GLYCOPROCESSING IN CLASSICAL GALACTOSAEMIA

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Abstract

Classical galactosaemia is a disorder of galactose metabolism that results from a deficiency of galactose-1-phosphate uridyltransferase. Infants with galactosaemia usually present with an acute toxicity syndrome that is characterised by vomiting, failure to thrive, hepatitis, jaundice, and sepsis. The acute symptoms are resolved by removing galactose from the diet and this treatment has formed the basis of management of galactosaemia for over 60 years. However, as a group, the galactosaemic patients develop longer term problems that include impaired intellectual development, learning disabilities, poor motor function, ovarian failure, and occasionally ataxia and tremor. These long-term complications develop independent of either the severity of initial illness, the age at which diet was started, or the success of dietary control.

If the long-term complications are not related to the acute toxicity of galactosaemia, then other explanations must be sought in alterations of the cellular environment that occur secondary to transferase deficiency. The serum in untreated galactosaemia contains hyposialylated isoforms of several glycoproteins. This suggests a possible disturbance in glycoprotein synthesis, which could contribute to the long-term complications. The aim of this thesis was to examine N-glycosylation in transferase-deficient skin fibroblasts to determine what abnormalities of glycoprotein synthesis occur in galactosaemia. When the mature complex N-linked oligosaccharides from galactosaemic fibroblasts were examined by size-exclusion chromatography and anion-exchange high performance liquid chromatography, they were structurally complete. However, the galactosaemic fibroblasts consistently incorporated less [2-^{3}H]-mannose into protein than normal controls. This occurred whether the incorporation was corrected to the cell protein or to the incorporation of [^{35}S]-methionine. The decrease was observed in cellular and secreted proteins and was proportional to the concentration of galactose in the culture medium. The galactosaemic fibroblasts also incorporated less [2-^{3}H]-mannose into dolichol-linked oligosaccharides. This was accompanied by an accumulation of $Man_{3-5}GlcNAc_2$ dolichol-linked intermediates, and predominantly $Man_5GlcNAc_2$-sized oligosaccharides were transferred to protein. These abnormalities are very similar to those reported in fibroblasts from patients with the carbohydrate-deficient glycoprotein syndrome type I, and in cells starved of glucose.

It is proposed that galactose-1-phosphate interferes with the conversion of glucose to mannose intermediates that are required for the synthesis of dolichol-linked oligosaccharides. This may be one mechanism for hyposialylation of serum glycoproteins in untreated galactosaemia. It remains to be determined whether abnormal N-glycosylation contributes to the long-term complications in patients.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

SIGNED: **DATE:** 3.4.97

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List of abbreviations

AMP	adenosine 5'-phosphate
Asn	asparagine
ATP	adenosine 5'-triphosphate
BCA	bicinchoninic acid
BME	Basal Medium Eagle
BSA	bovine serum albumin
CDG syndrome	carbohydrate-deficient glycoprotein syndrome
cDNA	complementary deoxyribonucleic acid
CHO cell	Chinese hamster ovary cell
CT	computerised tomography
D-MEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
Dol-P-glucose	dolichol phosphorylglucose
Dol-P-mannose	dolichol phosphorylmannose
DQ	developmental quotient
EDTA	ethylenediaminetetraacetic acid
EEG	electroencephalogram
ER	endoplasmic reticulum
FBS	fetal bovine serum
FSH	follicle stimulating hormone
Gal	galactose
GALT	galactose-1-phosphate uridyltransferase
GDP-mannose	guanosine diphosphate mannose
GH	growth hormone
Glc	glucose
GlcNAc	N-acetylglucosamine
GlcNAc-PP-Dol	dolichol pyrophosphoryl-N-acetylglucosamine
GPI anchor	glycosylphosphatidylinositol anchor
HPLC	high performance liquid chromatography
IQ	intelligence quotient
LAMP-1	lysosome-associated membrane protein-1
LH	luteinising hormone
Man	mannose
MRI	magnetic resonance imaging
NAD	nicotinamide adenine dinucleotide
Neu	sialic acid
NMR spectroscopy	nuclear magnetic resonance spectroscopy
PBS	phosphate-buffered saline
PNGase F	peptide-N-4(N-acetyl-β-glucosaminyl)asparagine amidase F
RBC	red blood cell
RER	rough endoplasmic reticulum
RNase B	ribonuclease B
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEP	somatosensory evoked potentials
TBG	thyroxine-binding globulin
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
TSH	thyroid stimulating hormone
UDP-galactose	uridine diphosphate galactose
UDP-glucose	uridine diphosphate glucose
UDP-N-acetylglucosamine	uridine diphosphate N-acetylglucosamine
UTP	uridine 5'-triphosphate

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General introduction

Classical galactosaemia is an inherited disorder of galactose metabolism in humans that results from a deficiency of the enzyme galactose-1-phosphate uridyltransferase (GALT). As a consequence, children born with this disorder are unable to convert dietary galactose into glucose. The children usually present in the first weeks of life, following the introduction of milk, with an acute toxicity syndrome due to the accumulation of galactose intermediates, such as galactose-1-phosphate. The presentation is characterised by vomiting, failure to thrive, jaundice, hepatitis, hepatosplenomegaly, cataracts, and a significant mortality from bacterial sepsis. If undiagnosed, the disease can cause significant mental retardation and chronic liver disease. These problems are avoided by removing galactose from the diet and this treatment has formed the basis of management of galactosaemia[1] for over 60 years. However, recent retrospective reviews of the outcome for children and adults on treatment have revealed other significant longer term problems. As a group, the patients exhibit delayed and impaired intellectual development, specific learning disabilities, poor motor function, and growth delay. Several adult patients have also developed specific neurological disorders that are characterised by ataxia and tremor, and females with galactosaemia are invariably infertile because of premature ovarian failure. These long-term complications develop independent of either the severity of the initial presentation, the age at which the diet was started, or the strictness with which the dietary treatment was followed.

If the long-term complications are not related to the acute toxicity of galactosaemia, then other explanations must be sought in alterations of the cellular environment that occur secondary to transferase deficiency. One of the products of the GALT reaction is uridine diphosphate galactose (UDP-galactose), which is a donor of galactose during glycoprotein and glycolipid synthesis (Figure 1.1). A disturbance in UDP-galactose metabolism could affect glycoprotein and glycolipid synthesis and thereby contribute to the long-term complications in galactosaemia. The concentration of UDP-galactose in galactosaemic cells has therefore been of considerable interest and the current evidence indicates that there is a disturbance in the equilibrium of UDP-galactose and uridine diphosphate glucose (UDP-glucose). However, it is not known if this disturbance is enough to affect glycoprotein and glycolipid synthesis. Some evidence in the literature tentatively suggests that there are abnormalities of glycoprotein and glycolipid in galactosaemic cells and tissues. However, what form these abnormalities take and whether they are secondary to a deficiency of UDP-galactose, or some other cellular disturbance, has not been determined. The serum in untreated galactosaemia contains hyposialylated isoforms of several glycoproteins, in particular transferrin. This suggests a possible disturbance of N-glycosylation in galactosaemia, which could involve incomplete terminal galactosylation of N-linked oligosaccharides. This would be consistent with a deficiency of UDP-galactose, but there could be other explanations.

The aim of this thesis was to examine N-glycosylation in galactosaemia and to determine whether there were specific abnormalities in the synthesis of N-linked oligosaccharides. It is difficult to obtain serum from neonates with galactosaemia before they are treated. N-glycosylation was therefore examined in skin fibroblast cultures from four patients with galactosaemia. The experimental plan was to radiolabel the fibroblasts with [2-^{3}H]-mannose

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[1] The term *galactosaemia* refers to classical or transferase-deficiency galactosaemia, unless otherwise indicated.

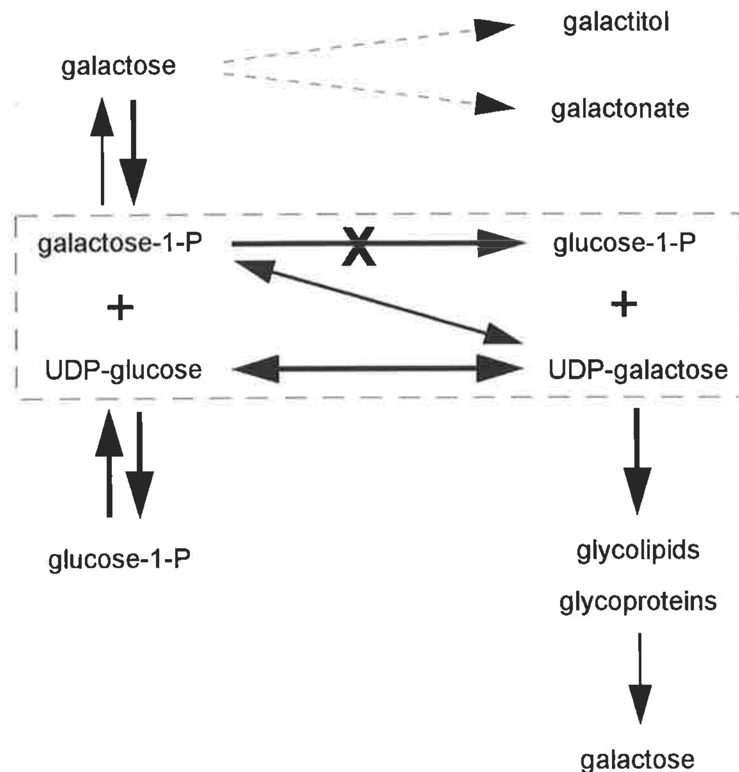

Figure 1.1. Galactose metabolic pathways. The box encloses the intermediates of the GALT reaction. The cross indicates the site of the metabolic block in classical galactosaemia (adapted from Segal, 1993).

and [^{35}S]-methionine and to recover the glycoproteins for analysis. [2-^{3}H]-Mannose has been widely used as a marker of N-glycosylation as it is incorporated specifically into the core mannose structure of N-linked oligosaccharides, whereas [^{35}S]-methionine labels the protein backbone. With these techniques it was possible to monitor glycoprotein synthesis in galactosaemic fibroblasts under various culture conditions. The fibroblasts were also exposed to media that contained galactose to replicate the toxic effects of galactose on galactosaemic tissues. The experimental work concentrated solely on N-glycosylation and did not attempt to examine either O-glycosylation or glycolipid synthesis.

The thesis is divided into nine chapters. Chapter 2 begins by introducing the disorders of galactose metabolism in humans and summarises their clinical and pathological features, diagnosis, and treatment. It also presents the clinical evidence for the long-term complications in classical galactosaemia and discusses each major complication in detail. The chapter then reviews the cellular metabolic disturbances that are associated with GALT deficiency and discusses how each might contribute to the long-term complications. The chapter concludes by examining the experimental evidence that suggests that glycoprotein synthesis in galactosaemia is disturbed, and then presents a hypothesis that implicates abnormal N-glycosylation in the long-term complications.

Chapter 3 introduces the terminology associated with normal N-glycosylation and highlights the steps in the N-linked oligosaccharide synthetic pathway. The chapter then reviews the human disorders of N-glycosylation, both primary and secondary, and examines the range of clinical and biochemical abnormalities that are associated with disturbed N-glycosylation. The carbohydrate-deficient glycoprotein (CDG) syndrome, which has many clinical and biochemical

features in common with galactosaemia, is particularly emphasised. The chapter then concludes with a review of the characteristics of transferase-deficient skin fibroblasts in culture, with particular reference to their use as a cellular model of N-glycosylation in galactosaemia.

Chapter 4 lists the materials and methods that were used in the experimental plans of Chapters 5, 6, 7, and 8. The general cell culture, radiochemical, and chromatographic techniques, which were common to all experimental plans, are presented first. They are then followed by six specific methods. Each of the specific methods, which relate to particular experimental plans, are presented in detail along with the reasons for choosing that method. Chapter 4 was designed as a reference section. The experimental plans in Chapters 5, 6, 7, and 8 do provide an overview of the particular experimental approaches used in those chapters. However, there are references, in the text, to specific methods in Chapter 4 for more detail as required.

Each of the experimental chapters begins by stating the aim of that particular chapter and then provides an outline of the experimental plan. Chapter 5, the first of the experimental chapters, examines the structure of N-linked oligosaccharides from galactosaemic fibroblasts. The aim of the chapter was to determine if the mature complex N-linked oligosaccharides were structurally complete. The fibroblast cultures were radiolabelled with [2-^{3}H]-mannose and the N-linked oligosaccharides were recovered from both the cellular and secreted proteins. The oligosaccharides were then analysed by size and charge with Bio-Gel P-4 size-exclusion chromatography and anion-exchange high performance liquid chromatography (HPLC). The methods were specifically designed to examine terminal galactosylation and sialylation of the N-glycans. The experiments were also performed with the galactosaemic fibroblasts at different stages of growth, and in various culture media, to determine what factors might influence the structure of the N-linked oligosaccharides.

In Chapter 6, there is a general examination of N-glycosylation in galactosaemic fibroblasts. The aim of the chapter was to determine the effect of galactose on total glycoprotein synthesis. The fibroblast cultures were exposed to media that contained increasing concentrations of galactose and were then radiolabelled, for six hours, with both [2-^{3}H]-mannose and [^{35}S]-methionine. The radioactivity was then determined in protein pellets that were precipitated with trichloroacetic acid (TCA). Both the cellular and secretory proteins were examined.

Chapter 7 examines the initial steps of N-glycosylation; the synthesis of dolichol-linked oligosaccharides and their transfer to protein in the lumen of the endoplasmic reticulum (ER). The aim of this chapter was to determine the effect of galactose on the initiation of N-linked oligosaccharide synthesis. The galactosaemic fibroblasts were preincubated in medium that contained galactose and were then radiolabelled with [2-^{3}H]-mannose for 30 minutes. The dolichol- and protein-linked oligosaccharides were then recovered and analysed by Bio-Gel P-4 size-exclusion chromatography.

The final experimental chapter, Chapter 8, examines the effect of galactose on the synthesis and processing of a specific glycoprotein in galactosaemic fibroblasts. Lysosome-associated membrane protein-1 (LAMP-1) is a membrane glycoprotein that is localised to the lysosomal membrane. LAMP-1 is heavily glycosylated with 18 N-linked glycans. The galactosaemic fibroblasts were preincubated in medium that contained galactose and were then radiolabelled with [^{35}S]-methionine. The LAMP-1 was then recovered by immunoprecipitation and analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The experiments used various chase times to monitor the processing of LAMP-1 in the ER and Golgi apparatus.

Each of the experimental chapters contains a discussion of the results obtained from that particular experimental plan. However, Chapter 9 provides a summary of the conclusions regarding N-glycosylation in transferase-deficient fibroblasts. The chapter then re-examines the evidence in the literature that suggests there is abnormal glycoprotein synthesis in galactosaemia, and reinterprets that evidence in light of the experimental results from this thesis. The chapter concludes with a discussion of how disturbances of N-glycosylation might contribute to the development of the long-term complications in classical galactosaemia.

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Background

2.1 Introduction

This chapter introduces the disorders of galactose metabolism in humans and summarises their clinical and pathological features, diagnosis, and treatment. It also presents the clinical evidence for the long-term complications in classical or transferase-deficiency galactosaemia and examines each major complication in detail. It then reviews the cellular metabolic disturbances that are associated with GALT deficiency and discusses how each might contribute to the long-term complications. The chapter concludes with a review of the experimental evidence that suggests there are disturbances of glycoprotein synthesis in galactosaemia, and then presents a hypothesis that implicates abnormal N-glycosylation in the long-term complications.

2.2 Galactose metabolism

The main source of galactose in the diet is from lactose in mammalian milk. Lactose is a disaccharide that is hydrolysed in the intestine by the microvillar enzyme lactase to produce glucose and galactose. Both monosaccharides are then readily absorbed into the portal venous system and transported to the liver where galactose is rapidly metabolised (Williams and Macdonald, 1982). The biochemical pathway that converts galactose to glucose is historically known as the Leloir pathway (Caputto *et al.*, 1948; Caputto *et al.*, 1950; Leloir, 1951; Kalckar *et al.*, 1953; and Munch-Petersen *et al.*, 1953). This pathway, which involves an epimerisation of the hydroxyl group of carbon 4, is outlined in Figure 2.1.

In the first step of the pathway, galactose is phosphorylated with adenosine 5'-triphosphate (ATP) by galactokinase (EC 2.7.1.6) to produce galactose-1-phosphate. Galactose-1-phosphate uridyltransferase (EC 2.7.7.12) then converts galactose-1-phosphate to UDP-galactose, while at the same time UDP-glucose is converted to glucose-1-phosphate. The final enzymatic step involves the conversion of UDP-galactose to UDP-glucose by UDP-galactose 4-epimerase (EC 5.1.3.2). This last step is reversible and uses nicotinamide adenine dinucleotide (NAD) as cofactor (Maxwell, 1957). The Leloir pathway therefore enables galactose to enter the glucose metabolic pathways. The UDP-glucose that is produced can serve as a substrate in the GALT reaction or in glycogen synthesis. The other intermediate in the pathway, UDP-galactose, is a donor of galactose during glycoprotein and glycolipid synthesis, particularly to the galactolipids of brain and nerve tissue (O'Brien and Sampson, 1965). Galactose, however, is not an essential component of food in humans as UDP-glucose pyrophosphorylase (EC 2.7.7.9) can synthesise UDP-glucose from glucose-1-phosphate and uridine 5'-triphosphate (UTP). When galactose is absent from the diet, the epimerase enzyme maintains an equilibrium between UDP-glucose and UDP-galactose of approximately 3 to 1, therefore ensuring that UDP-galactose is available for glycoprotein and glycolipid synthesis (Tsai *et al.*, 1970; and Gibson *et al.*, 1995a).

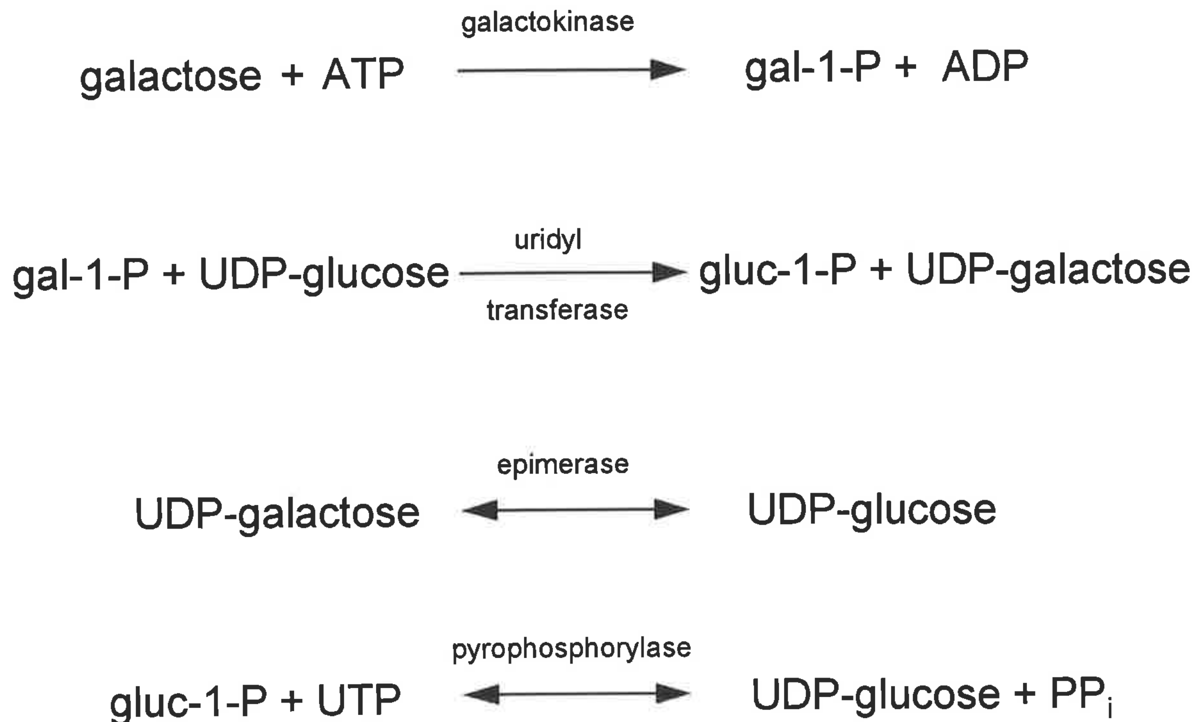


Figure 2.1. The metabolism of galactose by the Leloir pathway. The three enzymes of the pathway are galactokinase, galactose-1-phosphate uridyltransferase, and UDP-galactose 4-epimerase. An additional enzyme, UDP-glucose pyrophosphorylase, can synthesise UDP-glucose by an alternative mechanism. *Gal-1-P*, galactose-1-phosphate; *gluc-1-P*, glucose-1-phosphate; *UDP-glucose*, uridine diphosphate glucose; *UDP-galactose*, uridine diphosphate galactose; *UTP*, uridine 5'-triphosphate; and PP_i, pyrophosphate.

2.3 Disorders of galactose metabolism

There are three inherited disorders of galactose metabolism in humans. They are all autosomal recessive conditions and they result from either a deficiency of galactokinase, GALT, or UDP-galactose 4-epimerase, respectively. A deficiency of any of these enzymes disrupts the conversion of galactose to glucose and results in increased concentrations of galactose in the blood or galactosaemia. The clinical disorders that are associated with deficiencies of these enzymes are classified as galactokinase-deficiency, transferase-deficiency, and epimerase-deficiency galactosaemia.

Galactokinase-deficiency galactosaemia (MIM 230200)

Galactokinase deficiency is characterised clinically by cataracts that can appear in the first weeks of life. The disorder was first identified in a 44-year-old man with cataracts (Gitzelmann, 1965) whose 'galactose diabetes' had previously been noticed by Fanconi, (1933). Subsequently, two older siblings in the family were also found to have cataracts (Gitzelmann, 1967). Since then, several dozen patients with galactokinase deficiency have been described (for a review see Olambiwonnu *et al.*, 1974; and Segal and Berry, 1995). The patients excrete large amounts of galactose and galactitol in their urine and their ability to

metabolise [1-^{14}C]-galactose to $^{14}CO_2$ is significantly reduced (Gitzelmann *et al.*, 1974). They are usually identified either during investigation for cataract, from incidental galactosuria, or occasionally by neonatal programmes that are screening for transferase-deficiency galactosaemia (Thalhammer *et al.*, 1968; and Dahlqvist *et al.*, 1970). The main clinical finding is bilateral cataracts. Jaundice, liver disease, renal disease, or mental retardation are usually not features of this disorder, although two brothers have been reported with cataracts and mental retardation (Segal *et al.*, 1979). The cataracts are thought to be caused by the accumulation of galactitol in the lens, which damages the lens because of the osmotic effect (Kinoshita, 1965; and Gitzelmann *et al.*, 1967a). The incidence of galactokinase deficiency is about 1 in 40 000, however, the heterozygote frequency does differ between populations (Mayes and Guthrie, 1968; and Magnani *et al.*, 1982). The disorder is diagnosed by demonstrating absent galactokinase activity in erythrocytes and treated by removing galactose from the diet. This can resolve the cataracts if the diet is started early.

Epimerase-deficiency galactosaemia (MIM 230350)

UDP-galactose 4-epimerase deficiency occurs either as a partial defect or as a more severe generalised enzyme deficiency. The partial defect is benign and confined to a deficiency of the enzyme in erythrocytes only. The partial defect was first identified in a healthy infant who was detected incidentally by a neonatal screening programme (Gitzelmann, 1972). Gitzelmann later reported an additional eight cases from three families (Gitzelmann *et al.*, 1976). Three Japanese families with the partial defect have also been described (Oyanagi *et al.*, 1981). The children are able to metabolise galactose normally and the only metabolic consequence of ingesting galactose is an increase of erythrocyte galactose-1-phosphate, without any red cell abnormality. The children otherwise have normal growth and development. The epimerase activity is normal in liver, cultured skin fibroblasts, and activated lymphocytes. Most cases of this form of epimerase deficiency have been incidental findings of neonatal screening programmes that measure erythrocyte galactose-1-phosphate (Bowling and Brown, 1986). A Japanese screening programme estimated that their incidence of partial epimerase deficiency was 1 in 23 000 (Misumi *et al.*, 1981). The same programme later identified seven infants with epimerase deficiency from 265,019 neonates screened over 10 years (Inoue *et al.*, 1990).

In contrast, the severe form of epimerase deficiency represents a loss of enzyme activity from all tissues. Only two patients with this disorder have been described in detail. Holton *et al.*, (1981) identified a baby with a severe form of galactosaemia due to epimerase deficiency. The female proband was the offspring of Pakistani half-first-cousins and presented in the first week of life with vomiting, failure to thrive, jaundice, hepatomegaly, and hypotonia. There was a generalised aminoaciduria and galactosuria. The patient responded well to a galactose-restricted diet. However, at 19 months of age, the child's motor and intellectual development were delayed (Henderson *et al.*, 1983). Very small amounts of galactose in the diet caused large increases in the erythrocyte UDP-galactose, whereas the concentration of galactose-1-phosphate increased proportionately to the galactose intake. At seven years of age, the patient was physically well but had severe sensorineural deafness, little expressive speech, and an intelligence quotient (IQ) for the deaf of 85 (Segal and Berry, 1995).

The second patient was identified by a neonatal screening programme because of aminoacidaemia (Sardharwalla *et al.*, 1988). She was born to first-cousin parents and presented in the first week of life with poor feeding, vomiting, mild jaundice, and hepatomegaly. Investigations revealed liver dysfunction, galactosuria, and aminoaciduria. The erythrocyte galactose-1-phosphate and UDP-galactose concentrations were increased. A galactose-restricted diet was started at three weeks of age and the child responded quickly. The hepatomegaly regressed, liver functions returned to normal, and the cataracts, which were

present on slit-lamp examination, resolved. Despite satisfactory biochemical control and early treatment, clinical assessment at age 2 years 9 months showed that the child was severely mentally retarded with an IQ of 55, and had profound sensorineural deafness.

Two additional cases of generalised epimerase deficiency have been briefly reported (Besley *et al.*, 1995). The patients were a sibling and a cousin of the patient reported by Sardharwalla *et al.*, (1988). No clinical details were provided, however, both patients had markedly increased concentrations of erythrocyte galactose-1-phosphate, which decreased on galactose-restricted diets. Abnormal epimerase activity has also been reported in two other children who were only mildly or transiently symptomatic (Schulpis *et al.*, 1993; and Boleda *et al.*, 1995).

The clinical presentation in generalised epimerase deficiency is very similar to that of transferase-deficiency galactosaemia. However, sensorineural deafness is unique to epimerase-deficiency and none of the females with epimerase deficiency have yet developed ovarian failure (Holton, 1990). The patients with epimerase deficiency are dependent on some dietary galactose for the synthesis of UDP-galactose, which is required for glycoprotein and glycolipid synthesis. Treatment has therefore involved a galactose-restricted rather than a galactose-free diet. The epimerase enzyme catalyses two distinct reactions; the epimerisation of UDP-glucose to UDP-galactose, and of uridine diphosphate N-acetylglucosamine (UDP-N-acetylglucosamine) to UDP-N-acetylgalactosamine. This has been confirmed by purification of a bifunctional enzyme (Piller *et al.*, 1983) and by the loss of both enzyme activities in an epimerase-deficient mutant Chinese hamster ovary (CHO) cell line (Kingsley *et al.*, 1986a) and in a patient with generalised epimerase deficiency (Kingsley *et al.*, 1986c). When the mutant CHO cell line was grown in a glucose-based medium, it exhibited generalised defects in the synthesis of glycolipid and N- and O-linked carbohydrate chains of glycoprotein (Kingsley *et al.*, 1986b). It was therefore suggested that the dietary treatment of epimerase-deficiency galactosaemia should include small supplements of galactose and N-acetylgalactosamine.

Transferase-deficiency galactosaemia (MIM 230400)

This disorder of galactose metabolism is called either transferase-deficiency or classical galactosaemia and it results from a deficiency of GALT. The incidence of transferase-deficiency galactosaemia in Australia is 1 in 46 550 (Francis, 1991).

Early clinical descriptions

The first recognisable clinical description of classical galactosaemia appeared in 1908 when von Reuss reported an eight-month-old infant with mellituria who had hepatomegaly and severe failure to thrive (von Reuss, 1908). The mellituria disappeared when milk was removed from the diet. The infant died before further studies were possible and the post-mortem revealed cirrhosis of the liver. A metabolic basis for the disorder was not recognised until Goppert, (1917) identified a 29-month-old boy with failure to thrive, jaundice, hepatosplenomegaly, and mellituria and albuminuria. The boy was also severely mentally and physically delayed. Two siblings had died in early infancy from liver disease. The reducing substance in the boy's urine was identified as galactose and the extent of galactosuria was influenced by the amount of milk he was fed. He improved clinically once a milk-free diet was begun. He tolerated sucrose, maltose, glucose, and fructose but there was galactosuria after either oral lactose or galactose. Goppert, (1917) considered the boy was suffering from a familial liver disorder and that lactose needed to be replaced by another sugar in the diet.

Mason and Turner, (1935) then provided the first detailed clinical report of a patient with classical galactosaemia to appear in the English language. They described a black male infant who presented at 6 months of age with failure to thrive, poor feeding, lethargy, hepatosplenomegaly, moderate anaemia, intermittent mellituria, and albuminuria. The sugar in the urine was identified as galactose. When milk was replaced by a soybean preparation, which was free of lactose and galactose, the mellituria and albuminuria resolved. The patient also began to grow and the size of his liver and spleen decreased significantly. When he was challenged with an oral galactose load, there was considerable delay in removing galactose from the blood and this was associated with hypoglycaemia. Follow-up studies over several years continued to demonstrate galactose intolerance. However, his physical and mental development were normal on the diet. Mason and Turner, (1935) proposed that the primary defect was a reduced ability of the liver to convert galactose into glucose or glycogen.

In the following 20 years, more than forty cases were carefully detailed in the literature (for a review see Goldbloom and Brickman, 1946; Townsend *et al.*, 1951; Ritter and Cannon, 1955; and Komrower *et al.*, 1956). In 1956, the primary defect was finally identified as a deficiency of GALT in liver and erythrocytes (Isselbacher *et al.*, 1956; Kalckar *et al.*, 1956a; and Kalckar *et al.*, 1956b).

Acute clinical manifestations

The major symptoms of GALT deficiency appear in early infancy following the introduction of milk (for a review see Donnell *et al.*, 1960; Hsia and Walker, 1961; and Nadler *et al.*, 1969). Infants usually present with an acute toxicity syndrome that is characterised by failure to thrive, vomiting, diarrhoea, dehydration, jaundice, hepatomegaly, and lenticular cataracts. Additional features may include splenomegaly, ascites, growth retardation, lethargy, and hypotonia, and sometimes convulsions occur in association with hypoglycaemia. There is an increased mortality due to bacterial sepsis, particularly *Escherichia coli* (Kelly, 1971; Levy *et al.*, 1977; and Barr, 1992). The presentation may also be complicated by raised intracranial pressure and cerebral oedema (Huttenlocher *et al.*, 1970; Vogel *et al.*, 1976; Belman *et al.*, 1986; Welch and Milligan, 1987; and Perelmuter *et al.*, 1989), and very rarely, by serious lesions of the vitreous and retina (Buist *et al.*, 1995), and subcutaneous bleeding. If undiagnosed, the disease usually progresses to significant mental retardation and cirrhosis of the liver. Occasionally, infants may not present until they are several months of age. At that stage they usually have cataracts, hepatomegaly, and mental retardation. The infants that are identified by neonatal programmes that screen for galactosaemia, may have a milder clinical presentation.

Laboratory and pathological findings

At presentation, the laboratory findings include increased blood, tissue, and urine galactose, and increased intracellular galactose-1-phosphate (for a review see Komrower *et al.*, 1956; and Donnell *et al.*, 1960). In addition to the hypergalactosaemia there may be mild hypoglycaemia, especially following acute galactose loads. There is also evidence of renal damage with a generalised aminoaciduria and albuminuria (Holzel *et al.*, 1952; Hsia *et al.*, 1954; and Cusworth *et al.*, 1955), and a hyperchloraemic metabolic acidosis that may be secondary to the renal or gastrointestinal disturbances. The hyperbilirubinaemia is predominantly unconjugated (Levy *et al.*, 1975) and is usually associated with abnormal liver functions and increased activities of liver enzymes in the serum. In severe cases, the prothrombin time may be prolonged and the serum albumin decreased. Occasionally, there is also a haemolytic anaemia with a secondary reticulocytosis (Hsia and Walker, 1961; and Nadler *et al.*, 1969).

The histology of the liver shows an active process of cell damage and repair. The hepatocytes are also swollen and distorted with fat and glycogen, which are deposited to various degrees. As the disease progresses there is bile stasis, bile duct proliferation, pseudoacinar formation, and mild to moderate fibrosis. If left untreated, the disease may progress to cirrhosis (Donnell *et al.*, 1960; Smetana and Olen, 1962; Medline and Medline, 1972; Suzuki *et al.*, 1966; and Applebaum and Thaler, 1975).

Diagnosis

The diagnosis of classical galactosaemia is confirmed by finding absent GALT activity in erythrocytes and supported by the increased concentrations of either erythrocyte galactose-1-phosphate or blood and urine galactose. Various assays of erythrocyte GALT have been developed. The methods usually estimate either the UDP-glucose consumed or the UDP-galactose (or glucose-1-phosphate) produced in the reaction (for a review see Hsia, 1967). Earlier assays measured the *in vitro* or *in vivo* conversion of [^{14}C]-galactose to $^{14}CO_2$. However, these assays measured the activity of all the enzymes involved in galactose metabolism. Assays that are specific for GALT have also been applied to galactosaemic leucocytes (Mellman *et al.*, 1965; and Inouye *et al.*, 1968), liver (Anderson *et al.*, 1957; and Segal *et al.*, 1971), skin fibroblasts (Russell and DeMars, 1967), intestinal mucosa (Rogers *et al.*, 1970), and skeletal muscle (Bresolin *et al.*, 1993). Prenatal diagnosis is possible by measuring GALT activity in cultured amniocytes (Nadler, 1968; Fensom *et al.*, 1974; Fensom and Benson, 1975; and Monk and Holton, 1976) or biopsies of chorionic villi (Fensom *et al.*, 1979; and Rolland *et al.*, 1986). Prenatal diagnosis has also been attempted by fetal blood sampling (Fensom *et al.*, 1979). In those countries with neonatal programmes that screen for galactosaemia, the galactosaemic infants are identified relatively early and theoretically before the onset of life-threatening complications. Most of the screening programmes measure either galactose, galactose-1-phosphate, or GALT in dried blood-spots taken from neonates in the first week of life (for a recent review see Schweitzer, 1995). However, all the at-risk infants identified by these programmes still require definitive measurement of erythrocyte GALT.

Treatment

The treatment for the last 60 years has involved the removal of lactose and galactose from the diet. For infants, this has readily been achieved by excluding human or cow's milk and replacing it with soybean- or casein hydrolysate-based formulae. For the acutely unwell infant, removing galactose from the diet resolves the vomiting, jaundice, galactosaemia, galactosuria, albuminuria, and aminoaciduria within several days. The abnormal liver functions and hepatomegaly gradually resolve, and there is improved growth. The cataracts can regress and if small they will occasionally completely resolve.

The older galactosaemic children also exclude major galactose-containing foods from their diet but their treatment has required a greater understanding of potential sources of galactose. Milk, and products made with milk, still constitute the major sources of lactose and galactose. However, lactose is also widely used as an extender in many drug preparations (Kumar *et al.*, 1991) and is added to some processed foods. Cereals, fruits, legumes, nuts, seeds, tubers, and vegetables also contain galactose in various amounts (Acosta and Gross, 1995). Many fruit and vegetables contain galactose in a soluble or free form up to as much as 35.4 mg/100 g of fresh weight (Gross and Acosta, 1991). Papaya, date, bell pepper, tomato, and watermelon each contain over 10 mg of soluble galactose/100 g fresh weight. However, when Berry *et al.*, (1993) increased the galactose-rich fruit and vegetables in the diets of two patients with

transferase deficiency, they were unable to detect a significant effect on the concentration of erythrocyte galactose-1-phosphate or urinary galactitol.

Animals and plants, especially plant cell walls, also contain galactose that is bound in various glycosidic linkages (α1,6, β1,3, and β1,4) and as components of lipids (for a review see Acosta and Gross, 1995). The bioavailability of this bound galactose is not known. The affinity of human or intestinal bacterial galactosidases for the many galactose-containing polysaccharides in the cell walls of fruit and vegetables is also not known. It is therefore not possible to determine the potential absorption of this galactose from the gut. Several studies observed little effect from two common plant galactose-containing oligosaccharides, raffinose and stachyose, on the concentration of erythrocyte galactose-1-phosphate in a limited number of patients (Koch *et al.*, 1963; Gitzelmann and Auricchio, 1965; and Wiesmann *et al.*, 1995). It is certain that no patient with galactosaemia has ever been treated with a truly galactose-free diet. It is still recommended, however, that the diet is continued for life (Brandt, 1980).

Treatment monitoring

Schwarz *et al.*, (1956) originally noted that galactosaemic erythrocytes accumulated galactose-1-phosphate. This suggested that the enzyme defect was in the subsequent metabolism of galactose-1-phosphate and ultimately lead to the identification of the GALT deficiency. Two groups then suggested that galactose-1-phosphate could be used as a monitor of the adequacy of diet and to detect lapses in diet (Schwarz, 1960; and Donnell *et al.*, 1963). Since then, measuring the concentration of galactose-1-phosphate in erythrocytes has been the mainstay of monitoring in galactosaemia. Galactose-1-phosphate also accumulates in liver, kidney, and brain (Schwarz, 1960; and Tada, 1962), and lens (Gitzelmann *et al.*, 1967a). In untreated galactosaemia, the concentration of galactose-1-phosphate may be as high as 6.5 mmol/L red blood cells (RBC) (Gitzelmann, 1995). Once diet is started, the galactose-1-phosphate takes several months to reach a base line (Kaufman *et al.*, 1986), but even at that stage it remains above normal (0.1–0.2 mmol/L) (Donnell *et al.*, 1963; and Gitzelmann, 1995). In normal fasting children, galactose-1-phosphate is either undetectable or very low in erythrocytes (Donnell *et al.*, 1963; and Pesce and Bodourian, 1982). It is not known if the persistently increased galactose-1-phosphate in treated galactosaemia is due to either free and bound galactose in the diet, or endogenously produced galactose. The least amount of dietary galactose that will cause an increase in the erythrocyte galactose-1-phosphate concentration is also not known. Despite these problems, no significant alternative has been found for the monitoring of patients on treatment.

Clinical variants

The 'Negro' variant

In 1962, Segal *et al.*, developed an *in vivo* assay of the oxidation of intravenously administered [^{14}C]-galactose to $^{14}CO_2$ in expired air. They used the assay to study galactose oxidation in eight patients with galactosaemia. The patients were all mentally retarded and had cataracts. Each of the patients had no detectable GALT activity in their erythrocytes and the haemolysates were unable to oxidise [^{14}C]-galactose to $^{14}CO_2$. However, they found that two of the patients were able to oxidise intravenously administered [^{14}C]-galactose at a near-normal rate. The study was later extended to show the phenomenon in three of 12 galactosaemic patients (Segal *et al.*, 1965). The three patients were American Blacks who had presented during infancy with failure to thrive, liver disease, and cataracts. Liver biopsy specimens from

two of the patients were capable of metabolising galactose (Topper *et al.*, 1962; and Baker *et al.*, 1966). It has since been confirmed that intestinal mucosa (Rogers *et al.*, 1970) and liver (Segal *et al.*, 1971) in black galactosaemics have GALT activity about 10% of normal, despite the complete absence in erythrocytes. The residual hepatic enzyme presumably metabolises galactose but why these patients should develop acute symptoms as newborns is not known. A prevalent mis-sense mutation of the GALT gene, which results in a serine to leucine substitution at codon 135 (S135L), has recently been identified among black galactosaemics and proposed as a cause of this clinical variant of galactosaemia (Lai *et al.*, 1996).

The Duarte variant

In 1965, Beutler *et al.*, studied the activity of GALT in erythrocytes from normal adults to define the heterozygote frequency for classical galactosaemia. During family studies of those identified with 50% GALT activity, they incidentally discovered a common GALT variant that also results in reduced enzyme activity. They called it the Duarte variant. Approximately 10% of the normal population carry the Duarte allele, which is thought to be benign (Beutler *et al.*, 1966; and Mulley, 1982). Subjects who are heterozygous for the Duarte allele have approximately 75% of normal enzyme activity. Duarte homozygotes and galactosaemic heterozygotes have 50% and galactosaemic/Duarte compound heterozygotes approximately 25% of normal activity. On gel electrophoresis, the Duarte enzyme from erythrocytes appears as three bands that migrate faster than the normal enzyme band (Ng *et al.*, 1969; and Sparkes *et al.*, 1977).

The galactosaemic/Duarte compound heterozygotes have generated considerable interest as they are the most frequent cause of an abnormal result during screening of neonates for galactosaemia (Levy *et al.*, 1978; and Schwarz *et al.*, 1982). Adult compound heterozygotes, who were detected during family studies, were thought to be healthy (Beutler *et al.*, 1966; Gitzelmann *et al.*, 1967b; Levy *et al.*, 1978; and Kelly, 1979). However, there has been dispute whether the compound state is entirely benign during infancy (Levy *et al.*, 1978; Kelly, 1979; Schwarz *et al.*, 1982; and Ng *et al.*, 1987). Although most infants are asymptomatic, several presented with lethargy, poor feeding, and jaundice and they responded to a galactose-restricted diet (Kelly *et al.*, 1972; and Kelly, 1979). The level of erythrocyte galactose-1-phosphate in the infants at presentation usually correlates with the residual GALT activity and the extent of milk exposure (Gitzelmann and Bosshard, 1995). The infants also show higher than normal blood galactose and galactose-1-phosphate following oral galactose loads (Schwarz *et al.*, 1982) and they excrete increased galactose and galactitol in their urine (Schwarz *et al.*, 1985). Some centres have therefore recommended that these infants are treated with a galactose-restricted diet for between four months and two years. Gitzelmann and Bosshard, (1995) treat about 60% of their galactosaemic/Duarte compound heterozygotes with a low-lactose formula for four months, and they monitor the child's biochemical response to galactose. However, the final outcome for these patients is still unclear. Recent and conflicting studies have attempted to correlate premature ovarian failure, infertility, and ovarian cancer (Cramer *et al.*, 1989a; Cramer *et al.*, 1989b; Hagenfeldt *et al.*, 1989; Kaufman *et al.*, 1993; Cramer *et al.*, 1994a; Cramer *et al.*, 1994b; Cramer *et al.*, 1995; Herrinton *et al.*, 1996; and Sayle *et al.*, 1996), and presenile cataracts (Winder *et al.*, 1983; Burke *et al.*, 1988; and Endres and Shin, 1990) with carrier status for the galactosaemic and Duarte alleles.

Additional variants

Several other rare GALT variants have also been described. They differ in their enzyme activities, enzyme stabilities, and band patterns on gel electrophoresis, and they have various

clinical features. They include the Los Angeles (Ng *et al.*, 1973), Rennes (Schapira and Kaplan, 1969), Chicago (Chacko *et al.*, 1977), Indiana (Chacko *et al.*, 1971), Munster (Lang *et al.*, 1980), and Berne (Scherz *et al.*, 1976) variants.

2.4 Long-term complications in classical galactosaemia

A pattern emerges

It had long been recognised that if untreated, galactosaemia resulted in significant mental retardation. It was even recommended that mentally retarded children in institutions be screened to exclude undiagnosed galactosaemia (Bergren *et al.*, 1958). However, by 1961, Donnell *et al.*, had enough children with galactosaemia on treatment to permit a longitudinal study of their growth and development. Fifteen patients from the Children's Hospital in Los Angeles were studied. The children had been diagnosed with galactosaemia between the newborn period and 15 months of age. Their physical growth, once established on diet, was within normal limits for all but three of the children. However, intelligence testing showed that six of the children were in the low-normal to normal range, six were borderline, and three were mentally impaired. Only three of the children had an IQ over 100. The intellectual abilities of the parents and siblings of the children were normal. It was questioned whether over- or under-attention to the diet had contributed to the poor outcome. In the same year, Hsia and Walker, (1961) studied 45 children on treatment from 27 families. They found that approximately 50% of the children were more than one standard deviation below normal weight and height, which was significantly different to the unaffected siblings. Of 34 children, 20 were in the normal IQ range, seven were borderline, and seven were in the retarded but educable range. The ultimate mental development did not correlate with the age at starting diet. Hsia and Walker, (1961) were unable to draw definite conclusions regarding diet and outcome because of limited information on the success of dietary control in each case. Their overall impression was that 'once past early infancy, patients with galactosaemia generally enjoy good health'. The most unexpected finding was that almost two thirds of their patients had normal intelligence. Hsia and Walker, (1961) suggested that galactose-1-phosphate should be measured regularly in the children to monitor treatment outcomes more objectively.

In 1966, Fishler *et al.*, investigated the psychological profiles of 34 galactosaemic children who had been followed longitudinally over eight years. They found a personality profile (not apparent in children with other chronic metabolic disorders) that showed the children were excessively shy and anxious, and that they had personality difficulties, especially in early adolescence. Many of the older children had visual-perceptual limitations, regardless of intelligence, which caused specific learning disabilities. They also noted considerable variability in the ultimate intellectual ability of the children, which did not correlate with an index of dietary control. They later extended the study to include 45 patients followed longitudinally over 23 years (Fishler *et al.*, 1972) and confirmed a high incidence of visual-perceptual difficulties in the school-age group. The study also contained data from 11 sibling pairs where the younger siblings had been treated immediately from birth (Fishler *et al.*, 1972; and Fishler *et al.*, 1980). The two groups had a similar incidence of visual-perceptual problems and there was no significant difference in their mean IQ. This suggested that any delay in starting treatment had no influence on intellectual abilities, although the number of patients was small.

In 1969, Nadler *et al.*, extended the data from the Hsia and Walker (1961) study and reported the outcome in 55 patients from 39 families. They observed significant growth retardation in the children and a mean IQ that was significantly below their siblings, despite adequate

treatment over many years. They also found a poor correlation between IQ and actual achievement because many of the children had specific learning disabilities that affected their mathematical and spatial relationships. They suggested that the children needed closer supervision and additional teaching to help resolve these problems. They also found no correlation between the age of starting diet, or success of dietary control, and final intelligence.

In 1970, Komrower and Lee, reported the outcome in 60 galactosaemic children who were followed at the Royal Manchester Children's Hospital. They found that the group was physically healthy although eight had residual cataracts with impaired vision. Most of the children were below the 50th centile for height. They also found disturbed psychological profiles, which they could not conclude were either environmental or secondary to the disorder. The mean IQ in the children who were diagnosed and treated early was below the mean for the population and the IQ scores appeared to decrease with increasing age. In 1972, they reported the results of a battery of psychometric tests that they performed on the same children (Lee, 1972). Many of the children were emotionally disturbed, were particularly sensitive to criticism, and had a poor relationship with the adult world. They also had problems with coordination and fine motor tasks and showed perceptual difficulties that were probably related to their low intelligence. Many of the children had problems with their school work.

Kaufman *et al.*, (1979) then observed a new complication in treated galactosaemia. They briefly reported a high incidence of ovarian failure in galactosaemic females and proposed that galactose metabolites may be directly toxic to the ovaries. The ovarian failure was also noted by others (Hoefnagel *et al.*, 1979). Kaufman *et al.*, (1981) then evaluated the gonadal function in 18 female and 8 male patients with galactosaemia and found that 12 of the females had hypergonadotropic hypogonadism. This was manifested clinically by partial or complete failure of secondary sexual development and primary or secondary amenorrhoea. Ultrasound examination showed diminished or absent ovarian tissue. The ovarian dysfunction did not correlate with either the clinical course or the mean erythrocyte galactose-1-phosphate concentration in the patients. However, delay in diagnosis was associated with an increased risk of ovarian failure. The gonadal function in the males, which was assessed by pubertal development, serum follicle stimulating hormone (FSH), luteinising hormone (LH), and testosterone levels, was normal.

In 1982, Komrower, in the F.P. Hudson Memorial Lecture, reviewed the experience of the Los Angeles and Manchester groups in treating galactosaemia over thirty years. He concluded that the intellectual outcome for well-treated galactosaemic patients was worse than that of children with treated phenylketonuria and that there was clear evidence of visual-perceptual difficulties and problems with social adjustment. He considered that the less than favourable intellectual outcome, and the recently identified problem of female infertility, prompted the need for more extensive follow-up. This was a view echoed in an editorial in the Lancet of the same year (*Clouds over galactosaemia*; Editorial, 1982). The editorial called for both a thorough retrospective review of all known cases of galactosaemia and a multicentre prospective study to determine whether dietary and biochemical control correlated with intellectual and physical outcome. These proposals were supported by Komrower (Komrower, 1983).

Results of the collaborative studies

In 1987–88, Waggoner *et al.*, distributed a questionnaire to over 40 specialists in the United States and Europe who were treating patients with galactosaemia (Waggoner *et al.*, 1990). The questionnaire sought retrospective information on perinatal history, dietary management, biochemical parameters, and long-term outcome for their patients. The response provided data

on 350 patients aged between two weeks and 37 years, with a mean age of 9.5 years. There were equal numbers of males and females. The majority of patients in this study were from the United States and their data have also been reported elsewhere (Waggoner and Buist, 1993). One other retrospective study assessed the outcome in 134 patients who were monitored at Children's Hospitals within the Federal Republic of Germany (Schweitzer *et al.*, 1993). In this study, the authors personally examined 83 of the patients, while information on the remaining 51 came from a historical review of the case notes.

The results of the two collaborative studies were very similar. Although there was considerable variability in the outcome for individual patients, as a group, five common long-term complications emerged:

1. Impaired intellectual development with an apparent decline in IQ with age.
2. Specific learning disabilities that affect speech and language development.
3. Growth delay, more common among females than males.
4. Disturbed coordination, balance, and gait, with fine motor tremors and ataxia.
5. Ovarian failure, represented by hypergonadotropic hypogonadism.

There were two additional important findings from these studies. First, there was no correlation between any of the complications and either the severity of neonatal illness, the age at which diet was started, the strictness of dietary control, or the mean erythrocyte galactose-1-phosphate concentration on treatment. Even removing milk from the mother's diet during an affected pregnancy had no influence on the final outcome. There was, however, a greater incidence of developmental delay in those children who were not treated until after two months of age. This suggested a critical time beyond which damage to cerebral development was irreversible. And second, Waggoner *et al.*, (1990) included data on the outcome in 27 sibling pairs. The older siblings were identified because of symptoms or a positive screening result, whereas the younger siblings were treated within two days of birth. In this analysis, there was no significant difference in IQ scores between the siblings at various ages and the prevalence of speech and ovarian dysfunction was the same in both groups. This again suggested the acute neonatal illness did not contribute to the long-term complications.

The five common problems

1. Impaired intellectual development with an apparent decline in IQ with age

In Waggoner *et al.*, (1990) 45% of the patients aged 6 years or older were described as developmentally delayed. The mean IQ in those patients who were tested in successive age ranges (3–5 years, 6–9 years, and 10–16 years) also showed a significant decline with age. Schweitzer *et al.*, (1993) reported that 45% of their whole group had either a developmental quotient (DQ) or an IQ score less than 85, and they also noted a decline in score with age. The decline was similar when verbal and performance IQ were evaluated separately. Unfortunately many different DQ and IQ tests were applied to the children from different centres and this has complicated the analysis of the data. When Waggoner *et al.*, (1990) examined the scores from individual patients who had been tested at intervals with the same IQ test, there was no consistent decline in intelligence. It has recently been suggested that the apparent decline may be an artefact caused by increased educational opportunity or higher socio-economic status in the cohort of youngest patients (Kaufman *et al.*, 1995b). However, whether intelligence declines with age or not, clearly a significant proportion of patients with galactosaemia are intellectually delayed.

2. Specific learning disabilities that affect speech and language development

Waggoner *et al.*, (1990) reported problems with speech in 56% of the patients who were at least three years old. The majority of problems involved delayed vocabulary (92%) and articulation defects (90%), which were predominantly disordered articulation or verbal dyspraxia. The patients with speech problems had significantly lower DQ and IQ scores, at all ages, than those with normal speech. Schweitzer *et al.*, (1993) found 65% of the patients they examined over three years of age had speech abnormalities and many had required speech therapy. Speech problems were again more common in the children with the lowest IQ scores. They also observed problems with visual perception and calculation in 44% of the children older than three years. Waisbren *et al.*, (1983) also observed articulation problems in children with galactosaemia and they reported additional deficits in expressive language, particularly with immediate recall and word-retrieval skills. Nelson *et al.*, (1991) proposed that there was an association between galactosaemia and the specific articulation disorder, verbal dyspraxia. However, Kaufman *et al.*, (1995b), thought that the speech and language problems in galactosaemia resulted from a global set of cognitive impairments. Kaufman *et al.*, (1995b) administered a battery of detailed neuropsychologic tests to 45 of their own patients. They found that although most had deficits of cognitive functioning in one or more areas, the patients did not share a common pattern of strengths or weaknesses.

3. Growth delay, more common among females than males

Waggoner *et al.*, (1990) found that growth was severely delayed during childhood and early adolescence in many patients. However, it often continued through the late teens so that final adult height was normal. One-third of the females in their study were less than the 3rd centile for height at some stage between five and 16 years of age. For the males, only 12% were similarly affected. The growth disturbance was not related to early malnutrition as growth in early infancy was normal. They suggested that ovarian dysfunction may partly contribute to the problem, as the growth delay was more common in females. Schweitzer *et al.*, (1993) measured the height, weight, and head circumference of their patients. They did not find any delay in growth, however, the measurements were single rather than cumulative. Ten of 78 patients in their study were microcephalic. The microcephaly was associated with an increased incidence of neurologic disease.

4. Disturbed coordination, gait and balance, with fine motor tremors and ataxia

In Waggoner *et al.*, (1990), 18% of the patients were reported to have problems with coordination or gait. However, Waggoner and Buist, (1993) proposed that the reporting of motor problems by clinicians was probably much lower than that reported by the parents of galactosaemic children. In a separate unpublished survey, 35% of the parents thought that their children were delayed in fine or gross motor skills, especially running, balance, and handwriting (Waggoner and Buist, 1993). Waggoner and Buist proposed that galactosaemic children required standardised testing of their motor function. Schweitzer *et al.*, (1993) performed neurological examinations on 49 patients in their study. Severe clumsiness was present in 12 cases, 11 had an intention tremor, three mild ataxia, and three severe ataxia. The ataxias began at age 9–14 years. Earlier studies had recognised that even well-treated galactosaemic adults infrequently developed a triad of mental retardation, tremor, and cerebellar dysfunction (Jan and Wilson, 1973; Lo *et al.*, 1984; and Koch *et al.*, 1992), and occasionally extrapyramidal movement disorders (Bohles *et al.*, 1986; and Bundey *et al.*, 1996). Friedman *et al.*, (1989) reported two siblings who also developed seizures after the age

of 30 years. In a detailed review of 45 patients from their own clinic, Kaufman *et al.*, (1995b) found 12 patients with neurologic symptoms that included tremor, ataxia, and dysmetria.

5. Ovarian failure, represented by hypergonadotropic hypogonadism

It was assumed that ovarian function in galactosaemia was normal because of several reports of normal pregnancies (Roe *et al.*, 1971; and Tedesco *et al.*, 1972). However, Kaufman *et al.*, (1979) then observed a high incidence of hypergonadotropic hypogonadism in females with galactosaemia, which manifested clinically as partial or complete failure of secondary sexual development and various menstrual abnormalities. Of 18 women with galactosaemia, 12 had hypergonadotropic hypogonadism, five had primary amenorrhoea, six secondary amenorrhoea, and one oligomenorrhoea (Kaufman *et al.*, 1981). The women had either absent or diminished gonadal tissue, persistent elevations of serum FSH and LH, and low oestradiol. The secretion of androgens from the thecal stroma was also abnormally low (Kaufman *et al.*, 1987). Kaufman *et al.*, also gave luteinising release hormone stimulation tests to galactosaemic females between one and 12 years of age and found that seven of eight girls had an exaggerated release of gonadotropins, which suggested gonadal insufficiency (Kaufman *et al.*, 1986; and Kaufman *et al.*, 1988). The ovarian dysfunction in galactosaemia has since been confirmed by many other groups (Hoefnagel *et al.*, 1979; Steinmann *et al.*, 1981a; Steinmann *et al.*, 1981b; Robinson *et al.*, 1984; Schwarz *et al.*, 1984; Schwarz *et al.*, 1986; Fraser *et al.*, 1986; and Gibson, 1995).

In the Waggoner *et al.*, (1990) study, the mean age of menarche was 14 years. Of 34 women older than 17 years, eight had primary amenorrhoea. Most other women in this study developed oligomenorrhoea or secondary amenorrhoea within a few years of menarche and many were placed on hormone therapy. FSH was increased in 80% of 75 females during childhood and adolescence. In the females older than 15 years, 80% were reported to have ovarian failure because of absent or abnormal menstrual cycles, or increased FSH. Waggoner *et al.*, (1990) also reported a total of 14 pregnancies among nine of 37 women over the age of 17 years (for a review of pregnancy in galactosaemia see Gibson, 1995). Three of these women were known to have residual GALT activity or variant enzymes. Of the remainder, almost all developed menstrual irregularities before or after the pregnancy. In Schweitzer *et al.*, (1993) the majority of patients were prepubertal, however, they did observe delayed puberty in five of 11 females and one of 18 males older than 12 years. Most of the 11 females also had primary amenorrhoea and hypergonadotropic hypogonadism.

The majority of males with galactosaemia have normal pubertal development and two adult males had normal semen analysis (Kaufman *et al.*, 1986). Whether galactosaemic men can have children though has not been reported. Waggoner *et al.*, (1990) reported that the levels of serum testosterone, FSH, and LH were normal in 35 males except for one 13-year-old boy, and two other males were reported to have delayed puberty. The serum testosterone, LH, and FSH were normal in other studies of galactosaemic males (Kaufman *et al.*, 1981; and Kaufman *et al.*, 1986). However, two older men have been reported who had increased basal and stimulated levels of serum FSH (Steinmann *et al.*, 1981a; Steinmann *et al.*, 1981b; and Gitzelmann and Steinmann, 1984). Vogt *et al.*, (1980) observed testicular atrophy at post-mortem in a 55-year-old galactosaemic man who died from complications of liver cirrhosis. Crome, (1962) reported interstitial fibrosis and tubular atrophy in the testis of an eight-year-old galactosaemic boy who died from bronchopneumonia. Whether these morphological changes were directly related to the galactosaemia is not known.

Correlation between genotype and long-term outcome

In 1955, Holzel and Komrower, observed that many of the parents of children with galactosaemia had abnormal galactose tolerance. They concluded that the parents were heterozygotes for the galactosaemia gene and that the disorder was therefore transmitted in a recessive manner. Children with galactosaemia have no GALT activity but they can produce a protein that is immunologically similar to normal enzyme (Tedesco and Mellman, 1971; Tedesco *et al.*, 1975; and Reichardt, 1991). This suggested that the genetic defect in galactosaemia was a point mutation, which rendered the protein catalytically inactive. Since then, the normal sequences for both the human GALT complementary deoxyribonucleic acid (cDNA) (Reichardt and Berg, 1988; and Flach *et al.*, 1990) and GALT gene (Leslie *et al.*, 1992) have been established. The gene is organised into 11 exons that span 4 kb and exons 6, 9, and 10 represent evolutionally conserved regions in the gene. The cDNA encodes a polypeptide of 379 amino acids, with an estimated mass of 43 kDa, and the amino acid sequence shows an overall identity among humans, yeasts, and *E. coli* of 35%.

As soon as the normal sequences were identified, several groups began to directly sequence either polymerase-chain-reaction-amplified cDNA (Reichardt *et al.*, 1991; Reichardt and Woo, 1991; Reichardt *et al.*, 1992a; Reichardt *et al.*, 1992b) or genomic deoxyribonucleic acid (DNA) (Leslie *et al.*, 1992; and Elsas *et al.*, 1993) to identify candidate mutations in the GALT genes of patients with galactosaemia. More than 30 variations in the GALT gene sequence have now been described (for a review see Elsas *et al.*, 1995a; and Elsas *et al.*, 1995b). However, only nine have been evaluated in transfection experiments and therefore considered true mutations. One of the mutations, in exon 6, substitutes an arginine for a highly conserved glutamine at codon 188 (Q188R). This mutation is present in 60–70% of Caucasians with classical galactosaemia (Elsas *et al.*, 1993; Ng *et al.*, 1994; and Elsas *et al.*, 1995a). The mutation produced no detectable GALT activity in GALT-deficient yeast cells that were transformed with Q188R-containing human GALT cDNA (Fridovich-Keil and Jinks-Robertson, 1993). Another mis-sense codon, in which aspartic acid is substituted for asparagine at amino acid position 314 (N314D), is commonly associated with the Duarte and Los Angeles variant alleles (Elsas *et al.*, 1994; Lin *et al.*, 1994; and Podskarbi *et al.*, 1994). However, expression of N314D in a yeast system did not result in decreased enzyme activity (Fridovich-Keil *et al.*, 1995). Recent evidence suggests that N314D in the Duarte allele is linked to two regulatory mutations in introns D and E (Greber *et al.*, 1995; Podskarbi *et al.*, 1996).

Two studies have attempted to correlate genotype with long-term outcome in galactosaemia. Elsas *et al.*, (1993) screened 52 patients for the Q188R mutation and then rated their clinical outcome by their DQ/IQ scores, speech, motor and ovarian function, growth, and presence of cataracts. They obtained the patient information using a questionnaire. Any patient with two or more abnormalities was considered to have a poor outcome and those patients with no abnormality were considered to have a good outcome. Thirty-eight patients were classifiable by this means. Twenty patients had a good outcome and of these four were homozygous for Q188R. Eighteen patients had a poor outcome and of these 12 were homozygous for Q188R. This difference was significant ($\chi^2 = 8.464$; $p < 0.004$). Q188R homozygotes have no detectable GALT activity. Elsas *et al.*, (1993) concluded therefore that this was one of the factors that contributed to a poor long-term outcome. The data were later extended to include 52 classifiable patients and produced a similar correlation between poor outcome and homozygosity for Q188R (Elsas *et al.*, 1995a). However, there was no statistical association between the Q188R mutation and ovarian failure in the females of this group.

Kaufman *et al.*, (1994) performed a similar study in 41 patients who had no detectable GALT activity. The patients underwent extensive neuropsychologic examination. The females also

had clinical and biochemical assessments of their ovarian function. The Q188R mutation was present in 72% of the alleles. The mean broad cognitive scores were not statistically different in those patients who were homozygous, heterozygous, or negative for Q188R. The mutation also did not correlate with the presence of tremor, ataxia, or dysmetria (12 patients), and did not help distinguish those females with primary amenorrhoea (8 patients) from those with secondary amenorrhoea (14 patients). Kaufman *et al.*, (1994) concluded that the cognitive outcome, neurologic symptoms, and the time of onset of ovarian failure could not be explained by the Q188R status alone. Unfortunately, both of these studies were limited by relatively small numbers of patients and by differences in the way in which the outcomes were assessed. Kaufman *et al.*, (1994) compared each clinical variable separately whereas Elsas *et al.*, (1993) classified their patients into outcome groups. Elsas *et al.*, (1993) also did not state if their patients were completely deficient in GALT activity. In an even smaller study, Cleary *et al.*, (1995) observed no significant difference in the IQ scores between nine patients who were homozygous for Q188R and six who were Q188R heterozygotes.

A comprehensive interpretation of the correlation between genotype and phenotype will only be possible when the genotypes for all patients have been fully characterised. However, there already has been some suggestion, in a small number of patients, that the milder and more severe clinical forms of galactosaemia may be associated with milder and more severe gene defects, respectively (Gathof *et al.*, 1995; Sommer *et al.*, 1995; and Shin *et al.*, 1996). Ng *et al.*, (1994) also stressed the importance of accurately determining residual GALT activity with a sensitive multipoint radiochemical assay. They considered this was important when assessing long-term outcome in patients and when comparing the results of different molecular and biochemical studies.

Conclusions

The results of the two major retrospective studies, and the many smaller additional reports, have now clearly demonstrated that the long-term outcome in galactosaemia is poor and unrelated to either the success of dietary treatment or biochemical control as currently measured. The acute toxicity of early infancy is dramatically resolved by removing galactose from the diet. However, the longer term problems appear related to other factors. These factors could be present *in utero* or be ongoing, despite adequate dietary control. This suggests that their are other cellular disturbances secondary to a deficiency of GALT. Transferase-deficient cells use several alternative and abnormal galactose metabolic pathways. Several hypotheses have been proposed in the literature that implicate these pathways and their products in disturbed intracellular processes. These intracellular disturbances, and their possible adverse effects, are reviewed in the next section.

2.5 Abnormalities of cellular metabolism in classical galactosaemia

A deficiency of GALT results in several major intracellular disturbances. There is an accumulation of the GALT substrate, galactose-1-phosphate, and a decrease in the concentration of one of the products of the reaction, UDP-galactose. Alternative galactose metabolic pathways produce increased amounts of galactitol, galactonate, and galactose-6-phosphate (Figure 1.1, page 2). The synthesis or metabolism of myo-inositol, glycoprotein, and glycolipid are also disturbed. This section reviews each of these metabolic disturbances and discusses how they might contribute to the long-term complications in galactosaemia. The review concentrates primarily on the metabolic disturbances in patients with GALT deficiency. Although the review does include some experimental evidence from animal models of galactosaemia, it does not attempt to discuss all the metabolic disturbances in animals fed high-galactose diets. The glycoprotein and glycolipid abnormalities in galactosaemia, which are of particular interest to this thesis, are reviewed in the final section of this chapter (page 32).

Disturbed polyol metabolism

The acyclic polyols are sorbitol, galactitol, and mannitol; they are derived from glucose, galactose, and mannose, respectively. The predominant cyclic polyol is myo-inositol, which is synthesised from glucose-6-phosphate. The two enzymes of the polyol pathway are aldose reductase and sorbitol dehydrogenase, which catalyse the conversion of glucose to fructose, by way of sorbitol (for a review see Berry, 1995). The polyol pathway was first detected in placenta and seminal vesicles and produces the fructose in seminal fluid (Hers, 1960). In some cells, under certain conditions, the polyol pathway also produces sorbitol, which functions as an intracellular osmolyte in response to hypertonic stress (Burg, 1988). Polyol metabolism is disturbed in galactosaemia with increased production of galactitol and decreased tissue concentrations of myo-inositol.

Galactitol

The polyol pathway provides an alternative pathway for galactose metabolism in galactosaemia (Figure 1.1, page 2). Aldose reductase has a low affinity for galactose. However, if the intracellular concentration of galactose is increased, as in galactosaemia, the enzyme can convert galactose to galactitol. Galactitol then accumulates because it cannot be metabolised by sorbitol dehydrogenase. Patients with galactokinase deficiency were unable to metabolise $[^{14}C]$-galactitol to $^{14}CO_2$, which is consistent with galactitol being a metabolic end-product (Gitzelmann *et al.*, 1974). In 1964, during the routine investigation of two infants with classical galactosaemia, Wells *et al.*, noticed an additional non-reducing monosaccharide in their urine. The sugar was eventually identified as galactitol (Wells *et al.*, 1964). The galactitol in the urine then slowly decreased once a lactose-free diet was started. However, the clearance of galactitol was much slower than for galactose and it never completely returned to normal (galactitol is virtually undetectable in normal urine). Galactitol was also detected in the serum of one of the infants before treatment.

Soon after this report, galactitol was also identified in the brain of two infants who died from galactosaemia during the neonatal period (Wells *et al.*, 1965). In other analyses of post-mortem tissues from galactosaemic infants, galactitol was increased in all the tissues that were examined, except thyroid (Quan Ma *et al.*, 1966; and Perelmuter *et al.*, 1989). The concentration was particularly high in brain and skeletal muscle. A 17-year-old patient who was given oral $[^{14}C]$-galactose excreted $[^{14}C]$-galactitol in the urine. This confirmed that the

galactitol was derived directly from galactose (Egan and Wells, 1966). Roe *et al.*, (1973) then observed that galactitol was increased in the urine of all galactosaemic patients on treatment. The urinary excretion ranged from 150 to 750 mg/g creatinine (61–980 mg/24 h) and was not influenced by reducing the protein intake or by adding as much as 6 g/d of galactose to the diet. Roe *et al.*, (1973) concluded that galactitol was continuously produced in galactosaemia and that the base-line excretion in the urine was largely independent of dietary sources of galactose. This conclusion was recently supported by another study that observed little contribution from soluble galactose in fruit and vegetables, or from 200 mg/d of supplementary galactose, to the excretion of galactitol in the urine (Berry *et al.*, 1993). Newly formed galactitol was also excreted over several days, which suggested that galactitol accumulated in the tissues. Unfortunately, these studies also confirmed that galactitol was not sensitive enough to be a useful monitor of dietary galactose intake.

Several other groups have also measured the urinary excretion of galactitol in treated galactosaemia (Blau, 1972; Ng *et al.*, 1975) and following oral (Roe *et al.*, 1973; and Yamazaki *et al.*, 1991) and intravenous (Sitzmann *et al.*, 1977) galactose loads. The largest study, however, was part of the long-term follow-up of patients in Germany (Schweitzer *et al.*, 1993; and Jakobs *et al.*, 1995). In this study, the concentration of galactitol was measured in the plasma and urine of 75 patients on treatment. The plasma galactitol concentration ranged from 4.7 to 20 µmol/L (normal range 0.08–0.86) and urine galactitol from 45 to 900 mmol/mol creatinine (normal range 2–81). There was no correlation between the plasma galactitol and the concentration of erythrocyte galactose-1-phosphate, or between the urinary concentrations of galactitol and galactose (Jakobs *et al.*, 1995). The urine and plasma galactitol concentrations also did not correlate with long-term intellectual outcome. Kaufman *et al.*, (1981) also found no correlation between the concentration of galactitol in urine and ovarian failure.

Galactitol is implicated in the development of cataracts in galactosaemia. Gitzelmann *et al.*, (1967a) observed an increase in galactitol in the lens of a galactosaemic infant who died at 15 days of age. It had been known for several years that galactitol accumulated in the lens of galactose-fed rats and that it did not readily diffuse out (van Heyningen, 1959). Kinoshita, (1965) then proposed a model for galactose-induced cataracts that was based largely on an accumulation of galactitol in the lens. The osmotic swelling and disruption induced by the galactitol damaged the lens and lead to cataract formation. This model has since remained the most widely accepted explanation for cataract formation in classical galactosaemia. The model is also supported by a large body of experimental evidence from animal models of diabetic cataract in galactose-fed rats and dogs (for a review see Berry, 1995). Cataracts also occur in galactokinase-deficiency and epimerase-deficiency galactosaemia. In galactokinase deficiency, cataracts are the only clinical feature and the patients excrete large amounts of galactitol in their urine (Gitzelmann, 1967; Olambiwonnu *et al.*, 1974; and Sitzmann *et al.*, 1977). Cataracts were also detected in a transferase-deficient fetus at five months (Vannas *et al.*, 1975) and are occasionally seen at birth (Stambolian, 1988). This suggests that galactitol has an adverse effect on fetal lens development *in utero*. It is not known if galactokinase-deficient pregnancies accumulate galactitol, but cataracts have also been detected in these infants soon after birth. Patients with galactokinase deficiency do not develop brain, liver, or ovarian pathology. Galactitol has therefore not been considered a major factor in the development of these complications in transferase-deficiency galactosaemia (Gitzelmann and Steinmann, 1984).

Galactitol is increased approximately tenfold in the amniotic fluid of transferase-deficient pregnancies, even as early as 12 weeks (Holton, 1995). Galactitol was therefore proposed as method of prenatal diagnosis (Allen *et al.*, 1980; and Allen *et al.*, 1981) and Holton *et al.*, (1989) have reported their experience using amniotic fluid galactitol in 24 pregnancies at risk of galactosaemia. Galactitol was even increased in the amniotic fluid of two affected

pregnancies in which the mothers avoided milk products (Jakobs *et al.*, 1984; and Jakobs *et al.*, 1988). Galactitol was increased in the liver and lung of an affected fetus during the second trimester, although not to the same extent as in the liver of neonates who died from galactosaemia (Allen *et al.*, 1980).

Galactitol may contribute to the acute neurological problems in the neonatal period. Several infants with the acute toxicity syndrome had evidence of cerebral oedema on computerised tomography (CT) and cerebral ultrasound (Belman *et al.*, 1986; and Welch and Milligan, 1987), and at autopsy (Quan Ma *et al.*, 1966; and Suzuki *et al.*, 1966). Pseudotumour cerebri, with raised intracranial pressure, has also been reported in galactokinase-deficiency galactosaemia (Litman *et al.*, 1975). Huttenlocher *et al.*, (1970) proposed that accumulation of galactitol in the brain contributed to the development of acute cerebral oedema in these infants. Moller *et al.*, (1995) were unable to detect significant quantities of galactitol in the brains of six adult patients on treatment when they were examined with ^{1}H- and ^{31}P-nuclear magnetic resonance (NMR) spectroscopy.

Myo-inositol

Myo-inositol serves two main cellular functions. The first is in signal transduction, where G-protein hydrolysis of the polyphosphorylated form of phosphatidylinositol results in increased cytosolic Ca^{2+} and protein kinase C activity. This mechanism regulates many cellular functions including metabolism, secretion, contraction, neural activity, and cell proliferation (for a review see Berridge and Irvine, 1989). In the second, myo-inositol functions as an idiogenic osmole, particularly in brain and kidney (for a review see Berry, 1995). Disturbances in any of these functions could have significant effects on organ development.

During the investigations of the concentration of galactitol in the tissues of patients with galactosaemia, it was also observed that the concentration of myo-inositol was decreased. Both free and lipid-bound myo-inositol were decreased in the brains of three infants who died in the newborn period (Wells *et al.*, 1965; and Quan Ma *et al.*, 1966). The significance of this finding was unknown and the authors questioned whether it was related to the accumulation of galactitol. Galactitol also accumulated in the brain of rats fed high-galactose diets (Quan Ma and Wells, 1965). Wells and Wells, (1967) then reported a decrease in the concentration of free myo-inositol and phosphatidylinositol in the brain of fetal and young rats that were exposed to a maternal diet that contained 35% galactose. Brain slices from these rats also converted less [1-^{14}C]-glucose to myo-inositol. However, Wells *et al.*, (1969) were unable to replicate the decrease in inositol in similar experiments in older rats. This suggested that the effect of galactose occurred only during glial cell proliferation and active myelination. Warfield and Segal, (1978) then reported that isolated synaptosomes from the brain of newborn rats, exposed *in utero* to a maternal diet that contained 40% galactose, had normal concentrations of myo-inositol. They then proposed that the effect of galactose on inositol metabolism was confined to a component of the brain other than the nerve endings. However, they did observe a functional defect in phosphatidylinositol metabolism in the synaptosomes following stimulation of the acetylcholine receptor. Taken together, these studies suggested a possible disturbance in myo-inositol metabolism in galactosaemia and a mechanism by which galactose and its metabolites could damage the developing nervous system.

Since then, there have been many studies, predominantly in rats and chickens, of the effects of feeding animals galactose (for a review see Segal, 1995; and Segal and Berry, 1995). The fetal brain of the rat and chicken are very sensitive to the effects of galactose, more so than the adult brain. Fetal rats that are exposed to excess galactose have brains that are less developed, contain less DNA, and have reduced concentrations of glucose and free amino acids. The

chick develops an acute toxicity of the brain when exposed to galactose, which is associated with decreased brain ATP, glucose, and glycolytic intermediates (Kozak and Wells, 1969; and Kozak and Wells, 1971). The toxicity in the chick model may result from several factors including plasma hyperosmolality (Malone *et al.*, 1971; and Knull *et al.*, 1972), accumulation of galactose-1-phosphate, or reduced uptake of glucose into the brain (Knull *et al.*, 1972; and Malone *et al.*, 1972). Whether these animal models of galactose-induced neurotoxicity are similar to the human disease is not known. It is possible that the decrease in brain inositol in the fetal rat model was due to a general disturbance in brain metabolism. High-galactose diets are abnormal and the cellular disturbances could have been secondary to poor nutrition or protein restriction during critical stages in brain development (Editorial, 1970).

Unfortunately, there have been no further major studies of myo-inositol metabolism in patients with galactosaemia beyond the initial reports (Wells *et al.*, 1965; and Quan Ma *et al.*, 1966). Lott *et al.*, (1983) did briefly report that free and lipid-bound inositol were reduced to 50% of normal in the brain of an untreated 12-day-old galactosaemic infant. There have also been no studies of myo-inositol metabolism in galactosaemic cells in culture. Moller *et al.*, (1995) studied six adult patients with ^{1}H-NMR spectroscopy and reported that the free myo-inositol signal in the brain was normal. This suggested that the inositol pathway was normal, at least in treated adult patients, and that abnormal myo-inositol metabolism was an unlikely cause of the long-term neurological complications. Berry, (1995) suggested that only marked increases of blood and tissue galactose could result in physiologically relevant disturbances to myo-inositol metabolism. Therefore any effect from disturbed myo-inositol or phosphatidylinositol metabolism would have to occur during fetal development or in the neonatal period.

Galactonate

Several early studies observed that some galactosaemic patients were capable of metabolising significant amounts of [1-^{14}C]-galactose to $^{14}CO_2$ (Komrower *et al.*, 1956; Segal *et al.*, 1962; Segal *et al.*, 1965; and Baker *et al.*, 1966). These patients were American Blacks and were eventually proved to have residual GALT activity in the liver (Lai *et al.*, 1996). However, at the time, these observations lead to a search for alternative pathways of galactose metabolism.

Cuatrecasas and Segal, (1966c) proposed an alternative pathway of galactose metabolism, which involved the oxidation of galactose by way of β-ketogalactonate and xyulose. The xyulose was then metabolised by the pentose phosphate pathway. The theory of the galactonic acid pathway was supported by the observation that transferase-deficient patients were able to oxidise some [1-^{14}C]-galactose, but not [2-^{14}C]-galactose, to $^{14}CO_2$ (Cuatrecasas and Segal, 1966c; and Segal and Cuatrecasas, 1968). An NAD-requiring galactose dehydrogenase was then isolated and characterised in rat liver and a similar enzyme activity was reported in other mammalian livers, including humans (Cuatrecasas and Segal, 1966a; and Cuatrecasas and Segal, 1966b). However, this activity was not confirmed by others (Beutler, 1967; and Srivastava and Beutler, 1969).

Galactonate was isolated from the liver of a galactosaemic infant at post-mortem (Isselbacher, 1957). It was also identified in the urine of galactosaemic patients following oral galactose loads (Bergren *et al.*, 1972). Rancour *et al.*, (1979) also found galactonate in the urine and tissues, especially the liver, of rats that were fed a diet containing 40% galactose. Microsomes from the liver of rat, mouse, and cow also oxidised galactose to galactonate. Wada, (1986) also observed an increase in galactonolactone, the cyclised form of galactonate, in various tissues (highest in liver, lens, muscle, and urine) of guinea pigs fed a diet that contained 40% galactose. In general, the highest concentrations of galactonolactone were in the tissues that also accumulated galactitol. When the guinea pigs were given an aldose reductase inhibitor,

the concentration of galactitol in the tissues decreased but galactonolactone increased. This suggested that there was a close relationship between the reductive and oxidative pathways at toxic concentrations of galactose. Wada (1986) proposed that when the blood concentration of galactose was high the liver produced galactonolactone, which was then slowly released and excreted in the urine.

There has been no study of the urinary excretion of galactonate or galactonolactone in galactosaemic patients on treatment. There has also been no report of the concentration of galactonate in either amniotic fluid or fetal tissue from transferase-deficient pregnancies. Rakotomanga *et al.*, (1991) developed a method that simultaneously measures urinary galactitol and galactonolactone and suggested that it could be applied to galactosaemia.

Galactose-6-phosphate

In 1962, Inouye *et al.*, reported that galactosaemic erythrocytes produced small amounts of galactose-6-phosphate when exposed to galactose. They later reported that the galactose-6-phosphate was converted to 6-phosphogalactonic acid by glucose-6-phosphate dehydrogenase, in the presence of NADP. They proposed that this was an alternative pathway of galactose metabolism and that the galactose-6-phosphate was oxidised by the hexose monophosphate pathway (Inouye *et al.*, 1964). Galactose-1-phosphate can be converted to galactose-6-phosphate by muscle phosphoglucomutase (Posternak and Rosselet, 1954), however, this occurs at a much lower rate than with glucose-1-phosphate (Sidbury, 1957b). This mechanism has been suggested as a possible source of the galactose-6-phosphate in galactosaemia.

A hexose-6-phosphate dehydrogenase was detected in human liver (Ohno *et al.*, 1966) and in microsomes from rat liver (Srivastava and Beutler, 1969). The enzyme used NAD or NADP, and glucose-6-phosphate or galactose-6-phosphate as substrate. The product of the rat liver enzyme was identified as 6-phosphogalactonic acid. However, they were unable to show significant oxidation of [1-^{14}C]-6-phosphogalactonic acid to $^{14}CO_2$ in the rat liver fractions (Srivastava and Beutler, 1969). Srivastava and Beutler did not consider that galactose-6-phosphate and its oxidation by way of 6-phosphogalactonate was a physiologically significant pathway in galactosaemia.

Galactose-1-phosphate

It was the accumulation of galactose-1-phosphate in galactosaemic erythrocytes (Schwarz *et al.*, 1956) that originally helped identify the enzyme defect in classical galactosaemia (Kalckar *et al.*, 1956b). Galactose-1-phosphate is increased in other galactosaemic tissues, including liver, kidney, and brain (Schwarz, 1960; and Tada, 1962), and lens (Gitzelmann *et al.*, 1967a). It also accumulates in transferase-deficient skin fibroblasts (Mayes and Miller, 1973) and ovarian tissue (Xu *et al.*, 1989b) when they are exposed to galactose. It is also increased in the fetal liver and erythrocytes, and in cord blood at delivery (for a review see Holton, 1995). The highest tissue concentrations are found in affected infants soon after they are exposed to milk. The erythrocyte concentration then returns towards normal over several weeks to months once diet is started. However, despite adequate dietary control, the concentration of galactose-1-phosphate in erythrocytes remains persistently increased and the reasons for this are unknown. The concentration of galactose-1-phosphate is normal in galactokinase deficiency and therefore galactose-1-phosphate has been implicated in the toxicity of classical galactosaemia.

Endogenous production of galactose-1-phosphate

Several theories have been proposed that attempt to explain the persistent increase in galactose-1-phosphate in galactosaemic erythrocytes. Dietary sources of hidden galactose, particularly free galactose in fruit and vegetables, have been mentioned earlier and highlighted by Gross and Acosta, (1991). However, Berry *et al.*, (1993) reported that dietary galactose in fruit and vegetables had little influence on the concentration of galactose-1-phosphate in erythrocytes, or galactitol in urine. This suggested that endogenous galactose was largely responsible for the persistent production of galactose-1-phosphate by patients on treatment. Berry *et al.*, (1995) then studied endogenous galactose production in patients using continuous intravenous infusions of [1-^{13}C]-galactose. They analysed the steady-state flux of galactose in three adults with galactosaemia and in three normal adults. Both groups synthesised between 1.1 and 1.3 g of endogenous galactose per day, which greatly exceeded the free galactose intake of patients on galactose-restricted diets. Berry *et al.*, (1995) proposed that this endogenous galactose contributed to the persistent production of galactose-1-phosphate.

There are two pathways that could produce endogenous galactose. First, galactose is released during the catabolism of galactose-containing glycoprotein and glycolipid. And second, galactose-1-phosphate is produced by pyrophosphorolytic cleavage of UDP-galactose. The galactose-1-phosphate is then dephosphorylated to galactose. It is the second pathway that has interested researchers in galactosaemia for many years. The interest arose from a theory of self-intoxication, which proposed that galactose-1-phosphate was produced continuously from glucose.

Self-intoxication with galactose-1-phosphate

In 1957, Eisenberg *et al.*, administered [1-^{14}C]-galactose to a 24-year-old male with galactosaemia and detected small amounts of [^{14}C]-glucuronic acid in the urine. This demonstrated that some galactose was metabolised in GALT deficiency and they suggested that this indicated an alternative pathway of galactose metabolism. Isselbacher then identified a minor pathway of galactose-1-phosphate metabolism in rat and human liver (Isselbacher, 1957; and Isselbacher, 1958) that involved the enzyme UDP-galactose pyrophosphorylase (EC 2.7.7.10). The pathway is outlined in Figure 2.2. UDP-glucose pyrophosphorylase (EC 2.7.7.9) has a low specificity and probably catalyses the conversion of both UDP-glucose and UDP-galactose (Knop and Hansen, 1970). However, others have reported that separate pyrophosphorylases exist in human skin fibroblasts (Chacko *et al.*, 1972). The activity of pyrophosphorylase in human liver, with galactose-1-phosphate as substrate, was only 1–5% of the activity of GALT. This pathway therefore does not normally play a major role in hepatic galactose metabolism (Abraham and Howell, 1969; and Shin *et al.*, 1987).

In 1966, Gitzelmann then observed an unexplained rise in the concentration of galactose-1-phosphate in the blood of a galactosaemic baby who had been treated from birth with a galactose-free formula (Gitzelmann, 1969). He also noted that galactose-1-phosphate was even produced by infants who received only intravenous or oral glucose (Gitzelmann *et al.*, 1975). These unusual findings prompted him to investigate the source of the galactose-1-phosphate. He then reported that galactosaemic erythrocytes were capable of forming galactose-1-phosphate from UDP-galactose *in vitro* (Gitzelmann, 1969) and later proved that UDP-glucose pyrophosphorylase was the catalyst for this conversion (Gitzelmann and Hansen, 1974; and Gitzelmann *et al.*, 1975). Transferase-deficient skin fibroblasts also produced galactose-1-phosphate when cultured in medium that contained only glucose (Mayes and Miller, 1973), and they also exhibited UDP-galactose pyrophosphorylase activity (Chacko *et al.*, 1972; and Pourci *et al.*, 1985). This experimental evidence lead Gitzelmann to propose

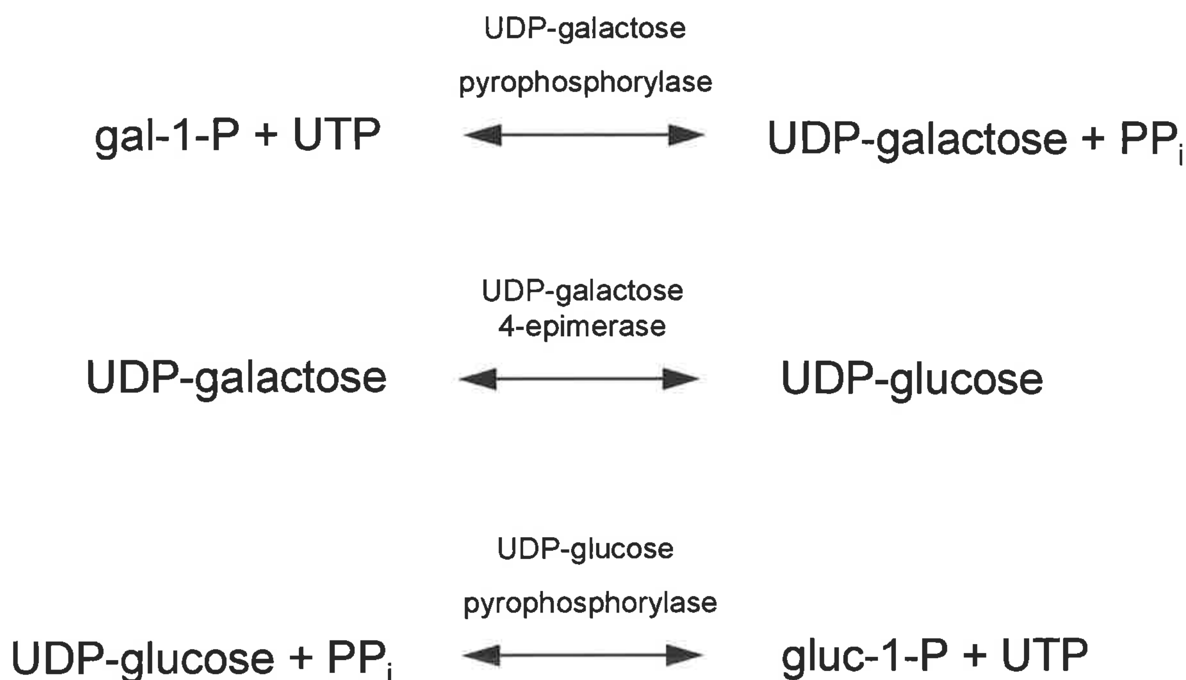


Figure 2.2. The pyrophosphorylase pathway. In this minor pathway, galactose-1-phosphate (*gal-1-P*) is converted to UDP-galactose by UDP-galactose pyrophosphorylase. It is then possible to convert UDP-galactose to glucose-1-phosphate (*gluc-1-P*) by way of UDP-galactose 4-epimerase and UDP-glucose pyrophosphorylase. Each of these enzymatic steps is reversible. *UTP*, uridine 5'-triphosphate; and PP_i, pyrophosphate.

that patients with galactosaemia were self-intoxicated by continuously producing galactose-1-phosphate from glucose-1-phosphate. This involved a reversal of the pyrophosphorylase pathway in Figure 2.2. Gitzelmann suggested that the activity of this pathway was supported by several clinical findings. First, cord blood from galactosaemic neonates contains galactose-1-phosphate even if the mother avoids galactose during the pregnancy. Second, galactosaemic women can produce lactose in their breast milk. Third, erythrocyte galactose-1-phosphate increases in galactosaemic women towards the end of pregnancy, with increasing lactosuria. Fourth, galactosaemic women give birth to healthy infants despite a strict galactose-free diet during pregnancy. And finally, erythrocyte galactose-1-phosphate concentrations take weeks to months to reach base-line levels despite rigid diet (Gitzelmann and Steinmann, 1984; and Gitzelmann, 1995). However, despite this theory and the supporting evidence, the origin of the galactose-1-phosphate in treated galactosaemia, and its rate of production, remain unknown (Segal, 1993; and Segal, 1995).

Adverse effects of galactose-1-phosphate

Enzyme inhibition

Galactose-1-phosphate was proposed as a factor in the development of human and experimental galactose cataract (Lerman, 1959a). The hexose monophosphate pathway is important in the energy metabolism of the lens (Kinoshita, 1955). The activity of a major

enzyme in this pathway, glucose-6-phosphate dehydrogenase, was decreased in the lens of rats that were fed a diet containing 30% galactose for two weeks. The enzyme was also inhibited *in vitro* by galactose-1-phosphate (Lerman, 1959b). Lerman suggested that this would disturb the hexose monophosphate shunt and proposed that galactose-1-phosphate contributed to the pathogenesis of experimental galactose cataract. However, these results were not confirmed by others (Weinberg and Segal, 1960; Korc, 1961; and Kinoshita, 1965) and the theory was soon replaced by galactitol (Kinoshita, 1965).

In 1956, Schwarz *et al.*, in the first study of the metabolism of galactosaemic erythrocytes, observed that their uptake of oxygen was reduced if the erythrocytes were exposed to galactose, *in vivo* or *in vitro*. This was associated with an increase in the erythrocyte concentration of galactose-1-phosphate and a decrease in the concentration of inorganic phosphate and phosphate ester. Schwarz *et al.*, (1956) proposed that galactose-1-phosphate disturbed cellular metabolism by inhibiting phosphoglucomutase. This involved either direct competition with glucose-1-phosphate for the enzyme, or depletion of the normal coenzyme, glucose-1,6-diphosphate, because of accumulation of galactose-1,6-diphosphate. Phosphoglucomutase in muscle can convert galactose-1-phosphate to galactose-6-phosphate, although at a slow rate (Posternak and Rosselet, 1954). Several groups then reported that phosphoglucomutase was inhibited by galactose-1-phosphate *in vitro* (Sidbury, 1957a; and Ginsburg and Neufeld, 1957), as long as glucose-1,6-diphosphate was not present in excess. It was proposed that the inhibition was due to depletion of the natural coenzyme because of the excessively slow reaction of galactose-1,6-diphosphate with the enzyme (Sidbury, 1957b; and Sidbury, 1960). No further studies were performed.

Galactose-1-phosphate has also been reported to inhibit glucose-6-phosphatase (K. J. Isselbacher; cited in Kalckar and Maxwell, 1958), liver glycogen phosphorylase (Maddaiah and Madsen, 1966), UDP-glucose pyrophosphorylase (Oliver, 1961), and cell surface galactosyltransferases (Roth *et al.*, 1971). Although there has been much speculation regarding the effect these inhibitions might have on cellular metabolism, the evidence is derived solely from studies of isolated enzymes *in vitro*. Whether these effects occur in galactosaemic cells in culture, or in galactosaemic patients, is unknown.

Energy deficits

Schwarz *et al.*, (1956), in their study of the metabolism of galactosaemic erythrocytes, observed that the total phosphate ester content (excluding galactose-1-phosphate) in the erythrocytes from a galactosaemic infant who had been exposed to galactose for eight days, was decreased by 25%. The inorganic phosphate concentration was also decreased by 70%. Schwarz *et al.*, (1956) suggested that the accumulation of galactose-1-phosphate in galactosaemic erythrocytes disturbed the distribution of intracellular phosphate and hence glucose metabolism. Similar abnormalities were reported in erythrocytes from another galactosaemic child (Komrower *et al.*, 1956). It was then proposed that the accumulation of galactose-1-phosphate contributed to the brain, liver, kidney, and lens pathology in galactosaemia by disturbing cellular energy metabolism (Schwarz *et al.*, 1956). This was supported by finding increased concentrations of galactose-1-phosphate in these tissues (Schwarz, 1960).

Penington and Prankerd, (1958) then observed a significant decrease in the concentration of ATP, and an increase in the inorganic phosphate, in the erythrocytes of a galactosaemic infant who was fed 50 g of lactose daily for one week. The concentrations returned to normal one week after stopping the lactose. However, when the same erythrocytes were exposed to 17 mmol/L of galactose *in vitro* for two hours, there was only a slight decrease in ATP.

Penington and Prankerd, (1958) suggested that the decrease represented either an impaired synthesis of ATP or enhanced breakdown of phosphate esters. Baar and Gordon, (1964) observed an increase in the efflux of potassium from galactosaemic erythrocytes when the erythrocytes were exposed to galactose *in vitro*. They suggested that this supported a deficiency of ATP, which is required for active transport of potassium by the erythrocyte membrane. Zipursky *et al.*, (1965), however, observed no effect of galactose on the turnover of ATP, 2,3 diphosphoglycerate, or inorganic phosphate, during short *in vitro* studies with [^{32}P]-orthophosphate in erythrocytes from two galactosaemic patients. They concluded that galactose-1-phosphate did not directly interfere with glycolysis. Unfortunately, the results from these various studies are conflicting. This appears to be due to variations in the experimental methods, especially the amount and time of galactose exposure, whether glucose was present in the medium, and the methods used to estimate the intracellular phosphate intermediates. It is therefore difficult to determine the extent of the galactose effect on energy metabolism in the erythrocytes.

Mayes and Miller, (1973) then reported that transferase-deficient skin fibroblasts in culture also accumulated galactose-1-phosphate. The intracellular concentration of galactose-1-phosphate reached a peak with two hours of adding galactose to the culture medium, but then slowly decreased with prolonged exposure. In pulse-chase studies, [1-^{14}C]-galactose was taken up by the fibroblasts and phosphorylated, however, over time it was dephosphorylated and secreted back into the medium as free galactose. Normal fibroblasts did not show this pattern. Mayes and Miller, (1973) suggested that this futile cycle of phosphorylation and dephosphorylation could contribute to the pathogenesis of galactosaemia by consuming ATP.

Several clinical studies have attempted to examine phosphate ester metabolism in galactosaemic patients who were given oral galactose loads. Kogut *et al.*, (1975) gave 50 g of oral galactose to three adult patients and observed a slight increase in the plasma uric acid and a decrease in the plasma inorganic phosphate concentrations within 1 to 5 hours of the load. The increase in urate was similar to that observed in healthy children following intravenous fructose loads, but much less than that seen in children with hereditary fructose intolerance (Kogut *et al.*, 1975). It was concluded that the greater increase in plasma urate with fructose represented the ability of fructokinase to phosphorylate fructose faster than galactose by galactokinase (Kogut *et al.*, 1975). Fructose-induced hyperuricaemia has been attributed to the breakdown of preformed hepatic adenine nucleotides (for a review see Gitzelmann *et al.*, 1995). The rapid phosphorylation of fructose by fructokinase results in an accumulation of fructose-1-phosphate and depletion of ATP and inorganic phosphate. Both ATP and inorganic phosphate are required for the stabilisation of adenosine 5'-phosphate (AMP). Their depletion results in degradation of AMP to inosine 5'-phosphate and then to uric acid. Forster *et al.*, (1975) also observed an increase in serum urate in five galactosaemic patients following an oral galactose load, and Levin *et al.*, (1963) observed a decrease in plasma inorganic phosphate following a 20 g oral galactose load in a galactosaemic child. These studies suggest that there is some degradation of ATP and AMP in galactosaemic patients, at least acutely after large galactose loads.

Kalderon *et al.*, (1992) administered an oral galactose load of 20 mg/kg to a galactosaemic patient on treatment and observed a change in the ^{31}P-NMR spectrum of the liver over 60 minutes that was consistent with accumulation of galactose-1-phosphate. The brain showed no change in its spectra over the same time period, which confirmed the importance of the liver as the initial site of galactose metabolism. Another patient in the same study, who was exposed to only 10 mg/kg of galactose, showed no change in the liver spectrum. In contrast to similar studies in hereditary fructose intolerance, none of the galactosaemic patients showed any decrease in hepatic ATP or intracellular phosphate (Oberhaensli *et al.*, 1987). Kalderon *et al.*, (1992) also administered intravenous galactose and ethanol to rats (ethanol inhibits UDP-

galactose 4-epimerase and blocks galactose metabolism) and measured the hepatic ^{31}P spectra. They observed an increase in galactose-1-phosphate and a small decrease in ATP in the rat livers (Kalderon *et al.*, 1992). However, the relative galactose load was 50 times that of the human study. Moller *et al.*, (1995) were unable to demonstrate either increased galactose-1-phosphate or decreased ATP, when they examined the brains of six treated galactosaemic patients with ^{31}P-NMR spectroscopy. They concluded that the energy metabolism in the brain was normal when on treatment.

Several other disturbances of nucleotide phosphate metabolism have been reported in galactosaemia. Forster *et al.*, (1975) exposed galactosaemic liver biopsies to galactose *in vitro* and observed an accumulation of galactose-1-phosphate. They also observed a significant decrease in the concentration of hepatic UTP. Depletion of UTP has been identified as the necrogenic lesion in the experimental liver injury induced by the galactose analogue, galactosamine (Decker and Keppler, 1974). The mechanism of damage involves phosphate trapping and accumulation of UDP-galactosamine. It was therefore proposed that the liver disease in galactosaemia could result from UTP depletion and galactose-induced phosphate trapping (Forster *et al.*, 1975). 2-Deoxy-2-fluoro-D-galactose also caused UTP depletion in rat hepatocytes (Grun *et al.*, 1990) and the experimental liver injury produced in rats by 2-deoxy-D-galactose was very similar to the liver injury in untreated galactosaemia. 2-Deoxy-D-galactose injury was therefore proposed as a potential model of the liver disease in galactosaemia (Smith and Keppler, 1977; and Lattke *et al.*, 1979). In these animal models of liver injury, depletion of hepatic UTP was associated with inhibition of RNA synthesis (Keppler *et al.*, 1974) and protein synthesis (Anukarahanonta *et al.*, 1973). RNA and protein synthesis were also inhibited acutely in rat liver following the administration of intravenous fructose (Maenpaa *et al.*, 1968; and Bode *et al.*, 1973). However, Forster and Keppler, (1975) in contrast to the hepatic study, were unable to demonstrate depletion of UTP in galactosaemic leucocytes that were exposed to galactose *in vitro*. The galactosaemic leucocytes were unable to accumulate UDP-galactose, presumably because of the GALT deficiency. Normal human leucocytes when exposed to galactose did accumulate UDP-galactose with a concomitant decrease in UTP, UDP, and UDP-glucose. This suggested that the epimerase enzyme was rate-limiting in normal galactose metabolism (Forster and Keppler, 1975). Gibson *et al.*, (1995a) also observed a transient increase in the concentration of UDP-galactose in the erythrocytes and leucocytes from normal adults following a 50 g oral load of galactose.

UDP-galactose deficiency

Soon after the enzyme defect was defined in galactosaemia it was recognised that a deficiency of GALT might affect the synthesis of UDP-galactose, one of the products of the GALT reaction. UDP-glucose 4-epimerase is capable of synthesising UDP-galactose in the absence of dietary galactose. However, UDP-galactose is a donor of galactose in glycoprotein and glycolipid synthesis and even a partial deficiency could have widespread effects. This lead to considerable interest in UDP-galactose as a possible mechanism for the long-term complications in galactosaemia.

In 1985, Shin *et al.*, then briefly reported that the concentration of UDP-galactose was decreased in the erythrocytes from 19 patients with galactosaemia. This was the first indication that there might be a disturbance in UDP-galactose metabolism in galactosaemia. Ng *et al.*, (1989) then suggested that if galactose-1-phosphate inhibited UDP-glucose pyrophosphorylase (Oliver, 1961) *in vivo*, then the cellular concentrations of both UDP-glucose and UDP-galactose should be decreased. They developed therefore a coupled enzymatic assay that measured both UDP-glucose and UDP-galactose. The assay converted

the UDP-glucose to UDP-gluconate with UDP-glucose dehydrogenase and then measured the fluorescence of the NADH that was produced. The UDP-galactose was estimated by adding UDP-galactose 4-epimerase to the same reaction and calculating the difference between the total and original UDP-glucose. Ng *et al.*, (1989) then measured both sugar nucleotides in various galactosaemic tissues and observed a significant decrease in the concentration of UDP-galactose in erythrocytes (n = 40), cultured skin fibroblasts (n = 6), and liver (n = 6). The concentration of UDP-galactose in the erythrocytes was approximately 35% of the normal mean. They later reported that UDP-galactose was also decreased in the cord blood of three galactosaemic neonates (Ng *et al.*, 1993a). The concentration of UDP-glucose in all the tissues, however, was normal. Ng *et al.*, (1989) proposed that the UDP-galactose deficiency contributed to the long-term complications in galactosaemia.

Ng *et al.*, (1989) also suggested that the UDP-galactose deficiency was directly related to the absence of GALT activity. In a related study of ovarian dysfunction in galactosaemia, they found three women with galactosaemia who had normal ovarian function. These women had some GALT activity (low-activity variants) and the concentration of UDP-galactose in their erythrocytes was normal (Kaufman *et al.*, 1988). Ng *et al.*, (1989) therefore proposed that the residual GALT activity protected the ovary from the damage associated with UDP-galactose deficiency. Normal ovarian tissue had higher activities of all the enzymes of galactose metabolism than normal testis and erythrocytes, with UDP-glucose pyrophosphorylase the most active (Xu *et al.*, 1989b). The normal ovarian concentrations of UDP-glucose and UDP-galactose were also increased. This suggested that galactose metabolism was important in ovarian tissue. Xu *et al.*, (1989b) proposed that a deficiency of UDP-galactose could specifically affect the ovary by disturbing glycoprotein and glycolipid synthesis. The same group also reported that the concentration of erythrocyte UDP-galactose in galactokinase deficiency was normal, therefore supporting their theory (Xu *et al.*, 1989a). The group then observed that adding uridine to galactosaemic erythrocytes (Ng *et al.*, 1989) and skin fibroblasts (Ng *et al.*, 1993a) *in vitro*, increased the concentrations of UDP-galactose and UDP-glucose. Oral uridine was then given to four galactosaemic patients for 48 hours and the concentration of UDP-galactose in their erythrocytes returned to normal. The concentration of galactose-1-phosphate remained unchanged (Kaufman *et al.*, 1989). This lead to a recommendation that uridine might prevent the long-term complications in galactosaemia (Ng *et al.*, 1989). The group then began an extended trial of uridine treatment in patients. The results of that trial, however, have not been formally reported.

The results in Ng *et al.*, (1989) caused considerable interest and concern as it had been considered that the epimerase enzyme maintained a normal equilibrium between UDP-glucose and UDP-galactose. Many patients and their families also learned of the deficiency of UDP-galactose and asked to be treated with uridine. However, two independent laboratories, using alternative methods of measurement, then reported different normal values for erythrocyte UDP-glucose and UDP-galactose to those published by Ng *et al.*, (1989) (Kirkman Jr, 1990; and Palmieri *et al.*, 1990). These reports lead to much correspondence regarding the purity of the UDP-glucose dehydrogenase and the validity of the extraction technique used by Ng *et al.* (1989) in their enzymatic assay (Kirkman Jr, 1991; Kirkman Jr and Clemons, 1993; and Ng *et al.*, 1993b). The normal concentrations of erythrocyte UDP-glucose and UDP-galactose reported by Ng *et al.*, (1989) were 3 to 4-fold higher than those observed by others using enzymatic (Kirkman Jr, 1992), HPLC (Palmieri *et al.*, 1991), ^{31}P-NMR spectroscopy (Wehrli *et al.*, 1992), and combined enzymatic and HPLC techniques (Gibson *et al.*, 1993). These other groups also reported no difference in the concentration of erythrocyte UDP-galactose between galactosaemic children and children with other metabolic disorders who were on protein-restricted diets (Berry *et al.*, 1992; and Kirkman Jr, 1992). However, the mean UDP-galactose concentration was significantly lower when the galactosaemic patients were compared with normal children and adults, although there was considerable overlap with the

normal range (Berry *et al.*, 1992; and Keevill *et al.*, 1993). When they extended their patient numbers, they observed that the ratio of UDP-glucose to UDP-galactose was increased in the majority of galactosaemic erythrocytes (Keevill *et al.*, 1993; and Gibson *et al.*, 1995b). This suggested that the epimerase enzyme did not maintain a normal equilibrium in galactosaemia.

Most authors then agreed that although UDP-galactose was decreased in galactosaemic erythrocytes, the deficiency was not as large as originally suggested by Ng *et al.*, (1989). HPLC was proposed as the preferred method for the estimation of UDP-hexose concentrations (for discussions see Holton *et al.*, 1993a; Holton *et al.*, 1993b; Kirkman, 1995; and Segal, 1995). When other cells types were examined, UDP-galactose, and its ratio with UDP-glucose, were reported as normal in galactosaemic leucocytes (Gibson *et al.*, 1994) and skin fibroblasts (Gibson *et al.*, 1994; and Keevill *et al.*, 1994). There was also no correlation between the UDP-hexose content of leucocytes and erythrocytes from the same blood sample (Palmieri *et al.*, 1993; and Gibson *et al.*, 1994). This suggested that the abnormalities of sugar nucleotide metabolism were confined to galactosaemic erythrocytes only.

Ng *et al.*, then reported their results using an HPLC method to measure UDP-hexose concentrations in galactosaemic erythrocytes and fibroblasts (Xu *et al.*, 1995a). Their method additionally incorporated alkaline phosphatase to remove AMP. AMP accumulated in their samples during storage and had a similar column retention time to UDP-galactose. With this method, their normal values for erythrocyte UDP-hexose were now similar to others using HPLC. However, they continued to observe a significant decrease in the concentration of UDP-galactose in those galactosaemic erythrocytes and fibroblasts that had no detectable GALT activity. There was no overlap of values with the normal range. Those erythrocytes or fibroblasts that had residual GALT activity ($> 0.1\%$ of normal) had normal concentrations of UDP-galactose. This dependence of UDP-galactose on the level of GALT activity had been noted previously (Ng *et al.*, 1989; and Schweitzer *et al.*, 1993). Xu *et al.*, (1995a) then proposed that it was necessary to correctly classify patients when interpreting their UDP-galactose levels. This required a sensitive radiochemical assay of GALT (Xu *et al.*, 1995b). Earlier studies had not reported GALT activities in their patients in such detail (Keevill *et al.*, 1993; and Gibson *et al.*, 1995b). Ng *et al.* concluded that this information was important when comparing clinical and biochemical outcomes in galactosaemia (Xu *et al.*, 1995b).

Patients with several other metabolic disorders, who are treated with low-protein and consequently low-lactose diets, also have decreased mean concentrations of erythrocyte UDP-galactose (Berry *et al.*, 1992; and Kirkman Jr, 1992). Gibson *et al.*, (1996) therefore supplemented the diets of six patients with maple syrup urine disease with galactose, and was able to normalise their concentrations of erythrocyte UDP-galactose. Gibson *et al.*, (1996) concluded that the amount of dietary galactose traversing the Leloir pathway contributed to the steady-state concentration of UDP-galactose and modulated the epimerase-mediated equilibrium. They proposed that a deficiency of GALT, and a galactose-free diet, would similarly influence the equilibrium in galactosaemia.

The debate over the extent of the UDP-galactose deficiency in galactosaemia and the mechanism for the disturbance in the epimerase equilibrium has occupied researchers in this area for the last 10 years. The interest has been stimulated by the possibility that a deficiency of UDP-galactose could disturb glycoprotein and glycolipid synthesis and therefore contribute to the long-term complications. However, whether there actually is a defect in glycoprotein and glycolipid synthesis in galactosaemia has not received as much attention. The evidence that suggests a disturbance in these pathways is reviewed in the next section.

2.6 Abnormalities of glycoprotein and glycolipid in classical galactosaemia

Post-mortem studies

Few studies have examined the pathology of the brain in galactosaemia in any detail. In 1962, Crome examined the brain from an eight-year-old boy with galactosaemia and significant mental retardation, who died from bronchopneumonia. The major histological change in the brain was marked gliosis of the white matter, which was accentuated around the ventricles and margin of the brain stem. The myelin fibres also showed diffuse pallor of staining particularly in the white matter of the cerebral hemispheres. The white matter of the internal capsule, brainstem, and cerebellum also contained many round lytic lesions. There was no significant loss of cortical neurones, however, the cerebellum showed a marked loss of Purkinje cells (also reported by Smetana and Olen, 1962; Suzuki *et al.*, 1966; and Haberland *et al.*, 1971) with less conspicuous loss of the granular layer. The total findings were consistent with a non-specific gliotic encephalomyelopathy. Crome (1962) was uncertain if these changes were representative of galactosaemia as the patient had significant hyperbilirubinaemia as a neonate (although the basal ganglia are primarily involved in residual kernicterus), and had been poorly controlled on diet. Lipid analysis of the brain revealed some loss of total cholesterol and phospholipid from both the cerebral cortex and white matter. However, the cerebroside and total hexosamine content of the white matter were probably normal.

Haberland *et al.*, (1971) examined the brain from a 25-year-old male with galactosaemia who remained undiagnosed until 13 years of age, and who suffered from severe mental retardation, recurrent hypoglycaemia, frequent seizures, and an extrapyramidal motor disorder. The patient died from bronchopneumonia. The brain was microcephalic and had reduced white matter, enlarged ventricles, glial fibrosis, and patchy pallor of myelin staining throughout the white matter. The entire cortex showed neuronal loss and in the cerebellar cortex there was cerebellar degeneration and diffuse Purkinje cell loss. Biochemical analysis of the brain revealed a reduction of cerebroside in the white matter, which was consistent with the histological findings. This suggested either abnormal or incomplete myelination. The other main biochemical abnormalities were a reduced hexosamine content of brain glycoprotein and glycosaminoglycans, and alterations in the distribution of glycoprotein in the white and grey matter (see also Brunngraber *et al.*, 1971). The sialic acid content of the brain glycoprotein was normal. The majority of gangliosides also did not differ significantly from control brains (Witting *et al.*, 1972). Unfortunately, the findings of both these post-mortem examinations suffer from two problems. First, it was not known to what extent the galactosaemia directly contributed to the histological and biochemical changes. And second, there were difficulties in obtaining appropriate controls for the lipid and glycoprotein comparisons. The control in Haberland *et al.*, (1971) was a normal eight-year-old girl and even the authors questioned whether the changes were age-related.

Smetana and Olen, (1962) examined the brains from seven children with galactosaemia who died between eight days and 50 months of age. Unfortunately, the tissues had been inadequately and poorly preserved and a complete study of the brains was not possible. However, significant changes were repeatedly observed. The dentate nucleus of the cerebellum and the olivary nuclei of the medulla showed nerve cell loss and gliosis, with selective necrosis of the Purkinje cells. There was also gliosis in the cerebral cortex and white matter. These abnormalities were present even in an infant who survived only a few days. There was no biochemical analyses of these brains.

Varma *et al.*, (1962) examined the brains from five children with galactosaemia and observed no disturbance in the glucose or galactose content of the brain gangliosides and cerebrosides. Unfortunately, the brains were preserved in formalin for various periods and these results have generally been discounted. Petry *et al.*, (1991) similarly examined the galactose content of the glycolipids from the brain of a galactosaemic infant who died at 12 days of age from *E. coli* sepsis. Two control brains were obtained from neonates who died of congenital heart disease and each brain was frozen and stored at -70°C immediately after death. The galactosaemic brain exhibited a 3 to 4-fold increase in glucosylceramide and a threefold decrease in galactosylceramide. GA2, a ganglioside that contains N-acetylgalactosamine, was also reduced to 12% of normal and GM3, formed by the addition of sialic acid to lactosylceramide, was increased sevenfold. Most other lipids were unaffected. Petry *et al.*, (1991) suggested that the results indicated a deficiency of galactose- and N-acetylgalactosamine-containing glycolipids in the brain, with an increase in the immediate precursors to these glycolipids. The cell membranes from a transferase-deficient lymphoblastic cell line exhibited a similar glycolipid imbalance (Petry *et al.*, 1991). This suggested that the glycolipid disturbance affected all tissues in galactosaemia and was continuously expressed. Petry *et al.*, (1991) concluded that the glycolipid disturbance most likely resulted from a deficiency of UDP-galactose and UDP-N-acetylgalactosamine.

Radiological studies

Several post-mortem examinations observed cerebral oedema in the infants who died of galactosaemia during the acute phase of the illness (Smetana and Olen, 1962; and Suzuki *et al.*, 1966). The early radiological studies of the brain in galactosaemia also observed diffuse cerebral oedema on CT scan, usually in galactosaemic infants who had signs of increased intracranial pressure (Belman *et al.*, 1986; and Perelmuter *et al.*, 1989). Marano *et al.*, (1987) also observed diffuse decreased attenuation throughout the white matter on CT scan of the brain in a five-week-old boy at diagnosis. At 19 months of age, a repeat scan showed significant recovery of the white matter attenuation. Marano *et al.*, (1987) proposed that the white matter changes was secondary to either cerebral oedema or loss of myelin. They also considered the abnormalities were part of the acute presentation and resolved with treatment. There were also brief reports of the CT findings of the brain in the older galactosaemic patients who had the triad of mental retardation, tremor, and cerebellar dysfunction (Jan and Wilson, 1973; Lo *et al.*, 1984; Bohles *et al.*, 1986; and Koch *et al.*, 1992). The common findings in these patients included white matter attenuation and atrophy of the cerebellum, brainstem, and basal ganglia (Lo *et al.*, 1984; Bohles *et al.*, 1986; and Friedman *et al.*, 1989). Choulot *et al.*, (1991) then reported an abnormal pattern of myelination on magnetic resonance imaging (MRI) of the brain in a boy with severe neurologic complications. Koch *et al.*, (1992) additionally reported abnormal myelination in two adult galactosaemic siblings who had progressive neurologic disease. These patients also had multiple foci of increased signal intensity, predominantly in the periventricular white matter on T2-weighted MR images.

It was not until 1992, however, that there was any systematic radiological evaluation of the brain in galactosaemic patients on treatment. Nelson Jr *et al.*, (1992) reported the MRI appearances in 67 cases. The patients ranged in age from one month to 42 years with a median age of 10 years. Twenty-four of the patients also had a repeat examination 1–4 years after the initial MRI. No congenital malformations were seen in any of the patients. The white matter signal intensity in the infants under one year of age appeared normal. However, 52 of 55 patients older than one year showed increased signal intensity in the peripheral white matter on intermediate- and T2-weighted images. At follow-up, the white matter abnormalities did not progress in the adults, however, seven of the eight infants under one year of age developed the

abnormal patterns of peripheral myelination. Basal ganglia or brain stem abnormalities were not seen in any patients. Twenty-two patients also had mild cerebral atrophy, eight had cerebellar atrophy, and 11 had multiple small hyperintense lesions in the cerebral white matter on T2-weighted images, which clustered about the horns of the lateral ventricles. Nelson Jr *et al.*, (1992) concluded that the abnormal myelin signal in the peripheral white matter probably represented an abnormality in the structure of myelin. They suggested that this was because of impaired synthesis of galactocerebrosides in myelin. A similar study in six adult patients also observed abnormal peripheral white matter signal intensity on T2-weighted images (Moller *et al.*, 1995). Patients with galactokinase-deficiency galactosaemia had normal appearances of the brain on MRI (Kaufman *et al.*, 1995a).

Nelson Jr *et al.*, later extended their study to include patient clinical data (Kaufman *et al.*, 1995b). It then became apparent that the MRI abnormalities did not correlate with the age of the patient at diagnosis, the severity of the illness at presentation, or the cognitive outcome. Kaufman *et al.*, (1995a) also assessed the integrity of the myelinated pathways by recording somatosensory evoked potentials (SEP) in 60 of these patients. SEP were obtained in the median and posterior tibial nerves and were abnormal in 17 of 60 patients who had median nerve, and in 26 of 34 patients who had posterior tibial nerve studies. The incidence of abnormal SEP was higher in infants than in the older children and adults. The majority of disturbances were due to central slowing rather than peripheral neuropathy. The SEP abnormalities did not correlate with the age at diagnosis, the severity of neurologic complications, the cognitive outcome, or the extent of white matter attenuation on MRI. However, there was a correlation with the severity of neonatal illness and with the presence of focal white matter lesions on MRI. Kaufman *et al.*, (1995a) concluded that the cognitive outcome and neurologic involvement in galactosaemia were related more to neuronal function, whereas the delayed SEP were secondary to defects in central myelination. They suggested that infants were possibly more affected during myelination of the brain and that the focal white matter lesions could be a marker of the severity of the dysmyelination. Kaufman *et al.*, (1995a) again proposed abnormal galactosylation of myelin as a possible mechanism.

Cell culture studies

Few studies have attempted to examine glycoprotein or glycolipid synthesis in transferase-deficient cells in culture. The only study of glycolipid from the cell membrane of a single lymphoblastic cell line has already been mentioned (Petry *et al.*, 1991; page 33). There have been several more studies of glycoprotein synthesis. In 1979, Tedesco and Miller radiolabelled five GALT-deficient skin fibroblast cultures with [^{35}S]-sulphate for 24 hours and then precipitated the cellular protein with TCA. They observed that the galactosaemic fibroblasts incorporated 65% less [^{35}S]-sulphate into protein than normal controls, when cultured in a medium that contained galactose for 4–6 days. Under the same experimental conditions, the incorporation of [^{3}H]-uridine was unaffected. They concluded that the reduced incorporation of [^{35}S]-sulphate was a direct result of the abnormal galactose metabolism, and not hexose starvation, as the [^{35}S]-sulphate incorporation in a hexose-free medium was normal. Tedesco and Miller (1979) drew no specific conclusion about the mechanism for the reduced incorporation, but others have suggested it represented a decrease in the galactose residues of glycoprotein that were available for sulphation (Segal, 1995).

Brown *et al.*, (1977) measured the incorporation of tritiated galactose, fucose, mannose, glucosamine, and N-acetylmannosamine (a sialic acid precursor) into an acid-precipitable fraction of cultured lymphocytes from three galactosaemic children. The lymphocytes were grown in RPM1 medium supplemented with 10% dialysed fetal bovine serum (FBS) and were radiolabelled over 20 hours. All the radiolabelled sugars were incorporated normally except

for galactose, which was incorporated at less than 7% of the controls. Similar results were obtained when the radiolabelling was repeated in glucose-free RPM1 medium. The galactosaemic lymphocytes were also sensitive to the effects of ricin. Ricin is a lectin that binds selectively to galactose residues in cell surface glycoproteins and inhibits protein synthesis. This suggested that the surface glycoproteins in the galactosaemic lymphocytes were structurally normal. In similar experiments, the stimulatory effects of the galactose and N-acetylgalactosamine-binding lectins *Abrus precatorius* agglutinin and *Phaseolus vulgaris* phytohaemagglutinin on the incorporation of [^{3}H]-thymidine were also normal (Brown *et al.*, 1977). Brown *et al.*, (1977) concluded that the glycoproteins were structurally complete in GALT-deficient lymphocytes. They also suggested that the epimerase enzyme produced enough UDP-galactose for glycoprotein synthesis. Friedman *et al.*, (1975) also observed normal incorporation of [1-^{14}C]-glucose into TCA-precipitable material from one galactosaemic fibroblast culture during a 24 hour incubation, and Xu *et al.*, (1989b) reported that the incorporation of [1-^{14}C]-glucose into TCA-insoluble material from ovarian tissue slices of one galactosaemic female was normal.

In 1990, Dobbie *et al.*, in a different approach, directly measured the total cell carbohydrate content of six galactosaemic fibroblast cultures. The monosaccharides were identified by gas chromatography. The amount of galactose or mannose recovered from the fibroblast extracts was not significantly different to control values. However, the ratio of galactose to mannose, and sialic acid plus galactose to mannose, was significantly reduced in the galactosaemic fibroblasts ($p < 0.05$). They concluded that the results indicated a defect in terminal galactosylation and sialylation of glycoprotein and that this was consistent with a deficiency of UDP-galactose.

In 1992, Ornstein *et al.*, then suggested that if there was a defect in terminal galactosylation in galactosaemia, the cells should contain increased acceptor sites for galactose. They measured the ability of skin fibroblast extracts from nine galactosaemic children to serve as acceptors for galactose. Their assay used UDP-[^{3}H]-galactose as the donor and a purified milk galactosyltransferase. They observed a 50% increase in the galactose acceptor activity in the galactosaemic cell extracts. The mean acceptor activity in the nine galactosaemic extracts was 0.146 versus 0.098 pmol/15 min/µg cell protein in seven normal controls ($p < 0.005$). However, three of the galactosaemic extracts had results that were within the normal range and two of the controls had acceptor levels that were clearly in the galactosaemic range. The activity of N-acetylglucosamine:UDP-galactose galactosyltransferase was also significantly higher ($p < 0.01$) in the galactosaemic extracts. Ornstein *et al.*, (1992) suggested the results indicated that the glycoconjugates from galactosaemic fibroblasts were in a hypogalactosylated state. However, the study did not determine if these were N-linked or O-linked N-acetylglucosaminide acceptor sites. They also suggested that the increased UDP-galactose galactosyltransferase activity was a compensation either for low levels of UDP-galactose or inhibition of the enzyme by galactose metabolites. In a later analysis of the data, Ornstein *et al.*, found no correlation between the galactose acceptor level and residual GALT activity or the Q188R mutation (Segal, 1995).

Abnormal serum proteins

Non-enzymatic galactosylation

The β-chain of haemoglobin A can be non-enzymatically glycosylated to produce a group of glycated haemoglobins known as haemoglobin A_1. Hyperglycaemia increases the formation of haemoglobin A_1 and glycated protein is implicated in the pathophysiology of diabetes mellitus

(Cerami *et al.*, 1979). Howard *et al.*, (1981) also detected increased haemoglobin A_1 in two newly diagnosed infants with galactosaemia (24.1% and 23.8%; normal range 6.3–8.2%). With exclusion of galactose from the diet, the levels of haemoglobin A_1 fell to near normal over several weeks, which suggested that the increased blood galactose was directly responsible. The levels of haemoglobin A_1 in patients with good dietary control were normal (Howard *et al.*, 1981). Haemoglobin A_1 was therefore proposed as a possible measure of dietary compliance in galactosaemia. Urbanowski *et al.*, (1982) subsequently observed that other serum proteins were affected when they identified galactosylated serum albumin in a 13-day-old boy with untreated galactosaemia. The concentration of galactose in the blood in untreated galactosaemia is usually less than glucose. However, Bunn, (1982) reported that galactose had a less stable ring structure than glucose and was therefore more reactive in forming adducts with protein. It was then proposed that non-enzymatic galactosylation of protein in untreated galactosaemia could contribute to the pathophysiology of the disorder (Urbanowski *et al.*, 1982).

More recently, Landing *et al.*, (1993) observed increased binding of biotinylated lectins to galactose and galactosamine residues in a variety of galactosaemic tissues. The formalin-fixed tissues were obtained from eight children with galactosaemia who were mostly untreated. Landing *et al.*, (1993) suggested that the lectin-staining patterns were consistent with non-enzymatic galactosylation and proposed that non-enzymatic galactosylation of protein was widespread in untreated galactosaemia. Several of the liver biopsies, from patients on treatment, also had evidence of non-enzymatic galactosylation in the cytoplasm of the hepatocytes.

Hyposialylated isoforms

In 1992, Spaapen *et al.*, examined the serum from two infants with untreated galactosaemia and observed a shift in the mobility of transferrin towards the cathode on isoelectric focusing. This suggested the presence of hyposialylated isoforms of transferrin. Jaeken *et al.*, (1992) then reported that the isoform patterns for serum β-N-acetylhexosaminidase and α-fucosidase were also abnormal in these infants. The isoform patterns for the enzymes returned to normal one week after starting the diet. At that time, these authors were also studying patients with the CDG syndrome, a new disorder of N-glycosylation (Jaeken *et al.*, 1991). The CDG syndrome is characterised by abnormalities of the nervous system, liver, gastrointestinal tract, kidneys, and gonads, and the serum from these patients also contains hyposialylated isoforms of glycoprotein. The CDG syndrome and classical galactosaemia have many clinical similarities, which lead the authors to propose that classical galactosaemia was a secondary 'Golgi disorder' (Jaeken *et al.*, 1992).

Since then, several other groups have also reported the presence of hyposialylated glycoprotein in the serum in untreated galactosaemia. Shin *et al.*, (1993) observed extra cathodal bands of serum α_1-antitrypsin in six untreated patients, which then normalised with galactose-restriction. The α_1-antitrypsin isoforms in the serum of 33 patients on treatment were normal (Shin *et al.*, 1993). Winchester *et al.*, (1995) observed a pattern of serum transferrin isoforms in an untreated galactosaemic infant that was similar to the pattern in the CDG syndrome. The isoforms were normal when retested three weeks later on diet. Van Pelt *et al.*, (1996b) quantified the serum transferrins in five untreated galactosaemic infants. Although the total transferrin concentration was decreased, the hyposialylated transferrins were increased. The concentrations of the transferrins in 30 galactosaemic patients on treatment were normal. Van Pelt *et al.*, (1996b) proposed that galactose or its metabolites interfered with N-glycosylation of various glycoproteins. Only one study to date has examined the structure of the glycoconjugates from the serum in untreated galactosaemia. Charlwood *et al.*, (1995)

examined the N-glycans of transferrin from untreated galactosaemic serum with matrix-assisted laser desorption ionisation time-of-flight mass spectrometry. They briefly reported that the glycans contained less sialic acid than normal. Prestoz *et al.*, (1997) recently reported that the sera from three galactosaemic women with ovarian failure contained hyposialylated isoforms of FSH.

The concentrations of certain serum glycoproteins can also be decreased in untreated galactosaemia. Spaapen *et al.*, (1992) reported a decrease in the concentration of thyroxine-binding globulin (TBG) in untreated galactosaemic serum that was similar to the CDG syndrome. Neonatal programmes that screen for hypothyroidism occasionally observe low serum thyroxine concentrations in neonates with untreated galactosaemia, which can be associated with transient increases in thyroid stimulating hormone (TSH) (Berger *et al.*, 1983; and von Petrykowski, 1984). Campbell and Kulin, (1984) observed a decreased serum thyroxine and an increased T_3-resin uptake in a male galactosaemic infant, which they proposed was secondary to decreased serum TBG. The thyroid functions returned to normal soon after starting a galactose-free diet. Van Pelt *et al.*, (1996b) also reported a decrease in the total transferrin concentration in the serum of five galactosaemic infants at presentation. It is not known if the decreased serum TBG and transferrin concentrations in untreated galactosaemia are secondary to the liver dysfunction or abnormal glycoprotein synthesis.

The South Australian experience

The Department of Chemical Pathology of the Women's and Children's Hospital, Adelaide has examined the serum transferrin isoforms from two galactosaemic infants. The infants were identified by the South Australian Neonatal Screening Programme and small aliquots of plasma were obtained for analysis from samples collected during the routine monitoring of the infants. The transferrin isoforms were analysed by isoelectric focusing in polyacrylamide gels (ampholyte range 4.0–6.5) and immunoprecipitated on cellulose acetate membranes. The method is outlined in Appendix I (page 152). Normal serum transferrin contains predominantly two biantennary sialylated N-linked glycans and appears as a tetrasialotransferrin band on isoelectric focusing. Minor bands of penta- and trisialotransferrin are also usually present.

The galactosaemic infants were both identified at age one week. *Patient 1* was female and at presentation had feeding difficulties, significant jaundice, a biochemical hepatitis, and required an exchange transfusion. The hyperbilirubinaemia was predominantly unconjugated (total bilirubin 685 μmol/L, conjugated 72 μmol/L). Her serum contained asialo- and disialotransferrins in addition to the normal tetrasialotransferrin band (Figure 2.3). The isoforms then returned to normal over eight days after starting a galactose-free diet. As might be expected, the hyposialylated transferrins virtually disappeared from the serum immediately following the exchange transfusion. However, they were again apparent in the serum 24 hours later (Figure 2.3). The transferrin isoforms also remained abnormal beyond the resolution of the hyperbilirubinaemia and the biochemical hepatitis.

Patient 2, a male, also had feeding difficulties, jaundice, and a biochemical hepatitis at presentation. The hyperbilirubinaemia was again predominantly unconjugated (total bilirubin 307 μmol/L, conjugated 39 μmol/L) and he was treated with phototherapy. His serum contained asialo-, monosialo-, and disialotransferrins in addition to the normal band (Figure 2.4, page 39). The isoform pattern remained unchanged over the next three days despite dietary treatment. Cord blood from this infant also contained bands of hyposialylated transferrin. It was reported that normal cord serum contained about twice the normal amount of carbohydrate-deficient transferrin (Stibler and Kristiansson, 1991), predominantly asialo-,

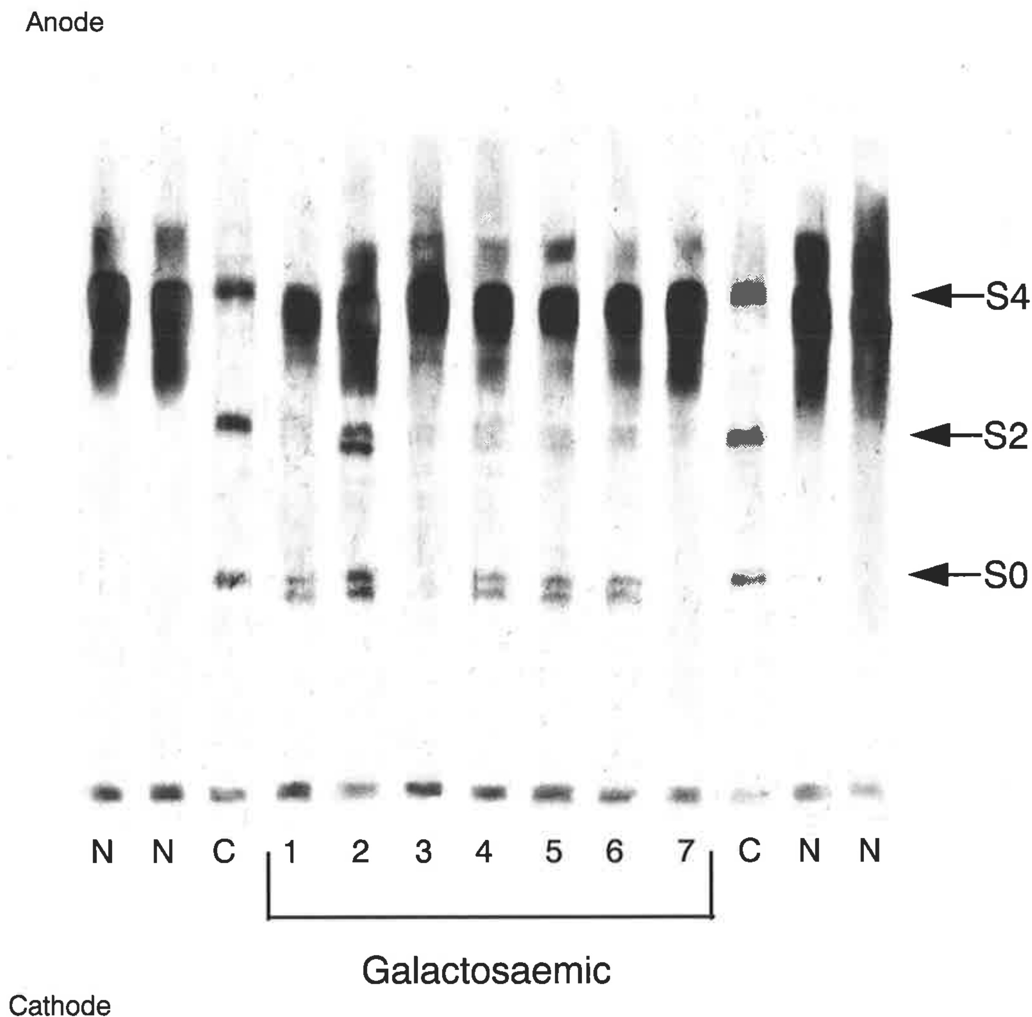


Figure 2.3. Transferrin isoforms from the plasma of an untreated galactosaemic infant (*Patient 1*) analysed by isoelectric focusing with immunodetection. *Sample 1* was obtained when the patient was one week of age and immediately before treatment was started. *Sample 2* was the same as *sample 1* but loaded with double the volume. *Sample 3* was taken six hours later, immediately following an exchange transfusion. *Samples 4, 5*, and *6* were from days 1, 2, and 3 post-treatment, respectively, and *sample 7* was from day 8 post-treatment. Control plasma was included from a patient with the CDG syndrome type I (*C*) and from several normals (*N*). *S0, S2*, and *S4* refer to asialo-, disialo-, and tetrasialotransferrin, respectively. The doublet banding of *S0* and *S2* in the patient was due to microheterogeneity of the protein, which was occasionally seen in normal controls.

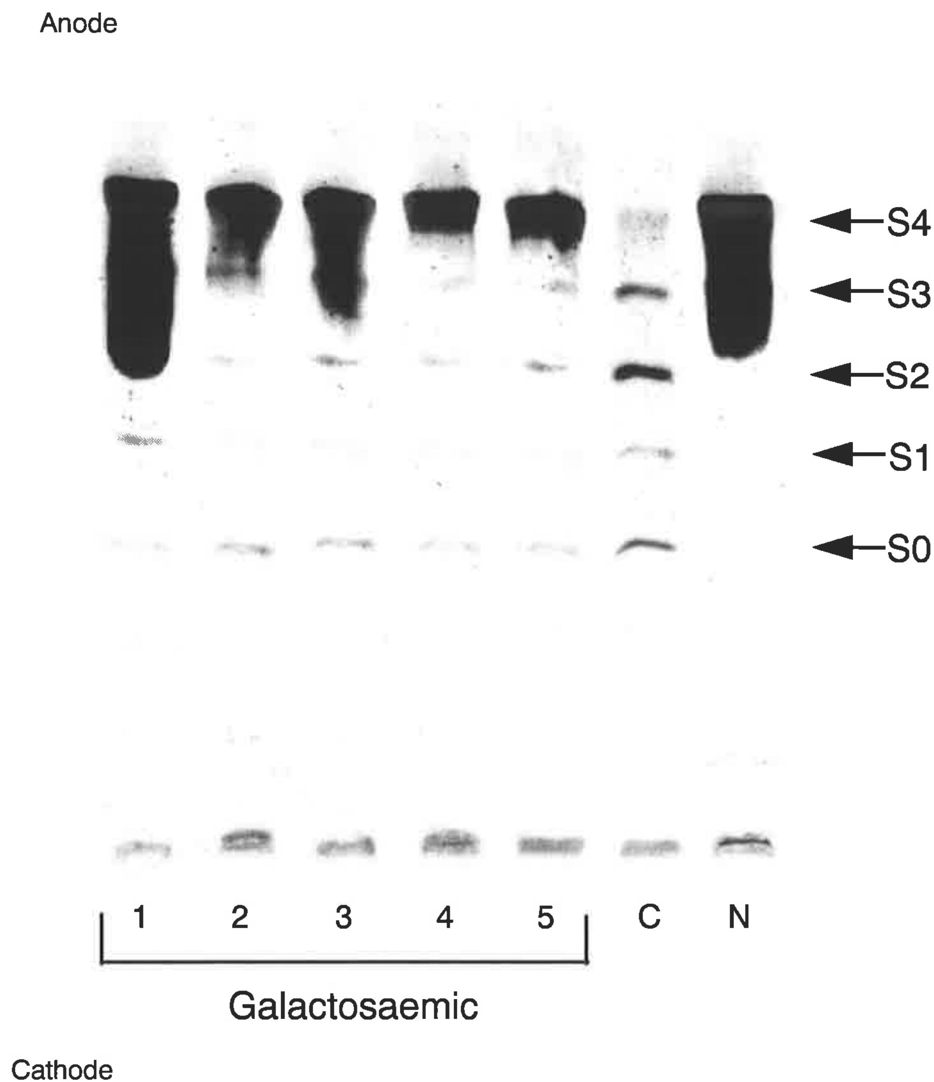


Figure 2.4. Transferrin isoforms from the plasma of an untreated galactosaemic infant (*Patient 2*) analysed by isoelectric focusing with immunodetection. *Sample 1* was from cord blood obtained at birth. *Sample 2* was taken when the patient was one week of age and immediately before treatment was started. *Samples 3, 4*, and *5* were from days 1, 2, and 3 post-treatment, respectively. Control plasma was included from a patient with the CDG syndrome type I (*C*) and one normal (*N*). *S0, S1, S2, S3*, and *S4* refer to asialo-, monosialo-, disialo-, trisialo-, and tetrasialotransferrin, respectively. Total transferrin measured in *samples 1, 2, 3, 4*, and *5* was 2.0, 1.4, 1.2, 1.2, and 1.3 g/L, respectively.

monosialo-, and disialotransferrins, and that the level returned to normal over the first week of life (van Pelt *et al.*, 1996a). However, the total transferrin concentration remained unaffected. The mechanism for the increase in hyposialylated transferrin in normal cord blood is not known. The total transferrin concentration in the cord serum from *patient 2* was normal (2.0 g/L), but at presentation one week later it was decreased and remained so during the initial days of treatment (Figure 2.4, page 39).

2.7 Conclusions and hypothesis

The cause of the long-term complications in classical galactosaemia is not known. The complications are also not influenced by the current treatments available to patients. The evidence from the post-mortem, radiological, and experimental studies suggests that there are abnormalities of glycoprotein and glycolipid synthesis in galactosaemia. Many of the studies have proposed that these abnormalities are consistent with a deficiency of UDP-galactose. However, the evidence for a defect in galactosylation is only presumptive. UDP-galactose deficiency is only one of several metabolic disturbances in GALT deficiency that could affect glycoprotein or glycolipid synthesis.

The increase in hyposialylated transferrin in the serum of untreated galactosaemic infants suggests a specific defect in N-glycosylation of protein. The synthesis and processing of N-linked oligosaccharides occurs in the cytoplasm, the lumen of the ER, and the Golgi apparatus, and components of this pathway could be affected by a disturbance in galactose metabolism. Galactosaemia and the CDG syndrome, which is a disorder of N-glycosylation, also have many clinical and biochemical similarities. This suggests that similar pathological mechanisms may be present in galactosaemia. It is therefore proposed that there are abnormalities of N-glycosylation in classical galactosaemia, and that these abnormalities could contribute to the long-term complications.

The next chapter introduces the terminology associated with N-glycosylation and outlines the steps in the normal pathway. It also reviews the clinical and biochemical features of the human disorders of N-glycosylation, particularly the CDG syndrome, and concludes with a discussion of the choice of an appropriate cellular model to study N-glycosylation in galactosaemia.

3

N-glycosylation of protein

3.1 Introduction

The aim of this chapter is to introduce the terminology associated with protein glycosylation and to specifically outline the steps involved in the synthesis and processing of N-linked oligosaccharides. The chapter then reviews the known human disorders of N-glycosylation, both primary and secondary, and examines the range of clinical and biochemical abnormalities that are associated with disturbed N-glycosylation. The CDG syndrome, a disorder that has many clinical and biochemical features in common with transferase-deficiency galactosaemia, is particularly emphasised. The chapter then concludes with a review of the characteristics of transferase-deficient skin fibroblasts in culture and examines the use of fibroblasts as a cellular model for the study of N-glycosylation in galactosaemia.

3.2 The process of N-glycosylation

The summary of the synthesis, trafficking, and processing of N-linked glycoprotein in this section was complied from extensive reviews of the subject by Staneloni and Leloir, 1982; Kornfeld and Kornfeld, 1985; Lehrman, 1991; Kobata, 1992; Lis and Sharon, 1993; Hirschberg and Snider, 1987; Daniel *et al.*, 1994; and Rothman, 1994.

Glycoproteins are proteins to which carbohydrates are covalently linked through glycosidic bonds and they are found widely distributed in all living organisms. Glycosylation is a common modification of proteins in higher organisms where glycoproteins function as enzymes, hormones, antibodies, structural proteins, carrier proteins, mucins of epithelial secretions, membrane transport proteins, and receptors. Proteoglycans, which are a specific class of glycoproteins with distinctive carbohydrate structures, are important constituents of the extracellular matrix of animal connective tissues.

Carbohydrates are attached to protein in three main ways:

1. An N-glycosidic bond to the side-chain of asparagine, where the asparagine is part of a triplet motif, asparagine-X-serine/threonine (where X can be any amino acid except proline).
2. An O-glycosidic bond to the side-chain of serine, threonine, hydroxylysine or hydroxyproline.
3. As part of a glycosylphosphatidylinositol (GPI) membrane anchor.

In N-linked and O-linked glycosylation there is considerable diversity in the composition of the oligosaccharides that can be attached at each potential glycosylation site. This leads to site microheterogeneity. Each glycoprotein may also have multiple sites for N- and O-linked glycosylation and not all sites will necessarily be fully occupied. Therefore, for a given eukaryotic glycoprotein, there will usually not be a single structural form but rather a set of glycosylated variants of a common polypeptide. These glycoforms differ in the number, location, and composition of their oligosaccharide structures.

Synthesis and processing of N-linked oligosaccharides

Asparagine-linked, or N-linked, oligosaccharide structures are composed of three main types, termed high mannose, hybrid, and complex. Typical examples of these oligosaccharide structures are shown in Figure 3.1. They all share a common pentasaccharide core structure Manα1–3(Manα1–6)Manβ1–4GlcNAcβ1–4GlcNAc-Asn (where Man represents mannose; GlcNAc, N-acetylglucosamine; and Asn, asparagine), but differ in their outer branches. The high mannose oligosaccharides typically have two to six additional mannose residues linked to the pentasaccharide core. The typical complex-type oligosaccharide contains two outer branches with a sialyllactosamine sequence. However, the structure can be modified either by the addition of extra branches on the α-linked mannoses to give tri- and tetraantennary structures, or by the addition of extra sugars to the core. Often, a fucose is linked α1,6 to the innermost N-acetylglucosamine residue, and a bisecting N-acetylglucosamine is linked β1,4 to the β-linked mannose residue. The outer chain sialyllactosamine structure can also be modified by the addition of extra sugars, usually fucose or galactose, or additional polysialic acid. Another important outer branch variant consists of repeating lactosamine disaccharides $(Gal\beta1–4GlcNAc\beta1–3)_n$ (where Gal represents galactose) that were first discovered on erythrocyte membrane glycoproteins. These polylactosamine oligosaccharides may be substituted in various ways and have been shown to carry the ABH and Ii blood group antigens. The hybrid N-linked oligosaccharides are so named because they contain features of both high mannose and complex-type oligosaccharides. Most hybrid structures contain a bisecting N-acetylglucosamine linked β1,4 to the β-linked mannose of the core structure.

All N-linked oligosaccharides have a common pentasaccharide core because they arise from the same biosynthetic precursor, a dolichol-linked oligosaccharide, which is transferred to nascent peptide chains in the lumen of the ER. The variability in the outer-chain structures is then introduced by a series of glycosidases and glycosyltransferases, which process the glycoproteins as they traverse the ER and Golgi apparatus.

Synthesis and transfer of dolichol-linked oligosaccharides

N-glycosylation in the ER involves the attachment of a dolichol-linked oligosaccharide, $Glc_3Man_9GlcNAc_2$-PP-Dol (where Glc represents glucose), to asparagine acceptors in nascent protein. The process begins with the synthesis of dolichol pyrophosphoryl-N-acetylglucosamine (GlcNAc-PP-Dol). This is then extended by a series of glycosyltransferase reactions, which add single sugar residues, to yield the mature dolichol-linked oligosaccharide. When complete, $Glc_3Man_9GlcNAc_2$ is transferred from the dolichol to a peptide acceptor within the lumen of the ER (Figure 3.2, page 44).

The topography of the synthesis of dolichol-linked oligosaccharides is still debated. However, the process is thought to start on the cytoplasmic side of the ER membrane with the synthesis of $Man_5GlcNAc_2$-PP-Dol. This involves the transfer of two N-acetylglucosamine residues from UDP-N-acetylglucosamine to dolichol phosphate to form $GlcNAc_2$-PP-Dol. This structure is then extended by the addition of five mannose residues, which are derived from guanosine diphosphate mannose (GDP-mannose). $Man_5GlcNAc_2$-PP-Dol is then ‘flipped’ from the cytoplasmic to the lumenal face of the ER membrane by a process that is probably enzyme-mediated. The next seven sugars (four mannose and three glucose residues) are then added to the oligosaccharide within the lumen of the ER by specific mannosyl- and glucosyltransferases. The sugars in these reactions are derived from dolichol phosphorylmannose (Dol-P-mannose) and dolichol phosphorylglucose (Dol-P-glucose). Dol-P-mannose and Dol-P-glucose are synthesised on the cytoplasmic face of the ER in reactions

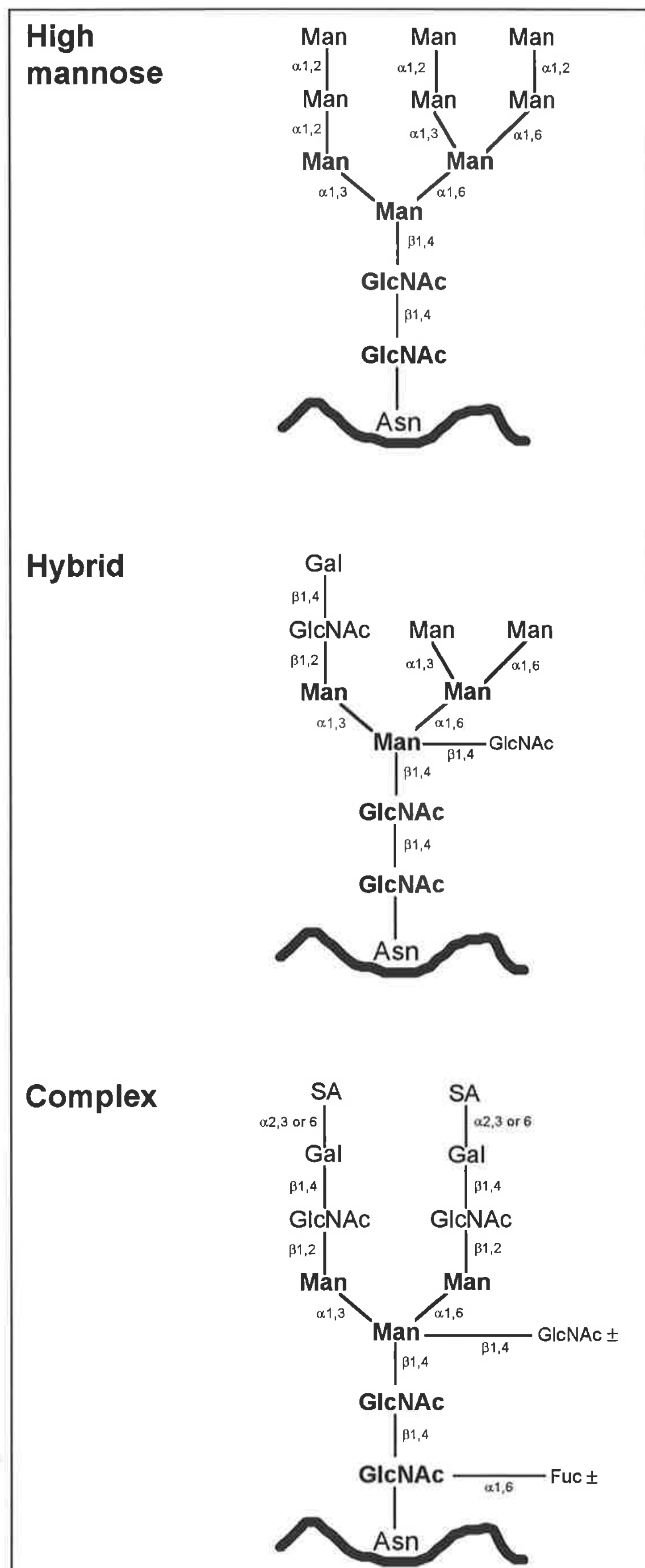


Figure 3.1. Structures of the three main types of asparagine-linked oligosaccharides: high mannose, hybrid, and complex. The bold area in each oligosaccharide structure represents the pentasaccharide core common to all N-linked glycans. *GlcNAc*, N-acetylglucosamine; *Man*, mannose; *Gal*, galactose; *SA*, sialic acid; and *Fuc*, fucose (adapted from Kornfeld and Kornfeld, 1985).

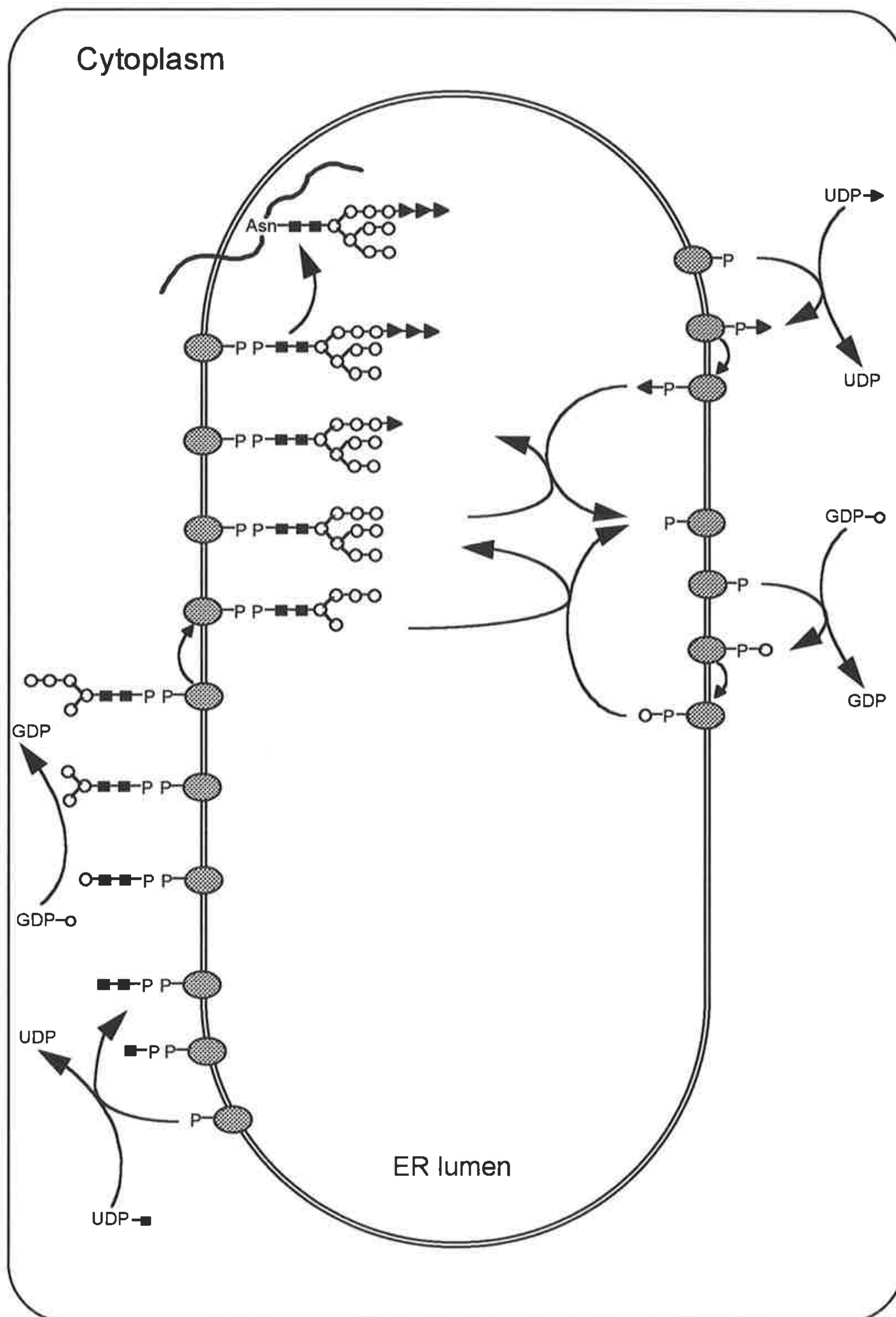


Figure 3.2. Assembly and transfer of dolichol-linked oligosaccharides in the rough endoplasmic reticulum (*ER*). The symbols represent: [dolichol phosphate symbol], dolichol phosphate; ■, N-acetylglucosamine; O, mannose; ▶, glucose; UDP, uridine 5'-diphosphate; and GDP, guanosine 5'-diphosphate (adapted from Hirschberg and Snider, 1987; and Narimatsu, 1994).

that involve dolichol phosphate and GDP-mannose and UDP-glucose. The dolichol-linked hexoses are then flipped into the ER lumen to serve as mannose and glucose donors to complete the mature dolichol-linked oligosaccharide, $Glc_3Man_9GlcNAc_2$-PP-Dol. It is also possible that UDP-glucose crosses the ER membrane and acts directly as a glucose donor within the ER.

Following completion, $Glc_3Man_9GlcNAc_2$ is transferred to asparagine acceptors in the lumen of the ER by N-oligosaccharyltransferase. The released dolichol pyrophosphate is converted back to dolichol phosphate by a pyrophosphatase and then reused in further dolichol-oligosaccharide synthesis. The efficiency of N-glycosylation depends on the supply of mature dolichol-linked oligosaccharides, the activity of N-oligosaccharyltransferase, and the availability of accessible Asn-X-Ser/Thr sequences in the protein acceptor. The initial enzyme in the pathway, UDP-N-acetylglucosamine:dolichol phosphate N-acetylglucosamine-1-phosphate transferase, is also subject to several forms of regulation.

N-linked oligosaccharide processing in the endoplasmic reticulum

The initial steps in processing of the high mannose oligosaccharide precursor begin in the lumen of the ER and may occur cotranslationally (Figure 3.3). Processing begins with the removal of the terminal glucose from $Glc_3Man_9GlcNAc_2$-Asn by α-glucosidase I. The two remaining glucoses are then removed by α-glucosidase II and then one α1,2-linked mannose is removed by ER α-mannosidase I or II to produce $Man_8GlcNAc_2$-Asn. The trimmed glycoprotein is then transported to the cis Golgi cisternae by vesicles that bud from the rough ER (RER) and fuse with the Golgi membranes. The extent of processing within the ER, however, can differ depending upon the specific glycoprotein. The deglucosylation steps are also reversible and considered to be part of the quality control mechanisms that recognise incompletely or inappropriately folded proteins within the ER.

N-linked oligosaccharide processing in the Golgi apparatus

Glycoproteins traverse the cis, medial, and trans Golgi cisternae by vesicular transport. During their transit they encounter a series of membrane-bound glycosidases and glycosyltransferases, which are aligned within the lumen of the Golgi (Figure 3.3). The processing enzymes have rigid specificities for specific oligosaccharide chain orientations and substrates. The order in which the glycoproteins are exposed to the processing enzymes also partly determines the final oligosaccharide structure. It is the interaction of these various enzymes with specific glycoproteins that gives rise to the variety of high mannose, hybrid, and complex N-linked oligosaccharides. Although it is useful to model the enzymes as resident within specific Golgi compartments, their activities are usually found in several compartments.

The $Man_8GlcNAc_2$ oligosaccharides that reach the cis Golgi cisternae are trimmed further by Golgi α-mannosidase I to produce $Man_5GlcNAc_2$-Asn (Figure 3.3). Those oligosaccharides that are destined to become complex N-linked oligosaccharides then have two further outer mannose residues removed by Golgi α-mannosidase II. Chain elongation then begins in the medial cisternae with the addition of N-acetylglucosamine to $Man_5GlcNAc_2$-Asn by N-acetylglucosaminyltransferase I. A further N-acetylglucosamine is then added in β1,2-linkage to the terminal α1,6 mannose of $GlcNAcMan_3GlcNAc_2$-Asn by N-acetylglucosaminyltransferase II. The complex N-linked oligosaccharides are then completed in the trans Golgi cisternae by the addition of galactose and sialic acid to each branch. This is catalysed by galactosyltransferase and sialyltransferase. Fucose may also be added to the

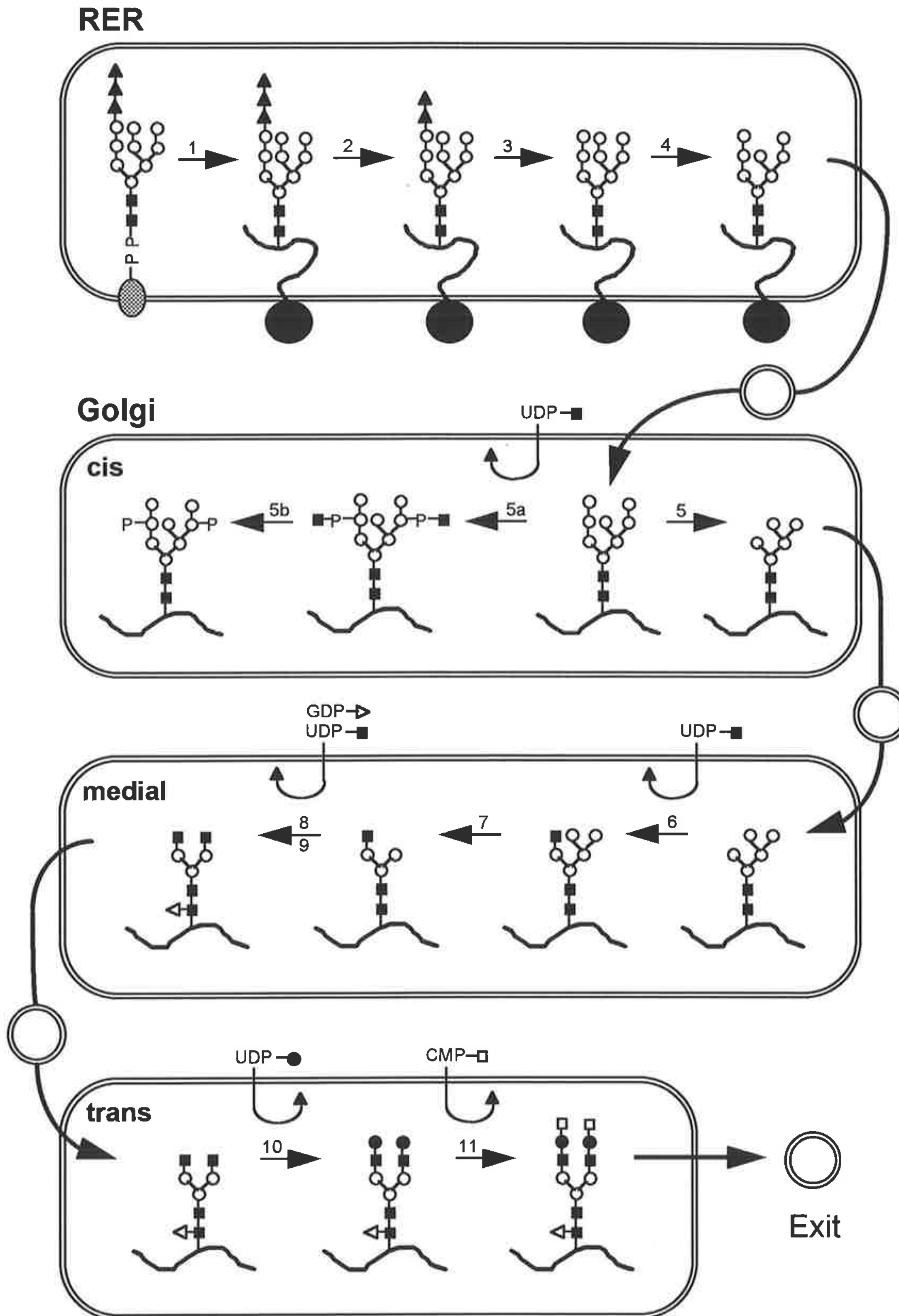


Figure 3.3. Pathway of N-linked oligosaccharide processing within the rough endoplasmic reticulum (*RER*) and Golgi apparatus. The enzymatic steps are catalysed by: (*1*) N-oligosaccharyltransferase, (*2*) α-glucosidase I, (*3*) α-glucosidase II, (*4*) ER α-1,2-mannosidase, (*5*) Golgi α-mannosidase I, (*5a*) N-acetylglucosaminylphosphotransferase, (*5b*) N-acetylglucosamine-1-phosphodiester α-N-acetylglucosaminidase, (*6*) N-acetylglucosaminyltransferase I, (*7*) Golgi α-mannosidase II, (*8*) N-acetylglucosaminyltransferase II, (*9*) fucosyltransferase, (*10*) galactosyltransferase, (*11*) sialyltransferase. The symbols represent: ⬭-P, dolichol phosphate; ■, N-acetylglucosamine; O, mannose; ▲, glucose; ▷, fucose; ●, galactose; and □, sialic acid (adapted from Kornfeld and Kornfeld, 1985; and Goochee and Monica, 1990).

innermost N-acetylglucosamine by fucosyltransferase in the medial cisternae. The completed glycoproteins are then transported out of the Golgi apparatus.

The extent of terminal branching of the complex N-linked oligosaccharides is determined by the activity of N-acetylglucosaminyltransferase III, which attaches a bisecting N-acetylglucosamine residue to the core β-mannose. This blocks further branching of the chain. If a bisecting residue is not attached, then N-acetylglucosaminyltransferase IV and V can attach additional N-acetylglucosamines to the α1,3 and α1,6 mannoses, respectively, to produce N-linked glycans with three or four outer branches. N-linked oligosaccharides can also be modified by phosphorylation of mannose residues, sulphation of mannose and N-acetylhexosamine, and O-acetylation of sialic acid. A subset of glycoproteins, the lysosomal enzymes, undergo a highly specific mannose phosphorylation catalysed by N-acetylglucosaminylphosphotransferase and N-acetylglucosamine-1-phosphodiester α-N-acetylglucosaminidase (Figure 3.3). This occurs in the cis Golgi cisternae and provides the enzymes with mannose-6-phosphate residues, which are required for targeting of the enzymes from the trans Golgi network to the lysosome by way of the mannose-6-phosphate receptor.

The activity of the Golgi enzymes is linked to the availability of intralumenal pools of sugar nucleotides. Nucleotide sugars are synthesised in the cytoplasm and then enter the Golgi lumen by specific antiporters that recognise the nucleotide and its sugar. At the same time the antiporter recycles the nucleoside monophosphate from the Golgi lumen to the cytoplasm. The products of the glycosylation reactions, however, are nucleoside diphosphates, except for cytidine monophosphate sialic acid (Figure 3.3). The nucleoside diphosphates are therefore cleaved by lumenal diphosphatases before they are transported across the Golgi membrane by the antiporters.

3.3 The disorders of N-glycosylation in humans

In recent years, several disorders have been identified in children that result from defects in N-glycosylation. The study of these disorders has confirmed the importance of protein glycosylation to normal cell and organ function. The disorder that has received most attention is the CDG syndrome. This syndrome has many clinical and biochemical features that are similar to classical galactosaemia, particularly an increase in hyposialylated isoforms of serum glycoproteins. Galactosaemia has therefore been proposed as a secondary Golgi disorder (Jaeken *et al.*, 1992). The study of the disorders of N-glycosylation has also suggested possible mechanisms for the abnormal glycoprotein synthesis in galactosaemia.

Carbohydrate-deficient glycoprotein syndrome

In 1980, Jaeken *et al.*, reported a new clinical disorder in twin sisters that was characterised by multiple organ disease and apparently unrelated biochemical abnormalities. The twins had severe psychomotor retardation, and cortical and cerebellar hypotrophy. In the blood, there were fluctuating levels of serum prolactin, FSH, and growth hormone (GH), a partial TBG deficiency, and increased serum arylsulphatase A activity. Jaeken *et al.*, later observed an increase in hyposialylated isoforms of transferrin in the twin's sera and proposed that they had a generalised defect in glycoprotein synthesis (Jaeken *et al.*, 1984). Carbohydrate analysis of the purified serum transferrin showed that it was deficient in sialic acid, galactose, and N-acetylglucosamine. This suggested a possible defect in Golgi function (Jaeken *et al.*, 1987; and Stibler and Jaeken, 1990). An assay for carbohydrate-deficient transferrin was then developed and used as a screening test for the disorder (Jaeken, 1989). Since then, over 120 patients

have been reported worldwide (for a review see Jaeken *et al.*, 1991; Jaeken and Carchon, 1993; and Jaeken *et al.*, 1993a).

Clinical features of CDG syndrome type I

The CDG syndrome currently includes four possible phenotypes that are distinguished by their clinical features and their patterns of serum transferrin isoforms. The vast majority of patients have a type I phenotype. The type II, III, and IV variants, which are similar to the type I phenotype, are each represented by only two patients and their clinical and biochemical features will not be described (Ramaekers *et al.*, 1991; Jaeken *et al.*, 1993b; Stibler *et al.*, 1993; and Stibler *et al.*, 1995). Other minor variants have also been proposed (Eyskens *et al.*, 1994; Charlwood *et al.*, 1996; and Skladal *et al.*, 1996).

During infancy, the CDG syndrome type I is characterised by psychomotor retardation, hypotonia, squint and nystagmus, feeding difficulties, failure to thrive, and abnormal adipose tissue distribution (for a review see Jaeken and Carchon, 1993). Hepatomegaly is often present, and sometimes pericardial effusion and cardiomyopathy. After infancy, the neurologic disease predominates with variable cerebellar ataxia, peripheral neuropathy, and intellectual retardation. Stroke-like episodes, seizures, and retinopathy may also develop. Other features include joint contractures, thoracic deformities, and particularly in females, hypogonadism. One fifth of the children die in the first years of life usually from sepsis, cardiac failure, or liver failure.

On radiological investigation, the patients usually exhibit marked cerebellar and brainstem atrophy, ventricular dilatation, small cysts in the white matter, and occasionally supratentorial cortical atrophy (Jaeken *et al.*, 1991; Petersen *et al.*, 1993; Stibler *et al.*, 1994; and Holzbach *et al.*, 1995). Holzbach *et al.*, (1995) also observed a reduction in N-acetylaspartate in white matter on ^{1}H-NMR spectroscopy, which indicated a loss of neuroaxonal tissue. The patterns of myelination of the brain were also abnormal in the type II and III patients (Ramaekers *et al.*, 1991; Stibler *et al.*, 1993; and Holzbach *et al.*, 1995). Common post-mortem findings in the brain include olivopontocerebellar hypoplasia, loss of neurones, and gliosis in the cerebral cortex, basal ganglia, and thalamus (Horslen *et al.*, 1991; Stromme *et al.*, 1991; and Eyskens *et al.*, 1994). The peripheral nerves have decreased myelin, multivacuolar inclusions in the Schwann cells (Nordborg *et al.*, 1991), and abnormal SEP (Itoh *et al.*, 1993). The liver pathology is characterised by fibrosis, steatosis, and glycogen storage, and the hepatocytes have lysosomal inclusions (Conradi *et al.*, 1991).

Biochemical features of CDG syndrome type I

The characteristic biochemical feature of the CDG syndrome is a deficiency of carbohydrate on glycoprotein. This is represented by an increase in hyposialylated isoforms of serum glycoproteins on isoelectric focusing. Analysis of serum transferrin, which forms the basis of screening tests for this disorder, shows that approximately half of the transferrin in the type I phenotype is present as disialo- and asialotransferrin (Kristiansson *et al.*, 1989; and Stibler *et al.*, 1991). However, all N-linked glycoproteins in the serum show an increase in isoforms with higher isoelectric points (Harrison *et al.*, 1992; and Yuasa *et al.*, 1995). This includes transport proteins, lysosomal and other enzymes, coagulation factors and inhibitors, and complement factors (for a review see Hagberg *et al.*, 1993; and Jaeken *et al.*, 1993a). Many of the glycoproteins are also reduced in concentration or activity including TBG, haptoglobin, transferrin, transcortin, apolipoprotein B, and certain coagulation factors. This results in a wide range of biochemical abnormalities, especially of thyroid function (Heyne *et al.*, 1994;

and Macchia *et al.*, 1995) and haemostasis (Okamoto *et al.*, 1993; van Geet and Jaeken, 1993; and Iijima *et al.*, 1994).

The levels of several hormones, including prolactin, FSH, LH, TSH, and insulin, fluctuate in the serum, and there is an increase in GH (de Zegher and Jaeken, 1995). Kristiansson *et al.*, (1995) observed normal patterns of sialylation for the serum FSH, LH, and TSH, and erythropoietin, which are synthesised in the pituitary and kidney, respectively. They suggested therefore that only the secretory glycoproteins from the liver were affected in the CDG syndrome. However, Jaeken *et al.*, (1993a) proposed that membrane-bound glycoproteins were also affected as the fluctuating hormone levels suggested that there were abnormalities of the glycoprotein receptors. Transferrin, isolated from liver, also had an abnormal pattern of isoforms (Stibler *et al.*, 1991), however, hexosaminidase A and fucosidase from liver and intestinal mucosa were normal (Jaeken and Kint, 1987). In experiments using lectin-affinity chromatography, Winchester *et al.*, (1995) observed altered glycosylation of intracellular lysosomal enzymes in skin fibroblasts from patients with the CDG syndrome type I.

The cause of CDG syndrome

CDG syndrome type I results from a deficiency of cytoplasmic phosphomannomutase (EC 5.4.2.8), which is required for the reversible conversion of mannose-6-phosphate to mannose-1-phosphate (van Schaftingen and Jaeken, 1995). It was originally noted that serum transferrin from these patients was deficient in terminal sialic acid, galactose, and N-acetylglucosamine (Stibler *et al.*, 1991). Later analysis by electrospray ionisation mass spectrometry and SDS-PAGE reported that the transferrin was missing one or both sialylated complex N-glycans (Wada *et al.*, 1992; Yamashita *et al.*, 1993a; and Yamashita *et al.*, 1993b). The N-glycans that were present, however, were structurally normal (Winchester *et al.*, 1995). This suggested a defect in N-linked oligosaccharide transfer. However, studies of N-oligosaccharyltransferase (Knauer *et al.*, 1994) and dolichol phosphate (Yasugi *et al.*, 1994) in CDG fibroblasts were normal.

It was then reported that CDG fibroblasts synthesised dolichol-linked oligosaccharides at a reduced rate and transferred either truncated or no N-glycans to protein (Powell *et al.*, 1994; and Krasnewich *et al.*, 1995). This suggested a defect in dolichol-linked oligosaccharide synthesis and lead to an analysis of the enzymes that convert glucose to mannose (Panneerselvam and Freeze, 1995). Van Schaftingen and Jaeken, (1995) then reported that phosphomannomutase was deficient in fibroblasts, liver, and leucocytes from six patients with the CDG syndrome type I. Phosphomannomutase converts mannose-6-phosphate to mannose-1-phosphate, which is then used to synthesise GDP-mannose. GDP-mannose is required for the synthesis of Dol-P-mannose and dolichol-linked oligosaccharides, and also GPI anchors. GPI anchors attach a range of proteins to membranes, including receptors, enzymes, coat proteins, and cell-adhesion molecules (for a review see Cross, 1987; Low, 1989; McConville and Ferguson, 1993; and Stevens, 1995). Phosphomannomutase deficiency would therefore affect a wide range of cellular and tissue processes. Adding mannose, but not glucose, to the culture medium of CDG fibroblasts corrected the abnormality of dolichol-linked oligosaccharide synthesis and the underglycosylation of protein (Panneerselvam and Freeze, 1995; and Panneerselvam and Freeze, 1996).

In the only study of cotranslational glycosylation in CDG cells, Marquardt *et al.*, (1995) observed normal glycosylation of three viral glycoproteins that were expressed by type I fibroblasts infected with influenza and Semliki Forest viruses. The Semliki Forest virus spike glycoproteins, E1 and p62, were also bound normally by calnexin, which suggested correct terminal glycosylation of the proteins (Marquardt *et al.*, 1996). Although the viral

glycoproteins appeared structurally normal, Marquardt *et al.* observed dilatation of the ER and a delay in transport of the glycoproteins from the ER to the medial Golgi cisternae. They concluded this was due to accumulation of abnormal protein in the ER (Marquardt *et al.*, 1995). CDG fibroblasts also exhibited secondary effects on proteoglycan expression and synthesis, which could contribute to the dysmorphism of CDG syndrome patients (Gu and Wada, 1995; and Gu and Wada, 1996). The gene for CDG syndrome type I has been linked to chromosome 16p13.3–12 (Martinsson *et al.*, 1994), although genetic heterogeneity among families has been reported (Matthijs *et al.*, 1996).

CDG syndrome type II is associated with a deficiency of Golgi N-acetyl-glucosaminyltransferase II (Jaeken *et al.*, 1994). This enzyme attaches N-acetylglucosamine to the α1,6 mannose of N-linked oligosaccharides. The N-glycans in CDG syndrome type II are therefore extended only on the α1,3 mannose. This results in an accumulation of incomplete antennae, which for serum transferrin, produces a disialotransferrin isoform with little or no asialo- or tetrasialotransferrin (Ramaekers *et al.*, 1991; and Jaeken *et al.*, 1994). Two unrelated patients with the CDG syndrome type II had point mutations in the catalytic domain of the gene that encodes N-acetylglucosaminyltransferase II (Tan *et al.*, 1996).

The biochemical bases of the CDG syndrome types III and IV are currently unknown. However, it is likely that they will also represent specific defects in N-glycosylation.

Disorders associated with partial defects of N-glycosylation

Several disorders of N-glycosylation are confined to specific pathways, cells, or tissues. These disorders are considered to represent partial defects of N-glycosylation. Patients with I-cell disease are deficient in N-acetylglucosaminylphosphotransferase, a Golgi enzyme required for the correct targeting of acid hydrolases to the lysosome (Reitman *et al.*, 1981). Primary release disorder, a hereditary bleeding disorder, is associated with a deficiency of platelet membrane sialyltransferase (Wu *et al.*, 1980) while a familial goitre with hyposialylated thyroglobulin is caused by sialyltransferase deficiency in the thyroid (Grollman *et al.*, 1992). Congenital dyserythropoietic anaemia type II (or HEMPAS) is a rare genetic anaemia that results from heterogeneous abnormalities of the erythrocyte membrane polylactosamines (Fukuda, 1990). The patients have a deficiency of either Golgi α-mannosidase II or N-acetylglucosaminyltransferase II. HEMPAS patients also have abnormal serum glycoproteins, which suggests that the defect is not confined to erythroid cells (Fukuda, 1990). Erythrocytes from patients with the CDG syndrome type II, however, do not show the serology typical of HEMPAS, which suggests that the genetic lesions responsible for these two disorders are different (Charuk *et al.*, 1995).

Secondary CDG syndromes in metabolic disease

Abnormal isoforms of glycoprotein have been observed in the serum of children with several metabolic disorders, particularly in the untreated state. The disorders include transferase-deficiency galactosaemia, epimerase-deficiency galactosaemia, hereditary fructose intolerance, and glycogen storage disease type Ib. Each of these disorders is associated with an accumulation of intracellular hexose phosphates.

Classical galactosaemia has been proposed as a secondary Golgi disorder because the serum glycoprotein abnormalities are similar to those in the CDG syndrome (Jaeken *et al.*, 1992). Galactosaemia and the CDG syndrome are both multisystem disorders that involve the nervous system, eyes, skeleton, intestine, liver, kidneys, gonads, and immune system (Jaeken and

Carchon, 1993). Some of the radiological and pathological changes are also similar, particularly in the brain. Two infants with generalised UDP-galactose 4-epimerase deficiency also had patterns of hyposialylated transferrins in their sera that were similar to the CDG syndrome (Besley *et al.*, 1995). The transferrin isoforms returned to normal on treatment as the erythrocyte galactose-1-phosphate concentration decreased. Epimerase-deficiency galactosaemia is also clinically similar to the CDG syndrome and could be a secondary Golgi disorder. In both forms of galactosaemia, the glycoprotein abnormalities in the serum resolve soon after the diet is started. The pattern of serum transferrin isoforms in untreated galactokinase deficiency has not been reported.

Adamowicz and Pronicka, (1996) also observed an increase in hyposialylated transferrin in the serum of a 15-month-old boy with untreated hereditary fructose intolerance. The pattern was similar to that in untreated galactosaemia and also returned to normal after starting treatment. Hereditary fructose intolerance results from a deficiency of fructose-1-phosphate aldolase in liver, kidney cortex, and small intestine (for a review see Gitzelmann *et al.*, 1995). Chronic exposure to dietary fructose results in vomiting, diarrhoea, failure to thrive, jaundice, hepatomegaly, ascites, haemorrhages, and lethargy. The biochemical features are those of liver failure and proximal renal tubular dysfunction. Untreated, the patients may develop cirrhosis of the liver, although many patients develop peculiar feeding habits that protect them from continued fructose exposure. Except for an absence of cataracts, the clinical presentation in the acute phase is very similar to transferase-deficiency galactosaemia. However, as aldolase activity is normal in brain, the patients do not suffer additional organ damage. It has been proposed that the toxicity in hereditary fructose intolerance results from the accumulation of fructose-1-phosphate (Maenpaa *et al.*, 1968; Bode *et al.*, 1973; Kogut *et al.*, 1975; and Gitzelmann *et al.*, 1995).

Glycogen storage disease type Ib is associated with a defect in the hepatic microsomal glucose-6-phosphate transport system. Heyne and Henke-Wolter, (1989) reported that six patients with this disorder had abnormal isoforms of α_1-antitrypsin in their serum. They observed an increase in sialylation of the α_1-antitrypsin, which suggested a shift from the usual biantennary to more triantennary complex N-glycans. They proposed that the changes resulted from a limited availability of glucose, or glucose derivatives, for normal N-glycosylation.

The abnormalities of the serum glycoproteins in these metabolic disorders may not be directly related to the primary enzyme defect. Similar glycoprotein changes are observed in other liver diseases. Hug *et al.*, (1982) observed increased cathodal bands of α_1-antitrypsin in the serum of a child who had a fatty liver because of cytomegalovirus infection, and Attenburrow, (1985) saw a similar pattern in two cases of neonatal hepatitis (one cytomegalovirus-related and the other idiopathic). The α_1-antitrypsin abnormalities reverted to normal with resolution of the liver disease. An increase of carbohydrate-deficient transferrin in the serum is also used as a marker of alcoholic liver disease (for a review see Stibler, 1991). It has been proposed that acetaldehyde inhibits various Golgi glycosyltransferases (Stibler and Borg, 1991; Kawahara *et al.*, 1993; and Xin *et al.*, 1995). This delays the processing of transferrin and produces hyposialylated transferrins in the serum (Matsuda *et al.*, 1991). Two-dimensional gel electrophoresis and Western blot analysis showed that many serum glycoproteins, in addition to transferrin, exhibit microheterogeneity in alcoholic liver disease (Wang *et al.*, 1993; and Gravel *et al.*, 1996). Carbohydrate-deficient transferrin is also occasionally increased in primary biliary cirrhosis and chronic active hepatitis (Stibler, 1991).

3.4 Galactosaemic fibroblasts as a cellular model of N-glycosylation

Skin fibroblasts from patients with classical galactosaemia are unable to oxidise galactose (Krooth and Weinberg, 1960), lack GALT activity (Russell and DeMars, 1967), and have been established as a useful cellular model of the metabolic defect in galactosaemia (Tedesco and Mellman, 1969). Several studies have also characterised the growth and metabolism of transferase-deficient fibroblasts under various culture conditions. These studies confirmed that galactosaemic fibroblasts were an appropriate cellular model to study N-glycosylation in galactosaemia. The results from those studies also helped in designing the experimental conditions used in this thesis. This section reviews the characteristics of galactosaemic fibroblasts in culture.

Galactose oxidation

Galactosaemic fibroblasts can oxidise small amounts of galactose to CO_2. Krooth and Weinberg, (1961) initially reported that the fibroblasts were unable to metabolise [1-^{14}C]-galactose to $^{14}CO_2$, but their incubation period was short and glucose was also present in the medium. Guerroui *et al.*, (1988) reported similar results with a three hour incubation. However, if the cells were incubated for 48 hours in a hexose-free medium, then they produced $^{14}CO_2$ from [1-^{14}C]-galactose at approximately 39% of the rate of normal fibroblasts (Friedman *et al.*, 1975). The ability of transferase-deficient fibroblasts to oxidise galactose depends on the time of incubation (Petricciani *et al.*, 1972), the concentration of [1-^{14}C]-galactose (Binder *et al.*, 1972), and the presence or absence of glucose in the medium (Petricciani *et al.*, 1972). These result suggested that galactosaemic fibroblasts had alternative pathways to oxidise galactose and that the pathways became saturated with galactose at much lower concentrations than occurred in normal fibroblasts. Galactosaemic fibroblasts oxidised [1-^{14}C]-glucose to $^{14}CO_2$ at a rate that was similar to normal fibroblasts (Krooth and Weinberg, 1961; and Guerroui *et al.*, 1988).

Galactose incorporation

Galactosaemic fibroblasts can incorporate galactose into TCA-insoluble macromolecules. Russell and DeMars, (1967) reported that the fibroblasts incorporated [1-^{14}C]-galactose into TCA-insoluble fractions at half the rate of control fibroblasts, at all stages of growth. Friedman *et al.*, (1975) observed a similar rate of incorporation, which was linear over 24 hours. Kadhom *et al.*, (1994) reported that the level of incorporation of [1-^{14}C]-galactose in 11 galactosaemic fibroblast cultures was 20–57% of normal, when expressed as a ratio with the incorporation of L-[4,5-^{3}H]-leucine into protein. Rozen *et al.*, (1977) observed slightly higher levels of incorporation in three fibroblast cultures when the [^{14}C]-galactose was expressed as a ratio with the incorporation of [^{14}C]-glucose. How the galactose was incorporated into the macromolecular fraction is not known. Most authors suggested either residual GALT activity or the UDP-galactose pyrophosphorylase pathway. The incorporation of [1-^{14}C]-galactose was reduced by lowering the pH, which suggested that the activity of the alternative pathway was influenced by the pH of the growth medium (Hill and Puck, 1973; Friedman *et al.*, 1974; and Hill, 1976). Galactokinase-deficient fibroblasts incorporated [^{14}C]-galactose at less than 10% of normal, which also suggested that the galactose had to be converted to galactose-1-phosphate before it could be incorporated (Friedman *et al.*, 1975; and Hill, 1976). Galactosaemic fibroblasts incorporated [^{14}C]-glucose into TCA-insoluble material at a rate that was similar to normal fibroblasts (Friedman *et al.*, 1975; Hill, 1976; and Rozen *et al.*, 1977).

Growth in galactose

Transferase-deficient skin fibroblasts will not grow in culture medium that contains galactose as the sole hexose. Krooth and Weinberg, (1961) exposed the fibroblasts to media that contained either glucose, galactose, or was hexose-free. The fibroblasts were cultured in the various media for 9–18 days and growth was assessed by measuring the change in total protein. The control fibroblasts grew equally well in either glucose or galactose. The galactosaemic fibroblasts also grew well in glucose, however, they grew only slowly in the galactose medium and at a rate that was similar to their growth in hexose-free medium. More recent studies have confirmed these results (Pourci *et al.*, 1990; and Wolfrom *et al.*, 1993). Pourci *et al.*, (1990) observed that galactosaemic fibroblasts died within three days when they were subcultured into a medium that contained galactose and dialysed FBS. The time course of death was similar to that of galactosaemic and control fibroblasts subcultured into a hexose-free medium. However, if the galactosaemic fibroblasts were grown to a stationary phase in glucose, and then exposed to galactose, they remained viable for several weeks in culture (Tedesco and Miller, 1979; and Pourci *et al.*, 1990). Galactosaemic fibroblasts grew in medium that contained galactose if the medium was supplemented with non-dialysed serum (Wolfrom *et al.*, 1993), small amounts of glucose (Krooth and Weinberg, 1961; and Wolfrom *et al.*, 1993), additional glutamine (Wolfrom *et al.*, 1993), or inosine (Pourci *et al.*, 1990). This suggested that there was a minimum requirement for growth in culture and that normal growth was possible, despite the accumulation of galactose-1-phosphate.

When normal skin fibroblasts were grown in galactose they exhibited a clear flattened cytoplasm and were arranged in regular parallel arrays. Galactosaemic fibroblasts, however, exhibited slightly increased granularity and moderate steatosis, and appeared long, narrow, and dark. The cells were also irregularly bundled (Wolfrom *et al.*, 1993). Only two limited studies have examined the appearance of galactosaemic skin fibroblasts under electron microscopy (Miller *et al.*, 1968; and Johnson *et al.*, 1976). Miller *et al.*, (1968) exposed a galactosaemic fibroblast culture to a medium that contained 5.5 mmol/L galactose, 1 mmol/L sodium pyruvate, and dialysed human serum. After 72 hours, the fibroblasts showed dilatation of the RER, widespread cytoplasmic degeneration, and autophagic vacuoles. Miller *et al.*, (1968) suggested that the abnormalities resulted from accumulation of galactose-1-phosphate and a deficiency of ATP. Johnson *et al.*, (1976) examined a single fibroblast culture in a medium that contained 25 mmol/L galactose, without added serum, and found no dilatation of the ER. They did observe autophagic vacuoles and increased electron-dense areas after five days of culture. However, some of these changes were also seen when the fibroblasts were exposed to a glucose medium for the same period of time. The galactosaemic fibroblasts showed more marked changes when they were grown in a hexose-free medium for five days. Johnson *et al.*, (1976) concluded that the cellular changes in the galactose medium resulted from partial hexose deficiency.

Intracellular biochemistry

Transferase-deficient skin fibroblasts in culture exhibit metabolic and biochemical abnormalities that are similar to galactosaemic patients. When galactosaemic fibroblasts were exposed to galactose, they rapidly accumulated galactose-1-phosphate, which reached a plateau by 2–4 hours (Mayes and Miller, 1973; and Tedesco and Miller, 1979). They also accumulated galactose-1-phosphate when they were exposed to media that contained only glucose, or glucose and galactose (Mayes and Miller, 1973; and Schaub *et al.*, 1979). The concentration of intracellular galactose-1-phosphate was higher during the stationary growth phase than the

logarithmic phase, and increasing the concentration of galactose in the medium from 5 to 25 mmol/L did not increase the galactose-1-phosphate further (Pourci *et al.*, 1990). In contrast, normal fibroblasts have minimal concentrations of galactose-1-phosphate whatever the medium or growth phase (Mayes and Miller, 1973; Schaub *et al.*, 1979; Tedesco and Miller, 1979; and Pourci *et al.*, 1990). Galactosaemic fibroblasts also accumulated galactitol when they were exposed to galactose, and secreted it into the medium (Schaub *et al.*, 1979).

When galactosaemic fibroblasts were exposed to galactose over prolonged periods, Mayes and Miller, (1973) observed a gradual decrease in the galactose-1-phosphate concentration. The level reached half the maximum concentration after eight days of galactose exposure. However, Tedesco and Miller, (1979) observed an increase in galactose-1-phosphate as the fibroblast sheet deteriorated after 12 days in galactose medium. Mayes and Miller, (1973) also reported that galactosaemic fibroblasts were capable of hydrolysing galactose-1-phosphate and secreting the galactose back into the medium. In pulse-chase experiments with [1-^{14}C]-galactose, they observed that over 87% of the radioactivity could be chased from the intracellular pool back into the medium. This suggested a futile cycle of phosphorylation and dephosphorylation. A similar phenomenon was observed in galactosaemic erythrocytes (Donnell *et al.*, 1967). Normal fibroblasts did not exhibit this cycle (Mayes and Miller, 1973).

Wolfrom *et al.*, (1993) examined the ability of galactosaemic fibroblasts to consume hexose from the culture medium. As expected, the fibroblasts consumed galactose at 55% of the rate of normal fibroblasts. However, they also consumed glucose at only 70% of the rate of normal. The reason for the reduced consumption of glucose is not known. The galactosaemic fibroblasts produced lactate at a normal rate, which suggested that the glycolytic pathway was not impaired (Wolfrom *et al.*, 1993).

4

Materials and methods

4.1 Introduction

This chapter outlines the general and specific materials and methods used in Chapters 5, 6, 7, and 8. Common tissue culture media, radiochemicals, and chromatographic techniques were used in most of the experimental plans and are therefore only briefly mentioned. The specific methods are presented in more detail along with the reasons for choosing each particular method. The specific methods are largely derived from standard techniques in the literature. However, the radiolabelling of cells in culture can be greatly influenced by variations in the tissue culture procedure. The specific methods are therefore presented in full to avoid confusion about the exact procedures followed during culture, radiolabelling, harvest, and preparation of extracts for analysis. Where a method required development or validation of its performance, then that information is also included here. This chapter was designed as a reference section. The experimental plans in Chapters 5, 6, 7, and 8 do provide an overview of the particular experimental approaches used in those chapters. However, there are references, in the text, to specific methods in Chapter 4 for more detail as required.

4.2 General methods

Tissue culture

All media, solutions, and reagents were stored in accordance with the manufacturer's instructions. The skin fibroblast cultures were prepared from cell lines stored in liquid nitrogen and were revived immediately before experimentation. The fibroblasts were revived in Basal Medium Eagle (BME) with Earle's salts and 10% (v/v) FBS. The medium was changed weekly. On subculture, the fibroblasts were usually passaged 1 in 2 into 75 cm^2, or 1 in 4 into 25 cm^2 polystyrene tissue culture flasks. The cultures were incubated at 37°C in 5% CO_2 and 90% humidity in a Forma Scientific water-jacketed incubator, model 3250. Whenever possible, the cells used in the experiments were between the third and sixth passages. The cell lines were regularly tested for mycoplasma by the Hoechst stain technique (Chen, 1977).

Cell lines

The skin fibroblast cell lines were obtained from the Cell Culture Laboratory, Department of Chemical Pathology, Women's and Children's Hospital, Adelaide. The controls included true normals as well as cells that were being investigated for a range of metabolic disorders, other than galactosaemia. Four GALT-deficient skin fibroblast cell lines were available for study and their biochemical characteristics and clinical data are summarised in Table 4.1. The mutation analysis of the galactosaemic fibroblast DNA was by restriction enzyme analysis (Ng *et al.*, 1994). The fibroblast GALT activity was estimated by a sensitive radiochemical assay that measured the conversion of [^{14}C]-galactose-1-phosphate to UDP-[^{14}C]-galactose (Monk and Holton, 1976).

Table 4.1. Characteristics of the galactosaemic skin fibroblast cultures.

Cell culture	Designation	Sex	Remarks	Genotype[a]	GALT[b] activity
SF 3313	*G1*	Male	Presented at two months of age with vomiting, hepatitis, and cataracts. Mild developmental delay.	X/X	8.7
SF 1392	*G2*	Female	GM 00727A. Human Genetic Mutant Cell Repository, Camden, NJ.	Q188R/Q188R	0.4
SF 3342	*G3*	Female	Sibling of *G1*.	X/X	6.8
SF 2623	*G4*	Female	Diagnosed by neonatal screening. Mild developmental and speech delay.	Q188R/X	2.8

[a] Fibroblast DNA was screened for Q188R and N314D only; X is unknown.
[b] Fibroblast GALT activity was measured in pmol/min/mg of protein (normal range 180–500).

Media and solutions

1. AUTOPOW Basal Medium Eagle (modified) with Earle's salts, without L-glutamine and sodium bicarbonate
Cat. no. 11-000-22
ICN Biomedicals, Costa Mesa, CA, USA

2. Dulbecco's Modified Eagle Medium with L-glutamine, 1000 mg/L D-glucose, and 110 mg/L sodium pyruvate
Cat. no. 11885-035
GIBCO BRL, Life Technologies Inc., Grand Island, NY, USA

3. Dulbecco's Modified Eagle Medium with L-glutamine, without D-glucose and sodium pyruvate
Cat no. 11966-017
GIBCO BRL, Life Technologies Inc., Grand Island, NY, USA

4. Dulbecco's Modified Eagle Medium with L-glutamine and 1000 mg/L D-glucose, without L-cystine, L-methionine, and sodium bicarbonate
Cat. no. D-3916
Sigma Chemical Co., St. Louis, MO, USA

5. Dulbecco's phosphate-buffered saline, without calcium and magnesium
Cat. no. 09832471
CSL Ltd., Parkville, VIC, Australia

6. Fetal bovine serum (not inactivated)
Cat. no. 09702301
CSL Ltd., Parkville, VIC, Australia

7. Trypsin-versene solution — Cat. no. 09601901, CSL Ltd., Parkville, VIC, Australia

8. Sodium bicarbonate 7.5% (w/v) solution — Cat. no. 16-883-49, ICN Biomedicals, Costa Mesa, CA, USA

9. L-glutamine 2.92% (w/v) solution — Cat. no. 09871901, CSL Ltd., Parkville, VIC, Australia

10. Benzylpenicillin sodium for injection — CSL Ltd., Parkville, VIC, Australia

Materials and reagents

1. Polystyrene tissue culture flasks 25 cm^2 and 75 cm^2 — Cat. nos. 25100-25 and 25110-75, Corning Inc., Corning, NY, USA

2. Teflon cell scrapers, 23 cm — Cat. no. 179693, Nunc Inc., Naperville, IL, USA

3. Minisart syringe filter holders 0.2 µm pore size — Cat. no. SM 17597 K, Sartorius AG, Göttingen, Germany

4. Selby dialysis membranes 10 and 25 mm flat widths — Cat. nos. 453103 and 453105, Selby Scientific and Medical, Notting Hill, VIC, Australia

5. D-glucose, anhydrous, M_r 180.16 — Cat. no. 783, Ajax Chemicals, Auburn, NSW, Australia

6. D-galactose, anhydrous, M_r 180.16 — Cat. no. G-6404, SigmaUltra, Sigma Chemical Co., St. Louis, MO, USA

7. Sodium pyruvate, cryst., M_r 110.1 — Cat. no. 5510, Calbiochem, San Diego, CA, USA

Experimental media

The skin fibroblast cultures were established in AUTOPOW BME (modified) with Earle's salts. Following preparation, each litre of BME contained 100 mL FBS, 20 mL 7.5% (w/v) sodium bicarbonate, 10 mL 2.92% (w/v) L-glutamine, and 50 mg benzylpenicillin. The D-glucose concentration was 5.5 mmol/L. When required for experimentation, the cells were then subcultured into Dulbecco's Modified Eagle Medium (D-MEM) with 10% (v/v) dialysed FBS, and grown to the required density. The rates of growth of the control and galactosaemic fibroblasts in this medium were similar and confluence was usually achieved within 3 to 4 days. The dialysed FBS was prepared by dialysing 100 mL of serum against three changes of 1 L of phosphate-buffered saline (PBS) over 48 hours, in a 4°C cold cabinet. The FBS was then passed through a 0.2 µm syringe filter and stored at -20°C until used. The concentration of glucose in the dialysed serum was less than 0.2 mmol/L.

Specific experimental media were prepared by adding various amounts of D-galactose, D-glucose, and sodium pyruvate to glucose-deficient D-MEM with 10% (v/v) dialysed FBS.

These media were used for preincubation, radiolabelling, and chase procedures. If radiochemicals were added to the medium during preparation, then the medium was passed through a 0.2 µm syringe filter and used immediately. On those occasions when the radiochemicals were added directly to the medium in the tissue culture flask, small aliquots of medium were taken to confirm that the radioactivity in each flask was similar. If the radiolabelling period was short and the experimental medium contained galactose, then the fibroblasts were preincubated for four hours in that medium to allow the cells time to adjust to the change in hexose. This also allowed the intracellular galactose-1-phosphate to reach a stable concentration in the galactosaemic fibroblasts (Mayes and Miller, 1973; and Tedesco and Miller, 1979). The four hour preincubation was also used to limit the acute effects of galactose exposure, such as altered ATP and inorganic phosphate levels (Penington and Prankerd, 1958; and Kogut *et al.*, 1975) and disturbed sugar nucleotide concentrations (Gibson *et al.*, 1995a).

Radiochemicals

1. Mannose, D-[2-^{3}H(N)]- in ethanol:water 9:1 specific activity 777.0 GBq/mmol — Cat. no. NET-570 NEN Research Products, Du Pont Co., Boston, MA, USA

2. Methionine, L-[^{35}S]- in 50 mmol/L Tricine-HCl (pH 7.4) specific activity 43.5 TBq/mmol — Cat. no. NEG-009A NEN Research Products, Du Pont Co., Boston, MA, USA

3. [^{35}S]-Protein Labeling Mix in 50 mmol/L Tricine-HCl (pH 7.4) specific activity 43.5 TBq/mmol — Cat. no. NEG-072 NEN Research Products, Du Pont Co., Boston, MA, USA

4. Tritiated sodium borohydride (NaB[^{3}H]$_4$) specific activity 488.4 GBq/mmol — Cat. no. NET-023H NEN Research Products, Du Pont Co., Boston, MA, USA

5. Optiphase 'HiSafe' 3 liquid scintillation cocktail — Cat. no. SC/9205/21 Wallac UK, Milton Keynes, England

6. Wallac 1409 liquid scintillation counter
   Each sample or fraction that contained isotope was counted for two minutes with a protocol that included DPM counting mode, external standard chemical quench correction, and chemiluminescence and colour correction.

Radiochemical preparation

1. D-[2-^{3}H]-mannose (in ethanol:water 9:1) was dried under a stream of nitrogen in a fume hood and then resuspended in PBS. The solution was prepared immediately before use.

2. L-[^{35}S]-methionine and [^{35}S]-Protein Labeling Mix were thawed and used directly from the Tricine-HCl buffer.

3. NaB[^{3}H]$_4$ was prepared by dissolving 3.7 GBq (100 mCi) in 200 µL of ice-cold 0.01 mol/L NaOH. The solution was then stored immediately at -80°C in 10 µL (or 185 MBq) aliquots.

Low pressure column chromatography

All gel filtration media were prepared according to the manufacturer's recommendations. All buffers were prepared with HPLC-grade water and vacuum degassed through 0.45 µm Millipore filters. Samples were made up to appropriate volumes in buffer and then centrifuged at 6000 x g for one minute immediately before they were applied to the columns.

Gel filtration media

1. Bio-Gel P-2 gel, fine, 45–90 µm — Cat. no. 150-4114, Bio-Rad Laboratories, Hercules, CA, USA
2. Bio-Gel P-4 gel, fine, 45–90 µm — Cat. no. 150-4124, Bio-Rad Laboratories, Hercules, CA, USA
3. Sephacryl S-200, superfine, 40–105 µm — Cat. no. 17-0871-01, Pharmacia AB, Uppsala, Sweden
4. Bio-Beads SM-2 macroporous beads 300–1180 µm — Cat. no. 152-3920, Bio-Rad Laboratories, Hercules, CA, USA
5. Sephadex G-15, 60–181 µm — Cat. no. 17-0020-01, Pharmacia AB, Uppsala, Sweden

Columns and accessories

1. Econo-Column 1.0 x 5 cm — Cat. no. 737-1007
2. Econo-Column 1.0 x 50 cm — Cat. no. 737-1052
3. Econo-Column 1.5 x 50 cm — Cat. no. 737-1552
4. Econo-Column 1.0 x 120 cm — Cat. no. 737-1093
5. Econo-Column flow adaptor, 1.0 cm — Cat. no. 738-0015
6. Poly-Prep polypropylene columns, 0.8 x 4 cm — Cat. no. 731-1550, Bio-Rad Laboratories, Hercules, CA, USA
7. Peristaltic pump P-1 — Cat. no. 19-4610-01
8. Fraction collector FRAC-200 — Cat. no. 19-8600-01, Pharmacia AB, Uppsala, Sweden
9. Millipore HA 0.45 µm filters — Cat. no. HATF 047-00, Millipore Corp., Bedford, MA, USA

Column calibration

All columns were calibrated with bovine serum albumin (BSA) and [^{14}C]-glucose. The elution volumes of the calibrants were then used to calculate the exclusion (V_O) and inclusion (V_T) volumes for the columns. BSA was detected either by the absorbance change at 280 nm or with a bicinchoninic acid (BCA) microassay for protein (Smith *et al.*, 1985).

Column standardisation

The Bio-Gel P-4 240 x 1 cm sizing column was standardised with a range of oligosaccharide standards. Several tritium-labelled high mannose N-linked alditols were purchased from Oxford GlycoSystems Ltd. (Abingdon, Oxford, England). Tritiated oligomannose standards were also prepared from bovine ribonuclease B (RNase B). Sialylated complex N-linked oligosaccharide standards were prepared by radiolabelling N-linked alditols derived from bovine fetuin. The preparation of these tritiated oligosaccharide standards and the standardisation data for the Bio-Gel P-4 column are included in Appendix 2 (page 154).

4.3 Specific methods

Method 1: Radiolabelling, recovery, and size analysis of fibroblast N-linked oligosaccharides

Introduction

The cultured skin fibroblasts were radiolabelled with D-[2-^{3}H]-mannose over 24 hours according to the techniques of Yurchenco *et al.*, 1978; Varki, 1994a; and Varki, 1994b. Following uptake into the cell, mannose is phosphorylated to mannose-6-phosphate, converted to mannose-1-phosphate, and then enters the GDP-mannose pool. The radiolabel is then incorporated into the pentasaccharide structure common to high mannose, hybrid and complex N-linked oligosaccharides, either directly or from Dol-P-mannose. Some label is also incorporated into the mannose residues of GPI anchors, and presumably also nuclear mannoproteins. Radiolabelling with D-[2-^{3}H]-mannose is very specific because any metabolism of [2-^{3}H]-mannose only yields [2-^{3}H]-fucose or unlabelled fructose-6-phosphate, regardless of the length of the labelling period (Varki, 1991; and Varki, 1994a). The incorporation of [2-^{3}H]-mannose depends on the hexose concentration of the culture medium, as glucose and galactose directly competing with mannose for uptake into the cell. The incorporation can be enhanced if glucose is replaced with pyruvate in the culture medium.

After the radiolabelling procedure, a protein pellet was prepared from both the cells and medium to recover cellular and secretory N-linked oligosaccharides. The glycolipids were removed by solvent extraction and the protein pellet was digested extensively with trypsin (Finne and Krusius, 1982; Tulsiani and Touster, 1983; and Beeley, 1985). The tryptic glycopeptides were then desalted on a Bio-Gel P-2 column and the N-linked oligosaccharides were cleaved from the peptide backbone with peptide-N-4(N-acetyl-β-glucosaminyl)asparagine amidase F (PNGase F). PNGase F cleaves all types of asparagine-bound glycans and is the preferred enzyme for complete removal of N-linked oligosaccharides (Tarentino *et al.*, 1985; Alexander and Elder, 1989; and Plummer and Tarentino, 1991). The enzyme is a glycoamidase that cleaves the bond between the asparagine residue of the protein and the N-acetylglucosamine residue of the oligosaccharide, provided that the amino- and carboxyl-groups of the asparagine are in a peptide linkage. The oligosaccharide must also contain, at a minimum, a chitobiose core (Keesey 1987). Although PNGase F will deglycosylate protein in its native form, the rate and completeness of deglycosylation can be increased considerably if the protein is denatured. This was accomplished either by digesting the protein with trypsin to produce specific glycopeptides, or by heating it in the presence of sodium dodecyl sulphate

(SDS; as in Method 5, page 72) (Keesey, 1987; and Varki, 1994a). PNGase F was also added in large excess. The enzymatic digests were performed under sterile conditions to prevent bacterial degradation of the glycoproteins.

The N-linked oligosaccharides were then separated by size with a Bio-Gel P-4 240 x 1 cm column following standard techniques (Yamashita *et al.*, 1982; Tulsiani and Touster, 1983; and Kobata, 1994). Size-exclusion chromatography is widely used in the separation and analysis of oligosaccharides and is effective in fractionating a wide range of oligosaccharides in a short time. Appropriate internal and external standards were used to determine the effective size of the oligosaccharides. The column was eluted with ammonium formate, rather than distilled water, to fractionate both charged and uncharged N-linked oligosaccharides.

Materials and reagents

1. Trypsin from bovine pancreas, lyophilised 100 mg/mL solution in water (pH 5.0) and stored at -20°C — Cat. no. 109 827 Boehringer Mannheim GmbH, Mannheim, Germany

2. Trypsin digest buffer — 40 mmol/L Tris-HCl (pH 8.0), 20 mmol/L $CaCl_2$, 0.02% (w/v) NaN_3

3. PNGase F, recombinant, *E. coli* 50U in 0.25 mL sodium phosphate buffer with 50% (v/v) glycerol — Cat. no. 1365 177 Boehringer Mannheim GmbH, Mannheim, Germany

4. PNGase F digest buffer — 0.25 mol/L sodium phosphate (pH 7.35), 10 mmol/L disodium ethylene-diaminetetraacetic acid (Na_2EDTA), 10 mmol/L 2-mercaptoethanol, 0.02% (w/v) NaN_3

5. Ystrom ultrasonic probe — Technic International Inc., Westwood, NJ, USA

Method

A. Radiolabelling procedure

The skin fibroblasts cultures were established in BME with 10% (v/v) FBS. The cells were then subcultured into either 25 cm^2 or 75 cm^2 polystyrene tissue culture flasks and grown in D-MEM with 10% (v/v) dialysed FBS to either 50%- or near-confluence. At the appropriate stage of growth, the cells were washed twice with 5 mL of prewarmed glucose-deficient D-MEM and then placed in 2–5 mL of a modified D-MEM that contained 10% (v/v) dialysed FBS, 3.7 MBq/mL D-[2-^{3}H]-mannose (100 µCi/mL), and various concentrations of D-glucose, D-galactose and sodium pyruvate, as required by the experimental procedure. The fibroblasts were then radiolabelled for 24 hours.

B. Cell harvest

The medium was removed and the fibroblasts were washed twice with 2.5 mL of ice-cold PBS. The medium and PBS washes were combined and centrifuged at 400 x g for three minutes. The supernatant was then passed through a 0.2 µm syringe filter and stored at -20°C for recovery later of the secreted protein. The fibroblasts were harvested with a cell scraper in 3 x 2 mL of ice-cold PBS and centrifuged at 400 x g for three minutes. The cell pellet was then washed a further three times with 5 mL of ice-cold PBS, then resuspended in 300 µL sterile water and sonicated for approximately 30 seconds. A small aliquot (10 µL) of the suspension was removed for estimation of the protein with a BCA microassay. The suspension was then rapidly frozen in a methanol/dry ice slurry and lyophilised overnight. The sample was then extracted three times with 1.5 mL of chloroform/methanol/water 10:10:3 (v/v/v). During each extraction, the sample stood at room temperature for 15 minutes and was centrifuged at 1000 x g for 10 minutes. The remaining protein pellet was dried under a stream of nitrogen.

C. Trypsin digest

The protein pellet was resuspended in 400 µL of trypsin digest buffer with brief sonication. Trypsin (2 mg) was added and the sample was incubated in a 37°C water bath for 24 hours. The digest was then continued with further additions of 1 mg of trypsin at 24 and 48 hours. The incubation was stopped at 72 hours by placing the sample in a 100°C water bath for five minutes. The sample was then centrifuged at 1500 x g for five minutes and the supernatant was desalted on a Bio-Gel P-2 50 x 1.5 cm column in 0.1 mol/L NH_4COOH at 20 mL/h. The tryptic glycopeptides from the cell pellet and medium of each cell culture eluted from the Bio-Gel P-2 column as a single peak near the void (Figure 4.1). The fractions that contained the glycopeptides were pooled and lyophilised three times to remove traces of ammonium formate.

D. PNGase F digest

The recovered glycopeptides were resuspended in 100 µL of PNGase F digest buffer and transferred to a 1 mL Eppendorf tube. Between three and five units of PNGase F were added and the sample was incubated in a 37°C water bath for 18 hours. The digest was stopped by placing the sample in a 100°C water bath for five minutes. The released oligosaccharides were then separated on a Bio-Gel P-4 240 x 1 cm column in 0.1 mol/L NH_4COOH at either 4 or 8 mL/h. Samples were loaded in 200–400 µL total volume and either 255 x 0.6 mL or 175 x 1 mL fractions were collected. One hundredth of each fraction was counted for two minutes in 4 mL of scintillation fluid. Over 90% of the radiolabelled N-glycans were resolved into discrete oligosaccharide peaks following the PNGase F digest (Figure 4.2, page 64). The oligosaccharides were then recovered as seven separate peaks, which were designated *peaks 1–7*. Each peak was pooled and lyophilised 2–3 times in preparation for anion-exchange HPLC.

E. Recovery of secreted glycoproteins

The media, including the initial PBS washes, were thawed and dialysed three times against 1 L of 0.1 mol/L NH_4COOH over 48 hours, in a 4°C cold cabinet. The solution that remained was lyophilised twice and the protein pellet was then digested with trypsin and PNGase F, following the same methods as for the cell pellet.

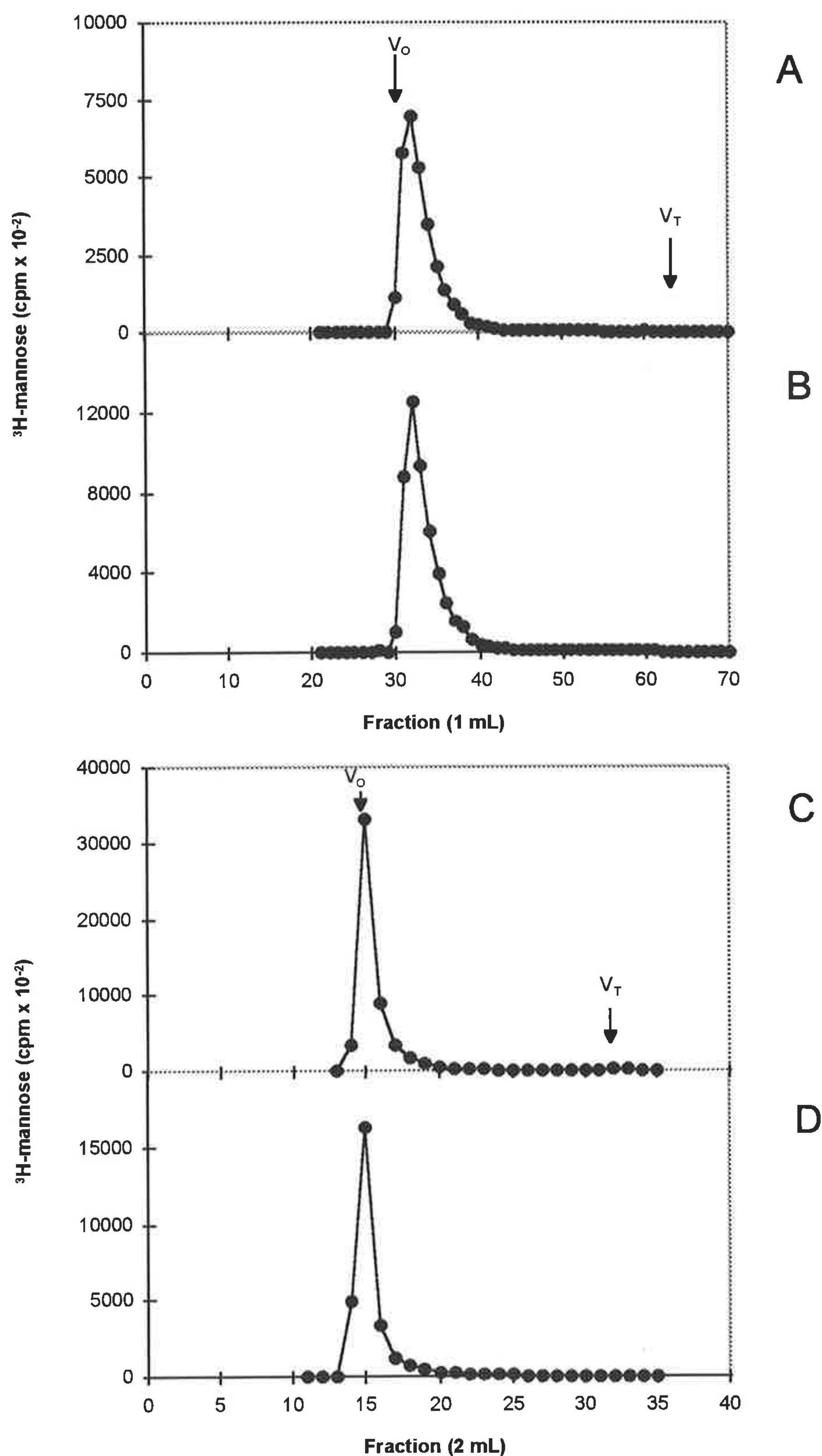


Figure 4.1. Tryptic glycopeptides from the cell pellets (*A* and *B*) and media (*C* and *D*) of a representative control (*A* and *C*) and galactosaemic (*B* and *D*) fibroblast culture were desalted on a Bio-Gel P-2 50 x 1.5 cm column in 0.1 mol/L NH_4COOH at 20 mL/h. 70 x 1 mL (cell pellets) or 35 x 2 mL (media) fractions were collected. The glycopeptides were recovered as a single peak near the void. The exclusion volume (V_O) for the column was 30 mL and inclusion volume (V_T) 64 mL.

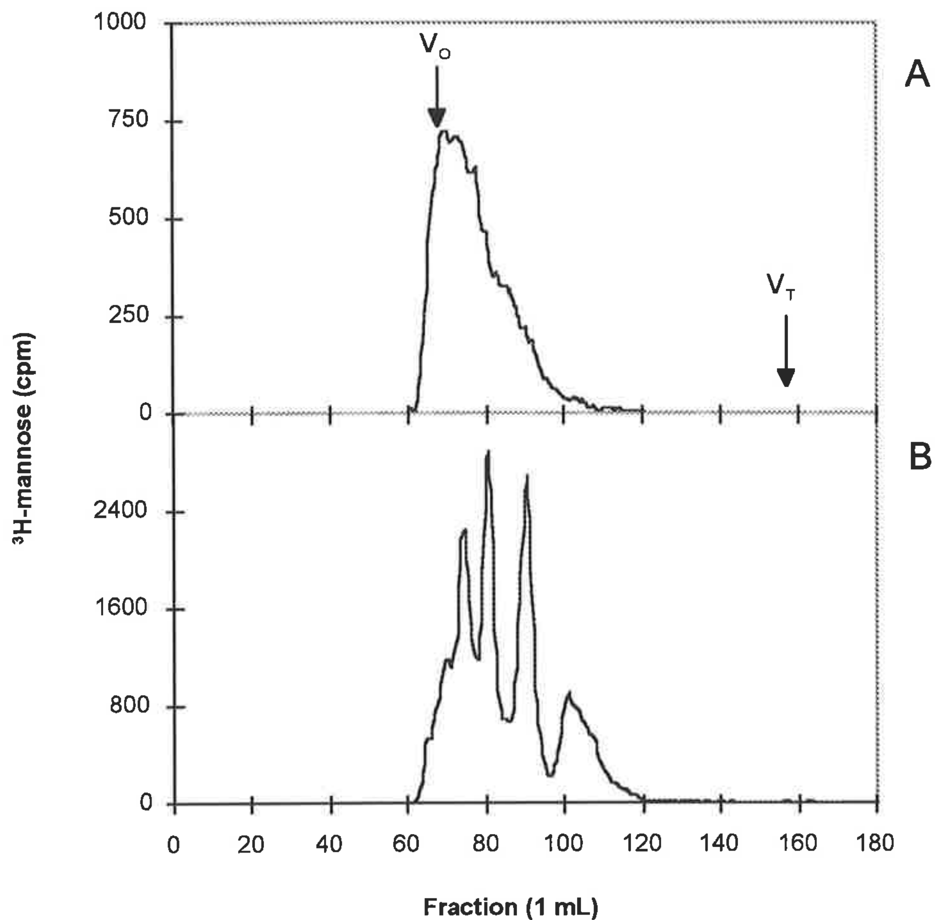


Figure 4.2. Tryptic glycopeptides (20 000–50 000 cpm) from the medium of a representative control fibroblast culture were sized on a Bio-Gel P-4 240 x 1 cm column in 0.1 mol/L NH_4COOH at 8 mL/h before (*A*) and after (*B*) PNGase F digestion to release the N-linked oligosaccharides. The majority of the glycopeptides eluted as a broad peak near the void whereas the free oligosaccharides were resolved into several discrete peaks that eluted later. The exclusion volume (V_O) for the column was 64 mL and inclusion volume (V_T) 159 mL.

Method 2: Anion-exchange HPLC of fibroblast N-linked oligosaccharides

Introduction

The cellular and secretory N-linked oligosaccharides that were recovered in Method 1 (page 60) were separated by the Bio-Gel P-4 240 x 1 cm column into seven individual peaks (Figure 5.1, page 85). The fractions from each of these peaks (*peaks 1–7*) were pooled and then each peak was analysed by anion-exchange HPLC to determine the extent of terminal sialylation of the complex N-glycans. The method was developed from standard techniques of the separation of anionic oligosaccharides by HPLC (Baenziger and Natowicz, 1981; Green and Baenziger, 1986; and Baenziger, 1994). Many N-linked oligosaccharides carry a charge either from terminal sialic acid or from phosphate and sulphate residues, which are attached to specific sugars within the structure. Sialic acid is present to various degrees in complex and hybrid N-linked oligosaccharides, and phosphate forms part of the mannose-6-phosphate signal that targets lysosomal enzymes to the lysosome. Although sulphate is attached to N-acetylgalactosamine residues in specific N-linked oligosaccharides, it is more commonly found in proteoglycans (Table I in Varki, 1994b).

Anion-exchange HPLC separates anionic oligosaccharides according to the number of their negatively charged species. Neutral oligosaccharides are not retained whereas the anionic oligosaccharides are eluted from the column by an increasing phosphate gradient. The technique cannot differentiate the charge from various anionic species. However, the extent of sialylation can be estimated by removing the sialic acid with neuraminidase and comparing the change in elution (Baenziger, 1994). Neuraminidase from *Arthrobacter ureafaciens* will hydrolyse terminal N- or O-acetylneuraminic acids that are α2,3-, α2,6-, or α2,8-linked to oligosaccharides. With this enzyme, the desialylation of N-linked oligosaccharides is almost complete (Uchida *et al.*, 1979).

The HPLC column was calibrated with sialylated bovine fetuin N-linked alditols (Dionex Corp., Sunnyvale, CA, USA). The Dionex N-linked alditols were recovered from bovine plasma fetuin with PNGase F. The alditol mixture contained predominantly a trisialylated complex N-linked glycan with lesser amounts of di- and tetrasialylated forms. The alditols are recommended for the standardisation of anion-exchange HPLC of sialylated oligosaccharides (Townsend *et al.*, 1989; and Hardy and Townsend, 1994).

Materials and Reagents

1. Beckman Altex Gradient Liquid Chromatograph, model 334
2. LKB 2158 Uvicord SD detector with 206 nm filter
3. Pharmacia FRAC-200 fraction collector
4. Cole-Palmer Instrument Co. chart recorder

5. Dynamax-300A 4.6 x 250 mm column 12 µm particle size	Cat. no. 83-603-C Ranin Instrument Co. Inc., Woburn, MA, USA
6. Dynamax C8 4.6 x 15 mm guard columns 8 µm particle size	Cat. no. 83-301-G Ranin Instrument Co. Inc., Woburn, MA, USA

7. Sialylated bovine fetuin N-linked alditols — Cat. no. 043064
Dionex Corp., Sunnyvale, CA, USA

8. Neuraminidase, *A. ureafaciens* — Cat. no. 269 611
Boehringer Mannheim GmbH,
Mannheim, Germany

Method

A: Sample preparation

Between 20 000 and 50 000 cpm of the N-linked oligosaccharides were resuspended in 50 µL of sterile water and stored at -20°C until analysis. Immediately before injection, the samples were thawed and the total volume was made up to 250 µL with 2.5 mmol/L KH_2PO_4 (pH 4.0). The samples were then passed through 3 mm Acrodisc 0.45 µm filters.

B: Anion-exchange HPLC programme conditions

All chemicals and water were of HPLC grade. The sample loop volume was 250 µL and the column flow rate 1 mL/min at 800–900 psi. Buffer A (2.5 mmol/L KH_2PO_4) and buffer B (250 mmol/L KH_2PO_4) were titrated to pH 4.0 with phosphoric acid. The gradient elution was programmed over 17.5 minutes. Following injection of the sample, there was a linear increase from 2.5 to 125 mmol/L KH_2PO_4 from 0 to 12.5 minutes, which was followed by a further linear increase from 125 to 250 mmol/L KH_2PO_4 over the next 2.5 minutes. The elution was isocratic for the last 2.5 minutes. The standard test procedure for the column was performed before each analytical run, and 5 µg of fetuin N-linked alditol standard was analysed at the beginning and end of each analytical run. The standard was detected with a UV detector with a 206 nm filter. The samples were collected into 70 x 250 µL fractions (0.25 min/fraction) and each fraction was counted in 4 mL of scintillation fluid.

C: Neuraminidase digest

Between 20 000 and 50 000 cpm of the N-linked oligosaccharides were resuspended in 50 µL of 0.1 mol/L sodium acetate (pH 5.5). Neuraminidase (50 mU or 5 µL) was then added and the sample was incubated in a 37°C water bath for 24 hours. A control sample was similarly treated except the neuraminidase was replaced with 5 µL of sterile water. At the end of the incubation, the sample volume was made up to 250 µL with 2.5 mmol/L KH_2PO_4 (pH 4.0). The sample was then passed through a 0.45 µm filter and injected directly onto the anion-exchange HPLC column. The sodium acetate buffer, when diluted to the final volume of 250 µL, did not interfere with the performance of the column (Figure 4.3). At least one representative control and galactosaemic sample from each of *peaks 1–7* were digested with neuraminidase to determine the proportion of sialylated N-linked oligosaccharides. Over 85% of the N-glycans from the control and galactosaemic samples were recovered in the void volume of the column after they were digested with neuraminidase. This showed that the charge on the anionic oligosaccharides was predominantly from sialic acid (Figure 4.4, page 68).

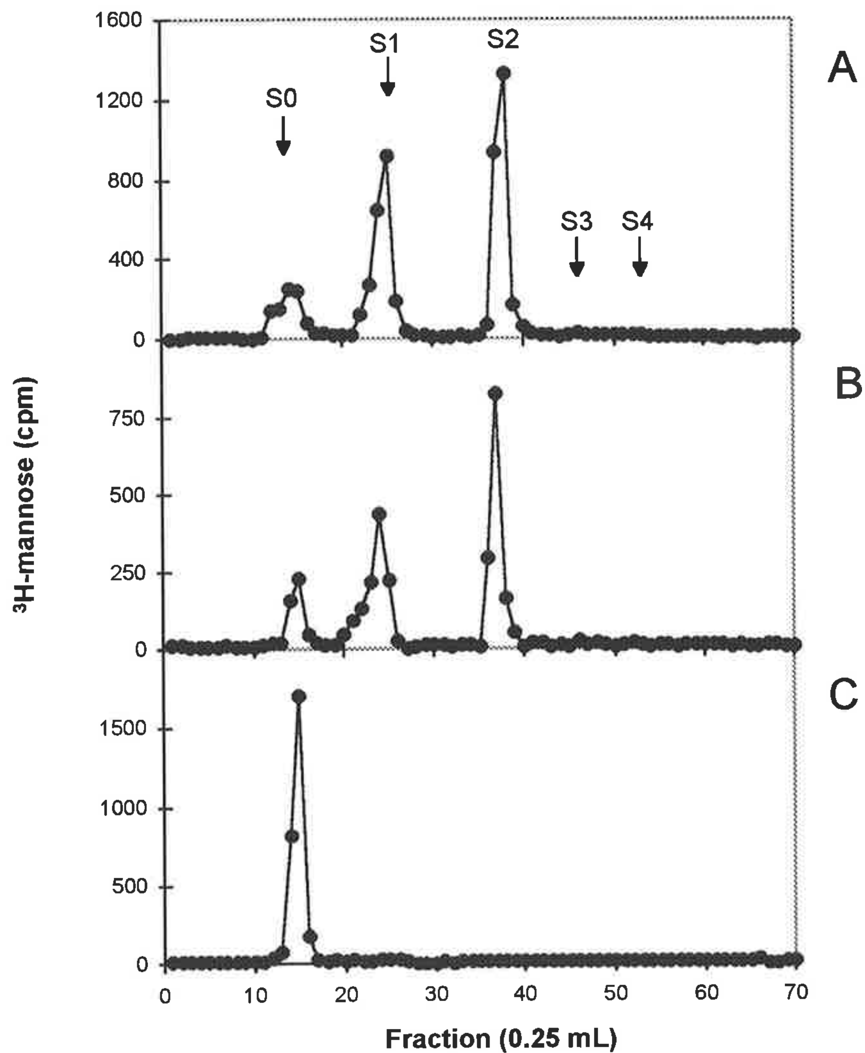


Figure 4.3. N-linked oligosaccharides (20 000 cpm) from a representative control sample separated by anion-exchange HPLC. ***A***, sample untreated; ***B***, sample incubated for 24 hours at 37°C in 0.1 mol/L sodium acetate buffer (without neuraminidase) and then injected directly onto the column; and *C*, sample incubated under the same conditions, but with neuraminidase, and then injected directly onto the column. The void (*S0*) eluted at 3.5 mL, disialylated fetuin N-linked alditol standard (*S2*) at 9.5 mL, trisialylated (*S3*) at 11.5 mL, and tetrasialylated (*S4*) at 13.5 mL.

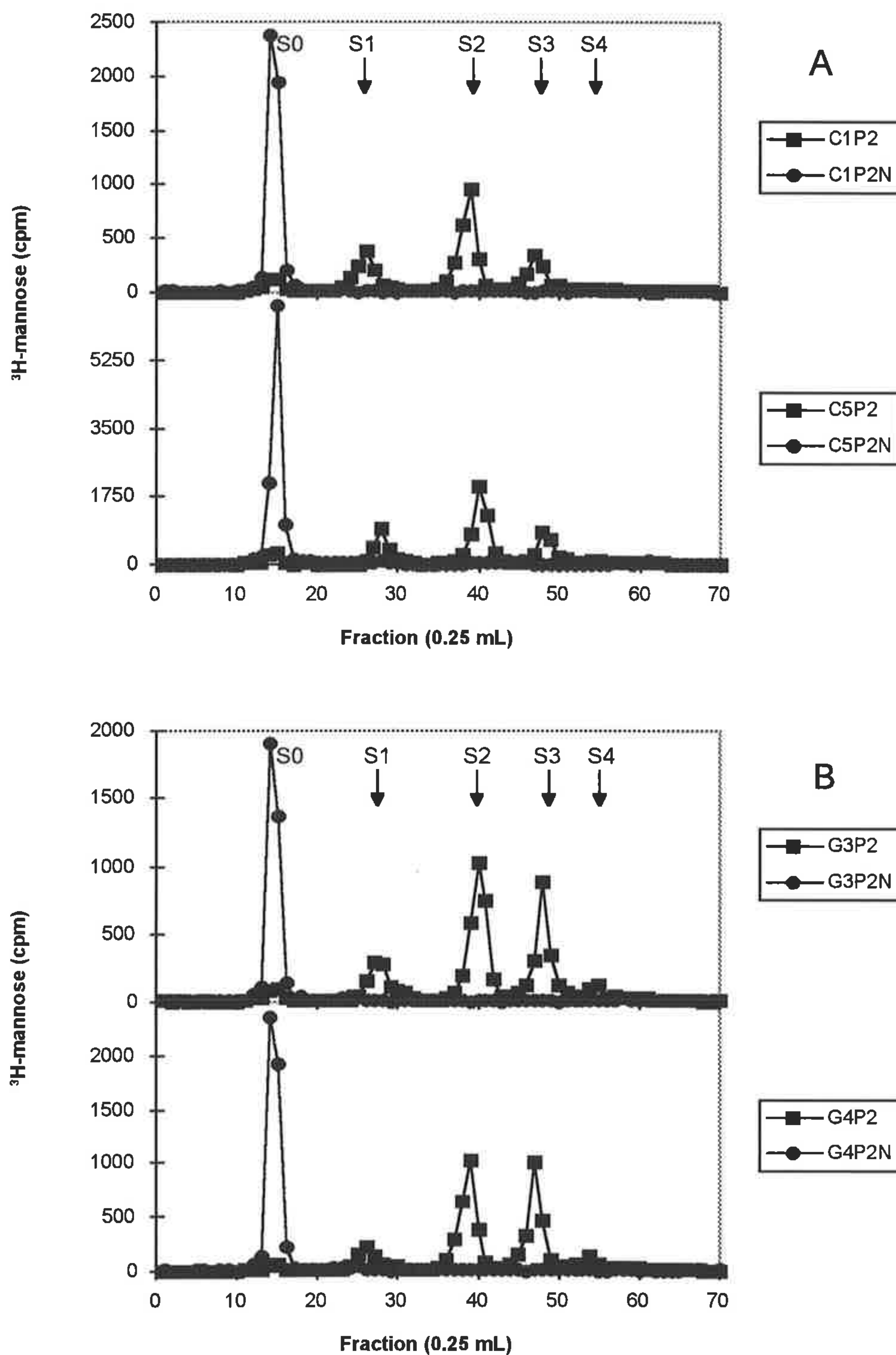


Figure 4.4. Anion-exchange HPLC profiles of cellular N-linked oligosaccharides (20 000 cpm) from *peak 2* (*P2*) of ***A***, two control (*C1P2* and *C5P2*); and ***B***, two galactosaemic (*G3P2* and *G4P2*) fibroblast cultures before and after neuraminidase (*N*) digestion. Over 85% of the N-glycans from the control and galactosaemic samples were recovered in the void following desialylation (*C1P2N, C5P2N, G3P2N,* and *G4P2N*). The void (*S0*) eluted at 3.5 mL, disialylated fetuin N-linked alditol standard (*S2*) at 9.5 mL, trisialylated (*S3*) at 11.5 mL, and tetrasialylated (*S4*) at 13.5 mL.

Method 3: The specificity of the incorporation of D-[2-^{3}H]-mannose into fibroblast N-linked oligosaccharides

Introduction

D-[2-^{3}H]-mannose is a relatively specific radiolabel. Even over prolonged radiolabelling periods the majority of label remains associated with mannose, while a lesser amount is converted to [2-^{3}H]-fucose (Yurchenco *et al.*, 1978; Varki, 1991; and Varki, 1994a). However, the extent of conversion depends on the cell type and the experimental conditions. It was therefore necessary to determine the specificity of the incorporation of [2-^{3}H]-mannose in the galactosaemic fibroblasts. The radiolabelled N-linked oligosaccharides were hydrolysed with acid and the monosaccharides were then separated by paper chromatography, according to established techniques (Colombo *et al.*, 1960; Lehnhardt and Winzler, 1968; Yurchenco *et al.*, 1978; and Beeley, 1985). The results showed that the incorporation and distribution of [2-^{3}H]-mannose were similar in the control and galactosaemic N-linked oligosaccharides (Figure 4.5).

Method

A: Acid hydrolysis of oligosaccharides

The samples consisted of 25 000 cpm of radiolabelled cellular N-linked oligosaccharides from *peak 2* of a representative control (*C2*) and galactosaemic (*G4*) fibroblast culture (see Method 1, page 60). The samples were lyophilised, resuspended in 50 µL of water, and then 50 µL of a 40% (w/v) fresh suspension of Dowex 50W x2 (H^+), 200–400 mesh (Sigma Chemical Co., St. Louis, MO, USA) in 0.02 mol/L HCl was added. The samples were placed in glass tubes, which were flushed with nitrogen and sealed with Teflon screw caps, and then incubated in a heating block at 100°C for 40 hours. The hydrolysates were then passed through minicolumns that contained 100 µL of a 20% (w/v) suspension of Bio-Rad AG 2-x8 (HCO_3^-), 200–400 mesh (Bio-Rad Laboratories, Hercules, CA, USA). The glass tubes were washed with 0.4 mL of water and this was also passed through the minicolumns. The columns were then eluted with 1 mL of 50% (v/v) methanol in water and the combined washes and eluate were dried in a rotary evaporator. The monosaccharides were resuspended in 50 µL of water. The recovery of the counts was complete.

B: Descending paper chromatography of monosaccharides

Aliquots (10 000 cpm) of the monosaccharides were separated on Whatman 3 MM paper, 57 cm long, in pyridine/ethyl acetate/water 1.0:3.6:1.15 (v/v/v), upper organic phase, over 18–20 hours. The monosaccharides were recovered in 40 x 1 cm fractions, eluted from the paper into 1 mL of water, and counted in 4 mL of scintillation fluid. The chromatograms were standardised against a range of unlabelled monosaccharides (10 µg of each), which were run concurrently and detected with alkaline $AgNO_3$ stain.

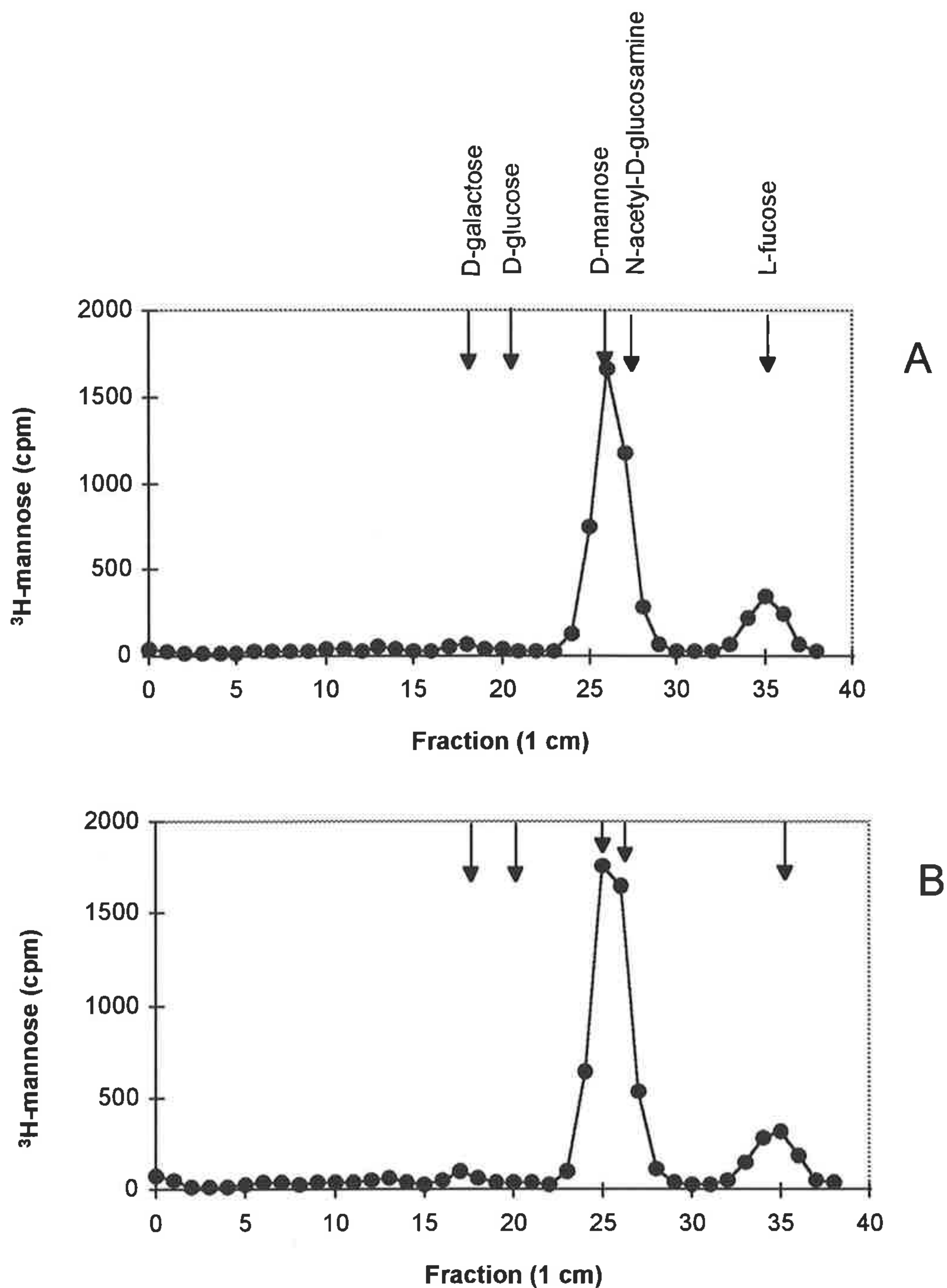


Figure 4.5. Approximately 10 000 cpm of radiolabelled monosaccharide from the cellular N-linked oligosaccharides from *peak 2* of a representative ***A***, control (*C2*) and ***B***, galactosaemic (*G4*) fibroblast culture were separated by descending paper chromatography. The distribution of radioactivity was similar in the control and galactosaemic samples with 70% of the total tritiated counts associated with mannose and 15% with fucose. Non-specific background accounted for the remaining radioactivity. R_f values for the monosaccharide standards (*arrows*) were: *L-fucose*, 1.0; *N-acetyl-D-glucosamine*, 0.79; *D-mannose*, 0.74; *D-glucose*, 0.61; and *D-galactose*, 0.52.

Method 4: Incorporation of D-[2-^{3}H]-mannose into fibroblast glycoprotein relative to total protein synthesis

Introduction

Monitoring the rate of incorporation of D-[2-^{3}H]-mannose into glycoprotein has been used as a an approach to screen for abnormalities in the synthesis and processing of N-glycans in cells (Varki, 1991). The assessment can be improved considerably if the incorporation is corrected to the incorporation of L-[^{35}S]-methionine into protein over the same period. This technique was used successfully in studying N-linked oligosaccharide synthesis in skin fibroblasts from patients with the CDG syndrome type I (Powell *et al.*, 1994; and Panneerselvam and Freeze, 1996). In the following method, the fibroblast cultures were radiolabelled with [2-^{3}H]-mannose and [^{35}S]-methionine for six hours, and the proteins from the cellular and secreted pools were then precipitated with TCA. The radioactivity incorporated into these protein fractions was then measured and expressed per µg protein and as a ratio of ^{3}H to ^{35}S. The method was adapted from Powell *et al.*, (1994). With this technique, it was possible to determine the effect of galactose on total N-glycan and protein synthesis in galactosaemic fibroblasts.

Method

A: Radiolabelling procedure

The skin fibroblasts cultures were established in BME with 10% (v/v) FBS. The fibroblasts were then subcultured into 25 cm^2 tissue culture flasks and grown to near-confluence in D-MEM with 10% (v/v) dialysed FBS. The cells were then washed twice with 5 mL of prewarmed glucose-deficient D-MEM and then preincubated for four hours in a modified D-MEM that contained 10% (v/v) dialysed FBS and either 2.5 mmol/L sodium pyruvate or 2.5 mmol/L D-glucose, and various concentrations of D-galactose, as required by the experimental procedure. The fibroblasts were then radiolabelled in the same medium for six hours by adding small aliquots of D-[2-^{3}H]-mannose (3.7 MBq/mL) and L-[^{35}S]-methionine (0.74 MBq/mL or 20 µCi/mL) directly to the medium.

B: Cell harvest and protein precipitation

The culture medium was removed and the fibroblasts were washed with 1 mL of ice-cold PBS. The medium and the wash were combined and centrifuged at 400 x g for three minutes. The supernatant was passed through a 0.2 µm syringe filter and then saved for analysis of the secreted glycoproteins. The fibroblasts were washed a further two times with 4 mL of ice-cold PBS and then harvested with 1 mL of trypsin-versene solution. The cells were centrifuged at 400 x g for three minutes and the pellet washed twice with 5 mL of ice-cold PBS. The pellet was resuspended in 1 mL of sterile water, sonicated for 30 seconds, and then 10 µL of 10% (w/v) SDS (0.1% final concentration) was added and mixed. A 25 µL aliquot of the sample was removed for estimation of the protein with a BCA microassay. To the remaining sample, 10 µL of 10% (w/v) BSA (1 mg) was added and then 250 µL of ice-cold 50% (w/v) TCA (10% final concentration). The sample was mixed and placed on ice for 15 minutes then centrifuged at 6000 x g for 15 minutes, and the supernatant was discarded. The precipitate

was washed with a further 1 mL of ice-cold 10% (w/v) TCA and then digested overnight in 250 µL of 1 mol/L NaOH. The radioactivity was then measured in a 25 µL aliquot. The incorporation of [2-^{3}H]-mannose and [^{35}S]-methionine were corrected for the protein content.

C: Media protein precipitation

To a 1 mL aliquot of the filtered medium, 250 µL of ice-cold 50% (w/v) TCA was added, and the sample was mixed and placed on ice for 15 minutes. The sample was then centrifuged at 6000 x g for 15 minutes and the supernatant was discarded. The precipitate was then washed four times with 1 mL of ice-cold 10% (w/v) TCA. The sample was mixed and centrifuged again during each wash step. The precipitate was then digested overnight in 250 µL of 1 mol/L NaOH and the radioactivity was measured in a 25 µL aliquot. The results were corrected for the total volume of the medium and the cell protein content.

Method 5: Radiolabelling, recovery, and size analysis of fibroblast dolichol-linked oligosaccharides

Introduction

Dolichol-linked oligosaccharides are the precursors of N-linked oligosaccharides. The oligosaccharides are synthesised in pyrophosphate-linkage to dolichol on the ER membrane (Figure 3.2, page 44). When completed, the oligosaccharides are transferred to asparagine residues of nascent protein within the lumen of the ER (Kornfeld and Kornfeld, 1985; and Hirschberg and Snider, 1987). Dolichol-linked oligosaccharides can be selectively labelled in the mannose, N-acetylglucosamine, or glucose residues with radiolabelled mannose, glucosamine, or galactose, respectively (Li *et al.*, 1978). However, D-[2-^{3}H]-mannose is most commonly used to label dolichol-linked oligosaccharides. The cell cultures are usually radiolabelled at 50–70% of confluence as the level of incorporation decreases with higher cell densities (Rosner *et al.*, 1982). The glucose concentration in the medium is also kept low to enhance mannose uptake. A complete absence of glucose from the medium, however, disturbs the equilibrium of the pathway and results in an accumulation of truncated dolichol-linked oligosaccharides (Gershman and Robbins, 1981; Rearick *et al.*, 1981a; and Chapman and Calhoun, 1988). Dolichol-linked oligosaccharides are also assembled very rapidly so short incubation times are required to isolate the intermediates in the pathway. Incorporation of [2-^{3}H]-mannose into $Glc_3Man_9GlcNAc_2$ reaches a steady-state equilibrium within 15–20 minutes. When fibroblasts are labelled for 30 minutes, the major intermediate observed is $Glc_3Man_9GlcNAc_2$, with lesser amounts of $Man_8GlcNAc_2$ and $Man_5GlcNAc_2$ (Hubbard and Robbins, 1979; and Hubbard and Robbins, 1980).

In the following method, the skin fibroblast cultures were radiolabelled with [2-^{3}H]-mannose for 30 minutes and the dolichol-linked oligosaccharides were then recovered in sequential lipid extractions. The oligosaccharides were then released from the dolichol by mild acid hydrolysis (Radin, 1969; Rosner *et al.*, 1982; and Powell *et al.*, 1994). The cell pellet was first extracted with chloroform/methanol 2:1 (v/v) to recover the smaller dolichol-linked intermediates. The pellet was then washed several times with water to remove degraded free oligosaccharides (Cacan *et al.*, 1987; and Cacan *et al.*, 1989) and low-molecular-weight intermediates (Panneerselvam and Freeze, 1996). The mature dolichol-linked oligosaccharides were then recovered in a chloroform/methanol/water 10:10:3 (v/v/v) extraction. Dolichol-linked oligosaccharides smaller than $Man_5GlcNAc_2$ usually partition in the chloroform/methanol 2:1 extract (Chapman *et al.*, 1979a; and Rosner *et al.*, 1982), although some intermediates can

appear in both solvent extracts (Rearick *et al.*, 1981b). The residual protein pellet contained the protein-bound oligosaccharides that were recently transferred to protein within the ER.

The oligosaccharides in the 2:1 and 10:10:3 solvent extracts were released from the dolichol by mild acid hydrolysis and were then separated by size on a Bio-Gel P-4 240 x 1 cm column (Rosner *et al.*, 1982). The residual protein pellet was solubilised in 0.6% (w/v) SDS and the protein-bound N-glycans were recovered with PNGase F, and similarly analysed (Varki, 1994a). Similar techniques were previously applied to the study of abnormalities in dolichol-linked oligosaccharide synthesis in skin fibroblasts from patients with the CDG syndrome type I (Powell *et al.*, 1994; Krasnewich *et al.*, 1995; and Panneerselvam and Freeze, 1996).

Reagents

1.	PNGase F, recombinant, *E. coli* lyophilisate in sodium phosphate buffer	Cat. no. 1365 193 Boehringer Mannheim GmbH, Mannheim, Germany

Method

A: Radiolabelling procedure

The skin fibroblasts were subcultured, at similar cell densities, into 8 cm sterile glass Petri dishes and grown in 5 mL of D-MEM with 10% (v/v) dialysed FBS to 50–70% of confluence (40–48 h). The cells were then washed twice with 4 mL of prewarmed glucose-deficient D-MEM and then preincubated for four hours in 4 mL of a modified D-MEM that contained 10% (v/v) dialysed FBS and either 2.5 or 5.5 mmol/L of D-glucose, and various concentrations of D-galactose, as required by the experimental procedure. The cells were then washed three times with 4 mL of prewarmed glucose-deficient D-MEM, and were then radiolabelled for 30 minutes in 2 mL of a modified D-MEM that contained 10% (v/v) dialysed FBS, 0.5 mmol/L D-glucose, and 3.7 MBq/mL of D-[2-^{3}H]-mannose. The glass dishes were turned every 10 minutes during the incubation to ensure even distribution of the radiolabel.

B: Cell harvest

The medium was removed and the glass dish was washed twice, quickly, with ice-cold PBS. The dish was then flooded with 4 mL of chloroform/methanol 2:1 (v/v) at 0°C and the fibroblasts were harvested with a Teflon cell scraper. The cellular material was then transferred to a 12 mL conical glass tube and stored at -20°C for two days, until extracted.

C: Extraction procedure

The sample was brought to room temperature for 15 minutes, with gentle mixing, and then centrifuged at 1000 x g for 10 minutes. The pellet was extracted a further two times with 2 mL of chloroform/methanol 2:1 (v/v) and the extracts were pooled (8 mL in total). The pellet was then dried under a stream of nitrogen, resuspended in 1 mL of sterile water, and then centrifuged at 1000 x g for 10 minutes. The pellet was then washed 3–4 times with 1 mL of water until the radioactivity in the washes was minimised. The pellet was then extracted once with 2 mL, and then twice with 1 mL, of chloroform/methanol/water 10:10:3 (v/v/v), and the

extracts were pooled (4 mL in total). The residual protein pellet was dried under a stream of nitrogen and resuspended in 500 µL of 0.6% (w/v) SDS. A 10 µL aliquot was taken for estimation of the protein with a BCA microassay and a 25 µL aliquot was used to determine the incorporation of [2-^{3}H]-mannose. The solubilised protein was then stored at -20°C.

D: Clean-up procedure for the chloroform/methanol extract

The chloroform/methanol 2:1 extracts (8 mL in total) were washed once with 2 mL of 4 mmol/L $MgCl_2$ and then a further three times with 1.5 mL of methanol/water/1 mol/L $MgCl_2$/chloroform 94:94:0.8:6 (v/v/v/v). During each wash step, the extracts were mixed for 10 minutes on a rocker bed and centrifuged at 400 x g for two minutes.

E: Mild acid hydrolysis procedure

The chloroform/methanol 2:1 and chloroform/methanol/water 10:10:3 extracts were evaporated to dryness under a stream of nitrogen in a heating block at 30–40°C. The residues were resuspended in 0.5 mL of n-propanol, 1 mL of 0.1 mol/L HCl was added, and the glass tubes were sealed with a Teflon screw cap and placed in a heating block at 100°C for 20 minutes. The samples were then dried under a stream of nitrogen in a heating block at 60–70°C (approx. 20 min), and the free oligosaccharides were resuspended in 400 µL of water. A 10 µL aliquot was used to determine the incorporation of [2-^{3}H]-mannose. The recovery of the radiolabel was complete. The oligosaccharides were sized on a Bio-Gel P-4 240 x 1 cm column with an internal standard of 100 µg BSA, 500 cpm [^{3}H]-mannose, and 10 µg L-fucose.

F: Recovery of protein-bound oligosaccharides

The protein-bound oligosaccharides were recovered from the residual protein pellet according to Varki (1994a). An aliquot of the sample (125 µL or 1/4) was taken and the volume was made up to 500 µL with water and 50 mmol/L 2-mercaptoethanol. The sample was then heated at 100°C for 3–5 minutes to denature the protein and then 500 µL of 0.2 mol/L Tris-HCl (pH 8.0), 1% (v/v) Nonidet P-40, and 20 mmol/L Na_2EDTA was added. Two units of PNGase F (10 µL) were added and the sample was then incubated in a 37°C water bath for 18 hours. The incubation was stopped by placing the sample in a 100°C water bath for three minutes. Control digests (1–5% of the original sample) were similarly treated, except Tris-HCl buffer was substituted for the enzyme.

The sample volume was made up to 2 mL with running buffer and the sample was then loaded onto a Sephacryl S-200 40 x 1.5 cm column in 0.1% (w/v) SDS/50 mmol/L Tris-HCl (pH 7.0), at a flow rate of 1 mL/min. Forty-five 2 mL fractions were collected and the fractions that contained the free oligosaccharides were pooled (Figure 4.6A). Saturated KCl (0.01 vol) was added to the pooled fractions, which were placed on ice for two hours, and then centrifuged at 1750 x g for 10 minutes to precipitate the SDS. The supernatant was then passed slowly over a 2 mL Biobead SM-2 column and the column was washed five times with 2 mL of water. The initial fractions, which contained the oligosaccharides, were pooled and lyophilised. The oligosaccharides were then resuspended in 1 mL of ice-cold water and desalted on a Bio-Gel P-2 50 x 1.5 cm column in water at 20 mL/h. Forty 2 mL fractions were collected and the oligosaccharides were recovered from near the void (Figure 4.6B). The oligosaccharides were then lyophilised, resuspended in 400 µL of water, and then sized on a Bio-Gel P-4 240 x 1 cm column with an internal standard of 100 µg BSA, 500 cpm [^{3}H]-mannose, and 10 µg L-fucose.

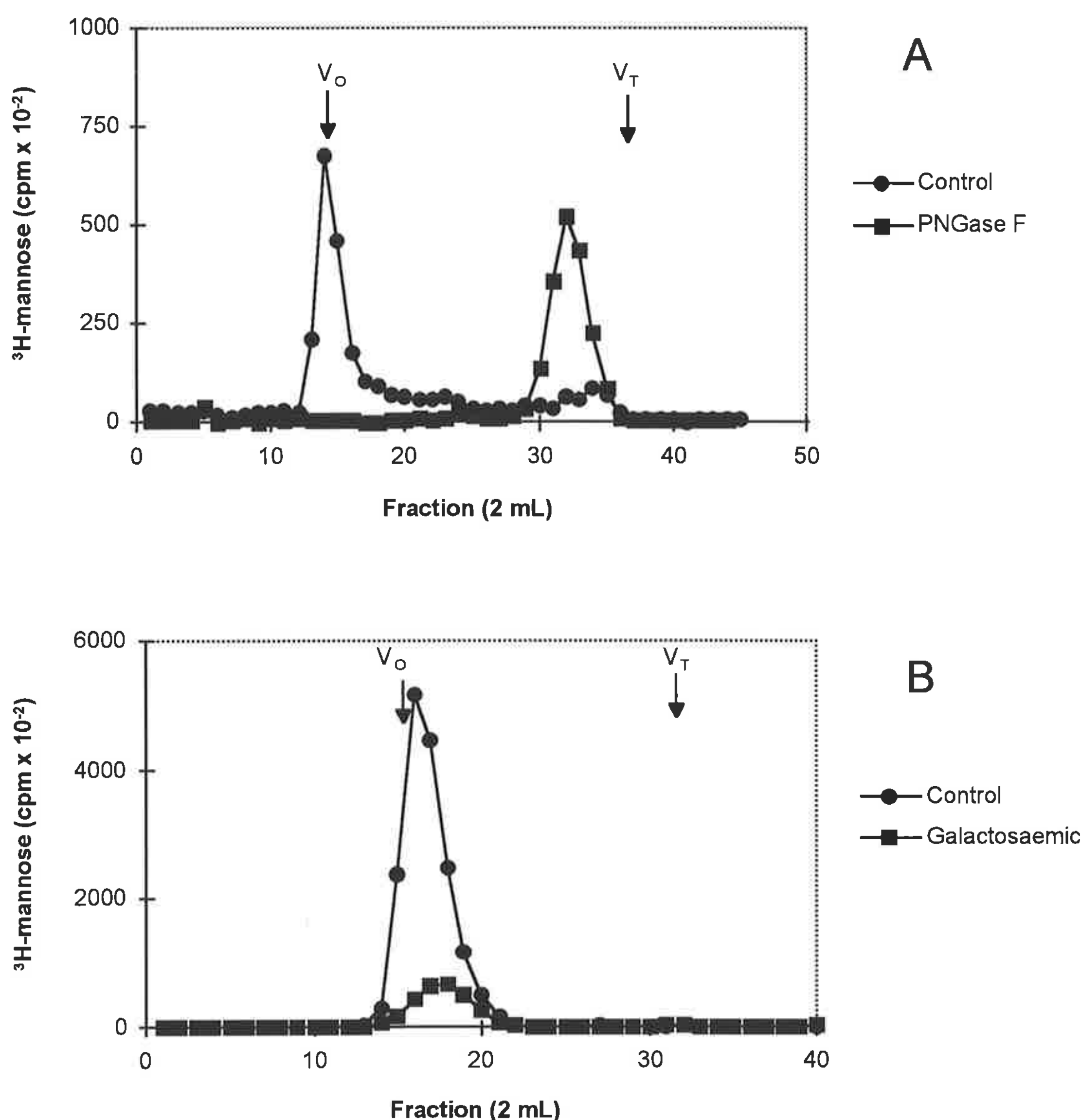


Figure 4.6. ***A,*** recovery of N-linked oligosaccharides from the residual protein pellet of a representative fibroblast culture. The protein-bound oligosaccharides in the control digest (*Control*) eluted from the Sephacryl S-200 40 x 1.5 cm column near the void. After the same sample was digested with PNGase F (*PNGase F*), the free oligosaccharides were recovered in later fractions. The column was eluted with 0.1% (w/v) SDS/50 mmol/L Tris-HCl (pH 7.0) at a flow rate of 1 mL/min. The exclusion volume (V_O) for the column was 34 mL and the inclusion volume (V_T) 75 mL. ***B,*** N-linked oligosaccharides from the residual protein pellet of a representative control and galactosaemic fibroblast culture were desalted on a Bio-Gel P-2 50 x 1.5 cm column in water at 20 mL/h. The oligosaccharides eluted as a single peak near the void and were recovered for size analysis. The exclusion volume (V_O) for the column was 30 mL and the inclusion volume (V_T) 64 mL.

Method 6: Radiolabelling, immunoprecipitation, and electrophoresis of LAMP-1 from fibroblasts

Introduction

LAMP-1 was chosen as a model glycoprotein to examine N-glycosylation in galactosaemic fibroblasts. LAMP-1 is one of the major sialoglycoproteins found in the lysosome (for a review see Fukuda, 1991). It is a type I membrane protein that is localised to the lysosomal membrane (Chen *et al.*, 1985a), but it also shuttles between lysosomes, endosomes and the plasma membrane (Carlsson and Fukuda, 1992; and Akasaki *et al.*, 1995). It can account for 0.1–0.2% of the total protein within a cell (Chen *et al.*, 1985b). Although the exact function of LAMP-1 is unknown, it has been implicated in the presentation of carbohydrate ligands to selectins (Do *et al.*, 1990; and Sawada *et al.*, 1993), in tumour cell metastasis (Chen *et al.*, 1988; and Heffernan *et al.*, 1989), and as part of the inflammatory response to bacteria (Dahlgren *et al.*, 1995; and Karlsson *et al.*, 1996). The major portion of LAMP-1 resides on the lumenal side of the lysosome and is heavily glycosylated. Human LAMP-1 contains 416 amino acid residues and 18 N-linked glycans. The N-glycans are clustered in two domains that are separated by a hinge region (Carlsson *et al.*, 1988; and Fukuda *et al.*, 1988). The hinge also contains a cluster of O-glycans that may protect this region from lysosomal proteases (Carlsson *et al.*, 1993). Near the carboxy terminus, there is a transmembrane domain of 24 hydrophobic amino acids, and a short cytoplasmic tail that contains a tyrosine structural motif, which is important in targeting lysosomal membrane proteins to the lysosome (Guarnieri *et al.*, 1993).

LAMP-1 is synthesised in the ER as a 90 kDa high mannose precursor and is then transported to the trans-Golgi network from the ER within 15–30 minutes. Pulse-chase labelling experiments with [^{35}S]-methionine have indicated that the majority of LAMP-1 follows a direct pathway to late-endosomes and subsequently lysosomes within 60–90 minutes of synthesis. Approximately 25% of LAMP-1, however, is transported to the lysosome by an alternative pathway that involves cell surface expression and the endocytic pathway (Chen *et al.*, 1985b; Chen *et al.*, 1986; D'Souza and August, 1986; Carlsson and Fukuda, 1992; and Akasaki *et al.*, 1995). Many different cell lines, including fibroblasts, express LAMP-1. The apparent molecular mass ranges from 105 to 140 kDa depending upon the cell type and stage of differentiation (Chen *et al.*, 1985a; and Chen *et al.*, 1985b). The heterogeneity of size results from differences in the oligosaccharide composition of the glycoprotein. LAMP-1 is extensively modified by N-glycans and contains sialylated complex-type, high mannose-type, and polylactosamine-type N-linked oligosaccharides (D'Souza and August, 1986; and Carlsson *et al.*, 1988). Over half the mass of LAMP-1 is due to the N-glycans as the molecular mass of the core polypeptide is only 40 kDa (Carlsson *et al.*, 1988). Five of the N-glycans can be potentially modified to longer poly-N-acetyl-lactosamine chains (Carlsson and Fukuda, 1990).

LAMP-1 has served as a useful model of the biosynthesis of poly-N-acetyl-lactosamines and of the processing of polylactosamine-containing glycoproteins. LAMP-1 was therefore selected as a potential model of the effects of galactose on N-glycosylation in galactosaemic fibroblasts. In the following method, the skin fibroblast cultures were radiolabelled with [^{35}S]-methionine for between 5 and 30 minutes and then chased for up to one hour. The radiolabelling procedures incorporated various concentrations of galactose in the culture medium. The fibroblasts were then lysed and the LAMP-1 was immunoprecipitated with anti-LAMP-1 antibody. The rabbit anti-LAMP-1 polyclonal antibody was kindly provided by S. R. Carlsson, Department of Medical Biochemistry and Biophysics, Umea University, Sweden (Dahlgren *et al.*, 1995). The LAMP-1 was then recovered and analysed by 10% SDS-PAGE, and visualised

by autoradiography. Tunicamycin was added to one of the control cultures to determine the size of the LAMP-1 nascent protein and to confirm that the polyclonal antibody recognised deglycosylated LAMP-1. Tunicamycin is a false sugar nucleotide that competitively inhibits the first enzymatic step in the dolichol-linked oligosaccharide pathway (the synthesis of GlcNAc-PP-Dol) and thereby inhibits N-glycosylation (Schwarz and Datema, 1982; and Elbein, 1984).

Materials and reagents

1. Tunicamycin, stock solution 1 mg/mL in 0.1 mol/L NaOH — Cat. no. T-7765, Sigma Chemical Co., St. Louis, MO, USA

2. Lysis buffer — 10 mmol/L Tris-HCl (pH 7.0), 0.15 mol/L NaCl, 1% (v/v) Nonidet P-40, 4 mmol/L Na_2EDTA, 0.02% (w/v) NaN_3, 1 mmol/L phenylmethylsulfonyl fluoride

3. Protein A Sepharose CL-4B — Cat. no. 17-0780-01, Pharmacia AB, Uppsala, Sweden

4. Protein A Sepharose bound to rabbit immunoglobulin
   Filter sterilise 1 mL of pre-immune rabbit sera and mix with 1 mL of packed protein A Sepharose on a rocker bed overnight at 4°C. Wash the Sepharose three times with 0.25 mol/L NaCl/20 mmol/L Tris-HCl (pH 7.0), and resuspend it in 1 mL of the same solution.

5. Affi-gel 10, coupled to gelatin at 1 mg/mL — Cat. no. 153-6099, Bio-Rad Laboratories, Hercules, CA, USA

6. Rabbit anti-LAMP-1 polyclonal antibody

7. Wash buffer — 20 mmol/L Tris-HCl (pH 7.0), 0.15 mol/L NaCl, 0.5% (v/v) Nonidet P-40, 4 mmol/L Na_2EDTA, 0.02% (w/v) NaN_3

8. Reducing sample buffer — 20 mmol/L Tris-HCl (pH 7.0), 10% (v/v) glycerol, 1% (w/v) SDS, 5% (v/v) 2-mercaptoethanol,

9. Liqui-gel, 40% (w/v) acrylamide/bis (37.5:1) — Gradipore Ltd, Pyrmont, NSW, Australia

10. Ammonium persulphate — Cat. no. 161-0700, Bio-Rad Laboratories, Hercules, CA, USA

11. N,N,N',N'-tetramethyl-ethylenediamine (TEMED) — Cat. no. 161-0801, Bio-Rad Laboratories, Hercules, CA, USA

12. [^{14}C]-Methylated protein standards M_r range 14 300–220 000 — Cat. no. CFA 626, Amersham Int. plc, Little Chalfont, Bucks, England

13. 'Amplify' — Cat. no. NAMP 100

Amersham Int. plc, Little Chalfont, Bucks, England

14. Kodak Scientific Imaging Film XAR 5 — Cat. no. 1651454, Eastman Kodak Co., Rochester, NY, USA

Method

A: Radiolabelling procedure

The skin fibroblasts were grown to near-confluence in 75 cm^2 tissue culture flasks in BME with 10% (v/v) FBS. The cells were then washed twice with 5 mL of prewarmed PBS and then preincubated for four hours in 3 mL of a modified D-MEM (without L-cystine and L-methionine) that contained 10% (v/v) dialysed FBS and various concentrations of galactose, as required by the experimental procedure. [^{35}S]-Protein Labeling Mix (3.7 MBq/mL) was then added directly to the medium and the fibroblasts were radiolabelled for either 5 or 30 minutes. The medium was then removed and the cells were washed twice with 5 mL of prewarmed chase medium. The fibroblasts were then chased for various times in 5 mL of a modified D-MEM that contained 10% (v/v) dialysed FBS and the same galactose concentrations as used in the preincubation medium. Tunicamycin (10 µg/mL) was added to the medium of one control culture, one hour before radiolabelling, and also during the chase.

B: Cell harvest

All the harvest procedures were done on ice or at 4°C. The chase medium was removed and the cells were washed three times with 5 mL of ice-cold PBS. The fibroblasts were then harvested with a Teflon cell scraper in an additional 5 mL of ice-cold PBS and the cells were then centrifuged at 400 x g for five minutes. The pellet was washed a further two times with 10 mL of ice-cold PBS, resuspended in 1 mL of lysis buffer, and then freeze-thawed six times in a methanol/dry ice slurry. The sample was then centrifuged at 6000 x g for five minutes and the supernatant was recovered for the immunoprecipitation of LAMP-1.

C: Immunoprecipitation of LAMP-1

All the immunoprecipitation steps were done at 4°C. The 1 mL of cellular supernatant fluid was added to 50 µL of packed protein A Sepharose bound to pre-immune rabbit sera, and then mixed on a rocker bed for between four hours and overnight. The sample was then centrifuged at 800 x g for one minute and the supernatant was added to 100 µL of packed gelatin-coupled Affi-gel, and mixed on a rocker bed for between four hours and overnight. The sample was then centrifuged at 800 x g for one minute and the supernatant was added to 5 µL of rabbit anti-LAMP-1 polyclonal antibody, and mixed on a rocker bed for between four hours and overnight. Next, 50 µL of packed protein A Sepharose was added, and the sample was mixed on a rocker bed for between four hours and overnight. The sample was then centrifuged at 800 x g for one minute and the supernatant was removed. The protein A Sepharose was then washed ten times with wash buffer and a further three times with 0.25 mol/L NaCl/20 mmol/L Tris-HCl (pH 7.0). The radioactivity was checked in successive steps to monitor the effectiveness of the washes. The LAMP-1 was then eluted from the protein A Sepharose by heating it in a 100°C water bath for three minutes with 100 µL of reducing sample buffer. The

sample was then centrifuged at 800 x g for one minute and the radioactivity was measured in a 5 µL aliquot of the supernatant. The LAMP-1 was then analysed by 10% SDS-PAGE and visualised with autoradiography.

D: Gel preparation

A 10% running gel was prepared from 7.5 mL of 40% (w/v) acrylamide/bis (37.5:1), 11.2 mL of 1 mol/L Tris-HCl (pH 8.8), 0.3 mL of 10% (w/v) SDS, and 11.2 mL of water. The solution was degassed for 15 minutes and then 100 µL of freshly made ammonium persulphate (100 mg/mL) and 20 µL of TEMED were added and mixed immediately. The gel was poured into a Bio-Rad model P-II casting stand with 20 x 18 cm and 20 x 16 cm glass plates and a 1.5 mm spacer. The gel was left to set for 30 minutes under a layer of isopropanol. The isopropanol was then removed and replaced with a wash solution of 4.7 mL of water, 2.8 mL of 1 mol/L Tris-HCl (pH 8.8), and 0.075 mL of 10% (w/v) SDS, and the gel was left for between 30 minutes and overnight.

A 5% stacking gel was prepared from 1.25 mL of 40% (w/v) acrylamide/bis (37.5:1), 1.25 mL of 1 mol/L Tris-HCl (pH 6.8), 0.1 mL of 10% (w/v) SDS, and 7.45 mL of water. The solution was degassed for 15 minutes and then 50 µL of freshly made ammonium persulphate (100 mg/mL) and 10 µL of TEMED were added and mixed immediately. The wash solution was then removed from the running gel, a comb was positioned, and the stacking gel was poured and allowed to set for one hour.

E: Gel electrophoresis

The 4 L of running buffer (pH 8.3) contained 12.12 g of Tris base, 57.68 g of glycine, and 4 g of SDS. The [^{14}C]-methylated protein standard (1 µL) was added to 100 µL of reducing sample buffer, and 5 µL of a tracker dye (Bromphenol Blue in reducing sample buffer) was added to each sample and the standard. The samples and standard were then heated in a 100°C water bath for three minutes. The gel slab was then attached to a Bio-Rad Protean™ II electrophoresis apparatus, the reservoir was filled with running buffer, the comb was carefully removed from the gel, and the samples were loaded into the wells using a 100 µL glass syringe. The electrophoresis was performed with a Pharmacia EPS 500/400 power supply at a constant current (40 mA) and with the voltage set at 400 V. The electrophoresis was stopped shortly before the tracker dye reached the bottom of the gel.

F: Gel autoradiography

After the gel was removed from the electrophoresis apparatus, it was fixed in propan-2-ol/water/glacial acetic acid 25:65:10 (v/v/v) for 30 minutes with constant shaking, and then soaked in Amplify for 30 minutes with shaking. The gel was then floated onto a sheet of filter paper, it was covered with cellophane wetted with Amplify, and then it was dried in a Bio-Rad Model 483 gel drier at 80°C for two hours. The dried gel was then placed in an autoradiography cassette, with intensifying screens and Kodak Scientific Imaging Film, and left at -80°C for between one and three days. The film was developed using X-ray film processing equipment and the autoradiograph images were scanned in a Microtek ScanMaker III reflectance scanner. The LAMP-1 molecular mass was estimated from a plot of electrophoretic mobility (mm) versus log molecular mass of the [^{14}C]-methylated protein standards.

5

The structure of N-linked oligosaccharides from galactosaemic fibroblasts

5.1 Introduction

In Chapter 2, much of the evidence in the galactosaemic literature suggested that there was a defect in terminal galactosylation, and hence sialylation, of complex N-glycans in classical galactosaemia. This evidence has been widely used to implicate abnormal glycoprotein synthesis in the long-term complications in galactosaemia and has been supported by the partial deficiency of cellular UDP-galactose. However, to date, the evidence has only implied a defect in N-glycosylation as no structural studies of N-glycans from galactosaemic cells or tissues have been done. The aim of this chapter was to recover N-linked oligosaccharides from galactosaemic fibroblasts and to examine their structure for possible defects in terminal galactosylation. The experimental plan was to radiolabel the galactosaemic fibroblasts with [2-^{3}H]-mannose and to recover the labelled mature N-glycans from the cellular and secreted proteins. The N-glycans were then examined by size and charge with Bio-Gel P-4 size-exclusion chromatography and anion-exchange HPLC. If the complex N-glycans were terminally incomplete then they should be smaller than normal, and have less charge due to incomplete sialylation. The experiments were also performed in various culture media, and at different stages of growth, to determine what factors might disturb the processing of N-linked oligosaccharides in galactosaemic fibroblasts.

5.2 Experimental plan

A: Five control (designated *C1, C2, C3, C4,* and *C5*) and four galactosaemic (designated *G1, G2, G3,* and *G4*; Table 4.1, page 56) fibroblast cultures were grown to near-confluence in 75 cm^{2} flasks. They were then radiolabelled with [2-^{3}H]-mannose for 24 hours in a modified D-MEM that contained 10% (v/v) dialysed FBS, 5 mmol/L sodium pyruvate, and 0.5 mmol/L D-glucose. The proteins from the cell pellet and medium were recovered separately and then digested with trypsin. The tryptic glycopeptides were desalted on a Bio-Gel P-2 column and the N-linked glycans were then released with PNGase F (Method 1, page 60). The free oligosaccharides were then separated on a Bio-Gel P-4 240 x 1 cm column. The individual peaks that eluted from this column (*peaks 1–7*; Figure 5.1, page 85) were recovered and analysed further by anion-exchange HPLC (Method 2, page 65).

B: One control (*C2*) and one galactosaemic (*G1*) fibroblast culture were grown to either 50%- or near-confluence in 25 cm^{2} flasks. They were then radiolabelled with [2-^{3}H]-mannose for 24 hours in a modified D-MEM that contained 10% (v/v) dialysed FBS and various concentrations of D-glucose, D-galactose, and sodium pyruvate: (*1*) 5.5 mmol/L glucose and 1.0 mmol/L pyruvate, (*2*) 5.5 mmol/L glucose and 5.5 mmol/L galactose, (*3*) 5.5 mmol/L galactose and 1.0 mmol/L pyruvate, (*4*) 2.5 mmol/L pyruvate, or (*5*) 5.5 mmol/L galactose.

The N-linked oligosaccharides from the cellular and secreted proteins were again recovered and analysed (Method 1, page 60; and Method 2, page 65).

5.3 Results of Experimental plan A

The control and galactosaemic fibroblasts exhibited slight morphological differences when they were cultured in either BME or D-MEM. The control fibroblasts grew in regular parallel arrays whereas the galactosaemic fibroblasts usually showed much more irregular bundling and increased granularity. However, there were no significant differences in either the time it took the cultures to reach the desired densities or in the total protein content of the cells. The growth characteristics of the galactosaemic fibroblasts were therefore similar to previous reports (Pourci *et al.*, 1990; and Wolfrom *et al.*, 1993).

The fibroblasts were radiolabelled in a culture medium that contained predominantly pyruvate to ensure adequate uptake and incorporation of [2-^{3}H]-mannose. Table 5.1 lists the total radiolabel incorporated by each fibroblast culture into the cell pellet, and the percent of the radiolabel that was finally recovered as free N-linked oligosaccharide. Similar amounts of radiolabel were recovered from each cell culture at each of the recovery steps. After the protein pellets were digested with trypsin there always remained an insoluble residue that had the characteristics of proteoglycan. No significant radiolabel was associated with this residual pellet (Table 5.1). The radiolabelled tryptic glycopeptides from each extract eluted from the Bio-Gel P-2 column as a single peak near the void (Figure 4.1, page 63). No significant amounts of either free [2-^{3}H]-mannose or labelled low-molecular-weight molecules were ever detected with the tryptic glycopeptides.

Table 5.1. Total incorporation of [2-^{3}H]-mannose into the cell pellets of five control and four galactosaemic fibroblast cultures, and the percent of radiolabel recovered in various fractions.

Cell culture	Total [2-^{3}H]-mannose incorporated (cpm)	Residual pellet [2-^{3}H]-mannose (cpm)	Tryptic [2-^{3}H]-glycopeptides recovered from Bio-Gel P-2 column (cpm)	[2-^{3}H]-N-linked oligosaccharides recovered from Bio-Gel P-4 column (cpm)
C1	14 321 494	94 593 (0.7)[a]	11 143 790 (78)	7 128 840 (50)
C2	5 730 352	23 275 (0.4)	4 506 830 (79)	3 110 270 (54)
C3	8 273 286	55 532 (0.7)	8 028 980 (97)	5 206 130 (63)
C4	3 204 403	17 456 (0.5)	2 885 330 (90)	1 962 970 (61)
C5	3 485 904	41 049 (1.2)	3 000 673 (86)	1 721 770 (49)
G1	5 587 979	105 446 (1.9)	4 903 440 (88)	3 141 750 (56)
G2	8 872 018	258 756 (2.9)	8 546 270 (96)	5 587 700 (63)
G3	6 469 479	102 244 (1.6)	5 683 820 (88)	5 144 720 (80)
G4	4 760 444	68 859 (1.4)	2 979 060 (63)	2 094 010 (44)

[a] Percent of total [2-^{3}H]-mannose incorporated.

The Bio-Gel P-4 column was assembled from two Bio-Rad 120 x 1 cm low pressure columns connected in tandem, and was fitted with flow adaptors. The column was standardised against a range of complex N-glycan and oligomannose standards (Appendix 2, page 154). The performance characteristics of the column were stable over the two years of its use. However, as the flow rate was changed from 4 to 8 mL/h during this time, there were minor changes in the size and number of fractions collected, and in the exclusion and inclusion volumes. Each sample was separated by the column into approximately 200 fractions. For clarity, the representative figures in this chapter are therefore presented without the individual data points.

The N-linked oligosaccharides from the cellular pellets were consistently resolved by the Bio-Gel P-4 column into seven peaks, in both the control and galactosaemic fibroblasts (Figure 5.1, page 85). *Peaks 2–6* were clearly defined whereas *peaks 1* and *7* were the shoulders to the oligosaccharide profiles. *Peak 1* appeared near or in the void volume of the column. *Peaks 2* and *3* eluted from the column in the same position as tri- and disialylated fetuin N-linked alditol standards, respectively. The uncharged oligomannose standards eluted much later from the column; *peaks 6* and *7* were similar in size to $Man_8GlcNAc_2$ and $Man_5GlcNAc_2$ standards, respectively. Each of the peaks was recovered separately for analysis later by anion-exchange HPLC. The same peak numbering, *peaks 1–7*, was also used during the analysis of that data.

The Bio-Gel P-4 chromatographic profiles of the cellular N-linked oligosaccharides from the five control and four galactosaemic fibroblasts cultures are displayed in Figure 5.2A and B (page 87). The profiles were very consistent in their appearance for both the control and galactosaemic fibroblasts. There were no additional or missing peaks in the galactosaemic profiles, however, *peaks 1, 2,* and *3* were proportionally increased. This was reflected by a 38% mean increase in the proportion of total radioactivity that was associated with *peaks 1–3* in the galactosaemic as opposed to the control profiles (Table 5.2).

Table 5.2. The fractions of the total cellular [2-^{3}H]-N-linked oligosaccharides that appeared in *peaks 1–7* of five control (*C1, C2, C3, C4,* and *C5*) and four galactosaemic (*G1, G2, G3,* and *G4*) fibroblast cultures.

Peak	C1	C2	C3	C4	C5	mean (sd)
1	0.048	0.044	0.041	0.051	0.066	0.050 (0.010)
2	0.097	0.073	0.080	0.074	0.060	0.077 (0.013)
3	0.075	0.071	0.105	0.087	0.089	0.086 (0.013)
4	0.183	0.140	0.153	0.156	0.139	0.154 (0.018)
5	0.250	0.279	0.267	0.231	0.282	0.262 (0.021)
6	0.254	0.318	0.275	0.317	0.253	0.283 (0.033)
7	0.093	0.074	0.079	0.083	0.111	0.088 (0.015)
Peak	**G1**	**G2**	**G3**	**G4**		
1	0.076	0.085	0.058	0.063		*0.071 (0.012)
2	0.107	0.140	0.108	0.134		**0.122 (0.017)
3	0.093	0.089	0.111	0.115		*0.102 (0.013)
4	0.132	0.121	0.140	0.134		*0.132 (0.008)
5	0.221	0.171	0.206	0.182		**0.195 (0.023)
6	0.287	0.289	0.292	0.279		0.287 (0.006)
7	0.084	0.104	0.084	0.093		0.091 (0.010)

* Significantly different from the mean of the control fibroblasts: * $p < 0.05$, ** $p < 0.01$.

The N-linked oligosaccharides from the media of two representative control (*C4* and *C5*) and galactosaemic (*G1* and *G4*) fibroblast cultures were analysed in a similar fashion. The Bio-Gel P-4 chromatographic profiles of the media N-glycans were generally similar in appearance to the cellular profiles (Figures 5.3 and 5.2, pages 88 and 87). However, *peaks 6* and *7* were considerably reduced in the media profiles in both the control and galactosaemic cultures. This is consistent with an absence of small high mannose oligosaccharides in the mature secreted glycoproteins. The two galactosaemic cultures showed an increase (approximately 30%) in the proportion of total counts that appeared in *peaks 1–3*, similar to that seen in the cellular N-linked oligosaccharides. The profiles of the media N-linked oligosaccharides showed no other major differences.

The galactosaemic fibroblasts appeared to be producing an increased proportion of larger, and hence more branched, N-linked oligosaccharides than the control fibroblasts. This was an unexpected finding. The change in the oligosaccharide profiles could have resulted from an altered distribution of [2-^{3}H]-mannose within the galactosaemic fibroblasts, with more radiolabel associated with the larger N-glycans, and hence more signal. Therefore, the specificity of the incorporation of [2-^{3}H]-mannose into the N-linked oligosaccharides was determined. The cellular N-glycans from *peak 2* of a representative control (*C2*) and galactosaemic (*G4*) culture were hydrolysed with acid and the monosaccharides were then separated on paper chromatography (Method 3, page 69). The distribution of radiolabel was the same in the control and galactosaemic N-glycans, with 70% of the tritiated counts associated with mannose and 15% with fucose (Figure 4.5, page 70). This suggested that the radiolabel was incorporated into the larger N-glycans in a similar manner by the control and galactosaemic fibroblasts. The pattern of incorporation was also similar to previous studies in other cell lines (Yurchenco *et al.*, 1978).

When the individual oligosaccharide peaks from the chromatographic profiles were analysed by anion-exchange HPLC, it became apparent that the larger N-glycans in the galactosaemic fibroblasts were more heavily sialylated. Representative HPLC profiles of the cellular oligosaccharides from two control (*C2* and *C5*) and two galactosaemic (*G1* and *G2*) fibroblast cultures are displayed in Figures 5.4A and B (page 90), and 5.5A and B (page 92), respectively. In all the control and galactosaemic samples, *peak 1* contained predominantly tetra- and trisialylated N-glycans; *peak 2*, tri-and disialylated N-glycans; *peak 3*, disialylated N-glycans; and *peak 4*, monosialylated N-glycans. The patterns of sialylation for these peaks were therefore compatible with the standardisation data that was obtained from the fetuin N-linked alditols on the Bio-Gel P-4 column (Appendix 2, page 154). *Peaks 5, 6,* and *7* did not contain any anionic or sialylated oligosaccharides, in either control or galactosaemic samples, and were therefore not included in the data analysis of the sialylated oligosaccharides. The proportions of counts that appeared in each of the sialylated peaks were combined and then expressed as a fraction of the total counts in *peaks 1–4*, for each cell culture. It then became apparent that the galactosaemic fibroblasts produced approximately 35% more sialylated complex N-linked oligosaccharides (tetra-, tri-, and disialylated N-glycans) than the control fibroblasts (Figure 5.6A, page 93). The secreted N-linked oligosaccharides from the galactosaemic fibroblasts exhibited a similar increase in the complexity of sialylation (Figure 5.6B, page 93). The anion-exchange HPLC profiles of the secreted N-linked oligosaccharides from *C4, C5, G1,* and *G4* are displayed in Figure 5.7 (page 94). As *peaks 1–4* from the media samples were pooled and analysed together, the HPLC profiles for each sample in Figure 5.7 represent the total pattern for *peaks 1–4*.

The increase in the proportion of sialylated N-glycans in the galactosaemic fibroblasts was similar to the increase in the proportion of larger N-glycans (Table 5.2, page 82). Therefore, under the culture conditions of Experimental plan A, the galactosaemic fibroblasts produced approximately 30–40% more sialylated complex N-linked oligosaccharides than the control

fibroblasts. It was not clear whether the increase in size and sialylation represented a specific abnormality in N-glycosylation, or was a general characteristic of galactosaemic fibroblasts in culture. The changes could have been secondary to minor differences in the growth characteristics of the cells or a response to the particular experimental conditions. There was, however, no evidence for a functional defect in terminal sialylation. However, the fibroblasts had not been stressed with galactose and any minor abnormalities of galactosylation may not have been apparent. The same experimental procedures were therefore repeated in one control (*C2*) and one galactosaemic (*G1*) fibroblast culture, but on this occasion they were exposed to various culture media, some of which contained galactose (Experimental plan B, page 80). The radiolabelling procedures were also performed during rapid growth and at confluence to determine if the growth characteristics of galactosaemic fibroblasts could influence the pattern of N-linked oligosaccharides produced. The results of Experimental plan B are provided on page 95.

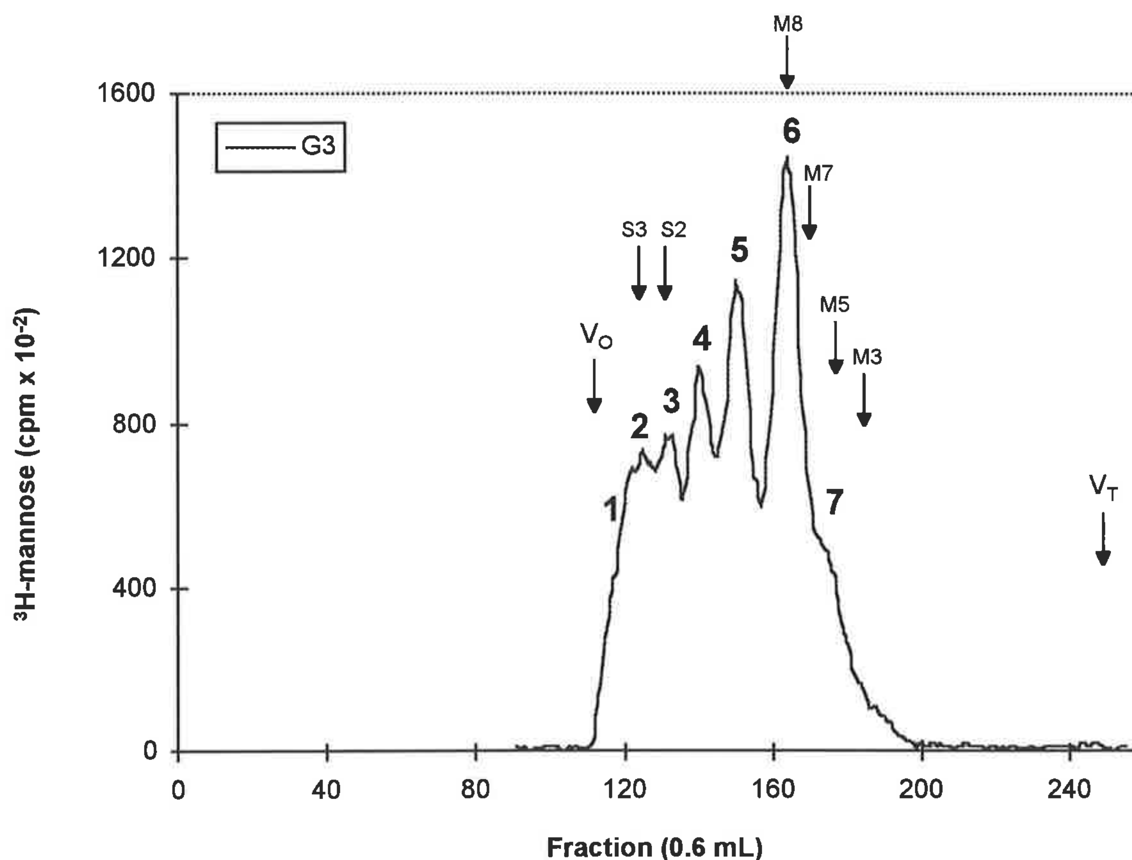


Figure 5.1. The N-linked oligosaccharides from the cellular protein of the fibroblast cultures were separated on a Bio-Gel P-4 240 x 1 cm column into seven individual peaks: *peak 1* (fractions 112–120), *peak 2* (fractions 121–128), *peak 3* (fractions 129–136), *peak 4* (fractions 137–145), *peak 5* (fractions 146–158), *peak 6* (fractions 159–173), and *peak 7* (fractions 174–193). The column was eluted with 0.1 mol/L NH_4COOH at 4 mL/h, and 255 x 0.6 mL fractions were collected. Similar peaks were recovered from each of the control and galactosaemic cultures, lyophilised, and prepared for analysis by anion-exchange HPLC. A range of tritiated oligosaccharide standards are shown (*arrows*): tri- and disialylated fetuin complex N-glycans (*S3* and *S2*), $Man_8GlcNAc_2$ (*M8*), $Man_7GlcNAc_2$ (*M7*), $Man_5GlcNAc_2$ (*M5*), and $Man_3GlcNAc_2$ (*M3*). The exclusion volume (V_O) for the column was 68 mL and inclusion volume (V_T) 149 mL.

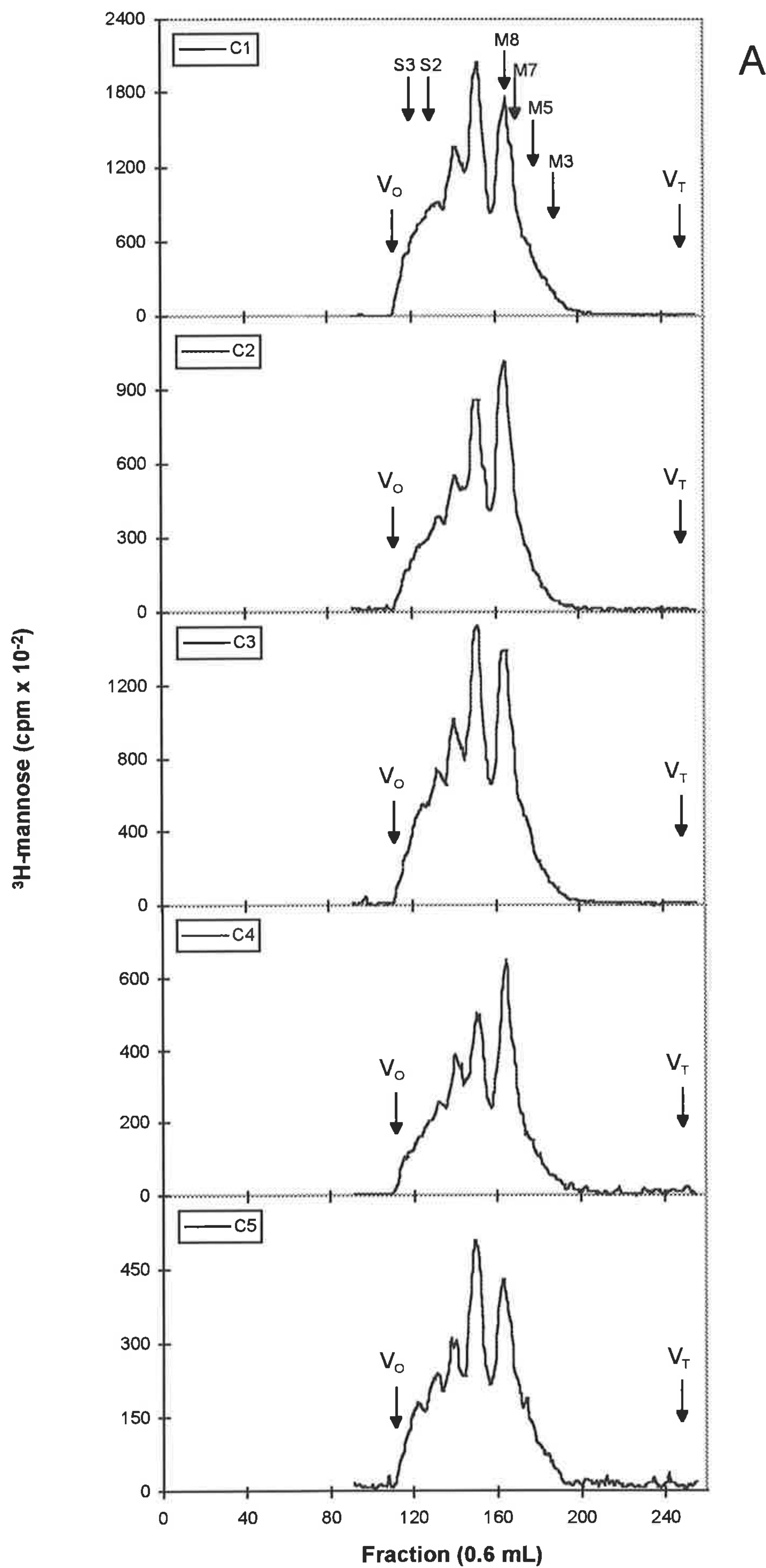


Figure 5.2 (A and B). *continued on next page*

***Figure 5.2** contd.*

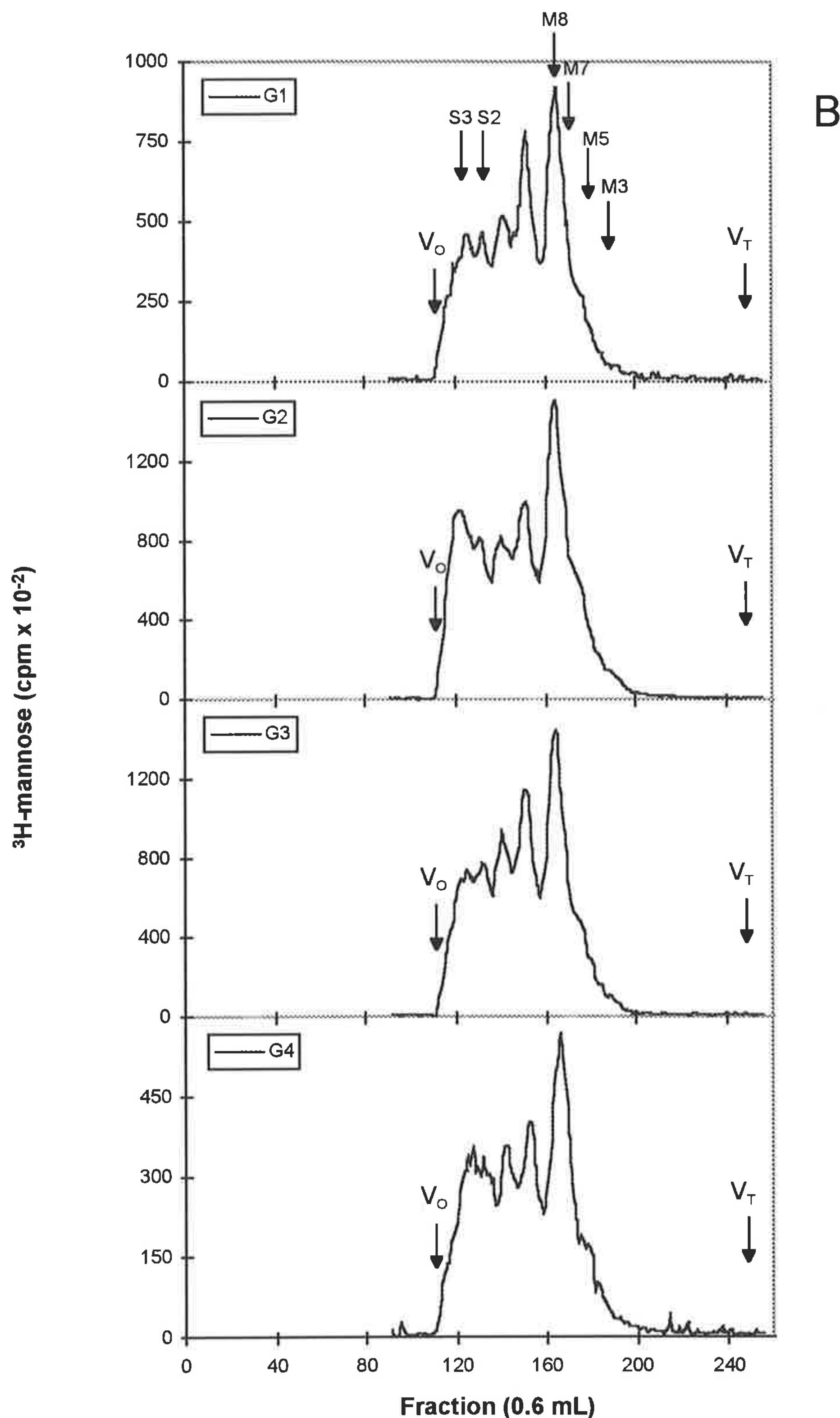


Figure 5.2 (A and B). Size analysis of [2-^{3}H]-mannose-labelled cellular N-linked oligosaccharides from ***A***, five control (*C1, C2, C3, C4,* and *C5*); and ***B***, four galactosaemic (*G1, G2, G3,* and *G4*) fibroblast cultures. The oligosaccharides were fractionated on a Bio-Gel P-4 240 x 1 cm column in 0.1 mol/L NH_4COOH at 4 mL/h. 255 x 0.6 mL fractions were collected and 6 µL of each fraction was counted in 4 mL of scintillation fluid. A range of tritiated oligosaccharide standards are shown (*arrows*): tri- and disialylated fetuin complex N-glycans (*S3* and *S2*), $Man_8GlcNAc_2$ (*M8*), $Man_7GlcNAc_2$ (*M7*), $Man_5GlcNAc_2$ (*M5*), and $Man_3GlcNAc_2$ (*M3*). The exclusion volume (V_O) for the column was 68 mL and inclusion volume (V_T) 149 mL.

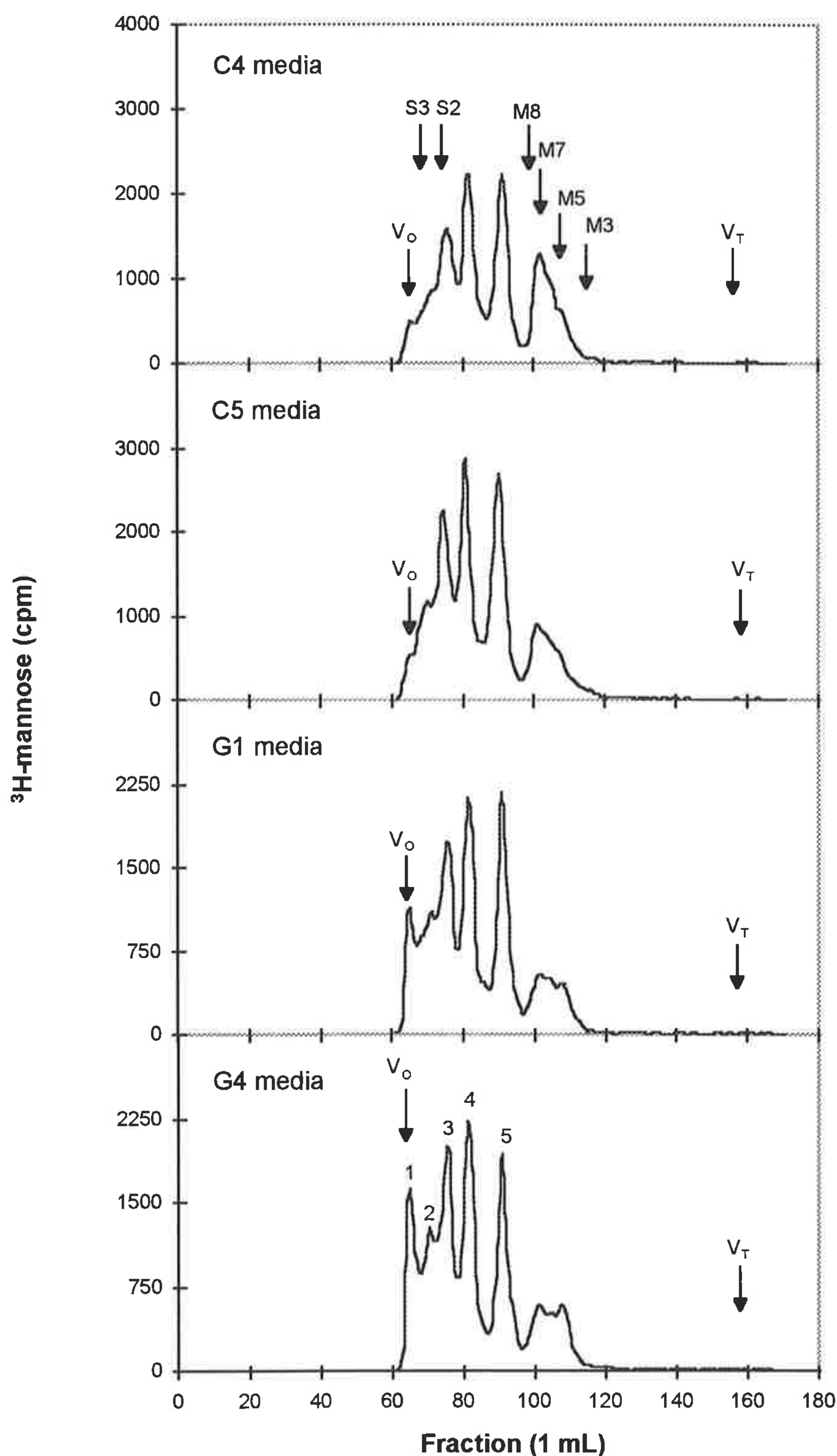


Figure 5.3. Size analysis of [2-^{3}H]-mannose-labelled N-linked oligosaccharides from the media of two control (*C4* and *C5*) and two galactosaemic (*G1* and *G4*) fibroblast cultures. The oligosaccharides were fractionated on a Bio-Gel P-4 240 x 1 cm column in 0.1 mol/L NH_4COOH at 8 mL/h. 175 x 1 mL fractions were collected and 50 000 cpm of each sample counted in total. Fractions 64–87 (*peaks 1–4*) of each sample were recovered, lyophilised, and prepared for analysis by anion-exchange HPLC. *Peaks 1, 2, 3, 4,* and *5* correspond in size to *peaks 1–5* of the cellular N-linked oligosaccharide profiles (Figure 5.1, page 85). A range of tritiated oligosaccharide standards are shown (*arrows*): tri- and disialylated fetuin complex N-glycans (*S3* and *S2*), $Man_8GlcNAc_2$ (*M8*), $Man_7GlcNAc_2$ (*M7*), $Man_5GlcNAc_2$ (*M5*), and $Man_3GlcNAc_2$ (*M3*). The exclusion volume (V_O) for the column was 65 mL and inclusion volume (V_T) 159 mL.

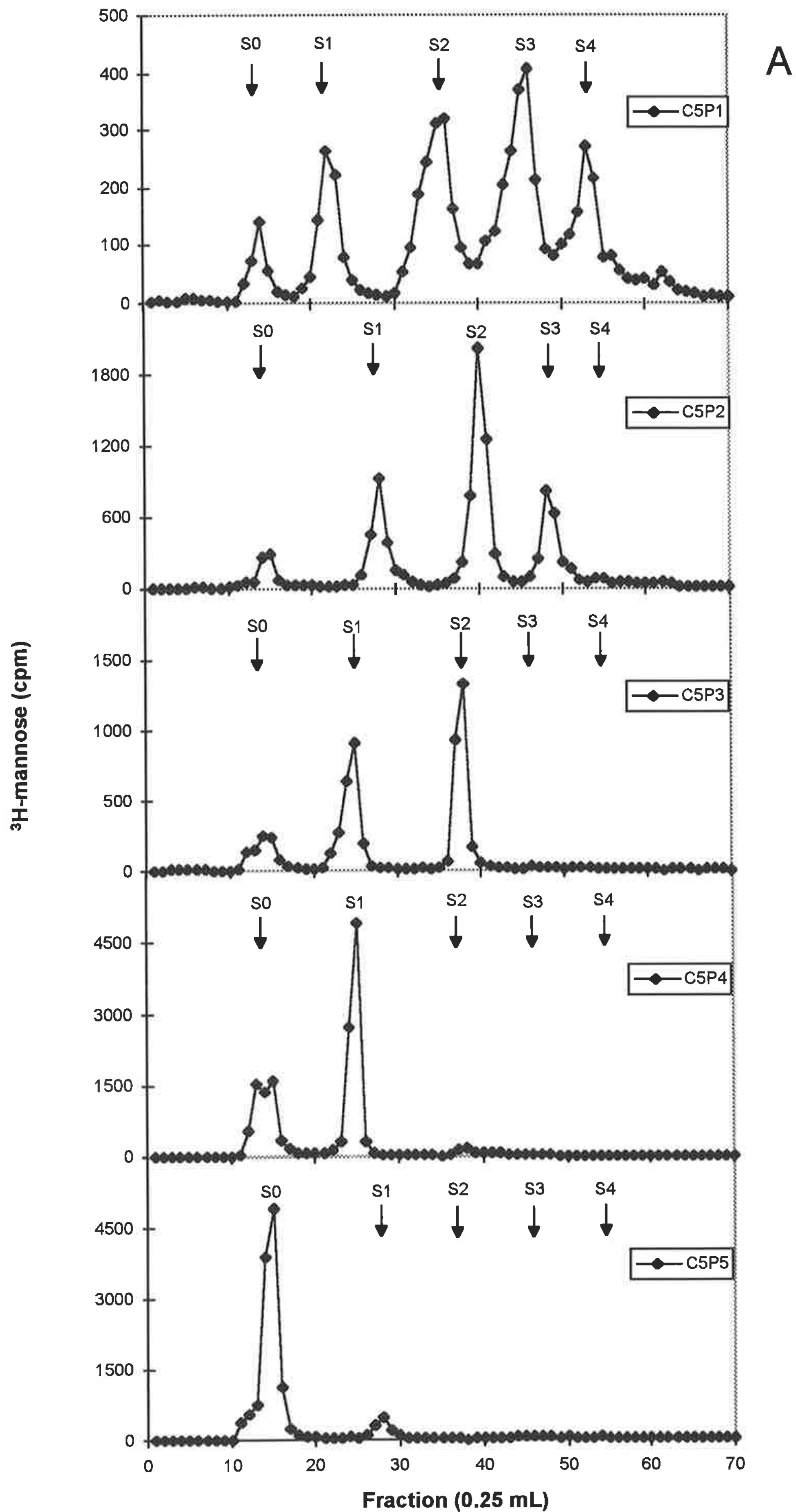


Figure 5.4 (A and B). *continued on next page*

Figure 5.4 contd.

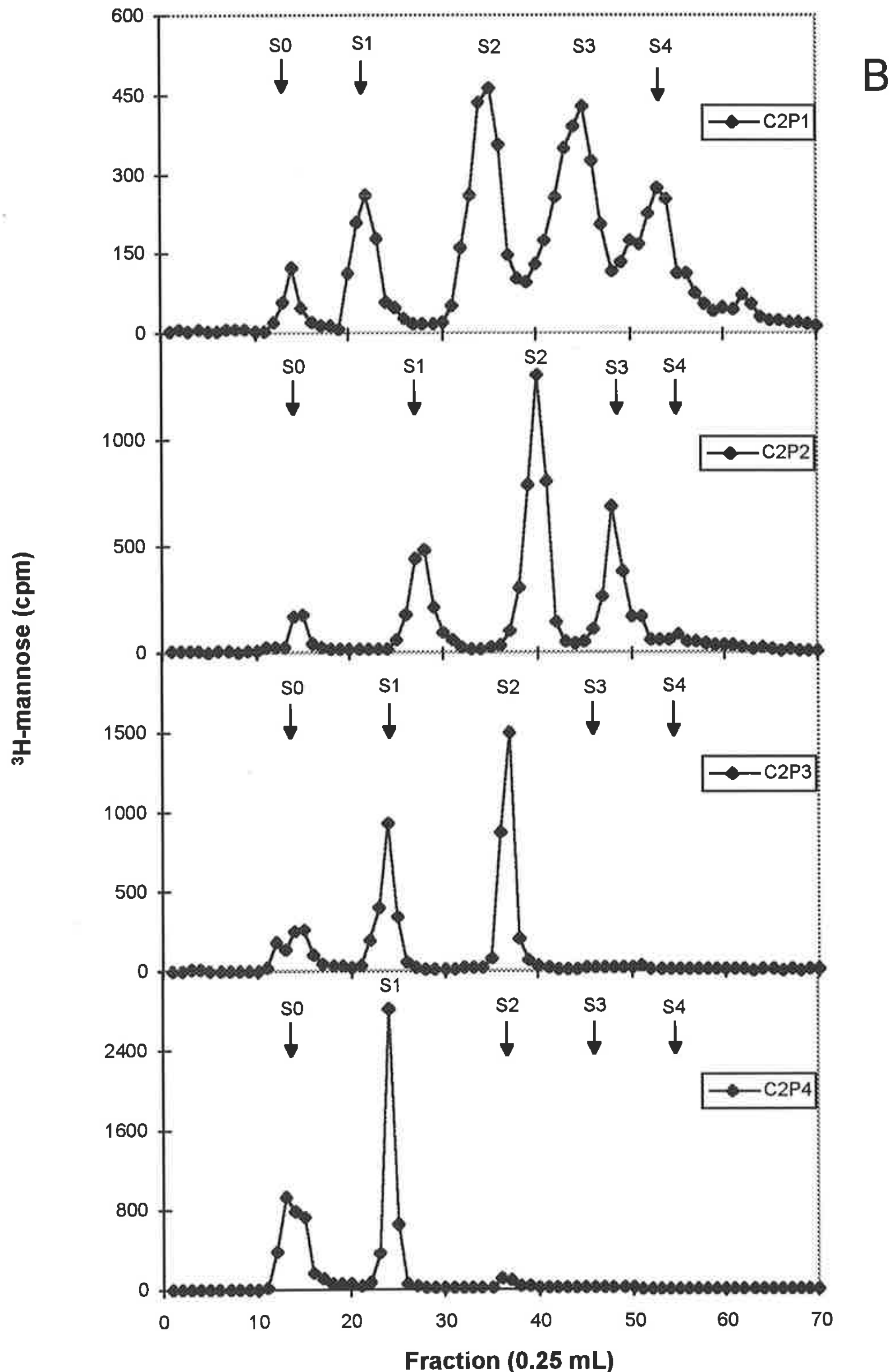


Figure 5.4 (A and B). Anion-exchange HPLC separation of cellular N-linked oligosaccharides from two representative control fibroblast cultures; *A*, *C5 peaks* (*P*) *1, 2, 3, 4,* and *5*; and ***B***, *C2 peaks* (*P*) *1, 2, 3,* and *4*. Approximately 15–20 000 cpm of each sample was separated on the column at a flow rate of 1 mL/min. 70 x 0.25 mL fractions were collected. Slight variations in peak elution volumes are secondary to changes in the programme conditions for the column between runs. A range of sialylated fetuin N-linked alditol standards are shown (*arrows*): *S0, S1, S2, S3,* and *S4* represent 0, 1, 2, 3, and 4 sialic acid residues, respectively.

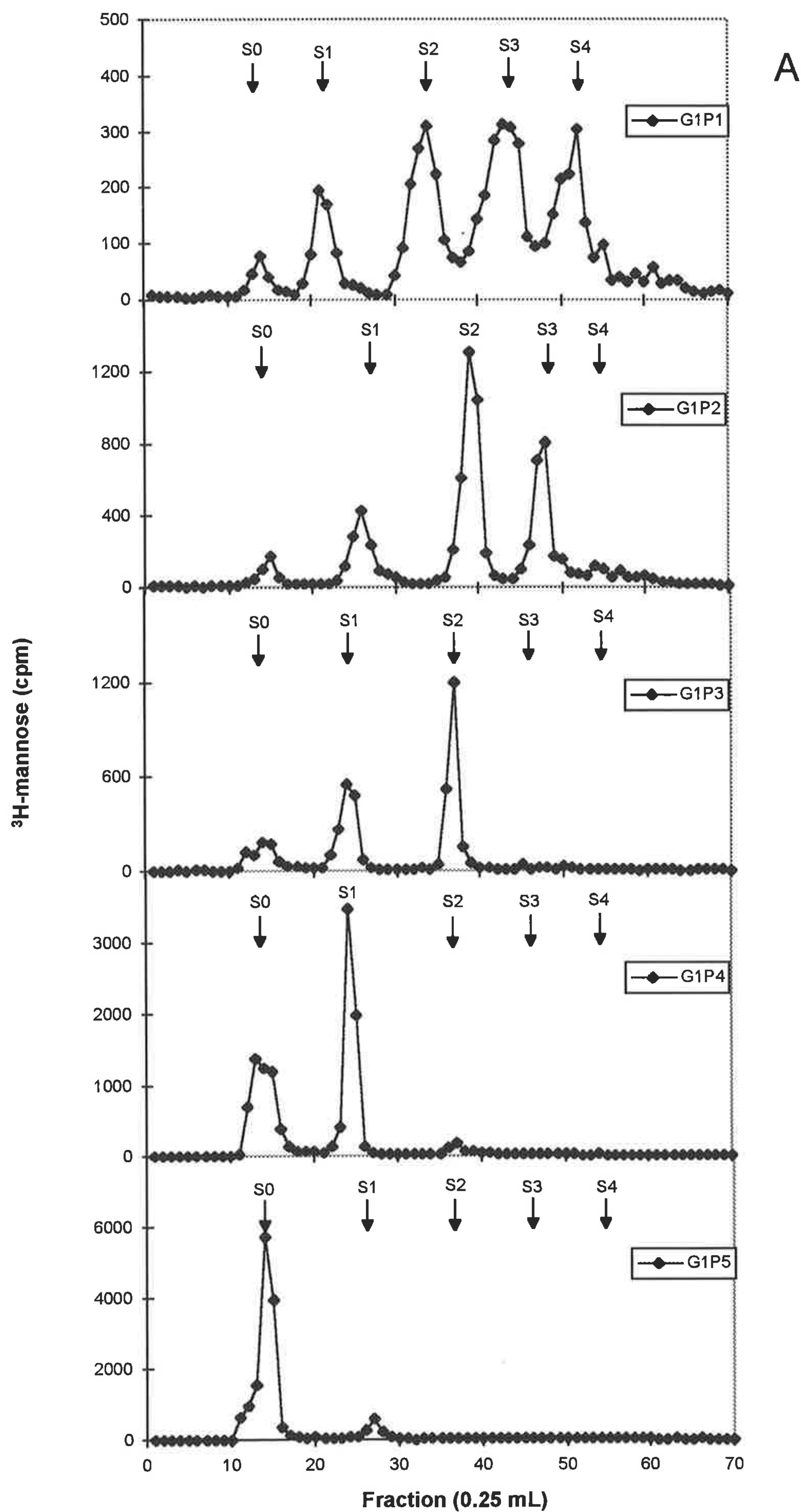


Figure 5.5 (A and B). continued on next page

***Figure 5.5** contd.*

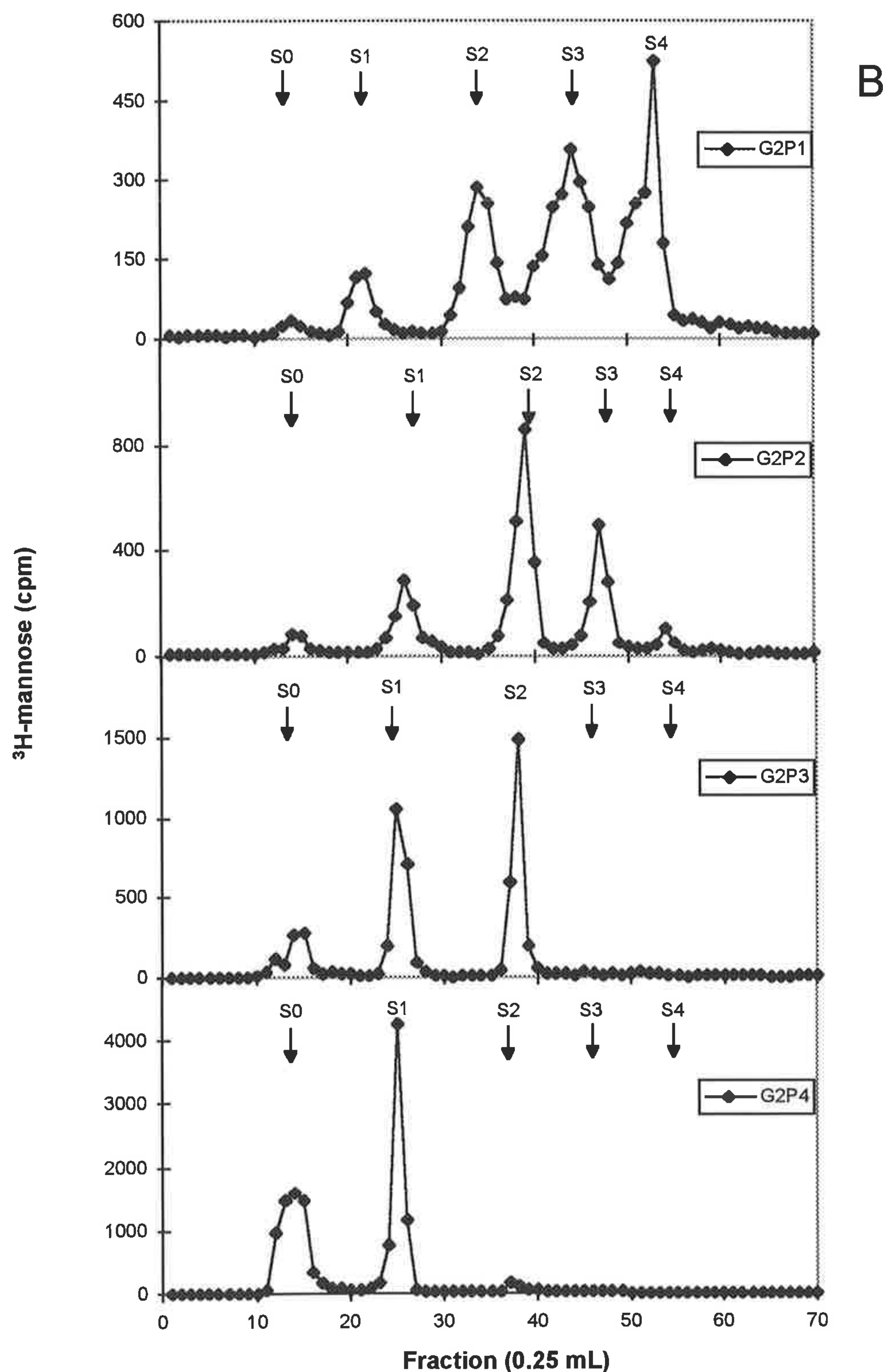


Figure 5.5 (A and B). Anion-exchange HPLC separation of cellular N-linked oligosaccharides from two representative galactosaemic fibroblast cultures; ***A***, *G1 peaks* (*P*) *1*, *2*, *3*, *4*, and *5*; and ***B***, *G2 peaks* (*P*) *1*, *2*, *3*, and *4*. Approximately 15–20 000 cpm of each sample was separated on the column at a flow rate of 1 mL/min. 70 x 0.25 mL fractions were collected. Slight variations in peak elution volumes are secondary to changes in the programme conditions for the column between runs. A range of sialylated fetuin N-linked alditol standards are shown (*arrows*): *S0*, *S1*, *S2*, S3, and *S4* represent 0, 1, 2, 3, and 4 sialic acid residues, respectively.

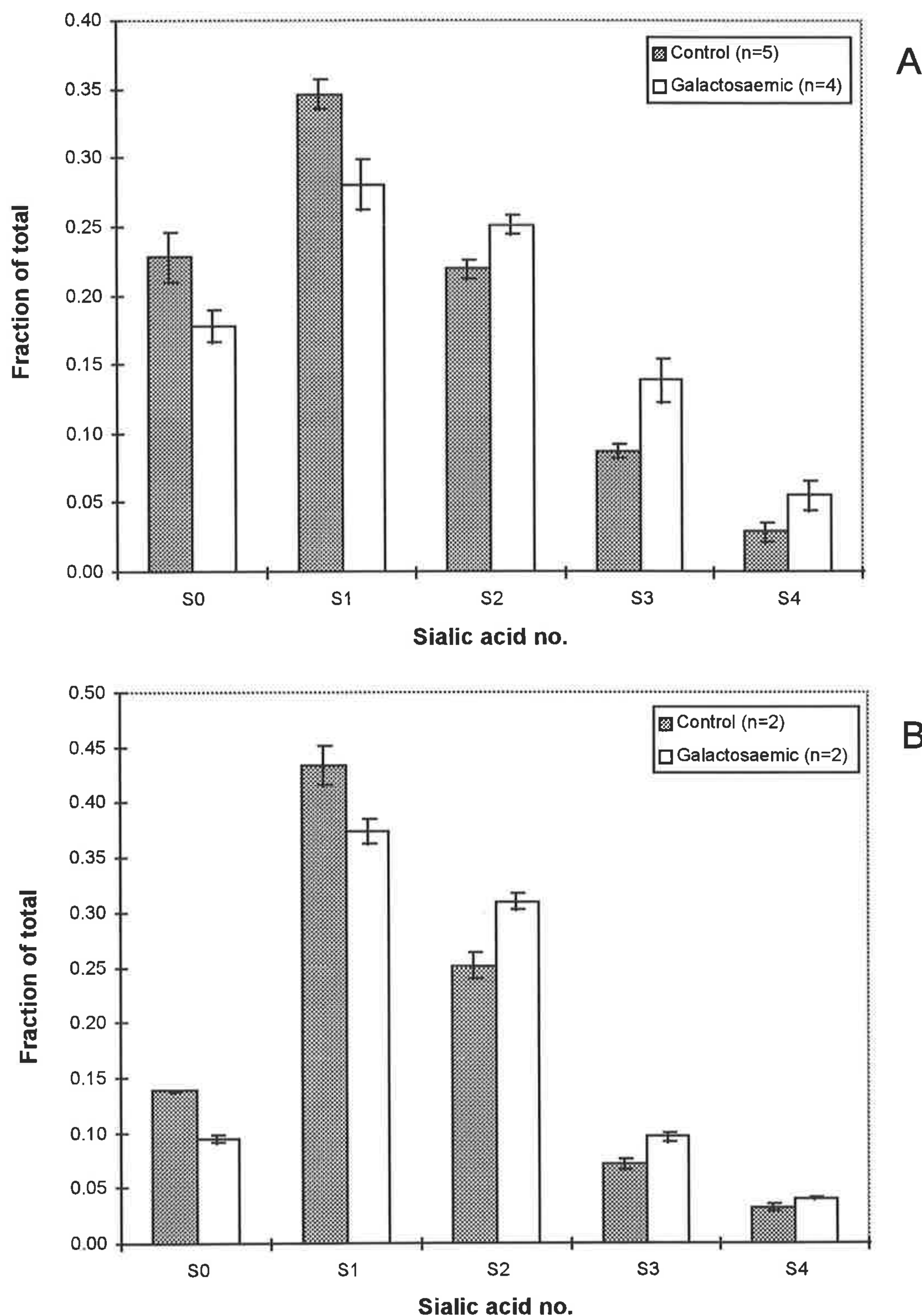


Figure 5.6. Anion-exchange HPLC patterns of sialylation of ***A***, cellular N-linked oligosaccharides recovered from *peaks 1–4* of five control (*C1, C2, C3, C4,* and *C5*) and four galactosaemic (*G1, G2, G3,* and *G4*) fibroblast cultures. Although each peak was analysed separately, the proportions of counts that appeared in each of the sialylated peaks were combined, and expressed as a fraction of the total counts in *peaks 1–4*, for each cell culture. Each bar in the figure represents the mean with 95% confidence interval ($p < 0.002$ at each level of sialylation); and ***B***, secreted N-linked oligosaccharides from *peaks 1–4* of the media from two control (*C4* and *C5*) and two galactosaemic (*G1* and *G4*) fibroblast cultures. The recovered peaks were analysed together and the proportion of counts in each sialylated peak was expressed as a fraction of the total counts in *peaks 1–4*. Each bar represents the mean, with range. *S0, S1, S2,* S3, and *S4* represent 0, 1, 2, 3, and 4 sialic acid residues, respectively.

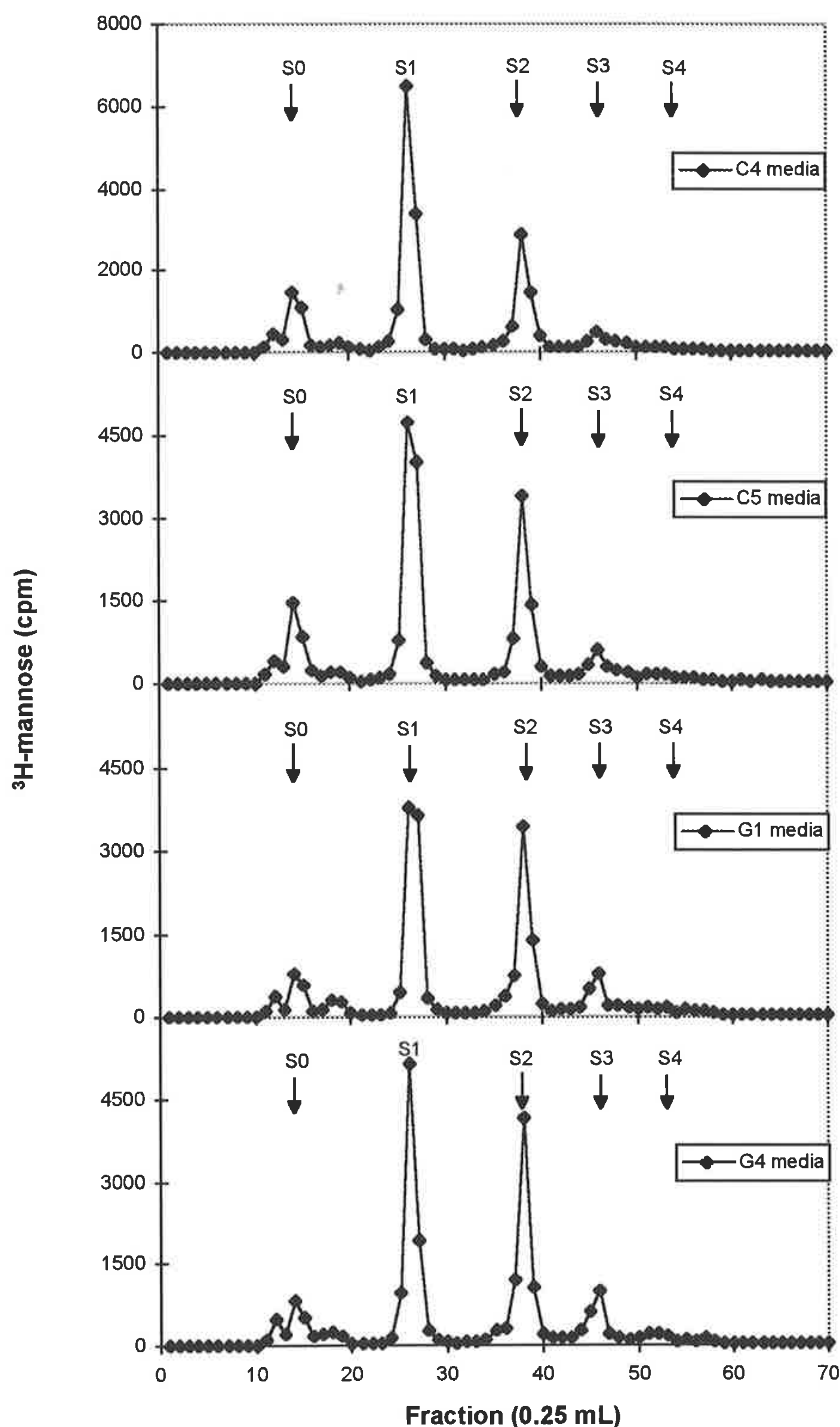


Figure 5.7. Anion-exchange HPLC separation of secreted N-linked oligosaccharides from *peaks 1–4* of the media of two control (*C4* and *C5*) and two galactosaemic (*G1* and *G4*) fibroblast cultures. The fractions from *peaks 1–4* were pooled and approximately 50 000 cpm of each sample was separated on the column and at a flow rate of 1 mL/min. 70 x 0.25 mL fractions were collected. A range of sialylated fetuin N-linked alditol standards are shown (*arrows*): *S0, S1, S2,* S3, and *S4* represent 0, 1, 2, 3, and 4 sialic acid residues, respectively.

5.4 Results of Experimental plan B

The radiolabelling procedures in this section were performed in culture medium that contained various concentrations of hexose: (*1*) 5.5 mmol/L glucose and 1.0 mmol/L pyruvate, (*2*) 5.5 mmol/L glucose and 5.5 mmol/L galactose, (*3*) 5.5 mmol/L galactose and 1.0 mmol/L pyruvate, (*4*) 2.5 mmol/L pyruvate, or (*5*) 5.5 mmol/L galactose. This influenced the incorporation of [2-^{3}H]-mannose due to competition for uptake into the cell. It was therefore not always possible to directly compare the level of incorporation among the cell cultures. However, several consistent findings were observed. The fibroblasts cultured in medium that contained only pyruvate incorporated the most radiolabel, and regardless of medium, the growing cells incorporated approximately three times more radiolabel per µg protein than the same cell cultures at confluence. The total cell protein was increased between 1.5 and 2.5 times in the cultures at confluence as opposed to during growth.

The Bio-Gel P-4 chromatographic profiles of the cellular and secreted N-linked oligosaccharides from the control (*C2*) and galactosaemic (*G1*) fibroblast cultures are displayed in Figures 5.8, 5.9, and 5.10 (pages 98, 100, and 101). The oligosaccharide profiles for the control and galactosaemic cultures at 50% of confluence are displayed in Figure 5.8A and B (page 98), and at confluence in Figure 5.9A and B (page 100), respectively. The secreted N-linked oligosaccharides were only recovered from the medium of those fibroblasts, at 50% of confluence, which were cultured in 5.5 mmol/L glucose and 5.5 mmol/L galactose, or 5.5 mmol/L galactose and 1.0 mmol/L pyruvate (Figure 5.10, page 101).

The Bio-Gel P-4 profiles of the cellular N-linked oligosaccharides in this section were similar to those in Experimental plan A and the same *peaks 1–7* were identified. However, several new features were apparent. First, the patterns of N-linked oligosaccharides produced by the control fibroblast cultures were similar, regardless of the composition of the medium, although the patterns in glucose as opposed to galactose media did show some minor variations, which were consistent at each stage of growth. Second, there was an increase in the proportion of counts in *peaks 1–3* in both the control and galactosaemic fibroblasts during growth as opposed to confluence (mean increase 25% and 46%, respectively). And third, the patterns of N-linked oligosaccharides produced by the galactosaemic fibroblasts were similar to the control, at both stages of growth, when they were cultured in 5.5 mmol/L glucose and 1.0 mmol/L pyruvate, or 5.5 mmol/L glucose and 5.5 mmol/L galactose. However, in the media that contained galactose, galactose and pyruvate, or pyruvate only, the galactosaemic fibroblasts produced an increased proportion of smaller N-linked oligosaccharides. These oligosaccharides ranged in size from $Man_3GlcNAc_2$ to $Man_5GlcNAc_2$ (*peak 7*) and were particularly evident during rapid growth (Figure 5.8B, page 98). Similar-sized oligosaccharides were also produced by the control in the pyruvate-only medium (Figure 5.8A, page 98). The increase in the proportion of smaller oligosaccharides was also associated with a relative decrease in the sizes of *peaks 5* and *6*. These changes resolved when the fibroblasts were at confluence, except the smaller oligosaccharides were still evident in the galactosaemic culture exposed to the galactose-only medium (Figure 5.9B, page 100). The smaller oligosaccharides were not detected in the profiles of N-glycans recovered from the secreted proteins of the galactosaemic cultures (Figure 5.10, page 101).

It was not always possible to directly compare the proportion of counts that appeared in *peaks 1–3* of the cellular N-linked oligosaccharides from the control and galactosaemic fibroblasts. The differences in the patterns made comparison of the proportions difficult. However, there was a mean increase of 19% in the proportion of counts in *peaks 1–3* in the galactosaemic fibroblasts as opposed to the control fibroblasts at 50% of confluence. This difference was not significant at confluence.

In Experimental plan A, the sialylated oligosaccharides eluted from the Bio-Gel P-4 column in *peaks 1-4*. The fractions from *peaks 1–4* in Experimental plan B were therefore pooled and analysed by anion-exchange HPLC. The profiles of sialylated cellular N-linked oligosaccharides from the control and galactosaemic fibroblasts at 50% of confluence are displayed in Figure 5.11A and B (page 103), and at confluence in Figure 5.12A and B (page 105). The profiles were very similar for each of the cell cultures, at each stage of growth, regardless of the composition of the experimental medium. The proportion of counts that appeared in each of the sialylated peaks were again expressed as a fraction of the total counts in *peaks 1–4*, for each cell culture. These fractions were then compared within and between the cell lines, for each of the experimental conditions. In both the control and galactosaemic fibroblasts, there was a shift towards more sialylated (tetra-, tri-, and disialylated) complex N-glycans in the growing cell cultures as opposed to the cultures at confluence (Figure 5.13A, page 106). This increase was consistent, except for the control fibroblasts cultured in the galactose-only medium where the degree of sialylation did not increase during growth. The control fibroblasts that were grown in 5.5 mmol/L galactose and 1.0 mmol/L pyruvate were also partially affected. This suggested that galactose, without glucose, partially inhibited certain growth responses in normal fibroblasts. The growth response in the galactosaemic fibroblasts, however, was not influenced by the composition of the medium (Figure 5.13B, page 106).

Although the control and galactosaemic fibroblasts both produced an increased proportion of sialylated oligosaccharides during growth, the galactosaemic fibroblast response was always greater. This was true at 50%-confluence and at confluence (Figure 5.14, page 107). The galactosaemic response was also independent of the composition of the medium. The shift in sialylation appeared greatest in the galactosaemic fibroblasts at 50%-confluence in the galactose-only medium. However, this in part reflected the lack of growth response of the control fibroblasts in the same medium. At confluence, the patterns of sialylated oligosaccharides that were produced by the galactosaemic fibroblasts were not influenced by the composition of the experimental medium (Figure 5.14, page 107).

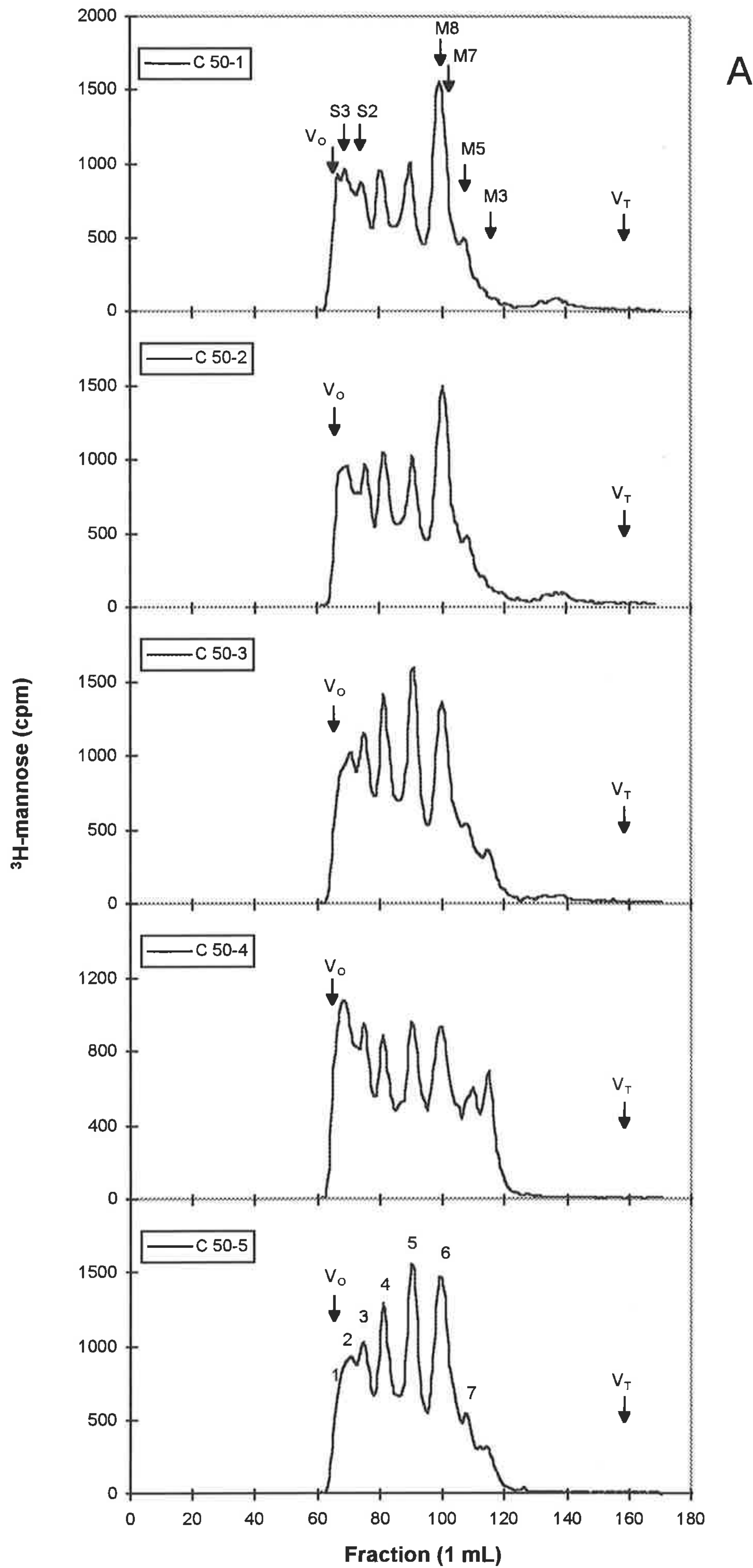
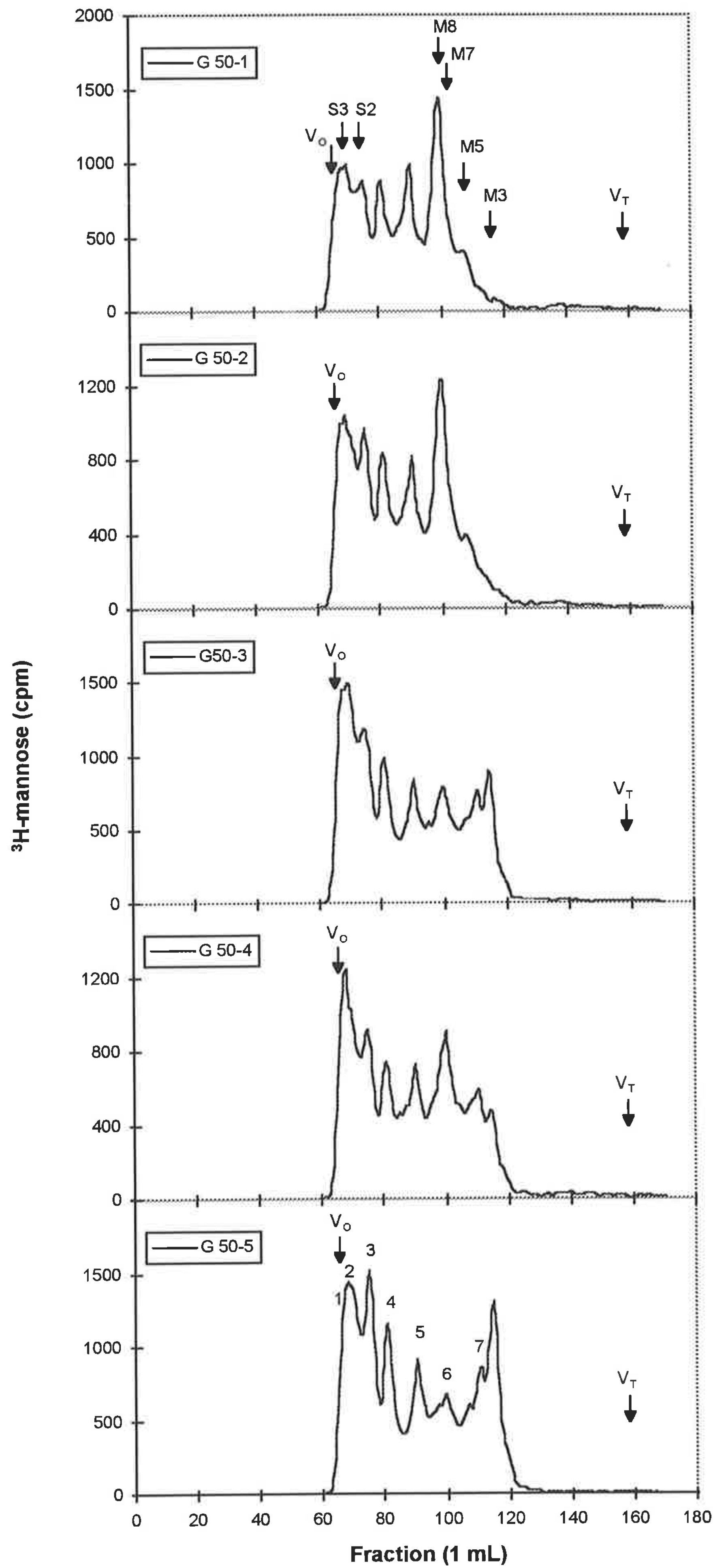


Figure 5.8 (A and B). *continued on next page*

***Figure 5.8** contd.*



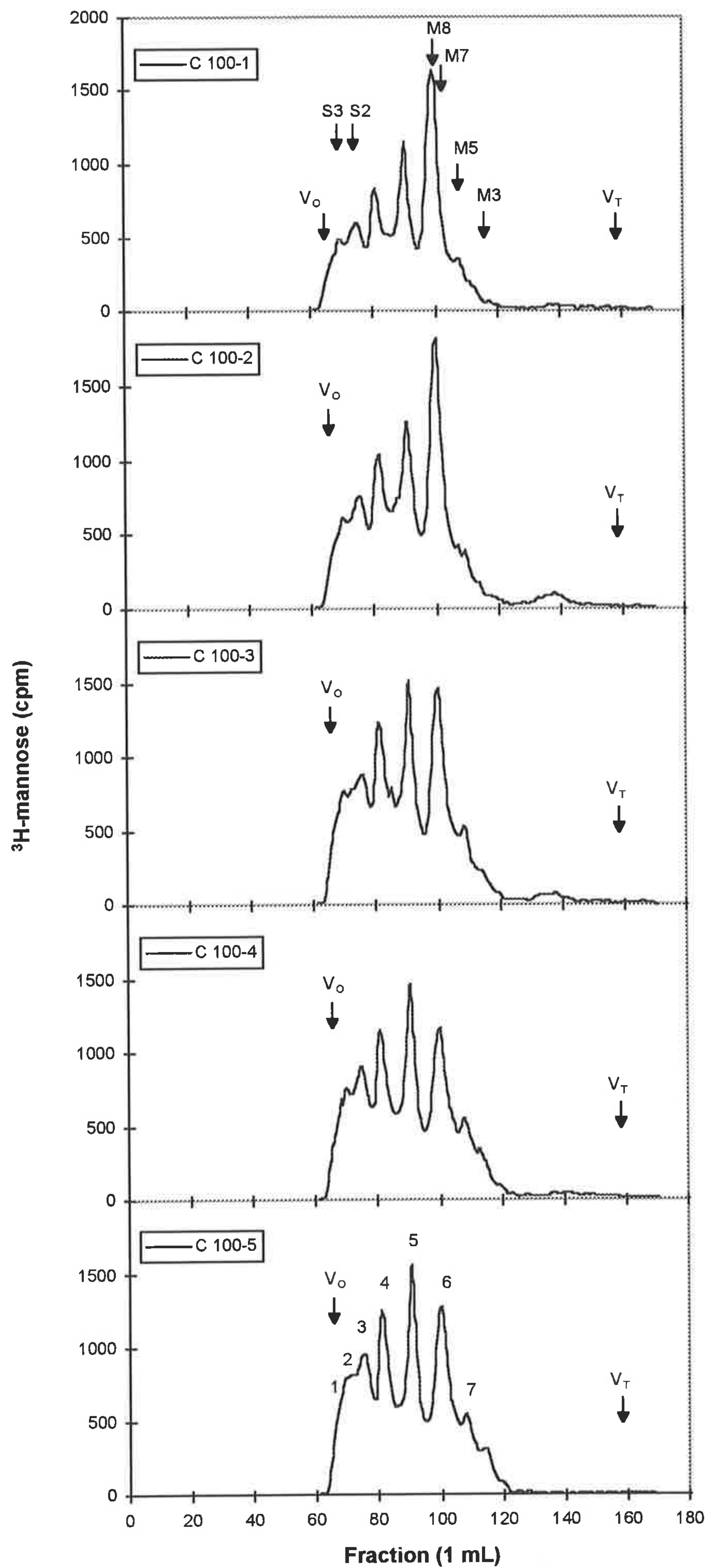
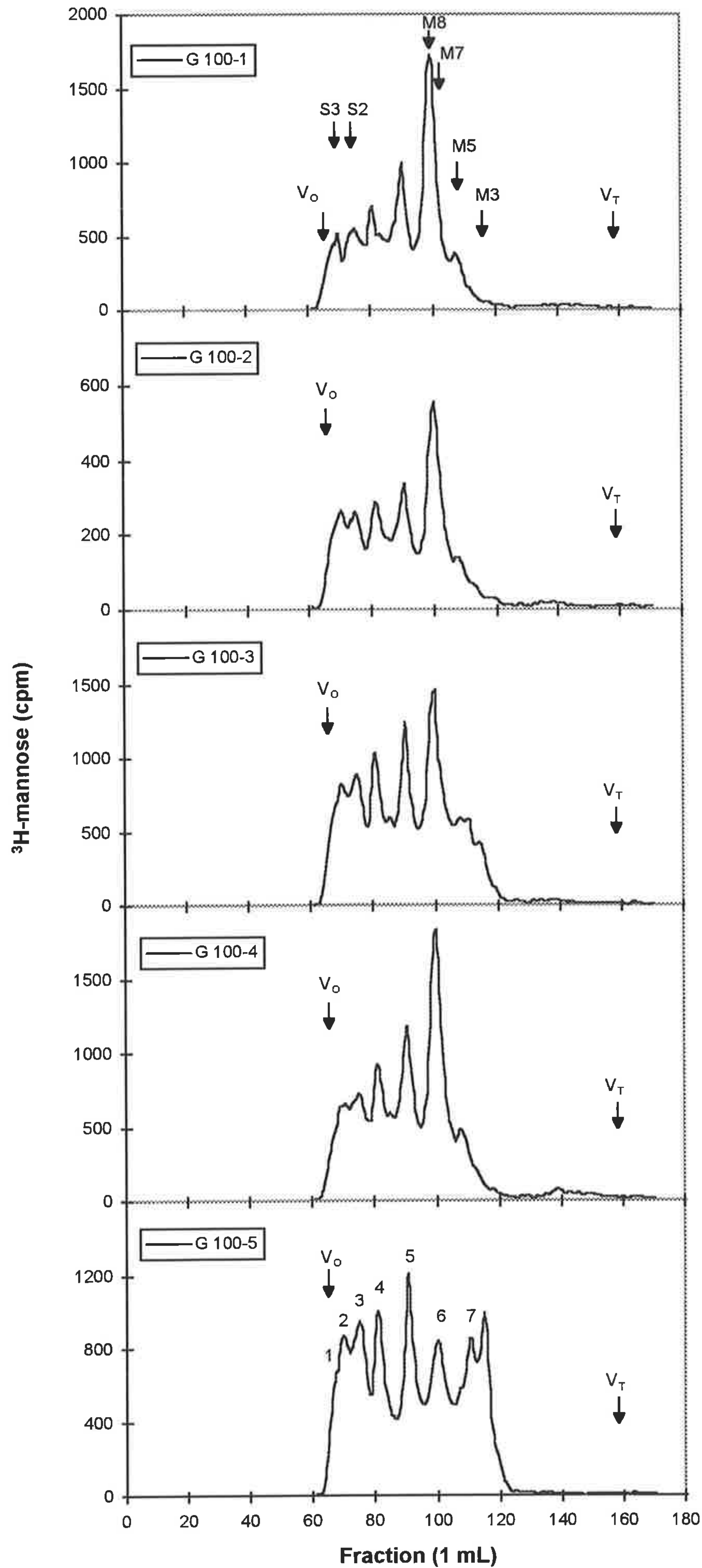


Figure 5.9 (A and B). *continued on next page*

***Figure 5.9** contd.*



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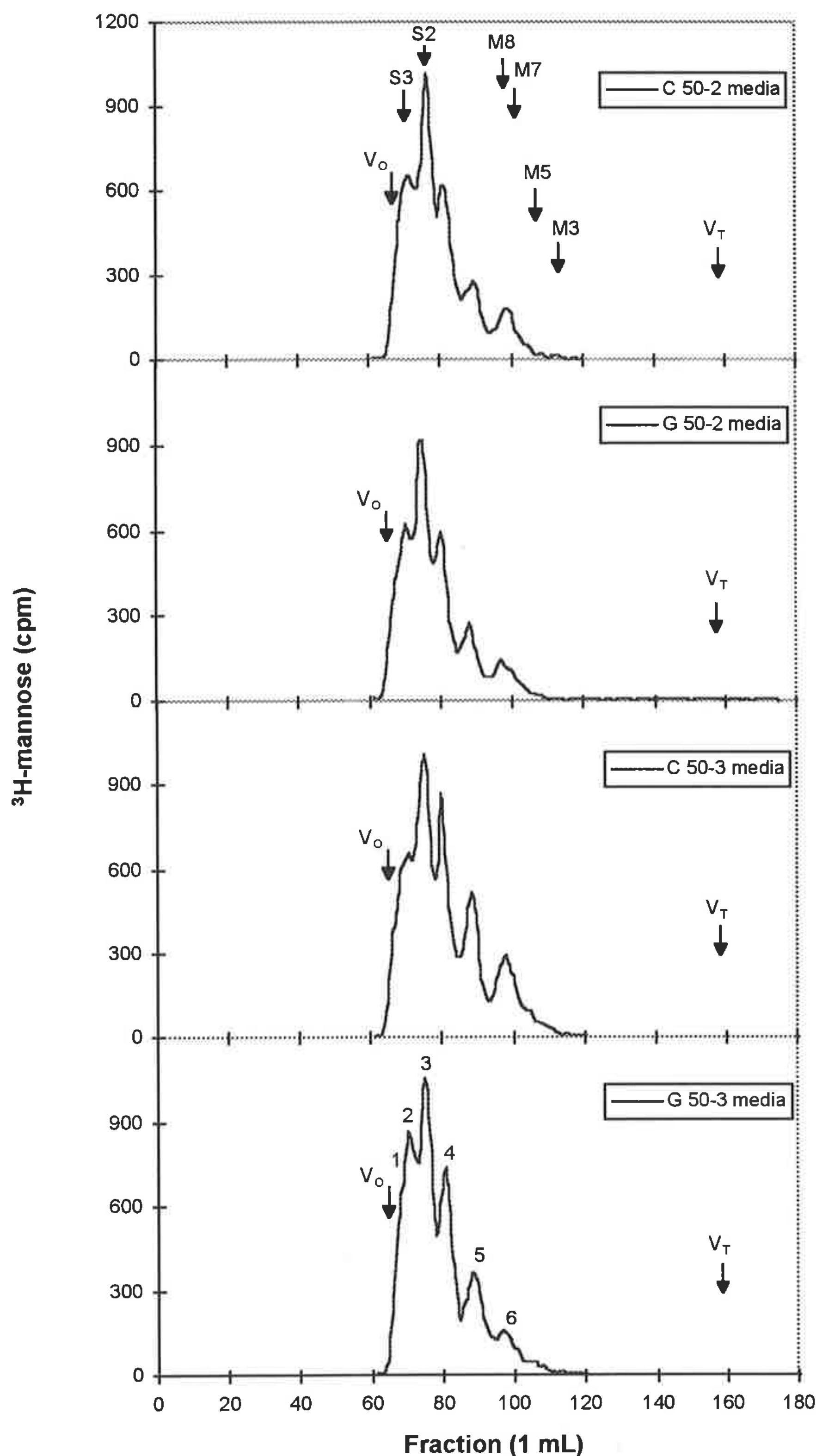


Figure 5.10. Size analysis of radiolabelled N-linked oligosaccharides from the media of a control (*C2*) and galactosaemic (*G1*) fibroblast culture. The cultures were radiolabelled at 50% of confluence (*50*) in medium that contained either 5.5 mmol/L glucose and 5.5 mmol/L galactose (*C 50-2* and *G 50-2*), or 5.5 mmol/L galactose and 1.0 mmol/L pyruvate (*C 50-3* and *G 50-3*). The oligosaccharides were fractionated on a Bio-Gel P-4 240 x 1 cm column in 0.1 mol/L NH_4COOH at 8 mL/h. 175 x 1 mL fractions were collected and 50 000 cpm of each sample was counted in total. A range of tritiated oligosaccharide standards are shown (*arrows*): tri- and disialylated fetuin complex N-glycans (*S3* and *S2*), $Man_8GlcNAc_2$ (*M8*), $Man_7GlcNAc_2$ (*M7*), $Man_5GlcNAc_2$ (*M5*), and $Man_3GlcNAc_2$ (*M3*). The exclusion volume (V_O) for the column was 65 mL and inclusion volume (V_T) 159 mL.

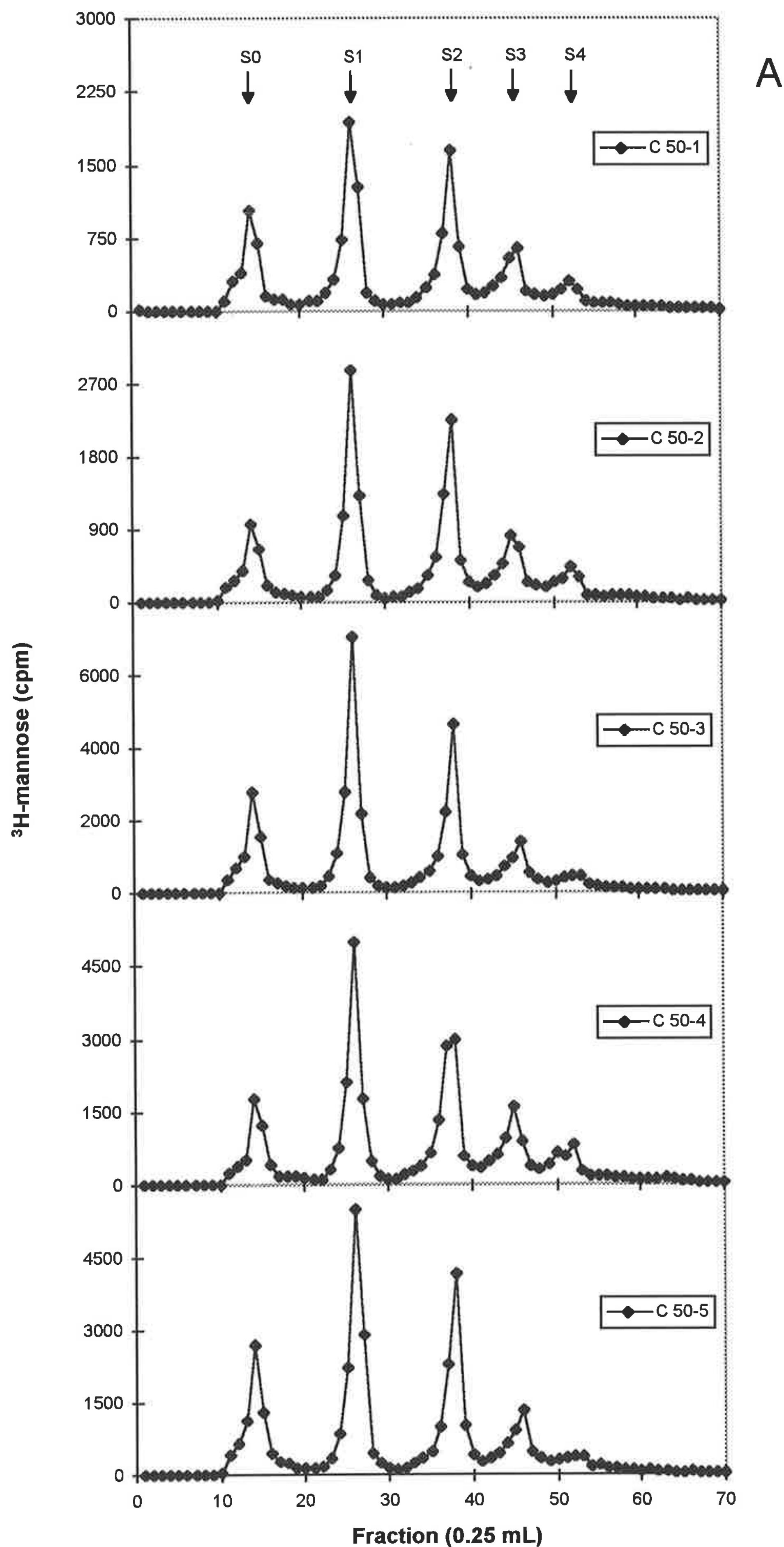
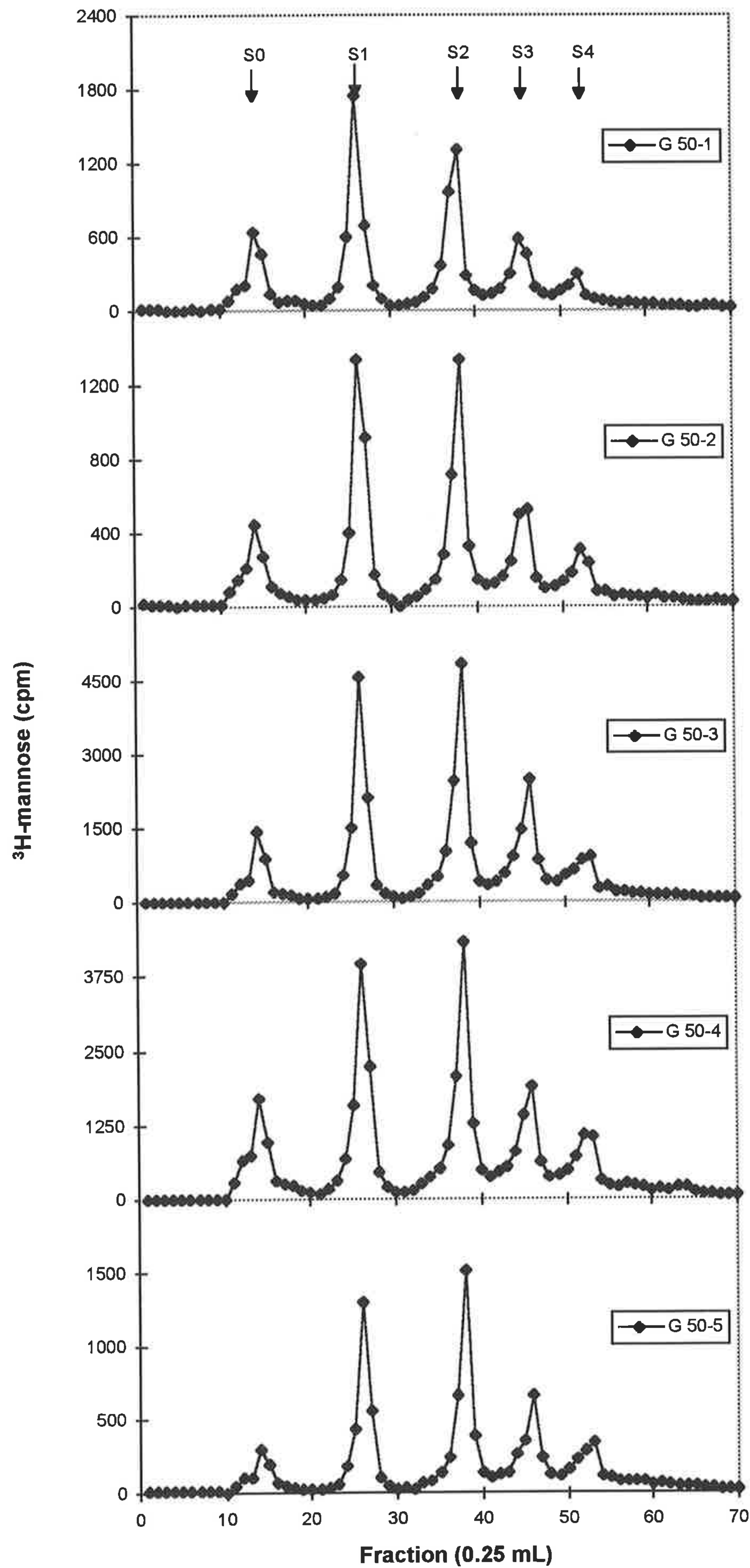


Figure 5.11 (A and B). *continued on next page*

***Figure 5.11** contd.*



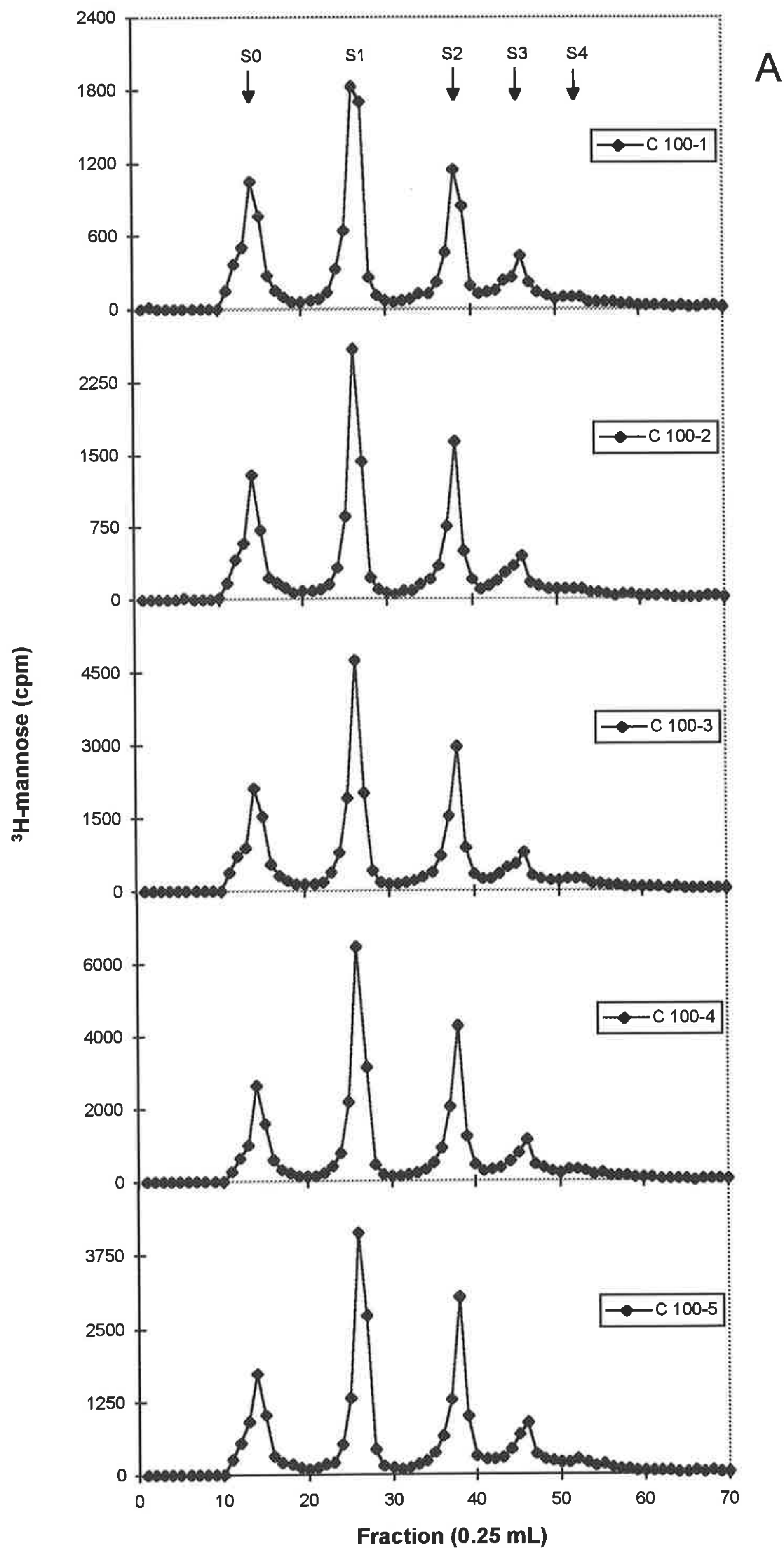
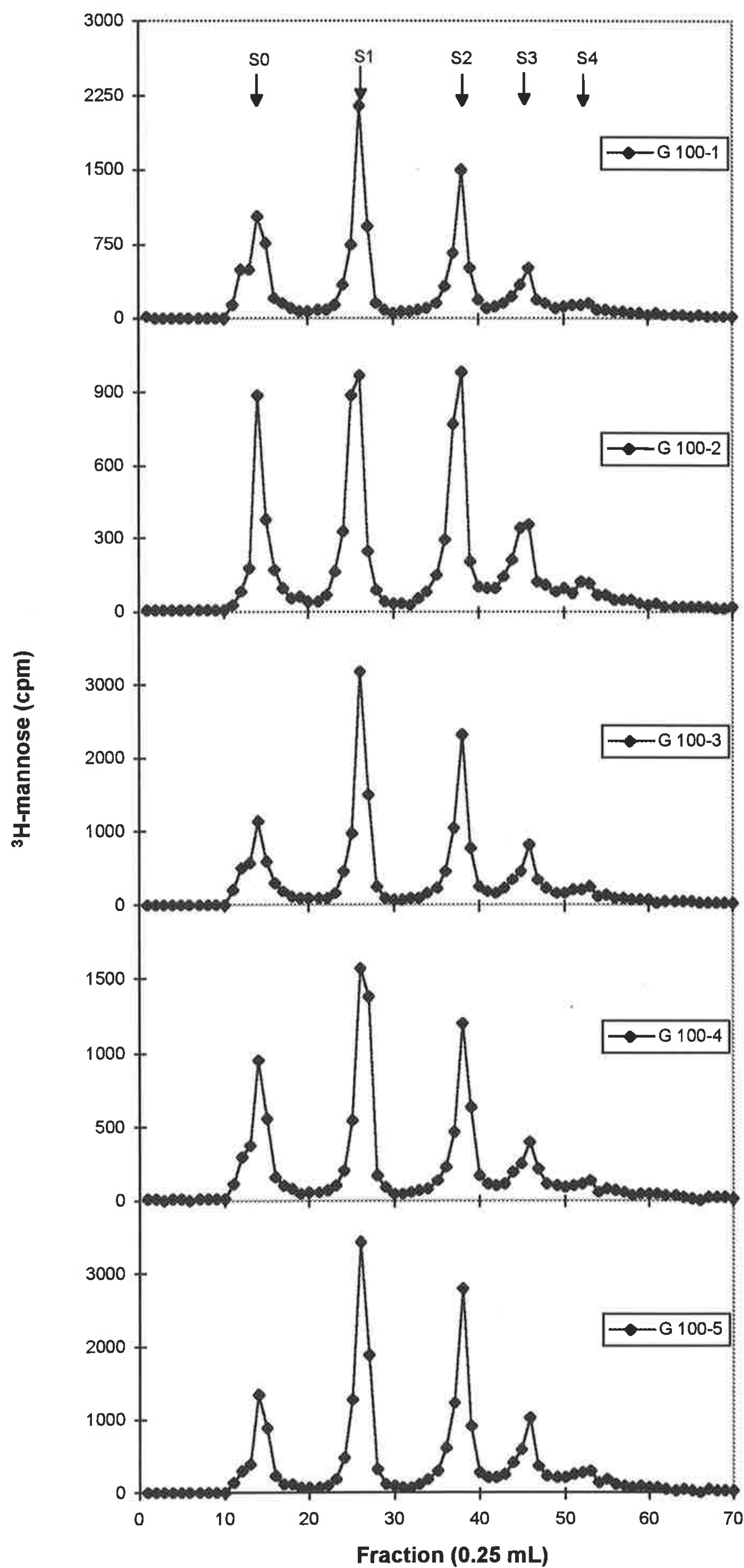


Figure 5.12 (A and B). *continued on next page*

***Figure 5.12** contd.*



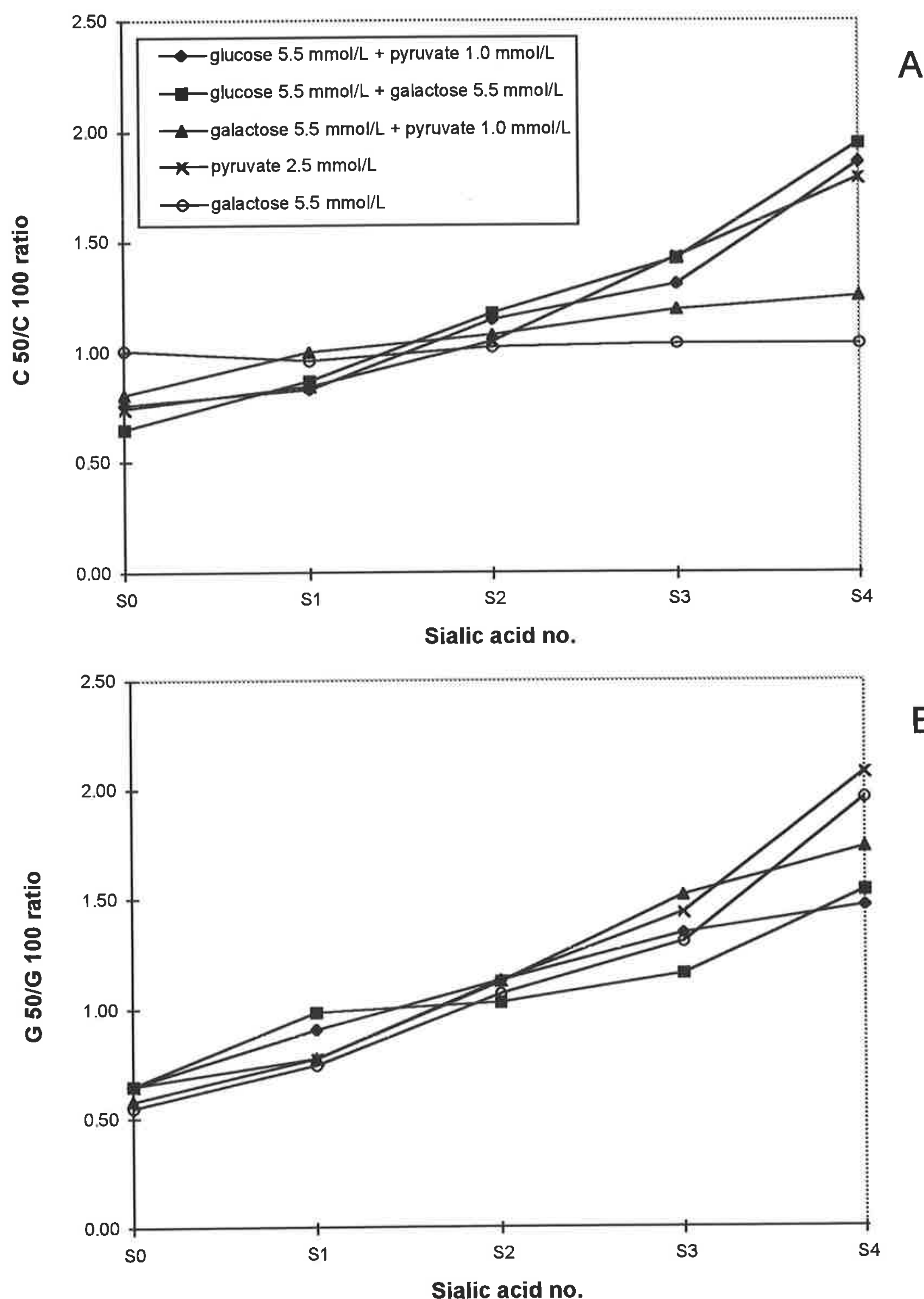


Figure 5.13. Anion-exchange HPLC patterns of sialylation of cellular N-linked oligosaccharides from ***A***, a control (*C2*) and ***B***, a galactosaemic (*G1*) fibroblast culture. The cultures were radiolabelled at 50%- and 100%-confluence in medium that contained either (***1***) 5.5 mmol/L glucose and 1.0 mmol/L pyruvate, (***2***) 5.5 mmol/L glucose and 5.5 mmol/L galactose, (***3***) 5.5 mmol/L galactose and 1.0 mmol/L pyruvate, (***4***) 2.5 mmol/L pyruvate, or (***5***) 5.5 mmol/L galactose. The proportion of counts that appeared in each sialylated peak were expressed as a fraction of the total counts in *peaks 1–4*, and then as a ratio to the same cell line at different stage of growth (*C50/C100 ratio* and *G50/G100 ratio*). To help in the interpretation, the data points within each experimental medium have been artificially joined with a line. *S0, S1, S2,* S3, and *S4* represent 0, 1, 2, 3, and 4 sialic acid residues, respectively.

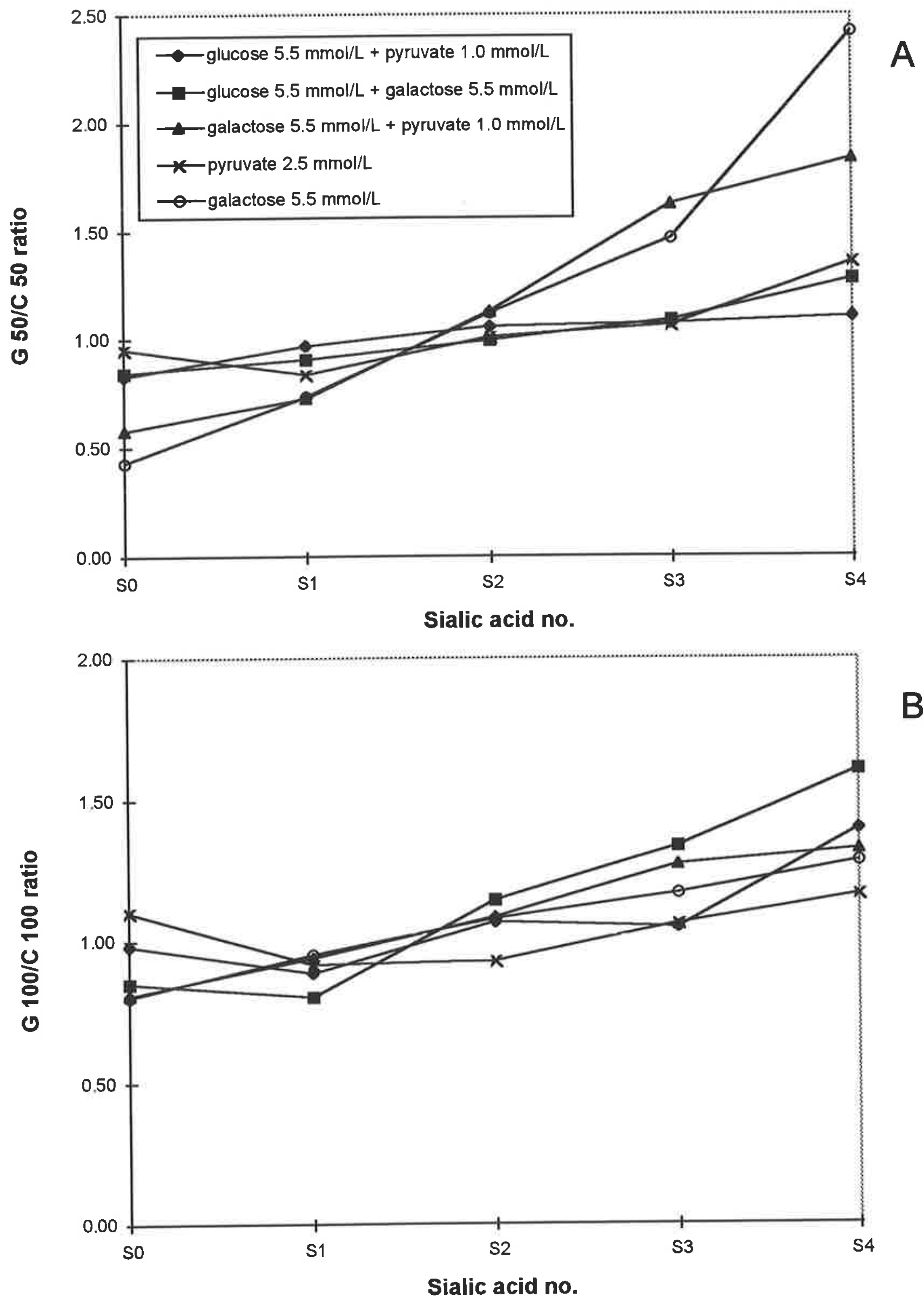


Figure 5.14. Anion-exchange HPLC patterns of sialylation of cellular N-linked oligosaccharides from a control (*C2*) and a galactosaemic (*G1*) fibroblast culture. The cultures were radiolabelled at ***A***, 50%-confluence and ***B***, 100%-confluence in medium that contained either (***1***) 5.5 mmol/L glucose and 1.0 mmol/L pyruvate, (***2***) 5.5 mmol/L glucose and 5.5 mmol/L galactose, (***3***) 5.5 mmol/L galactose and 1.0 mmol/L pyruvate, (***4***) 2.5 mmol/L pyruvate, or (***5***) 5.5 mmol/L galactose. The proportion of counts that appeared in each sialylated peak were expressed as a fraction of the total counts in *peaks 1–4*, and then as a ratio of the galactosaemic to control results at the same stage of growth (*G50/C50 ratio* and *G100/C100 ratio*). To help in the interpretation, the data points within each experimental medium have been artificially joined with a line. *S0, S1, S2,* S3, and *S4* represent 0, 1, 2, 3, and 4 sialic acid residues, respectively.

5.5 Discussion

The galactosaemic fibroblasts produced an increased proportion of larger, more heavily sialylated, complex N-linked oligosaccharides than the control fibroblasts. This change in the pattern of cellular and secretory N-glycans was observed during rapid growth and at confluence, and was not influenced by the hexose content of the culture medium. Even when cultured in galactose for 24 hours, the galactosaemic fibroblasts continued to produce an increased proportion of sialylated oligosaccharides. The experimental results in this chapter therefore do not support a functional defect in terminal galactosylation or sialylation of N-linked oligosaccharides in galactosaemic fibroblasts. It is still possible that a small population of terminally incomplete N-glycans were produced, but they were not readily apparent in the oligosaccharide profiles. A partial defect in galactosylation would theoretically produce populations of incomplete N-glycans, such as $GlcNAc_2Man_3GlcNAc_2$ or $(NeuGal)GlcNAc_2Man_3GlcNAc_2$ (where Neu represents sialic acid). $GlcNAc_2Man_3GlcNAc_2$ would elute from the Bio-Gel P-4 column with a relative size of $Man_7GlcNAc_2$ (Kobata, 1994), and $(NeuGal)GlcNAc_2Man_3GlcNAc_2$ would appear as a monosialylated peak on anion-exchange HPLC. However, there was no obvious accumulation of $Man_7GlcNAc_2$-sized oligosaccharides in the Bio-Gel P-4 profiles and the proportion of monosialylated oligosaccharides was decreased. When the galactosaemic cultures were exposed to galactose-only medium they also produced relatively less intermediate-sized oligosaccharides (Figure 5.8B, page 98). It appears unlikely therefore that there was a significant accumulation of terminally incomplete N-glycans in the galactosaemic fibroblasts.

The control and galactosaemic fibroblasts both produced an increased proportion of sialylated N-glycans during rapid growth in culture. This suggested that the increased proportion of sialylated oligosaccharides in the galactosaemic fibroblasts, and in the controls, could be a specific response to certain growth stimuli. However, growth-dependent changes in the patterns of radiolabelled N-linked oligosaccharides do not necessarily imply that the carbohydrate structures of individual glycoproteins are altered. The changes could also reflect a shift in the types of glycoprotein synthesised, or possibly a difference in the rate of cellular turnover of glycoprotein. Considerable evidence suggests that the types of glycoprotein, and hence N-linked oligosaccharides, synthesised by fibroblasts are influenced by the stage of growth of the cell culture.

Muramatsu *et al.*, (1976) examined the patterns of glycopeptides that were produced by human fibroblasts under different stages of growth. They radiolabelled the fibroblasts with [2-^{3}H]-mannose over 24 hours, either in the growing state when cell contacts were sparse, or in the confluent non-growing state. The cell surface glycoproteins were then removed by brief pronase digestion and the remaining cellular material was extensively digested with pronase. The cellular glycopeptides were then characterised by paper chromatography, size-exclusion and affinity chromatography, and glycosidase digestion. Muramatsu *et al.*, (1976) observed that the growing cells predominantly produced large neutral oligomannose cores ($Man_{7-8}GlcNAc_2$), whereas the confluent cells produced an increased proportion of sialylated glycopeptides. The increase in sialylated glycopeptides in the confluent fibroblasts is at odds with the results presented in this chapter. However, this is possibly explained by differences in the types of glycoprotein examined. Muramatsu *et al.* (1976) did not include the cell surface glycoproteins in their analysis. The same authors previously reported that surface glycoproteins from fibroblasts were more enriched in acidic oligosaccharides (Ceccarini *et al.*, 1975).

Human fibroblasts display different patterns of cell surface oligosaccharides depending upon their cell density and stage of growth (Mann *et al.*, 1987). Initiation of growth and cellular

spreading were both associated with a change in the type of oligosaccharides expressed, which suggested a relationship between cell surface oligosaccharides and the mechanisms that controlled growth (Mann *et al.*, 1988; and Mann *et al.*, 1992). Many growth-regulating interactions occur between membrane glycoproteins that contain N-linked oligosaccharides (Wieser *et al.*, 1985), which suggests that cell surface N-linked oligosaccharides are directly involved in growth control (Codogno *et al.*, 1985; and Hubbard, 1988). Whereas normal fibroblasts show a density-dependent regulation of their cell surface oligosaccharides, growth transformed cells do not (Mann *et al.*, 1988). The ability of tumour cells to metastasise and invade is linked to the expression of larger, more branched N-linked oligosaccharides. Several rodent and human tumour cell lines have increased β1,6-branched and sialylated N-linked oligosaccharides on their surface (Dennis and Laferte, 1989; and Fernandes *et al.*, 1991) and the ability of the tumour cells to metastasise and invade basement membranes is directly related to these specific N-glycans (Dennis, 1988; Dennis *et al.*, 1989b; Yagel *et al.*, 1989; Demetriou *et al.*, 1995; and Le Marer and Stehelin, 1995). A large proportion of these N-glycans were associated with the polylactosamine chains of a membrane glycoprotein that was structurally similar to LAMP-1 (Heffernan *et al.*, 1989; and Heffernan *et al.*, 1993). Many oncogenes also confer their metastatic potential by inducing an increase in β1,6-branched and sialylated surface N-linked oligosaccharides (Dennis *et al.*, 1989a; Santer *et al.*, 1989; and Easton *et al.*, 1991). Removing these oligosaccharides from the tumour cell surface resulted in a decrease in tumour cell proliferation in culture (Vanderelst and Dennis, 1991; Korczak *et al.*, 1994; and Takano *et al.*, 1994). The increased expression of β1,6-branched and sialylated surface N-glycans therefore contributed directly to relaxed growth control.

It is likely therefore that the increased proportion of sialylated complex N-glycans in the growing control and galactosaemic fibroblasts represents a shift in the types of glycoprotein synthesised due to the growth response of the cells in culture. However, the response of the galactosaemic fibroblasts was greater at all stages of growth. The experimental plans attempted to produce galactosaemic cultures that were at the same cell density and stage of growth as the control cultures. However, there were minor differences in the growth characteristics of the cells. Under light microscopy, the galactosaemic fibroblasts usually exhibited slightly increased granularity of the cytoplasm and the cellular sheets were less ordered. This disturbance in the arrangement of the cells could have influenced the growth interactions of the fibroblasts, and hence the types of surface glycoproteins produced (although it could equally be said that the altered patterns of glycoproteins lead to the disturbed growth). The profiles of oligosaccharides produced by the galactosaemic fibroblasts were largely unaffected by the composition of the medium. This suggests that the mechanism for the increased sialylation was inherent within the galactosaemic fibroblasts and not a feature of the particular culture conditions. For whatever reason, the stimulus to growth appears more active or upregulated in the galactosaemic fibroblasts, at every stage of growth. This could be related to intracellular abnormalities associated with GALT deficiency, although substituting galactose for glucose in the experimental medium did not increase the proportion of sialylated oligosaccharides (Figure 5.14, page 107).

There are, however, several other possible explanations for the change in the pattern of oligosaccharides in the galactosaemic fibroblasts. The increased branching and sialylation of the N-glycans could represent an increase in the activity of specific glycosyltransferases within the Golgi apparatus. The extent of branching of N-linked oligosaccharides is controlled by Golgi N-acetylglucosaminyltransferases IV and V, which transfer N-acetylglucosamine to the α1,3 and α1,6 mannoses of the pentasaccharide core, respectively. This produces structures with three or four outer branches (Schachter, 1984; and Schachter, 1986). The number of branches a specific N-glycan will have is determined before the antennae is completed, by an interaction of the branching enzymes, their level of expression, and their substrate specificities (Schachter, 1986). The increase in β1,6-branched N-glycans in tumour cells was directly

dependent on increased expression of N-acetylglucosaminyltransferase V (Easton *et al.*, 1991; Yousefi *et al.*, 1991; and Heffernan *et al.*, 1993) and the increased sialylation of tumour surface oligosaccharides was related to increased expression of sialyltransferases (Easton *et al.*, 1991; and Le Marer *et al.*, 1992). It is possible therefore that the activities of these enzymes are also increased in galactosaemic cells. Ornstein *et al.*, (1992) reported that the activity of N-acetylglucosamine:UDP-galactose galactosyltransferase in galactosaemic fibroblasts was increased. However, the activities of the other Golgi glycosyltransferases in galactosaemic cells have not been determined. Rosenwald *et al.*, (1989) observed an increase in β1,6-branched N-glycans in a CHO cell mutant that had a defect in dolichol phosphate synthesis. The CHO cells produced truncated dolichol-linked oligosaccharides that were transferred directly to protein. It appeared that the branching mechanism within the medial Golgi was influenced by factors that affected even the earliest steps in N-glycosylation (Rosenwald *et al.*, 1989). The metabolic disturbances in the cytoplasm in galactosaemia could have similar effects. Roth *et al.*, (1971) reported that galactose-1-phosphate partially inhibited UDP-galactose galactosyltransferases. However, whether galactose-1-phosphate, or any other galactose intermediates, accumulate in the lumen of the Golgi is not known.

The galactosaemic fibroblasts produced a population of small N-linked oligosaccharides when they were grown in media without glucose (Figure 5.8B, page 98). These oligosaccharides were similar in size to the $Man_{3-5}GlcNAc_2$ oligomannose standards. The presence of these oligosaccharides was not related to a deficiency of energy as the same small oligosaccharides were also present in the control fibroblasts when they were grown in 2.5 mmol/L pyruvate (Figure 5.8A, page 98). When cells are cultured in media without glucose they synthesis truncated dolichol-linked oligosaccharides, predominantly $Man_5GlcNAc_2$, which can be transferred directly to protein for further processing (Gershman and Robbins, 1981). This disturbance can be overcome by mannose, and partially by galactose, but not by adding pyruvate to the culture medium. The disturbance also occurs only during rapid growth and not at high cell densities (Gershman and Robbins, 1981). In the experiments in this chapter, the $Man_{3-5}GlcNAc_2$-sized oligosaccharides were also observed only during growth and only in those fibroblasts grown in media without glucose. The control fibroblasts did not produce the smaller oligosaccharides when they were grown in galactose-containing media as presumably the galactose partially substituted for glucose. Galactosaemic fibroblasts, however, cannot metabolise significant quantities of galactose and this would equate to a glucose-deficient state. The $Man_{3-5}GlcNAc_2$-sized oligosaccharides were present in the galactosaemic fibroblasts when they were grown in 5.5 mmol/L galactose, 5.5 mmol/L galactose and 1.0 mmol/L pyruvate, and in 2.5 mmol/L pyruvate. The accumulation appeared the greatest in 5.5 mmol/L galactose and the small N-glycans were still present at confluence in this medium (Figure 5.9B, page 100). The presence of the small N-glycans was also associated with a relative deficiency of *peaks 5* and *6*. These changes were not observed in the control fibroblasts in galactose-containing media. This suggests that galactose, in part, directly contributed to the disturbances in the galactosaemic fibroblasts. This could have involved competition between galactose metabolites and residual glucose intermediates, which enhanced the mechanisms that produce truncated oligosaccharides in glucose starvation. Despite the accumulation of these small N-glycans, the proportion of sialylated complex N-glycans produced by the galactosaemic fibroblasts remained increased.

The results in this chapter did not explain the increase in hyposialylated transferrin in the serum in untreated galactosaemia. The mature N-glycans synthesised by the galactosaemic fibroblasts appeared structurally complete. Therefore a different approach was required to exclude other possible defects in N-glycosylation. The next experimental chapter examines total glycoprotein synthesis in galactosaemic fibroblasts in a more general manner.

6

Glycoprotein synthesis in galactosaemic fibroblasts

6.1 Introduction

The experimental evidence in Chapter 5 suggested that there was no functional disturbance in the terminal completion of N-linked oligosaccharides in galactosaemic fibroblasts. However, this did not exclude all possible defects in N-glycosylation in galactosaemia. The aim therefore of this chapter was to screen the galactosaemic fibroblasts for other defects in N-glycan synthesis or processing. The experimental plan was to radiolabel the skin fibroblasts with [2-^{3}H]-mannose and [^{35}S]-methionine. [2-^{3}H]-Mannose selectively labels the N-glycans whereas [^{35}S]-methionine is incorporated into the protein backbone. The cellular and secretory proteins were then precipitated separately with TCA and the radioactivity was measured in the precipitates. The [2-^{3}H]-mannose incorporated into the N-glycans was then corrected to the total protein synthesised. The method was used to screen the galactosaemic fibroblasts for the effects of galactose on total N-glycan and protein synthesis. A similar method was successfully used in the study of N-glycosylation in fibroblasts from patients with the CDG syndrome type I (Powell *et al.*, 1994; and Panneerselvam and Freeze, 1996).

6.2 Experimental plan

A: Four control (designated *C1, C2, C3,* and *C4*) and four galactosaemic (designated *G1, G2, G3,* and *G4*; Table 4.1, page 56) fibroblast cultures were grown to near-confluence in D-MEM with 10% (v/v) dialysed FBS. The fibroblasts were then preincubated for four hours in a modified D-MEM that contained 10% (v/v) dialysed FBS and either ***(a)*** 2.5 mmol/L sodium pyruvate and 5.5 mmol/L D-galactose or ***(b)*** 2.5 mmol/L D-glucose and 5.5 mmol/L D-galactose. The fibroblasts were then radiolabelled in the same medium for six hours by adding small aliquots of D-[2-^{3}H]-mannose (3.7 MBq/mL) and L-[^{35}S]-methionine (0.74 MBq/mL) directly to the medium. The cells and medium were then harvested separately and the radioactivity was measured in protein pellets that were precipitated with TCA (Method 4, page 71).

B: One of the control (*C1*) and one of the galactosaemic (*G1*) fibroblast cultures were also grown, in duplicate, to near-confluence in D-MEM with 10% (v/v) dialysed FBS. The fibroblasts were then preincubated for four hours in a modified D-MEM that contained 10% (v/v) dialysed FBS, 2.5 mmol/L sodium pyruvate, and either 0, 1.0, 2.5, or 5.5 mmol/L of D-galactose. The fibroblasts were then radiolabelled in the same medium with D-[2-^{3}H]-mannose (3.7 MBq/mL) and L-[^{35}S]-methionine (0.74 MBq/mL) for six hours. The cells and medium were then harvested separately and the radioactivity was measured in protein pellets that were precipitated with TCA (Method 4, page 71).

6.3 Results

Experimental plan A

The results of the incorporation of [2-^{3}H]-mannose and [^{35}S]-methionine into the TCA-precipitates from the cellular and media extracts are presented, for each cell culture, in Table 6.1 (page 114). The results are then summarised, according to the experimental medium, in Table 6.2 (page 115), where the data are presented as the mean of the four cell cultures with the standard error of the mean. The means were analysed by Student's *t* test with a one-tailed distribution. The radioactivity that was incorporated into each TCA-precipitate was expressed per µg of cellular protein, which was measured in duplicate. In the eight control cultures, the total cell protein ranged from 264 to 418 µg, with a mean of 354 µg. In the eight galactosaemic cultures, the protein ranged from 159 to 437 µg, with a mean of 289 µg.

The results from the control fibroblasts were consistent regardless of the experimental media. The mean incorporation of either [2-^{3}H]-mannose or [^{35}S]-methionine in the pyruvate- and glucose-based media were not significantly different. This was true for both the cellular and secreted proteins (Table 6.2, page 115). The ratio of ^{3}H to ^{35}S was also similar in the cell and media precipitates regardless of the experimental medium, and approximately half as much radiolabel was recovered in the media pellets as opposed to the cell pellets. This suggested that the radiolabelling procedures and the recovery techniques were consistent among cell lines and that the incorporation was similar in cellular and secreted proteins.

The galactosaemics fibroblasts showed more variability in their results (Table 6.1, page 114). However, there were several consistent findings (Table 6.2, page 115). There was a significant reduction in the incorporation of [2-^{3}H]-mannose, per µg of protein, into the cell pellet in both the pyruvate- (33% of control mean; $p < 0.01$) and glucose-based (50% of control mean; $p < 0.01$) media. Similar reductions were also observed in the [2-^{3}H]-mannose incorporated into the media pellets (38% and 61% of control mean, respectively; $p < 0.05$). This reduction was reflected in the ^{3}H/^{35}S ratio for both the cell and media pellets, which were 57% and 27% of control mean ($p < 0.05$ and $p < 0.01$), respectively in the pyruvate-based medium, and 61% and 55% of control mean ($p < 0.05$), respectively in the glucose-based medium. If the results of the incorporation of [2-^{3}H]-mannose in the fibroblasts from both experimental media are pooled, then the decrease in [2-^{3}H]-mannose in the galactosaemic fibroblasts becomes highly significant in both the cell ($p < 0.001$) and media ($p = 0.001$) protein pellets. Despite this reduction, the media to cell ratio for ^{3}H remained very similar to the ratio in the control fibroblasts.

In the glucose-based medium, the galactosaemic fibroblasts incorporated [^{35}S]-methionine at a similar rate to the controls. However, in the pyruvate-based medium, the mean incorporation of [^{35}S]-methionine in the cell pellet was reduced to 55% of the control mean ($p < 0.01$). This was not associated with a similar reduction in the media pellet (Table 6.2, page 115). This resulted in higher and more variable media to cell ratios for [^{35}S]-methionine in the galactosaemic fibroblasts in the pyruvate-based medium. As a group, the galactosaemic fibroblasts also showed much more variability in their response to the different experimental media than the control fibroblasts (Table 6.1, page 114). *G3* and *G4* performed consistently for both radiolabels in both media. *G1* appeared to be most affected in the pyruvate-base medium, and *G2* gave low to near-normal results for both radiolabels, in both media. Although the experiments were not repeated to see if the galactosaemic fibroblasts performed consistently in this manner, *G1* produced similar results in Experimental plan B.

It was concluded that the galactosaemic fibroblasts incorporated less [2-^{3}H]-mannose than control fibroblasts, and that the effect was possibly greater in pyruvate-based medium. The reduced incorporation was also associated with a decrease in the ^{3}H/^{35}S ratio in both cellular and secreted protein. The galactosaemic fibroblasts also incorporated less [^{35}S]-methionine into cellular protein when they were exposed to medium that contained pyruvate and galactose. However, there was no obvious defect in their ability to secrete glycoprotein into the media.

Experimental plan B

The aim of Experimental plan B was to determine if the concentration of galactose in the medium influenced the incorporation of [2-^{3}H]-mannose in the galactosaemic fibroblasts. The fibroblasts were again radiolabelled at near-confluence but in medium that contained either 0, 1.0, 2.5, or 5.5 mmol/L D-galactose. Increasing the concentration of galactose above 5 mmol/L does not increase the galactose-1-phosphate concentration further (Pourci *et al.*, 1990). In this experimental procedure, the control and galactosaemic fibroblasts again showed similar growth characteristics. The total cell protein in the eight control cultures ranged from 207 to 309 µg (mean 262 µg), and in the eight galactosaemic cultures, from 207 to 325 µg (mean 265 µg). The results of the incorporation for each radiolabel are presented in Table 6.3 (page 116). The means of the incorporation in the duplicate cultures, at each concentration of galactose, are then displayed for [2-^{3}H]-mannose in Figure 6.1 (page 117), and for [^{35}S]-methionine in Figure 6.2 (page 118).

When the medium contained no galactose, there was no difference in the incorporation of [2-^{3}H]-mannose or [^{35}S]-methionine in the control and galactosaemic fibroblasts. This suggested that there was no defect in the uptake and incorporation of these radiolabels in the galactosaemic fibroblasts. Unfortunately, the incorporation of [2-^{3}H]-mannose in both the control and galactosaemic fibroblasts was influenced by the hexose concentration of the medium. This indicated competition between mannose and galactose for cellular uptake. However, as the concentration of galactose in the medium increased, the galactosaemic fibroblasts consistently incorporated less [2-^{3}H]-mannose and [^{35}S]-methionine than the control (Figures 6.1 and 6.2, pages 117 and 118). This occurred in both the cellular and secreted proteins. To remove the effect of the hexose concentration on the results, the mean incorporations for both radiolabels in the galactosaemic fibroblasts were then expressed as a percent of the mean control results (Figure 6.3, page 119). This showed that there was a proportional decrease in the incorporation of both [2-^{3}H]-mannose and [^{35}S]-methionine in the galactosaemic fibroblast as the concentration of galactose increased. The effect was observed in both the cellular and secreted proteins, and was greater for [2-^{3}H]-mannose. At 5.5 mmol/L of galactose, the incorporation of [2-^{3}H]-mannose per µg protein was reduced to 35% and 20% of control mean in the cell and media pellets, respectively, and for [^{35}S]-methionine to 49% and 63%, respectively. These results were similar to those observed for the *G1* cell line in the same medium in Experimental plan A (Table 6.1, page 114). The ^{3}H/^{35}S ratio also decreased to 72% and 31% of control mean in the cell and media pellets, respectively, which again compared favourably with the results obtained in Experimental plan A. The reduction in the ^{3}H/^{35}S ratio was always greater in the secreted protein because there was less effect on the incorporation of [^{35}S]-methionine in that protein pool.

It was concluded that the reduction in the incorporation of [2-^{3}H]-mannose and [^{35}S]-methionine in the galactosaemic fibroblasts was influenced by the concentration of galactose in the medium. The galactose also disturbed the ratio of ^{3}H to ^{35}S. When there was no galactose in the medium, the galactosaemic fibroblasts performed similarly to control fibroblasts.

Table 6.1. Incorporation of [2-^{3}H]-mannose and [^{35}S]-methionine into TCA-precipitates from control and galactosaemic fibroblast cultures, in media of different composition.

	Cell pellets			Media pellets			Media/cell ratio	
	^{3}H (cpm/μg protein)	^{35}S (cpm/μg protein)	^{3}H/^{35}S ratio	^{3}H (cpm/μg protein)	^{35}S (cpm/μg protein)	^{3}H/^{35}S ratio	^{3}H	^{35}S
Control								
C1a[a]	6 812	1 288	5.29	3 409	675	5.05	0.50	0.52
C1b	11 488	1 799	6.39	5 184	586	8.84	0.45	0.33
C2a	11 146	1 508	7.39	4 544	664	6.84	0.41	0.44
C2b	10 023	1 491	6.72	3 973	856	4.64	0.40	0.57
C3a	9 489	1 623	5.85	5 795	691	8.39	0.61	0.43
C3b	9 065	1 867	4.86	5 835	788	7.41	0.64	0.42
C4a	13 267	1 189	11.16	6 026	558	10.79	0.45	0.47
C4b	8 861	1 374	6.45	3 838	523	7.34	0.43	0.38
mean a	10 178	1 402	7.42	4 943	647	7.77	0.49	0.46
mean b	9 859	1 633	6.10	4 708	688	7.06	0.48	0.43
Galactosaemic								
G1a	2 004	816	2.46	471	384	1.23	0.24	0.47
G1b	7 221	1 982	3.64	4 616	629	7.34	0.64	0.32
G2a	5 166	861	6.00	3 950	1 048	3.77	0.76	1.22
G2b	6 813	1 159	5.88	3 586	846	4.24	0.53	0.73
G3a	1 843	472	3.91	1 208	1 284	0.94	0.66	2.72
G3b	2 765	875	3.16	1 609	1 140	1.41	0.58	1.30
G4a	4 289	958	4.48	1 903	779	2.44	0.44	0.81
G4b	2 955	1 299	2.28	1 668	659	2.53	0.56	0.51
mean a	3 325	777	4.21	1 883	874	2.09	0.52	1.31
mean b	4 939	1 329	3.74	2 870	818	3.88	0.58	0.71

[a] The four control (*C1, C2, C3,* and *C4*) and four galactosaemic (*G1, G2, G3,* and *G4*) fibroblast cultures were preincubated and radiolabelled in a modified D-MEM that contained either (***a***) 2.5 mmol/L sodium pyruvate and 5.5 mmol/L D-galactose or (***b***) 2.5 mmol/L D-glucose and 5.5 mmol/L D-galactose.

Table 6.2. Summary of the incorporation of [2-^{3}H]-mannose and [^{35}S]-methionine into TCA-precipitates from control and galactosaemic fibroblast cultures, in media of different composition.

	Controls (n = 4)		**Galactosaemics (n = 4)**	
	2.5 mmol/L pyruvate + 5.5 mmol/L galactose	2.5 mmol/L glucose + 5.5 mmol/L galactose	2.5 mmol/L pyruvate + 5.5 mmol/L galactose	2.5 mmol/L glucose + 5.5 mmol/L galactose
Cell pellets				
^{3}H cpm/μg protein	10 178 (1 363)[a]	9 859 (599)	**3 325 (830)	**4 939 (1 203)
^{35}S cpm/μg protein	1 402 (99)	1 633 (119)	**777 (106)	#1 329 (235)
^{3}H/^{35}S ratio	7.42 (1.32)	6.10 (0.42)	*4.21 (0.73)	*3.74 (0.77)
Media pellets				
^{3}H cpm/μg protein	4 943 (606)	4 708 (483)	*1 883 (748)	*2 870 (741)
^{35}S cpm/μg protein	647 (30)	688 (79)	874 (193)	818 (117)
^{3}H/^{35}S ratio	7.77 (1.22)	7.06 (0.88)	**2.09 (0.65)	*3.88 (1.29)
Media/cell ratio				
^{3}H	0.49 (0.04)	0.48 (0.06)	0.52 (0.12)	0.58 (0.02)
^{35}S	0.46 (0.02)	0.43 (0.05)	1.31 (0.50)	0.71 (0.21)

[a] Mean (standard error of the mean).
* Significantly different to the control fibroblasts in the same medium; * $p < 0.05$, ** $p < 0.01$.
[#] Significantly different to the galactosaemic fibroblasts in the other medium; $p < 0.05$.

Table 6.3. Incorporation of [2-^{3}H]-mannose and [^{35}S]-methionine into TCA-precipitates from a control and a galactosaemic fibroblast culture, in media with increasing concentrations of galactose.

	Control				Galactosaemic			
	^{3}H (cpm/μg protein)	mean	^{35}S (cpm/μg protein)	mean	^{3}H (cpm/μg protein)	mean	^{35}S (cpm/μg protein)	mean
Cell pellets								
0[a]	19 599[b]		2 626		25 649		2 718	
	23 720	21 660	2 407	2 516	21 672	23 661	3 622	3 170
1	8 326		3 509		11 161		2 478	
	11 106	9 716	3 777	3 643	2 610	6 885	2 142	2 310
2.5	15 727		2 730		7 767		2 022	
	15 478	15 603	3 020	2 875	6 302	7 035	1 939	1 981
5.5	10 059		3 160		3 266		1 644	
	8 443	9 251	3 162	3 161	3 179	3 222	1 435	1 540
Media pellets								
0	13 586		966		15 218		982	
	13 202	13 394	723	844	12 878	14 048	1 116	1 049
1	4 291		824		7 953		989	
	6 009	5 150	714	769	1 000	4 477	651	820
2.5	14 154		945		4 618		772	
	11 521	12 838	989	967	3 429	4 024	742	757
5.5	6 901		813		1 394		623	
	5 502	6 201	735	774	1 047	1 221	359	491

[a] Fibroblasts were preincubated and radiolabelled in a modified D-MEM that contained 2.5 mmol/L sodium pyruvate and either 0, 1.0, 2.5, or 5.5 mmol/L D-galactose.
[b] Results are shown for duplicate cultures, with the mean, at each media galactose concentration.

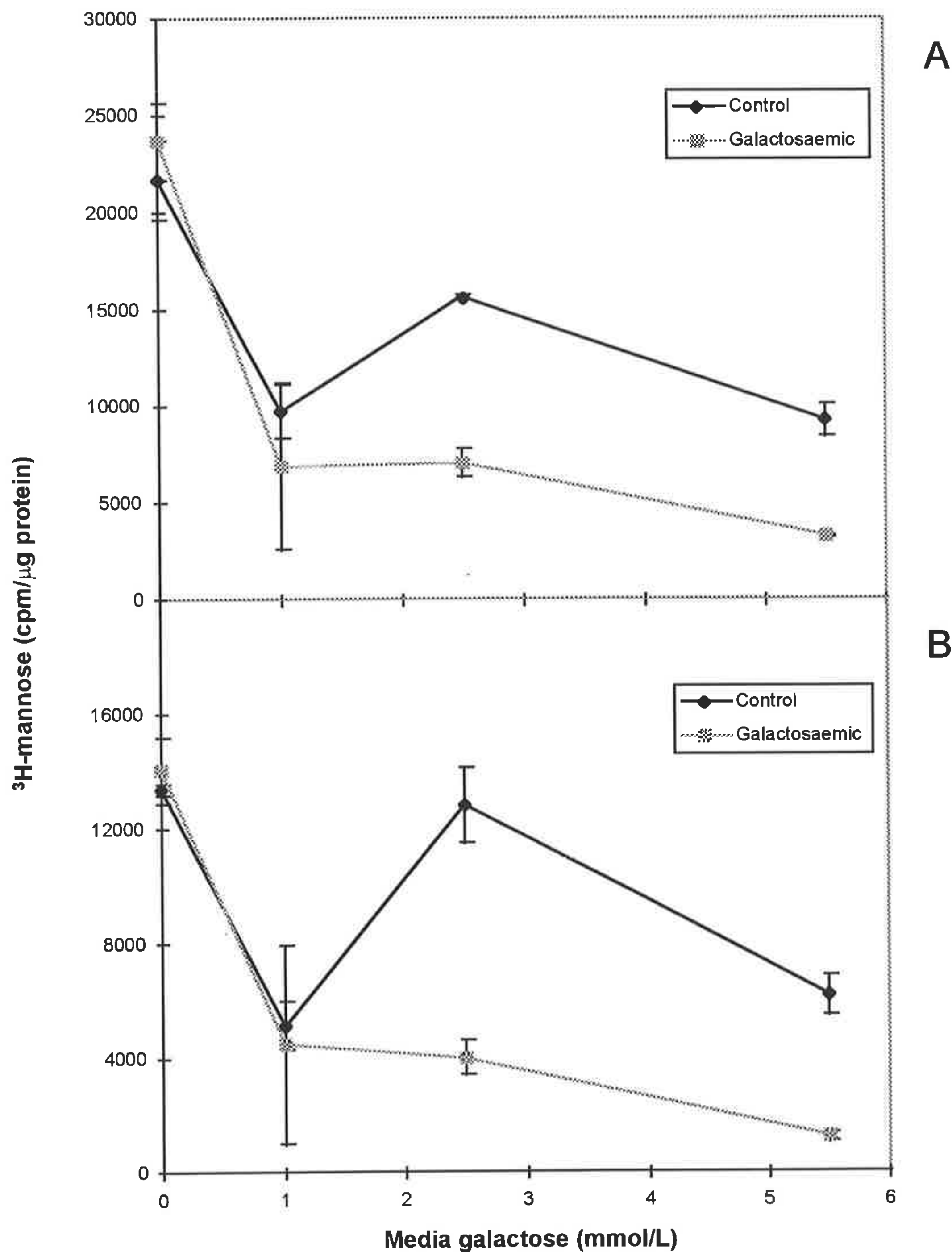


Figure 6.1. Incorporation of [2-^{3}H]-mannose into TCA-precipitates from *A*, the cell pellets; and *B*, the media of a control (*C1*) and a galactosaemic (*G1*) fibroblast culture, in a medium with an increasing concentration of galactose. The fibroblasts were preincubated and radiolabelled in a modified D-MEM that contained 2.5 mmol/L sodium pyruvate and either 0, 1.0, 2.5, or 5.5 mmol/L D-galactose. The results are expressed as the mean of duplicate cell cultures, with the range.

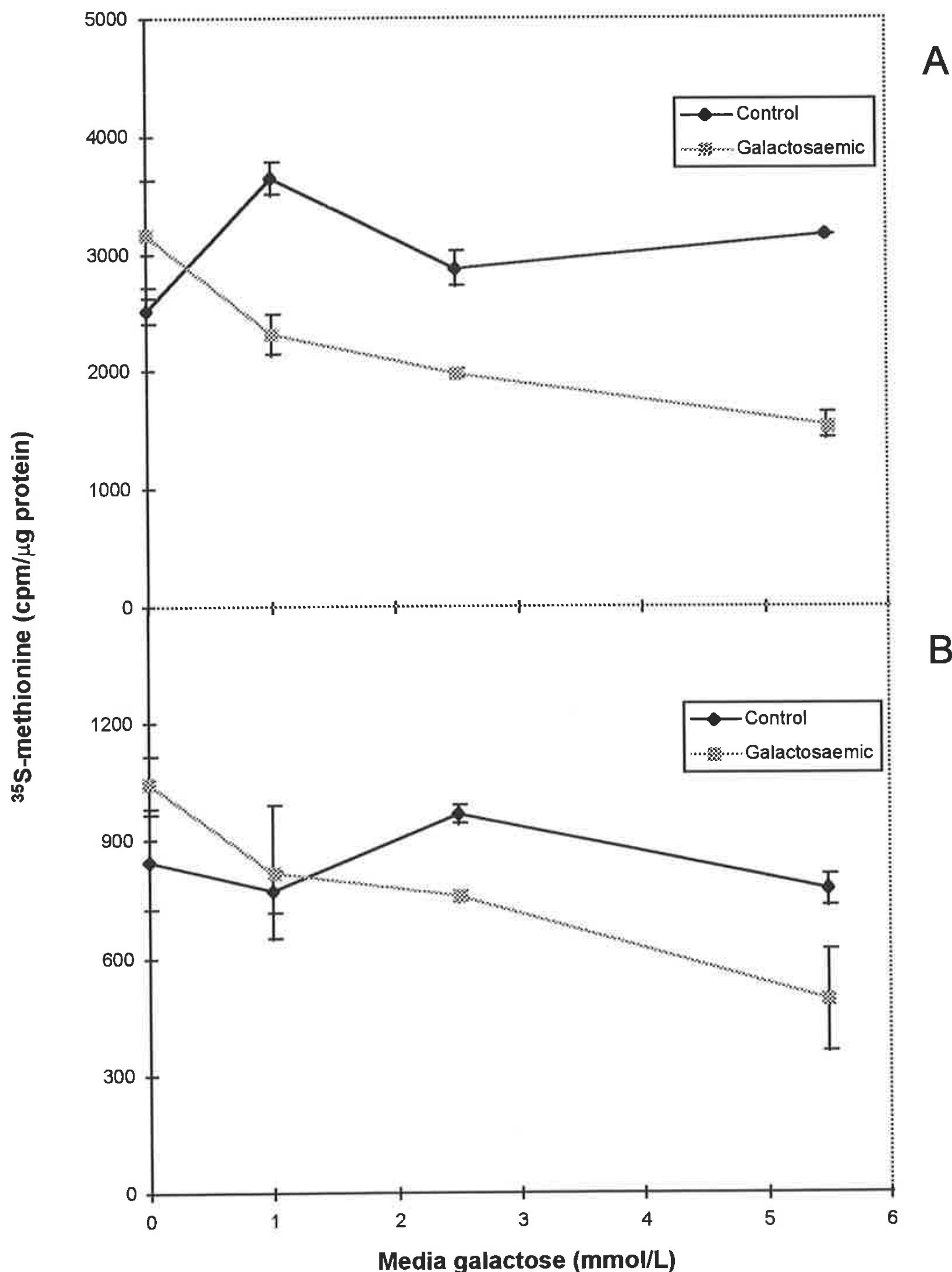


Figure 6.2. Incorporation of [^{35}S]-methionine into TCA-precipitates from *A*, the cell pellets; and ***B***, the media of a control (*C1*) and a galactosaemic (*G1*) fibroblast culture, in a medium with an increasing concentration of galactose. The fibroblasts were preincubated and radiolabelled in a modified D-MEM that contained 2.5 mmol/L sodium pyruvate and either 0, 1.0, 2.5, or 5.5 mmol/L D-galactose. The results are expressed as the mean of duplicate cell cultures, with the range.

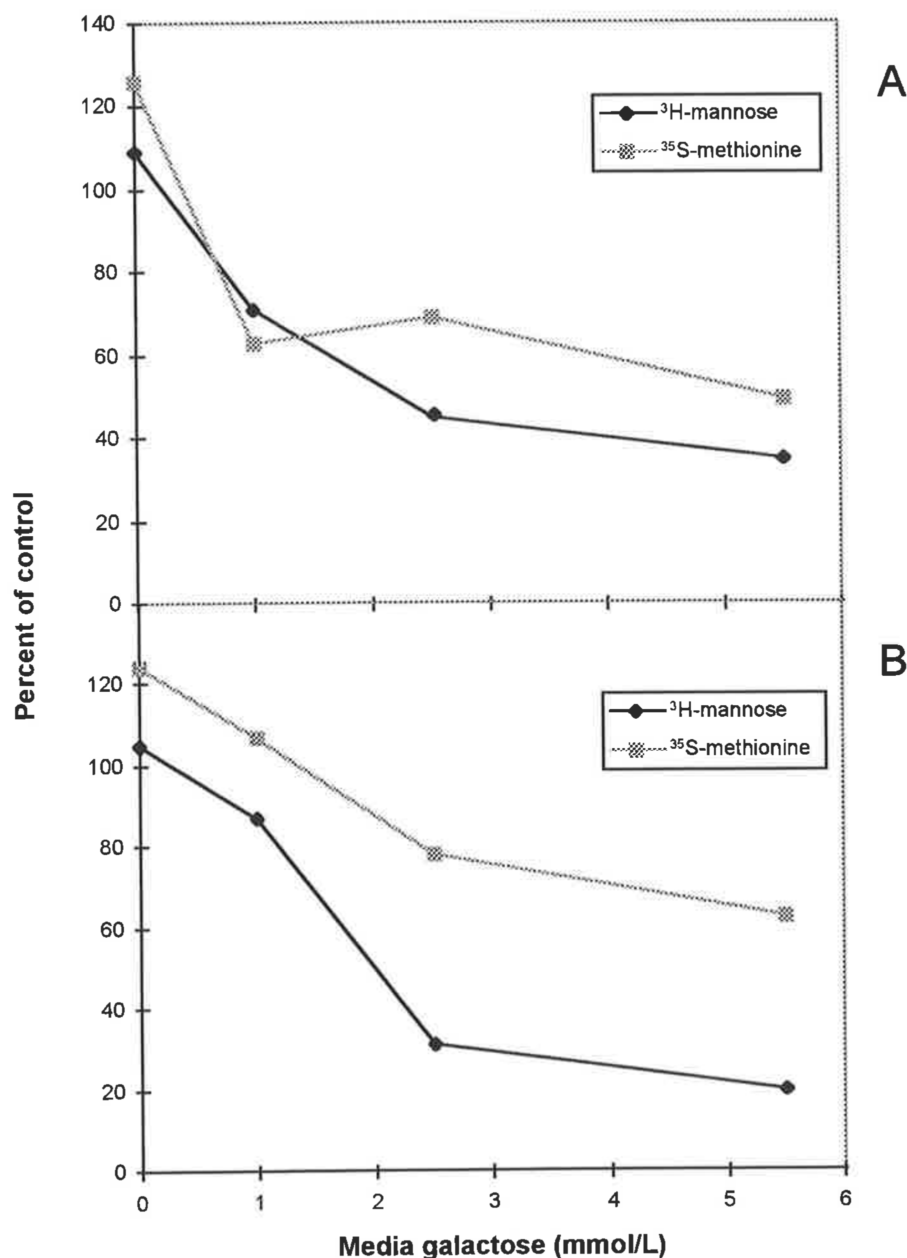


Figure 6.3. Incorporation of [2-^{3}H]-mannose and [^{35}S]-methionine into TCA-precipitates from *A*, the cell pellets; and *B*, the media of duplicate control and galactosaemic fibroblast cultures. The mean galactosaemic results are expressed as a percent of the mean control results, for each radiolabel, at each concentration of galactose in the medium. The fibroblasts were preincubated and radiolabelled in a modified D-MEM that contained 2.5 mmol/L sodium pyruvate and either 0, 1.0, 2.5, or 5.5 mmol/L of D-galactose.

6.4 Discussion

When the galactosaemic fibroblasts were exposed to galactose in the medium they incorporated less [2-^{3}H]-mannose into glycoprotein. There are several possible explanations for this result.

To incorporate [2-^{3}H]-mannose into N-linked oligosaccharides requires transport of the mannose into the cell by membrane sugar transporters, its incorporation and equilibration within the mannose phosphate and GDP-mannose pools, and finally its incorporation into dolichol-linked oligosaccharides. It is therefore possible that the decreased incorporation in the galactosaemic fibroblasts was secondary to either a decreased uptake or utilisation of mannose. Wolfrom *et al.*, (1993) reported that galactosaemic fibroblasts in culture use approximately 30% less extracellular glucose than normal skin fibroblasts. This has been observed by others (Tedesco and Miller, 1979). Wolfrom *et al.*, (1993) suggested that the kinetics of hexose transport in the galactosaemic fibroblast membranes were probably normal (Guerroui S; cited by Wolfrom *et al.*, 1993), and that the reduction was secondary to a decreased use of UDP-glucose, a result of the block in the Leloir pathway. A similar disturbance might also affect the uptake and utilisation of mannose. However, Wolfrom *et al.*, (1993) observed the deficit in glucose utilisation when the galactosaemic fibroblasts were in a medium without galactose (although the 10% human serum they used was not dialysed). In Experimental plan B, the incorporation of [2-^{3}H]-mannose was normal when the galactosaemic fibroblasts were grown in medium without galactose. This suggests that the pathway for uptake and utilisation of mannose was intact under these conditions. When galactose was added to the medium, the reduction in the incorporation of [2-^{3}H]-mannose was also greater than the 70% of normal reported by Wolfrom *et al.* (1993) for glucose.

Mayes and Miller, (1973) described a futile cycle of phosphorylation and dephosphorylation of galactose in galactosaemic fibroblasts in culture. This cycle was not observed in normal fibroblasts. It is possible therefore that the reduced [2-^{3}H]-mannose incorporation was secondary to any effects this futile cycle might have on mannose uptake. Presumably the effect would be proportional to the concentration of galactose in the medium, as observed in Experimental plan B. However, the decreased incorporation of [2-^{3}H]-mannose was also associated with a decrease in the incorporation of [^{35}S]-methionine into cellular protein, in the pyruvate-based medium. This suggests that the disturbances are linked as it is unlikely that the uptake of [^{35}S]-methionine would be affected by a disturbance in galactose phosphorylation.

Once inside the cell, mannose is phosphorylated to mannose-6-phosphate by hexokinase, and is then converted to mannose-1-phosphate by phosphomannomutase. Mannose-1-phosphate is then used to synthesise GDP-mannose and ultimately Dol-P-mannose, which are required for dolichol-linked oligosaccharide synthesis. It is also possible therefore that a deficiency of cellular ATP reduced the phosphorylation of [2-^{3}H]-mannose and thereby reduced its incorporation. The reduced ATP levels would also affect protein synthesis and reduce [^{35}S]-methionine incorporation. This mechanism could explain the changes seen for both radiolabels in this chapter. However, the evidence for a decrease in cellular ATP in galactosaemic erythrocytes exposed to galactose, *in vivo* or *in vitro*, is conflicting (Komrower *et al.*, 1956; Schwarz *et al.*, 1956; Penington and Prankerd, 1958; and Zipursky *et al.*, 1965). Galactosaemic patients do show minor increases in plasma urate following oral galactose loads and this suggests that there is some cellular ATP breakdown, at least acutely, following a galactose load (Forster *et al.*, 1975; and Kogut *et al.*, 1975). The extent of ATP breakdown also depends on the amount of galactose given (Kalderon *et al.*, 1992). Although galactosaemic fibroblasts accumulate galactose-1-phosphate when they are exposed to galactose, it is not known if this is associated with a decrease in ATP (Mayes and Miller, 1973;

and Tedesco and Miller, 1979). To avoid this possible complication, the galactosaemic fibroblasts in Experimental plan A and B were radiolabelled only after they had been preincubated for four hours in galactose-containing media. The radiolabels were also added directly to the medium to minimise any further metabolic changes.

If the radiolabels were taken up and distributed normally within the galactosaemic fibroblasts, then the altered patterns of [2-^{3}H]-mannose and [^{35}S]-methionine incorporation could represent an abnormality in N-glycan or N-linked glycoprotein synthesis. There are several possible mechanisms: either there was a reduction in the amount of total N-linked glycoprotein synthesised; or a change in the type of glycoprotein synthesised; or a change in the relative amounts of high mannose, hybrid and complex N-linked oligosaccharides; or the N-glycans were partly absent or truncated. In Chapter 5, the galactosaemic fibroblasts produced mature complex N-glycans that were remarkably similar to control fibroblasts, apart from an increase in complexity. They did accumulate smaller $Man_{3-5}GlcNAc_2$-sized oligosaccharides when they were exposed to media without glucose, but this occurred predominantly during rapid growth (Figure 5.8B, page 98). Taken together, these findings do not support a major change in the type of glycoprotein synthesised, or in the relative amounts of high mannose, hybrid and complex N-glycans, in the galactosaemic fibroblasts. When the galactosaemic fibroblasts were exposed to galactose, they incorporated [2-^{3}H]-mannose at approximately 30–60% of normal (Table 6.2, page 115). This was the same whether the incorporation was expressed per µg of protein or as a ratio with [^{35}S]-methionine, and was observed in both cellular and secreted proteins. Cellular protein synthesis was also reduced, as measured by [^{35}S]-methionine, in the medium that contained pyruvate and galactose. It is probable therefore that the galactosaemic fibroblasts synthesised less N-linked glycoprotein or that the N-glycans attached to protein were partly absent or truncated. In similar experiments in fibroblasts from patients with the CDG syndrome type I, the fibroblasts incorporated [2-^{3}H]-mannose at approximately 10–35% of normal, when corrected to protein content or [^{35}S]-methionine (Powell *et al.*, 1994; and Panneerselvam and Freeze, 1996). CDG type I fibroblasts are presumed to be unable to incorporate mannose into GDP-mannose (van Schaftingen and Jaeken, 1995). This causes abnormalities in dolichol-linked oligosaccharide synthesis and incomplete N-glycosylation (Powell *et al.*, 1994; Krasnewich *et al.*, 1995; and Panneerselvam and Freeze, 1996). It is possible that there is a similar defect in dolichol-linked oligosaccharide synthesis in galactosaemic fibroblasts, which could explain the reduced incorporation of [2-^{3}H]-mannose. The next chapter therefore examines dolichol-linked oligosaccharide synthesis in galactosaemic fibroblasts.

7

Dolichol-linked oligosaccharide synthesis in galactosaemic fibroblasts

7.1 Introduction

In Chapter 6, it was observed that galactosaemic fibroblasts incorporated less [2-^{3}H]-mannose into N-linked glycans when they were cultured in medium that contained galactose. This suggested a possible disturbance in dolichol-linked oligosaccharide synthesis, which could explain the hyposialylated glycoproteins in the serum in untreated galactosaemia. The aim therefore of this chapter was to examine dolichol-linked oligosaccharide synthesis in galactosaemic fibroblasts. The experimental plan was to determine the rate of incorporation of [2-^{3}H]-mannose into the dolichol-linked oligosaccharides, and to monitor the transfer of those oligosaccharides to protein in the ER. The dolichol- and protein-linked oligosaccharides were also recovered and analysed by size-exclusion chromatography, to determine the types of oligosaccharide intermediate produced by the galactosaemic fibroblasts.

The skin fibroblasts were radiolabelled with [2-^{3}H]-mannose over 30 minutes. The dolichol-linked oligosaccharides were then recovered from the cell pellet by sequential solvent extractions (Method 5, page 72). Before the radiolabelling procedure, the fibroblasts were preincubated for four hours in medium that contained glucose and galactose. This allowed the fibroblasts to adjust to the galactose, and the galactosaemic fibroblasts to accumulate galactose-1-phosphate. The fibroblasts were then washed several times to remove excess hexose and they were then radiolabelled in a medium that contained 0.5 mmol/L D-glucose. Complete glucose starvation disturbs the equilibrium of the synthetic pathway and produces an increased proportion of truncated dolichol-linked oligosaccharides (Gershman and Robbins, 1981). The small amount of glucose, however, still permits sufficient incorporation of [2-^{3}H]-mannose to label the dolichol-linked oligosaccharides. It was not possible therefore to include galactose in the radiolabelling medium as this would have considerably reduced the uptake of [2-^{3}H]-mannose. It was anticipated though that the experimental design would still permit study of the effect of intracellular galactose intermediates on dolichol-linked oligosaccharide synthesis in galactosaemic fibroblasts.

7.2 Experimental plan

Two control (*C1* and *C2*) and two galactosaemic (*G1* and *G4*; Table 4.1, page 56) fibroblast cultures were subcultured into 8 cm glass Petri dishes and grown to 50–70% of confluence (40–48 hours) in D-MEM with 10% (v/v) dialysed FBS. The fibroblasts were then preincubated for four hours in a modified D-MEM that contained 10% (v/v) dialysed FBS and either (***a***) 2.5 mmol/L D-glucose and 5.5 mmol/L D-galactose, (***b***) 5.5 mmol/L D-glucose and 10 mmol/L D-galactose, or (***c***) 5.5 mmol/L D-glucose. The fibroblasts were then washed with glucose-deficient D-MEM, and then radiolabelled for 30 minutes in a modified D-MEM that

contained 10% (v/v) dialysed FBS, 0.5 mmol/L D-glucose, and 3.7 MBq/mL D-[2-^{3}H]-mannose (Method 5, page 72).

The cells were harvested directly in chloroform/methanol 2:1 (v/v). The cell pellet was then sequentially extracted with chloroform/methanol 2:1 (v/v), water, and chloroform/methanol/water 10:10:3 (v/v/v). The dolichol-linked oligosaccharide intermediates, which were recovered from the 2:1 and 10:10:3 extracts, were then released from the dolichol by mild acid hydrolysis. The protein-bound oligosaccharides in the residual protein pellet were recovered with PNGase F (Method 5, page 72). The oligosaccharides from each of these fractions were then separated on a Bio-Gel P-4 240 x 1 cm column in 0.1 mol/L NH_4COOH, at a flow rate of 8 mL/h. Each sample was loaded on the column with an internal standard of 100 µg BSA, 500 cpm [^{3}H]-mannose, and 10 µg of L-fucose. The column was standardised against a range of oligomannose standards (Appendix 2, page 154).

7.3 Results

The results of the incorporation of [2-^{3}H]-mannose into the oligosaccharides of the chloroform/methanol 2:1 extract, chloroform/methanol/water 10:10:3 extract, and residual protein pellet are listed in Table 7.1 (page 126). The three recovered fractions were designated the '2:1 extract', the 'DLO fraction', and the 'protein fraction', respectively. The results in Table 7.1 are grouped according to the composition of the preincubation medium. The incorporation of the [2-^{3}H]-mannose in each of the fractions was corrected to the protein content of the residual pellet.

The data in Table 7.1 were collected in two separate radiolabelling experiments. In the first experiment, the fibroblasts were preincubated in D-MEM that contained 2.5 mmol/L glucose and 5.5 mmol/L of galactose. In the second experiment, the preincubation medium contained 5.5 mmol/L glucose and 10 mmol/L of galactose. In the second experiment, one of the control (*C1*) and one of the galactosaemic (*G4*) cultures were also preincubated in a medium that contained only 5.5 mmol/L glucose. The concentration of galactose in the preincubation medium in the second experiment was increased to determine if this disturbed normal dolichol-linked oligosaccharide synthesis. The ratio of galactose to glucose, however, was maintained at 2 to 1 to avoid any effects due to altering the availability of glucose to the cells. One control and one galactosaemic culture were also radiolabelled after they were preincubated in a glucose-only medium. This was to determine if galactose adversely affected either the uptake of the radiolabel, its incorporation into dolichol-linked oligosaccharides, or the equilibrium of the dolichol-linked oligosaccharide pathway. The procedural differences between experiment one and two resulted in some variation in the total radiolabel incorporated into the different fractions. The results of the incorporation were therefore recalculated as a percent of the mean normal control value, at each concentration of galactose in the medium, to allow comparison of the results between the experiments (Figure 7.1, page 127).

The control fibroblasts performed in a consistent manner regardless of the composition of the preincubation medium (Table 7.1, page 126). The galactosaemic fibroblasts, however, consistently incorporated less [2-^{3}H]-mannose into the DLO and protein fractions when the preincubation medium contained galactose. The incorporation was reduced to approximately 10–35% of mean normal control in both the DLO and protein fractions, and was similar whether the preincubation medium contained 5.5 or 10 mmol/L of galactose (Figure 7.1, page 127). When the galactosaemic culture (*G4*) was preincubated in glucose-only medium, the level of incorporation increased to approximately 40% of normal in both fractions. The control fibroblasts (*C1*), however, incorporated similar amounts of radiolabel, into each fraction, whether the preincubation medium contained 5.5 mmol/L glucose, or 5.5 mmol/L

glucose and 10 mmol/L galactose. Adding galactose to the preincubation medium did not therefore affect the uptake or distribution of [2-^{3}H]-mannose within the control fibroblasts. Although the galactosaemic fibroblasts consistently incorporated less [2-^{3}H]-mannose, the ratio of the radiolabel in the protein fraction to that in the DLO fraction (protein/DLO ratio; Table 7.1) was similar in the control and galactosaemic fibroblasts. This suggests that there was no delay in the transfer of oligosaccharide from dolichol to protein in the ER. The incorporation of [2-^{3}H]-mannose into the 2:1 extract was similar in both the control and galactosaemic fibroblasts (Table 7.1, page 126).

The oligosaccharides in the DLO fractions and 2:1 extracts were then released from the dolichol by mild acid hydrolysis and recovered as free oligosaccharide. The protein-bound oligosaccharides were then cleaved from the residual protein pellet with PNGase F, and the majority of the radiolabel was recovered as free oligosaccharide (Figure 4.6, page 75). Similar amounts of radiolabelled oligosaccharide, from each of the fractions, were then analysed on a Bio-Gel P-4 240 x 1 cm column to determine the size of the oligosaccharide intermediates. Each sample was resolved by the column into 175 fractions. For clarity, the representative figures in this chapter are therefore presented without the individual data points. The Bio-Gel P-4 chromatographic profiles of the oligosaccharides from the DLO fractions of the control and galactosaemic fibroblasts are displayed in Figures 7.2 and 7.3, respectively (pages 128 and 129). The results for the medium that contained 5.5 mmol/L galactose are presented first (Figure 7.2) followed by the profiles from the medium with 10 mmol/L galactose (Figure 7.3). The profiles of the oligosaccharides from the 2:1 extracts and the protein fractions are then presented in a similar manner in Figures 7.4, 7.5, 7.6, and 7.7, respectively (pages 130, 131, 132, and 133). Each figure directly compares the oligosaccharide profiles from the two control (*C1* and *C2*) and two galactosaemic (*G1* and *G4*) cultures, within each fraction. Finally, the profiles of the oligosaccharides from the glucose-only medium are displayed for the control (*C1*) in Figure 7.8 (page 134), and for the galactosaemic (*G4*) culture in Figure 7.9 (page 135).

The control fibroblasts produced profiles of oligosaccharides that were very similar regardless of the composition of the preincubation medium. The DLO fraction contained a single major peak, which was consistent in size with mature $Glc_3Man_9GlcNAc_2$. There were also minor peaks of $Man_8GlcNAc_2$ and $Man_5GlcNAc_2$ (Figures 7.2, 7.3, and 7.8; pages 128, 129, and 134). The 2:1 extract also contained several smaller dolichol-linked intermediates, particularly peaks of $Man_5GlcNAc_2$ and $Man_3GlcNAc_2$ (Figures 7.4, 7.5, and 7.8; pages 130, 131, and 134). The N-linked oligosaccharides from the protein fraction produced a single major broad peak, which was consistent with $Man_9GlcNAc_2$ to $Glc_{1-2}Man_9GlcNAc_2$-sized oligosaccharides (Figures 7.6, 7.7, and 7.8; pages 132, 133, and 134). There was also lesser amounts of several smaller intermediates in the protein fraction, which ranged in size down to $Man_5GlcNAc_2$.

The galactosaemic fibroblasts, however, produced quite different profiles of oligosaccharides. The reduced incorporation of [2-^{3}H]-mannose was associated with an accumulation of truncated oligosaccharides in both the DLO and protein fractions. The DLO fraction contained some mature $Glc_3Man_9GlcNAc_2$, however, there was an increase in the proportion of smaller intermediates, which ranged in size predominantly from $Man_3GlcNAc_2$ to $Man_5GlcNAc_2$ (Figures 7.2 and 7.3, pages 128 and 129). The 2:1 extract contained an increased proportion of $Man_{2-3}GlcNAc_2$-sized oligosaccharides (Figures 7.4 and 7.5, pages 130 and 131). The accumulation of smaller dolichol-linked intermediates was reflected in the oligosaccharides that were recovered from the protein fraction. The protein pellet contained some N-linked oligosaccharides of $Glc_{1-2}Man_9GlcNAc_2$ size, however, the major peak was consistent with $Man_5GlcNAc_2$. Slightly larger N-linked intermediates were also present in lesser amounts (Figures 7.6 and 7.7, pages 132 and 133).

The profiles of the oligosaccharides from the two galactosaemic cultures also showed more variability than the control fibroblasts. Both *G1* and *G4* appeared most affected after they had been preincubated in the medium that contained 10 mmol/L galactose. The *G4* cell line also produced profiles of oligosaccharides from the DLO and protein fractions that were consistently more abnormal than those from *G1*. *G4* also incorporated the least [2-^{3}H]-mannose into these fractions (Figure 7.1, page 127). The variability within the galactosaemic fibroblasts, and the differences with the control fibroblasts, were not related to either the cell passage number, cell protein content (the mean control and galactosaemic protein contents were 171 µg and 138 µg, respectively), or variations in the radiolabelling conditions. However, the measured GALT activity in the *G1* cell line was slightly more than *G4* (Table 4.1, page 56).

The control fibroblast, *C1*, produced profiles of dolichol- and protein-linked oligosaccharides that were similar whether the preincubation medium contained glucose or galactose. This confirmed that preincubation in the galactose did not affect the equilibrium of normal dolichol-linked oligosaccharide synthesis. However, the oligosaccharides that were synthesised by the galactosaemic fibroblast, *G4*, in the glucose-only medium were not entirely normal. The 2:1 extract still contained an accumulation of smaller dolichol-linked intermediates, particularly $Man_3GlcNAc_2$, and the protein fraction still contained a significant peak of $Man_5GlcNAc_2$-sized oligosaccharides (Figure 7.9, page 135). Although this experiment was performed on only one occasion, it is interesting that these changes were reflected in the reduced incorporation of [2-^{3}H]-mannose into the DLO and protein fractions in this cell line (Figure 7.1, page 127). This suggests that the synthesis of dolichol-linked oligosaccharides in the galactosaemic fibroblasts may even be disturbed in the absence of galactose.

Table 7.1. Incorporation of [2-^{3}H]-mannose into dolichol-linked and protein-linked oligosaccharides in control and galactosaemic fibroblast cultures that were preincubated in media with different concentrations of galactose.

Cell culture and media	2:1 extract[a] (cpm/μg protein)	DLO fraction[b] (cpm/μg protein)	protein fraction (cpm/μg protein)	protein/DLO ratio
2.5 mmol/L glucose + 5.5 mmol/L galactose				
C1[c]	2 870	476.9	10 109	21.2
C2	595	245.5	11 329	46.1
G1	2 522	88.6	3 945	44.5
G4	2 448	34.1	1 189	34.9
5.5 mmol/L glucose + 10 mmol/L galactose				
C1	928	791	4 226	5.3
C2	670	956	4 501	4.7
G1	939	124	1 098	8.8
G4	828	133	466	3.5
5.5 mmol/L glucose				
C1	700	747	4 217	5.6
G4	1 692	265	1 758	6.6

[a] Oligosaccharides recovered from the chloroform/methanol 2:1 extract.

[b] Oligosaccharides recovered from the chloroform/methanol/water 10:10:3 extract.

[c] The radiolabelling procedure that preincubated the fibroblasts in 2.5 mmol/L glucose and 5.5 mmol/L galactose was performed as a separate experiment to the remainder of the data. Different control fibroblast cultures (*C1* and *C2*) were also used.

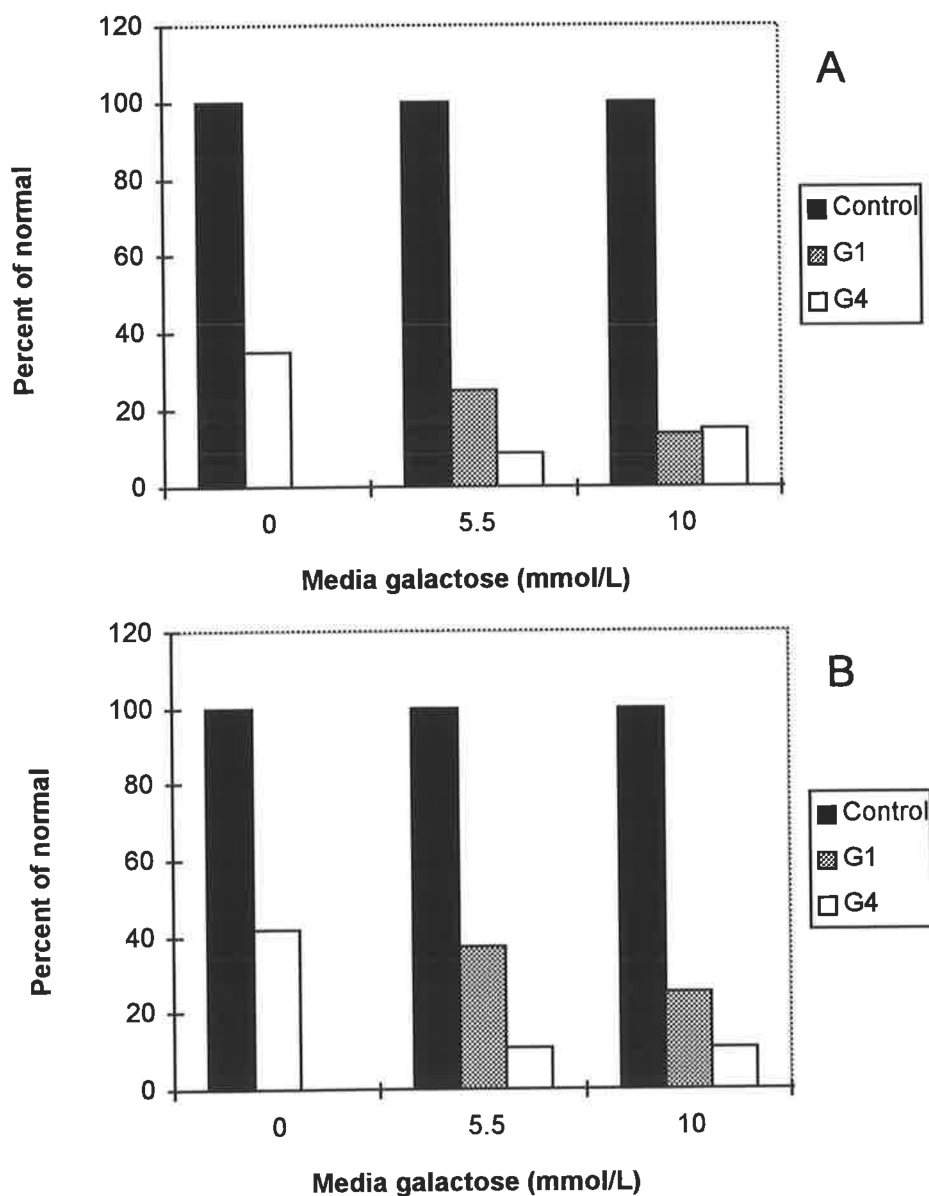


Figure 7.1. Incorporation of [2-^{3}H]-mannose into ***A***, the chloroform/methanol/water 10:10:3 extract; and ***B***, the protein pellet of two galactosaemic fibroblast cultures (*G1* and *G4*). The incorporation of radiolabel (cpm/µg protein) was expressed as a percent of normal, with the mean of the two control cultures arbitrarily set at 100%, for each concentration of galactose in the preincubation medium (data taken from Table 7.1, page 126).

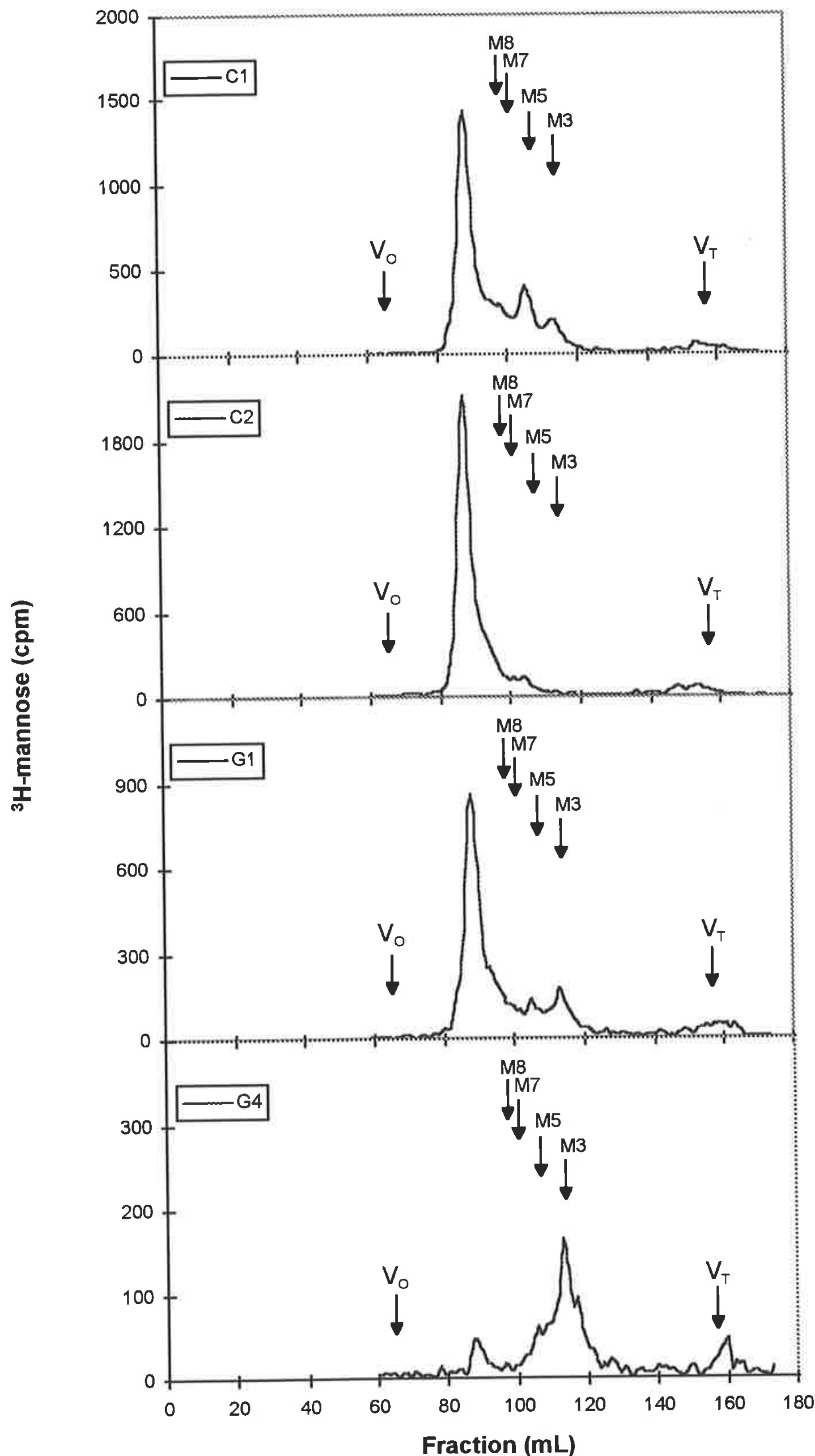


Figure 7.2. Dolichol-linked oligosaccharides recovered from the chloroform/methanol/water 10:10:3 extract of two control (*C1* and *C2*) and two galactosaemic (*G1* and *G4*) fibroblast cultures. The cultures were preincubated in 2.5 mmol/L D-glucose and 5.5 mmol/L D-galactose. 20 000 cpm of oligosaccharide from each extract were analysed on a Bio-Gel P-4 240 x 1 cm column in 0.1 mol/L NH_4COOH, at a flow rate of 8 mL/h. 175 x 1 mL fractions were collected. A range of tritiated oligomannose standards are shown (*arrows*): $Man_8GlcNAc_2$ (*M8*), $Man_7GlcNAc_2$ (*M7*), $Man_5GlcNAc_2$ (*M5*), and $Man_3GlcNAc_2$ (*M3*) peaks eluted at 98, 100, 106, and 115 mL, respectively. The exclusion volume (V_O) for the column was 65 mL and inclusion volume (V_T) 158 mL.

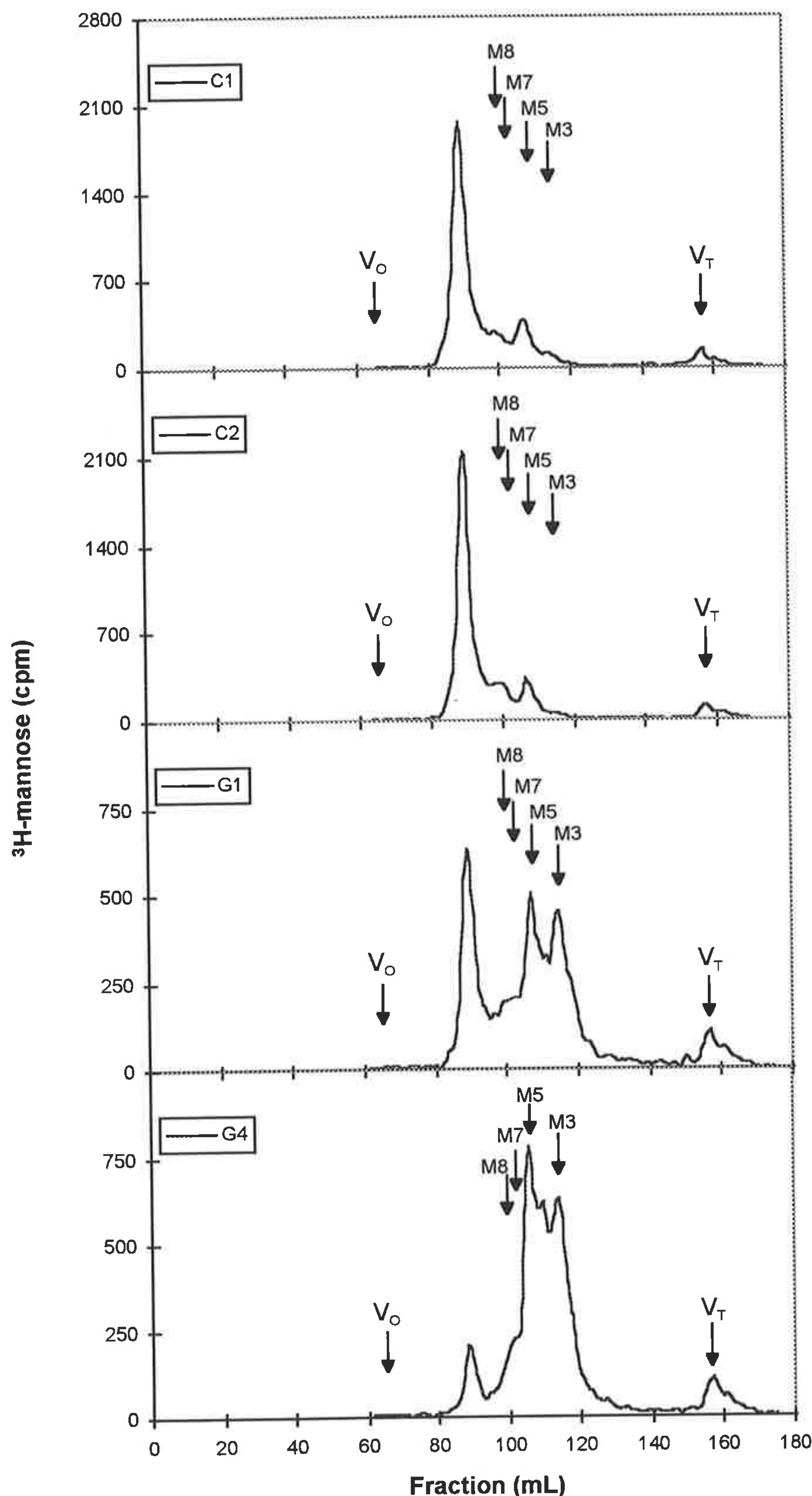


Figure 7.3. Dolichol-linked oligosaccharides recovered from the chloroform/methanol/water 10:10:3 extract of two control (*C1* and *C2*) and two galactosaemic (*G1* and *G4*) fibroblast cultures. The cultures were preincubated in 5.5 mmol/L D-glucose and 10 mmol/L D-galactose. 20 000 cpm of oligosaccharide from each extract were analysed on a Bio-Gel P-4 240 x 1 cm column in 0.1 mol/L NH_4COOH, at a flow rate of 8 mL/h. 175 x 1 mL fractions were collected. A range of tritiated oligomannose standards are shown (*arrows*): $Man_8GlcNAc_2$ (*M8*), $Man_7GlcNAc_2$ (*M7*), $Man_5GlcNAc_2$ (*M5*), and $Man_3GlcNAc_2$ (*M3*) peaks eluted at 98, 100, 106, and 115 mL, respectively. The exclusion volume (V_O) for the column was 65 mL and inclusion volume (V_T) 158 mL.

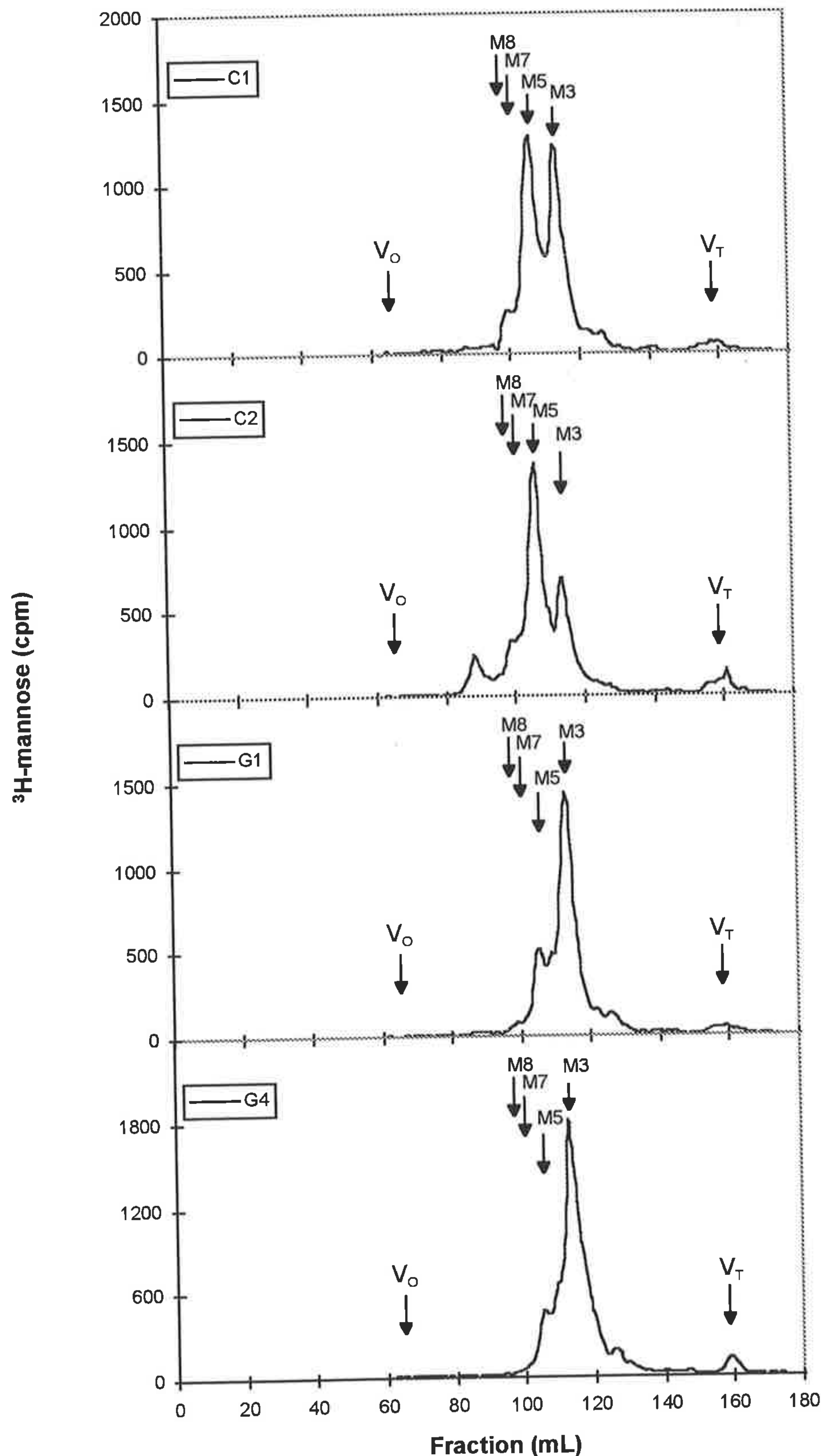


Figure 7.4. Dolichol-linked oligosaccharides recovered from the chloroform/methanol 2:1 extract of two control (*C1* and *C2*) and two galactosaemic (*G1* and *G4*) fibroblast cultures. The cultures were preincubated in 2.5 mmol/L D-glucose and 5.5 mmol/L D-galactose. 20 000 cpm of oligosaccharide from each extract were analysed on a Bio-Gel P-4 240 x 1 cm column in 0.1 mol/L NH_4COOH, at a flow rate of 8 mL/h. 175 x 1 mL fractions were collected. A range of tritiated oligomannose standards are shown (*arrows*): $Man_8GlcNAc_2$ (*M8*), $Man_7GlcNAc_2$ (*M7*), $Man_5GlcNAc_2$ (*M5*), and $Man_3GlcNAc_2$ (*M3*) peaks eluted at 98, 100, 106, and 115 mL, respectively. The exclusion volume (V_O) for the column was 65 mL and inclusion volume (V_T) 158 mL.

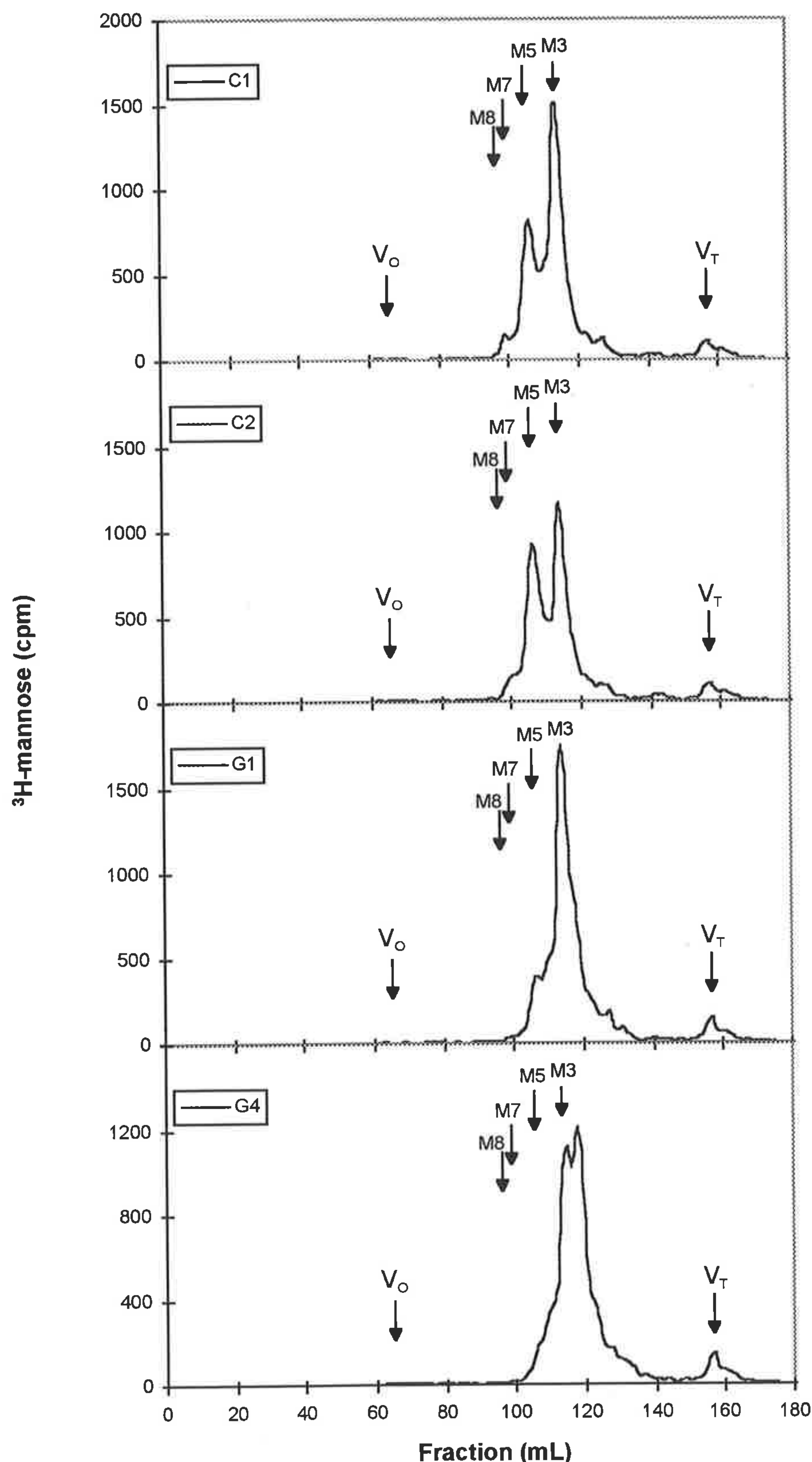


Figure 7.5. Dolichol-linked oligosaccharides recovered from the chloroform/methanol 2:1 extract of two control (*C1* and *C2*) and two galactosaemic (*G1* and *G4*) fibroblast cultures. The cultures were preincubated in 5.5 mmol/L D-glucose and 10 mmol/L D-galactose. 20 000 cpm of oligosaccharide from each extract were analysed on a Bio-Gel P-4 240 x 1 cm column in 0.1 mol/L NH_4COOH, at a flow rate of 8 mL/h. 175 x 1 mL fractions were collected. A range of tritiated oligomannose standards are shown (*arrows*): $Man_8GlcNAc_2$ (*M8*), $Man_7GlcNAc_2$ (*M7*), $Man_5GlcNAc_2$ (*M5*), and $Man_3GlcNAc_2$ (*M3*) peaks eluted at 98, 100, 106, and 115 mL, respectively. The exclusion volume (V_O) for the column was 65 mL and inclusion volume (V_T) 158 mL.

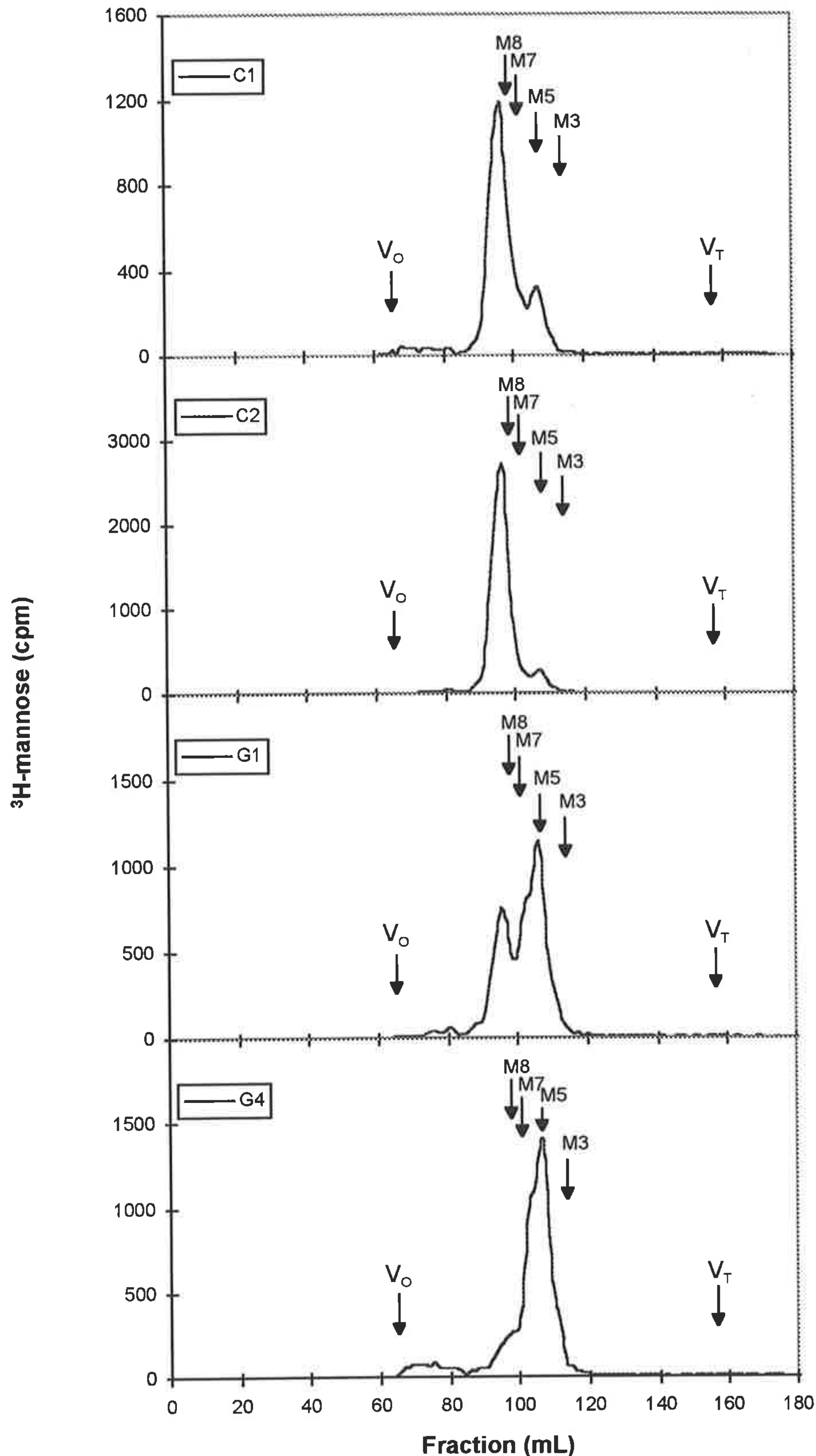


Figure 7.6. N-linked oligosaccharides recovered from the residual protein pellet of two control (*C1* and *C2*) and two galactosaemic (*G1* and *G4*) fibroblast cultures. The cultures were preincubated in 2.5 mmol/L D-glucose and 5.5 mmol/L D-galactose. 20 000 cpm of oligosaccharide from each pellet were analysed on a Bio-Gel P-4 240 x 1 cm column in 0.1 mol/L NH_4COOH, at a flow rate of 8 mL/h. 175 x 1 mL fractions were collected. A range of tritiated oligomannose standards are shown (*arrows*): $Man_8GlcNAc_2$ (*M8*), $Man_7GlcNAc_2$ (*M7*), $Man_5GlcNAc_2$ (*M5*), and $Man_3GlcNAc_2$ (*M3*) peaks eluted at 98, 100, 106, and 115 mL, respectively. The exclusion volume (V_O) for the column was 65 mL and inclusion volume (V_T) 158 mL.

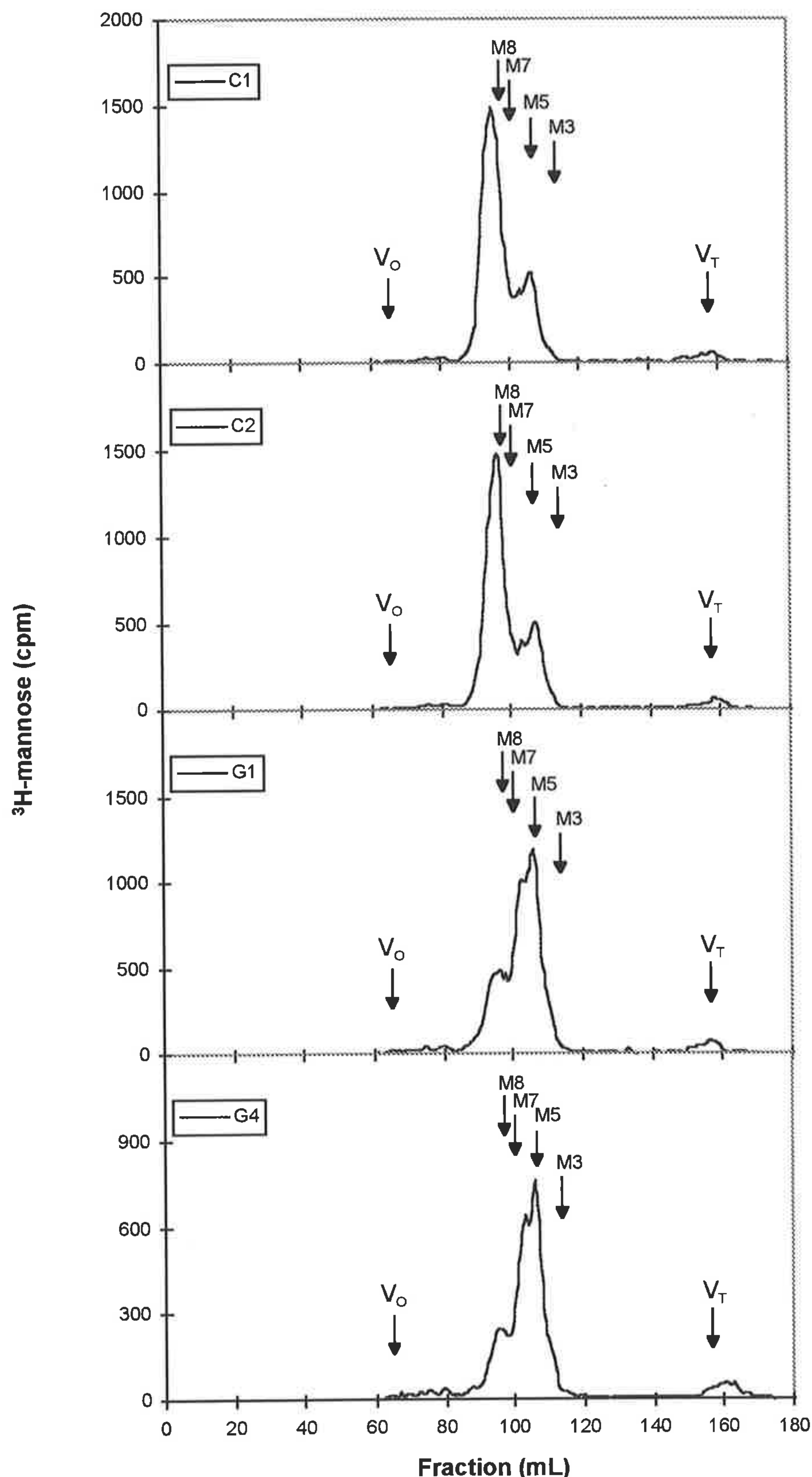


***Figure* 7.7.** N-linked oligosaccharides recovered from the residual protein pellet of two control (*C1* and *C2*) and two galactosaemic (*G1* and *G4*) fibroblast cultures. The cultures were preincubated in 5.5 mmol/L D-glucose and 10 mmol/L D-galactose. 20 000 cpm of oligosaccharide from each pellet were analysed on a Bio-Gel P-4 240 x 1 cm column in 0.1 mol/L NH_4COOH, at a flow rate of 8 mL/h. 175 x 1 mL fractions were collected. A range of tritiated oligomannose standards are shown (*arrows*): $Man_8GlcNAc_2$ (*M8*), $Man_7GlcNAc_2$ (*M7*), $Man_5GlcNAc_2$ (*M5*), and $Man_3GlcNAc_2$ (*M3*) peaks eluted at 98, 100, 106, and 115 mL, respectively. The exclusion volume (V_O) for the column was 65 mL and inclusion volume (V_T) 158 mL.

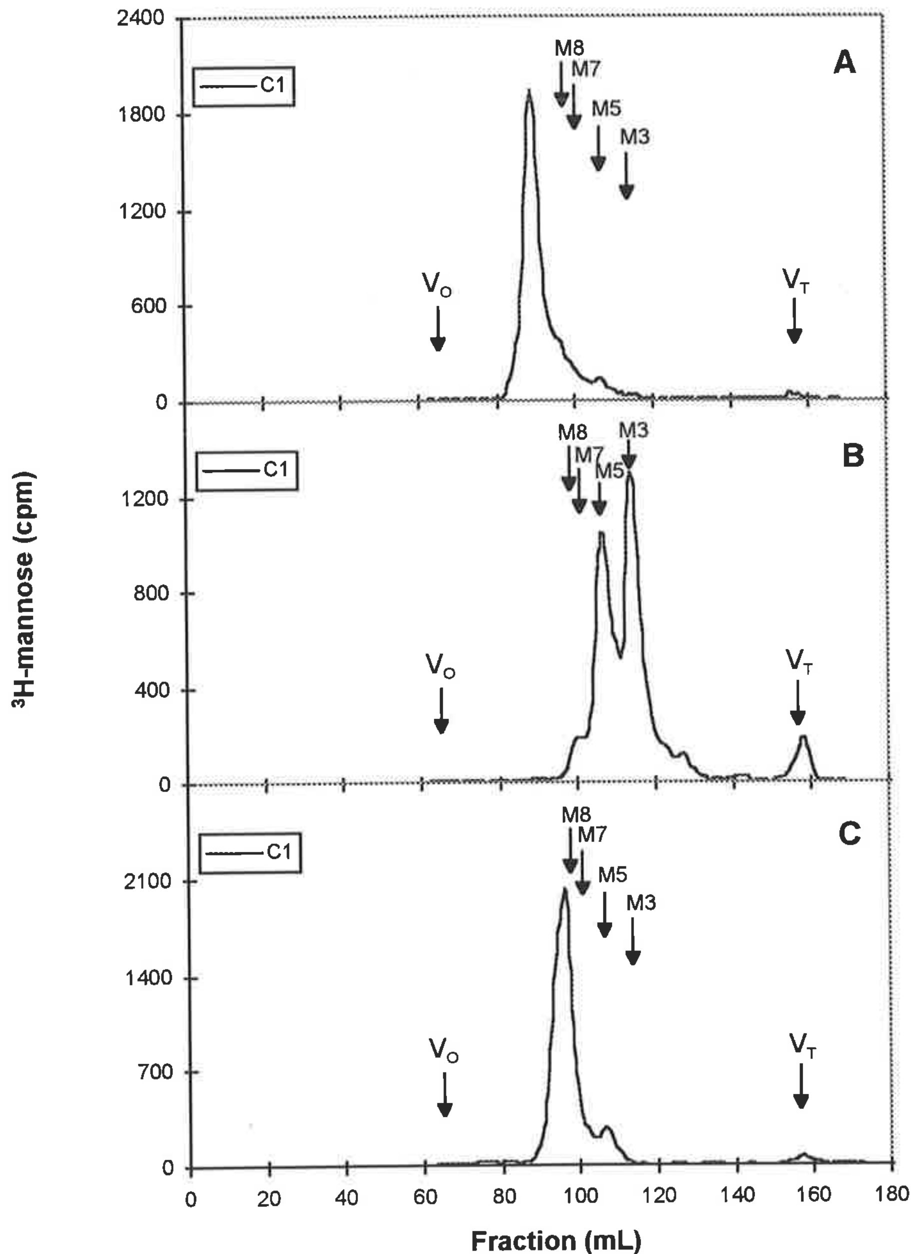


Figure 7.8. Oligosaccharides recovered from ***A***, the chloroform/methanol/water 10:10:3 extract; ***B***, the chloroform/methanol 2:1 extract; and ***C***, the residual protein pellet of a control (*C1*) fibroblast culture preincubated in 5.5 mmol/L D-glucose. 20 000 cpm of oligosaccharide from each extract were analysed on a Bio-Gel P-4 240 x 1 cm column in 0.1 mol/L NH_4COOH, at a flow rate of 8 mL/h. 175 x 1 mL fractions were collected. A range of tritiated oligomannose standards are shown (*arrows*): $Man_8GlcNAc_2$ (*M8*), $Man_7GlcNAc_2$ (*M7*), $Man_5GlcNAc_2$ (*M5*), and $Man_3GlcNAc_2$ (*M3*) peaks eluted at 98, 100, 106, and 115 mL, respectively. The exclusion volume (V_O) for the column was 65 mL and inclusion volume (V_T) 158 mL.

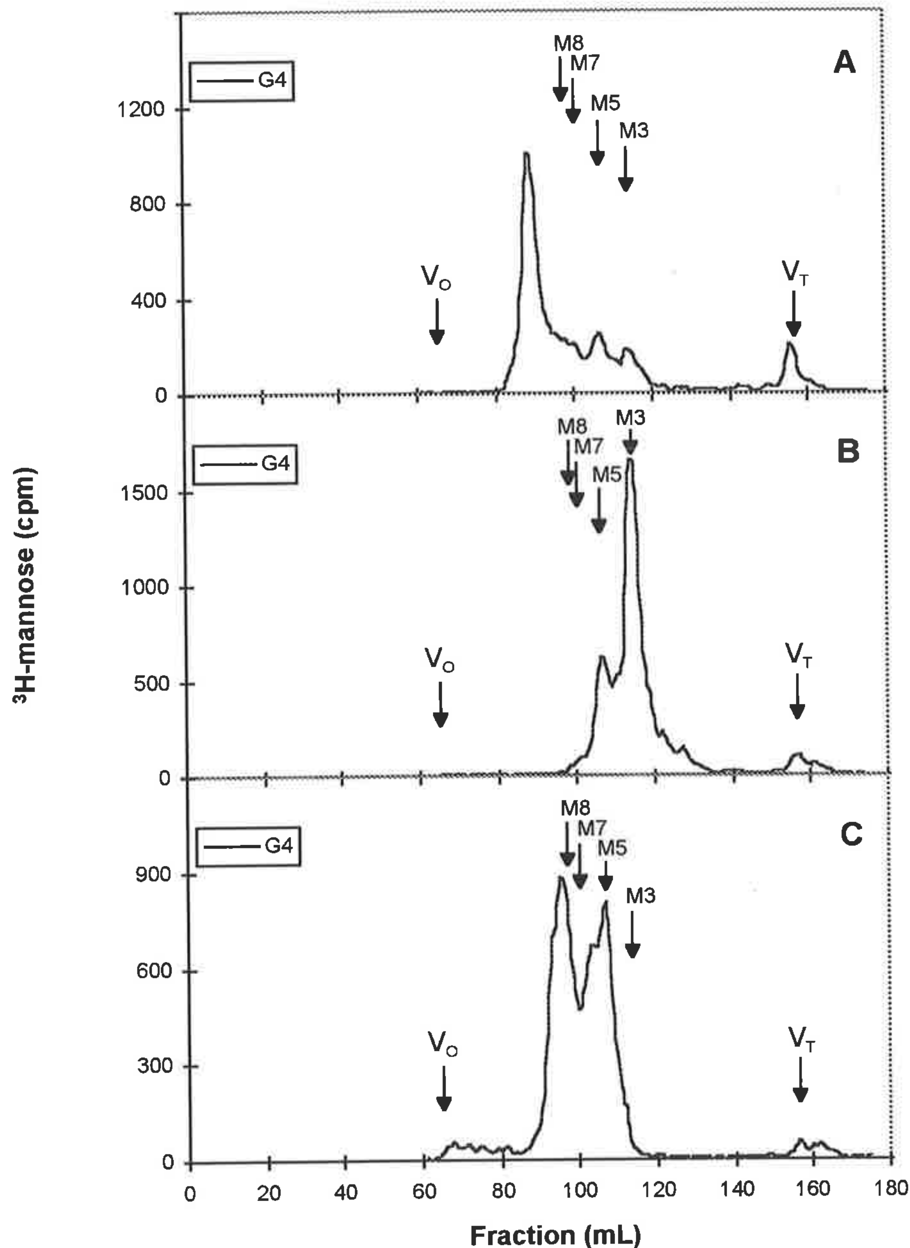


Figure 7.9. Oligosaccharides recovered from ***A***, the chloroform/methanol/water 10:10:3 extract; ***B***, the chloroform/methanol 2:1 extract; and ***C***, the residual protein pellet of a galactosaemic (*G4*) fibroblast culture preincubated in 5.5 mmol/L D-glucose. 20 000 cpm of oligosaccharide from each extract were analysed on a Bio-Gel P-4 240 x 1 cm column in 0.1 mol/L NH_4COOH, at a flow rate of 8 mL/h. 175 x 1 mL fractions were collected. A range of tritiated oligomannose standards are shown (*arrows*): $Man_8GlcNAc_2$ (*M8*), $Man_7GlcNAc_2$ (*M7*), $Man_5GlcNAc_2$ (*M5*), and $Man_3GlcNAc_2$ (*M3*) peaks eluted at 98, 100, 106, and 115 mL, respectively. The exclusion volume (V_O) for the column was 65 mL and inclusion volume (V_T) 158 mL.

7.4 Discussion

Dolichol-linked oligosaccharides are synthesised in a series of glycosylation reactions that occur on the membrane of the ER. The sugars required for the synthesis of the oligosaccharide are provided by UDP-N-acetylglucosamine, GDP-mannose, Dol-P-mannose, and Dol-P-glucose. Once completed, $Glc_3Man_9GlcNAc_2$ is transferred to an asparagine residue of protein within the lumen of the ER. The first enzymatic step in the pathway, dolichol phosphate:UDP-N-acetylglucosaminyltransferase, is the rate-limiting step in dolichol-linked oligosaccharide synthesis (Lehrman, 1991). The synthesis of dolichol-linked oligosaccharides is also closely linked to the cotranslation of protein (Schmitt and Elbein, 1979; Pan and Elbein, 1990; and Konrad and Merz, 1994). The dolichol-linked oligosaccharide pathway has been well characterised in various cell lines, including human skin fibroblasts, and the intermediates produced by the different cells are similar (for a review see Rosner *et al.*, 1982).

The majority of dolichol-linked oligosaccharide intermediates are short-lived with half-lives of only a few minutes. After 30 minutes of radiolabelling with [2-^{3}H]-mannose, the major intermediate observed is $Glc_3Man_9GlcNAc_2$. Lesser amounts of smaller intermediates can also be identified, predominantly $Man_8GlcNAc_2$ and $Man_5GlcNAc_2$ (Li *et al.*, 1978; Chapman *et al.*, 1979a; and Hubbard and Robbins, 1980). The dolichol-linked intermediates are harvested directly in chloroform/methanol 2:1 to prevent degradation of the oligosaccharides during recovery (Rosner *et al.*, 1982). Dolichol-linked oligosaccharides that are smaller than $Man_5GlcNAc_2$ are usually recovered in the 2:1 extract, while the larger intermediates are extracted in chloroform/methanol/water 10:10:3.

In the experiments in this chapter, the control skin fibroblasts produced profiles of dolichol- and protein-linked oligosaccharides that were similar to those reported in the literature for fibroblasts (Hubbard and Robbins, 1980; and Panneerselvam and Freeze, 1996). The predominant oligosaccharide that was recovered from the 10:10:3 extract was consistent in size with $Glc_3Man_9GlcNAc_2$, with smaller peaks of $Man_8GlcNAc_2$ and $Man_5GlcNAc_2$. The 2:1 extract also contained some $Man_5GlcNAc_2$ and $Man_3GlcNAc_2$. $Man_3GlcNAc_2$ is usually not observed as significant intermediate in normal skin fibroblasts. However, the fibroblasts in this experiment were grown in D-MEM that was supplemented with dialysed FBS. The dialysis of the FBS may have removed factors that were important in determining the equilibrium of DLO synthesis under normal conditions. The major protein-bound oligosaccharides in the control fibroblasts were similar in size to $Man_9GlcNAc_2$ and $Glc_{1-2}Man_9GlcNAc_2$. This was consistent with removal of the terminal glucose residues once the oligosaccharide had been transferred to protein in the ER.

The galactosaemic fibroblasts, however, produced an increased proportion of smaller or truncated dolichol-linked and protein-linked oligosaccharides. This was associated with a significant decrease (10–35% of normal) in the [2-^{3}H]-mannose incorporated into these fractions. The reduced incorporation was similar to that observed in Chapter 6, where the galactosaemic fibroblasts also incorporated less [2-^{3}H]-mannose into the cellular and secreted proteins (Table 6.2, page 115). These results therefore suggest an abnormality of dolichol-linked oligosaccharide synthesis in the galactosaemic fibroblasts, particularly when they are exposed to galactose in the culture medium. Even when there was no galactose in the medium there was still some indication that the disturbance was partially present. The accumulation of $Man_5GlcNAc_2$-sized oligosaccharides within the protein fraction suggests that $Man_5GlcNAc_2$ may have been transferred directly from dolichol to protein in the ER. The rate of transfer of the oligosaccharide, however, remained similar to that in the control fibroblasts. When the incorporation data were recalculated to account for the smaller size of the intermediates in the galactosaemic fibroblasts, the rate of transfer remained the same.

Several mutant mammalian cell lines produce truncated dolichol-linked intermediates. The cell lines have specific defects in dolichol-linked oligosaccharide synthesis. Class E Thy-1-negative mutant mouse lymphoma cells (Trowbridge and Hyman, 1979; and Chapman *et al.*, 1979b) and B4-2-1 mutant CHO cells (Stoll *et al.*, 1982) lack normal mature dolichol-linked oligosaccharides and they accumulate $Man_5GlcNAc_2$ intermediates. The mouse lymphoma cells are also unable to produce GPI anchors (Fatemi and Tartakoff, 1988). Both these cell lines have reduced mannosylphosphoryldolichol synthase activity and are unable to synthesise Dol-P-mannose (Chapman *et al.*, 1980; and Stoll *et al.*, 1982). This leads to an accumulation of truncated dolichol-linked intermediates and the transfer of either $Man_5GlcNAc_2$, or the glucosylated form of $Man_5GlcNAc_2$, to protein. N-oligosaccharyltransferase can transfer $Glc_3Man_5GlcNAc_2$ to protein, although under normal conditions this is a minor pathway (Kornfeld *et al.*, 1979). Another related CHO cell mutant, F2A8, which is unable to synthesise both Dol-P-mannose and dolichol phosphate, produces $Man_5GlcNAc_2$ linked to polyprenol (Stoll and Krag, 1988; and Stoll *et al.*, 1988). Lec9 CHO cells, which are defective in polyprenol reductase activity, also accumulate a polyprenol-linked $Man_5GlcNAc_2$, which is transferred directly to protein (Rosenwald *et al.*, 1989; and Rosenwald and Krag, 1990). Processing inhibitor-resistant CHO cells accumulate dolichol-linked $Man_5GlcNAc_2$ due to an *in vivo* block in mannosyltransferase VI function (Lehrman and Zeng, 1989; and Zeng and Lehrman, 1990).

These mutant cell lines also produce mature N-linked oligosaccharides that differ in their final structures. F2A8 CHO cells underglycosylated the G protein of vesicular stomatitis virus (Stoll and Krag, 1988) whereas B4-2-1 CHO cells lacked normal high mannose oligosaccharides on their mature glycoproteins (Stoll *et al.*, 1982). Processing inhibitor-resistant CHO cells appeared to produce normal mature N-linked oligosaccharides (Lehrman and Zeng, 1989), while Lec9 CHO cells produced partially glycosylated proteins that had increased branching of the N-linked oligosaccharides (Rosenwald *et al.*, 1989). This highlights the heterogeneity of mature oligosaccharides that can be produced by mutant cell lines with similar disturbances of dolichol-linked oligosaccharide synthesis. The mutant cells also incorporated less [^{3}H]-mannose into their dolichol-linked and protein-linked oligosaccharide fractions (Stoll *et al.*, 1982; Stoll and Krag, 1988; and Rosenwald *et al.*, 1989).

Skin fibroblasts from patients with the CDG syndrome type I also produce truncated dolichol-linked oligosaccharides. The CDG type I fibroblasts incorporated 3 to 10-fold less [2-^{3}H]-mannose into dolichol-linked and protein-linked oligosaccharides than normal fibroblasts. They also produced an increased proportion of $Man_{4-6}GlcNAc_2$-sized oligosaccharides, which were transferred to protein (Powell *et al.*, 1994; Krasnewich *et al.*, 1995; and Panneerselvam and Freeze, 1996). The CDG syndrome type I is associated with a deficiency of phosphomannomutase, which would affect the conversion of glucose to mannose. Presumably this leads to inadequate GDP-mannose and therefore disturbs dolichol-linked oligosaccharide synthesis (van Schaftingen and Jaeken, 1995). In some experiments, the CDG type I fibroblasts occasionally synthesised predominantly normal-sized dolichol-linked oligosaccharides (Powell *et al.*, 1994; and Panneerselvam and Freeze, 1996). The cause for this variability was not identified but it appeared unrelated to the cell passage number, cell density, or labelling conditions. Adding exogenous mannose, but not glucose, to the CDG type I fibroblasts in culture corrected both the decreased [2-^{3}H]-mannose incorporation and the size of the dolichol-linked intermediates (Panneerselvam and Freeze, 1996).

Many cultured cells will also produce truncated dolichol-linked oligosaccharides if they are starved of glucose (Turco, 1980; Gershman and Robbins, 1981; Rearick *et al.*, 1981a; and Chapman and Calhoun, 1988). Although the effect is influenced by cell type, cell density, and duration of starvation, the cells accumulate predominantly $Man_2GlcNAc_2$, $Man_5GlcNAc_2$, and

$Glc_3Man_5GlcNAc_2$ intermediates. This results in abnormal N-glycosylation (Stark and Heath, 1979; Turco, 1980; and Turco and Pickard, 1982). The disturbance can be resolved by adding small amounts of glucose or mannose to the culture medium, but not other sugars, pyruvate, or glutamine (Turco, 1980; and Gershman and Robbins, 1981). How glucose starvation disturbs dolichol-linked oligosaccharide synthesis is not known. However, Chapman and Calhoun, (1988) proposed that glucose starvation leads to a deficiency of GDP-mannose, and therefore insufficient Dol-P-mannose to complete the synthesise of dolichol-linked oligosaccharides. This mechanism is similar to the defect in the CDG syndrome type I where the cells are unable to produce mannose from glucose. An additional factor that might inhibit Dol-P-mannose synthesis in glucose starvation is ATP depletion. Cells treated with carbonyl cyanide m-chlorophenylhydrazone, an uncoupler of oxidative phosphorylation, have decreased Dol-P-mannose and produce truncated dolichol-linked oligosaccharides (Datema and Schwarz, 1981). However, Chapman and Calhoun, (1988) reported that the level of ATP in glucose-starved CHO cells decreased by only 30% and was insufficient to explain the abnormalities of dolichol-linked oligosaccharide synthesis. The decrease in ATP in galactosaemic cells exposed to galactose would presumably also be insufficient to explain the disturbance in dolichol-linked oligosaccharide synthesis (Komrower *et al.*, 1956; Schwarz *et al.*, 1956; Penington and Prankerd, 1958; and Zipursky *et al.*, 1965).

The patterns of truncated dolichol-linked oligosaccharides produced by the galactosaemic fibroblasts are very similar to those observed in the CDG syndrome type I and in glucose starvation. This suggests that there may be similar mechanisms for the disturbance. The truncated oligosaccharides in the galactosaemic fibroblasts could therefore result from inadequate production of GDP-mannose or Dol-P-mannose, or from inhibition of specific transferase steps within the synthetic pathway. It is also possible that the disturbance was due to a deficiency of dolichol or dolichol phosphate, although the accumulation of significant amounts of $Man_{2-3}GlcNAc_2$ intermediates in the 2:1 extract makes this less likely. Galactosaemic cells and tissues accumulate several galactose metabolites, which have been implicated in the cellular disturbance in galactosaemia (Chapter 2, page 20). These metabolites are produced in the cytoplasm and if they were to affect dolichol-linked oligosaccharide synthesis, then this would most likely occur on the cytoplasmic side of the ER membrane. This would affect the synthesis of the $Man_5GlcNAc_2$ intermediate and is supported by the observed accumulation of $Man_{2-5}GlcNAc_2$ dolichol-linked intermediates in the galactosaemic fibroblasts. Galactose-1-phosphate inhibits several cytoplasmic enzymes *in vitro* (Chapter 2, page 26). It is therefore possible it inhibits specific glycosyltransferases situated on the outside of the ER membrane, which are involved in the addition of N-acetylglucosamine and mannose to the dolichol-linked precursors. Galactose-1-phosphate also inhibited phosphoglucomutase *in vitro* (Ginsburg and Neufeld, 1957; and Sidbury, 1957a). Sidbury, (1960) proposed that the inhibition was due to an accumulation of galactose-1,6-diphosphate, which depleted the enzyme of its normal cofactor glucose-1,6-diphosphate. Phosphomannomutase is 50-times less active in fibroblasts than phosphoglucomutase (van Schaftingen and Jaeken, 1995). It also requires glucose-1,6-diphosphate or mannose-1,6-diphosphate as a cofactor (Guha and Rose, 1985). Phosphomannomutase may be even more sensitive to galactose-1-phosphate and galactose-1,6-diphosphate than phosphoglucomutase. This could, as in the CDG syndrome type I and in glucose starvation, result in insufficient GDP-mannose and Dol-P-mannose to complete the synthesis of dolichol-linked oligosaccharides.

The dolichol-linked oligosaccharide pathway is also affected by several sugar analogues: 2-deoxy-D-glucose, 2-deoxy-2-fluoro-D-glucose, 2-deoxy-2-fluoro-D-mannose, D-glucosamine, and 2-deoxy-2-fluoro-D-galactose. These sugar analogues cause various cellular disturbances including inhibition of N-glycosylation. The analogues are usually metabolised to false sugars or false sugar nucleotides, which then interfere with dolichol-linked oligosaccharide assembly. They achieve this either by inhibiting the formation of Dol-P-mannose and Dol-P-glucose, by

trapping dolichol phosphate, or by trapping uridylate and depleting UTP (for a review see Datema *et al.*, 1980; Schwarz and Datema, 1980; Schwarz and Datema, 1982; Elbein, 1984; and Loch *et al.*, 1991). When cells are exposed to these sugar analogues in the culture medium, they incorporate less [^{3}H]-mannose into dolichol-linked oligosaccharides and they produce truncated dolichol-linked intermediates. The effects of the sugar analogues can be competitively overcome by adding glucose or mannose to the culture medium. In galactosaemia, there has been considerable interest in UDP-glucose and UDP-galactose metabolism. It is not known, however, whether galactosaemic cells accumulate any abnormal sugars or sugar nucleotides, which might disturb the synthesis of dolichol-linked oligosaccharides.

The results in this chapter indicated that galactosaemic fibroblasts synthesised truncated dolichol-linked oligosaccharides, which they transferred to protein in the ER. This suggested a disturbance in the synthesis of dolichol-linked oligosaccharides. This disturbance could be one explanation for the hyposialylated glycoproteins in the serum in untreated galactosaemia. It could also account for some of the clinical similarities between galactosaemia and the CDG syndrome. The next chapter examines the synthesis and processing of a model glycoprotein in galactosaemic fibroblasts. The aim was to determine if the disturbance in dolichol-linked oligosaccharide synthesis could be reproduced in a specific glycoprotein.

8

Synthesis and processing of a model glycoprotein in galactosaemic fibroblasts

8.1 Introduction

The results in the previous experimental chapters indicated that there were disturbances in the synthesis and processing of N-linked oligosaccharides in galactosaemic fibroblasts. Specifically, dolichol-linked oligosaccharide synthesis and transfer, and terminal sialylation of complex N-glycans. The aim of this chapter was to examine the synthesis and processing of a model glycoprotein in galactosaemic fibroblasts to determine if these changes could be reproduced in a specific protein. The experimental plan was to radiolabel the skin fibroblasts with [^{35}S]-methionine and to recover LAMP-1 for analysis by SDS-PAGE. LAMP-1 is a type I membrane protein and is a major sialoglycoprotein of the lysosomal membrane. LAMP-1 contains 18 N-linked glycans of which five are potential poly-N-acetyl-lactosaminoglycans. However, to date, no defects have been reported in membrane glycoproteins in galactosaemic cells or tissues. The abnormalities of glycosylation have been confined to the glycoproteins of serum, primarily transferrin and α_1-antitrypsin (Jaeken *et al.*, 1992; Spaapen *et al.*, 1992; Shin *et al.*, 1993; Winchester *et al.*, 1995; and van Pelt *et al.*, 1996b). This is similar to the CDG syndrome type I, where abnormalities of membrane glycoprotein have been proposed but not actually observed (Jaeken and Carchon, 1993). The abnormalities of the serum glycoproteins in the CDG syndrome type I also differ with the specific glycoprotein examined and the number of its potential N-glycosylation sites (Wada *et al.*, 1992; Yamashita *et al.*, 1993a; and Krasnewich *et al.*, 1995). Marquardt *et al.*, (1995 and 1996) also found no obvious structural abnormalities in three viral glycoproteins when they were expressed by CDG type I fibroblasts. Choosing an appropriate glycoprotein to model in galactosaemic fibroblasts therefore has potential difficulties. LAMP-1 was successfully used to study polylactosaminoglycan synthesis and processing. It was therefore considered a potential model for N-glycosylation in galactosaemic fibroblasts (see Method 6, page 76 for a review of the synthesis of LAMP-1).

The experimental plan used a short pulse-chase experiment to determine if the correct number and size of high mannose oligosaccharides were transferred to nascent LAMP-1 in the ER. Longer chase times were used to recover the mature LAMP-1 from the lysosomal membrane. The fibroblasts were also exposed to different concentrations of galactose in the culture medium, before and during the radiolabelling procedure, to examine the effect of galactose on the synthesis and processing of LAMP-1. The predominant N-linked oligosaccharides in LAMP-1 are sialylated complex N-glycans that require correct processing in the Golgi apparatus. The polylactosaminoglycans are also extended in the Golgi by specific glycosyltransferases. LAMP-1 was immunoprecipitated from the fibroblast extracts with a rabbit polyclonal anti-LAMP-1 antibody (Dahlgren *et al.*, 1995). The immunoprecipitation method was developed within the Department of Chemical Pathology, Women's and Children's Hospital, Adelaide.

8.2 Experimental plan

A: One control (*C1*) and one galactosaemic (*G4*; Table 4.1, page 56) fibroblast culture were grown to near-confluence in 75 cm^2 tissue culture flasks in BME with 10% (v/v) FBS. The fibroblasts were then preincubated for four hours in a methionine- and cystine-deficient D-MEM (5.5 mmol/L D-glucose) that contained 10% (v/v) dialysed FBS and 25 mmol/L D-galactose. The fibroblasts were then radiolabelled for five minutes in the same medium with [^{35}S]-Protein Labeling Mix (3.7 MBq/mL), and then chased for either 0, 5, or 15 minutes in complete D-MEM with 10% (v/v) dialysed FBS and 25 mmol/L D-galactose. The fibroblasts were then harvested with a cell scraper. The LAMP-1 was recovered by immunoprecipitation and then analysed by 10% SDS-PAGE, and detected by autoradiography (Method 6, page 76).

B: One control (*C1*) and one galactosaemic (*G4*) fibroblast culture were grown to near-confluence in 75 cm^2 tissue culture flasks in BME with 10% (v/v) FBS. The fibroblasts were then preincubated for four hours in a methionine- and cystine-deficient D-MEM (5.5 mmol/L D-glucose) that contained 10% (v/v) dialysed FBS and either 0, 5, 10, or 25 mmol/L of D-galactose. The fibroblasts were then radiolabelled for 30 minutes in the same medium with [^{35}S]-Protein Labeling Mix (3.7 MBq/mL), and then chased for one hour in complete D-MEM with 10% (v/v) dialysed FBS and the same range of concentrations of D-galactose. The cells were then harvested with a cell scraper. The LAMP-1 was recovered by immunoprecipitation and then analysed by 10% SDS-PAGE, and detected by autoradiography (Method 6, page 76).

8.3 Results

Experimental plan A

The autoradiograph of the LAMP-1 from the fibroblasts that were radiolabelled for five minutes, and then chased for up to 15 minutes, is displayed in Figure 8.1. The total [^{35}S]-methionine activity recovered in the high mannose precursor of LAMP-1 was 6194, 6568, and 6570 cpm in the control cultures, and 4076, 2690, and 3078 cpm in the galactosaemic cultures, at chase times of 0, 5, and 15 minutes, respectively. The estimated mean molecular mass of the high mannose LAMP-1 precursor was 81 kDa in the control and galactosaemic fibroblasts. The molecular mass also did not differ significantly across the range of chase times.

Experimental plan B

The autoradiograph of the LAMP-1 from the fibroblasts that were radiolabelled for 30 minutes, and then chased for one hour, is displayed in Figure 8.2 (page 143). The total [^{35}S]-methionine activity that was recovered in the mature LAMP-1 was 14 202, 22 544, 16 616, and 31 288 cpm in the control cultures, and 14 048, 21 298, 14 846, and 16 194 cpm in the galactosaemic cultures, at the media galactose concentrations of 0, 5, 10, and 25 mmol/L, respectively. The estimated molecular mass of the nascent LAMP-1 protein, in the control that was exposed to tunicamycin, was 39 kDa (there was no galactose in this medium). This control also synthesised a small amount of normal-sized LAMP-1, despite the tunicamycin, which suggested that N-glycosylation was not completely inhibited. The size of the mature LAMP-1, from both the control and galactosaemic fibroblasts, did not differ significantly as the concentration of galactose in the culture medium increased. The mean range of the molecular mass for the mature LAMP-1 in the control fibroblasts was 72–102 kDa ($n = 3$), and in the galactosaemic fibroblasts, 73–106 kDa ($n = 4$).

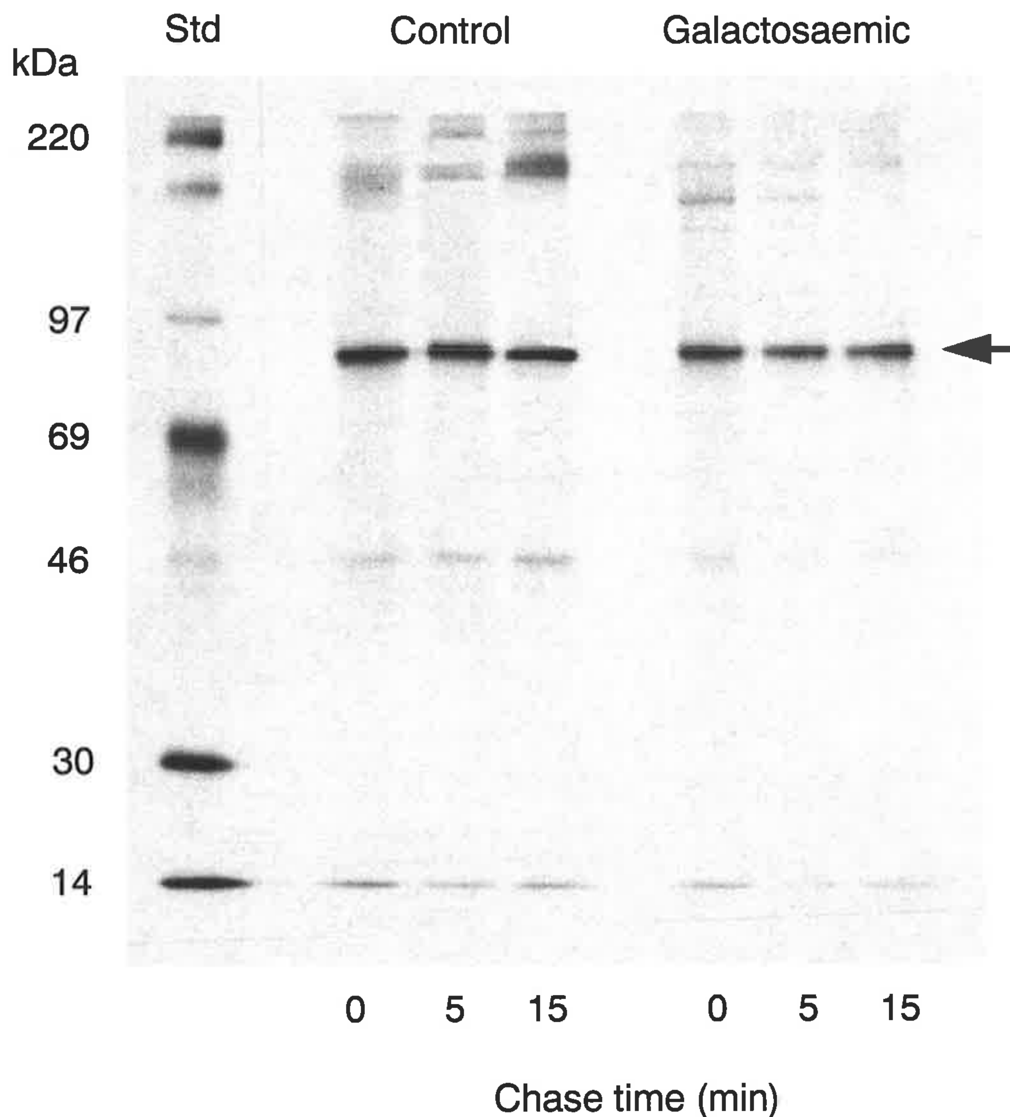


Figure 8.1. Autoradiograph of a 10% SDS-PAGE analysis of the high mannose precursor of LAMP-1 from skin fibroblasts. The fibroblasts were radiolabelled with [^{35}S]-methionine and the LAMP-1 was recovered by immunoprecipitation. The control (*C1*) and galactosaemic (*G4*) fibroblast cultures were preincubated for four hours in medium that contained 25 mmol/L D-galactose, radiolabelled for five minutes, and then chased for either 0, 5, or 15 minutes. The [^{14}C]-methylated protein standards (*std*) ranged from 14 to 220 kDa. The mean molecular mass of the high mannose precursor of LAMP-1 (*arrow*) was 81 kDa in both the control and galactosaemic cultures. The autoradiograph was scanned in a Microtek ScanMaker III reflectance scanner.

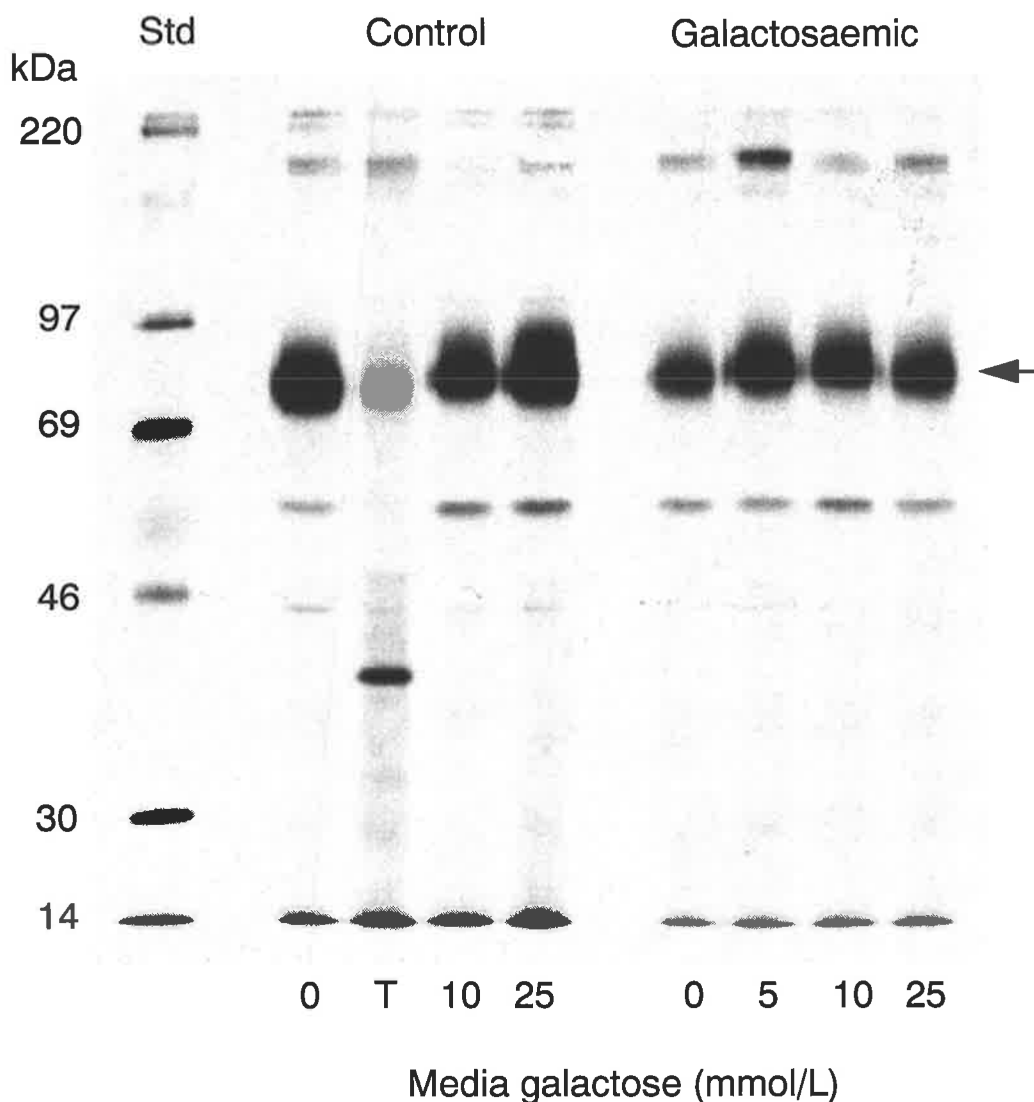


Figure 8.2. Autoradiograph of a 10% SDS-PAGE analysis of mature LAMP-1 from skin fibroblasts. The fibroblasts were radiolabelled with [^{35}S]-methionine and the LAMP-1 was recovered by immunoprecipitation. The control (*C1*) and galactosaemic (*G4*) fibroblast cultures were preincubated for four hours in medium that contained either 0, 5, 10, or 25 mmol/L of D-galactose, radiolabelled for 30 minutes, and then chased for 1 hour. In one of the control fibroblast cultures (*T*), tunicamycin (10 µg/mL) was added to the medium. The mean range of the molecular mass for the mature LAMP-1 (*arrow*) in the control fibroblasts was 72–102 kDa, and in the galactosaemic fibroblasts, 73–106 kDa. The 39 kDa band, in the control culture exposed to tunicamycin, was consistent with nascent LAMP-1 protein. The [^{14}C]-methylated protein standards (*std*) ranged from 14 to 220 kDa. The autoradiograph was scanned in a Microtek ScanMaker III reflectance scanner.

8.4 Discussion

In the pulse-chase radiolabelling experiments in this chapter, human skin fibroblast LAMP-1 was synthesised as a 39 kDa polypeptide that was then processed to an 81 kDa high mannose precursor within the lumen of the ER. These results are therefore similar to those published for LAMP-1 in pulse-chase experiments in human leucocytes (Carlsson *et al.*, 1988). The high mannose precursor of LAMP-1 was then processed in the Golgi apparatus to a mature LAMP-1, which ranged in molecular mass from 72 to 102 kDa. This size is smaller than that reported for LAMP-1 in several other cells and tissues (Chen *et al.*, 1985b). However, others have also observed a smaller mature LAMP-1 in human skin fibroblasts (approximately 80–100 kDa; personal communication from P. J. Meikle and D. A. Brooks, Department of Chemical Pathology, Women's and Children's Hospital, Adelaide). This suggests that human fibroblast LAMP-1 contains smaller or fewer polylactosaminoglycans. Most of the heterogeneity in the size of mature LAMP-1 is due to variation in the amount of polylactosaminoglycan (Carlsson *et al.*, 1988).

The galactosaemic fibroblasts showed no abnormality in the synthesis or processing of LAMP-1, even when they were exposed to 25 mmol/L galactose in the culture medium. The high mannose precursor of LAMP-1 was assembled in the ER without delay, and it appeared to receive the correct number and size of high mannose N-glycans. There was also no delay in processing of the glycoprotein to mature LAMP-1 in the Golgi apparatus. However, only one time point was used in monitoring the longer chase, and it is possible that minor delays in trafficking between the ER and Golgi apparatus were missed. The size of the mature LAMP-1 was also not influenced by the concentration of galactose in the medium. The synthesis of LAMP-1 was therefore not affected by the disturbance in dolichol-linked oligosaccharide synthesis observed in Chapter 7. The total radioactivity incorporated in the galactosaemic fibroblast LAMP-1, particularly during the short pulse-chase experiment, was approximately half that of the control fibroblasts. This is similar to the decrease in [^{35}S]-methionine that occurred in the cellular and secretory proteins, when the galactosaemic fibroblasts were exposed to galactose (Figure 6.3, page 119). However, there were many steps in the LAMP-1 recovery procedure and any differences in the final radioactivity must be considered cautiously.

The experimental results in this chapter did not support an abnormality either in dolichol-linked oligosaccharide synthesis or in terminal completion of complex N-glycans. However, the apparently normal results can be interpreted in other ways. It is possible that the synthesis and processing of LAMP-1 in the galactosaemic fibroblasts was either not affected by the abnormalities or the changes were minor and not detected by the pulse-chase method. There is no evidence that membrane glycoproteins are abnormal in patients with untreated galactosaemia. The abnormalities have been confined to glycoproteins from the secretory pathway of the liver. This is similar to the CDG syndrome, except in the CDG syndrome there are increased and fluctuating levels of FSH, LH, prolactin, insulin, and growth hormone in the serum (Jaeken *et al.*, 1980; de Zegher and Jaeken, 1995; and Kristiansson *et al.*, 1995). The isoforms of the serum FSH and LH in the CDG syndrome were normal on isoelectric focusing (Kristiansson *et al.*, 1995). This suggested that the membrane glycoprotein receptors for these hormones were probably abnormal (Jaeken and Carchon, 1993). Skin fibroblasts from a patient with the CDG syndrome type I (deficient in phosphomannomutase activity) exhibited no delay in the synthesis of the high mannose precursor of LAMP-1. The fibroblasts were examined in a pulse-chase labelling experiment that was similar to the experiments in this chapter (personal communication from D. A. Brooks, Department of Chemical Pathology, Women's and Children's Hospital, Adelaide). The high mannose precursor of LAMP-1 was also the correct size. However, there was a minor delay in the subsequent processing of the LAMP-1 to the mature form. This result is similar to that obtained by Marquardt *et al.*, (1995)

in their study of the processing of three viral glycoproteins in CDG type I fibroblasts. They observed a delay in the transport of the viral glycoproteins out of the ER, which they suggested was due to a disturbance in ER function.

It appears therefore that LAMP-1 may not be a useful model of N-glycosylation in human skin fibroblasts. It is possible that membrane glycoproteins are not affected by disorders of N-glycosylation. However, it is more likely that the quality control mechanisms within the ER are capable of discriminating between specific glycoproteins. These glycoproteins are probably retained within the ER until they are fully completed. This would result in reduced synthesis but could account for the apparently normal processing.

9

General discussion

Summary

Patients with classical galactosaemia have been treated with galactose-restricted diets for over 60 years. However, it is only in the last decade that the long-term complications in these patients have been well recognised. A galactose-restricted diet dramatically resolves the acute toxicity syndrome in the neonatal period. It will also prevent the development of severe mental retardation and chronic liver disease. However, the diet has done little to prevent the development of longer term problems such as impaired intellectual development, specific learning disabilities, growth delay, tremors and ataxia, and ovarian failure. It is now clear that these complications are not related to either galactose exposure during pregnancy, delays in starting dietary treatment, the severity of neonatal illness, or the strictness of dietary control. The evidence from MRI studies of the brain, and from hormonal testing in female patients, suggests that the brain and ovarian dysfunctions can be present at or soon after birth. This suggests some of the complications may originate *in utero*. The problem for clinicians treating patients with galactosaemia is in determining what cellular mechanisms contribute to the development of the long-term complications.

In classical galactosaemia, there are several disturbances of cellular metabolism including the accumulation of alternative galactose intermediates. It is not known, however, to what extent these intermediates influence other cellular pathways. The serum from infants with untreated galactosaemia contains hyposialylated isoforms of glycoprotein. The ratio of UDP-glucose to UDP-galactose in galactosaemic erythrocytes is also disturbed. These observations lead to a theory that implicated a deficiency of UDP-galactose in the abnormalities of the serum glycoproteins and suggested that this contributed to the long-term complications. Most of the studies that have examined glycoprotein synthesis in galactosaemia have therefore been directed towards confirming this theory.

This thesis presented a hypothesis that there were abnormalities of N-glycosylation in classical galactosaemia, and that these abnormalities could contribute to the long-term complications. The aim of the thesis was to characterise and model N-glycosylation in skin fibroblasts from patients with galactosaemia. In the initial experimental plan, the fibroblasts were radiolabelled with [2-^{3}H]-mannose and the N-linked oligosaccharides were recovered for analysis by size and charge. However, there was no obvious functional defect in the terminal completion of the complex N-linked glycans, which appeared structurally intact. This did not support a major disturbance in terminal galactosylation as suggested by the UDP-galactose theory. The next experimental plan therefore examined N-glycosylation in a more general manner. It was then observed that the galactosaemic fibroblasts incorporated less [2-^{3}H]-mannose into glycoprotein than control fibroblasts, and that the ratio of the incorporation of [2-^{3}H]-mannose to [^{35}S]-methionine was reduced. These results lead to further investigations that identified a disturbance in the synthesis and processing of dolichol-linked oligosaccharides. When the galactosaemic fibroblasts were exposed to galactose in the culture medium they incorporated less [2-^{3}H]-mannose into dolichol- and protein-linked oligosaccharides. This was associated with the production of truncated dolichol-linked oligosaccharides, which were transferred to protein within the ER. The initial steps in the synthesis of dolichol-linked oligosaccharides

occur on the cytoplasmic face of the ER membrane. These enzymatic steps could therefore be disturbed by an accumulation of galactose intermediates in the cytoplasm, such as galactose-1-phosphate, galactitol, or galactonate. The galactose metabolites could also compete with and disturb the glucose pathways which provide intermediates for the synthesis of dolichol-linked oligosaccharides. One proposed mechanism could involve the inhibition of phosphomannomutase by galactose-1-phosphate, in a manner similar to that reported for phosphoglucomutase.

It is anticipated that the serum glycoproteins in untreated galactosaemia may contain N-glycans that are partly absent or truncated. Patients with untreated epimerase-deficiency galactosaemia and hereditary fructose intolerance also have hyposialylated transferrin in their serum (Besley *et al.*, 1995; and Adamowicz and Pronicka, 1996). These patients accumulate galactose-1-phosphate and fructose-1-phosphate (and fructose-1,6-diphosphate), respectively. This suggests that a range of phosphorylated hexoses may interfere with the synthesis of dolichol-linked oligosaccharides. It is therefore proposed that galactose-1-phosphate interferes with the conversion of glucose to mannose intermediates that are required for the synthesis of dolichol-linked oligosaccharides. This may be one mechanism for the hyposialylation of serum glycoproteins in untreated galactosaemia. It remains to be determined whether abnormal N-glycosylation contributes to the long-term complications in patients.

Reinterpretation of the experimental evidence

Some of the experimental evidence in the literature, which suggested that glycoprotein synthesis in galactosaemia was abnormal, could be consistent with a defect in dolichol-linked oligosaccharide synthesis.

Dobbie *et al.*, (1990) measured the carbohydrate content of cell extracts from six galactosaemic fibroblast cultures. They observed a decrease ($p < 0.05$) in the mean ratios of galactose to mannose, and sialic acid plus galactose to mannose. The total mannose content of the control and galactosaemic extracts, however, were similar. Dobbie *et al.*, (1990) concluded that the results supported a defect in terminal completion of N-glycans. Similar results to these were reported when the carbohydrate content of serum transferrin was analysed in the CDG syndrome type I (Table 3, Stibler *et al.*, 1991). This initially suggested that the CDG syndrome was similarly associated with a defect in terminal completion of N-glycans, while it was later proved to have a defect in dolichol-linked oligosaccharide synthesis. Therefore the results from the galactosaemic cell extracts must also be viewed with caution. The decreased ratio of galactose to mannose could also be consistent with a disturbance in the ratio of high mannose to complex N-linked oligosaccharides, or an accumulation of truncated oligomannose structures with fewer mature N-glycans.

Tedesco and Miller, (1979) observed a decrease in the [^{35}S]-sulphate incorporated into TCA-precipitates from galactosaemic fibroblasts. The radiolabelling procedures were very similar to those used in Chapter 6. Confluent fibroblasts cultures were exposed to different experimental media for six days, and then radiolabelled with $H_2{}^{35}SO_4$ for 24 hours. The cellular protein was then precipitated with TCA. The galactosaemic fibroblasts incorporated 65% less [^{35}S]-sulphate in a medium that contained 5.5 mmol/L D-galactose than in a medium with 5.5 mmol/L D-glucose. The effect was not due to a deficiency of energy as the incorporation of [^{3}H]-uridine was normal, as was the incorporation of [^{35}S]-sulphate in a hexose-free medium. The galactosaemic fibroblasts incorporated normal amounts of [^{35}S]-sulphate when they were grown in a medium that contained glucose and galactose. Galactokinase-deficient fibroblasts were unaffected in all experimental media, which suggested the disturbance in [^{35}S]-sulphate incorporation was due to galactose-1-phosphate. These results are very similar to those in

Chapter 6, which measured the incorporation of [^{35}S]-methionine into cellular protein. The galactosaemic fibroblasts incorporated approximately 50% less [^{35}S]-methionine than the controls when they were radiolabelled in a medium that contained 5.5 mmol/L galactose and 2.5 mmol/L pyruvate (Table 6.2, page 115). The effect was not observed in medium that contained glucose and galactose. The reduced incorporation of [^{35}S]-methionine suggested a decrease in the amount of protein synthesised. Segal, (1995) proposed that the reduced incorporation of [^{35}S]-sulphate, observed by Tedesco and Miller, reflected less galactose residues in glycoprotein that were available for sulphation. However, a decrease in total protein synthesis could equally account for the change. It is interesting that glucose modulated the effect of galactose in both experiments. This suggests that galactose intermediates are competing or interfering with the normal glucose metabolic pathways.

Brown *et al.*, (1977) studied peripheral blood lymphocytes from three patients with galactosaemia. The lymphocytes were labelled with different tritiated hexoses for 20 hours, either in a normal medium (without galactose) or in a hexose-free medium. The radioactivity was then measured in protein pellets that were precipitated with acid. The lymphocytes incorporated normal amounts of tritiated mannose, fucose, glucosamine, and N-acetylmannosamine (a precursor of sialic acid) in both experimental media. Brown *et al.*, (1977) were also unable to detect any disturbance in the pattern of cell surface oligosaccharides, which were examined with a galactose-binding lectin. These results are consistent with those in Chapters 5 and 6, in which the mature N-linked oligosaccharides produced by the galactosaemic fibroblasts were structurally normal and the incorporation of [2-^{3}H]-mannose into TCA-precipitates was also normal, if the medium did not contain galactose.

Ornstein *et al.*, (1992) reported that cellular extracts from nine galactosaemic fibroblasts had increased vacant sites for galactose attachment. They incubated the cell extracts with UDP-[^{3}H]-galactose and a purified human milk galactosyltransferase, and observed a 50% increase in galactose acceptor activity in the galactosaemic extracts. They proposed that this was consistent with an increase in the N-acetylglucosamine sites in glycoconjugates available for galactosylation. A similar increase in galactose acceptor activity has recently been reported in a total serum extract from three females with galactosaemia and ovarian failure (Prestoz *et al.*, 1997). In Chapter 5, however, there was no evidence for a functional defect in terminal completion of the mature N-glycans in the galactosaemic fibroblasts. Ornstein *et al.*, (1992) suggested that the increased acceptor activity could also be explained by an increase in other galactose acceptors, such as O-linked terminal N-acetyglucosaminides. Galactosaemic lymphoblast membranes also contained an increased proportion of glucosylceramide, which could have acted as an additional galactose acceptor in these assays (Petry *et al.*, 1991). Skin fibroblasts from patients with the CDG syndrome type I exhibited abnormalities in the synthesis of the small proteoglycans, decorin and biglycan (Gu and Wada, 1995; and Gu and Wada, 1996). This suggests that there could be similar disturbances in galactosaemia. However, there has been no study of O-glycosylation in galactosaemic cells or tissues.

Skin fibroblasts from patients with the CDG syndrome type I show dilatation of the RER (Marquardt *et al.*, 1995). The dilatation was present in most of the fibroblasts when they were examined under electron microscopy. The Golgi apparatus appeared structurally normal. Marquardt *et al.*, (1995) suggested that the dilatation reflected an accumulation of abnormal or incompletely processed proteins in the lumen of the ER. Only two studies have examined galactosaemic fibroblasts with electron microscopy, and unfortunately the results are conflicting. Miller *et al.*, (1968) examined subconfluent fibroblast cultures in various media. After 72 hours in 5.5 mmol/L galactose, the galactosaemic fibroblasts exhibited dilatation of the ER with loss of intralumenal electron density, and autophagic vacuoles in the cytoplasm. These changes were not observed in the fibroblasts in hexose-free medium. In a similar

experiment, Johnson *et al.*, (1976) studied a galactosaemic fibroblast culture over five days. They did not observe dilatation of the ER but did observe autophagic vacuoles when the cells were exposed to a galactose-only medium. However, the fibroblasts in this experiment were confluent before they were exposed to galactose. Galactosaemic fibroblasts are less affected by galactose when in a stationary growth phase (Tedesco and Miller, 1979; and Pourci *et al.*, 1990). Marquardt *et al.*, (1995) also reported that the dilatation of the RER in CDG type I fibroblasts was greater when the cells were subconfluent and in a growth phase. Unfortunately, it is not possible to conclude from the limited evidence whether ER morphology and function are similarly disturbed in galactosaemic fibroblasts.

Reinterpretation of the long-term complications

The long-term complications in classical galactosaemia primarily involve two organs, the brain and the ovary. A disturbance in dolichol-linked oligosaccharide synthesis in these organs would influence the synthesis of N-linked oligosaccharides and also GPI membrane anchors, which derive their mannose residues from Dol-P-mannose (Stevens, 1995). These mechanisms have been proposed in the pathophysiology of the organ dysfunction in the CDG syndrome type I (van Schaftingen and Jaeken, 1995). In galactosaemia, the post-mortem examinations of the brain have consistently observed a lack of myelination of the white matter (Crome, 1962; Haberland *et al.*, 1971; and Smetana and Olen, 1972). A delay in peripheral myelination was also observed on MRI of the brain in patients on treatment (Nelson Jr *et al.*, 1992; and Moller *et al.*, 1995). Other specific findings have included lytic lesions that cluster around the lateral ventricles, selective loss of the Purkinje cells of the cerebellum, and cerebellar hypoplasia. The limited biochemical analyses of the brain in galactosaemia have suggested that there are disturbances in the composition and distribution of glycoprotein in the grey and white matter (Brunngraber *et al.*, 1971; and Haberland *et al.*, 1971), and that there is a reduction of galactose-containing glycolipids (Petry *et al.*, 1991).

Delay in myelination, with a generalised diminution in white matter, is also seen in several other inherited metabolic disorders, including pyridoxine-dependent seizures, glutaric aciduria type 1, maple syrup urine disease, phenylketonuria, infantile Refsum's disease, and Menke's disease (Shuman *et al.*, 1978; Hara and Taketomi, 1986; Treacy *et al.*, 1992; and Kolodny, 1993). The dysmyelination in Menke's disease was associated with a decrease in the major lipid components of myelin, including free cholesterol, phospholipids, sulfatide, and galactosylceramide (Hara and Taketomi, 1986). Galactosylceramide is the major glycolipid in myelin (O'Brien and Sampson, 1965), and the amount of galactosylceramide in normal human brain increases with myelination during the first year of life (Poduslo and Jang, 1984; Martinez, 1986; and Kinney *et al.*, 1994). Any disturbance in myelination would therefore alter the galactolipid composition of the brain. It is possible that the decrease in galactose-containing glycolipids in the galactosaemic brain studied by Petry *et al.*, (1991) reflects a delay in myelination rather than abnormal galactosylation. The delayed myelination would also disturb the glycoprotein content of the myelin sheath and this could account for the reduced hexosamine content of glycoprotein, and the altered distribution of glycoprotein, within galactosaemic brain (Brunngraber *et al.*, 1971; and Haberland *et al.*, 1971). Other major lipid components of myelin, such as cholesterol and phospholipid, have also been reported as decreased in galactosaemic brain (Crome, 1962; Lott *et al.*, 1982; and Lott *et al.*, 1983). This again suggests that a delay in myelination could account for the biochemical changes that have been observed.

Normal myelination is closely linked to the synthesis of specific glycoproteins within myelin, such as myelin basic protein and proteolipid protein. Mutant mice that lack either myelin basic protein (shiverer mutant) or proteolipid protein (jimpy mutant) exhibit dysmyelination and a

reduction in the number of mature oligodendrocytes (Campagnoni *et al.*, 1987; Campagnoni, 1988; and Skoff, 1995). The brains of these mutant mice also have significantly reduced galactosylceramide (Theret *et al.*, 1988). This confirms the importance of the myelin glycoproteins to normal myelination. The abnormalities in N-glycosylation in galactosaemia could affect the synthesis or function of these specific glycoproteins, although it is unlikely that a defect in N-glycosylation would affect a single protein in myelin. Myelin basic protein is also significantly O-glycosylated (Cruz *et al.*, 1984; and Persaud *et al.*, 1988).

The developing rat brain exhibits a large increase in dolichol-linked oligosaccharide synthesis and N-glycosylation (Crick and Waechter, 1994). The glycoproteins in brain that contain the most N-glycosylation sites are involved in cell to cell interactions. These interactions are important in oligodendrocyte differentiation and also in myelination (Lai *et al.*, 1987; Terkelsen *et al.*, 1989; Bhat and Silberberg, 1990; Walsh and Doherty, 1991; Rosen *et al.*, 1992; and Malek Hedayat and Rome, 1994). The cell adhesion molecules, which are often members of the immunoglobulin supergene family of glycoproteins, recognise each other through lectin interactions (Zhou *et al.*, 1990; and Walsh and Doherty, 1991). Many of these glycoproteins are also attached to the cell surface by GPI anchors (Morris, 1992; Moss and White, 1992; Rosen *et al.*, 1992; and Rougon *et al.*, 1994). Any disturbance in N-glycosylation would most likely affect the cell adhesion molecules, and this could be important during critical stages in development of the brain. The development of the cerebellum in rodents requires expression of a particular surface glycoprotein, Thy-1, which is linked to the neuronal surface by a GPI anchor (McKenzie and Fabre, 1981; Morris, 1985; Bolin and Rouse, 1986; Saleh and Bartlett, 1989; Walsh and Doherty, 1991; and Morris, 1992). Cerebellar hypoplasia is a feature of galactosaemia and the CDG syndrome. However, patients with the CDG syndrome type I do not exhibit delayed myelination on MRI (Holzbach *et al.*, 1995). Only those few patients with the type II and III phenotypes have been reported to show dysmyelination (Ramaekers *et al.*, 1991; Stibler *et al.*, 1993; and Holzbach *et al.*, 1995). This suggests that if N-glycosylation of cell adhesion molecules is disturbed in these disorders, then the effects may differ among the different phenotypes.

The ovarian dysfunction in galactosaemic females is characterised by hypergonadotropic hypogonadism (Kaufman *et al.*, 1981). The increased levels of FSH and LH have been observed even in infancy (Gibson, 1995) and can fluctuate over time, in a manner that is similar to the resistant ovary syndrome (Fraser *et al.*, 1986; and Twigg *et al.*, 1996). The ovarian follicles are usually reported as reduced in number, or completely absent, depending on the technique used to examine the ovary (Gibson, 1995). It is not known, however, whether this represents a congenital deficiency of oocytes or accelerated depletion of a normal number of oocytes. The ovary has increased activities of the enzymes involved in galactose metabolism (Xu *et al.*, 1989). Female rat fetuses exposed to maternal diets that contained 50% galactose had decreased numbers of oocytes at birth (Chen *et al.*, 1981), and adult mice fed a diet that contained 50% galactose exhibited a delay in oocyte maturation (Swartz and Mattison, 1988). The maturation delay was reversed if the galactose was removed from the diet. These studies suggest that the ovary is particularly sensitive to the effects of galactose, particularly during embryonic and follicular development. The extent of dolichol-linked oligosaccharide synthesis and N-glycosylation in the ovary is not known. However, the functioning of the ovary, like the brain, requires an interaction of cell adhesion molecules, which are particularly important during the development of the ovary, and during differentiation and maturation of the follicles. The cell adhesion molecules that are known to be involved in these processes in the ovary include Thy-1 and neural-cell adhesion molecule (Presl and Bukovsky, 1986; Mayerhofer *et al.*, 1991; Moller *et al.*, 1991; and Bukovsky *et al.*, 1995).

Avenues for future research

Future research needs to determine whether disturbances in N-glycosylation or GPI anchor synthesis contribute to the long-term complications in galactosaemia. The range of N-linked and O-linked oligosaccharides from galactosaemic serum need to be examined to determine the extent of the glycosylation defects in untreated galactosaemia. One group has already begun to characterise the serum transferrin isoforms by mass spectrometry (Charlwood *et al.*, 1995; and Winchester *et al.*, 1995). However, there may be different glycosylation defects depending on the particular glycoprotein studied, as reported for the CDG syndrome type I (Krasnewich *et al.*, 1995). It is also possible that there may be several disturbances to the pathway of N-glycosylation in galactosaemia.

The search for secondary glycosylation disorders also needs to be extended. The hyposialylated serum transferrin in epimerase-deficiency galactosaemia and hereditary fructose intolerance suggests that secondary disturbances of N-glycosylation may not be uncommon, particularly when associated with abnormal hexose metabolism. It has not been reported whether the serum in untreated galactokinase-deficiency galactosaemia contains hyposialylated glycoproteins. Patients with Lowe syndrome, who have a deficiency of Golgi phosphatidylinositol 4,5-bisphosphate 5-phosphatase activity (Suchy *et al.*, 1995), have many clinical and neuroradiological similarities to classical galactosaemia. Lowe syndrome could also have a secondary disturbance in Golgi function.

Van Schaftingen and Jaeken, (1995) proposed that the disturbance in N-glycosylation in the CDG syndrome type I was due to a deficiency of GDP-mannose. However, GDP-mannose has not yet been directly measured in CDG cells. It is now important to measure GDP-mannose in galactosaemic cells, and to determine if the abnormalities in dolichol-linked oligosaccharide synthesis in galactosaemic fibroblasts can also be corrected by adding mannose to the culture medium (Panneerselvam and Freeze, 1996). If confirmed, the activity of the enzymes that convert glucose to mannose would need to be examined, particularly phosphomannomutase. It is also possible that galactosaemic cells accumulate abnormal sugar nucleotides or dolichol sugars, such as UDP-galactosamine, dolichol phosphorylgalactose, or even dolichol phosphorylgalactosamine. The sugar nucleotides in galactosaemic cells, beyond UDP-glucose and UDP-galactose, have not been examined.

It is possible that the disturbances in N-glycosylation in galactosaemia are confined to glycoproteins from the secretory pathway of the liver, or that the hyposialylated serum glycoproteins are only markers of some other underlying metabolic disturbance. In that case, the glycosylation disturbance may not be directly involved in the long-term complications. It is also possible that the disturbances in N-glycosylation may differ between cell types and tissues. The secretory pathway in galactosaemic fibroblasts therefore needs to be examined with other glycoprotein models, including GPI-anchored proteins, to determine the extent of the glycosylation disturbance. It may then be possible to determine if the abnormalities of N-glycosylation in galactosaemia contribute directly to the long-term complications.

Appendices

Appendix 1

Isoelectric focusing of serum transferrin isoforms

Introduction

Serum transferrin isoforms were detected with a method developed from Ohno *et al.*, (1992).

Materials and reagents

1. Acrylamide/bis 40% (w/v) solution (29:1) — Cat. no. 161-0146, Bio-Rad Laboratories, Hercules, CA, USA
2. Pharmalyte ampholytes (pH 4.0–6.5) — Cat. no. 17-0452-01, Pharmacia AB, Uppsala, Sweden
3. Ammonium persulphate — Cat. no. A-3678, Sigma Chemical Co., St. Louis, MO, USA
4. TEMED — Cat. no. T-8133, Sigma Chemical Co., St. Louis, MO, USA
5. Anti-human transferrin antibody (goat) — Cat. no. 81913, Incstar Corp., Stillwater, MN, USA
6. Sepraphore 111 cellulose acetate strips 5.7 x 12.7 cm — Cat. no. 62092, Gelman Sciences, Ann Arbor, MI, USA
7. Coomassie Brilliant Blue R-200 — Cat. no. 161-0400, Bio-Rad Laboratories, Hercules, CA, USA
8. Staining solution
   Dissolve 0.5 g $CuSO_4.6H_2O$ in 60 mL water and add 10 mL glacial acetic acid. Dissolve 0.2 g Coomassie Brilliant Blue R-200 in 30 mL methanol. Combine the two solutions.
9. Destaining solution
   Mix 600 mL water with 300 mL methanol and 100 mL glacial acetic acid.
10. Final destaining solution
    Mix 90 mL destaining solution with 10 mL glycerol.

Method

A: Sample preparation

The serum or plasma (5 µL) samples were mixed with 15 µL 0.2 mmol/L ferric citrate and 30 µL water and were then left at room temperature for at least 15 minutes.

B: Gel preparation

The gels were prepared at least one day before use and stored at 4°C. To prepare one gel, 7.9 mL of water was mixed with 1.7 mL of acrylamide/bis solution, 2 g of glycerol, and 0.8 mL of Pharmalyte solution, and degassed for 15 minutes. Then, 120 µL of ammonium persulphate (30 mg/mL) and 5 µL of TEMED were added and mixed well. The gel was then poured into 10 x 12.5 cm glass plates, with a 0.8 mm template, and left to set at room temperature for 60 minutes.

C: Gel electrophoresis

The electrophoresis system (Bio-Rad Bio-Phoresis™ Horizontal Electrophoresis Cell) was cooled to 2°C. The wicks (as supplied and cut to 9.5 cm lengths) were wetted and blotted with cathode solution (0.5 mol/L NaOH) or anode solution (1 mol/L H_3PO_4) and then layered onto the gel in the isoelectric focusing tank. The electrode system was placed on the wicks and the gel was pre-focused for 20 minutes at 500 V/5 W, with an E-C Apparatus Corporation EC 500-90 power supply. The electrode system was then removed and a Bio-Rad sample mask was placed onto the gel, and 1 or 4 µL of sample was loaded. The samples were then subjected to electrophoresis for 30 minutes at maximum power (5 W) and maximum voltage (1300 V). The sample mask was then removed and the electrophoresis was continued for a further 120 minutes at the same settings. The gel was removed from the electrophoresis tank and the immunoprecipitation steps were begun immediately.

D: Immunoprecipitation and staining

The cellulose acetate membrane (cut to 5.7 x 10 cm) was soaked in 1 mL of 0.9% (w/v) NaCl and 200 µL of anti-human transferrin antibody. The soaked membrane was layered onto the gel surface and the gel was incubated at room temperature for 90 minutes in a humidified container. The membrane was then washed in three changes of 200 mL of 0.9% (w/v) NaCl over two hours, with constant shaking. The membrane was then briefly washed with water and then transferred to 50 mL of staining solution for 15 minutes at room temperature, with occasional mixing. The membrane was then transferred to 100 mL of destaining solution for 15 minutes, and the washing repeated until the membrane was destained. Finally, the membrane was washed in 10 mL of final destaining solution to prevent the membrane from cracking on drying. The membranes were then scanned in a Microtek ScanMaker III reflectance scanner.

Appendix 2

Preparation of tritiated oligosaccharide standards from RNase B and fetuin, and standardisation of the Bio-Gel P-4 sizing column

Introduction

The Bio-Gel P-4 240 x 1 cm sizing column was standardised with a range of oligosaccharide standards. Several tritiated high mannose N-linked alditols were purchased from Oxford GlycoSystems Ltd. Tritiated oligomannose standards were also prepared from bovine RNase B by PNGase F cleavage and direct reduction with $NaB[^3H]_4$ (Hardy and Townsend, 1994; and Varki, 1994a). RNase B contains a range of oligomannose structures from $Man_5GlcNAc_2$ to $Man_9GlcNAc_2$ (Liang *et al.*, 1980; and Hardy and Townsend, 1994). Sialylated complex N-linked oligosaccharide standards were prepared by radiolabelling N-linked alditols that were derived from bovine fetuin. The alditols were first treated by mild periodate oxidation and then reduced with $NaB[^3H]_4$ (Varki, 1994a). Bovine fetuin contains predominantly a sialylated complex triantennary structure, with lesser amounts of sialylated bi- and tetraantennary structures (Townsend *et al.*, 1989; and Hardy and Townsend, 1994).

Each tritiated standard (25 000–50 000 cpm) was loaded on the Bio-Gel P-4 240 x 1 cm column with an internal standard of 100 µg of BSA, 500 cpm of $[^3H]$-mannose, and 10 µg of L-fucose. The column was eluted with 0.1 mol/L NH_4COOH at 8 mL/h and 175 x 1 mL fractions were collected (Figures A2.1 and A2.2, pages 156 and 157).

Reagents

1. Ribonuclease B (EC 3.1.27.5) from bovine pancreas — Cat. no. R-7884, Sigma Chemical Co., St. Louis, MO, USA

2. Fetuin N-linked alditols, bovine — Cat. no. 043064, Dionex Corp., Sunnyvale, CA, USA

3. Tritium-labelled N-linked alditols
   - $Man_3GlcNAc_2$ — Cat. no. T-002300
   - $Man_5GlcNAc_2$ — Cat. no. T-002500
   - $Man_7GlcNAc_2$ — Cat. no. T-002700

   Oxford GlycoSystems Ltd, Abingdon, Oxford, England

Method

A: Preparation of RNase B oligosaccharides

The RNase B (5 mg) was digested with PNGase F and the free oligosaccharides were then recovered according to the procedure in Method 5 (page 72). During each of the recovery steps, the oligosaccharides were detected with a sensitive resorcinol colorimetric assay (Monsigny *et al.*, 1988). In this assay, the hexoses were heated at 90°C in 75% (v/v) sulphuric

acid with resorcinol to form a chromophore with maximum absorbance at 430 nm. The oligosaccharides were then desalted twice on a Bio-Gel P-2 column before they were radiolabelled.

B: Direct reduction of RNase B-derived oligosaccharides with $NaB[^3H]_4$

The RNase B-derived oligosaccharides (75 µg) were resuspended in 100 µL of 0.2 mol/L sodium borate buffer (pH 9.5), and 10 µL of 18.5 MBq/µL $NaB[^3H]_4$ (0.5 mCi/µL) in 0.01 mol/L NaOH was added. The sample was then incubated for two hours at room temperature in a fume hood. Following the radiolabelling, 100 µL of 1 mol/L $NaBH_4$/0.2 mol/L sodium borate buffer (pH 9.5) was added and the incubation was continued for a further two hours to complete the reduction. Following this, 3 mL of 1 mol/L acetic acid in methanol was added and the sample was dried in a 30°C heating block under a stream of nitrogen. This step was repeated, and then 3 mL of methanol was added, and the was sample dried again. The radiolabelled products were resuspended in 200 µL water and then separated on a Sephadex G-15 50 x 1 cm column in water, at 15 mL/h. Forty 1 mL fractions were collected and the oligosaccharides were recovered from near the void (Figure A2.3, page 158). The tritiated oligosaccharides were then lyophilised, resuspended in 500 µL water, and stored at -20°C.

C: Mild periodate oxidation of fetuin N-linked alditols and reduction with $NaB[^3H]_4$

The fetuin N-linked alditols (20 µg) were resuspended in 100 µL of 0.2 mmol/L sodium periodate and 100 µL of 10 mmol/L sodium acetate in a conical glass tube, and incubated on ice for 20 minutes. Then, 10 µL of 10 mmol/L glycerol was added and the sample was incubated at 37°C for 20 minutes. Following this, 100 µL of 0.2 mol/L sodium borate buffer (pH 9.5) and 10 µL of 18.5 MBq/µL $NaB[^3H]_4$ in 0.01 mol/L NaOH were added, and the sample was incubated for one hour at room temperature in a fume hood. Next, 100 µL of 0.2 mol/L $NaBH_4$/0.2 mol/L sodium borate buffer (pH 9.5) was added, and the incubation was continued for 30 minutes at room temperature to complete the reduction. Following this, 3 mL of 1 mol/L acetic acid in methanol was added, and the sample was dried in a 30°C heating block under a stream of nitrogen. This step was repeated, and then 3 mL of methanol was added, and the sample was dried again. The radiolabelled products were resuspended in 200 µL water and separated on a Sephadex G-15 50 x 1 cm column in water, at 15 mL/h. Forty 1 mL fractions were collected and the oligosaccharides were recovered from near the void (Figure A2.3, page 158). The tritiated oligosaccharides were then lyophilised, resuspended in 500 µL water, and stored at -20°C.

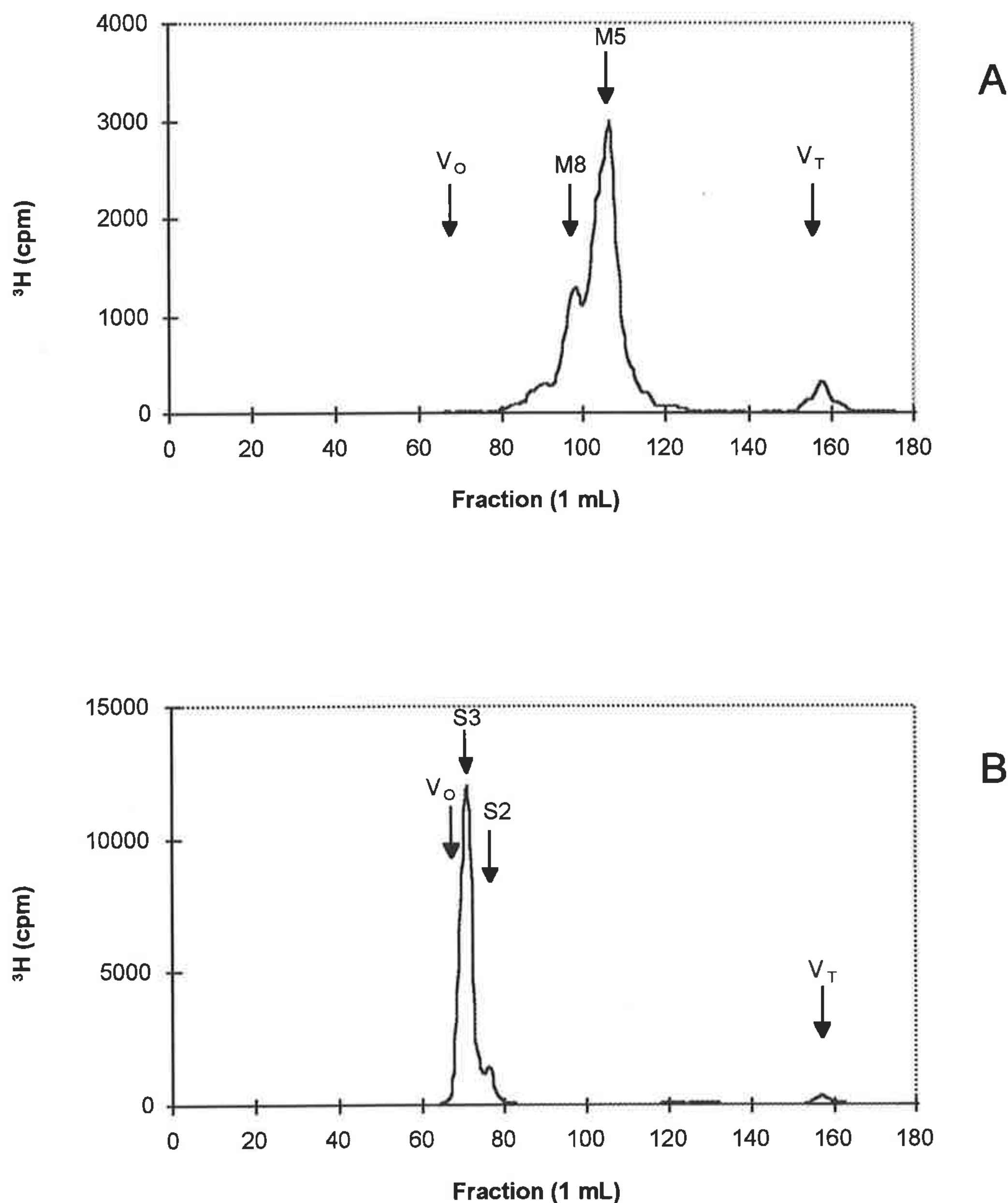


Figure A2.1. ***A***, tritiated RNase B N-linked oligosaccharides; and ***B***, tritiated fetuin N-linked alditols analysed on a Bio-Gel P-4 240 x 1 cm column in 0.1 mol/L NH_4COOH at 8 mL/h. 175 x 1 mL fractions were collected. Each oligosaccharide standard (50 000 cpm) was loaded with an internal standard of 100 µg of BSA, 500 cpm of [^{3}H]-mannose, and 10 µg of L-fucose. The RNase B N-linked oligosaccharides contained a range of oligomannose structures, with $Man_5GlcNAc_2$ (*M5*) and $Man_8GlcNAc_2$ (*M8*) predominant. The *M5* peak eluted at 106 mL and *M8* at 98 mL. The Dionex fetuin N-linked alditols contained predominantly a triantennary (*S3*) sialylated complex-type N-glycan, with lesser amounts of bi- (*S2*) and tetraantennary structures (*S4* appeared in the void volume). The *S3* peak eluted at 71 mL and *S2* at 76 mL. The exclusion volume (V_O) for the column was 65 mL and inclusion volume (V_T) 158 mL.

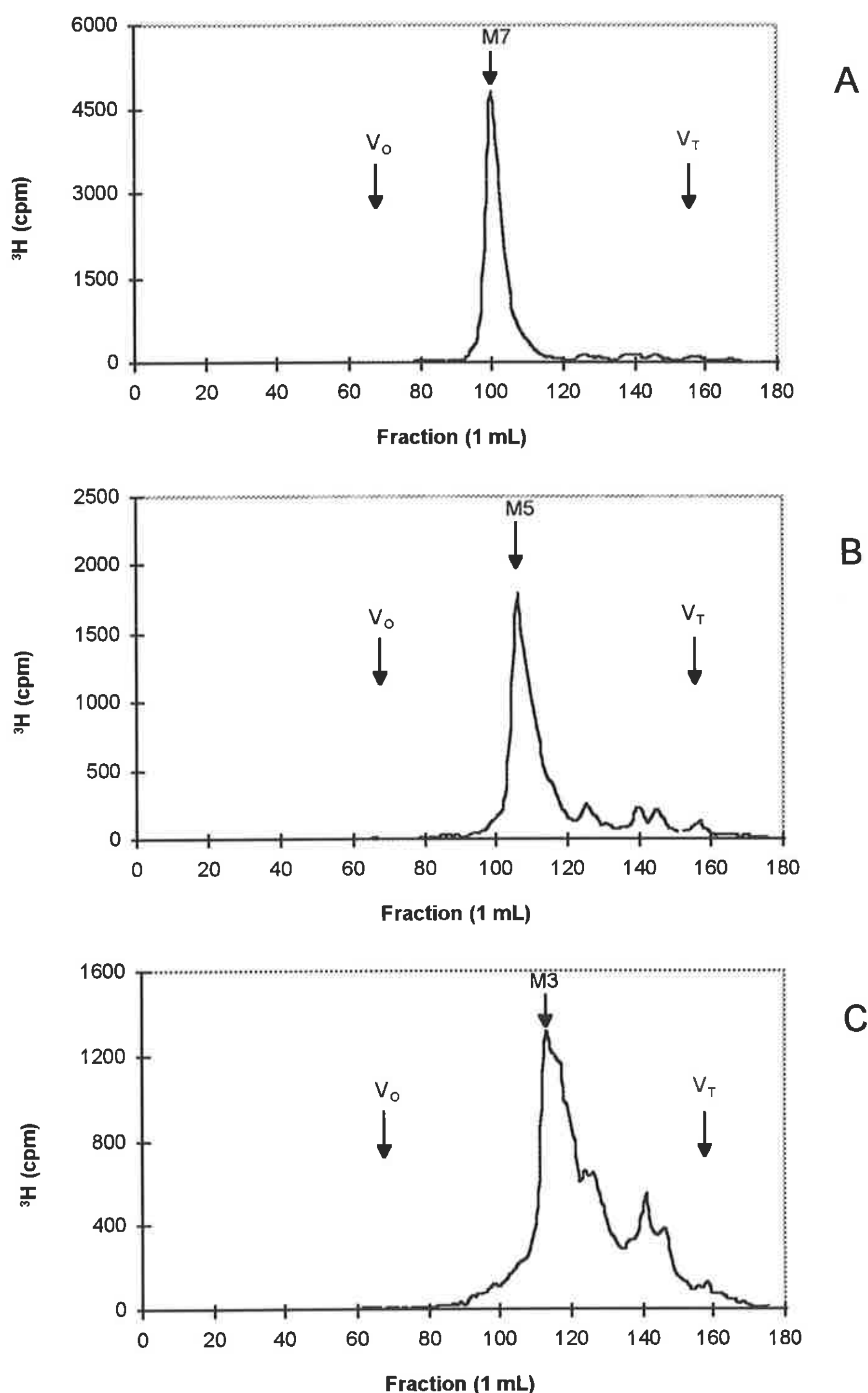


Figure A2.2. Tritiated N-linked alditols from Oxford GlycoSystems, ***A***, $Man_7GlcNAc_2$ (*M7*); ***B***, $Man_5GlcNAc_2$ (*M5*); and ***C***, $Man_3GlcNAc_2$ (*M3*) analysed on a Bio-Gel P-4 240 x 1 cm column in 0.1 mol/L NH_4COOH at 8 mL/h. 175 x 1 mL fractions were collected. Each oligosaccharide (25 000–50 000 cpm) was loaded with an internal standard of 100 µg of BSA, 500 cpm of [^{3}H]-mannose, and 10 µg of L-fucose. The *M7*, *M5*, and *M3* peaks eluted at 100 mL, 106 mL, and 114 mL, respectively. The *M3* standard also contained several smaller breakdown products. The exclusion volume (V_O) for the column was 65 mL and inclusion volume (V_T) 158 mL.

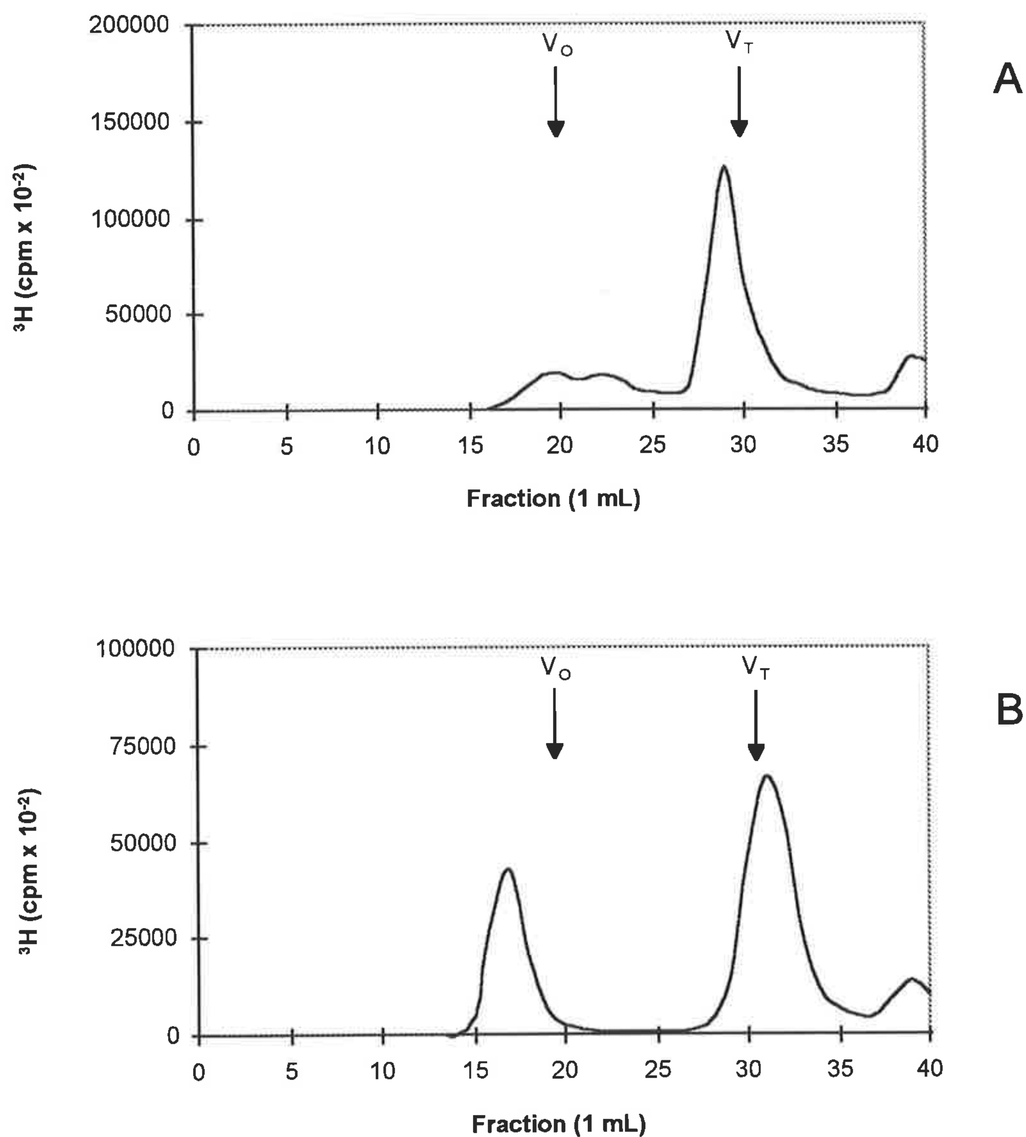


Figure A2.3. ***A***, tritiated RNase B N-linked oligosaccharides (fractions 17–24); and ***B***, tritiated fetuin N-linked alditols (fractions 15–20) were recovered from near the void, following separation on a Sephadex G-15 50 x 1 cm column in water at 15 mL/h. The low-molecular-weight breakdown products of $NaB[^3H]_4$ eluted in later fractions. The exclusion volume (V_O) for the column was 20 mL and inclusion volume (V_T) 30 mL.

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Addendum

In response to questions raised by the second Examiner, the following comments are provided to help clarify certain aspects of the results in this thesis.

1. In Chapter 2, the normal controls in Figures 2.3 and 2.4 were not age-matched. Although it is reported that normal cord serum contains about twice the normal amount of hyposialylated transferrins, the isoelectric focusing and immunodetection method used in this study was not sensitive enough to detect this physiological increase (the transferrin isoform patterns for five normal cord sera and five normal neonatal sera were not shown).

2. In Chapter 4, only four GALT-deficient skin fibroblast cell lines were obtained for the study. At the time of commencing the study these were the only established GALT-deficient fibroblast lines known to be available within Australia and reflects the relative rarity of the disorder (an incidence of 1 in 46 550 in Australia) and that fibroblast cultures are not routinely required or obtained from children with galactosaemia. In similar studies in patients with the CDG syndrome, N-linked oligosaccharide synthesis and structure were examined in fibroblast cultures from only two or four patients, and these patients were from two families.

3. In Chapter 6, mention is made of the variability of the results obtained from the galactosaemic fibroblasts in Experimental plan A. The control fibroblasts in this experiment performed in a very consistent manner regardless of the experimental conditions. This indicated that the methods used were very reproducible. Although the results from the galactosaemic fibroblasts were more variable, the reduction in the incorporation of [2-^{3}H]-mannose was a consistent finding. The reduction in the incorporation of [2-^{3}H]-mannose, and disturbance of the ^{3}H/^{35}S ratio, were observed for each cell line in two different media, and in both the cellular and secreted proteins. One of the galactosaemic fibroblasts (*G1*) also performed in a similar manner in the same media in Experimental plan B, when its results in Experimental plan A were compared to the same control fibroblast (*C1*). Experimental plan B was also performed using duplicate cell cultures.

The experiments in Chapter 6 were designed to screen for possible disturbances in total N-glycosylation in galactosaemic fibroblasts. As such, it was considered more useful to perform the experiments under different media conditions rather than repeat the same experiment more than once in the same cell line. Having identified a possible disturbance in [2-^{3}H]-mannose incorporation, this information was then used to direct the more specific examination of dolichol-linked oligosaccharide synthesis in Chapter 7. Moreover, the results in Chapter 7 were consistent with the conclusions drawn in Chapter 6. Thus, Chapter 6 connects the work in Chapters 5 and 7 and indicates the logic for the change in research direction.

4. There are typographical errors on page 33, paragraph 2, line 9 ('changes was' should read 'changes were'), and on page 112, paragraph 3, line 1 ('galactosaemics fibroblasts' should read 'galactosaemic fibroblasts').