
**Aberrant protein glycosylation in the
carbohydrate-deficient glycoprotein
syndromes and galactosaemia**

by

Joanne Charlwood

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Biochemistry Unit
Division of Biochemistry and Genetics
Institute of Child Health
University of London

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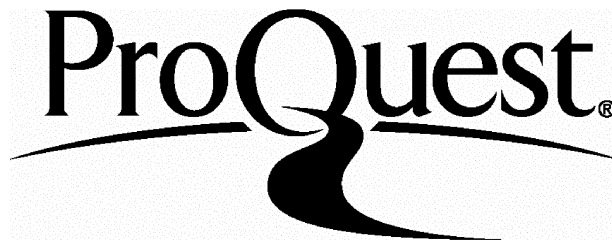
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Abstract

The carbohydrate-deficient glycoprotein syndromes (CDGS) and galactosaemia are autosomal recessive disorders of carbohydrate metabolism. They have been used in this study as tools to elucidate the mechanisms and consequences of defective glycosylation within the cell.

This study demonstrates that seven of the eight CDGS type 1 patients studied have a deficiency of phosphomannomutase (PMM), the enzyme responsible for the conversion of mannose-6-phosphate into mannose-1-phosphate. This is believed to lead to a deficiency of GDP-mannose, dolichol-P-mannose and the lipid-linked-oligosaccharide precursor. The net effect of a deficiency of PMM is non-occupancy of glycosylation sites as demonstrated in the thesis by structural analysis of the glycans released from purified serum transferrin and cellular glycoproteins. The same non-occupancy of glycosylation sites has been found in the eighth CDGS type 1 patient who has normal phosphomannomutase activity. The development of the PMM assay has allowed both the identification of variants and the prenatal diagnosis of CDGS type 1 for couples at risk. Lysosomal enzymes have been used as model glycoproteins in which to study glycoprotein synthesis and targeting in cells with a defect in glycosylation.

In galactosaemia a deficiency of galactose-1-phosphate uridyl transferase (GALT) leads to a build up of galactose-1-phosphate, which can be measured in red blood cells and is used to monitor the progress of treatment of patients on a galactose-free diet. The present investigation demonstrates that untreated patients have defective galactosylation of complex glycans in serum transferrin and cellular glycoproteins. Although the percentage of normally galactosylated glycans increases with treatment, some treated patients with low galactose-1-phosphate levels still have a large percentage of agalactosylated glycans in their serum transferrin. This discovery could explain the long term complications associated with galactosaemia and has implications in its treatment.

Preface

Part of the work presented in this thesis has been published

Papers

1. Charlwood JK, Mian N, Johnson A, Clayton P, Keir G, Winchester B. (1997) A case of the carbohydrate deficient glycoprotein syndrome type 1 (CDGS type 1) with normal phosphomannomutase activity. *Journal of Inherited Metabolic Disease* **20**: (in press).
2. Charlwood JK, Mian N, Clayton P, Young E, Winchester B. (1997) Prenatal diagnosis of phosphomannomutase deficiency by amniocentesis and chorionic villus sampling *Prenatal Diagnosis* (in press).
3. Charlwood JK, Clayton P, Keir G, Mian N, Winchester B (1997) Defective galactosylation of serum transferrin in galactosaemia. *Glycobiology* (in press).
4. Masaguer CF, Bleriot Y, Charlwood J, Winchester B, Fleet G (1997) 6C-Butylglucoses from glucuronolactone: Suppression of silyl migration during borohydride reduction of lactols by cerium (III) chloride: Inhibition of phosphoglucomutase. *Tetrahedron Letters* (in press).
5. Bleriot Y, Masaguer CF, Charlwood J, Winchester B, Lane AL, Crook S, Watkin DJ, Fleet G (1997) 6R-, and 6S,-6C-Methylglucose from D-glucuronolactone: efficient synthesis of a seven carbon fucose analogue: inhibition of some enzymes of primary metabolism. *Tetrahedron Letters* (in press).

Abstracts

1. Charlwood JK, Mian N, Johnson A, Clayton P, Keir G, Winchester B. (1995) Altered protein glycosylation in CDGS type 1 and galactosaemia. *Abstracts of the 33rd Annual Symposium of the Society for the Study of Inborn Errors of Metabolism, Toledo* P250
2. Charlwood JK, Mian N, Johnson A, Clayton P, Keir G, Winchester B. (1996). Carbohydrate deficient glycoprotein syndrome type 5? *Journal of Inherited Metabolic Disease* **19**(supp): 92.

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List of Abbreviations

2-AB	2-Aminobenzamide
ADP	Adenosine diphosphate
Asn	Asparagine
ATP	Adenosine triphosphate
Bis-Tris	2-[Bis-(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol
BSA	Bovine serum albumin
CDG1	CDGS gene region on chromosome 16
CDGS	Carbohydrate deficient glycoprotein syndrome
CDT	Carbohydrate deficient transferrin
CHAPS	(3-[(3-Cholamidopropyl)dimethylammonio]-1-propane-sulfonate)
CHO	Chinese hamster ovary
CNS	Central nervous system
CSF	Cerebral spinal fluid
Cys	Cysteine
Da	Daltons
DAPI	4,6 Diamidino-2-phenylindole
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dpi	Dots per inch
DTT	Dithiothreitol
ECL™	Enhanced chemiluminescence
EDTA	Ethylene diamine tetra acetic acid
EPO	Erythropoietin
ER	Endoplasmic reticulum
ESI	Electrospray ionisation
FACS	Fluorescent activated cell sorter
FSH	Follicle stimulating hormone
Gal	Galactose
GalNAc	N-Acetylgalactosamine
GALT	Galactose-1-phosphate uridyl transferase
GDP	Guanosine diphosphate
Glc	Glucose
GlcNAc	N-Acetylglucosamine
Glc-1-P	Glucose-1-phosphate
Glc-1,6-P	Glucose-1,6-bisphosphate
Glu	Glutamine
GPI	Glycosylated phosphatidyl inositol
GU	Glucose unit equivalent
HEMPAS	Hereditary erythroblastic multinuclearity with a positive acidified serum lysis test
Hepes	4-(2-Hydroxyethyl)-1-piperazinepropanesulphonic acid
HFI	Hereditary fructose intolerance
HPLC	High pressure liquid chromatography
HSA	Human serum albumin
[I]	Inhibitor concentration

List of Abbreviations

I ₅₀	Concentration of inhibitor required to produce 50% inhibition
IEF	Isoelectric focusing
K _a	Association constant
K _i	Dissociation constant in the presence of inhibitor
K _m	Michaelis constant
LAMP	Lysosome associated membrane protein
LLO	Lipid-linked oligosaccharide
LPL	Lipoprotein lipase
Lys	Lysine
MALDI-TOF	Matrix assisted laser desorption ionisation time of flight
Man	Mannose
Man-1-P	Mannose-1-phosphate
Man-6-P	Mannose-6-phosphate
Man-1,6-P	Mannose-1,6-bisphosphate
MDBK	Madin-Darby bovine kidney
MRA	Mycoplasma removal agent
mRNA	Messenger ribonucleic acid
MUGS	4-Methylumbelliferyl-β-D-acetylglucosamine-6-sulphate
MV	McIlvaine citrate-phosphate buffer
MTT	(3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADP	Nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NDP	Nicotinamide diphosphate
NeuAC	Sialic acid
NMR	Nuclear magnetic resonance
OPCA	Olivopontocerebellar atrophy
OsGn2	Oligosaccharide GlcNAc ₂
OsGn1	Oligosaccharide GlcNAc ₁
PBS	Phosphate buffered saline
PGI	Phosphoglucose isomerase
PGM	Phosphoglucomutase
pI	isoelectric point
PMI	Phosphomannose isomerase
PMM	Phosphomannomutase
PMM1	Phosphomannomutase gene on chromosome 22
PMM2	Phosphomannomutase gene on chromosome 16
PMS	Phenazine methosulfate
PMSF	Phenylmethylsulfoxide
Pro	Proline
psi	Pounds per square inch
Q	Glutamine
R	Arginine
Rivanol	6,9-Diamino-2-ethoxyacridine lactate
SATO	Serum free mixture added to cell culture medium
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis

List of Abbreviations

Ser	Serine
SRP	Signal recognition particle
TBS	Tris buffered saline
TCA	Trichloroacetic acid
TCR	T-cell receptor complex
TEA	Triethanolamine
TEMED	N,N,N',N'-Tetramethylethylenediamine
Thr	Threonine
t-PA	Tissue plasminogen activator
Tris	2-Amino-2-hydroxymethylpropane-1,3-diol
Trp	Tryptophan
Tyr	Tyrosine
UDP	Uridine diphosphate
UV	Ultra violet
v	Initial rate of reaction
Val	Valine
Xaa	Amino acid

Introduction

1.1 General introduction

The role of aberrant glycosylation in the pathogenesis of human disease is becoming an important area of research. Abnormal glycosylation is a feature of many diseases including cancer, rheumatoid arthritis, tuberculosis, alcoholism, Crohn's disease, HIV, I-cell disease and hereditary erythroblastic multinuclearity with a positive acidified serum lysis test (HEMPAS) (Kornfeld, 1986; Rademacher *et al.*, 1988; Fukuda, 1990; Stibler, 1991; Axford *et al.*, 1992; Varki, 1993; Edge, 1994; Dwek *et al.*, 1996). The carbohydrate deficient glycoprotein syndrome (CDGS) type 1 and galactosaemia provide two more examples of genetic diseases in which there is aberrant glycosylation of glycoproteins and are the subject of this thesis. CDGS type 1 is a primary disorder of glycoprotein biosynthesis in which the enzyme defect lies in the synthesis of the N-glycans of glycoproteins (section 1.5). Galactosaemia is a secondary carbohydrate deficient glycoprotein syndrome as the primary enzyme defect (galactose-1-phosphate uridyl transferase) leads indirectly to a defect in the processing of N-linked glycans (section 1.6).

The biosynthesis and catabolism of N-linked glycans is discussed in the first part of this chapter, followed by a review of CDGS, galactosaemia and other disorders of glycosylation. The aims of the thesis are presented at the end of the introduction chapter.

1.2 Glycoproteins

Glycoproteins are proteins which have an oligosaccharide covalently attached to the polypeptide chain. Glycosylation is just one example of protein modification, but there are many ways in which a protein can be modified. The simplest type of protein modification is proteolytic cleavage; others include phosphorylation, methylation, acetylation and ADP-ribosylation. Such modifications allow the proteins to have

additional functions, for example regulation of biological activity (see section 1.2.4). (Wold, 1981; Voet and Voet, 1990; Mathews and Van Holde, 1990).

Three common types of covalent attachment of glycans to proteins have been described 1) the co-translational formation of a β -N-glycosidic bond between the anomeric carbon of GlcNAc and the amide bond of asparagine (N-linked glycosylation, section 1.2.1); 2) the post-translational formation of an α -O-glycosidic bond between the anomeric carbon of N-acetylgalactosamine (GalNAc) or mannose and the hydroxyl group of serine or threonine (O-linked glycosylation, section 1.2.2); 3) the formation of a GPI anchor attached to the C-terminal amide via ethanolamine phosphate (section 1.2.3) (For reviews see Kornfeld and Kornfeld, 1985; Ferguson and Williams, 1988; Schachter, 1994).

1.2.1 N-linked Glycosylation

The sequon Asn-Xaa-Ser/Thr (where Xaa is any amino acid except proline) is necessary for N-linked glycosylation, but this is not always sufficient. Many possible glycosylation sequons are not glycosylated as they do not possess the necessary three dimensional conformation (Kasturi *et al.*, 1997). There are three classes of N-linked glycans; high mannose, complex and hybrid. They all have a common pentasaccharide ($\text{Man}_3\text{GlcNAc}_2$) core structure because they are synthesised from a common precursor (Kornfeld and Kornfeld, 1985) (see section 1.3). Complex glycans contain no other mannose residues apart from those present in the common core. They show the largest amount of structural variation. High mannose glycans contain only additional α -mannosyl residues whereas hybrid glycans show characteristics of both high mannose glycans and complex glycans (Figure 1.1). N-linked glycosylation varies with cell type; for example, yeast is only able to produce high mannose structures and mannan chains; insect cells lack some glycosyltransferases and therefore terminal glycosylation can differ from that found in mammalian cells, which can synthesise high mannose, complex and hybrid structures (Goochee *et al.*, 1992).

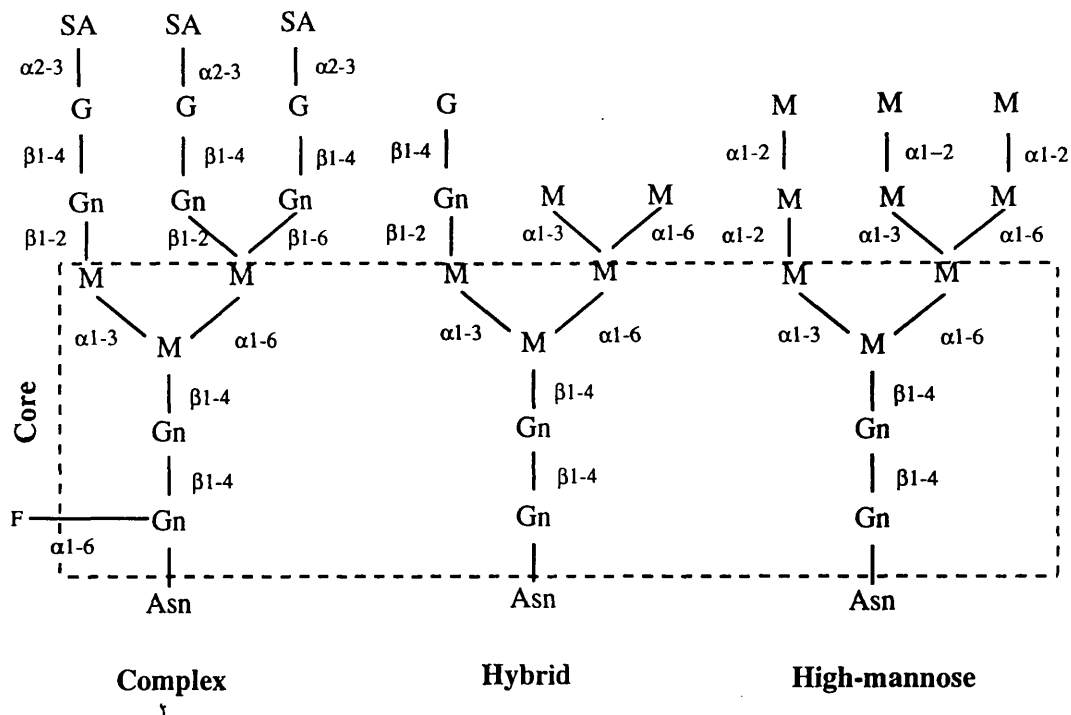


Figure 1.1: The three subgroups of N-linked glycans.

The boxed area shows the pentasaccharide core that is common to all N-linked glycans. (Adapted from Kornfeld and Kornfeld, 1985). M; mannose, Gn; N- acetylglucosamine, G; galactose, SA; sialic acid, F; fucose.

1.2.2 O-linked Glycosylation

O-linked glycans are most commonly found in cell surface glycoproteins and mucins, but they can be present on certain serum glycoproteins (e.g. erythropoietin) and also nuclear glycoproteins (Jentoft, 1990). O-linked glycans are synthesised by both prokaryotes and eukaryotes, whereas N-linked glycans are mainly synthesised by eukaryotes. (Rasmussen, 1991). This suggests that O-linked glycans evolved before N-linked glycans, however N-linked glycans have been studied in greater detail (Rademacher *et al.*, 1988; Geisow, 1991). In mammalian cells O-linked glycosylation differs from N-linked glycosylation in that it takes place post-translationally in the Golgi (Roth *et al.*, 1994) rather than co-translationally in the ER. O-linked glycans also differ from N-linked glycans as they are synthesised in a stepwise manner without the formation of a common precursor or the involvement of a lipid carrier (Geisow, 1991).

The first step in the synthesis of an O-linked glycan is the addition of GalNAc to a serine or threonine residue catalysed by UDP-GalNAc polypeptide: N-acetylgalactosaminyltransferase (EC. 2.4.1.41). Glycosyltransferases then add on the individual sugars in a stepwise manner, to create the 6 core classes;

Core 1: Gal β 1-3GalNAc-Ser/Thr

Core 2: GlcNAc β 1-6 (Gal β 1-3)GalNAc-Ser/Thr

Core 3: GlcNAc β 1-3GalNAc-Ser/Thr

Core 4: GlcNAc β 1-6(GlcNAc β 1-3)GalNAc-Ser/Thr

Core 5: GalNAc α 1-3GalNAc-Ser/Thr

Core 6: GlcNAc β 1-6GalNAc-Ser/Thr

The cores are elongated by the addition of galactose, N-acetylgalactosamine, N-acetylglucosamine and fucose and terminated by the addition of sialic acid or sulphate. The glycosyltransferases that synthesise O-linked glycans are thought to be different from those which synthesise N-linked glycans. Like N-linked glycans, mucin type O-linked glycans also show considerable heterogeneity in their oligosaccharide structures. They can vary in size from a single galactose residue to chains of up to a thousand disaccharide units but have less branching than N-linked glycans (For reviews see Schachter and Brockhausen, 1992; Schachter 1994).

Although a consensus sequence of amino acids for O-linked glycans has not yet been determined (Wang *et al.*, 1992; Nishimori *et al.*, 1994), it has been determined that the glycans tend to cluster in certain domains of the polypeptide backbone which are rich in serine and threonine residues. These regions are around 600-1200 amino acids long in mucins, but only 20 to 70 amino acids long in other glycoproteins (Jentoft, 1990). This clustering of O-linked glycans results in chain stiffening and they tend to look like bottle brushes due to their extended conformation. In contrast, N-glycosylated glycoproteins tend to be tightly folded globular proteins which are relatively fixed and compact in structure. (For reviews see Voet and Voet, 1990; Mathews and van Holde, 1990; Schachter and Brockhausen, 1992; Schachter, 1994).

1.2.3 Glycosylphosphatidylinositol (GPI) anchored proteins

Another type of covalent attachment of carbohydrates to polypeptides is *via* the GPI anchor. The function of a GPI anchor is to locate the protein at the plasma membrane. GPI-anchored proteins can have many functions, for example they can be hydrolytic enzymes (e.g. alkaline phosphatase, acetylcholine esterase), adhesion molecules (e.g. N-CAM) or receptors (Ferguson and Williams, 1988). Like N-linked glycans, GPI-anchored glycans are synthesised as precursors in the endoplasmic reticulum (Figure 1.2). In the first step GlcNAc is added to phosphatidylinositol, this is followed by deacetylation of GlcNAc to produce GlcN-PI. Three mannose residues are subsequently added via dolichol-P-Man. Finally the phosphoethanolamine is added to the third mannose to complete the core structure. In mammalian cells GPI anchors can be modified by the acetylation of inositol and the addition of phosphoethanolamines to the first and second mannose residues. The GPI is then transferred *en bloc* to the polypeptide chain. It has been suggested that GPI anchors are synthesised in excess of the requirements of the cell. Evidence of *en bloc* transfer of a precursor lies in the fact that the GPI anchor is found attached to the polypeptide immediately after synthesis of the polypeptide. Although the synthesis of the GPI anchor precursor is thought to be analogous to the synthesis of the N-linked oligosaccharide precursor, currently little is known about the glycosyltransferases responsible for the assembly of the GPI anchor precursor. The recipient polypeptide contains a signal sequence that causes it to be translocated into the ER during translation, and another sequence at the C-terminus signals the linkage of a GPI anchor. The C-terminal sequence is similar to the N-terminal signal sequence in that there is no definitive primary sequence of amino acids that are recognised, but a 20-30 amino acid sequence which is predominately hydrophobic. The signal sequence is cleaved following translocation of the polypeptide and replaced by the addition of the GPI anchor. (For reviews see Ferguson and Williams, 1988; Doering *et al.*, 1990; Ferguson, 1992; Takeda and Kinoshita 1995; Stevens, 1995).

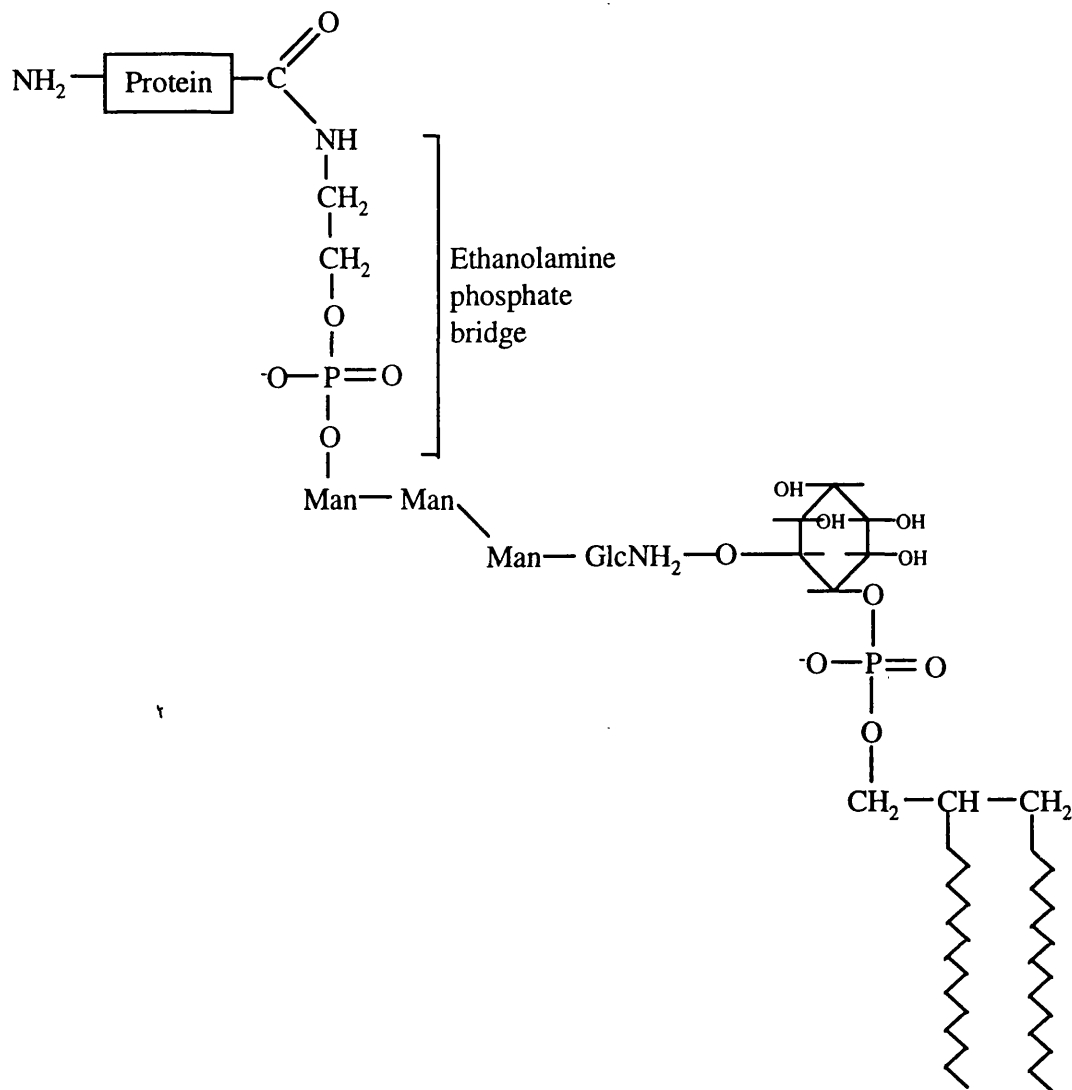


Figure 1.2: The core structure of a GPI anchor.

1.2.4 Function of oligosaccharides conjugated to proteins

Many functions have been suggested for the carbohydrates attached to proteins including; solubility, stabilisation, protection from proteolytic digestion, receptor interactions, intracellular trafficking, clearance and turnover, recognition and developmental regulation. (For reviews see Parekh *et al.*, 1989; Paulson, 1989; Goochee and Monica, 1990; Elbein, 1991a; Rasmussen, 1991; Kobata, 1992; Goochee *et al.*, 1992; Varki, 1993; Dwek, 1995a and 1995b; Gahmberg and Tolvanen, 1996).

1.2.4.1 Solubility and stability

The presence of oligosaccharides on proteins often affects the solubility of the protein. Sugar chains with their hydrophilic hydroxyl groups are more soluble in aqueous solutions than proteins. The presence of oligosaccharide chains can also prevent aggregation of the protein and hence promote stability. This has been demonstrated in both erythropoietin (Tsuda *et al.*, 1990) and interferon- β (Conradt *et al.*, 1987), in which the non-glycosylated forms are much less soluble and more prone to aggregation than the glycosylated forms. The glycan chains also provide the protein with increased protection against heat denaturation by stabilising the conformation of the protein and increased protection against proteolytic digestion by making the susceptible sites inaccessible to proteases. For example deglycosylated erythropoietin was found to be more susceptible to heat denaturation than the glycosylated counterpart (Tsuda *et al.*, 1990).

1.2.4.2 *In vivo* circulatory half lives

The presence of certain sugars on the oligosaccharide chain can determine the *in vivo* half-life of the glycoprotein. For example the asialoglycoprotein receptor, which is expressed on liver hepatocytes, binds and internalises glycoproteins in which the terminal sialic acid is lost and a galactose residue is exposed. Desialylated erythropoietin has a very much decreased biological activity *in vivo* because it is recognised by the asialoglycoprotein receptor and rapidly cleared from the circulation (Lodish, 1991; Yamaguchi *et al.*, 1991). In the kidneys proteins are filtered by glomerular tubules which have a cut off around 30 kDa - 40 kDa for excretion in urine. The oligosaccharides on glycoproteins can alter the molecular weight significantly so that when glycosylated they are not cleared, but when they are not glycosylated they are cleared by this filtration

mechanism (Rasmussen, 1991). Erythropoietin (EPO) which is glycosylated with tetra-antennary chains has greater activity *in vivo* than EPO which is glycosylated by biantennary chains, because the biantennary EPO is cleared from the circulation by the filtration mechanism in the kidney. Being larger in size, the tetra-antennary EPO escapes this clearance system (Yamaguchi *et al.*, 1991).

1.2.4.3 Secretion and intracellular transport

The effect of glycosylation on secretion of glycoproteins has been studied using tunicamycin, an inhibitor of N-linked glycosylation (Elbein, 1991b); site directed mutagenesis of the N-linked sequon (Busca *et al.*, 1995); and cell lines defective in O-linked and N-linked glycosylation (Stanley, 1984; Brandli, 1991). It has been found that in certain proteins, for example β -hexosaminidase (Weitz and Proia, 1992) and lipoprotein lipase (LPL) (Busca *et al.*, 1995), the specific glycosylation sites must be occupied to allow secretion of the glycoprotein. LPL which is not glycosylated at Asn 43 has been found to accumulate in the endoplasmic reticulum (Busca *et al.*, 1995). The glycans attached to the polypeptide play an important role in the correct folding of the glycoprotein and its intracellular transport (reviewed by Helenius, 1994). For example lysosomal enzymes normally acquire a phosphorylated mannose, which is recognised by the mannose-6-phosphate receptors which facilitate their transport from the Golgi to the lysosome. If there is no glycosylation of the polypeptide then there would be no mannose present for phosphorylation and hence no signal for the transport to the lysosome (Kornfeld and Kornfeld, 1985). Incorrectly folded glycopeptides may fail to associate correctly with the other subunits required for their functional activity and will be recognised and arrested in the ER by chaperones (Helenius, 1994). Non-glycosylated polypeptides which have decreased solubility tend to aggregate with each other to form precipitates in the endoplasmic reticulum, which in itself causes further disruption of the glycosylation machinery. This has been demonstrated in the case of deglycosylated LPL, which causes the retention of other glycoproteins in the endoplasmic reticulum, as well as retention of LPL itself (Busca *et al.*, 1995).

1.2.4.4 Biological activity

The absence of glycans often causes a decrease in the specific activity *in vivo* of the glycoprotein. Mostly this is explained by clearance mechanisms, as in the case of EPO. Desialylated and deglycosylated EPO has an increased enzyme activity *in vitro*, but *in vivo* activity is greatly reduced (Tsuda *et al.*, 1990). Tissue plasminogen activator (t-PA) also shows decreased activity *in vivo*, but increased activity *in vitro* when not glycosylated at Asn 184 (Rasmussen, 1991). One exception is clotting Factor IV, where the loss of sialic acid causes complete loss of the enzyme activity *in vitro* (Chavin and Weidner, 1984).

1.3 Biosynthesis of N-linked glycans

N-glycosylation is the result of the formation of a covalent bond between an oligosaccharide precursor and a polypeptide. The reaction takes place in the endoplasmic reticulum and is catalysed by oligosaccharyltransferase (Kornfeld and Kornfeld, 1985).

1.3.1 Synthesis of the lipid-linked oligosaccharide

The first step in the biosynthesis of N-linked glycans is the synthesis of the lipid-linked oligosaccharide (LLO) precursor. The oligosaccharide precursor is assembled on dolichol phosphate which is anchored in the membrane of the endoplasmic reticulum. Monosaccharide units are added to dolichol phosphate in a stepwise fashion, each step being catalysed by a specific glycosyltransferase, to form the common precursor. Two GlcNAc residues are added first via UDP-GlcNAc, and this is followed by the addition of five mannose residues via GDP-Man. All of these reactions occur on the cytoplasmic face of the endoplasmic reticulum. The growing oligosaccharide chain is then flipped into the lumen of the endoplasmic reticulum, and four more mannose residues are then added via dolichol-P-Man. Finally three glucose residues are added via dolichol-P-glucose. The oligosaccharide precursor is then co-translationally transferred *en bloc* from dolichol pyrophosphate to the Asn residue on the recipient polypeptide by oligosaccharyltransferase (Figure 1.3) (For reviews see Kornfeld and Kornfeld, 1985; Abeijon and Hirschberg, 1992; Verbert, 1995).

1.3.2 Synthesis of the polypeptide chain

Translation of mRNA for secreted, lysosomal and membrane proteins is initiated on ribosomes in the cytoplasm, but it is not completed until the ribosome attaches to the endoplasmic reticulum. The signal peptide is translated first and this is recognised by the signal recognition particle (SRP), which mediates the formation of a complex between the signal peptide, SRP and the SRP receptor (also located on the cytoplasmic face of the endoplasmic reticulum). Once the complex has docked, the SRP is displaced, releasing the block on translation, and translation and elongation continues in the ER. The nascent polypeptide chain emerges in the ER via a putative translocation tunnel and the signal peptide is cleaved off, and Asn-Xaa-Ser/Thr sequons are glycosylated (if they are glycosylated) as they emerge in the lumen of the ER. (Voet and Voet, 1990; Mathews and van Holde, 1990; Austen and Westwood, 1991; Andrews and Johnson, 1996).

1.3.3 Oligosaccharyltransferase

Peptide recognition studies for oligosaccharyltransferase have shown that the percentage of possible sequons actually glycosylated is very low (4.2% for Asn-Xaa-Ser and 11.3% for Asn-Xaa-Thr) suggesting that primary structure is not the only factor determining the recognition by oligosaccharyltransferase (Kaplan *et al.*, 1987; Kasturi *et al.*, 1997). It has also been shown that if Xaa is Cys, Trp or Pro then N-glycosylation is rare. Using both native and denatured proteins it has been shown that accessibility of the glycosylation sequon is an important factor in determining whether or not a site is occupied by an N-linked glycan. With respect to the lipid-linked oligosaccharide, it has been found that the complete precursor (Glc₃Man₉GlcNAc₂) is most efficiently transferred, with shorter oligosaccharides being less efficiently transferred. Oligosaccharyltransferase exhibits a broad specificity for the oligosaccharide lipid; the presence of the three glucose residues on the lipid-linked precursor promotes transfer, but is not a requirement for transfer, as non-glycosylated oligosaccharides can be transferred albeit less efficiently. The K_m of oligosaccharyltransferase for the peptide substrate is found to be altered by the lipid-linked oligosaccharide, the K_m for the same peptide being 10-fold lower when Glc₃Man₉GlcNAc₂-PP-dolichol is the substrate than when GlcNAc₂-PP-dolichol is the substrate.

1; addition of N-acetylglucosamine-1-P to dolichol-1-P by UDP N-acetylglucosamine: dolichyl phosphate N-acetylglucosamine-1-phosphate transferase, **2**; addition of the second N-acetylglucosamine, **3**; addition of 5 mannose residues from GDP-mannose by mannosyl transferases, **4**; addition of 4 mannose residues from dolichol-P-mannose by mannosyl transferases, **5**; addition of 3 glucose residues from dolichol-P-glucose by glucosyl transferases, **6**; addition of the oligosaccharide precursor to the polypeptide chain by oligosaccharyl transferase. **M**; mannose, **G_n**; N- acetylglucosamine, **G**; glucose. (Adapted from Mathews and van Holde, 1990).

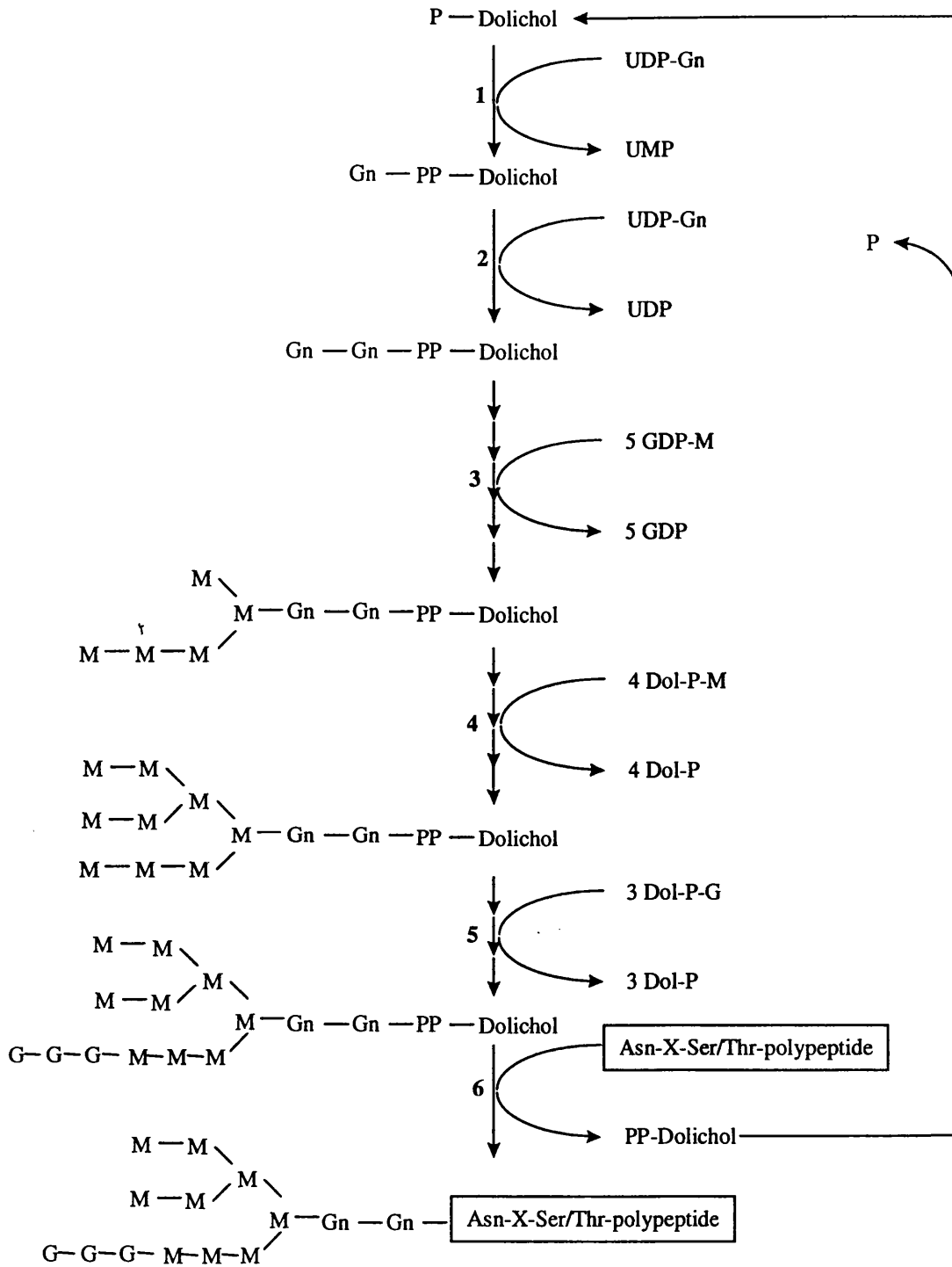


Figure 1.3: The scheme for the synthesis of the dolichol-linked oligosaccharide precursor.

Gn; GlcNAc, **M;** mannose, **G;** glucose, **P;** phosphate.

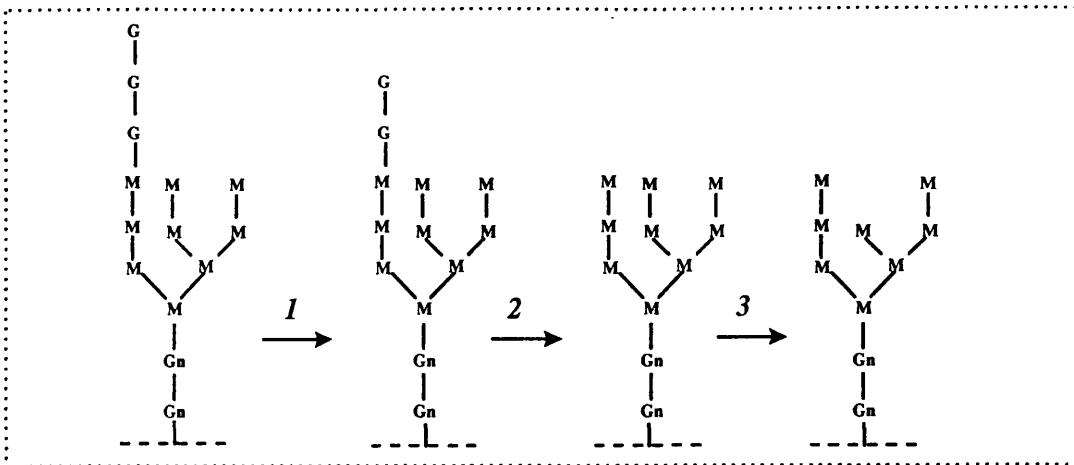
Oligosaccharyltransferase is believed to be located on the luminal face of the endoplasmic reticulum membrane. The evidence for this is that the lipid-linked oligosaccharide does not bind to concanavalin A in intact microsomes, and newly synthesised glycoproteins are not degraded by trypsin. Furthermore the free oligosaccharides released from their dolichol carrier become trapped inside the ER. Oligosaccharyltransferase is believed to be part of a protein complex, which is closely associated with the endoplasmic reticulum membrane, and includes ribophorins I and II (Kelleher *et al.*, 1992; Andrews and Johnson, 1996). (For reviews see Lau *et al.*, 1983; Kornfeld and Kornfeld, 1985; Kaplan *et al.*, 1987; Verbert, 1995).

1.3.4 Processing of N-linked oligosaccharides

Following the insertion of a high mannose oligosaccharide ($\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$) at the N-glycosylation site, the oligosaccharide is processed. The processing begins in the endoplasmic reticulum with the removal of the three glucose residues by α -glucosidases I and II (Moremen *et al.*, 1994) (Figure 1.4). The $\text{Man}_9\text{GlcNAc}_2$ chain can be reglucosylated by UDP-Glc:glycoprotein glucosyltransferase if it is attached to an unfolded glycoprotein. Calnexin is a chaperone that binds glycoproteins which are misfolded and therefore transport incompetent. Calnexin recognises such proteins by their monoglucosylated chains. When the chains are properly folded and become transport competent they are released by calnexin following the removal of the glucose by α -glucosidase II. This recycling and retention pathway provides a quality control system in the ER for misfolded glycoproteins (Herbert *et al.*, 1995). Only when the glycoprotein is properly folded it is released from this recycling pathway and transported from the ER to Golgi for further processing (Figure 1.4).

Transfer from each compartment occurs via vesicles. **1, 2**; α -glucosidases, **3, 4**; α 2-mannosidases, **5**; N-acetylglucosamine transferase I, **6**; α 3/6-mannosidase II, **7**; N-acetylglucosamine transferase II, **8**; core α 6-fucosyltransferase, **9**; galactosyltransferase, **10**; sialyltransferase, **I**; N-acetylglucosaminyl phosphotransferase, **II**; N-acetylglucosamine-1-phosphodiester α -N-acetylglucosaminidase. **M**; mannose, **Gn**; N-acetylglucosamine, **Gal**; galactose, **SA**; sialic acid, **G**; glucose, **F**; fucose. (Adapted from Kornfeld and Kornfeld, 1985).

Endoplasmic Reticulum:



Golgi:

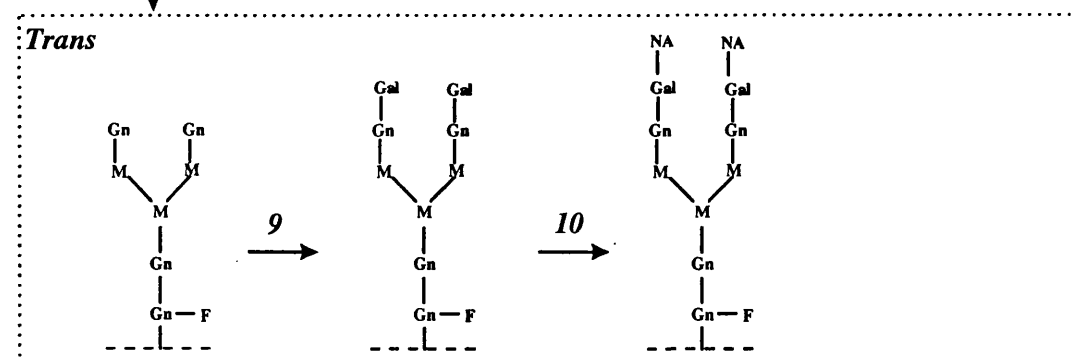
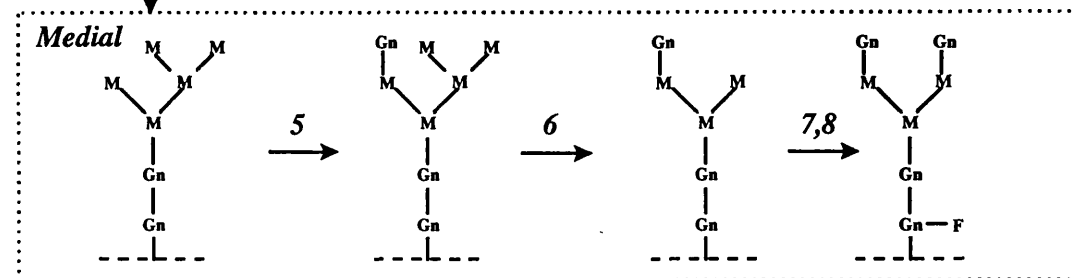
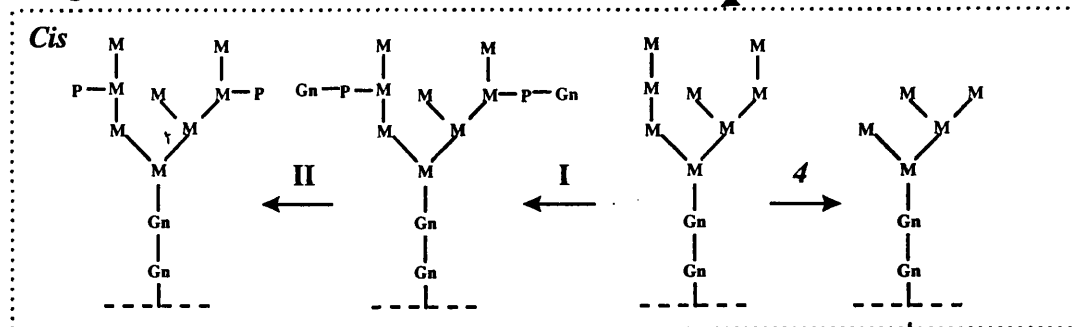


Figure 1.4: Schematic pathway of N-linked oligosaccharide processing in the endoplasmic reticulum and Golgi.

Gn; GlcNAc, M; mannose, Gal; galactose, F; fucose.

The next trimming event occurs when a mannose residue is removed by ER α -mannosidase before the glycoprotein leaves the endoplasmic reticulum. The glycoprotein (except for ER-resident glycoproteins) is then transported to the Golgi apparatus in a vesicle. In the *cis* Golgi, the lysosomal enzymes are specifically phosphorylated by N-acetylglucosaminylphosphotransferase and N-acetylglucosamine-1-phosphodiester α -N-acetylglucosaminidase (Kornfeld, 1986). This phosphorylation of mannose provides the lysosomal enzyme with a specific transport mechanism via the mannose-6-phosphate receptor. All other glycoproteins move through the Golgi stack from the *cis* to the *trans* cisternae and three more mannose residues are removed by Golgi- α -mannosidase I (Moremen *et al.*, 1994). GlcNAc transferase I adds on the first GlcNAc and then α -mannosidase II removes two mannose residues if a complex glycan is to be formed (Schachter, 1991b). The remaining glycan structure can then be acted on by GlcNAc transferase II, which adds on a GlcNAc residue to the α 1-6-mannose arm. This glycan is the substrate for galactosyltransferase and GlcNAc transferase IV and V (to produce tri- and tetra-antennary glycans). After the addition of galactose residues, sialic acid is added by sialyltransferase and some glycans are also core-fucosylated (Figure 1.4). Unlike proteins and nucleic acids whose sequences are genetically determined, there is no template for the assembly of glycan chains. As many stages of the biosynthesis of N-linked glycans produce substrates for more than one of the transferase enzymes (Schachter, 1991b) the expression of these enzymes can influence the type of sugars that are added and the branching of the glycan, ultimately determining the structure of the final product. Other factors influencing the final structure of the glycan include the supply of the activated sugars, the accessibility and in certain cases the sequence or 3-D structure of the polypeptide (Camphausen *et al.*, 1995). This aspect of N-glycosylation accounts for the micro-heterogeneity which is observed and the occurrence of glycoforms (For reviews see Schachter, 1984; Kornfeld and Kornfeld, 1985; Schachter, 1991a and 1991b; Shaper and Shaper, 1992, Moremen *et al.*, 1994).

1.4 Catabolism of N-linked glycans

There are two major pathways involved in the degradation of glycoproteins. One pathway occurs in the lysosome, where ageing proteins are degraded and the

oligosaccharides are broken down into their constituent sugars and released for recycling (Winchester, 1996). The second pathway is in the endoplasmic reticulum, where newly synthesised proteins are degraded. Much less is known about this pathway, but it is thought to act as a method of quality control (Cacan *et al.*, 1996).

1.4.1 Degradation of glycoproteins in the lysosome

Lysosomes are single membrane organelles containing phosphatases, proteases, nucleases, lipases and glycosidases. Together these enzymes have the ability to digest both foreign biological material and recycle intracellular material (Becker and Deamer, 1991).

The degradation of N-linked glycans in the lysosome is a highly ordered and bi-directional process. In the first steps the glycoprotein is digested by proteases to produce the glycoasparagines (Figure 1.5). Then α -fucosidase can act on complex glycans to remove the core fucose. Aspartyl-N-acetylglucosaminidase then cleaves the Asn-GlcNAc bond at the reducing end, followed by endo-N-acetylglucosaminidase which cleaves the reducing GlcNAc. Simultaneously at the non-reducing end α -neuraminidase cleaves terminal sialic acids, followed by the action of β -galactosidase, β -hexosaminidase and lastly the α - and β -mannosidases. High mannose glycans are also digested in a similar manner by the action of specific mannosidases (For review see Winchester, 1996).

1.4.1.1 Lysosomal glycosidases

Lysosomal glycosidases have acidic pH-optima and carry out a highly specific hydrolytic cleavage reaction (Winchester, 1996). They are glycoproteins and hence are synthesised in the rough endoplasmic reticulum. In the *cis* Golgi they undergo specific phosphorylation of mannose residues. This reaction is carried out by N-acetylglucosaminylphosphotransferase (EC. 2.7.8.17) and N-acetylglucosamine-1-phosphodiester α -N-acetylglucosaminidase (EC. 3.1.4.45). They are then recognised by the mannose-6-phosphate receptors, concentrated in the *cis* Golgi, and then transported into the lysosome via vesicles. These reactions are unique to lysosomal enzymes (Kornfeld and Kornfeld, 1985).

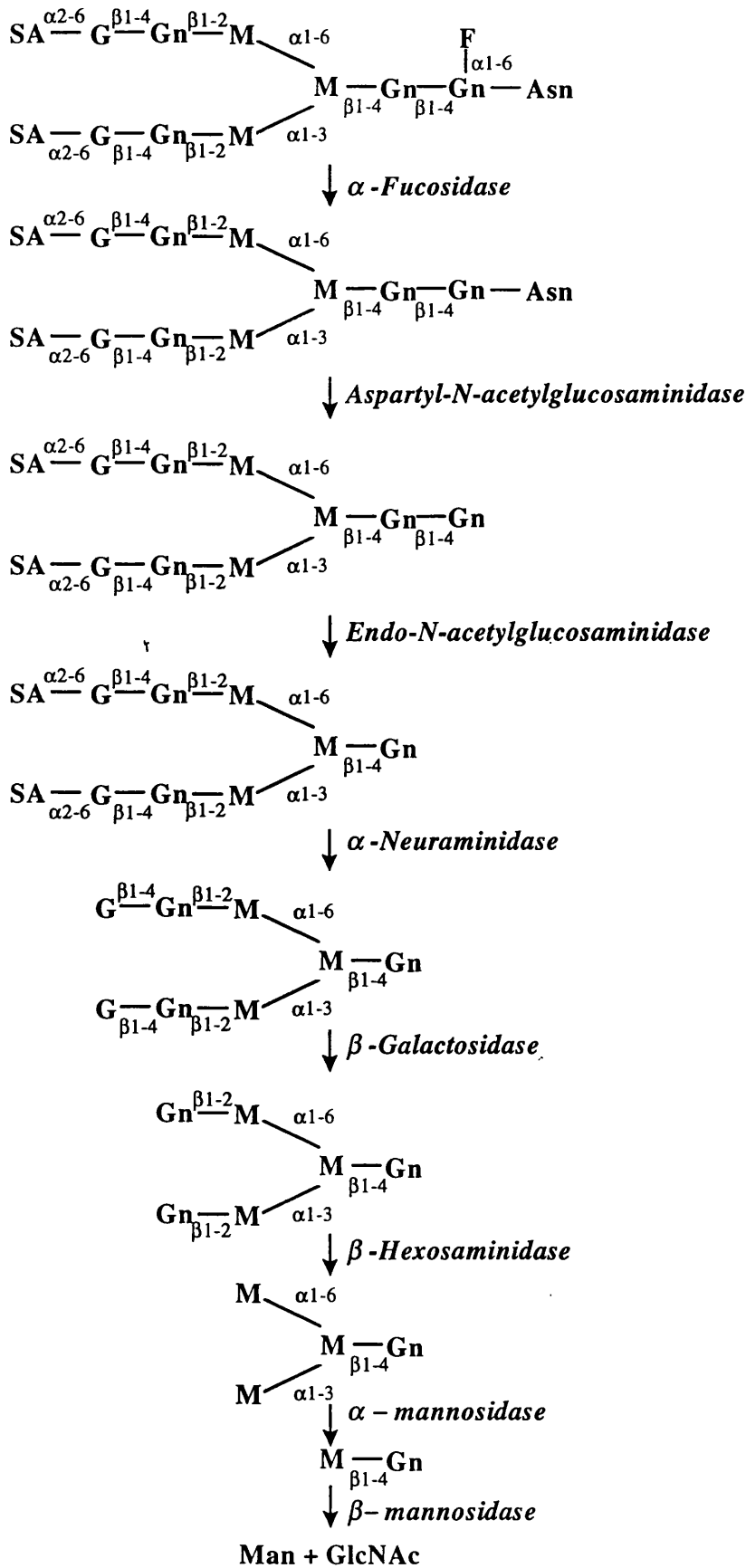


Figure 1.5: Lysosomal degradation of a complex N-linked glycan chain. (Adapted from Winchester, 1996).

Many lysosomal enzymes have been purified and cloned. The major feature in their amino acid sequence occurs in the signal peptide. It has been demonstrated that they have between 15 and 40% sequence identity with each other. The identity between species is much greater (Winchester 1996). Nonetheless, they have a common motif that is recognised by N-acetylglucosaminylphosphotransferase, which catalyses the transfer of GlcNAc-1-phosphate to the mannose residue on the oligosaccharide chain. It also has a recognition domain which specifically recognises lysosomal enzymes. As no simple sequence common to all lysosomal enzymes has been found it is thought that the recognition domains binds to a specific conformation which is common to all lysosomal enzymes (Kornfeld and Mellman, 1989). In support of this hypothesis it has been demonstrated that whilst deglycosylated lysosomal enzymes are potent inhibitors of N-acetylglucosaminylphosphotransferase activity, their peptide fragments or denatured polypeptides, are not. This suggests that the recognition is dependent on the conformation of the protein (Lang *et al.*, 1984).

The following section discusses the structures and function of two lysosomal enzymes; β -hexosaminidase and α -fucosidase, in detail as these two enzymes were used as cellular glycoproteins for the study of aberrant glycosylation in CDGS type 1 and galactosaemic fibroblasts (Chapter 6).

1.4.1.2 β -Hexosaminidase (EC 3.2.1.52)

β -Hexosaminidase is located predominantly in the lysosomes, but a small proportion is also secreted. It catalyses the release of non-reducing terminal N-acetylglucosamine or N-acetylgalactosamine from glycoproteins and glycolipids. β -Hexosaminidase is composed of two polypeptide subunits, α and β , which form non-covalently bound dimers; $\alpha\beta$, $\beta\beta$ and $\alpha\alpha$. This gives rise to catalytically active isoenzymes referred to as β -hexosaminidase A, β -hexosaminidase B and β -hexosaminidase S, respectively. β -Hexosaminidase A and B are the predominant forms in fibroblasts. The β subunit is located on chromosome 5 and the α subunit is located on chromosome 15. Both subunits are synthesised as precursor polypeptides which are processed by proteolytic enzymes. They are synthesised with a signal peptide which is cleaved following translocation into

the endoplasmic reticulum. The polypeptide chains then undergo N-glycosylation and folding. The mannose residues are phosphorylated in the Golgi by N-acetylglucosaminylphosphotransferase (Neufeld, 1989). They are recognised by the mannose-6-phosphate receptor which targets the molecule to the lysosome. Once the enzymes reach the lysosome the mannose-6-phosphate is removed (Kornfeld and Kornfeld, 1985). The association of α and β subunits to form β -hexosaminidase A occurs about 1 h after the synthesis of the α polypeptide. The association of β and β subunits to form β -hexosaminidase B is faster, taking only 15 minutes. Association of $\alpha\beta$ and $\beta\beta$ subunits is necessary for the catalytic activities of β -hexosaminidase A and B and also for transport to the lysosomes or for secretion from the cell. Unassociated α chains can leave the ER, but are not catalytically active in cultured fibroblasts (Proia *et al.*, 1984; Sonderfeld-Fresko and Proia, 1988).

The α polypeptide subunit has three N-glycosylation sites at Asn 115, 157 and 295. Loss of all three oligosaccharides results in an aberrantly folded protein which is retained in the endoplasmic reticulum. If only one oligosaccharide is removed there is still a substantial amount of enzyme activity. However, removal of two oligosaccharides causes a decrease in enzyme activity. The Asn 295 site on the α chain is the site of preference for phosphorylation of the mannose residues in the oligosaccharide (Weitz & Proia, 1992).

The β subunit contains five potential N-glycosylation sites at Asn 84, 142, 190, 327, and 497. Normally Asn 84, 190 and 327 are fully glycosylated, Asn 142 is partially glycosylated and Asn 427 is not glycosylated (Neufeld, 1989; Sonderfeld-Fresko & Proia, 1989). However the asparagine at position 427 has been shown to be essential for both the expression and stability of β -hexosaminidase B by site-directed mutagenesis. It has been demonstrated that the glycans present at Asn 84 and Asn 327 on the β chain are phosphorylated in preference over the other 3 sites, as mutations at these two sites caused a reduction in the amount of enzyme transported to the lysosome and an increase in the amount secreted (Sonderfeld-Fresko & Proia, 1989). About 90% of the oligosaccharide chains isolated from bovine lysosomal β -hexosaminidase are of the

oligomannose type and the remaining chains are disialylated biantennary structures. No signal was found with ^{31}P NMR and thus it is assumed that the oligomannose chains are dephosphorylated in the lysosome (Overdijk *et al.*, 1985).

1.4.1.3 α -Fucosidase (EC 3.2.1.51)

α -Fucosidase is a lysosomal hydrolase that catalyses the cleavage of α -fucose from glycoproteins and glycolipids. It is expressed in lysosomes in the liver, kidney, placenta spleen, fibroblasts and leukocytes. The pH / activity profile shows that it has two pH-optima, one between pH 4.5 and 5.0 and another at pH 5.5. These two isoforms are considered to be due to alternative splicing of the α -fucosidase gene (Durand *et al.*, 1982). The human α -fucosidase gene is located on chromosome 1p34 (Durand *et al.*, 1982) and mutations in the sequence have been shown to be responsible for fucosidosis. The translated polypeptide contains 461 amino acids including 22 amino acids of signal peptide and four possible glycosylation sequons (Occhiodoro *et al.*, 1989). In cultured lymphoid cells, α -fucosidase consists of four subunits each of which has a molecular weight of 52 kDa. Each subunit also has four N-linked glycosylation sites. The glycosylated subunits have a molecular mass of around 60 kDa depending on whether they contain high mannose or complex carbohydrates (DiCioccio and Mahoney, 1990). Oligomannoside and disialylated biantennary chains exist in a ratio of 3:1 in the mature enzyme (Beem *et al.*, 1987).

1.4.2 Degradation of newly synthesised glycoproteins in the endoplasmic reticulum

The cell requires a pathway for the removal of unassembled or misfolded proteins from the ER. Chaperones provide the quality control and the ER degradative pathway provides the mechanism for recycling the components. If there were no such pathway, then proteins which were not transport competent would accumulate in the ER and it would not be able to function properly (Klausner and Sitia, 1990). An example of protein misfolding which causes disturbance of the function of the ER is that of the mutant human lipoprotein lipase which is not glycosylated at Asn 43 (Busca *et al.*, 1995). This

mutant protein causes many other proteins to accumulate within the ER although they themselves are not defective.

The existence of a degradative pathway within the ER has been demonstrated by following the biosynthesis of the T-cell receptor complex (TCR) (Lippincott-Schwartz *et al.*, 1988). The TCR protein complex is made up of seven polypeptide chains which are all synthesised at different rates. This results in an excess of some of the polypeptides and hence they have to be degraded. Theoretically they could be transported to the lysosome and degraded there but it would be more energy-efficient to degrade them *in situ*. To demonstrate that they are not degraded within lysosomes, the T-cells were incubated with lysosomal enzyme inhibitors. Although lysosomal degradation was blocked there was no decrease in the rate of degradation of these polypeptides, indicating the presence of an alternative degradation pathway either in the ER or close to it as the polypeptides were shown not to enter the Golgi apparatus. This was demonstrated by retention of high mannose carbohydrates and Endo-H sensitivity. The oligosaccharide attached to TCR subunits that enter the Golgi are processed into complex glycans and become Endo-H resistant (Lippincott-Schwartz *et al.*, 1988).

The release of water soluble oligosaccharides in conjunction with protein N-glycosylation is another indication of degradation of glycoproteins within the ER (Villers *et al.*, 1994). There are two classes of water soluble oligosaccharides present; phosphorylated and neutral oligosaccharides. The phosphorylated oligosaccharides have been shown to derive from pyrophosphorylase action on the lipid-linked oligosaccharide in order to bypass the synthesis of dolichol-phosphate (Cacan *et al.*, 1992). The non-glucosylated phosphorylated oligosaccharides are dephosphorylated and found as oligosaccharides with two GlcNAc residues at the non-reducing end (OsGn2) within the water soluble oligosaccharide material. The neutral oligosaccharides can either possess two GlcNAc residues (OsGn2) or only one GlcNAc residue (OsGn1). Villers *et al.* (1994) hypothesised that OsGn2 is produced by hydrolysis of the oligosaccharide precursor by oligosaccharyltransferase, whereas OsGn1 is produced via the degradation of newly synthesised glycoproteins (Figure 1.6). This was shown to be the case by the use of pulse-chase experiments using [³H]-mannose in a glycosylation mutant CHO cell line which is unable to synthesise Man-PP-dolichol. It was found that OsGn1 was only

produced after a lag period, suggesting that some precursor synthesis was required before its release. When protein synthesis was inhibited by the addition of cycloheximide there was a decrease in the amount of mannose incorporation into glycoproteins, but an increase in the amount of mannose incorporated into OsGn2. Mannose incorporation into OsGn1 also decreased by the same order of magnitude as that for glycoprotein synthesis. The total mannose incorporation was constant. The authors have hypothesised that there is competition between the two activities of oligosaccharyltransferase, and that OsGn1 derives from the glycoprotein (Villers *et al.*, 1994). However, cytosolic chitobiase activity was later found in Madin-Darby bovine kidney (MDBK) cells and is responsible for the conversion of OsGn2 to OsGn1 (Cacan *et al.*, 1996). Hydrolytic activity of oligosaccharyltransferase is probably required for the regulation of the quality and quantity of the lipid-linked oligosaccharide (LLO). Excess LLO are hydrolysed and released as OsGn2 for recycling. The species can be either glucosylated or non-glucosylated and can vary in size from $\text{Man}_3\text{GlcNAc}_2$ to $\text{Man}_9\text{GlcNAc}_2$ (Cacan *et al.*, 1992).

Degradation of the newly synthesised glycoproteins was observed in the presence of the lysosomal inhibitor leupeptin (Villers *et al.*, 1994), confirming previous results indicating (Lippincott-Schwartz *et al.*, 1988) that the degradation must take place in the ER or a compartment close to it. There was also evidence for the involvement of transport processes between protein synthesis and degradation. At 39°C it was found that the amount of mannose in glycoproteins decreased during the chase, whereas it increased in OsGn1. However, at 16°C, where intracellular trafficking is effectively blocked, the amount of mannose was constant in both the glycoproteins and OsGn1, thus it was deemed that there was no degradation without transport (Villers *et al.*, 1994). It would make sense that there would have to be some sort of transport or segregation of proteins which are to be degraded because newly synthesised proteins which are in the process of being folded and assembled would be optimal targets for degradation as well, so they would have to be kept separate from those which are destined for degradation (Klausner and Sitia, 1990).

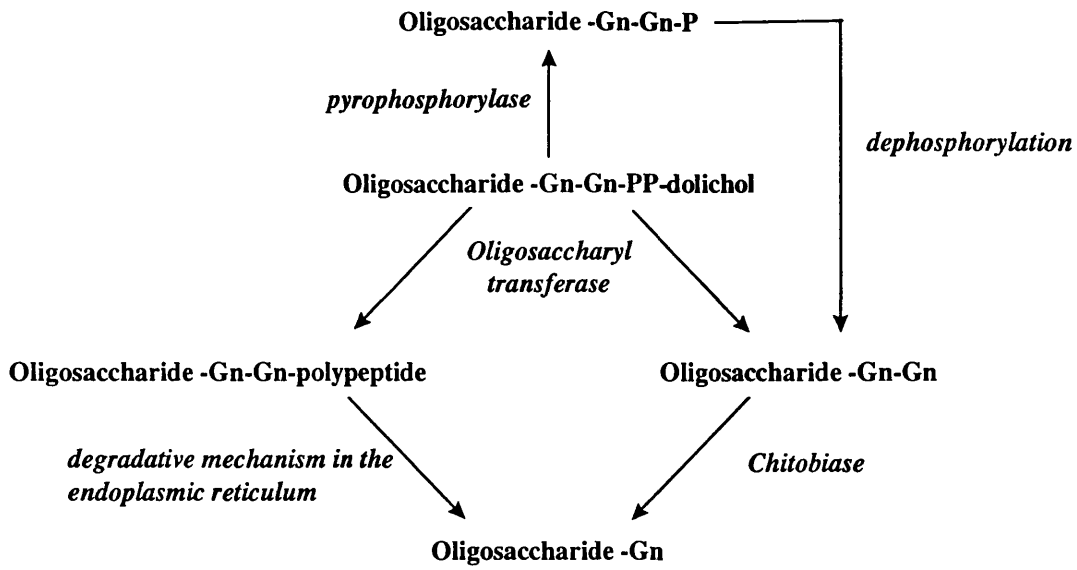


Figure 1.6: Proposed mechanism for the formation of OsGn1 and OsGn2.

(Cacan et al., 1992; Villers et al., 1994).

1.5 The Carbohydrate-Deficient Glycoprotein

Syndromes (CDGS)

1.5.1 Introduction

The carbohydrate-deficient glycoprotein syndromes (CDGS) are a class of genetic diseases in which the biochemical defects occur in the protein glycosylation pathway. At present these syndromes are subdivided into four categories; CDGS types 1, 2, 3 and 4, based on their differing glycosylation defects and clinical symptoms. All patients suffering from these syndromes display severe defects of the nervous system, as a result of altered glycosylation of glycoproteins. The focus of this thesis is CDGS type 1, the most common type of CDGS. Two other genetic disorders galactosaemia and hereditary fructose intolerance are now considered to be secondary carbohydrate-deficient glycoprotein syndromes (sections 1.6 and 1.7).

1.5.2 The history of the carbohydrate-deficient glycoprotein syndromes

CDGS was first reported in 1980 (Jaeken *et al.*, 1980), and by 1984 it was established that there was undersialylation of serum proteins in these patients (Jaeken *et al.*, 1984). Diagnosis of CDGS is made by demonstrating an abnormal pattern of serum transferrin by isoelectric focusing (IEF) followed by immunodetection. The major transferrin isoform in CDGS type 1 was found to be disialotransferrin, and so the disease was initially called 'disialotransferrin developmental deficiency syndrome' (Kristiansson *et al.*, 1989). However, there were found to be clinical similarities between 'disialotransferrin developmental deficiency syndrome' and olivopontocerebellar atrophy (OPCA) of neonatal onset (Horslen *et al.*, 1991) and eventually both diseases were called 'carbohydrate deficient glycoprotein syndrome' as a number of serum glycoproteins are affected (Jaeken *et al.*, 1991). OPCA with neonatal onset was considered to be the severest form of CDGS type 1.

Over the last five years much biochemical and molecular research has been carried out and the level of knowledge about CDGS has vastly increased (summarised in Figure 1.7). CDGS types 1, 2, 3 and 4 were identified by their different patterns of tetra-, di-, mono- and asialotransferrins on IEF gels (Jaeken *et al.*, 1993b; Stibler *et al.*, 1993, 1995).

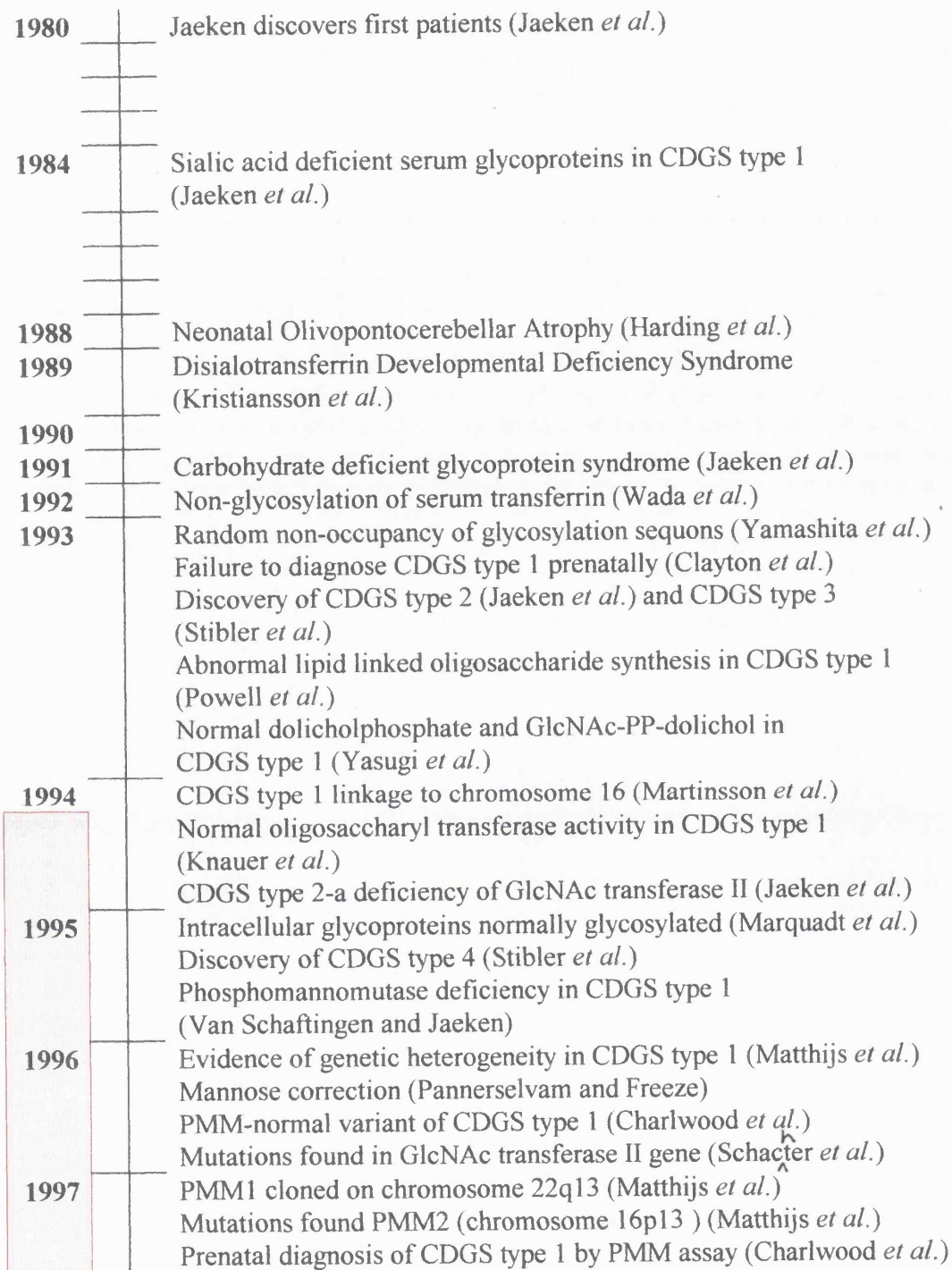


Figure 1.7: Key research findings on the carbohydrate-deficient glycoprotein syndromes. Red box; duration of this Ph.D. project (October 1994-September 1997).

Each CDGS has its own characteristic IEF pattern (section 1.5.4). The enzymic defect of N-acetyl-glucosaminyltransferase II (GlcNAc transferase II) in CDGS type 2 was characterised first (Jaeken *et al.*, 1993b and 1994), followed by demonstration of a deficiency of PMM in most CDGS type 1 patients at the end of 1995 (Van Schaftingen and Jaeken, 1995). The basic defects in CDGS types 3 and 4 are still unknown.

1.5.3 The clinical picture of the carbohydrate-deficient glycoprotein syndromes

The clinical symptoms of CDGS type 1 vary according to age and have been divided into four stages. (For general reviews see; Blennow *et al.*, 1991; Hagberg *et al.*, 1993; Jaeken and Carchon, 1993; Jaeken *et al.*, 1993; Jaeken *et al.*, 1997).

Stage I: Pregnancy and birth is normal for most CDGS patients. However neonates present within the first few days of life with feeding difficulties and failure to thrive, psychomotor retardation, delayed head control (floppiness), growth retardation, developmental delay and failure of major organs such as liver, kidney and heart. Physical signs are the presence of abnormal fat pads on the buttocks, long fingers and toes, peau d'orange skin, facial dysmorphism and inverted nipples. The British patients tend to be the most severely affected and most have died within their first year of life. However as a whole, CDGS type 1 patients have a mortality rate of 20% (Harding *et al.*, 1988; Blennow *et al.*, 1991; Horslen *et al.*, 1991; Petérsen *et al.*, 1993; Jaeken and Carchon, 1993; Jaeken *et al.*, 1993b; Hagberg *et al.*, 1993).

Stage II: In the 3 - 10 year age group mental retardation is the predominant feature and most patients reach an IQ level of between 40 and 60 at this stage of life. 'Stroke-like episodes' characterised by convulsions, coma, blindness and hemiplegia (paralysis of one side of the body) are also common. Most patients recover within hours or days of the episode, but in some cases hemiplegia is permanent due to cerebral infarctions occurring during the episode. Although the patients show developmental delay, they can sit up at 2 years and stand with support at 4 years of age. Walking with a rollator is achieved by a few children only. They can also learn to operate a wheelchair, feed themselves and most can be toilet-trained (Blennow *et al.*, 1991; Hagberg *et al.*, 1993).

Stage III: In teenage life there is no longer threat of internal organ failure and stroke-like episodes. The patients have a happy appearance and can communicate with siblings and parents. They differ in their abilities to express themselves, but some can draw and write. Generally, growth is retarded and skeletal deformities lead to a compressed thoracic spine, and limbs appear not to be in proportion with the rest of the body. The fat pads seen earlier in life disappear. Severe weakness of the legs and lower motor neurone atrophy dominate the neurological manifestations.

Stage IV: This stage has been noted in a CDGS woman of 48 years who has the appearance of a 70 year old woman, and thus it has been suggested that premature ageing occurs in these patients. (Jaeken *et al.*, 1991). None of the adult CDGS females have been reported to have passed puberty, but the males appear to have passed puberty. The neurological state remains static.

The clinical pictures of CDGS types 2, 3 and 4 are similar to CDGS type 1 and are summarised in table 1.1 (Jaeken *et al.*, 1993; Stibler *et al.*, 1993; Stibler *et al.*, 1995).

1.5.4 Biochemical investigations of serum glycoproteins and diagnosis of CDGS

1.5.4.1 Transferrin as a biochemical marker of disease

The carbohydrate-deficient glycoprotein syndromes are diagnosed by detection of abnormal isoforms of serum transferrin (Jaeken *et al.*, 1984; Stibler and Jaeken, 1990; Stibler and Cederberg, 1993). Analysis of transferrin in cerebrospinal fluid has also been used to diagnose cerebrospinal fluid (CSF) leakage from the nose or ear after traumatic fracture, surgery, infection and intra- and extra-cranial tumours. An increased proportion of asialotransferrin in CSF is used to diagnose such leakage. This is of great importance as in these conditions patients are at risk of contracting meningitis (Keir *et al.*, 1992). Aberrant forms of transferrin are detected by isoelectric focusing of serum, followed by immunodetection for transferrin.

Clinical Symptoms	Type of CDGS			
	1	2	3	4
Failure to thrive	+	-		
Skeletal				
Microcephaly (abnormally small head)	+/- ^a	-		+ ^a
Prominent maxilla (jawbone)	-	+		
Chest deformity	+/-	+		
Contractures (shortening of muscle tissue)	+/- ^b			+/-
Long fingers and toes	+	- ^c		-
Adducted thumbs	-			+
Other dysmorphic			+ ^d	
Fat pads	+			
Inverted nipples	+	- ^e		-
Antimongoloid palpebral fissures (wide spaced eyes)	+			-
Rotated / dysplastic ears	-	+		+
Variable skin pigment	-		+	
Neurological				
Nuchal hypotonia (decreased muscle tone in neck)	+	+	+	+/-
Psychomotor retardation	+	++	++	++
Hypertonia (increased muscle tone) of limbs	-	-	-	+
Oculomotor apraxia (abnormal eye movement)	+			
Seizures	-	-	++	++
Ophthalmological				
Pigmentary retinopathy (deposit of pigment causing disease of the eye)	+/-			
Optic atrophy (eye wasting)	-		+	
Cardiorespiratory				
Pericardial effusion (fluid in the cavity between the double membrane which surrounds the heart)	+			
Cardiomyopathy (chronic disorder of heart muscle)	+			
Septal defect (defect between the cavities in the heart)	-	+		
Chest infections	+	+		
Hepatic				
Ascites (fluid in the peritoneal cavity)	+			
Hepatomegaly (enlargement of the liver)	+/-	-	+/-	

Table 1.1: Clinical features of young infants with carbohydrate deficient glycoprotein syndrome.

a; may become apparent during first year of life, b; slightly limited extension of knee and abduction of hips only, c; abnormal positioning of toes, d; details of facial dysmorphism not given, e; wide spaced nipples.

The discovery of the carbohydrate deficient glycoprotein syndromes (CDGS) was a serendipitous finding by Jaeken *et al.* (1984) based on the similarity in the isoelectric focusing (IEF) patterns of serum transferrin from these patients and those observed in alcohol abuse cases (Stibler, 1991; Jeppsson *et al.*, 1993). The transferrin was described as sialic acid-deficient or carbohydrate-deficient transferrin (CDT). A change in the IEF pattern of a glycoprotein reflects a change in the overall charge of the molecule, in this case due to the number of sialic residues. Similar variations in IEF patterns were also noted in a large number of serum glycoproteins from these patients, e.g. α 1-antitrypsin, (Clayton *et al.* 1993), antithrombin III, α 1-acid glycoprotein, α 1-antichymotrypsin, α 1-B glycoprotein, ceruloplasmin, complement C1 esterase inhibitor, orosomucoid and others (for complete list see; Harrison *et al.*, 1992; Yuasa, 1995; Henry *et al.*, 1997). Variations in the serum transferrin IEF pattern and in clinical features of patients led to the classification of CDGS into four types (Jaeken *et al.*, 1993; Stibler *et al.*, 1993; Stibler *et al.*, 1995).

1.5.4.2 Transferrin

Transferrin is a monomeric glycoprotein which reversibly binds iron and transports it to the bone marrow and the liver. It is involved in the recycling of iron from the catabolism of haemoglobin back into the hematopoietic tissues, and hence has a role in the regulation of iron absorption. Transferrin also transfers iron to many other cells, including fibroblasts. Diferric transferrin binds to the transferrin receptor, which is concentrated in coated pits and a vesicle is formed as the diferric-transferrin-receptor-ligand complex is internalised. The acidic pH of the early endosome causes the dissociation of the iron from transferrin and subsequent transport of iron to the cytoplasm, where it is incorporated into heme in the case of hematopoietic tissue or is re-utilised for metabolic processes in other cells. The apotransferrin-transferrin receptor complex is then recycled back to the plasma membrane. The transferrin receptor has its greatest affinity for diferric transferrin, but can bind monoferric and apotransferrin with less affinity. This cycle can be repeated around 300 times before degradation of the transferrin receptor. Transferrin itself has a half life of eight days. (For reviews see Spik *et al.*, 1975; Spik *et al.*, 1985; Putman, 1980; MacGillivray *et al.*, 1983; de Jong *et al.*, 1990; Thorstensen and Romslo, 1990).

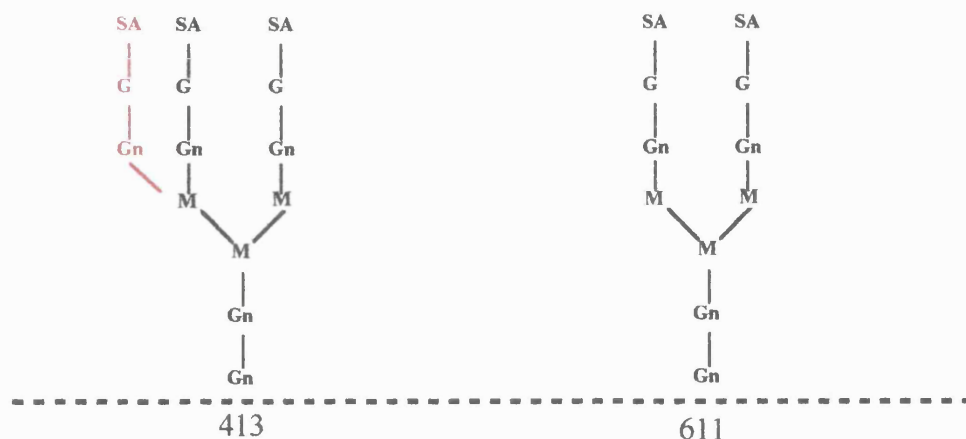


Figure 1.8: The structure of tetrasialotransferrin and pentasialotransferrin.

Sugars shown in black make up tetrasialotransferrin and the addition of the three sugars shown in red (at either glycosylation site) make up pentasialotransferrin. SA; sialic acid, G; galactose, M; mannose, Gn; N-acetylglucosamine.

Transferrin is mainly synthesised by hepatocytes in the liver, but is also synthesised in the brain, in capillary endothelial cells and oligodendroglial cells and during lactation, in the mammary gland. It is composed of 679 amino acids and has two N-glycosylation sites at Asn⁴¹³Lys Ser and Asn⁶¹¹Val Thr. The protein is divided into two homologous domains called the N-terminal domain and the C-terminal domain. Each globular domain has one metal binding site, which has its highest specificity for iron ($K_a 10^{22} M^{-1}$), although it does bind other metal ions. Each metal binding site is independent of the other (de Jong *et al.*, 1990).

The N-linked glycans of transferrin vary in structure and can be bi-, tri-, or tetra-antennary, although the major form is biantennary. All the branches are capped with terminal sialic acid residues, and hence each isoform of transferrin differs in net negative charge (Figure 1.8). This means that the different isoforms can be separated into 9 distinct bands on an IEF gel as they vary in pI from pH 5 to pH 6 (a-, mono-, di-, tri-, tetra-, penta-, hexa-, hepta- and octasialotransferrin) (de Jong *et al.*, 1990). The biological role of the N-linked glycans on transferrin has been well characterised. They enhance the water solubility of transferrin but are not required for the stability of the molecule, possibly because there are 16 intramolecular disulphide bonds holding it in a stable conformation. The N-linked glycans are also not required for the uptake and

release of iron, but are required for interactions with the cell membranes in the process of iron exchange (For reviews see Spik *et al.*, 1975; Spik *et al.*, 1985; Putman, 1980; MacGillivray *et al.*, 1983; de Jong *et al.*, 1990; Thorstensen and Romslo, 1990).

1.5.4.3 Isoelectric focusing of serum transferrin in the diagnosis of CDGS

Isoelectric focusing (IEF) is an electrophoretic technique in which a pH gradient is set up between an anode and a cathode. The pH increases from the anode to the cathode and the pH gradient is maintained by ampholytes. Proteins which are at a pH lower than their pI migrate towards the cathode and proteins at a pH above their pI migrate towards the anode. At their pI, proteins are in the zwitterion form and do not possess any net charge and thus they are 'focused' i.e. they remain at that pH. In this way narrow bands of separated proteins are formed. A difference in pI of 0.001 pH unit between two proteins is enough for complete separation. Detection of the different isoforms is achieved by immunoblotting (Hackler *et al.*, 1995).

In normal serum, transferrin migrates on an IEF gel to form one major band, corresponding to tetrasialotransferrin and a minor band of pentasialotransferrin (Figure 1.9). CDGS type 1 is characterised by a decrease in tetrasialotransferrin and an increase in disialotransferrin. There is also a prominent asialotransferrin band, which is not seen in normal transferrin. CDGS type 2 is characterised by an increase in disialotransferrin and monosialotransferrin and a decrease in tetrasialotransferrin. CDGS type 3 shows an appreciable amount of tetrasialotransferrin and an increase in trisialo-, disialo- and monosialotransferrin in roughly equal amounts. In CDGS type 4 there is an appreciable amount of tetrasialotransferrin, an increased amount of disialotransferrin and a small amount of trisialotransferrin. (Figure 1.9, Stibler *et al.*, 1991 and 1995).

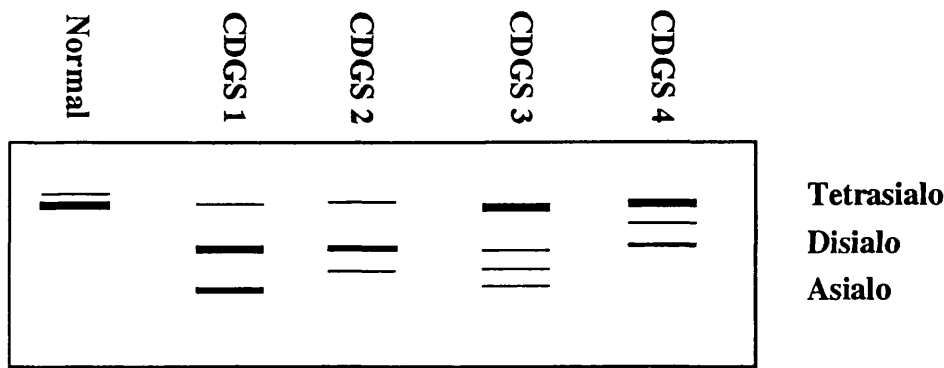
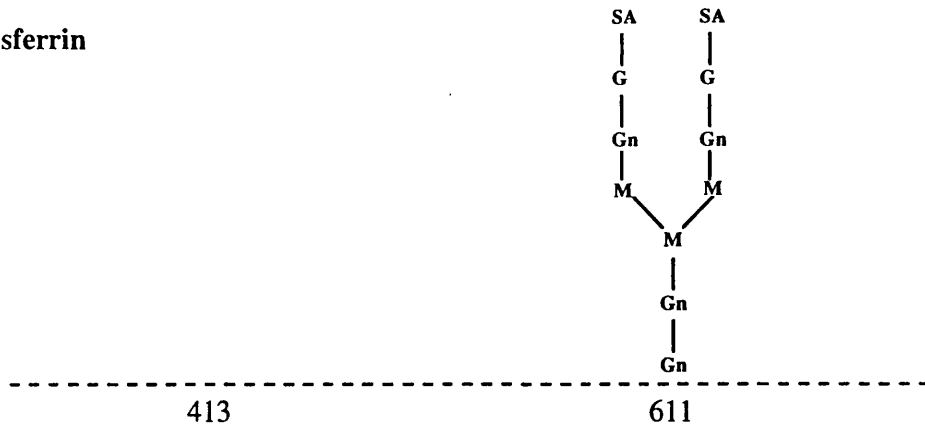


Figure 1.9 : Schematic representation of isoelectric focusing of serum followed by immunodetection of transferrin isoforms in normal and CDGS patients of each type (Stibler *et al.*, 1995).

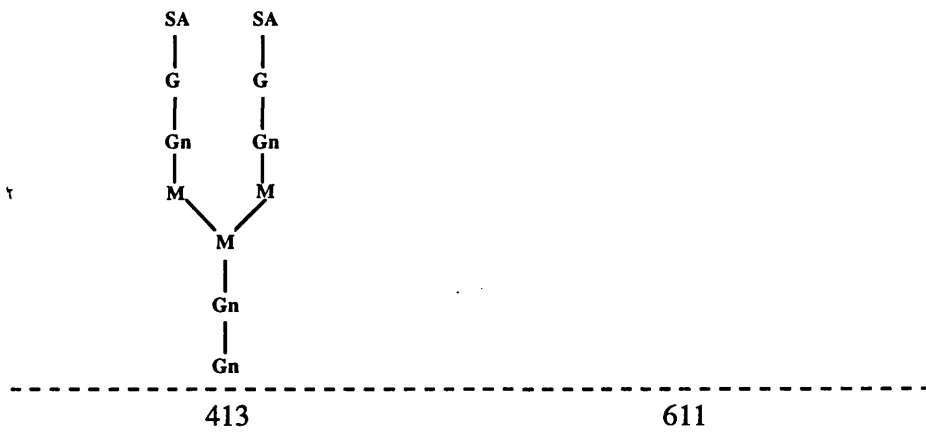
1.5.4.4 Structural analysis of transferrin in CDGS patients

The earlier studies on the monosaccharide composition of N-linked glycans in CDGS type 1 transferrin suggested that CDGS type 1 transferrin lacked the terminal structure NeuAc-Gal-GlcNAc (Stibler and Jaeken, 1990). Analysis of CDGS type 1 transferrin by SDS-PAGE and electrospray mass spectrometry demonstrated that the disialo and asialo isoforms were smaller in their molecular mass by 2,200 Da and 4,400 Da respectively compared with tetrasialotransferrin. This mass difference corresponds to the loss of one complete disialylated N-linked glycan for disialotransferrin and two N-linked glycans for asialotransferrin. Subsequently, it was shown that 93% of the glycans of CDGS type 1 transferrin are of the mature disialylated biantennary glycan as found in the normal transferrin (Wada *et al.*, 1992; Yamashita *et al.*, 1993a, 1993b; Winchester *et al.*, 1995; Iourin *et al.*, 1996) (Figure 1.10). The N-linked glycans of CDGS type 1 transferrin have been characterised by lectin affinity chromatography (Yamashita *et al.*, 1993a) and HPLC / capillary zone electrophoresis after fluorescently labelling the glycans (Iourin *et al.*, 1996).

Disialotransferrin



Disialotransferrin



Asialotransferrin

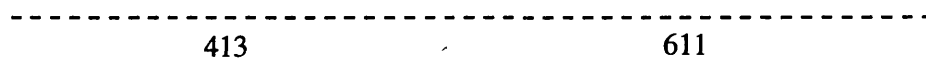


Figure 1.10 Abnormal isoforms of transferrin found in the serum of CDGS type 1 patients.

SA; sialic acid, G; galactose, M; mannose, Gn; N-acetylglucosamine

The studies by Yamashita *et al.* (1993b) also demonstrated that the lack of occupancy of the N-glycosylation sites in disialotransferrin molecules was a random process. Whilst cleavage of normal transferrin with trypsin produced two glycopeptides (T41 and T67), a similar tryptic digest of transferrin from CDGS type 1 patients produced a mixture of the two peptides in equal amounts of the glycosylated and unglycosylated forms. These observations provide evidence that non-occupancy of N-glycosylation sequons in CDGS type 1 transferrin is random. It was nevertheless determined from the earlier IEF studies that the absence of N-linked glycans in asialo and disialotransferrin in CDGS type 1 was not due to the removal of these glycans by endogenous N-glycanase. Had that been the case, then either both or one of the asparagine residues would have been converted to an aspartic acid which would cause the protein to display a different IEF pattern (Yamashita *et al.*, 1993b).

Transferrin from CDGS type 2 patients has also been analysed by ^1H NMR and ESI mass spectrometry (Jaeken *et al.*, 1994). In this case one of the biantennary arms of the glycan at both the N-glycosylation sites was found to be truncated. The differences in molecular mass between normal and CDGS type 2 disialotransferrin suggested that 2 moles of $\alpha\text{-NeuAc-(2}\rightarrow\text{6)-}\beta\text{-D-Gal(1}\rightarrow\text{4)-}\beta\text{-D-GlcNAc}$ were absent from each transferrin molecule. These results suggested that the basic defect was due to a deficiency of GlcNAc transferase II, which was shown to be the case (Jaeken *et al.*, 1994) (Figure 1.11). Mutations in the GlcNAc transferase II gene in the two patients have also been reported (Schachter *et al.*, 1996).

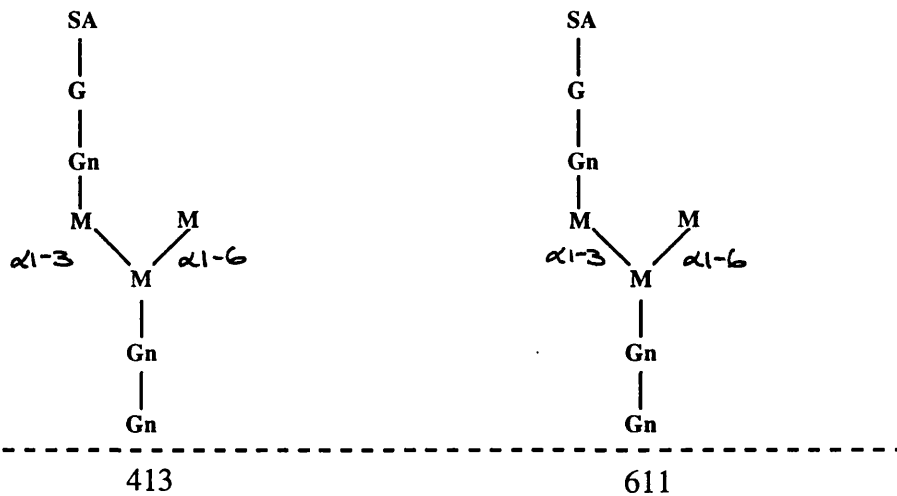


Figure 1.11 Abnormal glycoform of transferrin found in the serum of CDGS type 2 patients.

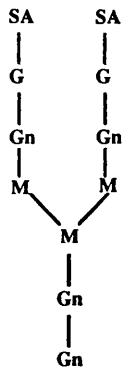
SA; sialic acid, G; galactose, M; mannose, Gn; N-acetylglucosamine

1.5.4.5 α 1-Antitrypsin

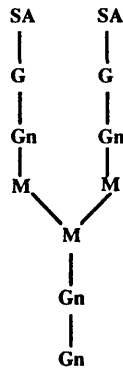
α 1-Antitrypsin is another serum glycoprotein that has also been used as a biochemical marker of CDGS. α 1-Antitrypsin is the most abundant protease inhibitor in serum with a normal concentration of around 1.3 g/l. It is synthesised in the liver and has a half life of about 6 days. Its presence in serum is required to protect tissues, in particular the lung, from damage by proteases. It is composed of a single polypeptide chain of 394 amino acids and has three N-glycosylation sites. There is no propeptide, but it does contain a signal peptide. It has a highly ordered structure, containing 30% α -helices and 40% β -sheets. α 1-Antitrypsin inhibits trypsin by binding in its active site and blocking the access of the protein substrate. The trypsin- α 1-antitrypsin complex does not dissociate under physiological conditions thereby rendering trypsin inactive (Carrell *et al.*, 1982; Brantly *et al.*, 1988).

In normal α 1-antitrypsin, asparagines 46, 83 and 247 are occupied with disialylated biantennary glycans, but the presence of triantennary glycans has also been demonstrated under certain physiological conditions. This heterogeneity leads to complex banding patterns on IEF gels.

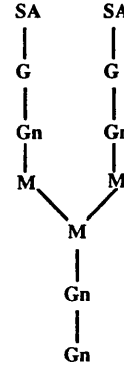
Isoform I



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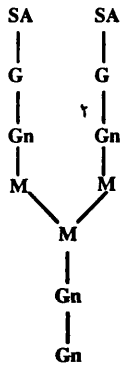


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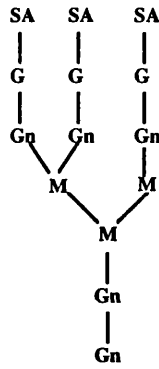


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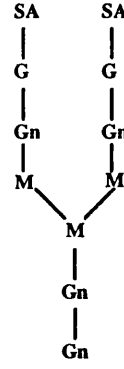
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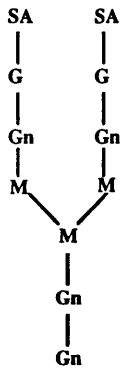


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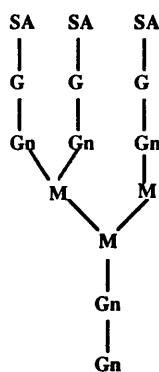


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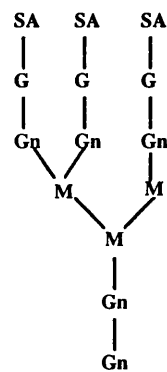
Isoform III



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46/247

Figure 1.12 Structures of the three isoforms of α 1-antitrypsin.

SA; sialic acid, G; galactose, M; mannose, Gn; N-acetylglucosamine

* This finding has not been confirmed in other patients and may have arisen due to the experimental procedures used.

In isoform I all 3 sites are occupied by biantennary chains, in isoform II, one site (usually Asn 83) is occupied by a triantennary glycan, and in isoform III two sites are occupied with triantennary glycans (Figure 1.12). The pattern is further complicated by micro heterogeneity due to post secretory modifications of the polypeptide chain (Carrell *et al.*, 1982).

α 1-Antitrypsin deficiency causes a predisposition to lung damage and has also been connected with renal disease, arthritis and malignancies. It is most commonly caused by single point mutations. The Z variant is created by a change of glutamic acid 342 to a lysine. The S variant is created by a change of glutamic acid 264 to a valine. Both glutamic acids form a salt bridge with lysine (Glu³⁴²-Lys²⁹⁰ and Glu²⁶⁴-Lys³⁸⁷) in normal α 1-antitrypsin, which becomes disrupted in the S and Z variants. In the S variant the α -helix is disrupted, which causes aggregation and intracellular degradation in the endoplasmic reticulum resulting in diminished secretion of α 1-antitrypsin into the serum. In the Z variant, there is interference with either cleavage or attachment of oligosaccharides leading to accumulation of α 1-antitrypsin in the hepatocytes. The Z variant contains immature high mannose glycans, because the glycans are not processed (Brantly *et al.*, 1988).

1.5.4.6 Structural analysis of α 1-antitrypsin in CDGS patients

The structure of the N-linked glycans of α 1-antitrypsin from two Danish CDGS type 1 patients has been determined (Krasnewich *et al.*, 1995). It was found that the α 1-antitrypsin from patients did not show under-occupancy of N-glycosylation sites. The structural analysis of the glycans revealed that only monosialylated glycans were present compared with disialylated glycans in the normal α 1-antitrypsin. The authors concluded that these two patients had a different defect from the patients in the earlier studies on transferrin by Wada *et al.* (1992) and Yamashita (1993a and 1993b), because they had truncated glycans instead of a lack of complete glycans.*

α 1-Antitrypsin had previously been shown to be undersialylated in CDGS type 1 patients (Clayton, 1993; Harrison *et al.*, 1992), therefore it is a useful diagnostic marker to

distinguish between all types of CDGS patients and other patients with unrelated liver disorders affecting their serum transferrin patterns (e.g. biliary atresia).

1.5.5 Discovery of the enzyme defect causing CDGS type 1

The quest for the discovery of the basic enzyme defect causing CDGS type 1 started in 1991 (Stibler *et al.*, 1991). At that time it was thought that the glycans of transferrin were missing only the three terminal sugars; sialic acid, galactose and N-acetylglucosamine. Hence the first enzymes to be investigated were the glycosyltransferases and glycosidases involved in the biosynthesis and catabolism of complex glycans. However, the activities of eleven of these enzymes (sialidase, β -galactosidase, N-acetylglucosaminidase, α -mannosidase, sialyltransferase, galactosyltransferase, N-acetylglucosaminyltransferase, cathepsin, cathepsin B1, cathepsin D and cathepsin L) were all found to be normal in CDGS type 1 fibroblasts.

After the discovery that the glycans of serum transferrin from CDGS type 1 patients were in fact disialylated biantennary glycans and that the disialo and asialo transferrin isoforms were formed by the random non-occupancy of the glycosylation sites (Wada *et al.*, 1992; Yamashita *et al.*, 1993), oligosaccharyltransferase became the prime suspect as the major cause of CDGS type 1. However, at the end of 1994 Knauer *et al.* (1994) were able to show that CDGS type 1 fibroblasts had normal oligosaccharyltransferase activity when dolichyldiphosphate N-N' diacetylchitobiose and a hexapeptide were used as the substrate and acceptor respectively. Both of these two components are not the natural substrates for oligosaccharyltransferase and so the results were treated with caution (see chapter 6 for discussion). Thus alternative hypotheses were proposed as to the cause of CDGS type 1. If the defect was not due to a deficiency of oligosaccharyltransferase itself then it is possible that the defect could arise from a deficiency of the substrate i.e. lipid-linked oligosaccharide precursor (LLO). The LLO is made up from its component activated sugars and is transferred *en bloc* to the polypeptide by oligosaccharyltransferase and hence the defect could lie in either the synthesis of the activated sugars or in the assembly of the precursor (section 1.3.1). A defect anywhere in the synthesis of the LLO would most likely result in the random non-occupancy of glycosylation sites due to a lack of substrate for oligosaccharyltransferase.

There were many candidate enzymes available for investigation. Yasugi *et al.*, (1994), ruled out the possibility that the defect occurred in the first step in the assembly of the precursor by demonstrating that there were normal amounts of dolichol phosphate present in CDGS type 1 fibroblasts. Hence a defect in the synthesis of dolichol phosphate was discounted. Secondly they showed that the activity of UDP-N-acetylglucosamine:dolichyl phosphate N-acetylglucosamine-1-phosphate transferase was also normal in CDGS type 1 fibroblasts. The authors concluded that on the basis of these results the synthesis of GlcNAc-PP-dolichol was normal in the CDGS type 1 fibroblasts (Yasugi *et al.*, 1994).

Powell *et al.* (1994) took an alternative approach and demonstrated that there was indeed a defect in the synthesis of the LLO. They showed that when CDGS type 1 fibroblasts were labelled with a 60 minute pulse of [³H]-mannose and [³⁵S]-methionine, there was no difference in the rate of protein synthesis, but there was a 3-10 fold decrease in the rate of incorporation of mannose into proteins. They also found that truncated LLOs were present in some of the CDGS cell lines. However, Krasnewich *et al.* (1995) found that the truncated LLOs were made only when a 60 minute pulse was used and that the LLO in CDGS type 1 fibroblasts were of the normal size when analysed after a 120 minute pulse of [³H]-mannose. Panneerselvam and Freeze (1995, 1996a), discovered that addition of mannose to the cell culture medium of CDGS type 1 fibroblasts resulted in a correction of the defect in the synthesis of the LLO. When the CDGS type 1 fibroblasts were incubated in 1 mM mannose immediately prior to the 60 minute labelling period the LLOs which were synthesised were of the normal size. However, the effect was not long lasting as cells which had been grown in the presence of mannose for 10 days prior to the labelling synthesised truncated LLOs when mannose was removed for 30 minute prior to labelling. The authors interpreted these results to mean that the enzyme defect in CDGS type 1 was either due to a deficiency of phosphomannose isomerase (PMI), hexokinase or phosphoglucose isomerase (PGI), i.e. in the pathway producing mannose-6-phosphate from glucose (Figure 1.13). The activities of these three enzymes were measured in CDGS type 1 fibroblasts and all were found to be within the normal range (Panneerselvam and Freeze, 1995). Finally at the end of 1995 the mystery was solved as Van Schaftingen and Jaeken (1995) discovered

that the enzyme defect was in fact in the next step in the pathway and they reported that their CDGS type 1 patients had a deficiency of phosphomannomutase (PMM). PMM is the enzyme that converts mannose-6-phosphate to mannose-1-phosphate prior to the synthesis of the GDP-Man and dolichol-P-Man, both of which are required for the synthesis of the LLO.

1.5.6 Phosphomutases

Phosphomutases are a family of enzymes which catalyse the transfer of sugar phosphates. Phosphomannomutase catalyses the interconversion of mannose-1-phosphate and mannose-6-phosphate, via the cofactor mannose-1,6-bisphosphate. Phosphoglucomutase carries out the same interconversion on glucose-1-phosphate and glucose-6-phosphate using glucose-1,6-bisphosphate as a cofactor. There are also specific phosphomutases which carry out similar reactions on ribose and N-acetylglucosamine phosphates (Rose, 1986).

Both phosphomannomutase and phosphoglucomutase require the bisphosphate cofactor for the activation of the enzyme. The reaction mechanism of phosphoglucomutase has been well studied and at this stage in our understanding, phosphomannomutase is thought to act in a similar manner. The enzyme is phosphorylated in the first step by the sugar-1,6-bisphosphate and a mixture of the two sugar phosphates is produced. The substrate is then phosphorylated in the 6-position and donates the 1-phosphate for regeneration of the active enzyme (Figure 1.14) (Rose, 1986 and Oesterhelt *et al.*, 1997).

1.5.6.1 Phosphomannomutase (EC 5.4.2.28)

Phosphomannomutase was first cloned in yeast as Sec53 and mutations in this gene result in defects in the N-glycosylation and secretory pathway in the cell, which is a lethal phenotype in yeast (Kepes and Scheckman, 1988). Although Sec53 is essentially a cytoplasmic protein, it is found to exist in two pools in wild type yeast cells; a soluble fraction and a membrane bound fraction. The membrane bound fraction is thought to be attached to the cytoplasmic surface of the endoplasmic reticulum by protein-protein interaction. It can be solubilised by the addition of 0.5 M KCl and Triton X-100 or 0.15 M KCl and CHAPS to the homogenisation buffer.

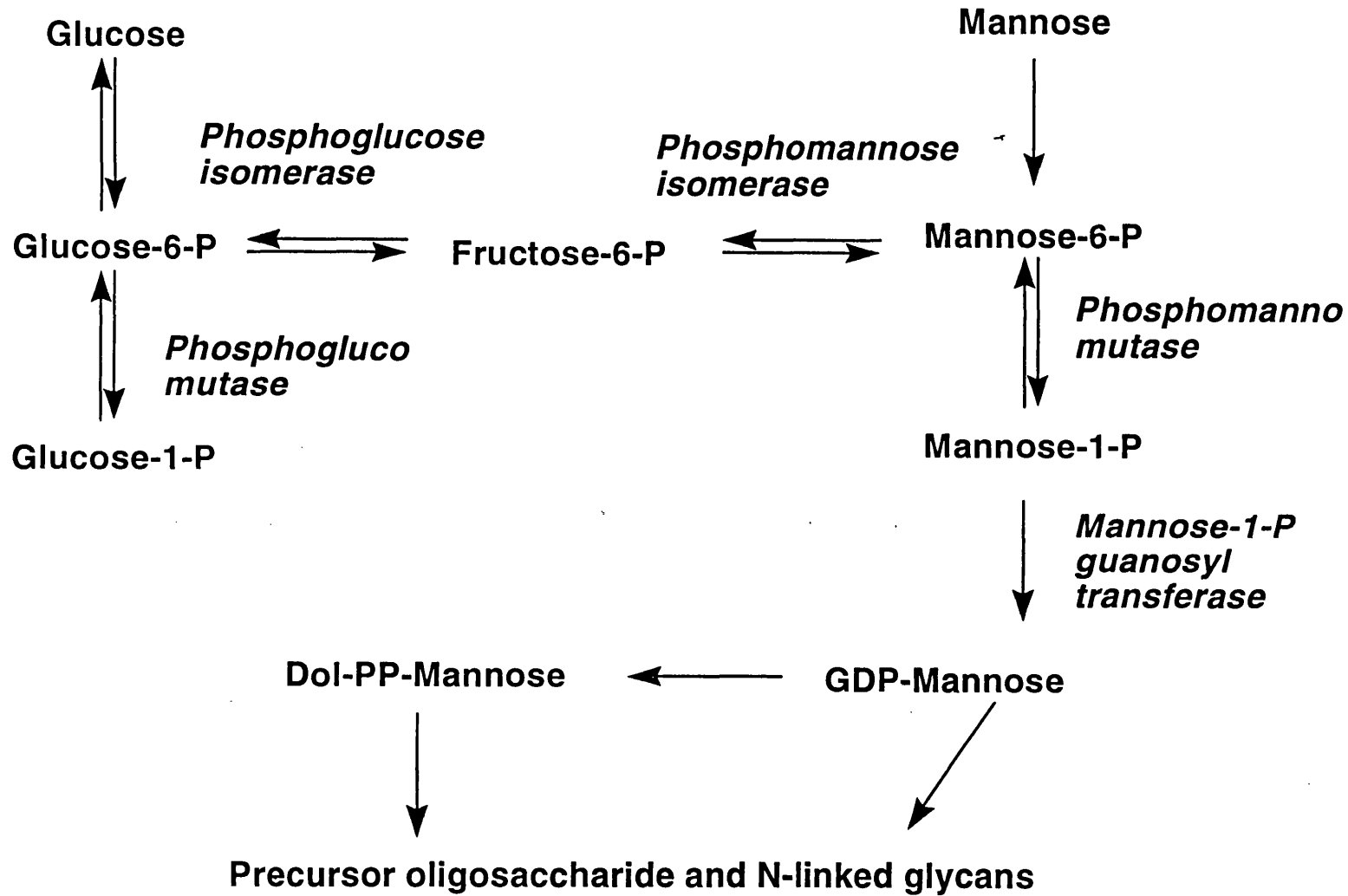
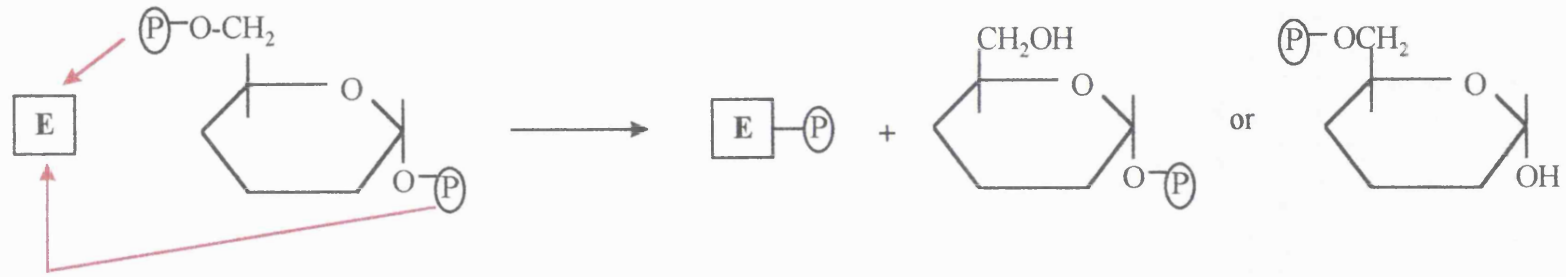


Figure 1.13 : The reactions involved in the synthesis of mannose-1-phosphate from glucose and mannose.

Activation step:



Reaction:

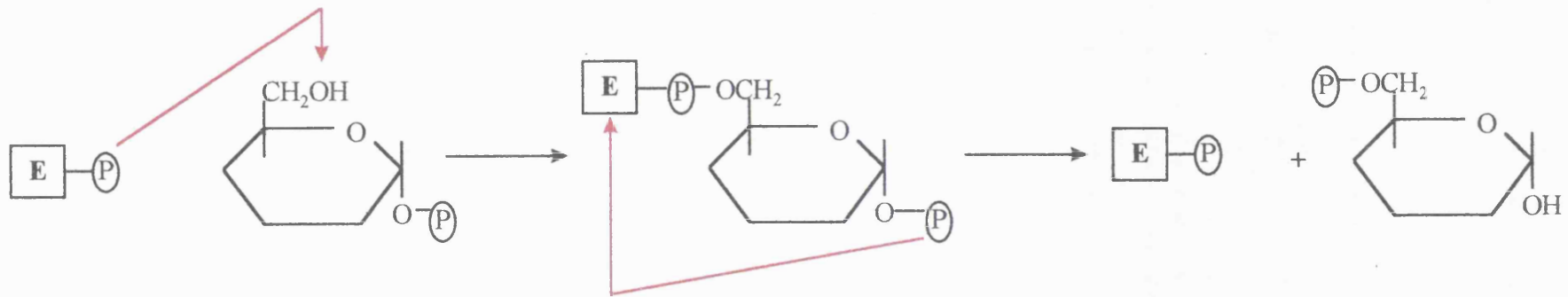


Figure 1.14: Proposed reaction mechanism for phosphoglucosyltransferase and phosphomannosyltransferase.

(figure adapted from Oesterhelt et al., 1997).

Although the amino acid sequence of Sec53 suggested a 29 kDa protein, the molecular mass of Sec53 on Sepharose gel filtration was found to be approximately 55 kDa (Ruohola and Ferro-Novick, 1987).

The studies on mammalian phosphomannomutase have been limited. In rabbit brain, two pools of PMM activity were found eluting from an anion exchange column, however the more tightly binding fraction was only present in small quantities and was not characterised (Guha and Rose, 1985). This suggests that there are two isoforms of PMM. Recently two human phosphomannomutase genes PMM1 and PMM2 have been cloned and sequenced (Matthijs *et al.*, 1997a and 1997b). PMM1 and PMM2 have been mapped to chromosome 22p13 and chromosome 16p13 respectively. PMM1 mRNA was found in human heart, brain, liver, kidney and pancreas. The human PMM1 gene has been expressed in *E.coli* and the enzyme was shown to convert mannose-1-phosphate to mannose-6-phosphate. Recombinant PMM1 was found to have a molecular mass of 29 kDa (Matthijs *et al.*, 1997a). PMM2 has 66% homology at the amino acid level with PMM1, but as yet it has not been expressed and so it is not known whether it actually converts mannose-6-phosphate to mannose-1-phosphate. Matthijs *et al.* (1997b) suggest that the reaction mechanism of PMM1 is different from that of phosphoglucomutase as PMM1 does not contain the phosphorylation consensus sequence found in other phosphomutases.

1.5.6.2 Phosphomannomutase deficiency in CDGS patients

Phosphomannomutase catalyses the production of mannose-1-phosphate from mannose-6-phosphate in the pathway leading to the production of GDP-Man (Figure 1.13). A deficiency of phosphomannomutase would in theory lead to a decrease in the amount of mannose-1-phosphate, GDP-Man and dolichol-P-Man. Mannose-6-phosphate would probably not accumulate as there are other pathways available for its utilisation. The dolichol-linked oligosaccharide precursor contains nine mannose residues all of which are obtained from either GDP-Man or dolichol-P-Man. If either or both of these substrates are decreased, then a slower rate of synthesis of the precursor would be expected. It has been shown by Panneerselvam and Freeze (1996a) that there is reduced GDP-Man, but not dolichol-P-Man, and the rate of synthesis of the lipid-linked oligosaccharide

precursor is indeed decreased in CDGS type 1 (PMM deficient) fibroblasts. The lipid-linked oligosaccharide precursor is the major substrate for oligosaccharyltransferase in the protein N-glycosylation reaction. Hence when there is a limited supply of this substrate some glycosylation sequons will not become glycosylated. This phenomenon would lead to the under-glycosylation of glycoproteins as observed in CDGS type 1 patients.

The genetic locus of CDGS type 1A (PMM-deficient) has been assigned to chromosome 16p13 (Martinsson *et al.*, 1994). The genetic locus of PMM-normal CDGS type 1 is elsewhere (Matthijs *et al.*, 1996,1997a). Eleven missense mutations have been found in PMM2 in 16 PMM-deficient CDGS type 1 patients (Matthijs *et al.*, 1997b). As yet there are no reports of expression of either the wild type human PMM2 or mutant PMM2 with which to confirm that these mutations are actually disease causing mutations.

1.5.6.3 Phosphoglucomutase (EC 5.4.2.2)

Phosphoglucomutase is the enzyme which forms the link between catabolism of glycogen with the glycolytic degradation pathway. In the reverse direction it contributes to the formation of UDP-Glc for the biosynthesis of glycoproteins (Boles, 1994). PGM exists as at least five isoenzymes; PGM1, PGM2, PGM3, PGM4, and PGM5, which are encoded by five distinct genes on chromosomes 1, 4, 6, unassigned and 9 respectively (Drago *et al.*, 1991; Edwards *et al.*, 1995). They are thought to have arisen by duplication of a common ancestral gene. The proteins are all single monomers having a molecular weight of around 60 kDa and they have diverged to have different substrate specificity by point mutations (Whitehouse *et al.*, 1992). PGM1 is immunologically distinct from PGM2 and PGM3 as antibodies against PGM1 do not cross-react with PGM2 or PGM3, however they do cross react with PGM4 (Drago *et al.*, 1991). PGM1 activity dominates in most cells, except for red blood cells where PGM2 is highly active and the lactating mammary gland where PGM4 is the most active (Putt *et al.*, 1993).

The complete sequence of human PGM1 is known (Whitehouse *et al.*, 1992) and the crystal structure of the enzyme from rabbit muscle has been identified (Lin *et al.*, 1986; Dai *et al.*, 1992). Rabbit muscle PGM1 has four domains, all of which contribute to the

active site cleft. Activation of the enzyme occurs via phosphorylation of serine 116, which lies in the bottom of the cavity between the protein domains, and the phosphates associate with arginines 426, 514 and 292. The bivalent metal ion activator region is also found in the cavity, and so the enzyme has a physiological requirement for Mg^{2+} . The reaction is thought to take place within the confines of the cavity (Lin *et al.*, 1986, Dai *et al.*, 1992). PGM1 consists of primary and secondary isoenzymes and is highly polymorphic and hence has been widely used in forensic science (Whitehouse *et al.*, 1992). PGM1 can be distinguished as two alloenzymes by starch gel electrophoresis, they are designated PGM1*1 and PGM1*2. PGM1*2 has a greater anodal electrophoretic mobility (March *et al.*, 1993). Isoelectric focusing of PGM1 further divides these alloenzymes into acidic enzymes (+) and basic enzymes (-). The molecular basis of the different isoenzymes of PGM1 seen on starch gel electrophoresis has been attributed to two point mutations. A change from Arg to Cys at residue 220 causes the PGM1*1/2 polymorphism, whereas a change from Tyr to His at residue 419 causes the PGM1 +/- polymorphism. It is thought that these polymorphisms have arisen by intragenic recombinations (Whitehouse *et al.*, 1992, March *et al.*, 1993; Putt *et al.*, 1993, Takahashi and Neel, 1993).

The phosphoglucomutases have broad specificity and can act on many sugar phosphates including mannose-1-phosphate. PGM2 has the highest phosphomannomutase activity (Putt *et al.*, 1993). Indeed, in mammals it was assumed until recently that phosphoglucomutase did interconvert mannose-1-phosphate and mannose-6-phosphate in the glycosylation pathway (Ray and Peck, 1972). Phosphomannomutase can also convert glucose-6-phosphate to glucose-1-phosphate as PGM double mutants still have some residual activity (Whitehouse *et al.*, 1992). PGM and PMM are however distinct enzymes as PGM genes have not been found on chromosome 16 or chromosome 22. However as PGM2 is known to have considerable phosphomannomutase activity and there is residual activity of phosphomannomutase in CDGS type 1 patients, the expression of the PGM isoenzymes in these patients requires further investigation.

Phosphoglucomutase is unusual in that it is a cytoplasmic protein which contains an oligosaccharide containing mannose, glucose and phosphate. It is believed that this post-

translational modification is important for modulating the function of PGM just as phosphorylation modulates other enzymes (Marchase, 1993).

1.5.7 Evidence for a glycosylation defect in CDGS type 1 fibroblasts

Marquardt *et al.* (1995) studied the glycosylation capacity of three intracellular glycoproteins to see whether the random non-occupancy of glycosylation sequons that has been demonstrated in serum transferrin from CDGS type 1 patients occurs in skin fibroblasts proteins from these patients. The CDGS type 1 fibroblasts were transfected with haemagglutinin from the influenza virus, E1 or p62 from the Semliki Forest virus. These glycoproteins were chosen because they are known to form irreversible covalent aggregates and be retained within the ER when their correct glycosylation was inhibited. The three viral glycoproteins were detected by SDS-PAGE followed by immunodetection. The co-translational glycosylation and folding of these three glycoproteins were found to be normal in the CDGS type 1 fibroblasts. However studies of the CDGS type 1 fibroblasts by electron microscopy revealed that the ER in these cells was dilated but that the Golgi had no morphological changes. Dilation of the ER is generally caused by protein retention, but no such retention could be demonstrated. Thus this group of researchers failed to find a model intracellular glycoprotein with which to study the effects of the enzyme defect causing CDGS type 1. A study by another group on the secretion of decorin (a small proteoglycan) from CDGS type 1 fibroblasts revealed that CDGS type 1 fibroblasts secreted less decorin than normal fibroblasts, but the glycosylation of decorin was indistinguishable from normal (Gu and Wada, 1995). Both of these studies concentrated on the mature glycoprotein whereas Powell *et al.* (1994) found evidence for aberrant glycosylation of intracellular glycoproteins by studying their biosynthesis. Using labelling experiments they were able to demonstrate that the truncated LLO produced in the CDGS type 1 fibroblasts were actually transferred to the polypeptide, as the glycoprotein fraction of CDGS type 1 fibroblasts also showed smaller glycans (Panneerselvam and Freeze 1996a). The water-soluble fraction of oligosaccharides also contained smaller oligosaccharides than seen in the control cell lines. However when they labelled the cells for three days and then studied the glycans released from the glycoprotein fraction of the CDGS type 1 fibroblasts by lectin affinity chromatography they found that the glycans had the normal expected

structures. The authors concluded that although the glycans transferred had been truncated, they were processed normally. The proteins were correctly glycosylated with regard to structure, but they were not able to quantitate glycosylation site occupancy (Powell *et al.*, 1994).

1.6 Classical Galactosaemia

Classical galactosaemia is an inherited disorder of galactose metabolism resulting from a deficiency of the enzyme galactose-1-phosphate uridyl transferase (GALT, EC. 2.7.12). It has an incidence in Europe of 1 in 55,000. GALT converts galactose-1-phosphate and UDP-Glc into UDP-Gal and glucose-1-phosphate (Figure 1.15). A deficiency of GALT thus leads to an accumulation of galactose-1-phosphate, galactose and galactitol (Segal, 1989). The GALT gene is located on chromosome 9 and is organised into 11 exons. In the Caucasian population 70% of patients have a missense mutation in exon 6 (Q188R, glutamine to arginine). The gene encodes for a 379 amino acid polypeptide and the active enzyme is a homodimer composed of 43 kDa subunits (Elsas *et al.*, 1995).

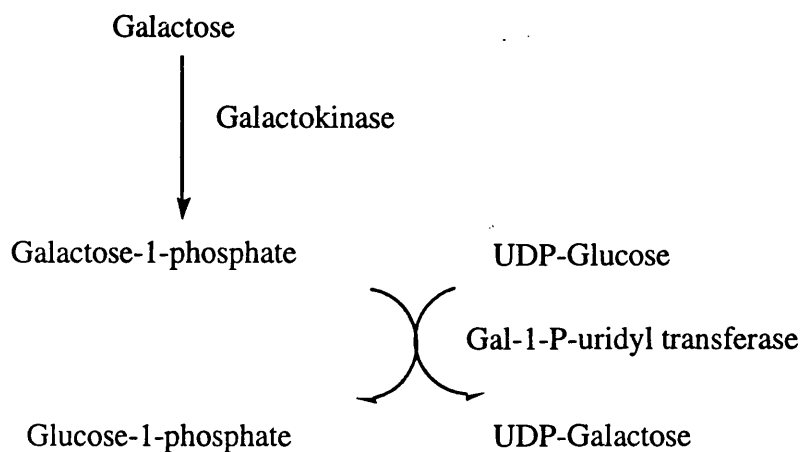


Figure 1.15: The metabolism of galactose.

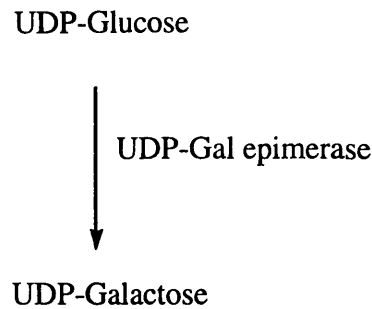


Figure 1.16: Conversion of UDP-Glc to UDP-Gal.

In the absence of galactose, UDP-Gal can be synthesised from UDP-Glc by the enzyme UDP-Gal epimerase (Figure 1.16). In galactosaemic patients it has been suggested that epimerase maintains the levels of UDP-Gal required (Dobbie *et al.*, 1990), i.e. the UDP-Glc : UDP-Gal ratio is maintained at 3:1. UDP-Gal is required for the synthesis of glycoproteins as well as galactolipids and mucopolysaccharides. Low concentrations of UDP-Gal are postulated to cause defective synthesis of complex carbohydrates, and hence galactosaemia can now be classed as a secondary carbohydrate-deficient glycoprotein syndrome.

1.6.1 Clinical picture and diagnosis

Patients normally present the clinical symptoms within the first few weeks of life. The clinical phenotype is characterised by failure to thrive, diarrhoea and dehydration, vomiting, jaundice, hepatomegaly, hypoglycaemia and cataracts. The cataracts are caused by high concentrations of galactitol in the eye. In the brain there are decreased amounts of cerebroside, glycosaminoglycans and hexosamines when compared with the controls. If untreated, the disease progresses to cirrhosis of the liver and mental retardation. A diagnosis can be made on the basis of a high level of galactose-1-phosphate in red cells and a low GALT activity (Segal, 1989, Komrower, 1982). CDGS-like isoelectric focusing patterns of serum transferrin can also aid diagnosis (Jaeken *et al.*, 1992).

1.6.2 Treatment

The only treatment available to date has been the complete removal of lactose and galactose from the diet. Such treatment removes the acute toxicity effects of galactose-1-phosphate and so many of the clinical symptoms disappear within a few days. However, there are some long term complications such as developmental delay, speech abnormalities and dysfunction of the ovaries. In babies, the galactose-free diet is maintained by replacing human or cow milk with soya milk. In children all galactose containing food stuffs must be omitted from the diet, which includes all products made from milk e.g. chocolate, and some fruit and vegetables which are particularly high in galactose. Most patients aim to keep their daily intake of galactose at around 125 mg per day (Gross and Acosta, 1991). This diet must be maintained for life if the patients are to remain symptom-free. Supplementing the diet with uridine to increase the production of UDP-Gal has been suggested, but no significant improvement has been seen as yet (Komrower, 1982, Kaufman *et al.*, 1989; Segal, 1995a).

1.6.3 Long term complications

There are five major long term complications associated with galactosaemia:

1) Intellectual development

A study of 134 German galactosaemic patients revealed that 47% of them had an IQ < 85. The IQ of the patients was found to decline with age. The percentage of patients having an IQ < 85 was 83% in patients 12 years and above, 56% in patients between 7 and 12 years of age and 12% in patients under 6 years of age (Schweitzer *et al.*, 1993). Similar results have been found in Norwegian (Hansen *et al.*, 1996), American (Waggoner *et al.*, 1990) and British patients (Cleary *et al.*, 1995). It has also been demonstrated that there is no correlation between IQ and age of diagnosis, galactose-1-phosphate levels or genotype (Cleary *et al.*, 1995).

2) Speech disorders

Over 60% of patients have some kind of speech disorder including reduced vocabulary, verbal dyspraxia and stuttering (Schweitzer *et al.*, 1993, Holton and Leonard, 1994).

Hansen *et al.*, 1996). Hansen *et al.* (1996) recommended speech evaluation of patients at an early age so that help could be offered.

3) Ovarian dysfunction

It has been found by many groups that 80% of female galactosaemic patients have ovarian dysfunction, whereas only 5% of males have delayed puberty. The age of onset of ovarian damage has been found as early as one year of age and as late as 29 years (after pregnancy). Once established, ovarian failure persists and cannot be treated. The testis, in contrast, is more resistant to effects of abnormal galactose metabolism as the majority of males undergo normal puberty and semen tests show normal volume and morphology of sperm (Kaufman *et al.*, 1986, Waggoner *et al.*, 1990). The ovarian dysfunction is believed to be initiated *in utero* and maintained by galactose-1-phosphate accumulation, although post mortem of a five day old galactosaemic revealed normal numbers of oocytes (Schweitzer *et al.*, 1993; Kaufman *et al.*, 1986; Holton 1995; Segal, 1995b). It has recently been proposed that the biochemical mechanism behind the ovarian dysfunction is defective galactosylation of follicle stimulating hormone (FSH) in galactosaemic patients. Increased neutral FSH isoforms disrupt receptor binding and along with galactose-1-phosphate accumulation lead to the disruption of normal ovarian function (Prestoz *et al.*, 1997). It has also been suggested that GALT itself may participate actively in galactose metabolism in the ovary, and thus the enzyme defect itself is responsible (Komrower *et al.*, 1982).

4) Delayed growth

Growth is more often retarded in female than male patients. Schweitzer *et al.* (1993) reported that 11% of patients had decreased body weight, 8% decreased body length, and 13% decreased head circumference. Waggoner *et al.* (1990) also found that 20% of patients had some growth retardation.

5) Impaired motor function and balance

Many patients have been reported to have ataxia, impaired balance, difficulty with running and handwriting, clumsiness and intention tremor (Waggoner *et al.*, 1990; Schweitzer *et al.*, 1993; Holton and Leonard, 1994).

These five complications do not correlate with age of diagnosis, severity of neonatal symptoms or galactose-1-phosphate levels. When age of diagnosis and implementation of the galactose-free diet was compared with the occurrence of developmental delay, speech problems, ovarian dysfunction and height, no correlation was found. Treatment during pregnancy was also found to have no effect on the occurrence of long term complications in the galactosaemic babies. Galactose-1-phosphate levels were not found to correlate with the occurrence of long-term complications but it was observed that women with normally functioning ovaries seemed to have lower baseline galactose-1-phosphate levels. Also there was no correlation between residual GALT activity and the likelihood of long term complications. Neither was there any correlation with genotype (Waggoner *et al.*, 1990; Schweitzer *et al.*, 1993; Cleary *et al.*, 1995; Segal, 1995b).

1.6.4 Disturbances in galactose metabolism in galactosaemia

A deficiency of GALT leads to an accumulation of galactose-1-phosphate and a postulated decrease in UDP-Gal. There are alternative pathways that come into play to cope with the excess galactose-1-phosphate and these result in increases in galactitol, galactonate and galactose-6-phosphate concentration in many tissues (Segal, 1995a).

1.6.4.1 Galactose-1-phosphate accumulation

Galactose-1-phosphate accumulates in the red blood cells and also in many other tissues including liver, kidney, ovarian tissue and brain. Measurement of the level of galactose-1-phosphate in red blood cells can be used to monitor treatment. Treatment with a galactose-free diet leads to a gradual decrease in red blood cell galactose-1-phosphate levels until they reach a baseline that is specific for each patient (Segal, 1989; Gitzelman, 1995). The level of galactose-1-phosphate in a galactosaemic patient is never as low as in a normal person, no matter how good the diet. There are several possible explanations for this observation. There could be hidden sources of galactose in the diet that lead to increased galactose-1-phosphate levels or it could be due to endogenous production of galactose, leading to self intoxication. It has been reported that certain fruits and vegetables have high amounts of galactose, but including these in the diet did not show a significant increase in the galactose-1-phosphate levels (Gross and Acosta, 1991; Berry *et al.*, 1993). Berry *et al.* (1995) proposed that endogenous production of galactose was

the reason behind the raised levels of galactose-1-phosphate in the well-treated galactosaemic patient. In their study they found that both normal adults and galactosaemic patients on a galactose-free diet produced 1.1-1.3 g of galactose per day. Therefore no matter how good the diet is, a normal level of galactose-1-phosphate will never be achieved. This endogenous production of galactose is disheartening for patients who restrict their dietary intake of galactose to less than 100 mg / day, when their bodies are making 10 times that much anyway.

The pathways causing the production of galactose include the degradation of glycoproteins and glycolipids containing galactose and the cleavage of UDP-Gal to produce galactose-1-phosphate. These pathways lead to the self-intoxication theory proposed by Gitzelman (1995). There is much evidence from galactosaemic fibroblasts for this hypothesis as they have been found to accumulate galactose-1-phosphate when maintained on medium containing only glucose as the carbon source. They also have UDP-Gal pyrophosphorylase activity which allows them to produce galactose-1-phosphate from glucose-1-phosphate.

Galactose-1-phosphate is thought to exert its pathophysiological effects by competitive inhibition of phosphoglucomutase, glucose-6-phosphatase, glucose-6-phosphate dehydrogenase and galactosyltransferase (Segal, 1995a). Inhibition of phosphoglucomutase would disturb the entry of glucose-6-phosphate into the glycolytic pathway, whilst inhibition of galactosyltransferase would disturb the biosynthesis of complex glycoproteins. The effects of galactose-1-phosphate accumulation on glycoprotein biosynthesis have not been studied extensively *in vivo* (Dobbie *et al.*, 1990; Ornstein *et al.*, 1992; Lewis, 1997). One of the aims of this project is to determine the extent of the agalactosylation of glycoproteins isolated from both serum and fibroblasts of galactosaemic patients.

1.6.4.2 Galactitol and galactonate accumulation

When present at high concentrations galactose can be reduced to galactitol by aldolase reductase which has a low affinity for galactose. Aldolase reductase is most active in the eye lens, but activity has also been found in the kidneys and in Schwann cells of the

peripheral nervous system. The build up of galactitol in the eye causes osmotic swelling and disruption of the lens leading to the formation of cataracts which are seen in untreated galactosaemic patients and even in some new born babies. Excess galactitol is excreted by the kidney and can be measured in the urine of untreated galactosaemic children. A ten-fold increase in the galactitol levels in amniotic fluid is detected in the affected fetus, even if the mother is on a galactose-free diet. (Segal, 1989; Segal, 1995b).

Alternatively galactose can be oxidised into galactonate, which is excreted into the urine of untreated galactosaemic patients and can be used to monitor dietary compliance.

When galactosaemic patients are treated with aldolase reductase inhibitors as treatment for cataracts, galactonate is found to accumulate in the lens and serum (Segal, 1989).

1.6.5 UDP-Gal deficiency controversy

It has been hypothesised that a deficiency of GALT leads to a deficiency of the product of the GALT reaction; UDP-Gal. On the other hand, UDP-Gal can also be synthesised from UDP-Glc via the epimerase. Thus UDP-Gal concentrations in tissues from galactosaemic patients were measured by several groups, as a deficiency of UDP-Gal has wide ranging implications for the synthesis of glycoconjugates. Using an enzyme-linked assay procedure, Ng *et al.* (1989) found that there was a significant decrease in the concentration of UDP-Gal in galactosaemic erythrocytes, fibroblasts and liver. They also reported a decrease in UDP-Glc concentrations in galactosaemic liver. By adding uridine *in vitro* they were able to demonstrate a correction of the UDP-Gal deficiency and suggested administration of uridine to patients as an effective treatment of the long term effects of galactosaemia. The clinical trial has been ongoing but early indications are that there is no significant benefit (Holton and Leonard, 1994, Segal, 1995a). The results of Ng *et al.* (1989) were contrary to the findings of the other groups who used different methods for the measurement of UDP-Gal concentrations (Kirkman, 1992, Berry *et al.* 1992, Gibson *et al.* 1993, and 1994; Holton *et al.*, 1993).

The controversy has arisen from the technical difficulties in measuring UDP-Gal accurately. UDP-Gal and UDP-Glc are unstable at room temperature. Keevill *et al.* (1993) reported a loss of 22% of the UDP-Gal and 13% of the UDP-Glc in red blood

cells after storage for 24 hours at room temperature. It was also noted that significant amounts of both UDP-Gal and UDP-Glc were lost during TCA precipitation if sample preparation took longer than thirty minutes. The original studies of Ng *et al.*, (1989), involved an enzyme-linked assay in which UDP-Gal was converted to UDP-Glc by the epimerase, and the total amount of UDP-Glc was estimated by converting it to UDP-glucuronate by UDP-Glc dehydrogenase and following the production of NADPH with time. The UDP-Gal concentration was estimated by carrying out the reactions in the presence and absence of epimerase. Kirkman (1992) using a similar assay system but with different concentrations and higher purity of UDP-Glc dehydrogenase, found that the UDP-Gal concentration was only 25% of that in normal patients compared to the amounts reported by Ng *et al.*(1989). Kirkman (1992) reported that the Sigma UDP-Glc dehydrogenase used by Ng *et al.* (1989) might be contaminated. Ng *et al.* (1993) responded that the results were similar irrespective of whether the Sigma preparation or the Boehringer preparation was used. However, when Ng *et al.* (1993) repeated the experiment at the concentration employed by Kirkman (1992) lower values were obtained. This was said to be due to quenching. At this point the National Institute of Child Health and Human Development (USA) who were running the clinical trial of uridine administration decided to step in and demanded an independent decision on the matter (Holton *et al.* 1993). It was found that the HPLC method developed by Gibson (1993) and the Kirkman (1992) enzyme method gave rise to the same results in normal controls, whereas the method of Ng *et al.*, (1989) gave at least a two-fold increase in the UDP-Gal levels. As these results (Gibson, 1993; Kirkman, 1992) also compared favourably with results obtained by ³¹P NMR it was decided that the HPLC method was the method that should be used.. Ng *et al.* repeated the experiments and still found a significant decrease in the UDP-Gal levels in red blood cells from galactosaemic patients (Ng *et al.*, 1993).

The finding that erythrocyte UDP-Gal concentrations are significantly decreased in patients with galactosaemia has now been confirmed by many groups (Kirkman, 1992 and 1993; Keevill *et al.*, 1993 and 1994, Holton *et al.*, 1993, Gibson *et al.*, 1994). It is also accepted now that incubation of cultured erythrocytes from galactosaemic patients

with uridine does increase the UDP-Gal level to normal concentrations and that the uridine trial can continue.

As erythrocytes carry out limited metabolic functions, it is important to establish the levels of UDP-Gal in other cell types. Ng *et al.* (1989) and Ornstein *et al.* (1992) reported that liver and fibroblasts also showed a significant decrease in UDP-Gal concentration. However these results were obtained using the flawed enzyme assay. Using the new HPLC method Gibson *et al.* (1994) and Keevill *et al.* (1994) have found that there was no decrease in UDP-Gal concentration in fibroblasts from patients with galactosaemia. The same is found to be true for leukocytes, so it would seem that the decreased UDP-Gal levels are a unique phenomenon of erythrocytes. (For discussion see Holton *et al.*, 1993, Kirkman, 1995, Segal, 1995b and Shin 1995).

1.6.6 Evidence for agalactosylation of glycoproteins in patients with galactosaemia

There is increasing evidence for a defect in galactosylation of both serum and cellular glycoproteins in patients with galactosaemia. The molecular basis for this is not known but there are two possibilities that need to be considered. Firstly, it could be due to a deficiency of UDP-Gal, as this activated sugar is required in the synthesis of complex glycoconjugates. Alternatively, it could be due to inhibition of galactosyltransferase by galactose-1-phosphate. The first possibility is unlikely as it has been shown that UDP-Gal levels are only decreased in red blood cells and not in fibroblasts (Holton *et al.*, 1993, Segal, 1995b). Inhibition of galactosyltransferase by galactose-1-phosphate has been demonstrated *in vivo* (Segal, 1995b) making this mechanism the most plausible.

Whatever the basis of the defective galactosylation it is the most likely explanation for the long term complications which are seen with the well treated galactosaemic patients (Segal, 1995b). This is probably the case for the ovarian dysfunction as abnormal glycosylation of follicle stimulating hormone has been clearly demonstrated (Prestoz *et al.*, 1997).

The first suggestion that there maybe a defect in galactosylation of glycoproteins in patients with galactosaemia was made by Haberland *et al.* (1971). An autopsy was

performed on a 25 year old galactosaemic patient and decreased cerebroside levels indicative of abnormal or incomplete myelination were found. An abnormal distribution of glycoproteins within the grey and the white matter was also found and it was hypothesised that this was caused by failure to synthesise complex glycoconjugates correctly.

The serum lysosomal enzymes, β -hexosaminidase and α -fucosidase, like serum transferrin, have been shown to be undersialylated in serum of galactosaemic patients (Jaeken *et al.*, 1992; Winchester *et al.*, 1995). Following treatment on a galactose-free diet the isoelectric focusing patterns of these serum glycoproteins return to normal. Although a decrease in sialic acid has been demonstrated by IEF and a decrease in galactose content proposed, structural studies of the glycans of these serum glycoproteins has not been carried out.

Studies of the glycoproteins of fibroblasts from galactosaemic patients have been more informative. The major study in this area was that of Ornstein *et al.* (1992) in which it was demonstrated that proteins from galactosaemic fibroblasts accepted 50% more galactose than those from control fibroblasts. In this experiment cell homogenates were incubated with purified UDP-galactosyltransferase and radiolabelled UDP-Gal. As more radiolabel was incorporated into the galactosaemic homogenates, it was concluded that they contained more exposed GlcNAc than controls. The galactose acceptor sites could have been on O-linked or N-linked glycans. Earlier work by Dobbie *et al.* (1990) showed that the galactose/mannose ratio in proteins from galactosaemic fibroblasts was lower than normal indicative of under-galactosylation. It is interesting to note that both of these sets of experiments were performed on fibroblasts that had been grown on glucose only medium, thus extrapolating to the treated patient.

Prestoz *et al.* (1997) repeated Ornstein's (Ornstein *et al.*, 1992) galactose acceptor experiments on serum glycoproteins. They were able to show that serum from galactosaemic patients accepted 66% more galactose than control serum. The patients used in this study were all well treated female patients who were between 9 and 21 years old. Using FSH as a model hormone glycoprotein they were also able to show that

abnormal under sialylated isoforms of FSH could be detected in the serum of these patients and hypothesised that this was a contributing factor to the ovarian dysfunction in these and other patients.

More research is required to establish the structural basis of the undersialylated isoforms of serum glycoproteins detected by isoelectric focusing and the molecular mechanism of the agalactosylation, be it a deficiency of UDP-Gal or inhibition of galactosyltransferase.

1.7 Other disorders of protein glycosylation

1.7.1 Hereditary Fructose Intolerance

Hereditary fructose intolerance (HFI) is another example of a secondary carbohydrate-deficient glycoprotein syndrome. It is caused by a deficiency of fructose-1-phosphate aldolase (aldolase B) (Gitzelman *et al.*, 1989). Aldolase B cleaves fructose-1-phosphate into D-glyceraldehyde and dihydroxyacetone phosphate in the liver, kidney and small intestine. Aldolases A and C perform similar reactions in the muscle and brain respectively. These aldolases consist of four subunits and have an overall molecular mass of around 160 kDa. There is a switch from expression of aldolase A and C in fetal liver, kidney and small intestine to aldolase B in later life.

1.7.1.1 Clinical picture, diagnosis and treatment

A child with hereditary fructose intolerance is asymptomatic during breast feeding but as soon as weaning occurs with the inclusion of vegetables and fruit in the diet, the symptoms become apparent. They usually consist of poor feeding, vomiting, hepatomegaly, jaundice and haemorrhage. If left untreated, it can result in death. Babies fed on cow milk formula containing either fructose or sucrose instead of breast feeding show these symptoms immediately. Diagnosis is achieved by measurement of fructose-1-phosphate aldolase B activity in a liver biopsy, as fibroblasts express only aldolase A. Treatment with a fructose- and sucrose-free diet alleviates the symptoms within a few days. Unlike galactosaemic patients, they have no intellectual impairment and lead a normal healthy life (Gitzelman *et al.*, 1989).

1.7.1.2 Fructose-1-phosphate accumulation and aberrant glycosylation

Many of the toxic effects of fructose in HFI are attributed to depletion of ATP and inorganic phosphates. However, the build up of fructose-1-phosphate can lead to the inhibition of phosphomannose isomerase and phosphorylase a (Gitzelman *et al.*, 1989).

It has been recently reported that serum transferrin from patients with HFI is also aberrantly glycosylated like that of CDGS type 1 patients (Adamowicz, 1996). On a normal diet 31.7 % of the serum transferrin from an HFI patient consisted of asialo-, monosialo-, and disialotransferrin isoforms, compared to 2.3 - 8.6 % for controls and 62.1 % for CDGS type 1. After four months treatment with a fructose-free diet, this value returned to that of the controls. The abnormal transferrin pattern was not found in the parents of this patient. HFI is now considered to be a secondary form of CDGS (Adamowicz, 1996).

Jaeken *et al.* (1996) have shown that fructose-1-phosphate is a competitive inhibitor of phosphomannose isomerase (EC 5.3.1.8). Phosphomannose isomerase converts fructose-6-phosphate to mannose-6-phosphate in the pathway depicted in Figure 1.13. When glucose is the only available carbon source, the PMI reaction is vital in the production of mannose-6-phosphate, GDP-Man and dolichol-P-Man for the synthesis of glycoproteins. Patients on a fructose free diet show normal glycosylation of serum transferrin (Adamowicz *et al.*, 1996). It appears that a depletion in the production of fructose-1-phosphate as a result of removal of dietary fructose allows normal PMI activity and the production of the mannose-1-phosphate precursor required for the synthesis of GDP-Man .

1.7.2 Hereditary erythroblastic multinuclearity with a positive acidified serum lysis test (HEMPAS)

HEMPAS is an autosomal recessive disorder in which the main symptoms include life-long anaemia, liver cirrhosis, hepatomegaly, splenomegaly, diabetes and gall stones. The major biochemical feature apart from the positive acidified serum lysis test is the abnormal glycosylation of the erythrocyte band 3 glycoproteins. Band 3 glycans are normally composed of a biantennary trimannosyl core with a polylactosamine side chain.

However, in HEMPAS patients, the band 3 glycans do not contain a polylectosamine side chain on the α 1-6 branch (Fukuda *et al.*, 1987). The absence of a polylectosamine side chain in the band 3 glycoprotein causes clustering because of increased hydrophobicity. This abnormal distribution of band 3 may be the cause of the morphological changes that are seen in the erythrocyte membranes (Fukuda, 1990; Fukuda *et al.*, 1990). The aberrant glycosylation of band 3 could arise from either a deficiency of α -mannosidase II or N-acetylglucosaminyl transferase II (Fukuda, 1990). Deficiencies of N-acetylglucosaminyl transferase II (Fukuda *et al.*, 1987) and α -mannosidase II (Fukuda *et al.*, 1990) have been reported, demonstrating that HEMPAS is a heterogeneous genetic disorder. It has also been noted that mice with a deficiency of α -mannosidase II have a clinical phenotype that resembles HEMPAS (Chui *et al.*, 1997).

The enzyme deficiency in HEMPAS patients has also been found to affect the glycosylation of serum glycoproteins. Analysis of the glycans released from serum transferrin revealed that many were of the high mannose and hybrid type rather than disialylated biantennary glycans. α 1-Acid glycoprotein was similarly aberrantly glycosylated (Fukuda *et al.*, 1992). Therefore it is likely that the aberrant glycosylation of serum glycoproteins leads to the liver cirrhosis in many patients. Although it would seem that HEMPAS maybe closely related to CDGS type II, HEMPAS differs in that both O-linked and N-linked oligosaccharides are affected (Tomita and Parker, 1994). CDGS type II erythrocytes do not show the typical polylectosamine pattern of HEMPAS erythrocytes and the clinical phenotypes of the two disorders are not similar (Charuk *et al.*, 1995).

1.7.3 Rheumatoid arthritis

There is a change in the population of the oligosaccharides attached to IgG in patients with rheumatoid arthritis (RA) (Parekh *et al.*, 1985; Rademacher *et al.*, 1988; Axford *et al.*, 1992). Normal IgG has one conserved glycosylation site at Asn 297 in the Fc region and it is also glycosylated at non-conserved positions in the variable region (Fab). There are no disialylated glycans and only small amounts of monosialylated glycans and glycans bisected by a GlcNAc residue on the glycan in the Fc region. This is in contrast to the glycans present on the Fab region in which there are many disialylated biantennary

glycans, monosialylated and bisected glycans. The oligosaccharides present in IgG in RA have a decreased amount of galactose and an increased amount of ^{terminal} N-acetylglucosamine. The same agalactosylated IgG is also observed in the juvenile form of RA and in patients with tuberculosis and Crohn's disease (Rademacher *et al.*, 1988). The molecular basis of the agalactosylated glycans is thought to be a deficiency of a specific galactosyltransferase that transfers galactose to agalactosyl IgG (Axford *et al.*, 1987). However, the presence of agalactosyl IgG in Crohn's disease and tuberculosis suggests that mycobacterium could play a role in the aetiology of RA (Rademacher *et al.*, 1988). It is interesting to note that serum IgA1 from RA patients does not contain agalactosylated oligosaccharides. Thus it would seem that the agalactosylation defect is limited to IgG. It is not known whether there is an increase in the amount of agalactosylated IgA1 in Crohn's disease or in tuberculosis (Field *et al.*, 1994).

1.74 Alcoholism

The carbohydrate-deficient transferrin isoforms that are found in chronic alcoholism are similar to those found in CDGS type 1 in that the disialo and asialo isoforms are formed by the loss of one and two disialylated biantennary glycans respectively (Landberg *et al.*, 1995). The proportions of the disialotransferrin and asialotransferrin isoforms are greater in CDGS type 1 than in chronic alcoholism (Stibler *et al.*, 1991b). However the underlying mechanisms of non-occupancy of glycosylation sequons in chronic alcoholism remain to be elucidated. Ethanol is oxidised in the liver to produce acet-aldehyde and so it is possible that acet-aldehyde may inhibit the glycosyltransferases, the nucleotide sugar transporters or oligosaccharyltransferase (Marinari *et al.*, 1993). Although acet-aldehyde has been shown to inhibit galactosyltransferase (Guasch *et al.*, 1992), it must also be a more potent inhibitor of one of the enzymes which acts earlier in the biosynthetic pathway because the glycans that are attached to serum transferrin from alcoholic patients are processed normally (Landberg *et al.*, 1995).

1.8 Aims of the thesis

The three major aims of this thesis are;

- 1) to determine the molecular basis of CDGS type 1 at the level of the enzyme defect and the aberrant glycosylation of serum transferrin.
- 2) to develop a diagnostic procedure for the postnatal and prenatal diagnosis of CDGS type 1.
- 3) to determine the structural basis for the aberrant glycosylation of serum proteins observed in galactosaemia.

Materials and Methods

2.1 Patients

2.1.1 CDGS type 1 patients

Eight CDGS type 1 patients were studied in this project (Table 2.1). Fibroblasts were available from six of the patients and lymphoblastoid cells from the other two (VB and TB). The patients were diagnosed as CDGS type 1 by Dr Peter Clayton, Great Ormond Street Hospital, on the basis of their clinical symptoms (Table 1.1). The diagnosis was confirmed by isoelectric focusing of serum transferrin by Dr Geoffrey Keir, Institute of Neurology, University of London. The patients with the severe neonatal form of CDGS type 1 all died within the first year of life (EW, DC, KT, CB, JB and NH). VB and TB are less severely affected and are still alive.

Although patient NH had many of the clinical features listed in Table 1.1, he did not have oculomotor apraxia (roving eye movements) suggesting that there was less severe CNS involvement (Charlwood *et al.*, 1997). There was only a very small amount of serum available from patient NH as he was difficult to bleed, being so small and sick. However his ascites fluid was kept and used for transferrin analysis, as the transferrin profiles from the ascites fluid and serum from this patient and controls are the same by isoelectric focusing.

2.1.1.1 Families offered prenatal diagnosis of CDGS type 1

During the course of this investigation two CDGS type 1 families requested prenatal diagnosis. This work was in collaboration with T. Martinsson, Sweden, who carried out the molecular genetic linkage analysis.

Family 1

The index case of family 1 was patient DC (Table 2.1). Amniocentesis was performed on the mother of family 1 at 16 weeks gestation. The amniotic fluid was stored at -20°C

until the β -hexosaminidase and β -glucuronidase activities were determined (sections 2.7.4 and 2.7.5). The cultured amniocytes were grown to confluency in Ham's F10 medium containing 12% (v/v) fetal calf serum, harvested by trypsinisation and stored at -70°C (section 2.3)

Family 2

The index case of family 2 was patient KT (Table 2.1). Chorionic villus sampling was carried out on the mother of family 2 at 10 weeks gestation. The chorionic villus biopsy was dissected to remove maternal contamination and frozen at -70°C . Cultured chorionic villi cells were grown, harvested and stored as above.

2.1.2 Galactosaemic patients

All the galactosaemic patients were diagnosed as having classical galactosaemia i.e. galactose-1-phosphate uridyl transferase deficiency by the Enzyme Laboratory, Great Ormond Street Hospital, London (Table 2.2). They all presented shortly after birth and treatment on a galactose-free diet commenced within the first few weeks of life. The galactose-1-phosphate concentration in the packed red blood cells was measured by the Enzyme Laboratory and the remaining serum was used for the analysis of serum transferrin isoforms. Fibroblast cell lines from two additional patients were kindly provided by Dr Tony Fensom, Guys Hospital, London.

2.2 Cell culture

All cell culture reagents were obtained from Sigma unless otherwise stated. Sterile disposable plasticware (e.g. tissue culture flasks, centrifuge tubes and pipettes) were purchased from Falcon. Fibroblast cell lines were established from a skin biopsy, cultured amniocytes from amniotic fluid and cultured chorionic villi cells from chorionic villi biopsy of patients. The fibroblasts investigated in this project were supplied either as frozen ampoules, which had been stored in liquid nitrogen or as growing cells. Cultured chorionic villi, amniocytes and lymphoblastoid cells were grown by the Enzyme Laboratory and were supplied as harvested cell pellets.

Patient	Sex	Material available	Notes
EW	female	fibroblasts	Severe - died within the first year of life.
DC	male	fibroblasts, plasma	Severe - died age 11 weeks (Clayton <i>et al.</i> , 1992)
CB	female	fibroblasts, amniocytes, amniotic fluid, plasma	Severe - died within the first year of life. Prenatal diagnosis attempted. (Clayton <i>et al.</i> , 1993)
JB	male	fibroblasts	Severe- died age 3 months (Hutchesson <i>et al.</i> , 1995)
KT	female	fibroblasts	Severe - died age 6 months (Clayton <i>et al.</i> , 1992)
VB	female	lymphoblastoids, plasma	Not severe - alive, age 10 years sister of TB
TB	male	lymphoblastoids, plasma	Not severe- alive, age 12 years brother of VB
NH	male	fibroblasts, ascites fluid, plasma	Severe- died age 3 months (Charlwood <i>et al.</i> , 1996, 1997)

Table 2.1 CDGS type 1 patients investigated in this study.

Patient	Samples	Length of treatment (weeks)	Galactose-1-phosphate ($\mu\text{mol/g}$)
G1	fibroblasts	N/A	N/A.
LV	fibroblasts	N/A	N/A
TR	plasma	0 38	4.0 0.31
NA	plasma	0 3	2.84 1.09
BR	plasma	6	0.34
RE	plasma	42	0.22
SO	plasma	0	8.75
TI	plasma	50	0.15
NI	plasma	42	0.5

Table 2.2: Galactosaemic patients investigated in this study.

Galactose-1-phosphate levels were measured in red blood cells. (N/A =not applicable)

2.2.1 Tissue culture medium

2.2.1.1 Ham's F10 with fetal calf serum

Ham's F10 medium (500 ml) was supplemented with 70 ml of fetal calf serum (Imperial Laboratories), 5 ml of penicillin/streptomycin (1 mg/ml) and 73 mg of L-glutamine and sterilised by filtration through a 0.22 μm Millex-GV Millipore filter. The medium was stored at 4⁰C for up to 1 month.

2.2.1.2 Ham's F10 with SATO mixture

SATO mix was added to Ham's F10 medium instead of fetal calf serum when serum-free medium was required.

SATO mix

SATO mix was composed of 1.3% (w/v) BSA rat-ocyte 4, 4.4 mM putrescine, 20 mM thyroxine, 20 mM triiodothyroxine, 90 mM progesterone and 0.1 mM sodium selenite in water. The solution was sterilised by filtration through a 0.22 μm Millipore filter and stored at -20⁰C.

Ham's F10 medium (100 ml) was supplemented with 2 ml of insulin (0.5 mg/ml), 1 ml of transferrin (10 mg/ml), 2.22 ml of SATO mix, 0.5 ml of 200 mM glutamine and 1 ml of penicillin/streptomycin (1 mg/ml) and sterilised by filtration through a 0.22 μm Millipore filter. Insulin and transferrin were dissolved in sterile water and stored at -20⁰C. The complete medium was stored at 4⁰C for up to 1 week.

2.2.2 Cell culture conditions

Fibroblasts, cultured chorionic villi cells and amniocytes were grown as monolayers in tissue culture flasks that had a surface area of either 25 cm² or 75 cm². Normally the cells were grown in Ham's F10 culture medium supplemented with fetal calf serum, but for secretion experiments serum-free medium (Ham's F10 with SATO) was used. 10 ml of medium was added to a 75 cm² flask and 4 ml of medium was added to a 25 cm² flask. Flasks were incubated at 37⁰C in 5% (v/v) CO₂.

2.2.2.1 Mycoplasma detection

Fibroblasts, cultured chorionic villi cells and amniocytes were routinely screened for mycoplasma contamination and affected flasks treated with Mycoplasma Removal Agent (MRA, ICN) at 1 ml per 100 ml cell culture medium for 5 days. Cells were grown for 4 days on a glass coverslip in a cell culture dish (25 cm²) containing cell culture medium. The cells were fixed by adding 5 ml of methanol: acetic acid (1:3) for 2 min. This solution was decanted and 5 ml of methanol: acetic acid (1:3) was added for a further 5 min. This was repeated twice more, and then the cells were left to air-dry. The coverslips were stained by incubation with 50µg/ml DAPI in PBS for 30 min. They were rinsed twice with water and mounted onto a microscope slide. The mycoplasma were visualised under a fluorescent microscope with a 53/44 barrier filter and a BG-3 exciter filter at a magnification of 500 X.

2.2.3 Reconstitution of cells stored in liquid nitrogen

Ampoules containing fibroblasts were removed from liquid N₂ and thawed at 37°C. The cells were centrifuged at 1000 x g for 5 min. and the medium was removed by aspiration. The cells were then re-suspended in 4 ml of cell culture medium and transferred into a 25 cm² flask.

2.2.4 Trypsinisation of cells

Trypsin buffer 6 g/l NaCl, 3 g/l sodium citrate, in sterile water pH 7.8

Trypsin solution 0.125% (w/v) trypsin in trypsin buffer

DMSO-medium 750 µl DMSO in 10 ml Ham's F10 with fetal calf serum

The trypsin buffer was sterilised in an autoclave at 120°C for 30 min, and the working trypsin solution and DMSO medium were filtered through a 0.22 µm Millipore filter. All of the above solutions were stored at 4°C.

When the fibroblasts, cultured amniocytes or cultured chorionic villi cells had reached confluency, as judged by phase contrast microscopy, they were trypsinised for harvesting, sub-culturing or storage in liquid nitrogen. The medium was removed by aspiration and 4 ml of trypsin buffer was added. The flask was rinsed and the trypsin

buffer removed. Trypsin solution (2 ml) was added to the flask which was incubated at 37°C for 10 min. For sub-culturing, the detached cells were transferred into two new flasks and all three flasks were fed with 10 ml of cell culture medium. For harvesting or storage in liquid nitrogen, the detached cells were transferred to a sterile centrifuge tube. The flask was washed out with 2 ml of medium and this was also added to the tube. The flask was then discarded. The cells were centrifuged at 1000 x g for 10 min. The medium was then removed. Harvested cells were washed twice with phosphate buffered saline (PBS) and after the final wash 50 µl of water was added and the cells were stored at -20°C or -70°C. Cells for storage in liquid nitrogen were re-suspended in 2 ml of DMSO-medium and transferred into an ampoule, which was stored overnight at 4°C and placed in the liquid nitrogen cylinder the following morning.

2.2.5 Collection of β -Hexosaminidase activity secreted into fibroblast cell culture medium

Fibroblasts were grown to confluence under routine cell culture conditions (section 2.2). The cells were washed in PBS and incubated in serum-free medium (Ham's F10 + SATO, section 2.2.1). Immediately after the addition of the medium a 0.5 ml aliquot was withdrawn from each flask and stored at -20°C. After 5 days, the spent medium was removed and centrifuged at 1000 x g for 5 min. The supernatant was stored at -20°C. The cells were harvested from the flask by trypsinisation and pelleted (section 2.2). In the mannose supplementation experiments, 5 mM mannose was added to the cell culture medium and fibroblasts treated as above. β -Hexosaminidase activity was measured in the cell culture medium (100 µl- undiluted) as described in section 2.7.2.1 and 2.7.2.2, and related to the protein concentration of the fibroblast pellet.

2.3 Purification of serum transferrin

Serum transferrin was used as a model serum glycoprotein for studying aberrant glycosylation in CDGS type 1 and galactosaemia (Chapter 4). Transferrin was purified from serum and plasma samples on arrival (frozen and fresh) by adaptation of the method described by Steinbuch and Nierwlarowski (1960).

Serum (0.5 ml) was added to 0.2 ml of deionised water and the solution was vortexed. All other proteins except for transferrin and the immunoglobulins were precipitated by the addition of 0.3 ml of Rivanol (3%, w/v, 6,9-diamino-2-ethoxyacridine lactate, Sigma). The suspension was centrifuged at 10,000 x g for 10 min in an MSE benchtop centrifuge. The yellow supernatant was collected and 0.43 ml of NaCl (25%, w/v) was added. The mixture was vortexed and centrifuged at 10,000 x g for 10 min. The clear supernatant was collected and 0.8 ml of saturated ammonium sulphate was added per ml of supernatant. The solution was vortexed and allowed to stand for 10 min. It was then centrifuged for 10 min at 10,000 x g. The supernatant was collected and dialysed extensively against water. It was then freeze dried overnight and stored at 4⁰C until analysis by electrophoresis, anion-exchange chromatography or mass spectrometry.

2.4 Electrophoresis

Isoelectric focusing of serum with immunodetection for either serum transferrin or α 1-antitrypsin was used to confirm diagnosis of CDGS type 1 and galactosaemia (Chapter 4). SDS-PAGE was used to separate purified serum transferrin isoforms (Chapter 4), α -fucosidase isoforms (Chapter 6) and *in vitro* translation products (Chapter 6). Starch gel electrophoresis was used to separate isoenzymes of phosphoglucomutase (Chapter 3).

The gels and autorads were scanned on a Hewlett Packard desktop scanner (600 by 600 dpi resolution) and analysed by GelWorks (UVP).

2.4.1 Isoelectric focusing of serum on the PhastSystem

Electrophoresis reagents for the PhastSystem were purchased from Pharmacia; pI and molecular weight markers were from BDH and other chemicals were from Sigma. The methods were adapted from Hackler *et al.* (1995).

Isoelectric focusing was carried out using the PhastSystem (Pharmacia) with ready made gels. Serum samples were diluted (1/50) in distilled water and applied to a Phast IEF gel with a 0.5 μ l applicator. The samples were applied at the cathode. The conditions used

to run the IEF gels are shown in Table 2.3. The pI values of the transferrin isoforms were estimated by comparison with the coloured pI markers described in Table 2.4 (BDH coloured isoelectric focusing markers product no. 44270 26).

A: IEF 3-9

Step	Max Voltage (V)	Current (mA)	Power (W)	Temp (°C)	Volthour (Vh)
1	2000	2.5	3.5	15	75
2	200	2.5	3.5	15	15
3	2000	2.5	3.5	15	410

B: IEF 4-6.5

Step	Max Voltage (V)	Current (mA)	Power (W)	Temp (°C)	Volthour (Vh)
1	2000	2.0	3.5	15	75
2	200	2.0	3.5	15	15
3	2000	5.0	3.5	15	410

Table 2.3: Conditions used for IEF on the PhastSystem.

A; IEF pH range 3-9 (serum transferrin), and **B;** IEF pH range 4-6.5 (serum α 1-antitrypsin). Pre-focusing takes place during step 1, the sample is applied during step 2 and focused during step 3. The voltage and power values are the maximum values, and the current applied is constant.

Protein	pI
C-Phycocyanin (<i>A. nidulans</i>)	4.75, 4.85
Azurin (<i>P.aeruginosa</i>)	5.65
Trifluoroacetylated Myoglobin Met (porcine)	5.9
Myoglobin Met (porcine)	6.45
Myoglobin Met (equine)	7.3
Myoglobin Met (sperm whale)	8.3
Cytochrome C (horse heart)	10.6

Table 2.4: Coloured pI markers used in IEF gels.

2.4.1.1 Immunodetection of transferrin on IEF gels

Sample transfer to a nitrocellulose membrane was performed by diffusion blotting. A nitrocellulose sheet was soaked in buffer; 25 mM Tris, 192 mM glycine in 20% (v/v) methanol (pH 8.3), for 5 min. The nitrocellulose was then laid on top of the gel, making sure there were no air bubbles, for 20 min. A petri dish lid was placed over the gel to prevent the nitrocellulose sheet from drying out. Distilled water was used to moisten the nitrocellulose prior to removal from the gel.

The nitrocellulose sheet was then incubated with 100 ml of 2% (w/v) Marvel in saline for 1 hour. After washing with water, the sheet was incubated with 100 ml of 0.2% (w/v) Marvel and 50 μ l of goat anti-transferrin IgG antibodies (200 μ g/ml, INCSTAR) overnight. After thorough washing, the nitrocellulose sheet was incubated with 100 ml of 0.2% (w/v) Marvel and 50 μ l of rabbit anti-goat antibody conjugated to horse radish peroxidase (200 μ g/ml, DAKO) for 2 hours. The blot was washed thoroughly with distilled water and incubated with 20 ml of 4-chloro-1-naphthol in methanol (1 mg/ml), 100 ml of 0.1M sodium acetate buffer, pH 4 and 100 μ l hydrogen peroxide (30%). The reaction was stopped by washing with water.

2.4.1.2 Immunodetection of α -1-antitrypsin on IEF gels

α -1-antitrypsin was detected on a nitrocellulose sheet as described for the detection of transferrin (section 2.4.3.1), except that the first antibody incubated with the membrane was goat anti- α 1-antitrypsin (200 μ g/ml, INCSTAR).

2.4.2 SDS-PAGE.

SDS-PAGE was carried out in a Hoeffer SE 600 gel tank. 7.5% gels were used to analyse transferrin isoforms (Chapter 4) and α -fucosidase (Chapter 6). 15% gels were used to analyse the *in vitro* translation reactions (Chapter 6). The methods are based on those described by Laemmli (1970).

2.4.2.1 Solutions

4 X Separating gel buffer:	1.5 M Tris-HCl, 0.4% (w/v) SDS, pH 8.8.
4 X Stacking gel buffer:	0.5 M Tris-HCl, 0.4% (w/v) SDS, pH 6.8.
Monomer solution:	30.8% (w/v) acrylamide, 2.7% (w/v) bisacrylamide.
Tank buffer:	0.025 M Tris, 0.192 M glycine, 0.1% (w/v) SDS, pH 8.3
Transfer buffer:	0.025 M Tris, 0.192 M glycine
TBS-Tween:	100 mM Tris-HCl, 0.9% (w/v) NaCl, 1% (v/v) Tween
2 X SDS-sample buffer:	10% (v/v) glycerol, 2% (w/v) SDS, 0.02% (w/v) bromophenol blue, 12.5% (v/v) stacking gel buffer and 5% (v/v) β -mercaptoethanol.

2.4.2.2 Gels

	4%	7.5%	15%
	Stacking Gel	Separating Gel	Separating Gel
Monomer solution	0.665 ml	3.75 ml	7.5 ml
Stacking / separating gel buffer	1.25 ml	3.75 ml	3.75 ml
H ₂ O	3.05 ml	7.5 ml	3.7 ml
10% Ammonium persulphate	25 μ l	75 μ l	75 μ l
TEMED	5 μ l	5 μ l	5 μ l

Table 2.5: Composition of SDS-PAGE gels

Gels were cast between glass plates (18 cm x 18 cm or 18 cm x 9 cm) with 0.75 mm spacers. The separating gel mix (15 ml, Table 2.5) was poured in first and a layer of water was placed on top. It was allowed to stand for 1 hour before the water was removed and the stacking gel mix (5 ml, Table 2.5) was applied on top. A comb was placed in the stacking gel and it was allowed to set for 20 min. before removal of the comb and application of the samples. The samples were dissolved in sample buffer and boiled for 2 min. at 100°C. The current was set at 30 mA for two 0.75 mm gels for

migration through the stacking gel and at 60 mA for the separating gel. The circulating water bath was set at 15°C.

2.4.2.3 Molecular weight markers

Pre-stained molecular weight markers were used to calculate the molecular weights of the protein samples (Table 2.6). These markers were also transferred onto the nitrocellulose membrane and were still visible.

Protein	Apparent molecular mass (kDa)
Ovotransferrin	105
Bovine serum albumin	98
Ovalbumin	53
Carbonic anhydrase	33
Soybean trypsin inhibitor	25

Table 2.6: Pre-stained molecular weight markers used on SDS-PAGE gels.
(BDH product no. 443932W)

2.4.2.4 Immunodetection of α fucosidase on SDS-PAGE gels

This method was adapted from Johnson *et al.* (1992). Proteins were transferred electrophoretically from SDS-PAGE gels (7.5%) to a nitrocellulose membrane using the Hoeffer TE 22 Transfer tank in transfer buffer (section 2.4.2.1) at 20 volts overnight. The nitrocellulose membranes were blocked for 1 hour in 5% (w/v) Marvel in TBS-Tween and then incubated with a 1 in 1000 dilution of goat anti- α fucosidase antibody (Johnson *et al.*, 1992) overnight in 0.2% (w/v) Marvel in TBS-Tween followed by 2 hours with horse radish peroxidase-conjugated rabbit anti goat IgG antibody (2 μ g/ml, DAKO) in 0.2% Marvel in TBS-Tween. α -Fucosidase was detected by chemiluminescence using the Amersham ECL detection Kit (RPN 2106). The membranes were incubated in the substrate solution (luminol) for 1 min. The membrane was wrapped in cling film and exposed for 30 seconds to autoradiography film (Kodak X-OMAT-AR).

2.4.2.5 Silver staining

This method was adapted from Merril *et al.* (1981).

2.4.2.5.1 Solutions

10 x Oxidiser: 2g potassium dichromate + 410 µl nitric acid in 200 ml water

10 x Silver Stain: 4.077g AgNO₃ in 200 ml water

Developer: 40.06g Na₂CO₃ + 0.25 µl formaldehyde in 500 ml water

2.4.2.5.2 Staining procedure

Gels were fixed twice with methanol:acetic acid: water (40:10:50) for 5 min. They were then fixed twice with ethanol: acetic acid:water (10:5:85) for 15 min. Gels were incubated for 5 min in oxidising solution (1 in 10 dilution of stock) and washed twice with water. Silver reagent (1 in 10 dilution of stock) was added for 20 min followed by washing twice with water. Each gel was developed (1 in 10 dilution of stock solution of developer) until the bands could be seen clearly. The reaction was stopped with 5% (v/v) acetic acid. The gels were then washed in water and stored in methanol:glycerol:water (10:5:85) before drying between cellulose sheets (Promega).

2.4.2.6 Autoradiography

The dried gels which contained [³⁵S]-methionine labelled samples were placed in autoradiography cassettes and exposed to film overnight (Kodak X-OMAT-AR).

2.4.3 Starch gel electrophoresis of phosphoglucomutase activity

The following methods have been described by Harris and Hopkinson (1976).

2.4.3.1 Preparation of starch gel and electrophoresis

Running buffer; 0.1M Tris, 0.1 M maleic anhydride, 0.01 M EDTA, 0.01M MgCl₂ at pH 7.4.

Potato starch (38.5g) was added to 350 ml of a 1 in 10 dilution of the running buffer and boiled for 1 min. The bubbles were removed by a vacuum pump and the gel was immediately poured into the gel mould (30 cm x 15 cm x 1 cm). When the gel had set, 0.5 mm square tabs of Whatman No.1 filter paper which had been soaked in the samples

were inserted in the gel. Electrophoresis was carried out at 200 volts and 60 mA overnight at 4^oC.

2.4.3.1 Detection of phosphoglucomutase activity

The starch gel was cut in half in thickness using fish wire and one half was stained for phosphoglucomutase activity by overlaying with a gel containing the substrate mixture. The overlay gel was composed of 0.5 M Tris-HCl (pH 8) (20 ml), glucose-1-phosphate (0.1g), 0.2 M MgCl₂ (2 ml), 1 mg/ml glucose-6-phosphate dehydrogenase (200μl), 5 mg/ml NADP (2 ml), 5 mg/ml MTT (2 ml), 5 mg/ml PMS (1 ml) and 2% agar (w/v, heated to 60^oC) (20 ml). The gel and overlay were incubated in the dark at 37^oC until dark bands were seen and the gel was photographed.

2.5 Medium pressure chromatography

Medium pressure chromatography was used to isolate the isoforms of transferrin prior to analysis by SDS-PAGE or release of the N-glycans (Chapter 4) and also to investigate the glycosylation of fibroblast β-hexosaminidase (Chapter 6).

The Biologic medium pressure chromatography system consisting of BioRad gradient pumps, system controller, fraction collector, UV and conductivity monitors was used for all the medium pressure ion-exchange and lectin affinity columns (BioScale Q2 and Econo columns). The chromatograms were analysed using the Biologic (version 1.1) program. The columns and standards (protein standard for anion exchange - 125-0561) were obtained from BioRad and the other reagents were obtained from Sigma. All buffers were degassed by sonication and vacuum for 20 min. prior to use in the chromatography system.

2.5.1 Anion-exchange chromatography of transferrin

Transferrin samples were dialysed against 20 mM Tris-HCl, pH 7.4 and 1 ml of the dialysate was applied to the BioScale Q2 column equilibrated in the same buffer. Transferrin was eluted with a gradient of 0.2 M NaCl over 36 ml at a flow rate of 2 ml/min and 2 ml fractions were collected. The absorbance at 280 nm was monitored. The

transferrin isoforms were identified by isoelectric focusing of the protein components in each fraction followed by immunodetection for transferrin (section 2.4.1).

2.5.2 Anion-exchange chromatography of fibroblast β -hexosaminidase

Cell pellets were thawed and sonicated for 15 seconds at 10 Hz using an MSE SoniPrep 150. The sonicates were diluted in 1 ml of 25 mM Bis-Tris buffer, pH 6. Fibroblast cell culture medium was concentrated to 1 ml in a MINICON B15 concentrator and dialysed against 25 mM Bis-Tris buffer at 4^oC overnight. All samples were centrifuged for 10 minutes at 10,000 x g and the supernatant removed and kept on ice before injection of 1 ml onto a BioScale Q2 column equilibrated in Bis-Tris buffer pH 6. The proteins were eluted with a linear gradient of 0 - 0.5M NaCl over 36 ml at a flow rate of 2 ml/min. Fractions (1 ml) were collected and assayed for β -hexosaminidase activity as described in section 2.5.3.[†]

2.5.3 β -Hexosaminidase activity in column fractions

50 μ l of each fraction was incubated with 50 μ l of 4-methylumbelliferyl-2-acetamido-2-deoxy- β -D-glucopyranoside (1.1 mg/ml in MV 4.5) for 30 minutes at 37^oC with shaking. The reaction was stopped by adding 150 μ l of 0.25 M glycine buffer pH 10.4 with 0.01% Triton X-100 (v/v). The fluorescence was measured at an excitation wavelength of 365 nm and an emission wavelength of 450 nm (see section 2.7.1 for further details).

2.5.4 Lectin affinity chromatography of fibroblast β -hexosaminidase

2.5.4.1 Solutions

Low salt buffer: 0.1M NaCl, 0.1 mM CaCl₂, 0.1 mM MnCl₂, 0.1 mM MgCl₂, in 10 mM sodium phosphate buffer, pH 6.8.

High salt buffer: 1M NaCl, 0.1 mM CaCl₂, 0.1 mM MnCl₂, 0.1 mM MgCl₂, in 10 mM sodium phosphate buffer, pH 6.8.

Elution buffer: 0.5M methyl- α -D-mannopyranoside in the high salt buffer.

A low pressure Econo column (1 ml bed volume) was filled with concanavalin A-Sepharose (Pharmacia). The column was equilibrated with low salt buffer. Cell sonicates were prepared as previously described (section 2.5.2), diluted in 1 ml of low salt buffer and applied to the column. After washing the column with 5 ml of low salt buffer, the column was washed with high salt buffer. Bound glycoproteins were eluted with a linear gradient of the elution buffer over 10 ml. The flow rate was 0.2 ml/min and 1 ml fractions were collected. Each fraction was assayed for β -hexosaminidase activity (section 2.5.3).

2.6 Assays of enzymes involved in interconversion and utilisation of sugar phosphates

The enzyme assays described in this section were used for the diagnosis of CDGS type 1 and for the inhibitor studies described in Chapters 3 and 7.

All the chemicals and enzymes were obtained from Sigma, except for the mannose-1,6-bisphosphate which was synthesised as described in section 2.6.3. The assays were performed using a Perkin Elmer LS 50 fluorimeter with four 500 μ l quartz cuvettes, which were thermostatted at 30⁰C by a circulating water bath. The fluorimeter was calibrated each day with a fresh standard solution of NADH, whose absorbance at 340 nm was also measured. The methods described in this section were adapted from Van Schaftingen and Jaeken (1995) (PMM, PGM, PMI, PGI, hexokinase) or Szumilo *et al.*, (1993) (mannose-1-phosphate guanosyl transferase).

2.6.1 Sample preparation

Pellets of cultured fibroblasts, amniocytes and chorionic villi cells were thawed, sonicated in 50 μ l of water for 10 s at 6 Hz (MSE-SoniPrep 150) and the resultant homogenates centrifuged for 5 min at 10,000 x g. The chorionic villus biopsy was thawed, homogenised in 50 μ l water in a 1 ml glass homogeniser and centrifuged as above. The supernatants were diluted to a final volume of 100 μ l for the measurement of protein concentration and for the enzyme assays.

2.6.2 Protein determination

This method is based on that described by Smith *et al.* (1985). The protein concentration of the cell homogenates was determined by adding 1 ml of bicinchoninic acid (Sigma B9643) to 10 μ l of cell sonicate and 40 μ l of distilled water. After incubation at 37°C for 10 minutes, 20 μ l of 4% CuSO₄.5H₂O was added and this was incubated for a further 20 minutes at 37°C. The absorbance at 562 nm was measured. The protein concentration was calculated from the standard curve constructed using bovine serum albumin (BSA) over a range of 0.1 mg/ml to 1 mg/ml.

For measurement of protein concentration below this range the micro protein method was used. In this procedure the reaction was performed at 60°C for 45 min. and the BSA standard was prepared over the range 0.01 mg/ml to 0.1 mg/ml.

2.6.3 Synthesis of mannose-1,6-bisphosphate

This cofactor was synthesised according to the method of Van Schaftingen and Jaeken (1995) by incubating 0.3 mM mannose 1-phosphate with 0.2 mM glucose-1,6-bisphosphate, 5 μ g/ml yeast glucose-6-phosphate dehydrogenase, 10 μ g/ml muscle phosphoglucomutase, 0.25 mM NADP, 5 mM MgCl₂ and 1 mM dithiothreitol in 50 mM Hepes buffer pH 7.1 for 1 hour at 30°C. The total reaction volume was 1 ml. The reaction was stopped by boiling for 5 min. and then centrifuged at 10000 x g for 3 min. and a clear supernatant was obtained.

2.6.4 Assay of phosphomannomutase, phosphoglucomutase, phosphomannose isomerase, phosphoglucose isomerase and hexokinase activity

The assays for measurement of phosphomannomutase (EC 5.4.2.28, PMM), phosphoglucomutase (EC 5.4.2.2, PGM), phosphomannose isomerase (EC 5.3.1.8, PMI), phosphoglucose isomerase (EC 5.3.1.9, PGI) and hexokinase activity in cell extracts are all enzyme-coupled assays with the glucose-6-phosphate dehydrogenase/NADPH system as the final step. The reaction conditions for each assay are summarised in Table 2.7. For each assay the incubation temperature was 30°C and

the total reaction volume was 500 μ l. The production of NADPH was measured at an excitation wavelength of 340 nm and an emission wavelength of 460 nm.

The following stock solutions were made up fresh each time in Hepes buffer, pH 7.1:

<i>Hepes Buffer, pH 7.1</i>	50 mM Hepes, 5 mM MgCl ₂ , pH 7.1
<i>NADP</i>	2.5 mM
<i>Glucose-6-phosphate dehydrogenase</i>	100 μ g/ml (20 μ g/ml for PMI assay)
<i>Phosphoglucose isomerase</i>	100 μ g/ml
<i>Phosphomannose isomerase</i>	35 μ g/ml
<i>Mannose-1-phosphate (Man-1-P)</i>	2 mM
<i>Glucose-1-phosphate (Glc-1-P)</i>	5 mM
<i>Mannose-6-phosphate (Man-6-P)</i>	5 mM
<i>Fructose</i>	10 mM
<i>Glucose</i>	10 mM
<i>Mannose-1,6-bisphosphate (Man-1,6-P)</i>	10 μ M
<i>Glucose-1,6-bisphosphate (Glc-1,6-P)</i>	10 μ M
<i>ATP</i>	10 mM

Initially NADP (50 μ l) and glucose-6-phosphate dehydrogenase (50 μ l) were added to each reaction tube. Subsequently 50 μ l of the appropriate other components (Table 2.7), unless otherwise indicated, were added and the total volume made up to 500 μ l with Hepes buffer. For the PMM and PMI reactions the cell extracts were added to the reaction tube, prior to the addition of the substrate, and the reaction mixture was pre-incubated as indicated (Table 2.7). The assay was started by the addition of the substrate. The pre-incubation period was required to remove endogenous substrates from the assay system. For the other reactions (PGM, PGI and hexokinase), the addition of the cell extract was used to start off the timed reaction. Each enzyme was assayed in the presence and absence of substrate, and a reagent blank was also measured. The final concentrations of each component in the reaction mixture are described in sections 2.6.4.1-2.6.4.5. The rate of reaction was determined by measuring the fluorescence at 2 min. or 10 min. intervals for the time periods described (Table 2.7) and by plotting a

graph of fluorescence against time. The linear part of the line was used to calculate the change in fluorescence over time.

Enzyme assay	Cell extract (μl)	Substrate	PGI	PMI	Cofactor	Pre-incubation (min)	Time (min)
		50 μl added (* = 25 μl)					
PMM	25	Man-1-P*	Yes	yes	Man-1,6-P	20	60
PGM	5	Glc-1-P	no	no	Glc-1,6-P	2	10
PMI	25	Man-6-P	yes	no	no	20	20
PGI	5	Fructose	no	no	no	2	10
Hexokinase	5	Glucose	no	no	ATP	2	10

Table 2.7: Summary of enzyme assay conditions for PMM, PGM PMI, PGI and hexokinase

To each reaction mixture 50 μl of NADP and 50 μl of glucose-6-phosphate dehydrogenase were added in addition to 50 μl of the other components (substrate*, PGI, PMI, and cofactor). The cell extracts were added prior to the addition of the substrate for the PMM and PMI assays.* 25 μl of mannose -1-phosphate was used instead of 50 μl .

2.6.4.1 Phosphomannomutase

Final concentration of components; 0.25 mM NADP, 1 μM mannose-1,6-bisphosphate, 10 $\mu\text{g/ml}$ phosphoglucose isomerase, 3.5 $\mu\text{g/ml}$ phosphomannose isomerase, 10 $\mu\text{g/ml}$ glucose-6-phosphate dehydrogenase in 50 mM Hepes pH 7.1, 5 mM MgCl_2 with and without 0.1 mM mannose-1-phosphate.

2.6.4.2 Phosphoglucomutase

Final concentration of components; 0.25 mM NADP, 1 μM glucose-1,6-bisphosphate, 10 $\mu\text{g/ml}$ glucose-6-phosphate dehydrogenase in 50 mM Hepes pH 7.1, 5 mM MgCl_2 with and without 0.5 mM glucose-1-phosphate.

2.6.4.3 Phosphomannose isomerase

Final concentration of components; 0.25 mM NADP, 10 µg/ml phosphoglucose isomerase, 2 µg/ml glucose-6-phosphate dehydrogenase in 50 mM Hepes pH 7.1, 5 mM MgCl₂ with and without 0.5 mM mannose-6-phosphate.

2.6.4.4 Phosphoglucose isomerase

Final concentration of components; 0.25 mM NADP, 10 µg/ml glucose-6-phosphate dehydrogenase in 50 mM Hepes pH 7.1, 5 mM MgCl₂ with and without 1 mM fructose.

2.6.4.5 Hexokinase

Final concentration of components; 0.25 mM NADP, 1 mM ATP, 10 µg/ml glucose-6-phosphate dehydrogenase in 50 mM Hepes pH 7.1, 5 mM MgCl₂ with and without 1 mM glucose.

2.6.5 Glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase (Sigma 5885) was diluted 1 in 1000 in Hepes buffer. 50 µl was assayed in the presence of 0.5 mM glucose-6-phosphate, 0.25 mM NADP and 1µM glucose-1,6-bisphosphate for 10 min. and 30 °C. The total reaction volume was 500 µl.

2.6.6 Mannose-1-phosphate guanosyltransferase

Cell extracts (25 µl) were assayed for mannose-1-phosphate guanosyltransferase activity in the presence of 0.5 mM NADP, 0.1 mM ADP, 1 mM glucose, 1 mM sodium pyrophosphate, 2.5U hexokinase, 2.5U NDP kinase, 2.5U glucose-6-phosphate dehydrogenase in 50 mM Tris buffer pH 7.5, 5 mM MgCl₂ with and without 0.2 mM GDP-mannose (added after a 20 min pre-incubation) for 1 hour at 30°C. The total reaction volume was 500 µl.

2.7 Assays of lysosomal enzymes

The lysosomal enzymes β -hexosaminidase (intracellular, secreted, plasma and amniotic fluid and in column fractions), α -mannosidase (intracellular) and β -glucuronidase (plasma, amniotic fluid) were assayed using the appropriate synthetic fluorogenic 4-methylumbelliferyl-glycoside substrates. The reactions were stopped by the addition of glycine buffer, pH 10.4 and the fluorescence of the 4-methylumbelliferone liberated was measured at 450 nm using an excitation wavelength of 365 nm. All the enzyme assays were performed on a Perkin Elmer LS 50 fluorimeter with either the sipper attachment (1 ml Eppendorf tubes) or the plate reader attachment. The fluorimeter was calibrated with a 1 mM solution of 4-methylumbelliferone. All the substrates and reagents were obtained from Sigma.

2.7.1 Buffers and substrates

Stopping buffer for sipper mode: 0.25 M glycine / NaOH pH 10.4

Stopping buffer for well plates: as above plus 0.01% Triton X-100

β -Hexosaminidase assay

McIlvaine citrate-phosphate buffer (MV 4.5): 0.1 M citric acid ,0.2 M Na₂HPO₄, pH 4.5 (McIlvaine, 1921)

Substrate: 4-methylumbelliferyl-2-acetamido-2-deoxy- β -D-glucopyranoside (1.1 mg/ml in MV 4.5)

α -Mannosidase assay

McIlvaine citrate-phosphate buffer (MV): 0.1 M citric acid ,0.2 M Na₂HPO₄, pH range 3-7 (McIlvaine, 1921)

Substrate: 4-methylumbelliferyl- α -D-mannopyranoside (4 mM) in water

β -Glucuronidase assay

Formate buffer: 0.1M formate buffer, pH 3.25

Substrate: 4-methylumbelliferyl- β -D-glucuronide trihydrate (7.4 mM) in formate buffer

2.7.2 β -Hexosaminidase activity in fibroblasts

(Methods adapted from O'Brien (1970)). Cell pellets were thawed and stored on ice. The pellets were sonicated at 10 Hz for 15 seconds in a MSE SoniPrep 150 and then diluted 100 fold in 0.2 % (w/v) HSA/MV 4.5.

2.7.2.1 Total β -hexosaminidase

100 μ l of the substrate was added to 100 μ l diluted cell extract and the reaction mixture was incubated for 10 minutes at 37°C. The reaction was stopped by adding 1 ml of stopping buffer.

2.7.2.2 β -Hexosaminidase A activity in fibroblasts by heat inactivation

Aliquots of diluted cell extracts (100 μ l) were put in six Eppendorf tubes. Three were stored at 4°C whilst the other three were heated for 1 hour at 50°C. All the samples were then assayed for β -hexosaminidase activity as described in 2.7.2.1. The percentage of β -hexosaminidase A was taken as 1-(heated/unheated).

2.7.2.3 β -Hexosaminidase A activity in fibroblasts by specific substrate

The proportion of β -hexosaminidase A was measured directly using 4-methylumbelliferyl- β -D-N-acetylglucosamine-6-SO₄ (MUGS) as the substrate instead of 4-methylumbelliferyl-2-acetamido-2-deoxy- β -D-gluco-pyranoside. 5 μ l of cell extract was added to 45 μ l of MV buffer pH 4 and 150 μ l of MUGS (3 mg/ml in MV pH 4). The reaction was incubated for 30 min at 37°C and stopped by the addition of 1 ml of stopping buffer.

2.7.3 β -Hexosaminidase activity in amniotic fluid and plasma

Amniotic fluid or plasma (5 μ l) was diluted in 95 μ l of 0.2% (w/v) human serum albumin (HSA) in MV pH 4.5. 100 μ l of substrate was added and incubated for 15 min at 37°C. The reaction was stopped by adding 1 ml of stopping buffer.

2.7.4 α -Mannosidase activity in fibroblasts

The reactions were carried out in 96 well plates. α -Mannosidase activity was measured over the range pH 3 - 7 in the presence and absence of 2 mM cobalt chloride. 25 μ l of cell extract was added to 50 μ l of MV buffer pH 3-7 and 25 μ l of substrate. After incubation with shaking at 37^oC for 4 hours, the reaction was stopped by the addition of 150 μ l of stopping buffer.

2.7.5 β -Glucuronidase activity in amniotic fluid and plasma

Amniotic fluid or plasma (10 μ l) was diluted in 90 μ l of H₂O in an Eppendorf tube. 100 μ l of substrate was added and the mixture incubated for 40 min at 37^oC. The reaction was stopped by the addition of 1 ml of stopping buffer and the fluorescence read.

2.8 Analysis of glycans by HPLC

The N-linked glycans released from purified transferrin and glycoproteins from fibroblasts from patients with galactosaemia and CDGS type 1 were derivatised by reaction with 2-aminobenzamide and analysed by HPLC (Chapters 4 and 6). The HPLC system consisted of 2 Shimadzu LC 10AS HPLC pumps, a SIL-9A autosampler and a RF 535 fluorescence HPLC monitor. The GlycoSep-N column (Oxford Glycosystems I4728) was heated at 30^oC in a column heater. The data was collected and analysed by the Class VP- Chromatography Data System (Shimadzu Europe). The reagents for glycan release and labelling were all obtained from Oxford Glycosystems as were the standards. The ammonium formate was HPLC grade from BDH. The buffers were all degassed by sonication and vacuum for 20 min. prior to use.

2.8.1 Preparation of N-glycans of transferrin for HPLC

Serum (1.5 ml) or ascites fluid (6 ml) was used for the purification of transferrin as described in section 2.3. The transferrin isoforms were separated by anion-exchange chromatography (section 2.5.1) and the fractions were freeze dried. The transferrin isoforms were reconstituted in 20 μ l of 50 mM ammonium bicarbonate buffer, pH 8.4 and digested with 5 μ l of trypsin (1 mg/ml in buffer, Sigma) for 5 hours at 37^oC. The

reaction was stopped by boiling for 3 min. 250 µl of recombinant N-glycanase F (1U, Oxford Glycosystems E5006) was added to the trypsin digest, which was incubated for a further 18 hours at 37 °C. 700 µl of acetonitrile was added and the suspension centrifuged on a microcentrifuge at 10,000 x g for 3 min.

2.8.2 Preparation of N-glycans from fibroblasts for HPLC

Free, lipid-linked or protein-bound monosaccharides and oligosaccharides from fibroblasts were separated into four fractions; dolichol-P-monosaccharides, water soluble oligosaccharides, dolichol-PP-oligosaccharides and protein-bound oligosaccharides, and analysed by HPLC. A summary of the extraction procedure is shown in Figure 5.1. The extraction methods were adapted from Powell *et al.* (1994).

2.8.2.1 Isolation of dolichol-monosaccharides - *fraction 1*

Fibroblast cell pellets (approx. 1 million cells) were re-suspended in 5 ml of chloroform:methanol (2:1 v/v) and vortexed to disperse the clumps. The suspension was centrifuged at 10,000 x g for 5 min. The supernatant was collected and the cells washed twice more with 5 ml chloroform:methanol (2:1 v/v). The supernatants were pooled and the solvent removed under nitrogen. The monosaccharides were released by acid hydrolysis (section 2.8.2.2).

2.8.2.2 Release of monosaccharides and oligosaccharides by mild acid hydrolysis

Fractions 1 and 3 were hydrolysed by incubating them in 200 µl of 0.1 M HCl in tetrahydrofuran for 2 hours at 50°C. The solvent was removed under nitrogen and the oligosaccharides dissolved in water and freeze dried before labelling with the 2-AB labelling kit (section 2.8.3). The released sugars were analysed on the GlycoSep-N column (section 2.8.4).

2.8.2.3 Isolation of water-soluble oligosaccharides - *fraction 2*

The residual pellet from fraction 1 was dried under nitrogen and re-suspended in 1 ml of water. The pellet was sonicated three times for 10s at 10 Hz. and 5 ml of water was added to the sonicate. The suspension was vortexed before centrifugation at 10,000 x g

for 5 min. The pellet was washed twice more with 5 ml water and the supernatants were combined. The water soluble oligosaccharides were freeze-dried and labelled with the 2-AB labelling kit directly (section 2.8.3) and analysed on the GlycoSep N column (section 2.8.4).

2.8.2.4 Isolation of lipid-linked oligosaccharides - fraction 3

The residual pellet from fraction 2 was re-suspended in 5 ml of chloroform:methanol:water (10:10:3 v/v/v), vortexed and centrifuged at 10,000 x g for 5 min. This was repeated twice and the supernatants combined. The lipid linked oligosaccharides were dried under nitrogen and the oligosaccharides released by acid hydrolysis (section 2.8.2.2).

2.8.2.5 Isolation of protein-bound oligosaccharides - fraction 4

The final pellet from fraction 3 was re-suspended in 100 µl of 20 mM sodium phosphate buffer, pH 8 containing 0.1% (w/v) SDS and vortexed (Kuster and Harvey, 1997). 5 ml of trypsin (1 mg/ml in buffer) was added and the suspension incubated at 37°C for 5 hours before the addition of 250 µl N-glycanase F (1 unit) to release the glycans from the glycopeptides. The reaction was stopped after 18 hours by the addition of 700 µl of acetonitrile. The suspension was centrifuged at 10,000 x g for 3 min and the supernatant freeze-dried. The released glycans were labelled with the 2-AB labelling kit (section 2.8.3), and analysed on the GlycoSep N column (section 2.8.4).

2.8.3 Labelling of released glycans with 2-aminobenzamide

The glycans (sections 2.8.1 and 3.8.2) were freeze dried in Eppendorf tubes and labelled with the Signal™ 2-AB labelling kit (K-404). The labelling mixture was prepared by adding 150 µl of glacial acetic acid to 350 µl of DMSO. 100 µl of this solvent was added to the Eppendorf containing the 2-aminobenzamide (2-AB) dye. This mixture was then added to the glass vial containing the reducing agent (sodium borohydride) to make the complete labelling reagent. 10 µl of labelling reagent was added to each sample and incubated for two hours at 65°C. The free 2-AB label was separated from the 2-AB labelled glycans by using a GlycoClean S column which absorbs hydrophilic glycans. The

columns were primed by sequential washing with 1 ml water, 5 ml 30% (v/v) acetic acid and 1 ml acetonitrile. The sample was then loaded onto the column and incubated for 15 min. The Eppendorf tubes were then rinsed with 100 μ l of acetonitrile and this was added to the column. The free label was washed away with 1 ml of acetonitrile and 5 x 1 ml of water:acetonitrile (4:96). The 2-AB labelled glycans were eluted with 3 x 0.5 ml water and eluants freeze dried. The glycans were reconstituted in water:acetonitrile (30:70) and analysed on the GlycoSep-N HPLC column (2.8.4).

2.8.4 HPLC with the GlycoSep-N column

20 μ l of a solution of 2-AB labelled glycans were analysed by high-performance liquid chromatography on the GlycoSep-N column at 30°C. The separated 2-AB labelled glycans were detected by monitoring the effluent at an excitation wavelength of 330 nm and emission wavelength 420 nm. The columns were eluted at a flow rate of 0.4 ml/min with a 70 min gradient of 35% to 100% 250 mM ammonium formate buffer, pH 4.4 in acetonitrile.

2.8.3.1 Calibration of the GlycoSep-N column

The column was calibrated with 2-AB-labelled dextran hydrolysate and authentic oligosaccharide standards obtained from Oxford Glycosystems (MAN5, M3N2, NGA2FB, HYBRID, NGA2, NGA3, NGA4, A1 and A2, Figure 2.1 and 2.2) using the method described by Guile *et al.* (1996). The elution times of the authentic standards were converted into glucose unit equivalents (GU) by calibration of the column with a dextran hydrolysate (Figure 2.3A and 2.3B). The GU values of glycans are representative of the affinity of the glycan for the column matrix and hydrophilicity of the glycan. Although the glucose units are not absolute parameters, the values obtained in this study were comparable with those obtained by Guile *et al.* (1996). The incremental value in the retention of each type of glycosyl residue was determined by calculating the GU values for each of the standards and then estimating the GU values of the glycosyl residue by subtraction (Table 2.8).

Monosaccharide	Formula	Glucose units
Mannose (α 1-3/6)	(MAN5-M3N2)/2	0.95
GlcNAc	(NGA2-M3N2)/2	0.53
Bisecting GlcNAc	HYBRID-(MAN5+GlcNAc)	0.47
Core fucose (α 1-6)	NGA2FB-NGA2B	0.34
Galactose	(NA2-NGA2)/2	0.9
Sialic acid	A2-A1	1.18

Table 2.8 Incremental values for monosaccharide units.

The deduced retention times of other structures were then calculated from the sequential addition of the individual residues to the core M3N2 to give the glucose unit equivalent for the complete structure. The retention time was then calculated from the equation of the line obtained for the standards (Guile *et al.*, 1996). These data are shown in table 2.9. For example the retention time of NM3N2 was calculated by the addition of the GU for M3N2 plus the GU for a peripheral GlcNAc ($4.09 + 0.53 = 4.69$ GU, which is equivalent to a retention time of 28 min).

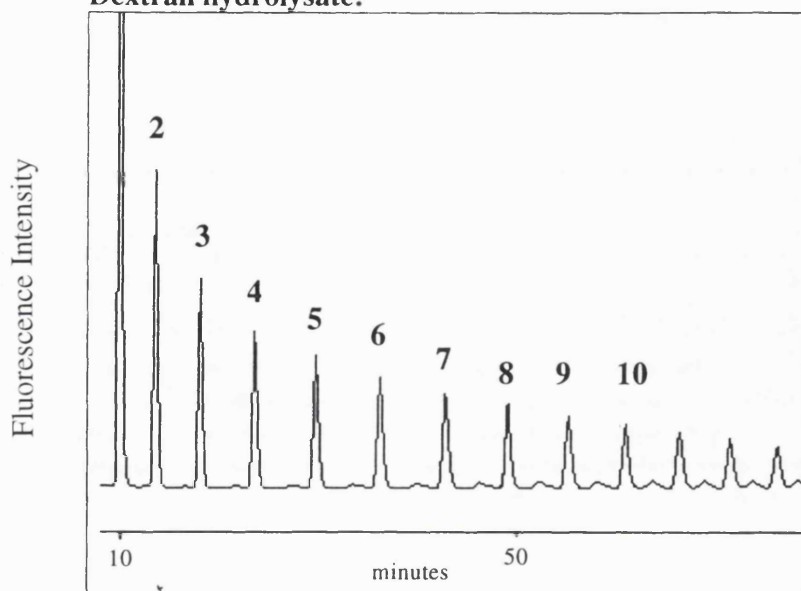
*Man 5	*HYBRID	*M3N2	NM3N2
*NGA2	NGA2B	*NGA3	*NGA4
NA2	NA2B	NA3	NA4
*A1	*A2	A2F	*NGA2FB

Figure 2.1: Key to nomenclature of structures of glycans used for HPLC on the GlycoSep-N column

* Indicates retention time of authentic 2-AB labelled oligosaccharide standards

Figure 2.2:

Dextran hydrolysate:



A2 and A1:

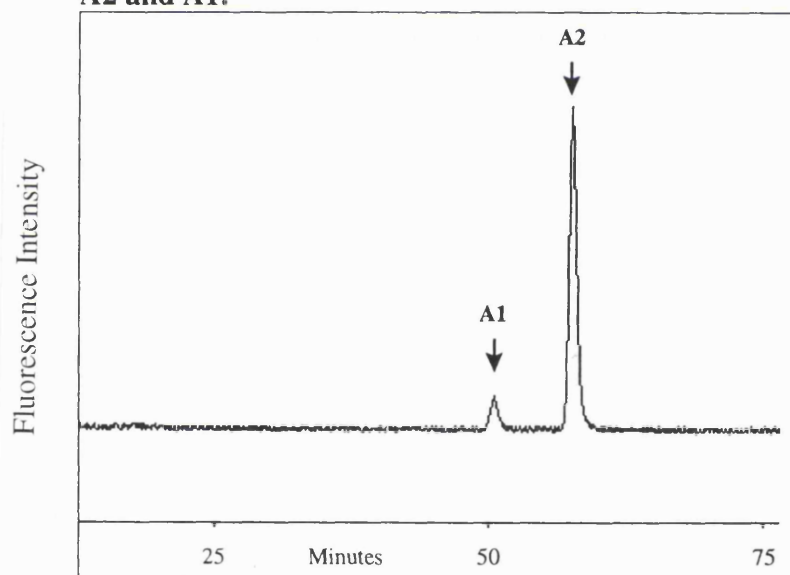


Figure 2.2:

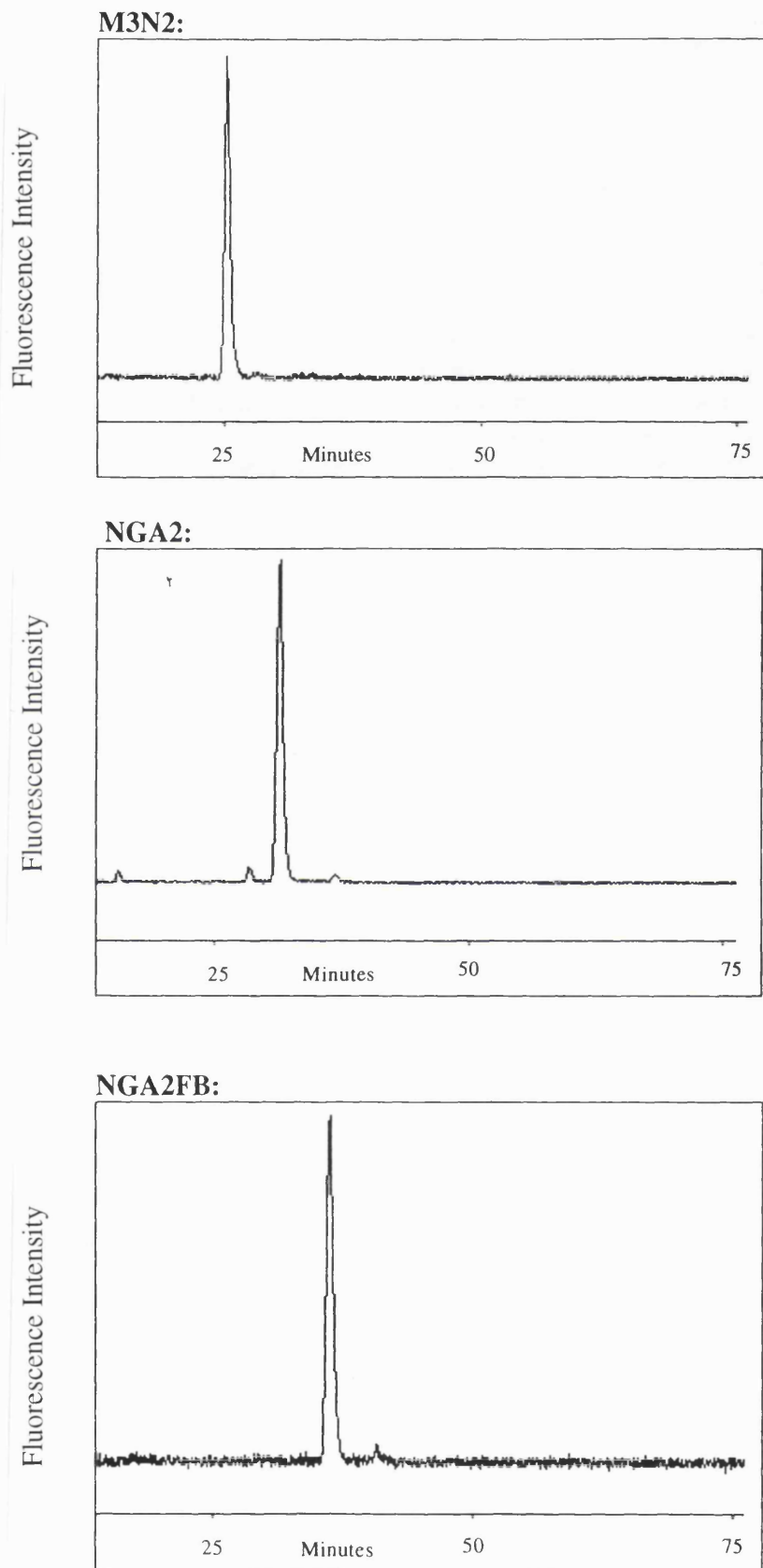
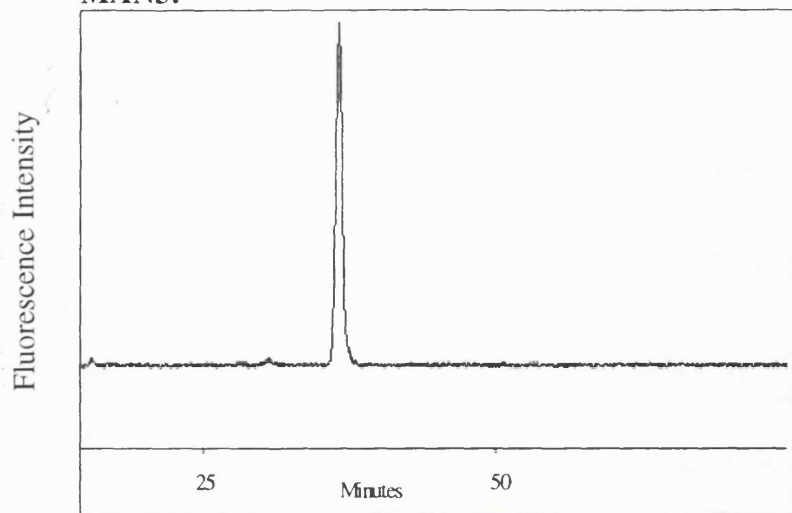
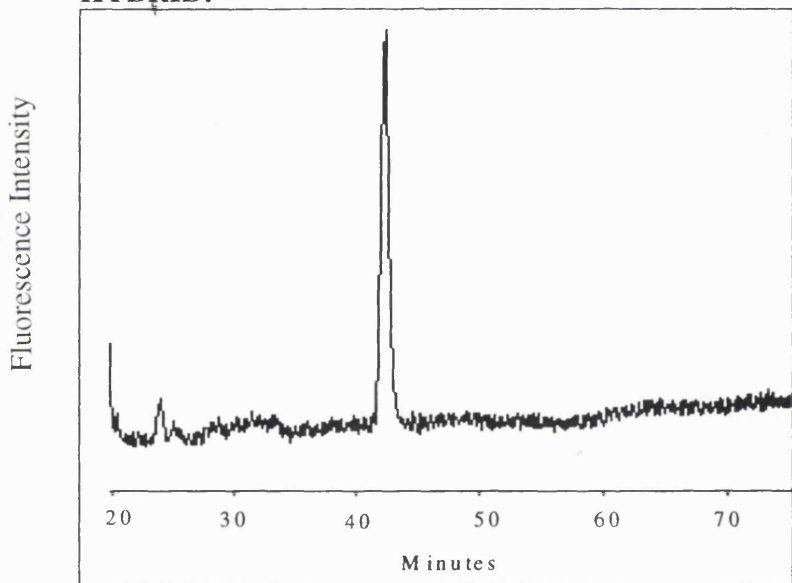


Figure 2.2:

MAN5:



HYBRID:



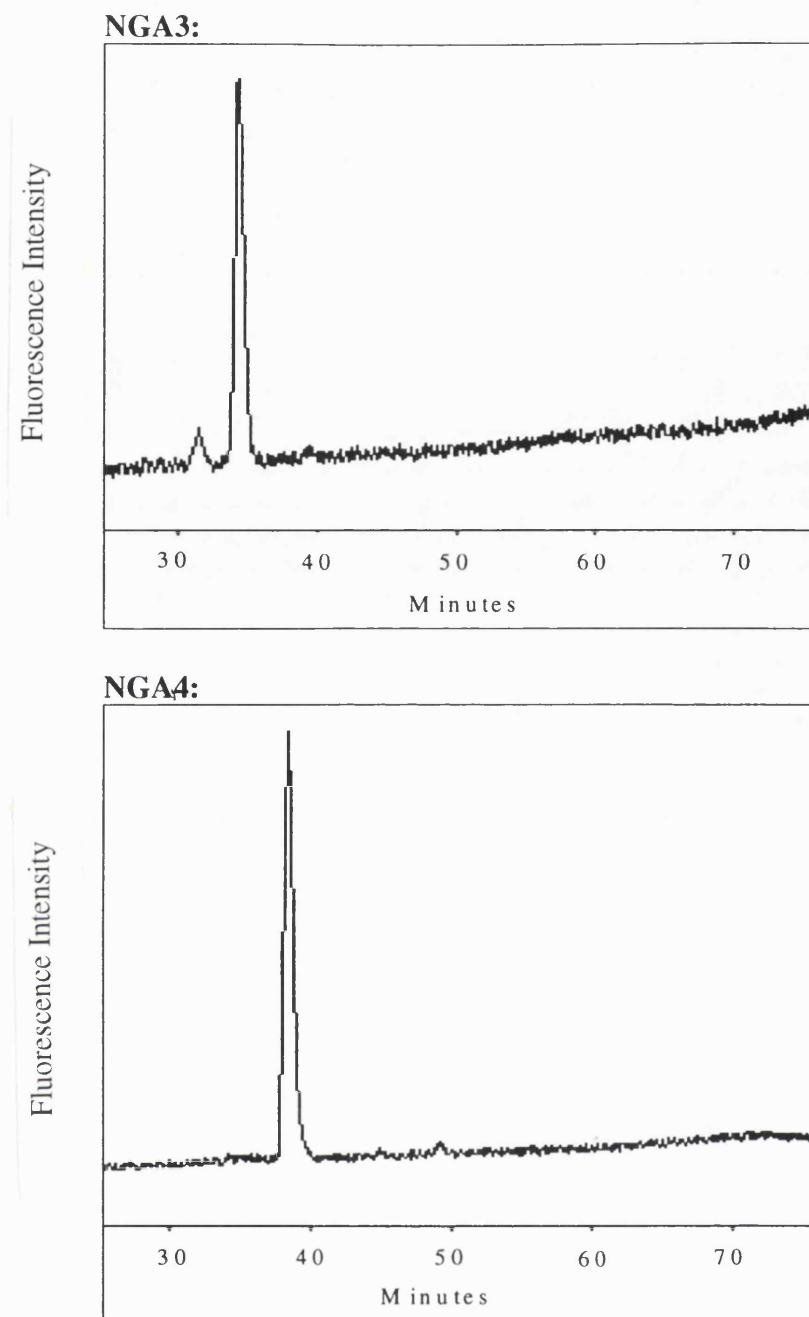


Figure 2.2: Authentic 2-AB standards eluting from the GlycoSep-N column.

The authentic standards were eluted from the GlycoSep-N column with a gradient from 35% to 100% 250 mM ammonium formate buffer pH 4.4 in acetonitrile. Numbers indicate number of glucose units. (Shown on page 88).

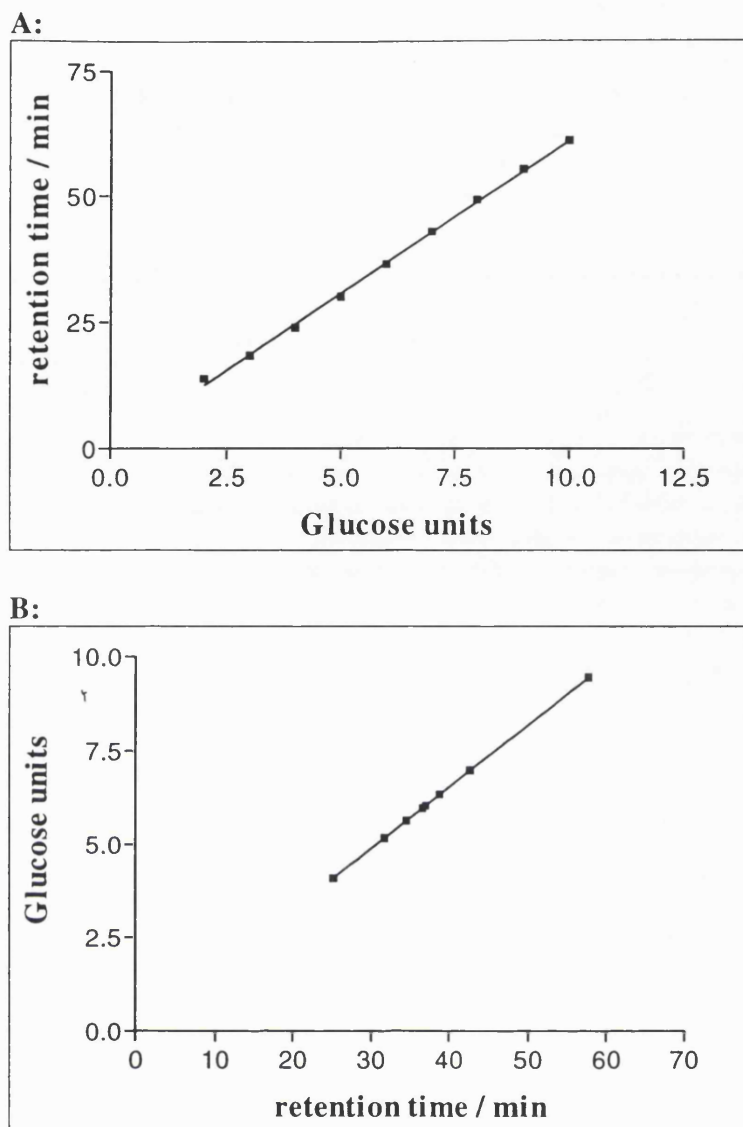


Figure 2.3. Calibration of the GlycoSep-N column with 2-AB labelled standards. Retention time versus glucose units for 2-AB labelled standards on the GlycoSep N column. **A**; dextran hydrolysate, **B**; M3N2, MAN5, HYBRID, NGA2, NGA3, NGA4, NGA2FB, and A2.

Structure	Glucose units	Retention time
M3N2	4.09	25.2
NM3N2	4.62	28.0
MAN4	5.04	30.2
MAN5	5.98	37.0
MAN6	6.93	42.0
MAN7	7.88	47.4
HYBRID	6.98	42.7
NGA2	5.16	31.7
MAN8	8.83	53.2
MAN9	9.78	58.9
NGA2F	5.50	33.0
NGA2FB	5.97	36.6
NA2	7.04	42.6
NA2B	7.51	45.0
NGA2B	5.63	33.6
NGA3	5.63	34.5
NA3	8.33	50.1
NGA4	6.34	38.8
NA4	9.94	60.0
A1	8.26	50.5
A2	9.45	57.7
A2F	9.79	58.9

Table 2.9: Authentic and deduced retention times of 2-AB labelled glycans.

Structures in bold denote standards used in the calibration of the column.

2.9 *In vitro* translation and glycosylation

2.9.1 Subcellular fractionation and preparation of microsomes

A Beckman XL-90 Ultracentrifuge with SW50.1 and 70 Ti rotors was used to prepare microsomes from fibroblasts. The Sepharose CL-2B column was purchased from Pharmacia and the *in vitro* translation kit from Promega. The [³⁵S]-methionine was obtained from ICN. All other reagents were from Sigma.

2.9.1.1 Buffers

Buffer A: 50 mM TEA, 250 mM sucrose, 50 mM potassium acetate, 6 mM magnesium acetate, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, pH 7.5

Buffer B: 50 mM TEA, 250 mM sucrose, 1 mM DTT, pH 7.5

Buffer C: 50 mM TEA, 1.5 mM magnesium acetate, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, pH 7.5

Three different methods of subcellular fractionation were used to prepare microsomes from fibroblasts. The organelles present in each of the fractions obtained were identified by assaying the activity of marker enzymes. The translation activity and glycosylation capacity was determined by *in vitro* translation of α -factor in the presence of the microsomal fraction.

2.9.1.2 Homogenisation of fibroblasts

Cells were disrupted either by shearing in an homogeniser or by nitrogen cavitation. Cells were lysed by 40 strokes in a 1 ml Dounce homogeniser. For nitrogen cavitation, a suspension of the cells was placed in the nitrogen cavitation bomb and nitrogen was applied at 50 psi for 10 min. The pressure was released gently and the cells collected. The cavitation bomb was then disconnected from the nitrogen gas supply and flushed out with 1 ml of buffer A (section 2.9.1.1). Samples of the lysed and the unbroken cells were spotted onto microscope slides and a drop of trypan blue was added to each sample. The slides were then visualised under a phase contrast microscope and the percentage of broken cells was estimated by counting the number of cells that had taken up the trypan blue dye.

2.9.1.3 Method 1: Preparation of microsomes by gel filtration followed by ultracentrifugation

This method was based on that described by Walter and Blobel (1983). Fibroblasts were harvested, washed twice in buffer A, re-suspended in buffer A and homogenised as in section 2.9.1.2. The homogenate was centrifuged in the Ti 70 fixed angle rotor at 12,000 x g (12800 rpm) for 10 min at 4°C. The void volumes of two gel filtration (Sephadex CL-2B) columns were measured using Blue dextran (10 mg/ml) and measuring the absorbance of the effluent at 625 nm. The supernatants were then applied to the gel filtration columns which had been equilibrated with buffer C and the microsomes were eluted overnight at 25 ml/hr. The void volume was collected (fractions 4-15 inclusive) and centrifuged on a 1.3M sucrose cushion in buffer A at 140,000 x g for 2 hours at 4°C. The resulting pellets were re-suspended in 1.5 ml of buffer B and centrifuged on the Ti 70 fixed angle rotor for 30 min at 40,000 x g. The pellets were re-suspended in 100 µl of buffer B and stored at -20°C.

2.9.1.4 Method 2: Preparation of microsomes by differential centrifugation

As the samples were found to be diluted too much by gel filtration it was decided that differential centrifugation should be investigated as an alternative method for the purification of microsomes from fibroblasts. Lysed cells were centrifuged at 12,000 x g (12,800 rpm) in the Ti 70 fixed angle rotor for 10 min at 4°C. The supernatant was collected, and the pellet re-suspended in buffer A. This suspension was then re-applied to the nitrogen cavitation bomb for a second time and centrifuged as above. The pooled supernatants were added to 8 ml of buffer A and loaded on top of a 5 ml sucrose cushion (1.3M sucrose in buffer A). The tubes were centrifuged at 140,000 x g (28k rpm) for 2 hours at 4°C. The resulting pellet was re-suspended in 200 µl of buffer B and stored at -70°C in 50 µl aliquots.

2.9.1.5 Method 3: Preparation of crude microsomes by centrifugation

This method was based on that described by Knauer *et al.* (1994). After nitrogen cavitation of the cell pellets and removal of cell debris by centrifugation, the supernatant was centrifuged in the SW 50.1 swing out rotor for 30 min at 66,000 x g. The resulting

pellet was re-suspended in 200 μ l of buffer B and stored at -70°C in 50 μ l aliquots.

These microsomes were active and so this method was used to prepare microsomes from both normal and PMM-deficient CDGS type 1 fibroblasts (Chapter 6).

2.9.1.6 Assay of marker enzymes for organelles

For each marker enzyme 50 μ l of each fraction (supernatant or re-suspended pellet) was assayed in a microtitre well plate. 50 μ l of the appropriate substrate was added and the plate was incubated for 2 to 4 hours at 37°C with shaking. The reaction was stopped by the addition of 150 μ l of 0.25M glycine/NaOH, pH 10.4 containing 0.01% (v/v) Triton X-100. The fluorescence was measured using an excitation wavelength of 365 nm and emission wavelength of 450 nm on a Perkin Elmer LS 50.

Substrates

Lysosomal marker; β -hexosaminidase

3 mM 4-methylumbelliferyl-2-acetamido-2-deoxy- β -D-glucopyranoside in phosphate-citrate buffer pH 4.5

Endoplasmic reticulum marker; neutral α -glucosidase

0.375 mM 4-methylumbelliferyl- α -glucopyranoside in 0.25M sodium cacodylate buffer, pH 7

Golgi marker; α -mannosidase

6 mM 4-methylumbelliferyl α -D-mannopyranoside in phosphate-citrate buffer pH 5.6

2.9.2 *In vitro* translation

The *in vitro* translation kit (Promega) consisted of rabbit reticulocyte lysate (L4960), amino acid mixture (minus methionine) (L9961), core glycosylation control mRNA (α factor) (Y4071) and canine pancreatic microsomal membranes (Y4041).

The following reagents were added to each sterile microcentrifuge tube (0.5 ml): nuclease-treated rabbit reticulocyte lysate (17.5 μ l), 1 mM amino acid mixture (minus methionine) (0.5 μ l), [^{35}S] methionine (1,200 Ci/mmol) at 10 mCi/ml (2.0 μ l), α -factor

mRNA at 0.1 µg/µl (1.0 µl), and appropriate amounts of membranes and ribonuclease-free water to make a total volume of 35 µl. The tubes were incubated at 30 °C for 1 hour and then stored on ice. 10 µl of each reaction mixture was removed and added to 20 µl of sample buffer (2.4.2) for SDS-PAGE analysis. The tubes were boiled for 2 min and 5 µl of each sample was analysed on a 15% separating gel (2.4.2) with a 4% stacking gel (2.4.2). The gel was silver stained directly and dried in a frame (2.4.2). This method is described in the Promega technical manual #TM231.

2.10 Matrix assisted laser desorption time of flight mass spectrometry (MALDI-TOF)

Samples of purified transferrin were dialysed extensively against water and freeze-dried. For analysis by MALDI-TOF MS they were re-dissolved in sinapinic acid (10 mg/ml) 60% water:acetonitrile (6:4) containing 0.1% TFA. The samples were analysed on a Micromass TofSpec E MALDI-TOF mass spectrometer, in linear mode. The mass spectrometer was calibrated with BSA. A N₂ UV laser was used which produces a 4ns pulse width at wavelength of 337 nm and each pulse has an energy of 180 microjoules. For data acquisition, the laser energy was 50%, the source was 25000 V and the focus was 23500 V. The mass suppression was set at 500 Da. This work was carried out by Dr Andrew Johnson, Institute of Child Health.

2.11 Genetic analysis

Genomic DNA was extracted from whole blood in EDTA, fibroblasts, cultured amniotic cells, cultured chorionic villi cells and frozen chorionic villi. DNA from the fetus, index case and the parents was haplotyped using microsatellite markers on both sides of the CDGS 1 gene (Martinsson *et al.*, 1994; Bjursell *et al.*, 1997). The markers were D16S495, AFMA284WD5, GATAP6084, D16S768, D16S406, D16S519 and D16S500. This work was carried out by Tommy Martinsson, Jan Wahlstrom and Cecilia Bjursell University of Gothenburg, East Hospital, Gothenburg, Sweden.

2.12 Statistical analysis

Statistical analysis was performed using both the Mann-Whitney U test and the small sample t-test. The Mann-Whitney U test involves no assumptions about the distribution of the population and can be used for groups with as little as three samples in each group. The values from each group are ranked and the U value is calculated by determining how many test values preceded the control values. The small sample t-test does assume that the distribution of the population is normal. With such small sample numbers it is not possible to know whether this is true or not (Siegel, 1959; Swinscow, 1988). In the tests performed on the data presented in this thesis, both the Mann-Whitney U test and small sample t-test were in agreement.

Phosphomutases in fibroblasts from CDGS and galactosaemic patients

3.1 Introduction

The aim of the work described in this chapter was to determine whether there was a deficiency of phosphomannomutase (PMM) in the eight British CDGS type 1 patients, as had been found in other European patients (Van Schaftingen and Jaeken, 1995). A reliable method for the determination of PMM activity in both fibroblasts and lymphoblastoid cells was developed based on the fluorimetric measurement of NADPH generated in an enzyme-coupled assay. The activities of two other enzymes on the same pathway (Figure 3.1); phosphomannose isomerase and phosphoglucose isomerase, were also measured in the CDGS type 1 patients.

Human phosphomannomutase activity was subsequently found to be the product of two genes; PMM1 and PMM2 (Matthijs *et al.*, 1997a and 1997b). Therefore evidence for two PMM isoenzymes was sought. PMM-deficient CDGS type 1 patients lack only the PMM2 gene product and not the PMM 1 gene product, so the residual PMM activity in these patients is believed to be due to PMM1 (Matthijs *et al.*, 1997b). The enzyme activity-pH profile of phosphomannomutase in fibroblasts from CDGS type 1 patients and controls were obtained to see whether the two isoenzymes could be distinguished by the pH-dependence of their activities. As phosphoglucosyltransferase is also known to catalyse the interconversion of mannose-1-phosphate and mannose-6-phosphate (Weller, 1979), the isoenzymes of PGM in red blood cells and fibroblasts from CDGS type 1 patients were analysed to see if there was any difference in CDGS type 1.

3.2 Basis of the assay

The phosphoglucosyltransferase and phosphomannomutase assays used in this study were based on those described by Van Schaftingen and Jaeken (1995). The strategy for the measurement of PMM activity is as follows; mannose-1-phosphate (the substrate) is converted to mannose-6-phosphate by the action of PMM in the presence of the

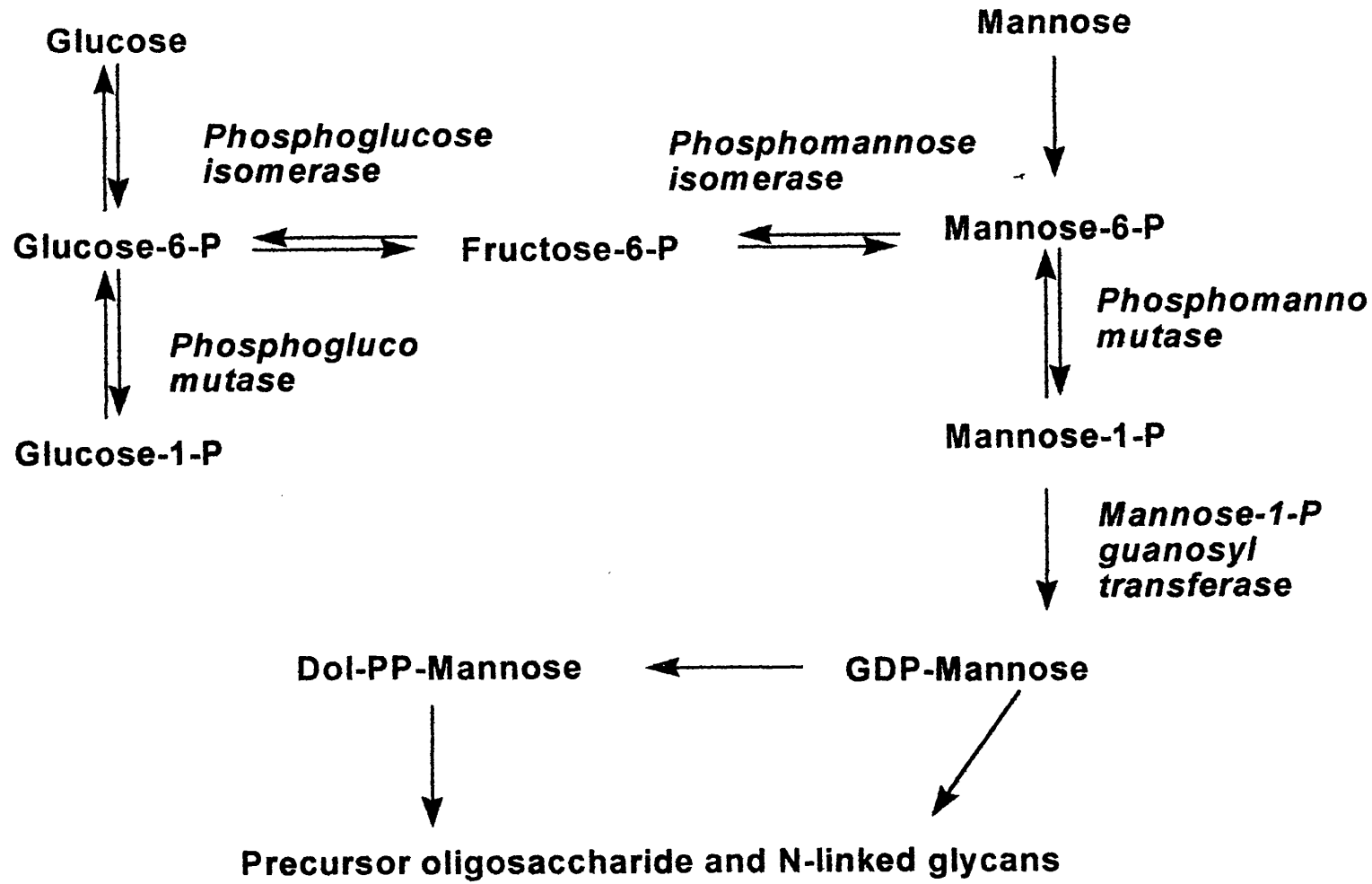


Figure 3.1: The reactions involved in the synthesis of mannose-1-phosphate from glucose and mannose.

cofactor mannose-1,6-bisphosphate. The product, mannose-6-phosphate, is converted to fructose-6-phosphate by phosphomannose isomerase (PMI) and then to glucose-6-phosphate by phosphoglucose isomerase (PGI). Finally, glucose-6-phosphate dehydrogenase is used to oxidise glucose-6-phosphate to 6-phosphogluconate in the presence of NADP. The rate of the PMM reaction can be determined by measuring the rate of production of NADPH if all the linking enzymes and cofactors are present in excess making the PMM reaction the rate-limiting step (Figure 3.2).

Similarly, PGM activity is measured by using glucose-1-phosphate as the substrate and glucose-1,6-bisphosphate as the cofactor and measuring the rate of NADPH production by the action of glucose-6-phosphate dehydrogenase on the product of the PGM reaction, glucose-6-phosphate.

3.3 Optimisation of the assay

3.3.1 Sensitivity

The original methods were made more sensitive by following the production of NADPH fluorimetrically rather than spectrophotometrically, using an excitation wavelength of 340 nm and an emission wavelength of 460 nm. The fluorescent readings were plotted against time and the linear part of the line was taken as the gradient for the calculation of enzyme activity. The fluorimeter used 500 μ l quartz cuvettes which allowed the assay to be performed with half the volume used previously (Van Schaftingen and Jaeken, 1995). The temperature of the assay (30°C) was controlled by a circulating water bath (section 2.6).

3.3.2 Enhancement of phosphomannomutase activity by mannose-1,6-bisphosphate

The cofactor mannose-1,6-bisphosphate was synthesised in a large quantity (4 ml), and stored at -20°C as 200 μ l aliquots (section 2.6.3). The concentration of mannose-1,6-bisphosphate was determined by calculating the concentration of NADPH produced (again by linking the reaction to glucose-6-phosphate dehydrogenase) and assuming that this related directly to the production of mannose-1,6-bisphosphate.

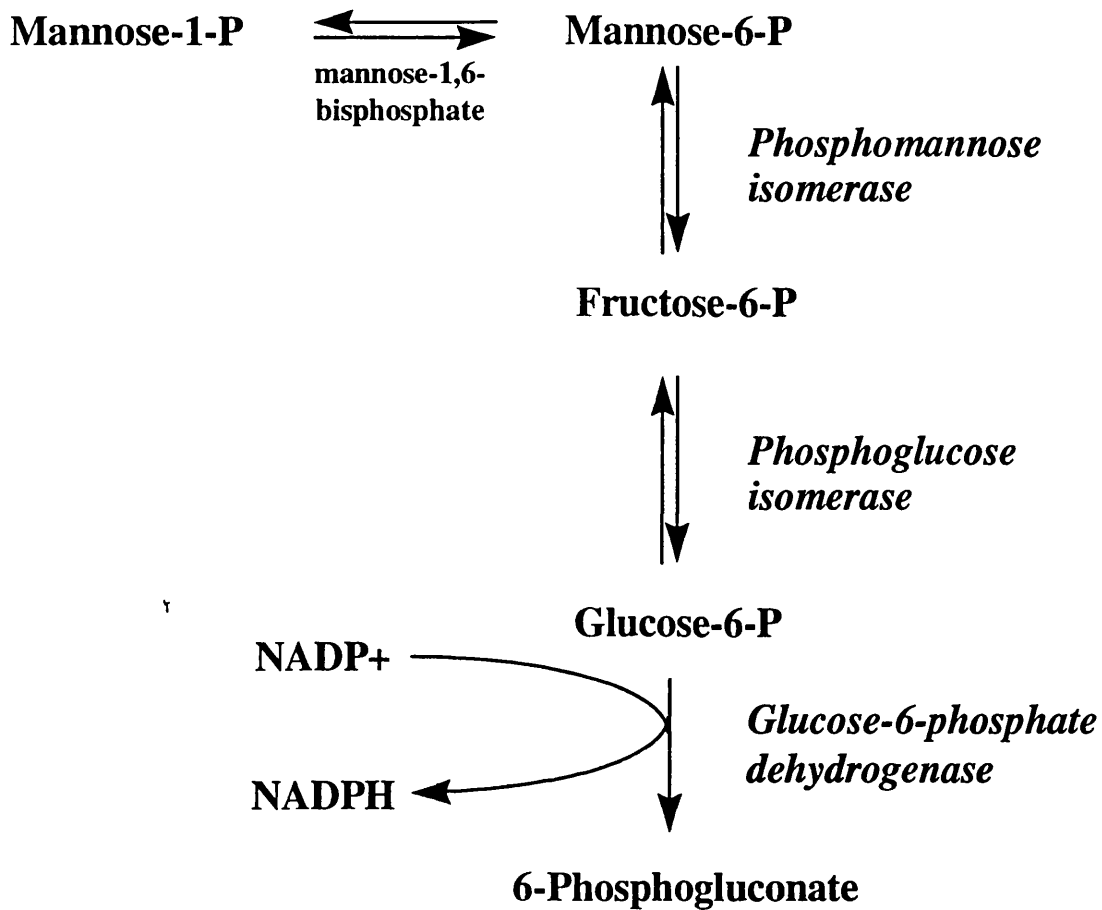
Phosphomannomutase

Figure 3.2: Measurement of phosphomannomutase activity in fibroblasts via an enzyme-coupled assay system.

The cell extracts are incubated with mannose-1,6-bisphosphate, phosphomannose isomerase, phosphoglucose isomerase, glucose-6-phosphate dehydrogenase and NADP. The reaction is initiated by the addition of mannose-1-phosphate and the rate of reaction determined by fluorimetric measurement of the production of NADPH.

It is probable that some mannose-6-phosphate was produced, resulting in an over estimation of the amount of mannose-1,6-bisphosphate present in the mixture. Thus a pre-run was introduced to the PMM assay procedure to allow for this and deplete the endogenous mannose-1-phosphate and mannose-6-phosphate (section 2.6.4). The concentration of mannose-1,6-bisphosphate used in this investigation was calculated to be 10 μM . This cofactor preparation gave a 25% increase in PMM activity over the same concentration of glucose-1,6-bisphosphate, and as the same batch was used throughout this investigation the reproducibility of the results was not compromised.

3.3.3 Effect of protein concentration on the activity of phosphomannomutase

One of the problems with the assay used by Van Schaftingen and Jaeken (1995) was the amount of protein required for the phosphomannomutase assay. Their assay system was developed for protein concentrations of between 3 and 10 mg/ml. This is not practical in the diagnostic setting, where the average protein concentration from a 25cm² flask of fibroblasts is between 1 and 2 mg/ml. Even less protein is often obtained from cultured amniocytes and chorionic villi cells, so it was necessary to modify the assay to make it reliable at lower protein concentrations.

The specific activity of PMM was measured over a range of protein concentrations to determine the amount of protein for a reliable assay. There was a linear relationship between the protein concentration and the NADPH produced during the reaction over a protein concentration range of 0.7 mg/ml to 3 mg/ml (Figure 3.3). However, below 0.7 mg/ml the fluorescence was not proportional to the protein concentration. Dilution of the sample appeared to inactivate the PMM activity, so human serum albumin (HSA) and non-fat dried milk (Marvel) were investigated as protective agents of the enzyme activity at lower enzyme concentrations. The addition of 0.1% HSA or Marvel decreased the PMM activity by 20% and 56% respectively (at 1.5 mg/ml protein concentration), showing that neither of these two reagents could protect the phosphomannomutase activity at low enzyme concentrations. It was concluded that for fibroblasts the protein concentration of each sample should be between 1 and 3 mg/ml, and where possible all

samples in one batch should be diluted to the same protein concentration (usually about 2 mg/ml, depending on the size of the smallest cell pellet).

3.3.4 Stability of phosphomannomutase and phosphoglucomutase activities in frozen cell pellets

Initially fibroblasts were harvested by trypsinisation, frozen and stored at -20°C until required (section 2.2). It was discovered that the PMM and PGM activities decreased with a storage period of greater than 3 months at -20°C . Therefore cell pellets were stored at -70°C at which temperature the activity of PMM and PGM was not affected even after storage for one year. All CDGS type 1 cell lines and controls were harvested and stored in exactly the same manner. The data presented in this chapter were obtained using freshly frozen cells which had been stored for less than 1 month at -20°C or -70°C . No difference was found for the PMM or PGM activities under these two sets of conditions. For the diagnosis of a PMM-deficient CDGS type 1 patient, PMM and PGM activities were always measured in the same sample at the same time. The PGM activity was used as quality control of the samples as it had been reported (Van Schaftingen and Jaeken, 1995) that the PGM activity in CDGS type 1 fibroblasts is within the normal range for fibroblasts. The PGM/PMM ratio was determined for each patient and cell pellets with low or no PGM activity were discarded.

3.4 Phosphomannomutase activity in fibroblasts and lymphoblastoid cells

3.4.1 Phosphomannomutase activity in CDGS type 1 patients

The activity of PMM was determined in the fibroblasts of six British CDGS type 1 patients (EW, DC, JB, KT, CB and NH). Five of the six CDGS type 1 patients (EW, DC, JB, KT and CB) had less than 10% of the activity of PMM compared with normal fibroblasts (Table 3.1 and Figure 3.4). The PGM activities in the fibroblasts from these CDGS type 1 patients were within the normal range and the PGM/PMM ratios were elevated. PMM activity was also measured in lymphoblastoid cells derived from two siblings with CDGS type 1; VB and TB. They had PMM activities of 0.04 and 0.24 nmol/min/mg protein respectively, compared to a mean control value of 3.0 nmol/min/mg

protein. These data confirmed the findings of Van Schaftingen and Jaeken (1995) that most CDGS type 1 patients have a marked deficiency of PMM.

	PMM nmol/min/mg	PGM nmol/min/mg	PGM/PMM ratio
Control mean n=23	1.67 SD = 0.58	38.3 SD = 21.7	26.0 SD = 17.6
Control range	1.03 - 3.82	11.6 - 92.9 ^{\$}	7 - 76 ^{\$}
CDGS mean n=10	0.12* SD = 0.05	41 SD = 22.4	453* SD = 413
CDGS range	0.05 - 0.18	9.1 - 75.5	70 - 1510

Table 3.1: Phosphomannomutase and phosphoglucomutase activity in the fibroblasts from PMM-deficient CDGS type 1 patients and controls.

The PMM activity for each cell line was determined on at least two separate occasions (n = number of determinations). There were 10 control cell lines and 5 CDGS type 1 cell lines. * significantly different at the 95% confidence level (small sample t-test). \$ = outlier N.B. The PMM activity of patient NH is not included in the data presented in this table.

	PMM nmol/min/mg	PGM nmol/min/mg	PGM/PMM ratio
Control mean n=23	1.67 SD = 0.58	38.3 SD = 21.7	26.0 SD = 17.6
Control range	1.03 - 3.82	11.6 - 92.9	7 - 76
Galactosaemic mean (n = 4)	1.5 SD = 0.3	34 SD = 12.7	24 SD = 14
Galactosaemic range	1.1 - 1.9	19.0 - 52.0	13 - 48

Table 3.2: Phosphomannomutase and phosphoglucomutase activity in the fibroblasts from patients with galactosaemia and controls.

The PMM activity for each cell line was determined on at least two separate occasions (n = number of determinations). There were 10 control cell lines and 2 galactosaemic cell lines.

The PMM activity in fibroblasts from the eighth patient (NH) was 1.76 and 1.23 nmol/min/mg protein (duplicate measurements) compared to a control range of 1.03-3.82 nmol/min/mg protein i.e. well within the normal reference range (Figure 3.4) (Charlwood *et al.*, 1996 and 1997). This patient therefore did not have a deficiency of phosphomannomutase. This patient had a serum transferrin isoelectric focusing pattern consistent with a diagnosis of CDGS type 1, but some clinical features distinct from classical CDGS type 1 (section 2.1). This patient appeared to represent a variant form of CDGS type 1 (Charlwood *et al.*, 1996). Subsequently Jaeken's group also reported that 20% of the European CDGS type 1 patients had normal PMM activity (Jaeken *et al.*, 1997). For the remainder of this thesis NH will be referred to as a PMM-normal CDGS type 1 patient. The details of his clinical presentation are described in Chapter 2, and the diagnosis of CDGS type 1 by IEF and immunodetection for transferrin are detailed in Chapter 4, along with full characterisation of the glycans of his serum transferrin (Charlwood *et al.*, 1997).

3.4.2 Phosphomannomutase activity in galactosaemic patients

The activity of PMM in fibroblasts from patients with galactosaemia was within the normal range for controls (Table 3.2), demonstrating that the activity of PMM is not affected in this secondary carbohydrate-deficient glycoprotein syndrome. A deficiency of PMM is a unique feature of CDGS type 1 patients.

3.5 Measurement of activities of other enzymes involved in the biosynthesis of glycosylation precursors

Inhibition of phosphomannose isomerase (PMI) by fructose-1-phosphate has been found to be the cause of the CDGS-like transferrin pattern seen on IEF of serum from hereditary fructose intolerance (HFI) patients (Jaeken *et al.*, 1996). On the basis of this observation it was considered possible that a deficiency of PMI in the PMM-normal CDGS type 1 patient could be the cause of CDGS type 1. The same argument could also be applied to phosphoglucose isomerase (PGI) and mannose-1-phosphate guanosyl transferase as they are in the same pathway as PMM leading to the production of GDP-mannose and dolichol-P-mannose (Figure 3.1).

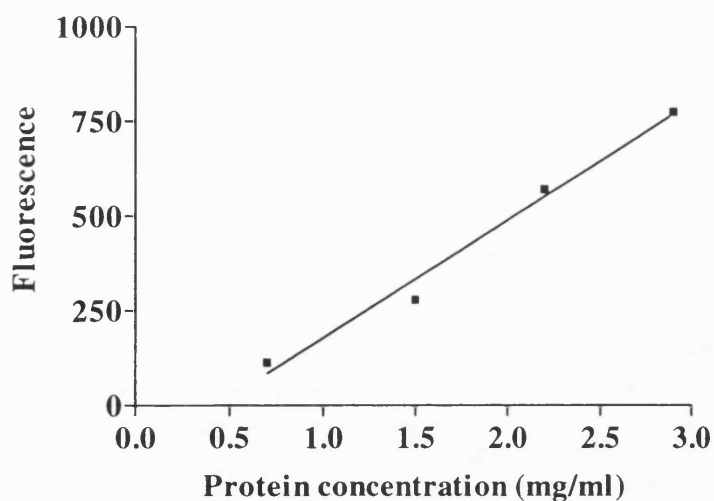


Figure 3.3: Effect of protein concentration on phosphomannomutase activity.

Net change in fluorescence (arbitrary units) plotted against protein concentration in the range of 0.7 mg/ml to 3 mg/ml for control fibroblasts.

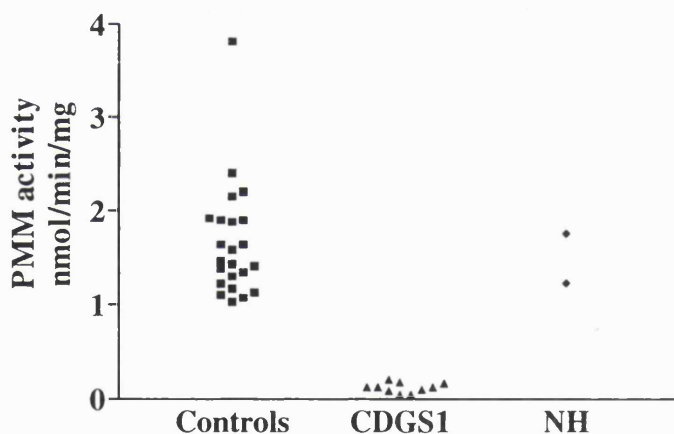


Figure 3.4: Comparison of PMM activities in fibroblasts from controls, PMM-deficient CDGS type 1 patients and patient NH (PMM-normal CDGS type 1).

For each cell line the PMM activity was determined on at least two separate occasions.

There were 10 control cell lines and 5 PMM-deficient CDGS type 1 cell lines.

	Control	PMM-normal CDGS type 1	PMM-deficient CDGS type 1
Phosphoglucomutase (nmol/min/mg)	12 - 93 (n = 20)	30, 66	9 - 75 (n = 10)
Phosphomannose isomerase (nmol/min/mg)	14-23 (n = 4)	14, 12	11, 24
Phosphoglucose isomerase (nmol/min/mg)	689-839 (n = 3)	773	ND

Table 3.3. Phosphoglucomutase, phosphomannose isomerase and phosphoglucose isomerase activity in fibroblasts from the PMM-normal CDGS type 1 patient (NH), PMM-deficient CDGS type 1 patients and controls.

The number of determinations of the phosphoglucomutase activity are indicated, with each cell line being assayed on at least two separate occasions (number of control cell lines = 10, and CDGS type 1 cell lines = 5). The PMI activity was only measured once for each control cell line and each PMM-deficient CDGS type 1 cell line, but twice for the PMM-normal CDGS type 1 cell line. The PGI activity was measured only once for each control and PMM-normal cell line, and was not determined for the PMM-deficient CDGS type 1 fibroblasts.

A block at any one of the steps is likely to lead to a reduced production of GDP-mannose and possibly a phenotype similar to CDGS type 1. Therefore the activities of these three enzymes were determined in the fibroblasts of the PMM-normal CDGS type 1 patient.

The activities of PGI and PMI were found to be within the normal range in the PMM-normal CDGS type 1 fibroblasts (Table 3.3). The activity of PGM was measured as a control enzyme for each assay. The activity of PMI in the PMM-deficient CDGS type 1 patients was also found to be within the normal range (Table 3.3).

The method used to assay the mannose-1-phosphate-guanosyl transferase activity (Szumilo *et al.*, 1993) was based on an enzyme-coupled reaction with the conversion of NADP to NADPH (section 2.6.6). Unfortunately there was no distinguishable difference between the amount of fluorescence produced in the presence or absence of the substrate in the reaction mixture. An alternative method for the measurement of this enzyme activity in fibroblasts needs to be devised. Thus the cause of protein underglycosylation in the PMM-normal CDGS type 1 patient remains elusive.

3.6 Isoenzymes of phosphomannomutase and phosphoglucomutase

3.6.1 Activity/pH profile of phosphomannomutase in fibroblasts from CDGS type 1 patients

Phosphomannomutase is thought to exist as two isoenzymes in humans as there are two genes encoding PMM; PMM1 and PMM2 (Matthijs *et al.*, 1997a and 1997b). One way of differentiating between two isoenzymes is by their different pH-optima. This often arises from different compartmental localisations e.g. Golgi and lysosomal α -mannosidases have different pH-optima (Daniel *et al.*, 1994).

Figure 3.5A:

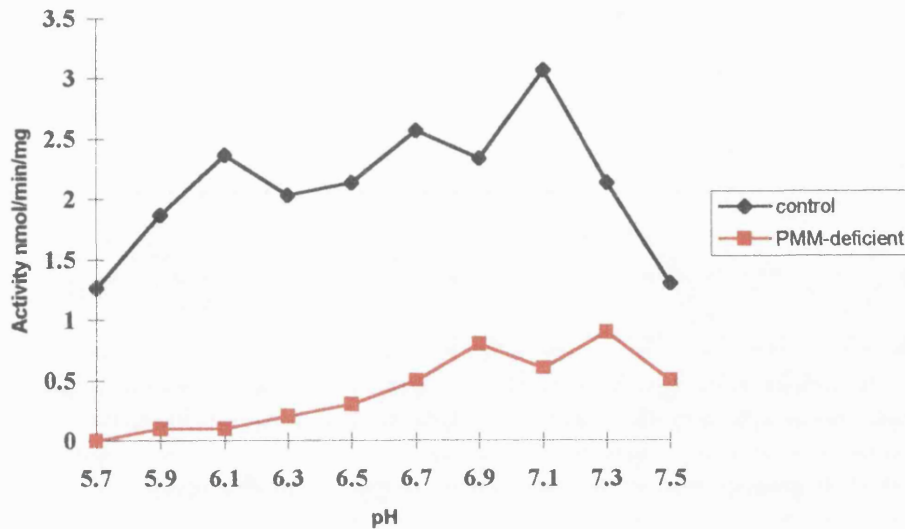


Figure 3.5B:

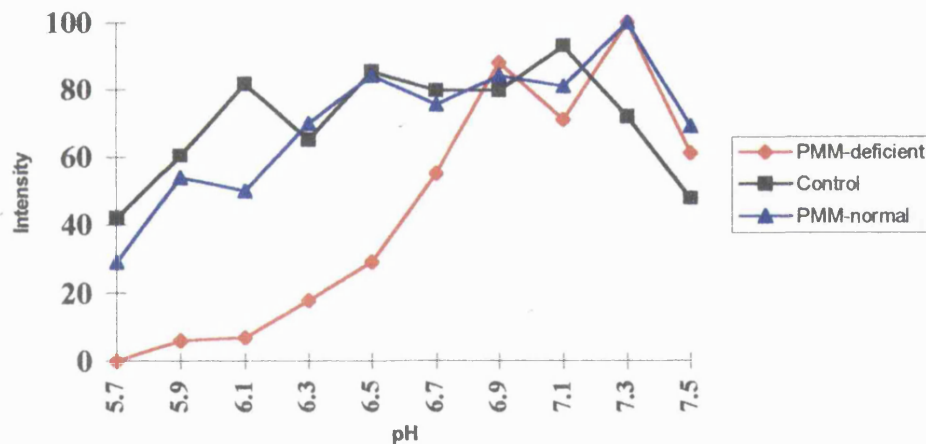


Figure 3.5: pH dependence of phosphomannomutase activity in fibroblasts

Black; normal fibroblasts, **Blue**; fibroblasts from PMM-normal CDGS type 1 patient and **Red**; fibroblasts from PMM-deficient CDGS type 1 patient. **A**: Specific activity of PMM (nmol/min/mg protein) versus pH, **B**: Data normalised as percentage of highest activity. The specific activities of PMM are higher than those reported in Table 3.1 because these assays were performed using a protein concentration that was above the range covered by the standard protein concentration curve (section 2.6.2) and so could only be estimated to be around 6 mg/ml.

The pH-optima of phosphomannomutase in control, PMM-normal and PMM-deficient CDGS type 1 fibroblasts were determined using the Hepes buffer system over the pH range, 5.7 to 7.5 (Figure 3.5). It was found that PMM was active over this pH range in the fibroblasts from controls and the PMM-normal CDGS type 1 patient. The PMM activity in the PMM-deficient CDGS type 1 fibroblasts was markedly decreased over the whole range of pH, but particularly at the lower range of pH (Figure 3.5A). It was considered that this residual activity was predominantly due to PMM1.

It was expected that the two gene products, PMM1 and PMM2, might have different pH-optima and that they could be distinguished by comparing the enzyme activity-pH profiles for the PMM-deficient CDGS type 1 cells and the normal cells, as the PMM-deficient samples would be lacking the PMM2 gene product (Matthijs *et al.*, 1997b). When the data was normalised as a percentage of the highest activity it was observed that there was a greater decrease in PMM activity at the lower pH range in the PMM deficient fibroblasts (Figure 3.5B). It is possible therefore that PMM2 has a lower pH-optimum than PMM1. However, it would seem that the majority of the PMM activity in fibroblasts is due to PMM2, as the highest residual PMM1 activity (at pH 7.3) in the PMM2 deficient fibroblasts is only 25% of the total activity (Figure 3.5A). The pH activity profile of PMM in the fibroblasts from the PMM-normal patient was very similar to that of the normal controls showing that there is not a deficiency of PMM1 or PMM2 in this case (Figure 3.5B).

3.6.2 Analysis of the isoenzymes of phosphoglucomutase by starch gel electrophoresis

The isoenzymes of PGM can be separated by starch gel electrophoresis and detected by staining for PGM activity (Harris and Hopkinson, 1976). This technique was carried out on extracts of red blood cells and fibroblasts from CDGS type 1 patients.

A typical analysis of the PGM activity in red blood cells by starch gel electrophoresis is shown in Figure 3.6A. The two uppermost bands are defined as PGM2 and the lower bands as PGM1. The pattern is complicated by polymorphism of PGM1.

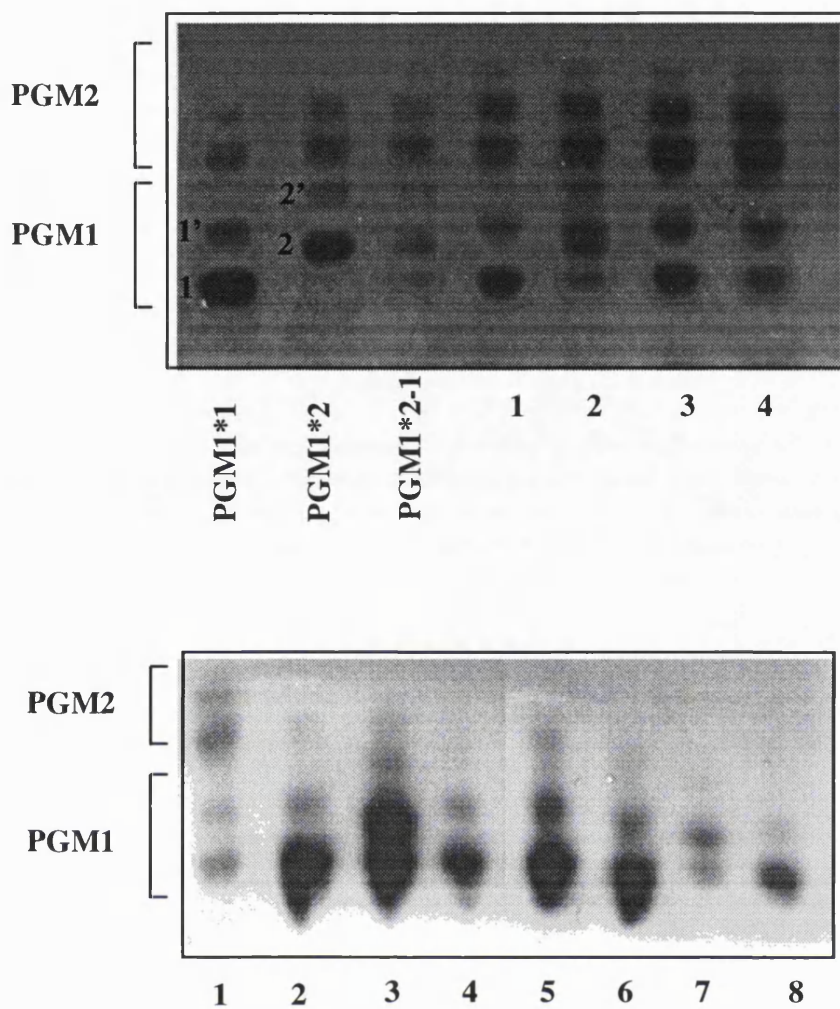


Figure 3.6 Starch gel electrophoresis of PGM isoenzymes.

A: *Phosphoglucomutase activity in red blood cells from controls and PMM-deficient CDGS type 1 patients.*

Lane 1; control (PGM1*1), lane 2; patient TB (PGM1*2-1), lane 3; patient VB (PGM1*1), lane 4; control (PGM1*2-1).

B: *Phosphoglucomutase activity in fibroblasts from PMM-deficient and PMM-normal CDGS type 1 and galactosaemic patients.*

Lane 1; control red blood cells (PGM1*1), lane 2; control (PGM1*1), lane 3; patient NH (PGM1*2-1), lane 4; patient CB (PGM1*1), lane 5; patient KT (PGM1*1), lane 6; galactosaemic patient (PGM1*1), lane 7, patient JB (PGM1*2-1) and lane 8, control (PGM1*1).

The isoenzymes of PGM1 can be separated into two alleles PGM1*1 and PGM1*2 and each of these appears as two bands on the gel (Whitehouse *et al.*, 1992). Thus in an individual heterozygous for the PGM1 polymorphism, four bands are seen in the PGM1 region of the gel. The two PMM-deficient CDGS type 1 patients (TB and VB) had a normal profile of PGM isoenzymes and were found to be PGM1*2-1 and PGM1*1 respectively (lanes 2 and 3). The controls (lanes 1 and 4) were PGM1*1 and PGM1*2-1 respectively (Figure 3.6A).

The PGM isoenzymes in fibroblasts were also investigated by starch gel electrophoresis. PGM2 is expressed mostly in red blood cells and so the PGM2 bands in fibroblasts were hardly visible, but the PGM1 bands were clearly seen (Figure 3.6B). For all CDGS type 1 patients (PMM-deficient and PMM-normal), the PGM1 isoenzymes were comparable with controls, as were the PGM isoenzymes from galactosaemic fibroblasts. The PMM-normal CDGS type 1 patient was PGM1*2-1, as was the PMM-deficient CDGS type 1 patient JB. All the other control, CDGS type 1 and galactosaemic patients were found to be PGM1*1. It was concluded that the PGM isoenzymes and activities in PMM-deficient and PMM-normal CDGS type 1 patients were normal. Also there was no correlation between the PGM1 polymorphism and the residual activity of PMM or the severity of CDGS type 1 disease, as the less severe CDGS type 1 patients had the PGM1*1 and PGM1*2-1 genotype as did the more severe CDGS type 1 patients.

3.7 Effect of inhibitors on phosphomannomutase and phosphoglucomutase activity

3.7.1 Effect of galactose-1-phosphate on phosphomannomutase activity

As galactose-1-phosphate accumulates in galactosaemia it is possible by analogy with HFI, that the cause of the aberrant glycosylation in galactosaemia may be inhibition of PMM by galactose-1-phosphate. This hypothesis was tested by measuring PMM activity in fibroblasts in the presence of galactose-1-phosphate. It was found that galactose-1-phosphate did not inhibit PMM, but it did inhibit both PGM and glucose-6-phosphate dehydrogenase (Table 3.4). Galactose-1-phosphate is known to inhibit PGM, glucose-6-phosphate dehydrogenase and glycogen phosphorylase (Gitzelman, 1995) but inhibition

of PMM activity had not been tested previously. The non-inhibitory effect of galactose-1-phosphate on PMM has been confirmed by Jaeken *et al.* (1996).

3.7.2 Effect of mannose and glucose derivatives on phosphomannomutase and phosphoglucomutase activities

A model system for PMM-deficient CDGS type 1 could be created by inhibition of the PMM activity in HepG2 cells, which synthesise transferrin. In order to achieve this specific inhibitors of PMM activity are required. Several synthetic derivatives of mannose phosphates were tested for inhibition of PMM activity. Derivatives of glucose were also tested for inhibition of PGM activity in order to gain further understanding of the reaction mechanisms of PMM and PGM (Figure 3.7). The inhibition of glucose-6-phosphate dehydrogenase activity was also tested as this enzyme is a linking enzyme in both enzyme assays, as the inhibition of glucose-6-phosphate dehydrogenase could lead to false positive results.

The glucose derivatives tested for inhibition of phosphoglucomutase were; 6R-6C-methylglucose, 6S-6C-methylglucose, 6R-6C-butylglucose, 6S-6C-butylglucose and 6R-azide.

6R-6C-Methylglucose showed the greatest inhibition of phosphoglucomutase; 34% at 1 mM (Table 3.4). There was no detectable inhibition of the linking enzyme glucose-6-phosphate dehydrogenase, so the inhibition observed was due to direct inhibition of PGM. Values of 4 mM and 0.5 mM were found for I_{50} and K_i respectively (Figures 3.8A and B). 6S-6C-Methylglucose only inhibited PGM by 10% at 1 mM concentration. 6R-6C-Butylglucose, 6S-6C-butylglucose and the 6R-azide only showed inhibition at 5 mM concentration. As the 6R-azide also inhibited glucose-6-phosphate dehydrogenase the inhibition of PGM by this inhibitor cannot be interpreted using this method of analysis. However, 6R-6C-butylglucose and 6S-6C-butylglucose did not inhibit glucose-6-phosphate dehydrogenase so the PGM results of these four inhibitors are reliable (Table 3.4). As all of these inhibitors were even less potent than 6R-6C-methylglucose, they were not investigated any further.

Inhibitor	mM	PGM	PMM	Glucose-6-phosphate dehydrogenase
galactose-1-phosphate	1	80	0	15
6S-6C-methylglucose	1	10	0	0
6R-6C-methylglucose	1	34	0	1
6C-methylmannose	5	100	100	31
6R-azide	5	28	ND	18
6S-6C-butylglucose	5	30	ND	2
6R-6C-butylglucose	5	49	ND	4
1-methyl castanospermine	10	38	ND	ND
swainsonine	0.8	0	0	ND

Table 3.4: Inhibition of phosphoglucomutase (PGM), phosphomannomutase (PMM), and glucose-6-phosphate dehydrogenase activities by synthetic galactose-1-phosphate and glucose and mannose analogues.

ND = not determined. Activity was measured in presence and absence of analogue.

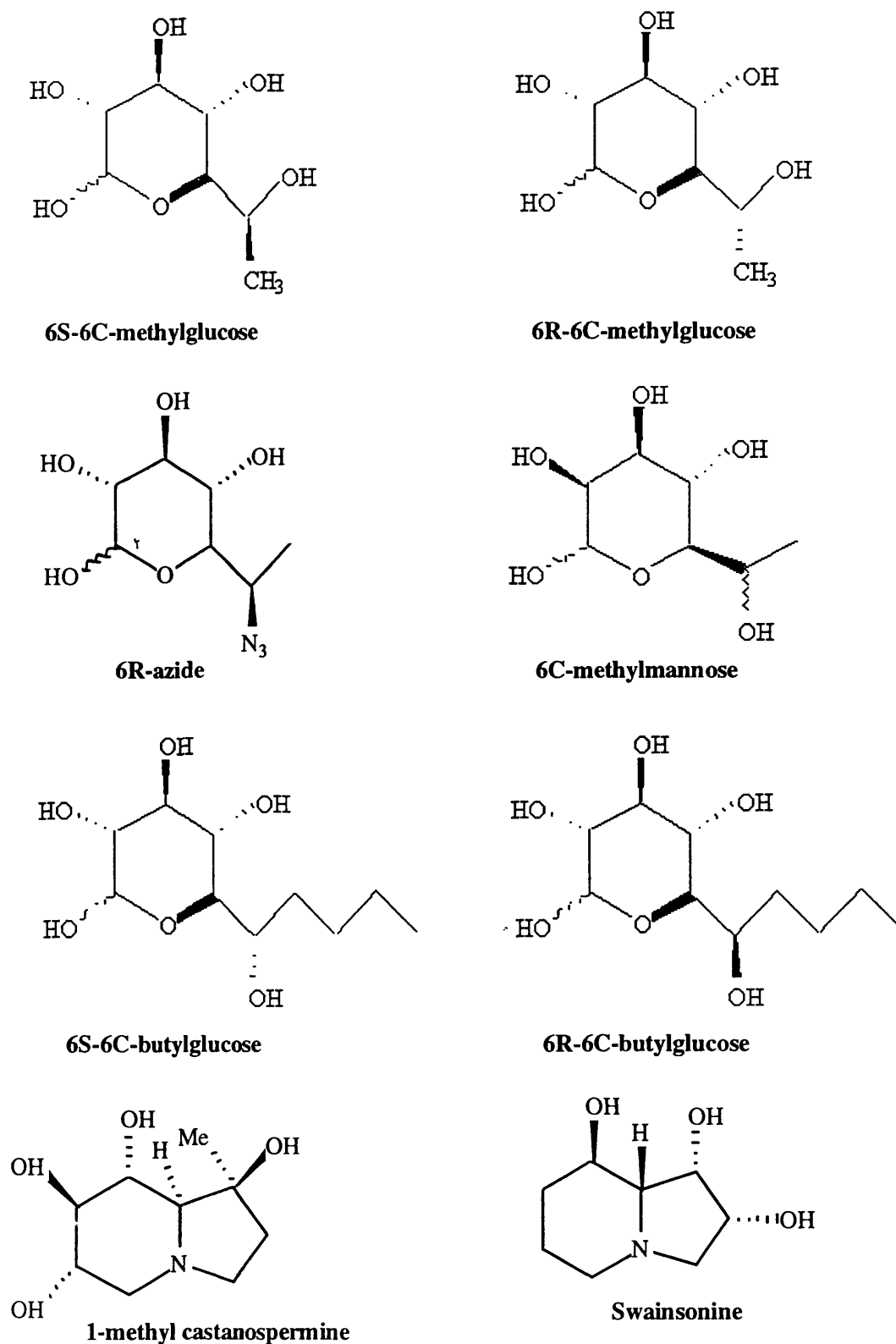


Figure 3.7 Structures of analogues used for the inhibition of phosphoglucomutase and phosphomannomutase activities.

The configuration at carbon 6 was not defined for 6C-methylmannose. This inhibitor completely inhibited both the phosphoglucomutase and phosphomannomutase activity at a concentration of 1 mM. However, it also inhibited glucose-6-phosphate dehydrogenase by 31% explaining the inhibition of both PMM and PGM. Thus the effect of 6C-methylmannose could not be studied using this method of analysis.

1-Methyl-castanospermine is a bicyclic analogue of 6R-6C-methylglucose. As castanospermine is an inhibitor of α - and β -glucosidases (Winchester and Fleet, 1992), 1-methyl-castanospermine was tested as a potential inhibitor of PGM activity. At 10 mM concentration 1-methyl-castanospermine inhibited PGM by 38% compared to 80% by the monocyclic analogue (Table 3.4). Swainsonine is a potent inhibitor of both lysosomal and Golgi α -mannosidases (Winchester and Fleet, 1992), but it did not inhibit PMM activity (Table 3.4).

6R-6C-Methylglucose was the best inhibitor of PGM activity. As it was a better inhibitor than 6S-6C-methylglucose it suggests that the R configuration is required for optimal inhibition of PGM. The substitution of butyl groups for methyl groups to make 6R-6C-butylglucose and 6S-6C-butylglucose confirmed that the R configuration caused greater inhibition than the S configuration, but increasing the size of the alkyl groups decreased the inhibition. The 6R-azide analogue was not a good inhibitor of PGM although it had the R configuration. 1-Methyl-castanospermine also has the R configuration at the comparable C6 position to 6R-6C-methylglucose, but it was not a good inhibitor of PGM activity. The presence of the nitrogen instead of the oxygen might cause a decrease in the inhibition of PGM or it could be because it was bicyclic and therefore sterically hindered.

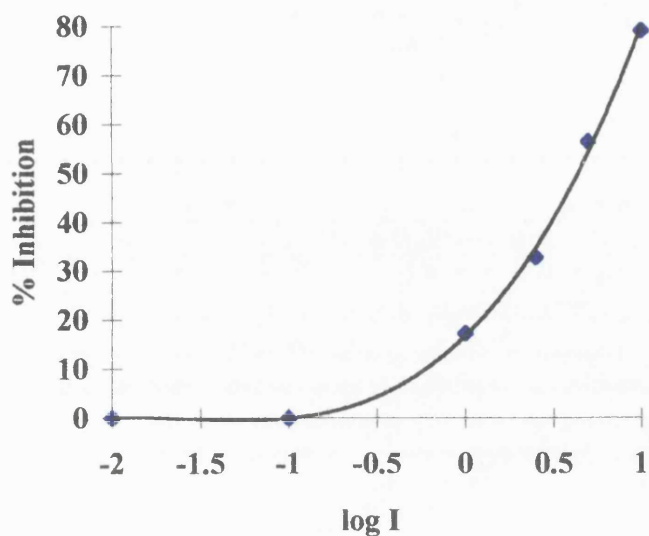


Figure 3.8A: Dose curve for inhibition of PGM by 6R-6C-methylglucose

The log of the inhibitor concentration was plotted against the percentage inhibition of PGM activity and the inhibitor concentration at which 50% inhibition occurred was determined.

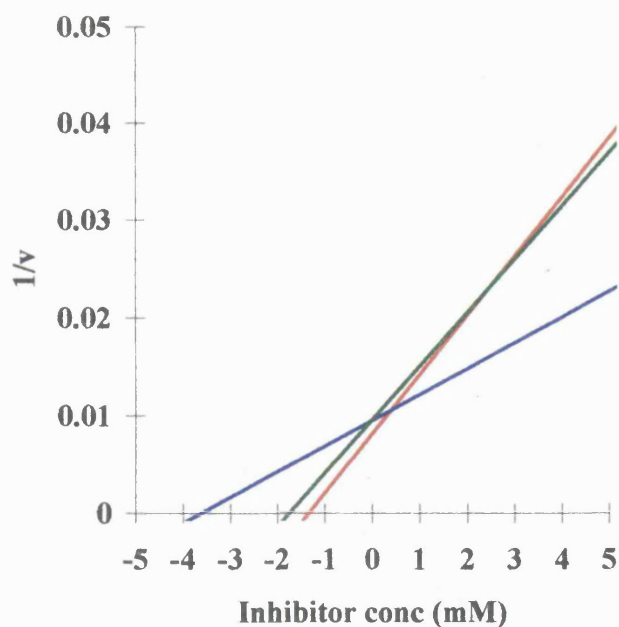


Figure 3.8B: Determination of K_i by Dixon procedure for inhibition of PGM by 6R-6C-methylglucose. The lines of best fit are shown. **Blue line;** 1 mM glucose-1-phosphate, **red line;** 0.25 mM glucose-1-phosphate, **green line;** 0.5 mM glucose-1-phosphate. The activity of PGM in the presence of inhibitor 165 was determined at 3 different substrate concentrations and the point of intersection of the red and green lines with the blue was used to determine the K_i of 6R-6C-methylglucose.

3.8 Assays of lysosomal enzymes in serum from the PMM-normal CDGS type 1 patient

As CDGS type 1 patients have raised levels of some serum lysosomal enzymes, the β -hexosaminidase and β -glucuronidase activities in the serum of patient NH were assayed to support the diagnosis of CDGS type 1 (section 2.7). The β -hexosaminidase activity was markedly raised but the β -glucuronidase activity was within the normal range (Table 3.5). These results are consistent with a diagnosis of CDGS type 1 for this patient (Clayton *et al.*, 1993).

	β -Glucuronidase (nmol/hr/ml)	β -Hexosaminidase (μ mol/hr/ml)
Control (n = 3)	154 - 286	0.85 - 1.82
PMM-normal CDGS type 1	211	2.63

Table 3.5: β -Hexosaminidase and β -glucuronidase activities in serum of controls and the PMM-normal CDGS type 1 patient.

The activity of the lysosomal enzymes was determined once in the same serum sample for each patient or control sample.

3.9 Discussion

3.9.1 A deficiency of phosphomannomutase is the major, but not the only cause of CDGS type 1.

During the course of this Ph.D. project, Van Schaftingen and Jaeken (1995) reported that some CDGS type 1 patients had a marked deficiency of phosphomannomutase in both fibroblasts and leukocytes. Therefore the PMM activity was measured in all the available British CDGS type 1 patients. The seven CDGS type 1 patients who had a deficiency of phosphomannomutase included those with the severe neonatal form of the disease (EW, CB, KT, JB and DC) as well as the two older children (VB and TB). There was however no correlation between the residual activity of PMM and the severity of the disease. There are two possible explanations for this. The residual activity could be due

to the PMM1 isoenzyme (Matthijs *et al.*, 1997b). Alternatively the cell sonicates used also contained PGM which has about a 30 fold greater activity than PMM, and as PGM can also convert mannose-6-phosphate to mannose-1-phosphate, this could be the reason that residual activity converting mannose-6-phosphate to mannose-1-phosphate is seen.

In order to assess whether the residual activity of PMM related to the PGM phenotype, the PGM isoenzymes for each patient were investigated by starch gel electrophoresis. Of the five severe patients, four of them had the PGM1*1 phenotype and the other one had the PGM1*2-1 phenotype. Of the two older patients one had the PGM1*1 phenotype and the other one was PGM1*2-1. All of the patients had normal PGM2 isoenzymes. There was no correlation between the PGM phenotypes and the PMM residual activity or the severity of the disease.

One CDGS type 1 patient had normal PMM activity (Charlwood *et al.*, 1996). Later on Jaeken *et al.* (1997a) also found that 20% of the CDGS type 1 patients investigated in a larger study had normal phosphomannomutase activity. More recent studies by us (Charlwood *et al.*, 1997) and others (Matthijs *et al.*, 1997a, 1997b; Jaeken *et al.*, 1997b) have led to the classification of CDGS type 1 patients into two classes; PMM-deficient or CDGS type 1A and PMM-normal or CDGS type 1B. It has been reported that the PMM-normal CDGS type 1 patients have less severe nervous system involvement than the PMM-deficient patients, although the clinical phenotype and transferrin patterns in each group are very similar (Charlwood *et al.*, 1996 and 1997, Jaeken *et al.*, 1997b). It has been shown that the PMM-deficient CDGS type 1 locus maps to chromosome 16, and patients have mutations in the PMM2 gene, but the PMM-normal CDGS type 1 locus does not map to chromosome 16 (Matthijs *et al.*, 1996, 1997a).

The diagnosis of CDGS type 1 in the PMM-normal patient was supported by the observation that he had increased activity of β -hexosaminidase in his serum which is another marker of CDGS type 1 (Clayton *et al.*, 1993). Unfortunately linkage analysis could not be performed on our PMM-normal CDGS type 1 patient's family as there are no unaffected siblings for comparison of the affected and unaffected haplotypes.

An attempt was made to elucidate the enzyme defect in the PMM-normal patient by measuring the activity of other enzymes on the same pathway. Both phosphomannose isomerase and phosphoglucose isomerase activities in fibroblasts of the PMM-normal patient were found to be within the normal range. An attempt to measure the activity of mannose-1-phosphate guanosyl transferase in fibroblasts from this patient using an enzyme-linked assay system was not successful. A deficiency of mannose-1-phosphate guanosyl transferase in PMM-normal CDGS type 1 could lead to a decreased production of GDP-Man and dolichol-P-Man and thus the same under-occupancy effect as is seen in the PMM-deficient CDGS type 1 patients. Another possibility was that the PMM-normal CDGS type 1 patient had a deficiency of PMM1 or an abnormal form of PMM not detectable by the standard assay conditions. Therefore the activity of PMM was measured over the range of pH 5.7 to 7.5. The activity-pH profile was normal indicating that a deficiency of PMM was not the cause of CDGS type 1 in this patient.

If the PMM-normal patient also shows non-glycosylation of serum glycoproteins, other candidate enzymes for the cause of CDGS type 1 in this patient would be oligosaccharyl transferase or any of the enzymes involved in the synthesis of dolichol or the lipid linked oligosaccharide precursor as investigated originally in the PMM-deficient CDGS type 1 patients (Stibler *et al.*, 1991a; Knauer *et al.*, 1994; Yasugi *et al.*, 1994; Panneerselvam and Freeze, 1995).

3.9.2 Studies on potential inhibitors of phosphoglucomutase and phosphomannomutase

Prior to the observation of Adamowicz and Pronicka (1996) that fructose-1-phosphate accumulation in HFI patients caused CDGS-like glycosylation defect in serum transferrin, we had speculated that the aberrant glycosylation observed in untreated galactosaemic serum transferrin might be due to inhibition of PMM by galactose-1-phosphate. Galactosaemic patients have an abnormal isoelectric focusing pattern of serum transferrin when untreated, which returns to the normal pattern after treatment on a galactose-free diet, as observed in HFI patients. However, galactose-1-phosphate was found not to inhibit PMM, and these results were later confirmed by Jaeken *et al.* (1996).

Previous observations that galactose-1-phosphate was an inhibitor of glucose-6-phosphate dehydrogenase and phosphoglucomutase activity (Gitzelman, 1995) were confirmed in these studies.

The use of derivatives or analogues of mannose as inhibitors of PMM activity proved to be unsuccessful in creating a chemically induced phenocopy of CDGS type 1, since none of these compounds was found to have a direct effect on the PMM activity in fibroblasts. The idea was to inhibit PMM activity in HepG2 cells, (transformed hepatocytes which synthesise transferrin), and then to characterise the isoforms of transferrin produced to investigate whether or not protein under-glycosylation defect can be reproduced.

On the other hand, glucose derivatives to inhibit PGM activity were found to be more successful. It was found that efficient inhibition of PGM activity required the substitution at C6 to be in the R configuration. The compounds became less effective as the size of the alkyl group increased. These findings are of potential use to synthesise another set of compounds which might inhibit PMM activity. The mannose equivalent of 6R-6C-methylglucose (i.e. 6R-6C-methylmannose) would be good candidate to test in future studies.

3.10 Conclusion

There are two variants of CDGS type 1; PMM-deficient and PMM-normal. Their clinical phenotypes are very similar and the two groups can only be distinguished by their PMM activities or by genetic studies. The molecular basis for CDGS type 1 in the PMM-normal patient is still unknown and requires further investigation. The question of whether or not the basis of the aberrant glycosylation of serum glycoproteins is the same in both groups is the subject of the next Chapter.

Structural analysis of the N-glycans of serum glycoproteins in CDGS and galactosaemic patients.

4.1 Introduction.

It has been established in Chapter 3 that there are two variants of CDGS type 1; PMM-deficient and PMM-normal. Although aberrant glycosylation of serum transferrin has been demonstrated in PMM-normal CDGS type 1 patients, it is not known whether it is due to under-occupancy of glycosylation sequons as in the case of the PMM-deficient CDGS type 1 patients (Wada *et al.*, 1992; Yamashita *et al.*, 1993a and 1993b; Iourin *et al.*, 1996), or whether there is a processing defect as in the case of CDGS type 2 (Jaeken *et al.*, 1993b and 1994; Schachter *et al.*, 1996). In this chapter transferrin is used as a model serum glycoprotein to investigate whether British PMM-deficient patients and the PMM-normal CDGS type 1 patient both have under-occupancy of glycosylation sites as described in the European and Japanese patients studied previously (Wada *et al.*, 1992; Yamashita *et al.*, 1993a and 1993b). This chapter also describes the analysis of the glycans from transferrin from patients with galactosaemia. Agalactosylation of serum glycoproteins from galactosaemic patients has been demonstrated previously by the observation that they are much better acceptors of galactose than those from normal controls, but no structural studies have been carried out to support this conclusion (Segal, 1995a; Prestoz *et al.*, 1997). The second aim of this chapter is to demonstrate conclusively that there is agalactosylation of complex glycans in patients with galactosaemia.

Serum transferrin is an ideal model glycoprotein for studying defects in N-glycosylation since it can be easily purified and it contains two disialylated biantennary glycans (Spik *et al.*, 1975) which can be used to investigate both non-occupancy of glycosylation sites and defects in the processing of complex glycans. The strategy for analysis of the glycans of serum transferrin is summarised in Figure 4.1. The aberrant glycosylation of transferrin, due to differences in sialic acid content, can be identified by isoelectric focusing (IEF) of serum and immunodetection of transferrin. Serum transferrin from

CDGS type 1 and galactosaemic patients can be purified by treatment with Rivanol followed by anion-exchange chromatography. The isolated isoforms were analysed by SDS-PAGE and MALDI-TOF to determine their molecular mass, and glycans were released enzymatically and labelled with a fluorescent tag for structural analysis by HPLC.

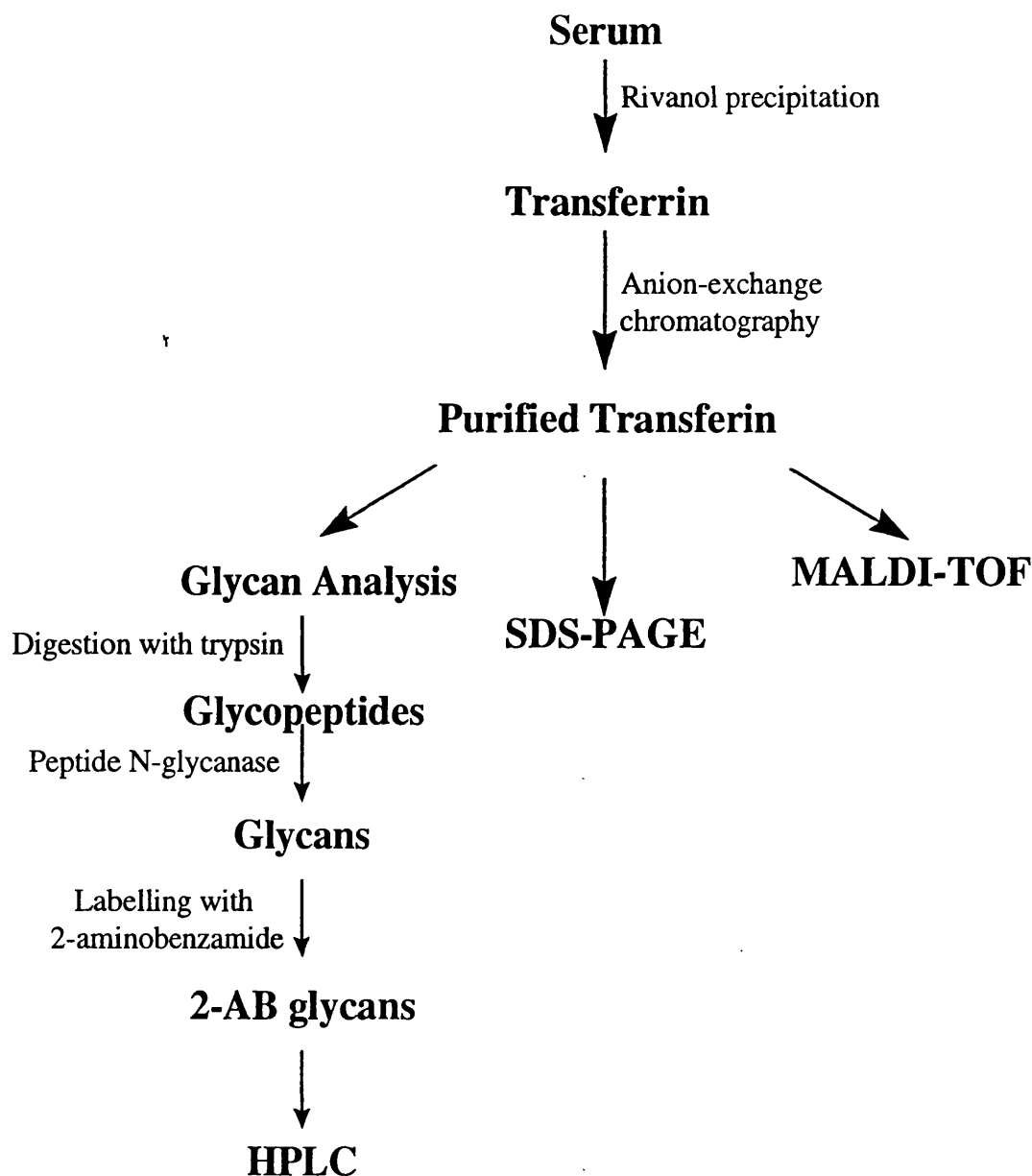


Figure 4.1: Experimental strategy for the analysis of the glycans from serum transferrin.

4.2 Results

4.2.1 Isoelectric focusing of serum transferrin

Serum samples from patients with CDGS type 1 (PMM-deficient and PMM-normal), and from patients with galactosaemia before and after treatment on a galactose-free diet (section 2.1) were analysed by isoelectric focusing using the PhastSystem followed by immunodetection for transferrin (section 2.4.1).

4.2.1.2 CDGS type 1 patients

The major isoform of transferrin in normal serum samples was found to be tetrasialotransferrin which had an estimated pI of 5.5 (Figure 4.2, lanes 1,2 and 6). However, there was a decrease in the relative amount of tetrasialotransferrin with a corresponding increase in the amount of disialotransferrin (pI 5.7) and asialotransferrin (pI 5.9) in the serum from the PMM-deficient CDGS type 1 patient (lane 5). There was also an increase in the amount of disialotransferrin in the PMM-normal patient (lanes 3 and 4) compared to that in the control, but the proportion of asialotransferrin was less than that found in the PMM-deficient CDGS type 1 patient. This pattern was however consistent with a diagnosis of CDGS type 1 (Stibler *et al.*, 1991a; Hagberg *et al.*, 1993; Jaeken *et al.*, 1993a).

4.2.1.2 Galactosaemic patients

Less acidic isoforms of serum transferrin were also seen in the sample from the untreated galactosaemic patient (Figure 4.3, lane 5). The major isoforms in this sample were tetrasialo-, disialo- and asialotransferrin, however, after treatment (lanes 3 and 4) the transferrin pattern returned to that seen in the normal control serum (lanes 1 and 6). The relative proportions of the transferrin isoforms were different from those found in CDGS type 1 (lane 2), the serum from the untreated galactosaemic patient having a greater proportion of tetrasialotransferrin. The sample in lane 4 was of a patient who had been on a galactose-free diet for three weeks and the sample in lane 3 was taken 9 months post-treatment, by which time the transferrin pattern was normal in this patient. These results confirm that in galactosaemic patients the initiation of a galactose-free diet

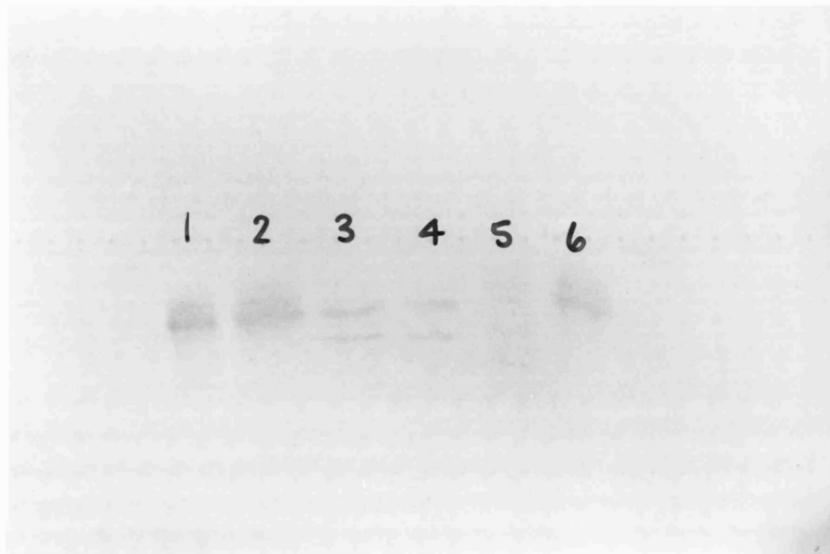


Figure 4.2: Isoelectric focusing of serum with a pH gradient of 3-9 and immunodetection of transferrin.

Lanes 1,2 and 6; controls, lane 5; CDGS type 1 (PMM-deficient), and lanes 3 and 4; CDGS patient NH (PMM-normal).



Figure 4.3: Isoelectric focusing of serum with a pH gradient of 3-9 and immunodetection of transferrin.

Lanes 1 and 6; controls, lane 2; CDGS type 1 (PMM-deficient), lanes 3 and 4; treated galactosaemic patient (9 months and 3 weeks post-treatment respectively), and lane 5; untreated galactosaemic patient.

appears to correct the aberrant glycosylation of serum transferrin (Winchester *et al.*, 1995). The serum transferrin from parents of patients with CDGS type 1 and galactosaemia were also analysed by isoelectric focusing and immunodetection and in all cases they were found to have a normal pattern.

4.2.2 Isoelectric focusing of serum α 1-antitrypsin

α 1-Antitrypsin is another serum glycoprotein that can be used as a biochemical marker of under glycosylation in CDGS type 1 patients (Krasnewich *et al.*, 1995). Serum samples from patients with CDGS type 1 (PMM-deficient and PMM-normal), and patients with untreated galactosaemia were analysed by isoelectric focusing using the PhastSystem followed by immunodetection of α 1-antitrypsin (section 2.4.1) (Figure 4.4). Normally α 1-antitrypsin consists of 3 isoforms; I, II and III (Figure 1.12), where isoform III is the most heavily sialylated, containing two triantennary glycans (Carrell *et al.*, 1982).

4.2.2.1 CDGS type patients

The serum α 1-antitrypsin from both PMM-deficient (Figure 4.4, lane 6) and PMM-normal (lanes 1 and 4) CDGS type 1 patients had abnormal isoforms when compared to the controls (lanes 3,5 and 7). There was an increase in the amount of the less sialylated isoforms I and II of α 1-antitrypsin in serum from CDGS type 1 patients. There were also some extra bands, which were less acidic, corresponding to partially non-glycosylated isoforms of α 1-antitrypsin in the CDGS type 1 patients. These results confirm earlier observations made by Clayton *et al.* (1993) and Krasnewich *et al.* (1995). The PMM-normal and PMM-deficient CDGS type 1 patients had similar IEF pattern of isoforms of serum α 1-antitrypsin.

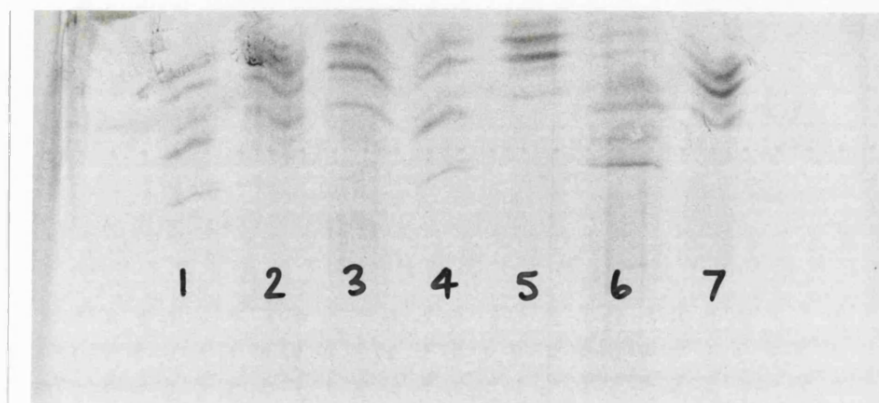


Figure 4.4: Isoelectric focusing of serum with a pH gradient of 4-6.5 and immunodetection of α 1-antitrypsin.

Lanes 3, 5 and 7; controls, lane 6; CDGS type 1 (PMM-deficient), lanes 1 and 4; CDGS patient NH (PMM-normal), lane 2; untreated galactosaemic.

4.2.2.2 Galactosaemic patients

The serum α 1-antitrypsin from a patient with untreated galactosaemia (Figure 4.4, lane 2) did not show the undersialylated isoforms of α 1-antitrypsin like that seen in the CDGS type 1 serum. This was contrary to observations made on serum transferrin, where sialic acid-deficient isoforms were clearly seen in the untreated galactosaemic patient. It seems that in contrast to CDGS type 1, the serum transferrin was under-glycosylated and serum α 1-antitrypsin was normally glycosylated in galactosaemic patients.

4.2.3 Anion-exchange chromatography of transferrin

Serum¹ transferrin was purified using Rivanol, which precipitates the majority of serum proteins except the immunoglobulins, which were subsequently removed by precipitation

¹ As only 200 μ l of serum but 60 ml of ascitic fluid was available from patient NH, transferrin was purified from ascitic fluid. The transferrin isoforms in serum and ascitic fluid were shown to be identical by IEF.

with saturated ammonium sulphate (section 2.3). The transferrin in the remaining supernatant was then analysed by anion-exchange chromatography on a BioScale Q2 column using a 0-0.2 M NaCl linear gradient in 20 mM Tris buffer pH 7.4. The absorbance of the effluent was monitored at 280 nm and 2 ml fractions were collected. The identity of the transferrin isoform in each peak was determined by IEF followed by immunodetection for transferrin. The breakthrough peak contained immunoglobulins and the wash at the end of the gradient contained residual albumin. The identity of the peaks was determined by calibration of the column with authentic standards and isoelectric focusing. The proportion of each transferrin isoform present was calculated by integrating the area under the relevant peak.

4.2.3.1 CDGS type 1 patients

Figure 4.5A shows the anion-exchange chromatographic profile for normal serum transferrin. The major peak corresponded to tetrasialotransferrin and the less tightly and more tightly bound isoforms corresponded to pentasialotransferrin and trisialotransferrin, respectively. There were negligible amounts of any of the other transferrin isoforms. A typical profile for transferrin from a PMM-deficient CDGS type 1 patient is shown in Figure 4.5B. There was a decreased proportion of tetrasialotransferrin and an increase in the proportions of disialo- and asialotransferrins. The anion-exchange chromatographic profile of transferrin isoforms from the PMM-normal CDGS type 1 patient is shown in Figure 4.5C, where there was again an increase in the proportion of disialotransferrin.

4.2.3.2 Galactosaemic patients

The anion-exchange chromatographic profile of transferrin purified from serum from an untreated galactosaemic patient is shown in Figure 4.5D. There was a marked increase in asialotransferrin. Less sialylated transferrin isoforms corresponding to trisialo-, disialo- and monosialotransferrin were present, but they were not resolved into individual peaks under the conditions used. These isoforms were identified by IEF with immunodetection for transferrin.

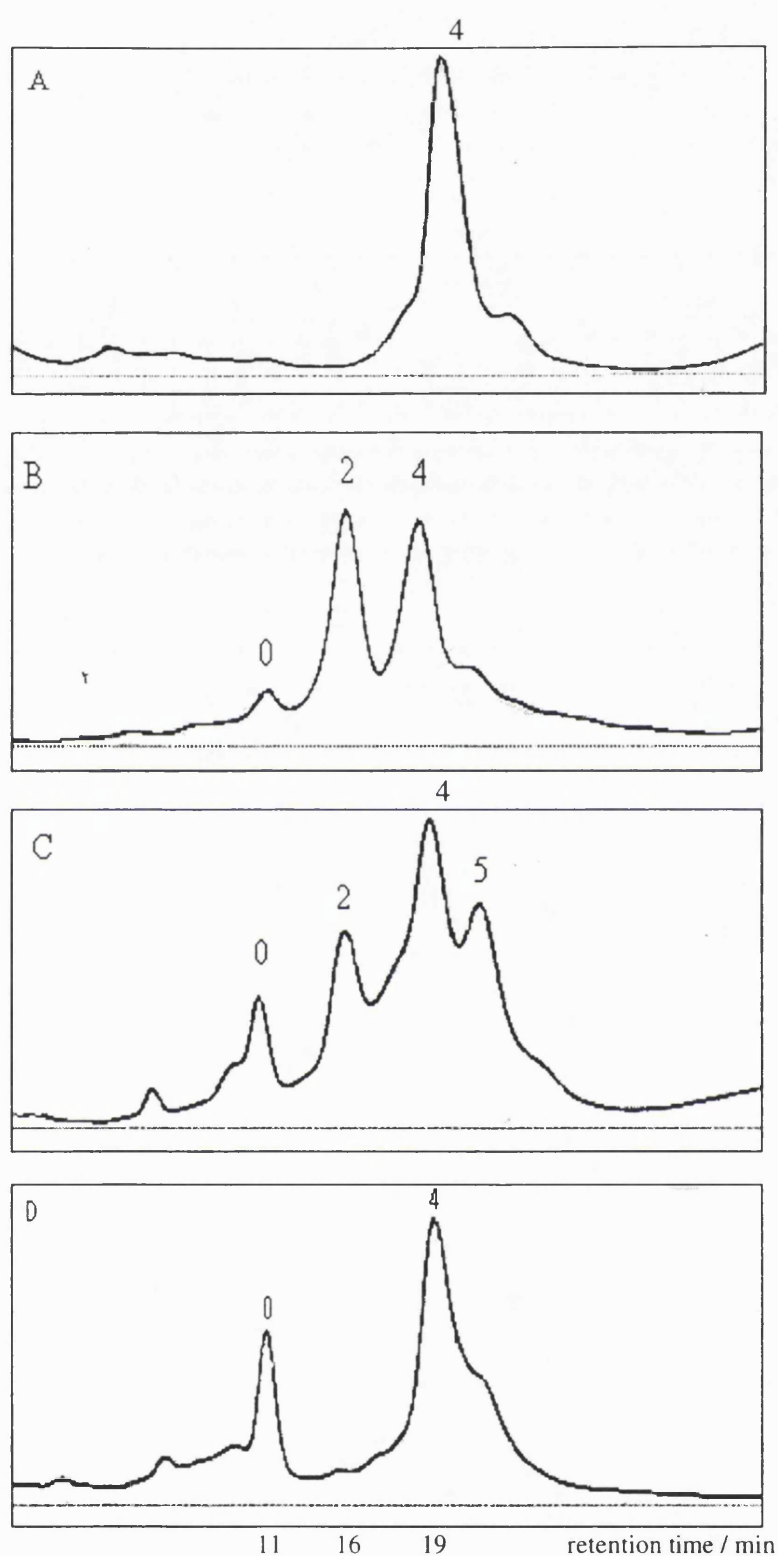


Figure 4.5: Anion-exchange chromatography of Rivanol-purified transferrins. A; normal serum transferrin, B; serum transferrin from PMM-deficient CDGS type 1 patient, C; ascites fluid transferrin from PMM-normal CDGS type 1 patient, D; serum transferrin from an untreated galactosaemic patient. 0; asialo-, 2; disialo-, 4; tetrasialo- and 5; pentasialo- transferrin.

4.2.3.3 Comparison of transferrin isoforms from CDGS type 1 and galactosaemic patients

The anion-exchange chromatographic profiles are comparable with the patterns seen on IEF with immunodetection of transferrin. Anion-exchange chromatography allows determination of the relative proportions of each isoform of transferrin by integration of the peak area. The proportion of each isoform of transferrin on IEF gels can be determined by scanning the Western blot and analysing with GelWorks. However this technique does not provide an accurate and reliable analysis because of the small size of the PhastGels and the limit to the amount of serum which can be analysed.

The quantitative variations in the proportion of each transferrin isoform between the PMM-deficient and PMM-normal CDGS type 1 are not discernible (Table 4.1). In a previous study of PMM-deficient CDGS type 1 patients, one patient had only 4% of the total transferrin in the asialotransferrin glycoform, whilst another patient had 25% of the total transferrin as the asialotransferrin glycoform (Iourin *et al.*, 1996). The proportion of the asialotransferrin isoform in the PMM-normal CDGS type 1 patient falls within this reference range. Such variations may be due to the fact that abnormal transferrin isoforms are a secondary effect of CDGS type 1 and not the primary defect. The calculated proportions of each isoform of transferrin from a galactosaemic patient were also within the same range as previously found for CDGS type 1 patients (Iourin *et al.*, 1996).

	Tetrasialo isoforms	Disialo isoforms	Asialo isoform	Other isoforms
Normal	86	-	-	14
PMM-deficient CDGS 1	36	21	14	29
PMM-normal CDGS 1	40	40	8	12
Untreated Galactosaemic	30	11	41	18

Table 4.1. Proportion of each isoform of transferrin in controls, CDGS patients and patients with untreated galactosaemia.

n = 1 for each patient studied.

4.2.4 SDS-PAGE of purified transferrin

Transferrin isoforms were prepared by treatment with Rivanol and anion-exchange chromatography, dialysed against water and freeze dried. They were then analysed by SDS-PAGE (7.5%) followed by silver staining (section 2.4.2, Figure 4.6). The transferrin purified from normal serum had one band on SDS-PAGE with a molecular mass of 80 kDa (lanes 1 and 2). Transferrin from a PMM-deficient CDGS type 1 patient had three bands corresponding to molecular masses of 80 kDa, 77.5 kDa, and 75 kDa (lane 6), whereas the transferrin from the PMM-normal CDGS type 1 patient had two major bands, with molecular masses of 80 kDa and 77.5 kDa (lane 3). This pattern was *qualitatively* similar to that from PMM-deficient CDGS type 1 patients. The transferrin from a patient with untreated galactosaemia also had three bands with similar molecular masses to those of the transferrin from the PMM-deficient CDGS type 1 patient (lanes 4 and 5). This gel was scanned and analysed using GelWorks which allows the determination of the molecular masses of the bands by reference to standards, and can give an indication of whether bands in different lanes have the same molecular mass (using the band matching tool). The transferrin isoforms from the untreated galactosaemic patient were slightly larger than the transferrin isoforms from the PMM-deficient CDGS type 1 patient. These results suggest that the isoforms are not identical.

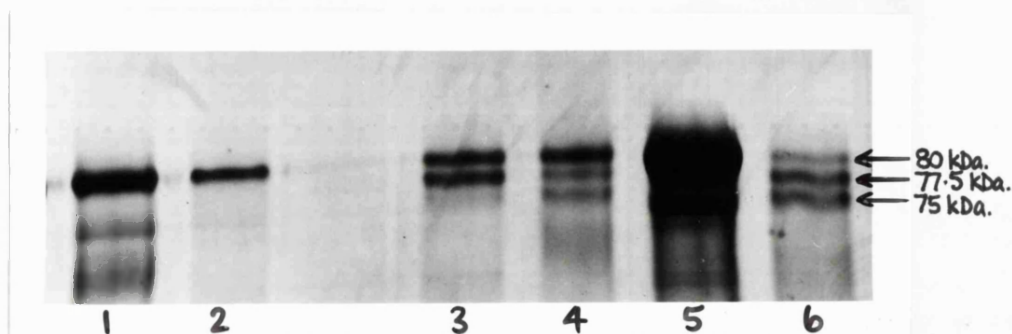


Figure 4.6: SDS PAGE of purified transferrin with silver staining.

Lanes 1 and 2; normal, lane 3; CDGS patient NH (PMM-normal), lanes 4 and 5; untreated galactosaemia, lane 6; CDGS type 1 (PMM-deficient). Arrows indicate masses estimated using GelWorks.

SDS-PAGE analysis of transferrin allowed the different isoforms to be separated according to their molecular mass. It can be concluded that the basis of the change in overall charge observed on IEF is due to a change in the structure of the glycans attached to transferrin. Similar changes are also observed in many serum glycoproteins from CDGS type 1 patients, indicating that the changes in charge are due to the glycans and not to the polypeptides (Harrison *et al.*, 1992; Yuasa *et al.*, 1995; Henry *et al.*, 1997). The difference in molecular mass between the tetrasialo and disialotransferrin isoforms of CDGS type 1 patients was about 2.5 kDa, corresponding to the loss of a complete glycan chain. However, the difference between the tetrasialo and disialotransferrin isoforms from the galactosaemic patient was less than this suggesting loss of a few sugar residues, rather than the whole glycan. Analysis of transferrin isoforms by SDS-PAGE cannot provide information on the structures of the N-linked glycans of transferrin. The data only suggest that both PMM-deficient and PMM-normal CDGS type 1 patients have transferrin isoforms which have one or both glycans missing compared with normal tetrasialotransferrin. On the other hand in the case of untreated

galactosaemia patients the data indicates the presence of truncated glycans. More specific characterisation of the N-linked glycans was required to prove this hypothesis.

4.2.5 Analysis of transferrin by matrix assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF).

Rivanol-purified transferrin was also analysed by matrix assisted laser desorption ionisation time of flight mass spectrometry.

Normal transferrin had a molecular mass of 78 kDa on the MALDI-TOF, which corresponds to the tetrasialotransferrin isoform. A small amount of asialotransferrin at 74 kDa, was seen as a shoulder. The peak at 66.5 kDa was albumin. (Figure 4.7A).

Transferrin from a PMM-deficient CDGS type 1 patient showed two major isoforms at 78 kDa and 76 kDa corresponding to tetrasialo and disialotransferrin (Figure 4.7B).

Transferrin from the PMM-normal CDGS patient consisted of a mixture of different isoforms ranging from 79.5 kDa to 77.3 kDa, but this was not resolved by MALDI-TOF (Figure 4.7C). The transferrin from the untreated galactosaemic patient gave a broad peak (around 78 kDa) which was not resolved into different isoforms (Figure 4.7D).

Analysis of transferrin by MALDI-TOF was not particularly successful as the data did not provide any more information than that already provided by SDS-PAGE. The main problem was from the small amount of sample that was available for analysis.

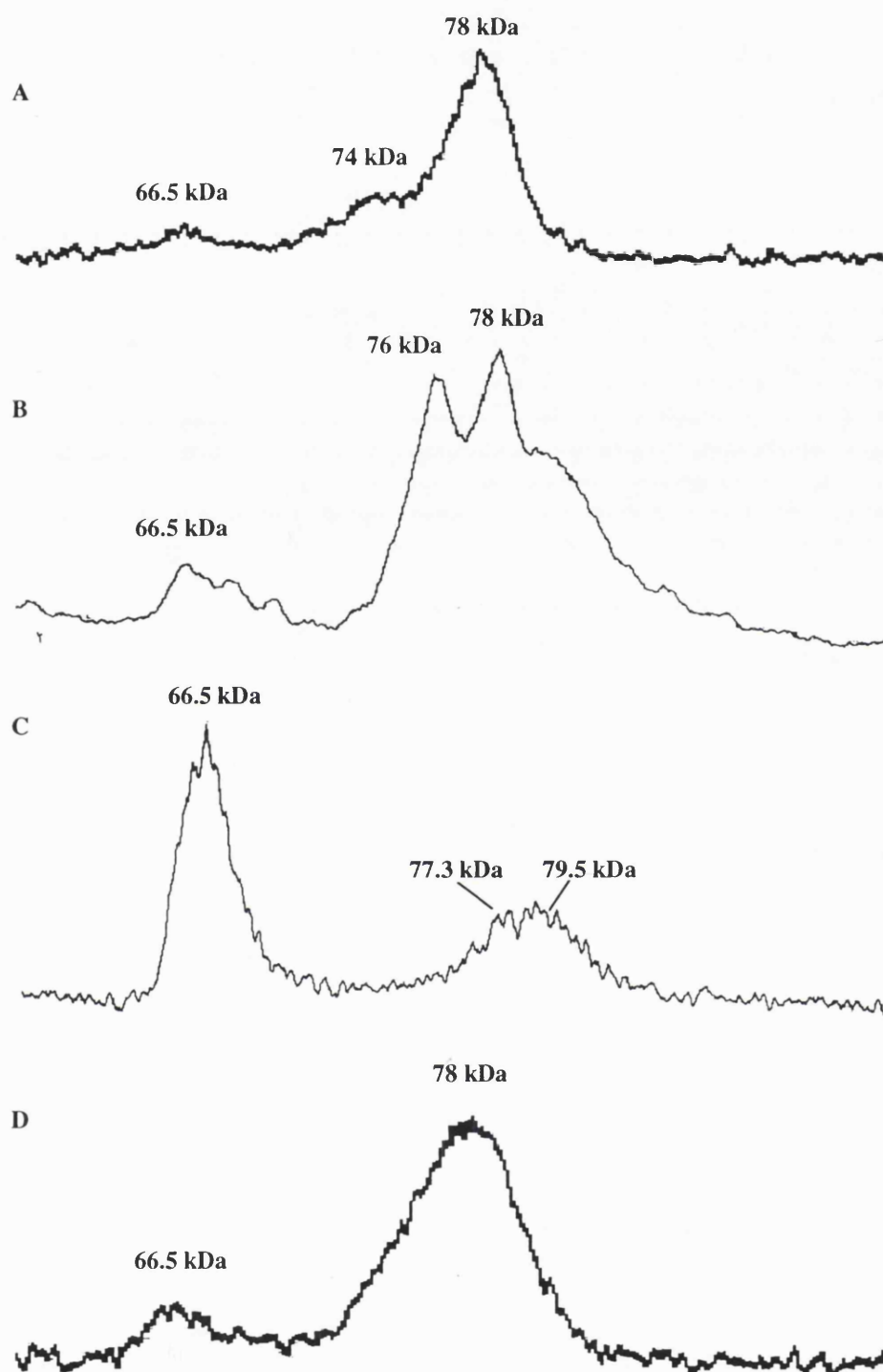


Figure 4.7: Matrix assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF) of transferrin.

A; normal, B; PMM-deficient CDGS type 1 patient C; PMM-normal CDGS type 1 patient and D; untreated galactosaemic patient.

The best way to use the MALDI-TOF for this type of analysis would be to trypsinize transferrin first and then analyse the resulting glycopeptides. Trypsin-digested transferrin would produce smaller fragments which would be easier to resolve by MALDI-TOF mass spectrometry. However, as this methodology was not available in our Laboratory, the N-linked glycans released from transferrin from controls, CDGS type 1 and galactosaemic patients were analysed by HPLC.

4.2.6 Characterisation of the N-linked glycans of transferrin by HPLC

The N-linked glycans of purified transferrin isoforms were released by sequential digestion with trypsin and peptide N-glycanase (section 2.8.1). The released glycans were labelled with 2-aminobenzamide (2-AB) and analysed by chromatography on a GlycoSep-N column using a gradient from 35% to 100% of 250 mM ammonium formate pH 4.4 in acetonitrile for elution. The fluorescence of the effluent was monitored at an excitation wavelength of 330 nm and emission at 420 nm. The GlycoSep-N column was calibrated with 2-AB standards from Oxford Glycosystems as described in Chapter 2 (Section 2.8).

4.2.6.1 CDGS type 1 patients

The predominant glycan found in normal serum transferrin (Figure 4.8A), which had a retention time of 58 min. was identified as the disialylated biantennary structure (A2) by co-chromatography with an authentic standard. The glycans of transferrin from PMM-deficient CDGS type 1 patients also had the A2 structure (Figure 4.8B). No unusual carbohydrate structures were seen in this transferrin, confirming previous work by Wada *et al.* (1992) that the disialotransferrin and asialotransferrin isoforms must be formed by the complete absence of glycan chains. The transferrin from the PMM-normal CDGS type 1 patient was analysed for each of three isoforms; tetrasialo- (Figure 4.8C), disialo- (Figure 4.8D) and asialotransferrin (Figure 4.8E). Both the tetrasialo- and disialotransferrin isoforms contained only the A2 structure. The asialotransferrin was shown not to be glycosylated as no peaks were detected except for a very small amount of A2. Co-chromatography of the glycan from disialotransferrin of the PMM-normal CDGS type 1 patient and the authentic 2-AB labelled standard in equimolar amounts

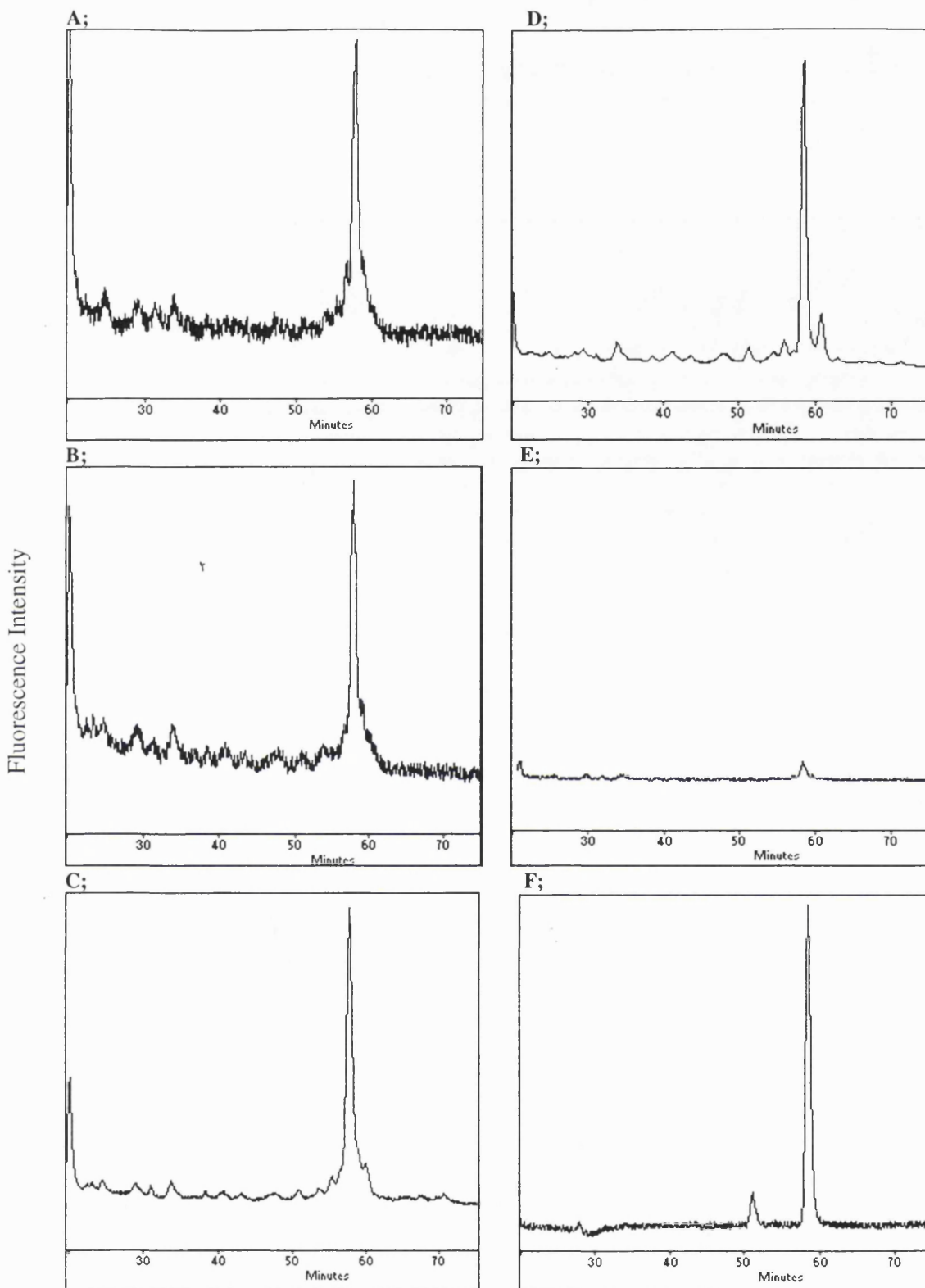


Figure 4.8. Analysis by HPLC of fluorescently labelled glycans released from transferrin from CDGS type 1 patients.

A; total normal transferrin, **B;** total transferrin from PMM-deficient patient, **C;** tetrasialotransferrin from PMM-normal patient, **D;** disialotransferrin from PMM-normal patient, **E;** asialotransferrin from PMM-normal patient, **F;** co-chromatography of **D** and the authentic 2-AB-A2.

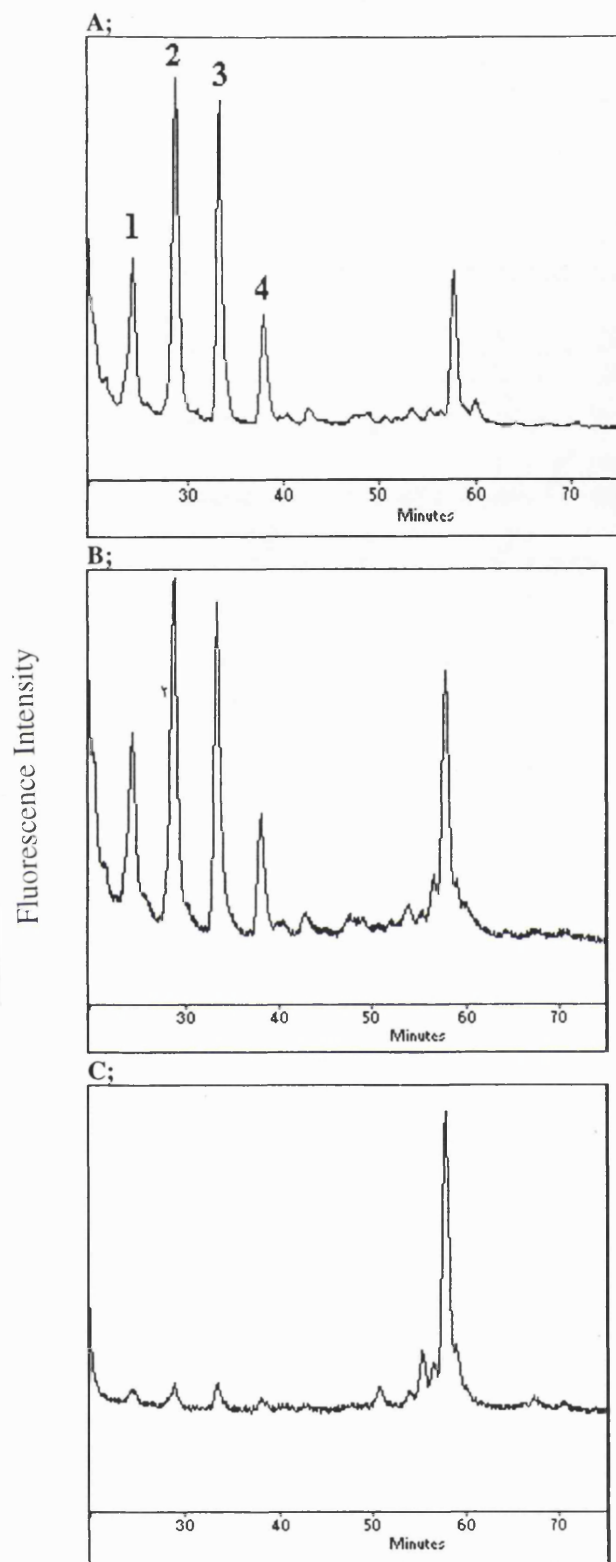


Figure 4.9. Analysis by HPLC of fluorescently labelled glycans released from transferrin from galactosaemic patients.

A; total transferrin from untreated galactosaemic patient ; **B;** total transferrin from galactosaemic patient -3 weeks post treatment, **C;** total transferrin from galactosaemic- 9 months post treatment.

confirmed the identity of the A2 peak (Figure 4.8F). It was concluded that the aberrant transferrin glycoforms of CDGS type 1 patients with and without the PMM deficiency were similar. Whether the non-occupancy of the glycosylation sites was random or not, could not be determined by this method.

4.2.6.2 Galactosaemic patients

Instead of the single glycan peak seen in the HPLC chromatogram for normal transferrin (Figure 4.8A), a range of truncated glycans (1-4) was observed when the glycans from transferrin of untreated patients with galactosaemia were analysed (Figure 4.9A). During treatment on a galactose-free diet the proportion of the A2 glycan increased and the amount of the truncated glycans decreased, until mainly normal disialylated biantennary glycans were seen (Figure 4.9B and C). The proportion of each truncated structure found in transferrin from an untreated galactosaemic patient is shown in Table 4.2.

Structure	1 (M3N2)	2 (NM3N2)	3 (NGA2B)	4 (NGA4)
% of total glycans	17	32	29	9

Table 4.2: Agalactosylated structures as a percentage of the total glycans in untreated galactosaemic transferrin.

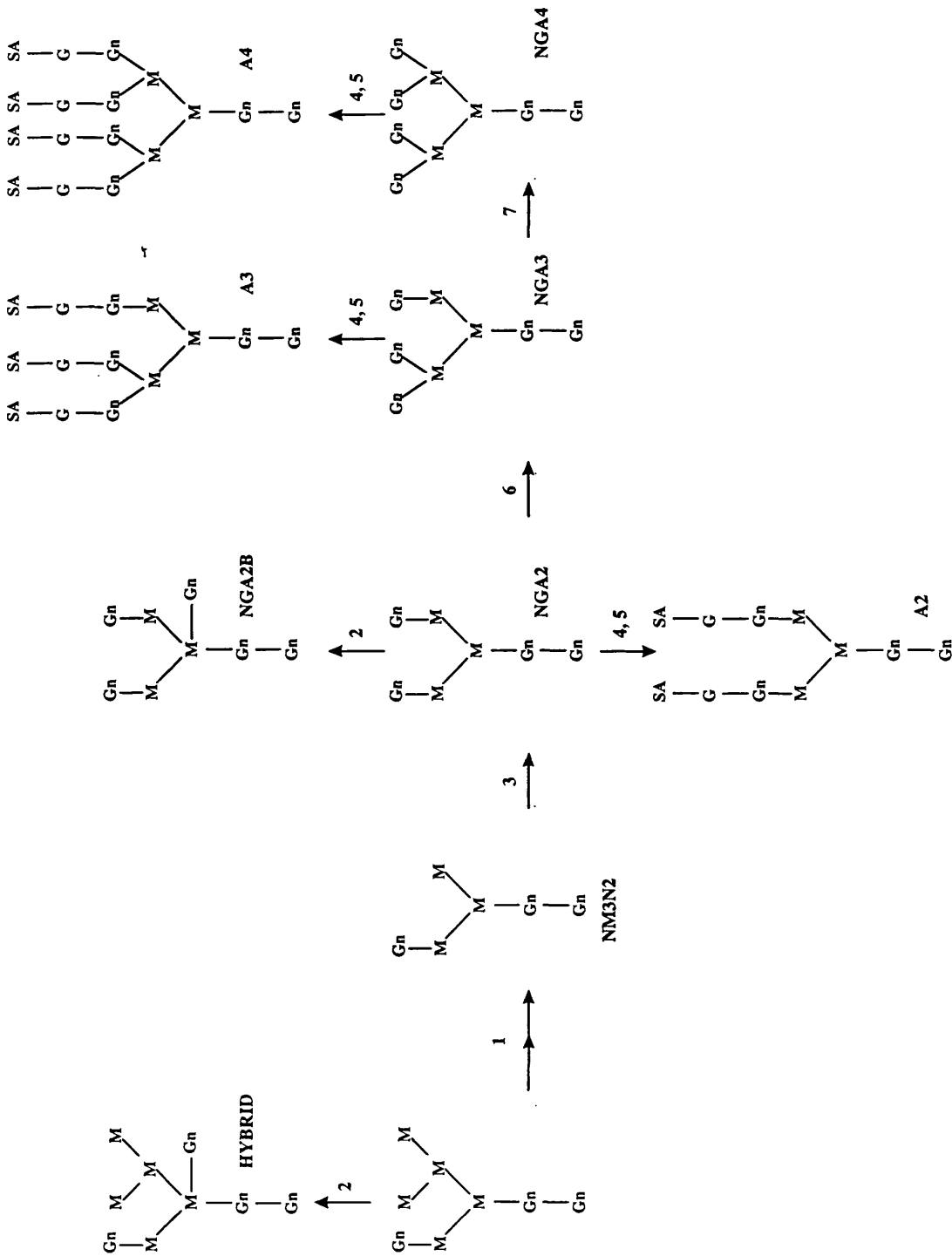
The truncated glycans were identified either by co-chromatography with an authentic standard (M3N2 and NGA4) or by calculation of the glucose unit equivalents and by deduction (NM3N2 and NGA2B), when authentic standards were not available.

(Calibration of the GlycoSep column is described in Chapter 2, section 2.8 based on the method of Guile *et al.*, 1996).

Component 1 was shown to be the core pentasaccharide (M3N2), by co-chromatography with the authentic standard. The structure of component 2 was deduced to be the core pentasaccharide with an additional GlcNAc attached to one of the peripheral mannose residues (NM3N2). It had a retention time consistent with the addition of GlcNAc to component 1, or the loss of a GlcNAc in a terminal position from NGA2. Component 3

Figure 4.10: Processing of N-linked glycans in patients with galactosaemia.

Enzymes: **1**; α 3/6-mannosidase II, **2**; GlcNAc transferase III, **3**; GlcNAc transferase II, **4**; galactosyltransferase, **5**; sialyltransferase, **6**; GlcNAc transferase IV, **7**; GlcNAc transferase V. **M**; mannose, **G**; galactose, **Gn**; GlcNAc, **SA**; sialic acid.



had a retention time slightly less than that of the authentic NGA3 standard, but was consistent with the calculated retention time of NGA2B. Component 4 co-chromatographed with the authentic NGA4 standard. Except for 1, these structures (2, 3 and 4) are all biosynthetic intermediates in the glycosylation pathway (Figure 4.10), and they all terminate with a N-acetylglucosamine residue, which is normally capped by galactose and sialic acid. This increase in exposed GlcNAc residues would explain the increase in galactose acceptor capacity of glycoproteins from the serum of patients with galactosaemia (Prestoz *et al.*, 1997). Also it is interesting to note that structure 2 has previously been identified in serum transferrin from HEMPAS patients (Fukuda *et al.*, 1992).

As authentic standards of two of these structures (NGA2B and NM3N2) were not available, the identity of 2 and 3 would need to be confirmed by either mass spectrometry or by digestion of the glycans with β -hexosaminidase and then reapplying them to the HPLC column, where they should co-elute with M3N2. If they did not co-elute with M3N2 after β -hexosaminidase digestion then they were not correctly identified. Unfortunately neither of these two methods could be used because of the very limited supply of sample from untreated galactosaemic patients.

The effectiveness of treating galactosaemic patients with a galactose-free diet is usually monitored by measuring the concentration of galactose-1-phosphate in red blood cells (Gitzelman, 1995). As treatment also leads to the formation of normally glycosylated serum transferrin, the correlation between the red cell galactose-1-phosphate concentration and the percentage of truncated glycans present in serum transferrin in patients with galactosaemia was investigated. As shown in Table 4.3, there was variation between the galactose-1-phosphate concentration and the percentage of normal disialylated biantennary glycans present in the glycans purified from serum transferrin. There is no direct correlation, except that a trend can be seen. The samples from untreated galactosaemic patients (galactose-1-phosphate concentration greater than 1 $\mu\text{mol/g}$ in red blood cells) contain 13 % normal disialylated glycans, whereas the treated galactosaemic patients contain between 18 and 82% disialylated biantennary glycans.

However, some patients who were on treatment have only a small proportion of normal glycans, which is surprising. Thus the length of treatment was compared to the percentage of disialylated biantennary glycans. Again there was no direct correlation, as the patient who had 82% normal disialylated biantennary glycans had been treated for 9 months, but a patient who had been treated for a year still had only 28 % normal glycans. Thus the occurrence of long term complications in patients with galactosaemia may be related to how long the glycans remain truncated. A patient whose glycans return to normal more quickly may have a better prognosis than one whose glycans remain truncated for extended periods of time.

	Length of treatment weeks	Galactose-1-phosphate ($\mu\text{mol/g}$)	% disialylated biantennary glycans
Untreated	0 [*]	2.8	13
	0 ^s	4.0	13
	0	8.75	13
Treated	3 [*]	1.1	18
	6	0.34	21
	24	0.5	20
	38 ^s	0.3	82
	42	0.22	58
	50	0.15	28

Table 4.3: The relationship between treatment and level of galactose-1-phosphate in red blood cells and proportion of disialylated biantennary glycans on serum transferrin in patients with galactosaemia.

* and \$ indicate measurements on the same patients

4.3 Discussion

4.3.1 Aberrant glycosylation in CDGS type 1 patients

There was a decrease in the proportion of tetrasialotransferrin and a corresponding increase in the proportions of disialo- and asialotransferrins in the serum of CDGS type 1 patients. The proportions of each isoform of transferrin were quantified by anion-exchange chromatography of Rivanol-purified transferrin. Although a difference in the proportions of the disialo- and asialo- isoforms was found between the PMM-deficient

and PMM-normal patients, this was not significant as there have been previous reports of CDGS type 1 patients with as little as 4% and as much as 25% asialotransferrin (Iourin *et al.*, 1996; Charlwood *et al.*, 1997). There seems to be no correlation between the amount of under-glycosylated transferrin and the severity of the disease. NH was a very severe CDGS type 1 patient and died at the age of three months, but surprisingly he had a greater amount of disialotransferrin and a very small amount of asialotransferrin compared with the less severely affected CDGS type 1 patient (VB). Iourin *et al.* (1996) studied the transferrin isoforms of the severely affected PMM-deficient CDGS type 1 patients (DC, EW) and they too had a greater proportion of glycosylation sequons occupied than patient VB. One might have expected that in such patients the clinical condition would be less severe, but this was not the case for either the PMM-normal or the PMM-deficient CDGS type 1 patients.

Characterisation of the glycans released from purified transferrin demonstrated that in both PMM-deficient and PMM-normal CDGS type 1 patients the glycans present on the occupied N-glycosylation sites in transferrin were of the disialylated biantennary structure, as is found in normal transferrin. This means that the abnormal forms of transferrin that are detected by IEF, anion-exchange chromatography and SDS-PAGE arise from non-glycosylation of N-glycosylation sequons. It has previously been shown that for PMM-deficient CDGS type 1 patients this non-glycosylation phenomenon is random (Yamashita, 1993a). Although a detailed structural analysis of N-glycans from $\alpha 1$ -antitrypsin was not carried out, it can be deduced from the IEF data that in both PMM-normal and PMM-deficient CDGS type 1 patients the isoforms of $\alpha 1$ -antitrypsin were less sialylated. This is presumably due to the non-occupancy of one or more of the three sites in the molecule. In contrast, in the galactosaemic patients there was no evidence of aberrantly glycosylated isoforms of $\alpha 1$ -antitrypsin.

Although the precise mechanism of under-glycosylation in CDGS type 1 is not understood yet, a deficiency of mannose-1-phosphate, GDP-Man and dolichol-P-Man has been implicated in PMM-deficient variants of CDGS type 1 (Van Schaftingen and Jaeken, 1995). A similar mechanism has been proposed for hereditary fructose

intolerance (HFI) patients, who also show aberrant glycosylation of serum transferrin. Inhibition of phosphomannose isomerase by fructose-1-phosphate in HFI patients is also believed to cause a deficiency of mannose-1-phosphate, GDP-Man and dolichol-P-Man (Adamowicz and Pronicka, 1996, Jaeken *et al.*, 1996). However, the structure of the N-linked glycans of serum transferrin from HFI patients have not been investigated.

4.3.2 Aberrant glycosylation of serum transferrin in patients with galactosaemia

Isoelectric focusing of serum transferrin obtained from galactosaemic patients, before and during treatment with a galactose-free diet, showed that the abnormal pattern seen before treatment reverted to a normal pattern following treatment.

Analysis of the glycans released from transferrin from the untreated galactosaemic patients indicated that many of the chains were truncated and terminated in GlcNAc residues and there was only a small proportion of correctly processed glycans. The key intermediate in the biosynthetic pathway, NGA2 (Figure 4.10) is a substrate for 3 enzymes; galactosyltransferase, N-acetylglucosaminyltransferase III and IV. If there is inhibition of galactosyltransferase or a deficiency of the UDP-Gal then N-acetylglucosaminyltransferase III, IV and V may act preferentially leading to the formation of NGA2B or NGA3 or NGA4 (Figure 4.10). One of the major glycans found in the galactosaemic patients was NGA2B. This is formed by the action of N-acetylglucosaminyltransferase III on NGA2. Bisecting the glycan chain prevents further branching, so in the untreated galactosaemic patient NGA2 would be the final structure if N-acetylglucosaminyltransferase III acted before N-acetylglucosaminyltransferase IV and V. There was also a large amount of NM3N2 present, which is the substrate for N-acetylglucosaminyltransferase II. A small amount of NGA4 was seen, and NGA3 appeared as a shoulder on peak 3. NGA3 is formed by branching of NGA2 by N-acetylglucosaminyltransferase IV, and NGA4 is formed by the action of N-acetylglucosaminyltransferase V (Schachter, 1984; 1991a; 1991b; 1995a and 1995b). NGA2, NGA2B, NGA3 and NGA4 are all substrates for galactosyltransferase. The build up of these structures is indicative of a block in the transfer of galactose to the glycan chain by galactosyltransferase. This suggested that there was either a lack of UDP-galactose, inhibition of galactosyltransferase by galactose-1-phosphate (or another

derivative of galactose) or defective transport of UDP-galactose into the Golgi. This blockage not only results in non-galactosylation of the biantennary glycan but also leads to increased branching of the glycan. It is also interesting to note that there were no mono-sialylated structures indicating that it is an all or none effect with the addition of galactose. It was not possible from the present studies to determine which of these three possibilities was the cause of non-galactosylation of the glycans.

The origin of the core pentasaccharide (1 M3N2) is interesting because it is not normally an intermediate in the biosynthesis or processing pathways for glycoproteins in most cell types (Kornfeld and Kornfeld, 1985). However, there is evidence that in nonerythroid cells there is a distinct α -mannosidase (III) that can remove two mannose residues from the $\text{Man}_5\text{GlcNAc}_2$ processing intermediate to produce compound 1 without the prior action of N-acetylglucosaminyltransferase I (Bonay and Hughes, 1991; Chui *et al.*, 1997). Compound 1 was not seen in the transferrin from normal serum or from CDGS type 1 serum, and thus is unique to the galactosaemic serum. The mechanism of its formation is unclear and requires further investigation. It can be speculated that it may have been formed by the action of β -hexosaminidase, which is also present in serum.

Prior to this study there was strong evidence for a galactosylation defect in patients with galactosaemia. Ornstein *et al.* (1992) showed that fibroblasts from galactosaemic patients were better acceptors of galactose from radiolabelled UDP-galactose than normal fibroblasts, suggesting that the glycoproteins in these cells were under-galactosylated. Interestingly these fibroblasts were grown on medium containing glucose only. This work has recently been confirmed in serum by Prestoz *et al.* (1997). Until now undergalactosylation of glycoproteins has been assumed to be responsible for the observed increased capacity for acceptance of galactose (Dobbie *et al.*, 1990; Ornstein *et al.*, 1992; Segal *et al.*, 1995a and Prestoz *et al.*, 1997). The present investigation is the first analysis of the N-glycan structures on serum transferrin from untreated galactosaemic patients. It provides the first direct evidence for agalactosylation of serum proteins in galactosaemia.

The analysis of the glycans from serum transferrin in untreated and treated galactosaemic patients revealed that for some patients with a low concentration of galactose-1-phosphate in red blood cells the proportion of normal disialylated biantennary glycans was high, but in others it was as low as 20%. It is known that the concentration of galactose-1-phosphate drops within a few days of commencing the galactose-free diet (Segal, 1989). However, it has been shown in alcoholics that at least 10 days abstinence is required before the transferrin patterns start to return to normal (Stibler, 1991). The transferrin pattern as observed by agarose gel electrophoresis in patients with galactosaemia returns to normal after 3 weeks treatment (Winchester *et al.*, 1995). The present results suggest that in some patients it may take up to 9 months of treatment for the transferrin pattern to return to normal. The variations in the rate at which the transferrin returns to normal may be explained by compliance with diet. It is interesting to note that for patients with hereditary fructose intolerance it can also take several months of treatment on a fructose-free diet for the transferrin pattern to return to normal (Adamowicz and Pronicka, 1996).

It has been suggested that measurement of the concentration of galactose-1-phosphate in red blood cells is not informative for the rest of the body organs (Segal, 1995b). There have been many studies on the intellectual impairment of galactosaemic patients, but no correlation has been found between the concentration of galactose-1-phosphate, genotype or age of onset of treatment (Waggoner *et al.*, 1990; Schweitzer *et al.*, 1993; Cleary *et al.*, 1995). Analysis of the glycan chains of transferrin thus may give a better indication of the effectiveness of the treatment.

Although the level of galactose-1-phosphate in red blood cells drops with treatment, it is never normal. If the residual galactose-1-phosphate concentration is causing inhibition of galactosyltransferase, then it would be expected that there would be a direct correlation between the galactose-1-phosphate concentration and normal disialylated biantennary glycans. This hypothesis seems unlikely in the light of the present results, which seem to favour a decrease in the amount of UDP-galactose galactosylation of the glycans. The activity of UDP-Gal epimerase and pyrophosphorylase in the cell probably controls the level of UDP-Gal. Some patients may have a greater amount of epimerase activity and

thus a larger capacity for UDP-galactose production than others and this may explain their differing responses to treatment. The activity of epimerase has not been measured in galactosaemic patients, but the concentrations of UDP-galactose have been determined, although not agreed upon (see section 1.6.5 and references therein). Until the situation concerning UDP-galactose concentrations in galactosaemic patients is clarified, the underlying mechanism of non-galactosylation of complex glycans will remain elusive. Future work on these patients would include a long term study of the rate at which the transferrin glycans return to normal in each patient and their overall outcome. Such studies may reveal that glycan analysis of serum transferrin may be a better indicator of dietary compliance than measurement of galactose-1-phosphate concentration.

4.4 Conclusion

In this chapter it has been demonstrated that the molecular basis for the aberrant glycoforms of serum transferrin in both PMM-deficient and PMM-normal CDGS type 1 patients is non-occupancy of glycosylation sequons. The glycans of transferrin have the normal disialylated biantennary structure, but there is under-occupancy of the glycosylation sites leading to the production of disialotransferrin and asialotransferrin. Although the enzyme defect in these two groups of patients is different, the same abnormalities in glycosylation of serum glycoproteins are seen and hence the clinical phenotypes of these two groups are very similar.

It has also been demonstrated that in serum glycoproteins from galactosaemic patients there is under-galactosylation of N-linked glycans leading to the formation of less sialylated isoforms of transferrin. It has been speculated that this under-galactosylation could be responsible for the poor outcome of galactosaemic patients on a galactose-free diet. It is suggested that determination of truncated glycans may be a better predictor of outcome for galactosaemic patients than measurement of the galactose-1-phosphate concentration in red blood cells.

Structural analysis of lipid-linked and protein N-linked oligosaccharides in CDGS and galactosaemic fibroblasts

5.1 Introduction

In the previous chapter it was demonstrated that the basis of the glycosylation defect in serum glycoproteins from PMM-normal and PMM-deficient CDGS type 1 patients was partial non-occupancy of N-glycosylation sequons. In this chapter the glycosylation of cellular glycoproteins from these patients is described. Previous studies on fibroblasts from CDGS type 1 patients that had been transfected with viral glycoproteins showed that the expressed glycoproteins were normally glycosylated although some variation in the secretion was noted (Marquardt, 1995). These studies suggested that perhaps, only serum glycoproteins are aberrantly glycosylated in CDGS type 1. However, evidence for defective glycosylation in CDGS type 1 fibroblasts has been observed by studying the synthesis of lipid-linked oligosaccharides in these cell lines (Powell *et al.*, 1994; Panneerselvam and Freeze, 1996a). A decreased rate of synthesis of lipid-linked oligosaccharide precursors and transfer of truncated oligosaccharides to glycoproteins was found in CDGS type 1 fibroblasts. Panneerselvam and Freeze (1996a) also claimed that the presence of mannose in the cell culture medium could correct this defect. Another group of workers performed similar experiments and found that there was no accumulation of truncated lipid-linked oligosaccharides in CDGS type 1 fibroblasts, but there was an accumulation of dehydrodolichol (Ohkura *et al.*, 1997). Further investigations are required to clarify the situation.

The glycans released from serum transferrin from untreated galactosaemic patients were agalactosylated, demonstrating that there was defective transfer of galactose to complex glycans (Chapter 4). In contrast to the situation in CDGS type 1 patients, there is convincing evidence that cellular glycoproteins are similarly affected in galactosaemic fibroblasts. Ornstein *et al.* (1992) demonstrated that there was an increased ability to accept galactose by the glycoproteins extracted from galactosaemic fibroblasts. They

concluded that the glycoproteins found in galactosaemic fibroblasts have a higher proportion of glycan chains that terminate in GlcNAc residues than the glycoproteins extracted from normal fibroblasts. However no structural studies were performed to support this hypothesis.

The work in this chapter seeks to answer two questions:

- 1) Can the non-occupancy of glycosylation sequons found in serum transferrin from CDGS type 1 patients be attributed to a defect in the synthesis of the lipid-linked oligosaccharide (LLO)?
- 2) Can abnormal glycosylation be demonstrated in cellular glycoproteins in CDGS type 1 and galactosaemia?

5.2 Experimental plan

Fibroblasts were harvested and extracted with chloroform/methanol (2:1) to obtain the lipid-linked oligosaccharides (smaller than $\text{Man}_5\text{GlcNAc}_2$) and monosaccharides. The monosaccharides and oligosaccharides were released from the lipid by mild acid hydrolysis (*fraction 1*). The pellet was then sonicated in water and the water-soluble oligosaccharides were isolated in the supernatant (*fraction 2*). The larger lipid-linked oligosaccharides were extracted from the pellet in chloroform/methanol/water (10:10:3) and the oligosaccharides released by mild acid hydrolysis (*fraction 3*). The final pellet was digested with trypsin and peptide N-glycanase to release N-linked oligosaccharides from the cellular glycoproteins (*fraction 4*). See Figure 5.1 for the summary of the experimental plan, and section 2.8.2 for the description of the methods used. The mono- and oligosaccharides obtained in each of the fractions were labelled by reductive amination using 2-aminobenzamide (2-AB). The samples were analysed by HPLC on a GlycoSep-N column and fluorescent monitoring of the eluant (section 2.8.4). The oligosaccharides were identified by co-chromatography of authentic standards or by deduction. This experiment was repeated using the galactosaemic fibroblasts which had been grown in the presence of 5 mM galactose for 3 days prior to harvesting.

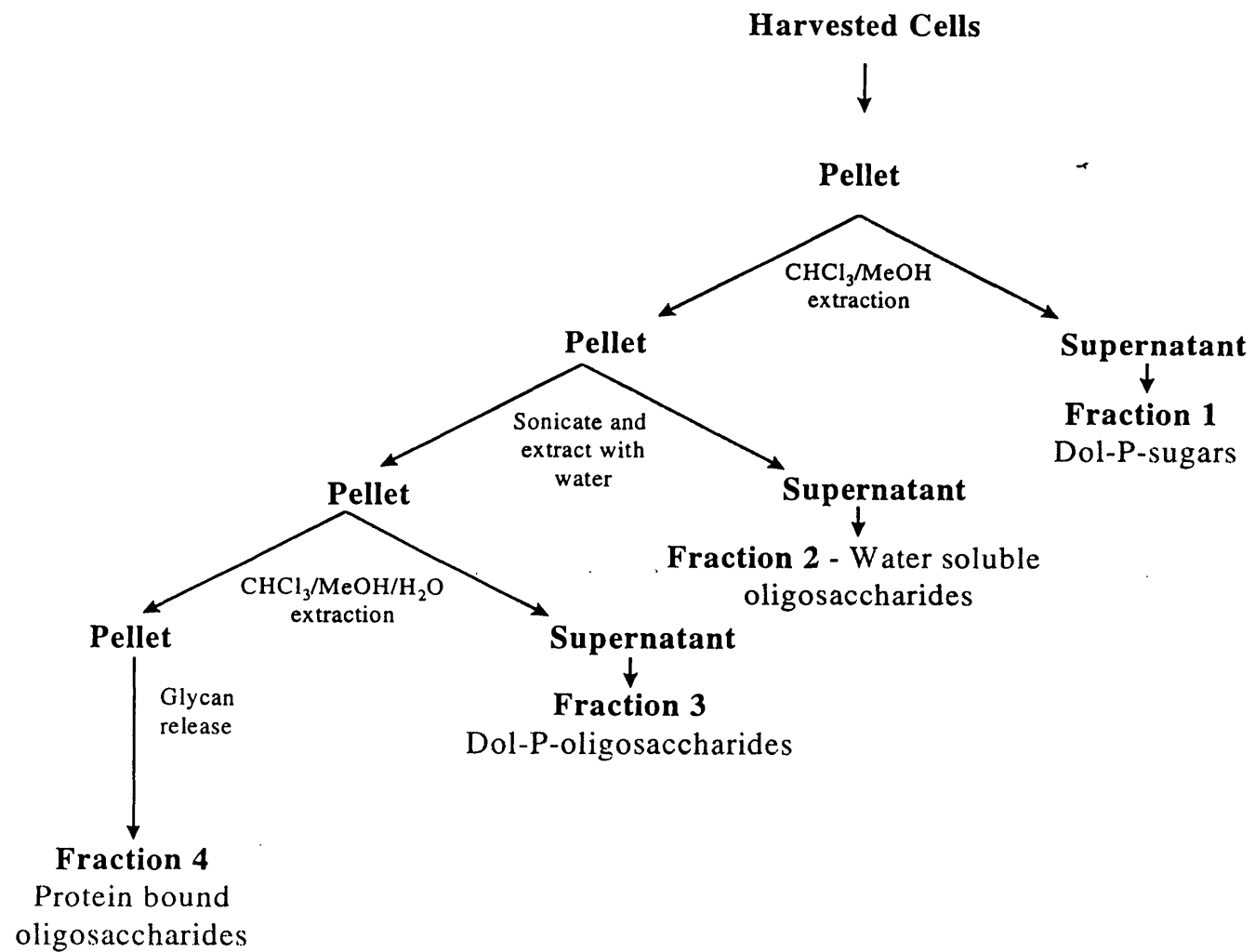


Figure 5.1: Isolation of lipid-linked, water-soluble and protein-bound oligosaccharides

The experimental method used in this chapter differed from those described because the oligosaccharides were analysed in the steady state. Previously pulse-chase labelling with [³H]-mannose was used to monitor the oligosaccharides (Powell *et al.*, 1994; Panneerselvam and Freeze, 1996a; Lewis, 1997).

5.3 Results

5.3.1 Analysis of dolichol-linked-monosaccharides (fraction 1)

Fraction 1 was expected to contain dolichol-P-mannose, dolichol-P-glucose and lipid-linked oligosaccharides in the first steps in the synthesis of the LLO (Figure 1.3). The HPLC profile of the released sugars in fraction 1 showed one large peak of monosaccharide (which was probably predominantly glucose) which obscured all other components extracted. This was a general problem found with analysing extracts from fibroblasts using this methodology. It was not possible to remove the glucose by dialysis prior to extraction of the fibroblasts. It was also found that by analysing pure glucose and mannose derivatives that they co-eluted on the GlycoSep-N column under the conditions used for separation of oligosaccharides. Thus identification and measurement of dolichol-P-mannose and dolichol-P-glucose in fraction 1 was not possible. Therefore, fraction 1 from CDGS type 1 or galactosaemic fibroblasts was not analysed. To determine the amount of dolichol-P-mannose in CDGS type 1 and galactosaemic fibroblasts the cells would have to be labelled with [³H]-mannose so that biosynthetic intermediates could be detected specifically.

5.3.2 Analysis of water-soluble oligosaccharides (fraction 2)

The water-soluble oligosaccharides extracted from normal fibroblasts were identified as high mannose oligosaccharides ranging from Man₃ to Man₉ with either one or two GlcNAc residues at the reducing end (Figure 5.2). Based on previous studies by Villers *et al.* (1994) it can be concluded that the oligosaccharides extracted in this fraction represent a pool of oligosaccharides released from the endoplasmic reticulum. They were all of the high-mannose type and did not exhibit processing that would have taken place in the Golgi. The water-soluble oligosaccharides were most likely derived from

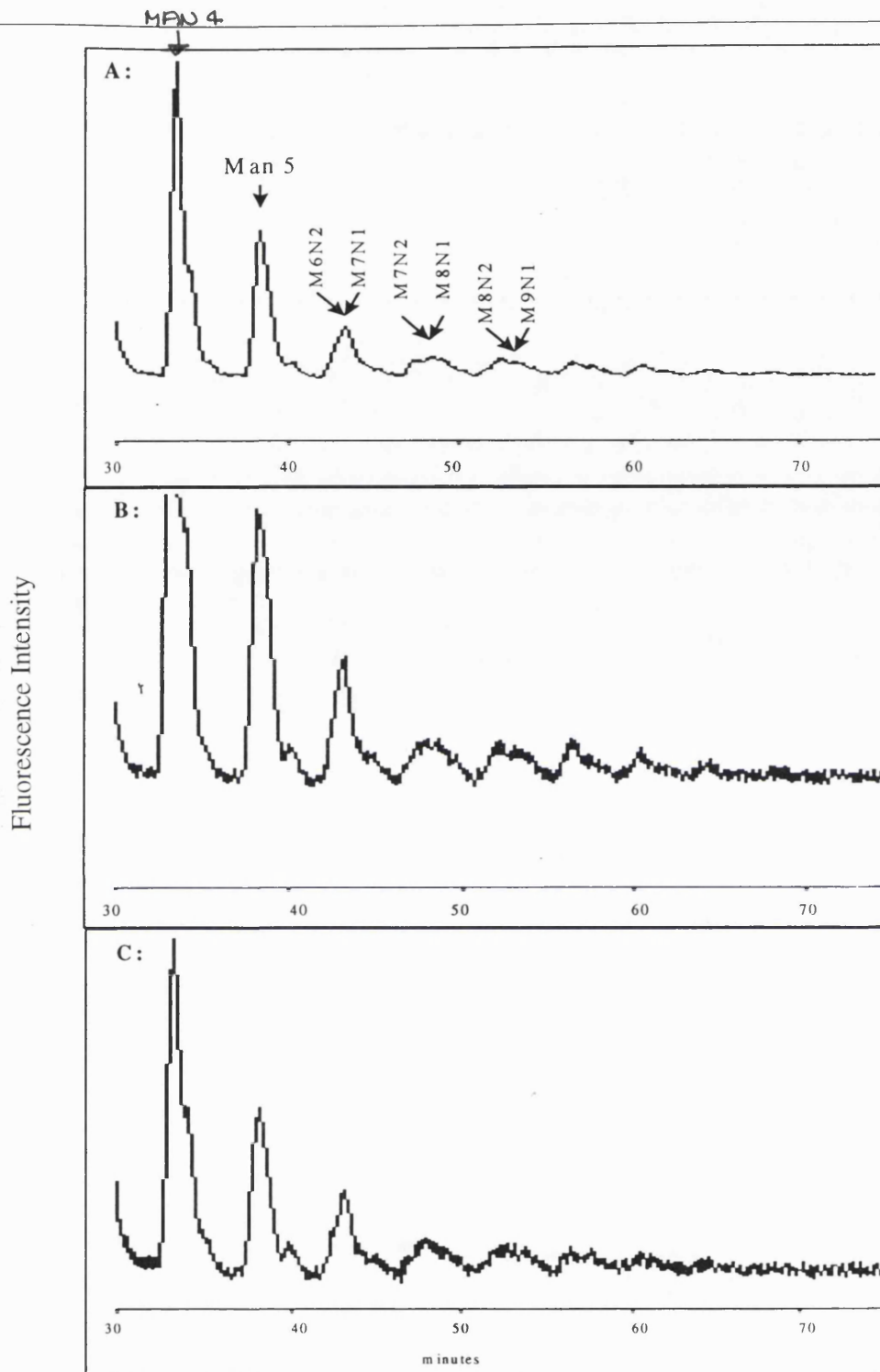


Figure 5.2: HPLC of water-soluble oligosaccharides (fraction 2)

A; normal, **B;** PMM-deficient CDGS type 1, and **C;** galactosaemic fibroblasts eluting from the GlycoSep-N HPLC column. The HPLC profile for PMM-normal CDGS type 1 fibroblasts is identical to profile **B**. Arrows indicate identification of oligosaccharides by co-chromatography or deduction.

hydrolysis of the LLO or by degradation of newly synthesised glycoproteins (section 1.4 and references therein). The water-soluble oligosaccharides which have one GlcNAc residue at the reducing end were probably derived from newly synthesised glycoproteins, and those with two GlcNAc residues were derived by hydrolysis of LLO by oligosaccharyltransferase (Cacan *et al.*, 1992; Villers *et al.*, 1994).

5.3.2.1 PMM-deficient and PMM-normal CDGS type 1 fibroblasts

There was no difference in the types or relative proportions of the water-soluble oligosaccharides found in the fibroblasts from CDGS type 1 (both PMM-deficient and PMM-normal) patients (Figure 5.2). It would have been expected that the amount of water-soluble oligosaccharides might have been decreased in CDGS type 1 fibroblasts because they are all derived from the LLO. If there were limiting amounts of LLO for transfer to glycoproteins, then there should be a corresponding decrease in the amount of complete LLO hydrolysed by oligosaccharyl transferase. However this was not found to be the case. The present studies were qualitative and not quantitative so it is possible that although the proportions released of each oligosaccharide remained constant, there could have been a decrease in the total water-soluble oligosaccharides in the CDGS type 1 patient fibroblasts. However, the present results are in contrast to those reported by Panneerselvam and Freeze (1996a) as they found the water-soluble oligosaccharides in CDGS type 1 fibroblasts were truncated compared to those found in normal fibroblasts.

5.3.2.2 Galactosaemic fibroblasts

The pattern of water-soluble oligosaccharides found in the fibroblasts from galactosaemic patients was similar to that seen in control and CDGS type 1 fibroblasts. It was predicted that the oligosaccharides extracted from the galactosaemic fibroblasts would have been comparable in structure and proportions to those from normal fibroblasts, because the defect in the galactosaemic fibroblasts lies in the *trans*-Golgi network and the water-soluble oligosaccharides were extracted from the endoplasmic reticulum.

These data suggest that the water-soluble oligosaccharides are not affected by the enzyme deficiencies in either the CDGS type 1 or the galactosaemic patient fibroblasts.

5.3.3 Analysis of lipid-linked oligosaccharides

The oligosaccharides extracted from normal fibroblasts into fraction 3 were identified as a series of high mannose oligosaccharides from $\text{Man}_3\text{GlcNAc}_2$ to $\text{Man}_9\text{GlcNAc}_2$ (Figure 5.3). This is in contrast to the water-soluble oligosaccharide fraction in which the $\text{Man}_5\text{GlcNAc}_1$ to $\text{Man}_9\text{GlcNAc}_1$ series was also present. The oligosaccharides identified were the structures expected to be found as biosynthetic intermediates in the LLO pathway (Figure 1.3). The oligosaccharides from the PMM-deficient and PMM-normal CDGS type 1 as well as from the galactosaemic fibroblasts were the same as those found in control fibroblasts (Figure 5.3).

The ratio of $\text{Man}_5\text{GlcNAc}_2$: $\text{Man}_9\text{GlcNAc}_2$ for each cell line was calculated by integrating the peak areas and the results are shown in Table 5.1. If there was an increase in the proportion of $\text{Man}_5\text{GlcNAc}_2$ in either the CDGS type 1 or galactosaemic fibroblasts, then the $\text{Man}_5\text{GlcNAc}_2$: $\text{Man}_9\text{GlcNAc}_2$ ratio would be increased compared to the controls. There was no increase in the $\text{Man}_5\text{GlcNAc}_2$: $\text{Man}_9\text{GlcNAc}_2$ ratio in the CDGS type 1 (PMM-deficient and PMM-normal) fibroblasts, when compared to controls (Table 5.1) nor was there an increase in the ratio in the galactosaemic fibroblasts. These results are in contrast to previous findings (Powell *et al.*, 1994; Krasnewich *et al.*, 1995; Panneerselvam and Freeze, 1996a; Lewis, 1997).

Fibroblasts cell line	Ratio $\text{Man}_5\text{GlcNAc}_2$: $\text{Man}_9\text{GlcNAc}_2$
Control (n = 4)	1.4, 2.3, 2.8, 4.8
PMM deficient CDGS type 1	2.3, 4.3
PMM normal CDGS type 1	1.2
Galactosaemic	2.4

Table 5.1: Ratio of $\text{Man}_5\text{GlcNAc}_2$: $\text{Man}_9\text{GlcNAc}_2$ in control, CDGS type 1 and galactosaemic fibroblasts.

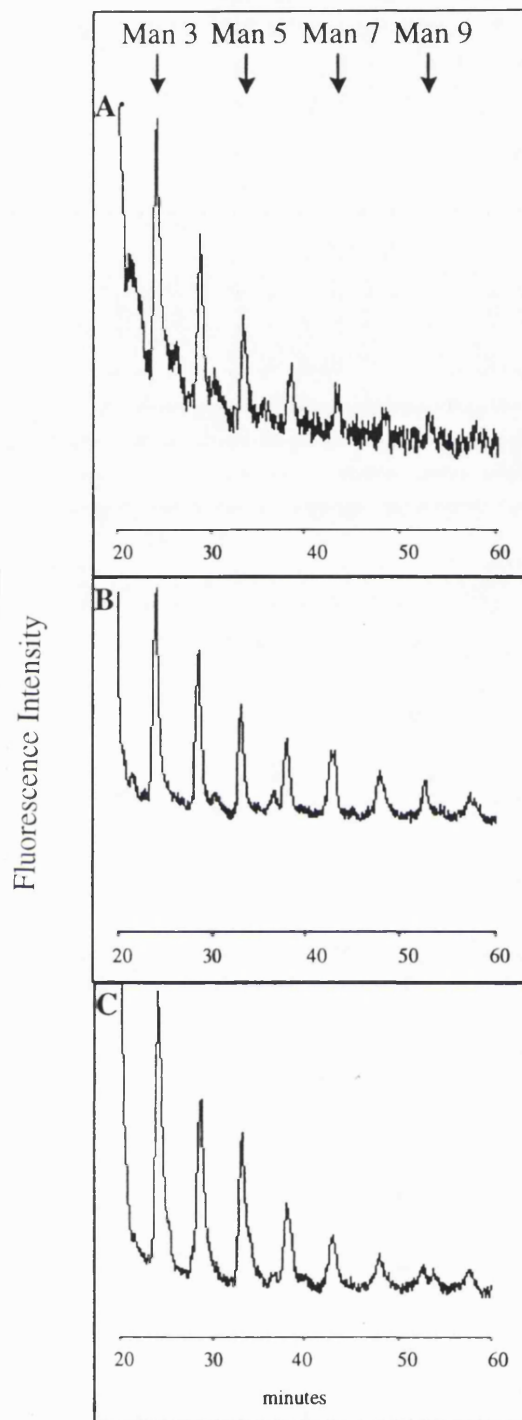


Figure 5.3: HPLC of lipid-linked oligosaccharides (fraction 3)

A; normal, **B;** CDGS type 1 (PMM-deficient) fibroblasts, and **C;** galactosaemic fibroblasts eluting from the GlycoSep HPLC column. The HPLC profile for PMM-normal CDGS type 1 fibroblasts is identical to profile **B**. Arrows indicate identification of oligosaccharides by co-chromatography or deduction.

5.3.4 Analysis of protein bound N-linked oligosaccharides

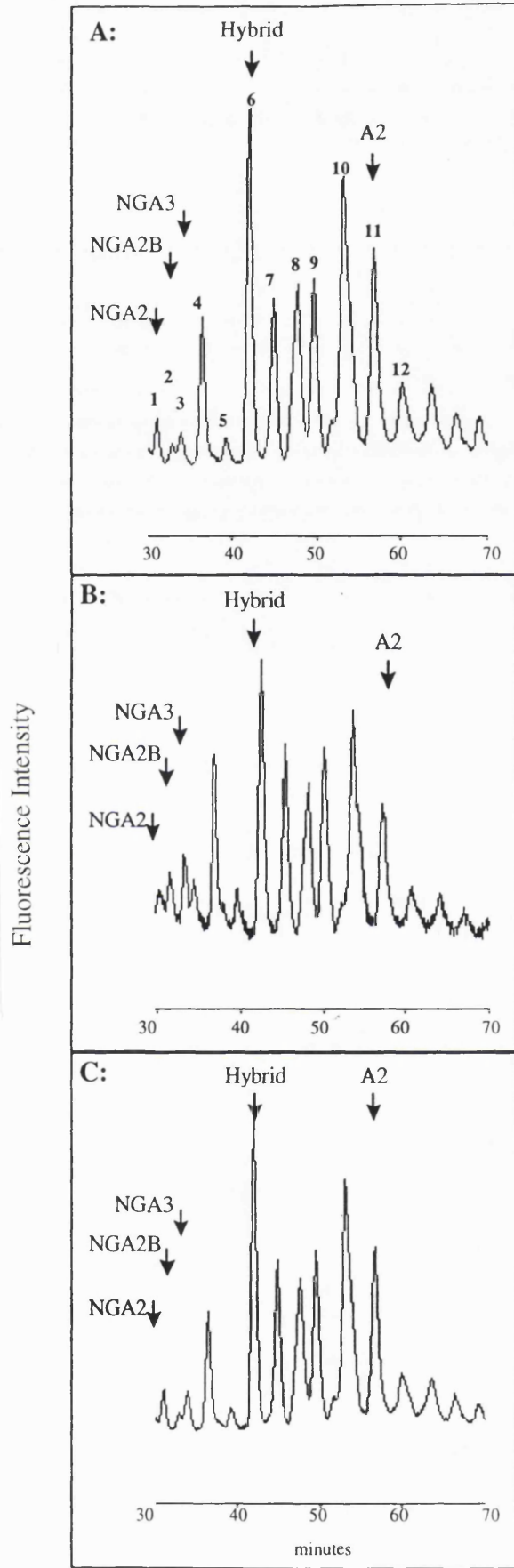
The HPLC profile of the N-linked oligosaccharides released by N-glycanase digestion from cellular proteins in normal fibroblasts is shown in Figure 5.4A. The oligosaccharides were identified either by co-chromatography with authentic standards or by deduction (Table 5.2). The positions of the oligosaccharides of interest (NGA2, NGA2B, NGA3, HYBRID and A2) are marked in Figure 5.4.

Peak Number	Structure
1	NGA2
2	NGA2B
3	NGA3
4	MAN5
5	NGA4
6	HYBRID
7	MAN6
8	NA2B
9	MAN7
10	A1
11	A2
12	NA4

Table 5.2 Identification of oligosaccharides released into fraction 4

Figure 5.4: HPLC of protein-bound N-linked oligosaccharides (fraction 4)

A; normal, **B;** PMM-deficient CDGS type 1, and **C;** galactosaemic fibroblasts fed on glucose-only medium. The HPLC profile for PMM-normal CDGS type 1 fibroblasts is identical to profile **B**. For identification of oligosaccharides **1-12** see table 5.2. Arrows indicate the retention time of the authentic standards.



5.3.4.1 PMM-deficient and PMM-normal CDGS type 1 fibroblasts

The HPLC profiles of the glycans released from cellular glycoproteins from PMM-deficient and PMM-normal CDGS type 1 fibroblasts were identical to those obtained for the normal fibroblasts (Figure 5.4B). There was no difference in the oligosaccharides present nor in the relative proportions of any of the oligosaccharides. These results suggest that in CDGS type 1 fibroblasts the glycans produced are mature as was seen in serum transferrin. Again, the method of analysis used did not allow determination of whether the glycoproteins were under-glycosylated as exact quantitation was not possible since there was no internal standard. However, it was observed that for the CDGS type 1 fibroblasts when the same number of cells were taken through the fractionation and labelling procedure, the intensity of the peaks on the HPLC was decreased compared to the galactosaemic and control fibroblasts. These observations indicate that there may be a reduced amount of oligosaccharides in the glycoprotein fraction in the CDGS type 1 fibroblasts.

5.3.4.2 Galactosaemic fibroblasts

The HPLC profile of the glycans released from cellular glycoproteins extracted from galactosaemic fibroblasts that had been grown on a glucose-only medium was similar to that for control fibroblasts (Figure 5.4C). Although there was no difference in the range of oligosaccharides present, there was a small increase in the relative proportions of the truncated glycans NGA2, NGA2B and NGA3 when compared with the controls (Table 5.3). These three oligosaccharides were also observed in the transferrin purified from serum from untreated galactosaemic patients (Chapter 4). There was a further increase in the proportion of these truncated glycans in the galactosaemic fibroblasts that had been fed on 5 mM galactose for 3 days prior to harvesting (Table 5.3). However, these truncated oligosaccharides make up only a small proportion of the total glycans released and hence a more specific glycoprotein marker is required. These results do confirm Ornstein's (1992) finding that there was a greater proportion of galactose acceptors in galactosaemic fibroblasts, and the present analysis has allowed us to identify these oligosaccharides as NGA2, NGA2B and NGA3. They are also in agreement with recent work by Lewis (1997), who found an increase in the proportion of smaller oligosaccharides bound to cellular proteins in galactosaemic fibroblasts.

Fibroblast cell line	Percentage of truncated glycans	
	<i>no galactose</i>	<i>5 mM galactose</i>
Normal control	2.5-2.8	1.4-2.1
Galactosaemic	*3.0-3.7	*3.2-5.0

Table 5.3: Effect of galactose on the proportion of truncated N-linked glycans on glycoproteins in galactosaemia

* significant difference (Mann-Whitney U test)

5.4 Discussion

5.4.1 Studies on fibroblasts from CDGS type 1 patients

Although the glycosylation defects in the CDGS type 1 serum glycoproteins are well established (section 1.5.4), there have been conflicting reports on the glycosylation defects in the fibroblasts of the CDGS type 1 patients. Comprehensive studies by Powell *et al.* (1994), Krasnewich *et al.* (1995) and Panneerselvam and Freeze (1996a) found that the lipid linked oligosaccharides (LLO) produced in CDGS type 1 fibroblasts were truncated. However it had been noted by one group (Krasnewich *et al.*, 1995) that these truncated LLOs were only observed when the cells were labelled for 1 hour with [³H]-mannose, as after a two hour labelling time normal sized LLOs were synthesised. An alternative interpretation of these results is that they indicate that in CDGS type 1 fibroblasts there is a decrease in the rate of synthesis of the LLO. As no evidence of accumulation of truncated LLO was found it is not likely to be an enzyme deficiency in the synthesis the LLO itself, but perhaps in the amount of substrate available in the cell.

The results presented in this chapter in which the LLOs were analysed in the steady state, instead of the rate of synthesis of the LLO, confirmed the hypothesis. The structures of the LLO in the CDGS type 1 fibroblasts in the steady state were similar to that found in normal fibroblasts and no unusual structures were detected. The relative proportions of each structure present were also similar to that found in the normal fibroblasts. Thus these results suggest that the previously observed truncated LLO (Powell *et al.*, 1994;

Krasnewich *et al.*, 1995; Panneerselvam and Freeze, 1996a) were due to the different rate of synthesis of the LLO in the CDGS type 1 and the normal fibroblasts. The difference between the previous findings and the present investigation can be explained by the difference in the methods that were used.

Ohkura *et al.* (1997) re-investigated the structures of the LLO in the CDGS type 1 fibroblasts, as they too believed that the truncated LLOs were found as a result of the labelling conditions used and were not indicative of a blockage in the pathway of LLO synthesis. To prove their hypothesis they took a similar approach to the one used in this Chapter. In their case steady state labelling conditions were obtained by incubating the cells with [³H]-mannose for 3 hours instead of the 1 hour used by the other groups (Powell *et al.*, 1994; Krasnewich *et al.* 1995; Panneerselvam and Freeze, 1996a). In their experiments the LLO in the CDGS type 1 fibroblasts had the same structures as found in the normal fibroblasts, thus confirming the findings reported in this Chapter. These authors also repeated the experiment exchanging [³H]-mannose with [³H]-GlcNAc and again no truncated LLO were found and in both cases, only full size LLO were transferred to the proteins. In light of these developments it can be concluded that the rate of synthesis of the LLO is decreased and this is the most probable cause of the under glycosylation of sequons seen in the CDGS type 1 glycoproteins. The decreased rate of synthesis of the LLO is likely to be caused by the deficiency of PMM. In the CDGS type 1 fibroblasts there is a decreased rate of synthesis of mannose-1-phosphate, and so this in turn must lead to decreased rate of synthesis of GDP-mannose and dolichol-P-mannose, both of which are required for the synthesis of the LLO (Chapter 1, Figure 1.3).

It was also observed by Ohkura *et al.* (1997) that the decreased levels of LLO were only observed in the S phase of the cell cycle. The levels of LLO in CDGS type 1 and control fibroblasts were the same in the G1 and G2 phases showing that the difference in levels in CDGS type 1 fibroblasts occurs only in the S phase. The decreased rate of synthesis of LLO only during certain phases of the cell cycle has implications as to the classes of glycoprotein affected. It would be expected that glycoproteins synthesised during the G1 and G2 phases would be normally glycosylated as the amount of LLO available for transfer to the growing polypeptide chain would be sufficient for complete glycosylation,

whereas glycoproteins synthesised during the S phase would suffer from non-occupancy of glycosylation sequons because of a deficiency of the fully formed oligosaccharide precursor.

It was also found that there was an increase in the amount of dehydrololichol and a decreased amount of dolichol in CDGS type 1 fibroblasts (Ohkura *et al.*, 1997). Dolichol is produced from dehydrololichol by the enzyme dehydrololichol reductase. An accumulation of dehydrololichol in CDGS type 1 (PMM deficient) fibroblasts suggests that there could be a block in the synthesis of dolichol or alternatively it could be the result of a feedback mechanism within the cell due to the lack of utilisation of dolichol as a result of impairment of GDP-mannose production. A deficiency of dehydrololichol reductase activity has not been shown in CDGS type 1 cell lines as yet.

The methodology of Powell *et al.* (1994) and Panneerselvam and Freeze (1996a) only provides a narrow window of time in the glycosylation pathway and does not represent what happens in the steady state. The difference between their results and the present findings can be explained by the fact that with the radiolabelling experiments the rate of production of the oligosaccharide precursor was followed. As there was reduced incorporation of mannose into the cells a decrease in the rate of production of the oligosaccharide precursor is to be expected. This explanation would account for the production of smaller oligosaccharides after a 1 hour chase and normal sized oligosaccharides after a 2 hour chase. Thus in the steady state the presence of the oligosaccharides in the correct proportions is to be expected. The same principle can be applied to the newly synthesised glycoprotein fraction. Using the steady state conditions, the present study has shown that the glycans from CDGS type 1 fibroblasts were the same species and present in the same proportions as was found in normal fibroblasts, whereas Powell *et al.* (1994) and Panneerselvam and Freeze (1996a) found smaller glycans in their glycoprotein fraction suggesting that smaller glycans were transferred to the protein.

5.4.2 Studies on fibroblasts from galactosaemic patients

It has been shown in this investigation that, as in CDGS type 1 fibroblasts, the pattern of lipid-linked oligosaccharides in galactosaemic fibroblasts was similar to that in normal cells and that the $\text{Man}_5\text{GlcNAc}_2:\text{Man}_9\text{GlcNAc}_2$ ratio was within the range found in normal fibroblasts. Interestingly, Lewis (1997) has found that galactosaemic fibroblasts incorporate less [^3H]-mannose than normal fibroblasts and that their lipid-linked oligosaccharides are smaller than those found in normal fibroblasts. Again, the same problem applies with galactosaemic fibroblasts as with CDGS type 1 fibroblasts, as the methods of Lewis *et al.* (1997) were essentially those used by Panneerselvam and Freeze (1996a). However, in contrast to the CDGS type 1 fibroblasts, the glycans from the glycoprotein fraction of galactosaemic fibroblasts showed an increase in the proportion of truncated glycans compared to the normal fibroblasts. This truncation could arise from under galactosylation as previously suggested by Dobbie *et al.* (1990), Ornstein *et al.* (1992) and Lewis (1997). The increase in truncated glycans on glycoproteins was seen in galactosaemic fibroblasts that had been grown on medium containing glucose as the only carbon source (comparable with a galactose-free diet) and also in galactosaemic fibroblasts that were fed on the same medium supplemented with 5 mM galactose (comparable with an untreated patient). It would seem that just removing galactose from the diet or the cell culture medium does not allow normal processing of complex glycans. This may have important implications for the mechanism of the long term complications associated with the treated galactosaemic patients (see Chapter 8).

5.5 Summary

Using direct analysis of the various oligosaccharide pools of the cell under steady state metabolic conditions and without the use of radiolabelled sugars, the data presented in this Chapter show that the oligosaccharide pools originating from the LLO in CDGS type 1 and galactosaemic fibroblasts were comparable with controls. However, this method did not allow quantitation of the amount of each oligosaccharide present. It would seem that a decrease in the rate of synthesis of LLO produces truncated LLO in some CDGS type 1 and galactosaemic cell lines when a 60 min pulse-chase strategy is used (Powell *et al.*, 1994; Panneerselvam and Freeze, 1996a; Lewis, 1997). It has also

been demonstrated that in CDGS type 1 fibroblasts (PMM-deficient and PMM-normal) the glycoprotein fraction contains oligosaccharides which have the same oligosaccharide structures as those seen in the normal fibroblasts. In contrast, in the galactosaemic fibroblasts the glycoprotein fraction contains a greater proportion of glycans which are agalactosylated whether grown in the presence or absence of galactose. In the next chapter the synthesis and processing of lysosomal enzymes as model glycoproteins in CDGS type 1 and galactosaemic fibroblasts is discussed.

Glycosylation of glycoproteins in CDGS type 1 and galactosaemic fibroblasts

6.1 Introduction

Serum transferrin was used as a model glycoprotein to investigate the aberrant glycosylation resulting from the genetic defects in CDGS type 1 and galactosaemic patients (Chapter 4). From these studies it was concluded that the CDGS type 1 defect is expressed as partial occupancy of glycosylation sites by oligosaccharides in the endoplasmic reticulum, whilst the galactosaemic defect is expressed as reduced galactosylation of glycans in the *trans* Golgi network. These conclusions were supported by analysis of the N-linked oligosaccharides released from glycoproteins extracted from fibroblasts from CDGS type 1 and galactosaemic patients (Chapter 5). The N-linked oligosaccharides of glycoproteins from CDGS type 1 fibroblasts were of the normal complex and high mannose types, whereas the glycoproteins from galactosaemic fibroblasts contained a high proportion of agalactosylated oligosaccharides. Apart from an earlier study by Marquardt *et al.* (1995) on the recombinant expression of viral glycoproteins by CDGS type 1 fibroblasts, there have been no reports in the literature of an intracellular glycoprotein in fibroblasts that exhibits the same characteristic aberrant glycosylation as serum transferrin.

The aim of the work presented in this chapter was to carry out quantitative and qualitative assessment of the N-glycosylation ability of cultured fibroblasts from CDGS type 1 and galactosaemic patients in an attempt to demonstrate aberrant glycosylation of cellular glycoproteins in these two disorders. Two different approaches were adopted. In the first, the capacity of microsomal fractions prepared from control and CDGS type 1 fibroblasts to glycosylate yeast α -factor (*S. cerevisiae*) was tested in a cell-free system. The microsomes mimic the endoplasmic reticulum component of the cell. The α -factor polypeptide contains six N-glycosylation sites, of which three are occupied at any one time. The α -factor polypeptide was radiolabelled with [³⁵S]-methionine and the resulting products of the translation/glycosylation reactions were analysed by SDS-PAGE followed by autoradiography.

The second approach involved assessment of the N-glycosylation of two lysosomal enzymes in intact fibroblasts from CDGS type 1 and galactosaemic patients. As lysosomal enzymes are relatively abundant in fibroblasts they were considered suitable candidates for such studies. β -Hexosaminidase and α -fucosidase were chosen as reporter molecules in this investigation, as serum β -hexosaminidase and α -fucosidase have been shown to be aberrantly glycosylated in both CDGS type 1 patients and galactosaemic patients (Jaeken *et al.*, 1992; Clayton *et al.*, 1993; Winchester *et al.*, 1995). The structures of these two lysosomal enzymes has been reviewed in section 1.4.1.

6.2 *In vitro* translation and glycosylation of α -factor

Commercially available cell-free *in vitro* translation kits can be used to express mRNA and to characterise the translated protein. Rabbit reticulocyte lysate and canine pancreatic microsomes, the two main components of the kit, provide the necessary precursors and enzymes for the *in vitro* translation of mRNA and subsequent glycosylation (i.e. tRNA, ribosomes, dolichol-PP-oligosaccharides and oligosaccharyltransferase). In the present investigation this system was used to compare the glycosylation capacity of microsomal fractions prepared from CDGS type 1 fibroblasts and normal fibroblasts by using them in place of canine microsomes. Microsomes are commonly used to investigate both co-translational and post-translational processing because they are capable of protein synthesis, glycosylation and lipid synthesis (Walter and Blobel, 1983).

6.2.1 Preparation of microsomes

6.2.1.1 Homogenisation of fibroblasts

The efficiency of homogenisation was investigated by comparing the use of a 1 ml glass homogeniser and a nitrogen cavitation bomb (section 2.9). Trypan blue-uptake studies revealed that 80-90% of the fibroblasts which had been subjected to nitrogen cavitation were lysed compared with 20% in the untreated sample. Only 60% of the fibroblasts which had been homogenised in the glass homogeniser were lysed. It was concluded that nitrogen cavitation was the more efficient method for homogenising the fibroblasts, and it was used in all subsequent experiments.

6.2.1.2 Purification of microsomes

The cell homogenates prepared in 6.2.1.1 contained other organelles such as the nucleus, mitochondria and lysosomes as well as microsomes, so a method of separation was required in order to purify the microsomal component of the homogenate. This was attempted by various combinations of gel filtration and ultracentrifugation (Table 6.1). In each method of preparation of microsomes, the components of each fraction were identified by measuring the activity of various markers i.e. Golgi α -mannosidase, β -hexosaminidase (marker for lysosomes) and neutral α -glucosidase (marker for ER) (section 2.9). The microsomes prepared using methods 1 and 2 were not able to glycosylate α -factor, but the ones prepared by method 3 (Knauer *et al.*, 1994) were active, so this method was used to prepare active microsomes from control and PMM-deficient CDGS type 1 fibroblasts.

Method	Centrifugation	Gel filtration	Sucrose cushion	Centrifugation	Active microsomes
1	12,000 x g, 10 min.	yes	yes	40,000 x g, 30 min.	no
2	12,000 x g, 10 min.	no	yes	no	no
3	12,000 x g, 10 min.	no	no	66,000 x g, 30 min.	yes

Table 6.1: Comparison of the methods used to prepare microsomes from fibroblasts

The full methods are described in section 2.9.

6.2.2 *In vitro* translation and glycosylation of α -factor by microsomes extracted from fibroblasts

The ability of the microsomes (prepared by method 3) to glycosylate α -factor was tested by performing translation / glycosylation reactions *in vitro* and analysing the products by SDS-PAGE followed by autoradiography (section 2.9, Figure 6.1). The sample in lane 6 is the material obtained after translation of α -factor without the microsomes. The single

band with a molecular weight of 18 kDa is the unglycosylated α -factor polypeptide. Lane 1 contains the translation products when the reaction was carried out in the presence of canine microsomes. Two major bands were seen, one at 18 kDa and one at 30 kDa. The 30 kDa band is the glycosylated α -factor. By comparing the bands in lanes 4 and 5 (fibroblast microsomes without mRNA) with those in lanes 2 and 3 (fibroblast microsomes with mRNA), it was seen that there were two extra bands in lanes 2 and 3 at 18 kDa and 30 kDa corresponding to α -factor and glycosylated α -factor respectively. It was concluded that the crude membranes prepared from control fibroblasts and PMM-deficient CDGS type 1 fibroblasts were glycosylation-competent. Thus the membrane preparations for both CDGS type 1 and controls contained the necessary components for glycosylation i.e. oligosaccharyltransferase and dolichol-linked oligosaccharides. These preparations were then used to investigate the activity of oligosaccharyltransferase and also the amount of dolichol-linked oligosaccharide present in CDGS type 1 fibroblasts.

The effects of time, temperature, concentration of mRNA and concentration of membranes on the percentage of glycosylation of α -factor were then investigated. The conditions of the *in vitro* translation reaction are described in Table 6.2. The percentage of glycosylated α -factor was determined by integrating the area in each band using GelWorks (Figures 6.2 and 6.3).

Saturation of the glycosylation of α -factor was seen after 1 hour in the normal membranes, but after 2 hours in the CDGS type 1 membranes (Figure 6.4). A greater percentage of α -factor was glycosylated in the CDGS type 1 membranes. In both cases there was not more than 50% glycosylation of α -factor.

In both CDGS type 1 microsomes and controls, the maximum glycosylation of α -factor was achieved at 0.05 mg/ml mRNA. The CDGS type 1 microsomes consistently glycosylated a greater percentage of α -factor than the controls (Figure 6.5). The concentration of mRNA did not make much difference to the percentage of glycosylation.

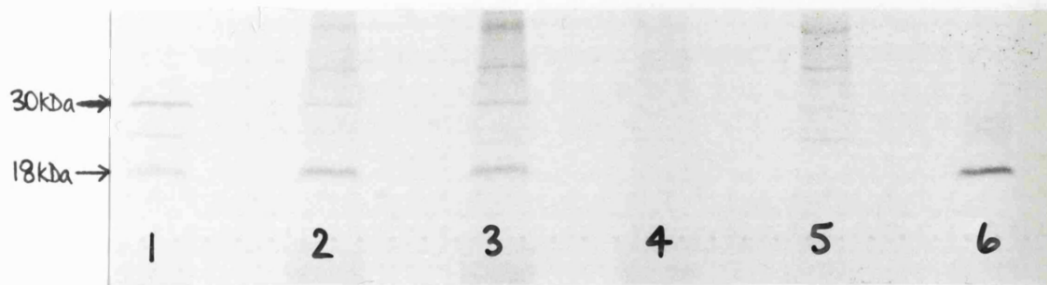


Figure 6.1: *In vitro* translation of α -factor by canine microsomes and crude membranes from control and PMM-deficient CDGS type 1 fibroblasts. SDS-PAGE (15%) followed by autoradiography. lane 1; canine microsomes in the presence of α -factor mRNA, lane 2; crude membranes from control fibroblasts in the presence of α -factor mRNA, lane 3; crude membranes from PMM-deficient CDGS type 1 fibroblasts in the presence of α -factor mRNA, lane 4; crude membranes from control fibroblasts, lane 5; crude membranes from PMM-deficient fibroblasts, lane 6; *in vitro* translation without canine microsomes.

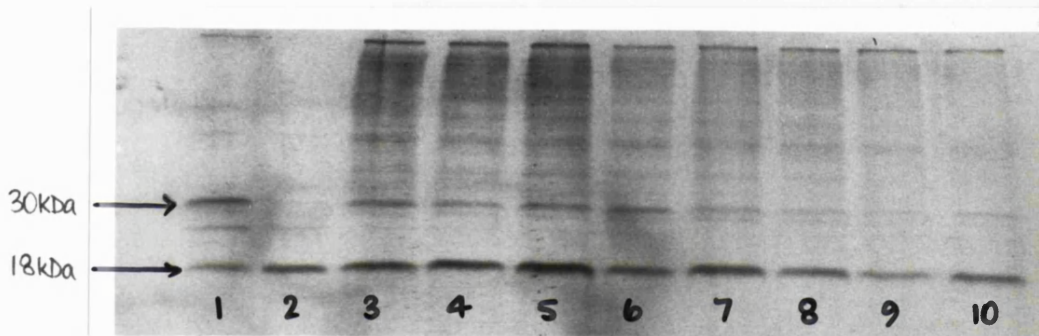


Figure 6.2: *In vitro* translation reactions for crude membranes prepared from control fibroblasts.

SDS-PAGE (15%) followed by autoradiography. For lane details see table 6.2.

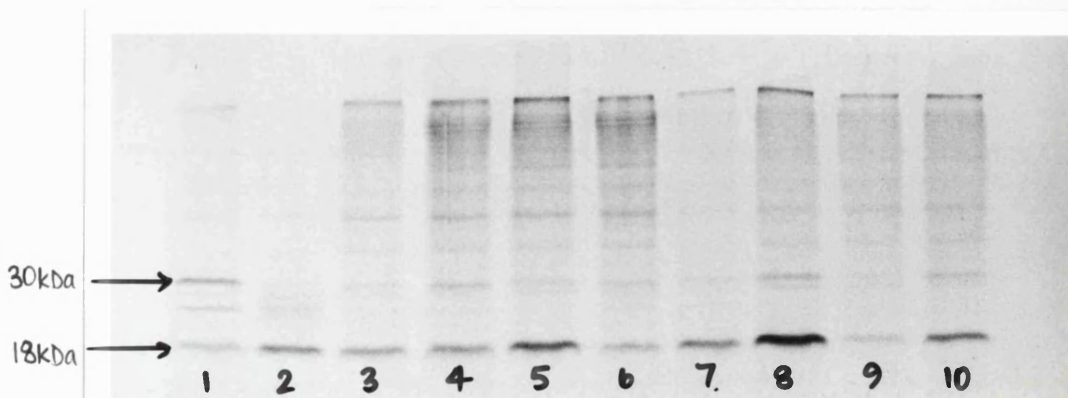


Figure 6.3: *In vitro* translation reactions for crude membranes prepared from PMM-deficient CDGS type 1 fibroblasts.

SDS-PAGE (15%) followed by autoradiography. For lane details see table 6.2.

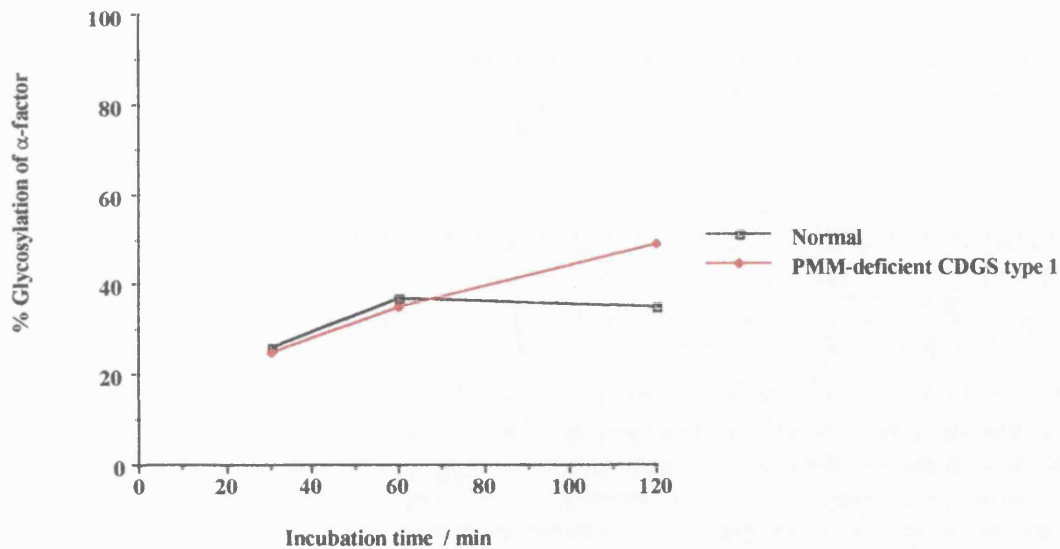


Figure 6.4: Effect of the time of incubation on the glycosylation of α -factor in control and CDGS type 1 membranes.

Black line; microsomes from control fibroblasts, **Red line;** microsomes from PMM deficient CDGS type 1 fibroblasts. The percentage glycosylation of α -factor was determined by analysing the reaction products by SDS-PAGE with autoradiography and integrating the intensity of the relevant bands using GelWorks.

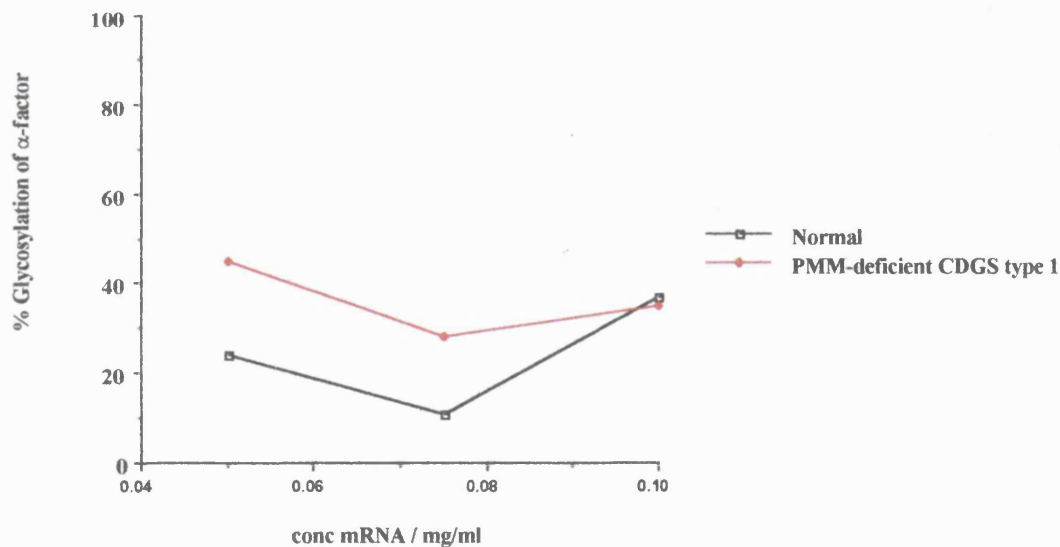


Figure 6.5: Effect of the mRNA concentration on the glycosylation of α -factor in control and CDGS type 1 membranes.

Black line; microsomes from control fibroblasts, **Red line;** microsomes from PMM deficient CDGS type 1 fibroblasts. The percentage glycosylation of α -factor was determined by analysing the reaction products by SDS-PAGE with autoradiography and integrating the intensity of the relevant bands using GelWorks.

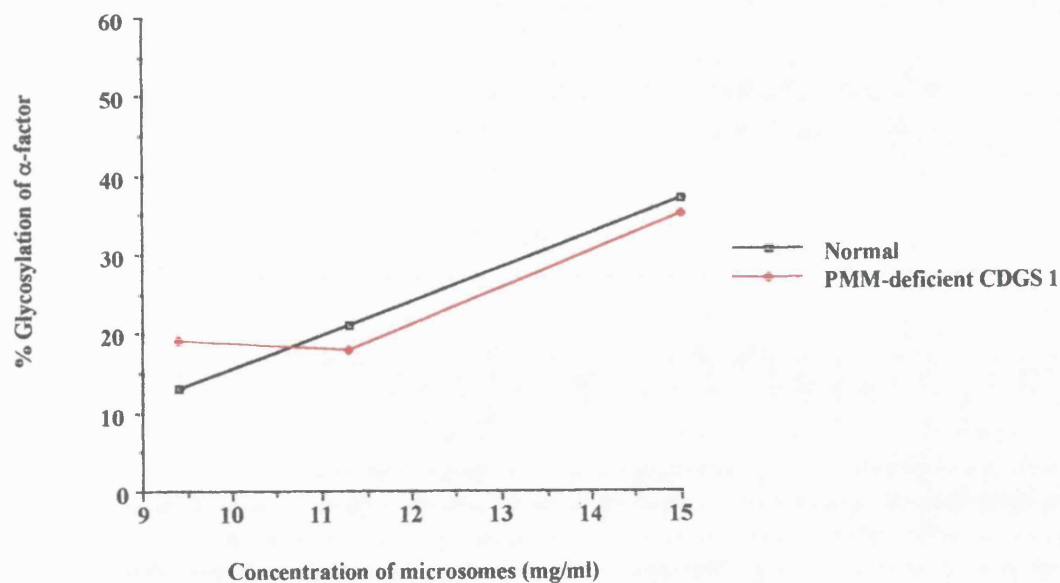


Figure 6.6: Effect of the membrane concentration on the glycosylation of α -factor in control and CDGS type 1 membranes.

Black line; microsomes from control fibroblasts, **Red line;** microsomes from PMM deficient CDGS type 1 fibroblasts. The percentage glycosylation of α -factor was determined by analysing the reaction products by SDS-PAGE with autoradiography and integrating the intensity of the relevant bands using GelWorks.

In both CDGS type 1 and normal microsomes the percentage glycosylation of α -factor increased with increasing membrane concentration (Figure 6.6) and also with increasing temperature but again both normal and CDGS type 1 profiles were similar.

Lane	Sample	Temp (°C)	Time (hours)	Membrane concentration (mg/ml)	mRNA concentration (mg/ml)
1	Canine microsomes	30	1	20	0.1
2	α -factor	30	1	none	0.1
3	crude membranes	30	1	15	0.1
4	crude membranes	37	1	15	0.1
5	crude membranes	30	0.5	15	0.1
6	crude membranes	30	2	15	0.1
7	crude membranes	30	1	9.4	0.1
8	crude membranes	30	1	11.3	0.1
9	crude membranes	30	1	15	0.05
10	crude membranes	30	1	15	0.075

Table 6.2: Composition of the *in vitro* translation reactions for the investigation of the effects of time, temperature and mRNA concentration on the glycosylation of α -factor.

From these results it was concluded that there was no defect in the oligosaccharyltransferase activity in the PMM-deficient CDGS type 1 fibroblasts¹, confirming the results of Knauer *et al.* (1994), furthermore these results indicate that there was sufficient precursor oligosaccharide for the glycosylation of α -factor. Since there were no faster running bands, this indicates there were no truncated LLOs. This contrasts with the results of Powell *et al.* (1994), Panneerselvam and Freeze (1996a), who observed both truncated and decreased amounts of LLOs in PMM-deficient CDGS type 1 fibroblasts. However, the present results are consistent with the findings of Ohkura *et al.* (1997).

6.2.3 Discussion

The *in vitro* translation/glycosylation experiments described here were performed before the discovery that a deficiency of phosphomannomutase was the major cause of CDGS

¹ At the time these experiments were performed it was not known that the defect causing CDGS type 1 in this cell line was due to a deficiency of phosphomannomutase.

type 1 (Van Schaftingen and Jaeken, 1995). These experiments were designed to determine whether there was a decreased capacity for glycosylation due to a deficiency of oligosaccharyltransferase or the lipid-linked oligosaccharide precursor in CDGS type 1 fibroblasts. It was found that the glycosylation activity of oligosaccharyl-transferase in CDGS type 1 fibroblasts was comparable to oligosaccharyltransferase activity in normal fibroblasts, and concluded that this was not the major cause of CDGS type 1. This conclusion was confirmed by Knauer *et al.* (1994) who had measured the oligosaccharyltransferase activity in CDGS type 1 fibroblasts directly with synthetic substrates. Our system had the advantage that it used the native dolichol-linked oligosaccharide precursor and that the polypeptide substrate had up to six glycosylation sequons whereas Knauer *et al.*, used hexapeptides as substrates. However the results were the same with both systems.

6.3 Lysosomal enzymes as cellular markers of glycosylation in fibroblasts

6.3.1 Intracellular and secreted β -hexosaminidase activities

The intracellular and secreted activities of β -hexosaminidase were measured in cultures of fibroblasts from controls, PMM-deficient and PMM-normal CDGS type 1 patients and patients with galactosaemia (sections 2.2.5 and 2.7). The intracellular activity of β -hexosaminidase was found to be similar in control, PMM-deficient CDGS type 1, PMM-normal CDGS type 1 and galactosaemic fibroblasts (Table 6.3). Statistical analysis (Mann-Whitney test and small sample t-test) provided no evidence of a difference in the intracellular enzyme activities in control vs. PMM-deficient CDGS type 1; control vs. PMM-normal CDGS type 1 or control vs. galactosaemia.

The amount of secreted β -hexosaminidase relative to cellular protein was found to be similar in control and galactosaemic fibroblasts, but much reduced in the PMM-deficient CDGS type 1 and PMM-normal CDGS type 1 fibroblasts (Table 6.3). Statistical analysis of the results showed a significant decrease (at the 95% confidence level) in the activity of the secreted enzyme between the controls and the CDGS type 1 patient fibroblasts. These values are prefixed with a "*" in Table 6.3b. There was no statistical evidence for a decrease in the amount of secreted enzyme in the galactosaemic fibroblasts.

6.3.1.3 Other lysosomal enzyme activities

The intracellular activity of another lysosomal enzyme, α -mannosidase (section 2.7), was measured in the control, CDGS type 1 and galactosaemic fibroblasts. There was no difference in the intracellular α -mannosidase activity between the CDGS type 1 or the galactosaemic fibroblasts and the controls. The amount of α and β -mannosidase, β -glucosidase, and α -fucosidase secreted into the cell culture medium of control, CDGS type 1 and galactosaemic fibroblasts was also measured but the activities of all these enzymes were too low for interpretation. It is not known on the basis of these results whether decreased secretion of β -hexosaminidase in CDGS type 1 fibroblasts is a unique or a universal finding among the lysosomal enzymes. However the decreased secretion of β -hexosaminidase is an interesting finding in view of the fact that Gu and Wada (1995) found that CDGS type 1 fibroblasts secrete less decorin than normal fibroblasts but that it is glycosylated normally. The decreased secretion of β -hexosaminidase is the only fibroblast marker of defective glycosylation in CDGS type 1 fibroblasts described so far.

6.3.1.2 Mannose supplementation

Panneerselvam and Freeze (1996a) have shown that the aberrant glycosylation seen in CDGS type 1 fibroblasts can be corrected by supplementation of the cell culture medium with mannose. As a decreased amount of the secreted enzyme was found in the CDGS type 1 fibroblasts, mannose was added to the cell culture medium to see whether it could restore the secretion of β -hexosaminidase. Fibroblasts were grown in the presence of 5 mM mannose for 5 days and the intracellular and secreted β -hexosaminidase activities were measured at the end of this period. There was no significant difference in the activities of either the intracellular enzyme (Table 6.3a) or the secreted enzyme (Table 6.3b) between fibroblasts grown in the absence or presence of mannose for the control, CDGS type 1 or galactosaemic fibroblasts. It was concluded that supplementation of the cell culture medium with 5 mM mannose did not increase the amount of β -hexosaminidase secreted into the medium in CDGS type 1 fibroblasts.

	β -Hexosaminidase activity ($\mu\text{mol/hr/mg}$)			
	no mannose		5 mM mannose	
	<i>Mean</i>	<i>Range</i>	<i>Mean</i>	<i>Range</i>
Controls (n = 5)	5.9 SD = 2.3	3.7 - 9.8	5.2 SD = 1.3	3.5 - 6.7
PMM-deficient (n = 9)	3.8 SD = 1.1	2.5 - 5.6	3.5 SD = 0.9	2.2 - 4.9
PMM-normal (n = 3)	2.9 SD = 0.5	2.6 - 3.3	2.9 SD = 0.5	2.5 - 3.3
Galactosaemia (n = 6)	3.1 SD = 1.0	1.9 - 4.8	2.9 SD = 1.3	0.9 - 4.8

Table 6.3a. Intracellular β -hexosaminidase activity in fibroblasts.

n = number of determinations. There were 3 control, 5 PMM-deficient, 1 PMM-normal and 2 galactosaemic cell lines.

	β -Hexosaminidase activity ($\mu\text{mol/hr/mg protein}$)			
	no mannose		5 mM mannose	
	<i>Mean</i>	<i>Range</i>	<i>Mean</i>	<i>Range</i>
Controls (n = 5)	4.2 SD = 1.0	3.7 - 5.9	3.8 SD = 0.8	2.4 - 4.3
PMM-deficient (n = 9)	* 2.3 SD = 0.8	1.1 - 3.4	2.5 SD = 1.2	1.5 - 4.7
PMM-normal (n = 3)	* 2.5 SD = 1.5	1.0 - 3.9	2.2 SD = 0.7	1.8 - 3.0
Galactosaemia (n = 6)	2.8 SD = 0.8	1.8 - 3.7	3.3 SD = 1.0	2.0 - 4.8

Table 6.3b: β -Hexosaminidase activity secreted by fibroblasts.

* values are significantly different from the control values at the 95% confidence level.
n = number of determinations. There were 3 control, 5 PMM-deficient, 1 PMM-normal and 2 galactosaemic cell lines.

6.3.2 Ion-exchange chromatography of intracellular and secreted β -hexosaminidase

The intracellular and secreted β -hexosaminidase isoenzymes in control, CDGS type 1 and galactosaemic fibroblasts were investigated by anion-exchange chromatography in order to determine any differences in the amount of sialic acids present on the glycans.

6.3.2.1 Intracellular β -hexosaminidase

The intracellular β -hexosaminidase activity was separated into the two isoenzymes, β -hexosaminidase A and B (Figure 6.7). The second peak of activity was confirmed as β -hexosaminidase A by comparison with the elution profile of β -hexosaminidase activity from fibroblasts from a patient with Tay Sachs disease, in which β -hexosaminidase A is absent (O'Brien *et al.*, 1970). Comparison of the elution profiles for the intracellular β -hexosaminidase from control, PMM-deficient CDGS type 1, PMM-normal CDGS type 1 and galactosaemic fibroblasts revealed no differences (Figure 6.7). This data demonstrated that there was no difference in the overall charge or sialylation of the intracellular β -hexosaminidase isoforms in the PMM-deficient, PMM-normal CDGS type 1 or galactosaemic patient fibroblasts compared with the control cells.

6.3.2.2 Secreted β -hexosaminidase

As a decrease in the amount of secreted β -hexosaminidase had been observed in the CDGS type 1 fibroblasts, the secreted β -hexosaminidase was also analysed by anion-exchange chromatography to see whether there was any difference in the sialylation of the isoforms. The secreted β -hexosaminidase was separated into β -hexosaminidase B and A, but the secreted β -hexosaminidase B bound to the column more tightly than the intracellular β -hexosaminidase B. The elution profiles of the enzyme activities from PMM-deficient CDGS type 1, PMM-normal CDGS type 1 and galactosaemic fibroblasts were similar to that of normal cells (Figure 6.8). It was concluded that no changes in the sialylation of β -hexosaminidase could be detected in either the CDGS type 1 fibroblasts or the galactosaemic fibroblasts by anion-exchange chromatography. Similarly the elution profile of the secreted β -hexosaminidase from fibroblasts of CDGS type 1, incubated for 5 days in medium supplemented with 5 mM mannose showed no difference compared to controls.

Cell extracts were applied to a BioScale Q2 column equilibrated in 25 mM Bis-Tris buffer, pH 6 and eluted with a linear gradient of 0 M to 0.5M NaCl (Red line). Each fraction collected was assayed for β -hexosaminidase activity. **A**; β -hexosaminidase A, **B**; β -hexosaminidase B.

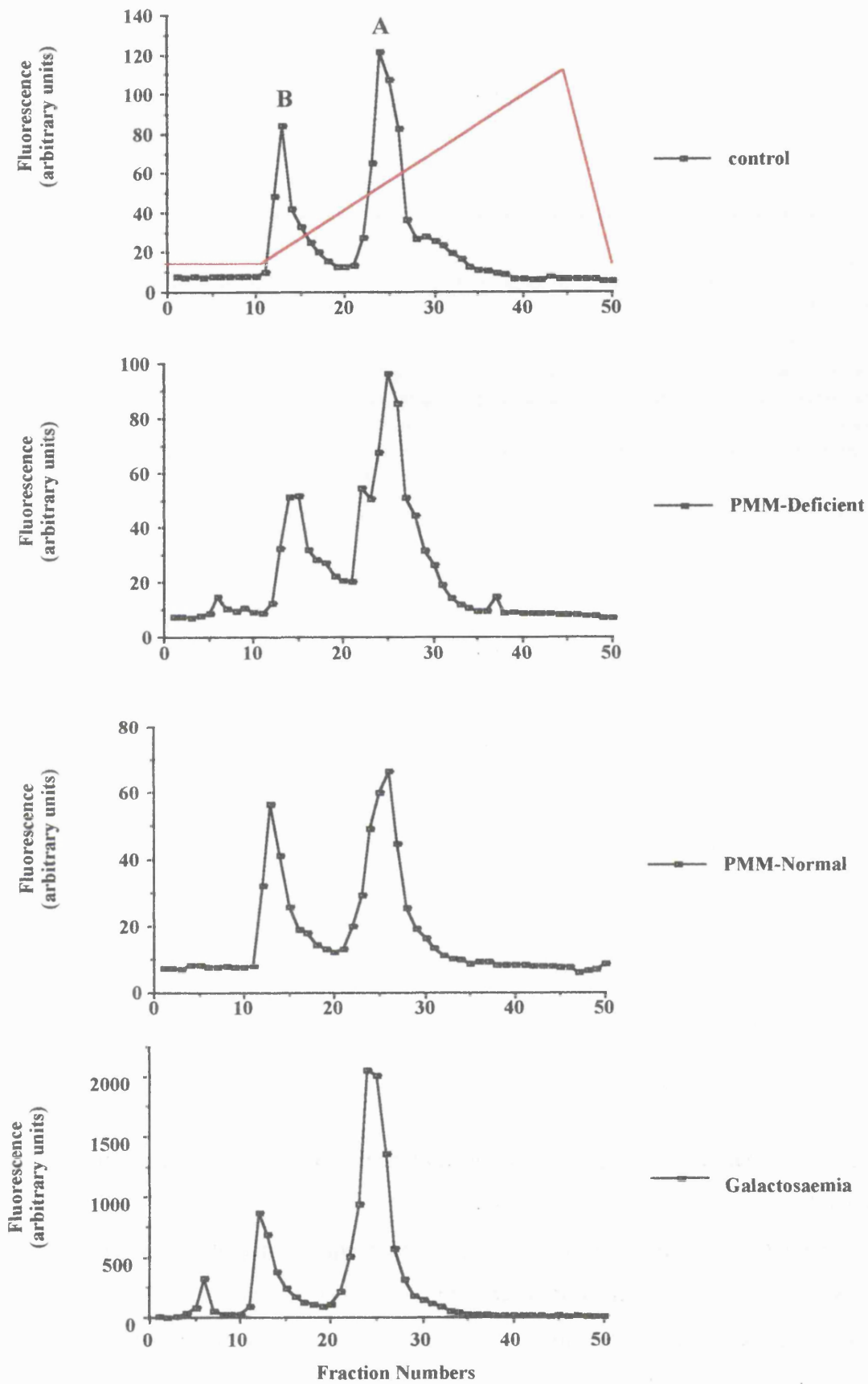
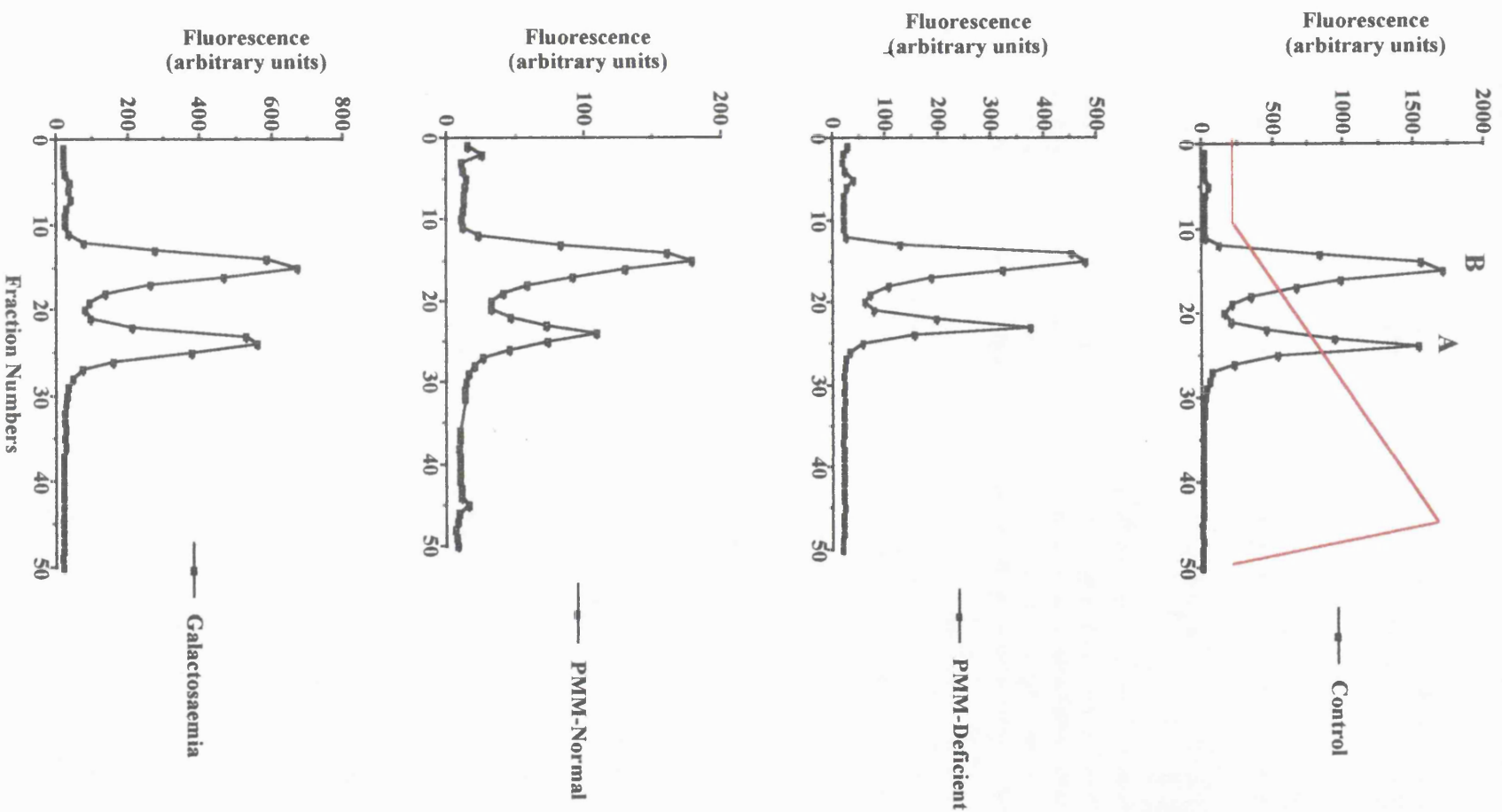


Figure 6.7: Anion-exchange chromatography of intracellular β -hexosaminidase.

Figure 6.8: Anion-exchange chromatography of β -hexosaminidase secreted by fibroblasts.

Cell extracts were applied to a BioScale Q2 column equilibrated in 25 mM Bis-Tris buffer, pH 6 and eluted with a linear gradient of 0 M to 0.5M NaCl (Red line). Each fraction collected was assayed for β -hexosaminidase activity. **A**; β -hexosaminidase A, **B**; β -hexosaminidase B.



6.3.2.3 Relative proportions of β -hexosaminidase A and B

The relative proportions of intracellular and secreted β -hexosaminidase A and B were determined by heat inactivation of β -hexosaminidase A and anion-exchange chromatography (Table 6.4). There were no differences in the relative proportions of the intracellular and secreted β -hexosaminidase A and B amongst control, PMM-deficient CDGS type 1 and galactosaemic fibroblasts. The percentage of intracellular β -hexosaminidase A in the PMM-normal CDGS type 1 fibroblasts measured by ion-exchange chromatography was just outside the reference range for control fibroblasts, but the percentage of extracellular β -hexosaminidase A was within the normal reference range.

It has previously been observed that if two of the glycosylation sites of the α subunit are not occupied then there is a decrease in the amount of intracellular and extracellular β -hexosaminidase A activity (Weitz and Proia, 1992). However as there was no significant decrease in the percentage of β -hexosaminidase A in CDGS type 1 or galactosaemic fibroblasts it can be concluded that the glycosylation of the α subunit is complete. Overall these results suggest that the N-glycosylation status of the component enzyme subunits was similar in control, CDGS type 1 and galactosaemic fibroblasts.

	Intracellular isoenzyme A % of total enzyme		Extracellular isoenzyme A % of total enzyme	
	Heat Inactivation	Ion-exchange chromatography	Heat Inactivation	Ion-exchange chromatography
Control (n = 3)	58 (52 - 63)	72 (66 - 82)	49 (38 - 59)	34 (20 - 42)
PMM-deficient (n = 3)	59 (55 - 64)	67 (66 - 67)	48 (44 - 51)	37 (34 - 43)
PMM-normal (n = 1)	ND	62	ND	36
Galactosaemia (n = 2)	59, 63	67, 75	51, 57	26, 44

Table 6.4: Percentage of β -hexosaminidase activity due to β -hexosaminidase A.

The main value is the mean and the range is shown in brackets. ND = not determined.
n = number of cell lines used.

Figure 6.9: Chromatography of intracellular β -hexosaminidase concanavalin-A-Sepharose.

Cell sonicates were applied to a 1 ml column containing concanavalin-A-sepharose, equilibrated in low salt buffer. The column was washed with 5 ml high salt buffer and eluted with a linear gradient of 0.5M methyl α -D-mannopyranoside (over 10 ml). The arrow indicates the start of the gradient. Each 1 ml fraction collected was assayed for β -hexosaminidase activity.

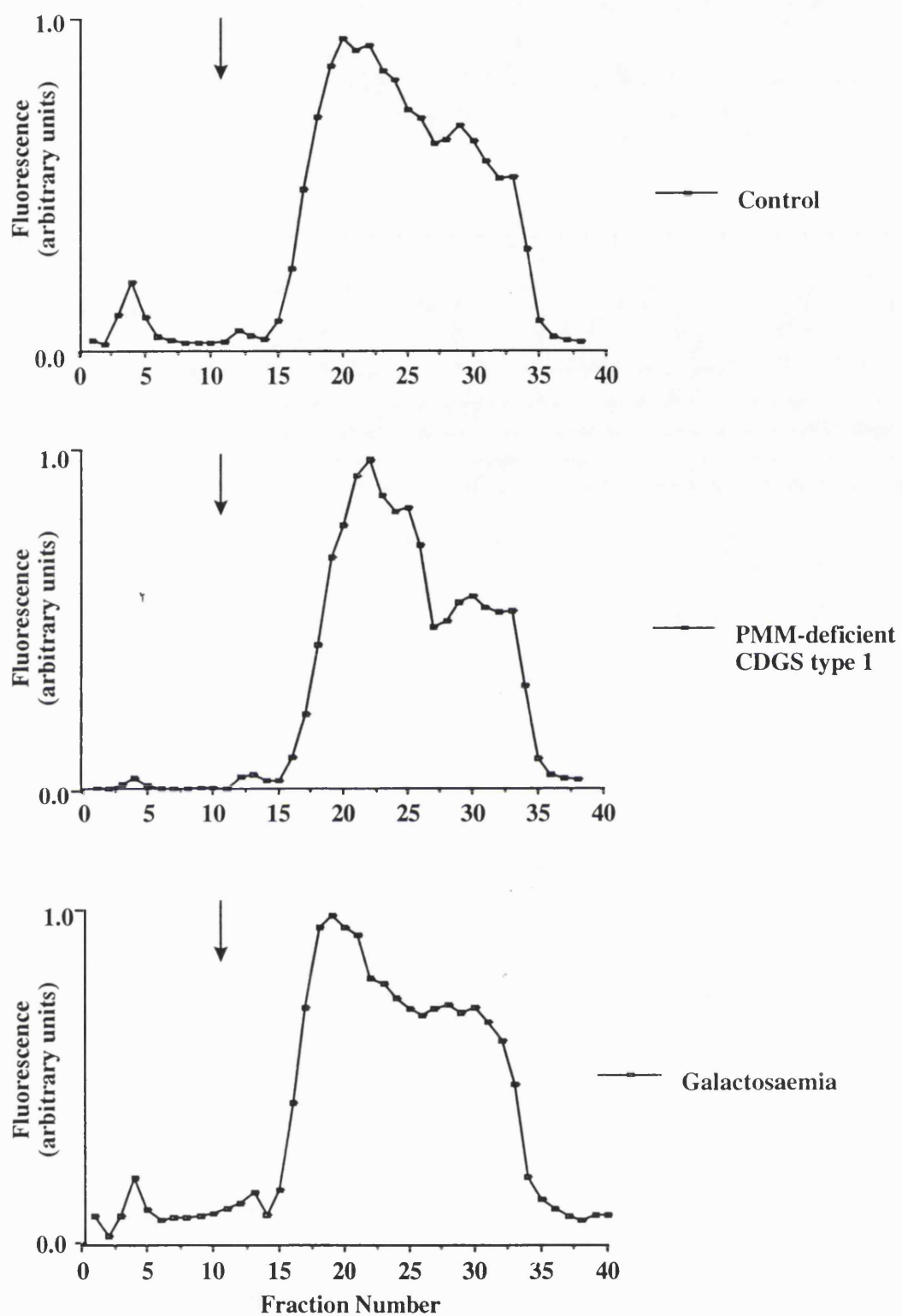


Figure 6.9: Chromatography of intracellular β -hexosaminidase concanavalin-A-Sepharose.

6.3.3 Lectin affinity chromatography of intracellular and secreted β -hexosaminidase

As only ten percent of the glycans of intracellular β -hexosaminidase are sialylated (Overdijk *et al.*, 1985) any differences in glycosylation between control, CDGS type 1 and galactosaemic fibroblasts may have been too subtle to detect by anion-exchange chromatography. Therefore the intracellular forms of the enzyme were also analysed by lectin affinity chromatography using concanavalin-A Sepharose (Figure 6.9). The majority of the β -hexosaminidase from normal fibroblasts bound to the concanavalin-A Sepharose column and was eluted as two components; the weakest binding components eluting between fractions 15 and 27 and the more strongly binding pool between fractions 27 and 35. The same chromatographic profile was seen for the β -hexosaminidase from CDGS type 1 (both PMM-normal and PMM-deficient) and galactosaemic fibroblasts. It can be concluded that any change in the glycosylation of the intracellular β -hexosaminidase in CDGS type 1 or galactosaemic fibroblasts does not affect its binding to concanavalin A.

6.3.4 Western blotting of α -fucosidase

The glycosylation status of intracellular α -fucosidase in control, PMM-deficient CDGS type 1, PMM-normal CDGS type 1 and galactosaemic fibroblasts was analysed by Western blotting (section 2.4.2) using anti- α -fucosidase antibodies. Five bands with molecular weights of 87, 54, 53, 47 and 33 kDa were detected in the control samples. Staining the same gel with coomassie blue revealed that the most abundant proteins in the cell extracts had molecular weights of 87 and 33 kDa. Fucosidosis patients were found to have no bands or reduced intensity of the bands at 47, 53 and 54 kDa, which confirmed that these three bands were due to α -fucosidase and the two other bands were non-specific binding of the anti- α -fucosidase antibody. The molecular weights of these three bands agreed with previous observations (Johnson *et al.*, 1992) for the molecular weight of α -fucosidase. The band at 47 kDa is the un-glycosylated α -fucosidase and the slower running bands at 53 and 54 kDa are glycosylated α -fucosidase (Johnson *et al.*, 1992). The same three bands were detected in the PMM-deficient CDGS type 1, PMM-normal CDGS type 1 and galactosaemic fibroblasts (Figure 6.10). It was concluded that

there was no detectable defect in glycosylation of α -fucosidase in CDGS type 1 or galactosaemic fibroblasts.

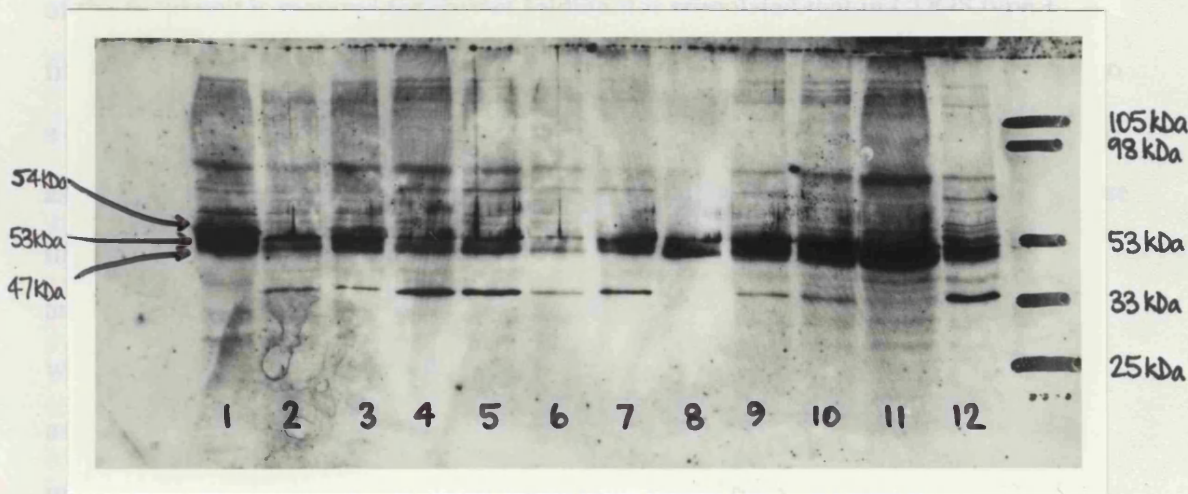


Figure 6.10: SDS-PAGE (7.5%) of cellular proteins followed by immunodetection of α -fucosidase.

Lane 1; control (P), lane 2; PMM normal CDGS type 1 (NH), lane 3; PMM-deficient CDGS type 1 (CB), lane 4; control (R), lane 5 and 6; galactosaemia (G1, LV), lane 7; control (K), lanes 8 to 11; PMM deficient CDGS type 1 (DC, KT, JB, EW), lane 12; control (P). The bands corresponding to the markers are indicated on the right of the gel and those due to α -fucosidase are indicated on the left.

6.3.5 Discussion

6.3.5.1 Lysosomal enzymes as cellular markers of aberrant glycosylation in CDGS fibroblasts

There was no difference in the specific activity of intracellular β -hexosaminidase in fibroblasts from controls, PMM-deficient CDGS type 1 and PMM-normal CDGS type 1 patients. There was, however, a significant decrease in the amount of β -hexosaminidase secreted into the medium from CDGS type 1 fibroblasts compared to that secreted by the controls. This observation suggests that the cell preferentially targets the correctly glycosylated β -hexosaminidase to the lysosome. In normal cells the secretion of the enzyme may be due to excess production and may not be vital to the survival of the cells. It is this pool that is decreased in the CDGS type 1 cells. It is known that in certain

patients with Tay-Sachs disease incorrectly folded α subunits aggregate in the endoplasmic reticulum and are degraded (Weitz and Proia, 1992). Thus as glycosylation of the α -subunit is required for correct folding it is speculated that in CDGS type 1 fibroblasts, the non-glycosylated α subunits aggregate in the ER and this in turn leads to a decrease in the amount of α subunits available. Any correctly folded α subunits would associate with a β subunit, be recognised by N-acetylglucosaminylphosphoryl transferase in the Golgi and be targeted to the lysosome. This would explain the decrease in the amount of enzyme secreted into the medium in CDGS type 1 cells. If this phenomenon were limited to the α subunit alone, then one would expect to see a decrease in the amount of β -hexosaminidase A only. However determination of the percentage of intracellular and extracellular β -hexosaminidase A by anion-exchange chromatography and heat inactivation revealed that there was not a decrease in the proportion of β -hexosaminidase A.

The anion-exchange chromatographic profiles of intracellular and extracellular β -hexosaminidase were comparable in control, PMM-deficient and PMM-normal CDGS type 1 fibroblasts. Had there been under-glycosylation of one or more N-glycosylation sites resulting in decreased sialylation in the constituent α and β subunits, this would have been detected as an anodal shift in the elution profiles of β -hexosaminidase A and B.

Similarly, a decrease in the glycosylation of β -hexosaminidase A and B would be expected to decrease the affinity for concanavalin A. Concanavalin A binds most strongly to oligomannose glycans, but will also bind hybrid and complex glycans. Glycans which have bisecting GlcNAc residues or terminal galactose and sialic acids bind less strongly to concanavalin A than oligomannose glycans. For example, NA2B, NA2BF, A2B, A2BF, NA3, NA3F, A3, A3F, A4, A4F, NA4 and NA4F do not bind to concanavalin A. NGA2B, NGA2BF, NA2, A2, A2F and hybrid structures bind weakly to concanavalin A, and NGA2, NGA2F and oligomannose glycans bind strongly to concanavalin A (Narasimhan *et al.*, 1979). Thus for normal fibroblast β -hexosaminidase the peak eluting between fraction 15 and 27 would contain the disialylated biantennary glycans and the

peak eluting between fractions 27 and 35 would contain the oligomannose glycans. It was predicted that the CDGS type 1 β -hexosaminidase activity might not bind as tightly to concanavalin A as there would be fewer glycans on each enzyme to bind due to underglycosylation. A shift to less tightly binding structures in CDGS type 1 fibroblasts was not seen though.

As no differences were detected using any of the chromatographic procedures, this would suggest that the enzyme molecules that are transported to the lysosomes or the medium were assembled from fully N-glycosylated constituent α and β subunits. Unlike serum transferrin, which is found in normal, partially glycosylated and non-glycosylated forms in both variants of CDGS type 1, β -hexosaminidase does not appear to be underglycosylated in PMM-deficient or PMM-normal CDGS type 1 fibroblasts. The glycosylation status of β -hexosaminidase could be determined rigorously by purifying the enzyme from fibroblasts and analysing the glycans in a similar way to that used for serum transferrin. However, this would require large scale cultures and radiolabelling of the glycans.

The observation that a lysosomal enzyme (β -hexosaminidase) was normally glycosylated in CDGS type 1 fibroblasts was supported by studies of another lysosomal enzyme, α -fucosidase. Western blot analysis of intracellular α -fucosidase showed that there was no glycosylation defect in either the PMM-deficient or the PMM-normal CDGS type 1 fibroblasts. Non-glycosylation of α -fucosidase would have been detected by a decrease in the molecular mass of the immunodetected subunits of the enzyme. Earlier studies by DiCioccio and Mahoney (1990) have shown that incubation of lymphoblastoid cells with tunicamycin (an inhibitor of N-linked glycosylation) resulted in a decrease in the molecular weight of α -fucosidase from 58 kDa to 52 kDa. The SDS-PAGE conditions used were the same as those used to analyse the isoforms of transferrin and so the resolution should have been sufficient to identify a shift in molecular mass.

The results obtained in fibroblasts are in contrast to those found for serum, in that in many CDGS type 1 patients there is an increase in the amount of β -hexosaminidase

present in serum and that under-sialylated forms of β -hexosaminidase have been observed on isoelectric focusing gels (Jaeken, 1992; Clayton *et al.*, 1993). However they confirm previous observations that β -hexosaminidase and α -fucosidase are normally glycosylated in other tissues such as liver and intestinal mucosa from CDGS type 1 patients (Jaeken and Carchon, 1993). The observation that there is decreased secretion of β -hexosaminidase in CDGS type 1 fibroblasts is comparable with the results of Gu and Wada (1995). They found that CDGS type 1 fibroblasts secreted less decorin into the cell culture medium, but the structure of the decorin was normal. It would seem that there is a difference between the effects of CDGS type 1 in the liver *in vivo* and in fibroblasts in culture as it has been observed that the precursors of serum glycoproteins are aberrantly glycosylated in the liver of CDGS type 1 patients (Henry *et al.*, 1997).

It has been suggested previously that the continual presence of mannose in the culture medium can increase the intracellular pool of dolichol-linked oligosaccharides in CDGS type 1 fibroblasts and correct the glycosylation defect in CDGS type 1 (Panneerselvam and Freeze, 1996a). In the present investigation culture of CDGS type 1 fibroblasts in medium containing 5 mM mannose for 5 days did not increase the level of secreted β -hexosaminidase, the only cellular marker of aberrant N-glycosylation capacity of the CDGS type 1 fibroblasts.

6.3.5.2 Lysosomal enzymes as cellular markers of aberrant glycosylation in galactosaemic fibroblasts

There were no differences in the intracellular or extracellular β -hexosaminidase activities between normal and galactosaemic fibroblasts. Unlike the CDGS type 1 fibroblasts, there was no decrease in the secretion of β -hexosaminidase, which supports the hypothesis that decreased secretion is due to non-glycosylation. In galactosaemic fibroblasts the defective galactosylation of glycans would only affect the complex glycans and so up to that step in the biosynthetic pathway β -hexosaminidase subunits would be synthesised normally. As only 10% of the glycans are complex glycans one would not expect to see any major effect on glycosylation of β -hexosaminidase in galactosaemic fibroblasts, and this was indeed the case. Tighter binding to concanavalin A Sepharose and shorter

retention times on ion-exchange chromatography due to undergalactosylation might have been expected, but were not observed.

Interestingly, α -fucosidase was found to be of normal size by Western blotting and therefore presumed to be glycosylated normally. As it has been hypothesised that the aberrant glycosylation of glycoproteins seen in galactosaemic patients is caused by a block in the addition of galactose to carbohydrates, one might have expected to see a change in the size of the α -fucosidase. Although 25% of the glycans of α -fucosidase are complex glycans, this may have been too small a fraction of glycans to be affected by a partial defect or it may not have been seen on the gel as the resolution was not good enough. Thus the overall conclusion based on the present findings is that in galactosaemic fibroblasts these two lysosomal enzymes are correctly glycosylated and thus cannot be used as markers of aberrant glycosylation in galactosaemic fibroblasts. The results presented in this chapter confirm the findings of Lewis (1997), who found that lysosome-associated membrane protein (LAMP) is also normally glycosylated in galactosaemic fibroblasts.

6.4 Conclusion

The only cellular marker indicating a defect in glycosylation discovered from this series of experiments is a decrease in the secretion of β -hexosaminidase into the culture medium of CDGS type 1 fibroblasts. This defect was not correctable by supplementation of the cell culture medium by the addition of 5 mM mannose. There was no such decrease in secretion of β -hexosaminidase into the cell culture medium of galactosaemic fibroblasts, demonstrating that this phenomenon is unique to CDGS type 1 fibroblasts. It can be concluded that the cell reduces its glycosylation capacity so that essential proteins are protected and only correctly glycosylated proteins are transported.

Prenatal diagnosis of CDGS type 1 by measurement of phosphomannomutase activity and genetic linkage analysis

7.1 Introduction

Seven out of eight of the British CDGS type 1 patients have a deficiency of phosphomannomutase, which can be demonstrated in the fibroblasts of these patients to confirm the diagnosis of CDGS type 1 (Chapter 3). The possibility of assaying PMM activity in fetal samples to diagnose PMM-deficient CDGS type 1 prenatally in conjunction with genetic linkage analysis is explored in this Chapter. Pregnancies in three families have been studied. The first family was offered prenatal diagnosis after amniocentesis at 16 weeks gestation and the second family after chorionic villus sampling at 10 weeks gestation. The third family was a retrospective enzymatic study of the patient CB, who was the subject of the first failed prenatal diagnosis of CDGS type 1 by analysis of fetal blood transferrin (Clayton *et al.*, 1993). Amniocentesis had been performed on the mother at 16 weeks gestation, and the amniotic fluid and cultured amniocytes stored for later use.

7.1.1 Previous experience of prenatal diagnosis of CDGS type 1

Preliminary biochemical diagnosis of CDGS is based on the presence of asialo- and disialoisoforms instead of the normal predominant tetrasialoisoform of serum transferrin in neonates (section 1.5.4.2). Before the discovery of the deficiency of phosphomannomutase in CDGS type 1, prenatal diagnosis was attempted using IEF of fetal blood transferrin (Clayton *et al.* 1993). It was demonstrated that the transferrin in fetal blood samples consisted mainly of the tetrasialoisoform as was found in neonatal blood, hence it was thought that diagnosis of CDGS type 1 by IEF of transferrin should be possible (Stibler and Kristiansson, 1991). Fetal blood was obtained from a fetus at risk of CDGS type 1 at 19 weeks gestation and was analysed by IEF with immunodetection of transferrin and α 1-antitrypsin. Both transferrin and α 1-antitrypsin

from the fetal blood were found to be indistinguishable from the proteins in fetal blood from a normal fetus by IEF with immunodetection. It was concluded that this fetus was unaffected by CDGS type 1. However, the plasma β -hexosaminidase activity was found to be elevated although the IEF pattern was again identical to that found in normal fetal blood. The baby girl was born with the typical clinical picture of CDGS type 1 (patient CB). Neonatal blood was analysed at 10, 19, 30 and 32 days postnatally. The abnormal IEF patterns of transferrin, α 1 antitrypsin and β -hexosaminidase were not observed until the third week of life (Clayton *et al.*, 1993). The erroneous transferrin results could have resulted from maternal contamination (Stibler and Kristiansson, 1991), but this is not the case for α 1 antitrypsin and β -hexosaminidase because neither of these two enzymes can cross the placenta (Clayton *et al.*, 1993).

A similar failure to prenatally diagnose CDGS type 1 was also reported by Stibler and Skovby (1994), who examined fetal blood transferrin and α 1-fetoprotein. The twin babies born had the clinical symptoms of CDGS type 1, although no difference in the IEF gels with immunodetection for either fetal serum transferrin or α 1-fetoprotein was observed. The carbohydrate-deficient transferrin was found in blood spotted on Guthrie cards on the first day of life (Stibler and Skovby, 1994).

The general consensus of opinion was that prenatal diagnosis of CDGS type 1 could only be carried out when the enzyme defect or the genetic localisation of CDGS type 1 had been elucidated (Clayton *et al.*, 1993; Stibler and Skovby, 1994). With the discovery of the phosphomannomutase deficiency (Van Schaftingen and Jaeken, 1995) and proven linkage of CDGS type 1 to polymorphic markers on chromosome 16 (Martinsson *et al.*, 1994) it was felt that prenatal diagnosis by a combination of these two methods could be offered to families at risk of PMM-deficient CDGS type 1. We were fortunate in that cultured amniocytes from the previous unsuccessful prenatal diagnosis of CDGS type 1 (Clayton *et al.*, 1993) had been stored at -70°C and were available for analysis. During this study two requests were made for prenatal diagnosis of CDGS type 1. This work was done in collaboration with Tommy Martinsson, Jan Wahlstrom and Cecilia Bjursell University of Gothenburg, East Hospital, Gothenburg, Sweden, who carried out the genetic linkage analysis.

7.2 Results

The optimisation and validation of the original PMM and PGM assays (Van Schaftingen and Jaeken, 1995) for cultured cells is described in Chapter 3. The methods are described in section 2.6. As Van Schaftingen and Jaeken (1995) have reported that PMM is expressed in amniocytes, the modified procedure was applied to measure PMM activity in chorionic villus, cultured chorionic villi cells and cultured amniocytes.

Phosphomannomutase activity was measured in cultured amniocytes from three pregnancies not at risk for CDGS type 1 to establish the normal reference range for PMM activity in cultured amniocytes (section 2.6). The specific activity was found to be similar to that of fibroblasts (Table 7.1). This is not surprising as cultured amniocytes are fibroblastic cells derived from the skin cells of the fetus. Cultured chorionic villi cells had a similar specific PMM activity to that in fibroblasts (Table 7.3). PMM activity was also measured directly in a chorionic villus biopsy which was found to have a specific activity of 4.4 nmol/min/mg protein. Thus as phosphomannomutase activity is expressed and can be measured in cultured amniocytes, chorionic villi cells and directly in chorionic villi, it was felt that prenatal diagnosis could be offered to CDGS type 1 families with a proven deficiency of phosphomannomutase by measurement of phosphomannomutase activity in the fetal samples.

7.2.1 Demonstration of phosphomannomutase deficiency in the index cases of families 1 and 2

Due to the presence of two variants of CDGS type 1, PMM-deficient and PMM-normal, it was considered important to first establish a deficiency of PMM in the fibroblasts of the index case of each family before prenatal diagnosis could be offered. The phosphomannomutase activities were 0.12 and 0.17 nmol/min/mg protein in the fibroblasts from the index cases of families 1 and 2 respectively, compared with a range of 1.1 to 2.6 nmol/min/mg protein for controls (n=8). The corresponding PGM/PMM ratios were 383, 86 and 11-48, respectively. The PMM activity of patient CB was 0.18 nmol/min/mg protein and the PGM/PMM ratio was 146 (data from Chapter 3). In

families 1 and 2 linkage to chromosome 16 had also been established prior to the investigation, and hence genetic linkage analysis would be informative.

7.2.2 Family 1

The index case of family 1 was patient DC (section 2.1). Amniocentesis was performed on the mother of family 1 at 16 weeks gestation. The amniotic fluid and cultured amniotic cells from a proven case of PMM-deficient CDGS type 1 (patient CB, family 3) were used as positive controls (Clayton *et al.*, 1993). The PMM activity and the PGM/PMM ratio in the cultured amniocytes from family 1 were within the range expected for cultured amniocytes from a fetus unaffected by CDGS type 1 (Table 7.1). The PMM activity was slightly below the activity found in the normal cultured amniocytes because this fetus was likely to be a carrier of CDGS type 1. The PMM activity in the proven case of CDGS type 1 cultured amniocytes was about 15% of that of the control and the PGM/PMM ratio was elevated compared to the controls (Table 7.1). These results suggested that the fetus of family 1 was not likely to be affected by CDGS type 1.

However as misdiagnosis had been made in prior attempts at prenatal diagnosis of CDGS type 1 (Clayton *et al.*, 1993; Stibler and Skovby, 1994), further biochemical studies were carried out to confirm this conclusion. The amniotic fluid supernatant from this pregnancy was assayed for the lysosomal enzymes, β -hexosaminidase and β -glucuronidase (section 2.7), as these had been previously found to be elevated in the amniotic fluid from a proven case of PMM-deficient CDGS type 1 (Clayton *et al.*, 1993). The activities of both enzymes in the amniotic fluid of the present pregnancy were found to be within the normal range whereas the activities in the amniotic fluid of the fetus affected with CDGS type 1 were elevated (Table 7.2). This observation supported the conclusion that the fetus was not affected with CDGS type 1. Elevation of the lysosomal enzymes β -hexosaminidase and β -glucuronidase in the amniotic fluid is a useful marker to support the diagnosis of CDGS type 1.

The diagnosis was also confirmed by genetic linkage analysis. The microsatellite markers D16S495, AFMA284WD5, GATAP6084/ D16S768, D16S406, D16S519, D16S500 were used to track the inheritance of the CDGS type 1 gene in the fetus. The disease-carrying alleles were identified by comparison of the alleles of the mother, father and the index case (Figure 7.1a). Genetic linkage analysis showed that this baby had inherited one normal copy of the CDG1 region from the mother and one disease-carrying copy of this region from the father (Figure 7.1a). These results indicated that this fetus was likely to be a carrier of CDGS type 1 and not affected.

All the data obtained indicated that the fetus was not affected by CDGS type 1. On the basis of the biochemical and genetic findings the pregnancy went to term. The male baby showed no clinical signs of CDGS type 1 at birth and he had a normal isoelectric focusing pattern of serum transferrin confirming that he was indeed unaffected by CDGS type 1.

7.2.3 Family 2

The index case of family 2 was patient KT (section 2.1). A chorionic villus biopsy was obtained at 10 weeks gestation from the mother of family 2. The biopsy was assayed directly for PMM and PGM activity. The specific activity of PMM was found to be decreased, and the PGM/PMM ratio was elevated compared with controls suggesting an affected fetus (Table 7.3). The assays were repeated on the cultured chorionic villi cells which confirmed that this fetus was likely to be affected with CDGS type 1.

Family 1:

<u>Cultured Amniocytes</u>	Controls (n = 3)	Pregnancy 1	CDGS type 1
Phosphomannomutase (nmol/min/mg protein)	1.0 - 1.5	0.94 , 0.86	0.18
PGM /PMM Ratio	13 -18	26, 15	67

Table 7.1: Phosphomannomutase activity and PGM/PMM ratios in cultured amniocytes. n = number of samples

Family 1:

<u>Amniotic fluid</u>	Controls (n = 3)	Pregnancy 1	CDGS type 1
β -Hexosaminidase (nmol/hr/ml)	463-744	665	1662
β -Glucuronidase (nmol/hr/ml)	48-74	69	195

Table 7.2: β -Hexosaminidase and β -glucuronidase activities in amniotic fluid.

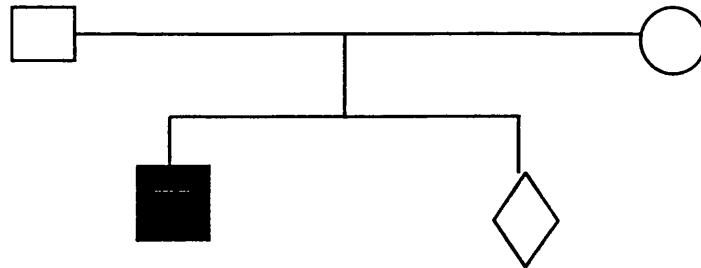
n = number of samples

Family 2:

	Phosphomannomutase (nmol/min/mg protein)	PGM /PMM Ratio
<u>Chorionic villus biopsy:</u>		
Controls (n = 4)	0.9-4.9	5-21
Family 2	0.24, 0.28	63, 73
<u>Cultured chorionic villi:</u>		
Controls (n = 5)	0.9-1.5	6-16
Family 2	0.37	53

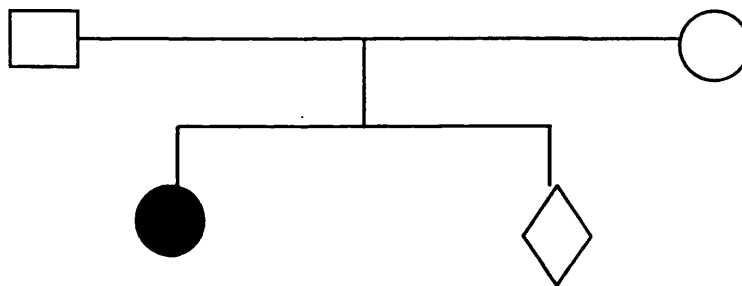
Table 7.3: Phosphomannomutase activity and PGM/PMM ratio in chorionic villi and cultured chorionic villi cells. n = number of samples

Figure 7.1A: Family 1



D16S495	2		1	1		2	1		2	1		1
AFMA284WD5	1		1	1		1	1		1	1		1
GATAP6084/D16S768	1		2	1		1	3		1	3		1
D16S406	3		1	2		3	3		3	3		2
D16S519	2		1	3		2	3		2	3		3
D16S500	3		2	1		3	3		3	3		1

Figure 7.1B: Family 2



D16S768	1		2	1		3	1		3	3		2
D16S406	2		4	2		3	2		3	3		1
D16S500	4		2	4		3	4		3	3		1

Figure 7.1: Analysis of haplotypes of families obtained using microsatellite markers on both sides of the CDG1 gene

(Bjursell *et al.*, 1997) A; family 1, B; family 2.

DNA analysis confirmed that this fetus had inherited exactly the same haplotypes for the CDG1 gene region as the female index case in this family (Figure 7.1b). By the time of this investigation, the linkage with PMM-deficient CDGS type 1 gene had been narrowed down and so only three markers D16S768, D16S406 and D16S500 were required to determine the haplotypes of the fetus (Bjursell *et al.*, 1997). On the basis of these results the mother chose to have the pregnancy terminated. Fibroblasts were grown from the termination products and assayed for PMM activity. The fetal fibroblasts had a phosphomannomutase activity of 0.1 nmol/min/mg protein (controls 1.1 to 2.6 nmol/min/mg protein (n=8)) consistent with a diagnosis PMM-deficient CDGS type 1.

7.3 Discussion

7.3.1 The use and development of the phosphomannomutase assay for the prenatal diagnosis of CDGS type 1

These results demonstrate that phosphomannomutase activity can be measured reliably in chorionic villus samples, cultured chorionic villus cells and cultured amniocytes for the prenatal diagnosis of CDGS type 1. The fetus in family 1 was predicted to be unaffected as the PMM activity in cultured amniocytes was just below the normal reference range and there was no elevation of the lysosomal enzyme activities in the amniotic fluid. These predictions were confirmed by genetic linkage analysis and a healthy baby boy was born. The fetus in family 2 was predicted to be affected with CDGS type 1 on the basis of a deficiency of PMM in chorionic villi and cultured chorionic villus cells. These results were confirmed in fibroblasts from the termination products and by genetic linkage analysis.

A deficiency of PMM activity was demonstrated retrospectively in the cultured amniocytes from a pregnancy which had previously been tested for CDGS type 1 by analysis of transferrin in fetal blood (Clayton *et al.*, 1993). This demonstrates that the assay of PMM activity in chorionic villi or amniocytes can usefully contribute to the prenatal diagnosis of CDGS type 1 in families in which there is a proven deficiency of PMM in the index case.

The use of a fluorogenic assay for the measurement of PMM activity has allowed greater sensitivity than found with the original method of Van Schaftingen and Jaeken (1995). This was vital in the development of the assay for prenatal diagnosis as the protein concentration of the samples was much lower than that used in the conventional assay on fibroblasts. In this situation it is also important to relate the PMM activity to the PGM activity. The PGM/PMM ratio gives greater confidence to the results at the lower protein concentrations as any inaccuracy in the measurement of the protein concentration is effectively cancelled out. As already demonstrated in Chapter 3, the specific activity of PMM is only constant at a protein concentration between 0.7 mg/ml and 3 mg/ml. The most likely reason for this is in the accuracy of the protein assay itself. It is also very important that the protein concentration of the prenatal samples are all exactly the same for the controls and the tests so that a reliable comparison can be made. In these two examples the protein concentrations of the samples were either all set at 0.5 mg/ml for cultured cells or 0.9 mg/ml for direct chorionic villi. Although the protein concentration for the cultured cells was below the ideal range, the size of the sample dictated that 0.5 mg/ml was the maximum protein concentration that could be used. As the protein concentration of all samples tested at this time was 0.5 mg/ml, the results are comparable with each other, but not with other results obtained at the higher protein concentrations. Ideally, the protein concentration would have been around 1 mg/ml, but this was unfortunately not possible, unless more cells were grown, which would have delayed the results by another 3 weeks. This option was not available in the case the gestational age would be too great for termination.

When carrying out a prenatal diagnosis for an autosomally recessive disorder there is a 25% chance of finding an affected fetus, a 25% chance of a normal fetus and a 50% chance of a carrier. It has been reported that carriers or heterozygotes of CDGS type 1 have between 50 and 100% of the normal PMM activity (Jaeken *et al.*, 1997b) and hence a lower than normal PMM activity will be expected in 50% of the prenatal diagnoses due to the child being a carrier. This was demonstrated in family 1, where the PMM activity

of the fetus was a little lower than the normal range (Table 7.1). From these results and the genetic analysis, it is likely that this baby is a carrier of CDGS type 1.

7.3.2 Lysosomal enzymes as markers for the prenatal diagnosis of CDGS type 1

The activities of the lysosomal enzymes, β -hexosaminidase and β -glucuronidase were measured in the amniotic fluid supernatant to support the diagnosis of fetus 1. The activities of these two enzymes have been reported to be elevated in CDGS type 1 serum as well as in amniotic fluid from an affected pregnancy (Clayton *et al.*, 1993). The activities of these enzymes in the amniotic fluid of the pregnancy in family 1 were within the normal range and the birth of a normal baby boy ensued. Thus these two lysosomal enzymes provide another biochemical tool for use in the prenatal diagnosis of CDGS type 1.

7.3.3 Genetic analysis of CDGS type 1

At the time that these prenatal diagnoses were performed, the PMM2 gene had not been cloned, thus the only method of genetic analysis was one of linkage to the markers in the region of the PMM gene. In the future when the mutations for each family have been found in the PMM2 gene, prenatal diagnosis for CDGS type 1 can be performed by mutation analysis directly. However, until then the PMM assay of fetal samples can be used in conjunction with linkage analysis for families where the mutations are not known.

7.3.4 Variants of CDGS type 1

As discussed in Chapter 3, not all the patients who have the clinical picture and the characteristic isoelectric focusing pattern of transferrin of CDGS type 1 have a deficiency of phosphomannomutase. Prenatal diagnosis cannot be performed in these families using the present method of biochemical analysis. If these families showed linkage to chromosome 16 then prenatal diagnosis could be performed by genetic analysis, but a recent report has suggested that the PMM-normal patients map to another chromosome

and not to chromosome 16 (Matthijs *et al.*, 1996, 1997a). This demonstrates that there is heterogeneity in CDGS type 1, which one has to be aware of when deciding on which method of analysis is most suitable for a particular family. At the present time prenatal diagnosis for PMM-normal CDGS type 1 patients has not been established as the basic biochemical defect has not been elucidated. It is not known whether the lysosomal enzymes, β -hexosaminidase and β -glucuronidase, are elevated in the amniotic fluid of the PMM-normal CDGS type 1 fetus. This is a possibility as β -hexosaminidase is elevated in the serum of the PMM-normal CDGS type 1 patient (Table 3.5).

7.4 Conclusion

These results show that measurement of phosphomannomutase activity in chorionic villi or amniocytes can be used in conjunction with genetic linkage analysis for prenatal diagnosis of CDGS type 1 in families where a deficiency of phosphomannomutase has been demonstrated in the index case. Measurement of the ratio PGM/PMM and marker lysosomal enzymes in amniotic fluid provide additional support for the diagnosis. The availability of genetic linkage analysis and the direct assay of PMM activity avoid the need to rely on secondary phenomena such as altered glycosylation of transferrin or α 1 antitrypsin for the prenatal diagnosis of CDGS type 1. However prenatal diagnosis can still not be performed for those families in which there is not a deficiency of PMM. It is a matter of some urgency to establish the enzymic defect or genetic linkage for these families.

General discussion

8.1 CDGS type 1

8.1.1 There are at least two enzyme defects that lead to the clinical symptoms associated with CDGS type 1

On the basis of the studies reported in this thesis and those of others it is clear that the CDGS type 1 phenotype does not arise solely from one enzyme defect. Recent evidence suggests there may be three variants of CDGS type 1. The major variant is characterised by a deficiency of PMM (type 1A); 45 out of a total of 54 patients investigated worldwide have this deficiency (Jaeken *et al.*, 1997b). The remainder are PMM-normal.

The PMM-normal and PMM-deficient CDGS type 1 patients who were the subject of this study have the common feature that in each case the abnormal serum transferrin isoforms represent partial or complete non-occupancy of N-glycosylation sequons (Charlwood *et al.*, 1997). The third group of patients is that reported by Krasnewich *et al.*, (1995). These two Danish patients differ from the other patients because they show normal occupancy of glycosylation sites, but the structures of the glycans present in α 1-antitrypsin are truncated compared to those found in normal serum α 1 antitrypsin. It is not known whether these patients also have a deficiency of PMM.

8.1.2 PMM-deficient CDGS type 1

The significance of the PMM-deficiency in CDGS type 1 has not been unequivocally established. The more widely held view seems to be that the PMM-deficiency is the basis of the CDGS type 1A phenotype (Van Schaftingen and Jaeken, 1995; Charlwood *et al.*, 1996; Jaeken *et al.*, 1997a, 1997b; Matthijs *et al.*, 1997b). In support of this, the parents of PMM-deficient patients display between 50% - 100% PMM activity in their leukocytes (Van Schaftingen and Jaeken, 1995), which is as expected for a recessive disorder. Eleven missense mutations have been found in the PMM2 gene of CDGS type 1 patients. Eight of these mutations were in conserved amino acids and at least seven were found to change the normal amino acid for one that was very different in either size or charge, thus destabilising the protein (Matthijs *et al.*, 1997b). Both of the mutations in

the PMM2 gene were found in 16 patients, only one in 7 patients and no mutations were found in the remaining 10 patients studied. The expression of PMM2 is high in the pancreas and the liver, but its expression is much lower in the brain. These results are somewhat unexpected as the clinical picture of CDGS type 1 is very much dominated by degeneration of the nervous system. It is most likely that the PMM2 gene product is developmentally regulated in different tissues. The deficiency of PMM2 in the brain during fetal development may be responsible for the neurological impairment. On the other hand the PMM2 gene remains active in the pancreas and liver during postnatal development. The PMM2 gene has also been shown to code for a protein that has PMM activity (Matthijs *et al.*, 1997b). The fact that the deficiency of PMM can be detected in chorionic villi, shows that it is very likely to be a primary defect, because secondary defects in glycosylation such as found in serum transferrin only become apparent after birth.

The addition of mannose to the cell culture medium has been reported to improve the synthesis of the glycans in CDGS type 1 fibroblasts (Panneerselvam and Freeze, 1996a). However, injection of mannose into patients did not result in either correction of the aberrant glycosylation or improvement of clinical symptoms. (Jaeken, 1996). This finding is not unexpected as transport of mannose into the cell occurs before the involvement of PMM in the synthesis of GDP-mannose and dolichol-P-mannose. This result suggests that the observations of Panneerselvam and Freeze (1996a) may have been due to experimental conditions.

In contrast to the above, Ohkura *et al.* (1997) have reported that Japanese CDGS type 1 patients who have a deficiency of PMM also show accumulation of dehydrodolichol in their fibroblasts. This is indicative of a defect in dehydrodolichol reductase, which converts dehydrodolichol into dolichol. This enzyme activity has not however been measured in the fibroblasts of CDGS type 1 patients. A deficiency of dehydrodolichol reductase would lead to a decrease in the amount of dolichol and hence to a decrease in the amount of LLO and dolichol-P-mannose being synthesised. A decrease in the rate of synthesis of LLO has been observed by several groups (Powell *et al.*, 1994; Krasnewich *et al.*, 1995; Panneerselvam and Freeze, 1996a; Ohkura *et al.*, 1997) and a decrease in

the amount of dolichol-P-mannose in CDGS type 1 fibroblasts has been observed by Ohkura *et al.* (1997). Yamashita and Ohno (1996) have reported a decrease in the amount of GPI-anchored proteins in serum from CDGS type 1 patients suggesting that there was a deficiency of dolichol-P-mannose. Dolichol-P-mannose is a common precursor in the synthesis of both N-linked glycans and GPI anchors.

Ohkura *et al.* (1997) have also argued that if a deficiency of PMM is the defect causing CDGS type 1 then an accumulation of dolichol-PP-GlcNAc₂ would be observed due to a deficiency of the substrate GDP-mannose (see Figure 1.3). Dolichol-PP-GlcNAc₂ accumulation was not detected in their study nor in the present investigation (Chapter 5 - fraction 1). However it is possible that dolichol-PP-GlcNAc₂ is degraded and recycled and accumulation would not occur. Ohkura *et al.* (1997) have also reported that the ratio of mannose-6-phosphate to mannose-1-phosphate was the same in CDGS type 1 fibroblasts as in controls. This indicates that the PMM activity *in vivo* is sufficient to maintain the normal ratio.

8.1.3 PMM-normal CDGS type 1

The PMM-normal CDGS type 1 patient had a clinical phenotype that was similar to that of the severe neonatal PMM-deficient CDGS type 1 patients. He presented with failure to thrive and growth retardation. He had long fingers and toes and marked head lag. However, he differed in that there were no abnormal eye movements which is a characteristic feature of the other CDGS type 1 patients. The IEF pattern of his serum transferrin was also slightly different, in that there was a greater amount of disialotransferrin and a smaller amount of asialotransferrin than seen in many of the other CDGS type 1 patients. A diagnosis of CDGS type 4 was suspected on the basis of the IEF pattern of serum transferrin (Figure 1.9), but the clinical phenotype of this patient most closely matched that of CDGS type 1 (Table 1.1). Therefore he was diagnosed as a possible variant of type 1 (Charlwood *et al.*, 1997).

The structural basis for the aberrant glycosylation of serum proteins in the PMM-normal patient was also found to be non-occupancy of N-glycosylation sequons, as is the case for the PMM-deficient patients (Chapter 4). The PMM-normal CDGS type 1 fibroblasts

were indistinguishable from PMM-deficient CDGS type 1 fibroblasts with regard to synthesis of LLO, protein-linked N-glycans and secretion and synthesis of lysosomal enzymes (Chapters 5 and 6). Therefore it is likely that the enzyme defect in the PMM-normal patient is located at a different step in the LLO's biosynthetic pathway.

8.1.3.1 Possible causes of CDGS type 1 in the PMM-normal patient

Several possible causes of CDGS type 1 in the PMM-normal patient were investigated in this study. The PMM activity-pH profile demonstrated that this patient did not have a deficiency of PMM1 or PMM2, nor did he have an abnormal form of PMM. The activities of two other candidate enzymes, phosphomannose isomerase and phosphoglucose isomerase, were found to be within the normal range in the fibroblasts from the PMM-normal patient. Thus alternative causes must be considered.

A partial deficiency of oligosaccharyltransferase could be the cause of CDGS type 1 in the PMM-normal patient as this would lead to non-occupancy of glycosylation sites. However, this would seem to be less likely for this patient than it was for the other CDGS type 1 patients, as the transferrin of the PMM-normal patient had a greater proportion of the available glycosylation sequons occupied. The activity of oligosaccharyltransferase in fibroblasts from the PMM-normal patient should be determined in the future using the method of Knauer *et al.* (1994) (Figure 8.1).

Another possibility is that the defective enzyme is mannose-1-phosphate guanosyltransferase, which would block the synthesis of GDP-mannose. Unfortunately attempts to measure the activity of this enzyme using an enzyme-linked assay were unsuccessful, but an alternative method involving the use of radiolabelled GDP-mannose should be used in future studies (Szumilo *et al.*, 1993) (Figure 8.1).

Alternatively any enzyme involved in the biosynthesis of the lipid-linked oligosaccharide, or the production or transport of UDP-GlcNAc, GDP-mannose, dolichol-P-mannose, UDP-glucose and dolichol-P-glucose could be defective in the PMM-normal patient.

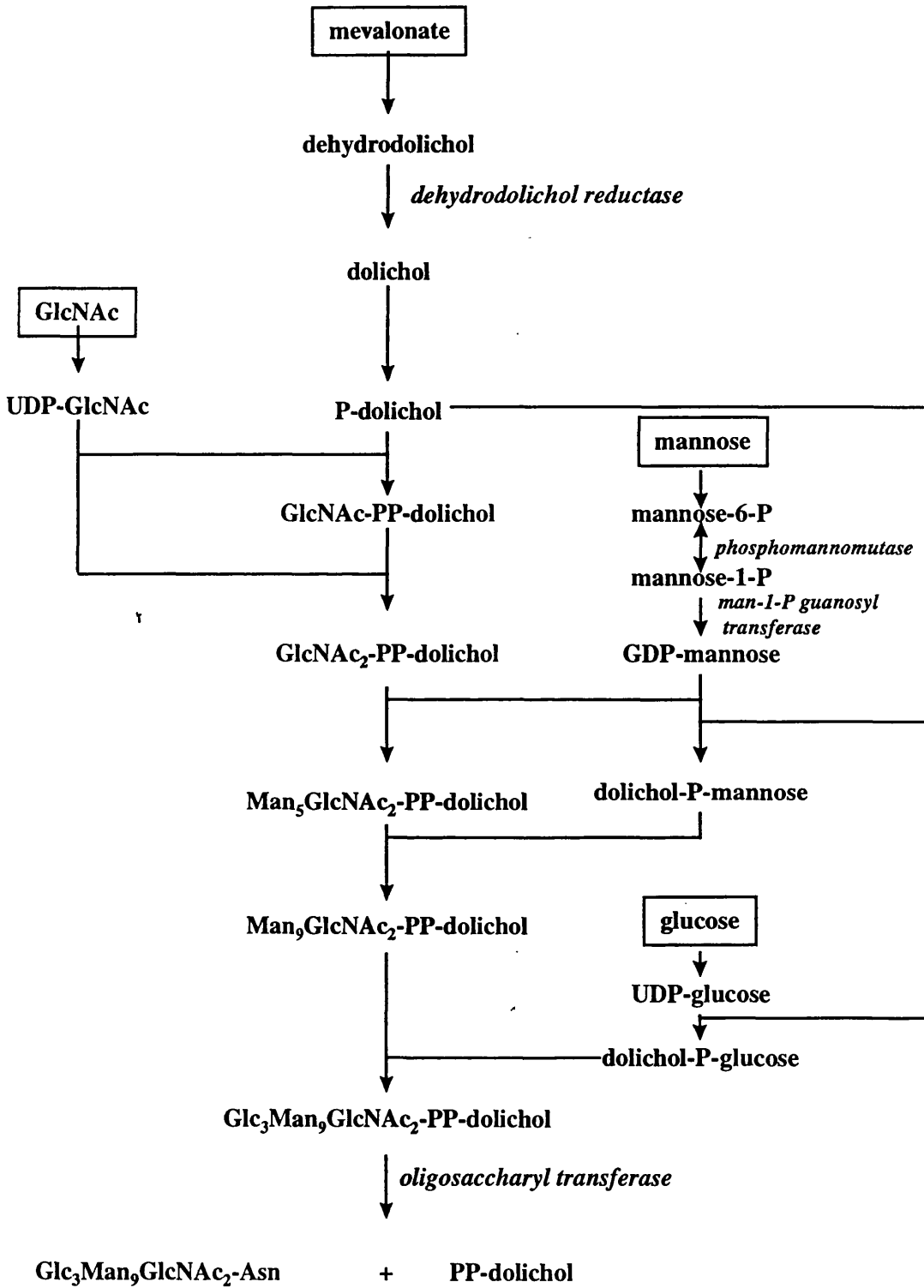


Figure 8.1: Pathways involved in the synthesis of N-linked glycans.

Mannose-1-phosphate guanosyltransferase, oligosaccharyl transferase and dehydrodolichol reductase are candidate enzymes for the cause of CDGS type 1 in the PMM-normal patient.

These five activated sugars are required for the synthesis of the lipid-linked precursor and so a limiting supply of any one of them would have the same consequences as the limiting supply of GDP-mannose and dolichol-P-mannose. Equally, the defect could be in the synthesis of the lipid-linked carrier itself dolichol. This hypothesis has already been suggested for the PMM-deficient CDGS type 1 patients (Ohkura *et al.*, 1997; Figure 8.1). However none of the intermediates involved in the synthesis of the lipid-linked oligosaccharide precursor was found to accumulate in the PMM-normal CDGS type 1 fibroblasts (Chapter 5).

8.1.4 Mannose metabolism in CDGS type 1

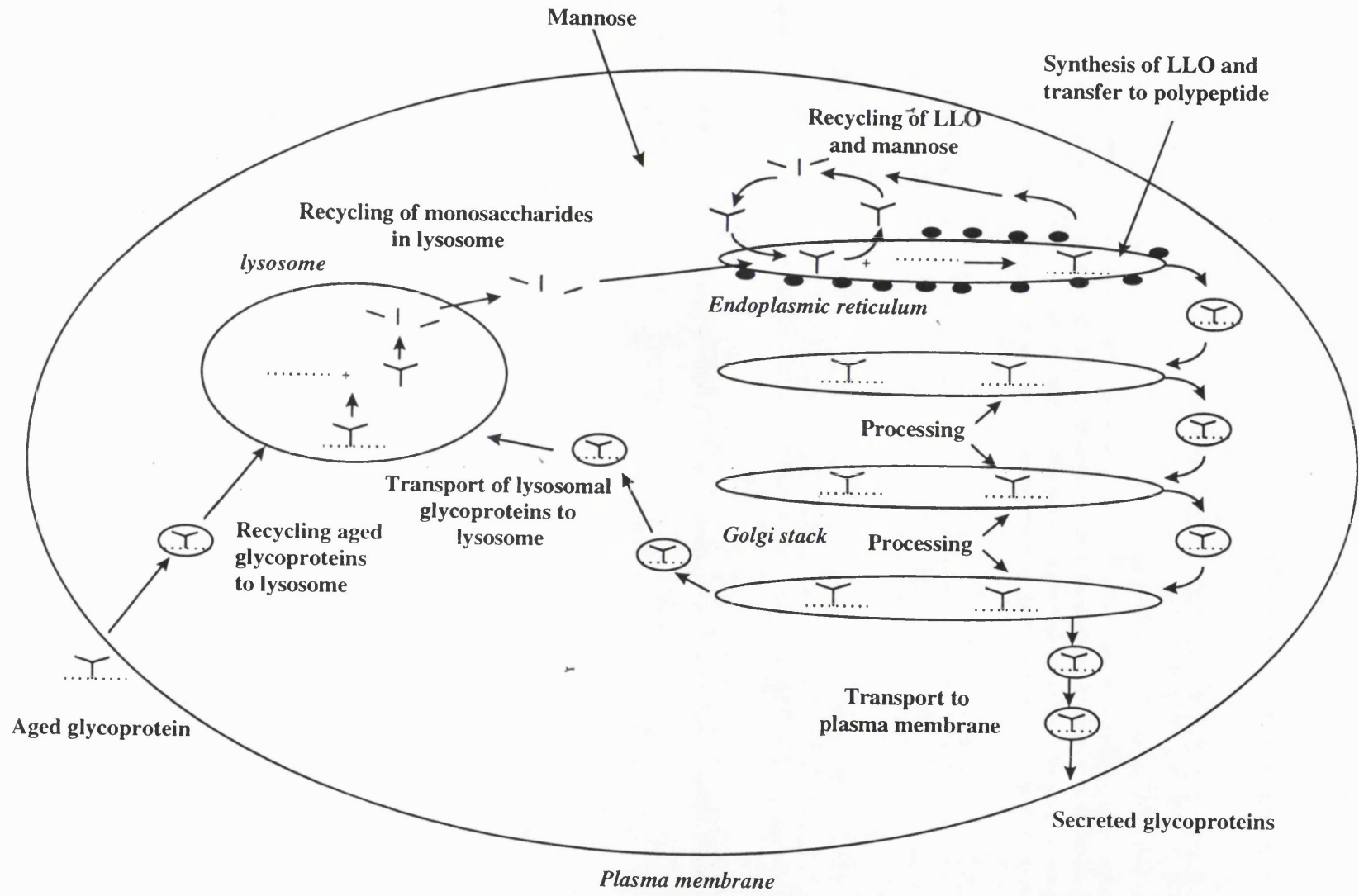
There is a decreased rate (3-10 fold) of mannose incorporation into PMM-deficient CDGS type 1 fibroblasts (Powell *et al.*, 1994, Krasnewich *et al.*, 1995, Panneerselvam and Freeze, 1996a). It is not known whether a similar situation exists in the PMM-normal CDGS type 1 patients.

There are three possible explanations for this observation;

- 1) The deficiency of PMM in CDGS type 1 fibroblasts causes a decrease in the flux through the pathway converting exogenous mannose to GDP-mannose (Figure 8.1). A decrease in the rate of conversion of mannose-6-phosphate to mannose-1-phosphate would cause an increase in the amount of mannose-6-phosphate. A feedback mechanism might be in place to restrict the amount of mannose that is transported into the cell.
- 2) There may be impaired mannose entry into the cell due to a defect in a mannose transporter (Panneerselvam and Freeze, 1996a, b). It has already been established that the glucose transporter can transport mannose into the cell and the activity of this transporter depends on its N-linked glycosylation (Feugeas *et al.*, 1990). Thus a defect in glycosylation could cause a secondary defect in mannose transport. The glucose transporter was found to have only a 10% decrease in activity in CDGS type 1 fibroblasts compared with normal fibroblasts. There is also a specific mannose transporter in fibroblasts, but its activity in CDGS type 1 fibroblasts has not yet been established (Panneerselvam and Freeze, 1996a, b).

Figure 8.2: Mannose metabolism in relation to protein glycosylation.

Mannose for N-linked glycosylation can be acquired by 1) transport into the cell, 2) synthesis from glucose *via* PMI, 3) degradation of LLO in the endoplasmic reticulum, 4) trimming and processing of high mannose chains in Golgi or 5) degradation of aged glycoproteins in the lysosome.



3) The concentration of intracellular mannose in CDGS type 1 fibroblasts is higher than that found in normal cells and thus the rate uptake of the [³H]-mannose in CDGS type 1 is reduced (Krasnewich *et al.*, 1995).

A similar decrease in mannose incorporation has also been observed in CHO cell lines defective in the assembly of LLO (due to reduced amounts of dolichol phosphate) (Stoll and Krag, 1988) and fibroblasts from patients with galactosaemia (Lewis, 1997). Again the underlying mechanisms are not known. Cells starved of glucose also show a decrease in the rate of synthesis of LLO and underglycosylation of glycoproteins, but addition of glucose to the cell culture medium corrects these defects (Goochee and Monica, 1990). On the other hand CDGS type 1 fibroblasts are reportedly corrected by the addition of mannose, but not glucose to the cell culture medium (Panneerselvam and Freeze, 1996a). Thus the mechanism leading to the decreased pool of available mannose in CDGS type 1 fibroblasts and glucose-starved cells must be different.

Previously it was thought that most of the mannose that is required in the cell is synthesised from glucose via PMI (Figure 1.13) because plasma glucose levels are much higher than plasma mannose levels (Panneerselvam and Freeze, 1996b). However mannose can also be recycled by degradation of the LLO and of newly synthesised glycoproteins in the endoplasmic reticulum (see section 1.4.2 and references therein) or by lysosomal degradation of aged glycoproteins (see section 1.4.1 and references therein) (Figure 8.2). These two mechanisms are now believed to account for 80% of the intracellular pool of mannose (Panneerselvam and Freeze, 1996a). If there is a decrease in the number of glycans attached to the proteins, then there will be a decrease in the amount of mannose that is released into the cytoplasm from the lysosome. There will in turn be a decrease in the amount of mannose available for incorporation into the LLO and hence a decrease in the number of glycosylation sites that are occupied by a glycan (Figure 8.2). However Panneerselvam and Freeze (1996a) have shown that more free mannose can be incorporated into the cells and also into LLO by incubating the cells with exogenous mannose. The mannose must be continually present for the increased incorporation to be maintained (Panneerselvam and Freeze, 1996a). This observation

suggests that the addition of exogenous mannose can force the system to produce GDP-mannose and dolichol-P-mannose by maximising the available PMM activity.

8.1.4.1 Mannose trial in CDGS type 1 patients

On the basis of the mannose feeding experiments, a clinical trial was initiated. The CDGS type 1 patients were given mannose either orally or intravenously in the hope that some of their clinical symptoms might be alleviated if their glycoprotein synthesis returns to normal. Jaeken (1996) expressed some concern about this hypothesis commenting that he believed that the mannose would not be taken up into the cells and it would all be removed from the body. The data presented in Chapter 6 indicates that feeding patients with mannose would not correct the synthesis of the glycoproteins as no enhancement of the secretion of β -hexosaminidase was observed in CDGS type 1 cells which were grown in the presence of mannose. Recent reports suggest that feeding the CDGS type 1 patients with mannose increased the concentration of mannose in their plasma (Alton *et al.*, 1997), but it did not alleviate their clinical symptoms. At high doses side effects such as dizziness and variable pallor were noted (Jaeken, 1997).

8.1.5 Avenues of future research in to CDGS type 1

The enzyme defect in the PMM-normal CDGS type 1 patient needs to be elucidated so that postnatal and prenatal diagnosis can be offered to British and other families around the world, who are now thought to make up about 18% of the CDGS type 1 families (Jaeken *et al.*, 1997).

Future investigations should include the identification of the mutations in PMM2 in the seven PMM-deficient CDGS type 1 patients and also linkage analysis of the PMM-normal CDGS type 1 patient. These analyses would allow both carrier detection and prenatal diagnosis to become possible. Any correlation between the mutations and the severity of the disease could be identified. Five of the British patients in this study had a very severe neonatal phenotype, whereas the other two are less severe and more characteristic of the other European patients. It would be expected that patients TB and VB (less severe) would be the most likely patients to have one of the common mutations (R141H, R162W, F119L) reported in the European families and that the other five

PMM-deficient patients would have novel mutations (Matthijs *et al.*, 1997b). Patient NH would not be expected to have any mutations in PMM2.

There are three possible ways in which to demonstrate that mutations in PMM2 cause CDGS type 1;

- 1) Correction of the defects seen in the synthesis of LLO by expression of wild-type PMM2 gene in PMM2-deficient cell lines.
- 2) Inhibition of PMM in HepG2 cells to create a chemically induced phenocopy of PMM-deficient CDGS type 1. This system has the advantage that HepG2 cells synthesise transferrin and so the underglycosylation of transferrin should be demonstrable when PMM is inhibited in these cells. Normal glycosylation of transferrin should be restored when the inhibitor is removed.
- 3) Creation of a mutant mouse which is deficient in PMM2. The serum transferrin of this mouse could then be compared with normal mouse transferrin. It would also be valuable for experimental forms of therapy.

As no specific cellular glycoprotein has been found to be underglycosylated in fibroblasts in this investigation or others (Marquardt *et al.*, 1995, Gu and Wada, 1995), further studies in this area are also required. Four components were found to have altered coordinates compared to those in normal fibroblasts when extracts of proteins from PMM-deficient CDGS type 1 fibroblasts were analysed by 2D gel electrophoresis (Kevin Mills, personal communication). The identification of these proteins would be the next step in the discovery of a specific glycoprotein marker in fibroblasts with CDGS type 1. Other potential cellular markers for aberrant glycosylation are the membrane-bound glycoproteins. An attempt was made in this project to analyse the glycosylation of the transferrin receptor in CDGS type 1 fibroblasts, but unfortunately it was unsuccessful. GPI-anchored proteins could also be analysed in CDGS type 1 fibroblasts, as their synthesis involves the use of dolichol-P-mannose (section 1.2.3). Thus it is likely that they would be affected by a deficiency of PMM in CDGS type 1 fibroblasts. Alkaline phosphatase is a GPI-anchored protein that is found both in serum and fibroblasts. In normal serum it is exclusively coupled to a GPI-anchor, but in CDGS type 1 serum a proportion of it is not coupled to a GPI-anchor, demonstrating that GPI-anchored

proteins are also affected by the genetic defect ((Yamashita and Ohno, 1996). The amount of alkaline phosphatase attached to the surface of fibroblasts *via* the GPI anchor can be quantitated by FACS (fluorescent activated cell sorting) using antibodies against alkaline phosphatase.

There are several potential forms of therapy for CDGS type 1 which could be explored. The first is to provide the patients with the metabolite which is missing, in this case mannose-1-phosphate. Mannose-1-phosphate cannot be given directly to patients because it would not be taken up into the cells where it is required. However, mannose-1-phosphate could be chemically modified to an ester that would be transported into the cell and acted on by an esterase to produce mannose-1-phosphate *in situ*. This compound could be used to increase the amount of GDP-mannose and dolichol-P-mannose produced within the cells and thus correct the aberrant glycosylation of glycoproteins. Alternatively an enzyme replacement therapy could be applied to the treatment of CDGS type 1 patients. Unfortunately patients cannot be treated by simply injecting PMM into the blood stream as PMM would probably not be transported across the blood-brain barrier nor is it likely that it would be targeted to the correct cells. Thus either bone marrow transplant or gene therapy would be required to provide a source of cells synthesising PMM. This type of therapy would have to be given at a very early age before the onset of the clinical symptoms because nervous system degeneration cannot normally be reversed. This would be very difficult to achieve with the severe patients who commonly have symptoms at birth. Treatment for CDGS type 1 may take many years to develop, therefore at this time the best way forward would be prevention by carrier detection and prenatal diagnosis.

8.2 Galactosaemia

8.2.1 Further evidence for a defect in the galactosylation of glycoproteins in galactosaemia

Prior to this investigation there were only speculative reports of a defect in the galactosylation of glycoproteins in galactosaemia. It had been shown that cellular (Ornstein *et al.*, 1992) and serum (Prestoz *et al.*, 1997) glycoproteins from

galactosaemic patients were better acceptors of galactose than glycoproteins obtained from normal fibroblasts or serum. Dobbie *et al.*, (1990) had also been able to demonstrate that there was a decrease in the galactose/mannose ratio in galactosaemic fibroblasts compared to normal fibroblasts. It was hypothesised that these glycoproteins terminate in GlcNAc residues. This has been confirmed in this project by demonstrating that the glycans purified from serum transferrin from galactosaemic patients were truncated and terminate predominantly in GlcNAc residues at the non-reducing end. Similar structural analysis of the glycans released from intracellular glycoproteins from galactosaemic fibroblasts also revealed that there is an increase in the proportion of N-linked glycans terminating in GlcNAc residues or truncated glycans.

When the patients are treated on a galactose-free diet the proportion of the truncated glycans attached to serum transferrin was found to be decreased but did not return to normal in several of the children studied. There was no correlation between the length of treatment or the red cell galactose-1-phosphate concentration and the percentage of the disialylated biantennary glycans. Similarly, the galactosaemic fibroblasts which were grown on medium containing only glucose still showed an increase in the proportion of truncated glycans compared to the normal fibroblasts, although it was lower than that observed when the cells were fed 5 mM galactose.

Although the present results have confirmed that there is a defect in the galactosylation of glycoproteins in galactosaemic patients, this did not provide an explanation of the underlying mechanism of agalactosylation. It can be speculated that as there is no correlation between the red-cell galactose-1-phosphate concentration and the agalactosylation of serum transferrin, inhibition of galactosyl transferase is not the underlying cause of the observed agalactosylation. On the basis of these results it would seem that a deficiency of the substrate, UDP-galactose, is the most likely cause of the agalactosylation of serum glycoproteins in galactosaemia.

8.2.2 Poor-outcome of galactosaemic patients on a galactose-free diet.

It has been well documented that the treatment of galactosaemic patients with a galactose-free diet, even in the womb, does not prevent all the symptoms of this disorder

(Segal, 1995b; Waggoner *et al.*, 1990). Treated patients suffer from developmental delay, ovarian dysfunction and speech impairment (see section 1.6.3). Segal (1995b) has proposed a chronic galactose intoxication model to explain this poor outcome. Galactose can be produced endogenously by the breakdown of glycoproteins or by the breakdown of UDP-galactose which is produced from UDP-glucose. It has recently been discovered that endogenous production of galactose can be as much as 1 g per day (Berry *et al.*, 1995). Thus, no matter how good the galactose-free diet may be, it is difficult to overcome the problem of galactose produced endogenously. The findings of Ornstein *et al.* (1992) and also the present investigation support this theory, as a defect in galactosylation of cellular glycoproteins has been demonstrated in fibroblasts that had been fed on medium containing only glucose. In this case the only source of galactose was endogenous production. It has also been found that some of the N-glycans in serum transferrin from treated patients having low galactose-1-phosphate concentration were truncated. The presence of these abnormal glycans could explain the poor outcome in these patients on a galactose-free diet.

A long term study (over 15 years or more) of the glycans of serum transferrin starting before the commencement of treatment and continuing at regular intervals would reveal any correlation between the long term complications and the glycosylation defects in serum transferrin. This would provide clinicians with a clearer picture of the prospects for the galactosaemic patients. Galactosaemia is considered to be a treatable metabolic disease, and so prenatal diagnosis is not normally offered to families. However as it becomes increasingly evident that the treatment is not entirely satisfactory, clinicians may wish to reconsider the case on the basis of such a study.

8.3 The role of glycosylation in disease

Altered glycosylation of glycoproteins is not a unique feature of the carbohydrate-deficient glycoproteins syndromes and galactosaemia. It has been reported in many human diseases including HFI, HEMPAS, Crohn's disease, cancer, rheumatoid arthritis and chronic alcoholism (Rademacher *et al.*, 1988; Fukuda, 1990; Stibler, 1991; Adamowicz *et al.*, 1996; Dwek *et al.*, 1996). The liver is one of the most affected organs

in CDGS, galactosaemia, HFI, HEMPAS and alcoholism probably because it is the major organ for the synthesis and degradation of serum glycoproteins.

Comparison amongst the different disorders (Table 8.1) reveals that the serum transferrin in both alcoholic and CDGS type 1 patients contains asialo- and disialo- isoforms which are created by the underoccupancy of the glycosylation sites (Landberg *et al.*, 1995). By analogy with CDGS type 1, it is probable that the synthesis of the lipid-linked oligosaccharide (LLO) precursor is defective. As abnormal glycans are not seen on serum transferrin from alcoholic patients it would seem that the defect lies in the synthesis or transport of one of the activated sugars required for the assembly of the LLO.

The molecular mechanism of the aberrant glycosylation seen in untreated HFI patients has been shown to be due to inhibition of PMI by fructose-1-phosphate. Thus, by analogy with the PMM deficiency in CDGS type 1, the transferrin isoforms in serum from untreated HFI patients may also be created by under glycosylation of the glycosylation sites.

A defect in the galactosylation of glycoproteins occurs in both galactosaemia (Chapter 4) and in rheumatoid arthritis. In galactosaemia serum glycoproteins are affected, leading to disease of the liver. In rheumatoid arthritis, only IgG has been found to be agalactosylated so far (Parekh *et al.*, 1985, Field *et al.*, 1994). This is thought to be due to a deficiency of galactosyl transferase in B-cells (Axford *et al.*, 1987). Other disorders of glycoprotein processing include HEMPAS and CDGS type 2 (Fukuda *et al.*, 1990 ; Jaeken *et al.*, 1994). In HEMPAS a deficiency of GlcNAc transferase II or α -mannosidase II leads to an increased proportion of high mannose or hybrid glycans (Fukuda *et al.*, 1990), and in CDGS type 2, a deficiency of N-acetylglucosaminyl transferase II leads to the production of truncated glycans (Jaeken *et al.*, 1994, Charuk *et al.*, 1995).

Many of the basic functions of the carbohydrates have already been elucidated (section 1.2.4 and references therein), however, complete understanding of the importance of the

structure and proportion of different glycoforms and their specific roles in many of the basic functions are far from being understood. The discovery and use of glycosylation mutants have proved invaluable in determining the role of the carbohydrates in the structure/function relationships of individual glycoproteins, cellular membranes and organelles (Stanley, 1984,; Brandli, 1991). Glycosylation mutants have been used to delineate the pathways involved in the biosynthesis and regulation of glycoproteins. Before any glycosylation mutant can successfully be used as a tool, its biochemical characteristics must be characterised fully. The majority of mammalian glycosylation mutants discovered so far are defective in N-linked glycosylation. Most steps in the biosynthetic pathway are represented by a glycosylation mutant (Stanley, 1984). In this thesis three more glycosylation mutant cell lines (PMM-normal CDGS type 1 fibroblasts, PMM-deficient CDGS type 1 fibroblasts and galactosaemic fibroblasts) have been identified and biochemically characterised with a view to understanding how the cell can prioritise the synthesis of glycoproteins and overcome defects in glycosylation in order to survive. Further exploitation of these and other mutant cell lines will allow the determination of the factors that are required for the correct biosynthesis and compartmentalisation of glycoproteins. The elucidation of the biochemical mechanisms involved in these human diseases and their relationship to the clinical symptoms will eventually lead to further understanding of the role of the carbohydrates *in vivo*.

Disease	Enzyme defect	Glycosylation defect
CDGS type 1A	phosphomannomutase	non-occupancy of glycosylation sequons of many serum glycoproteins
CDGS type 1B	unknown	non-occupancy of glycosylation sequons of many serum glycoproteins
CDGS type 2	N-acetylglucosaminyltransferase II	glycans not extended on the mannose α 1-3 branch.
Galactosaemia	galactose-1-phosphate uridyl transferase	agalactosylation of N-linked glycans
Hereditary fructose intolerance	fructose-1-phosphate aldolase	unknown
Rheumatoid arthritis	B-cell galactosyltransferase	agalactosylation of IgG
Alcoholism	none	non-occupancy of glycosylation sequons in serum transferrin
HEMPAS	α -mannosidase II/ GlcNAc transferase II	truncated glycans

Table 8.1: Comparison of some human disorders in which glycosylation is affected.

References

- Abeijon C, Hirschberg CB (1992) Topography of glycosylation reactions in the endoplasmic reticulum. *TIBS* **17**;32-36
- Adamowicz M, Pronicka E (1996) Carbohydrate deficient glycoprotein syndrome-like transferrin isoelectric focusing pattern in untreated fructosaemia. *European Journal of Pediatrics* **155** (4);347-348
- Alton G, Kjaergaard S, Etchison JR, Skovby F, Freeze HH (1997) Oral ingestion of mannose elevates blood mannose levels: A first step towards a potential therapy for carbohydrate-deficient glycoprotein syndrome type 1. *Biochemical and Molecular Medicine* **60** (2);127-133
- Andrews DW, Johnson AE (1996) The translocon: more than a hole in the ER membrane? *TIBS* **21**;365-369
- Austen BM, Westwood OMR (1991) *Protein Targeting and Secretion* (edited by Rickwood D). IRL Press
- Axford JS, Sumar N, Alavi A, Isenberg DA, Young A, Bodman KB, Roitt IM (1992) Changes in normal glycosylation mechanisms in autoimmune rheumatic disease. *Journal of Clinical Investigation* **89**;1021-1031
- Axford JS, Lydyard PM, Isenburg DA, Mackenzie L, Hay FC, Roitt IM (1987) Reduced B-cell galactosyltransferase activity in rheumatoid arthritis. *The Lancet* **2**;1486-1488
- Becker WM, Deamer DW (1991) *The World of the Cell. (Second edition) Benjamin Cummings*
- Beem EP, Lisman JJW, Van Steijn GJ, Van Der Wal CJ, Trippelwitz LAW, Overdijk B, Van Halbeek H, Mutsaers JHGM, Vliegenthart JFG (1987) Structural analysis of the carbohydrate moieties of α -fucosidase from human liver *Glycoconjugate Journal* **4**;33-42
- Berry G, Nissim I, Lin Z, Mazur A, Gibson JB, Segal S (1995) Endogenous synthesis of galactose in normal men and patients with hereditary galactosaemia. *The Lancet* **346**;1073-1074
- Berry GT, Palmieri M, Gross KC, Acosta B, Henstenburg JA, Mazur A, Reynolds R, Segal S (1993) The effect of dietary fruits and vegetables on urinary galactitol excretion in galactose-1-phosphate uridyl transferase deficiency. *Journal of Inherited Metabolic Disease* **16**;91-100
- Berry GT, Palmieri MJ, Heales S, Leonard JV, Segal S (1992) Red blood cell uridine sugar nucleotide levels in patients with classic galactosaemia and other metabolic disorders. *Metabolism* **41**;783-787

- Bjursell C, Stibler H, Wahlstrom J, Kristiansson B, Skovby F, Stromme P, Blennow G, Martinsson T (1997) Fine mapping of the gene for carbohydrate-deficient glycoprotein syndrome, type 1 (CDG1): Linkage disequilibrium and founder effect in Scandinavian families. *Genomics* **39**;247-253
- Blennow G, Jaeken J, Wiklund LM (1991) Neurological findings in the carbohydrate-deficient glycoprotein syndrome. *Acta Paediatr. Scand. Suppl.* **375** (5);14-20
- Boles E, Liebetrau W, Hofmann M, Zimmermann FK (1994) A family of hexosephosphate mutases in *Saccharomyces cerevisiae*. *European Journal of Biochemistry* **220**;83-96
- Bonay P, Hughes RC (1991) Purification and characterisation of a novel broad-specificity (α 1-2, α 1-3 and α 1-6) mannosidase from rat liver. *European Journal of Biochemistry* **197**;229-238
- Brandli AW (1991) Mammalian glycosylation mutants as tools for the analysis and reconstitution of protein transport. *Biochemical Journal* **276**;1-12
- Brantly M, Nukiwas T, Crystal RG (1988) Molecular of basis of alpha-1-antitrypsin deficiency. *The American Journal of Medicine* **84** (Suppl 6A);13-31
- Busca R, Pujana A, Pognonec P, Auwerx J, Deeb SS, Reina M, Vilaro S (1995) Absence of N-glycosylation at asparagine 43 in human lipoprotein lipase induces its accumulation in the rough endoplasmic reticulum and alters this cellular compartment. *Journal of Lipid Research* **36**;939-951
- Cacan R, Dengremont C, Labiau O, Kmiecik D, Mir A-M, Verbert A (1996) Occurrence of a cytosolic neutral chitobiase activity involved in oligomannoside degradation: a study with Madin-Darby bovine kidney (MDBK) cells. *Biochemical Journal* **313**;597-602
- Cacan R, Villers C, Belard M, Kaiden A, Krag SS, Verbert A (1992) Different fates of the oligosaccharide moieties of lipid intermediates. *Glycobiology* **2** (2);127-136
- Camphausen RT, Yu H-A, Cumming DA (1995) Chapter 5 Biosynthesis 6: The role of polypeptide in the biosynthesis of protein linked oligosaccharides. *Glycoproteins (edited by Montreuil J, Schachter H, Vliegenthart JFG) Elsevier Science* 391-414
- Carrell RW, Jeppsson J-O, Laurell C-B, Brennan SO, Owen MC, Vaughan L, Boswell DR (1982) Structure and variation of human α 1-antitrypsin. *Nature* **298**;329-334
- Charlwood JK, Mian N, Johnson A, Clayton P, Keir G, Winchester B (1996) Carbohydrate deficient glycoprotein syndrome type 5? *Journal of Inherited Metabolic Disease* **19** (Suppl 1);92
- Charlwood JK, Clayton P, Johnson A, Keir G, Mian N, Winchester B (1997) A case of the carbohydrate-deficient glycoprotein syndrome type 1 (CDGS type 1) with normal phosphomannomutase activity. *Journal of Inherited Metabolic Disease* **20**, in press

- Charuk JHM, Tan J, Bernardini M, Haddad S, Reithmeier RAF, Jaeken J, Schachter H (1995) Carbohydrate deficient glycoprotein syndrome type II. An autosomal recessive N-acetylglucosaminyltransferase II deficiency different from typical hereditary erythroblastic multinuclearity, with a positive acidified serum lysis test (HEMPAS). *European Journal of Biochemistry* **230**;797-805
- Chavin SLI, Weidner SM (1984) Blood clotting factor IX. *The Journal of Biological Chemistry* **259** (6);3387-3390
- Chui D, Oh-Eda M, Liao L-F, Panneerselvam K, Lal A, Marek KW, Freeze HH, Moremen K, Fukuda MN, Marth JD (1997) Alpha-mannosidase II deficiency results in dyserythropoiesis and unveils an alternate pathway in oligosaccharide biosynthesis. *Cell* **90**;157-167
- Clayton P, Winchester B, Di Tomaso E, Young E, Keir G, Rodbeck C (1993) Carbohydrate-deficient glycoprotein syndrome: normal glycosylation in the fetus. *The Lancet* **341**;956
- Clayton PT, Winchester BG, Keir G (1992a) Hypertrophic obstructive cardiomyopathy in a neonate with carbohydrate-deficient glycoprotein syndrome *Journal of Inherited Metabolic Disease* **15**;857-861
- Clayton P, Di Tomaso E, Prudden M, Keir G, Winchester B (1992b) Carbohydrate deficient glycoprotein syndrome: the spectrum of clinical abnormalities in early infancy Proceedings of the 30th annual symposium of the SSIEM, Leuven P180
- Cleary MA, Heptinstall LE, Wraith JE, Walter JH (1995) Galactosaemia: relationship of IQ to biochemical control and genotype. *Journal of Inherited Metabolic Disease* **18**;151-152
- Conradt HS, Egge H, Peter-Katatinic J, Reiser W, Siklosi T, Shaper K (1987) Structure of the carbohydrate moiety of human interferon- β secreted by a recombinant chinese hamster ovary cell line. *The Journal of Biological Chemistry* **262**;14600-14605
- Dai J-B, Liu Y, Ray WJ, Konno M (1992) The crystal structure of muscle phosphoglucomutase refined at 2.7 angstrom resolution. *The Journal of Biological Chemistry* **267** (9);6322-6337
- Daniel PF, Winchester B, Warren CD (1994) Mammalian alpha mannosidases- multiple forms but a common purpose? *Glycobiology* **4** (5);551-566
- de Jong G, van Dijk JP, van Eijk HG (1990) The biology of transferrin. *Clinica Chimica Acta* **190**;1-46
- DiCioccio RA, Mahoney CM (1990) Effect of glycosylation inhibitors and acidotropic amines on the synthesis, processing, and intracellular-extracellular distribution of α -L-fucosidase in B-lymphoblastoid cells. *Carbohydrate Research* **197**;217-226

-
- Dobbie JA, Holton JB, Clamp JR (1990) Defective galactosylation of proteins in cultured skin fibroblasts from galactosaemic patients. *Ann. Clin. Biochem.* **27**;274-275
- Doering TL, Masterson WJ, Hart GW, Englund PT (1990) Biosynthesis of glycosyl phosphatidylinositol membrane anchors. *The Journal of Biological Chemistry* **265** (2);611-614
- Drago GA, Hopkinson DA, Westwood SA, Whitehouse DB (1991) Antigenic analysis of the major human phosphoglucomutase isozymes: PGM1, PGM2, PGM3 and PGM4. *Ann. Hum. Genet.* **55**;263-271
- Durand P, Gatti R, Borrone C (1982) Fucosidosis. *Genetic Errors of Glycoprotein Metabolism* (edited by Durand P, O'Brien JS) 49-87
- Dwek MV, Brooks SA, Streets AJ, Harvey DJ, Leatham AJC (1996) Oligosaccharide release from frozen and paraffin-wax-embedded archival tissues. *Analytical Biochemistry* **242**;8-14
- Dwek RA (1995a) Glycobiology: Towards understanding the function of sugars. *Biochemical Society Transactions* **23**;1-23
- Dwek RA (1995b) Glycobiology: More functions for oligosaccharides. *Science* **269**;1234-1235
- Edge C (1994) The role of glycosylation in disease. *Tools for Glycobiology, Oxford GlycoSystems* 30-31
- Edwards YH, Putt W, Fox M, Ives JH (1995) A novel human phosphoglucomutase (PGM5) maps to the centromeric region of chromosome 9. *Genomics* **30**;350-353
- Elbein AD (1991a) The role of N-linked oligosaccharides in glycoprotein function. *TIBTECH* **9**;346-352
- Elbein AD (1991b) Inhibitors of the addition, modification or processing of the oligosaccharide chains of the N-linked glycoproteins. *Biology of Carbohydrates* **3**;119-177
- Elsas LJ, Langley S, Steele E, Evinger J, Fridovich-Keil JL, Brown A, Singh R, Fernhoff P, Hjelm LN, Dembure PP (1995) Galactosaemia: A strategy to identify new biochemical phenotypes and molecular genotypes. *American Journal of Human Genetics* **56**;630-639
- Ferguson MAJ (1992) Glycosyl-phosphatidylinositol membrane anchors: the tale of a tail. *Biochemical Society Transactions* **20**;243-256
- Ferguson MAJ, Williams AF (1988) Cell-surface anchoring of proteins via glycosyl-phosphatidylinositol structures. *Ann. Rev. Biochem.* **57**;285-320
- Feugeas JP, Neel D, Pavia AA, Laham A, Goussault Y, Derappe C (1990) Glycosylation of the human erythrocyte glucose transporter is essential for glucose-transport activity. *Biochim. Biophys. Acta* **1030**;60-64

- Field MC, Amatayakul-Chantler S, Rademacher TW, Rudd PM, Dwek RA (1994) Structural analysis of the N-glycans from human immunoglobulin A1: comparison of normal human serum immunoglobulin A1 with that isolated from patients with rheumatoid arthritis. *Biochemical Journal* **299**;261-275
- Fukuda MN (1990) HEMPAS disease: genetic defect of glycosylation. *Glycobiology* **1** (1);9-15
- Fukuda MN, Gaetani GF, Izzo P, Scartezzini P, Dell A (1992) Incompletely processed N-glycans of serum glycoproteins in congenital dyserythropoietic anaemia type II (HEMPAS). *British Journal of Haematology* **82**;745-752
- Fukuda MN, Masri KA, Dell A, Luzzatto L, Morenen KW (1990) Incomplete synthesis of N-Glycans in congenital dyserythropoietic anaemia type II caused by a defect in the gene encoding α -mannosidase II. *Proc. Natl. Acad. Sci. USA* **87**;7443-7447
- Fukuda MN, Dell A, Scartezzini P (1987) Primary defect of congenital dyserythropoietic anaemia type II: Failure in glycosylation of erythrocyte lactosaminoglycan proteins caused by lowered N-acetylglucosaminyltransferase II. *The Journal of Biological Chemistry* **262** (15);7195-7206
- Gahmberg CG, Tolvanen M (1996) Why mammalian cell surface proteins are glycoproteins. *TIBS* **21**;308-311
- Geisow MJ (1991) The coming age of glycobiology. *TIBTECH* **9**;221-225
- Gibson JB, Reynolds A, Palmieri MJ, States B, Berry GT, Segal S (1994) Uridine diphosphate hexoses in leukocytes and fibroblasts of classic galactosaemics and patients with other metabolic diseases. *Pediatric Research* **36** (5);613-618
- Gibson JB, Reynolds RA, Rogers S, Palmieri MJ, Segal S (1993) Uridine diphosphoglucose content of human erythrocytes: Assessment by conversion to uridine diphosphoglucuronate. *The Journal of Pediatrics* **123** (6);906-914
- Gitzelmann R (1995) Galactose-1-phosphate in the pathophysiology of galactosaemia. *European Journal of Pediatrics* **154** (Suppl 2); S46-S49
- Gitzelmann R, Steinmann B, Van den Berghe G (1989) Disorders of fructose metabolism. *The Metabolic Basis of Inherited Diseases* (edited by Scriver CR, Beaudet AL, Sly WS, Valle D.) McGraw-Hill, New York 399-413
- Goochee CF, Monica T (1990) Environmental effects on protein glycosylation. *Biotechnology* **8**;421-427
- Goochee CF, Gramer MJ, Andersen DC, Bahr JB, Rasmussen JR (1992) The oligosaccharides of glycoproteins: Factors affecting their synthesis and their influence on glycoprotein properties. *Frontiers in Bioprocessing II* (edited by Todd P, Sikdar SK, Bier M). 199-240

-
- Gross KC, Acosta PB (1991) Fruits and vegetables are a source of galactose: Implications in planning the diets of patients with galactosaemia. *Journal of Inherited Metabolic Disease* **14**;253-258
- Gu J, Wada Y (1995) Aberrant expressions of decorin and biglycan genes in the carbohydrate-deficient glycoprotein syndrome. *The Journal of Biochemistry* **117**;1276-1279
- Guasch R, Renau-Piqueras J, Guerri C (1992) Chronic ethanol consumption induces accumulation of proteins in the liver Golgi apparatus and decreases galactosyltransferase activity. *Alcoholism: Clinical and Experimental Research* **16** (5);942-948
- Guha SK, Rose ZB (1985) The synthesis of mannose-1-phosphate in brain. *Archives of Biochemistry and Biophysics* **243** (1);168-173
- Guile GR, Rudd PM, Wing DR, Prime SB, Dwek RA (1996) A rapid high-resolution high-performance liquid chromatographic method for separating glycan mixtures and analysing oligosaccharide profiles. *Analytical Biochemistry* **240**;210-226
- Haberland C, Perou M, Brunngraber EG, Hof H (1971) The neuropathology of galactosaemia. *Journal of Neuropathology and Experimental Neurology* **30**;431-447
- Hackler R, Arndt T, Kleine TO Gressner AM (1995) Effect of separation conditions on automated isoelectric focusing of carbohydrate-deficient transferrin and other human isotransferrins using the PhastSystem. *Analytical Biochemistry* **230**;281-289
- Hagberg BA, Blennow G, Kristiansson B, Stibler H (1993) Carbohydrate-deficient glycoprotein syndromes: peculiar group of new disorders. *Pediatric Neurology* **9**;255-262
- Hansen TWR, Henrichsen B, Rasmussen RK, Carling A, Andreassen AB, Skjeldal O (1996) Neuropsychological and linguistic follow up studies of children with galactosaemia from an unscreened population. *Acta Paediatr.* **85**;1197-1201
- Harding BN, Dunger DB, Grant DB, Erdohazi M (1988) Familial olivopontocerebellar atrophy with neonatal onset: a recessively inherited syndrome with systemic and biochemical abnormalities. *Journal of Neurology, Neurosurgery and Psychiatry* **51**;385-390
- Harris H, Hopkinson DA (1976) *Handbook of Enzyme Electrophoresis in Human Genetics* 1
- Harrison HH, Harbison MD, Miller KL, Slonim AE (1992) Multiple serum protein abnormalities in carbohydrate-deficient glycoprotein syndrome: pathognomonic finding of two dimensional electrophoresis? *Clinical Chemistry* **38** (7);1390-1392
- Hebert DN, Foellmer B, Helenius A (1995) Glucose trimming and reglucosylation determine glycoprotein association with calnexin in the endoplasmic reticulum. *Cell* **81**;425-433
-

- Helenius A (1994) How N-linked oligosaccharides affect glycoprotein folding in the endoplasmic reticulum. *Molecular Biology of the Cell* **5**;253-265
- Henry H, Tissot J-D, Messerli B, Markert M, Muntau A, Skladal D, Sperl W, Jaeken J, Weidinger S, Heyne K, Bachmann C (1997) Microheterogeneity of serum glycoproteins and their liver precursors in patients with carbohydrate-deficient glycoprotein syndrome type 1: Apparent deficiencies in clusterin and serum amyloid P. *Journal of Laboratory and Clinical Medicine* **129** (4);412-421
- Holton JB (1995) Effects of galactosaemia in utero. *European Journal of Pediatrics* **154** (Suppl 1);S77-S81
- Holton JB, Leonard JV (1994) Clouds still gathering over Galactosaemia. *The Lancet* **344**;1242-1243
- Holton JB, De la Cruz F, Levy HL (1993) Galactosaemia: The uridine diphosphate galactose deficiency - uridine treatment controversy. *The Journal of Pediatrics* **123** (6);1009-1014
- Horslen SP, Clayton PT, Harding BN, Hall NA, Keir G, Winchester B (1991) Olivopontocerebellar atrophy of neonatal onset and disialotransferrin developmental deficiency syndrome. *Archives of Disease in Childhood* **66**;1027-1032
- Hutchesson A, Gray RGF, Spencer DA, Keir G (1995) Carbohydrate deficient glycoprotein syndrome; multiple abnormalities and diagnostic delay. *Archives of Disease in Childhood* **72**;445-446
- Iourin O, Mattu TS, Mian N, Keir G, Winchester B, Dwek RA, Rudd PM (1996) The identification of abnormal glycoforms of serum transferrin in carbohydrate deficient glycoprotein syndrome type 1 by capillary zone electrophoresis. *Glycoconjugate Journal* **13**;1031-1042
- Jaeken J (1997) CDGS Research. <http://www.cdgs.com>
- Jaeken J (1996) CDGS Research. <http://www.cdgs.com>
- Jaeken J, Carchon H (1993) The carbohydrate-deficient glycoprotein syndromes: An overview. *Journal of Inherited Metabolic Disease* **16**;813-820
- Jaeken J, Matthijs G, Barone R, Carchon H (1997a) Carbohydrate deficient glycoprotein (CDG) syndrome type I. *Journal of Medical Genetics* **34**;73-76
- Jaeken J, Artigas J, Barone R, Fiumara A, de Konning TD, Poll-The BT, de Rijk-van Andel JF, Hoffmann G, Mayatepek E, Pineda M, Vilaseca MA, Saudubray JM, Schluter B, Wevers R, Van Schaftingen E (1997b) Phosphomannomutase deficiency is the main cause of carbohydrate-deficient glycoprotein syndrome with type 1 isoelectric-focusing pattern of serum sialotransferrins. *Journal of Inherited Metabolic Disease* **20**, in press

- Jaeken J, Pirard M, Adamowicz M, Pronicka E, Van Schaftingen E (1996) Inhibition of phosphomannose isomerase by fructose-1-phosphate: an explanation for defective N-glycosylation in hereditary fructose intolerance. *Pediatric Research* **40** (5);764-766
- Jaeken J, Schachter H, Carchon H, De Cock P, Coddeville B, Spik G (1994) Carbohydrate deficient glycoprotein syndrome type II: A deficiency in Golgi localised N-acetylglucosaminyltransferase II. *Archives of Disease in Childhood* **71**;123-127
- Jaeken J, Carchon H, Stibler H (1993a) The carbohydrate-deficient glycoprotein syndromes: Pre-Golgi and Golgi disorders? *Glycobiology* **3** (5);423-428
- Jaeken J, DeCock P, Stibler H, van Geet C, Kint J, Ramaekers V, Carchon H (1993b) Carbohydrate deficient glycoprotein syndrome type II. *Journal of Inherited Metabolic Disease* **16**;1041
- Jaeken J, Kint J, Spaapen L (1992) Serum lysosomal enzyme abnormalities in galactosaemia. *The Lancet* **340**;1472-1473
- Jaeken J, Hagberg B, Stromme P (1991) Clinical presentation and natural course of the carbohydrate-deficient glycoprotein syndrome. *Acta Paediatr. Scand. Suppl.* **375** (5);6-13
- Jaeken J, van Eijk HG, van der Heul C, Corbeel L, Eeckels R, Eggermont E (1984) Sialic acid-deficient serum and cerebrospinal fluid transferrin in a newly recognised genetic syndrome. *Clinica Chimica Acta* **144**;245-247
- Jaeken J, Vanderschwere-Lodeweyd M, Casaer P, Snoeck L, Corbeel L, Eggermont E, Eeckels R (1980) Familial psychomotor retardation with markedly fluctuating serum prolactin, FSH and GH levels, partial TBG deficiency, increased serum arylsulfatase A and increased CSF protein: A new syndrome? *Pediatric Research* **14**;179
- Jentoft N (1990) Why are proteins O-glycosylated? *TIBS* **15**;291-294
- Jeppsson J-O, Kristensson H, Fimiani C (1993) Carbohydrate-deficient transferrin quantified by HPLC to determine heavy consumption of alcohol. *Clin. Chem.* **39** (10);2115-2120
- Johnson SW, Piesecki S, Wang R, Damjanov I, Alhadeff JA (1992) Analysis of purified human liver α -L-fucosidase by Western-blotting with lectins and polyclonal and monoclonal antibodies. *Biochemical Journal* **282**;829-834
- Kaplan HA, Welply JK, Lennarz WJ (1987) Oligosaccharyl transferase: the central enzyme in the pathway of glycoprotein assembly. *Biochimica et Biophysica Acta* **906**;161-173
- Kasturi L, Chen H, Shakin-Eshleman SH (1997) Regulation of N-linked core glycosylation: use of a site directed mutagenesis approach to identify Asn-Xaa-Ser/Thr sequons that are poor oligosaccharide acceptors. *Biochemical Journal* **323**;415-419

-
- Kaufman FR, Xu YK, Ng WG, Silva PD, Lobo RA, Donnell GN (1989) Gonadal function and ovarian galactose metabolism in classic galactosaemia. *Acta Endocrinologica* **120**;129-133
- Kaufman FR, Donnell GN, Roe TF, Kogut MD (1986) Gonadal function in patients with galactosaemia. *Journal of Inherited Metabolic Disease* **9**;140-146
- Keevill NJ, Holton JB, Allen JT (1994) UDP-Glucose and UDP-Galactose concentrations in cultured skin fibroblasts of patients with classical galactosaemia. *Journal of Inherited Metabolic Disease* **17**;23-26
- Keevill NJ, Holton JB, Allen JT (1993) The investigation of UDP glucose and UDP galactose concentration in red blood cells of patients with classical galactosaemia. *Clinica Chimica Acta* **221**;135-142
- Keir G, Zeman A, Brookes G, Porter M, Thompson EJ (1992) Immunology of transferrin in the identification of cerebrospinal fluid otorrhoea and rhinorrhoea. *Ann. Clin. Biochem* **29**;210-213
- Kepes F, Schekman R (1988) The yeast SEC53 gene encodes phosphomannomutase. *Journal of Biological Chemistry* **263**;9155-9161
- Kelleher DJ, Kreibich G, Gilmore R (1992) Oligosaccharyltransferase activity is associated with a protein complex composed of ribophorins I and II and a 48 kd protein. *Cell* **69**;55-65
- Kirkman HN (1995) Measurements of uridine diphosphate glucose and uridine diphosphate galactose—an appraisal. *European Journal of Pediatrics* **154 (Suppl 2)**;S72-S74
- Kirkman HN (1993) Reply. *The Journal of Pediatrics* **123**;1015-1016
- Kirkman HN (1992) Erythrocytic uridine diphosphate galactose in galactosaemia. *Journal of Inherited Metabolic Disease* **15**;4-16
- Klausner RD, Sitia R (1990) Protein degradation in the endoplasmic reticulum. *Cell* **62**;611-614
- Knauer R, Lehle L, Hanefeld F, von Figura K (1994) Normal N-oligosaccharyltransferase activity in fibroblasts from patients with carbohydrate-deficient glycoprotein syndrome. *Journal of Inherited Metabolic Disease* **17**;541-544
- Kobata A (1992) Structures and functions of the sugar chains of glycoproteins. *European Journal of Biochemistry* **209**;483-501
- Komrower GM (1982) Galactosaemia—thirty years on. The experience of a generation. *Journal of Inherited Metabolic Disease* **5 (Suppl 2)**;96-104
- Kornfeld S (1986) Trafficking of lysosomal enzymes in normal and disease states. *Journal of Clinical Investigation* **77**;1-6

- Kornfeld R, Kornfeld S (1985) Assembly of asparagine linked oligosaccharides. *Ann. Rev. Biochem.* **54**;631-664
- Kornfeld S, Mellman I (1989) The biogenesis of lysosomes. *Ann. Rev. Cell Biol.* **5**;483-525
- Krasnewich DM, Holt GD, Brantly M, Skovby F, Redwine J, Gahl WA (1995) Abnormal synthesis of dolichol-linked oligosaccharides in carbohydrate-deficient glycoprotein syndrome. *Glycobiology* **5** (5);503-510
- Kristiansson B, Andersson M, Tonnby B, Hagberg B (1989) Disialotransferrin developmental deficiency syndrome. *Archives of Disease in Childhood* **64**;71-76
- Kuster B, Harvey DJ (1997) Ammonium containing buffers should be avoided during enzymatic release of glycans from glycoproteins when followed by reducing terminal derivatisation. *Glycobiology* **7** (3); vii-ix
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**;680-685
- Landberg E, Pahlsson P, Lundblad A, Arnetorp A, Jeppsson J-O (1995) Carbohydrate composition of serum transferrin isoforms from patients with high alcohol consumption. *Biochemistry and Biophysics Research Communications* **210** (2);267-274
- Lang L, Reitman M, Tang J, Roberts RM, Kornfeld S (1984) Lysosomal enzyme phosphorylation. *The Journal of Biological Chemistry* **259** (23);14663-14671
- Lau JTY, Welply JK, Shenbagamurthi P, Naider F, Lennarz WJ (1983) Substrate recognition by oligosaccharyl transferase. *The Journal of Biological Chemistry* **258** (24);15255-15260
- Lewis BD (1997) Thesis-Glycoprocessing in classical galactosaemia. Women's and Childrens Hospital, North Adelaide, Australia.
- Lin Z-J, Konno M, Abad-Zapatero C, Wierenga R, Murthy MRN, Ray WJ, Rossmann MG (1986) The structure of rabbit muscle phosphoglucomutase at intermediate resolution. *The Journal of Biological Chemistry* **261** (1);264-274
- Lippincott-Schwartz J, Bonifacino JS, Yuan LC, Klausner RD (1988) Degradation from the endoplasmic reticulum: Disposing of newly synthesised proteins. *Cell* **54**;209-220
- Lodish HF (1991) Recognition of complex oligosaccharides by the multisubunit asialoglycoprotein receptor. *TIBS* **16**;374-377
- MacGillivray RTA, Mendez E, Shewale JG, Sinha SK, Lineback-Zins J, Brew K (1983) The primary structure of human serum transferrin. *The Journal of Biological Chemistry* **258** (6);3543-3553
- March RE, Putt W, Hollyoake M, Ives JH, Lovegrove JU, Hopkinson DA, Edwards YH, Whitehouse DB (1993) The classical human phosphoglucomutase (PGM1)

- isozyme polymorphism is generated by intragenic recombination. *Proc. Natl. Acad. Sci. USA.* **90**;10730-10733
- Marchase RB, Bounelis P, Brumley LM, Dey N, Browne B, Auger D, Fritz TA, Kulesza P, Bedwell DM (1993) Phosphoglucomutase in *Saccharomyces cerevisiae* is a cytoplasmic glycoprotein and the acceptor for a Glc-phosphotransferase. *The Journal of Biological Chemistry* **268**;8341-8349
- Marinari UM, Pronzato MA, Pizzorno R, Cottalasso D, Maloberti G, Domenicotti C, Gazzo P, Nanni G (1993) Acetaldehyde-induced impairment of protein glycosylation in liver Golgi apparatus. *Biochemistry and Molecular Biology International* **29** (6);1131-1138
- Marquardt T, Ullrich K, Zimmer P, Hasilik A, Deufel T, Harms E (1995) Carbohydrate deficient glycoprotein syndrome - glycosylation, folding and intracellular transport of newly synthesised glycoproteins. *European Journal of Cell Biology* **66**;268-273
- Martinsson T, Bjursell C, Stibler H, Kristiansson B, Skovby F, Jaeken J, Blennow G, Stromme P, Hanefeld F, Wahlstrom J (1994) Linkage of a locus for carbohydrate-deficient glycoprotein syndrome type 1 (CDG1) to chromosome 16 P, and linkage disequilibrium to microsatellite marker D16S406. *Human Molecular Genetics* **3** (11);2037-2042
- Mathews CK, van Holde KE (1990) *Biochemistry. Benjamin Cummings*
- Matthijs G, Legius E, Schollen E, Vandenberk P, Jaeken P, Barone R, Fiumara A, Visser G, Lambert M, Cassiman JJ (1996) Evidence for genetic heterogeneity in the carbohydrate deficient glycoprotein syndrome type 1 (CDG 1). *Genomics* **35**;597-599
- Matthijs G, Schollen E, Pirard M, Budarf ML, Van Schaftingen E, Cassiman J-J (1997a) PMM (PMM1), the human homologue of SEC53 or yeast phosphomannomutase, is localised on chromosome 22q13. *Genomics* **40**;41-47
- Matthijs G, Schollen E, Pardon E, Veiga-Da-Cunha M, Jaeken J, Cassiman JJ, Van Schaftingen E (1997b) Mutations in PMM2, a phosphomannomutase gene on chromosome 16p13, in carbohydrate-deficient glycoprotein type 1 syndrome (Jaeken syndrome). *Nature Genetics* **16**;88-92
- McIlvaine TC (1921) A buffer solution for colourimetric comparison. *The Journal of Biological Chemistry* **49**;183
- Merril CR, Goldman D, Sedman SA, Ebert MH (1981) Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science* **211**;1437-1438
- Moremen KW, Trimble RB, Herscovics A (1994) Glycosidases of the asparagine-linked oligosaccharide processing pathway. *Glycobiology* **4** (2);113-125

- Narasimhan S, Wilson JR, Martin E, Schachter H (1979) A structural basis for four distinct elution profiles on concanavalin A-Sepharose affinity chromatography of glycopeptides. *Can .J. Biochem.* **57**;83-96
- Neufeld EF (1989) Natural history and inherited disorders of a lysosomal enzyme, β -hexosaminidase. *The Journal of Biological Chemistry* **264** (19);10927-10930
- Ng WG, Xu YK, Kaufman FR, Donnell GN (1993) Measurement of uridine diphosphate hexoses in galactosaemia. *The Journal of Pediatrics* **123**;1015
- Ng WG, Xu YK, Kaufman FR, Donnell GN (1989) Deficit of uridine diphosphate galactose in galactosaemia. *Journal of Inherited Metabolic Disease* **12**;257-266
- Nishimori I, Johnson NR, Sanderson SD, Perini F, Mountjoy K, Cerny RL, Gross ML, Hollingsworth MA (1994) Influence of acceptor substrate primary amino acid sequence on the activity of human UDP-N-acetylgalactosamine:polypeptide N-acetylgalactosaminyltransferase. *The Journal of Biological Chemistry* **269** (23);16123-16130
- O'Brien J, Ikada S, Chen A, Fillerup D (1970) Tay Sachs Disease. *New England Journal of Medicine* **283**;15-20
- Occhiodoro T, Beckman KR, Morris CP , Hopwood JJ (1989) Human- α -L-fucosidase: Complete coding sequence from cDNA clones. *Biochemical and Biophysical Research Communications* **164** (1);439-445
- Oesterhelt C, Schnarrenberger C, Gross W (1997) The reaction mechanism of phosphomannomutase in plants. *FEBS Letters* **401**;35-37
- Ohkura T, Fukushima K, Kurisaki A, Sagami H, Ogura K, Ohno K, Hara-Kuge S, Yamashita K (1997) A partial deficiency of dehydrodolichol reduction is a cause of carbohydrate-deficient glycoprotein syndrome type 1. *The Journal of Biological Chemistry* **272** (11);6868-6875
- Ornstein KS, McGuire EJ, Berry GT, Roth S, Segal S (1992) Abnormal galactosylation of complex carbohydrates in cultured fibroblasts from patients with galactose-1-phosphate uridylyltransferase deficiency. *Pediatric Research* **31** (5);508-511
- Overdijk B, Beem EP, van Steijn GJ, Trippelvit LAW, Lisman JJW, Paz Parente J, Cardon P, Leroy Y, Fournet B, van Halbeek H, Mutsaers JHGM, Vliegenthart JFG (1985) Structural analysis of the carbohydrate chains of β -N-acetylhexosaminidase from bovine brain. *Biochemical Journal* **232**;637-641
- Panneerselvam K, Freeze HH (1995) Enzymes involved in the synthesis of mannose-6-phosphate from glucose are normal in carbohydrate deficient glycoprotein syndrome fibroblasts. *Biochemical and Biophysical Research Communications* **208** (2);517-522

- Panneerselvam K, Freeze HH (1996a) Mannose corrects altered N-glycosylation in carbohydrate-deficient glycoprotein syndrome fibroblasts. *Journal of Clinical Investigation* **97** (6);1478-1487
- Panneerselvam K, Freeze HH (1996b) Mannose enters mammalian cells using a specific transporter that is insensitive to glucose. *The Journal of Biological Chemistry* **271** (16);9417-9421
- Parekh RB, Dwek RA, Edge CJ, Rademacher TW (1989) N-glycosylation and the production of recombinant glycoproteins. *TIBTECH* **7**;117-121
- Parekh RB, Dwek RA, Sutton BJ, Fernandes DL, Leung A, Stanworth D, Rademacher TW, Mizuochi T, Taniguchi T, Matsuta K, Takeuchi F, Nagano Y, Miyamoto T, Kobata A (1985) Association of rheumatoid arthritis and primary osteoarthritis with changes in the glycosylation pattern of total serum IgG. *Nature* **316**;452-457
- Paulson JC (1989) Glycoproteins: what are the sugar chains for? *TIBS* **14**;272-276
- Petersen MB, Brostrom K, Stibler H, Skovby F (1993) Early manifestations of the carbohydrate-deficient glycoprotein syndrome. *Journal of Pediatrics* **122**;66-70
- Powell LD, Panneerselvam K, Vij R, Diaz S, Manzib A, Buist N, Freeze H, Varki A (1994) Carbohydrate deficient glycoprotein syndrome: Not an N-linked oligosaccharide processing defect but an abnormality in lipid-linked oligosaccharide biosynthesis? *Journal of Clinical Investigation* **94**;1901-1909
- Prestoz LLC, Couto AS, Shin YS, Petry KG (1997) Altered follicle stimulating hormone isoforms in female galactosaemia patients. *European Journal of Pediatrics* **156**;116-120
- Proia RL, d'Azzo A, Neufeld EF (1984) Association of α and β subunits during the biosynthesis of β -hexosaminidase in cultured human fibroblasts. *The Journal of Biological Chemistry* **259** (5);3350-3354
- Putman FW (1980) Chapter 6; Transferrin. *Plasma Proteins (edited by FW Putman)*. Plenum Press 265-315
- Putt W, Ives JH, Hollyoake M, Hopkinson DA, Whitehouse DB, Edwards YH (1993) Phosphoglucomutase 1: a gene with two promoters and a duplicated first exon. *Biochemical Journal* **296**;417-422
- Rademacher TW, Parekh RB, Dwek RA (1988) Glycobiology. *Annual Review of Biochemistry* **57**;785-838
- Rasmussen JR (1991) Glycosylation of recombinant proteins. *Biology of Carbohydrates* **3**;179-285
- Ray WJ, Peck EJ (1972) Phosphomutases. *The Enzymes (edited by Boyer PD)*. Academic Press, New York 3rd edition **6**;407-477

-
- Rose ZB (1986) The glucose bisphosphate family of enzymes. *TIBS* **11**;253-254
- Roth J, Wang Y, Eckhardt AE, Hill RL (1994) Subcellular localisation of the UDP-N-acetyl-D-galactosamine: polypeptide N-acetylgalactosaminyltransferase-mediated O-glycosylation reaction in the submaxillary gland. *Proc. Natl. Acad. Sci. USA* **91**;8935-8939
- Ruohola H, Ferro-Novick S (1987) Sec53, a protein required for an early step in secretory protein processing and transport in yeast, interacts with the cytoplasmic surface of the endoplasmic reticulum. *Proc. Natl. Acad. Sci. USA* **84**;8468-8472
- Schachter H (1995a) Chapter 5 Biosynthesis 2c: Glycosyltransferases involved in the synthesis of N-glycan antennae. *Glycoproteins (edited by Montreuil J, Schachter H, Vliegthart JFG). Elsevier Science* 153-199
- Schachter H (1995b) Chapter 5 Biosynthesis 4b: Substrate level controls for N-glycan assembly. *Glycoproteins (edited by Montreuil J, Schachter H, Vliegthart JFG). Elsevier Science* 281-286
- Schachter H (1994) Biosynthesis of N- and O-glycans. *Tools for Glycobiology, Oxford GlycoSystems* 9-11
- Schachter H (1991a) Enzymes associated with glycosylation. *Current Opinion in Structural Biology* **1**;755-765
- Schachter H (1991b) The 'yellow brick road' to branched complex glycans. *Glycobiology* **1** (5);453-461
- Schachter H (1984) Co-ordination between enzyme specificity and intracellular compartmentation in the control of protein-bound oligosaccharide biosynthesis. *Biol. Cell* **51**;133-146
- Schachter H, Brockhausen I (1992) The biosynthesis of serine (threonine)- N-acetylgalactosamine-linked carbohydrate moieties. *Glycoconjugates, Composition, Structure and Function (edited by Allen HJ, Kisailus EC). Marcel Dekker, New York* 263-332
- Schachter H, Tan J, Dunn J, Jaeken J (1996) Molecular basis of carbohydrate deficient glycoprotein syndrome type II. *Glycobiology* **6** (7);abstract C4
- Schweitzer S, Shin Y, Jakobs C, Brodehl J (1993) Long term outcome in 134 patients with galactosaemia. *European Journal of Pediatrics* **152**;36-43
- Segal S (1995a) Defective galactosylation in galactosaemia: is low cell UDP-galactose an explanation? *European Journal of Pediatrics* **154** (Suppl 2);S65-S71
- Segal S (1995b) Galactosaemia Unsolved. *European Journal of Pediatrics* **154** (Suppl 2);S97-S102

Segal S (1989) Disorders of galactose metabolism. *The Metabolic Basis of Inherited Diseases* (edited by Scriver CR, Beaudet AL, Sly WS, Valle D). McGraw-Hill, New York 453-480

Shaper JH, Shaper NL (1992) Enzymes associated with glycosylation. *Current Opinion in Structural Biology* **2**;701-709

Shin YS (1995) Nucleotide sugars: determination of cellular levels and discrepancies in results. *European Journal of Pediatrics* **154** (Suppl 2);S75-S76

Siegel S (1959) *Nonparametric statistics for the behavioural sciences* McGraw-Hill

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985) Measurement of protein using bicinchoninic acid. *Analytical Biochemistry* **150**;76-85

Sonderfeld-Fresko S, Proia RL (1989) Analysis of the glycosylation and phosphorylation of the lysosomal enzyme, β -hexosaminidase B, by site directed mutagenesis. *The Journal of Biological Chemistry* **264** (13);7692-7697

Sonderfeld-Fresko S, Proia RL (1988) Synthesis and assembly of a catalytically active lysosomal enzyme, β -hexosaminidase B, in a cell free system. *The Journal of Biological Chemistry* **263** (26);13463-13469

Spik G, Debyruyne V, Montreuil J, van Halbeek H, Vliegthart JFG (1985) Primary structure of two sialylated triantennary glycans from human serotransferrin. *FEBS Letters* **183** (1);65-69

Spik G, Bayard B, Fournet B, Strecker G, Bouquelet S, Montreuil J (1975) Complete structure of the two carbohydrate units of human serotransferrin. *FEBS Letters* **50**;296-299

Stanley P (1984) Glycosylation mutants of animal cells. *Ann. Rev. Genet.* **18**;525-552

Steinbuch N, Nierwlarowski S (1960) *Nature* **186**;87-88

Stevens VL (1995) Biosynthesis of glycosylphosphatidylinositol membrane anchors. *Biochemical Journal* **310**;361-370

Stibler H (1991) Carbohydrate-deficient transferrin in serum: A new marker of potentially harmful alcohol consumption reviewed. *Clinical Chemistry* **37** (12);2029-2037

Stibler H, Skovby F (1994) Failure to diagnose carbohydrate-deficient glycoprotein syndrome prenatally. *Pediatr. Neurol.* **11**;71

Stibler H, Cederberg B (1993) Diagnosis of the carbohydrate-deficient glycoprotein syndrome by analysis of transferrin in filter paper blood spots. *Acta Paediatr.* **82**;55-59

- Stibler H, Kristiansson B (1991) Analysis of transferrin and α 1-fetoprotein in amniotic fluid and neonatal serum: a possible means for indirect prenatal diagnosis of the carbohydrate-deficient glycoprotein syndrome. *Acta Paediatr. Scand. Suppl.* **375** (5);32-38
- Stibler H, Jaeken J (1990) Carbohydrate-deficient serum transferrin in a new systemic hereditary syndrome. *Archives of Disease in Childhood* **65**;107-111
- Stibler H, Stephani U, Kutsch U (1995) Carbohydrate-deficient glycoprotein syndrome - a fourth subtype. *Neuropediatrics* **26**;235-237
- Stibler H, Westerberg B, Hanefeld F, Hagberg B (1993) Carbohydrate-deficient glycoprotein syndrome (CDG) - a new variant, type III. *Neuropediatrics* **24**;51-52
- Stibler H, Jaeken J, Kristiansson B (1991) Biochemical characteristics and diagnosis of the carbohydrate-deficient glycoprotein syndrome. *Acta Paediatr. Scand. Suppl.* **375** (5);21-31
- Stoll J, Krag SS (1988) A mutant of Chinese hamster ovary cells with a reduction in levels of dolichyl phosphate available for glycosylation. *The Journal of Biological Chemistry* **263**;10766-10773
- Swinscow TDV (1988) *Statistics at square one. British medical Association*
- Szumilo T, Drake RR, York JL, Elbein AD (1993) GDP-mannose pyrophosphorylase. Purification to homogeneity, properties and utilisation to prepare photoaffinity analogues. *The Journal of Biological Chemistry* **268** (24);17943-17950
- Takahashi N, Neel JV (1993) Intragenic recombination at the human phosphoglucomutase 1 locus: Predictions fulfilled. *Proc. Natl. Acad. Sci. USA* **90**;10725-10729
- Takeda J, Kinoshita T (1995) GPI-anchor biosynthesis. *TIBS* **20**;367-371
- Thorstensen K, Romslo I (1990) The role of transferrin in the mechanism of cellular iron uptake. *Biochemical Journal* **271**;1-10
- Tomita A, Parker CJ (1994) Aberrant regulation of complement by the erythrocytes of hereditary erythroblastic multinuclearity with a positive acidified serum lysis test (HEMPAS). *Blood* **83** (1); 250-259
- Tsuda E, Kawanishi G, Ueda M, Masuda S, Sasaki R (1990) The role of carbohydrate in recombinant human erythropoietin. *European Journal of Biochemistry* **188**;405-411
- Van Schaftingen E, Jaeken J (1995) Phosphomannomutase deficiency is a cause of carbohydrate-deficient glycoprotein syndrome type 1. *FEBS Letters* **377**;318-320

-
- Varki A (1993) Biological roles of oligosaccharides: all theories are correct. *Glycobiology* **3** (2);97-130
- Verbert A (1995) Chapter 5 Biosynthesis 2b: From Glc₃Man₉GlcNAc₂-protein to Man₅GlcNAc₂-protein: transfer 'en bloc' and processing. *Glycoproteins (edited by Montreuil J, Schachter H, Vliegenthart JFG) Elsevier Science* 145-152
- Villers C, Cacan R, Mir A-M, Labiau O, Verbert A (1994) Release of oligomannoside-type glycans as a marker of the degradation of newly synthesised glycoproteins. *Biochemical Journal* **298**;135-142
- Voet D, Voet JG (1990) *Biochemistry. John Wiley & Sons*
- Wada Y, Nishikawa A, Okamoto N, Inui K, Tsukamoto H, Okada S, Taniguchi N (1992) Structure of serum transferrin in carbohydrate-deficient glycoprotein syndrome. *Biochemical and Biophysical Research Communications* **189** (2);832-836
- Waggoner DD, Buist NRM, Donnell GN (1990) Long term prognosis in galactosaemia: Results of a survey of 350 cases. *Journal of Inherited Metabolic Disease* **13**;802-818
- Walter P, Blobel G (1983) Preparation of microsomal membranes for cotranslational protein translocation. *Methods in Enzymology* **96**;84-93
- Wang Y, Abernethy JL, Eckhardt AE, Hill RL (1992) Purification and characterisation of a UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase specific for glycosylation of threonine residues. *The Journal of Biological Chemistry* **267** (18);12709-12716
- Watson JD, Gilman M, Witkowski J, Zoller M (1992) *Recombinant DNA. (Second edition) Scientific American Books, New York* 522
- Weitz G, Proia RL (1992) Analysis of the glycosylation and phosphorylation of the α -subunit of the lysosomal enzyme, β -hexosaminidase A, by site directed mutagenesis. *The Journal of Biological Chemistry* **267**(14);10039-10044
- Weller M (1979) Chapter 6; Enzymes which contain covalently bound phosphorous. *Protein Phosphorylation. Pion Limited, London.*
- Whitehouse DB, Putt W, Lovegrove JU, Morrison K, Hollyoake M, Fox M F, Hopkinson DA, Edwards YH (1992) Phosphoglucosyltransferase 1: Complete human and rabbit mRNA sequences and direct mapping of this highly polymorphic marker on human chromosome 1. *Proc. Natl. Acad. Sci. USA* **89**;411-415
- Winchester BG (1996) Chapter 7; Lysosomal metabolism of glycoconjugates. *Subcellular Biochemistry, Biology of the Lysosome (edited by Lloyd and Mason). Plenum Press, New York* **27**;191-238

- Winchester B, Fleet GWJ (1992) Amino-sugar glycosidase inhibitors: versatile tools for glycobiologists. *Glycobiology* **2** (3);199-210
- Winchester B, Clayton P, Mian N, di-Tomaso E, Dell A, Reason A, Keir G (1995) The carbohydrate-deficient glycoprotein syndrome: an experiment of nature in glycosylation. *Biochemical Society Transactions* **23**;185-188
- Wold F (1981) *In vivo* chemical modifications of proteins. *Ann. Rev. Biochem.* **50**;783-814
- Yamaguchi K, Akai K, Kawanishi G, Ueda M, Masuda S, Sasaki R (1991) Effects of site-directed removal of N-glycosylation sites in human erythropoietin on its production and biological properties. *The Journal of Biological Chemistry* **266** (30);20434-20439
- Yamashita K, Ohno K (1996) *Glycoproteins and Disease (edited by Montreuil J, Vlieghehart JFG, Schachter H). Elsevier Science Publishers, Amsterdam* 443-453
- Yamashita K, Ohkura T, Ideo H, Ohno K, Kanai M (1993a) Electrospray ionisation mass spectrometric analysis of serum transferrin isoforms in patients with carbohydrate-deficient glycoprotein syndrome. *Journal of Biochemistry* **114**;766-769
- Yamashita K, Ideo H, Ohkura T, Fukushima K, Yuasa I, Ohno K, Takeshita K (1993b) Sugar chains of serum transferrin from patients with carbohydrate deficient glycoprotein syndrome. *The Journal of Biological Chemistry* **268** (8);5783-5789
- Yasugi E, Nakasuji M, Dohi T, Oshima M (1994) Major defect of carbohydrate-deficient glycoprotein syndrome is not found in the synthesis of dolichylphosphate or N-acetylglucosaminyl pyrophosphoryl-dolichol. *Biochemical and Biophysical Research Communications* **200** (2);816-820
- Yuasa I, Ohno K, Hashimoto K, Iijima K, Yamashita K, Takeshita K (1995) Carbohydrate-deficient glycoprotein syndrome: electrophoretic study of multiple serum glycoproteins. *Brain and Development* **17**;13-19