoproteins, however, due to the complexity of the disease and the diverse functions of glycoproteins, a direct role for the carbohydrate of cell surface glycoproteins in the biology of cancer has not been established. A vast amount of research has been accomplished in this area and the following examples only serve to illustrate a small part of this field. The products of oncogenes, which generate the transformed properties of cells, are in some instances glycoproteins which require a specific carbohydrate residue to be active. The carbohydrate structure of N-linked oligosaccharides can be altered using a class of molecules known as glycoprotein processing inhibitors. The inhibitors block specific pathways of sugar removal in the synthesis of oligosaccharides. Modification of the cell surface asparagine-linked carbohydrate of gp140 (the oncogene product of feline sarcoma virus), using processing inhibitors, results in alteration of the transformed properties (Hadwiger *et al.*, 1986).

The relationship between metastatic potential and cell-matrix interactions, which involve glycoproteins of the cell surface and extracellular matrix, is another intriguing question presently being addressed (Liotta, 1986). Steck and Nicolson (1983) have found a relationship between metastases and a high molecular weight, extracellular matrix and cell surface glycoprotein (gp580) with a high content of glucosamine and galactosamine (Steck *et al.*, 1987) in mammary adenocarcinoma cells. However, its contribution to the metastatic potential of the cell is unknown. Nicolson (1984) indicates that in many studies involving metastases of cells through the blood, there is an increase of the sugar sequence NeuAc-Gal-GlcNac on the metastatic cells' surfaces. WGA resistant B-16 melanoma cells reduced in metastatic potential and tumorigenicity, exhibited decreased amounts of cell surface sialic acid and increased amounts of fucose (Tao and Burger, 1977, 1982).

Although there is some correlation between the alteration of certain cell-surface carbohydrate and glycoproteins, and tumorigenicity in some types of cancer cells, no

basic unifying link has been established. Definition of the function of metastatic and tumor-associated cell-surface glycoproteins and an explanation for alterations in cell-surface carbohydrate may explain some properties of tumor cells.

The function of glycoproteins as cell surface receptors is well documented, but arising from the new knowledge of the heterogeneity of oligosaccharides on glycoproteins is the important question of how the structure of the carbohydrate affects the functionality of the glycoprotein, and furthermore, does the oligosaccharide have a role itself in cell-cell and cell-substratum adhesion and recognition? The above examples have served to illustrate the role of glycoproteins in membrane adhesion and although there is increased awareness of the significance of oligosaccharides in targetting, recognition and the functional properties of glycoproteins, the precise functional role of oligosaccharides on most glycoproteins remains unknown. Recent advances in determining the structure of oligosaccharides using n.m.r. (nuclear magnetic reasonance) and mass spectrometry may aid immensely in correlating the exact structure of oligosaccharides with the functional properties of glycoproteins.

B. *Glycoproteins*

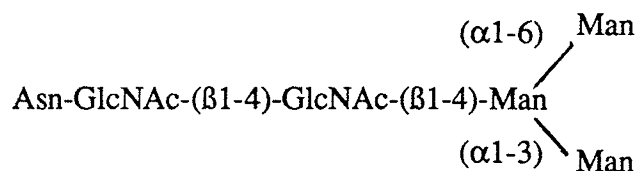
Glycoproteins, composed of covalently linked carbohydrate and protein, are ubiquitous in nature, existing in plant and animal cells and viruses. Their diversity in existence is seemingly matched by their diversity in function. The significance of the carbohydrate portion of the molecule has increasingly become a focus of attention. New techniques for analyzing the wide variety of carbohydrate structures present on glycoproteins have dramatically enhanced our current knowledge of oligosaccharide structure and the biosynthetic pathway of glycoprotein production.

1. Glycoprotein Structure

Glycoproteins are part of a large family of molecules, also comprised of proteoglycans, mucins and glycosaminoglycans. Their common feature is the presence of carbohydrate and protein, but the ratio and linkage that exists between these two components distinguishes the different structural groups. The carbohydrate of glycoproteins may be attached in either an N- or O-linkage to the protein. There are three major types of O-linkages: Ser(Thr)-GalNAc, found on many cell surface and secretable glycoproteins; Ser-xylose, found in glycosaminoglycans; and hydroxylysine-Gal, found in collagen and basement membranes. The Ser(Thr)-GalNAc linked oligosaccharides may contain from one up to eighteen or more residues comprised mainly of GalNAc, Gal, GlcNAc, sialic acid and fucose, and they are often classified according to their core structure. Their primary site of synthesis is the Golgi, where sugar residues are added sequentially to the oligosaccharide, directly attached to the protein.

The asparagine or N-linked glycoproteins involve a N-glycosylamine bond formed between the amide group of asparagine and the C-1 hydroxyl group of the reducing

N-acetylglucosamine residue of the oligosaccharide. All N-linked glycoproteins contain the same inner core arrangement of sugars with the following structure:



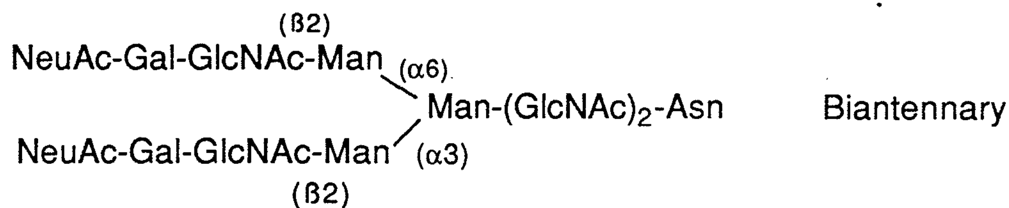
The outer sugar residues determine the classification of the oligosaccharide into one of two major categories: the high mannose oligosaccharides or the complex oligosaccharides. In complex oligosaccharides, a specific sequence of sugar residues comprised of N-acetylglucosamine (linked ß1-2, ß1-4, or ß1-6), galactose (ß1-4) and N-acetylneuraminic acid (α2-3 or α2-6) are attached to the core and referred to as the 'terminal triplet'. The multiple valence of the core mannose residues allows for the attachment of more than one terminal sequence per end mannose residue and allows for the formation of bi, tri and tetra antennary complex oligosaccharides (Figure 2). Several bisected structures with a ß1-4 GlcNAc attached to the ß-linked mannose of the core also exist (Schachter *et al.*, 1985). Fucose is another sugar residue commonly found in complex oligosaccharides and may be linked α1-3 to the N-acetylglucosamine bond. Sialic acid may also exist in an α2-6 linkage to the GlcNAc of the terminal triplet. Poly-N-acetyllactosaminoglycans, with multiple GlcNAc-Gal units, are specialized N-linked oligosaccharides found in embryonic tissues (Li *et al.*, 1980). Several other structural variations of complex oligosaccharides of glycoproteins exist and are reviewed by Kornfeld and Kornfeld (1985), Schachter *et al.*(1985) and Montreuil (1982).

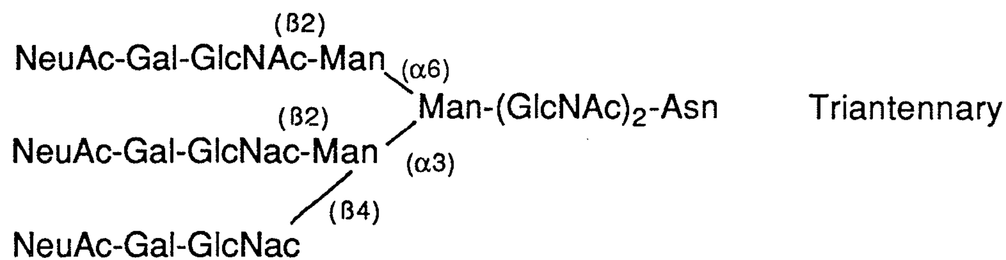
The second predominant group of carbohydrate structures found in N-linked glycoproteins are the high mannose oligosaccharides. Generally, they contain eight or nine branched mannose residues linked to the core N-acetylglucosamine residues,

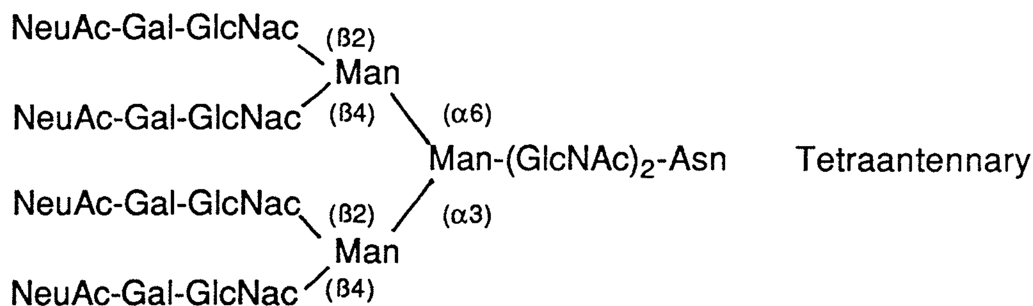
Figure 2.

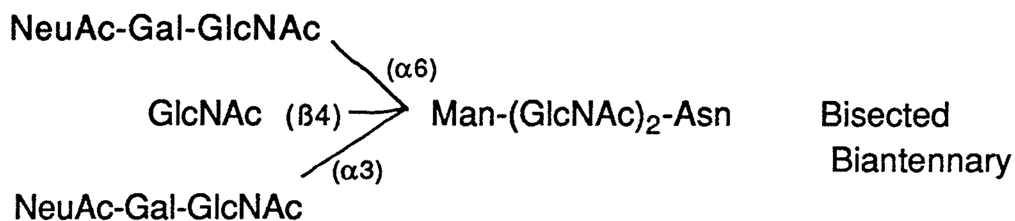
Three common structures found in the large groups of N-linked complex oligosaccharides are the biantennary, triantennary, and tetraantennary . Branching at the core mannose residues allows for the attachment of up to three terminal triplet sequences per mannose residue. The bisected biantennary oligosaccharide , with a GlcNAc in a (ß1-4) linkage to the interior mannose of the core, represents one of the several possible bisected complex structures.

Complex Oligosaccharides









although as few as five mannoses may exist (Figure 3). The high mannose oligosaccharides represent a 'less processed' form of carbohydrate, meaning they do not undergo the synthetic reactions which remove and add sugar residues to produce complex oligosaccharides. A GlcNAc intersected structure has been recently identified in *Dictyostelium discoideum* (Couso *et al.*, 1987) (Figure 3). Hybrid structures, with one arm of mannose residues and a second arm with complex oligosaccharide character, incorporate features of both high mannose and complex oligosaccharides, and are usually bisected by a single GlcNAc residue attached in a ß1-4 linkage to the ß1-4 linked core mannose residue (Figure 4).

Oligosaccharide chains are linked to the polypeptide chain not randomly but rather at very specific sites called asparagine sequons. The sequon is comprised of the amino acid sequence Asn-X-Ser (Thr) (Marshall, 1974) where X can be any amino acid with the exception of proline (Bause, 1979), however, not all sequons are glycosylated.

Techniques for defining the structures of N-linked oligosaccharides have advanced greatly from the standard methods of enzymatic digestion and derivatization coupled with chromatography, to the much more recent applications of n.m.r. and mass spectrometry. This will allow faster and more accurate identification of N-linked oligosaccharides.

2. N-linked Glycoprotein Synthesis

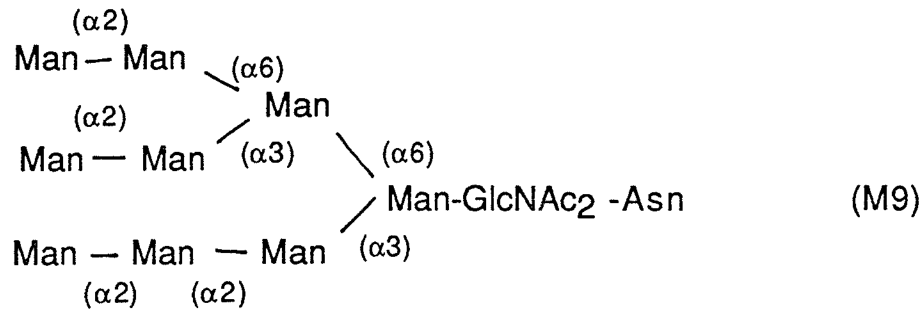
a. Protein Synthesis

Glycoproteins are synthesized initially as two entities, the protein and the oligosaccharide, which are then covalently attached and further modified to produce their mature form. The generally accepted model suggests a cotranslational mechanism for the attachment of oligosaccharide before the protein reaches its final

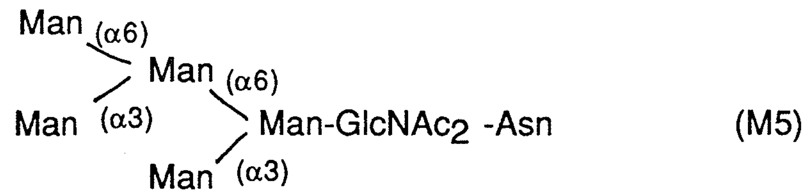
Figure 3.

Examples of high mannose oligosaccharide structures. The nine mannose residue (M9) oligosaccharide is commonly found on many glycoproteins. The five mannose oligosaccharide (M5) represents a processed form of the high mannose oligosaccharides. A GlcNAc intersected structure is a novel high mannose oligosaccharide.

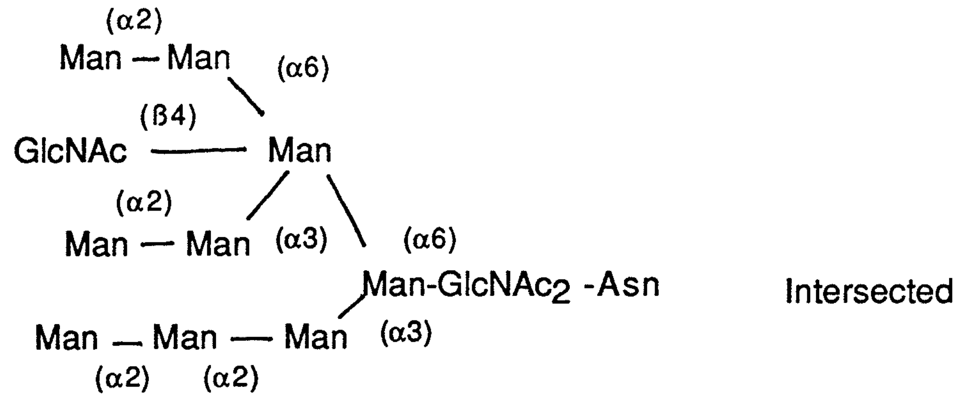
High Mannose Oligosaccharides



(M9)



(M5)

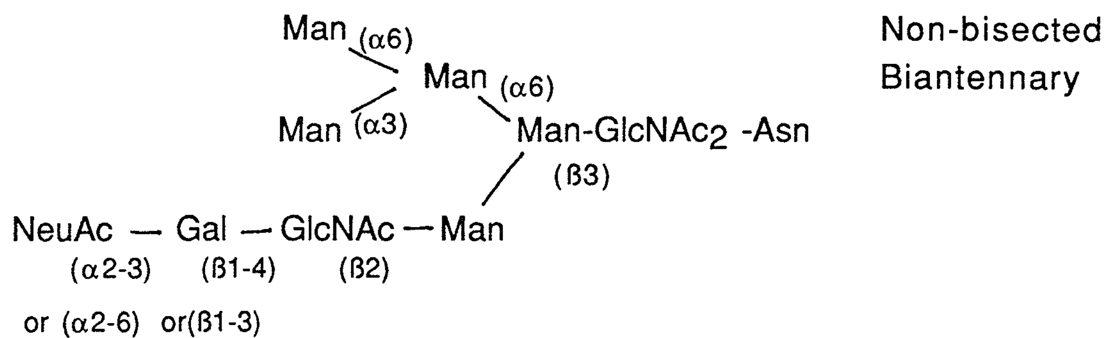


Intersected

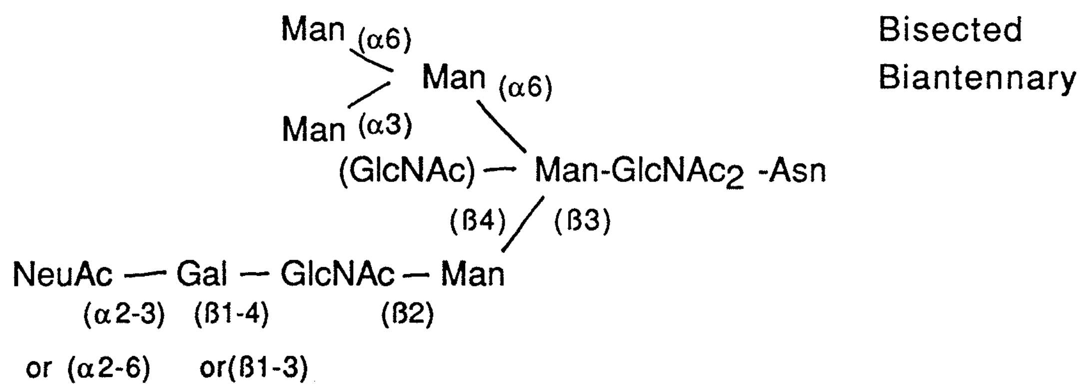
Figure 4.

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

Hybrid Oligosaccharides



Non-bisected
Biantennary



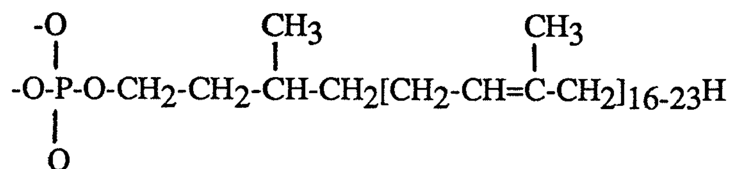
Bisected
Biantennary

conformation (Kornfeld and Kornfeld, 1985), however, post-translational attachment occurs in liver (Jamieson, 1977; Jamieson, 1983) and may exist for other glycoproteins as well (Kaplan *et al.*, 1987). Both pathways are possible because of the way the protein is synthesized and transported to the site of glycosylation.

The mechanism of transfer of nascent polypeptide from the ribosome into the RER, where glycosylation occurs, was originally proposed by Blobel and Dobberstein (1975 a,b) and has been recently reviewed (Hortsch and Meyer, 1986). An initial hydrophobic leader sequence of 20-40 amino acids on the nascent polypeptide acts as a signal peptide. A signal recongition particle (SRP) binds to the signal peptide (Walter and Blobel, 1982), which in turn is recognized and bound by a SRP receptor protein (Gilmore *et al.*, 1982) or docking protein (Meyer *et al* ., 1982) on the surface of the RER. The model suggests that a channel is then created through the membrane allowing vectoral transport of the nascent polypeptide into the lumen of the ER, or incorporation into the ER membrane. Signal peptidase cleaves off the signal portion of the protein during translation (Jackson and Blobel, 1977).

b. The Dolichol Cycle

Oligosaccharides are synthesized on lipid carrier molecules. Several lipids may act in this capacity (Richards and Hemming, 1972), however, the current weight of evidence suggests that the primary lipid carrier molecules are the dolichol phosphates. Dolichols are polyprenol alcohols made of a linear chain of 16-23 isoprene units with a saturated α-isoprene unit.



The formation of the oligosaccharide on the lipid carrier occurs in a step-wise manner known as the dolichol cycle (Sharon and Lis, 1981). The main product of the cycle is Glc_3-Man_9-$GlcNAc_2$-P-P-dolichol (Figure 5), and the carbohydrate portion transferred to protein is known as the G-oligosaccharide (Li *et al.*, 1978). The variability of oligosaccharide structure, found on mature glycoproteins, is introduced later in a series of modification steps called the processing reactions.

The first reaction in the dolichol cycle is the formation of GlcNAc-P-P-Dol from Dol-P and N-acetylglucosamine-1-P with UDP-GlcNAc as the sugar donor. This is followed by the addition of a second N-acetylglucosamine from UDP-GlcNAc to produce dolichol-pyrophosphoryl-di-N-acetylchitobiose (Struck and Lennarz, 1980). Five mannose residues are then added from the nucleotide sugar, GDP-mannose, to produce Dol-P-P-$GlcNAc_2$-Man_5 in a branching pattern (Prakash *et al.*, 1984). A further four mannose residues are transferred to the oligosaccharide from the substrate Dol-P-mannose by mannosyl transferases (Chapman *et al.*, 1980). These mannose residues are all attached in α1-2 linkages and result in the structure Dol-P-P-$GlcNAc_2$-Man_9. Theoretically, there may be as many as nine distinct mannosyl transferase enzymes, as each mannose differs in its environment relative to the other mannose residues. Many of these enzymes have been studied in partially purified or solubilized preparations from various sources. The mannosyl transferase which attaches mannose α1-3 to the ß-linked mannose has been purified and characterized (Jensen and Schutzbach, 1981).

The final addition of three glucose residues from the substrate Dol-P-glucose, completes the addition of sugar residues *via* the dolicol cycle. The three glucose residues form a linear structure (α1-2, α1-3, α1-3) linked to a specific terminal mannose residue of the Man_9-$GlcNAc_2$-P-P-dolichol (Liu *et al.*, 1979; Chapman *et*

al., 1979a). The presence of the glucose residues may inhibit degradation of the lipid-oligosaccharide by phosphodiesterase (Hoflack *et al.*, 1981).

The topography of the synthesis of the lipid-oligosaccharide and the sites of the enzymes and substrates involved in the tranfer of sugar residues to the oligosaccahride have been recently reviewed (Hirschberg and Snider, 1987). Studies suggest that Dol-P-P-GlcNAc$_2$-Man$_5$ is synthesized on the cytoplasmic side of the RER with transmembrane movement of the complex to the luminal face where mannose and glucose are added from the Dol-P-sugars (Snider *et al.*, 1980; Snider and Robbins, 1982; Snider and Rodgers, 1984) . This agrees with the inability of the nucleotide sugars to enter the lumen of the RER, and the finding that Dol-P-Man and Dol-P-Glc, which are synthesized from the nucleotide sugars, must be transported into the RER (Snider and Rodger, 1984). Hanover and Lennarz (1982) have proposed that the formation of lipid-oligosaccharide occurs *via* a transmembrane, multi-enzyme complex.

c. Transfer of Oligosaccharide to Protein

The transfer of the oligosaccharide to the peptide chain is mediated by the enzyme oligosaccharyl transferase (Czichi and Lennarz, 1977), and the specific site of attachment on the protein is the asparagine sequon (Asn-X-Ser or Thr) (Marshall 1974). The properties of oligosaccharyl transferase have recently been reviewed (Kaplan *et al.*, 1987).

The conformation of the protein around the sequon may be of significance in glycosylation, because unfolded proteins and polypeptide fragments containing the tripeptide, Asn-X-Ser (or Thr), allow glycosylation *in vitro,* but native (folded) proteins are not glycosylated (Kronquist and Lennarz, 1978). This would favor the

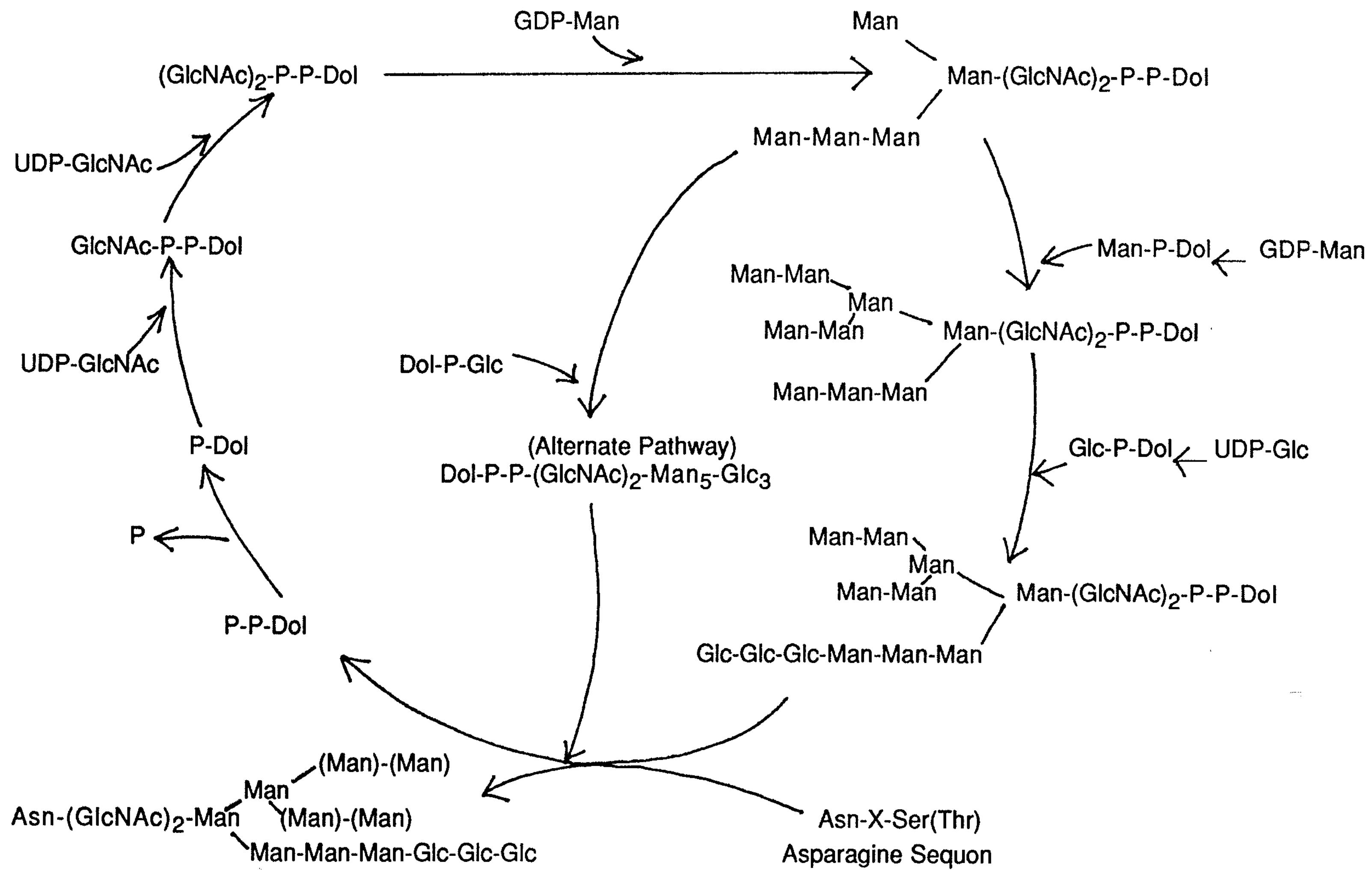
model of co-translational glycosylation as opposed to post-translational addition of oligosaccharides.

Studies with synthetic peptides with a sequon containing threonine, serine or cysteine indicate that glycosylation rates are dependent upon hydrogen bond formation between the hydroxyl group of the hydroxylamino acid of threonine or serine and the amide of the asparagine. This increases the nucleophilic character of the amide of asparagine, thus enhancing its reactivity with the Dol-P-P-oligosaccharide, and bonding capability with the oligosaccharide (Bause and Legler, 1981). Hydrogen bonding within the sequon is enhanced by the existence of the ß-turn conformation within the peptide chain and increases the chances of glycosylation (Bause, 1984). Lack of this conformation may be one reason why not all sequons are glycosylated. In thyroid microsomes, oligosaccharyl transferase also contained hydrolase activity, releasing Glc_3-Man_9-$GlcNAc_2$ from the dolichol carrier (Anumula and Spiro, 1983).

The "en bloc" transfer of the oligosaccharide is facilitated by the presence of the three terminal glucose residues, however, their presence is not essential. Oligosaccharide-lipid, containing one or two glucose residues, is transferred at a faster rate and to a greater extent than non-glucose containing lipid-oligosaccharide in fibroblast microsome preparations (Turco *et al.*, 1977), and oligosaccharides containing three glucose residues are transferred ten times more effectively than a Glc_2-oligosaccharide (Murphy and Spiro, 1981). Yeast mutants, deficient in terminal glucose and mannose residues, however, glycosylated protein at a much slower rate (Huffaker and Robbins, 1983; Runge *et al*., 1984; Runge and Robbins, 1986). In F9 teratocarcinoma cells, non-glucosylated Man_7-$GlcNAc_2$ was transferred to protein (Romero and Herscovics, 1986b).

Figure 5.

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



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

A nine mannose-containing oligosaccharide is also not a requirement for the glycosylation of protein. An alternate pathway (Figure 5) allows the transfer of Glc_3-Man_5-$GlcNAc_2$ in glucose starved Chinese hamster ovary cells (Rearick *et al*., 1981). Similarly, a mutant mouse lymphoma cell line (class E Thy-1^-), defective in the formation of Dol-P-Man, also transferred a Glc_3-Man_5-$GlcNAc_2$ to protein (Kornfeld *et al.*, 1979). Energy depletion of influenza virus-infected chick embryo cells resulted in a reduction in the formation of Dol-P-Man, and the resulting oligosaccharides contained only up to five mannose residues. Glycosylation did occur but complex oligosaccharides were not produced (Datema and Schwarz, 1981)

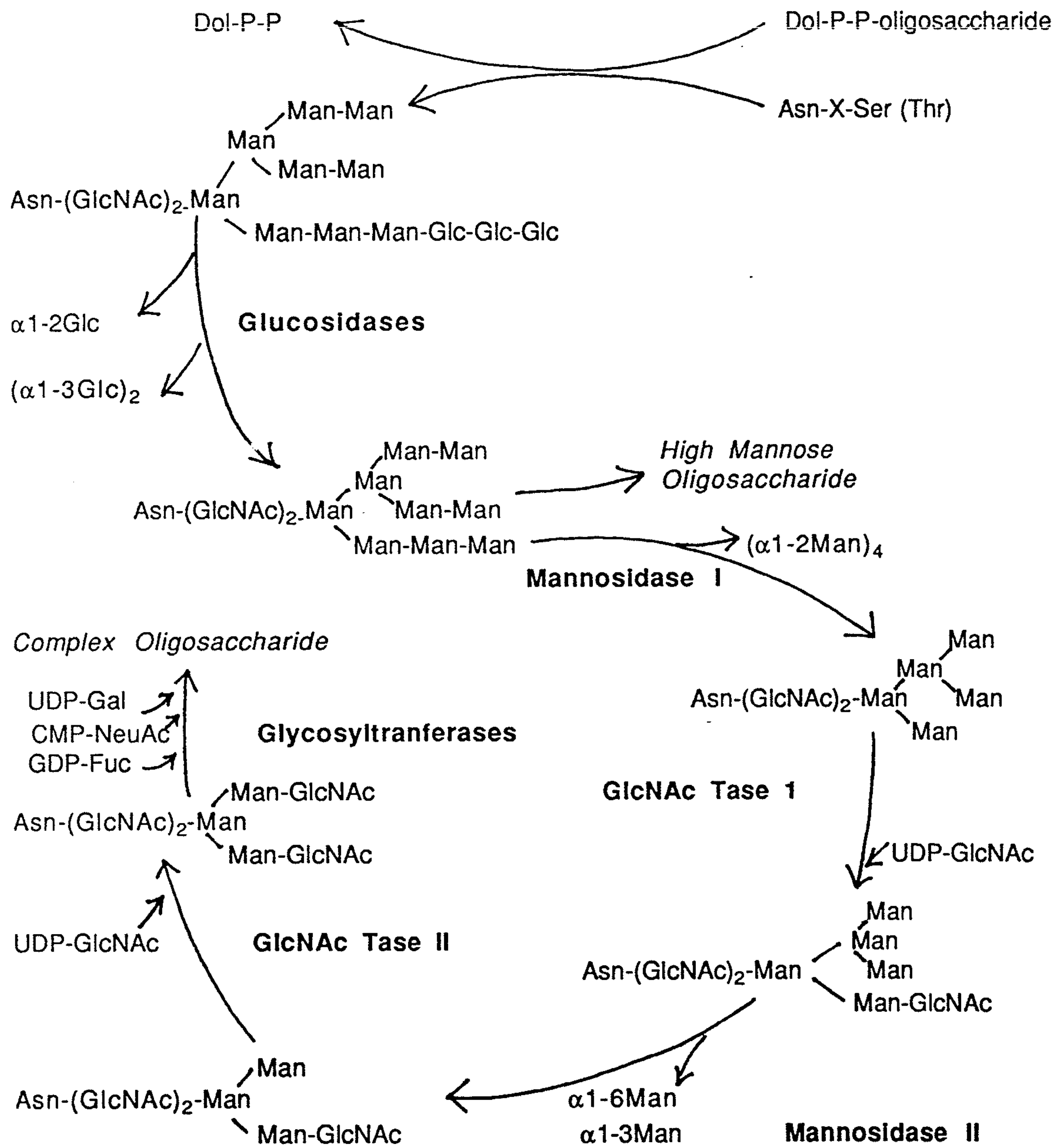
d. The Processing Reactions

The processing reactions are a series of enzymatic modifications of the oligosaccharide after transfer to the protein (Figure 6). The diversity of the N-linked oligosaccharides arises in the processing pathway and results in a variety of complex oligosaccharides, as well as the high mannose oligosaccharides. The processing reactions may be closely associated with sorting of glycoproteins for destination within the cell or for secretion.

The modification of the protein-linked oligosaccharide (Glc_3-Man_9-$GlcNAc_2$) begins with the removal of the three terminal glucose residues. Two enzymes are involved: glucosidase I which removes the exterior (α1-2 linked) glucose residue, and glucosidase II which removes the two remaining (α1-3 linked) glucose residues (Turco and Robbins, 1979; Grinna and Robbins, 1979; Kornfeld *et al.*, 1978; Ugalde *et al.*, 1979; Ugalde *et al.*, 1980; Michael and Kornfeld, 1980; Hubbard and Robbins, 1980; Hunt, 1979, 1980a and b; Etling *et al.*, 1980). Both glucosidases have been localized to the rough and smooth endoplasmic reticulum by immunoelectron microscopy (Lucocq *et al.*, 1986). Partially purified glucosidase I from *S. cerevisiae*

Figure 6.

The processing reactions remove the three glucose residues resulting in the formation of the high mannose oligosaccharide (M9). The oligosaccharide may remain as a high mannose oligosaccharide or be further modified by mannosidase I and II, and the transferase enzymes which add the sugar residues found in the hybrid and complex oligosaccharides. (Figure source- Jamieson, 1983)



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

has a pH optimum of 6.8 and no metal ion requirement (Kilker *et al.*, 1981; Bause *et al.*, 1986). Glucosidase I has also been partially purified by affinity chromatography from calf liver microsomes and has a pH optimum of 6.2 (Hettkamp *et al.*, 1984). Partially purified rat liver glucosidase II has a broad pH optimum between 6.0 and 7.5, and its activity towards oligosaccharides containing only one or two glucose residues indicates that it is distinct from glucosidase I (Burns and Touster, 1982). Glucosidase II has also been purified from rat kidney (Brada and Dubach, 1984), and a human neutral glucosidase gene, which has a gene product biochemically similar to glucosidase II of rat and pig, has been localized to the long arm of chromosome 11 (Martiniuk *et al.*, 1985).

The first two glucose residues are removed rapidly, whereas the mannose-linked glucose residue is removed up to 20 to 30 minutes later (Kornfeld *et al.*, 1978; Hubbard and Robbins, 1979). The initial stages of processing may also act as a sorting signal for secreted and intracellular glycoproteins. However, the α and ß subunits of the secretable glycoprotein, thyroid-stimulating hormone, was found to have a much faster rate of removal of the three glucose residues (less than 10 min) compared with non-secretable glycoproteins (up to 120 min) (Ronin *et al.*, 1984).

The next steps of the processing reactions are critical because they distinguish between oligosaccharides which will remain as high mannose oligosaccharides (Man_{5-9}), and oligosaccharides which will be processed to complex chains. The factors which determine whether or not an oligosaccharide is to be further processed are not understood, but conformation of the protein may be involved. Blockage in processing of MOPC-46B K chain oligosaccharide to a complex oligosaccharide was achieved by incorporation of analogues of leucine and isoleucine which alter protein conformation (Green, 1982). Accessibility of the oligosaccharides to processing enzymes may result in modification to complex oligosaccharides, or lack of

accessibility may result in retention of oligosaccharides in the high mannose form (Hsieh *et al.*, 1983; Faye *et al.*, 1986; Shao and Wold, 1987; Shao *et al.*, 1987). It is unlikely that high mannose and complex glycoproteins follow different physical processing pathways because many glycoproteins contain both types of oligosaccharides.

The specificity of the processing enzymes themselves may also be a factor in determining which oligosaccharides become complex or high mannose. Oligosaccharides of pancreatic ribonuclease B, which are normally only of the high mannose form, were processed to complex oligosaccharides when added to liver Golgi membranes, indicating a differential recognition of the oligosaccharide by liver and pancreatic enzymes (Williams and Lennarz, 1984). Furthermore, glycosylation of the HLA-DR antigen was dependent on the cell line transfected with the DR gene (Neel *et al.*, 1987). Transient re-glucosylation of protein-bound oligosaccharide in RER have been reported in calf thyroid (Parodi *et al.*, 1983), rat liver and *Phaseolus vulgaris* (Parodi *et al.*, 1984). The significance of this is not known, however, it may prevent mannosidase degradation of oligosaccharides in the RER.

The reactions of the processing pathway which are essential for the formation of complex oligosaccharides, and which may also partially process the high mannose oligosaccharides, are the removal of α1-2 linked mannose residues. Evidence for α-mannosidase activity in the RER, which removes the terminal α1-2 linked mannose residues, began to accumulate with recognition of glycoproteins containing Man_{6-8}-$GlcNAc_2$ oligosaccharides prior to Golgi processing (Godelaine *et al.*, 1981; Hercz and Harpaz, 1980; Zilberstein *et al.*, 1980). Subsequently, a rat liver α-mannosidase has been partially purified from endoplasmic reticulum (ER) (Bischoff and Kornfeld, 1983). This enzyme cleaved the artificial substrate *p*-nitrophenyl-α-mannoside, but did not bind to con A-Sepharose, which allowed separation from Golgi and

lysosomal mannosidases. A mannosidase which produces Man_{6-8}-$GlcNAc_2$ oligosaccharides has also been partially purified from calf liver, and is probably an ER enzyme (Schweden *et al.*, 1986). The use of the energy inhibitor CCCP (carbonyl cyanide *m*–chlorophenylhydrazone) to block migration of glycoproteins from the RER to the Golgi results in increased amounts of Man_8 and Glc_3-Man_8 oligosaccharides on nascent VSV (Vesicular stomatitis virus) polypeptides in HeLa cells (Atkinson and Lee, 1984) and Man_8 oligosaccharides on rotavirus glycoproteins in Ma104 cells (Kabcenell and Atkinson, 1985). An accumulation of Man_{6-8} oligosaccharides on immunoglobulin A in the RER of plasmacytoma cells (Hickman *et al.*, 1984), further emphasized the existence of an RER α-mannosidase. Recent evidence indicated that the first mannose to be removed in the processing of Man_9 oligosaccharides of IgM in MOPC 104E cells is the most terminal mannose residue of the middle branch, and the most probable location of this modification is the RER (Brown and Hickman, 1986). The same Man_8 structure has been identified on α_1-acid glycoprotein from rat liver RER (Silvanovich, 1987). This initial processing of a mannose residue parallels the processing of the Man_9 to Man_8 oligosaccharides in yeast, however, further modifications in yeast allow the addition of several mannose residues resulting in polymannans (Byrd *et al.*, 1982). A Golgi endo-α-mannosidase activity, which removes a glucosylmannose disaccharide from Glc_1-Man_9-$GlcNac_2$ resulting in a Man_8-$GlcNAc_2$ structure, was recently reported (Lubas and Spiro, 1987), however, its significance is presently unknown.

The remainder of the α1-2 linked mannose residues are removed by mannosidase I of the Golgi after translocation of the glycoprotein from the RER, and all subsequent processing reactions occur within the Golgi apparatus. Dewald and Touster (1973) first reported the existence of a Golgi mannosidase activity in rat liver, which was later distinguished from lysosomal or cytosolic mannosidase activity based on several

characteristics (Tulsiani *et al.*, 1977). Rat liver Golgi mannosidase activity includes at least two distinct enzymes; mannosidase IA (Tabas and Kornfeld, 1979) and mannosidase IB, which both cleave α1-2 linked mannose residues, but have different physical properties (Tulsiani *et al.*, 1982b). A phospholipid-dependent α1-2 mannosidase has been reported in rat liver, however, its relation to the Golgi mannosidases is unknown (Forsee and Schutzbach, 1981). Interestingly, processing of the α1-2 linked mannose residues was much slower in oligosaccharides which become complex carbohydrate compared with oligosaccharides which remain as high mannose structures (Brown and Hickman, 1986).

The next reaction in the formation of complex oligosaccharides is the addition of an N-acetylglucosamine residue from UDP-GlcNAc by N-acetylglucosaminyl transferase I (GlcNAc Tase I) in a ß1-2 linkage to the α1-3 linked mannose of the trimannosyl core of the Man_5 oligosaccharide. This results in GlcNAc-Man_5-$GlcNAc_2$ (Harpaz and Schachter, 1980a). The enzyme has been purified from a variety of sources (Schachter *et al.*, 1983), and has been localized to the medial cisternae of the Golgi in hepatocytes (Dunphy *et al.*, 1985). Following the addition of the GlcNAc residues, mannosidase II can remove the two branching terminal mannose residues (α1-3 and α1-6 linked), resulting in GlcNAc-Man_3-$GlcNAc_2$ (Tulsiani *et al.*, 1982b). This reaction is highly dependent upon the prior action of GlcNAc Tase I (Harpaz and Schachter, 1980b). Mannosidase II is the last degradative enzyme of the processing reactions and all further modifications to the oligosaccharide chain involve the addition of sugar residues found in complex oligosaccharides.

The transferase enzymes and the possible pathways of modifications can result in the huge variety of known complex oligosaccharides. Addition of one sugar residue at a specific site on the growing oligosaccharide may consequently route the oligosaccharide into different avenues of processing. Competition among transferase

enzymes for the appropriate oligosaccharide structure results in the variety of complex oligosaccharides. A recent, thorough review of the formation of complex oligosaccharides is found in Schachter *et al.* (1985) and the following is only a brief summary of the transferase enzymes required in the formation of N-linked complex oligosaccharides.

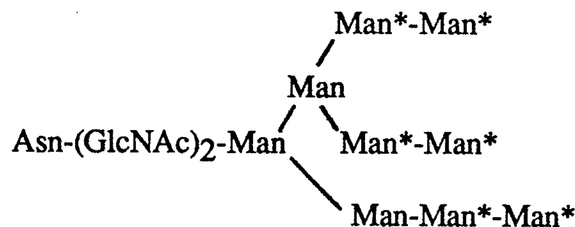
The addition of a ß1-2 linked GlcNAc residue to the α1-6 linked core mannose residue involves GlcNAc transferase II (GlcNAc Tase II). The resulting $GlcNAc_2$-Man_3-$GlcNAc_2$ oligosaccharide is a substrate for several enzymes. GlcNAc Tase III addition of a ß1-4 linked GlcNAc residue to the ß1-4 linked mannose results in a bisected structure and is thus named the bisecting GlcNAc (Narasimham, 1982). Addition of the bisecting GlcNAc to the Man_5-$GlcNAc_2$ oligosaccharides prevents further processing by GlcNAc Tase II, mannosidase II, α1-6 fucosyl transferase and GlcNAc Tase IV, resulting in the formation of the hybrid structure (Harpaz and Schachter, 1980a,b; Schachter *et al.*, 1983). If the oligosaccharide is non-bisected, GlcNAc Tase IV can add a second GlcNAc residue to the α1-3 linked mannose and this leads to the formation of triantennary complex oligosaccharides (Gleeson and Schachter, 1983). GlcNAc Tase V adds a second GlcNAc residue (ß1-6 linkage) to the α1-6 linked mannose resulting in a structure with four branches (tetraantennary) (Cummings *et al.*, 1982). Galactosyl transferase adds ß1-4 or ß1-3 linked galactose residues to the terminal GlcNAc residues on the α1-3 and α1-6 linked mannose residues but not to the bisecting GlcNAc. The addition of N-acetylneuraminic acid from CMP-NeuAc to the terminal galactose residues by α2-3 or α2-6 sialyl transferase completes the terminal triplet sugars of complex oligosaccharides (reviewed in Beyer *et al.*, 1981). Both galactosyl transferase (Roth and Berger, 1982) and sialyl transferase are located in the *trans* region of the Golgi. The addition of fucose by fucosyl transferase in either an α1-3 or α1-6 attachment to the Asn-linked

GlcNAc can occur, but only after GlcNAc Tase I has acted and if fucose is not present on the bisected structures (Longmore and Schachter, 1982). Sulfation of complex oligosaccharides is another modification which occurs in lysosomal enzymes of human fibroblasts (Braulke *et al.*, 1987), slime molds (Herscovics, 1987), and some pituitary hormones (Baenziger, 1985).

The ultimate structure of the complex oligosaccharide following processing within the Golgi is highly dependent on the specificity of the processing enzymes and the competition between processing enzymes for the various oligosaccharide substrates. The controlling factors which influence the final oligosaccharide structure are summarized by Schachter *et al.* (1983) as the following: a) competition for a common substrate; b) requirement for specific sugar residues on a substrate; and c) inhibition of enzyme action by the presence of key glycosyl residues.

e. Mannose-6-phosphate Modification

A unique oligosaccharide modification is found in lysosomal enzymes. Specialized processing of the high mannose oligosaccharides creates mannose-6-phosphate moieties which are required for the targetting of lysosomal enzymes to the lysosomes via mannose-6-phosphate receptors (reviewed in Sly and Fischer, 1982; Jamieson, 1983; and Kornfeld and Kornfeld, 1985; Kornfeld, 1986, 1987) (Figure 7). The six α1-2 linked mannose residues of a high mannose oligosaccharide are potential acceptors (*) of the phosphate group



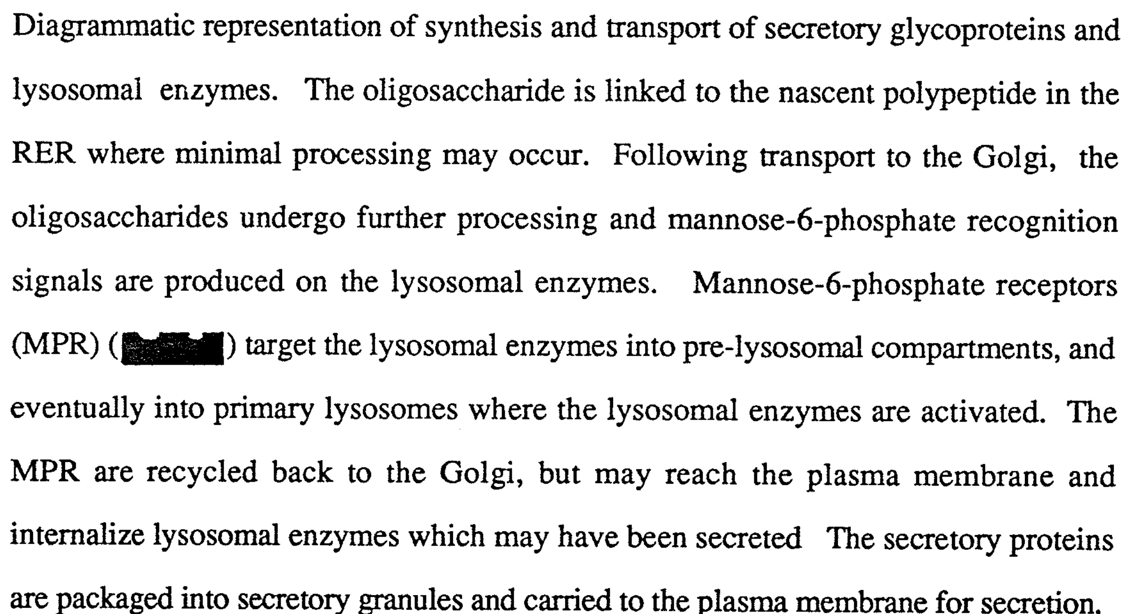
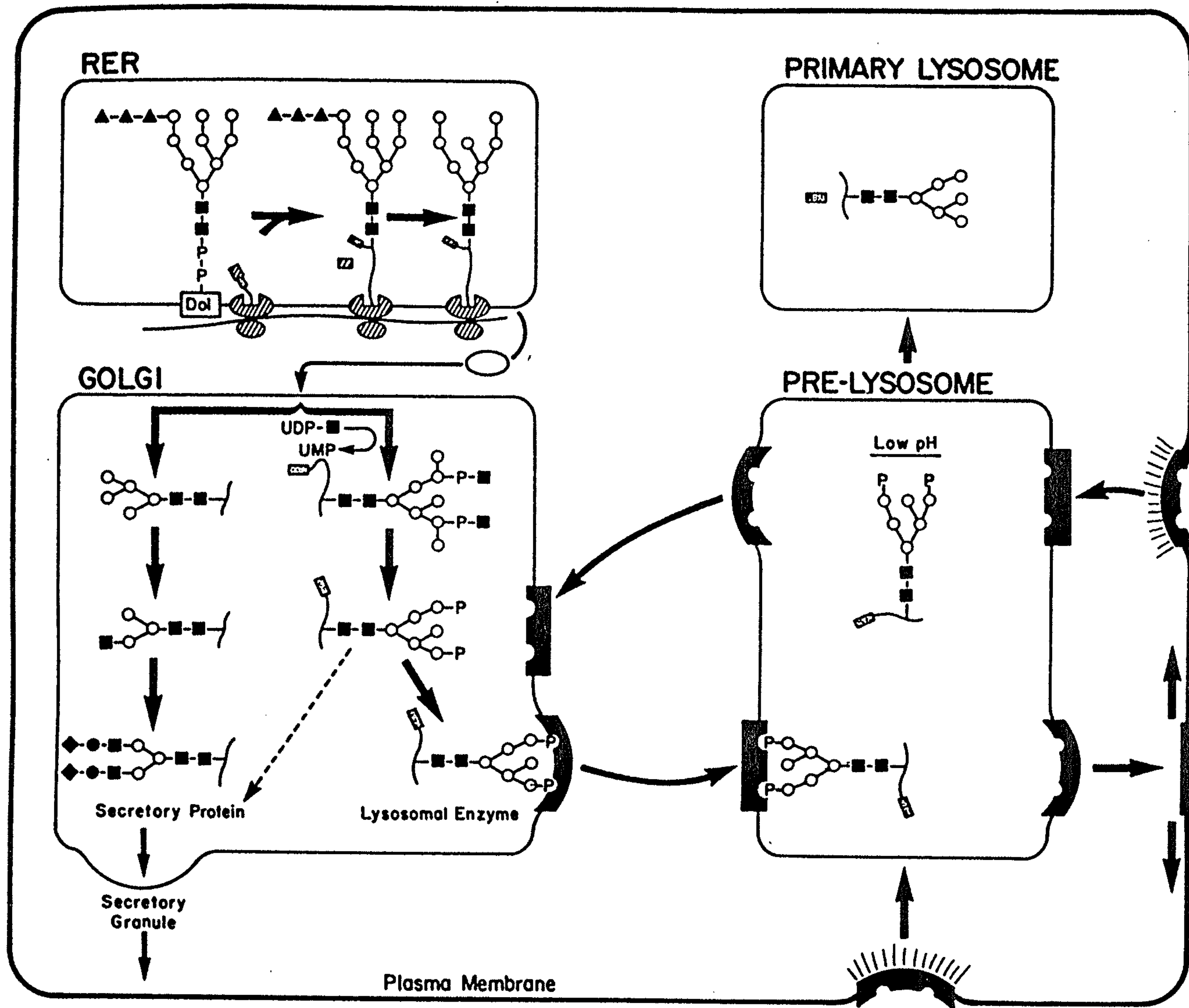


Figure 7.

Diagrammatic representation of synthesis and transport of secretory glycoproteins and lysosomal enzymes. The oligosaccharide is linked to the nascent polypeptide in the RER where minimal processing may occur. Following transport to the Golgi, the oligosaccharides undergo further processing and mannose-6-phosphate recognition signals are produced on the lysosomal enzymes. Mannose-6-phosphate receptors (MPR) ( ) target the lysosomal enzymes into pre-lysosomal compartments, and eventually into primary lysosomes where the lysosomal enzymes are activated. The MPR are recycled back to the Golgi, but may reach the plasma membrane and internalize lysosomal enzymes which may have been secreted The secretory proteins are packaged into secretory granules and carried to the plasma membrane for secretion. (Source of diagram - Kornfeld, 1986, 1987)


RER
Dol
PRIMARY LYSOSOME
GOLGI
PRE-LYSOSOME
UDP-
UMP
Low pH
Secretory Protein
Lysosomal Enzyme
Secretory
Granule
Plasma Membrane

(Couso *et al.*, 1986), however, usually only one or two phosphate groups are found per oligosaccharide (Varki and Kornfeld, 1980b; Natowicz *et al.*, 1982). Limited phosphorylation can occur on glucose containing high mannose oligosaccharides and truncated Man_5 oligosaccharides of Thy-1 mutants (Gabel and Kornfeld, 1982).

Two enzymes are responsible for the formation of the mannose-6-phosphate recognition marker. The first, UDP-N-acetylglucosamine: glycoprotein N-acetylglucosamine-1-phosphotransferase, utilizes UDP-GlcNAc as a substrate and attaches a phosphate-GlcNAc to the mannose residue through a phosphodiester linkage (Reitman and Kornfeld, 1981a; Hasilik *et al.*, 1981). The second enzyme, α–N-acetylglucosaminyl phosphodiesterase, removes the GlcNAc residue leaving a mannose-6-phosphate group (Varki and Kornfeld, 1980a; Varki and Kornfeld, 1981; Waheed *et al.*, 1981). The enzymes have been localized to the Golgi and can be separated into different regions of the Golgi through continuous sucrose gradient fractionation (Goldberg and Kornfeld, 1983). The phosphotransferase enzyme may distinguish between lysosomal and non-lysosomal oligosaccharides through the recognition of a specific peptide sequence (Reitman and Kornfeld, 1981a), or the conformation of the protein may influence activity (Lang *et al.*, 1984). Denatured lysosomal enzymes lose their ability to serve as acceptors and this also emphasizes the importance of protein conformation (Reitman and Kornfeld, 1981b). More recent studies with simple eukaryotes indicate that protein-dependent oligosaccharide conformation, specific to lysosomal enzymes, may be required for phosphotransferase recognition (Lang *et al.*, 1986).

C. *Glycosylation Mutants and Glycosylation Inhibitors*

The significance of glycoprotein carbohydrate, with regard to the relationship between specific oligosaccharide structures and specialized functions, has only begun to be understood within the last two decades due to the enhanced knowledge of glycoprotein biosynthesis, and due to the development of new techniques for determining oligosaccharide structure. As the discoveries of new and different oligosaccharide structures continue, there are several questions regarding functional significance which must be addressed. The most important question is to determine whether carbohydrate residues of membrane glycoproteins serve only in a general capacity for the prevention of proteolytic digestion (Olden *et al.*, 1982b; Knudsen, 1985) and the establishment of antigenic determinants, or do they also play a more prominent role in the functional aspects of the plasma membrane glycoproteins. Diversity in the structure of N-linked oligosaccharides occurs in the processing reactions and better knowledge of the regulation of enzymes within that pathway may clarify unknown entities, such as the requirement for fucosylation, the significance of hybrid structures, effects of replacing complex oligosaccharides with high mannose oligosaccharides and vice versa, and the existence of microheterogeneity in some glycoproteins.

At present glycoprotein biochemists have two types of tools which have had considerable impact on our understanding of carbohydrate biosynthesis and function. The selection and characterization of mutant cells with altered N-linked oligosaccharides using plant lectins was introduced in the early 1970's, and a large number of mutants (or variants) have since been isolated and classified (reviewed by Wright, 1979; Wright *et al.*, 1980; Stanley, 1980; Stanley, 1984; Briles, 1982). A second means of establishing a role for N-linked oligosaccharides is through the

removal or modification of the oligosaccharides using antibiotic molecules, such as tunicamycin, and a new class of sugar analogues, the glycoprotein processing inhibitors.

1. Lectin-resistant Mutants

The selection and characterization of mutants or variants which are resistant to the cytotoxic effects of plant lectins, have proven to be a powerful resource in defining the pathway of N-linked glycoprotein biosynthesis, as well as establishing a functional role for carbohydrates. The lectins recognize specific carbohydrate residues. To produce the cytotoxic effect, the lectin must bind to the appropriate membrane carbohydrate, and be internalized to disrupt the cell's normal metabolism by a means specific to each type of lectin. Lectins commonly used for the selection of mutants are concanavalin A (con A) from jack bean meal with an affinity for mannose and glucose residues, WGA (wheat germ agglutinin) which binds complex carbohydrates containing GlcNAc and sialic acid, and PHA (phytohemagglutinin) from *Phaseolus vulgaris* which binds complex oligosaccharides containing GlcNAc and galactose.

Cells resistant to the cytotoxic effect of the lectin are cloned and characterized for phenotypic alterations and assessed for stability. Mutagens may be added to the selection culture to increase the frequency of lectin resistant mutants (Wright, 1973; Stanley, 1975). The term 'mutant' is often used loosely, as most lectin-resistant cell lines are better termed variants because their genetic mutation has not been defined at the molecular level. However, Stanley (1984) equates mutant to variant, because of the mutation-like events, the stability under non-selective conditions, hybridization studies revealing the recessive nature of the lectin-resistant cells, and a deficiency in one enzyme which is the basis for glycosylation defects in many of the variant cells.

Lectin resistant cells must be characterized to confirm that their resistance is due to alterations in cell surface oligosaccharide and not a result of blockage in internalization of the lectin or modification of the target molecule. A series of lectin binding studies, and the measurement of incorporation of various sugars into lipid-oligosaccharides and glycoproteins, will often indicate the relative position of the defect in the pathway of oligosaccharide synthesis and structural analysis will confirm that the lectin resistance is due to altered oligosaccharide structure. Glycosylation mutants have been classified into twenty-one distinct groups (Stanley, 1984) based upon the structural modification of the oligosaccharide, and for some the enzymatic alteration is also known.

The elucidation of the enzymatic defect is often dependent upon the current understanding of the biosynthetic pathway, but studies with lectin resistant mutants have also contributed greatly to defining some aspects of oligosaccharide synthesis. The classic LecR 1 phenotype, which has been independently selected from CHO cell lines using a variety of different lectins (ricin, WGA, PHA and LCA), contains oligosaccharides lacking the terminal triplet, GlcNAc-Gal-NeuAc (Briles, 1982; Stanley, 1984). Studies undertaken to find the enzymatic defect revealed the existence of two distinct GlcNAc transferase enzymes (now known as GlcNAc Tase I and GlcNAc Tase II, Narasimham *et al.*, 1977), with the LecR 1 cells deficient in one of these activities (Gottlieb *et al.*, 1975; Stanley *et al.*, 1975). Characterization of the mutants showed that the addition of GlcNAc by GlcNAc Tase I is a critical step in the processing reaction and a lack of this enzyme resulted in $GlcNAc_2$-Man_5 oligosaccharides deficient in complex sugars (Tabas and Kornfeld, 1978).

Many lectin-resistant cells are altered in such cellular properties as morphology, adhesion, and temperature sensitivity (Wright *et al.*, 1980). The effects of lectin resistance on intracellular targetting of lysosomal enzymes (Krag and Robbins, 1982)

and the correlation with altered fusion capacity in L6 myoblasts (Parfett *et al.*, 1981 and 1983; Cates *et al.*, 1984), are more specific examples of the results of modifications of N-linked oligosaccharides.

The selection of glycosylation mutants is not restricted to the use of plant lectins. The 'suicide technique' uses radioactive sugar added to the medium to select for variants with a reduced incorporation of the labelled sugar. Monoclonal antibodies or nontoxic lectins may be conjugated to toxins or complement to select for cells with altered cell surface carbohydrate. The class E complementaion group of mutant lymphoma cells was originally selected because of a lack of the cell surface Thy-1 antigen, but the deficiency was found to be a defect in the synthesis of high mannose oligosaccharides (Trowbridge and Hyman, 1979; Chapman *et al.*, 1979b). The loss of α1-2 mannosyl tranferase activity was first suspected because no high mannose oligosaccharides beyond five mannose residues were produced, but further studies revealed that the defect was blockage in the formation of dolichol-P-mannose (Chapman, 1980).

The study of N-linked glycosylation-defective cell types selected by lectin-resistance and other means have contributed greatly to the understanding of asparagine-linked oligosaccharide biosynthesis. However, there are problems associated with this type of study. A limited variety of lectins restricts the number of lectin-resistant phenotypes. The stability of the mutant must be established and characterization of the mutant's biochemical defect is necessary. Although techniques for determining oligosaccharide structures have greatly advanced, the time required to select and characterize a lectin-resistant variant and the possibility of the resulting variant having an identical phenotype to previous variants, have diverted

attention to a more specific means of altering N-linked oligosaccharides using glycosylation and glycoprotein processing inhibitors (Spearman *et al.*, 1987).

2. Glycosylation Inhibitors

a. Tunicamycin

The study of structure-function relationships for glycoprotein carbohydrate was given a great boost in the early 1970's with the discovery of the antibiotic tunicamycin from *Streptomyces lysosuperficus* nov. sp. (Takatsuki *et al.*, 1971). Tunicamycin blocks the addition of N-acetylglucosamine-1-P to dolichol-phosphate, the first step in the dolichol cycle (Tkacz and Lampen, 1975), resulting in protein free of asparagine-linked carbohydrate. The inhibitory effect of tunicamycin is due to its structural analogy to UDP-2-acetamido-2-deoxyglucose, but many major isomers of tunicamycin exist which vary in the length of the fatty acid chain.

The application of tunicamycin inhibition to several cell systems has revealed the requirement for glycosylation among proteins varies greatly. Extracellular secretion or intracellular targetting of some glycoproteins is highly dependent on the carbohydrate content of the molecule. However, some glycoproteins, such as human interferon (Mizrahi *et al.*, 1978) and glycophorin A (Gahmberg *et al.*, 1980) are transported normally without the presence of N-linked carbohydrate. The susceptibility to proteolytic digestion, the biological activity and the physical properties of some glycoproteins are altered when produced in the presence of tunicamycin, but there are several glycoproteins which exhibit no effect when glycosylation is prevented (reviewed in Gibson *et al.*, 1980; Olden *et al.*, 1985). Tunicamycin has also been used to study cellular differentiation and has been shown to prevent compaction in embryogenesis (Ivatt, 1984), block myoblast fusion (Gilfix and Sanwal, 1980) and affect neuronal differentiation (Richter-Landsberg and Daskin, 1983), findings which

emphasize the requirement for N-linked carbohydrate in these biological processes. Several other effects of tunicamycin are reviewed in Elbein (1987a,b).

Tunicamycin has contributed tremendously to our understanding of glycoprotein biosynthesis and the function of asparagine-linked glycans, but these studies are limited to the total removal of N-linked oligosaccharide and do not allow the examination of subtle differences in oligosaccharide structure. It is apparent, however, that oligosaccharides may serve many varied functions, but in order to fully recognize the significance of the numerous structures in relation to their function, it is necessary to utilize more specific tools to modify oligosaccharides rather than totally remove them.

b. Glycoprotein Processing Inhibitors

A new and exciting class of compounds has emerged within the last seven years, which has allowed glycoprotein biochemists to test the effects of blocking specific sites of sugar removal along the processing pathway. The glycoprotein processing inhibitors are compounds that are either sugar analogues or naturally occuring alkaloids that mimic sugar structure. Their effects on a huge array of glycoproteins have been studied, with some very surprising results. Colonization of metastatic cells, immunochemistry, and viral infectivity in the presence of the glycoprotein processing inhibitors have also been a focus of study.

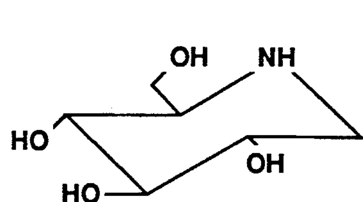
i. Glucosidase Inhibitors

The initial steps of the processing reactions are the removal of the α1-2 linked glucose by glucosidase I and the two α1-3 linked glucose residues by glucosidase II. At present, there are five major inhibitors of glucosidase processing enzymes which have been characterized by their ability to inhibit both enzymes or either one.

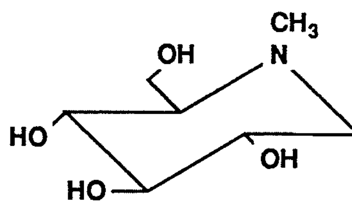
Figure 8.

Structures of the glycoprotein processing inhibitors. 1-Deoxynojirimycin, N-methyldeoxynojirimycin and castanospermine inhibit the processing α-glucosidases. 1–Deoxymannojirimycin is an inhibitor of mannosidase I, and swainsonine prevents processing by mannosidase II.

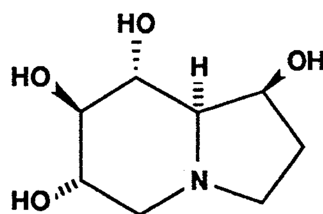
Glucosidase Inhibitors



1-deoxynojirimycin

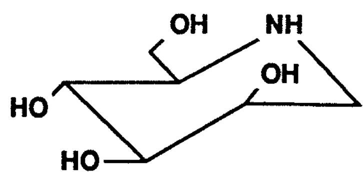


N-methyldeoxynojirimycin

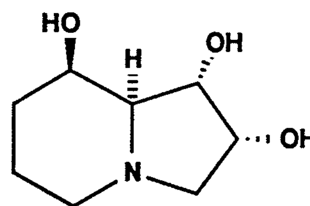


castanospermine

Mannosidase Inhibitors



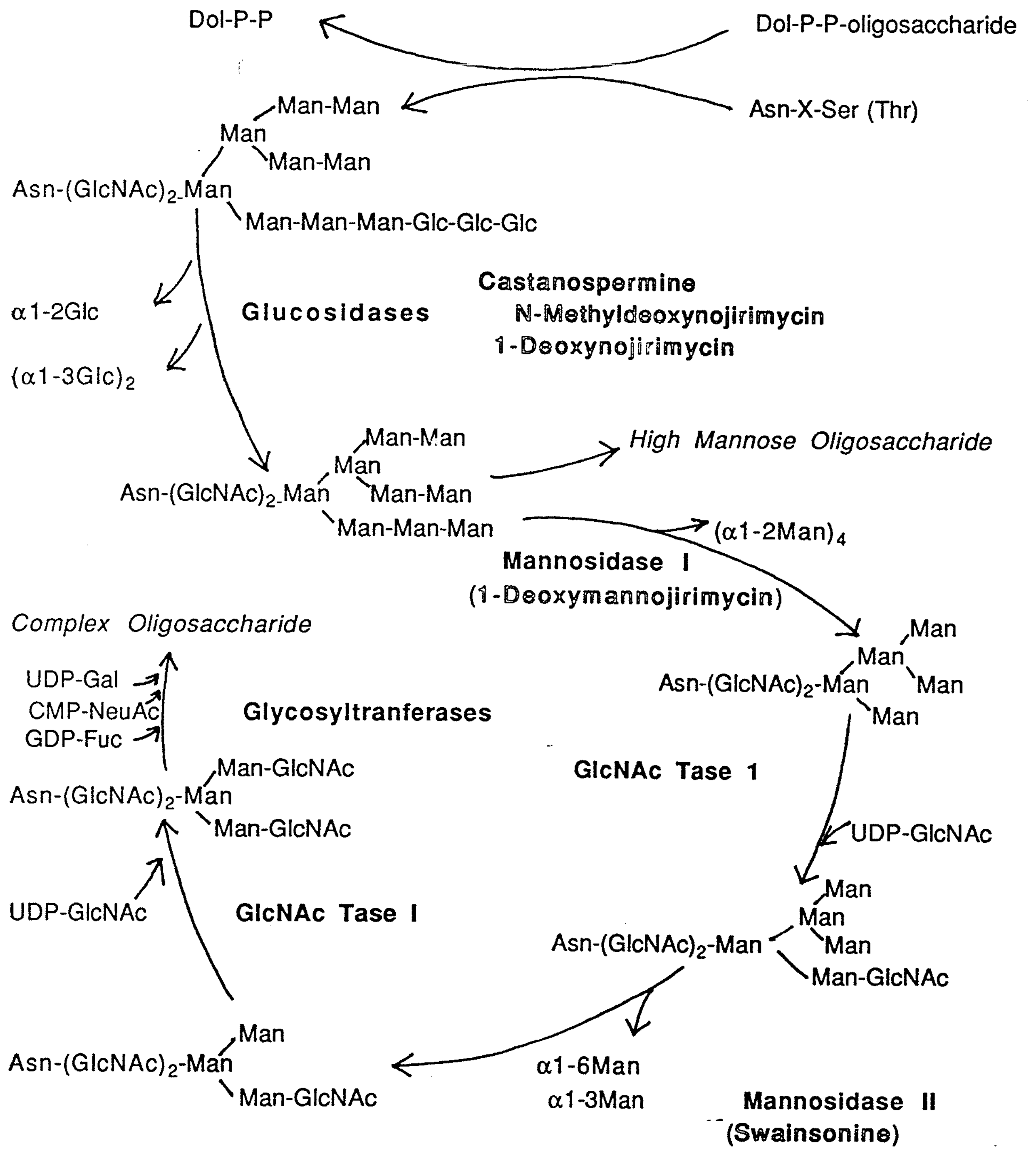
deoxymannojirimycin



swainsonine

Figure 9.

The site of inhibition of the glycoprotein processing inhibitors within the processing pathway. (Figure source- Jamieson (1983) with modifications).



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

1-Deoxynojirimycin is the reduced form of the antibiotic nojirimycin, produced by some strains of *Streptomyces*. As an analogue of glucose with an NH group substituted for the ring oxygen (Figure 8), it can be produced biosynthetically by a strain of *Bacillus* (Frommer *et al.*, 1979). Initially it was found that both nojirimycin and deoxynojirimycin inhibited intestinal α-glucosidases (Schmidt *et al*., 1979), but it was not until 1982 that they were first described as inhibitors of the processing reactions of N-linked oligosaccharides. Partially purified glucosidase I and glucosidase II from *Saccharomyces cerevisiae* were inhibited 50% with deoxynojirimycin at concentrations of 20 μM and 2 μM, respectively, and calf pancreas microsomal processing glucosidases were also inhibited (Saunier *et al.*, 1982). Deoxynojirimycin also decreased the percentage of complex oligosaccharides and resulted in high mannose oligosaccharides with an increased resistance to α-mannosidase activity, signifying the retention of glucose residues. In contrast to the inhibition of yeast enzymes, deoxynojirimycin was a much more effective inhibitor of glucosidase I than of glucosidase II in calf liver microsomal preparations (Hettkamp *et al.*, 1982). Deoxynojirimycin was approximately twenty times more effective an inhibitor than nojirimycin. These two initial studies defined deoxynojirimycin as a potent inhibitor of the processing glucosidases which led to its practical use as a modifier of N-linked oligosaccharides. The discrepancy regarding more effective inhibition of glucosidase I or II, may be due to the differences between the yeast and mammalian enzymes (Fuhrmann *et al.*, 1985).

N-methyl-1-deoxynojirimycin (Figure 8), another inhibitor of glucosidase processing, is synthesized by the monomethylation of deoxynojirimycin with methyl iodide (Murai *et al.*, 1977). Glycoproteins from fowl plague virus particles produced in the presence of methyldeoxynojirimycin had Glc_3-Man_7-$GlcNAc_2$ as the predominant form of oligosaccharide (Romero *et al.*, 1983). This indicates a

preferential inhibition of glucosidase I by methyldeoxynojirimycin. Comparison of deoxynojirimycin and its methylated derivative in rat liver microsomes found methyldeoxynojirimycin to be a slightly better inhibitor of glucose removal at 10 μM compared to the 18 μM required by deoxynojirimycin for complete inhibiton (Romero *et al.*, 1983). A comparison of the major structures produced in intestinal epithelial cells, exposed to 5 mM deoxynojirimycin and 2 mM methyldeoxynojirimycin, showed 70% of the endo H released oligosaccharides to contain three glucose residues in the presence of methyldeoxynojirimycin, whereas deoxynojirimcyin treated cells produced oligosaccharides containing predominantly one and two glucose residues (Romero *et al.*, 1985a).

Castanospermine (1,6,7,8-tetrahydroxy-octahyroindolizine, Figure 8), a plant alkaloid isolated from *Castanospermum australe*, is another inhibitor of the processing glucosidases. Because of its similarity in structure to swainsonine, a plant alkaloid which inhibits mannosidase II activity, castanospermine was tested for its inhibitory effect on a variety of glycosidase enzymes (Saul *et al.*, 1983). It was found to be a potent inhibitor of almond emulsin ß-glucosidase and lysosomal glucosidase in fibroblast extracts. The inhibitory activity of castanospermine on the processing enzymes was first studied in influenza infected MDCK cells (Pan *et al.*, 1983). Castanospermine treated cells resulted in a conversion from 30% high mannose to 80-90% high mannose oligosaccharides, identified by endo H sensitivity. Characterization of the glycopeptides using enzymatic treatments coupled with methylation analysis, revealed the major structure to be Glc_3-Man_7-GlcNAc which indicated that the primary site of inhibition is glucosidase I. The Glc_3-Man_7-GlcNAc oligosaccharide was also the major structure in mouse lymphoma cells (Palamarczyk and Elbein, 1985) and plant glycoproteins (Hori *et al.*, 1984) in the presence of castanospermine. The preferential inhibition of the processing glucosidase may be

due to the requirement for deprotonation of castanospermine to be active. With a pK of 6.09, the slightly acidic environment (pH 6.0) of the Golgi allows the active form to exist (Saul *et al.*, 1984).

Bromoconduritol, (6-bromo-3,4,5-trihydroxy-cyclohex-1-ene), also inhibits glucosidase II activity, and results in the formation of primarily Glc_1-Man_{7-9}-GlcNAc oligosaccharides in fowl plague virus formation (Datema *et al.*, 1982). However, the usefulness of bromoconduritol as a modifier of oligosaccharide structure is limited, because of its instability in aqueous solution (half life of 15 min., 39°C, pH 7.3) (Fuhrmann *et al.*, 1985). A pyrrolidine alkaloid (2,5-dihydroxymethyl-3,4-dihydroxy-pyrrolidine) isolated from the plant *Lonchocarpus sericeus*, also inhibited glucosidase I activity in influenza virus (Elbein *et al.*, 1984b), but it is not as effective as other glucosidase inhibitors.

One very important observation, which is evident in the oligosaccharide structures resulting from the glucosidase inhibitors, is that cleavage of mannose residues can occur without the removal of all the terminal glucose residues. This suggests that the RER mannosidase, and possibly the Golgi mannosidase I, are able to remove up to three mannose residues without the prerequisite of glucose removal.

Several studies concentrating on metabolism of specific glycoproteins, or viral products and maturation, emphasized the variation in effects of the glucosidase inhibitors (Table 1), which appears to depend on the glycoproteins, the cell (and viral) system and the inhibitor. Since many glycoproteins are destined for extracellular secretion, or are transported to specific sites such as the lysosomes or plasma membrane, most studies have measured the effects of the processing inhibitors on cellular transport of the modified glycoproteins.

Deoxynojirimycin caused significant decreases in the secretion of α_1-proteinase inhibitor (Gross *et al.*, 1983), α_1-antitrypsin and α_1-antichymotrypsin (Lodish and

Table 1 Effects of 1-deoxynojirimycin, N-methyldeoxynojirimycin, and castanospermine on secretion, maturation or cell-surface expression of glycoproteins.

Glycoprotein	Cell Type	Inhibitor	Property and Effect	Reference
viral glycoproteins (chick embryo)	fowl-plague virus	MdN (2mM)	no effect on maturation and infectivity	Datema *et al.*, (1982)
α_1-proteinase inhibitor	rat hepatocytes	dN (5mM)	decreased secretion	Gross *et al.*, (1983)
HA1 and HA2 (avian influenza virus)	chick embryo fibroblasts	MdN (2mM) BC (4.8mM)	no effect on tryptic cleavage	Bosch *et al.*, (1984)
RS viral glycoproteins	chick embryo fibroblasts	MdN (2mM) BC (2.4mM)	no effect on maturation and infectivity	Bosch and Schwarz, (1984)
α_1-antitrypsin α_1-antichymotrypsin	human hepatoma	dN (10mM)	decreased rate of secretion	Lodish and Kong (1984)
complement C3 transferrin	human hepatoma	dN (10mM)	marginal effect on rate of secretion	Lodish and Kong (1984)
cathepsin D ß-hexosaminidase	human fibroblasts	dN (5mM)	decreased transport to lysosomes	Lemansky *et al.*, (1984)
G protein (San Juan VSV)	chick embryo fibroblasts	dN (1mM) Csn (0.1mM)	decreased virion formation	Schlesinger *et al.*, (1984)
ß-hexosaminidase ß-galactosidase	*Aspergillus fumigatus*	Csn (0.010mM)	decreased secretion	Elbein *et al.*, (1984c)

continued next page...

Table 1 continued

Glycoprotein	Cell Type	Inhibitor	Property and Effect	Reference
α_1-antitrypsin caeruloplasmin antithrombin III	human hepatoma	Csn (2.5mM)	decreased secretion	Sasak *et al.*, (1985)
E1 and E2 MHV glycoproteins	Balb/c 3T3	Csn (4mM) MdN (4mM)	decreased transport and synthesis	Repp *et al.*, (1985)
v-*fms* oncogene product	transformed Fischer rat embryo	Csn (.13mM) MdN (2mM)	decreased cell surface expression	Nichols *et al.*, (1985)
thyroglobulin	porcine thyroid	dN (1-3mM)	no effect on secretion	Franc *et al.*, (1986)
IgD and IgM	hybridomas	dN (1mM)	blocks secretion (IgD) no effect (IgM)	Peyrieras *et al.*, (1983)
HLA-A,B,C	Daudi lymphoblastoid	dN (1mM)	internal degradation	Peyrieras *et al.*, (1983)
VSV-G protein	BHK	dN (2mM)	no effect on cell surface expression or infectivity	Burke *et al.*, (1984)
influenza virus hemagglutinin	MDCK	dN (2mM)	no effect on cell surface expression or infectivity	Burke *et al.*, (1984)

Abbreviations: dN, 1-deoxynojirimycin; MdN, N-methyldeoxynojirimycin; Csn, castanospermine; RS, Rous sarcoma; HA, viral hemagglutinin; VSV, vesicular stomatitis virus; MHV mouse hepatitis virus; HLA, human leukocyte antigen.

Kong, 1984), but had only a marginal effect on complement C3 and transferrin secretion (Lodish and Kong, 1984) and no effect on secretion of thyroglobulin (Franc *et al.*, 1986). The lysosomal enzymes, cathepsin D and ß-hexosaminidase, were retarded in their transport to the lysosomes in the presence of deoxynojirimycin, and resulted in the build-up of non-processed forms in the Golgi (Lemansky *et al.*, 1984). The differential effect of deoxynojirmycin on immunoglobulin secretion is also surprising. IgD secretion was blocked but IgM secretion was not affected (Peyrieras *et al.*, 1983). The effect of deoxynojirimycin on the cell surface expression of human histocompatability antigens, HLA-A, B, C and HLA-DR, appears to be dependent on the cell type under investigation. HLA-A, B and C were internally degraded at a much higher rate in Daudi lymphoblastoid cells in the presence of deoxynojirimycin, but were normally expressed on the cell surface of Raji lymphoblastoid cells. Deoxynojirmycin had no effect on HLA-DR expression in either type of cell (Peyrieras *et al.*, 1983). The surface expression of the major histocompatability antigens, I-A and H-2K in B lymphoma cells was not altered but an increase in thymocyte stimulation occurred (Powell *et al.*, 1985). Deoxynojirmycin had no effect on the tumor invasion of embryonic chick heart by drug-treated malignant mouse MO_4 cells (Marell *et al.*, 1985) and also did not alter the transformed phenotype of AEV-transformed fibroblasts containing the *erb* B oncogene (Schmidt *et al.*, 1985).

Castanospermine, the glucosidase I inhibitor, decreased the secretion of α_1–antitrypsin, ceruloplasmin, and antithrombin III in human hepatoma cells (Sasak *et al.*, 1985) and also decreased the secretion of ß-hexosaminidase and ß-galactosidase in the bacteria, *Aspergillus fumigatus* (Elbein *et al.*, 1984c). The *in vivo* effect in rat liver was the reduction of both neutral and acidic glucosidase activity, elevation of ß–hexosaminidase activity, a decrease in α-mannosidase activity and no effect on ß–galactosidase. The same study found an increase in glycogen levels in both

cytoplasm and lysosomes (Saul *et al.*, 1985). Castanospermine also blocks the uptake of mannose-terminated glycoproteins in macrophages (Chung *et al.*, 1984).

The use of virus infected cells to study glycoprotein metabolism had proven to be advantageous and several invesigators have coupled this with the use of processing inhibitors to define the role of oligosaccharides in viral glycoprotein function and virus formation. In both VSV, infected BHK cells and influenza virus infected MDCK cells, neither the cell surface expression of viral glycoprotein nor the viral infectivity were affected by deoxynojirimycin (Burke *et al.*, 1984). In the San Juan strain of VSV, the cell surface expression of G protein, a major viral glycoprotein, was also not affected by deoxynojirimycin or castanospermine treatment, but virion formation was significantly decreased (Schlesinger *et al.*, 1984). The effects of modification of oligosaccharides with methyldeoxynojirmycin in viral systems also appear to be dependent on the virus and cell system. No effect was reported for the viral glycoproteins of fowl plague virus infected chick embryo cells (Datema *et al.*, 1983). Also, virus maturation and infectivity of Rous sarcoma virus (Bosch and Schwarz, 1984) and the tryptic cleavage of viral hemagglutinins in avian influenza virus (Bosch *et al.*, 1984) were not affected by either methyldeoxynojirimycin or bromoconduritol. However, the envelope glycoproteins, E1 and E2, of mouse hepatitis virus A59 were reduced in their transport and synthesis (Repp *et al.*, 1985) and the *v-fms* oncogene product of McDonough feline sarcoma virus-transformed Fischer rat embryo cells was decreased in cell surface expression (Nichols *et al.*, 1985) by both methyldeoxynojirimycin and castanospermine.

ii. Mannosidase Inhibitors

Swainsonine

The plant alkaloid, swainsonine (8α-indolizidine-1,2,8-triol, Figure 8) inhibits both acidic lysosomal mannosidase and Golgi mannosidase II activities, and has proven to be very important for studying the effects of oligosaccharide alterations, as well as a model system for the genetic disease, mannosidosis. Swainsonine has been isolated from *Swainsona canescens*, a leguminous plant (Colegate *et al.*, 1979), *Astralagus lentiginosus*, spotted locoweed (Molyneux and James, 1982) and *Rhizoctonia leguminicola,* a fungus (Schneider *et al.*, 1983) and it has also been chemically synthesized (Suami *et al.*, 1984). Grazing animals which ingest plants containing swainsonine develop a disease called locoism (Molyneux and James, 1982). Locoism closely resembles a genetic disease called mannosidosis, which occurs in humans and Angus cattle and is caused by a defect in lysosomal mannosidase activity, and diagnosed by abnormal behavior of the animal (Huxtable and Dorling, 1982). The similarities of the diseases led to the finding that swainsonine reversibly inhibited acidic α-mannosidase activity in mammalian tissue, liver of lamprey eel, the seed of jack bean (Dorling *et al.*, 1980), and mouse liver homogenate (Molyneux and James, 1982).

Swainsonine was recognized by two groups as an inhibitor of oligosaccharide processing. Elbein *et al.* (1981), using influenza infected MDCK cells, reported an increase in high mannose oligosaccharides with a concurrent decrease in complex oligosaccharides on viral glycoproteins. Mannosidase, however, could not completely digest the isolated "high mannose" oligosaccharide which suggested a modified structure. Tulsiani *et al.* (1982a) first reported that swainsonine specifically inhibited rat liver Golgi mannosidase II by showing an accumulation of GlcNAc-

Man_5-GlcNAc in the presence of swainsonine and the substrates GlcNAc-Man_5-GlcNAc and UDP-GlcNAc. Because the blockage by swainsonine occurs at a point of processing which directly precedes the formation of complex oligosaccharides, it was not easy to predict the resulting structure. Methylation studies and sequential enzymatic digestion of VSV viral oligosaccharides, produced in the presence of swainsonine, revealed a replacement of complex oligosaccharides by a hybrid structure in which one branch had a terminal triplet and the other branch had three mannose residues (see Figure 4, Historical Review) (Kang and Elbein, 1983). Tulsiani and Touster (1983) also characterized the swainsonine-produced hybrid structures in human skin fibroblasts and rat liver Golgi by glycohydrolase susceptibility and the ability to adsorb to serotonin-Sepharose 4B, indicating the presence of sialic acid. The hybrid structure was confirmed with n.m.r. studies of oligosaccharides isolated from swainsonine-treated BHK (baby hamster kidney) cells. The oligosaccharide also contained α1-6 fucose and NeuAc linked α2-3 to galactose (Foddy *et al.*, 1986). Normal sulfation of hybrid oligosaccharides of influenza infected MDCK cells occurred (Merkle *et al.*, 1985).

The kinetics of uptake of swainsonine in normal human fibroblasts revealed a rapid internalization (within a minute) and temperature sensitivity. The uptake was not affected by mannose or mannose-6-P. This indicated entry by permeation rather than receptor mediated uptake, and swainsonine concentrated in the lysosomes (Chotai *et al.*, 1983). Swainsonine also binds tightly to Golgi membranes (Tulsiani and Touster, 1983). Swainsonine inhibition of rat liver lysosomal mannosidase, rat liver Golgi mannosidase II and jack bean mannosidase differed in the required inhibitor concentration and reversibility (Tulsiani *et al.*, 1985). Inhibition of jack bean mannosidase was irreversible; lysosomal mannosidase inhibition was reversible;

Golgi mannosidase II had two types of binding, one rapid and irreversible and the other slower and reversible. Greenaway *et al.* (1983) also found lysosomal mannosidase inhibition to be reversible.

Swainsonine inhibition of mannosidase II represents a unique tool for studying how the replacement of complex oligosaccharides with hybrid structures affects the function of specific glycoproteins or the properties and processes of the cell. Numerous glycoproteins in a variety of cell systems have been studied for the effects of swainsonine on their cell surface expression or secretion (Table 2). The alterations in oligosaccharide structure due to swainsonine have little or no effect on the transport and function of the glycoproteins studied, with two possible exceptions. Aminopeptidase N transport to the microvillar membrane of mucosa cells was decreased by 13%, although this was not considered a significant change (Danielson *et al.*, 1983). Also, swainsonine accelerated the secretion of glycoproteins in hepatoma cells (Yeo *et al.*, 1985). The decrease in the lag period by ten minutes was explained by the passage of glycoproteins through the Golgi more quickly. This is in contrast to another report which found swainsonine to have no effect on secretion of glycoproteins in human heptoma (Lodish and Kong, 1984). Generally, the conversion of complex oligosaccharides to hybrid structures has no significant effect on the production, transport and function of the majority of glycoproteins. The retention of one arm of the oligosaccharide with complex sugars may be sufficient for recognition as a complex oligosaccharide when required.

Studies of cellular processes in the presence of swainsonine have found some cellular functions which are much more dependent on the integrity of complex oligosaccharides. In these studies the glycoproteins involved are not specifically identified or known. Cell-cell interactions such as the adsorption of macrophages to bone (Bar-Shavit *et al* ., 1983) and the homing of lymphoma cells to liver and spleen

Table 2 Effects of swainsonine on secretion, cell surface expression and maturation of glycoproteins.

Glycoprotein	Cell Type	Swainsonine Concentration	Property and Effect	Reference
fibronectin	human fibroblast	0.006-0.6mM	no effect on synthesis or secretion	Arumughan and Tanzer (1983a)
aminopeptidase N	mucosal explants	0.01mM	13% inhibition of cell surface expression	Danielson *et al.*, (1983)
influenza hemagglutinin	calf kidney	0.06mM	no effect on titer	Elbein *et al.*, (1982)
VSV G protein	BHK	0.008-0.03mM	no effect on infectivity	Kang and Elbein (1983)
α_1-antitrypsin	hepatocytes	0.1mM	no effect on secretion	Gross *et al.*, (1983)
epidermal growth factor receptor	A-431	0.006mM	no effect on binding capacity or expression	Soderquist and Carpenter (1984)
α_1-antichymotrypsin transferrin complement C3	human hepatoma	0.1mM	no effect on secretion	Lodish and Kong, (1984)
IgM and IgD	mouse hybridoma	0.2mM	no effect on secretion	Peyrieras *et al.*, (1983)

continued next page...

Table 2 continued

Glycoprotein	Cell Type	Swainsonine Concentration	Property and Effect	Reference
HLA-A, B, C	human lymphoid	0.2mM	no effect on cell surface expression	Peyrieras *et al.*, (1983)
von Willebrand protein	human endothelial	0.006mM	no effect on multimer formation	Wagner *et al.*, (1985)
transferrin ceruloplasmin α_2-macroglobulin α_1- antitrypsin	human hepatoma	0.006mM	accelerated secretion	Yeo *et al.*, (1985)
insulin receptor and insulin-like growth factor receptor	IM-9	0.012mM	no effect on hormone binding or cell surface expression	Duronio *et al.*, (1986)
v-fms oncogene protein	tranformed Fischer rat embryo cells	58µM	no effect on cell surface expresssion	Nichols *et al.*, (1985)
mouse hepatitis virus gp E2	BALB/c 3T3	0.006mM	no effect on transport	Repp *et al.*, (1985)

Abbreviations: BHK, baby hamster kidney; VSV, vesicular stomatitis virus; HLA, human leukocyte antigen; gp, glycoprotein.

(Hooghe *et al* ., 1984) are significantly decreased by pre-treatment with swainsonine. Receptor mediated uptake of mannosyl-oligosaccharides (Arumugham and Tanzer, 1983b) and receptor mediated uptake of mannosyl-glycoproteins (Chung *et al.*, 1984) by macrophages were inhibited in the presence of swainsonine. A model describing the inhibition by swainsonine, suggested the interference of the Man_3 arm of the hybrid structure with the normal receptor recognition of the high mannose oligosaccharide (Chung *et al.*, 1984). The normal complex oligosaccharides on the cell surface would not interfere with the receptor binding. The degradation of internalized asialo fetuin in both rat liver parenchymal cells (Winkler and Segal, 1984a) and isolated lysosomes (Winkler and Segal, 1984b) was decreased in the presence of swainsonine.

Swainsonine may also be valuable in studying the immune system. Swainsonine inhibited the differentiation of human B lymphocytes into plasma (immunoglobulin-secreting) cells, suggesting that complex oligosaccharides are a requirement for this process (Tulp *et al.*, 1986). However, pretreatment of a B cell lymphoma (AKTB-1b) with swainsonine increased the cell's ability to stimulate thymocyte proliferation by 5-10 fold (Powell *et al.*, 1985). Con A-stimulated lymphocyte proliferation, suppressed by immunosuppressive factor from sarcoma tumors, was restored to normal levels with the addition of swainsonine (Hino *et al.*, 1985). In a related study with mice, swainsonine restored production of antibody to sheep red blood cells in the presence of tumor immunosuppressive factor (Kino *et al.*, 1985). The authors suggest that swainsonine may prove to be effective as a supplement to chemotherapy in increasing the immune response in cancer patients. In addition, swainsonine reduced the pulmonary colonization of B16-F10 murine melanoma cells in mice, although swainsonine did not decrease the tumorigenicity of the cells (Humphries, 1986). NIH 3T3 cells transfected with DNA containing the oncogene H-*ras*, lost the transformed

phenotype when treated with swainsonine (DeSantis *et al.*, 1987). However, swainsonine did not alter the transformed phenotype of AEV-transformed erythroblasts, nor the transport of the v-*erb* B oncogene glycoprotein (Schmidt *et al.*, 1985) and had no effect on the v-*fms* transformed phenotype (Nichols *et al.*, 1985). The infectivity of *Trypanosoma cruzi,* a protozoan which causes Chagas disease, was significantly decreased when either the host cells (peritoneal macrophage or rat heart myoblasts) or the protozoan were pre-incubated with swainsonine (Villalta and Kierszenbaum, 1985). Again, this represents a cell-cell association which is disrupted with swainsonine treatment. Obviously, further studies are required to determine the effectiveness of swainsonine in drug treatment, as well as possible toxicity, but these initial studies illustrate its potential.

Deoxymannojirimycin

The successful use of the nojirimycin derivatives, deoxynojirimycin and methyldeoxynojirimycin, as inhibitors of the processing glucosidases, led to the synthesis of the mannose analogue of deoxynojirimycin (1,5-dideoxy-1,5-imino-D-mannitol) (Figure 8) which was soon found to be an inhibitor of the processing enzyme, mannosidase I (Fuhrmann *et al.*, 1984). In that study, deoxymannojirimycin was compared to swainsonine for its capacity to alter the oligosaccharide structure of the secretable glycoproteins, IgD and IgM, from a mixture of hybridoma cells. Both the secreted and intracellular forms of the immunoglobulins had the same molecular weight in the presence of deoxymannojirimycin, in contrast to swainsonine which resulted in secreted forms with higher molecular weight. This suggested that deoxymannojirimycin acts at a stage of processing prior to the inhibition by swainsonine. IgM and IgD oligosaccharides produced in the presence of deoxymannojirimycin were not neuraminidase sensitive, but were endo H sensitive

and had elution patterns on Bio-Gel P4 identical to Man_{8-9}-GlcNAc oligosaccharides. Thus, it was concluded that deoxymannojirimycin inhibited mannosidase I of the processing reactions and also conversion of complex oligosaccharides to high mannose oligosaccharides of IgM and IgD, but did not alter their capacity for secretion.

Bischoff and Kornfeld (1984) confirmed the specificity of deoxymannojirimycin by studying its effect on various rat liver α-mannosidase activities. Golgi mannosidase I showed a 50% inhibition with 1-2 μM deoxymannojirimycin using Man_9-GlcNAc as the substrate, whereas Golgi mannosidase II was inhibited by only 14% at concentrations as high as 500 μM, using Man_5-GlcNAc as the substrate. Deoxymannojirimycin is a non-competetive inhibitor of Golgi mannosidase I with a K_i of 2 μM. Endoplasmic reticulum mannosidase activity was measured using *p*–nitrophenyl-α-D-mannoside (which is not a substrate for mannosidase I), in the presence of swainsonine to block lysosomal and mannosidase II activity. Interestingly, the ER mannosidase was not inhibited by deoxymannojirimycin. This may account for the presence of Man_8-GlcNAc structures on glycoproteins produced in the presence of deoxymannojirimycin.

Several studies have been done to determine the effect of converting complex oligosaccharides to high mannose oligosaccharides on the functional properties of specific glycoproteins. Rous sarcoma virus, grown in cultures treated with deoxymannojirimycin and swainsonine, produces viral glycoproteins of slightly different molecular weight, but the inhibitors did not affect proteolytic cleavage of the precursor glycoprotein to the mature form, nor the infectivity of the virus (Bosch *et al.*, 1985). Deoxymannojirimycin had little effect on the formation of mature virus particles, or the transport of glycoprotein E2 of mouse hepatitis virus (Repp *et al.*,

1985). Elbein *et al.* (1984a) also found that the release of virus, quantity of virus, and infectivity of virus, in two strains of influenza virus, were not affected by deoxymannojirimycin. However, differential effects of deoxymannojirimycin on the incorporation of ^{3}H-mannose into lipid-oligosaccharide appeared to be dependent on host cell type, virus strains and the length of incubation in the presence of the inhibitor.

In rat hepatocytes, the synthesis and secretion of the glycoproteins α_1-proteinase inhibitor and α_1-acid glycoprotein were not affected by deoxymannojirimycin (Gross *et al.*, 1985). The extracellular forms, however, became endo H sensitive and decreased in apparent molecular weight to become equivalent to the molecular weight of intracellular forms. This is consistent with the blockage of formation of higher molecular weight complex oligosaccharides. Analysis of the oligosaccharides revealed the major structure to be Man_9-GlcNAc with a small amount of Man_{7-8}-GlcNAc. Deoxymannojirimycin in intesintal epithelial cells increased the amount of Man_{7-9}-GlcNAc to 86% of the total oligosaccharide with a concomitant decrease in the amount of Man_5 oligosaccharide (Romero *et al* ., 1985b). The presence of Man_{7-8}-GlcNAc structures in both rat hepatocytes and intestinal epithelial glycoproteins supports the suggestion of an RER mannosidase I which is resistant to deoxymannojirimycin.

The effect of deoxymannojirimycin on the segregation and secretion of lysosomal enzymes was dependent on the cell type (Naureth *et al.*, 1985). Oligosaccharides of cathepsin D, arylsulfatase B and ß-hexosaminidase from HepG2 (hepatoma cell line) and human fibroblasts, increased in endo H sensitivity. Segregation and proteolytic digestion of cathepsin D to the mature form in the lysosomes was not affected by deoxymannojirimycin in fibroblasts, but in HepG2 cells the rate of secretion of cathepsin D was increased, and proteolytic maturation in the lysosomes was delayed.

The differential effect on cathepsin D in the two cell types was suggested to be due to a disruption of receptor-mediated transport specific for complex oligosaccharides in HepG2 cells, caused by the conversion of complex oligosaccharides to the high mannose structures. This is in contrast to the Man-6-P receptor transport of lysosomal enzymes in fibroblasts which would not be affected by deoxymannojirimycin.

Recently, it was reported that deoxymannojirmycin also inhibited differentiation of human B lymphocytes into plasma cells which secrete immunoglobulin (Tulp *et al.*, 1986), thus suggesting a requirement for glycoproteins containing complex oligosaccharides. Deoxymannojirimycin has not been as widely used as the other processing inhibitors in studying its effects on other cellular processes, but it is highly useful in determining the cellular requirements for complex oligosaccharides.

D. *Myogenesis*

Myogenesis is the development of muscle tissue during either embryogenesis or regeneration of damaged muscle tissue. Skeletal muscle is comprised of long, multinucleated cells know as myotubes, which are bundled into muscle fibers. Myotubes are biochemically and morphologically distinct from other types of cells. The formation of the multinucleated myotubes involves a complex process of differentiation of mono-nucleated pre-muscle cells, known as myoblasts, which fuse into multinucleated myotubes and undergo biochemical differentiation to produce muscle-specific proteins. In muscle tissue, the myoblasts believed to be involved in tissue regeneration and growth are satellite cells. They are surrounded by the basal lamina of the myofiber and are believed to undergo mitosis and eventual differentiation to myotubes (reviewed in Campion, 1984).

Contamination with other cell types (fibroblasts, nerve and connective tissue cells), and also the low level of differentiating cells in healthy adult tissue, cause difficulties in studying myogensis *in vivo*. These problems have been overcome somewhat by the use of primary cell cultures of myoblasts (reviewed by Konigsberg, 1979). However, obtaining myogenic cultures still requires elimination of contaminating, non-myogenic cell types, and primary myoblasts must be grown on plates pretreated with collagen or gelatin, and require media supplements of embryo extract to necessitate attachment and growth. A further problem with primary cell cultures is a restriction on passage number, which necessitates re-establishment of cultures for prolonged experiments. To overcome the problems associated with primary cell culture, permanent 'transformed' cell lines from myogenic cells of rat and mice have been established, which can be maintained in culture indefinitely and still maintain the capacity to differentiate.

The rat L6 myoblast cell line was selected by Yaffe (1968) from trypsinized newborn rat muscle and transformed using the carcinogen, 20(3) methylcholanthrene. Non-senescing rat myoblasts (L8 and L63) capable of fusion and terminal differentiation were later isolated (Richler and Yaffe, 1970). In culture, the rat myoblasts will grow and divide attached to the culture vessel, until they reach high density or confluence on the surface, and then withdraw from the cell cycle. Fusion and biochemical differentiation then begin and are facilitated by the reduction of serum supplements from 10% to 1% of the medium.

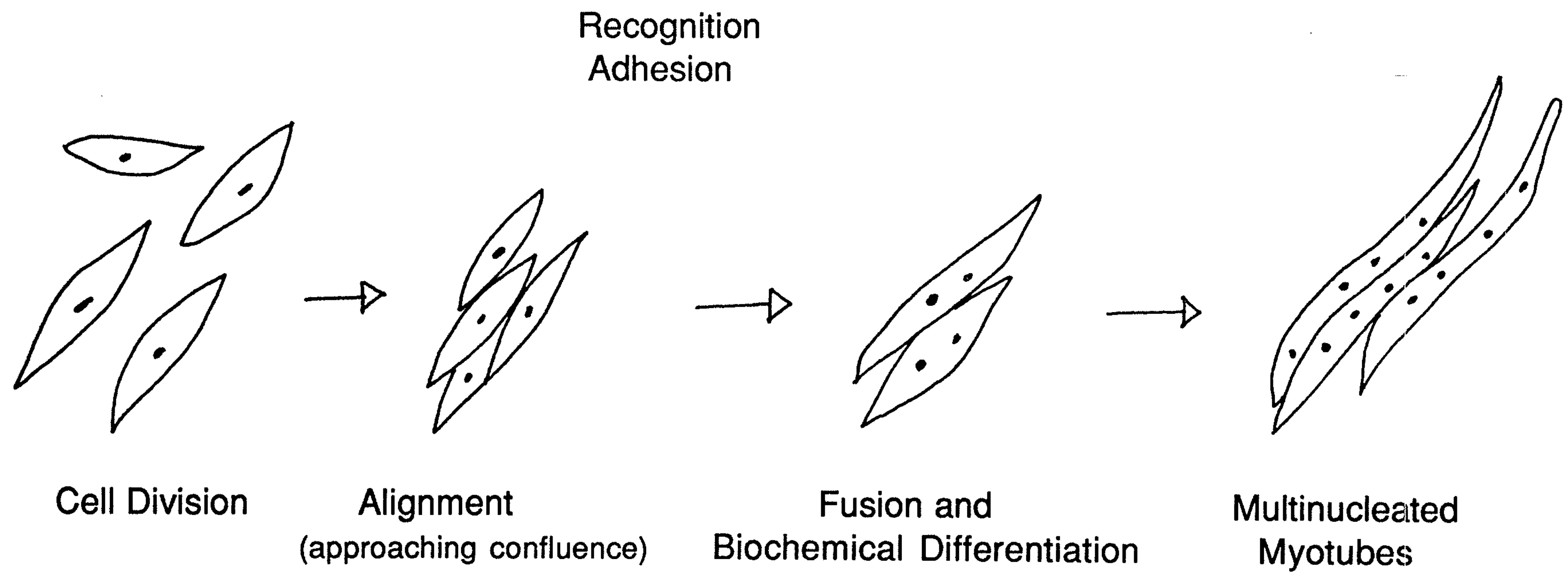
Variant myoblast cell lines, which are unable to fuse but still undergo biochemical differentiation (Spizz *et al.*, 1986), and also variants which lack both aspects of differentiation (Parfett *et al.*, 1981, 1983; Cates *et al.*, 1984; Creasey *et al.*, 1982; Creasey and Wright, 1984; Whatley *et al.*, 1976; Pearson, 1980; Ng, 1980) have been isolated from permanent cell lines. The ability of some variants to maintain biochemical differentiation, while losing their fusion capabilities, has indicated separate control over each system. The variants are important for comparison with the normally differentiating myoblasts and have aided in distinguishing factors which affect both or either one of the differentiation processes.

Fusion is also highly dependent on calcium concentration and synchronously fusing cultures can be produced by the reduction of calcium concentrations to below 1.4 mM, followed by replenishment to this critical concentration (Shainberg *et al.*, 1969). Based on calcium studies with primary myoblasts from chick, Knudsen and Horowitz (1977) have described several sequential events which define the fusion process (Figure 10). Following withdrawal from the cell cycle, myoblasts align with the long axes parallel. Then, with the appropriate medium conditions, myoblasts undergo cell-cell recognition, calcium-dependent adhesion, and membrane union leading to the formation of the multinucleated cell. Inhibition of membrane fusion,

Figure 10.

Differentiation of myoblasts in culture. As the myoblasts approach confluence, parallel and end to end alignment of the myoblasts occurs. Recognition and adhesion directly precede fusion of the myoblasts which coincides with biochemical differentiation to produce muscle-specific proteins. Fusion continues resulting in the formation of multinucleated myotubes.(Source of diagram - Merlie *et al.*, 1977)

In vitro Myogenesis



but not of myoblast aggregation, by con A and 20 mM Mg^{++} (Knudsen and Horowitz, 1978), shows the delineation between the series of events which comprise the fusion process. Membrane continuity occurs 20-30 minutes after the fusion process begins. However, the appearance of multinucleated cells requires one hour (Neff *et al.*, 1984). This delay may indicate that other structural changes within the cell are required before myotube formation is complete (Wakelam, 1985). Spontaneous contractions have also been reported in cultured myoblasts (Richler and Yaffe, 1970). The fusion process must involve a myoblast-specific recognition system because fusion with non-muscle cells does not normally occur. Heterotypic cells cultured with myoblasts reduced myoblast fusion capacity by interferring with fusion (Shimada, 1968).

Along with the morphological differentiation of myoblasts, numerous biochemical changes occur. In order for the muscle fiber to be functional, several muscle-specific proteins must be produced, such as muscle-specific creatine kinase, acetylcholine receptors, myosin, actin filaments, and myoglobulin. These will not be discussed further, but several other biochemical changes occur which can be associated with the fusion event itself (reviewed by Wakelam, 1985) and these will be dealt with in a later section.

1.Factors Affecting Fusion

The model proposed by Knudsen and Horowitz (1977) suggests that the fusion process is not a one step phenomenon but rather a series of highly integrated events. Aggregation and recognition must occur before membrane union is possible. Much support for this theory of the fusion process has come from work with various inhibitors or stimulators in attempts to define the natural effectors of myogenesis. In early studies, Yaffe (1971) found that myoblasts must undergo 'developmental

changes' in order to become fusion competent. He recognized that the pre-fusion changes are partially dependent upon the nutritional media.

A calcium concentration of 1.4 mM is required for fusion; below 1.4 mM alignment of myoblasts can occur but not fusion (Shainberg *et al.*, 1969). Both calcium-dependent and calcium-independent mechanisms for adhesion have been reported (Gibralter and Turner, 1985). The mechanism of calcium control is not yet known, however, an influx of calcium into the cell is required prior to fusion (David *et al.*, 1981). This may be controlled by prostaglandin E1 (David and Higginbotham, 1981), possibly through a voltage-dependent mechanism (Entwistle *et al.*, 1983, 1986) or by depolarization of the membrane through active acetylcholine receptors on myoblasts (Bevan *et al.*, 1985). Several different studies have identified possible sites of calcium control (Table 3) such as an ecto protein kinase (Lognonne and Wahrmann, 1986), calmodulin (Shainberg and Cahan, 1985; Bar-Sagi and Prives, 1983), and activation of the protease, CAF or calpain II (Schollmeyer, 1986a). Calcium control over myogenesis appears to be a very complex process and calcium influx may indeed activate or initiate a number of events which render myoblasts fusion-competent.

The requirement of primary myoblast cultures for chick embryo extract in growth and differentiation, and the necessity for a reduction in the serum content of myoblast cell lines to promote fusion, has prompted studies dealing with hormonal and growth factor control of myoblast differentiation. Media conditioned by myogenic cultures increased the onset of fusion in myoblasts (Konigsberg, 1971) suggesting production or removal of a factor affecting differentiation. A fusion-promoting activity was purified from conditioned media, however, it had minimal effect in serum-enriched media (Doering and Fischman, 1977). Early alignment was also attained by the addition of concentrated media from aligned myoblasts (Engel *et al.*, 1982).

Table 3 Factors Affecting Myoblast Fusion

Factor	Effect	Reference
calcium (1.4 mM)	required for fusion	Shainberg *et al.*, 1969
conditioned media	myoblast alignment	Engel *et al.*, 1982
FGF	stimulate proliferation	Linkhart *et al.*, 1980
	delay fusion	Gospodarowicz *et al.*, 1976
	reduce CPK mRNA	Spizz *et al.*, 1986
TGF-ß	inhibits differentiation	Florini *et al.*, 1986
somatomedians	stimulate differentiation	Ewton and Florini, 1981
human leukocyte interferon	stimulate primary human myotube formation	Fischer *et al.*, 1983
chick interferon	inhibit chick myotube formation	Tomita and Hasegawa, 1984
epinephrine; isoproterenol	induce myoblast fusion	Curtis and Zalin, 1981
aspirin; indomethacin	no inhibition of fusion	Steiner *et al.*, 1984
choroquine; indomethacin	inhibition of fusion	Entwistle and Zalin, 1984
prostaglandin E1	increases calcium influx and enhances fusion	Entwistle and Zalin, 1984
diazepam	inhibits fusion	Bandman and Strohman, 1979
3-deaza-adenosine	inhibits fusion	Scarpa *et al.*, 1984
zinc and vanadate	inhibits fusion	Carlsson, 1984

Abbreviations: FGF, fibroblast growth factor; TGF-ß, transforming growth factor-ß

Another theory has suggested that elimination of mitogenic agents, such as fibroblast growth factor (FGF), by myoblasts as culture densities increase, reduces proliferation and allows the differentiation process to begin (Linkhardt *et al.*, 1981; Linkhardt *et al.*, 1980; Gospodarowicz *et al.*, 1976), and also reduces levels of muscle-specific creatine kinase mRNA (Spizz *et al.*, 1986). Epidermal growth factor may also be involved in control of myoblast proliferation, and hence the ability to differentiate as well (Lim and Haushka, 1984). Similarily, transforming growth factor ß (TGF-ß) inhibits myoblast differentiation but is not mitogenic (Florini *et al.*, 1986). Other drugs or hormonal factors which have an effect on fusion are the somatomedins (human somatomedin C, insulin-like growth factor I and multiplication stimulating activity) (Ewton and Florini, 1981), human leukocyte interferon (Fisher *et al.*, 1983), partially purified chick interferon (Tomita and Hasegawa, 1984), epinephrine and isoproterenol (Curtis and Zalin, 1981), diazepam (Bandman and Strohman, 1979) and 3-deaza-adenosine (Scarpa *et al.*, 1984) (Table 3).

Prostaglandins may play a key role in the control of myogenesis. Addition of prostaglandin E1 to chick myoblasts enhanced fusion capacity possibly through a transient increase in cAMP levels (Zalin, 1977). Similar studies by David and Higginbottham (1981) came to the same conclusion, but a calcium influx was a co-requirement to prostaglandin stimulation. However, reports of the effects of inhibitors of prostaglandin synthesis on myoblast differentiation are conflicting (Steiner *et al.*, 1984; Entwistle and Zalin, 1984). In more recent studies, prostaglandins of the one series are reported not to enhance fusion by increasing cAMP levels as previously found, but rather to allow the influx of calcium required for fusion competent myoblasts (Entwistle *et al.*, 1986) and prostaglandin binding precedes cell-cell aggregation (Hausman and Berggrum 1987). It appears that PGE_1

may be essential in producing fusion-competent myoblasts, but this is probably not a primary event in the actual induction of fusion of myoblast membranes.

The large number and diversity of agents which have an effect on the process of myogenesis, emphasizes the very specific conditions which must exist for differentiation to occur. Although there appears to be a relationship between some effectors and events which result in fusion, much research is necessary to clarify the sequence of events at the biochemical level which render myoblasts fusion-competent. It is also unclear at what step of the fusion process some inhibitors are acting; fusion competence, adhesion or membrane fusion? Also, why do some agents affect fusion and not biochemical differentiation? The key event which initiates membrane fusion has not been defined. Receptor-mediated recognition is only one possibility.

2. Plasma Membrane Changes In Myoblast Fusion

The second major approach in the study of myoblast fusion has been to define and describe the changes which occur in the plasma membrane prior to or during the fusion process. This approach includes morphological studies using electron microscopy and biochemical studies of membrane lipid, protein and glycoprotein.

The development of freeze fracture EM (electron microscopy) allowed a three-dimensional view of membrane fusion, and resulted in defining membrane fusion as three related events: membrane apposition in which the two membranes form a pentalaminar structure; fusion, represented by the union of the membranes; and finally fission to form the cytoplasmic bridge (Fumagalli *et al.*, 1981). Particle-free regions may be sites of membrane fusion (Kalderon and Gilula, 1977) and may be related to electron opaque regions formed by the secretion of material from particle-free cytoplasmic vesicles (Engel *et al.*, 1985). Clustering of PMP's (plasma membrane intramembranous particle) in myoblasts which are redistributed uniformly following

fusion (Furcht and Wendelschafer-Crabb, 1978), may also indicate a necessity for particle-free regions for fusion. Vesicles produced from plasma membranes of myoblasts fused under appropriate conditions, but vesicles from mature myoblasts, taken just prior to fusion, had a higher fusion capacity (Dahl *et al.*, 1978). Other studies have described the formation of blebs (small protruding regions of membrane) prior to myoblast fusion, however, their significance is unknown (Peterson and de Harven, 1981; Engel and Przybylski, 1983).

3. Biochemical Changes In Membranes During Myogenesis

Presently, there is no direct evidence to implicate specific membrane lipids in the initiation of myoblast fusion (reviewed by Wakelam, 1985). No changes in lipid content (phospholipid, fatty acid and cholesterol) have been correlated with the various stages of myogenesis (Boland *et al.*, 1977). Asymmetry of the bilayer with regard to phospholipid content may be significant in the fusion of membranes, but total phospholipd content does not correlate with fusion potential (Wakelam, 1985). Modification of lipid content through various methods showed that the normal balance of lipids is necessary for fusion. For example, addition of cholesterol inhibits fusion (van der Bosch *et al.*, 1973) but inhibition of cholesterol synthesis also inhibits myoblast fusion (Cornell *et al.*, 1980).

Fusion is accompanied by a substantial increase in the breakdown of phosphatidyl inositol and other polyphosphoinositides and an increase in 1,2–diacylglycerol, the by-product of this reaction (Wakelam and Pette, 1982; Wakelam, 1983). Wakelam (1986) has suggested that the purpose of the metabolism of phosphinositides is to produce a more fluid membrane and also remove electrostatic bonds which stabilize lipid-protein interactions.

A variety of techniques have been used to analyze changes in membrane protein during myogenesis. One of the most common is the use of SDS-PAGE to separate proteins according to molecular weight in combination with radioactive iodination for the 'visualization' of the protein. While some report no distinct changes occuring during fusion (Curtis *et al.*, 1980; Cates and Holland, 1980), one study using two-dimensional electrophoresis of L6 cell surface proteins detected a 200,000-250,000 mw protein which increased during the formation of myotubes (Yoshioka and Sueoka, 1983). More interesting is the detection of transient proteins of 66,000 mw and lower molecular weight proteins (11,000, 12,000 and 14,000 mw) which appeared prior to fusion and are lost following it (Pauw and David, 1979). The production of monoclonal antibodies against chick muscle cells at different stages of myogenesis identified unique determinants during myotube formation but only approximately 5% of the total determinants change. More obvious variations were seen in the amounts of certain cell surface antigens (Grove *et al.*, 1985).

The major problem with this general approach in comparing myoblast and myotube cell surface proteins is that developmentally regulated proteins may appear in fusing myoblast/myotube populations, not because they are important to the fusion process but rather as a consequence of the concurrent biochemical differentiation process.

4. The Role of Glycoproteins in Myoblast Differentiation

Glycoproteins have proven to be extremely important in a number of cell surface phenomena. Because of the numerous other studies which have implicated plasma membrane glycoproteins in cell adhesion and receptor recognition, several investigators have considered the possibility of glycoproteins as participants in recognition and adhesion in myogenesis.

One of the first indications that glycosylated membrane components may be involved in myoblast fusion was evidence of a ß-D-galactoside-specific lectin activity (Gartner and Podleski, 1975), which increased as fusion competence increased (Gartner and Podleski, 1976). Its activity was measured by the ability to agglutinate erythrocytes, an effect which is inhibited in the presence of thiodigalactose and lactose. Purification from chick embryo muscle (Nowak *et al.*, 1977; Den and Malinzak, 1977) revealed a dimer with 15,000 mw subunits. Antibody to the lectin indicated its presence on the myoblast cell surface (Nowak *et al.*, 1977), however, its relevance to recognition or adhesion in the fusion process has been disputed. The inhibitory effect of thiodigalactose and lactose on the fusion of L6 myoblasts (Gartner and Podleski, 1977) was not found with chick embryonic myoblasts (Den *et al.*, 1976), and there are conflicting reports of inhibitory effects on fusion of L8 myoblasts (Kaufman and Lawless, 1980; Den and Malinzak, 1977). Differential effects of the purified lectin on chick primary myoblast fusion (Den and Chim, 1981; McBride and Przybylski, 1980) have also been reported. In view of these results, the relevance of this endogenous lectin in myoblast fusion remains questionable.

The inhibition of myoblast fusion in the presence of the plant lectin, con A, was a significant step in recognizing a possible role for glycoconjugates in myogenesis (Den *et al.*, 1975). This study also delineated between the importance of different types of carbohydrates, as WGA (wheat germ agglutinin) had more binding sites than con A, but had no effect on fusion. Since then, much work has been done using lectins to determine membrane topography of cell surface glycoconjugates during fusion. In microscopy studies, pre-fusion myoblasts exhibited an even distribution of con A sites with redistribution of binding sites in myotubes into patches and capped areas (Furcht and Wendelschafer-Crabb, 1977, 1978). However, other types of assays found con A receptors clustered prior to fusion, with dispersion to an even

distribution during membrane fusion, and followed by a return to the clustered state after the completion of fusion (Herman and Fernandez, 1982). Others report constant, even distribution of fluorescein-conjugated WGA, con A and LCA (*Lens culinaris* agglutinin) lectins throughout myogenesis, with only SBA (soy bean agglutinin) lectin aggregating during fusion (Sawyer and Akeson, 1986). Although these studies offer conflicting evidence regarding distribution of lectin binding sites, it can be concluded that some glycoproteins are mobile within the lipid bilayer during fusion.

Lectin binding has also been used in combination with gel electrophoresis to identify alterations in molecular weight patterns of glycoproteins during myotube formation. Neuraminidase-sensitive glycoproteins exhibited decreases in *Ricinus communis* agglutinin binding to two glycoproteins of 136,000 and 49,000 mw and shifting from a 115,000 mw to a 107,000 mw glycoprotein (Holland *et al.*, 1984). In another study several glycoproteins detected by con A either accumulated, had altered glycosylation or appeared during fusion (Senechal, 1982). One problem associated with lectin binding to either whole cells or separated membrane components is the necessity to determine whether changes in lectin binding patterns are due to altered translation, targetting of the glycoprotein to the cell surface, or changes in glycosylation. An alternative method is the use of monoclonal antibodies or antiserum to define glycoproteins which are involved in fusion. Using monoclonal antibodies, a cell surface antigen of approximately 38,000 mw common to myoblasts, muscle satellite cells and glia cells, was identified. This glycoprotein increases during myogenesis but it is not found on mature myotubes, indicating its possible significance in myoblast fusion (Wakshull *et al.*, 1983).

Most changes in glycosylation occur during the adhesion step of myotube formation (Senechal *et al.*, 1983) suggesting that cell surface glycoprotein may be of most significance in the very early stages of myoblast fusion when initial cell-cell

most significance in the very early stages of myoblast fusion when initial cell-cell contact occurs. Myoblast-secreted glycoprotein complexes, called adherons, promote calcium-dependent cell-cell adhesion in L6 myoblasts (Schubert and Lacorbiere, 1982). Calcium may affect conformation of cell surface glycoproteins; trypsin digestion and its subsequent inhibitory effect on fusion is partially blocked in the presence of calcium (Knudsen, 1985). Several components of the extracellular matrix of myoblast cultures are also glycoproteins. A number of studies have measured levels of fibronectin and collagen (Gardner and Fambrough, 1983; Holland *et al.*, 1984; Rao *et al.*, 1985; Leibovitch *et al.*, 1986), but there is no consistency between these reports regarding increases or decreases during fusion, and no direct evidence to indicate that they have a role in fusion.

Various drugs which affect glycosylation or transport of glycoproteins to the cell surface have been used to prove the necessity for proper glycosylation and positioning of glycoproteins for fusion. Tunicamycin, an inhibitor of N-linked glycosylation, blocks calcium-dependent adhesion and thus fusion (Knudsen, 1985). Other groups have also reported inhibition of myoblast fusion with tunicamycin in rat (Gilfix and Sanwal, 1980), quail (Olden *et al.*, 1981), and chick embryo myoblasts (Gweinn, 1980), but Knudsen's work (1985) was the first indication that the adhesion step was primarily affected. A partial reversal of tunicamycin-inhibited fusion by the addition of protease inhibitors was interpreted as meaning that the carbohydrate portion of the glycoprotein may be acting as a protection mechanism against proteolytic degradation (Olden *et al.*, 1982b; Knudsen, 1985). Monensin, a carboxylic ionophore which blocks secretion and movement of glycoproteins to the plasma membrane and then interferes with the glycosylation process, inhibits myoblast fusion in embryonic chick myoblasts (Den, 1985). Fusion can also be blocked by antiserum

against fusion-competent myoblasts and this inhibition can be reversed by the addition of a WGA-binding fraction of pectoral muscle detergent extract (Knudsen, 1985).

An inhibitor of a soluble metalloendoprotease activity, 1,10-phenathroline, also inhibited myoblast fusion (Couch and Strittmatter, 1984; Mundy and Strittmatter, 1985). During fusion, expression of a 105,000 mw glycoprotein was decreased and a 90,000 mw glycoprotein was newly expressed. The metalloendoprotease inhibitor blocked these changes suggesting that highly specific proteolysis of glycoproteins is a key step in inducing myoblast fusion. The 90,000 mw glycoprotein may act as a fusogenic agent in myoblast fusion (Rosenberg *et al.*, 1985). Metalloendoprotease inhibitors also blocked biochemical differentiation by preventing the expression of the muscle-specific proteins, creatine kinase, myosin heavy chain and alpha actin (Baldwin and Kayalay, 1986). Although this indicates that specific proteolysis of glycoproteins may be a necessary event in myoblast differentiation, these studies have not yet identified the precise involvement of the 90,000 mw glycoprotein in the myogenic process.

Myoblast differentiation appears to be coordinated with increased levels of biosynthetic enzymes required for the glycosylation of proteins. A non-fusing but biochemically differentiation-competent myoblast line, BC_3H, exhibits an increase in oligosaccharyl transferase activity during differentiation. The enzyme activity decreases when myoblasts are stimulated to proliferate with mitogens (Grant *et al.*, 1986). In mouse skeletal myoblasts, glucosyl transferase levels decreased upon differentiation, while mannosyl transferase and N-acetylglucosaminyl transferase activities increased with differentiation (Miller *et al.*, 1986). Denervation of rat skeletal muscle, which promotes increases in myoblast differentiation, resulted in an increase in plasma membrane galactosyl and sialyl transferase activities (Jeffrey and

5. Myogenic Mutants and Variants

Studying the biochemical changes in membranes during myogenesis has thus far identified several membrane components and some enzyme activities which change during myogenesis. Another approach to studying the myogenic process is the isolation of mutants or variant cell lines which are reduced in myogenic potential. Comparison of the mutant to wild type populations may identify alterations or defects in the metabolism of the myoblasts which hinder or block the myogenic process. Mutants with myogenic-defective phenotypes have been isolated using selection agents such as α-amanatin (an RNA-polymerase inhibitor) (Pearson, 1980), azacytidine (Whatley *et al.*, 1976; Ng, 1980), and hydroxyurea (Creasey *et al.*, 1982; Creasey and Wright, 1984).

The selection and characterization of lectin-resistant mutant myoblasts have yielded very interesting information regarding the glycoprotein and glycosylation requirements for myoblast fusion. A strong correlation between the loss of fusion capacity and con A resistance in four independently selected, lectin-resistant variants of L6 myoblasts (Parfett *et al.*, 1981), offered a good model system for studying the effects of altered glycosylation on myoblast differentiation. Besides their inability to fuse, the con A-resistant variants had reduced creatine phosphokinase activity indicating a lack of biochemical differention. A decrease in the incorporation of mannose from GDP-mannose into isolated lipid fractions suggested a loss of mannosyl transferase activity, which would result in decreased numbers of high mannose oligosaccharides on cell surface glycoproteins. Thus, a conclusion from this study was that the integrity of the high mannose oligosaccharides is necessary in myoblast differentiation. The deficiency in con A binding to the variants was attributed to a decrease in the number of high affinity con A binding sites, most likely the high mannose oligosaccharides of cell surface glycoproteins (Parfett *et al.*, 1983).

Separation of ^{14}C-mannose labelled glycoproteins by electrophoresis showed a decrease in mannose incorporation in a number of membrane glycoproteins, but the most prominent difference was a glycoprotein of 44,500 mw with reduced mannose incorporation and con A binding. This cell-surface glycoprotein may have an important role as a mediator or initiator of myoblast differentiation.

Confirmation of this work was found in very similar studies which identified two con A resistant, non-fusing L6 myoblast variants, RI and RII (Cates *et al.*, 1984). A three-fold decrease in the susceptibility of the RI variant to con A toxicity was attributed to altered glycoprotein metabolism. The RII variants, with a five-fold increase in con A resistance and a defect in the transfer of mannose from GDP-mannose to the lipid-linked form, appear to be very similar to the con A resistant variants isolated by Parfett *et al.* (1981). Another significant similarity was the identification of a plasma membrane glycoprotein of 46,000 mw with reduced con A binding capacity in the RI variants. RI and RII do not have identical genetic defects as RI/RII hybrids complement each other, shown by reappearance of the 46,000 mw glycoprotein and a partial regaining of fusion capacity and biochemical differentiation.

E. *Introduction to the Present Work*

Many oligosaccharides of N-linked glycoproteins exhibit specific functions, such as the mannose-6-phosphate recognition and targetting of lysosomal enzymes (reviewed in Sly and Fischer, 1982; Kornfeld and Kornfeld, 1985; Kornfeld, 1986, 1987), and binding of asialoglycoproteins to hepatic membranes through terminal galactose recognition (Ashwell and Morell, 1974; Hudgin *et al.*, 1974), and also non-specific functions like prevention of proteolysis (Olden, 1982b). There is growing evidence that N-linked carbohydrate is important in several other types of cellular processes such as cellular adhesion and recognition (see Historical Review, Section A). The positioning of the carbohydrate on the extracellular face of the membrane immediately raises questions regarding the significance of N–linked glycoproteins and their oligosaccharide moieties in cell-cell, cell-matrix, and cell-ligand interactions.

The L6 myoblasts have proven to be an excellent cell system for studying the relationship between cell surface oligosaccharide and differentiation. This thesis is designed to further develop the theory that implicates cell surface mannosylated glycoproteins in myoblast recognition/adhesion and differentiation, which was originally proposed in earlier studies with con A-resistant fusion-defective myoblasts (Parfett *et al.*, 1981, 1983). A second aspect of this work was to further explore and define the biochemical defects of the con A-resistant myoblasts with regard to altered glycoprotein synthesis and effects on the cellular phenotype.

The discovery of a series of compounds which can individually inhibit N-linked glycoprotein processing reactions in a specific way, have allowed manipulation of the oligosaccharide structures of glycoproteins in a variety of cell types. The work presented in this thesis represents the first study in which the processing inhibitors have been applied to the myoblast system to determine the effects of subtle, yet highly specific, alterations in oligosaccharide structure on the differentiation capacity of the

cells. The inhibition of glycoprotein processing reactions to alter oligosaccharide structure is a more advantagous approach than the isolation of carbohydrate-defective mutants, because it allows one to study the effects of changes in oligosaccharide structure in the L6 myoblasts without having to characterize a mutation in glycoprotein biosynthesis.

The processing inhibitors not only demonstrate the dependence of the fusion reaction on proper N-linked glycosylation of cell surface oligosaccharides, but also are utilized to show the significance of carbohydrate structure of other myoblast glycoproteins, such as the insulin receptor and lysosomal enzymes. The insulin receptor is representative of the many cell surface glycoproteins which may be affected in their function and cell surface translocation by altered oligosaccharide processing. The lysosomal enzymes are also sensitive to the glycoprotein processing inhibitors both directly through specific inhibition, and also indirectly by modification of their oligosaccharide. The targetting and transport of the lysosomal enzymes to the lysosomes is dependent on the formation of the Man-6-P recognition signal on high mannose oligosaccharides. The synthesis of this recognition signal may be sensitive to the lack of carbohydrate processing.

Con A-resistant myoblasts are unable to differentiate into myotubes and this has been correlated with a reduced incorporation of mannose from GDP-mannose into lipid oligosaccharide (Parfett *et al.*, 1981, 1983). Chromatography of the radioactively labelled oligosaccharides of the con A-resistant myoblasts confirms that only truncated high mannose oligosaccharides are produced. This defect in oligosaccharide structure may have wide ranging effects on the metabolism of the cell, and would especially affect glycoproteins which require proper glycosylation in order to be functional. Two such groups of glycoproteins are studied in the con A-resistant myoblasts to determine if altered glysoylation may affect their metabolism. The first group, lysosomal glycosidases, exhibit altered α-mannosidase and ß-hexosaminidase activities in the con

A-resistant myoblasts. This is consistent with studies of several other cell lines with defective glycoprotein metabolism which have alterations in lysosomal enzyme activities (Blaschuk *et al.*, 1980 a,b; Robbins, 1979; Robbins and Myerowitz, 1981; Krag and Robbins, 1982). The second group of glycoproteins studied in the con A-resistant myoblasts is comprised of galactosyl and sialyl transferase, which add the penultimate and terminal sugar residues (-Gal-NeuAc), respectively, to the complex oligosaccharides. Altered glycosylation of these enzymes may affect their activity, which in turn would altered the carbohydrate composition of the complex oligosaccharides. Both sialyl and galactosyl transferase activities are altered in the con A-resistant myoblasts. One theory has suggested that these transferases may play a role in cellular adhesion or interactions through their affinity for sugar residues on surfaces of adjacent cells (Roth *et al.*, 1971 a,b), however, there is evidence indicating that complex oligosaccharides are not a major factor in myoblast adhesion (Gilfix and Sanwal, 1984).

When studying any cellular process, it is important to apply our knowledge to disease mechanisms which may be related to the area under study. Duchenne muscular dystrophy is an X-linked recessive genetic disease which results in wasting of the muscle tissue and eventual death. The predominant theory suggests that the disease is a result of a generalized membrane defect resulting in 'leaky' membranes. Significant advances have been made in determining the genetic defect in Duchenne muscular dystrophy in the past year, and the protein product of the defective gene has been named dystrophin (Hoffman *et al.*, 1987). However, the function of the protein is not known. One study has indicated that a lack of fusion and biochemical differentiation contributes to the diseased state of the muscle tissue (Jasmin *et al.*, 1984). Since glycoproteins constitute a major portion of active functional membrane components, any minor alterations in structure or glycosylation could dramatically effect the integrity of the membrane and its functional properties. Lectin binding studies can detect gross

structural changes in carbohydrate content of the plasma membrane and in this study con A and WGA were utilized to determine if fibroblasts from Duchenne dystrophic patients had altered lectin binding properties. Since lysosomal enzymes are potentially destructive to the cell surface carbohydrate if allowed to leak from the cells, the activities of ß-hexosaminidase and α-mannosidase acitivities have also been determined in fibroblasts taken from Duchenne dystrophic patients and compared with normal age-matched controls.

EXPERIMENTAL

A. *Materials*

1. Materials for Cell Culture:

Plastic tissue culture plates, Lux Scientific Co.; calf and fetal bovine serum, GIBCO; α-minimal essential medium, Flow Lababoratories; penicillin G and streptomycin sulphate, Sigma Chemical Co.; Bactotrypsin, Difco.

2. Materials for Enzyme Assays and Ligand Binding Assays:

p-Nitrophenyl-glycopyranosides, 4-methylumbelliferyl-α-D-mannose, bovine fetuin, bovine insulin, Triton X-100, bovine serum albumin, α-methyl-D-mannoside, UDP-galactose and N–acetylglucosamine were obtained from Sigma Chemical Co.; concanavalin A and pronase B were obtained from Calbiochem; wheat germ agglutinin and endo-ß-N-acetylglucosaminidase H were obtained from Miles Scientific; glucose-6-phosphate dehydrogenase and hexokinase were obtained from Boerhinger Mannheim; Bio-Gel P–4 and P–10 and Bio-Rad Protein Assay Kit were obtained from Bio-Rad Laboratories; ACS liquid scintillation fluid was from Amersham Corp.; other chemicals were of reagent grade and were obtained from Fisher Scientific Co. The following radioisotopes were obtained from New England Nuclear Corp.: CMP–[4,5,6,7,8,9–^{14}C]–NeuAc (250 mCi/mmol); UDP–[U–^{14}C]–Gal (333 mCi/mmol); ^{125}Iodine (17 Ci/mg); L–[4,5–^{3}H]–leucine (345 mCi/mmol); 2-deoxy–D–[^{3}H]-glucose (7.1 Ci/mmol); [^{125}I]–insulin (2200 Ci/mmol) and D–[1–^{14}C]-mannose (57 mCi/mmol). Oligosaccharide standards (Glc_{1-3}–Man_9–GlcNAc) labelled with [^{14}C]–glucose were gifts from Dr. A. Herscovics, McGill University, Montreal.

3. Glycoprotein Processing Inhibitors:

Castanospermine was a gift from Dr. A.D. Elbein, U. of Texas, San Antonio, and Genzyme Corp., Boston Mass. and was also purchased from Calbiochem. Swainsonine was a gift from Dr. O. Touster, Vanderbilt University, and was also purchased from Calbiochem. 1–Deoxymannojirimycin was a gift from Dr. G. Kinast, A.G. Bayer, FRG. and was also purchased from Miles Scientific. N-Methyldeoxynojirimycin was a gift from Yoskiaki Aoyagi, Nippon Shinyaku, Co., Kyoto, Japan. and was also purchased from Miles Scientific. 1–Deoxynojirimycin was a gift from Drs. W. Frommer and D. Schmidt, A.G. Bayer, FRG. and was also purchased from Miles Scientific.

B. *Methods*

1. Cells and Growth Conditions

L6 myoblasts (clone 2 and clone 5), were obtained from J.A. Wright (University of Manitoba) as subclones of the L6 myoblasts originally isolated by Yaffe (1968). L6 variants, resistant to the cytotoxic effects of the plant lectin, concanavalin A (con A), were previously selected by a one step exposure to con A (Parfett *et al.*, 1981). Cells used in experiments were between 5 and 25 passages (one passage refers to growth from 1×10^5 to 4×10^6 cells per 100 mm plate) and cultures beyond 25 passages were discarded. Early passages (3-4) were checked for fusion competence, and passages 5-6 were frozen in liquid nitrogen or a -80°C freezer in medium containing 10% calf serum and 5% dimethylsulfoxide, and retrieved when fresh cultures were required. Cells were maintained in an atmosphere of 5% CO_2 on 100 mm plates in α–minimal essential medium (α-MEM) supplemented with 10% calf serum and antibiotics (100 units penicillin G per ml and 0.0685 mg/ml streptomycin sulphate). Culture medium was changed every 2-3 days until confluence was reached, upon which cells were replated following removal from the plates with 0.2% Bactotrypsin in phosphate buffered saline (PBS), pH 7.0 (containing NaCl 8.0 g/l, KCl 0.2 g/l, Na_2PO_4 1.15 g/l and KH_2PO_4 0.2 g/l).

In assays to determine myoblast fusion capacities, the cells were plated on 60 mm plastic plates with 4 ml of medium, or 30 mm plates with 2 ml of medium, at a density of 1×10^5 cells/ml. α-MEM containing 10% calf serum and antibiotics was replaced every second day until the cells reached confluence (covered the surface of the plate) and the medium was replaced with medium containing 1% calf serum to enhance and synchronize fusion. Disturbance of the cells during fusion decreased the fusion potential and therefore, the medium was not usually changed during the fusion assays, with the exception of drug reversal experiments (see below).

In assays to determine the effects of the glycoprotein processing inhibitors on fusion capacity, myoblasts were treated with trypsin and replated at a density of 4×10^5 cells per 60 mm plate with 4 ml of α-MEM supplemented with 10% calf serum and antibiotics. When the myoblasts had reached confluence and random fusion events had occurred, medium was removed and replaced with α-MEM containing 1% calf serum and the glycoprotein processing inhibitors at the following concentrations: 1–deoxynojirimycin (2.5 mM), N-methyldeoxynojirimycin (1.0 mM), castanospermine (0.1 mM), 1-deoxymannojirimycin (0.5 and 1.0 mM) and swainsonine (2.6 μM). The medium was not changed again during the incubation. The myoblasts were cultured for up to five days post-confluence, at which time the control cultures containing no inhibitors had reached approximately 80% fusion. In reversal experiments, which measured the effect of removing the processing inhibitors, the inhibitors were added to the cultures as described above and the cultures were incubated for two days (corresponding to Day 3 of fusion). Half of the plates were stained and quantitated for fusion. In the duplicate plates, the medium was replaced with α-MEM (1% calf serum) containing no processing inhibitors and the cultures were incubated for a further two days followed by quantitation of fusion at Day 5 of fusion.

The fusion index, or percentage of nuclei in myotubes, was determined in fixed and stained cultures according to Parfett *et al.* (1981). The medium was removed and the plates were washed with PBS. Dimethylsulfoxide : $ZnCl_2$ (10^{-3}M) (4 : 1, v:v) was added to plasmolyze the cells. The cells were fixed with methanol for 30 sec and then stained with methylene blue. The number of nuclei within a field of 2 mm diameter was counted using a Biostar inverted microscope (model 1820, American Optical) at 100x magnification. Nine random fields were counted per plate with 600-800 nuclei per field. A myotube was defined as a cell containing more than three nuclei.

Fibroblasts from Duchenne muscular dystrophy patients and age-matched normal donors were obtained from the Repository for Mutant Human cell strains, Montreal

Children's Hospital. The strains of fibroblasts from Duchenne dystrophic (DMD) patients were WG 348 (4.5 yrs), WG 840 (5.5 yrs), WG 502 (5.5 yrs), WG 448 (6yrs), WG 280 (7 yrs), WG 729 (13.5 yrs), WG 730 (15.5 yrs) and the fibroblasts from normal male donors were MCH 48 (5 yrs), MCH 40 (6 yrs), MCH 50 (6 yrs), MCH 52 (7 yrs), and MCH 57 (17 yrs). Three other cell strains were obtained from Dr. K. Wrogemann (University of Manitoba); GM 3871 (DMD-11 yrs) and GM 323 (normal-11 yrs). These were originally obtained from the Institute for Medical Research, Camden, N.J. and Anderson (12 yrs) was from a culture of skin fibroblasts from a local male with Duchenne muscular dystrophy.

The fibroblasts were maintained in α-MEM supplemented with 10% fetal bovine serum and antibiotics (100 units penicillin G per ml and 0.0685 mg/ml streptomycin sulphate). Cells were removed from plates with 0.1% trypsin and 0.02% EDTA in PBS. When the fibroblasts achieved rounded appearance, they were washed from the plate in PBS and divided equally onto two 100 mm culture plates or onto four 60 mm plates for assays. The fibroblast cultures were maintained for only 20 passages (a passage equals one doubling) and then discarded. Early passages were frozen in medium containing 10% fetal bovine serum and 5% dimethylsulfoxide, stored in liquid N_2 or a -80°C freezer, and retrieved when fresh cultures were required.

2. Glycosidase Assays

Con A-resistant variant cells and their parental L6 wild type myoblasts, and Duchenne dystrophic and normal fibroblasts were grown to confluence on 100 mm plates, medium was removed, and the cells were rinsed with 5 ml of PBS. Distilled water (0.5 ml) was added to each plate and the cells were removed by scraping with a rubber policeman. The cell suspension was sonicated for 60 sec (four 15 sec intervals) at 4°C at 60 W with a 3mm probe using an Artek Sonic Dismembrator, and then

centrifuged for one minute at 8000 g_{av} in an Eppendorf 3200 centrifuge to remove any remaining particulate matter. The supernatant (cell extract) was used for the assays.

In order to assay the glycosidase activity in the culture medium, cells were grown to near confluence and 24 hours before harvesting, the medium was removed and cells were washed with PBS. Medium containing heat-inactivated calf serum (2 hr at 60°C, Blaschuk *et al.*, 1980b) was added. Just prior to harvesting the cells, the medium was removed and centrifuged at 4300 g_{av} in a Sorvall RC 2-B centrifuge at 4°C and the supernatant was used in the extracellular enzyme assays.

Glycosidase activities were assayed according to the method of Blaschuk *et al.* (1980a,b). The assay contained: up to 30 µl of cell extract or 50 µl of extracellular medium; 0.1 M citric acid (adjusted to the required pH with Na_2HPO_4) and the appropriate substrate. For the measurement of α-mannosidase activity, 2.5 mM 4-methylumbelliferyl-α-D-mannoside was the substrate, and the total assay volume was 0.2 ml. The substrates for the measurement of the remaining glycosidase activities were 1.6 mM *p*-nitrophenyl-glycopyranosides (*p*-nitrophenyl-N-acetyl-ß-D-glucosaminide for ß-hexosaminidase; *p*-nitrophenyl-α-L-fucoside for fucosidase; *p*-nitrophenyl-ß-D-galactose for galactosidase and *p*-nitrophenyl-α-D-glucose for glucosidase) with a total assay volume of 0.5 ml. Assays were incubated at 37°C for up to 120 min and the reactions were stopped with 0.2 M glycine (adjusted to pH 9.5 with NaOH) to a final volume of 2.0 ml. After centrifugation of samples for 2 min at maximum speed in an Eppendorf 3200 centrifuge, the absorbance or fluorescence of the supernatants was determined. In assays with the *p*-nitrophenyl substrates, the absorbance of the product, *p*-nitrophenyl, was determined at 400 nm and converted to concentration units using the molar extinction coefficient of 1.77×10^4 M^{-1} cm^{-1}. For fluorescence assays with 4-methylumbelliferyl glycosides, the fluorescence of the product, 4-methylumbelliferone, was measured on a Carl Zeiss PMQ Spectrophotometer equipped with a ZFM 4 fluorescence attachment. The excitation

wavelength was 365nm (mercury vapour lamp) with an emission wavelength of 466 nm. Assays were compared to standards containing up to 10 μg of 4-methylumbelliferone in 0.2 M glycine-NaOH stopping buffer, pH 9.5. A unit of enzyme activity for both assay systems is defined as the amount of enzyme required to hydrolyze 1 nmol of the appropriate glycoside per min.

Protein was assayed using the Bio-Rad method for protein determination (Bio-Rad Technical Bulletin 1051) for the glycosidase assays and for all other protein determinations.

3. Sialyl Transferase and Galactosyl Transferase Assays

Wild type and con A-resistant L6 myoblasts (clone 5) were grown to near confluence on 100 mm plates (2-6 x 10^6 cells/plate) and cell extracts for the assays were prepared as described above for the glycosidase assays. For assay of sialyl and galactosyl transferase, acceptors were prepared from fetuin and α_1-acid glycoprotein. Sialic acid was removed by mild acid hyrolysis in 0.05 N H_2SO_4 at 80°C for 1 hour (Jamieson 1977; Turchen *et al.*, 1977; Kaplan *et al.*, 1983) followed by dialysis overnight and lyophilization. Galactose was removed with ß-galactosidase (Turchen *et al.*, 1977) isolated from Jack bean meal by the method of Li and Li (1972) . Asialo fetuin and asialo α_1-acid glycoprotein (6-14 mg/ml) were incubated with 8 mUnits of ß-galactosidase in McIlvaine buffer (citric acid-Na_2HPO_4), pH 4.0 for 24 hrs at 37°C. The reaction was stopped by boiling for 5 min, followed by dialysis overnight and lyophilization.

Sialyl and galactosyl transferase activities were assayed in systems adapted from the methods of Baxter and Durham (1979) and Toyama *et al.* (1983) using conditions optimized for the L6 myoblasts. The sialyl transferase system with asialo fetuin as the acceptor contained: 120 nCi CMP-[^{14}C]-NeuAc (250mCi/mmol); up to 200 μg acceptor; 125 mM MES buffer, pH 6.6 and up to 100 μl of sonicated cell extract in a

total assay volume of 200 µl. The sialyl transferase assay system using asialo α_1-acid glycoprotein as the acceptor contained 20 nCi CMP-[^{14}C]-NeuAc (4mCi/mmol), up to 200 µg acceptor, 125 mM MES buffer, pH 6.6 and up to 50 µl of cell extract in a total volume of 150 µl. The galactosyl transferase assay system with either asialoagalacto fetuin or asialoagalacto α_1-acid glycoprotein contained: 120 nCi UDP-[^{14}C]-Gal (6 mCi/mmol); up to 200 µg of acceptor; 125 mM MES buffer, pH 6.0; 5mM $MnCl_2$ and up to 100 µl of sonicated cell extract in a total assay volume of 200 µl. Endogenous activity was measured in the absence of the acceptor glycoproteins and was subtracted from the total activity. Incubations were for 4 hours at 37°C. The reactions were stopped by placing the tubes on ice and a total of 80 µl was spotted on two 2.5 cm Whatman paper discs (40 µl each). The discs were washed three times in ice cold 10% (w/v) trichloroacetic acid (10 min/wash), followed by a wash in 2:1 (v/v) ethanol:ether with a final wash in ether. After drying, the discs were counted using 10 ml ACS liquid scintillation fluid in an LKB 1215 Rackbeta II liquid scintillation counter.

4. Con A and WGA Binding Assays

Concanavalin A and WGA (wheat germ agglutinin) were iodinated with ^{125}Iodine [^{125}I] using the chloramine T method of Tanner and Anstee (1976). Con A (20 mg) was dissolved in 1 ml of saline solution (0.85% NaCl, 0.01 M NaN_3, 0.001 M $CaCl_2$, 0.001 M $MnCl_2$, 0.001 M $MgCl_2$, pH 7.0) with 0.3 M methyl-α-D-mannoside to protect the active site. WGA (3.0 mg) was dissolved in 1 ml of 0.05 M Tris-HCl and 0.15 M NaCl buffer, pH 8.6 with 6 mg of N-acetylglucosamine present to protect the active site. Labelled con A or WGA was separated from free ^{125}I on a Bio-Gel P10 column (30 x 1 cm) using the Tris-saline solution or PBS for elution.

For the myoblast lectin binding experiments, the con A binding assay used the method of Parfett *et al.* (1981) and the WGA binding assay followed the method of

Pohajdak *et al.* (1984). The L6 myoblasts (clone 5) and the respective con A-resistant variant cells were plated on 60 mm culture dishes at a density of 6 x 10^4 cells per ml in 4 ml of α-MEM supplemented with 10% calf serum and antibiotics. For assays in which the effects of the glycoprotein processing inhibitors were to be determined, the inhibitors, castanospermine (up to 0.1mM), swainsonine (up to 6μM), 1–deoxynojirimycin (up to 5mM), N-methyldeoxynojirimycin (up to 4mM), and 1–deoxymannojirimycin (up to 1mM), were added to the freshly plated cells from stock solutions made up in sterile PBS. At 16-20 hours prior to the binding assay, when the myoblasts were approaching confluence, the medium was removed and replaced with medium containing no calf serum and the processing inhibitors were added.

In reversal experiments, the myoblasts were cultured in the presence of the inhibitors for 3 days and the medium was replaced with medium containing 10% calf serum (with no processing inhibitors) and the cells were grown for a further 2 days. At 16-20 hours prior to the binding assays, the medium was also removed and replaced with medium containing neither calf serum nor the processing inhibitors.

The cultures were prepared for the binding assay by pre-cooling the plates to 4°C for 30 min. The medium was replaced with 1 ml of binding buffer (for con A assays: 0.85% NaCl, 0.01 M NaN_3, 0.001 M $CaCl_2$, 0.001 M $MnCl_2$, 0.001 M $MgCl_2$, pH 7.0; and for WGA: 10 mM HEPES buffered α-MEM, pH 7.2 with 0.1% NaN_3) and incubated at 4°C for 30 min. The buffer was removed and the cells were washed twice with 1 ml of the same solution. The assay was started by the addition of 0.5 ml binding solution containing 100 μg/ml of [^{125}I]-con A or [^{125}I]-WGA (2 x 10^5 cpm/ml) and the plates were incubated for 20 min at 4°C with occasional agitation. The medium was aspirated and the plates were washed 5 times with 1 ml of binding buffer. The cells were dissolved overnight in 1 ml of 10% Triton X-100. The contents of each plate were transferred to a scintillation vial using a rubber policeman, 10 ml of ACS cocktail was added and samples were counted in an 1215 LKB Rackbeta II liquid

scintillation counter. Duplicate plates containing 0.2 M methyl-α-D-mannoside (con A binding assays) or 0.2 M N–acetylglucosamine (WGA binding assays) were included in the assay to determine non-specific binding (typically 3-5% of the total binding activity) which was subtracted from the total binding. Duplicate plates were used for protein assays.

The assay system used to determine con A binding in fibroblasts from Duchenne dystrophic patients and age-matched controls, was similar to the myoblast binding assays except that the time of incubation was 1 hour at 4°C and the cells were solubilized overnight in 1 ml of 0.1 N NaOH at 37°C and then neutralized with 0.1 ml of 1N HCl. A hemocytometer was used to determine the number of cells per plate in duplicate plates of those used in the binding assay.

5. Insulin Binding Assays

Rat L6 wild type myoblasts (clone 5) and the con A-resistant cultures were grown to near confluence and removed by trypsin treatment and replated at a density of 2 x 10^5 cells on 35 mm plates in 2 ml of α-MEM supplemented with 10% calf serum and antibiotics. In experiments where glycoprotein processing inhibitors were used, the appropriate concentrations were added to the freshly plated cultures. When the cultures approached confluence (16-20 hrs prior to the assay), the medium was replaced with medium containing no calf serum and the processing inhibitors were added to the cultures at the original concentrations. To prepare the cultures for the binding assay, the plates were equilibrated at 4°C for 30 min followed by two washing steps with 2 ml of binding buffer (20 mM HEPES buffered α-MEM, pH 7.6). To begin the assay, 0.5 ml of binding medium containing 1% bovine serum albumin (BSA), bovine insulin at concentrations of 10^{-4} and 10^{-10} M and [^{125}I]-insulin (2 x 10^5 cpm/ml) were added and the plates were incubated for 4 hours at 4°C with occasional agitation. Following the incubation, medium was aspirated and plates were

washed 4 times with 2 ml of binding medium and then solubilized overnight in 1.0 ml of 0.1% Triton X–100. The remainder of the assay followed the procedure described for the lectin binding assays, except that a 100 μl aliquot was removed for protein determination. Non-specific binding was measured at 10^{-4} M insulin and was normally subtracted from the binding activity at 10^{-10} M insulin. In assays where insulin binding was studied in relation to fusion of the myoblasts, duplicate plates were stained and quantitated for fusion.

6. Creatine Phosphokinase Assay

Rat L6 myoblasts (clone 5) were plated on 60 mm plates as described for the fusion assays and when the cells had reached confluence and random fusion events were evident, the medium was replaced with medium containing 1% calf serum and glycoprotein processing inhibitors were added to the plates at appropriate concentrations. When control cultures, which contained no inhibitors, had reached 50% fusion (2-3 days post confluence), or 80% fusion (5 days post confluence), the medium was removed and the cells were washed twice with PBS. The cells were removed from the plates with the addition of 0.5 ml of 0.05 M glycylglycine buffer, pH 6.75 and scraped with a rubber policeman, followed by sonication for 15 sec as previously described. Creatine phosphokinase activity was measured according to the method of Shainberg *et al.* (1971), which is dependent on a linked enzyme system with glucose-6-phosphate dehydrogenase and hexokinase. Diadenosine phosphate (10μM) was added to inhibit adenylate kinase (Leinhard and Secemski, 1973). The rate of production of NADPH was measured at 340 nm in a Gilford Spectrophotometer. A mU of creatine phosphokinase activity is defined as the production of 1 nmol of NADPH per min at 20°C.

7. Incorporation of [^{3}H]-leucine

Wild type L6 myoblasts (clone 5) and their con A-resistant variants were plated as described for the fusion assays except on 35 mm plates with 2 ml of medium containing the glycoprotein processing inhibitors at the same concentrations used in the fusion assays. When the cultures approached confluence, the medium was replaced with 1 ml of α-MEM, and the glycoprotein processing inhibitors were added at the original concentrations. Upon the addition of 20 μCi of [4,5-^{3}H]-leucine, the cells were incubated for 2 hr at 37°C. The cells were washed with PBS, 0.5 ml of water was added per plate, and cells were removed from the plates by scraping with a rubber policeman. The cells were disrupted by sonication as previously described. Samples of 40 μl were spotted on 2.5 cm Whatman No. 1 paper discs and washed and counted as described for the glycosyl transferase assays. An aliquot of the sample was assayed for protein.

8. Uptake of 2-deoxy-D-[^{3}H]-Glucose

The uptake of 2-deoxy-D-[^{3}H]-glucose in wild type L6 myoblasts was measured according to the method of Standaert *et al.* (1984). Myoblasts were plated and grown to confluence as described for the insulin binding assays. The culture medium was removed and the cells were rinsed three times with 1 ml of Dulbecco's PBS (138 mM NaCl, 8.1 mM Na_2HPO_4, 0.5 mM $MgCl_2$, 1.5 mM KH_2PO_4, 2.6 mM KCl, and 0.1 mM $CaCl_2$, pH 7.4) containing 0.1% bovine serum albumin. The cells were pre-incubated for 10 min at 37°C with 900 μl of the above solution, followed by the addition of 20 μl of buffer containing insulin to attain concentrations of 10^{-6} M, 10^{-7}M, 10^{-9} M and 10^{-11} M. After a pre-incubation in the presence of insulin for 25 min, 80 μl of buffer containing 10 nmol of 2-deoxy-D-[^{3}H]-glucose (100 μCi/μmol) were added and the cultures were incubated for a further 5 min. The plates were rapidly washed with ice cold PBS (4 x 1ml) and lysed with 1 ml of Triton-X 100

overnight. The contents of the plates were transferred to vials, 10 ml of ACS scintillation fluid were added and the vials were counted in a scintillation counter.

9. Preparation of D-[1-^{14}C]-mannose-labelled Glycopeptides from L6 Myoblasts.

Wild type and con A-resistant myoblasts were plated at a density of 4 x 10^5 cells/plate on 100 mm plates and grown to confluence in medium containing 10% calf serum and antibiotics. Cells were incubated for an additional 24 hrs following the addition of 5 µCi of D-[1-^{14}C]-mannose (57 mCi/mmol) per plate (Romero *et al.*, 1985). The myoblasts were washed twice with 5 ml of PBS; 1 ml of water was added and the cells were removed from the plate by scraping with a rubber policeman.

The lipid-oligosaccharide was removed by three extractions of the cell extract with chloroform : methanol : water (10 : 10 : 3, v:v:v) (Sasak *et al.*, 1982). The collected aqueous layer, containing the cell protein, was partially evaporated under N_2 to remove the methanol and 100 µg of Pronase was added in 1 ml of phosphate buffered saline, pH 7.9 with10 mm $CaCl_2$. This was incubated for 3 days at 37°C in the presence of toluene and an additional 100 µg of Pronase was added every 24 hrs. The reaction was stopped by boiling for 3 min (Sasak *et al.*, 1982).

10. Fractionation of Glycopeptides and Oligosaccharides.

The glycopeptides prepared by Pronase B digestion of the D-[1-^{14}C]-mannose labelled myoblasts were separated on a Bio-Gel P4 (200-400 mesh) column (1.5 x 200 cm). Water was used as the liquid phase with a flow rate of 6 ml/hr; fractions of 2.5 ml were collected and 50 µl of each fraction was counted for radioactivity. The radioactive peaks were pooled as indicated in the Results section and lyophilized.

The pooled fractions were digested with endo-ß-N-acetylglucosaminidase H (endo H) according to the method of Sasak *et al.* (1982). The samples were dissolved in 50 mM citrate-phosphate buffer, pH 5.0 containing 5 mUnits of endo H and incubated for

3 days at 37°C in the presence of toluene. The reaction was stopped by boiling for 3 min and the samples were centrifuged for 5 min at maximum speed in a Fisher micro-centrifuge (Model 235A) to remove particulate matter.

The oligosaccharides were chromatographed on a guard column (0.46 x 5 cm) followed by a column (0.46 x 25 cm) packed with 5 µm particle size Aminospherisorb (Chromatography Sciences Co.) linked to a Perkin-Elmer Series 4 Liquid Chromatograph, using a modification of the method of Romero *et al.* (1985b). A sample of 100 µl was applied to the column which had been equilibrated in water : acetonitrile (60:40, v/v). A 14 min elution with water : acetonitrile (60:40) at a flow rate of 1.5 ml/min was followed by a linear gradient over 65 min to water : acetonitrile (60:40) and the elution was completed with 10 min of water : acetonitrile (60:40). Fractions of 1 ml were collected directly in scintillation vials and 4 ml of ACS scintillation fluid was added. The samples were counted in a LKB 1215 Rackbeta liquid scintillation counter. Standards of [^{14}C]-mannose and GlcNAc-Man_9-Glc_{1-3} containing a [^{14}C]-glucose label (a gift from Dr. A. Herscovics, McGill University, Montreal) were used as standards.

RESULTS

A. *Effect of glycoprotein processing inhibitors on L6 myoblast differentiation.*

1. The effect of glycoprotein processing inhibitors on fusion capacity of L6 myoblasts.

The involvement of high mannose oligosaccharides in myoblast fusion was previously demonstrated with the correlation of lack of fusion with defective mannose incorporation into high mannose oligosaccharides in con A-resistant L6 myoblasts (Parfett *et al.*, 1981, 1983). Through the use of glycoprotein processing inhibitors, manipulation of the N-linked oligosaccharide allows a study of the effect of changing oligosaccharide structure as an alternative to mutant selection. Five glycoprotein processing inhibitors were available for this study. 1–Deoxynojirimycin, N–methyldeoxynojirimycin, and castanopsermine are glucosidase inhibitors which block the removal of the three terminal glucose residues of the G oligosaccharide. Deoxymannojirimycin is a mannosidase I inhibitor which blocks removal of four mannose residues from the Man_9-oligosaccharide and swainsonine is a mannosidase II inhibitor which results in the formation of hybrid oligosaccharides.

Wild type L6 myoblasts in culture normally fuse to incorporate approximately 80% of the nuclei into myotubes within five days post confluence. With the addition of the glucosidase inhibitors to the media of confluent cultures, the fusion capacity of the wild type myoblasts (clone 5) was reduced to less than 20% at five days post confluence (Figure 11, panel A). Similarly, in the presence of the mannosidase II inhibitor, swainsonine, fusion of wild type myoblasts was less than 20% (Figure 11, panel B). However, addition of deoxymannojirimycin (0.5 mM), the mannosidase I inhibitor, had a minimal efffect on fusion; myoblasts reached a fusion index of 75%. Concentrations of 1.0 mM deoxymannojirimycin, which is known to effectively inhibit mannosidase I activity (Fuhrmann *et al.*, 1985), also had a negligible effect on myoblast

Figure 11

The effect of oligosaccharide processing inhibitors on L6 myoblast fusion. Cells were grown to confluence (4-5 days) and the medium was replaced with medium containing oligosaccharide processing inhibitors Panel A: fusion index in control (●) cultures and cultures treated with castanospermine (▲), 1-deoxynojirimycin (❑), and N-methyldeoxynojirimycin (Δ). Panel B: fusion index in control (●) cultures and cultures treated with swainsonine (■) and deoxymannojirimycin (O). The fusion index is the number of nuclei within myotubes as a percentage of the total nuclei. A myotube was defined as a cell containing 3 or more nuclei. Results represent the mean of 4-6 plates for each point and over 2500 nuclei were counted per plate; the S.D. was within ±8% for the control and 1-deoxymannojirimycin treated cultures, and ±10% for the other cultures.

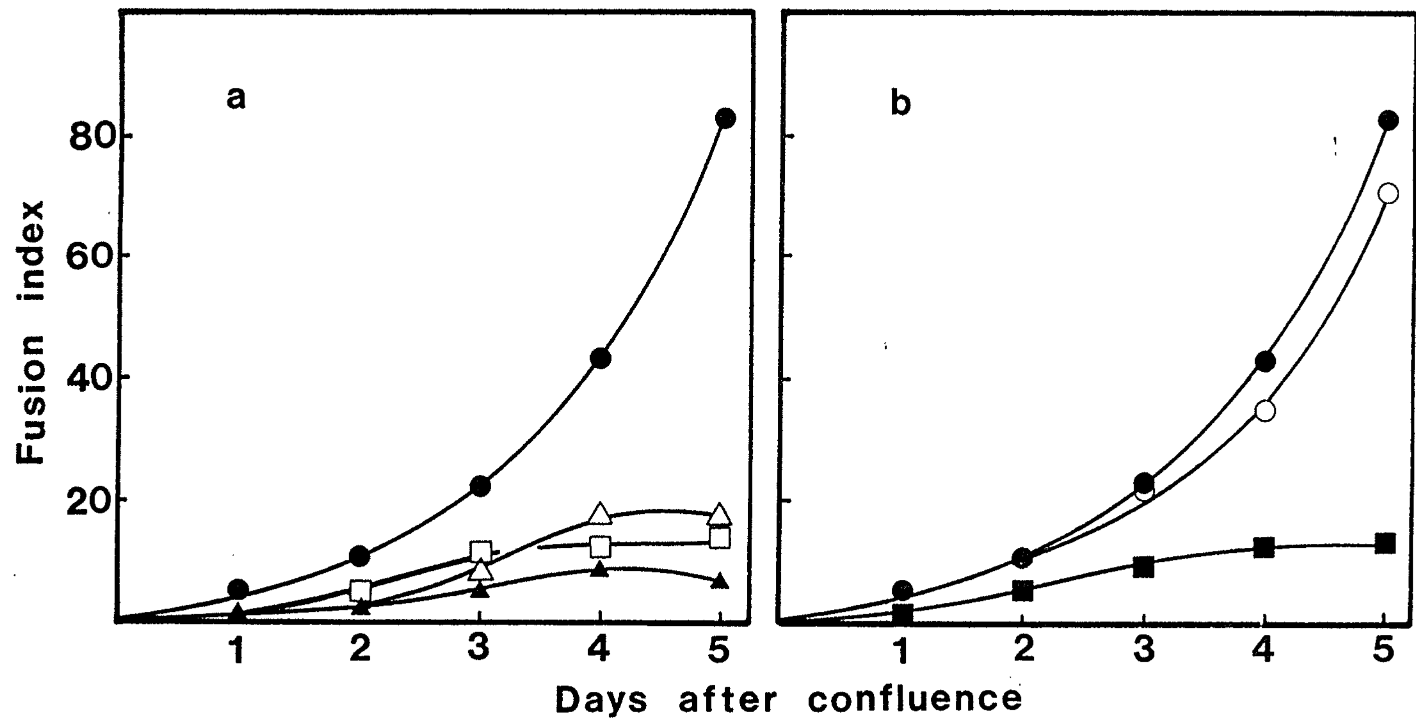

a
b
80
60
40
20
Fusion index
1
2
3
4
5
Days after confluence

Table 4 Effect of removing processing inhibitors on the fusion index of L6 myoblasts

Inhibitor	Fusion Index, %[a]	
	Day 3	Day 5
Control	30.8 ± 2.5	54.8 ± 4.6
N-Methyldeoxynojirimycin, 1mM	15.1 ± 1.9	45.4 ± 3.2
1-Deoxynojirimycin, 2.5mM	20.1 ± 2.1	47.3 ± 3.5
Castanospermine, 0.1mM	14.7 ± 1.7	40.3 ± 2.7
1-Deoxymannojirimycin, 0.5mM	27.7 ± 2.1	61.1 ± 3.1
Swainsonine, 2.6μM	20.7 ± 2.0	39.9 ± 2.6

[a] On day 1, the first day of confluence, the inhibitors are added. Values for Day 3 represent the fusion index of myoblasts which are two days post confluence. In a duplicate set of plates, the medium was replaced with fresh medium containing no inhibitors and the fusion index was determined after a further two days, which are represented by Day 5 values. The control values are less than the fusion index of other experiments because fusion is sensitive to the frequent medium changes which are necessary to match medium changes in inhibitor treated plates.

fusion capacity (data not shown). Con A-resistant myoblasts (clone 5) which are normally unable to fuse, remained incapable of fusion when cultured with the processing inhibitors.

The inhibition of fusion by the processing inhibitors was reversible (Table 4). After three days of post confluent culture in the presence of the processing inhibitors, fusion was reduced or not affected as described above. Replacement of the media with fresh media containing no inhibitors allowed a significant increase in fusion capacity in the cultures previously treated with the glucosidase and mannosidase II inhibitors. Control and reversal cultures did not reach the normal fusion capacity of approximately 80% because of delayed fusion due to the presence of the inhibitors, and sensitivity of fusion to the required medium changes.

2. Effect of glycoprotein processing inhibitors on creatine phosphokinase activity in L6 myoblasts.

Creatine phosphokinase (CPK) is a good marker enzyme for the measurement of biochemical differentiation which accompanies fusion. Differentiating myoblasts from control and glycoprotein processing inhibitor-treated plates were assayed for creatine phosphokinase activity when the control cultures had reached 50 and 80% fusion (Table 5). Although control levels of CPK activity were fairly low at 50% fusion, the myoblasts grown in the presence of methyldeoxynojirimycin, deoxynojirimycin, castanospermine and swainsonine had substantially lower CPK activities. CPK levels in deoxymannojirimycin-treated cultures remained comparable to control values. At 80% fusion in the control cultures, cultures treated with the glucosidase inhibitors were reduced in CPK activity by 40-60%, and CPK activities in swainsonine-treated cultures were reduced by 55%. However, deoxymannojirimycin treatment resulted in only a 20% decrease in CPK activity compared with control cultures. The

Table 5 Effect of oligosaccharide processing inhibitors on creatine phosphokinase activity in L6 myoblasts[a]

Inhibitor	Creatine phosphokinase activity, (mUnits/mg protein)	
	50% Fusion[b]	80% Fusion[b]
Control	12.0 ± 1.5	49.5 ± 1.9
N-Methyldeoxynojirimycin, 1mM	6.0 ± 0.5	20.1 ± 3.8
1-Deoxynojirimycin, 2.5mM	8.8 ± 1.1	28.9 ± 2.5
Castanospermine, 0.1mM	6.3 ± 0.5	20.5 ± 2.4
1-Dexoymannojirimycin, 0.5mM	12.3 ± 0.4	39.4 ± 1.5
Swainsonine, 2.6μM	8.6 ± 0.8	27.8 ± 0.6

[a] Control plates, together with corresponding test plates containing oligosaccharide processing inhibitors,were allowed to fuse;

[b] creatine phosphokinase activity was measured in all plates when corresponding control plates with no inhibitors had reached 50% and 80% fusion; creatine phosphokinase was assayed as described in Methods; pre-confluent myoblasts expressed 2-3mUnits/mg protein of creatine phosphokinase activity. Results are means from 2-4 determinations ±SD.

glycoprotein processing inhibitors appear to have similar effects on morphologic and biochemical differentiation processes.

3. Effects of glycoprotein processing inhibitors on con A and WGA binding capacities in L6 myoblasts.

The effects of glycoprotein processing inhibitors on the oligosaccharide structure of several glycoproteins a variety of cellular systems have been defined and documented (see section C. of Historical Review). However, it was necessary to correlate their effects on myoblast fusion and biochemical differentiation with alterations in the cell surface carbohydrate structure. An effective, yet relatively simple, method for measuring gross changes in cell surface oligosaccharide content is through the use of lectin binding assays. Con A, with a high affinity for mannose and a slightly lower affinity for glucose residues, will bind tightly to high mannose oligosaccharides on the cell surface, and also has a slight affinity for biantennary complex oligosaccharides . WGA (wheat germ agglutinin) has an affinity for N-acetylglucosamine and sialic acid residues and thus will effectively bind oligosaccharides only of the complex type.

Glycoprotein processing inhibitors were added to freshly plated cultures of con A-resistant and wild type L6 myoblasts and lectin binding capacities were measured when myoblasts had reached confluence on the plates. Castanospermine (up to 0.1 mM), methyldeoxynojirimycin (up to 4 mM) and deoxynojirimycin (up to 6 mM) all resulted in substantial increases in con A binding capacity in both wild type and con A-resistant myoblasts with concurrent decreases in WGA binding capacities (Figure 12). Deoxymannojirimycin (up to 1.0 mM) resulted in an increase in con A binding capacity by approximately 60% in the wild type and 40% in the variant, accompanied by a decrease in WGA binding capacity (Figure 13). Swainsonine (up to 5.6 μM) significantly increased the amount of con A bound to the wild type myoblasts,

Figure 12

The effect of the glucosidase processing inhibitors, castanospermine, 1–deoxynojirimycin and N–methyldeoxynojirimycin on con A and WGA binding in clone 5 wild type and con A resistant L6 myoblasts. Top panels: con A binding in wild type (●) and con A-resistant (■) myoblasts in the presence of the inhibitors. Bottom panels: WGA binding in wild type (●) and con A-resistant (■) myoblasts. Results are expressed as percentages of control values (with no inhibitor present) which represent 100%. Results are means from three plates for each point; the S.D. was within ±12%.

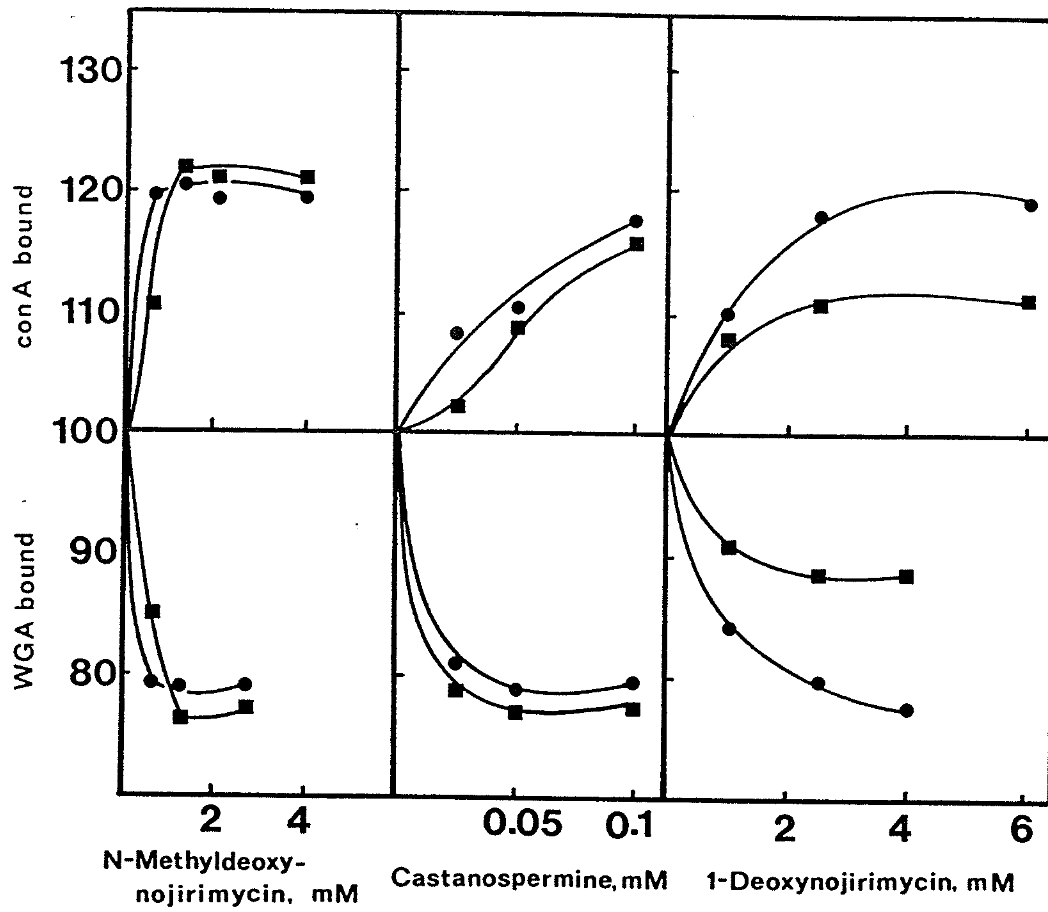
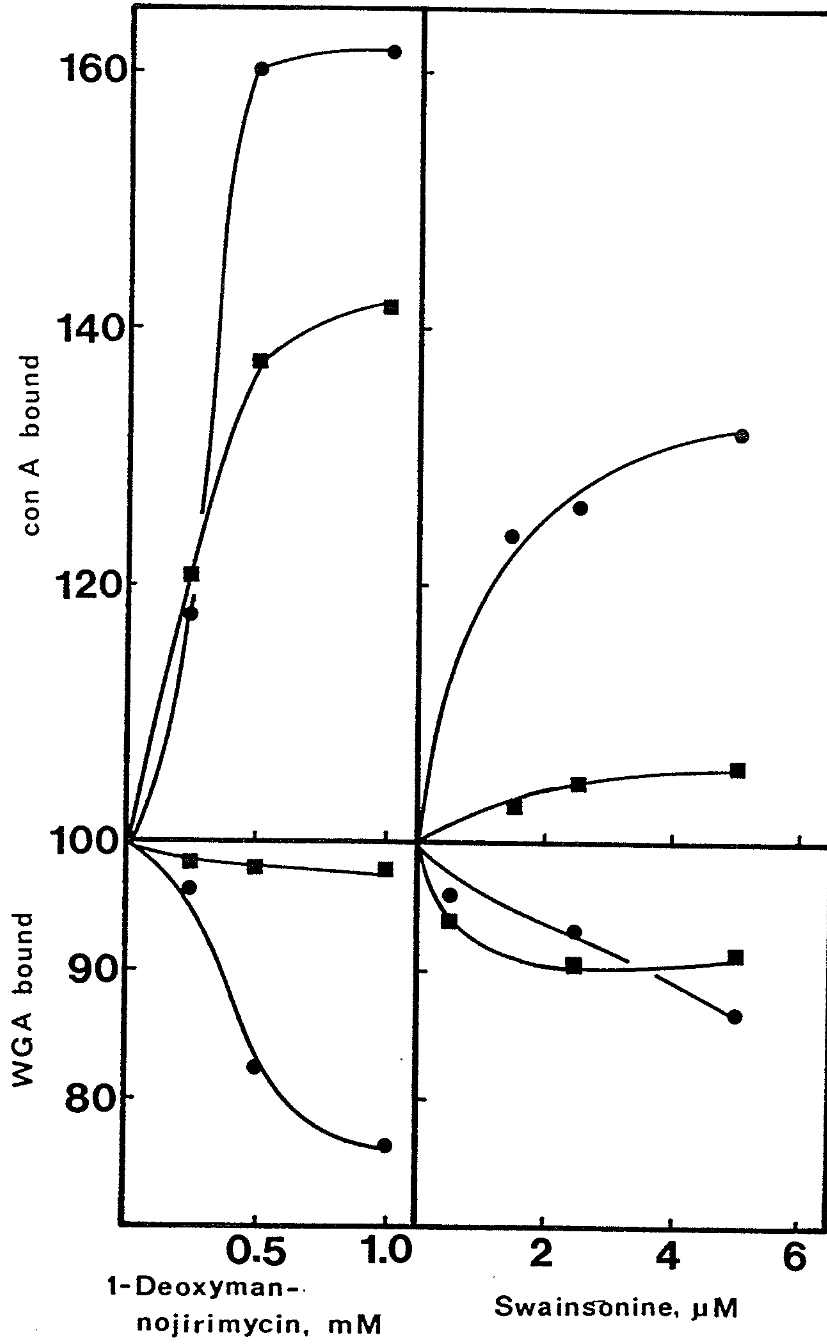

130
120
110
100
90
80
con A bound
WGA bound
2
4
0.05
0.1
2
4
6
N-Methyldeoxy-
nojirimycin, mM
Castanospermine, mM
1-Deoxynojirimycin, mM

Figure 13

The effect of the α-mannosidase processing inhibitors on con A and WGA binding to clone 5 wild type and con A-resistant L6 myoblasts. Top panels: con A binding to wild type (●) and con A-resistant (■) myoblasts in the presence of 1–deoxymannojirimycin and swainsonine. Bottom panels: WGA binding to wild type (●) and con A-resistant (■) myoblasts in the presence of 1-deoxynojirimycin and swainsonine. Results are expressed as a percentage of control values (with no inhibitor present). Results are means from three plates for each point; the S.D. was within ±12%.



160
140
120
100
90
80
con A bound
WGA bound
0.5
1.0
2
4
6
1-Deoxyman-
nojirimycin, mM
Swainsonine, μM

Table 6 Effect of removing processing inhibitors on binding of con A and WGA to L6 myoblasts

Inhibitor	con A bound[a]		WGA bound[a]	
	+inhibitor	reversal	+inhibitor	reversal
Control	100	100	100	100
N-methyldeoxynojirimycin, 1mM	120	104	70	99
1-Deoxynojirimycin, 2.5mM	117	99	82	102
Castanospermine, 0.1mM	118	99	83	93
1-Deoxymannojirimycin, 0.5mM	163	97	84	97
Swainsonine, 2.6μM	124	99	93	93

[a] Lectin binding is expressed as a percentage relative to controls without inhibitors which were taken as 100%. In the reversal experiments, cells were exposed to inhibitors for three days; inhibitors were removed for two days and lectin binding experiments were performed as in Methods. In the reversal experiments parallel plates were monitored for fusion. The results are means from three experiments; the S.D. was within ±5%.

however, the con A binding capacity of the variant was only increased by approximately 5% (Figure 13). Swainsonine treatment was also accompanied by a decrease in WGA binding capacity. Both con A and WGA binding returned to near control values following the removal of the inhibitors from the culture media (Table 6). Non-specific binding, measured in the presence of α-methylmannoside for con A binding and N-acetylglucosamine for WGA binding, were normally less than 15% of the total binding and have been subtracted from the values.

4. Effect of glycoprotein processing inhibitors on protein synthesis in L6 myoblasts.

The incorporation of [^{3}H]-leucine into protein in wild type and con A resistant-myoblasts was measured in the presence of the glycoprotein processing inhibitors (Table 7), to ensure that the decrease in differentiation was not due to an inhibition of protein synthesis which in itself may affect differentiation. Although the overall incorporation of radioactive label was lower in the con A-resistant variant, there appeared to be no substantial differences in inhibitor treated cultures as compared to control cultures. Thus the inhibition of myoblast fusion and creatine phosphokinase activity by the glycoprotein processing inhibitors cannot be explained by changes in the ability of the cells to synthesize protein.

Table 7 Incorporation of $[^3H]$-leucine into protein of wild type and con A-resistant L6 myoblasts in the presence of oligosaccharide processing inhibitors[a]

Inhibitor	$[^3H]$-leucine incorporation (pmoles/mg protein/hr)	
	wild type L6C15	con A-resistant L6C15
Control	11.4 ± 0.7	7.5 ± 0.9
N-Methyldeoxynojirimycin, 1mM	10.3 ± 1.7	9.5 ± 1.2
1-Deoxynojirimycin, 2.5mM	11.1 ± 0.2	9.6 ± 1.0
Castanospermine, 0.1mM	10.1 ± 1.0	8.2 ± 0.8
1-Deoxymannojirimycin, 0.5mM	9.9 ± 0.3	7.3 ± 0.6
Swainsonine, 2.6μM	10.1 ± 0.1	7.7 ± 0.5

[a] Results are means of five determinations ± SEM.

B. *Glycosidase activities in L6 myoblasts.*

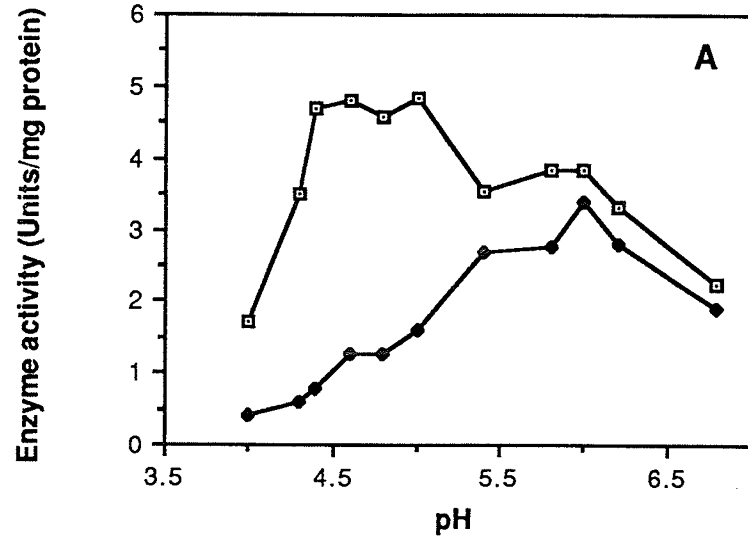
1. Acidic and neutral glycosidase activities in wild type and con A-resistant myoblasts.

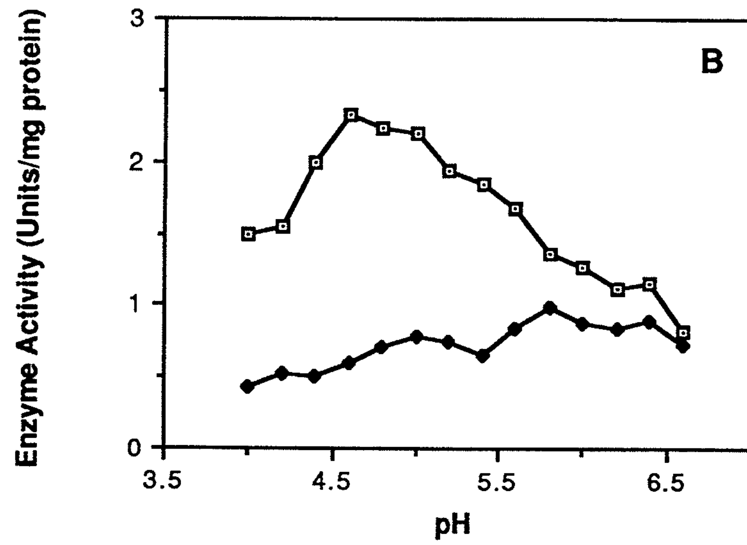
Lectin resistant mutants often display complex pleiotropic phenotypes with membrane-associated modifications in comparison with the corresponding parental wild type cell line (Wright, 1973; Ceri and Wright, 1978). The con A-resistant myoblasts have a reduced incorporation of mannose into lipid-sugars and lipid-oligosaccharides. This would affect all glycoproteins but those most susceptible to these changes in glycosylation would be glycoproteins dependent upon their high mannose oligosaccharides for structural or functional properties, such as the lysosomal enzymes which require the mannose-6-phosphate recognition signal on the high mannose oligosaccharides for targetting to the lysosomes. The basis for studying the glycosidases in the con A-resistant and wild type myoblasts was to determine if any differences in enzyme activity existed. Defective glycosylation in the con A-resistant myoblasts could result in changes in activity, and also secretion patterns of the lysosomal enzymes.

Wild type myoblasts of clone 2 and clone 5 and their respective con A-resistant variants (Parfett *et al* ., 1981) were assayed for several glycosidase activities. In initial studies, α-fucosidase (α-L-fucosidase, EC 3.2.1.51), ß-galactosidase (ß-D-galactosidase, EC 3.2.1.23), and ß-hexosaminidase (N-Acetyl-ß-D-glucosaminidase, EC 3.2.1.30) were assayed using *p*-nitrophenyl glycopyranoside derivatives in a colorimetric assay. α-Mannosidase (α-D-mannosidase, EC 3.2.1.24) assay required the use of a more sensitive assay due to very low activity and therefore 4–methylumbelliferyl-α-D-mannoside was used in a fluorimetric assay.

Figure 14

The effect of pH on α-mannosidase activity in clone 2 (Panel A) and clone 5 (Panel B), wild type (❑) and con A-resistant (◆) L6 myoblasts. One unit of enzyme activity represents the amount of enzyme required to hydrolyze 1 μmol of 4–methylumbelliferyl-α-D-mannosidase per minute. Results represent the mean of 2-4 assays and S.D. are with ±15%.


A
Enzyme activity (Units/mg protein)
0
1
2
3
4
5
6
3.5
4.5
5.5
6.5
pH

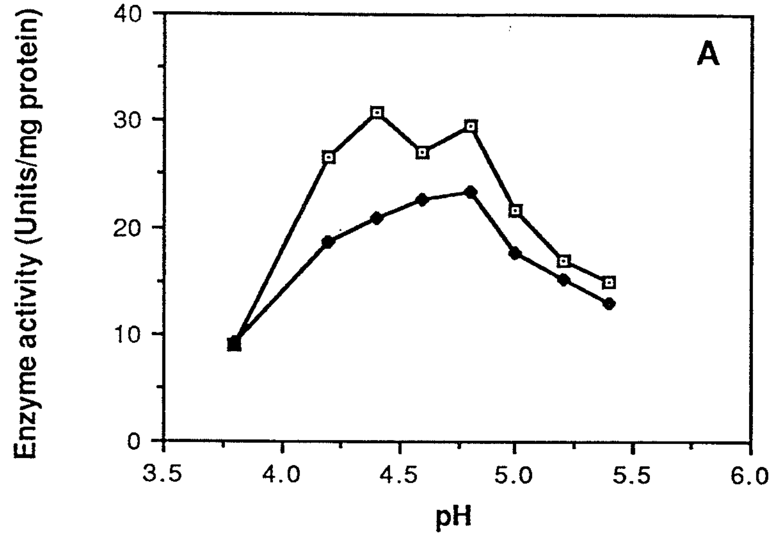

B
Enzyme Activity (Units/mg protein)
0
1
2
3
3.5
4.5
5.5
6.5
pH

The pH profile of mannosidase displayed the most dramatic differences between the wild type and con A-resistant myoblasts (Figure 14). In clone 2 myoblasts the wild type had two slightly overlapping, yet distinct, pH optima of activity at pH 4.6 and 6.0, which were designated as the acidic and neutral pH optima, respectively. The clone 5 wild type had an acidic pH optimum of 4.5, but a less obvious neutral pH optimum, which also may be due to a slight overlapping of two different activities. A substantial reduction in the acidic α-mannosidase activity of both clone 2 and clone 5 con A-resistant variants was observed, with only approximately 20% of the wild type activity at pH 4.6. Only slight reductions in the neutral α-mannosidase activity of the variant were apparent.

The pH profiles for ß-hexosaminidase had slightly reduced levels of activity for both clone 2 and clone 5 con A-resistant variants compared with their respective wild types (Figure 15). A fairly broad optimum range was evident, from pH 4.2 to 4.8 in clone 2 and from pH 4.2 to 4.6 in clone 5. This may indicate the presence of more than one ß-hexosaminidase activity, which was reported in earlier studies with CHO cells (Blaschuk *et al.*, 1980c) and their con A-resistant variants (Blaschuk *et al.*, 1980b). ß-Galactosidase pH profiles also showed an acidic pH optimum around pH 4.4 for both clone 2 and 5 (Figure 16). Levels of ß-galactosidase activity were much lower than ß–hexosaminidase activities. The pH profile of α-fucosidase activity had a broad pH optimum in wild type clone 2 and clone 5 with slightly higher peaks of activity around pH 4.5 and pH 6.2. Levels of α-fucosidase activity were slightly reduced in the con A-resistant variants compared with activity in the wild type myoblasts (Figure 17). α–Fucosidase activity was also much lower than ß-hexosaminidase activity. The activity of α-glucosidase was also measured over an acidic pH range and both clone 2 wild type and variant had very similar pH profiles with one distinct pH optimum at approximately pH 4.4 and a smaller optimum at pH 5.6 (Figure 18). Enzyme activities

Figure 15

The effect of pH on ß-hexosaminidase activity in clone 2 (Panel A) and clone 5 (Panel B) wild type (❑) and con A-resistant (◆) L6 myoblasts. One unit of enzyme activity represents the amount of enzyme required to hydrolyze 1 μmol of *p*-nitrophenyl-ß-D-N-acetylglucosamine per minute. Results respresent the mean of two assays and S.D. is within ±15%.


A
Enzyme activity (Units/mg protein)
40
30
20
10
0
3.5
4.0
4.5
5.0
5.5
6.0
pH

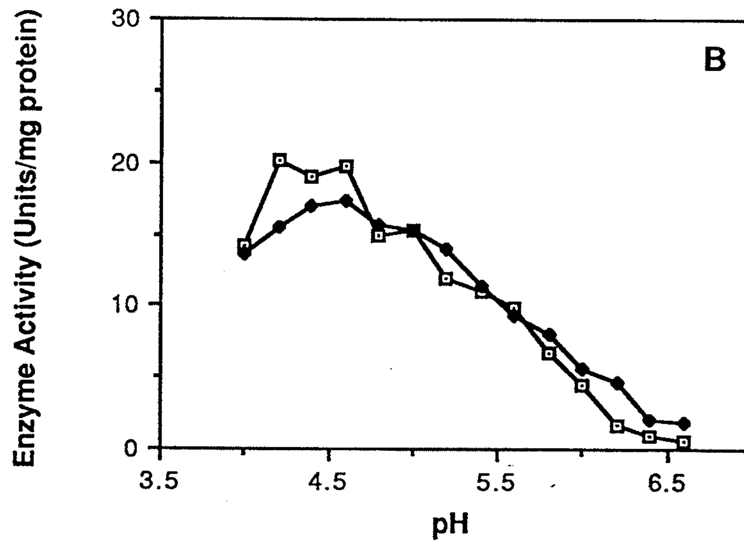
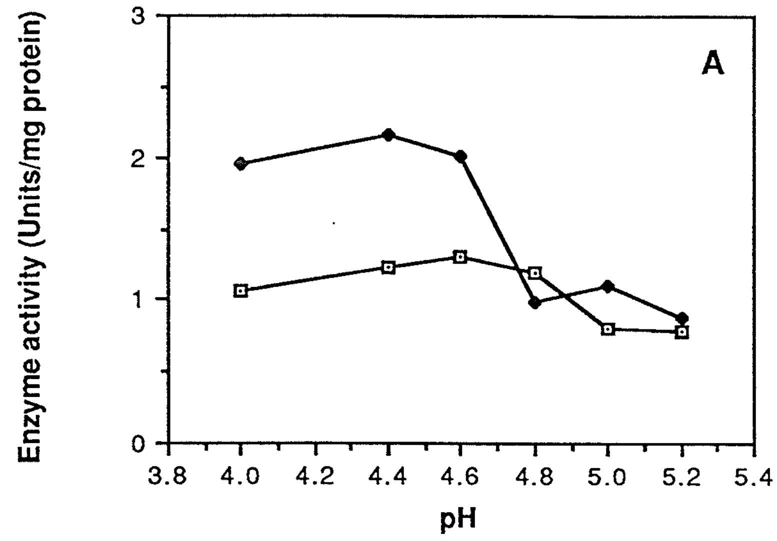

B
Enzyme Activity (Units/mg protein)
30
20
10
0
3.5
4.5
5.5
6.5
pH

Figure 16

The effect of pH on ß-galactosidase activity in clone 2 (Panel A) and clone 5 (Panel B) wild type (❑) and con A-resistant (◆) L6 myoblasts. One unit of enzyme activity represents the amount of enzyme required to hydrolyze 1μmol of *p*-nitrophenyl-ß-D-galactoside per minute. Results represent the mean of 2 assays and S.D. is within ±15%.


A
Enzyme activity (Units/mg protein)
pH
0
1
2
3
3.8
4.0
4.2
4.4
4.6
4.8
5.0
5.2
5.4

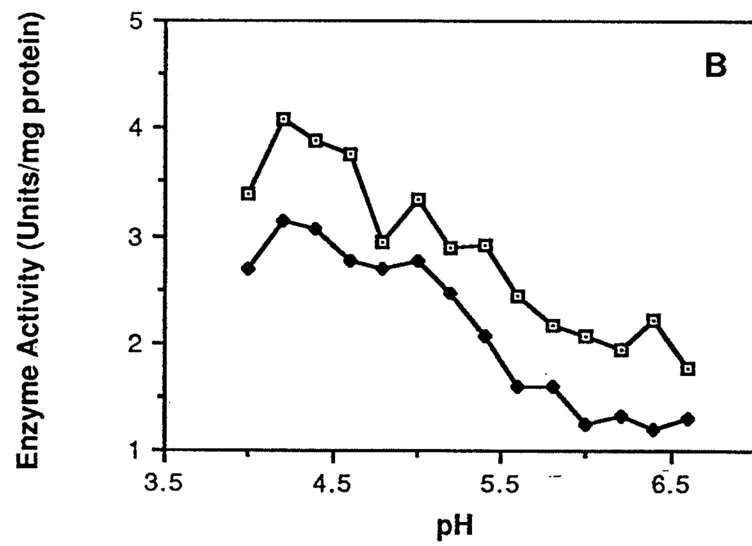
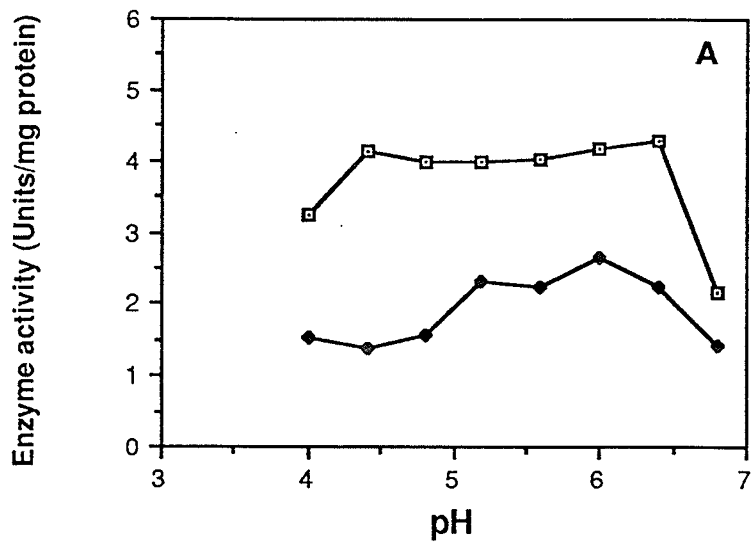

B
Enzyme Activity (Units/mg protein)
pH
1
2
3
4
5
3.5
4.5
5.5
6.5

Figure 17

The effect of pH on α-fucosidase activity in clone 2 (Panel A) and clone 5 (Panel B) wild type (❑) and con A-resistant (◆) L6 myoblasts. One unit of enzyme activity represents the amount of enzyme required to hydrolyze 1 μmol of *p*-nitrophenyl-α-D-fucoside per minute. Results represent the mean of two assays and S.D. is within ±15%.


A
Enzyme activity (Units/mg protein)
pH
0
1
2
3
4
5
6
3
4
5
6
7

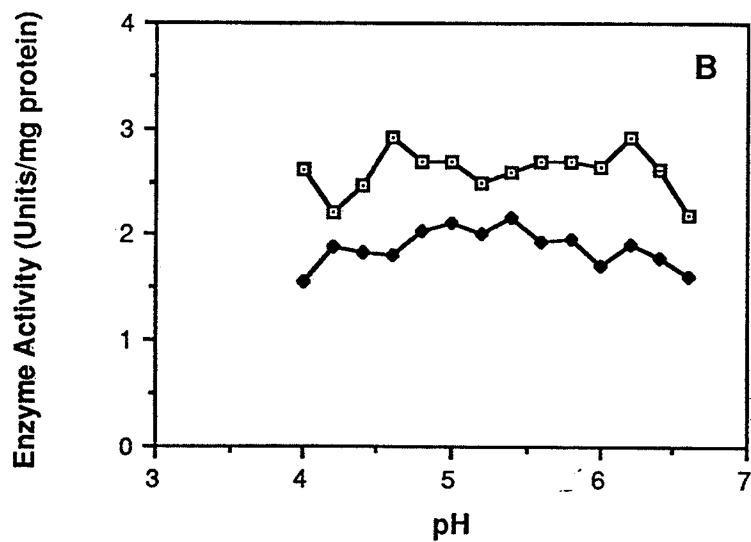
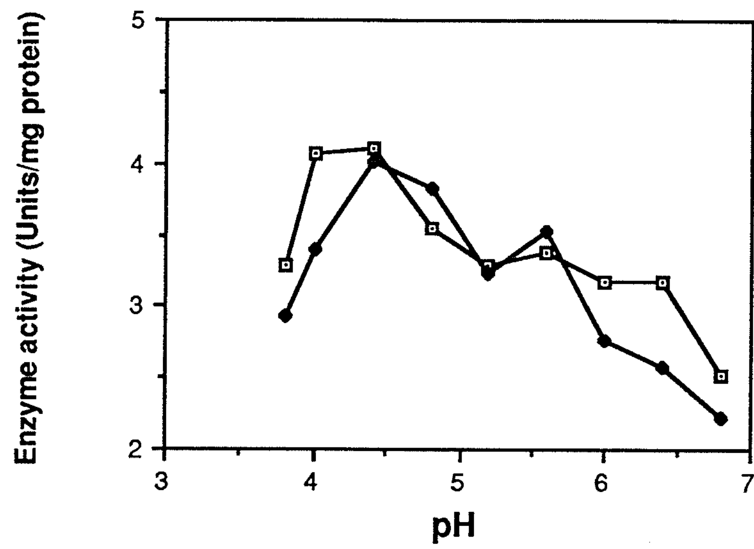

B
Enzyme Activity (Units/mg protein)
pH
0
1
2
3
4
3
4
5
6
7

Figure 18

The effect of pH on α-glucosidase activity in clone 2 wild type (❑) and con A-resistant (◆) L6 myoblasts. One unit of enzyme activity represents the amount of enzyme required to hydrolyze 1 μmol of *p*-nitrophenyl-α-D-glucoside per minute. Results represent the mean of 2 assays and S.D. is within ±15%.


Enzyme activity (Units/mg protein)
5
4
3
2
3
4
5
6
7
pH

were linear with respect to time up to 120 min. for ß-galactosidase, α-fucosidase, α–mannosidase and α-glucosidase, and up to 60 min. for ß-hexosaminidase, and linear with respect to cell protein up to 30 μg (data not shown).

Kinetic constants of pH 4.6 and 6.0 α-mannosidase activities, pH 4.4 ß–hexosaminidase activity, pH 4.4 ß-galactosidase activity, and pH 6.0 α-fucosidase activity were determined in clone 2 (Table 8) and clone 5 (Table 9) wild type and con A-resistant variants . The major differences also involved the mannosidase activities. In clone 2 myoblasts, the K_m values for both the acidic and neutral α-mannosidase activities of the variant were significantly larger than the wild type K_m values, indicating a decreased affinity for the substrate in the variant enzymes. The V_{max} values were also significantly decreased for both α-mannosidase activities of the variant compared with the wild type V_{max} values. In clone 5 myoblasts (Table 9), the K_m of the acidic mannosidase was again significantly larger in the variant with a reduced V_{max}. However, the K_m and V_{max} values of the neutral α-mannosidase were not substantially different in the clone 5 wild type and con A-resistant variant. ß–Hexosaminidase and α-fucosidase K_m and V_{max} values were comparable between both wild types and their respective con A-resistant variants. However, the K_m for ß–galactosidase was reduced by 50% in the clone 2 con A-resistant variant, with no significant change in the V_{max}. This was not evident in the clone 5 con A-resistant variant. The kinetic data indicate that the acidic α-mannosidase activity in the con A-resistant myoblasts is very different from the wild type activity. This may be due to a loss in activity of one or more enzymes.

There are several possibilities which could account for the differences in the kinetic constants, one of which could be the altered targetting of a lysosomal mannosidase. To investigate this, total extracellular and intracellular activites per plate were compared to

Table 8 Values of Km and Vmax for glycosidase activities in extracts of clone 2 wild type and con A-resistant myoblasts *

Enzyme Activity	Optimum pH	wild type K_m	wild type V_{max}	L6C12V1 K_m	L6C12V1 V_{max}
Acidic Mannosidase	4.6	7.0	14.0	18.5	4.0
Neutral Mannosidase	6.0	4.7	7.5	8.0	4.2
Hexosaminidase	4.4	1.1	37.0	0.9	45.0
Galactosidase	4.4	3.0	2.6	1.5	2.0
Fucosidase	6.0	0.5	4.1	0.6	3.2

* K_m values are in mM; V_{max} values are U/mg protein. One unit of enzyme activity is the amount of enzyme required to hydrolyze 1 μmole of substrate per minute. Results are means of 4 assays; standard deviations from the means are within ±15%.

Table 9 Values of K_m and V_{max} for glycosidase activities in extracts of clone 5 wild type and con A-resistant L6 myoblasts*

Enzyme Activity	Optimum pH	wild type		L6C15V1	
		K_m	V_{max}	K_m	V_{max}
Acidic Mannosidase	4.6	7.5	9.2	16.2	2.2
Neutral Mannosidase	6.0	2.7	2.4	1.7	1.1
Hexosaminidase	4.4	1.8	26.4	0.6	24.2
Galactosidase	4.4	0.4	6.3	0.3	4.2
Fucosidase	6.0	0.7	8.6	0.3	4.0

* Km values are in mM; Vmax values are U/mg protein. A unit of enzyme activity represents the amount of enzyme required to hydrolyze 1 μmol of substrate per minute. Results are means of 2 assays; standard deviations from the means were within ±15%.

determine if alterations in intracellular activites could be a result of enhanced secretion of the enzymes. Heat-inactivated serum was used for culturing the myoblasts (Blaschuk *et al.*, 1980a), to avoid interference from low levels of glycosidase activities known to be present in calf serum. Values for both neutral and acidic extracellular mannosidase activities of the clone 2 variant were not significantly different from the wild type values (Table 10). However, while the extracellular acidic mannosidase activity of the wild type represents only 11% of the intracellular activity, the extracellular acidic mannosidase activity of the variant represents 100% of the intracellular activity. Thus it appears that a greater percentage of intracellular mannosidase activity in the variants is secreted into the media. However, the total of intracellular and extracellular acidic mannosidase activity of the con A-resistant variant is still far less than the total activities of the wild type. Extracellular secretion is therefore not a major alternate pathway for the acidic mannosidase and not a likely cause of the loss of the activity in the con A-resistant variant.

Table 10 Activities of clone 2 wild type and con A-resistant myoblast intracellular and extracellular glycosidase enzymes*

	Wild type		L6Cl2V1	
Enzyme Activity	Intracellular	Extracellular	Intracellular	Extracellular
Acidic mannosidase	118.0 ± 15.0	13.0 ± 1.0	12.0 ± 4.0	12.0 ± 2.0
Neutral mannosidase	78.0 ± 6.0	2.7 ± 0.2	34.0 ± 11.0	2.8 ± 0.6
Hexosaminidase	825.0 ± 84.0	13.0 ± 4.0	797.0 ± 54.0	76.0 ±18.0
Galactosidase ¶	137.0 ± 11.0	---	106.0 ± 26.0	---
Fucosidase ¶	110.0 ± 17.0	---	75.0 ± 11.0	---

* Results are expressed as units of enzyme activity per 100 mm plate ± SD of the mean. One unit of enzyme activity is the amount of enzyme required to hydrolyze 1 µmol of substrate per minute. Results are from three assays.
¶ Extracellular galactosidase and fucosidase activities were not detectable.

2. Effect of processing inhibitors on glycosidase activities in L6 myoblasts.

The inhibitory effect of the oligosaccharide processing inhibitors on glycosidase activities has been well documented in several cell types (see Historical Review). Lysosomal glycosidases, as well as processing glycosidases, are directly inhibited by some of the inhibitors. For example, swainsonine inhibits lysosomal mannosidase acitivity (Dorling *et al* ., 1980; Molyneux and James, 1982) and castanospermine (Saul *et al.*, 1983) inhibits lysosomal α-glucosidase activity. Lysosomal enzymes are themselves glycoproteins and alterations in processing of their oligosaccharides, in the presence of the inhibitors, may affect targetting to the lysosomes, which is dependent on receptor recognition of phosphorylated high mannose oligosacccharides. The processing inhibitors may therefore have a two fold effect on lysosomal enzyme activity. Both con A-resistant CHO cells (Blaschuk *et al.*, 1980a) and con A-resistant myoblasts (Hellman *et al.*, 1983; described in previous section), which have defective oligosaccharide synthesis, also have alterations in lysosomal enzyme activities. Therefore, these studies were carried out to ensure that the processing inhibitors were inhibiting the activity of the neutral processing enzymes, but also to determine if the inhibitors were also affecting the lysosomal enzyme activities.

The effects of the glycoprotein processing inhibitors on acidic glycosidase activities (representative of lysosomal enzymes) and neutral glycosidase activities (representative of Golgi processing enzymes) were determined with two different types of assays for comparison. In the first group of assays the inhibitors, 1-deoxynojirimycin, N-methyldeoxynojirimycin, castanospermine, swainsonine and 1-deoxymannojirimycin were added directly to the enzyme assay tubes with the cell extract and substrate. In the second group, the same concentrations of inhibitors were added to newly plated cells and upon reaching confluence, the cells were harvested and assayed for glycosidase activity. The cells were washed extensively before harvesting to remove any adherent

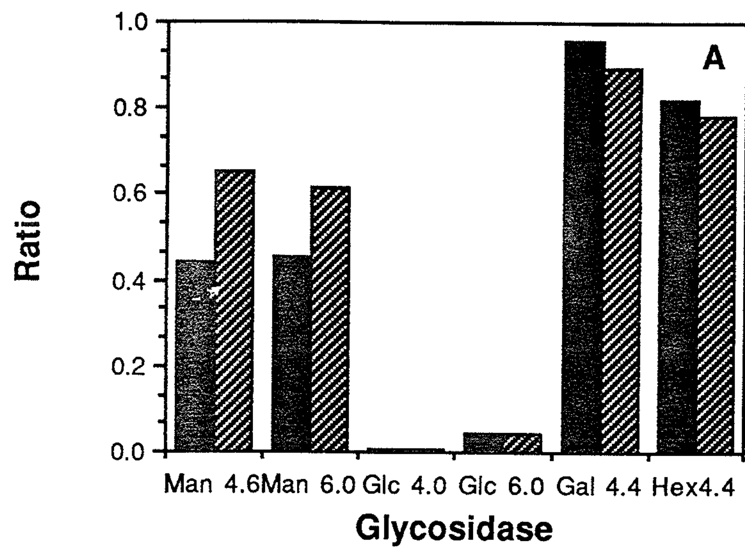
processing inhibitors, however, some inhibitor will still be present intracellularly. ß–Galactosidase (pH 4.4), ß-hexosaminidase (pH 4.4), and α-glucosidase (pH 4.0 and 6.0) activities were assayed using the glycopyranoside substrates of *p*–nitrophenyl. α-Mannosidase (pH 4.6 and 6.0) activities were assayed using 4-methyl-umbelliferyl-α-D-mannopyranoside. The pH optimum and assay conditions were as described above. The effects of the processing inhibitors on these enzyme activities were measured at several concentrations of the inhibitor, but the values given in the bar graphs represent the effect at only the concentration of the inhibitor used in the fusion inhibition assays. The data are presented as ratios to control values with no inhibitor present.

When added directly to the enzyme assay system, the glucosidase inhibitors, 1–deoxynojirimycin (2.5 mM) (Figure 19, panel A), castanospermine (0.1 mM) (Figure 20, panel A), N–methyldeoxynojirimycin (2.0 mM) (Figure 21, panel A), all reduced levels of α-glucosidase activity at both pH 4.0 and 6.0 to minimal levels (less than 20% of the control). N–Methyldeoxynojirimycin and 1–deoxynojirimycin also reduced acidic (pH 4.6) and neutral (pH 6.0) α-mannosidase activity by variable degrees. This may represent a cross-inhibitory effect in this assay system. The glucosidase inhibitors did not have a significant effect on either ß-hexosaminidase or ß–galactosidase activity.

Different effects on the glycosidase activities were observed when the myoblasts were cultured in presence of the inhibitors and then assayed for glycosidase activities (Figures 19-21, B panels). The α-glucosidase inhibitors all substantially inhibited both neutral (pH 6.0) and acidic (pH 4.0) α-glucosidase activities, as expected. However, the acidic α-mannosidase activity was also substantially reduced in both wild type and variant cells with all of the glucosidase inhibitors. A very interesting result was the large increase (70-80%) in the neutral mannosidase activity of the variant

Figure 19

The effect of deoxynojirimycin (2.5mM) on glycosidase activities in wild type (solid bars) and con A-resistant (striped bars) myoblasts (clone 5). Panel A: myoblasts were cultured with no inhibitor present but were assayed in the presence of deoxynojirimycin. Panel B: myoblasts were cultured with the inhibitor and then assayed for glycosidase activities. The abbreviations used for the enzyme activities and the pH at which they were assayed are: Man 4.6 - α-mannosidase, pH 4.6; Man 6.0 - α-mannosidase, pH 6.0; Glc 4.0 - α-glucosidase, pH 4.0; Glc 6.0 - α-glucosidase, pH 6.0; Gal 4.4 - ß-galactosidase, pH 4.4; Hex 4.4 - ß-hexosaminidase, pH 4.4. Results represent the mean of 2 assays and S.D. is within ±10%.


1.0
0.8
0.6
0.4
0.2
0.0
A
Ratio
Man 4.6
Man 6.0
Glc 4.0
Glc 6.0
Gal 4.4
Hex4.4
Glycosidase

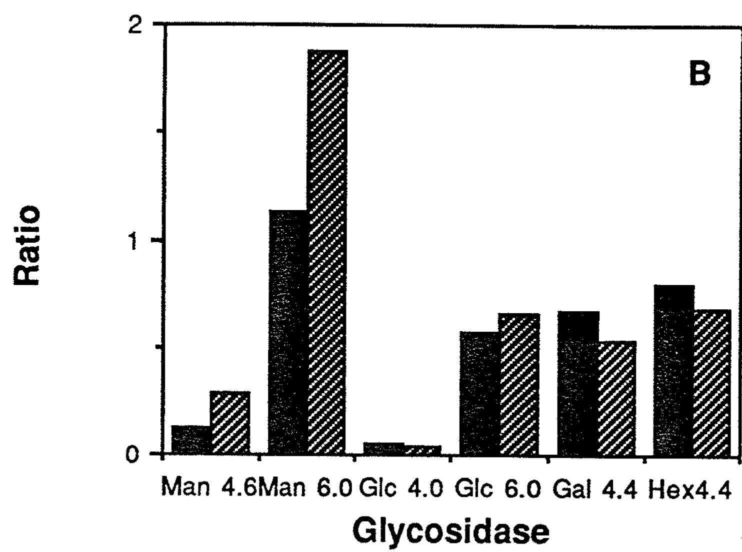
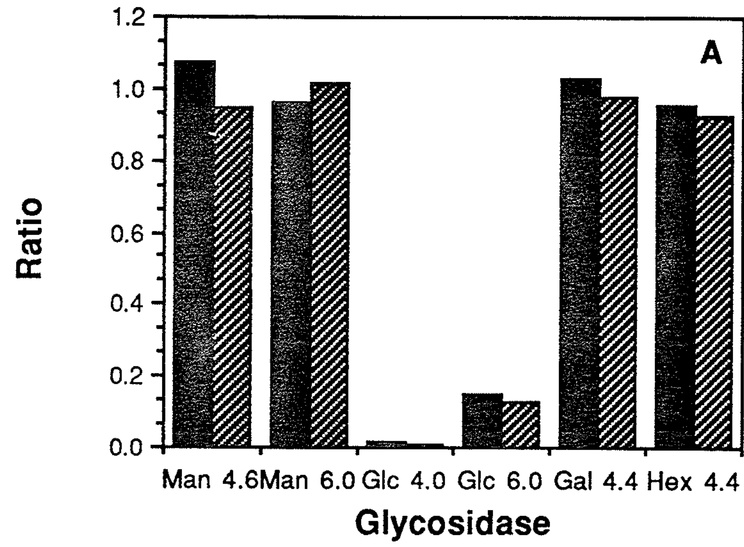

2
1
0
B
Ratio
Man 4.6
Man 6.0
Glc 4.0
Glc 6.0
Gal 4.4
Hex4.4
Glycosidase

Figure 20

The effect of castanospermine (0.1mM) on glycosidase activities in wild type (solid bars) and con A-resistant (striped bars) L6 myoblasts (clone 5). Panel A: myoblasts were cultured with no inhibitor present but castanospermine was present in the glycosidase assays. Panel B: myoblasts were cultured in the presence of the inhibitor and then assayed for glycosidase activities. The abbreviations used for the enzyme activities and the pH at which they were assayed are: Man 4.6 - α-mannosidase, pH 4.6; Man 6.0 - α-mannosidase, pH 6.0; Glc 4.0 - α-glucosidase, pH 4.0; Glc 6.0 - α-glucosidase, pH 6.0; Gal 4.4 - ß-galactosidase, pH 4.4; Hex 4.4 - ß-hexosaminidase, pH 4.4. Results represent the mean of 2 assays and S.D. was within ±10%.


A
Ratio
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Man 4.6
Man 6.0
Glc 4.0
Glc 6.0
Gal 4.4
Hex 4.4
Glycosidase

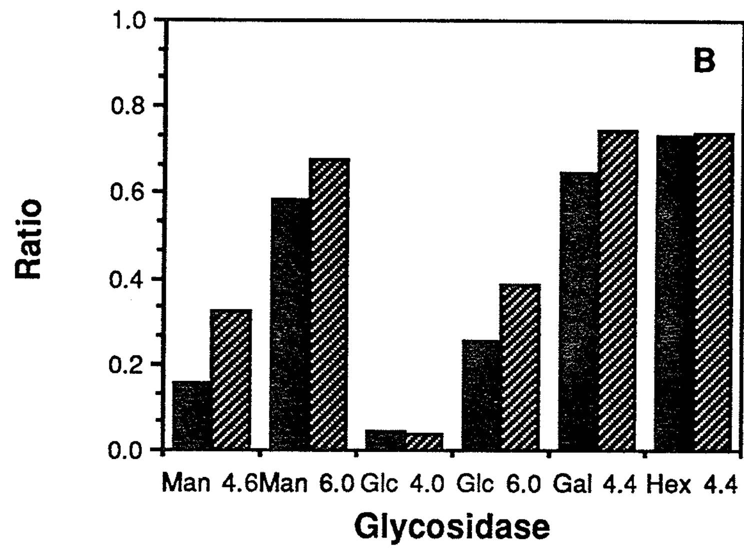
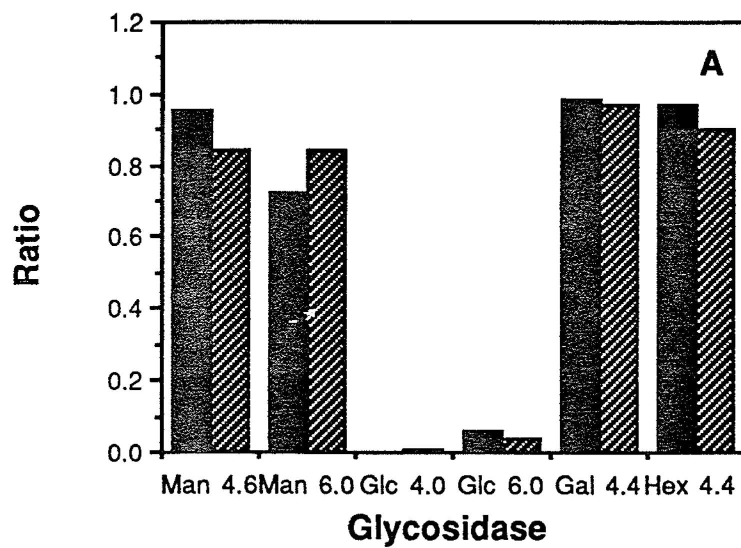
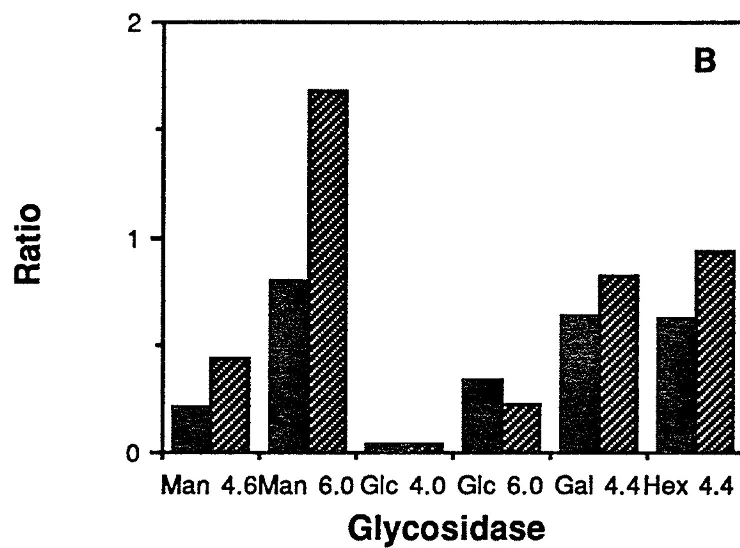

B
Ratio
1.0
0.8
0.6
0.4
0.2
0.0
Man 4.6
Man 6.0
Glc 4.0
Glc 6.0
Gal 4.4
Hex 4.4
Glycosidase

Figure 21

The effect of N-methyldeoxynojirimycin (1.0 mM) on glycosidase activities in wild type (solid bars) and con A-resistant (striped bars) myoblasts (clone 5). Panel A: myoblasts were cultured with no inhibitor present but were assayed in the presence of N-methyldeoxynojirimycin. Panel B: myoblasts were grown in the presence of the inhibitor and then assayed for glycosidase activities. The abbreviations used for the enzyme activities and the pH at which they were assayed are: Man 4.6 - α-mannosidase, pH 4.6; Man 6.0 - α-mannosidase, pH 6.0; Glc 4.0 - α-glucosidase, pH 4.0; Glc 6.0 - α-glucosidase, pH 6.0; Gal 4.4 - ß-galactosidase, pH 4.4; Hex 4.4 - ß-hexosaminidase, pH 4.4. Results represent the mean of 2 assays and S.D. was within ±10%.


1.2
1.0
0.8
0.6
0.4
0.2
0.0
Ratio
A
Man 4.6
Man 6.0
Glc 4.0
Glc 6.0
Gal 4.4
Hex 4.4
Glycosidase


2
1
0
Ratio
B
Man 4.6
Man 6.0
Glc 4.0
Glc 6.0
Gal 4.4
Hex 4.4
Glycosidase

myoblasts grown in the presence of 1-deoxynojirimycin and N–methyldeoxynojirimycin. This effect was not observed for castanospermine in the variant myoblasts. Also, neutral α-mannosidase activity of the wild type myoblasts was slightly inhibited by N-methyldeoxynojirimycin and castanospermine, whereas deoxynojirimycin had no effect. ß–Galactosidase and ß-hexosaminidase activities decreased up to 40% in wild type and variant myoblasts cultured with the glucosidase inhibitors.

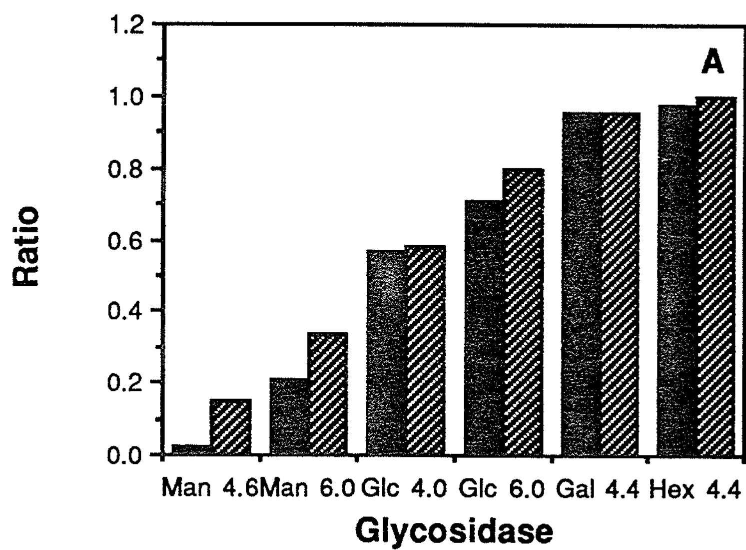
Swainsonine (2.6 μM), the mannosidase II and lysosomal mannosidase inhibitor, inhibited both neutral and acidic α-mannosidase activity, when added directly to the enzyme assay (Figure 22, panel A). Swainsonine also significantly inhibited acidic α–glucosidase acitivity and to a lesser extent neutral α-glucosidase activity. Again, this may represent a cross inhibitory effect in this assay system. ß-Hexosaminidase and ß–galactosidase activites were not significantly altered.

Wild type and variant myoblasts cultured in the presence of swainsonine (Figure 22, panel B) had reduced levels of acidic α-mannosidase activity and both neutral and acidic α–glucosidase activities. The neutral α-mannosidase activity of the wild type cells was also reduced (40%), but the neutral α-mannosidase activity of the con A-resistant variant increased by approximately 25%. Again, ß-hexosaminidase and ß-galactosidase activities were not significantly affected.

The mannosidase II inhibitor, deoxymannojirimycin, when added to the assay system, reduced the activity of neutral and acidic α-glucosidase (20-25%) and α-mannosidase, but had no effect on ß-hexosaminidase and ß-galactosidase activities. Glycosidase activities of myoblasts cultured in the presence of deoxymannojirimycin were not significantly affected with the exception of acidic α-mannosidase which was reduced by approximately 35% in the wild type. Also, ß-galactosidase was inhibited by 30%, but only in the variant myoblasts (Figure 23).

Figure 22

The effect of swainsonine (2.6 μM) on glycosidase activities in wild type (solid bars) and con A-resistant (striped bars) L6 myoblasts (clone 5). Panel A: myoblasts were cultured with no inhibitor present but swainsonine was present in the glycosidase assays. Panel B: myoblasts were cultured in the presence of the inhibitor and then assayed for glycosidase activities The abbreviations used for the enzyme activities and the pH at which they were assayed are: Man 4.6 - α-mannosidase, pH 4.6; Man 6.0 - α-mannosidase, pH 6.0; Glc 4.0 - α-glucosidase, pH 4.0; Glc 6.0 - α-glucosidase, pH 6.0; Gal 4.4 - ß-galactosidase, pH 4.4; Hex 4.4 - ß-hexosaminidase, pH 4.4. Results represent the mean of 2 assays and S.D. was within ±10%.


A
Ratio
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Man 4.6
Man 6.0
Glc 4.0
Glc 6.0
Gal 4.4
Hex 4.4
Glycosidase

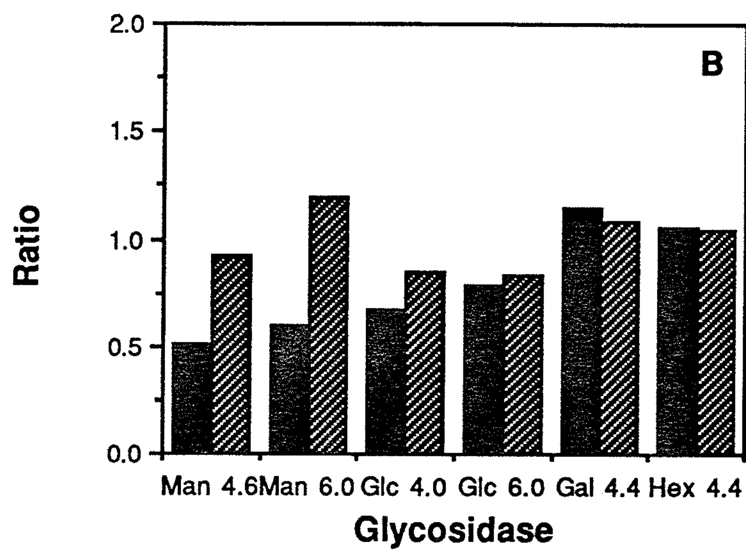
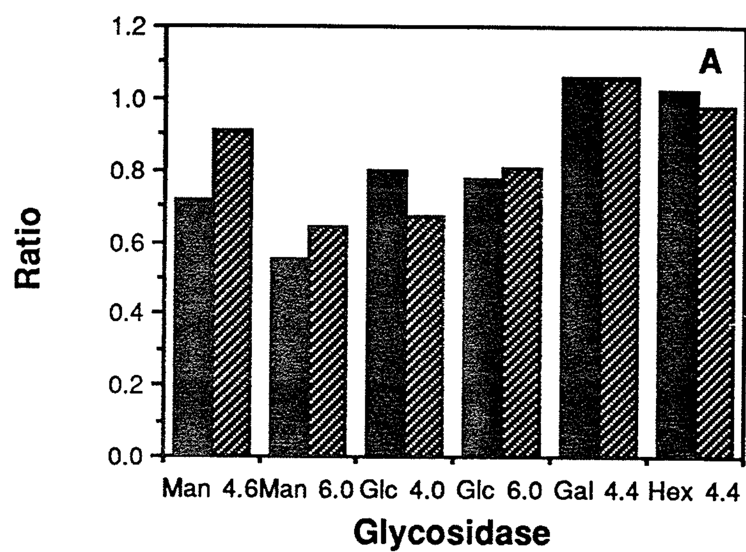
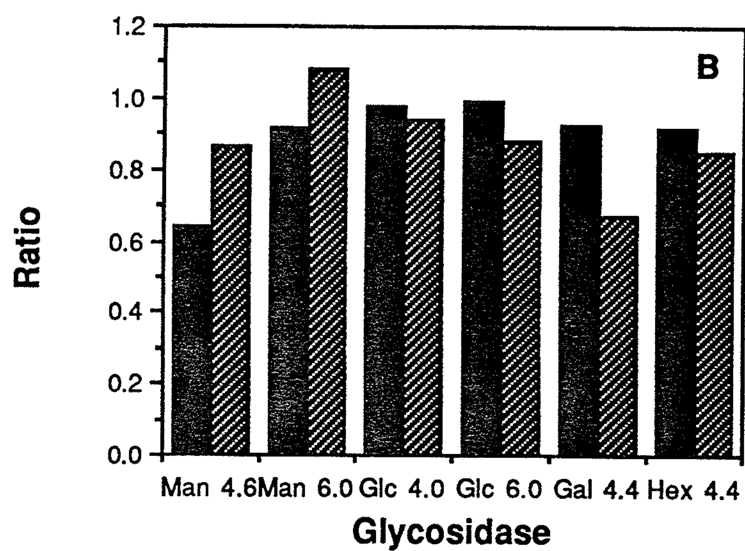

B
Ratio
2.0
1.5
1.0
0.5
0.0
Man 4.6
Man 6.0
Glc 4.0
Glc 6.0
Gal 4.4
Hex 4.4
Glycosidase

Figure 23

The effect of deoxymannojirimycin (0.5 mM) on glycosidase activities in wild type (solid bars) and con A-resistant (striped bars) L6 myoblasts (clone 5). Panel A: myoblasts were cultured with no inhibitor present but deoxymannojirimycin was added to the glycosidase assays. Panel B: myoblasts were cultured in the presence of the inhibitor and then assayed for glycosidase activities. The abbreviations used for the enzyme activities and the pH at which they were assayed are: Man 4.6 - α-mannosidase, pH 4.6; Man 6.0 - α-mannosidase, pH 6.0; Glc 4.0 - α-glucosidase, pH 4.0; Glc 6.0 - α-glucosidase, pH 6.0; Gal 4.4 - ß-galactosidase, pH 4.4; Hex 4.4 - ß-hexosaminidase, pH 4.4. Results represent the mean of 2 assays and S.D. was within ±10%.


A
Ratio
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Man 4.6
Man 6.0
Glc 4.0
Glc 6.0
Gal 4.4
Hex 4.4
Glycosidase


B
Ratio
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Man 4.6
Man 6.0
Glc 4.0
Glc 6.0
Gal 4.4
Hex 4.4
Glycosidase

It is important to note that the assay system uses artificial substrates and the activities measured will represent more than one enzyme activity. Also, mannosidase I will not cleave artificial substrates, and thus the activity measured could represent ER mannosidase, cytoplasmic mannosidase, lysosomal mannosidase and mannosidase II activities depending upon the pH of the assay system.

ß-Hexosaminidase and ß-galactosidase activities appear to be insensitive to the processing inhibitors added to the enzyme assay system. However, the inhibition of galactosidase and hexosaminidase activities (up to 40%) , when myoblasts are cultured with the glucosidase inhibitors, suggests that defective oligosaccharide processing affects the activity, integrity or transport of these lysosomal enzymes due to a lack of removal of the glucose residues.

The cross inhibitory effect of mannosidase inhibitors on glucosidase activities, and the glucosidase inhibitor on mannosidase activity, may be due to an overlap in specificity of the enzymes for the artificial substrate. This cross inhibition has not been observed by others when natural substrates are used.

C. *The effects of glycoprotein processing inhibitors on insulin binding in L6 myoblasts.*

The insulin receptor is an integral membrane glycoprotein composed of two α and two ß subunits linked through disulfide bonds. Both complex and high mannose oligosaccharides are present on the receptor (Heidenriech and Brandenburg, 1986), but the function of the oligosaccharides is presently unknown. However, lack of glycosylation in the presence of tunicamycin (Keffer and Demeyts, 1981) decreases insulin binding, and mutants with altered glycosylation have receptors with reduced affinity for insulin (Podskalny *et al.*, 1984). Because of the known effects of altered glycosylation on the insulin receptor, the effects of glycoprotein processing inhibitors on the insulin receptor were examined concurrently with the experiments studying fusion and glycosidase activities.

Preliminary experiments were necessary to establish optimal conditions for insulin binding in the L6 myoblasts. The presence of an active insulin receptor can be demonstrated by the uptake of deoxy-glucose, therefore the uptake of 2-deoxy-D-[^{3}H]glucose in the presence of insulin concentrations of 10^{-6}, 10^{-7}, 10^{-9} and 10^{-11} M was determined. An increase in 2-deoxy-glucose uptake by approximately 40-50% indicated that active insulin receptors were present on non-differentiating myoblasts (Table 11).

The pH profile of insulin binding in wild type myoblasts had an optimum at pH 7.6 (Figure 24). The amount of insulin bound was also proportional to the amount of cellular protein in non-differentiating wild type myoblasts (Figure 25).

In order to measure the effect of differentiation on insulin binding, L6 myoblasts were plated on sequential days to establish cultures which were at different stages of

Table 11 Uptake of 2-D-[^{3}H]-glucose in insulin-treated clone 5 wild type* L6 myoblasts

Insulin (M)	2-Deoxy-D-glucose (nmol/mg protein)
0	1.05 ± .20
10^{-11}	1.51 ± .08
10^{-9}	1.36 ± .02
10^{-7}	1.38 ± .02
10^{-6}	1.40 ± .14

* Wild type myoblasts were confluent but non-differentiating.

Figure 24

The effect of pH on insulin binding in clone 5 wild type L6 myoblasts. Results represent the mean of three assays and error bars represent the standard deviation. Non-specific binding at 10^{-4}M insulin was subtracted from the total binding.

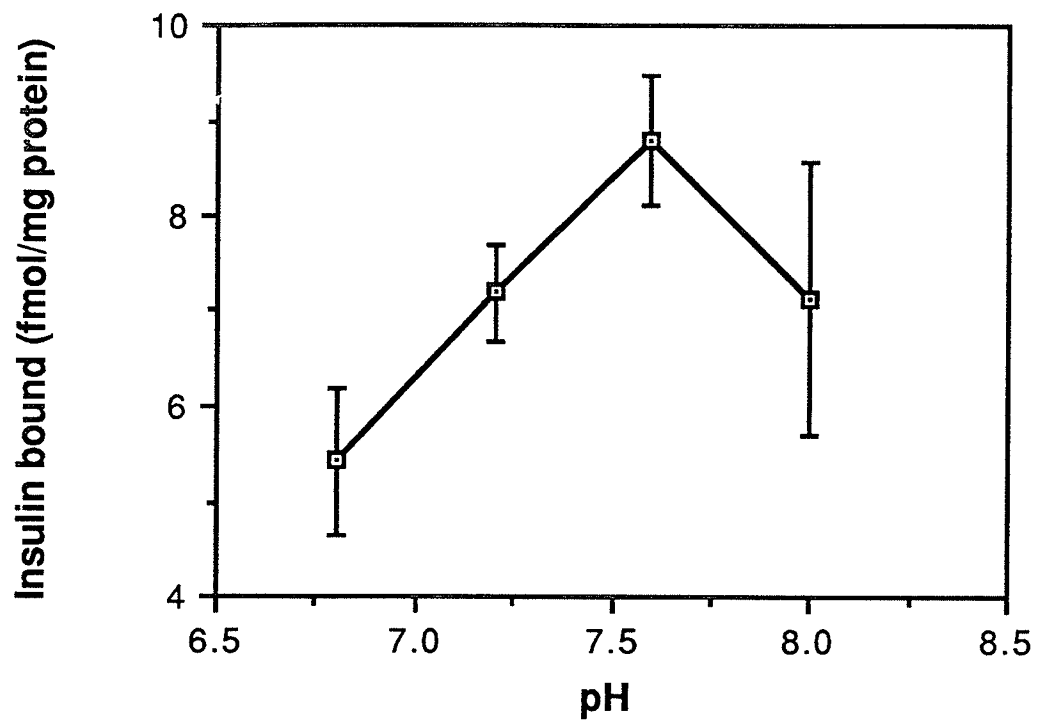
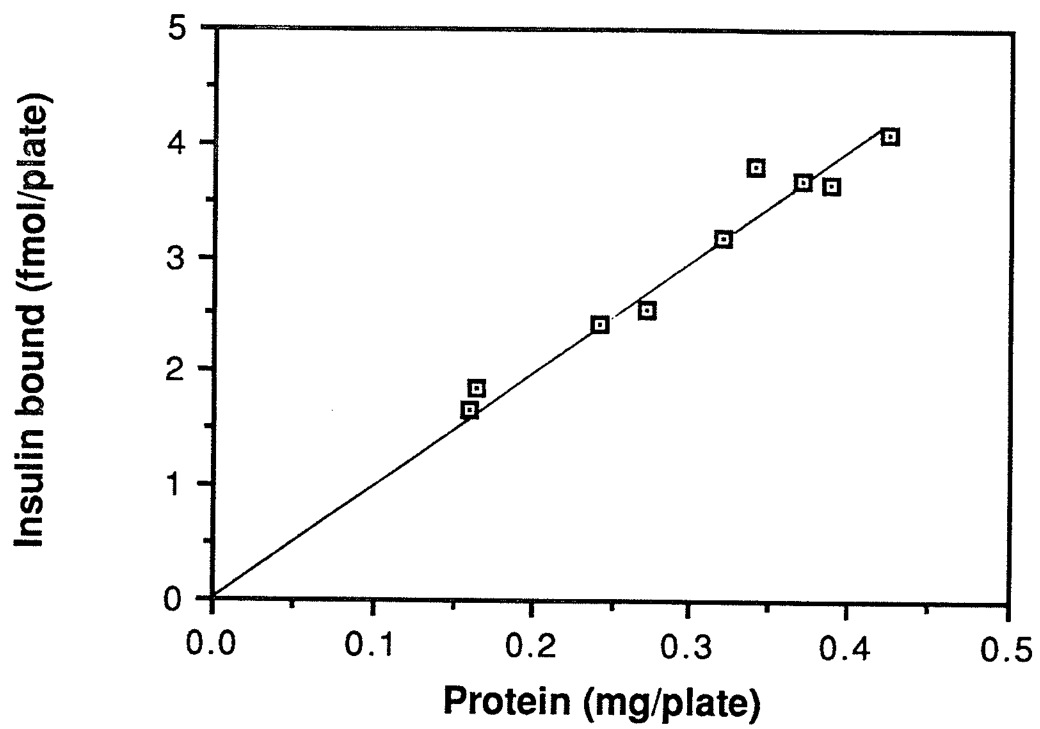

Insulin bound (fmol/mg protein)
10
8
6
4
6.5
7.0
7.5
8.0
8.5
pH

Figure 25

The effect of protein concentration on insulin binding in clone 5 wild type L6 myoblasts. Non-specific binding at 10^{-4}M has not been subtracted but represents less than 40% of the total binding.



Insulin bound (fmol/plate)
0
1
2
3
4
5
0.0
0.1
0.2
0.3
0.4
0.5
Protein (mg/plate)

differentiation. At the time of the binding assay, cultures ranged from confluent cultures with no apparent fusion, to cultures approaching maximum fusion by visual inspection. All plates were assayed at the same time under the conditions defined in Methods and duplicate plates were stained and counted to determine the fusion index.. The amount of insulin bound per plate increased in a fairly linearly manner from 0% to 76% fusion (Table 12). However, when the amount of insulin bound was normalized to the amount of protein per plate, no increase was apparent in bound insulin with respect to fusion. This may be due to the large increase in protein synthesis associated with biochemical differentiation which occurs in the early stages of fusion.

Three inhibitors of glycoprotein processing were used to study their effect on insulin binding in pre-fusion L6 myoblasts. To ensure that differences in insulin binding were not due to an effect of the inhibitors on differentiation, the myoblast cultures were used when confluent but not differentiating. Deoxymannojirimycin (the mannosidase I inhibitor), swainsonine (the mannosidase II inhibitor), and castanospermine (a glucosidase inhibitor), represented the three groups of inhibitors. Concentration ranges of the inhibitors similar to those used in the lectin binding studies (see above) were used. The effects of the inhibitors on insulin binding capacity are represented in the following figures as ratios to control values with no inhibitor present. Both deoxymannojirimycin (1.0mM) (Figure 26) and swainsonine (5.6μM) (Figure 27) increased insulin binding by approximately 50%. Deoxymannojirimycin increases the high mannose oligosaccharide structures present on myoblast cell surface glycoproteins as is evident from con A binding studies, while swainsonine results in the formation of hybrid oligosaccharides instead of complex oligosaccharides (Kang and Elbein, 1983), thereby also increasing mannose content of the oligosaccharides. Castanospermine (Figure 28) slightly reduced binding capacity by approximately 20%. Castanospermine results in high mannose oligosaccharides with terminal glucose

Table 12 Effect of differentiation on insulin binding in clone 5 wild type L6 myoblasts.

Fusion Index[a] (%)	Insulin Bound	
	fmol/plate	fmol/mg protein
0	0.42 ± 0.10	3.94 ± 1.73
9.7	0.67 ± 0.28	3.57 ± 1.23
21.7	1.12 ± 0.06	4.41 ± 0.46
55.3	1.05 ± 0.10	3.29 ± 0.43
76.0	1.27 ± 0.14	3.65 ± 0.42

[a] The fusion index represents the percentage of total nuclei which are found within myotubes.

Figure 26

The effect of deoxymannojirimycin on insulin binding in clone 5 wild type L6 myoblasts. Values are given as the ratio to control myoblasts with no inhibitor present. Non-specific binding has been subtracted and was less than 40% of total binding. Results are the mean of three assays and error bars represent the S.D.

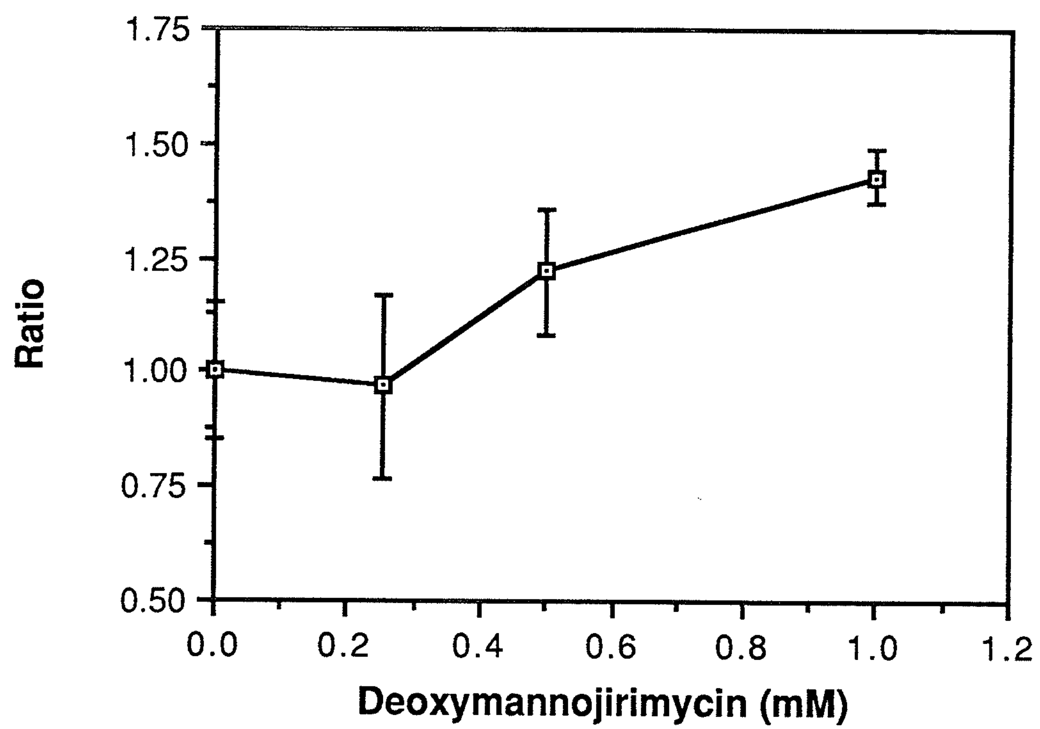

1.75
1.50
1.25
1.00
0.75
0.50
Ratio
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Deoxymannojirimycin (mM)

Figure 27

The effect of swainsonine on insulin binding in clone 5 wild type L6 myoblasts. Values represent the ratio of binding compared to control myoblasts with no inhibitor added. Non-specific binding has been subtracted and represents less than 40% of total insulin binding. Results are the mean of three assays and the error bars represent S.D.

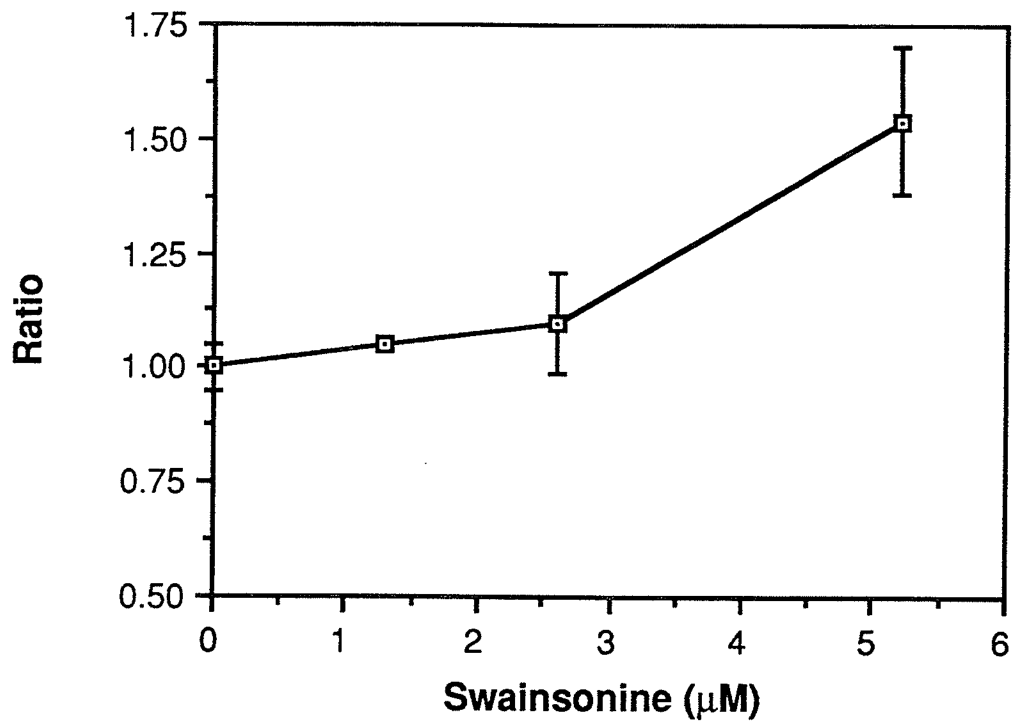
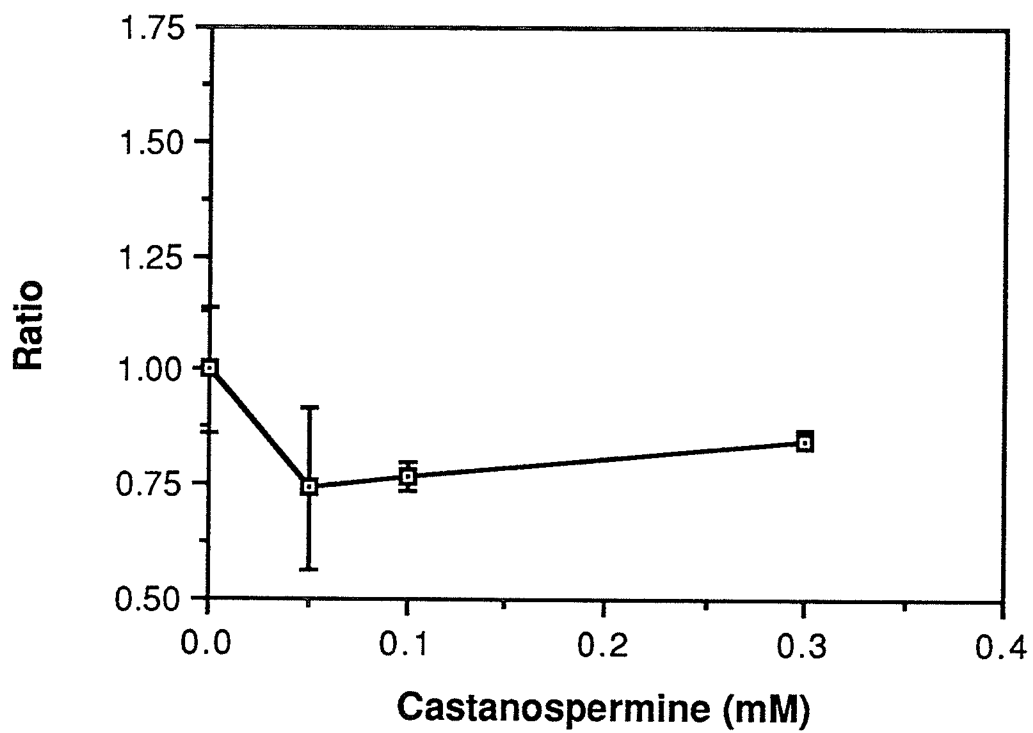

Ratio
1.75
1.50
1.25
1.00
0.75
0.50
0
1
2
3
4
5
6
Swainsonine (μM)

Figure 28

The effect of castanospermine on insulin binding in clone 5 wild type L6 myoblasts. The values are given as a ratio to control myoblasts with no inhibitor present. Non-specific binding has been subtracted and was less than 40% of total binding. Results are the mean of three assays and the error bars represent S.D.


1.75
1.50
1.25
1.00
0.75
0.50
Ratio
0.0
0.1
0.2
0.3
0.4
Castanospermine (mM)

residues replacing high mannose and complex oligosaccharides (Pan *et al.*, 1983). The high mannose character of cell surface glycoproteins such as the insulin receptor is increased, but oligosaccharides are in a less processed form. The reduction in insulin binding capacity could be due to a delay in transport of the components of the insulin receptor caused by the blockage in processing of the glucose residues in the Golgi. Similar observations have been noted for some lysosomal enzymes in fibroblasts (Lemansky *et al.*, 1984).

D. *High mannose oligosaccharides in clone 5 wild type and con A-resistant myoblasts.*

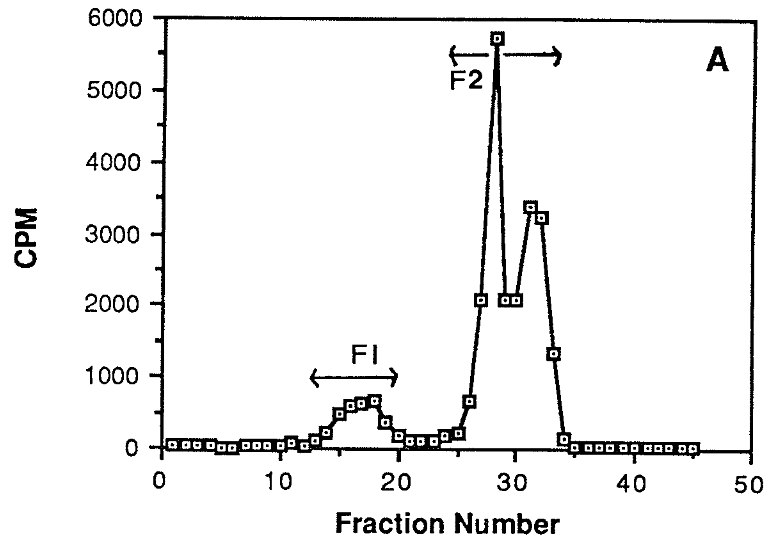
The incorporation of mannose from Dol-P-Man into lipid-oligosaccharide is significantly reduced in con A-resistant myoblasts (Parfett *et al.*, 1983). Normally, the addition of four mannose residues from Dol-P-Man results in the formation of Dol-P-P-$GlcNAc_2$-Man_9 from Dol-P-P-$GlcNAc_2$-Man_5. Loss of the transfer of Man from Dol-P-Man would be expected to result in truncated high-mannose oligosaccharides.

Oligosaccharides were labelled with [^{14}C]-mannose and glycopeptides were prepared from the cells by exhaustive digestion with Pronase. Fractionation of the digest on Bio-Gel P-4 produced two separate groups of glycopeptides (Figure 29). A small peak of higher molecular weight glycopeptides which eluted around fraction 13 was characteristic of the elution pattern of higher molecular weight oligosaccharides, probably of the complex type. In the wild type elution profile (panel A), two slightly overlapping larger peaks at fractions 28 and 31 represented glycopeptides of lower molecular weight, probably of the high mannose type. Glycopeptides from the con A-resistant variant produced a similar profile, with a small broad peak of higher molecular weight glycopeptides at fractions 13 to 18, but instead of two separate peaks of the lower molecular weight glycopeptides, one larger peak at fraction 28 had a shoulder around fraction 30 (Figure 29, panel B).

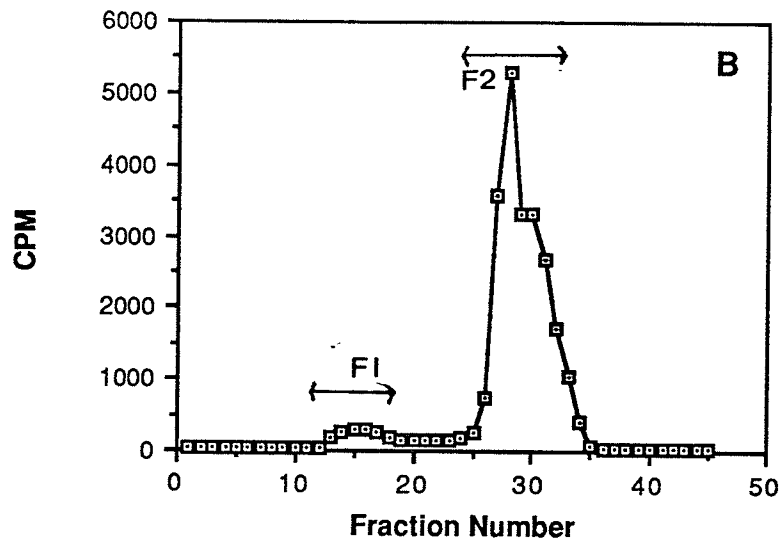
The peaks representing the higher (F1) and lower molecular weight glycopeptides (F2) were separately pooled and subjected to endo H digestion. Approximately equal amounts (based on radioactivity) of samples from the wild type and con A-resistant myoblasts, were applied to the h.p.l.c. column packed with Aminospherisorb and eluted using a gradient of acetonitrile and water (as described in Methods). Radioactive mannose and GlcNAc-Man_9-Glc_{1-3} were used as standards to calibrate the column.

Figure 29

Bio-Gel P-4 elution profile of glycopeptides from Pronase digests of D-[1-^{14}C]-mannose treated wild type (A) and con A-resistant (B) L6 myoblasts (clone 5). Fractions are 2.5 ml and 50 µl of each fraction were counted for radioactivity.



A
F2
F1
CPM
6000
5000
4000
3000
2000
1000
0
0
10
20
30
40
50
Fraction Number

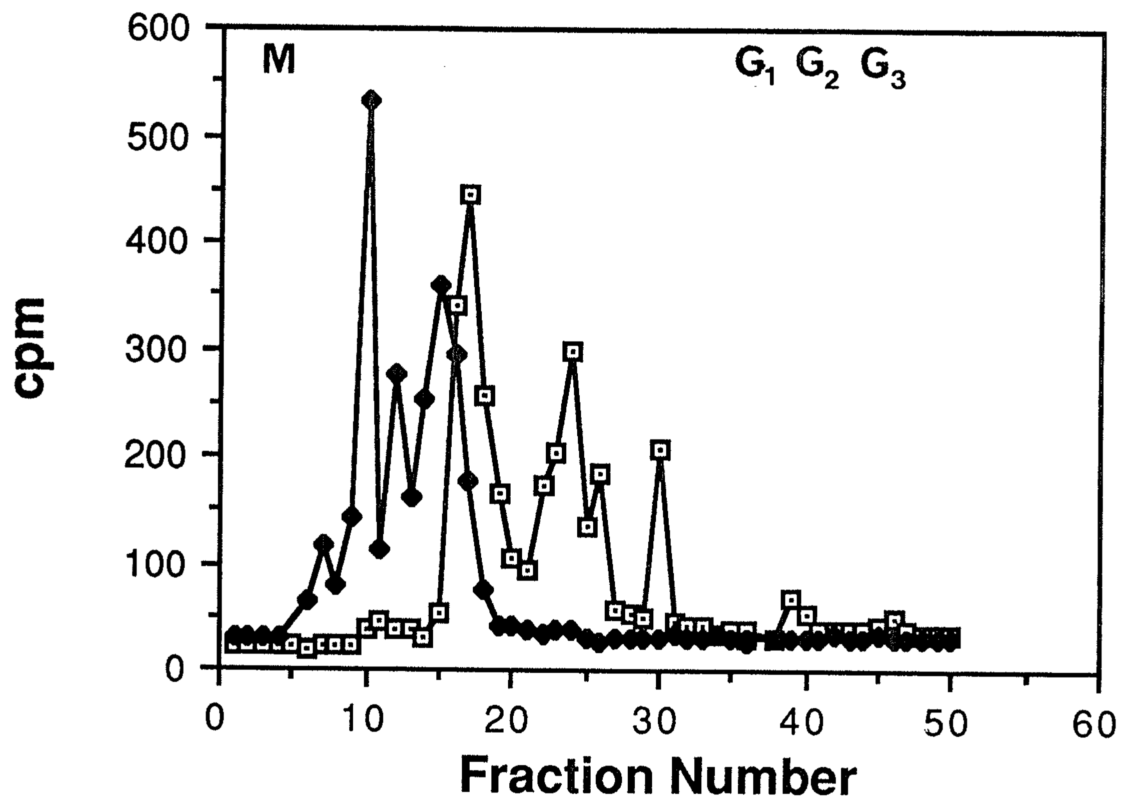


B
F2
F1
CPM
6000
5000
4000
3000
2000
1000
0
0
10
20
30
40
50
Fraction Number

The elution profile of the Fraction 2 wild type oligosaccharides contained six distinct peaks with the largest molecular weight peak at fraction 46 eluting at the GlcNAc-Man_9-Glc_3 standard and another small peak at fraction 39 which coincides with the elution of the GlcNAc-Man_9-Glc_2 standard (Figure 30). Another four distinct peaks of lower molecular weight are discernable at fractions 30, 26, 24, and 17 with a slight shoulder off the left of the peak at fraction 24. Oligosaccharides from the variant myoblasts eluted much earlier than the wild type oligosaccharides. Although oligosaccharide standards within the mid range of the elution profile were not available and also no further characterization of the oligosaccharide peaks was carried out, it is apparent that con A-resistant myoblasts have high mannose oligosaccharides of a much reduced size in comparison with the wild type. The lowest molecular weight oligosaccharide of the high mannose type normally found in nature is a GlcNAc-Man_5 structure and thus the lowest molecular weight oligosaccharide peak of the wild type myoblasts may represent an oligosaccharide of this size. Endo H digests of F1 of the wild type and variant did not separate on the column, suggesting a complex oligosaccharide character, resistant to endo H digestion.

Figure 30

Elution profile of h.p.l.c. separation of endo H digests of D-[1-^{14}C]-mannose labelled glycopeptides of wild type (❑) and con A–resistant (◆) L6 myoblasts (clone 5) using an Aminospherisorb column (5 μM, Chromatography Sciences Co.). The column was eluted with a gradient of acetonitrile and water as described in Methods. D-[1-^{14}C]-mannose (M), and GlcNAc-Man_9-Glc_1 (G_1), GlcNAc-Man_9-Glc_2 (G_2) and GlcNAc-Man_9-Glc_3 (G_3) radioactively labelled with [^{14}C]-glucose, were used as standards. Fractions were 1.0 ml volume and the total fraction was counted to determine radioactivity.


M
G1 G2 G3
cpm
600
500
400
300
200
100
0
0
10
20
30
40
50
60
Fraction Number

E. *Sialyl and galactosyl transferase activities in clone 5 wild type and con A-resistant myoblasts.*

Sialyl and galactosyl transferase enzymes are key enzymes in the formation of the terminal triplet (-GlcNAc-Gal-NeuAc) of complex oligosaccharides. Sialyl transferase adds the terminal sialic acid (NeuAc) residue to the oligosaccharide chain and galactosyl transferase adds the penultimate sugar. Several lectin-resistant mutants display alterations in these glycosyl transferase activities (Stanley, 1980, 1984), and therefore, a study was undertaken to determine if significant differences were present in sialyl and galactosyl transferase activities in the con A-resistant myoblasts.

1. Sialyl transferase

The pH profile of sialyl tranferase over a pH range of 5.4 to 7.2 gave a sharp peak of activity at pH 6.6 for both wild type and variant myoblasts (Figure 31). The amount of activity was measure over a period of five hours to determine an optimal incubation period (Figure 32). Although linear incorporation of the radioactive sugar into the glycoprotein acceptor was achieved over this time period, incubation for four hours was chosen because it was the shortest incubation time which gave reproducible results.

Kinetic activity was determined for two different glycoprotein acceptors which had the sialic acid removed by acid hydrolysis. The mechanism of sialylation is a bi-substrate reaction, involving the nucleotide sugar and the glycoprotein acceptor. Therefore, kinetic values determined for CMP-NeuAc at only one concentration of the glycoprotein acceptor (and *vice versa*), represent only apparent K_m and V_{max}. The asialo fetuin acceptor contains three N-linked and three O-linked oligosaccharides for the potential attachment of fifteen NeuAc residues (Nilsson *et al.*, 1979). Asialo α_1-acid

glycoprotein has only N-linked oligosaccharides with an average of 5.5 NeuAc acceptor positions per molecule (Kaplan *et al.*, 1983). Concentration plots for asialo fetuin and asialo α_1-acid glycoprotein and also for CMP-NeuAc using both acceptor glycoproteins are given in Figures 33 to 36 and kinetic constants are summarized in Table 13. No significant differences in kinetic plots for both asialo α_1-acid glycoprotein or CMP-NeuAc (using α_1-acid glycoprotein as the acceptor) were apparent when comparing wild type and con A-resistant variants results (Figures 33 and 34). Because the data for asialo a1-acid glycoprotein (Figure 34) represent on the first order region of the saturation curve, V_{max} values can only be defined as greater than the maximum activity measured, and K_m is defined as greater than the substrate concentration at one half of the maximum activity (Table 13). However, substantial differences were apparent between wild type and variant with respect to kinetic values for asialo fetuin and CMP-NeuAc (with asialo fetuin as acceptor) (Figures 35 and 36). Although the data for sialyl transferase activity of the variant using asialo fetuin was representative of only the first order region of the plot, the V_{max} is obviously significantly higher in the variant myoblasts in comparison with the wild type activity, and suggests that the K_m for the variant activity would also be larger than the wild type. The kinetic constants for CMP-NeuAc using asialo fetuin as the acceptor, were not significantly different between wild type and variant.

Because fetuin contains both N-linked and O-linked oligosaccharides, the difference in K_m between the wild type and variant for asialo fetuin might be due to a sialyl transferase activity specific for O-linked oligosaccharides. However, sialyl transferase measured in the presence of asialo bovine mucin, which contains oligosaccharides with O-linkages, showed negligible activity (data not shown).

Figure 31

The effect of pH on sialyl transferase activity in clone 5 wild type (❑) and clone 5 con A-resistant (◆) L6 myoblasts. One unit of enzyme activity represents the amount of NeuAc from CMP-NeuAc transferred to the acceptor glycoprotein, asialo fetuin, per hour. Results represent the mean of 2 assays and endogenous activity has been subtracted from the total activity.

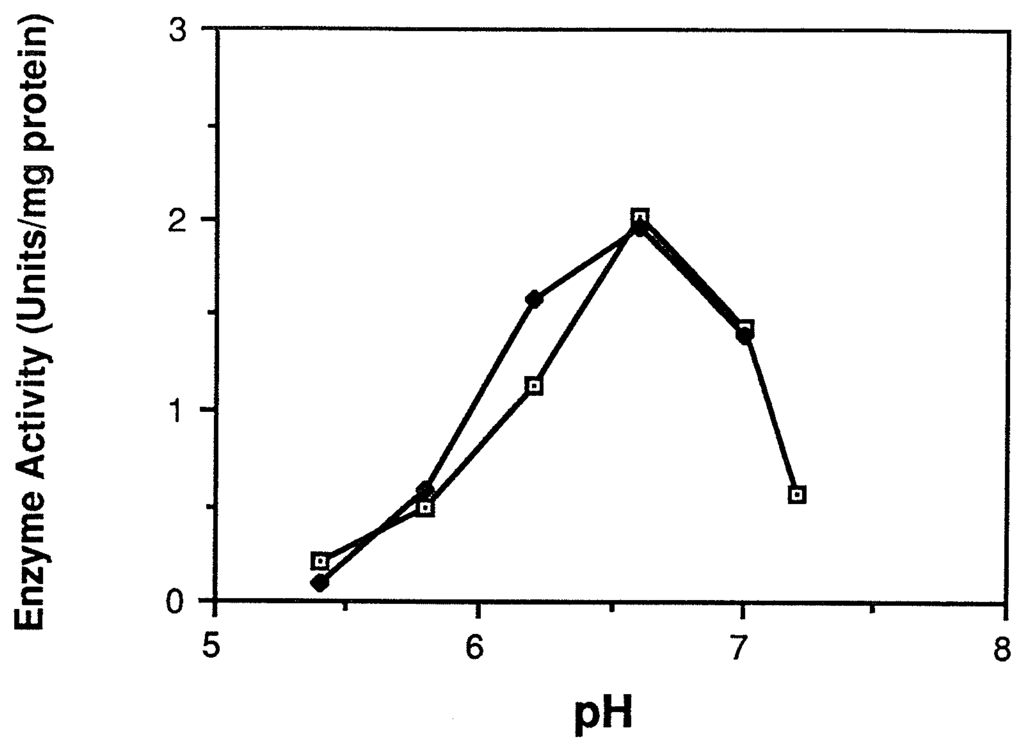

Enzyme Activity (Units/mg protein)
0
1
2
3
5
6
7
8
pH

Figure 32

The effect of time of incubation on sialyl transferase activity for wild type (❑) and con A-resistant (◆) L6 myoblasts (clone 5). Enzyme activity is expressed as pmol of NeuAc from CMP-NeuAc transferred to 200 μg asialo fetuin per mg of cell protein. Endogenous activity has been subtracted from the total activity. Results are the mean of 2 assays and S.D. is within ±20%.

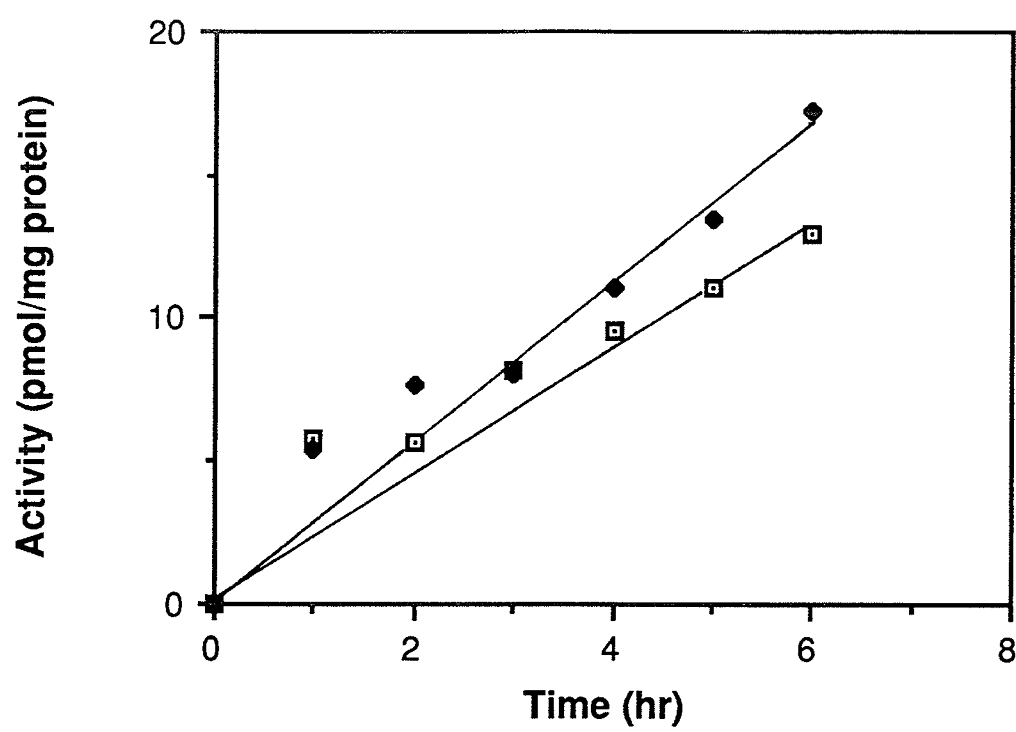

20
10
0
0
2
4
6
8
Activity (pmol/mg protein)
Time (hr)

Figure 33

The effect of CMP-NeuAc concentration on sialyl transferase activity in clone 5 wild type (❑) and clone 5 con A-resistant (◆) L6 myoblasts using asialo α_1-acid glycoprotein (200 µg) as the acceptor. Units of enzyme activity represent the amount of NeuAc transferred to the glycoprotein acceptor per hour. Endogenous activity has been subtracted from the total activity. Results are the mean of 2 assays and the S.D. is within ±20%.

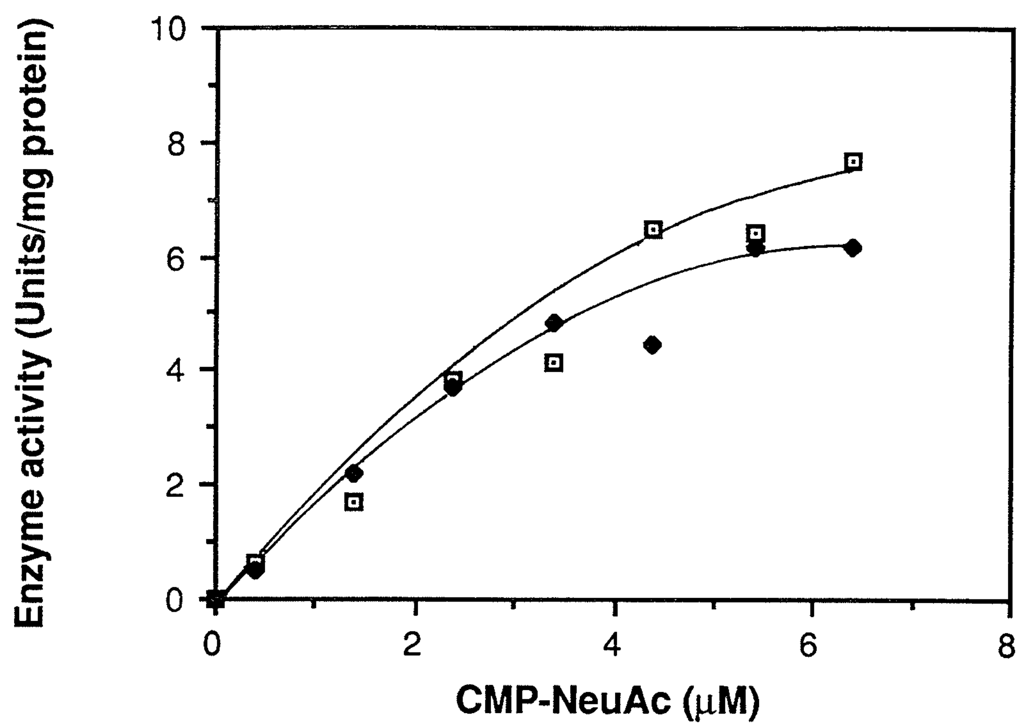

Enzyme activity (Units/mg protein)
10
8
6
4
2
0
0
2
4
6
8
CMP-NeuAc (μM)

Figure 34

Effect of asialo α_1-acid glycoprotein concentration on sialyl transferase activity in clone 5 wild type (❑) and clone 5 con A-resistant (◆) L6 myoblasts. Units of enzyme activity represent the amount of NeuAc transferred to the acceptor glycoprotein per hour. Endogenous activity has been subtracted from the total activity. Results are the mean of 3 assays and the S.D. is within ±20%.

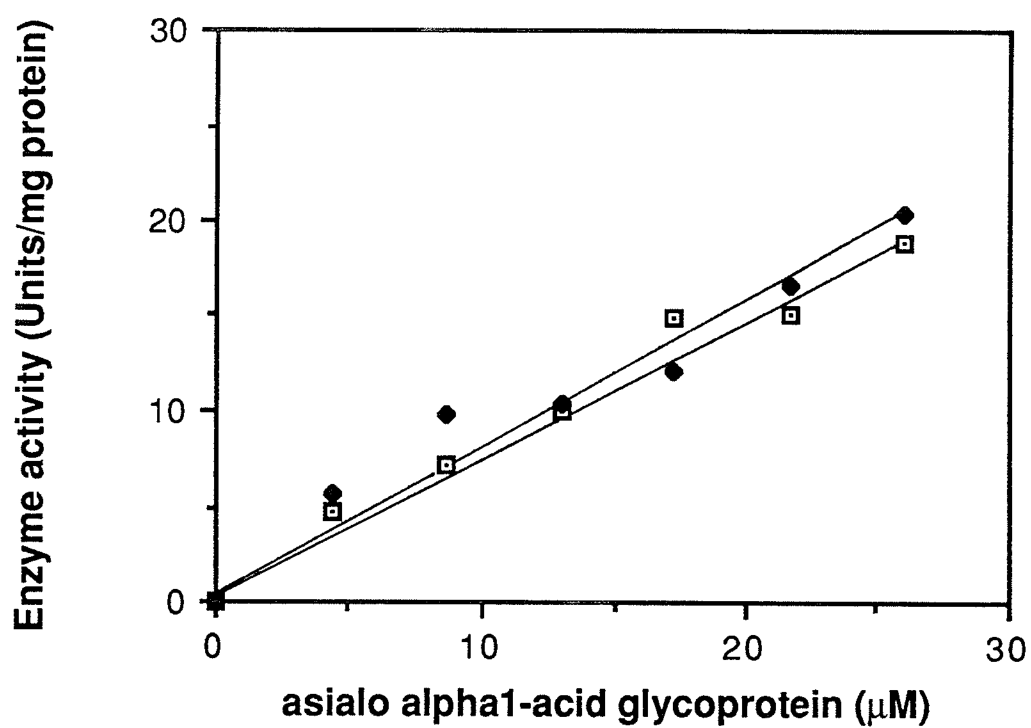

Enzyme activity (Units/mg protein)
30
20
10
0
0
10
20
30
asialo alpha1-acid glycoprotein (μM)

Figure 35

Effect of CMP-NeuAc concentration on sialyl transferase activity in clone 5 wild type (❑) and clone 5 con A-resistant (◆) L6 myoblasts in the presence of 200 μg of asialo fetuin. A unit of enzyme activity represents the amount of NeuAc transferred to asialo fetuin per hour. Endogenous activity has been subtracted from the total activity. Results are the mean of 2 assays and S.D. is within ±20%.

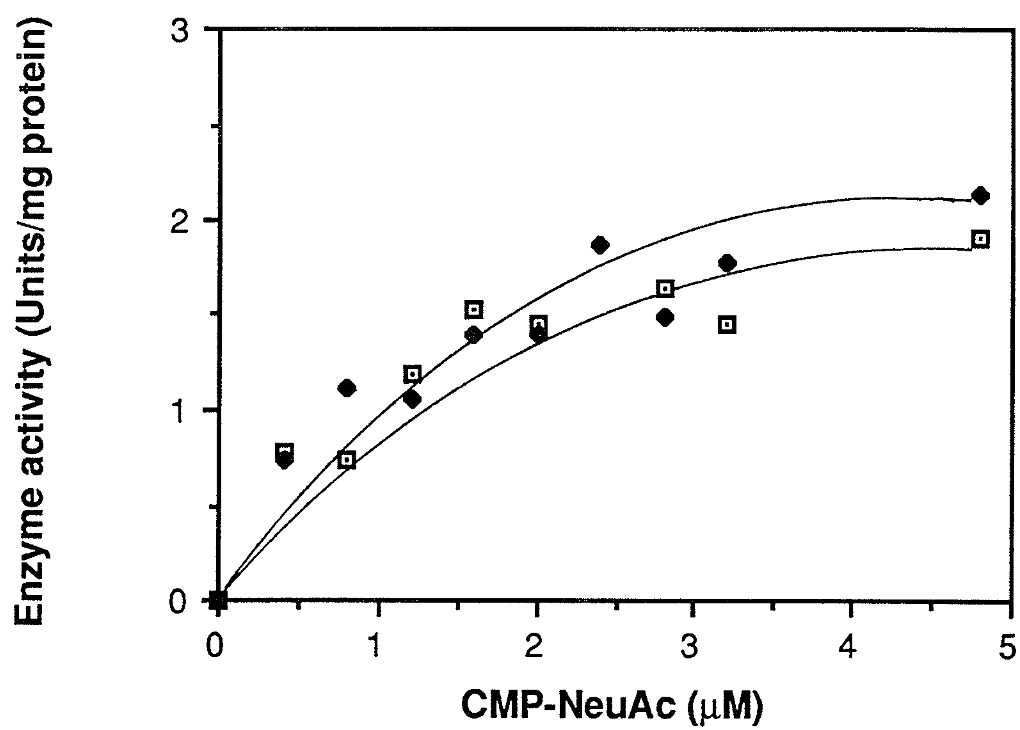

Enzyme activity (Units/mg protein)
CMP-NeuAc (μM)
0
1
2
3
4
5
0
1
2
3

Figure 36

Effect of asialo fetuin concentration on sialyl transferase activity in clone 5 wild type (❑) and clone 5 con A-resistant (◆) L6 myoblasts. A unit of enzyme activity represents the amount of NeuAc transferred to asialo fetuin per hour. Endogenous activity has been subtracted from the total activity. Results are the mean of 2 assays and S.D. is within ±25%.

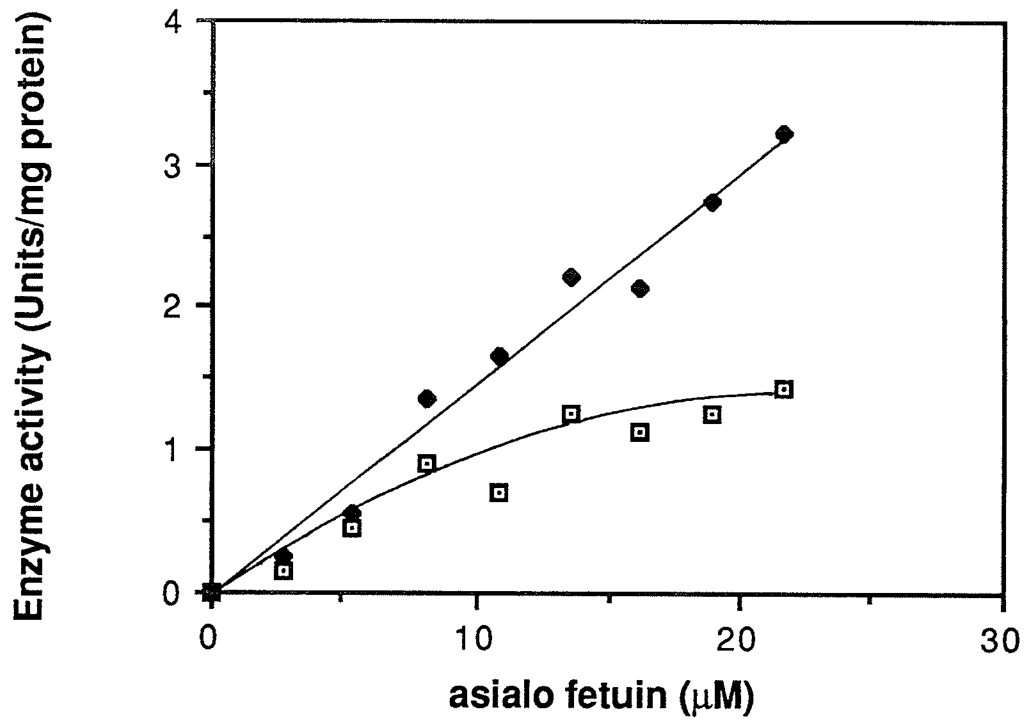

Enzyme activity (Units/mg protein)
4
3
2
1
0
0
10
20
30
asialo fetuin (μM)

Table 13 Kinetic Constants* for Sialyl Transferase Activity in L6 Clone 5 Wild Type and Con A-resistant Myoblasts.

	Wild Type		Con A-resistant Variant	
Substrate	K_m	V_{max}	K_m	V_{max}
Asialo Fetuin	23 ± 6	3.1 ± 0.7	>20	>3
CMP-NeuAc (asialo fetuin)	1.4 ± 0.5	2.3 ± 0.4	1.3 ± 0.7	2.3 ± 0.4
Asialo α_1-acid glycoprotein	>15	>20	>15	>20
CMP-NeuAc (asialo α_1-acid glycoprotein)	10 ± 5	20 ± 5	7 ± 5	10 ± 3

* The K_m and V_{max} values represent apparent K_m and apparent V_{max} because sialyl transferase is a bisubstrate enzyme. K_m units are µM and V_{max} units are pmol NeuAc transferred/mg cell protein/hr. K_m and V_{max} calculations are based Wilkinson (1961).

2. Galactosyl transferase

The pH profile of galactosyl transferase using asialoagalacto fetuin as the acceptor showed slight differences for wild type and variant myoblasts. The wild type activity had a sharp pH optimum at pH 6.2, whereas the variant activity was a broader curve with the optimum activity at pH 5.8 (Figure 37). A pH of 6.0 was chosen for further assays. Incorporation of galactose from UDP-Gal was linear up to 6 hours (Figure 38), however, activity at times of three hours or less gave inconsistent results, therefore 4 hours was chosen as an appropriate time of incubation.

Galactosyl transferase is also a bisubstrate enzyme requiring both asialoagalacto acceptor glycoprotein and the nucleotide sugar, UDP-Gal. Graphs of kinetic data for the acceptor glycoproteins, asialoagalacto fetuin and asialoagalacto α_1-acid glycoprotein, and for the nucleotide sugar using either of the acceptors are given in Figures 39 to 42 and kinetic constants are summarized in Table 14. No significant differences were observed in K_m values between the wild type and variant myoblasts for either asialoagalacto α_1-acid glycoprotein as the acceptor, or UDP-Gal with the same acceptor glycoprotein. V_{max} values were also similar for UDP-Gal, however, the V_{max} for asialoagalacto α_1-acid glycoprotein was slightly higher for the variant enzyme. As was found with the sialyl transferase data, the greatest differences between the kinetic constants of the wild type and variant enzyme acitivities were with respect to the glycoprotein acceptor fetuin. Althought the data for the sialyl transferase activity in the variant, with respect to asialoagalacto fetuin, were only in the first order region of the curve, the K_m and V_{max} values of the variant are substantially greater than the wild type values. The wild type K_m value for UDP-Gal, in the presence of asialoagalacto fetuin, was slightly greater than the variant but there was no significant differences between V_{max} values. Again, the large difference in the affinity of the galactosyl

transferase activity of the variant for asialoagalacto fetuin may be due to the presence of O-linked oligosaccharides; asialoagalacto α_1-acid glycoprotein contains only N-linked oligosaccharides.

Figure 37

The effect of pH on galactosyl transferase activity for clone 5 wild type (❑) and clone 5 con A-resistant L6 (◆) myoblasts. One unit of enzyme activity represents the amount of galactose from UDP-galactose transferred to 200 μg of asialoagalacto fetuin, per hour. The results are the mean of 2 assays and endogenous activity has been subtracted from the total activity.

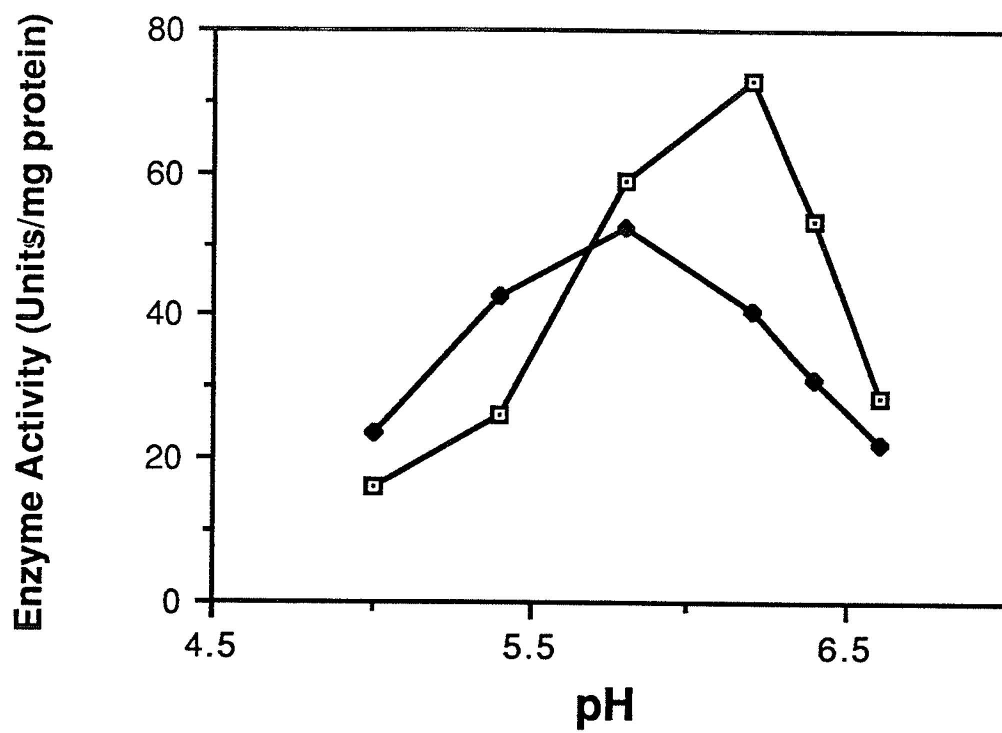

Enzyme Activity (Units/mg protein)
80
60
40
20
0
4.5
5.5
6.5
pH

Figure 38

The effect of time of incubation on galactosyl transferase for wild type (❑) and con A-resistant (◆) L6 myoblasts (clone 5). Enzyme activity is expressed as the amount of galactose transferred to 200 μg of asialoagalacto fetuin per mg of cell protein. Endogenous activity has been subtracted from the total activity. Results are the mean of 2 assays and S.D. is within ±20%.

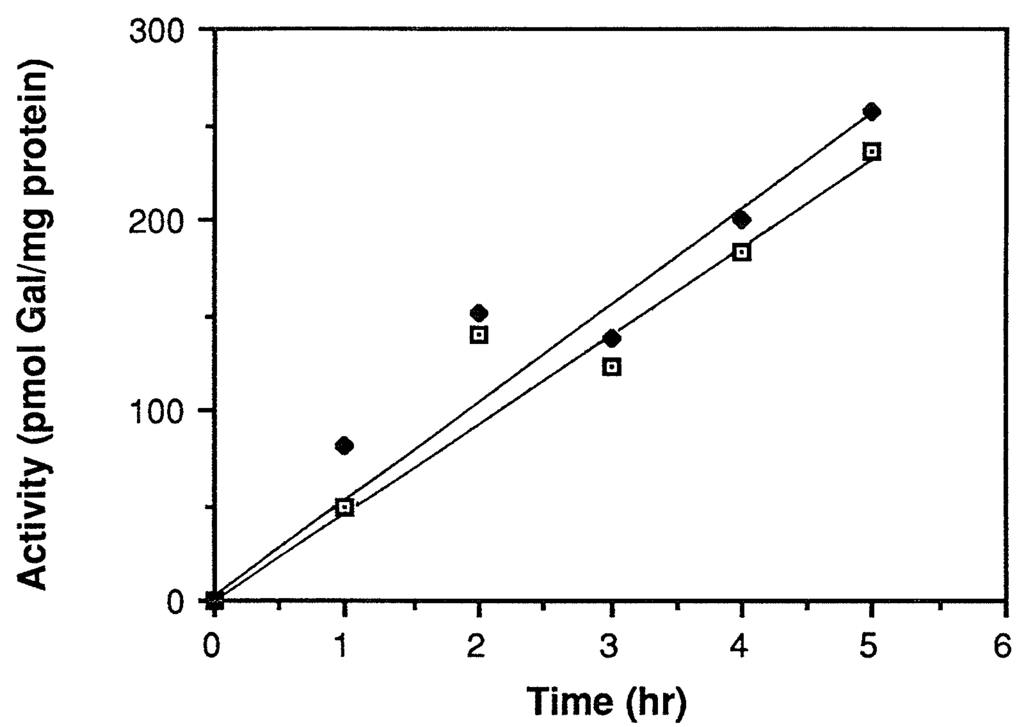

Activity (pmol Gal/mg protein)
300
200
100
0
0
1
2
3
4
5
6
Time (hr)

Figure 39

The effect of asialoagalacto α_1-acid glycoprotein concentration on galactosyl transferase activity in clone 5 wild type (❑) and clone 5 con-A resistant (◆) L6 myoblasts. A unit of enzyme activity represents the amount of galactose transferred to asialoagalacto α_1-acid glycoprotein per hour (agalacto α_1-acid glycoprotein refers to asialoagalacto α_1-acid glycoprotein). Endogenous activity has been subtracted from the total activity. Results represent the mean of 3 assays and S.D. is within ±20%.

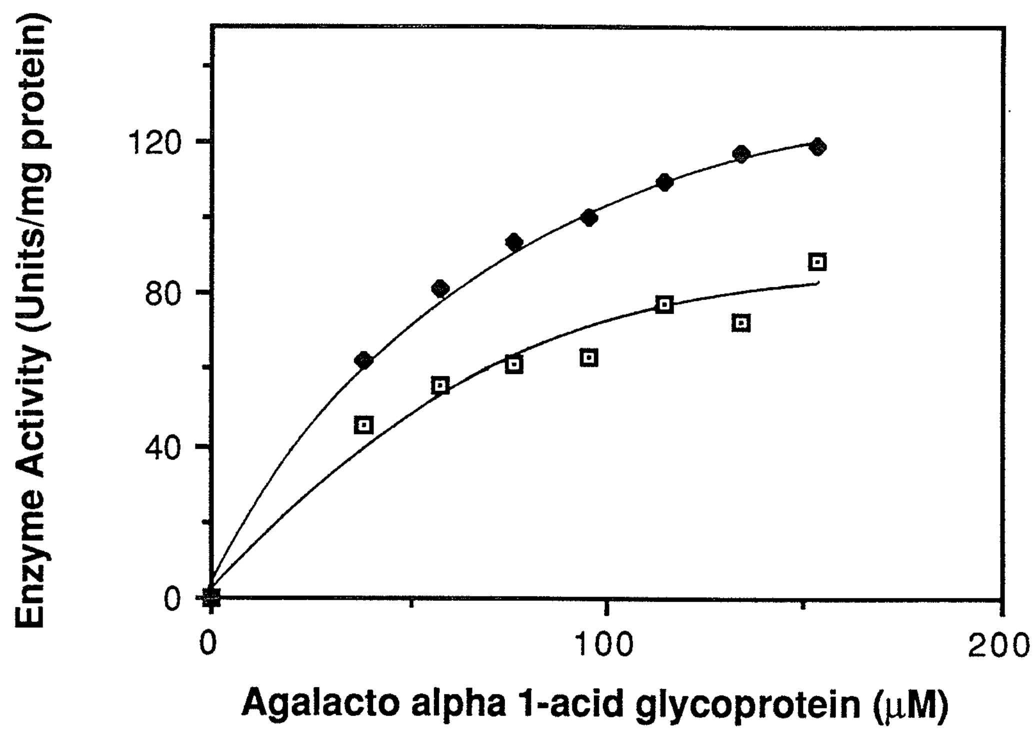

Enzyme Activity (Units/mg protein)
120
80
40
0
0
100
200
Agalacto alpha 1-acid glycoprotein (μM)

Figure 40

The effect of UDP-Gal concentration on galactosyl transferase activity in clone 5 wild type (❑) and clone 5 con A-resistant (◆) L6 myoblasts in the presence of 200 μg of asialoagalacto α_1-acid glycoprotein. A unit of enzyme activity represents the amount of galactose transferred to asialoagalacto α_1-acid glycoprotein per hour. Endogenous activity has been subtracted from the total activity. Results represent the mean of 3 assays and S.D. is within ±20%.

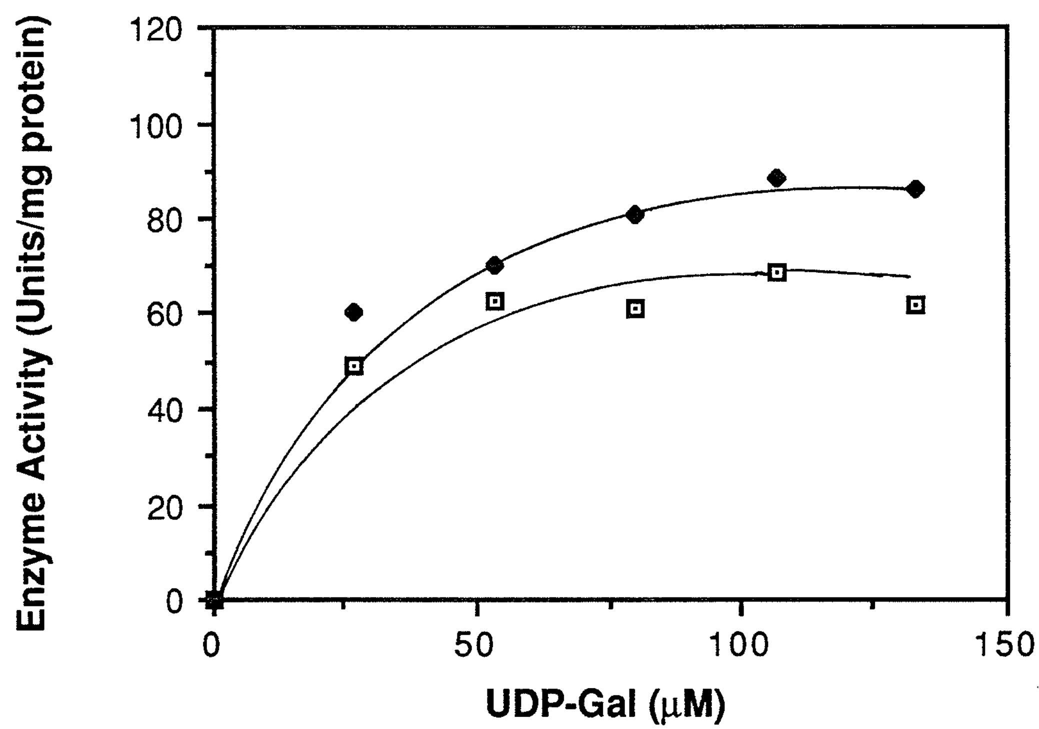
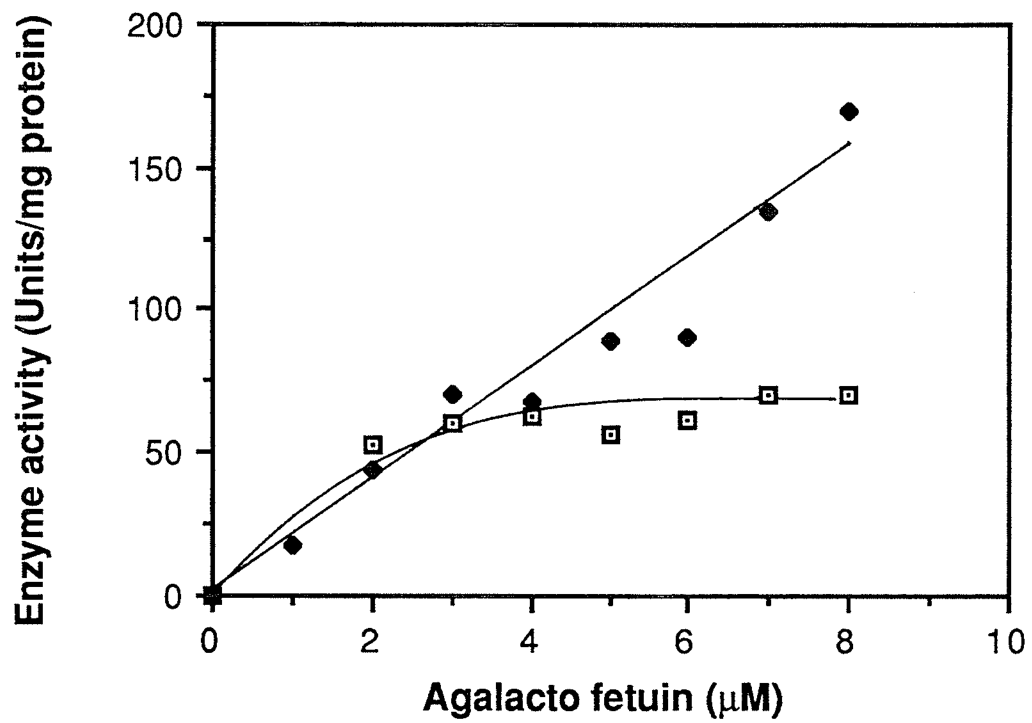

Enzyme Activity (Units/mg protein)
120
100
80
60
40
20
0
0
50
100
150
UDP-Gal (μM)

Figure 41

Effect of asialoagalacto fetuin concentration on galactosyl transferase activity in clone 5 wild type (❑) and clone 5 con A-resistant (◆) L6 myoblasts. A unit of enzyme activity represents the amount of galactose transferred to asialoagalacto fetuin per hour (Agalacto fetuin refers to asialoagalactofetuin) . Endogenous activity has been subtracted from the total activity. Results are the mean of 2 assays and S.D. is within ±20%.



200
150
100
50
0
0
2
4
6
8
10
Enzyme activity (Units/mg protein)
Agalacto fetuin (μM)

Figure 42

The effect of UDP-Gal concentration on galactosyl transferase activity in clone 5 wild type (❑) and clone 5 con A-resistant (◆) L6 myoblasts in the presence of 200 μg of asialoagalacto fetuin. A unit of enzyme activity represents the amount of galactose transferred to asialoagalacto fetuin per hour. Endogenous activity has been subtracted from the total activity. Results represents the mean of 2 assays and S.D. is within ±20%.

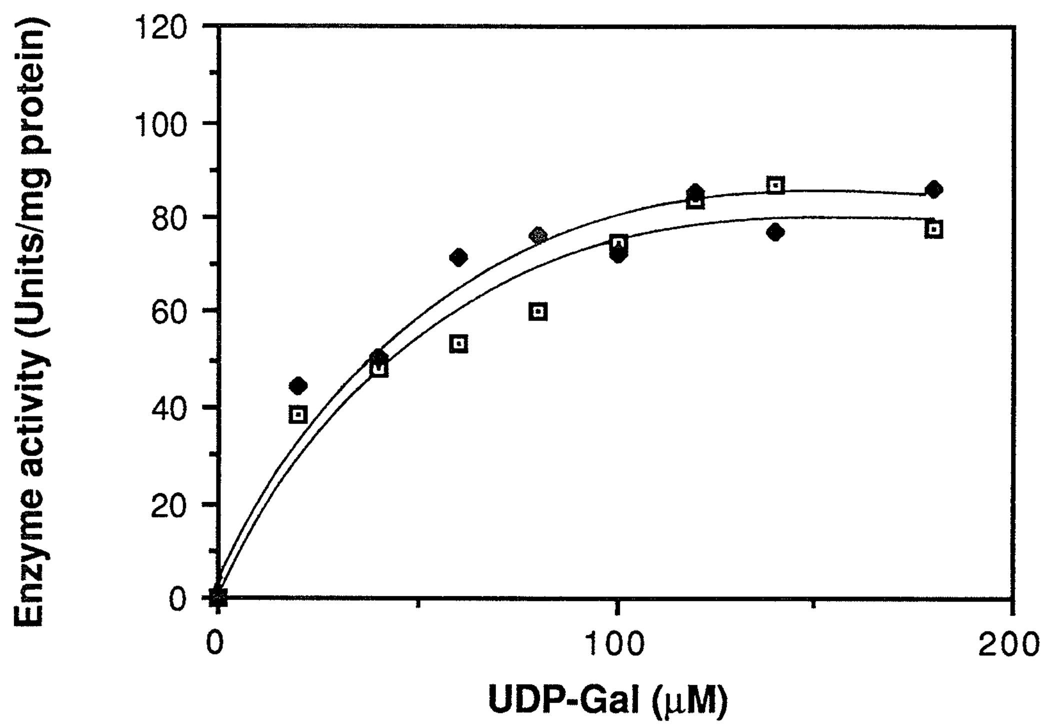

Enzyme activity (Units/mg protein)
120
100
80
60
40
20
0
0
100
200
UDP-Gal (μM)

Table 14 Kinetic Constants* for Galactosyl Transferase Activity in L6 Clone 5 Wild Type and Con A-resistant Myoblasts.

	Wild Type		Con A-resistant Variant	
Substrate	K_m	V_{max}	K_m	V_{max}
Agalacto fetuin	0.5 ± 0.3	68 ± 5	>4	>150
UDP-Gal (agalacto fetuin)	50 ± 10	110 ± 10	28 ± 7	99 ± 6
Agalacto α_1-acid glycoprotein	48 ± 7	121 ± 7	42 ± 4	162 ± 5
UDP-Gal (agalacto α_1-acid glycoprotein)	17 ± 5	102 ± 10	19 ± 3	115 ± 7

* The values for the kinetic constants represent the apparent K_m and apparent V_{max} because galactosyl transferase is a bisubstrate enzyme. Agalacto acceptors refer to asialoagalacto acceptors. K_m units are µM and V_{max} units are pmol Gal transferred/mg cell protein/hr. K_m and V_{max} calculations were based on Wilkinson (1961).

F. *Aspects of Glycoprotein Metabolism in Duchenne Dystrophic Fibroblasts*

1. Glycosidase activities in fibroblasts from Duchenne dystrophic patients and normal age-matched donors.

Duchenne muscular dystrophy is an X-linked, degenerative disease of muscle tissue. The genetic defect has not yet been established but several biochemical alterations are known (Rowland, 1980; Anand, 1983). Elevated serum levels of several enzymes from muscle tissue have led to the proposal of one current theory which suggests that a generalized membrane defect results in leakage of the enzymes from muscle tissue. Based on this theory, erythrocytes, lymphocytes and fibroblasts from Duchenne patients have been studied and biochemical alterations have been reported in these cell types (Anand, 1983). Also, muscle tissue is difficult to obtain and skin fibroblasts from skin biopsies are an alternative source of primary cells from Duchenne patients which can be grown in tissue culture.

Altered lysosomal hydrolase activities are associated with several degenerative genetic diseases, such as fucosidosis, mannosidosis, sialidosis, I-cell disease and pseudo-Hurler polydystrophy (Durand and O'Brien, 1982). To investigate the possibility of a defect in lysosomal enzyme activity in Duchenne dystrophic fibroblasts, two enzyme activities, α-mannosidase and ß-hexosaminidase, which displayed significant alterations in con A-resistant L6 myoblasts compared to the wild type, were studied in cultures of primary fibroblasts from male patients diagnosed with Duchenne muscular dystrophy and normal age-matched controls. The following table gives the dystrophic and normal cultures used in this study with the corresponding age of the donor in years, divided into comparison groups loosely based on the age of the donor.

Duchenne dystrophic		Normal	
Cell Line	Age (yrs.)	Cell Line	Age (yrs.)
WG 348	4.5	MCH 48	5
WG 840	5.5	MCH 40	6
WG 502	5.5	MCH 50	6
WG 448	6	MCH 52	7
WG 280	7		
GM 3871	11	GM 323	11
Anderson	12		
WG 729	13.5		
WG 730	15.5	MCH 57	17

The α-mannosidase activity was measured over a pH range of 4.0 to 6.6. The α-mannosidase activity in the fibroblasts from the youngest age group of DMD patients (age 4.5 to 7.0 years) had a pH optimum around pH 4.5 with activity slowly decreasing and plateauing around pH 6.0 (Figure 43 A). At pH 4.5 the activity ranged from approximately 0.7 to 1.2 units/mg protein. The normal fibroblasts of the corresponding age group had similar pH profiles with an optimum at pH 4.5, and dropping to a plateau at higher pH values (Figure 43 B). The range of values for mannosidase activity at pH 4.5 is similar to that for the dystrophic fibroblasts, with the exception of MCH 48 which had about twice the level of activity.

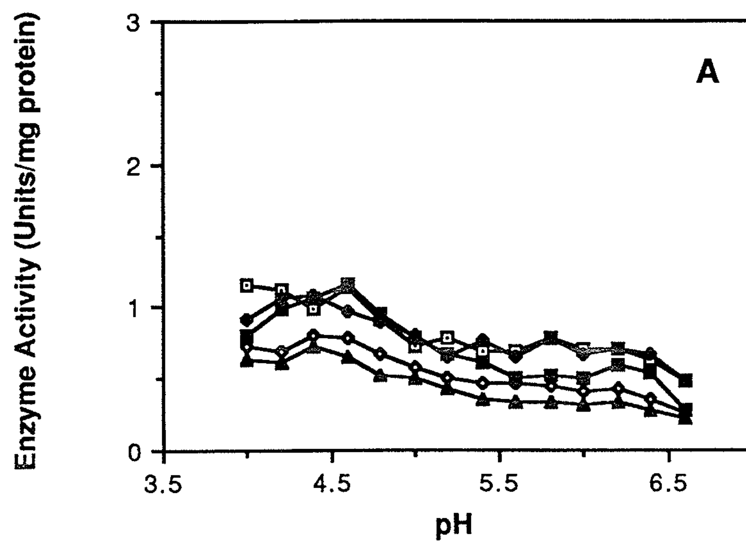
The pH profiles of mannosidase activities in the fibroblasts from slightly older patients (age 11 to 13.5 years) were very similar for normal and dystrophic fibroblasts and are presented on the same graph (Figure 44). Their pH optimum was around pH 4.4. The oldest age group (age 15.5 and 17 years) also had a pH optimum of 4.5 and a similar shape for the pH profile (Figure 45). All cultures of fibroblasts, dystrophic and normal, had mannosidase activities which fell within similar ranges of activity except for MCH 48.

The pH profiles of ß-hexosaminidase activity in Duchenne dystrophic fibroblasts had a pH optimum around pH 4.5 with a steady decrease in activity as the pH increased to pH 6.5 (Figure 46 A). A similar profile and pH optimum was apparent for ß-hexosaminidase activity of normal fibroblasts (Figure 46 B). The range of activity at pH 4.5 was 40 to 65 units/mg protein in the dystrophic fibroblasts and from 45 to 55 in the normal fibroblasts.

These results suggest that α-mannosidase and ß-hexosaminidase activites are not significantly different in fibroblasts from Duchenne dystrophic patients and normal age-matched donors.

Figure 43

The effect of pH on α-mannosidase activity in skin fibroblasts from Duchenne dystrophic patients and normal donors (age range: 4.5-7 yr.). Duchenne dystrophic fibroblasts (Panel A): WG 448 (❑), 6 yr.; WG 840 (◆), 5.5 yr.; WG 280 (▲), 7 yr.; WG 348 (◊), 4.5 yr.; WG 502 (■), 5.5 yr. Normal fibroblasts (Panel B): MCH 50 (❑), 6 yr.; MCH 48 (◆), 5 yr.; MCH 52 (■), 7 yr., MCH 40 (◇), 6 yr. A unit of enzyme activity is the amount of enzyme required to hydrolyze 1 μmol of 4-methylumbelliferyl-α-D-mannoside per minute. Results are the mean of 2-6 assays.


Enzyme Activity (Units/mg protein)
A
pH
3
2
1
0
3.5
4.5
5.5
6.5

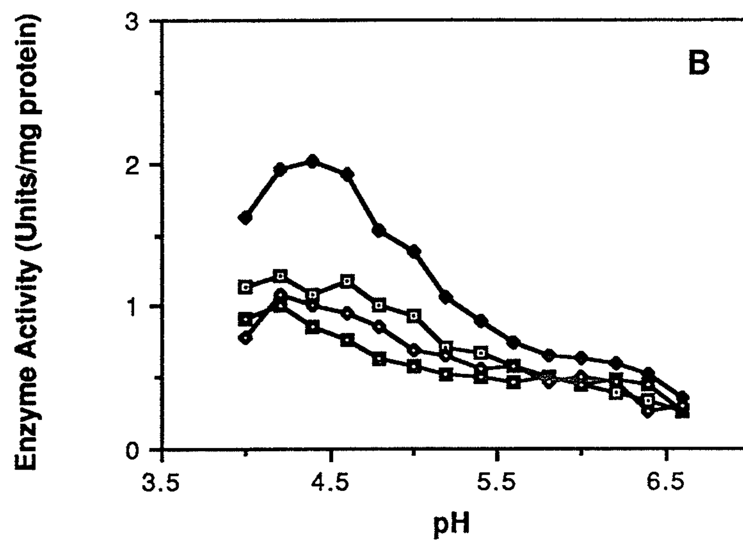

Enzyme Activity (Units/mg protein)
B
pH
3
2
1
0
3.5
4.5
5.5
6.5

Figure 44

The effect of pH on α-mannosidase activity in skin fibroblasts from Duchenne dystrophic patients and normal donors (age range: 11-13.5 yr.). Duchenne dystrophic fibroblasts: GM 3871 (◆), 11 yr.; Anderson (■), 12 yr.; WG 729 (◇), 13.5 yr. Normal fibroblasts: GM 323 (□), 11 yr. A unit of enzyme activity is the amount of enzyme required to hydrolyze 1 μmol of 4-methylumbelliferyl-α-D-mannoside per minute. Results are the mean of 2-4 assays.

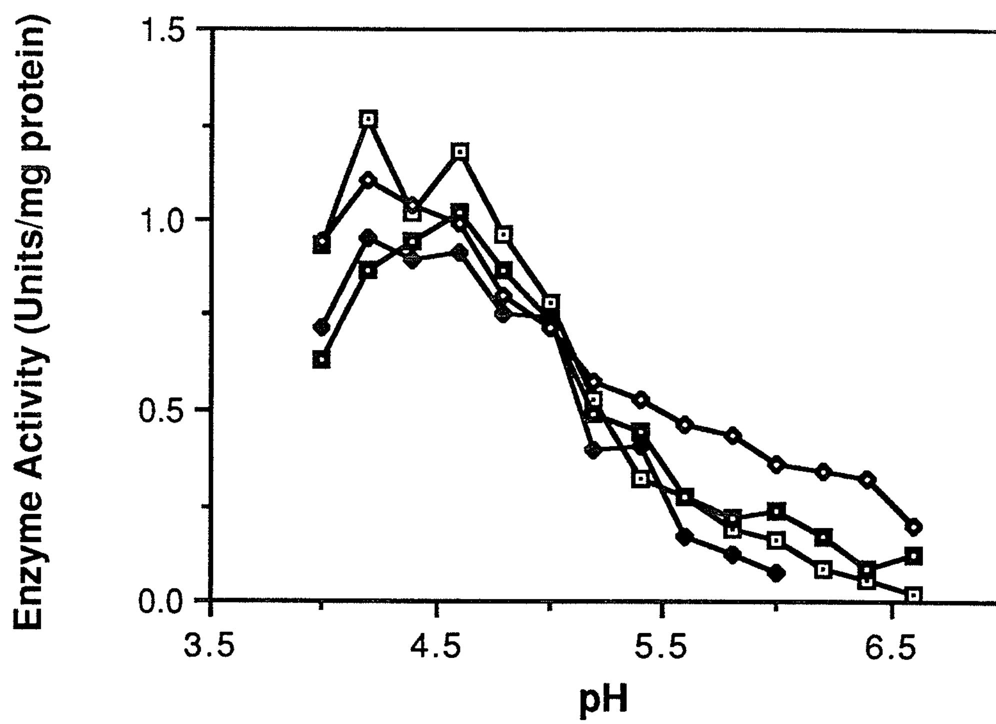

Enzyme Activity (Units/mg protein)
1.5
1.0
0.5
0.0
3.5
4.5
5.5
6.5
pH

Figure 45

The effect of pH on α-mannosidase activity in skin fibroblasts from Duchenne dystrophic patients and normal donors (age range: 15.5-17 yr.). Duchenne dystrophic fibroblasts: WG 730 (◆), 15.5 yr. Normal fibroblasts: MCH 57 (❑), 17 yr. A unit of enzyme activity is the amount of enzyme required to hydrolyze 1 μmol of 4-methylumbelliferyl-α-D-mannoside per minute. Results are the mean of 2-4 assays.

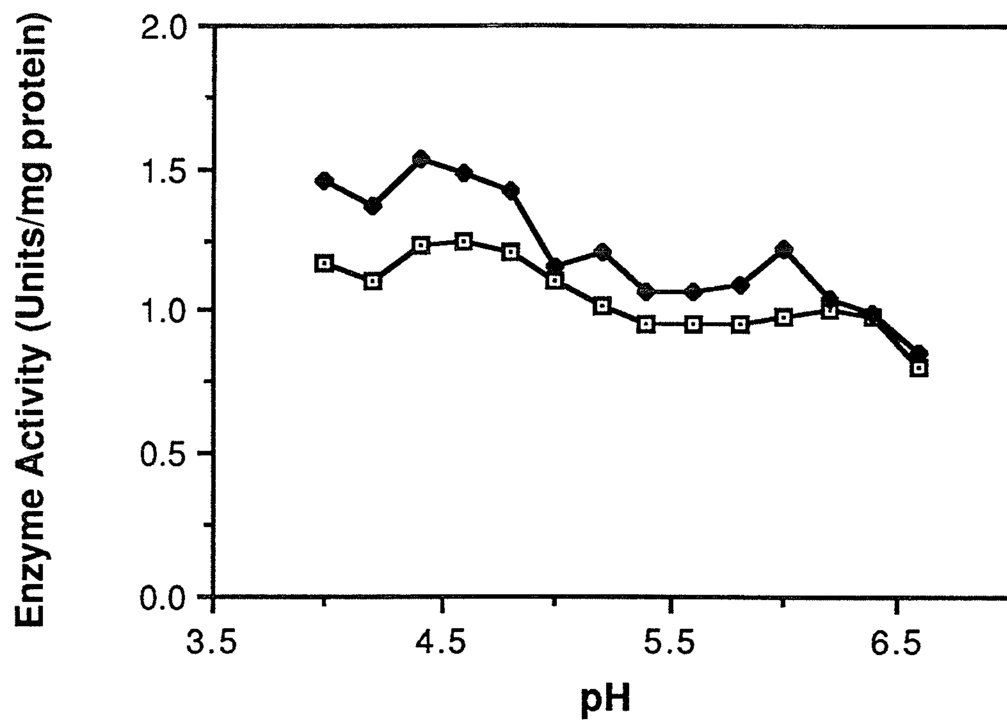
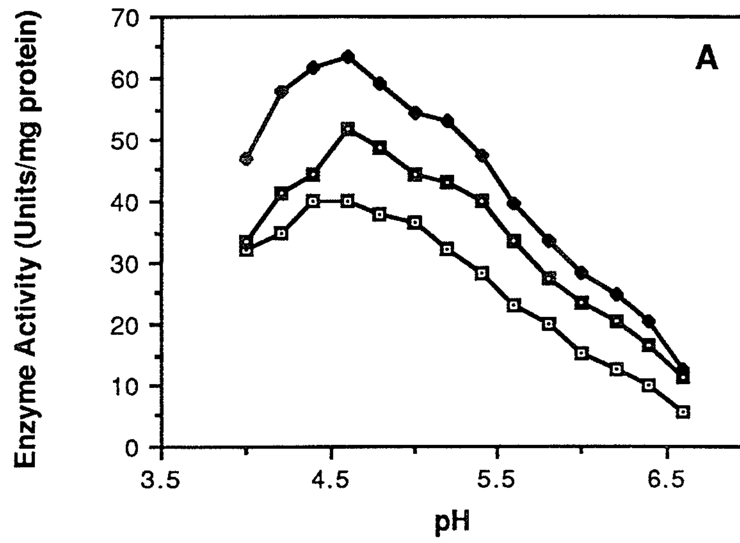
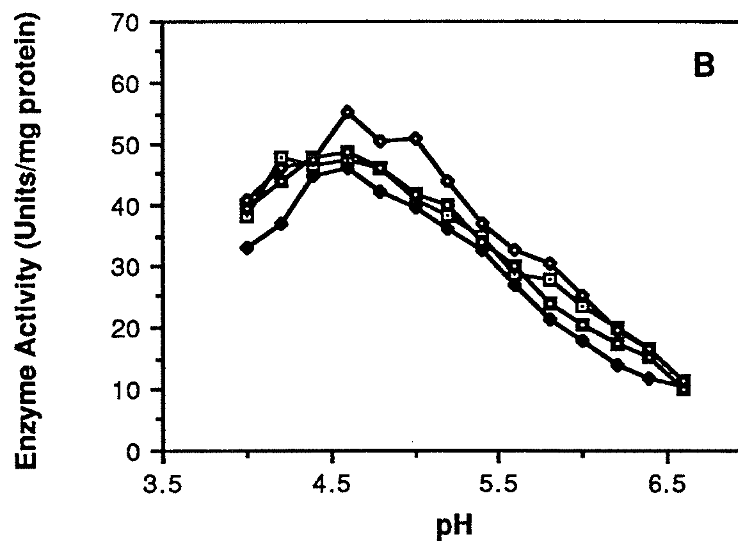

Enzyme Activity (Units/mg protein)
2.0
1.5
1.0
0.5
0.0
3.5
4.5
5.5
6.5
pH

Figure 46

The effect of pH on ß-hexosaminidase activity in skin fibroblasts form Duchenne dystrophic patients (panel A) and normal donors (panel B). Duchenne dystrophic fibroblasts: WG 348 (❑), 4.5 yr.; WG 502 (◆), 5.5 yr.; WG 280 (■), 7 yr. Normal fibroblasts: MCH 52 (❑), 7 yr.; MCH 48 (◆), 5 yr.; MCH 40 (■), 6 yr.; MCH 57 (◊), 17 yr. One unit of enzyme activity represents the amount of enzyme required to hydrolyze 1 µmol of *p*-nitrophenyl-ß-D-N-acetylglucosamine per minute. Results are the mean of 2-6 assays.


Enzyme Activity (Units/mg protein)
70
60
50
40
30
20
10
0
A
3.5
4.5
5.5
6.5
pH


Enzyme Activity (Units/mg protein)
70
60
50
40
30
20
10
0
B
3.5
4.5
5.5
6.5
pH

2. Con A binding in fibroblasts from Duchenne dystrophic patients and normal age-matched donors.

Con A binding to fibroblasts from Duchenne dystrophic patients and age-matched normal donors was compared using ^{125}-iodine labelled con A. The amount of con A bound was normalized to either the amount of protein per plate (Figures 47 and 49) or the number of cells per plate (Figures 48 and 50), to ensure that possible differences in binding capacitities were not due to differences in protein content of the normal and dystrophic cells. Two different age groups of 6 years (WG 448-dystrophic, MCH 50-normal), and 15.5 to 17 years (WG 730-dystrophic, MCH 57-normal) were investigated.

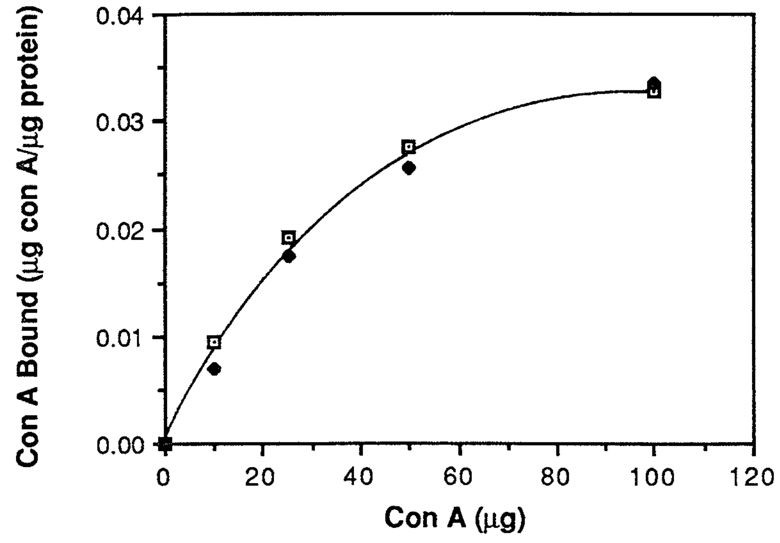
In the younger age group, the con A bound per protein is slightly reduced in the DMD fibroblasts (WG 448) compared to normal fibroblasts (MCH 50) (Figure 47 A). Scatchard analysis revealed positive cooperativity for both cell types (Figure 47 B). When the con A bound was normalized to the number of cells per plate, WG 448 had slightly less con A bound than the normal fibroblasts (MCH 50) (Figure 48 A) and also displayed positive cooperativity in Scatchard analysis (Figure 48 B).

The DMD fibroblasts from the older patients (WG 730) had a slightly larger amount of con A bound than the normal fibroblasts (MCH 57) when normalized to the amount of protein per plate (Figure 49). However, when con A binding is expressed per cell (Figure 50) the amount of con A bound for MCH 57 was 10% greater than WG 730. Scatchard analysis of both sets of data again showed positive cooperativity with small differences in binding capacities (Figure 49 and 50, B panels). Thus, there appear to be no significant or consistent differences between con A binding to the Duchenne dystrophic and normal fibroblasts used in these assays with respect to either con A bound per cell or per μg of protein. However, there may be differences in

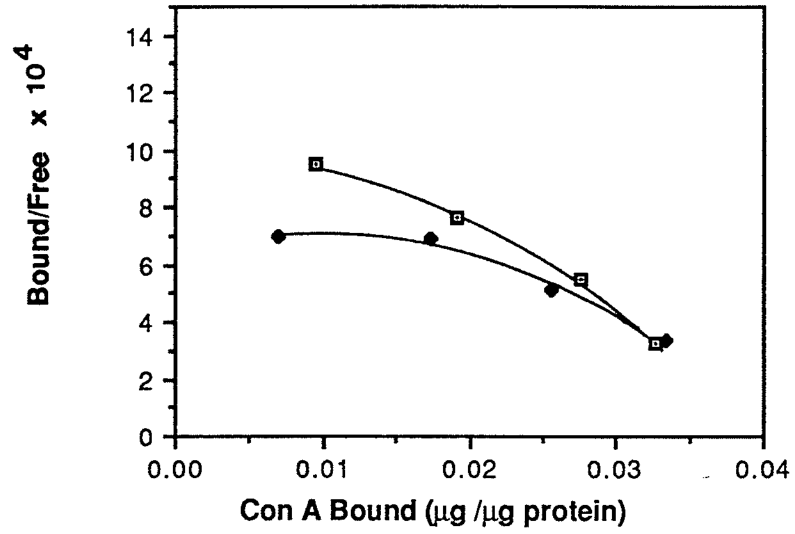
oligosaccharide structure between normal and dystrophic fibroblasts which is not detectable through lectin binding. Although iodinated lectin binding studies provide a highly sensitive method for measuring gross changes in cell surface oligosaccharide structure, they cannot detect subtle differences in carbohydrate structures.

Figure 47

The effect of various concentrations of con A on binding to skin fibroblasts from a 6 year old Duchenne dystrophic patient and a 6 year old normal donor. The amount of con A bound is normalized to the amount of protein per plate. WG 448 (❑), Duchenne dystrophic, 6 yr.; MCH 50 (◆), normal donor, 6 yr. Panel B is a Scatchard analysis of the data given in panel A. Results are the mean of three assays and S.D. is within ± 3%.



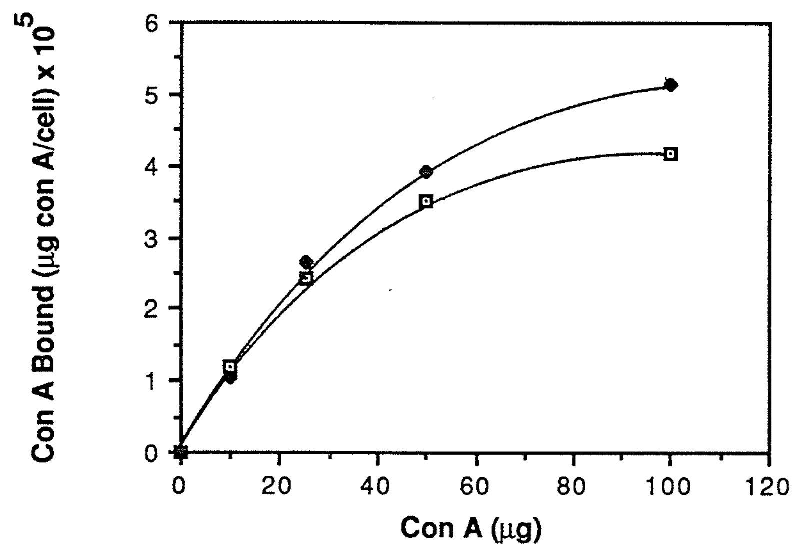
Con A Bound (μg con A/μg protein)
0.04
0.03
0.02
0.01
0.00
0
20
40
60
80
100
120
Con A (μg)



Bound/Free x 10^4
14
12
10
8
6
4
2
0
0.00
0.01
0.02
0.03
0.04
Con A Bound (μg /μg protein)

Figure 48

Effect of various concentrations of con A on binding to skin fibroblasts from a 6 year old Duchenne dystrophic patient and a 6 year old normal donor. The amount of con A bound is normalized to the number of cells per plate. WG 448 (❑), Duchenne dystrophic, 6 yr; MCH 50 (◆), normal, 6 yr. Panel B is a Scatchard representation of the data presented in panel A. Results are the mean of three assays and the S.D. is within ± 3%.


Con A Bound (μg con A/cell) x 10^5
0
1
2
3
4
5
6
0
20
40
60
80
100
120
Con A (μg)

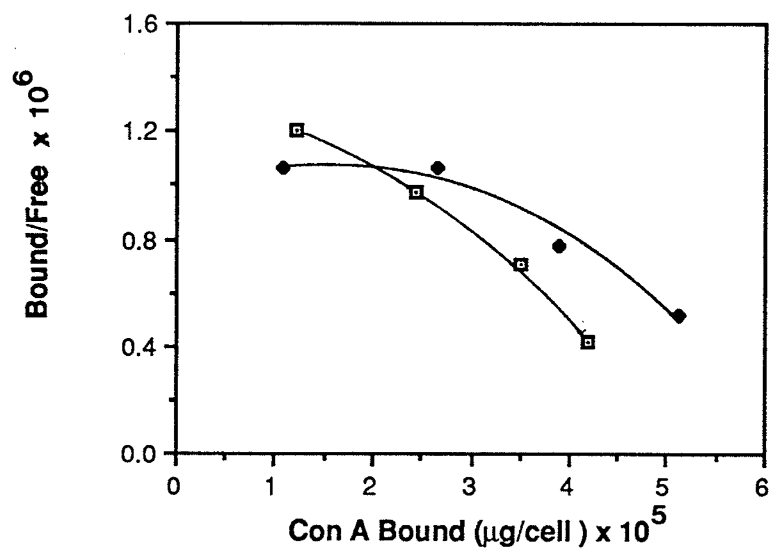
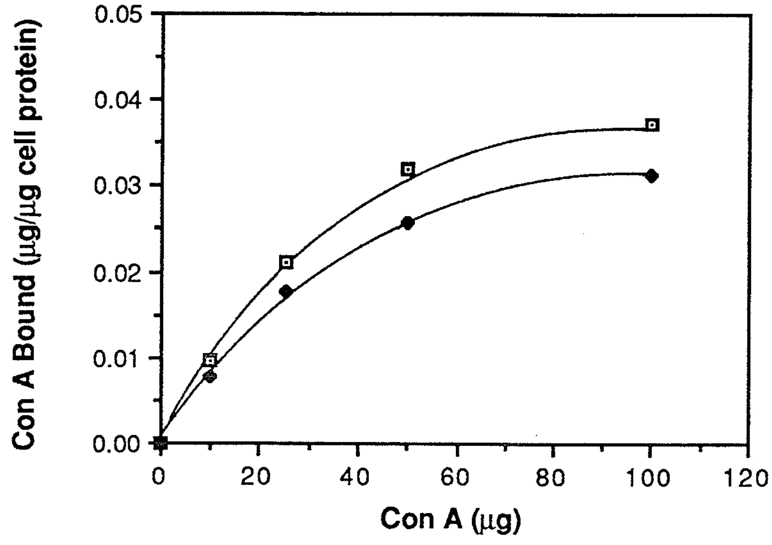

Bound/Free x 10^6
0.0
0.4
0.8
1.2
1.6
0
1
2
3
4
5
6
Con A Bound (μg/cell) x 10^5

Figure 49

The effect of various concentrations of con A on binding to skin fibroblasts from a 15.5 year old Duchenne dystrophic patient and a 17 year old normal donor. The amount of con A bound is normalized to the amount of protein per plate. WG 730 (❑), Duchenne dystrophic, 15.5 yr; MCH 57 (◆), normal donor, 17 yr. Panel B is a Scatchard analysis of the data in panel A. Results are the mean of three assays and the S.D. is within ± 3%.


Con A Bound (µg/µg cell protein)
0.05
0.04
0.03
0.02
0.01
0.00
0
20
40
60
80
100
120
Con A (µg)

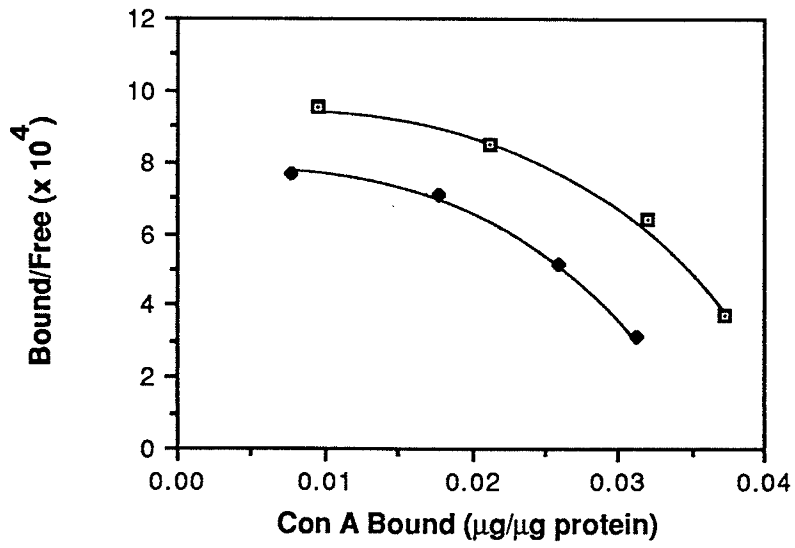
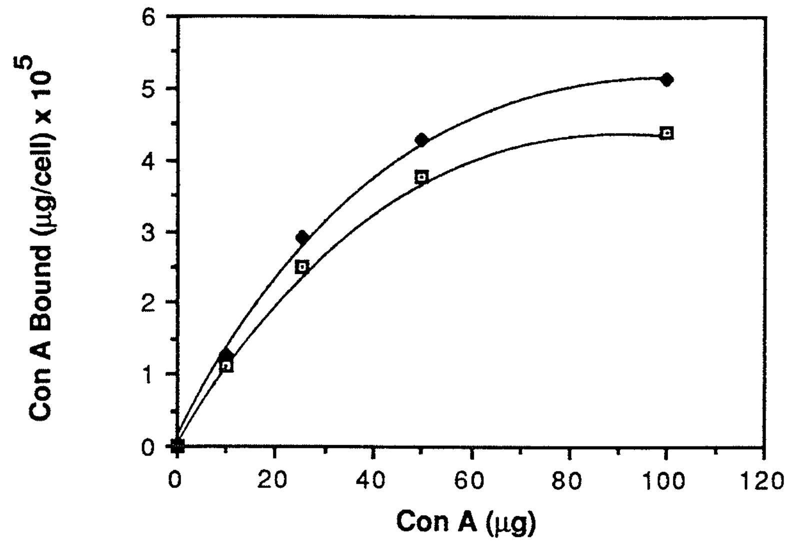
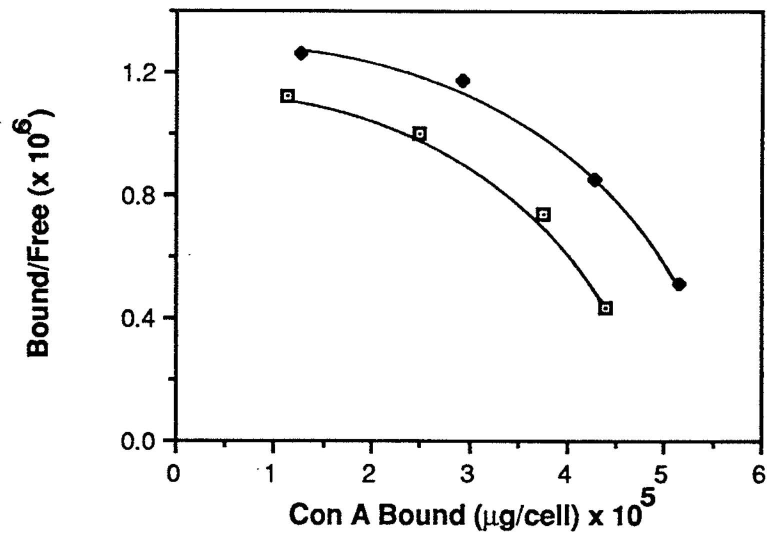

Bound/Free (x 10^4)
12
10
8
6
4
2
0
0.00
0.01
0.02
0.03
0.04
Con A Bound (µg/µg protein)

Figure 50

The effect of various concentrations of con A on binding to skin fibroblasts from a 15.5 year old Duchenne dystrophic patient and a 17 year old normal donor. The amount of con A bound is normalized to the number of cells per plate. WG 730 (❑), Duchenne dystrophic, 15.5 yr.; MCH 57 (◆), normal, 17 yr. Panel B is a Scatchard analysis of the data in panel A. Results are the mean of three assays and the S.D. is within ± 3%.



Con A Bound (μg/cell) x 10^5
Con A (μg)
0
1
2
3
4
5
6
0
20
40
60
80
100
120



Bound/Free (x 10^6)
Con A Bound (μg/cell) x 10^5
0.0
0.4
0.8
1.2
0
1
2
3
4
5
6

Table 15 Con A binding capacities* from Scatchard analysis of fibroblasts from Duchenne dystrophic patients and normal age-matched donors.

Cell Type	Binding Capacity*	
	(μg con A/cell) x 10^5	(μg con A/μg protein)
WG 448	5.0 ± 0.5	0.036 ± 0.005
WG 730	5.1 ± 0.5	0.045 ± 0.005
MCH 50	6.5 ± 0.5	0.044 ± 0.005
MCH 57	6.0 ± 0.5	0.036 ± 0.005

* values were extrapolated from Scatchard plots.

DISCUSSION

Discussion

How cells undergo differentiation to form unique cell types within multicellular organisms is one of the many intriguing questions in cell biology today. A large area of research has been devoted to determining factors which affect growth and differentiation of cells, including the understanding of embryonic development and the mechanism of replenishment of damaged tissue, and identifying factors which contribute to the lack of control of growth and differentiation in cancer.

Cell lines which retain the capacity to differentiate in culture, such as the L6 myoblast cell line are extremely useful in these studies. The selection of genetic mutants, which are unable to differentiate, allows direct comparison of the phenotype with the wild type to determine the effects of alterations in biochemistry on the differentiation process.

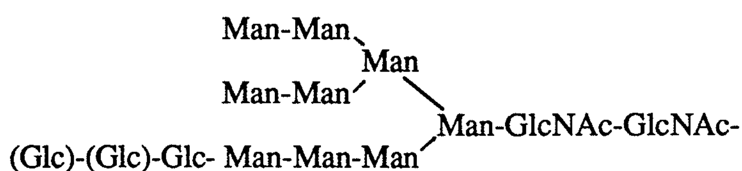
Undoubtedly, the plasma membrane plays a large role in cellular differentiation. Cell surface membrane receptors interact with extracellular components such as growth factors and other hormones which initiate cellular differentiation, but in some types of cellular differentiation, such as the fusion of L6 myoblasts, the plasma membranes are more intimately involved. Several studies have indicated a requirement for N-linked glycosylation during myoblast fusion (see Historical Review), and there is considerable evidence from the characterization of con A-resistant mutants to suggest that high mannose oligosaccharides are essential for L6 myoblast fusion to occur.

A. *The Effects of Oligosaccharide Processing Inhibitors on Rat L6 Myoblast Differentiation.*

The demonstration of inhibition of fusion and biochemical differentiation with specific glycoprotein processing inhibitors further develops the theory that cell surface

high mannose oligosaccharides are required for myoblast differentiation, and may even be directly involved in myoblast fusion (Parfett *et al.*, 1981, 1983). One current model for membrane fusion during myoblast differentiation suggests cell surface glycoproteins may be involved in recognition and adhesion of components of the adjacent myoblast membrane which precedes membrane union (Knudsen and Horowitz, 1977) (Figure 10). If this model is considered along with the correlation between loss of fusion capacity and defective mannosylation of glycoproteins in the con A-resistant myoblasts, it may be construed that high mannose containing glycoproteins (or a specific structure within this group) are directly involved in adhesion and/or recognition prior to membrane union. Two obvious possibilities are 1) the high mannose oligosaccharide acts as a ligand and is recognized by a specific receptor on the surface of an adjacent cell, or 2) a group of cell surface receptors, for an unidentified cell surface ligand, require high mannose oligosaccharides to ensure correct protein conformation either for optimum binding of the ligand and/or targetting and transport to the cell surface (Figure 51).

The glucosidase inhibitors, castantospermine, deoxynojirmycin and methyl-deoxynojirimycin, dramatically inhibit the ability of L6 myoblasts to undergo fusion and biochemical differentiation. These inhibitors cause a blockage in the removal of glucose residues from high mannose oligosaccharides, and at the same time reduce the formation of complex oligosaccharides. In wild type myoblasts the structures containing one to three glucose residues are:



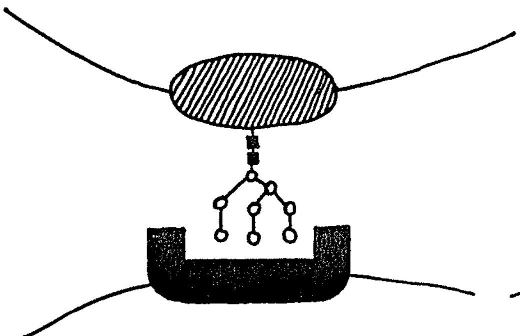
Results from the characterization of the oligosaccharide structures from the con A-resistant myoblasts indicate that instead of high mannose oligosaccharides,

Figure 51

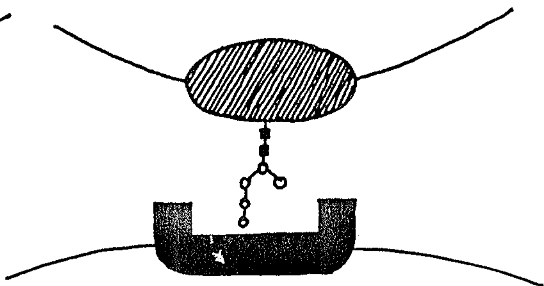
Diagrammatic representations of possible ligands and receptors on wild type and con A-resistant L6 myoblasts. Panel A represents normal recognition of the high mannose oligosaccharides by a receptor on adjacent wild type cells. Panel B represents the lack of receptor recognition of truncated high mannose oligosaccharides of the con A-resistant myoblasts. Panel C represents an alternative model in which a receptor molecule requires normal high mannose oligosaccharides for recognition of an unidentified ligand on adjacent wild type myoblasts. Truncated high mannose oligosaccharides in the con A-resistant myoblasts (Panel D) alter the conformation of the receptor molecule so recognition of the ligand cannot occur.

(■ - N-acetylglucosamine; O - mannose).

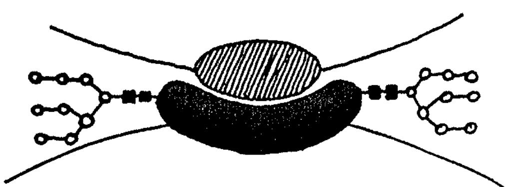
A



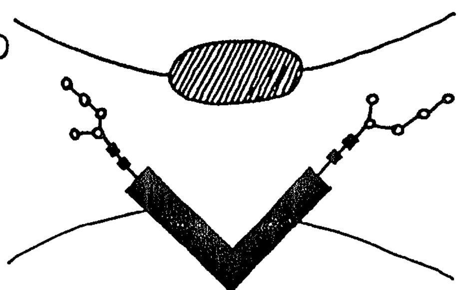
B



C



D

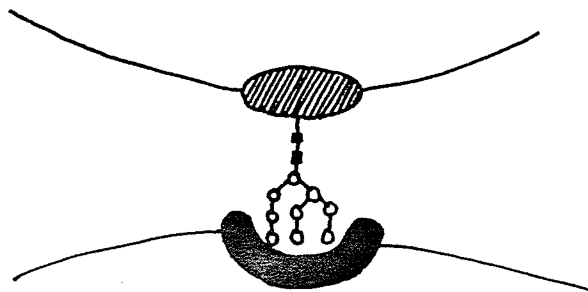


oligosaccharides of no greater than Man_5 are produced. In the presence of the glucosidase inhibitors, these would also contain terminal glucose residues.

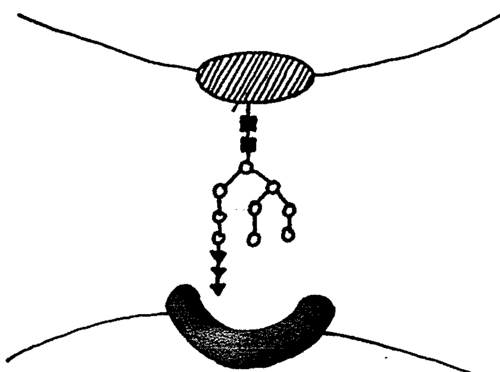
If the first model is considered, the decrease in myoblast fusion in the presence of the glucosidase inhibitors can be explained simply by a reduction in recognition of cell surface high mannose oligosaccharides, due to the retention of the glucose residues which interfere with receptor affinity (Figure 52). The glucose-containing high mannose oligosaccharide will simply not "fit" the receptor. However, there is another possible explanation for the inhibition of fusion in the presence of the glucosidase inhibitors. The blockage of the removal of the glucose residues in the processing reactions has been shown to result in an accumulation of unprocessed glycoproteins in the Golgi in fibroblast cells (Lemansky *et al.*, 1984). If this were also true for the myoblasts, the inhibition of fusion could be due to a decrease in the cell surface population of specific glycoproteins which contain the high mannose oligosaccharides necessary for recognition. Two independent studies have identified cell surface glycoproteins (either 44,500 mw or 46,000 mw) which are significantly decreased on the cell surface of con A-resistant, non-fusing myoblasts (Parfett *et al.*, 1983; Cates *et al.*, 1984). Also, the putative receptor itself may be a glycoprotein which is retained within the Golgi in an unprocessed form. Some secreted and membrane glycoproteins may overcome or bypass this blockage in glucose removal and follow the normal pathway of processing which may account for the low level of fusion which does occur. It is possible that glycoproteins, necessary for adhesion and/or recognition, remain in the ER and Golgi in an unprocessed form, causing a reduction in fusion capacity in glucosidase inhibitor-treated myoblasts. However, it is unlikely that the presence of the glucose residues are preventing transport of the high mannose containing glycoproteins to the cell surface, because a large increase in cell surface con A binding capacity of myoblasts was observed for all of the glucosidase inhibitors. If glycoproteins were being retained in the Golgi due to lack of processing, a decrease in

Figure 52

Diagrammatic representation of the possible interference of receptor recognition of high mannose oligosaccharides by glucose residues which remain on high mannose oligosaccharides in the presence of castanospermine, deoxynojirimycin and methyldeoxynojirimycin. The putative receptor may also be a glycoprotein which is dependent upon glucose removal to attain the correct conformation for recognition of the cell surface ligand. (■ - N–acetylglucosamine; ○ - mannose; ▼ - glucose).



CASTANOSPERMINE
DEOXYNOJIRIMYCIN
METHYLDEOXYNOJIRIMYCIN



con A bound at the cell surface would be expected, and this is not found. At the same time, the decrease in WGA binding capacity, in glucosidase inhibitor treated myoblasts, is indicative of a reduction in complex oligosaccharides. However, it is unlikely that complex oligosaccharides are necessary for the fusion event, because L6 myoblasts selected for resistance to WGA have complex oligosaccharides lacking galactose and sialic acid, yet retain normal fusion capacity (Gilfix and Sanwal, 1984). Thus, more credible explanations for the decreased fusion capactity in terms of the model, are a reduction in recognition of the high mannose oligosaccharides due to the presence of the glucose residues by a putative receptor, or inactivation of the receptor itself due to nonprocessed oligosaccharides, or both.

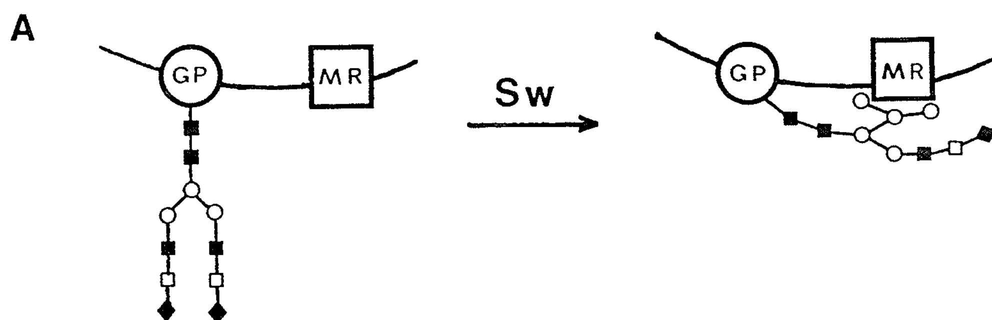
The inhibition of fusion by the mannosidase II inhibitor, swainsonine, is indeed surprising when the above model for involvement of mannosylated glycoproteins in fusion is considered. Swainsonine results in the conversion of complex oligosaccharides to hybrid oligosaccharides with one arm of a complex character and another arm retaining high mannose character (Tulsiani *et al.*, 1982a). However, the high mannose oligosaccharides are not affected by swainsonine. The conversion of complex oligosaccharides to hybrid oligosaccharides causes an increase in con A binding to the cell surface. Swainsonine would not initially be expected to affect myoblast fusion with respect to the above models (Figure 51), beause the high mannose oligosaccharides remain intact. However, a model has been proposed by Chung *et al.*, (1984) for the inhibition of recognition and uptake of high mannose oligosaccharides from ß-glucuronidase in the presence of swainsonine which may also explain swainsonine's effect on myoblast fusion. Their model for the inhibiton of recognition of high mannose oligosaccharides by macrophages suggests that the hybrid oligosaccharide of cell surface glycoprotein, with its partial high mannose character, may interfere with the normal recognition of high mannose oligosaccharides by the receptor (Figure 53A). Reversal of the swainsonine effect requires 24 hours,

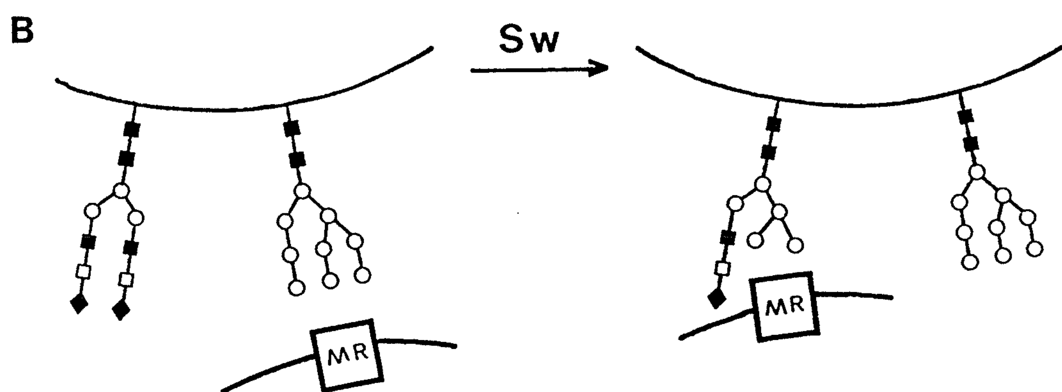
presumably for the synthesis of new receptors and possibly for *de novo* synthesis of mannosidase II. This model can be applied to inhibition of fusion in swainsonine treated myoblasts (Figure 53B). The mannose arm of hybrid oligosaccharides, near the putative cell surface receptor, may have a partial affinity for the receptor, and thus interfere with the normal recognition of high mannose oligosaccharides on adjacent cell surfaces.

The inhibition of swainsonine is not likely due to a blockage in transport through the ER and Golgi. Swainsonine does not affect the secretion or transport to the cell surface of most glycoproteins tested (see Historical review, Table 2). Another explanation for inhibition of fusion in the presence of swainsonine is a direct interference of swainsonine with the receptor recognition of the high mannose oligosaccharide on the cell surface. The receptor may have an affinity for swainsonine, thus causing competition between swainsonine and the high mannose oligosaccharide for the putative receptor. Swainsonine, at 1 μg/ml, inhibited the receptor-mediated internalization of high mannose oligosaccharides in macrophages by 40%. (Arumugham and Tanzer, 1983b). The concentrations of swainsonine used in the myoblast fusion experiment was 2.6 μM (0.5 μg/ml) which is slightly lower than the macrophage system, however, simple cell surface binding of swainsonine may account for the partial inhibition of fusion in the myoblasts. The inhibition of receptor-mediated uptake of high mannose oligosaccharides in macrophages by swainsonine, coupled with the inhibition of myoblast fusion by swainsonine, strongly suppports the model for fusion occuring via receptor recognition of a high mannose oligosaccharide on an adjacent cell. Radioactively labelled swainsonine would be required to measure the cell surface binding capacities of the myoblasts in order to test the possiblity of swainsonine interference with receptor recognition. This type of inhibition appears to be restricted to swainsonine, because mannose and structural analogues of mannose, α–methyl-mannose and 1-deoxymannojirimycin do not have an effect on fusion.

Figure 53

Diagrammatic representation of the model for the action of swainsonine on receptor recognition of high mannose oligosaccharides in macrophages (Panel A) and myoblasts (Panel B). The macrophage model (Chung *et al.*, 1984) shows the formation of a hybrid chain from complex oligosaccharides in the presence of swainsonine. The mannose arm of this chain interferes with the recognition between a mannose receptor (MR) and high mannose oligosaccharides on glycoproteins (GP) that are normally internalized by the cell. In the myoblast model the mannose arm of the hybrid chain interferes with the recogition process involving high mannose oligosaccharides of glycoproteins involved in the fusion process. (■ - N-acetylglucosamine; ○ - mannose; ❑ - galactose; ◆ - N-acetylneuraminic acid). Source of diagram - J.C. Jamieson.


A
GP
MR
Sw
GP
MR


B
Sw
MR
MR

Deoxymannojirimycin has a very minimal effect on myoblast fusion which is expected, if the theory of the importance of high mannose oligosaccharide in myoblast recognition is correct. This block in processing creates high mannose oligosaccharides from what would normally be complex oligosaccharides. Thus, oligosaccharides which are normally of the high mannose type are not affected. This finding does raise questions regarding the application of Chung's model to the myoblast recognition system. The question arises as to why hybrid oligosaccharides (produced in the presence of swainsonine) with only partial high mannose character can interfere with the high mannose receptor, whereas, high mannose oligosaccharides converted from complex oligosaccharides in the presence of deoxymannojirimycin exhibit no competitive effect? Thus, the model for direct inhibition of receptor recognition of high mannose oligosaccharides by swainsonine seems to be a more credible explanation.

Deoxymannojirimycin results in the presence of high mannose oligosaccharides of a Man_9–$GlcNAc_2$ structure but a Man_8-$GlcNAc_2$ structure are also possible. An ER α-mannosidase which can minimally process high mannose oligosaccharides (Bischoff and Kornfeld, 1983) and is not sensitive to deoxymannojirimycin (Bischoff *et al.*, 1986), has been identified . If this same activity is present in myoblasts, the Man_8-$GlcNAc_2$ structures may be more important than Man_9-$GlcNac_2$ structures in recognition and adhesion. This does not rule out recognition of other high mannose oligosaccharides ($Man_{6\text{-}7}$-$GlcNAc_2$) which may be present and involved in recognition in untreated myoblasts.

Creatine phosphokinase (CPK) activity, which is representative of several muscle specific enzymes which increase during differentiation, was also inhibited with the glucosidase processing inhibitors and swainsonine. Biochemical differentation can occur independently of the fusion reaction in fusion deficient mutants (Grant *et al.*, 1986). CPK is not a glycoprotein (Watts, 1973) and would therefore not be directly

affected by the processing inhibitors. However, the reduction in activity of CPK in myoblasts treated with swainsonine and the glucosidase inhibitors may indicate that fusion and biochemical differentiation are ultimately controlled by the same initial reaction which stimulates the cell to begin differentiating.

The lack of fusion in the glycoprotein processing inhibitor treated con A-resistant L6 myoblasts is expected. Based on their known sites of action, none of the processing inhibitors would allow the variants to regain the production of oligosaccharides containing more than five mannose residues.

B. *Effect of Processing Inhibitors on Insulin Binding in L6 Myoblasts*

The effect of the glycoprotein processing inhibitors on myoblast fusion and the glycosylation of cell surface glycoproteins, as shown with the lectin binding studies, prompted an investigation into the effect of processing inhibitors on another known cell surface glycoprotein, the insulin receptor. Preliminary studies with 2-deoxy-glucose uptake indicated that the L6 myoblast cells were responsive to insulin, however, they exhibited a minimal response to insulin when compared with other cell types, and there is even one report that has indicated that L6 myoblasts do not take up 2-deoxy-glucose in response to insulin (Klip *et al.*, 1983). Differentiation of myoblasts to myotubes does appear to cause an increase in insulin binding capabilities when expressed as insulin bound/plate but no increase with differentiation when the data was expressed as insulin bound/mg protein. This may be due to increases in amount of cellular protein as a result of the biochemical differentiation which accompanies myoblast fusion. Although others have indicated an increase in insulin binding capabilities following differentiation, they have not reported the fusion index of the myotubes and therefore, it is difficult to make a direct comparison between studies. One study has reported that insulin receptor concentrations increased prior to fusion (Beguinot *et al.*, 1986).

More dramatic changes in insulin receptor population may be evident prior to morphological differentiation.

The displacement of insulin binding capabilities by the lectins con A and WGA indicated the adipocyte insulin receptor contained oligosaccharides (Cuatrecasas and Tell, 1973), and treatment of lymphocytes with tunicamycin decreased insulin binding capacity to 16% of control levels (Keefer and De Meyts, 1981). However, it appears that the glycoprotein content and oligosaccharide structures differ for different cell types. In mouse liver cells, digestion with ß-galactosidase eliminated high affinity receptors but α-fucosidase, ß-hexosaminidase and neuraminidase had no effect (Caron *et al.*, 1978). In the same cells, con A inhibited insulin binding but WGA had no effect suggesting high mannose oligosaccharides on the receptor. A direct comparison of insulin receptors in brain and adipocytes indicated that both contained high mannose and complex oligosaccharides on α and ß-subunits, however, the complex oligosaccharides of adipocyte receptors were different structures than the brain receptors (Heindenreich and Brandenburg, 1986).

The glycoprotein processing inhibitors, castanospermine, deoxymannojirimycin and swainsonine, all affected insulin binding capacity in the L6 myoblasts. The increase in insulin binding capacity with both deoxymannojirimycin and swainsonine indicate that an increase in the high mannose character of the receptor may cause either an increase in the affinity of the receptor, or an increase in the total number of insulin receptors available on the cell surface. The effect of swainsonine on insulin binding capacity has been determined in IM-9 lymphocytes, but was found to cause no significant difference in either the affinity to the receptor or the autophosphorylation of the receptor (Duronio *et al.*, 1986). However, because the oligosaccharide content of neither the myoblast insulin receptor nor the lymphocyte insulin receptor is known, a direct comparison of these studies cannot be made. These results may indicate differences exist in the oligosaccharide content of these two receptors.

The decreased insulin binding capacity in the presence of castanospermine indicates the inhibition of glucose processing also affects the insulin receptor. Glucosidase inhibitors have been shown to inhibit cell surface expression and secretion of glycoproteins by blocking the exit from the Golgi (see Table 1, Historical review). Therefore, one possible explanation for the decrease in insulin binding capacity is a reduced expression of insulin receptors on the cell surface caused by retention of the unprocessed forms in the Golgi. The lack of processing may also prevent the insulin receptor from assuming the necessary conformation to have normal binding affinity. The insulin receptor is translated as one large polypeptide which undergoes proteolytic cleavage resulting in α and ß subunits (Hedo *et al.*, 1983). This is accompanied by oligosaccharide synthesis and processing. Another study with IM-9 lymphocytes has found that both castanospermine and deoxynojirimycin caused a 50% reduction in insulin receptors and the decrease was not due to increased degradation of the receptors (Arkaki *et al.*, 1987). They also suggest the decrease is due to a delay in oligosaccharide processing, but it does not affect cleavage of the proreceptor to α and ß subunits.

The effects of alterations in glycosylation on insulin binding capacities have also been identified in glycosylation mutants of CHO cells. B4-2-1 cells, which produce only truncated high mannose oligosaccharides (Man_5-$GlcNAc_2$), due to the inability to produce Dol-P-Man, have a much higher insulin binding capacity with increased receptor affinity (Podskalny *et al.*, 1984). Another mutant, Lec 1.3c, which lacks complex carbohydrate due to the loss of GlcNAc transferase I, had a 50% reduction in insulin binding capacity due to lower affinity of insulin for the receptor. Thus, altered glycosylation can have profound effects on insulin binding capacity. However, since the mutation is pleiotropic affecting all glycoproteins, the reduced affinity may be a consequence of the changes in other membrane glycoproteins with altered structure affecting the affinity of the receptor (Podskalny *et al.*, 1984).

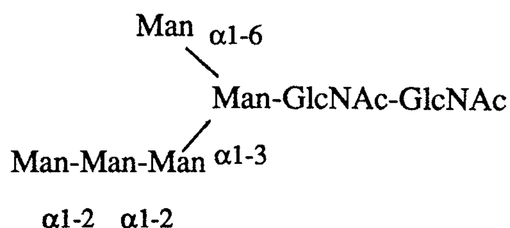
From the results it is evident that altering the carbohydrate structure of the insulin receptor does affect the insulin binding capacity, however, the results do not distinguish between a decrease in the number of receptors on the cell surface or a decrease in affinity of the receptors. Scatchard analysis of the binding capacity at several concentrations of insulin in the presence of the glycoprotein processing inhibitors would be necessary to distinguish between these two existing possibilities.

C. *Glycosidases in Con A-resistant Myoblasts*

Associated with the defect in oligosaccharide biosynthesis in the con A-resistant myoblasts, are alterations in the glycosidase activities. A substantial reduction in the intracellular acidic or lysosomal α-mannosidase activity is not accompanied by an equal increase in extracellular activity. However, extracellular ß-hexosaminidase activity is slightly increased in the variant myoblasts when compared with the wild type cells. Intracellular levels of ß–hexosaminidase activity are slightly lower at pH values around 4.25 to 4.75 which may account for the extracellular activity. The question that arises is what is the cause of the altered activities. Is it due to alterations in the oligosaccharide content of the lysosomal enzymes, caused by the defect in synthesis of high mannose oligosaccharides, or is it an independent genetic event (or events) which only affect specific glycosidase activities?

Several other lectin-resistant cell types exhibit alterations in their glycosidase activities. Of the three hexosaminidase activities (Hex I, II and III) found in wild type CHO cells, con A-resistant CHO cells (C^{R}-7) had lower intracellular levels of Hex I and III which corresponded with an increase in their extracellular activities (Blaschuk et al., 1980a). The C^{R}-7 CHO cells are defective in the formation of lipid-oligosaccharide intermediates of the dolichol cycle (Wright *et al.*, 1979, 1980). These cells also exhibit several other phenotypic alterations with respect to cell growth and morphology, which may be a result of the altered oligosaccharide synthesis (Ceri and Wright, 1978). No correlation was made with respect to the altered glycosidase activities and the defective glycoprotein synthesis. However, it was suggested that the increase in extracellular activity may be due to defective targetting of the hexosaminidase enzyme to the lysosomes as a result of decreased mannose-6-phosphate signal residues (Blaschuk *et al.*, 1980a).

Similar defects in lysosomal enzyme activities in both clone 2 and clone 5 con A-resistant myoblasts suggests that the enzyme defects are closely associated with the decrease in the addition of mannose from Dol-P-Man. The altered oligosaccharide synthesis of the con A-resistant myoblasts results in truncated high mannose oligosaccharides, containing oligosaccharides only as large as Man_5. These structures on the lysosomal enzymes probably do not result in faulty transport of the enzymes themselves. A mutant lymphoma cell line (Class E Thy-1$^-$) (Chapman *et al.*, 1979b), also with defective synthesis of Dol-P-Man, has Man_5-oligosaccharides with the following structure:



and produces complex oligosaccharides through an alternate processing pathway (Kornfeld *et al.*, 1979). The lysosomal enzymes produced in these cells have 4 mannose-containing carbohydrate chains with either a single phosphodiester or phosphomonoester group. Althought the Man-6-P recognition signals are significantly reduced, the phosphorylation of one mannose residue is sufficient to produce a recognition signal for proper transport to the lysosomes, because ß-galactosidase isolated from the Thy-1$^-$ mutants can be endocytosed normally (Gabel and Kornfeld, 1982). Couso *et al.* (1986), using rat liver, *Acanthamoeba castellani,* and *Dictyostelium discoideum*, have found that at least one α1-2 linked mannose residue is required for the phosphorylation of high mannose oligosaccharides of uteroferrin. Assuming the con A-resistant myoblasts have the same truncated high mannose oligosaccharide as the Thy-1$^-$ mutant, phosphorylation of the α1-2 linked mannose residues of the truncated oligosaccharide should occur. Thus, the altered oligosaccharide structure probably does not account for the altered glycosidase activities.

Several other glycosylation mutants have been isolated which have altered glycosidase activities. CHO mutants deficient in the addition of glucose residues to lipid-oligosaccharide, have decrease activities of several lysosomal enzymes (Krag and Robbins, 1982; Robbins, 1979). Another mutant of CHO cells, selected by its resistance to ω-(6-phospho)-pentamannose oligosaccharides linked to the lectin ricin, had decreased uptake of bovine testicular ß-galactosidase due to an increase in dissociation of the enzyme and receptor complex (Robbins *et al.*, 1981). The mutants also secreted higher than normal levels of several other lysosomal hydrolases including α–mannosidase, ß-hexosaminidase, ß-glucuronidase and α-fucosidase (Robbins and Myerowitz, 1981), however, the enzymes were compartmentalized normally into the lysosomes and the lysosmal enzymes from the mutants were internalized into the wild type cells. These results suggested the defect in the mutant was in the mannose-6-phosphate receptor, rather than the lysosmal enzymes themselves. This corresponds to the method used for selection, which would be expected to select for cells which do not recognize a phosphorylated mannose oligosaccharide. The same mutant was also found to have a defect in the formation of Dol-P-Man, but no correlation between this defect and the altered uptake of lysosomal enzymes has been established (Stoll *et al.*, 1982). However, they did establish that the truncated Man_5-oligosaccharide resulting from the Dol-P-Man defect, was phosphorylated and would be recognized by the Man-6-P receptor of the wild type cells. Thus, two separate mutants with similar defects in high mannose oligosaccharide structure have functionally phosphorylated lysosomal hydrolases. This would strongly suggest that the truncated high mannose oligosaccharide structure in the con A-resistant myoblasts is not, in itself, the main cause of altered lysosomal enzyme targetting, resulting in decrease intracellular mannosidase activity or increased extracellular ß-hexosaminidase activity.

A second possible explanation for the altered lysosomal enzyme activities is a defect in the phosphorylation mechanism of the lysosomal enzymes. This is not very

probable however, because it would result in decreases in all of the lysosomal enzymes, and would not be selective for the large decrease in acidic α-mannosidase activity. Defective phosphorylation of lysosomal enzymes would be equivalent to a known genetic disease called I-cell disease, which results in large inclusion bodies of undigested macromolecular material within the cell (Kornfeld, 1986).

The third and most likely explanation is a defect in the expression of α–mannosidase activity. The activity is not only decreased but appears to be completely absent (ie. no optimum activity at pH 4.6) and mannosidase activity at the acidic pH optimum in the variants appears to be residual activity from the neutral mannosidase activity. Either a mutation does not allow protein production of the lysosomal mannosidase enzyme, or the protein may be altered structurally such that its activity is destroyed. In a mutant of *Dictyostelium discoideum*, both lysosomal α–mannnosidase and α–glucosidase do not undergo normal cellular transport to the lysosomes because of a mutation which prevents their proteolytic processing in the RER (Woychik and Dimond, 1987). An antibody preparation specific for rat lysosomal α-mannosidase would be necessary to detect the presence or absence of the α-mannosidase protein in an inactive state. An attempt at isolation of lysosomal α-mannosidase enzyme from rat liver was made, however, the enzyme is difficult to separate from other lysosomal glycosidase activities, and α-mannosidase activity decreased as the fractionation proceeded because of the unstable nature of the enzyme.

D. *Effects of Oligosaccharide Processing Inhibitors on Myoblast Glycosidase Activities.*

The intent of these assays was to ensure that the processing inhibitors were inhibiting the processing reactions in the myoblasts, but also to determine if alterations in oligosaccharide structure would affect the lysosomal glycosidase activities within the

cells. Artificial substrates are normally used for the assays, because of the difficulty in preparing large amounts of natural substrates. Two types of assays are used: one in which the inhibitor is added directly to the enzyme assay tube, and the other in which the cells are grown in the presence of the inhibitor and then assayed for the glycosidase activities.

The glucosidase inhibitors, 1-deoxynojiirmycin, N-methyldeoxynojirimycin and castanospermine, are known inhibitors of the processing glucosidases of the Golgi, but they also directly inhibit lysosomal glucosidase activities, and this was demonstrated in assays in which the glucosidase inhibitors were added to the assay tubes. The concentrations of the inhibitors added directly to the assay were identical to the concentrations of the inhibitors in the media, but the intracellular concentration, or rather the intra Golgi and intra lysosomal concentrations in these experiments, could be significantly different due to variations in uptake of the inhibitors. Inhibition of the neutral glucosidase activities in cell extracts from inhibitor treated plates was generally much less than that found in assay systems with the inhibitor added directly to the cell extract.

Interestingly, the glucosidase inhibitor treated cultures have lower levels of ß-galactosidase and ß–hexosaminidase activities, but these enzyme activities should not be significantly inhibited by the processing inhibitors when added directly to the cell extracts. Thus, any inhibition of ß-galactosidase and ß-hexosaminidase during culture may be due either to 1) an accumulation of inhibitor at high concentrations within the lysosomes to a degree such that they will directly affect the activity of the lysosomal enzymes or, 2) an effect on the synthesis and transport of these enzymes to the lysosomes as a result of improper oligosaccharide processing (retention of glucose residues). Lysosomal enzymes, cathepsin D and ß-hexosaminidase, have been shown to be retained in the Golgi in fibroblasts treated with the glucosidase inhibitor, deoxynojirimycin (Lemansky *et al.*, 1984). In the same study, none of the Golgi-

accumulated oligosaccharides of the lysosomal enzyme contained the mannose-6-P recognition signal, but some enzyme escaped the Golgi and was normally processed with the recognition signal intact. When *Aspergillus fumigatus* is treated with castanospermine (100 μg/ml) ß-hexosaminidase decreases by 30-40%. In the same study a concentration of 2mg/ml of castanospermine was required to inhibit ß-hexosaminidase activity significantly(Elbein *et al.*, 1984c), 100 times the concentrations used in the present study with myoblasts. However, when castanospermine was injected into rats, ß-hexosaminidase was increased by 30% in the liver, ß-galactosidase was unchanged, and α-mannnosidase was inhibited by greated than 50% (Saul *et al.*, 1985). Again, altered glycosylation of the enzymes was suggested to affect lysosomal enzyme targetting.

A mutant mouse lymphoma cell line ($Pha^{R2.7}$) which is deficient in glucosidase II activity showed a significant reduction (50-80%) in the amount of Man-6-P formed (Gabel and Kornfeld, 1982). Only a single phosphate is added to the glucose containing high mannose oligosaccharide and is usually attached to the branch linked α1-6 to the ß-linked mannose. The mutant oligosaccharides would be analogous to the oligosaccharides produced in the presence of the glucosidase inhibitors in the myoblasts. Although the affinity of the lysosomal enzymes of the mutant for the Man-6-P receptor was not measured, $Thy\text{-}1^-$ mutants with only one Man-6-P residue had normal affinity for the receptor. Thus, phosphorylation of the lysosomal enzymes probably does occur in the glucosidase inhibitor treated myoblasts.

It has been suggested that the glucosidase inhibitors may act like lysosomotropic drugs (chloroquine and ammonium choride) by becoming protonated when they enter the intralysosomal space, raising the pH of the lysosomal compartments and thus resulting in destruction or disruption of lysosomal enzyme activation or targetting which requires low pH (Elbein *et al.*, 1984c). Another possible explanation given for the effect of glucosidase inhibitors on the lysosomal enzyme

activities is that abnormal glycosylation, *i.e.* retention of glucosylated high mannose oligosaccharides instead of complex and normal high mannose oligosaccharides, may influence the conformation of the protein and thus may affect both activity and transport of the enzyme.

One interesting effect of deoxynojirimycin and methyldeoxynojirimycin is the substantial increase in neutral mannosidase activity in con A-resistant myoblasts cultured in the presence of these inhibitors. The explanation for this is not readily apparent. The same enzyme activity in the wild type myoblasts is not significantly affected by the inhibitors and castanospermine does not appear to affect the variant neutral mannosidase activity. When comparing the pH optimum curves for mannosidase activity, the neutral mannosidase activity in the con A-resistant variants is slightly lower than the wild type level. The treatment with deoxynojirimycin and its methyl derivative may somehow stimulate this activity in the variant myoblasts. Because mannosidase I activity is not measured by the artificial substrates, and lysosomal mannosidase activity is low at pH 6.0, the activity measured in this assay may represent the ER mannosidase and mannosidase II activities, and possibly a cytosolic mannosidase. In order to distinguish which activity is affected it would be necessary to use natural oligosaccharide substrates specific for each enzyme.

Swainsonine is an inhibitor of both mannosidase II and lysosomal mannosidase activities. It binds directly to the active site of lysosomal mannosidase because of its similarity in steric structure to the natural substrate, mannose (Dorling *et al.*, 1980). Below 0.5 μM swainsonine is a competitive inhibitor, however, at concentrations greater than 0.5 μM, swainsonine is a non-competitive inhibitor of rat liver lysosomal mannosidase (Tulsiani *et al.*, 1985). Swainsonine caused increased plasma levels of lysosomal hydrolases in rats injected with the inhibitor (Tulsiani and Touster, 1983). Pigs injected with swainsonine had increased levels of lysosomal hydrolases in most tissues and plasma, but reduced plasma levels of glucosidase and

mannosidase (Tulsiani *et al.*, 1984). Macrophages exposed to swainsonine for 1-2 weeks showed increases in ß-hexosaminidase (Greenaway *et al.*, 1983).

Swainsonine did not significantly affect intracellular levels of ß-hexosaminidase nor ß-galactosidase activities in either the assay-treated or culture-treated experiments for both the wild type and con A-resistant myoblasts. This suggests that the presence of hybrid oligosaccharides instead of complex oligosaccharides has no effect on targetting or activity of these lysosomal enzymes. These results would be expected because the high mannose oligosaccharides, which are not affected, carry the Man-6-P recognition signal for targetting.

Both neutral and lysosomal mannosidase activities are substantially reduced in the presence of swainsonine for assay-treated and culture-treated cells, a finding which is also expected from the known inhibitory effect on these enzymes. However, inhibition of glucosidase activity was not expected. Cell extracts assayed in the presence of swainsonine had reduced levels of acidic glucosidase activity which may suggest that swainsonine is exhibiting cross-inhibitory activity of glucosidase at this concentration. Swainsonine may concentrate in the lysosomes (Tulsiani and Touster, 1983) and for myoblasts cultured in the presence of the inhibitor the concentration of the drug in the lysosomes may be significantly higher than that in the medium, and thus cause some type of non-specific inhibition of the lysosomal glucosidase activity. However, Dorling *et al.* (1980) found that concentrations of swainsonine (up to 200 μM) had no effect on α-glucosidase, as well as ß-galactosidase, ß-hexosaminidase and ß-glucuronidase. Further investigations with high concentrations of swainsonine in cell extract assays or with natural substrates of processing glucosidases may be necessary to demonstrate an effect of this inhibitor on these enzyme activities in the L6 myoblasts.

Deoxymannojirimycin appeared to have the least effect on both lysosomal and neutral glycosidase activities. Although significant reductions in both neutral and acidic mannosidase and glucosidase were evident in the treated cell extracts, ß-galactosidase

and ß-hexosaminidase activities were not affected. The neutral mannosidase activity represents several mannosidase activities, including ER mannosidase, Golgi mannosidase I and II and cytosolic mannosidase activity. Although deoxymannojirimycin inhibits mannosidase I, artificial substrates are poor substrates for this enzyme. Thus, in order to study the inhibition of this enzyme, the use of natural substrates would be required. In relation to this, miminal effects on most glycosidase activities were apparent for enzyme activities in deoxymannojirimycin treated cultures. Deoxymannojirimycin would not be expected to affect the transport and targetting of lysosomal enzymes significantly, because it does not affect the processing of the high mannose oligosaccharides which carry the Man-6-P recognition signal. However, in hepatoma cells treated with deoxymannojirimycin, proteolytic processing of cathepsin D was reduced, while there was a two-fold increase in secretion of the enzyme (Naureth *et al.*, 1985). These effects were not found in fibroblasts, which suggests that different cell types may have different types of lysosomal enzyme receptors.

E. *Sialyl and Galactosyl Transferase Activities in Wild Type and Con A-resistant L6 Myoblasts.*

Complex oligosaccharides are important in several aspects of cellular behaviour. Sialic acid, a highly charged molecule, may affect the conformation and/or interaction of the glycoprotein with other molecules. Galactosyl transferases and sialyl transferases catalyze the addition of the galactose and sialic acid residues to the penultimate and terminal positions of complex chains, respectively. Besides adding the terminal residues of the complex oligosaccharides within the Golgi complex, both sialyl and galactosyl transferase activities have been found on the cell surface of many cell types (Morin *et al.*, 1983; Cerven, 1977; Podolsky and Weiser, 1975; LaMont *et al.*, 1977). Galactosyl transferase is itself a glycoprotein with high mannose residues (Podolsky and Weiser, 1975), and thus may be affected by defective glycosylation in the con A-resistant myoblasts. Some preliminary experiments with rat liver α2-6 sialyl transferase also indicate that it is a glycoprotein (Jamieson, personal communication), and the amino acid sequence inferred from the nucleotide sequence indicates that there are three sequons for the addition of N-linked oligosaccharides (Weinstein *et al.*, 1987).

Galactosyl transferase is an enzyme which has been implicated in cell-cell interactions (Porter and Bernacki, 1975; Webb and Roth, 1974) in which cell surface galactosyl transferase is involved in binding to exposed galactose residues on adjacent cells. This phenomenon may be involved in several types of cell-adhesion processes. For example, galactosyl transferase on the mouse sperm surface recognizes and binds to GlcNAc residues on the egg (Lopez *et al.*, 1985). Sialyl transferase has also been found to be elevated in virally transformed cells (Bosmann, 1972), and in the serum and liver of inflamed rats (Kaplan *et al.*, 1983), mice and guinea pigs (Lammers and Jamieson, 1986). Some types of neoplastic cells release large amounts of glycosyl

transferases into serum (Kessel and Allen, 1975; Liu *et al.*, 1982; Frithz *et al.*, 1985), or exhibit elevated levels of intracellular enzymes (Baker *et al.*, 1985).

Altered complex carbohydrate formation in the con A-resistant myoblasts may affect cell-surface interactions and characteristics of the cell. Thus, it was important to determine if enzymes responsible for the formation of complex oligosaccharides were affected by the pleiotropic mutation known to exist in the con A-resistant myoblasts, which have altered cell surface properties. It has also been reported that denervation of rat skeletal muscle, which causes stimulation of myogenesis, resulted in an increase in cell membrane activities of both sialyl and galactosyl transferases (Jeffrey and Appel, 1978).

The differences in kinetic properties of both sialyl and galactosyl transferase activities between the wild type and con A-resistant myoblasts, using fetuin as the acceptor, are not evident using α_1-acid glycoprotein as the acceptor. This suggests that different enzyme activities are being measured with each acceptor. The "one enzyme-one linkage" hypothesis proposed by Hagopian and Eylar (1968) suggests that for each sugar residue, with unique linkage and environment, a different glycosyl transferase enzyme is required. Therefore, it is necessary to look at the oligosaccharide structures of the acceptor glycoproteins to distinguish differences in linkage structure to establish which of the enzyme activities may be altered in the con A-resistant myoblasts.

Several sialyl transferase activities exist (Beyer, 1981), and some have been purified from a variety of tissues. Although several of these enzyme are specific for O-linked oligosaccharides (Carlson *et al.*, 1973; Sadler *et al.*, 1979; Beyer *et al.*, 1981), two of these major N-linked enzymes have been purified from rat liver (Weinstein *et al.*, 1982a) and also from bovine colostrum (Paulson *et al.*, 1977). Two major groups of sialyl transferase activities have been defined based on the linkage of sialic acid to the carbohydrate: CMP-NeuAc:Galß1-4GlcNAc α2-6-sialyl transferase (known as α2-6 sialyl transferase) and CMP-NeuAc:Gal ß1-3(4) GlcNAc α2-3 sialyl transferase

(known as α2-3 sialyl transferase). Sialyl transferase enzymes for the α2-4 linkage must also exist because carbohydrate chains containing this type of linkage have been isolated. The α2-6 sialyl transferase adds sialic acid in a highly preferential manner to Gal linked ß1-4 to the GlcNAc (Weinstein *et al.*, 1982b) and prefers the branch with Galß1-4GlcNAcß1-2Manα1-3 (van den Eijnden *et al.*, 1980; Joziasse *et al.*, 1987).

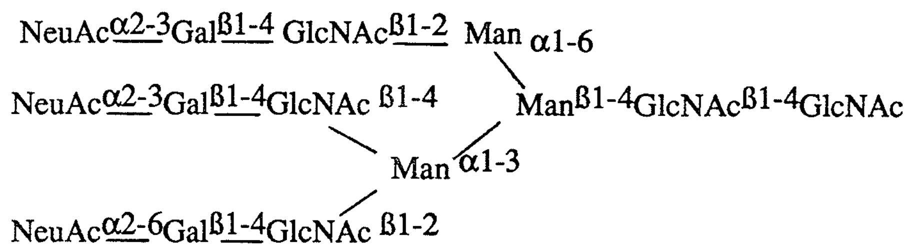
The α2-3 sialyl transferase prefers the ß1-3 linked galactose but will also add sialic acid to a ß1-4 linked galactose of N-linked oligosaccharides (Schachter *et al.*, 1985). Two other α2–3 sialyl transferase activities have also been described: one is specific for Galß1-4 Glc-ceramide (G_{M3}), and the other is specific for Galß1-3GalNac-R which is found in mucins and glycosphinogolipid (Schachter *et al.*, 1985). With new techniques for isolation and examination of oligosaccharide structure, it is likely that other linkages for sialic acid will be found.

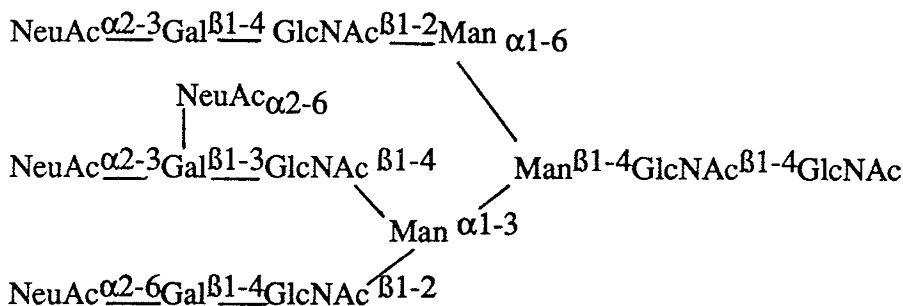
Only slight differences in kinetic properties of sialyl transferase activity were observed using asialo α_1-acid glycoprotein as the acceptor, however, when asialo fetuin was used as the acceptor glycoprotein, the K_m value of the con A-resistant myoblasts was much larger that the K_m value of the wild type myoblasts. The V_{max} was also significantly higher for the con A-resistant sialyl transferase activity using fetuin as the acceptor. The lower affinity of the enzyme using fetuin acceptor would suggest that fetuin is not as good an acceptor for the major population of sialyl transferase enzymes within the con A-resistant myoblasts. A comparison of the major oligosaccharide structures of fetuin and α_1-acid glycoprotein may suggest why the affinities for the substrates differ between the wild type and con A-resistant myoblasts and may suggest which enzyme activities could be altered or absent in the con A-resistant myoblasts.

Fetuin contains O-linked oligosaccharides, whereas rat α_1-acid glycoprotein does not. This immediately suggested that differences in enzyme activities may be due to altered O-linked sialyl transferase activities. However, results using asialo mucin

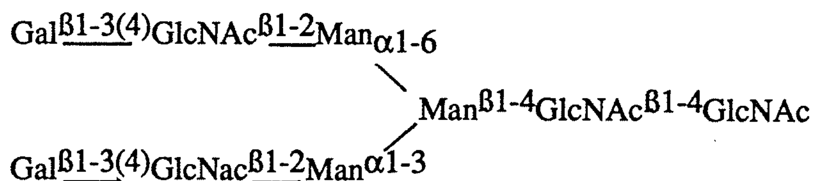
as the acceptor glycoprotein (data not shown) found an extremely low level of sialyl transferase activity specific for O-linked oligosaccharides.

Structural studies of the N-linked oligosaccharides of fetuin found that the predominant structures are triantennary oligosaccharides (Takasaki and Kobata, 1986) of the following structures:





The predominant oligosaccharides (80%) at the five glycosylation sites on rat α_1-acid glycoprotein are biantennary structures with Gal linked either both ß1-3, both ß1-4, or ß1-4 and ß1-3 on the Man α1-6 and Man α1-3 arms, respectively :



Rat α_1-acid glycoprotein also contains a small amount of triantennary chains. Although the linkages of sialic acid to the oligosaccharides were not determined individually, analysis of total sialic acid linkage found three types of linkages: NeuAcα2-3Gal, NeuAcα2-6Gal and Galß1-3(NeuAcα2-6)GlcNAc (Yoshima *et al.*, 1981).

Fetuin and rat α_1-acid glycoprotein contain N-linked oligosaccharides with both α2-3 and α2-6 linked sialic acid residues, and therefore differences in sialyl transferase kinetics may be a result of altered levels of either the α2-3 or α2-6 sialyl transferase activities in the con A-resistant myoblasts.

The kinetic studies of galactosyl transferase activities in the wild type and con A-resistant myoblasts show increased K_m values with respect to asialoagalacto fetuin, and no substantial differences were measured using asialoagalacto α_1-acid glycoprotein as the acceptor. Again the K_m value for galactosyl transferase using fetuin was much greater in the con A-resistant myoblasts when compared with the wild type, suggesting a decreased affinity for fetuin oligosaccharides. Fetuin and α_1-acid glycoprotein contain galactose in both ß1-3 and ß1-4 linkages and thus an altered activity in either one of these enzyme activities may result in altered kinetics for both substrates.

The one major difference between the acceptors, however, is that fetuin oligosaccharides are predominantly triantennary, whereas rat α_1-acid glycoprotein has predominantly biantennary oligosaccharides. If one considers the "one linkage, one enzyme" theory (Hagopian and Eylar, 1968), there may be a galactosyl transferase or sialyl transferase which is specific for the addition of galactose and sialic acid, respectively, to the third chain of triantennary oligosaccharides. However, this is unlikely because no distinction, thus far, has been made between sialyl and galactosyl transferase activities specific for the triantennary oligosaccharide. However, the specificities of sialyl transferase activities appears to pertain to more than just the terminal galactose residues, and this may partially account for differences in affinities

with different substrates. Complete sialylation of the biantennary oligosaccharides of transferrin containing ß1-2 linked GlcNAc oligosaccharides were observed, whereas under the same conditions only 50% of the tetraantennary oligosaccharides of human α_1-acid glycoprotein containing ß1-4, ß1-6 and ß1-2 linked GlcNAc were sialylated (Paulson *et al.*, 1978). Studies with bovine colostrum Galß1-4GlcNAc-R α2-6 sialyl transferase have found that sialic acid is always added initially to the galactose residue of Gal ß1-4GlcNAcß1-2Manα1-3 branch (Joziasse *et al.*, 1987), and that the GlcNAc residue within the core oligosaccharide (with Man ß1-4 linkage) is required for the branch specificity of α2-6 sialyl transferase.

Several galactosyl transferase enzymes exist (Beyer, 1981; Strous, 1986). Although the ß1-4 galactosyl transferase has been purified and characterized in a number of different cell types (Strous, 1986; Furukawa and Roth, 1985), an N-linked ß1-3 galactosyl transferase enzyme has not yet been purified. Such an activity must exist because several glycoproteins, including fetuin and α_1-acid glycoprotein, contain ß1-3 linked galactose. The differences in kinetic constants between wild type and con A-resistant myoblasts using fetuin, again may be due to an alteration of one or more galactosyl transferase enzymes which are not required for glycosylation of α_1-acid glycoprotein, or which have different affinities for α_1-acid glycoprotein.

The differences in kinetic properties between the wild type and con A-resistant myoblasts, for both sialyl and galactosyl transferase activities, may be a result of the defective oligosaccharide synthesis in the con A-resistant myoblasts. The fetuin acceptor may be able to detect subtle differences in the kinetic properties of the enzyme pools of sialyl and galactosyl transferase which are not evident with α_1-acid glycoprotein as an acceptor. The kinetic values represent more than one enzyme activity, and an alteration in one or more enzyme activities, or a defective enzyme, could result in the increased K_m values in the con A-resistant myoblasts. The amounts of α2-3 and α2-6 sialyl lactose produced, with lactose as an acceptor, can be used to

indicate alterations in the relative amounts of α2-3 and α2-6 sialyl transferase activities. Substrates specific for ß1-3 and ß1-4 galactosyl transferases would be required to show differences in these two enzyme activities between the wild type and con A-resistant myoblasts.

F. *Concanavalin A Binding and Glycosidase Activity in Fibroblasts from Duchenne Dystrophic Patients and Age-matched Controls*

Current theory favors a generalized cell membrane defect in Duchenne dystrophic patients and both fibroblasts and erythrocytes from Duchenne dystrophic patients are commonly used as a tissue source for study (Roses, 1980; Rowland, 1980; Anand, 1983). There is evidence suggesting that cell surface glycoprotein content are altered in DMD (Duchenne muscular dystrophy) fibroblasts resulting in lower cellular adhesion compared to normal fibroblasts (Jones and Witkowski, 1979). DMD fibroblasts treated with trypsin and allowed to recover in the presence of monensin, an ionophore which affects Golgi organization and glycoprotein metabolism, do not recover as quickly as normal cells (Jones *et al.*, 1985).

The comparison of [^{125}I]-con A binding capacity in fibroblasts from Duchenne dystrophic patients and age-matched controls did not reveal any major differences in con A binding capacity expressed as con A bound/cell or con A bound/µg protein. Only minor differences in binding capacities between DMD and control fibroblasts were evident. The con A bound was expressed both per cell and per µg protein because there are reports of differences in size and protein content between normal and dystrophic cells (Liechti-Gallanti *et al.*, 1981). Con A bound per protein was slightly higher in DMD fibroblasts for both age ranges, whereas, con A bound per cell was slightly lower than the age-matched control fibroblasts. Scatchard analysis found positive cooperativity in all binding studies, with only small differences between the extrapolated binding constants. The conclusion from these studies is that the cell surface carbohydrate, which has an affinity for con A, is not significantly different in the DMD fibroblasts tested compared to the control fibroblasts.

Several other groups have used lectins to determine if lectin binding capacities differed in sample tissues or cells from dystrophic patients. Bonilla *et al.* (1980) has

demonstrated focal alterations in con A (conjugated to horseradish peroxidase) binding patterns for DMD muscle cells. The same group has found that cultured muscle cells from dystrophic patients do not exhibit any abnormalities in con A binding (Heiman-Patterson *et al.*, 1982). Dystrophic fibroblasts also displayed no significant differences from normal cells in binding of fluorescein isothiocyanate conjugated con A (Newman, 1982). These discrepencies between studies may well be due to variable cell surface carbohydrate content dependent upon the culture conditions used and the cell type employed (Parfett *et al.*, 1983). Assays in which con A binding is visualized by conjugated stains are not as sensitive as the radiolabelled con A method used in this study.

The lack of significant differences in con A binding to the Duchenne dystropic fibroblasts in comparison to the control cells using the iodinated con A shows that membrane defects in fibroblasts if they exist, may not be detected by con A binding. This does not eliminate the possibility of cell surface carbohydrate alteration, but simply means that alterations could not be detected by the methods used. Another study using *Ricinus communis* I agglutinin (RCA I), a lectin which has an affinity for α– and ß-galactose, detected two cell surface glycoproteins of 370,000 mw and 54,000 mw which were altered or absent in muscle tissue from Duchenne dystrophic patients (Capaldi *et al.*, 1985). Since con A has little affinity for galactose residues, this alteration in glycoprotein content would not be detected in these binding studies.

Interestingly, recent studies using antibodies against protein from cDNA from the Duchenne dystrophic locus have identified a protein of 400,000 mw called dystrophin, which is absent in both Duchenne muscular dystrophy patients and a mouse model system (Hoffman *et al.*, 1987). This is very close in molecular weight to the surface glycoprotein of 370,000 mw shown to be missing above, but there is no evidence that the 400,000 mw protein is a glycoprotein, or a cell surface molecule. Dystrophin is thought to be a structural protein, however its exact function is not defined. The results

from the con A binding studies which show no defect in cell surface carbohydrate concur with this recent information regarding the genetic defect in Duchenne muscular dystrophy.

The second part of the study with the DMD and control fibroblasts was to determine if levels of activity of two lysosomal enzymes required for the degradation of carbohydrate were significantly altered in the dystrophic cells. Lysosomal enzymes are important in the recycling of endocytosed material within the cell, and deficiencies in activity of one or more of these enzymes can have serious consequences on cellular metabolism resulting in one of several known lysosomal storage diseases (Durand and O'Brien, 1982).

The discovery that muscle tissue is broken down by proteolytic enzymes of lysosomal origin in DMD patients has prompted several investigations into the levels of these enzyme activities in dystrophic tissue (Kar and Pearson, 1979; Gelman *et al.*, 1980; Davis *et al.*, 1982a,b; Doughty and Gruenstein, 1987). One study has also identified inclusion bodies (Wyatt and Cox, 1977), which are usually representative of alterations in lysosomal enzyme activity. However, others do not confirm this and could not distinguish normal and DMD fibroblasts based on this feature (Cullen and Parsons, 1977).

The activities of α-mannosidase and ß-hexosaminidase activities are not significantly nor consistently different between fibroblasts from DMD and age-matched control donors. The optimum activities of both enzymes were in the acidic pH range and were representative of lysosomal enzyme activities, similar to the pH optima found in the L6 myoblasts. One other report has also found no abnormal levels of ß-glucuronidase, ß-glucosidase, ß-galactosidase and N-acetylgalactosamine activities in fibroblasts from DMD patients (Gelman *et al.*, 1980). However, in the same study, the level of cathepsin C activity was markedly reduced in DMD fibroblasts. Considering these results and the current results with α-mannosidase and ß-hexosaminidase

activities, it appears that no general defect in lysosomal glycosidase activity exists in DMD fibroblasts, but that other specific lysosomal enzymes, such as cathepsin C, are altered.

G. *Summary and Future Considerations*

The preceding work has defined specific alterations in glycoprotein metabolism and function which occur with alterations in N-linked carbohydrate structure, either in a mutant defective in high mannose oligosaccharide synthesis, or through the use of glycoprotein processing inhibitors. The next step in these studies is to explore why alterations in carbohydrate structure have an affect.

The glycoprotein processing inhibitors represent extremely valuable tools for indirectly demonstrating the requirement for proper glycosylation of glycoprotein. This thesis has demonstrated that high mannose oligosaccharides are necessary for the differentiation of myoblasts, both morphologically and biochemically, and strongly supports the theory of receptor recognition of high mannose oligosaccharides in adhesion/recognition preceding myoblast fusion. However, it has not been ascertained whether the inhibition by the glucosidase inhibitors and swainsonine is due to a block in recognition, or to a decrease in cell surface receptor and/or high mannose oligosaccharide-containing glycoproteins. Another study has reported inhibition of myoblast fusion with methyldeoxynojirimycin and no effect on fusion with deoxymannojirimycin (Holland and Herscovics, 1986). The present work is much more extensive, using all three glucosidase processing inhibitors as well as swainsonine and deoxymannojirimycin. The inhibition of fusion by swainsonine is a very important result, because it only affects the structure of complex oligosaccharides and does not affect high mannose oligosaccharides. Also, most studies show that swainsonine has no effect on the secretion and transport of glycoproteins within the cell, whereas evidence for the inhibition of secretion by the glucosidase inhibitors does exist. This suggests that delayed or blocked transport of cell surface glycoproteins is not the cause of fusion inhibiton by swainsonine. The two possible effects of swainsonine, either direct competition by swainsonine, or the indirect effect through

competition of the hybrid oligosaccharides with the high mannose oligosaccharides for the putative cell surface receptor, do require further studies to establish if both or either are the cause of inhibition. Inhibition of fusion in the presence of hybrid oligosaccharides would demonstrate that the model proposed by Chung *et al.* (1984) for macrophages would be applicable to the myoblast system. The interference may be artificially introduced by adding glycoproteins containing hybrid oligosaccharides or the oligosaccharides alone to the cell medium. Sufficient quantities would have to be added to overcome the possiblity of degradation.

In order to test the second possible explanation for inhibition of fusion by swainsonine, radiolabelled swainsonine would be required to measure its binding capacity to the myoblast cell surface. If swainsonine does bind to the cell surface, it may be used as an affinity adsorbant as part of the purification procedure for the high mannose receptor. This could lead to the development of an antibody preparation which could be used to directly show the receptor's involvement in myoblast fusion.

Another avenue worth pursuing would be to measure the cell surface expression of the 44,500 mw glycoprotein which may be involved in the adhesion/recognition process. Isolation and characterization of the 44,500 cell surface glycoprotein, reduced in con A-resistant myoblasts (Parfett *et al.*, 1983), would be required for the preparation of an antibody to this glycoprotein. The antibody would be highly useful in determining what happens to this cell surface glycoprotein in the presence of the glycoprotein processing inhibitors.

The altered lysosomal α-mannosidase and ß-hexosaminidase activities, and altered kinetics for sialyl and galactosyl transferases, may or may not be secondary effects of the defective formation of the high mannose oligosaccharides in the con A-resistant myoblasts. Since all of these enzymes are themselves glycoproteins, alterations in carbohydrate structure may affect their activity by altering the enzyme conformation or transport within the cell. Further studies will be necessary to

determine whether the decrease in lysosomal α-mannosidase activity is due to altered protein structure or lack of translation of the protein, or if the defective high mannose oligosaccharide structure selectively affects acidic α-mannosidase activity and the translocation of the enzyme to the lysosomes. The increased extracellular secretion of ß-hexosaminidase may also be due to altered oligosaccharide processing and targetting. A third possibility is a defect in the formation of the Man-6-phosphate recognition system due to altered activities of the phosphotransferase and phosphodiesterase enzymes. An antibody against the wild type lysosomal α-mannosidase enzymes may distinguish if the protein itself is missing or altered in the con A-resistant myoblasts. The lysosomal enzymes also appear to be sensitive to alterations in oligosaccharide structure created by the glucosidase inhibitors and swainsonine. The effect of the inhibition of processing could also be measured with respect to alterations in extracellular secretion of the lysosomal enzymes which would reflect altered transport of the enzymes. Since α-mannosidase and α-glucosidase are directly affected by the processing inhibitors, ß-galactosidase and ß-hexosaminidase are good candidates for a detailed study.

The altered kinetics of sialyl and galactosyl transferase enzymes in the con A-resistant myoblasts with respect to the acceptor, fetuin, may be due to altered levels of the α2-6 and α2-3 sialyl transferases, and the ß1-3 and ß1-4 galactosyl transferases. Chromatography of sialyllactose derivatives can be used to distinguish relative differences in sialyl transferase activities. It would also be interesting to determine if the alterations in the enzyme activities have an effect on the sialic acid and galactose content of the con A-resistant myoblasts. Since complex oligosaccharides, particularly sialic acid, are significant in the expression of cell surface character, determination of the content of these sugar residues using lectin binding studies specific for them may prove relevant.

The glycoprotein processing inhibitors have demonstrated the requirement for high mannose glycosylation in the differentiation of myoblasts to myotubes (Spearman *et al.*, 1987). This work supports earlier studies (Parfett *et al.*, 1981, 1983) suggesting the requirement for high mannose oligosaccharides in the fusion of rat L6 myoblasts, but further develops the theory by suggesting that high mannose oligosaccharides are directly involved in the cell-cell recognition process. Recognition of the high mannose oligosaccharides on the cell surface may result in adhesion of the cells, but may also be a trigger for the cell surface receptor to initiate further intracellular events culminating in gene expression required for differentiation. The decrease in creatine phosphokinase activity may be a reflection of the decreased recognition capacity of the swainsonine and glucosidase inhibitor treated myoblasts. Several other muscle specific proteins could also be tested for the effect of the processing inhibitors.

The glycoprotein processing inhibitors have proven to be highly useful in the exploration of the role of oligosaccharides in muscle differentiation, and the studies presented in this thesis are among the first reports of the use of the processing inhibitors to study the well characterized biological phenomenon of membrane fusion in myoblasts. This study could be used as a model for studying several other types of cell-cell, cell-matrix or cell-ligand interaction which may require carbohydrate or specific carbohyrate structures, such as, adhesion to the extracellular matrix (integrins), neural cell adhesion (N-CAM), fertilization of ova by sperm, formation of desmosomes, gap junctions and other intercellular adhesion molecules. Also, the reduced insulin binding capacity in the presence of the inhibitors leads to the question of effect of altered carbohydrate structure on other hormone and growth factor receptors. Most certainly the glycoprotein processing inhbitors are powerful new tools for the biochemist in the quest to understand the significance of cell surface oligosaccharides and their relation to biological processes.

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