STRUCTURAL STUDIES ON OLIGOSACCHARIDES FROM MAMMALIAN GLYCOPROTEINS

Thesis submitted for the degree of

Doctor of Philosophy

by

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ABSTRACT: A method for the purification of human serum immunoglobulin A1 (IgA1), suitable for subsequent oligosaccharide analysis, was devised. The N- and O-linked glycans of normal human serum IgA1 were released from the protein and the structures determined by a combination of gas-chromatography mass spectrometry, nuclear magnetic resonance spectroscopy and enzymatic degradation. The glycans from serum IgA1 from normal humans and patients with rheumatoid arthritis were compared. Contrary to observations for immunoglobulin G (IgG), no significant alterations in the IgA1 glycans were encountered in the disease state, suggesting that the defect is IgG specific.

The antibodies HNK-1 and L2 bind to a number of glycoproteins important in neural cell adhesion and also recognise glycolipids with a sulphonylglucuronic acid residue. The saccharide from these glycolipids influences neural cell adhesion *in vitro* and is considered to be an important functional component of the cell adhesion glycoproteins. The N-linked glycans from human peripheral nerve myelin glycoprotein, Po, purified with an L2 monoclonal antibody, were released and their structures studied. The glycans were highly sulphated but a sequence analogous to the lipid glycan was not found Therefore the glycans present on Po are distinct from that of the glycolipids, indicating that the rôle of glycans in neural cell adhesion may be very complex. In addition, the glycans released from whole murine brain by hydrazinolysis were studied in order to begin to define the glycosylation of central nervous system glycoproteins.

The glycosylation of glycoproteins in the cerebrospinal fluid and serum of patients with multiple sclerosis (MS) were studied to determine if alterations in glycosylation occur in this autoimmune disease. Little evidence for MS-associated glycosylation disorders was found. Glycosylation of serum IgG from patients with glomerulonephritis was studied. A decrease in the levels of galactosylation occured in some of the patients, which may indicate that IgG glycosylation has a rôle in the pathogenesis of some renal disease.

ERATTA

Page 1, line 10; for has, read have

Figure 1.3a, structure e; GlcNAcβ1→2Galβ1→3 should read as GlcNAcβ1→3Galβ1→3, and should be refered to in the legend as a poly-N-acetyllactosamine containing oligosaccharide.

Page 9, last line; for keratin, read keratan

Page 11, penultimate line; for interection, read interaction

Page 12, line 25; for GlcNAC, read GlcNAc

Page 14, line 12; for Parekh *et al.* (1989a), read Parekh *et al.* (1989b)

Page 43, line 6; for elctrophoresis, read electrophoresis

Figure 2.5; upper limit NaCl concentration should read 2.5M, not 0.5M

Page 74, line 19; the citation of Gooi *et al.* (1987) should be replaced by Stoll, M.S., *et al.* (1988) Biochem. J. 256 661

Page 81, line 9; for decine, read decline

Figure 3.2, legend line 12; for possible CSF, read possible MS CSF

Page 121, line 11; for Stockert *et al.* 1982, read Stockert *et al.* 1981

Figure 5.9, legend lines 8 and 9; lanes 1 and 3 should read lane 2, and lanes 2 and 4 should read as lane 1

Page 150, line 21; replace Vliegendthart with Vliegenthart

Page 183, last two citations; replace Hakamori with Hakomori

Page A.11, line 5; replace permiation with permeation

Page A.19, line 6; replace trimetylsilane with trimethylsilane

Page A.41, line 11; replace permiation with permeation

Page A.44, line 4; replace Hakamori with Hakomori

Page A.45, last line; replace an with and

The following citations were omitted from the bibliography;

Biermann, C.J., and McGinnis, G.D., (1989) 'Analysis of Carbohydrates by GLC and MS', CRC Press, Boca Raton, Florida, USA

Gorter, A., *et al.* (1988) Immunology 64 207

Vliegenthart, J.F.J. *et al.* (1983) Adv. Carbohydr. Chem. Biochem. 41 209

for helen

Recitar! Mentre preso dal delirio
Non so più quel che dico e quel che faccio!
Eppur...è d'uopo...sforzati!
Bah, se'tu forse un uom!
Tu se'Pagliaccio!
Vesti la giubba e la faccia infarina.
La gente paga e rider vuole qua.
E se Arlecchin t'invola Colombina.
Ridi Pagliaccio, e ognun applaudirà!
Tramuta in lazzi lo spasmo ed il pianto;
In una smorfia il singhiozzo e il dolore...
Ridi Pagliaccio, sul tuo amore infranto!
Ridi del duol che t'avvelena il cor!

Leoncavallo (1892)

Nessun dorma! Nessun dorma!
Tu pure, o Principessa,
nella tua fredda stanza
guardi le stelle che tremano
d'amore e di speranza!
Ma il mio mistero è chiuso in me,
il nome mio nessun saprà!
No, no, sulla tua bocca lo dirò,
quando la luce splenderà!
Ed il mio bacio scioglierà
il silenzio che ti fa mia!
Dilegua, o notte! Tramontate, stelle!
Tramontate, stelle! All'alba vincerò!
Vincerò! Vincerò!

Adami and Simoni (1924)

ACKNOWLEDGEMENTS

Firstly, I should like to thank the Directors of the Glycobiology Unit, Oxford, Professor R. A. Dwek and Dr T. W. Rademacher for allowing me to carry out the research described in this thesis in their laboratories.

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For advice on methodology, experimental strategy and encouragement I am very grateful to Drs M. A. J. Ferguson, D. Ashford, J. Thomas, C. J. Edge and my supervisors Prof. R. A. Dwek and Dr T. W. Rademacher, as well as S. Amatyakul, P. M. Rudd, Drs P. Williams and D. R. Wing for advice throughout the experimental work.

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I am most of all indebted to Helen for putting up with me whilst this thesis was produced, especially during the final phases!

LIST OF ABBREVIATIONS

A	absorbance
ADP	adenosine diphosphate
ADCC	antibody-dependant cell cytotoxicity
AMLR	autologous mixed lymphocyte reaction
ATP	adenosine triphosphate
BSA	bovine serum albumin
C	prefix to denote the carboxy-terminus of a peptide
CAM	cell adhesion molecule
CD2 etc.	lympocyte surface antigens
cDNA	complementary DNA
Cer	ceramide
$C_H1, 2..$	immunoglobulin heavy chain constant domain 1, 2 etc
CIC	circulating immune complex
ConA	*canavalia ensiformis* concanavalin A lectin
cpm	counts per minute
CS	chondroitin sulphate
CSF	cerebrospinal fluid
DAB	diaminobutane
dal	dalton
DEAE	diethylaminoethane
DNA	deoxyribonucleic acid
Dol	dolichol
dpm	disintegrations per minute
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EIM	electron impact mode
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunoabsorbant assay
ER	endoplasmic reticulum
Fab	antigen binding fragment of an immunoglobulin comprising the variable and first constant domain
Fc	crystallising fragment of an immunoglobulin comprising the

	constant domains minus C_H1
FAB-MS	fast atom bombardment mass spectrometry
FID	flame ionisation detector
FPLC	fast protein liquid chromatography
FSH	follitropin, or follicle stimulating hormone
Go, 1, 2	complex N-linked oligosaccharides bearing zero, one or two non-reducing terminal β-galactose residues
GAG	glycosaminoglycan
GALT	gut-associated lymphoid tissue
GC	gas-chromatography
GCMS	gas-chromatography mass spectrometry
GDP	guanosine diphosphate
GM-CSF	granulocyte macrophage colony stimulating factor
GPI	glycosylphosphatidylinositol membrane anchor
gu	glucose unit
hCG	human chorionic gonadotrophin
HEV	high endothelial venule
HEBF	high endothelial venule binding factor
HIC	hydrophobic interaction chromatography
HLA	histocompatibility locus antigen
HPLAC	high performance liquid affinity chromatography
HPLC	high performance liquid chromatography
HS	heparan sulphate
HVE	high voltage electrophoresis
Ia	class two MHC antigen
IC	immune complex
I.D.	internal diameter
IEP	immunoelectrophoresis
IFN	interferon
IL-1, 2..	interleukin 1, 2 etc
Ig	immunoglobulin
KPi	potassium phosphate buffer
LH	leutropin or lutenising hormone
MAB	monoclonal antibody
MAG	myelin-associated glycoprotein
MGN	membranous glomerulonephritis

MPGN	membranoproliferative glomerulonephritis
MHC	major histocompatibility locus
MS	multiple sclerosis
MS	mass-spectrometry or mass-spectral
mw	molecular weight
m/z	mass to charge ratio
N-	prefix to denote the amino terminus of a peptide or oligosaccharide linkage to protein through Asn
N-CAM	neural-cell adhesion molecule
NHS	normal human serum
NK	natural killer cell
NMR	nuclear magnetic resonance spectroscopy
NPi	sodium phosphate buffer
O-	prefix to denote oligosaccharide linkage to protein through an amino acid hydroxyl function
PAGE	polyacrylamide gel electrophoresis
PAPS	3'-phosphoadenosine 5'phosphosulphate
PBS	phosphate buffered saline
PEG	polyethylene glycol
PG	proteoglycan
PMAA	partially methylated alditol acetate
PNP	paranitrophenol
PNS	peripheral nervous system
PTA	phoshotungstic acid
QAE	quaternary aminoethane
RA	rheumatoid arthritis
RER	rough endoplasmic reticulum
RF	rheumatoid factor
R_f	relative migration distance
R_T	relative retention time
SC	secretory component
SDM	site directed mutagenesis
SDS	sodium dodecyl sulphate
SLE	systemic lupus erythematosis
SGGL	sulphonylglucuronylneolactoglycolipid
SIM	selective ion monitoring

SS	Sjörgren's syndrome
T4, T8	lymphocyte subset surface antigens (CD4 and CD8 respectively)
T_{cs}	contrasupressor T-cell
TBS	tris-buffered saline
TCA	trichloroacetic acid
TGFβ	transforming growth factor β
TGN	trans Golgi network
TMS	trimethylsilane
TNF	tumour necrosis factor
tPA	tissue-type plasminogen activator
Tris	tris(hydroxymethyl)aminomethane
U	unit of enzyme activity
V	variable domain of an immunoglobulin
VSV	vesicular stomatitus virus
2H4	supressor-inducer T cell subset marker

UNITS

A	ampères
°C	degrees centigrade
Ci	curies
g	gravitational force
g	gramme
K	degrees kelvin
l	litre
M	molar
U	unit of enzyme acitvity, defined as the quantity of an enzyme which hydrolyses 1μmole of substrate in one minute
V	volts

PREFIXES

k-	kilo (10^{3})
c-	centi (10^{-2})
m-	milli (10^{-3})
μ-	micro (10^{-6})
n-	nano (10^{-9})
p-	pico (10^{-12})

AMINO ACID CODES

Amino acid	3 letter code	Single letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Asn and/or Asp	Asx	B
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Gln and/or Glu	Glx	Z
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

MONOSACCHARIDE CODES

Monosaccharide	Abbreviation	Other code
Fucose	Fuc	F
Galactose	Gal	G
Glucose	Glu	
Glucuronic acid	GlcA	
Mannose	Man	M
N-acetylgalactosamine	GalNAc	
N-acetylglucosamine	GlcNAc	N
N-acetylneuraminic acid	NeuNAc	SA
N-glycoylneuraminic acid	NeuGlc	SA
Xylose	Xyl	X

Sugar residues refered to in this thesis are in the D-pyranose configuration, except fucose, which is in the L-configuration. The subscript $_{ol}$ is used to indicate that the reducing terminal residue has been converted to the alditol. Linkages between monosaccharides are written in this thesis in the following format Galβ1→3GlcNAc.

CONTENTS

CHAPTER ONE

INTRODUCTION: STRUCTURE, FUNCTION AND BIOSYNTHESIS OF PROTEIN-LINKED CARBOHYDRATES

1.1 *Preamble*

The work described in this thesis is concerned with structural studies of protein-linked carbohydrates. The last two decades have witnessed a revolution in this field following the introduction of new technology which has allowed full structural elucidation to be performed as well as analysis of complex material of biological origin. Studies on proteins from many sources has highlighted the enormous diversification engendered by post-translational modification of a polypeptide, and has afforded a new perspective of the complexity of these systems. Indeed, proteins modified following the translation process are clear examples of several gene products having a bearing on the final molecular phenotype, and hence the function, of a given protein.

Specifically, two systems have been chosen for study; the immunoglobulins G and A, and the mammalian nervous system. The experimental work is centered on investigations of possible alterations in glycosylation that arise in autoimmune disease, and study of a carbohydrate determinant that occurs on neural glycoproteins which is believed to be involved in cell adhesion. This chapter provides a brief review of post-translational modification, focusing on the biosynthesis and function of N-linked carbohydrates. The reader is directed to appendix three for a discussion of

the biosynthesis of O-linked oligosaccharides including glycosaminoglycans. A brief overview of the technology commonly applied to structural elucidation is given in appendix two.

1.2 *The Post-Translational Modification of Proteins*

Following the synthesis of the polypeptide by the ribosome a number of covalent modifications can take place. These may happen whilst the protein is still bound to the ribosome, immediately after the protein has been synthesised, or at some point later in time. The distinction between post-translational or co-translational modification is unclear, as the precise timing of many of the modification processes with respect to protein synthesis is not known. As a working definition it is sufficient to consider post-translational modifications as those additions or alterations to the polypeptide that occur at the time of or shortly following protein synthesis. Whilst a host of post-translational modifications occur in prokaryotes, the discussion that follows is restricted to those found in eukaryotes.

Proteolytic modifications are common, such as cleavage of signal sequences and the processing of peptide hormones and enzymes from the inactive pro-state to the holo-form, e.g. insulin, chymotrypsin (Wright 1973). The amino terminal amino acid can be acetylated, which usually occurs co-translationally, or cyclised as in the case of conversion of glutamate to pyroglutamate. Amidation of the terminal carboxyl group occurs in peptides that are cleaved from larger precursors; glycine is degraded to leave only the amino group. Direct alterations to internal amino acids are found; e.g. many of the proteins in the blood clotting cascade contain

modified glutamic acid, γ-carboxyglutamic acid, which functions as a calcium chelator (Stenflo 1978). Hydroxylation of proline and lysine in collagen is performed co-translationally and is essential for the correct folding of the protein. Hydroxylysine also functions as a site for attachment of O-linked carbohydrate (Adams and Frank 1980). Methylated amino acids, specifically lysine, arginine and histidine have been found in some proteins e.g. ribosome proteins and histones. Methylation of glutamate appears to be involved in the chemotactic responses of lymphocytes.

Many proteins are phosphated and/or sulphated post-translational. The reversible addition of phosphate groups is a widely occuring modification used in the regulation of function (Cohen 1979), and forms the basis of one of the mechanisms of secondary signal transduction. Phosphorylation is brought about by the action of specific kinase enzymes and often has a dramatic effect on function. Phosphate acceptors are usually serines, threonines or tyrosines, and occasionally histidine and lysine.

The widespread occurrence of protein tyrosine sulphation has only recently been appreciated (Huttner 1988). An overall function for this modification has not been documented, but in at least one system the presence of sulphate appears to decrease the time a secretory protein spends in the Golgi apparatus (Huttner 1988).

Other modifications include the addition of the adenyl moiety of ATP or ADP-ribose from nicotinamide dinucleotide (Hayaishi and Ueda 1977), which is important in the control of DNA synthesis and repair, and the addition of ubiquitin, a 76 amino acid polypeptide, which is found linked to the side chain of a lysine in the primary polypeptide. This modification is highly prevalent amongst the histones.

An important class of modifications involves the addition of

hydrophobic moieties, i.e. fatty acids, to the polypeptide. Such modifications include myristoylation of the N-terminus, typically glycine, and the addition of palmitic acid either to serine in ether linkage, or to cysteine in thioether linkage (Scultz 1988). In addition a complex glycolipid, a glycosylphosphatidy-linositol lipid anchor (GPI-anchor), is added to the C-terminal residue of a number of proteins, for example the *Trypanosoma brucei* variant surface glycoprotein and human erythrocyte acetylcholine esterase (Ferguson and Williams 1988).

Glycosylation occurs in two distinct forms. O-linked, or mucin type glycans are commonly linked to serine or threonine residues, although other amino acids may be used, and are built up by the addition of monosaccharide units to the protein. The glycosaminoglycan chains (GAGs), e.g. chondroitin sulphate and heparin, are also O-linked to a serine. N-Linked, or serum type carbohydrate is linked exclusively to asparagine in the sequence Asn-x-Ser/Thr, called the *sequon.*

1.3 *Biosynthesis of N-linked Glycans*

1.3.1 The Biosynthetic Pathway

A complete oligosaccharide is constructed initially as a lipid-linked moiety with the isoprenaline derivative, dolichol phosphate (Dol-P), as the lipid portion. The immature oligosaccharide is transferred *en bloc* (figure 1.1). The completed saccharide for transfer is built up within the rough endoplasmic reticulum (RER). The first seven sugars are added directly from UDP-GlcNAc and GDP-Man, and the remaining seven from the nucleotide sugars via Man-P-Dol and Glc-P-Dol, as the immediate donors, by transfer of the

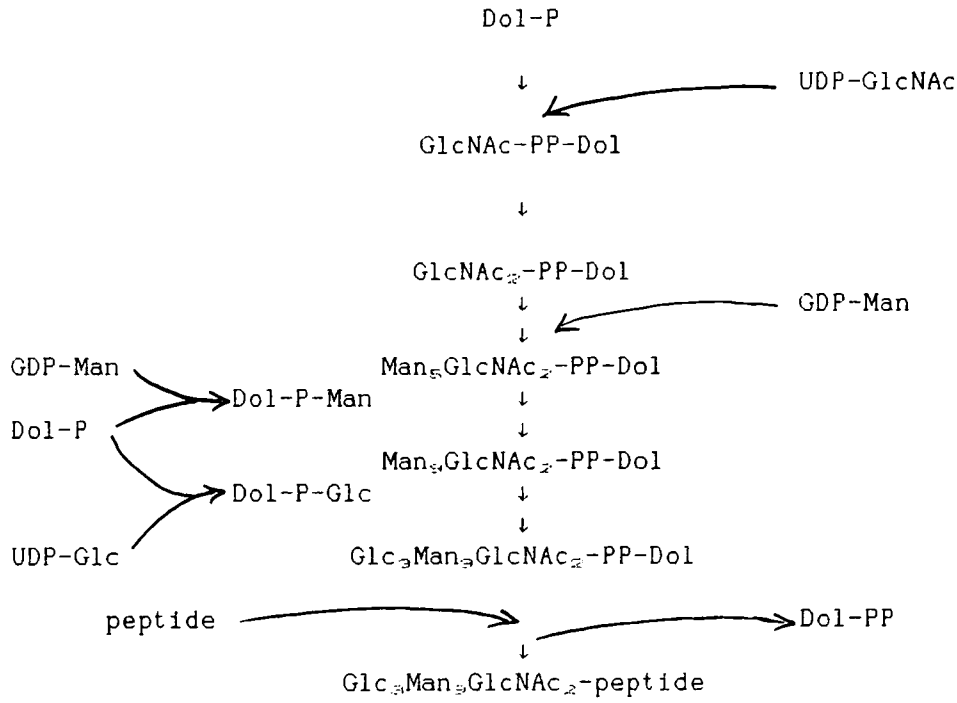


Figure 1.1 Schematic diagram illustrating the pathway of assembly of the lipid-linked precursor of N-linked oligosaccharides. The oligosaccharide is constructed by the addition of monosaccharides, and the completed structure is then transfered *en bloc* to the sequon, Asn-x-Ser/Thr. See text for further details. The figure is redrawn from Hirschberg and Snider (1987).

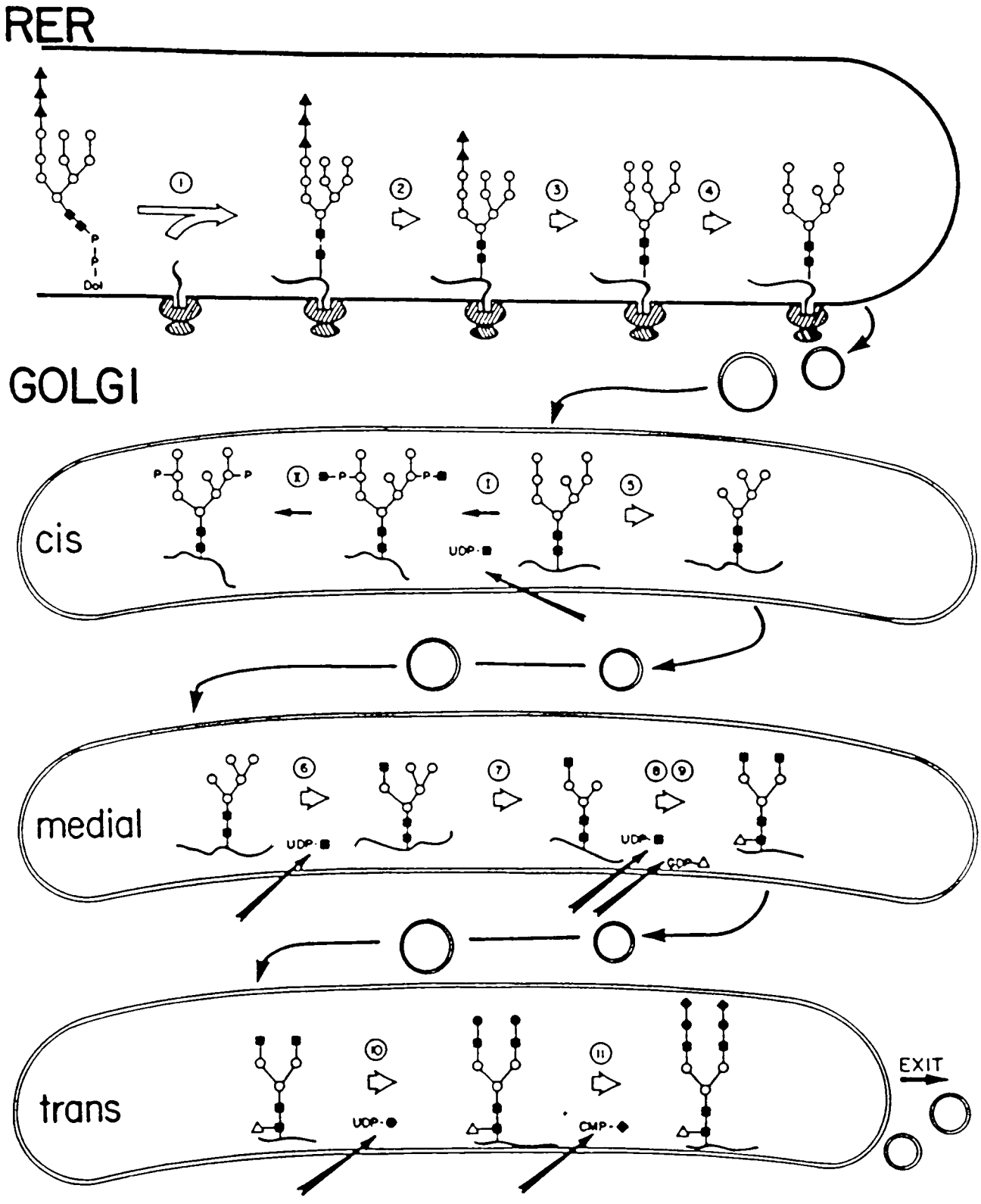


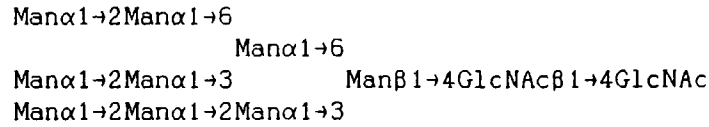
Figure 1.2 Schematic diagram of the pathway of oligosaccharide processing on newly synthesised glycoproteins. The reactions are catalysed by the following enzymes: (1) oligosaccharyltransferase, (2) α-glucosidase I, (3) α-glucosidase II, (4) RER α-mannosidase, (I) N-acetylglucosaminylphosphotransferase, (II) N-acetylglucosamine-1-phosphodiester α-N-acetylglucosaminidase, (5) Golgi α-mannosidase I, (6) N-acetylglucosaminyl-transferase I, (7) Golgi α-mannosidase II, (8) N-acetylglucosaminyl-transferase II, (9) fucosyltransferase, (10) galactosyltransferase, (11) sialyltransferase. The symbols represent: ■, N-acetylglucosamine; o, mannose; ▲, glucose; △, fucose; ●, galactose; ♦, sialic acid. The figure is taken from Kornfeld and Kornfeld (1985).

monosaccharide phosphate to Dol. The reducing terminus is constructed from UDP-GlcNAc and Dol to produce GlcNAc-PP-Dol (Hirschberg and Snider 1987). The lipid linked oligosaccharide must cross the endoplasmic reticulum (ER) membrane at least once during biosynthesis since the mannose and GlcNAc nucleotide sugars are located in the cytosol and several of the transferase enzymes responsible for this part of the synthesis are clearly located on the cytoplasmic face of the ER. Also the $Man_5GlcNAc_2$-PP-Dol intermediate can be localised to this face, and GDP-Man cannot be translocated into the RER lumen (Snider and Rogers 1984, Hanover and Lennarz 1982). All further intermediates can be recovered from the ER lumen which implies that translocation of the nascent chain occurs at this stage.

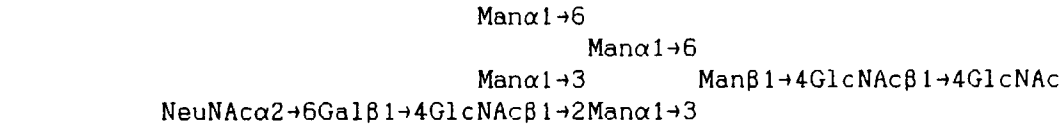
The fully constructed donor (figure 1.3b) is then transferred to the sequon by oligosaccharyltransferase, a multicomponent enzyme located with in the ER lumen. The cDNA of the sequon binding subunit has recently been isolated (Geetha-Habib *et al* 1988). In the mammalian system there appears to be an almost complete requirement for the $Glc_3Man_9GlcNAc_2$ structure to be present. However, some thy-1 negative mutant murine lymphomas are unable to make Man-P-dol, and thus cannot extend the Man_5 structure. Instead these cells translocate $Glc_3Man_5GlcNAc_2$ from dolichol to the polypeptide in what has been called the alternative pathway (Yamashita *et al.* 1983b). A strict reliance on glucosylated donor saccharides is not prevalent in some lower eukaryotes, e.g. yeast (Rumge *et al.* 1984), *Trypanosoma cruzi* and *Leshmania mexicana* (Parodi *et al.* 1983, Parodi and Martin-Barrientos 1984, Parodi *et al.* 1981).

All asparagine-linked oligosaccharide attachment sites examined so far conform to the tripeptide sequence N-Asn-x-Ser/Thr-C (Kornfeld and Kornfeld 1985, Hirshberg and Snider 1987). The amino and carboxyl termini must be

(a)



(b)



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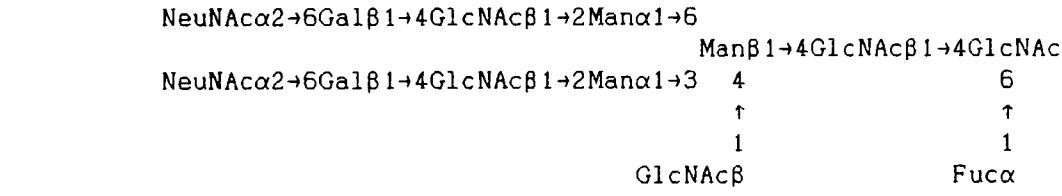

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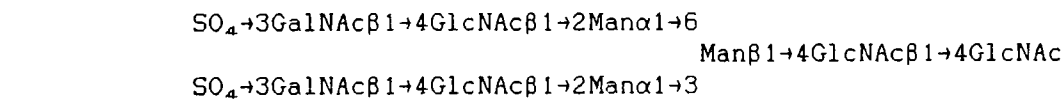

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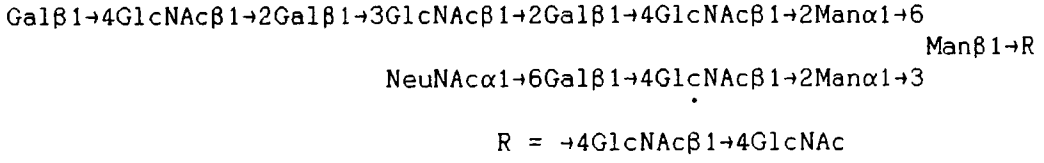
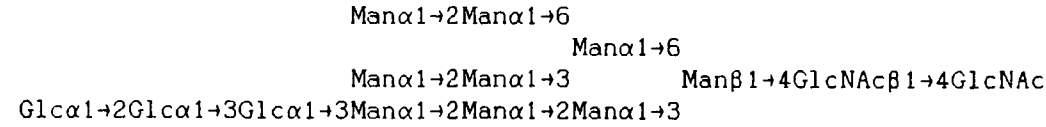


Figure 1.3a Examples of asparagine linked glycans. (a) an oligomannose structure (Man-9), (b) a hybrid, (c) a complex glycan of the biantennary type, (d) a complex sulphated biantennary glycan, and (e) a lactosamine containing oligosaccharide. The reducing terminal GlcNAc is shown at the right, and is in linkage to Asn in the glycoprotein.

(a)



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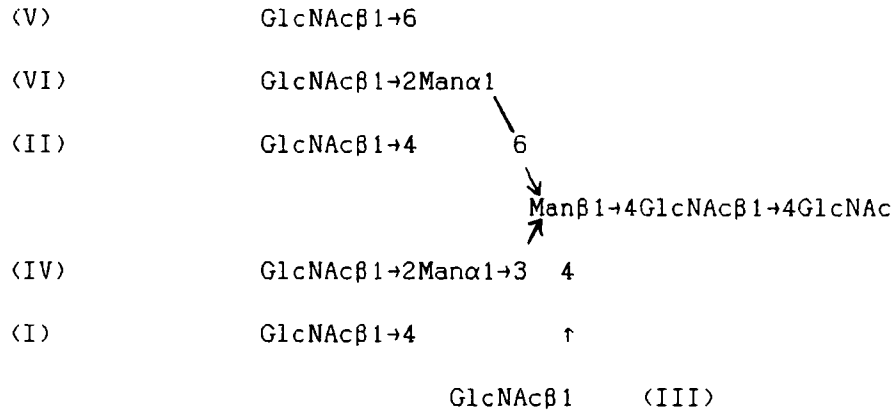


Figure 1.3b. (a) The complete oligosaccharide that is assembled as a lipid-linked moiety. This glycan is translocated to the sequon in the RER by the oligosaccharyltransferase, and is then subjected to the maturation pathway as a protein linked sugar. The structure of this glycan shows a remarkable conservation amongst the eukaryotes.
(b) The GlcNAc additions catalysed by the various Golgi N-acetylglucosaminyltransferases.

substituted (Welply *et al.* 1983). Overall only 30% of sequons are glycosylated (Kornfeld and Kornfeld 1985). Information from secondary structure prediction and X-ray crystallography has shown that the sequon is more often used in the β-turn conformation (Beeley 1987) so that the hydroxyl group of the serine or threonine can form a hydrogen bond with the asparagine amide (Bause and Liegler 1981, Bause 1983).

There is some debate about the precise timing of the addition of the oligosaccharide; it was originally considered to be a co-translational event, but recent data from studies of the heavy chain binding protein (BiP or HSP60) indicate that glycosylation may occur after considerable folding of the protein has taken place (Dorner *et al* 1987, Hendershot 1987). The observation that only those sequons that are destined to lie on the surface of the native glycoprotein become glycosylated, and not those that are ultimately buried lends weight to the post-translational theory, although folding of some of the N-terminal sequences can take place even whilst the polypeptide is still attached to the ribosome.

Factors controlling whether or not an individual sequon is used are only beginning to be understood. In ovalbumin there are three sequons of which two are on the surface. In the native protein only one site is ever used, but denaturation allows glycosylation of all three sites. One of the nonglycosylated sites is buried in the interior of the folded protein but the other two sequons juxtaposed at the surface of the protein (Glabe 1980). It has been proposed that the presence of a large oligosaccharide on the utilized sequon blocks addition of a second sugar chain, but Sheares (1988) has demonstrated that deleting the competent acceptor site by site-directed mutagenesis (SDM) does not allow glycosylation of the second surface sequon. Therefore the topology of the protein surface is playing

some, as yet undefined role, in discrimination between the two exposed sites.

The intracellular locations of the processing exoglycosidases and transferases are discrete (Kornfeld and Kornfeld 1985). Studies using immunocytochemistry and subfractionation of the Golgi saccules has allowed localisation of specific enzymes to different regions of the Golgi, e.g. N-acetylglucosaminyltransferase I to the medial Golgi (Dunphy and Rothman 1985), and galactosyltransferase to the trans Golgi. This information, taken together with structural studies on the mature glycans as isolated from glycoproteins has allowed a detailed description of the pathway to emerge.

The three glucose residues are removed by RER enzymes. α-Glucosidase I removes the terminal Glcα1→2 residue, and α-glucosidase II removes the remaining two residues, both in α1→3 linkage. Initial trimming of the oligomannose structure involves the removal of one Manα1→2 residue, which is classically considered to be the central terminal mannose in the structures shown in figure 1.2 and 1.3b. The Man_8 glycan is further processed on entry to the Golgi. All reactions take place within the lumen of the Golgi saccules (figure 1.2). It is important to realise that the glycans expressed on a mature protein can be derived from a cessation of processing at any point within the pathway, but the three glucose residues are rarely found on mature proteins. The glycoprotein traverses the Golgi saccules in an ordered fashion, from the cis, to the medial, and finally to the trans Golgi before exiting via the trans-Golgi network (TGN). Compartmentalisation of the various processing enzymes serves to control maturation of the oligosaccharide.

The Man_8 structure is a substrate for either Golgi α-mannosidase I or

N-acetylglucosaminylphosphotransferase. The latter adds GlcNAc-PO_4 to two of the penultimate mannose residues at the C6 hydroxyl group. Subsequent removal of the GlcNAc leaves an oligomannose structure bearing mannose-6-phosphate, which marks the protein as destined for the lysosome via recognition by the Golgi mannose-6-phosphate receptor. Action of Golgi mannosidase I prevents the addition of GlcNAc-PO_4 and produces a Man_8 structure. The Man_5 structure is an important determination point. The glycan is a substrate for N-acetylglucosaminyltransferase I, which can only act if the glycan has been trimmed to the Man_5 structure. Therefore any larger oligomannose glycans that reach this point are processed no further. The glycan generated by the action of N-acetylglucosaminyltransferase I is a substrate for N-acetylglucosaminyltransferase III, which adds a GlcNAcβ1→4 residue to the core β-mannose. These two enzymes appear to play a critical role in the subsequent fate of the glycan (see below). If the bisecting residue is added, then no further trimming of the Manα1→6 arm can occur, and the glycan is committed to becoming a hybrid structure. However, some hybrids have been demonstrated to have only four mannose residues, so removal of one of the terminal mannose residues must be possible. If Golgi α-mannosidase II acts, two further mannose residues are removed, and the complex glycan trimannosylcore is generated. This structure is a substrate for N-acetylglucosaminyltransferase II, which adds a second GlcNAc to the glycan.

Entry of the glycoprotein into the trans Golgi exposes the glycan to a further set of enzymes, specifically galactosyltransferases and sialyltransferases. Because galactose can be found in several linkages (β1→4/3, α1→3) it is clear that several transferases exist, and some of these have been characterised. The same is true for sialyltransferase and

late acting N-acetylglucosaminyltransferases. Sulphation and fucosylation also occur as late Golgi events. Most of the sulphate esters appear to be linked to the C3 or C6 positions of galactose or GlcNAc (see chapter two). Competition between the enzymes is an important factor in determining the final spectrum of structures, for example in the calf thyroid it is probable that sulphotransferase, Galα1→3 galactosyltransferase and SAα2→3 sialyltransferase all compete for C3 of the terminal Galβ1→3 (Spiro and Bhoyroo 1988). The late acting N-acetylglucosaminyl-transferases IV-VI serve to branch the glycan to generate tri-, tetra- and pentaantennary complex oligosaccharides (figure 1.3b). It is of relevance to note that N-acetylglucosaminyltransferases I-V cannot act on a substrate that has a bisecting GlcNAc residue (Nishikawa *et al.* 1988), and that the presence of this residue also blocks the action of Golgi α-mannosidase II and fucosyltransferase, and therefore the occurrence of bisected tri- and tetraantennary complex oligosaccharides suggests that N-acetylglucosaminyl-transferase can also act at a late point in biosynthesis. In yeasts the set of reactions leading to the production of complex oligosaccharides do not occur, but extension of the Man_8 core does take place. Mannose is added in α1→2,3 and 6 linkages, and the oligosaccharide can be highly branched and contain more that 100 residues.

Examples of the major types of oligosaccharides encountered on mature glycoproteins are shown in figure 1.3. It is usually considered that there are three classes of N-linked glycan, the oligomannose, the hybrid and the complex sugars, but lactosamine repeat structures may represent a fourth class as the biosynthesis of these oligosaccharides requires sequential action of transferases before capping, by e.g. sialyltransferase, takes place. Sulphation of lactosamines generates keratin sulphate

oligosaccharides. The factors that determine what type of oligosaccharide will be synthesised on a particular glycoprotein are considered in the next section.

1.3.2 *Control of Biosynthesis*

Determination of which glycan structure will be present on a mature glycoprotein is a complex phenomenon. Restrictions engendered by the protein and the biosynthetic machinery itself come together to control the final spectrum of oligosaccharides encountered on the mature glycoprotein. The contributary factors are discussed below.

Green and Baenziger (1988 a, b) were able to show that the glycans present on the α-chain of the pituitary hormones lutropin (LH) and follitropin (FSH) in humans, sheep and cattle differ widely in the respective ratios of sialylated to sulphated structures, with the ratio for FSH being ten fold greater than for LH. The α-chain of these two hormones is an identical polypeptide and both hormones are produced by the same cell. Therefore, in this case, the glycosylation of the α-chain was influenced by association with the hormone specific β-chain. Either the β-chain is able to alter the conformation of the common α-chain, so that it is different in FSH or LH, or the β-chain itself is influential in controlling the enzymatic processing of the nascent α-chain glycans, perhaps by restricting access. Dahms and Hart (1986) analysed the common β-chain of Mac1 and LFA-1, two members of a subset of the integrin family synthesised by the same murine macrophage cell line. The α-subunits are distinct polypeptides and associate with a common β-subunit before entry to the Golgi apparatus. The glycosylation was distinct for the two proteins,

and the spectrum of glycan structures on the same site derived from the two β-chains of the different integrins was different for four out of the total of five sequons. Similar mechanisms, as discussed for the glycohormones, could account for the different glycans produced on Mac1 and LFA-1.

Ovalbumin and ovomucoid are both synthesised within the hen oviduct. Ovalbumin contains mainly oligomannose and hybrid structures, whilst ovomucoid contains a large proportion of bisected and highly branched oligosaccharides that terminate in GlcNAc. Both of these glycoproteins are rich in bisected glycans. Schachter (1986) has proposed that the difference in the glycans may be due to differential sensitivity of the proteins towards the action of N-acetylglucosaminyl-transferase III, i.e. ovalbumin becomes a good substrate for this enzyme at the Man_5 level, leading to hybrid synthesis, whilst ovomucoid is not sensitive to the enzyme until the glycan is processed to a Man_3 structure, and the glycans are complex type. Therefore, both the glycan and the polypeptide are important in dictating the sensitivity of a glycoprotein to the processing enzymes.

A human IgGx (Hom), which had N-glycans at the heavy-chain Fc site, (Asn 297) and also on the Fab at Asn 107 of the light-chain was studied (Savvidou *et al.* 1984). It is important to bear in mind that the heavy chain is prevented from leaving the RER until it has associated with the light-chain (see section 1.4). All the oligosaccharides were biantennary complex, with core fucosylation, but the oligosaccaharides derived from the Fab site were mono- and disialylated bisected glycans whilst the sugars on the Fc were composed of 73% nonbisected and 27% bisected neutral oligosaccharides. It has been proposed that the Fab glycan is a better substrate for N-acetylglucosaminyl-transferase III because for interection with this enzyme the Manα1→6 arm must be bent back along the core of the

glycan. In the Fc this conformation is prevented by the polypeptide, and so the oligosaccharide is unable to act as a substrate for the transferase (Schachter 1986). The addition of the bisecting GlcNAc residue alters the number of conformations that the free oligosaccharide in aqueous solution can sample, so that the Manα1→6 arm becomes locked back along the core of the glycan (Homans *et al.* 1987). The presence of 27% bisected oligosaccharide on the Fc of the IgG in this case indicates that application of dogmatic rules for biosynthetic control may be inappropriate.

The presence of a membrane anchor can influence glycan processing. The addition of a transmembrane sequence to human chorionic gonadotrophin (hCG) resulted in an increase in the prevalence of lactosamine chains over soluble hCG expressed in the same cell line (Fukuda *et al* 1988). Therefore the location of the protein within the Golgi, i.e. soluble or membrane bound, can also affect glycan biosynthesis. The glycosylation of two vesicular stomatitus virus (VSV) serotypes in BHK cells was investigated (Hunt *et al.* 1983). For one serotype, complex oligosaccharides were found, but for a second serotype, one of the glycosylation sites bore predominantly oligomannose glycans. Therefore, alterations in the primary sequence of a protein or its location in the Golgi can result in a large change in the structures of oligosaccharides that are found on a mature glycoprotein.

The importance of biosynthetic control is clearly significant. For example it has been shown that the level of β1→4 linked GlcNAc on the outer arm (in the sequence GlcNACβ1→4[GlcNAcβ1→2]Manα1→3) of oligosaccharides can correlate with malignancy e.g. in choriocarcinoma, hCG shows this structure whereas in the normal state no β1→4 linked GlcNAc is observed on the

peripheral arms (Mizuochi T *et al* 1983). Alteration of the glycan sequence can influence the level of bioactivity, as was found for choriocarcinoma hCG (Nishimura *et al* 1981). Similarly, a direct association between metastasis and GlcNAcβ1→6 branches of complex N-linked glycans was observed in murine mammary carcinoma cells (Dennis *et al* 1987). Therefore alterations to the biosynthetic pathway as a result of oncogenesis can alter cell phenotype.

1.3.3 *Site Microheterogeneity and Tissue/Species Specific Glycosylation*

Structural information from many investigators has led to the realisation that all glycoproteins carry a set of glycans and that individual sites also carry populations of glycans (Anderson and Grimes 1982). This phenomenon is termed site microheterogeneity. As each individual molecule can carry only one structure at a given site heterogeneity is evident at the level of the individual glycoprotein molecule. The potential for variation is clearly enormous; if a glycoprotein were to have just one sequon and ten structures were found to be associated with the population then there would be ten distinct glycoproteins. But if the protein had the same level of heterogeneity over three sites, then theoretically 1000 individual variants could exist. Rademacher *et al* (1988c) have used the term *glycoform* to describe this heterogeneity, i.e. each of the possible combinations in the hypothetical three-site glycoprotein would be a glycoform. The overall population is called the *glycotype*. Therefore, when considering a glycoprotein at the molecular level it is necessary to specify the range of glycans present. This concept should not be restricted to N-linked glycans, but extended to

all post-translational modifications.

The functional importance of glycoforms is not completely clear The difficulty in determining the role of glycoforms is compounded by the obvious problem that N-linked glycans have a variety of functions that are not common to all systems, e.g in hCG the presence of an oligosaccharide is essential for function, whereas in another case, granulocyte-macrophage colony stimulating factor (GM-CSF), the presence of carbohydrate probably has a minimal role to play. The blood group system is an example of glycoform heterogeneity that shows differences between individuals and has important clinical ramifications.

Few studies have succeeded in correlating a detailed description of the glycan population with the function of a glycoprotein. Parekh *et al* (1989a in press) analysed the oligosaccharides and kinetic parameters for tissue type plasminogen activator (tPA) produced from several sources. The presence of an oligosaccharide at site 184 in type II tPA (and absent in type I) altered the binding of the protein to an affinity matrix as well as the lag time for fibrin cleavage by plasmin production. By removal of the sequons by SDM it was also shown that the presence of a glycan at Asn 184 has a profound influence on activity (Hansen *et al.* 1988).

Microheterogeneity clearly arises from the cessation of processing at different points along the maturation pathway. A number of factors may be important here; (1) the level of expression of the processing enzymes, under genetic (oncodevelopmental) control, (2) the conformation of the protein, especially around the glycosylation site, (3) the transit time of the protein through the Golgi apparatus, affecting the length of time in which a given enzyme may act, and (4) possible interactions with other proteins in the Golgi, either in the secretory pathway or resident in the

cisternae. It would appear unlikely that very tight control of the level of each glycan expressed on the mature glycoprotein can be maintained in the face of such a battery of variable factors, so that the *precise* structure of an oligosaccharide may not be of great importance, and a range of structures may be functionally equivalent. However, it is certainly the case that the glycosylation of individual glycoproteins can show remarkable conservation, and this appears to also hold true for complex mixtures, e.g. serum glycoproteins.

Species and tissue specific glycosylation is also observed. The fact that carbohydrate antigens can be used as oncodevelopmental markers clearly demonstrates both the specificity and variety of glycans that can be expressed, depending on the tissue and cell type (Feizi 1985). For example, in the rat the terminal sequence Galα1→3GlcNAc is encountered, but this is not observed in human N-linked glycans (Kobata 1984). Human and rat α1-acid glycoprotein show specific differences in their carbohydrates. The human α1-acid glycoprotein glycans are predominantly tri- and tetraantennary, whilst those from the rat protein are mainly biantennary and the glycans from the rat protein possess Fucα1→3 residues on the proximal GlcNAc residues and the Galα1→3GlcNAc saequence, both absent in the human glycoprotein. The glycans from γ-glutamyltranspeptidase produced by the kidney have a high incidence of bisecting GlcNAc residues, which are absent in the hepatic glycoprotein (Kobata 1984). This seems to be the case for several mammalian species, e.g. rat, cow and mouse. The N-glycans from IgA have been found to be very different in milk and serum (Pierce-Cretel *et al.* 1984, Baenziger and Kornfeld 1976a). The glycans of glycoproteins found in the mucosa or milk are much more complex than those for serum or tissue glycoproteins, and are larger, highly branched and contain a number of

blood group determinants (e.g. see Finne *et al.* 1989). The carbohydrate composition of thy-1 from rat lymphocytes and brain are different (Pink 1983). Site analysis showed that none of the glycoforms of thy-1 were common between the two tissues (Parekh *et al* 1987), and therefore thy-1 from brain and thymocytes can be considered to be a different glycoprotein. Therefore tissue or species specific glycosylation can produce different glycoforms.

Comparison of the glycans of transformed cells and their non-transformed progenitors has been used to analyse developmental changes in glycosylation. Transformation of BHK cells leads to a decrease in the incidence of oligomannose oligosaccharides, with an increase in the level of tetraantennary glycans on the cell surface due to a greater number of outer chains linked β1→6 to the Manα1→6 (Yamashita *et al.* 1984). An increase in the presence of sialic acid and alterations to the neutral glycan structures was noted as occuring on transformation (Atkinson and Hakimi 1980). In Nil8 fibroblasts transformed with Hamster sarcoma virus (HSV) there was an increase in the incidence of highly branched and polylactosamine structures compared to the parent cell line (Hubbard 1987). Analysis of the glycans recovered from the G protein of VSV grown in both Nil8 and transformed Nil8 cells showned that the changes affected some sequons more than others; glycans attached to Asn-179 of the G protein were similar in both cell lines, but at Asn-336, which in the Nil8 cells carried biantennary glycans, highly branched oligosaccharides were found in the Nil8 transformants. Much of the increased branching was due to the presence of a greater number of GlcNAcβ1→6Manα1→6 branches.

Glycosylation of the enzyme complex sucrase-isomaltase in HT-29 cells is altered on differentiation (Trugan *et al.* 1987). The $Man_9GlcNAc_2$

oligosaccharide accumulated in the undifferentiated but not differentiated cells, presumably reflecting induction of the relevant processing enzymes and/or accumulation of nucleoside sugars due to alterations in gene expression (Ogier-Denis 1988). The complexity of glycan structures increased with differentiation of murine haematopoitic cell lines as it was found that lactosamine structures were more branched in mature granulocytes in comparison to progenitor cells (Gallager *et al* 1987).

Microheterogeneity is hence a general feature of N-glycosylation. The factors that determine the range of structures found at an individual site are complex, and involve contributions from the polypeptide, the cell type, and the differentiation state of the cell. At present the functional importance of site heterogeneity is not fully appreciated, but evidence is emerging that the glycans can modulate function so that a glycoprotein may display a spectrum of activity which correlates with the glycotype. As with most post-translational modifications, the importance of this may not be universal, but in those cases where glycosylation is important, the heterogeneity may be crucial for function. The differences in glycoprotein glycans observed as isolated from various tissues is evidence that the functional demands made on a glycoprotein in separate tissues may vary, and that the carbohydrate moieties allow the same polypeptide to be utilised in more than one biological context.

1.4 The Functions of N-linked Glycans

Oligosaccharides have been proposed to have numerous roles in protein function, from physicochemical effects due to the chemical nature of the sugar chain, to roles in the binding of a glycoprotein to a receptor

molecule, where a specific sequence is important. Various aspects of N-linked oligosaccharides as functional components of a glycoprotein are considered below.

1.4.1 *Physicochemical and Kinetic Effects*

Solubility and stability of glycoproteins is influenced by the incidence and quality of glycosylation as carbohydrate chains are extremely hydrophilic, and can contribute significantly to the overall charge of a protein, e.g. glycophorin (Krotkiewski 1988). The addition of simple sugars to aqueous protein solutions has been observed to stabilise proteins as well as to favour self-association of the protein molecules (Arakawa and Timasheff 1982). The effect of covalently bound carbohydrates varies with the protein under study and is dependant on temperature (Lawson *et al.* 1983). Desialylation of an IgG protein (Grn) increased solubility in water, but similar treatment caused a mild decrease in solubility of an IgM (Hug). Removal of more carbohydrate by enzyme treatment decreased the solubility of both immunoglobulins. Thus the effect on solubility is a complex phenomenon, and depends on the nature of both the carbohydrate and the protein.

The effect of N-linked carbohydrate on aggregation has been studied recently for *Saccharomyces cerevisiae* invertase, which in the wild-type strain forms an octamer. The presence of N-linked glycans appears to facilitate oligomerisation (Esmon *et al.* 1987, Tammi *et al.* 1987). Removal of carbohydrate from human thyroxine binding globulin resulted in loss of stability and also affected the hydrodynamic properties of the protein (Grimaldi *et al.* 1985). The binding constant for the ligand thyroxine was

N-GLYCANS AS COMPONENTS OF PEPTIDE REGULATORY FACTORS

System	Reference
Granulocyte Macrophage Colony Stimulating Factor	
(i) Removal of N-glycans increases potency, but does not alter the effects or cell tropism	(Kaushansky *et al.* 1987)
Interferon β	
(i) The nonglycosylated protein is inactive due to insolubility	(Conradt *et al.* 1987)
(ii) The N-glycans are different when the protein is produced in different cell lines and this may affect function	(Kagawa *et al.* 1988)
Erythropoietin	
(i) Inactive when expressed in *E.coli* (non-glycosylated), but active when expressed in CHO cells (glycosylated). Desialylation of CHO produced factor inactivates it	(Sasaki *et al.* 1987)

Table 1.1 Some examples of the roles of N-glycans as components of peptide regulatory factors. No evidence is availible for a direct role of carbohydrates in the functions of these molecules (Metcalf 1989), but the glycan component appears to be important in controlling solubility and stability of the polypeptide.

only slightly altered. Thus it is well documented that carbohydrate can affect the properties of proteins in solution, and this effect is probably a primary function of glycosylation. In addition, protection from proteolysis by carbohydrates seems to be a general phenomenon, and probably arises by steric hinderance of enzymes to cleavage sequences in the glycoprotein. Evidence is accumulating that whilst the N-glycans of the small peptide lymphokines and growth factors are not involved directly in receptor binding, the presence of glycans is essential for maintainance of the native conformation of the peptide (Metcalf 1989 and see table 1.1).

Terminal galactose residues on O- and N-linked glycans have a profound influence on the half-life of serum glycoproteins by being ligands for the hepatic asialoglycoprotein receptor (Ashwell and Hartford 1982). The aquisition of complex glycans results in a great decrease in the clearance rate of a protein from the circulation (Gross *et al.* 1987, 1988). Exposure of cryptic α-galactose determinants may be a mechanism for the removal of aged or abnormal erythrocytes from the circulation in the human (Galili *et al.* 1986).

1.4.2 *Glycoconjugates in Receptor-Ligand Interactions*

Receptor-ligand interactions have been found to be influenced by carbohydrate (table 1.2). For the epidermal growth factor receptor, glycosylation itself is required for maturation of the receptor, and aquisition of ligand binding, whilst the precise glycan structure can influence the affinity of the receptor for its ligand. For the glycohormones hCG, LH and FSH, the presence of N-linked glycans is essential for bioactivity without affecting receptor binding. In this

GLYCOCONJUGATES AS MEDIATORS OF LIGAND INTERACTIONS

System	Reference
Epidermal Growth Factor Receptor	
(i) Culture of cells in the presence of tunicamycin ablates ligand binding	(Sleicker and Lane 1985)
(ii) Endoglycosidase H treatment of the mature receptor does not affect ligand binding, but glycosylation is important in receptor maturation	(Sleicker *et al.* 1986)
(iii) Absence of blood group A increases concentration of high affinity receptors and tyrosine kinase activity, and decreases half-life	(Defize *et al.* 1988)
Glycohormones	
(i) N-glycans essential for stimulation of adenylate cyclase, but not for receptor binding of hCG	(Calvo and Ryan 1985)
(ii) Similar effects in ovine lutropin	(Marijunath *et al.* 1982)
(iii) Alteration of glycans modulates bioactivity of follitropin	(Dahl *et al.* 1988)
Transforming Growth Factor β	
(i) Synthesised as latent complex bound to cell surface	(Sporn *et al* 1987)
(ii) Activated by neuraminidase or N-glycanase treatment	(Miyazono and Heldin 1989)
Interleukin 1 (IL 1)	
(i) N-linked oligosaccharides are involved in the interaction between IL 1 and uromodulin	(Sherblom *et al.* 1988)
β-Galactosyltransferase	
(i) Surface expressed β-galactosyltransferase acts as a receptor for glycoconjugates on ovum surface	(Shur and Neely 1988)
(ii) β-galactosyltransferase is important in murine morula compaction	(Bayna *et al.* 1988)
Microorganism adherance	
(i) *Leshmania donovanii*: macrophage adhesion	(Handman *et al.* 1987)
(ii) *E. coli* fimbral lectin binds to host cell glycolipids	(Kallenius *et al 1981*)
(iii) *Rhizobium* recognition by a legume lectin	(Bauer 1981, Dazzo *et al.* 1983)

Table 1.2 Examples of the involvement of glycoconjugates in ligand-receptor interactions. See text for discussion.

system the precise glycan structure may be important, as in malignancy both oligosaccharide structure and bioactivity are altered (Mizuochi *et al.* 1983). Transforming growth factor β (TGFβ) is retained at the cell surface as an inactive complex, but removing the N-linked oligosaccharides from other proteins in the complex activates the factor. It is possible that TGFβ is held in the complex by binding to the carbohydrate, or that deglycosylation results in a conformational change allowing the TGFβ to be released.

Glycoconjugates have been heavily implicated as mediators of cell adhesion, and in particular, in lymphocyte migration. A number of saccharides and exoglycosidase treatments can influence lymphocyte adherance to endothelia (table 1.3). The recent elucidation of the primary structure of a number of these molecules has added weight to the hypothesis that glycans are important ligands for the adhesion molecules, as the mammalian lectin domain appears to be a common feature of the polypeptide sequences available so far (Stoolman 1989).

There are a number of cases where the recognition of glycans is a problem. For example, autoimmunity in Chagas disease, a result of infection with *Trypanosoma cruzi*, is thought to be due to cross reaction between α-galactose residues on the parasite surface proteins and on basement laminin (Towbin *et al.* 1987). This example serves to illustrate that glycans are often highly immunogenic, and can elicit recognition of antigens which are otherwise not seen, i.e. breaking tolerance. This point is very important in the potential use of glycoproteins as clinical reagents, where a similar cross reaction may be elicited.

INVOLVEMENT OF GLYCOCONJUGATES IN LYMPHOCYTE MIGRATION

(i) Mannose, fucose and polyphosphated or polysulphated saccharides inhibit rat lymphocyte adhesion to endothelia	(Stoolman and Rosen 1983, Stoolman *et al.* 1984)
(ii) Neuraminidase treatment of HEV inhibits lymphocyte adhesion in mice	(Rosen *et al.* 1985)
(iii) Lymph node HEV antigen Mel 14, identified as a lymphocyte receptor, has a lectin domain	(Siegelman *et al.* 1989)
(iv) Neutrophil to endothelial adhesion molecule, ELAM-1, contains a lectin domain similar to Mel 14	(see Stoolman 1989)

Table 1.3 Some examples of data that implicate glycoconjugates as mediators of lymphocyte migration events. The putative glycan ligands in this system have not been identified, but at the time of writing this area is moving very rapidly, with the identification and cloning of a number of other molecules considered to be lymphocyte adhesion molecules.

1.4.3 *Protein Secretion and Intracellular Targeting*

The path that a protein takes through the Golgi is clearly influenced by the glycans that it carries. For example the presence of mannose-6-phosphate as part of an N-linked oligosaccharide of a protein in the Golgi will always target the glycoprotein to the lysozomal organelle due to interaction with the mannose-6-phosphate receptor (Kornfeld and Kornfeld 1985). However the addition of a phosphate group to the oligomannose glycan is dependant on some feature of the polypeptide, which facilitates interaction with the relevant processing enzymes, and therefore, whilst the recognition signal is carbohydrate, the presence of mannose-6-phosphate is controlled by the polypeptide and its interaction with the biosynthetic pathway.

Glycosylation may also be important for correct secretion. The heavy chain binding protein, BiP or HSP60 (Munro and Pelham 1986) is able to prevent the exit of a variety of proteins from the ER unless glycosylation has occurred (Dorner *et al* 1987). This phenomenon may be due to the availability of a BiP binding site on the polypeptide that becomes masked upon addition of the glycan. Chaparone proteins like BiP may be a common feature of several intracellular processes. (Verner and Schatz 1988). Deletion of Asn from the sequon in the β-chain of hCG reduced the rate of secretion of the glycoprotein (Matzuk and Boihme 1988). However, the effect of glycosylation on secretion cannot be considered to be a fundamental aspect of control as cells cultured in the presence of tunicamycin are still able to secrete proteins that are totally devoid of N-linked carbohydrate, albeit often at reduced efficiency. In the case of immunoglobulin G it has been reported that tunicamycin treatment results in

Fc tyrosine sulphation, and this alternate modification may compensate for loss of the carbohydrate (Baeuerle and Huttner 1984). Therefore, correct glycosylation ensures efficient secretion and targetting of proteins in the cell, probably partly due to solubility considerations as well as the ability to be recognised by the relevant proteins responsible for sorting of the glycoproteins into the correct compartment.

Therefore the N-glycans of a variety of proteins have been implicated as having biological function. It is possible that oligosacchardes have been exploited during evolution to assist in the roles of proteins, and this may explain why a single, or group of, function(s) cannot be universally ascribed to N-linked oligosaccharides. As the number of contexts in which a glycan can be functionally important has increased during evolution, the original reason why glycosylation provided a selective advantage may have become overshadowed by the more complex roles that have been aquired, as exemplified by the examples described above.

CHAPTER TWO

ANALYSIS OF THE HNK-1/L2 EPITOPE OF NEURAL GLYCOPROTEINS

2.1 *Preamble*

A number of cell adhesion molecules (CAMs) have been demonstrated to carry a carbohydrate epitope recognised by the HNK-1 (Leu-7) monoclonal antibody, originally raised against the surface of a subset of human natural killer cells (NK cells) (Abo and Balch 1981, Abo *et al.* 1982, Shachner *et al.* 1983, see table 2.1). The antibody recognises most central nervous system (CNS) neurones as well as a number of other cell types. The epitope appears to be associated with the process of neurone migration. The structure and function of the HNK-1 determinant are discussed below, emphasising that in glycolipids the presence of 3'-SO_4-Glucuronic acid appears to be crucial for binding to the antibody. The L2 series of monoclonals also react with the HNK-1 epitope (Kruse *et al.* 1984). Enzymatic removal of N-linked glycans has been found to result in a loss of HNK-1 reactivity against a glycoprotein previously bound by the antibody (e.g. Shashoa *et al.* 1986). As glucuronic acid has not reliably been shown to be a component of N-linked oligosaccharides, and much of the work reported in the literature is potentially contradictory, it is important to establish the structure of the HNK-1 epitope on a glycoprotein.

Two routes have been taken to approach the structural elucidation of the HNK-1/L2 epitope. The first was focused on a study of the N-linked

DISTRIBUTION OF THE HNK-1/L2 DETERMINANT AMONGST NEURAL CELL ADHESION MOLECULES

Cell Adhesion Molecule (CAM) or other	Adhesion Event Mediated	Epitope(s)	Reference*
NCAM (BSP-2/D2) (Neural Cell Adhesion Molecule)	N-N/A-N/A-A/A-ECM <HOMOPHILIC>	HNK-1/L2 (20%)	1
L1 (NILE/Ng-CAM E-cadherin)	N-N	HNK-1/L2 +/or L3	2
MAG (Myelin Associated Glycoprotein)	N-O/O-O/N-ECM	HNK-1/L2 +/or L3	3
J1 (Cytotactin/ Tenascin	N-A	HNK-1/L2	4,5
Po	Peripheral Myelin Compaction	HNK-1/L2 or L3	6
Chondroitin Sulfate Proteoglycan	N-ECM	HNK-1/L2	7

Table 2.1 Neural cell adhesion molecules in vertebrates.
The list of molecules is not exhaustive, and is restricted to those molecules that are well defined. Alternative terminology is given in parenthesis. The existence of several names for the same molecule reflects the fact that it was isolated from different species by independent workers, with identity only fully realised following primary sequence determination or detailed immunochemical analysis. The L3 epitope is not discussed in this thesis but several members of the L2 family also carry this putative carbohydrate determinant (Kücherer *et al.* 1987). The structural identity of L3 has not been established.

* References; the full citation can be found in the bibliography at the end of this thesis.

1 Niele and Schachner (1985)
2 Murray and Strecker (1984)
3 Kruse *et al.* (1984)
4 Kruse *et al.* (1985)
5 Faissner *et al.* (1985)
6 Bollensen and Schachner (1987)
7 Hoffman and Edelman (1987)

Abbreviations: N-N neurone to neurone adhesion; N-A neurone to astrocyte adhesion; A-A astrocyte to astrocyte adhesion; N-O neurone to oligodendrocyte adhesion; O-O oligodendrocyte to oligodendrocyte adhesion; N-ECM neurone to substratum/extracellular matrix adhesion.

glycans of the human myelin glycoprotein, Po. This protein was chosen for the following reasons; (1) it is the major glycoprotein in human peripheral myelin and can be isolated relatively easily, (2) it carries the HNK-1/L2 determinant (Bollensen and Schachner 1987), (3) it possesses a single N-linked glycosylation site, and thus affinity purification of Po with L2 antibodies will result in minimal contamination with unreactive glycans, (4) no O-glycosylation has been reported for this molecule, and (5) the glycoprotein has been implicated as a cell adhesion molecule. The second approach was to characterise the whole spectrum of N-linked oligosaccharides from murine central neural tissue in an attempt to delineate the gross features of neural glycans, especially with respect to sulphation of the saccharides. Murine brains were chosen as a source because (1) the mouse has the HNK-1 epitope, (2) material can be easily obtained, and (3) many defined CAMs have been studied in the mouse.

The N-glycans from murine brain were shown to carry several anionic moieties, and these were quantified. There was a high incidence of sulphated oligosaccharides. Metabolic labelling of murine brain N-glycans with $^{35}SO_4$ allowed a preliminary analysis of the sulphated glycans to be undertaken, and identified a glycosaminoglycan-like component in the hydrazine-released material. Composition data for this material is given. The characterisation of the N-linked glycans from L2 reactive Po is also presented. From the data on the Po glycans the following conclusions can be drawn; (1) the Po glycans are heavily sulphated, (2) the glycan population is highly heterogenous, consisting of oligomannose, hybrid, complex and repeat lactosamine containing glycans, and therefore there are many glycoforms of Po, and (3) glucuronic acid is not a component of the Po glycans, and thus in this case cannot be a part of the glycoprotein

determinant which binds to L2 antibodies. Possible explanations for the L2 reactivity of the Po N-glycans are discussed.

2.2 Introduction

2.2.1 *The HNK-1/L2 Epitope in Lipids*

Structural studies of the HNK-1/L2 epitope have been mainly based on the fact that the antibodies are able to bind to a specific glycolipid in addition to the glycoproteins listed in table 2.1. The glycolipids shown to carry the HNK-1 epitope were originally studied because some patients with demyelinating diseases were found to possess IgM paraproteins that bound to unusual glycolipids carrying a sulphated glucuronic acid (Chou *et al.* 1985). The HNK-1 antibody also bound to the same lipids, but with slightly different structural requirements. These lipids have been found in peripheral nerve (Chou *et al.* 1985) as well as central nervous system tissue (Chou *et al.* 1986). More recently Arita *et al.* (1987) have detected a series of these lipids with the following common structure;

$$SO_4\text{-}3\text{-}GlcA\beta1\rightarrow3Gal\beta1\rightarrow(4GlcNAc\beta1\rightarrow3Gal\beta1\rightarrow)_n4Glc\beta1\rightarrow1Ceramide$$

where GlcA is glucuronic acid. Lipids have been identified where n=1 or n=2, and both of these moieties can be bound by the IgM paraproteins. These lipids are termed sulphonylglucuronylneolactoglycolipids (SGGLs). The SGGLs are not the only glycolipids that IgM paraproteins from humans with neuropathies can react against; for example, Illyas *et al.* (1988) have found IgMs that react with glycolipids bearing terminal GalNAcβ residues.

Structural investigations into the SGGLs have been very thorough and thus there is excellent evidence that these glycans are capable of binding to the HNK-1/L2 antibodies.

Several groups have investigated the expression of HNK-1 reactivity in neural tissue. Chou *et al.* (1987) examined the expression of the SGGL septasaccharide (n=2) in dog, rat and human, and followed the temporal expression of the lipid in the rat. SGGL expression is developmentally regulated and is greatest when neurones are migrating (see below). In rats the SGGL is not seen in the adult cortex but is present in the peripheral nervous system (PNS). Expression of the SGGLs continues in the adult cerebellum of the mouse, and their absence appears to correlate with Purkinje cell degeneration as found in the mutants *lurcher* and *staggerer* (Chou and Jungalwala 1988).

2.2.2 *Expression of the HNK-1/L2 Epitope in Lipids and Proteins; Is the Epitope Structure the Same?*

Cross reactivity of HNK-1 with an IgM paraprotein determinant was first noted by Murray and Stecker (1984) where it was shown that IgM paraproteins from neuropathy patients bound to the same peptides as HNK-1 when myelin associated glycoprotein (MAG) was digested with proteases. All IgM paraproteins that show HNK-1 like activity against MAG also bind to the adhesion molecules N-CAM and L1 (Tucker *et al.* 1987). A considerable problem with investigating the HNK-1/L2 epitope on glycoproteins is the use of a number of monoclonals that are assumed to bind the same determinant, based on histochemical and cross-reactivity data (see Bon *et al.* 1987 for a list of these antibodies), but without a full characterisation of their

binding requirements. Therefore, whilst it has been a tacit assumption that the epitope as detected on the SGGLs with HNK-1 is also present on the glycoproteins that bind HNK-1 and L2, this has not been proven. For example, Schwarting *et al.* (1987) followed HNK-1 epitope expression in both glycolipids and glycoproteins from the mouse. They also used a further monoclonal, 4F4, which exhibited identical staining patterns to HNK-1 in immunohistochemical studies. The reactivity of glycoproteins with HNK-1 was taken as evidence that GlcA is a component of glycoprotein carbohydrate.

Treatment of HNK-1 reactive glycoproteins with 'Glusulase', a commercial preparation of β-glucuronidase and arylsulphatase from *Helix pomatia,* resulted in the loss of HNK-1 binding (Schwarting *et al.* 1987). However, as this preparation is highly impure other glycosidases may have been present, and the individual contributions of sulphate and GlcA to the HNK-1 epitope could not be assessed. Yamamoto *et al.* (1988) examined the expression of HNK-1 reactive material in the adult mouse central nervous system (CNS), where HNK-1 reactivity appeared to overlap with γ-aminobutyric acid containing neurones. Here the authors assume that 4F4 and HNK-1 antibody reactivity aginst a glycoprotein can be considered diagnostic for the presence of glucuronic acid and sulphate containing glycoconjugates. Dennis *et al.* (1988) observed binding of an L2 monoclonal antibody to acidic glycolipids in the CNS of *Calliphora vicina.* The antibody binding was not altered by mild methanolysis, but desulphation may not have been complete. Dennis *et al.* (1988) also showed that the HNK-1 epitope was present on glycoproteins of the neural tissue of *C. vicina.* As insects appear to produce N-linked oligosaccharides of the oligomannose type only, a structure similar to that on the SGGLs could not be responsible for the presence of the HNK-1 epitope in this case.

Apart from the molecules listed in table 2.1 and the *C. vicina* neural glyoproteins, the HNK-1 epitope has been found on a number of glycoproteins, e.g. acetylcholinesterase molecules from the electric organs of *Electrophorus electricus* and *Torpedo marmorata* (*Bon et al.* 1987), glycoproteins from rat and goldfish brain, PC12 cells and bovine chromaffin granules (Margolis *et al.* 1987). HNK-1 binding was also observed with chondroitin sulphate proteoglycans from rat brain, Swarm rat chondrocarcinoma cells and bovine nasal tissue. The epitope was not sensitive to chondroitinase ABC digestion, indicating that it was present on N- or O-linked carbohydrate (Margolis *et al.* 1987). Evidence for only one HNK-1 reactive glycoprotein possessing glucuronic acid as a constituent of N-linked glycans has been presented, the goldfish brain ependymins (Shashoa *et al.* 1986). Although N-glycanase treatment of the ependymins caused a loss of HNK-1 binding, this was not shown to correlate with removal of GlcA, and therefore the GlcA was not necessarily covalently linked to the glycoprotein.

Mikol *et al.* (1988) showed that HNK-1 reactive polypeptides in the human CNS show a decrease in molecular weight with age. E and L phytohaemagglutinin bound to these polypeptides and to proteolytic fragments generated from the reactive proteins. This implies that a triantennary glycan carries the epitope. The HNK-1 reactivity was sensitive to endoglycosidase F, but not endoglycosidase H, confirming the assignment of the HNK-1 reactive glycans as complex type oligosaccharides.

Despite a large number of studies on HNK-1 reactive glycoproteins the structure of the HNK-1 determinant on proteins has not been fully elucidated. Most of the studies cited above are consistent with the view that the epitope does not contain GlcA, but still reacts with HNK-1. To

unequivocally delineate the HNK-1 reactive N-linked glycans from a glycoprotein these saccharides must be subjected to detailed structural characterisation. Ideally, the glycoprotein chosen for study must not carry other carbohydrates, i.e O-linked mucins or glycosaminoglycans to circumvent the possibility that HNK-1 binding could be due to other carbohydrate structures.

2.2.3 Functional Role of the HNK-1/L2 Epitope

Schachner *et al.* (1985) showed that the monoclonal antibodies L2 and HNK-1 compete with each other for binding to postnatal cultured murine cerebellar cells. Sequential immunoprecipitation demonstrated that only some of the N-CAM and L1 molecules present on these cells carried the L2 epitope. Using mouse cerebellum explants it was shown that whilst L2 monoclonal antibody Fab fragments did not inhibit neurone to neurone adhesion, they did decrease binding of neurones to astrocytes and astrocytes to astrocytes (Schachner *et al.* 1985). Kunemund *et al.* (1988) have demonstrated that L2 monoclonal antibody Fab fragments prevent migration of cells from cerebellar explants of 6 day old mice as well as neurite extension. The glycan SO_4-3-GlcAβ1→3Galβ1→4GlcNAcβ1→3Gal (the L2 tetrasacchride) had the same effect as the L2 Fabs, whilst sulphatide, SO_4-3-Galβ1→1-Ceramide, and heparin could also inhibit both neurite extension and cell adhesion. As N-CAM possesses a heparin binding site that is involved in neural cell-substratum adhesion (Cole and Glasier 1986), this may in part explain the effects of heparin in this system. Both heparin and sulphatide show structural similarities to the L2 saccharide and desulphation of the L2 glycan or sulphatide abolished the activity of

these compounds. Not all sulphated glycans. e.g. chondroitin sulphate, were active in these experiments. The effect of the L2 saccharide was more pronounced in inhibition of cell to substrate adhesion when compared to cell to cell adhesion, which supports a role for the L2/HNK-1 determinant in cell migration. The HNK-1 antibody can inhibit the adhesion of embryonic mouse neurones to the extracellular matrix (Riopelle *et al.* 1986) and the L2 antibody binds to the neurone cell membrane at areas that are opposite to astrocyte processes (Yamamoto *et al.* 1988). The temporal and spatial regulation of the HNK-1 epitope are consistent with the proposal that the HNK-1 epitope is important in neurone migration (Schwarting *et al* 1987). Therefore *in vitro*, and probably *in vivo* the HNK-1/L2 determinant is influential in the migration of CNS neurones and the extension of neurites, and the presence of a sulphate ester appears essential for activity. The observation that sulphatide has similar effects to the L2 glycolipid indicates that the cellular receptor for the L2 glycan may recognise a simple monosaccharide sulphate.

Schwarting and Yamamoto (1988) have proposed a mechanism for the regulation of the HNK-1 epitope *in vivo*. The temporal regulation of the Lewis X (Le^x, Fucα1→3Galβ1→4GlcNAc-R) antigen shows similar temporal regulation to the SGGLs, in that Le^x glycolipids are only observed in embryonic tissue, but the Le^x determinant persists into the adult as a component of glycoproteins. Also the embryonic spatial expression appears to be complementary, and Le^x appears to be expressed predominantly on premigratory cortex cells, whilst the HNK-1 epitope is present on migrating cells. Both of these antigens could be derived from a common precursor saccharide, lactoneotetraose (LNT) and biosynthetic control of these lipid antigens could be tightly coupled.

2.2.4 *Po Glycoprotein*

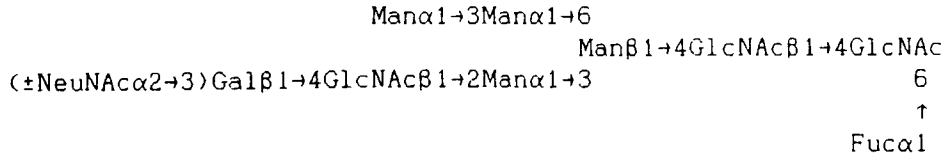
Primary polypeptide structures of the bovine (Sukamoto *et al.* 1987) and rat Po (Lemke and Axel 1985) proteins have been presented. The protein has a molecular weight of 29 000 daltons, an extracellular N-terminal domain, a hydrophobic transmembrane sequence, and a C-terminal cytoplasmic domain. The amino acid sequences are highly homologous, with only 15 differences over a total of 219 amino acids. The primary structure shows that the N-terminal domain of Po belongs to the same subset of the immunoglobulin superfamily as CD4 and the poly-Ig receptor (Lai *et al.* 1987, Lemke *et al.* 1988), and as this domain is external, the probable secondary structure of the protein is consistent with a role as an adhesion molecule (Edelman 1987). There is a single N-linked glycosylation site at Asn93 in both proteins. The N-terminal domain appears to be slightly hydrophobic, which may be offset in part by the presence of an oligosaccharide. The C-terminal domain is somewhat basic, and this has been proposed as a possible mechanism to facilitate binding to the opposite surface of the myelin lamellae via ionic interactions with acidic phospholipid head groups (Lemke and Axel 1985). In the rat there appears to be only one gene per haploid genome, and a single mRNA species was detected throughout development and adult life. The mRNA levels parallel the myelin synthesis rate. The gene has recently been isolated from rat and mouse (Lemke *et al.* 1988). The protein is acylated, but at an unknown site (Agrawal *et al.* 1983). Po expression is confined to peripheral myelin and is driven by a highly cell-specific promotor (Lemke *et al.* 1988).

Trapp (1981) observed that Po expression began in the developing rat at the point where Schwann cell membranes became intimately associated with

axon membranes, and that Po insertion into the myelin membrane of the rat and cat is associated with compaction (Trapp 1988, Willison *et al.* 1987). Synthesis of Po increases greatly from birth to about day 10 of age in the rat and is maximal at day 5. Incorporation of ^{32}phosphate and ^{3}H-fucose are greatest at this time (Wiggins *et al.* 1980) and incorporation of ^{3}H-leucine decreases markedly between days 10 and 25, reflecting the synthesis rate of myelin itself (Toews *et al.* 1987). ^{35}Sulphate could be incorporated into the protein before insertion into the membrane and was turned over with a ^{3}H-leucine label, demonstrating that sulphation is not transient. Phosphorylation, presumably by protein kinase C, of the polypeptide can occur when the protein is *in situ* in the membrane (Brunden and Poduslo 1987).

Bollensen and Schachner (1987) demonstrated that the HNK-1/L2 epitope was present on Po. In addition, Po can bind IgM paraproteins, indicating that the HNK-1 epitope is present (Martini *et al.* 1988). As the mammalian neural glycoproteins that carry the HNK-1/L2 determinant have all been shown to have a potential role in cell-adhesion, the occurence of this epitope strengthens the contention that Po is an adhesion molecule (Bollensen and Schachner 1987). In co-localisation studies Martini *et al.* (1988) could not correlate Po and L2 expression, casting some doubt on the role of the glycan, but not necessarily on the role of Po as a CAM.

Sakamoto *et al.* (1987) proposed a common N-linked oligosaccharide structure for bovine Po;



with heterogenous sulphation of the glycan. The positions of the sulphate esters on the glycan were not identified. Carbohydrate composition data for lapine Po were obtained by Ishaque *et al.* (1980) and were consistent with a similar *average* structure as that found on the bovine molecule. No evidence has been presented for O-linked glycosylation of Po.

Clearly, the studies cited here show that HNK-1 and L2 monoclonal antibodies are able to bind to the Po glycan, but as only an average structure has been proposed for the whole population of Po molecules present in myelin, it is not possible to make predictions about the determinant recognised by HNK-1. In a similar manner as discussed above for N-CAM, not all Po molecule may bind to HNK-1 or L2 antibodies, and therefore it is necessary to investigate specifically the L2 reactive fraction of Po to determine the structural requirements for binding of glycoprotein oligosaccharides to HNK-1 and L2 antibodies.

2.2.5 *N-linked Oligosaccharides Substituted With Sulphate Esters*

Although it has become evident that N-linked carbohydrates from a number of secretory and membrane glycoproteins are sulphated, the delineation of the structural and enzymatic requirements for sulphation are only beginning to be addressed. In the case of the pituitary glycohormones, a specific residue, terminal GalNAc, is required for sulphation (Green *et al.* 1986), but in most cases a novel residue is not present and it appears that the addition of sulphate occurs either in competition with other capping groups, i.e. sialic acid or α-galactose (Spiro and Bhoyroo 1988), or without competing directly with other substituents, e.g. the sequence identified by Roux *et al.* (1988) (table 2.2). Therefore the incidence of

DISTRIBUTION AND LINKAGE OF SULPHATE ESTERS AMONGST N-LINKED OLIGOSACCHARIDES

Linkage	Glycan Type[a]	Organism/Tissue/Cell	Reference
N,K,	Various	Viral envelope glycoproteins	Pinter and Compans (1975)
Mannose-6-SO_4-R	N,K,	D, discoidium	Freeze and Wolgaust (1986) Freeze *et al*, (1983)
Mannose-4-SO_4	Hybrid	Chick ovalbumin	Yamashita *et al*, (1983b)
N,K,	Various	Various mammalian cell line surface glycoproteins	Freeze and Varki (1986)
SO_4-6-GlcNAcβ-*	Complex	Bovine endothelia	Roux *et al* (1988)
Various	GAG chain containing	Bovine endothelia	Sundblad *et al*, (1988)
SO_4-6-GlcNAcβ-*	Complex	Porcine Thyroglobulin	Kamerling *et al*, (1988)
SO_4-3-Galβ1→4R	Complex	Porcine Thyroglobulin	Kamerling *et al*, (1988)
SO_4-3-GalNAcβ-R	Complex	Ovine, bovine and human LH and FSH	Green *et al* (1986) Green *et al*, (1988a,b)
SO_4-3-Galβ1→4-R	Complex	Bovine, human Thyroglobulin	Spiro and Bhoyroo (1988)
SO_4-GlcNAcβ1→R	Complex	Human endothelia	Heifertz *et al*, (1982)

Table 2,2 Examples of the distribution of sulphated N-linked oligosaccharides, Where known the linkage position is also given,
N,K, not known, R denotes the rest of the glycan, and the sequence shown is terminal,
* The sulphate is found to occur as part of the terminal sequence
NeuNAcα2→3(6)Galβ1→4(SO_4→6)GlcNAcβ1→4,
[a] i,e complex, hybrid, glycosaminoglycan (GAG),

sulphate esters is controlled by the absolute activity of a sulphotransferase and by competition between the sulphotransferase and other enzymes capable of catalysing additions to the same positions. For example, human thyroglobulin has a higher incidence of sulphated glycans than the calf glycoprotein, which is probably due to the absence of a competing α-galactosyltransferase in the human (Spiro and Bhoyroo 1988). Sulphate is added from 3'-phosphoadenosine 5'-phosphosulphate (PAPS), and some sulphotransferases have been characterised (Smith and Baenziger 1988, Kato and Spiro 1989). Some examples of the structures of sulphated glycans are given in table 2.2. It should be noted that in the mammal sulphate is primarily added to galactose and N-acetylglucosamine residues.

The HNK-1 epitope could clearly be carried by various glycans. No hard and fast rule for the structures of sulphated glycans can be drawn from the examples given in table 2.2 that set them apart from nonsulphated oligosaccharides. Also, the large number of proteins, from very different sources, that bind HNK-1 suggests that HNK-1 can recognise a variety of saccharides. It should be noted in this regard that the occurence of sulphate esters as constituents of otherwise standard N-linked glycans is consistent with much of the data discussed above, i.e. it has not been rigorously demonstrated that HNK-1/L2 binding N-glycans possess novel substituents, and that sulphation similarly is not reliant on the presence of unusual residues.

2.2.6 *Methodology; Removal of Sulphate and Phosphate Esters*

In order to elucidate the structures of the Po glycans and to appreciate the endemic distribution of sulphate esters in CNS N-glycans it

is necessary to be able to specifically remove these moieties. A number of methods for desulphation are available, but many of these have been used against glycosaminoglycans and glycolipids rather than N-linked oligosaccharides. Five methods were chosen for study and are detailed below (section 2.3.9). Alkaline treatment was rejected at the outset because the reaction involves the formation of an epoxide that results in epimerisation of the hydroxyls involved in the reaction (Percival 1980) altering the structure of the glycan. The kinetics of release of sulphate from glycans by chemical methods has been used to determine linkage position (Freeze and Wolgast 1986, Rees 1960). Enzymatic removal is not feasible at the present time because the enzymes that are commercially available are of low purity and are not characterised (Roy 1987), although a number of specific sulphatases have been characterised (e.g. Freeman and Hopwood 1987, Gibson *et al.* 1987, Wagh *et al.* 1987).

Removal of phosphomonoesters is less difficult as alkaline phosphatase can be used, but phosphodiesters are not sensitive to this enzyme. Mild acid hydrolysis has been used to remove one substituent from the phosphate (Mizuochi *et al.* 1981) and render it sensitive to alkaline phosphatase. However acid resistant phosphodiesters can occur as substituents of oligosaccharides (e.g. Freeze *et al.* 1983) and so this method is not universally applicable. The use of 50% aqueous hydrogen fluoride against phosphodiesters in oligosaccharides has been reported (Jansson *et al* 1981, Ferguson *et al.* 1988) and so the possibility of applying this method to N-linked oligosaccharides was investigated.

2.3 *Methods and Materials*

2.3.1 *Materials*

Human Po glycoprotein and human Po affinity purified on an L2 monoclonal antibody column were the gift of Prof. M. Schachner, Heidelberg. L2 reactive Po was prepared by the method of Bollensen and Schachner (1987) as described below. L2 monoclonal antibody (492) was also the gift of Prof. Schachner. Whole murine brain ^{3}H reduced oligosaccharides were isolated as described in appendix one by Dr D. R. Wing of the Glycobiology Unit.

Monosaccharides, glucose 6-sulphate, N-acetyl glucosamine 3- and 6-sulphates were obtained from Sigma, Bio-Gel P6 was obtained from Biorad. Oligosaccharides were produced in the laboratory from the following sources; the oligosaccharide 13.5

Galβ1→4GlcNAcβ1→2Manα1→6
Manβ1→4GlcNAcβ1→4GlcNAcol
Galβ1→4GlcNAcβ1→2Manα1→3

was obtained from human serum transferrin (Sigma), by hydrazinolysis and neuraminidase treatment. Fucα1→6GlcNAcol was obtained from ovine IgG by hydrazinolysis and exoglycosidase digestion. Radiolabelled monosaccharides wer prepared by the author. Anhydrous dioxane was the gift of Dr S. Petursson, of the Glycobiology Unit.

2.3.2 Purification of L2 Reactive Po From Peripheral Nerve Myelin

Sciatic nerves were obtained from human cadavers showing no evidence of neurological disease at autopsy, and frozen at -70°C, 16-24 hr after death. Nerve tissue was homogenised in hypotonic buffer (1mM $NaHCO_3$, 0.2mM $CaCl_2$,

0.2mM $MgCl_2$, 1mM spermidine, pH7.9) and washed several times with this buffer. A crude membrane fraction was obtained following the method of Rathjen (1982). The crude membranes were extracted with solubilisation buffer containing 0.25% deoxycholate (DOC), 20mM Tris-HCl, 150mM NaCl, 1mM EDTA, 1mM EGTA, pH8.3. The detergent extract was clarified by centrifugation at 100 000g at 4°C for 1 hr and the supernatant was passed sequentially over a column of monoclonal antibody to myelin-associated glycoprotein and then over a monoclonal L2 antibody column. The columns were washed with solubilisation buffer containing 0.3M NaCl and bound material was eluted with 0.1M diethylamine, 0.1% DOC, 150mM NaCl, 1mM EDTA, 1mM EGTA, pH 11.5, concentrated by pressure dialysis and dialysed against 20mM Tris-HCl, 0.1% DOC and 150mM NaCl pH 7.3.

The material that was not bound by the anti-MAG column, but was bound by the L2 column and subsequently eluted under the conditions described (section 2.3.6) was shown to be Po and L2 reactive by western blotting. The material was lyophilised and then sent to the author. The received material was analysed by coomassie stained SDS-PAGE by the author and a single band at 29kdal was detected.

2.3.3 *Isolation, Purification and Analysis of N-linked Oligosaccharides From Po*

The N-linked oligosaccharides were released, purified and radiolabelled as described in appendix one. Two preparations of Po purified as described above were investigated. The first contained 150µg of protein and the second circa 400µg. Analytical methods were performed as described in appendix one.

2.3.4 *Isolation of L2 Reactive and Total Po Sulphated Oligosaccharides*

A third preparation of L2 reactive Po, and a preparation of total Po made by acid extraction of human sciatic nerve followed by precipitation of Po with sodium hydroxide were received. Analysis by SDS-PAGE with silver staining revealed several high molecular weight bands in addition to the 29 kdalton Po band. On account of the fact that these bands did not stain with coomassie blue, but were heavily stained with silver, it is probable that these contaminants represent proteoglycans. Therefore the individual Po preparations were further purified by the author using preparative SDS-PAGE. Three hundred micrograms of the material were mixed with an equal volume (250µl) of SDS-PAGE sample buffer containing 0.1% β-mercaptomethanol and boiled for five minutes. The solution was loaded onto a 15% discontinuous polyacrylamide gel (15 x 20 x 0.5cm) (Laemmli 1970) and electrophoresis carried out at 150V/150mA at 4°C for 4 hours. The gel was then stained with coomassie blue and the band migrating at 29kdalton was cut out and chopped into small pieces. The protein was electroeluted with a Biotrap apparatus using electrophoresis buffer, following the manufacturer's instructions. The eluted protein was then dialysed against 5% methanol (aq) in a flow dialyser with a 6kdalton cutoff membrane, and lyophilised. The oligosaccharides were isolated and radiolabelled (appendix one) and treated with *A. ureafaciens* neuraminidase. The glycans that were still acidic following neuraminidase were neutralised by methanolysis, and methyl esters hydrolysed as described below (section 2.3.10). Neutral glycans were then analysed by Bio-Gel P4 chromatography.

2.3.5 *Preparation of ^{35}S-labelled Mouse Brain Oligosaccharides*

^{35}S-Metabolically labelled oligosaccharides were obtained by intracranial injection of 25μCi carrier free ^{35}S sodium sulphate (Amersham International, UK) in 1μl of 0.85% (w/v) NaCl into the third ventricle of each of 40 juvenile mice (20 male, 20 female). The animals were left for 18 hours before sacrifice and dissection and removal of the whole brain. The brains were prepared and the oligosaccharides isolated as described in appendix one.

2.3.6 *L2 Affinity Chromatography*

The L2(492) monoclonal was coupled to cyanogen bromide activated sepharose as described (appendix one), at a final coupling density of 4mg protein/ml of sepharose. The gel was packed into a glass column and equilibrated in phosphate buffered saline (PBS). The column was pumped with a peristaltic pump at 12ml/hr and 4ml fractions were collected. Oligosaccharides were loaded onto the column at room temperature and the sugars allowed to interact with the gel for 1hr by stopping the flow. The column was then washed with PBS at 4°C and then bound material was eluted with 0.1M triethylamine containing 1mM EDTA, 1mM EGTA, pH 11.5. Radioactivity was then determined in the fractions by scintillation counting. The bound and unbound oligosaccharides were pooled seperately and desalted by passage through a column of Bio-Gel P4 (200-400 mesh), 8ml bed volume equilibrated in water. The radioactivity was pooled, dried by rotary evaporation and re-N-acetylated by the addition of 200μl saturated sodium bicarbonate and two aliquots of 10μl each acetic anhydride over an hour

The solution was desalted by passage through a 1ml column of dowex AG50 (H^+ form), taken to dryness by rotary evaporation and then analysed by paper electrophoresis in pyridine acetate buffer as described. Repassage of the unbound material did not result in the retention of further radioactivity by the column.

2.3.7 *Bio-Gel P6 Gel Filtration*

Oligosaccharides were separated by gel filtration on a 100 x 1.5cm Bio-Gel P6 column (200 mesh). The column was maintained at 50°C and with a mobile phase of 0.1M sodium acetate pH 5.5, containing 0.1% chlorbutol, at a flow rate of 12ml/hour. The eluant was monitored with an ERMA refractive index monitor. Fractions of 1ml were collected and the radioactivity in the fractions was determined by scintillation counting. The column was calibrated with various saccharide standards.

2.3.8 *Dowex Fractionation*

Murine brain glycans were fractionated by chromatography on AG1-X2 resin (Cl^- form) (Biorad), by a modification of the procedure of McCarthy and Baker (1979). Two millilitres of resin was packed into a Pharmacia HR5/5 column and the column was equilibrated with water. Murine brain oligosaccharides were applied onto the column in water and a linear gradient from 0 to 4M NaCl over 100ml was applied immediately. The mobile phase was pumped at a flow rate of 13ml/hr and 5 minute fractions were collected. Radioactivity in the fractions was determined by scintillation

counting. The column was calibrated with ^{3}H-N-acetylglucosaminitol-3-sulphate.

2.3.9 Sulphate Ester Removal

Ten micromoles of N-acetylglucosamine-3-sulphate and 10μM of glucose-6-sulphate, with scyllo-inositol included as an internal standard, were subjected to desulphation by five different methods. All samples were made up in water and freeze dried overnight in glass tubes equipped with a valve in an evacuated dessicator with a liquid nitrogen trap. The dessicator was re-pressurised with dry argon and the reactivalves closed. The reagents were introduced with a glass syringe and hypodermic needle through the valve. For methods that used a pyridine salt of the sugar as the starting material (c-e, below) 15μl of pyridine was added before freeze drying.

(a) Dioxane solvolysis (Ishizaka 1982); 500μl of anhydrous dioxane (stored over sodium wire) was added and the tubes were incubated at 100°C for two hours in a reactitherm (Pierce). The dioxane was removed by rotary evaporation and the sample worked up (below).

(b) Mild methanolysis (Kantor and Schubert 1957) was performed by adding 500μl of dry 50mM methanolic-HCl and incubation at room temperature for 24 hours. The reagent was removed by rotary evaporation and the sugars worked up. In addition, a sample containing 10μM of N-acetyl-glucosamine-6-sulphate and scyllo-inositol was included for analysis.

(c/d) Solvolysis in dimethylsulphoxide (Nagasawa *et al.* 1987, Freeze *at al.* 1982); the pyridine salt of the sugar was incubated with 500μl of dimethylsulphoxide (DMSO) containing 10% (v/v) methanol (method c) or 10% water (method d) at 100°C for 2 hours. The reagent was removed by reduced

pressure evaporation and the sample worked up.

(e) Pyridine/dimethylsulphoxide solvolysis (Usov *et al.* 1971); To the pyridine salt of the sugar 1ml of pyridine:DMSO (v/v) was added and the sample incubated at 100°C for 14 hours. The reagent was removed by reduced pressure evaporation and the sample worked up.

Work up and analysis. The dried sample was taken up in water and dried down by rotary evaporation. Saturated sodium bicarbonate (500µl) was added with two additions of 25µl of acetic anhydride over an hour. The reaction was carried out at room temperature. This solution was then passed through a column equilibrated in water containing (top first) 100µl chelex 100 (Na^+ form), 1000µl AG50 (H^+ form), 100µl AG3 (OH^- form) and 100µl QAE sepharose. The sample was filtered tthrough a 0.25µm PTFE filter (Millipore, UK) and dried. The amount of $(TMS)_6$-scylloinositol, $(TMS)_4$-N-acetylglucosamine and $(TMS)_5$-glucose released by each method was determined by direct derivatisation with 15µl of Sigma-Sil A (Sigma) and GCMS as described in appendix one for composition analysis. A control set of monosaccharides were included that were passed through a resin column and derivatised with Sigma-Sil A, and then analysed by GCMS.

Following the results from the study of sulphate removal methods the methanolysis protocol (b) was followed routinely. The method was also applied to oligosaccharide 13.5, Fucα1→6GlcNAc$_{2,1}$, reduced monosaccharide-sulphates and bovine sialyllactitol. Hydrolysis of carboxylic acid methylesters formed during methanolysis was performed by incubation with 200µl of 200mM NaOH in 90% methanol for 2 hours at room temperature. Analysis of the products was made by high voltage electrophoresis in pyridine acetate buffer as described (appendix one).

2.3.10 *Analysis of the Charged Moieties of Murine Brain Oligosaccharides*

The relative incidence of charged moities in the ^{3}H reduced whole murine brain oligosaccharides was assessed by sequential treatment with neuraminidase, mild acid, alkaline phosphatase, HF and methanolysis. Brain oligosaccharides that had or had not been passed through AG3 (OH^- form) were analysed. Analysis was performed by high voltage elctrophoresis in pyridine acetate buffer. The neutral material at the origin, and the anionic material were eluted seperately and the radioactivity determined by scintillation counting. The ^{35}S labelled oligosaccharides were treated with the neuraminidase, alkaline phosphatase and HF under standard conditions (appendix one) with the following exceptions:; for the neuraminidase digestion 1 unit of the enzyme in 1ml of 0.1M sodium acetate buffer pH 5.0 was used. For removal of phosphomonoesters 1U of the enzyme was used in a reaction volume of 250µl.

2.4 *Results*

2.4.1 *Analysis of Methods for Sulphate and Phosphate Ester Removal*

Three of the methods, b, c and d, (table 2.3) were found to be effective in removal of sulphate esters from the monosaccharides, and were each tested under identical conditions on 300 picomoles of radiolabelled Po desialylated acidic oligosaccharides (table 2.4). Clearly methanolysis provided the most efficient neutralisation with complex saccharides and therefore this method was used in subsequent experiments.

Quantitation of the efficiency of desulphation of monosaccharides by

SURVEY OF PROCEDURES FOR CHEMICAL DESULPHATION

Method*	Conditions used	Monosaccharide§	Effect
A	Anhydrous Dioxane	GlcNAc3S	-
		Glc6S	-
B	50mM Methanolic HCl	GlcNAc3S	+
		Glc6S	+
C	DMSO with 10% MeOH	GlcNAc3S	+
		Glc6S	+
D	DMSO with 10% H_2O	GlcNAc3S	+
		Glc6S	-
E	DMSO/Pyridine	GlcNAc3S	-
		Glc6S	-

Table 2.3 A method was considered as negative if the GlcNAc-$(TMS)_4$ or Glc-$(TMS)_5$ peak area was 2% or less of the scylloinositol-$(TMS)_6$ peak area by flame ionisation detection. Scylloinositol-$(TMS)_6$ was the only carbohydrate detected in the control samples.
Charged monosaccharides are not detected by the GC system because they are too polar to chromatograph. In addition, passage through mixed bed resins removes most of the sulphated monosaccharides before derivatisation.
* Method letter refers to the methods described fully in the text (methods).
§ Monosaccharide abbreviations GlcNAc3S; N-acetylglucosamine-3-sulphate, Glc6S; glucose-6-sulphate.

EFFICACY OF DESULPHATION PROCEDURES ON Po OLIGOSACCHARIDES

Method[a]		Percent Neutralised
B	Methanolysis	58.8
C	DMSO with 10% MeOH	29.0
D	DMSO with 10% H_2O	23.0

Table 2.4

[a]Full details of the methods can be found in the text. Following the treatment the glycans were re-N-acetylated as described and analysed by high voltage electrophoresis. The incidence of neutral glycans was determined by eluting separately the radioactivity that was at the origin (neutral) and the radioactivity that had migrated (acidic) and calculating the percentage at the origin of the total radioactivity returned from the paper.

EFFICIENCY OF NEUTRALISATION OF MONOSACCHARIDE-SULPHATES AND MONOSACCHARIDE ALDITOL-SULPHATES BY METHANOLYSIS

Compound	Percent Neutralised
Glucose 6-sulphate	66.0
N-acetylglucosamine 6-sulphate	36.0
N-acetylglucosamine 3-sulphate	95.9
Glucitol 6-sulphate	83.6
N-acetylglucosaminitol 6-sulphate	62.6
N-acetylglucosaminitol 3-sulphate	65.5

Table 2.5

The efficiency of the neutralisation of the unreduced compounds determined by GCMS. The peak areas of the GC peaks from the flame ionisation detector were determined by integration and adjusted for response factors that had been determined independantly; scylloinositol-$(TMS)_6$ 1.0, N-acetylglucosamine-$(TMS)_4$ 0.65, and glucose-$(TMS)_5$ 0.97.

The reduced compounds were analysed by high voltage paper electrophoresis by quantitation of the amount of radioactivity that remained at the origin relative to the total returned following electrophoresis.

methanolysis by gas chromatography and paper electrophoresis showed that the efficiency of removal of a sulphate ester was dependant on the linkage position (table 2.5). No alteration in the Bio-Gel P4 retention time of oligosaccharide 13.5 was observed, and less than 10% of Fucα1→6GlcNAc$_{ol}$ was hydrolysed by methanolysis. Only 2.5% of the sialic acid was lost from bovine sialyllactitol on methanolysis. 86.0% was rendered neutral by methanolic-HCl treatment alone, indicating the formation of methyl esters on the carboxylic group, which were efficiently hydrolysed by base treatment. These data indicate that the methanolysis method as used here is a valid procedure for the efficient removal of sulphate esters from oligosaccharides during structural determination.

Hydrogen fluoride treatment of oligosaccharide 13.5 did not alter the retention time on Bio-Gel P4. GCMS of glucose-6-sulphate and N-acetylglucosamine-3-sulphate after HF treatment and derivatisation with Sigma-Sil A did not identify $(TMS)_5$-glucose and $(TMS)_4$-N-acetylglucosamine indicating that sulphate esters had not been removed with the HF. Therefore a combination of HF, methanolysis, alkaline phosphatase and desialylation allows the detemination of the presence and removal of the common anionic moieties encountered on N-linked glycans.

2.4.2 *Charge Analysis and Composition of Murine Brain N-linked Oligosaccharides.*

The results of sequential treatment of the murine brain oligosaccharides are presented in table 2.6. Analysis of oligosaccharides that had been passaged through AG3 resin in the presence of acetic acid demonstrated that severe depletion of the phosphated and sulphated

Table 2.6

RELATIVE INCIDENCE OF ANIONIC MOIETIES IN MURINE BRAIN N-LINKED OLIGOSACCHARIDES

Class[a]	Incidence			
	Total[b]		AG3 Depleted[b]	
	Percent	Ratio[c]	Percent	Ratio
(1)Naturally occurring neutral	12.9	1.0	21.0	1.0
(2)Neuraminidase sensitive sialic acid	22.6	1.8	54.0	2.6
(3)Neuraminidase resistant sialic acid	23.6	1.8	0.0	0.0
(8)Neutralised by alkaline phosphatase only	N.D.		2.9	0.1
(4)Neutralised with alkaline phosphatase after neuraminidase	10.0	0.8	4.1	0.2
(5)HF neutralised following neuraminidase and alkaline phosphatase	13.5	1.0	5.8	0.3
(6)Neutralised by methanolysis after HF treatment	20.2	1.6	5.9	0.3
(7)Neutralised by methanolysis alone	17.5	1.4	N.D.	
Uncharacterised	20.8	1.6	7.1	0.3

Table 2.6. The data set was obtained by treatment of the glycans as described in the text. Following each treatment the neutral and acidic glycans were separated by high voltage paper electrophoresis, eluted in water and the radioactivity determined. Therefore it must be born in mind that glycans carrying more than one class of charged moiety could not always be resolved i.e. an oligosaccharide that is both sulphated and phosphated would only be neutralised following methanolysis, and would remain in the acidic compartment after HF or alkaline phosphatase digestion. The sulphated glycans were analysed in the untreated brain and following HF treatment. Therefore the difference in the incidences of these two pools (6 and 7) provides an estimate of the sulphated oligosaccharides that also carry sialic acid and/or phosphate esters. The uncharacterised material is at least partially radiochemical contaminant from the reduction, and was not defined further.

§ Total refers to whole brain glycans and AG3 treated refers to the fraction of whole brain glycans that were unbound by an AG3 (OH^-) column run in 4M acetic acid.

N.D.; Not determined.

* Normalised to 1.0 relative to the naturally neutral oligosaccharides in each preparation.

** Numbers refer to the fractions discussed in the text (section 2.5.2). The anionic moieties that would have been removed from each of the fractions are as follows;

Class	Moieties Removed to Generate Neutral Material
(1)	None
(2)	Neuraminidase sensitive sialic acid (α2→3 and α2→6), following removal of (1)
(3)	All sialic acid (including α2→8, polysialic acids), following removal of (1)
(4)	Phosphomonoesters following removal of glycans neutralised by (1) and (2)
(5)	Phosphodiesters and polysialic acid, following removal of (1), (2) and (4)
(6)	All sulphate esters following removal of (1), (2) and (4)
(7)	Sulphate esters following removal of (1)
(8)	Phosphomonoesters following removal of (1)

MONOSACCHARIDE COMPOSITION OF WHOLE MURINE BRAIN N-LINKED OLIGOSACCHARIDES

Residue	Molar Percent	
	Murine	Rat[15]
Mannose	50.5	31.0
Galactose	19.3	17.5
N-acetylglucosamine	13.5	23.6
Neuraminic acid	4.4	14.0
Fucose	12.3	9.2
Xylose	Trace*	N.D.
N-acetylgalactosamine	Trace	4.6

Table 2.7 The monosaccharides were analysed as their TMS-methylglycoside derivatives by GCMS as described in appendix one. Quantitation was achieved by integration of the output of a flame ionisation detector. Peak areas were adjusted with response factors determined from whole bovine serotransferrin for mannose, galactose, neuraminic acid and N-acetylglucosamine, assuming the composition reported by Leclerq *et al.* (1987), and from a calibration experiment with equal quantities of $(TMS)_4$-methylmannoside and $(TMS)_3$-methylfucoside. The relative incidences were then normalised to 100% total incidence for the quantifiable residues.
[15] Data taken from Krusius and Finne (1977).

oligosaccharides occured, but a less dramatic alteration in the ratio of the naturally neutral to sialylated glycans was observed, indicating that these latter classes of glycan did not bind to the resin. Also, significantly less uncharacterised material was found in the AG3 treated oligosaccharides as compared to the untreated material, indicating that the radiolabel was associated with acidic material. The composition of the murine brain oligosaccharides was determined by GCMS (table 2.7). A representative high voltage radioelectrophoretogram of untreated ^{3}H-reduced whole murine brain oligosaccharides is presented in figure 2.1. It is clear that the glycans are highly heterogenous and show a considerable spectrum of anionic constituents.

2.4.3 *Analysis of ^{35}S Metabolically Labelled Murine Brain N-linked Oligosaccharides*

Metabolic labelling resulted in the production of oligosaccharides containing 2 x 10^{6} ^{35}S cpm following purification. A Folsch wash of an aliquot of the glycans in 0.5ml of water with 2.5ml of 2:1 (v/v) chloroform/methanol resulted in the partitioning of all the radioactivity into the aqueous phase demonstrating that the glycans were not contaminated with radioactivity associated with lipids. The radioactivity did not migrate when subjected to descending paper chromatography in butanol/ethanol/water, 4:1:1 (v/v/v) for 24 hours indicating that all of the radioactivity was associated with carbohydrate larger than a trisaccharide. ^{3}H-reduction of the oligosaccharides and analysis of the reducing terminus determined that GlcNAc was the only reducing terminal monosaccharide confirming that these oligosaccharides were originally N-

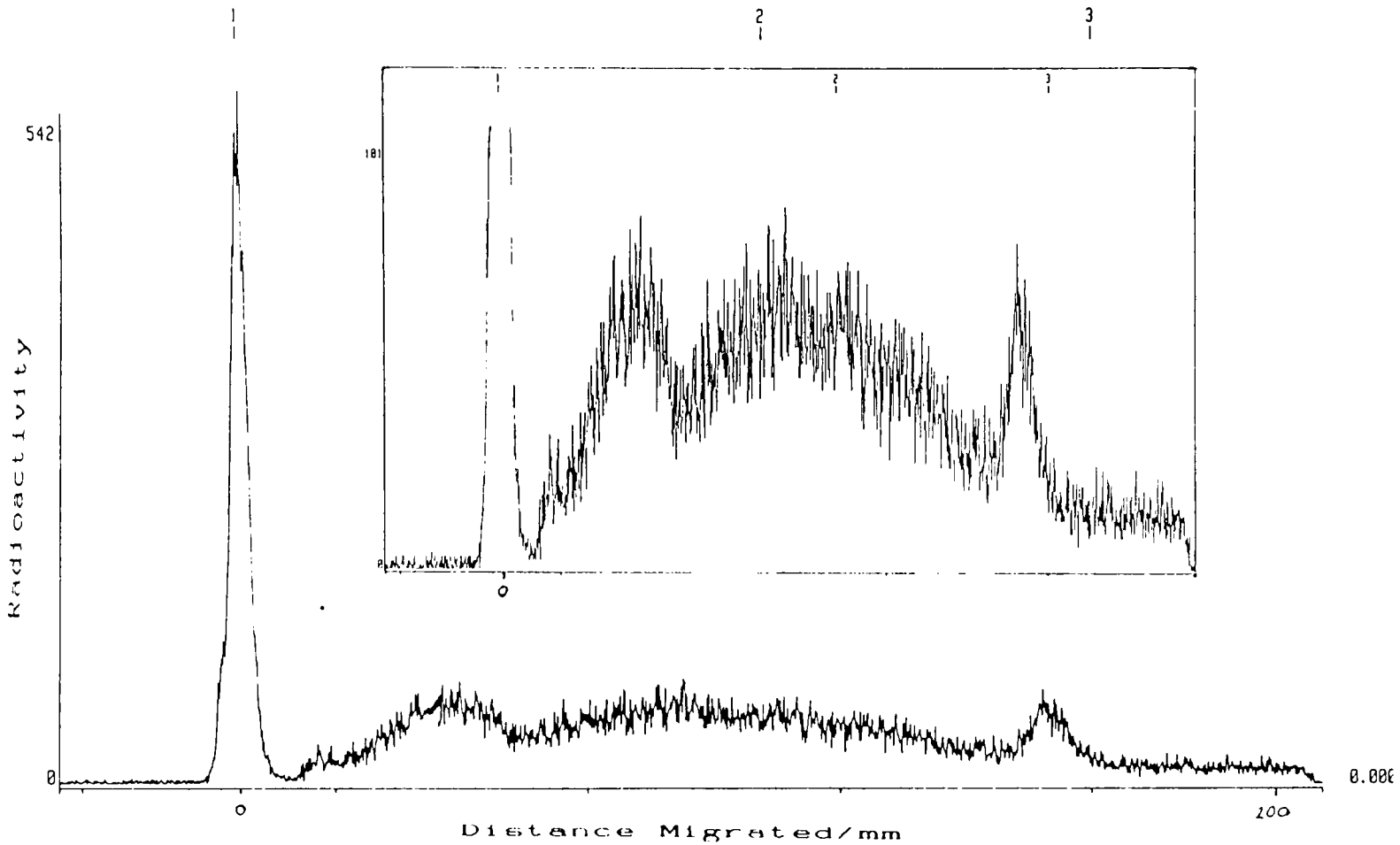


Figure 2.1 High voltage pyridine acetate radioelectrophoretogram of total ^{3}H-reduced murine brain N-linked oligosaccharides. The migration positions of the standard saccharides lactitol, sialyllactitol, and the dye bromophenol blue are indicated by 1, 2 and 3 respectively. The inset shows the acidic region of the electrophoretogram with an expanded vertical scale.

linked to protein.

High voltage electrophoresis of the total ^{35}S-labelled oligosaccharides visualised a highly heterogenous and highly mobile population of glycans indicative of the presence of much charged material (figure 2.2). This profile can be compared to that obtained with the ^{3}H-reduced murine brain oligosaccharides shown in figure 2.1. Bromophenol blue migrates to a position similar to a disialylbiantennary complex type oligosaccharide, therefore as much of the labelled material migrated beyond this marker, these fast moving glycans must carry several anionic groups. Passage of an aliquot of the ^{35}S-labelled oligosaccharides through AG3 (OH^- form) in the presence of 4M acetic acid resulted in a loss of 30% of the radioactivity, (figure 2.2) confirming the observation that sulphated spiecies were retained by the resin under these conditions. Treatment with neuraminidase, alkaline phosphatase and HF resulted in some loss of mobility of the whole brain glycans, but even following HF treatment a lot of the radioactivity retained a high mobility (figure 2.3), indicating that much of this material was still highly charged.

Bio-Gel P6 gel filtration of the untreated ^{35}S labelled glycans showed that a large proportion of the oligosaccharides ran in the void volume of the column (figure 2.4). Treatment of the glycans with neuraminidase, alkaline phosphatase and HF did not result in a significant alteration to the profile, indicating that a large proportion of the glycans carrying SO_4 are neither sialylated nor phosphated, which agrees with the electrophoresis data. Following HF treatment, which removed all sialic acids and phosphates, glycans were fractionated by AG1 chromatography (figure 2.5). Three fractions were resolved; A, unbound, B, slightly retarded and C, tightly bound and eluting as a broad peak between 1 and 2M

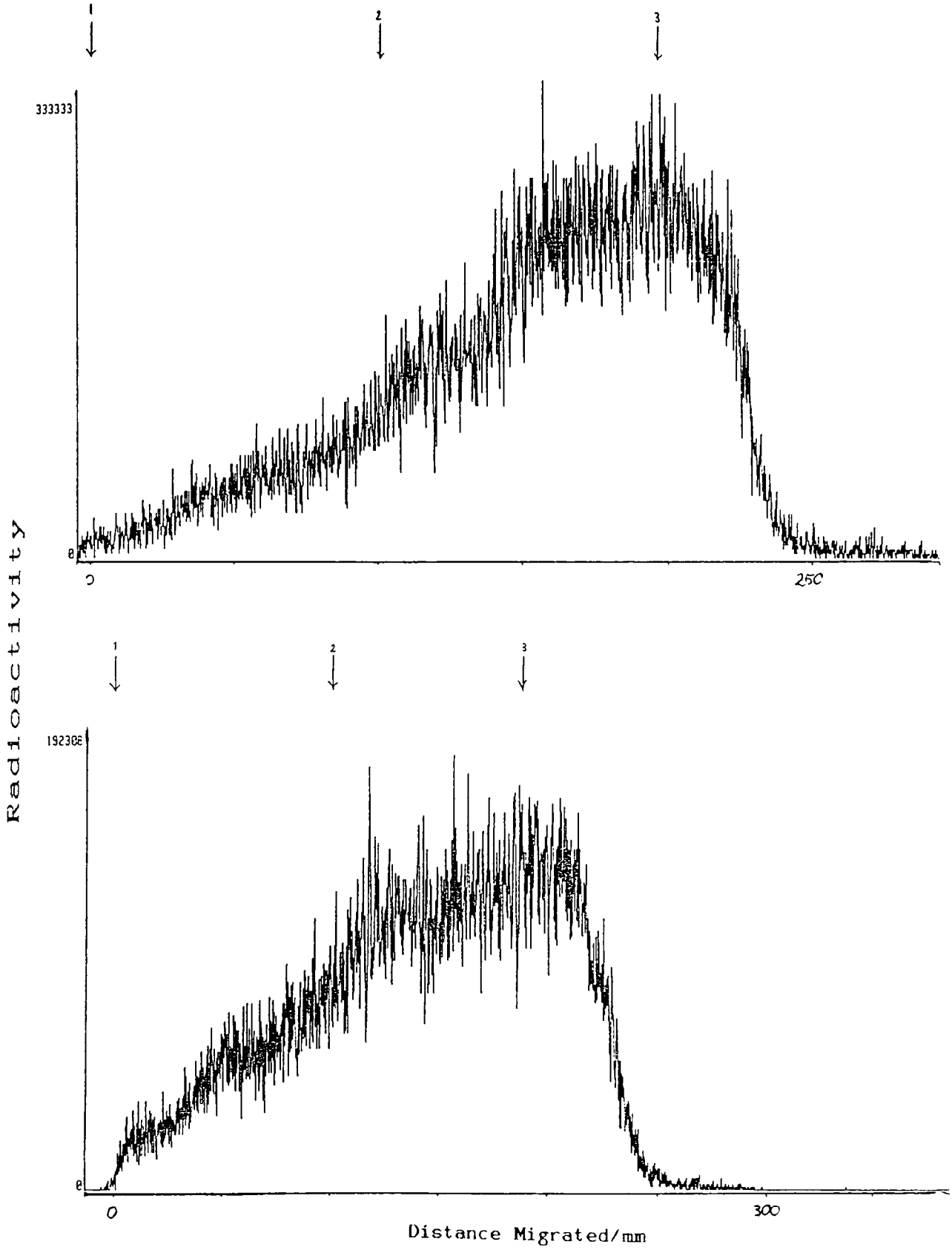


Figure 2.2 High voltage pyridine acetate radioelectrophoretogram of ^{35}S metabolically labelled murine brain oligosaccharides. The top panel shows the untreated glycans, and the lower panel the oligosaccharides not retained by AG3 resin. Standards are as given in figure 2.1.

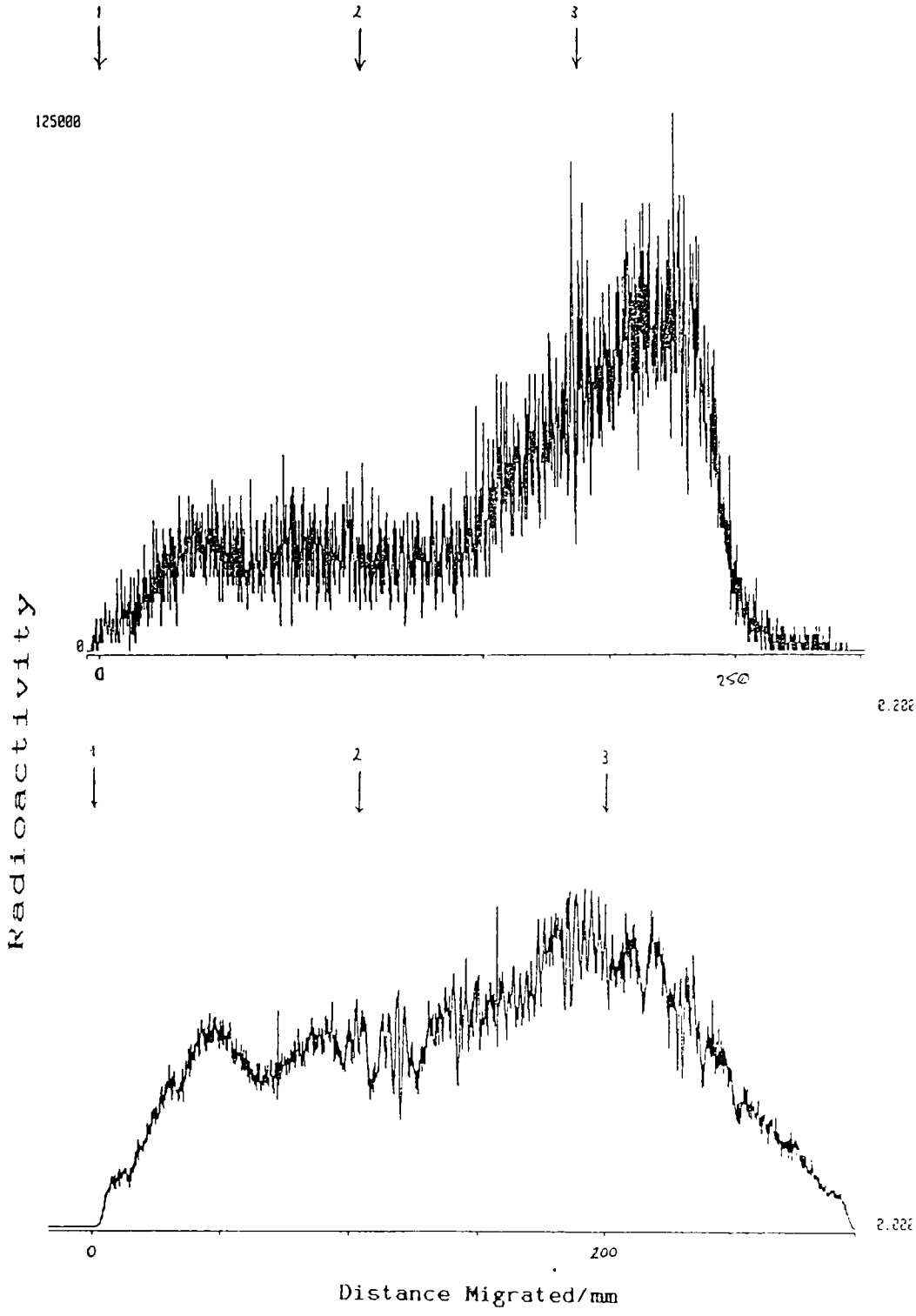


Figure 2.3 High voltage radioelectrophoretogram run in pyridine acetate buffer of ^{35}S metabolically labelled murine brain oligosaccharides. The top panel shows the glycans after desialylation with *Artherobacter ureafaciens* neuraminidase and the lower panel the oligosaccharides after HF treatment. Standards are as given in figure 2.1.

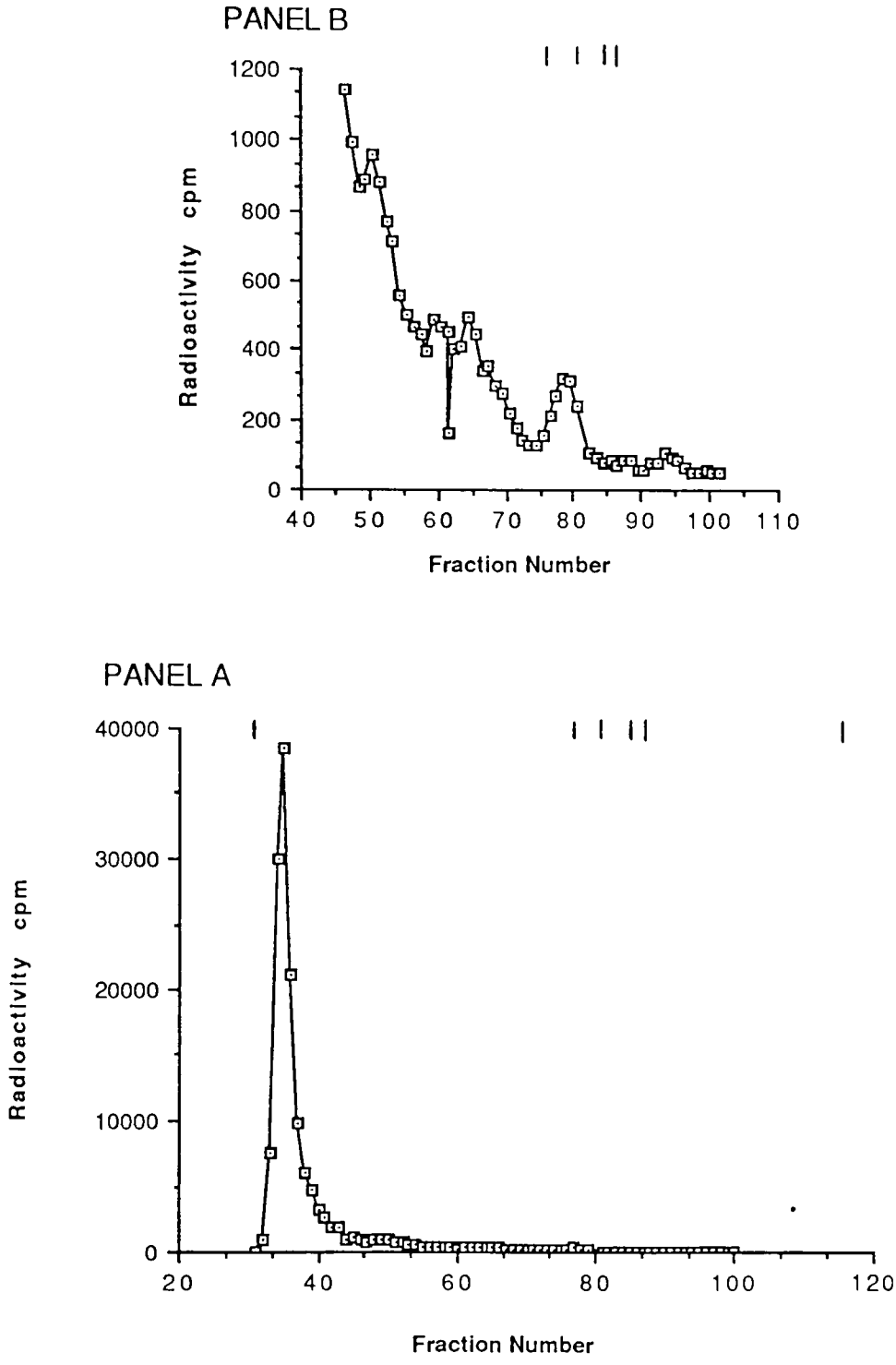


Figure 2.4 Bio-Gel P6 gel filtration chromatogram of ^{35}S metabolically labelled murine brain glycans. Panel B shows the region of fraction 40 to 110 with the vertical axix expanded. The elution positions of standard oligosaccharides, from left to right, for panel A are void volume, sialyllactitol, reduced sialic acic, oligosaccharide 13.5, N-acetylglucosaminitol-3-sulphate and reduced chitobiose, and panel B sialyllactitol, reduced sialic acid, oligosaccharide 13.5, and N-acetylglucosaminitol-3-sulphate.

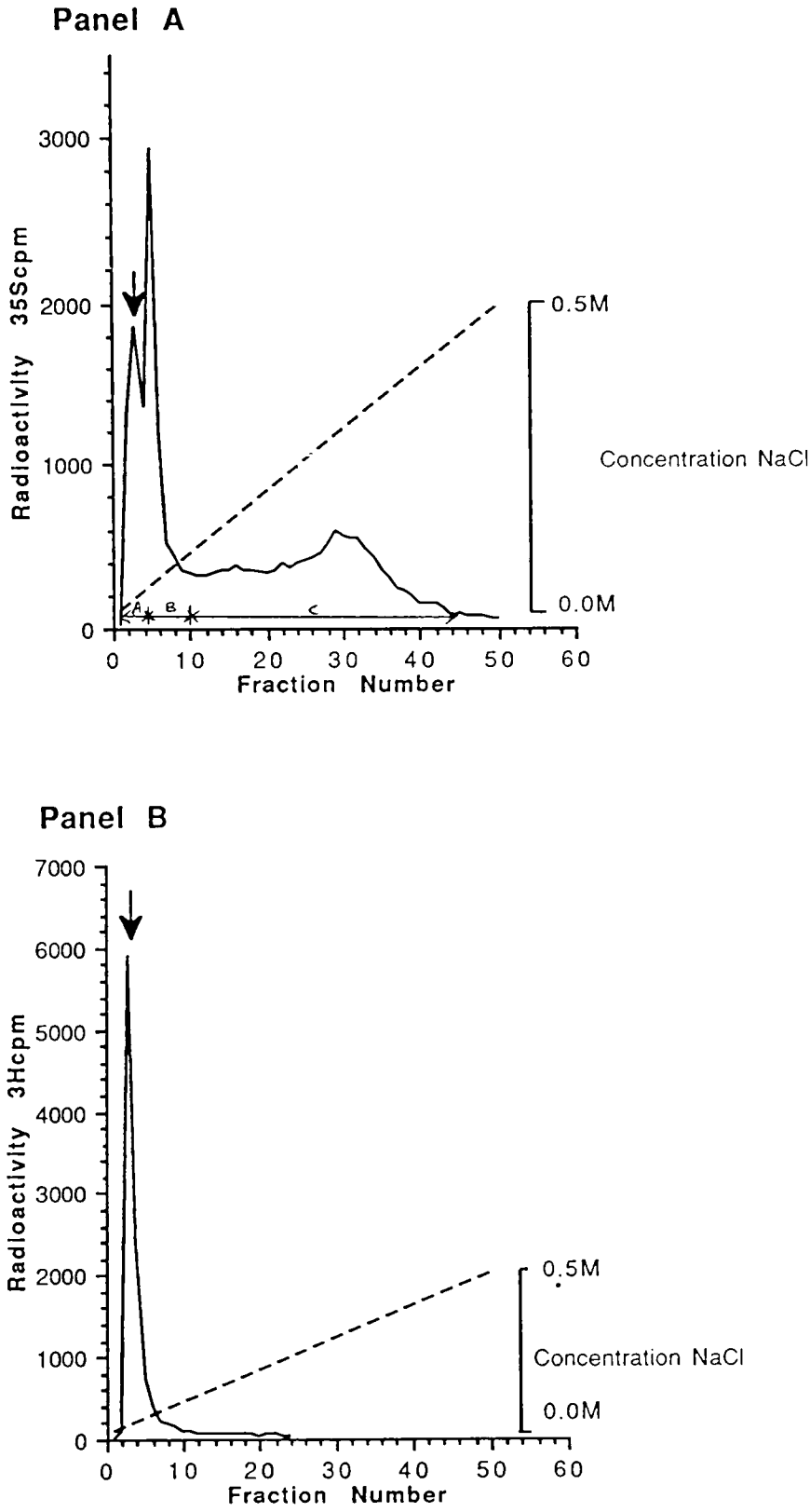


Figure 2.5 Ion exchange chromatograms of murine brain oligosaccharides on AG1 (Cl^- form) resin. Panel A shows the elution profile obtained with the ^{35}S metabolically labelled oligosaccharides and the panel B the ^{3}H reduced oligosaccharide elution profile. Both samples were treated with HF before chromatography (see text). The elution position of N-acetylglucosaminitol-3-sulphate is indicated by an arrow. The relative ^{35}S activity in the pools A, B, and C was determined as 19.6% in A, 27.8% in B and 52.6% in C. 88% of the applied radioactivity was recovered in pools A, B, and C. The NaCl gradient is shown by a dotted line.

NaCl, with a maximum at 1.5M. A total return of 85% of the applied radioactivity was obtained. N-acetylglucosaminitol 3-sulphate, and all ^{3}H-radioactivity from reduced murine brain glycans following HF digestion eluted with the same retention time as pool A. High voltage electrophoresis of pools A and B confirmed that these fractions contained the less highly charged species. When the fractions A, B and C were individually subjected to gel filtration on Bio-Gel P6, pools A and B were almost completely included in the column but pool C ran in the void volume (figure 2.6). These data indicate that pool C is highly charged and/or large. By virtue of behaviour on AG1 the glycans in pool C are concluded to be glycosaminoglycan (GAG) type, with similar overall charge to chondroitin sulphate or undersulphated keratan sulphate. The broad elution of the pool C saccharides from AG1 indicates that this material is heterogenous, and therefore may contain other GAGs beside chondroitin sulphate or keratin sulphate. Based on their behaviour on AG1 and Bio-Gel P6, pools A and B are probably predominantly sulphated N-linked oligosaccharides with hybrid or complex type structures. Because of the contribution of SO_4 to the hydrodynamic volume, these glycans cannot be carrying more than two or three sulphate esters. The oligosaccharides in pool C are clearly different being highly charged and probably of high molecular weight. Because of a lack of material the glycans could not be analysed further.

2.4.4 *Determination of the Charged Moieties on the Po Oligosaccharides*

High voltage electrophoresis of total Po oligosaccharides, isolated by L2 affinity chromatography, produced the profile shown in figure 2.7. Two separate preparations of Po oligosaccharides derived from different

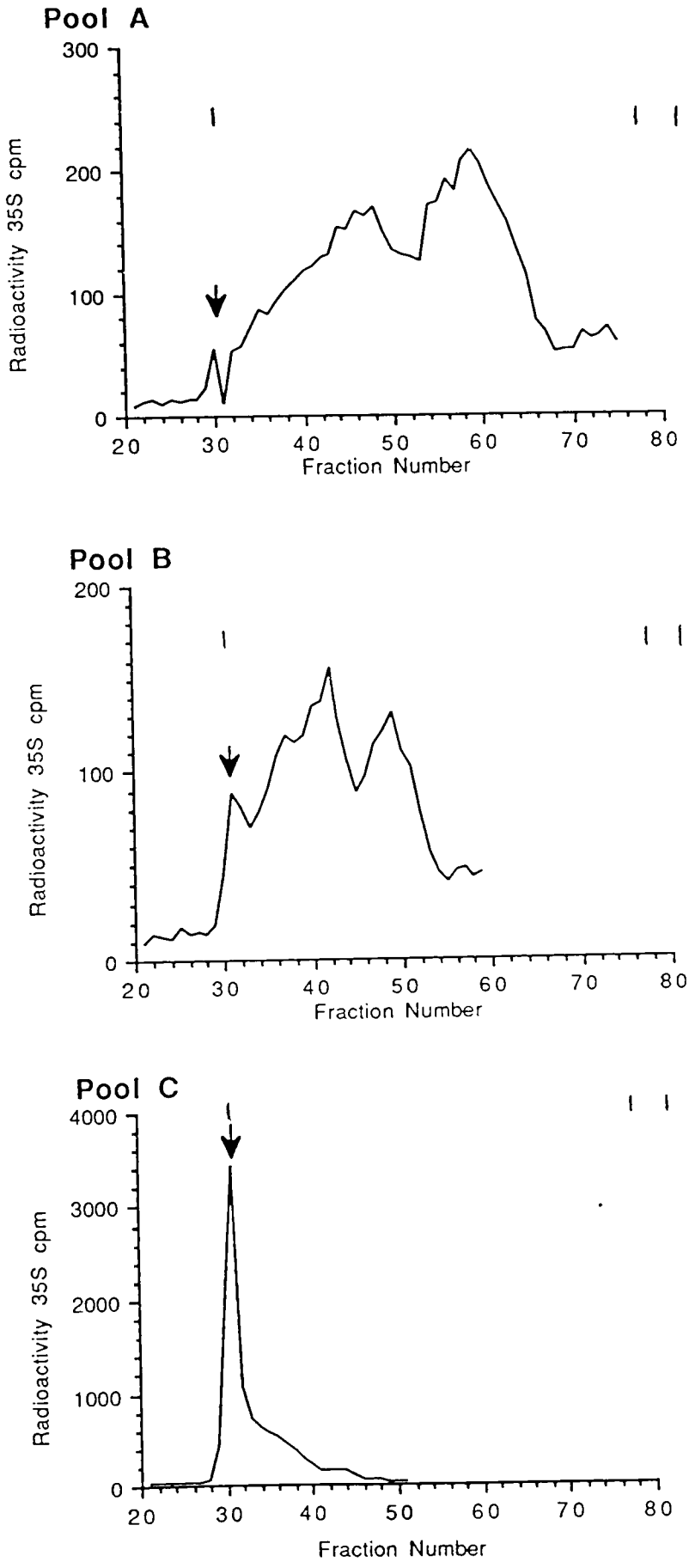


Figure 2.6 Bio-Gel P6 gel filtration of pools A, B, and C from the AG1 ion exchange column eluate. The elution positions, from left to right of the void volume, sialyllactitol and reduced sialic acid are indicated by vertical lines.

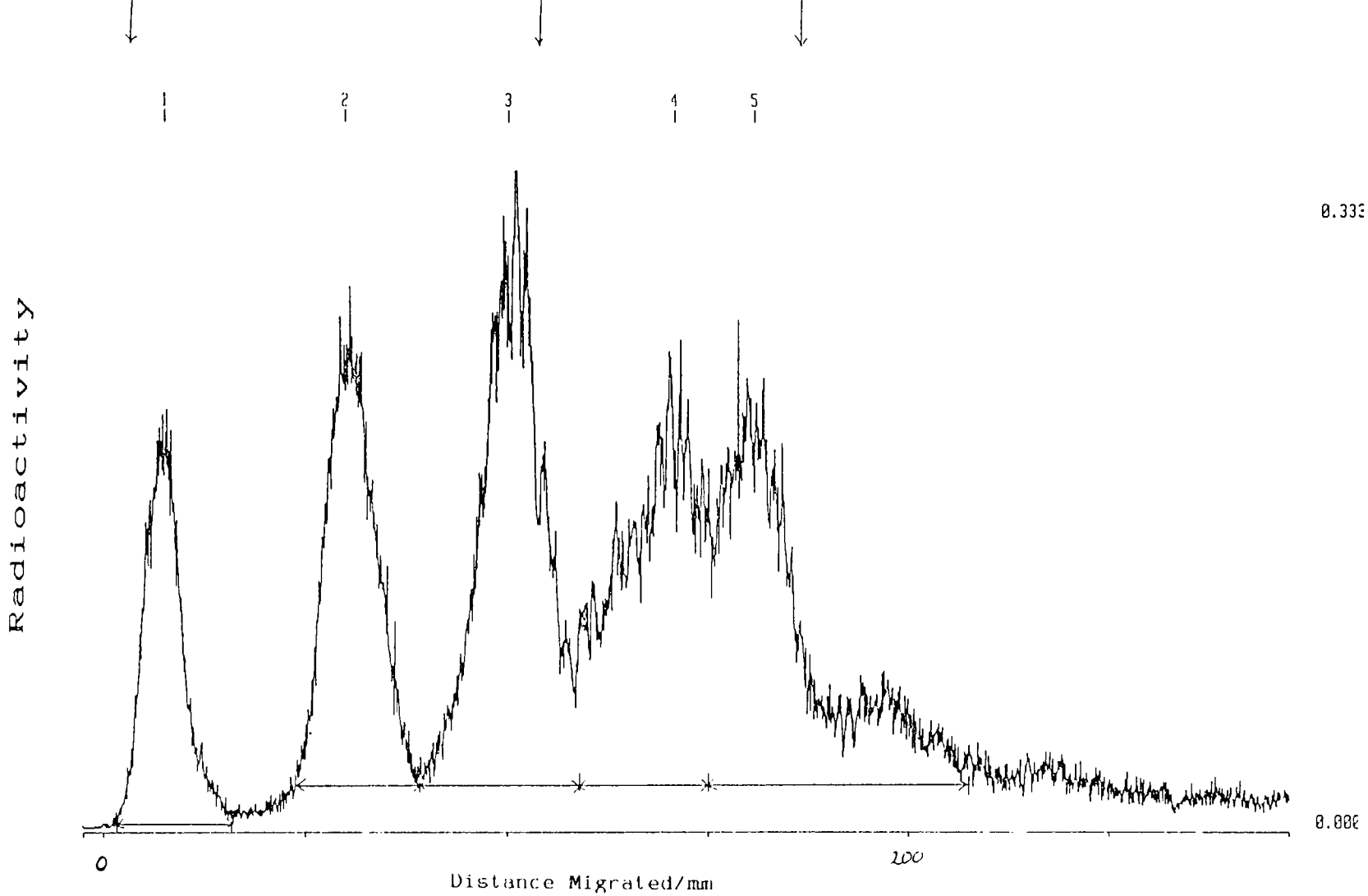


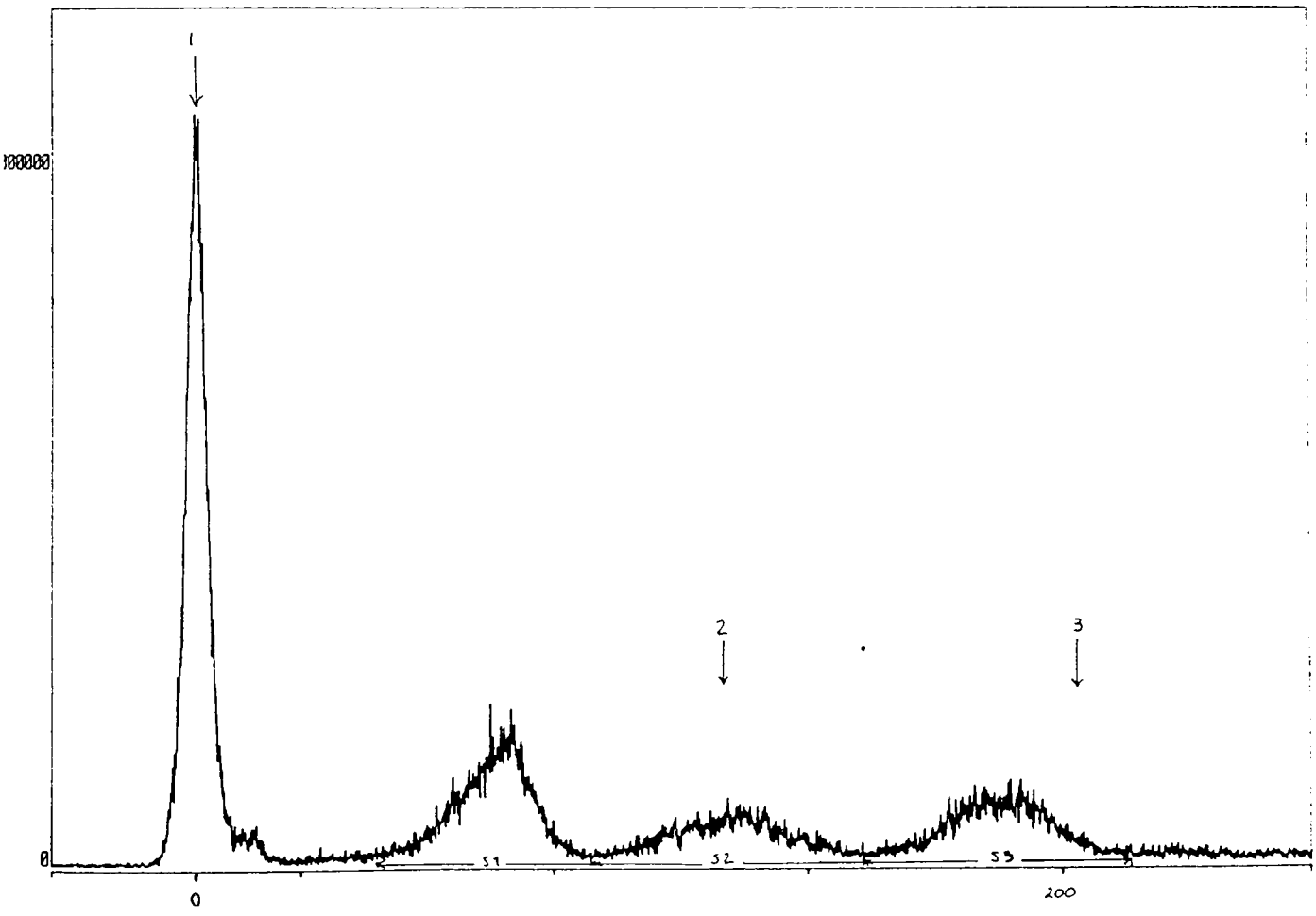
Figure 2.7 High voltage paper electrophoretogram of total Po oligosaccharides derived from the second preparation. The positions of the fractions refered to in table 2.8 are indicated. The migration positions of standard compounds are indicated as in figure 2.1.

preparations of the protein gave very similar profiles. The material that migrated only a small distance from the origin was not composed exclusively of neutral glycans because the peak maximum had clearly migrated to a different position to the lactitol standard. However, based on the later identification of an oligomannose glycan, some neutral oligosaccharides had to be present in this peak.

Digestion of the oligosaccharides from the first Po preparation with *Artherobacter ureafaciens* neuraminidase, followed by paper electrophoresis, generated the profile shown in figure 2.8. Following this treatment the neutral glycans remained at the origin. Three peaks of acidic material were still present and could not be neutralised by repeat treatment with neuraminidase. The oligosaccharides from the second preparation of Po were initially treated with Newcastle Disease Virus (NDV) neuraminidase which hydrolyses sialic acid residues linked α2→3 only. This did not give complete desialylation. Treatment of the products of NDV neuraminidase digestion with mild acid resulted in complete removal of sialic acid, but did not generate further neutral glycans. Therefore at least some sialic acid is present in α2→3 linkage, but that present on the glycans which also contain non-sialic acid anionic moieties is in α2→6 or α2→3 linkage. The radioactivity in the neutral and acidic material was determined, as well as in the individual neuraminidase resistant peaks (table 2.8) There was a clear difference in the relative amount of neutral to acidic material following removal of sialic acid for the two preparations, but conservation of the ratios of the acidic peaks following desialylation

Treatment with 50% aqueous hydrogen fluoride did not alter the mobility of the desialylated acidic saccharides on electrophoresis, demonstrating that no phosphate esters were present as substituents of the glycans. An

Figure 2.8 High voltage paper electrophoretogram of Po oligosaccharides following desialylation. The sulphated fractions are indicated by S1, S2 and S3. Standards are as in figure 2.1.



CHARGE ANALYSIS OF THE Po GLYCANS

Untreated Oligosaccharides

Preparation	Fraction Number				
	1	2	3	4	5
#1	11.7	21.2	31.9	21.6	13.5
	1.0	1.8	2.7	1.8	1.1
#2	9.6	17.3	25.0	21.8	26.4
	1.0	1.8	2.6	2.3	2.8

Desialylated

	Fraction Number			
	Neutral	S1	S2	S3
#1	52.0	25.1	10.6	12.3
		2.4	1.0	1.2
#2	18.9	42.4	17.9	20.8
		2.4	1.0	1.2

Table 2.8 The charge distributions of the various fractions visualised by high voltage electrophoresis of the Po glycans both untreated and following desialylation. For the fraction designation see figures 2.7 and 2.8. In each case the percent relative incidence is reported and below these are reported the ratio of these values normalised to 1.0 for the least abundant fraction, i.e. fraction 1 in the untreated material and S2 following desialylation. S1, S2 and S3 refer to the fractions proposed to carry one, two and three sulphate esters, respectively.
Note that there is absolute conservation of the relative incidence of the sulphated glycans with respect to each other, even though the relative amount of material that remains at the origin of a paper electrophoretogram following desialylation is different between the two preparations. In addition, the incidence of the more highly charged glycans is increased in #2 in comparison to #1, and this is most likely due to the higher proportion of sulphated oligosaccharides in #2.
Data for the untreated glycans were acquired from a radioelectrophoretogram by integration of the digitised data accumulated with a linear scanner as described in appendix one. The incidence of the neuraminidase treated fractions were obtained by elution of the electrophoresis paper with water prior to quantitation of the radioactivity present in each fraction.

aliquot of each peak was taken and digested with *Streptococcus plicatus* endoglycosidase H and re-analysed by paper electrophoresis. The mobility of the peaks did not alter and therefore the oligosaccharides were not sensitive to this enzyme. The individual peaks of the neuraminidase resistant acidic glycans were subjected to methanolysis resulting in the generation of neutral material when re-analysed by paper electrophoresis, which is indicative of the presence of sulphate esters (Kantor and Schubert 1967). Methanolysis of the more mobile peaks generated some material that migrated in a position identical to that of the less mobile peaks, i.e. peak S3 generated mainly neutral glycans, with some material comigrating with S1 and S2 as well as material that still migrated at the S3 position. Similarly, S2 products migrated as neutral, S1 and S2. The remaining acidic material was subjected to a second methanolysis as before, and more neutral material was generated. The neutralised material from the individual peaks was pooled; greater than 95% (S1), 80% (S2) and 75% (S3) of the radioactivity was neutralised by two sequential methanolysis treatments (table 2.9). Because resistance to neutralisation increased with greater mobility and some products are generated from the more mobile fractions which comigrate with the less charged fractions, it is concluded that the peaks represent a charged series. The efficiency of neutralisation by methanolysis is in agreement with this (table 2.9 and legend), and therefore it is concluded that S1 carries one sulphate ester, S2 carries two and S3 has three.

NEUTRALISATION OF THE SULPHATED Po OLIGOSACCHARIDES

Fraction	First treatment		Second treatment	
	Neutral	Total Acidic	Neutral	Total Acidic
	Percent			
S1	87.1	12.9	95.6	5.4
S2	72.4	27.6	84.0	16.0
S3	64.7	35.3	77.6	22.4

Percent neutralised with each treatment

	First Treatment		Second Treatment	
	Actual	Theoretical	Actual	Theoretical
	Percent			
S1	87.1	87.1	58.1	58.1
S2	72.4	75.9	42.0	33.8
S3	64.7	66.0	36.5	19.6

Table 2.9 Neutralisation of the desialylated acidic human Po oligosaccharides. The raw data are presented in the first half of the table and were obtained as follows. After treatment with methanolic-HCl of the individual fractions the products were separated by paper electrophoresis. The neutral and acidic glycans were separately eluted and the radioactivity quantitated. The acidic material was then subjected to a second treatment with methanolic-HCl and fractionated as before. The value of the percent neutral after this second treatment was added to that from the first to give the figures reported in that column as percent following a second treatment.

Table 2.9 continued.

The percentage neutralised by each treatment are shown in the second half of the table. The actual values were obtained by experiment as described. The theoretical values are the S1 empirical value squared or cubed for S2 and S3, respectively. If S1 and S2 carry one and two identical charges respectively, then treatment with methanolic-HCl will have an equal efficiency for the removal of each charge, but as the presence of one charge is enough to incorporate it into the acidic class, the efficiency of neutralisation for S2 will be $(0.871)^2$, i.e. 0.759. The actual and theoretical values for the first methanolic-HCl treatment compare favourably. Actual values for the second treatment are more efficient than theoretical, due to the fact that S2 is not all truly disulphated following the first methanolysis, i.e. some of the glycans will have been converted to monosulphated oligosaccharides, and similarly for the S3 fraction. The observation that the efficiency of the neutralisation of S1 is less than the previous methanolic-HCl treatment may be due to the fact that residual radiochemical contaminants were still present in this material which did not behave as sulphated oligosaccharides, and thus the residual acidic compartment is apparently over-represented.

2.4.5 *Bio-Gel P4 and Exoglycosidase Analysis of the Po Glycans.*

Structural characterisation was carried out on the oligosacchrides derived from the second Po preparation. Neutral oligosaccharides obtained by neuraminidase, and those in S1, S2 and S3 neutralised by methanolysis, were fractionated on Bio-Gel P4. Based on the efficiency of radiochemical incorporation, about 1 nanomole of oligosaccharides were recovered from each pool following neutralisation by neuraminidase and methanolysis. The details of the exoglycosidase digestion analysis are given below. In table 2.10 the fully characterised structures proposed from these data are shown, and in table 2.11 the classes of the glycans and their percent incidence are presented.

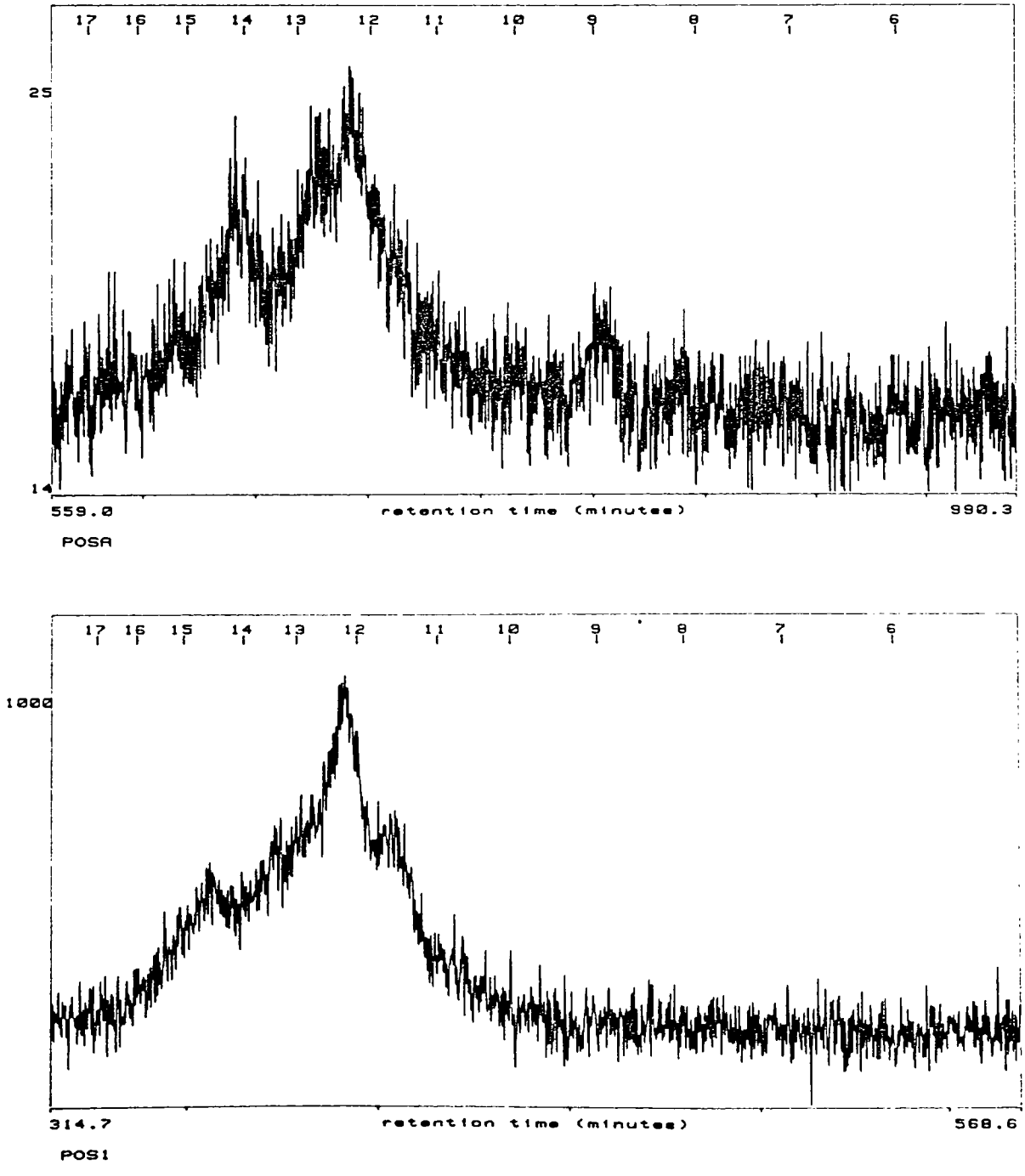
2.4.5.1 Structural Characterisation of the Unsulphated Po Oligosaccharides (POSA) by Sequential Exoglycosidase Digestion

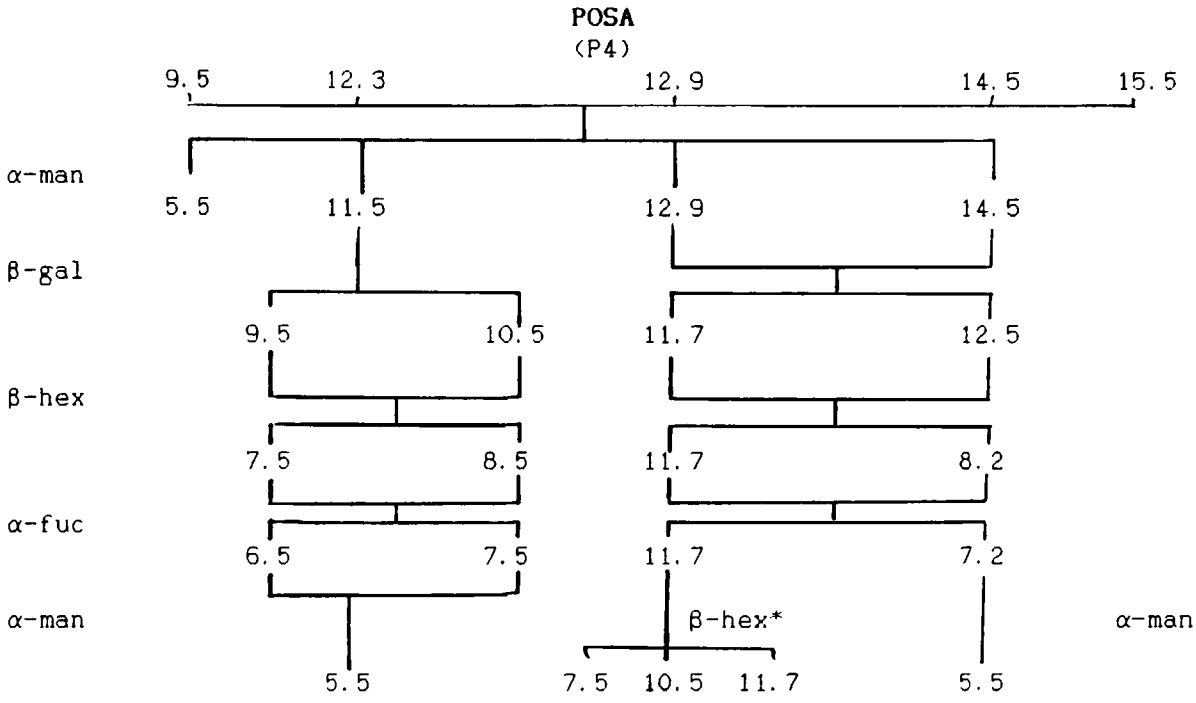
The Bio-Gel P4 profile of this material had peaks at 9.5, 12.3 (with a shoulder at 11.5), 12.9, 14.5 and 15.5 glucose units (gu) (Figure 2.9, scheme 2.1). Digestion of POSA with jack bean α-mannosidase resulted in peaks eluting at 5.5, 11.5 (with a shoulder at 10.5), 12.9 and 14.5gu. The peak at 5.5gu was concluded to have been derived from the 9.5gu peak, and was thus an oligomannose glycan, containing five mannose residues originally. It is proposed that the 11.5gu material was derived from the 12.3gu peak by loss of a single α-mannose residue while the two other peaks at 12.9gu and 14.5gu were not sensitive to the α-mannosidase. The three largest products from the α-mannosidase digestion were treated with bovine epididymal β-galactosidase. A peak generated at 10.5gu by this enzyme is

proposed to derive from the 11.5gu peak by loss of one β-galactose residue; a shoulder at 9.5gu was also observed, which was probably derived from the 10.5gu shoulder. Treatment of the combined 10.5 and 9.5gu peak with jack bean β-hexosaminidase produced a peak at 8.5gu and a smaller one at 7.5gu, consistent with the loss of one HexNAc from each residue. Treatment of the 7.5 and 8.5gu peaks with bovine epididymal α-fucosidase removed a further glucose unit and treatment with jack bean α-mannosidase converted both peaks to ones eluting at 5.5gu. These data indicate that the 12.9gu peak and the shoulder at 11.5gu were hybrid oligosaccharides, both with core fucosylation, and the larger saccharide bearing one more α-mannose residue than the smaller glycan.

The two peaks that eluted at 11.7 and 12.5gu after β-galactosidase treatment are proposed to have been derived by the loss of one and two β-galactose residues from the 12.9 and 14.5gu peaks, respectively. The combined 11.7 and 12.5gu peaks were digested with jack bean β-hexosaminidase, which generated material eluting at 8.2gu with the concomitant loss of most of the 12.5gu material. However the 11.7gu material was unaffected. The 8.2gu peak was digested with bovine epididymal α-fucosidase, which resulted in the loss of one fucose residue, and then by jack bean α-mannosidase, with the further loss of two mannose residues. The 11.7 gu peak could not be digested with jack bean β-hexosaminidase or bovine epididymal α-fucosidase, but treatment with *Streptococcus pneumoniae* β-hexosaminidase resulted in the movement of some of this material to 10.5gu and 7.5gu while some remained undigested. On the basis of these data it is proposed that the 14.5gu oligosaccharide is a core fucosylated, digalactosylated biantennary oligosaccharide, and that the 12.9gu peak is composed of a monogalactosylated biantennary oligosaccharide with a

Figure 2.9 Bio-Gel P4 gel filtration profiles of Po oligosaccharides from the second preparation. Upper panel; glycans neutralised by desialylation, lower panel; glycans from fraction S1, i.e. carrying one sulphate ester before methanolysis. Vertical lines across the top of the figure indicate the elution positions of isomaltose oligosaccharide oligomers corun as an internal standard.





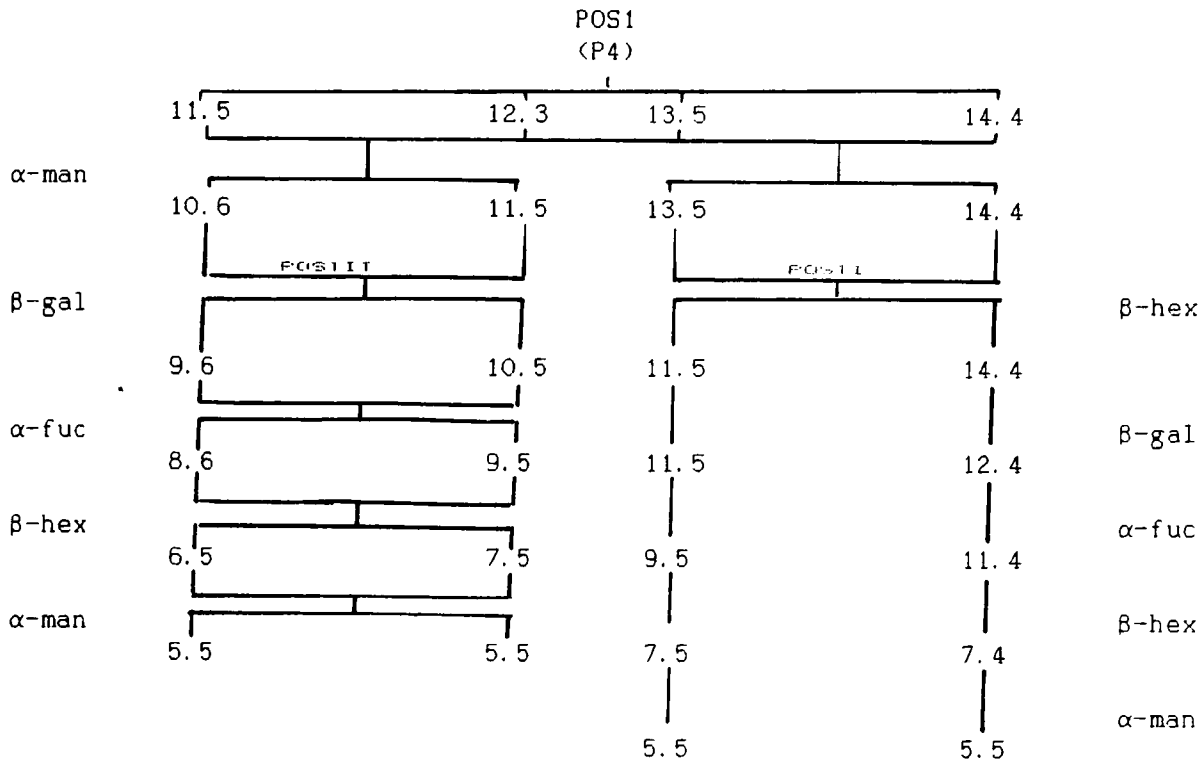
Scheme 2.1 Summary of exoglycosidase degradation of the POSA oligosaccharides. The figures refer to the elution position in glucose units, and α-man, β-gal, β-hex, β-hex* and α-fuc (jack bean α-mannosidase, bovine epididymal β-galactosidase, jack bean β-hexosaminidase, *Streptococcus pneumoniae* β-hexosaminidase and bovine epididymal α-fucosidase respectively) refer to the enzymes used for the analysis. The enzyme used is indicated at the left of the figure, and refers to all digestions performed at that line, except where otherwise indicated. A full discussion can be found in the text.

bisecting GlcNAc residue, because of the sensitivity of the latter glycan to *S. pneumoniae* β-hexosaminidase, and resistance to jack bean β-hexosaminidase.

2.4.5.2 Structural Characterisation of the Monosulphated Po Oligosaccharides (POS1) by Exoglycosidase Digestion

Peaks were observed at 11.5, 12.3, 13.5 and 14.4gu upon Bio-Gel P4 chromatography (figure 2.9, scheme 2.2). Treatment of the pooled glycans with jack bean α-mannosidase resulted in the production of peaks at 10.6 and 11.5gu (the major species), which were pooled as POS1II, and peaks at 14.4 and 13.5gu (pooled as POS1I). Treatment of POS1II with bovine epididymal β-galactosidase removed one glucose unit from each of the α-mannosidase products, bovine epididymal α-fucosidase removed a further one glucose unit, and subsequent treatment with jack bean β-hexosaminidase reduced the elution volume by a further two glucose units, resulting in two peaks at 7.5 and 6.5gu. Digestion of the 7 5 and 6.5gu peaks with jack bean α-mannosidase brought both to 5.5gu. Therefore POS1II is composed of hybrid oligosaccharides with the same heterogeneity in the mannose substituents as described for the POSA hybrid glycans.

The glycans designated as POS1I were treated with jack bean β-hexosaminidase which resulted in the movement of the 13.5gu peak to 11.5gu, but the 14.4gu peak did not move. These two peaks were individually treated with bovine epididymal β-galactosidase; the 14.4gu peak lost two glucose units, but the 11.5gu peak did not move. Digestion of the 14.4gu peak with bovine epididymal α-fucosidase removed one glucose unit, digestion with jack bean β-hexosaminidase removed a further four, and



Scheme 2.2 Summary of the exoglycosidase degradation of POS1. Abbreviations are as for scheme 2.1.

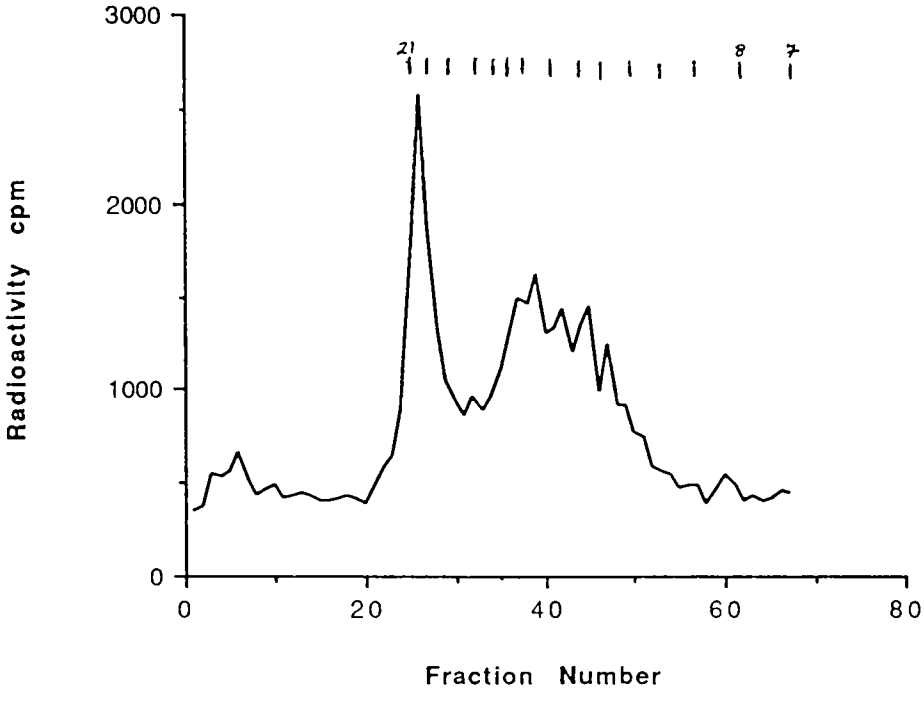
finally jack bean α-mannosidase removed two, resulting in a core that eluted at 5.5gu. Digestion of the 11.5gu peak with bovine epididymal α-fucosidase resulted in the movement of this material to 9.5gu. Subsequent digestion with jack bean β-hexosaminidase removed two glucose units and finally jack bean α-mannosidase digestion resulted in the loss of a further two glucose units, with the core glycan eluting at 5.5gu.

Therefore the 14.4gu oligosaccharide is proposed to be an analogous structure to the core fucosylated, digalactosylated biantennary seen in POSA. The 13.5 gu oligosaccharide is proposed to be an unusual structure, with no terminal galactose but two fucose residues. Based on the observation that jack bean β-hexosaminidase was only able to remove one HexNAc prior to bovine epididymal α-fucosidase digestion, it is concluded that one of the fucose residues of the 13.5 gu oligosaccharide is present on an arm HexNAc, rendering it resistant to removal by the first hexosaminidase digestion.

2.4.5.3. Structural Characterisation of the Disulphated Po Oligosaccharides (POS2) by Exoglycosidase Degradation

The Bio-Gel P4 profile of this fraction had a dominant peak with a hydrodynamic volume of 20.5gu. and an envelope that extended from 15.5 to about 10gu (Figure 2.10, scheme 2.3). The high molecular weight glycan, eluting at 20.5gu, was determined to be a repeat lactosamine containing oligosaccharide by the following analysis. Digestion with *Bacillus fragillis* endo-β-galactosidase resulted in the production of peaks at 13.5 and 10.5gu, as well as undigested material at 20.5gu. Treatment of the 13.5 and 10.5gu glycans with jack bean β-hexosaminidase removed two glucose

Gel Filtration Chromatogram of Po N-Linked Oligosaccharides, Fraction S2



Gel Filtration Chromatogram of Po N-Linked Oligosaccharides, Fraction S3

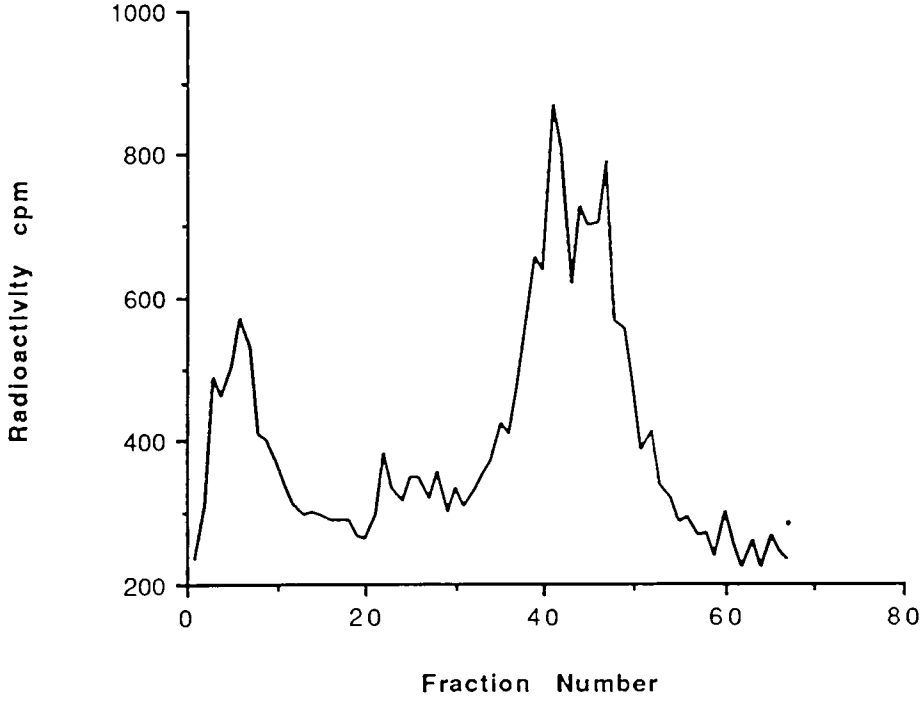
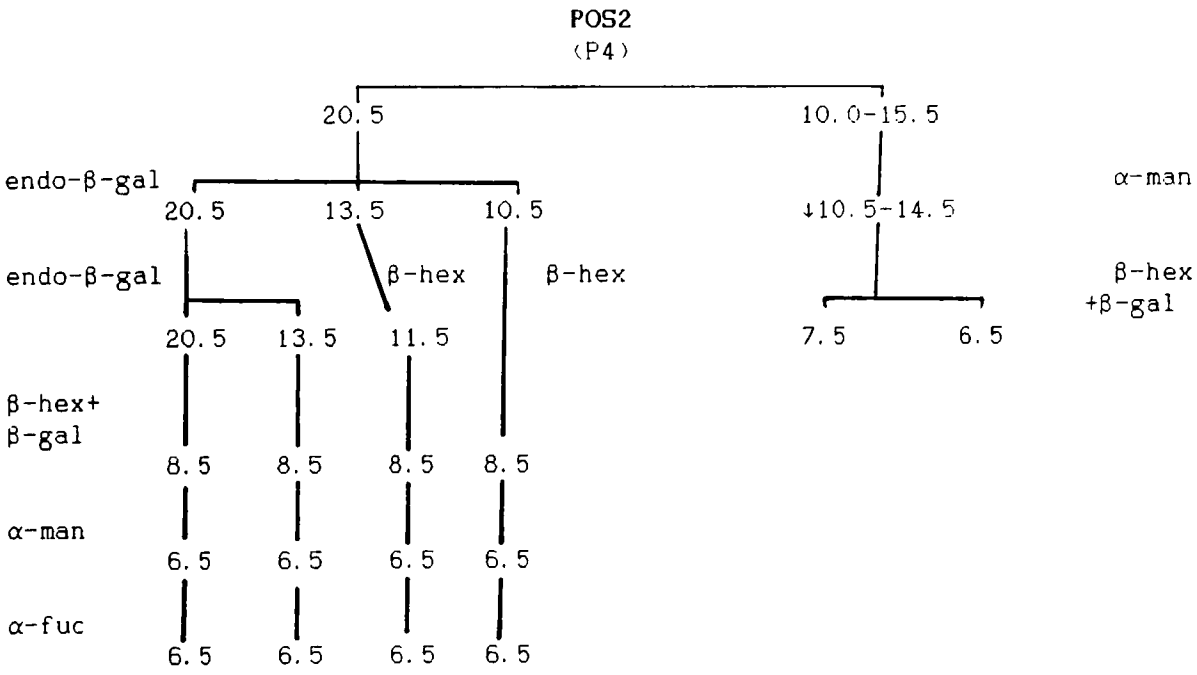
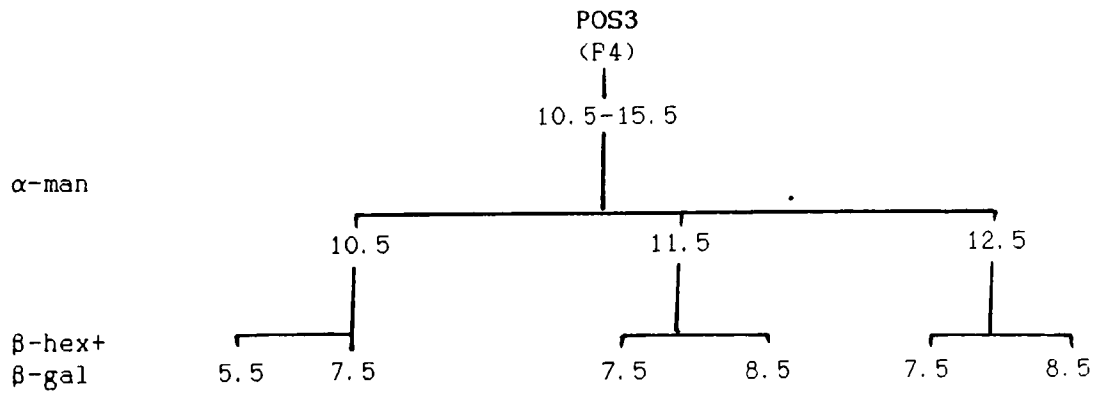


Figure 2.10 Bio-Gel P4 gel filtration profiles of Po oligosaccahrides from the second preparation. Upper panel; glycans from S2, i.e. carrying two sulphate esters before methanolysis, lower panel; glycans from S3, i.e carrying three sulphate esters before methanolysis. Internal isomaltose oligomer standards (dp 6-20) are indicated as for figure 2.9.



Scheme 2.3 Summary of exoglycosidase digestions of POS2. Abbreviations are as described for 2.1. Endo-β-galactosidase is indicated by endo-β-gal.



Scheme 2.4 Summary of the exoglycosidase digestions of POS3. Abbreviations are as described in scheme 2.1.

units from each, i.e. a single HexNAc thereby determining that the lactosamine repeat was present on one arm only (this can be concluded because the action of the endo-β-galactosidase generates a GlcNAc nonreducing terminus after cleavage of the glycan). Repeat digestion of the 20.5gu material with endo β-galactosidase generated more of the 13.5gu material. Digestion of this new 13.5gu and the residual 20.5gu material, as well as the 11.5gu glycan resulting from the jack bean β-hexosaminidase digestion, with a mixture of jack bean β-hexosaminidase and β-galactosidase reduced the size of all these glycans to 8.5gu. Therefore it is proposed that the heterogeneity observed on the original digestion was the result of cleavage at different Galβ1→4(3)GlcNAc linkages along the lactosamine chain. Four Galβ1→4(3)GlcNAc lactosamine units were present in this oligosaccharide based on the hydrodynamic volume of the intact glycan. In addition, the repeat lactosamine was unbranched as branching would prevent the production of both the 13.5 and 10.5gu products (Scudder *et al.* 1984). Treatment of the 8.5gu core with jack bean α-mannosidase resulted in the loss of only two glucose units, and subsequent digestion of the 8.5gu material with bovine epididymal α-fucosidase did not alter the elution volume. Even though the α-mannosidase removed only two residues from the 8.5gu core, it was concluded that the core of the glycan contained three α-mannose residues, and incomplete digestion was due to a low substrate concentration. Therefore the 20.5gu glycan was a repeat lactosamine oligosaccharide, with mannose only on the other proximal arm.

The remaining glycans from POS2, i.e. those eluting from P4 between 15.5 and 10gu, were treated with jack bean α-mannosidase, which resulted in the appearance of a peak at 10.5gu and the loss of some of the higher molecular weight material at 14.5gu. The alteration of the envelope upon α-

mannosidase digestion demonstrates that hybrid glycans were present in this fraction, however not all glycans appeared to show α-mannosidase sensitivity. Digestion of the products of α-mannosidase digestion with a mixture of jack bean β-hexosaminidase and jack bean β-galactosidase resulted in the collapse of this envelope to two major peaks at 7.5gu (30%) and 6.5gu (70%). The 6.5gu material must have been derived from hybrid glycans because one of the trimannosyl core Manα1→ residues has been removed. On the other hand the the 7.5 gu material could have been derived from complex oligosaccharides due to protection of the pentasaccharide core by GlcNAc substituents, or hybrid glycans incompletely digested with α-mannosidase. Because there was very little radioactivity present in the 7.5 and the 6.5 gu peaks the material could not be analysed further. An estimate of 30% complex oligosaccharide, from the incidence of the 7.5 gu product, provides a maximum limit for the level of this class of glycans in this fraction. Therefore POS2 contains predominantly hybrid glycans and some of these contain repeat lactosamines.

2.4.5.4 Structural Characterisation of the Trisulphated Po Oligosaccharides (POS3) by Exoglycosidase Analysis

These glycans appeared on Bio-Gel P4 as an envelope between 15.5gu and 10.0gu (scheme 2.4, figure 2.10). Treatment of the envelope with jack bean α-mannosidase resulted in a large shift in the profile with a major peak at 10.5gu (accounting for 50% of the radioactivity recovered fvrom Bio-Gel P4), a smaller one at 11.5gu and the residual material eluting at 12.5gu, clearly indicating that the vast majority of these oligosaccharides were hybrid oligosaccharides. The individual peaks were treated with jack bean

β-hexosaminidase and β-galactosidase, resulting in the complete removal of all proximal arm residues of the glycans, i.e. the 10.5gu yielded material at 7.5 and 5.5gu, the 11.5gu at 8.5 and 7.5gu, and the 12.5gu at 7.5 and 8.5gu. Clearly the oligosaccharides exhibit, for the most part, α-mannosidase sensitivity as well as sensitivity to β-galactosidase and hexosaminidase. Based on the fact that the minimum size for a complex class oligosaccharide is 11.5gu, and that 50% of the saccharides eluted at 10.5gu following α-mannosidase digestion, 50% of this fraction at maximum could be complex glycan. The true incidence of the complex class is likely to be much lower than this, based on the observation that the Bio-Gel P4 profile altered very greatly on digestion of the oligosaccharides with α-mannosidase. In addition, a fucosylated complex glycan would have a minimum hydrodynamic volume of 12.5gu, therefore the material eluting at 8.5 gu following galactosidase/hexosaminidase digestion cannot be complex oligosaccharide. Also, a high proportion of the oligosaccharides eluted with a hydrodynamic volume greater than 12.5gu, which were α-mannosidase sensitive because they became smaller on digestion with the mannosidase, and if all of these glycans eluted at 10.5gu following treatment with this enzyme, then a very large number of mannose residues would have had to have been present on these glycans. This is unlikely, in view of the observations made for the other fractions. In addition, all the complex glycans found in the other fractions are larger that 11.5gu. Therefore it is proposed that the incidence of hybrid oligosaccharides accounts for 70-80% of the glycans in POS3.

2.4.6 *L2 Affinity Chromatography*

Approximately 7 nanomoles of total murine brain ^{3}H reduced oligosaccharides and one nanomole of total ^{3}H-reduced oligosaccharides from the first Po preparation were fractionated on an L2 antibody-sepharose column. None of the radiolabelled Po oligosaccharides were retained by the column. Of the whole murine brain oligosaccharides, 0.14% of the radioactivity was retained by the column and subsequently eluted with the elution buffer. No radioactivity was retained by a sepharose 4B column run under the same conditions. Repassage of the unbound murine brain oligosaccharides through the same L2 column did not result in the retention of any radioactivity.

From preliminary experiments it was found that incubation for 1 hour in the elution buffer used in this experiment resulted in de-N-acetylation of the glycans, so that the oligosaccharides were re-N-acetylated as described in appendix one before analysis by high voltage electrophoresis. The electrophoretogram is shown in figure 2.11.

2.4.7 *Gel Filtration Analysis of Sulphated Oligosaccharides From Total and L2 Reactive Po*

Bio-Gel P4 chromatography of the sulphated glycans from total and L2 reactive human Po were obtained following desulphation of the oligosacharides as described in section 2.3.4. The profiles are clearly very similar for both preparations (figure 2.12).

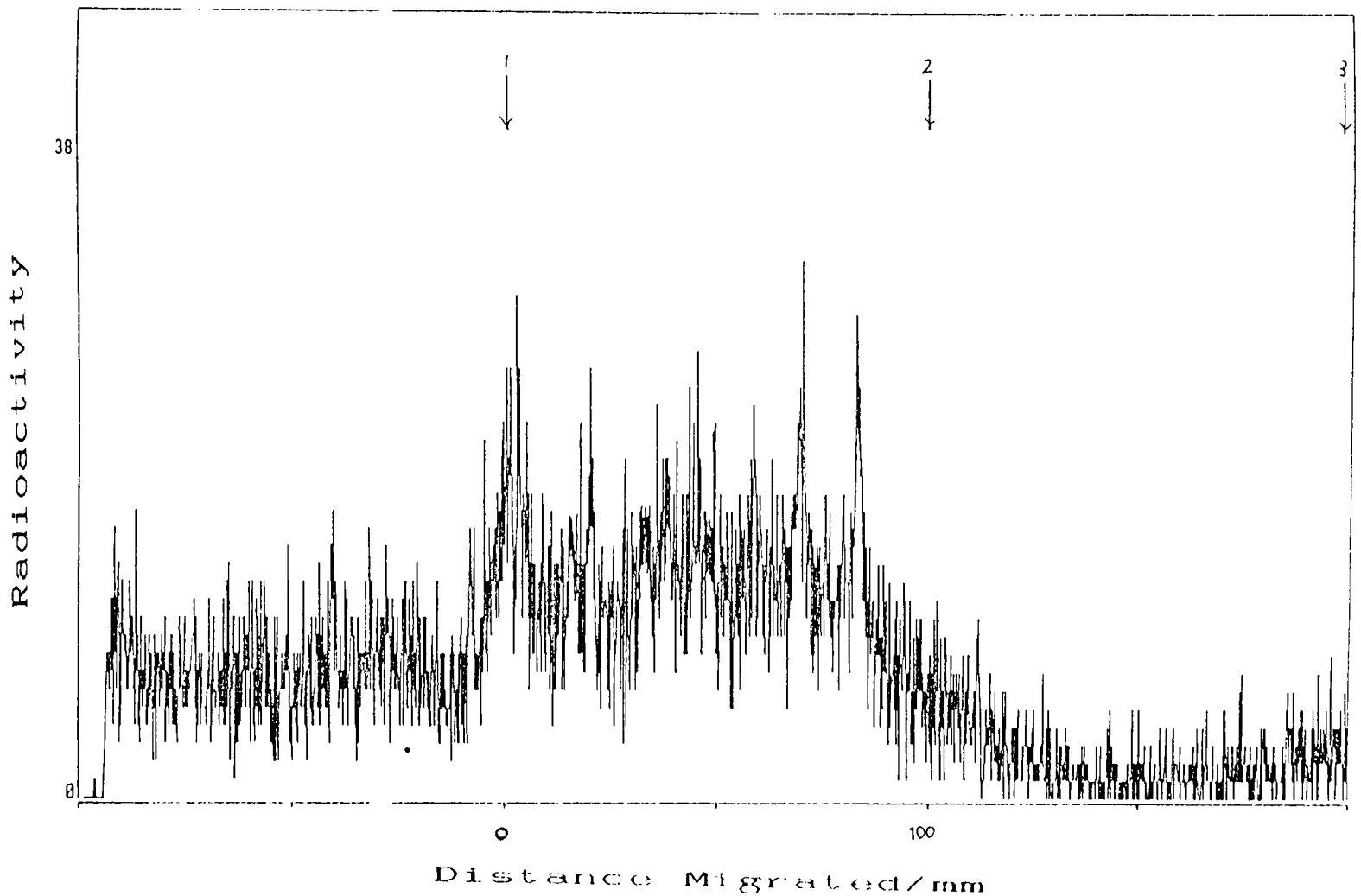
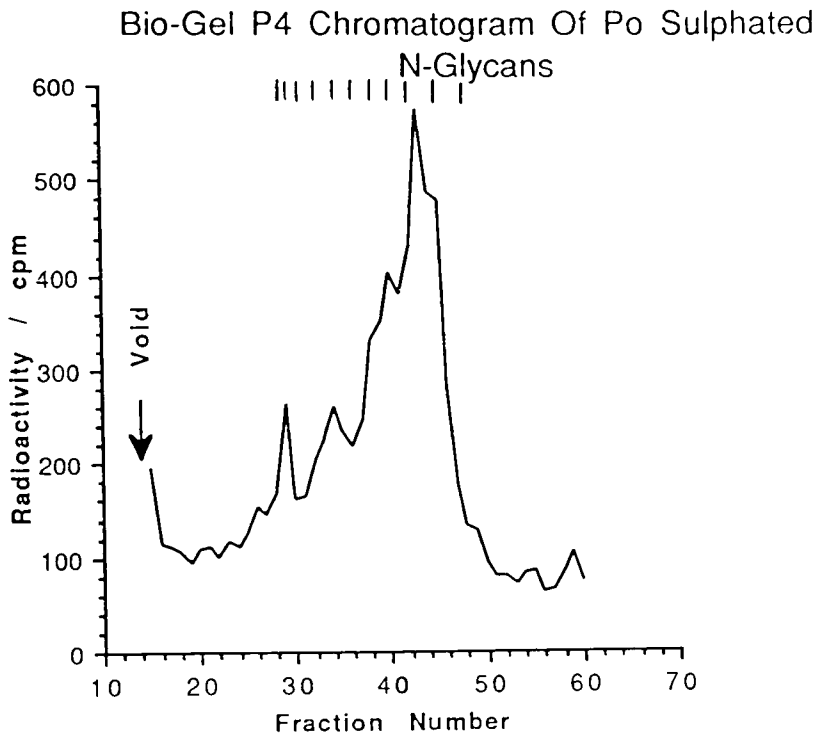


Figure 2.11 High voltage electrophoretogram of ^{3}H reduced oligosaccharides from whole murine brain retained by immobilised L2 monoclonal antibody. Standards are as indicated in figure 2.1.



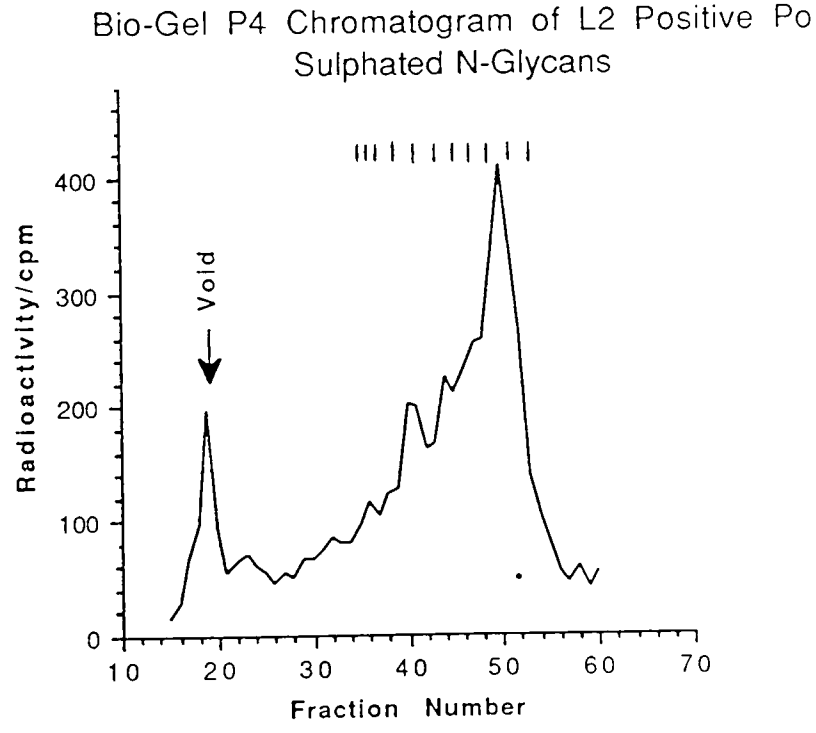


Figure 2.12 Bio-Gel P4 gel filtration chromatograms of Po sulphated oligosaccharides following methanolysis. Upper panel total Po sulphated oligosaccharides, lower panel; L2 reactive Po sulphated oligosaccharides. Note that the profiles are very similar. Internal standards of isomaltose oligosaccharides (dp 10-20) are indicated as for figure 2.9.

2.5 *Discussion*

2.5.1 *Removal of Sulphate and Phosphate Esters*

Several of the protocols for desulphation were effective in the removal of sulphate esters from monosaccharides (tables 2.3 and 2.5). It was found that the efficiency of the reaction depended on the linkage of the sulphate to the saccharide. Differential hydrolysis rates with both acid and alkali have been observed for a number of sulphated glycans, and suseptibility to hydrolysis depends on linkage position and the structure of the glycan (Rees 1963, Percival 1980). This phenomenon can be used to determine linkage positions (Freeze and Wolgast 1986). Methanolysis has been used extensively with acid stable glycans. During methanolysis the three position appears the most labile, and the presence of an N-acetyl group decreases the removal of a sulphate at C6, whilst reduction of the monosaccharide increased the sensitivity of the 6-linked sulphate to methanolysis (table 2.5). By careful manipulation of the sample minimal hydrolysis of glycosidic linkages was observed, even with sialylated glycans. This observation is in agreement with that of Yamashita *et al.* (1983), who also used mild methanolysis to desulphate N-linked glycans from ovalbumin.

Of the other methods, solvolysis in dimethylsulphoxide (Nagasawa *et al.* 1977, Freeze *et al.* 1982) was effective with monosaccharides, but less efficient when used on Po oligosaccharides. Dimethylsulphoxide with methanol could desulphate mannose-6-sulphate containing N-glycans from *Dictyostellium discoideum* (*Freeze et al.* 1982, Freeze and Wolgaust 1986). Therefore this method may only be applicable to some glycans.

Phosphodiesterases that act on complex carbohydrates are not available, but phosphomonoesters can be easily removed with alkaline phosphatase. Therefore using HF in combination with alkaline phosphatase provides an alternative to mild acid hydrolysis, which will only remove methyl groups from mannose-6-PO_4-CH_3 moieties (Mizuochi *et al.* 1981). It was found that 50% aqueous HF did not cleave glycosidic bonds in a reduced complex glycan, nor did it hydrolyse sulphate esters, and therefore this method is generally applicable to the study of N-linked glycans.

The demonstration that methanolysis and HF could be successfully applied to N-linked carbohydrates allowed the application of these protocols to the characterisation of the anionic moieties of whole murine brain and Po N-linked carbohydrates, as well as the selective removal of phosphate and sulphate esters from the whole brain glycans. It is anticipated that these methods will be applicable to other systems in the future.

2.5.2 *Anionic Moieties and Composition of Murine Brain N-linked Oligosaccharides*

Sequential neutralisation of N-linked glycans from whole murine brain allowed the glycans to be fractionated into seven defined groups; (1) naturally neutral, (2) glycans with neuraminidase sensitive sialic acid as the only anionic moiety, (3) oligosaccharides with neuraminidase insensitive sialic acids (polysialic acid and sialic acid in α2→8 linkage) (4) those rendered neutral by both neuraminidase and alkaline phosphatase, (5) oligosaccharides neutralised by HF treatment, following desialylation and alkaline phosphatase treatment, (6) those neutralised by methanolysis

following removal of HF neutralised glycans, and (7) those neutralised by methanolysis alone (table 2.6). Some of the radioactivity could not be neutralised by these treatments, and was not characterised further Alkaline phosphatase alone neutralised a similar proportion of the AG3 treated glycans as when used following neuraminidase digestion, indicating that the majority of the phosphomonoester containing oligosaccharides in this material did not contain neuraminidase sensitive sialic acid.

Passage through AG3 resin severely depleted the brain oligosaccharides of the sulphate and phosphate containing glycans, as well as saccharides rendered neutral by mild acid (table 2.6). The ratio of neuraminidase neutralised to naturally neutral glycans is similar (0.57 for the untreated oligosaccharides compared to 0.38 following AG3 passage). Therefore it is probable that the majority of the sialylated oligosaccharides were not also phosphated, sulphated or polysialylated, because such glycans would have been removed by the resin. In the untreated oligosaccharide preparation there is a significant incidence of sialylated and sulphated glycans. It is very likely that the isolation procedure for the brain oligosaccharides results in the inclusion of lysosomal and Golgi/reticulum glycans, and therefore some of the phosphated oligosaccharides may be derived from intracellular glycoproteins. However, the observation that the mild acid neutralisation could account for all of the glycans rendered neutral by HF treatment indicates that the level of phosphodiester glycans recovered was vey low (table 2.6). A large proportion of the glycans were neutralised by mild acid treatment, indicating that there is a significant amount of polysialic acid and/or acid labile anionic constituents in CNS N-linked glycans. Methanolysis of the untreated brain oligosaccharides neutralised a similar proportion of the glycans as was obtained following HF treatment

(which would remove all the sialic acid and phosphate esters) which suggests that the majority of the sulphated glycans carry only sulphate and no other anionic moiety. This is in agreement with the observation that AG3 treatment did not remove the neuraminidase sensitive sialic acid bearing glycans. The determination of the reducing terminus as only GlcNAcol indicates that significant levels of O-linked glycans were not present.

Therefore it can be concluded that the N-linked glycans from murine brain are heterogenous with respect to the anionic moieties that they contain, and in particular there is a high incidence of sulphated oligosaccharides (20%). This is in contrast to the observation that all of the glycans from human cerebrospinal fluid are neutralised by neuraminidase (chapter three), indicating that the sulphated oligosaccharides may be predominantly associated with membrane bound or intracellular glycoproteins in this case. The high degree of sulphation of the brain N-glycans is interesting in the light of the presence of sulphated oligosaccharides as potential participants in cell adhesion, e.g. N-CAM carries sulphated N-glycans (Sorkin *et al.* 1984), and sulphated saccharides can inhibit lymphocyte migration (Stoolman and Rosen 1983) and adhesion (Parish *et al.* 1988).

The composition of the N-linked oligosaccharide preparation is consistent with that expected for a large population of asparagine-linked glycans. The level of N-acetylglucosamine and sialic acid are low, but within experimental error as amino sugars give lower relative yields than neutral monosaccharides, and sialic acid-TMS derivatives elute late from the GC column and are also poorly recovered. A large amount of fucose was detected, which may reflect the presence of fucose substituents on arm GlcNAc residues as has been reported to be a common substituent in rat

brain glycopeptides (Krusius and Finne 1977). The composition again confirms that the oligosaccharides were originally N-linked to protein. A trace of xylose and GalNAc may indicate the presence of glycosaminoglycan (GAG) or mucin type O-link oligosaccharides, but the level of the former would be very low as no uronic acids were detected.

2.5.3 *Metabolically Labelled Murine Brain Oligosaccharides*

A preparation of $^{35}SO_4$ metabolically labelled N-linked oligosaccharides was obtained by *in vivo* injection of radiolabelled inorganic sulphate directly into the CNS. The radioactivity was shown to be associated with N-linked glycans by several criteria; (1) no radioactivity was soluble in chloroform; methanol, indicating no contamination with lipidic material, (2) all radioactivity remained at the origin of a descending paper chromatogram run under conditions where oligosaccharides do not migrate, (3) GlcNAc was identified as the sole reducing terminus residue, (4) the composition of identically prepared material not labelled *in vivo* was consistent with that expected for N-linked glycans (table 2.7). Taken together these data indicate that the ^{35}S-labelled material consists of N-linked oligosaccharides.

The sequential treatment of the ^{35}S labelled glycans with the reagents for neutralisation resulted in an alteration to the radioelectrophoretogram obtained following paper electrophoresis, but as expected the oligosaccharides remained very highly charged. Neuraminidase treatment resulted in the production of some less mobile glycans. Little alteration appears to have occured with the alkaline phosphatase digestion but a shift in the profile was seen with the HF treatment. This data indicates that the

sulphated glycans may also carry sialic acid and/or phosphodiesters. Because of the low resolution of the paper electrophoresis fractionation it is not possible to draw strong conclusions from this data. Initial attempts with ion exchange chromatography gave very poor yields, and were discontinued. The observation of neuraminidase and HF sensitivity of the ^{35}S glycans is contrary to the ^{3}H-reduced oligosaccharides, where only a small amount of the sulphated glycans appeared to bear other anionic moieties. This may be due to the fact that alterations in an HVE profile do not correlate with neutralisation, and that *in vivo* labelling can give a false impression of the amount of movement that is occuring, in molar terms.

Fractionation of the HF treated ^{35}S labelled oligosaccharides on AG-1 resin identified highly charged material that could be glycosaminoglycan. Whole murine brain oligosaccharides labelled by ^{3}H-reduction did not appear to contain this material even though both preparations had been produced identically. The reason for this discrepancy is probably due to the greater efficiency of incorporation of ^{35}S into the GAG-like glycans, which would be expected to have many more sulphation sites in comparison to standard N-linked glycans. If it is assumed that the pools A and B (figure 2.5) represent standard N-linked glycans, and carry on average two sulphate esters, and pool C is sulphated in a manner similar to GAGs, then this latter material could carry on average about 100 sulphate esters per chain. Considering the incidences of these fractions following AG1 chromatography, pool C would account for 2% of the sulphated glycans by molar incidence, and hence even less of a proportion of the total oligosaccharides, and this is consistent with the composition data.

There are two possible explanations for the presence of the GAG-like

glycans in the brain oligosaccharides; (1) the chains are true GAGs, O-linked to protein through xylose, or (2) they are N-linked to the protein through trimannose-chitobiose cores (Sundblad *et al.* 1988). In support of the latter possibility is the observation that whole tissues contain a huge amount of GAG chains, and because a very small amount of GAG type material was detected, it is difficult to envisage how such a small percent of the total GAG would copurify, i.e. if any GAG was to be obtained it would be reasonable to anticipate a higher representation than seen here. Small O-linked oligosaccharides can be released by hydrazine from the core protein, but this has not been shown for GAGs, and it may be very inefficient for these glycans. In their study, Sundblad *et al.* (1988) estimated that N-linked GAG chains accounted for approximately 0.6% of total CPAE-cell-surface N-glycanase released carbohydrate, and this level is similar to that encountered here. Whatever the linkage of the GAG-like material to protein, it is clearly a very minor component, and the vast majority of the glycans are standard N-linked oligosaccharides, based on the behaviour of both the ^{35}S and ^{3}H-labelled glycans on AG1 and the ^{35}S-labelled saccharides on gel filtration.

The observation of small levels of GAG chains in the N-glycan preparation may have important consequences for the use of such glycans in immunoassays. The undetectable GAGs, present with whatever core, in the ^{3}H labelled brain glycans are completely cryptic to composition and reducing terminus analysis, and were only detected by metabolic labelling. Going to these lengths to characterise material is clearly very time consuming, and also raises the possibility that other moieties may be present that were undetected here. This problem may be further compounded if the glycans are fractionated and the relative concentration of an undetected contaminant

increases if it copurifies with the material of interest. In particular, the whole brain oligosaccharides have been used in an ELISA system to detect L2 activity in the saccharides. The possible potency of a GAG (e.g heparin) in this system could be very high, so that a small level of contamination would be highly significant. It will be of interest to further characterise the GAG chains to determine their linkage to protein by N-glycanase release, and to assess their structure by enzymatic or chemical cleavage methods. The amount of material prevented enzymatic degradation of the GAGs from being performed here. If these GAG-type chains are N-linked to proteins then they may be of great importance functionally, as GAGs are known mediators of extracellular matrix adhesion (McDonald 1988) and of growth factor potency (Scott-Burden and Bühler 1988), and may engender a further level of complexity to the function of cell adhesion molecules if they are components of these proteins.

It is anticipated that the application of the methods for selective removal of anionic moieties from the whole brain oligosaccharides will be of use in characterising the HNK-1/L2 epitope of murine brain glycoproteins. Initial observations have found that the epitope is contained within the acidic oligosaccharides following neuraminidase treatment, which is in accordance with sulphate being an important determinant (M. Schachner and B. Schmitz, personal communication). Subfractionation of this material will allow the structural elucidation of the L2 reactive glycans from murine brain. Further, this method could be used to probe for the *in vivo* L2 oligosaccharide receptor, which may show a different spectrum of activity to that displayed by the monoclonal antibodies used here. Such an approach provides a new probe to study protein-carbohydrate interactions. Studies with ^{35}S *in vivo* labelled

oligosaccharides demonstrated practical possibility and provided some information on these glycans. As sulphated polylactosamine glycans have been found to be HNK-1 reactive (Gowda *et al* 1989), the GAG chains may also be important here.

2.5.4 *The Distribution of Sulphate Esters and Sialic Acids on the Human Po Glycans*

Resistance to neutralisation by neuraminidase and 50% aqueous HF, and neutralisation by methanolysis are all consistent with the presence of sulphate esters on the Po glycans. The absence of glucuronic acid substituents can be inferred from the fact that the glycans were all neutralised by neuraminidase or methanolysis, and could then be degraded enzymatically without the use of a glucuronidase. This observation is in agreement with the composition data of Sakamoto *et al.* (1987) for bovine Po and Ishaque *et al.* (1980) for lapine Po. The observation that the sulphated glycan population is highly conserved between the two preparations, based on the charge analysis (table 2.8) is consistent with the proposal that the L2 monoclonal antibody recognises sulphated oligosaccharides. In addition, the data from the susceptibility to methanolysis (table 2.9) are consistent with the proposal that all of the sulphate esters are present in the same or very similar linkage, and therefore the sulphate may provide a common determinant for all of these glycans. Also, the fractions S1, S2 and S3 contain one, two and three sulphate esters respectively. The observation that the sulphated glycans from the L2 reactive and the total Po preparations are very similar, based on gel filtration analysis (figure 2.12) implies that the L2 monoclonal antibody is not selective for a

restricted set of sulphated oligosaccharides from Po, but rather is sampling the entire population of sulphated glycans, and therefore is able to bind to a heterogenous group of oligosaccharides. The variability in the proportion of neuraminidase-neutralised glycans to sulphated glycans is quite dramatic (table 2.8) and is in sharp contrast to the conservation of the proportions of the sulphated fractions. An explanation for this observation is that the Po molecules bearing nonsulphated oligosaccharides are bound to the L2 affinity column by the Po molecules possessing sulphated glycans. The behavior of the Po glycoprotein, which is a membrane protein with a hydrophobic extracellular domain, a fatty acyl substituent and a strong propensity to aggregate even in the presence of detergents (Ishaque *et al.* 1980), is consistent with this explanation.

Sialic acid was determined to be the other charged moiety on the Po N-linked glycans, and was completely removed with *Artherobacter ureafaciens* neuraminidase. Treatment of the second Po preparation glycans with NDV neuraminidase, which hydrolyses N-acetylneuraminic acid in α2→3 linkage only, resulted in the neutralisation of some of the oligosaccharides. However the electrophoretogram of the treated oligosaccharides was not consistent with the complete loss of all sialic acid, i.e. the electrophoretogram was not identical to that obtained following *A. ureafaciens* neuraminidase digestion. Treatment of the products of the NDV neuraminidase digestion with mild acid did produce a profile similar to that obtained from the first Po preparation oligosaccharides with *A. ureafaciens* digestion, but did not generate further neutral material. Therefore it is proposed that the sialic acid present on the nonsulphated oligosaccharides is in α2→3 linkage, as these were neutralised by NDV neuraminidase, but that present on the sulphated glycans was in either α2→6

or α2→3 linkage. If α2→3 linked sialic acid is present on the sulphated oligosaccharides, the failure of NDV neuraminidase to desialylate these glycans may be due to inhibition of the action of the enzyme by the SO_4 group, which may be located in close proximity to the sialic acid glycosidic linkage. Methanolysis prior to desialylation would resolve this issue. From the results of the NDV neuraminidase digestion it is clear that some of the oligosaccharides carry both sulphate and sialic acid, but it cannot be determined if some sulphated glycans are devoid of sialic acid from the current data. However, the 13.5gu glycan from fraction S1 (table 2.10) could not carry a neuraminidase sensitive sialic acid, and so at least this glycan would be predicted to be sulphated only.

2.5.5 *Structural Analysis of the Human Po Oligosaccharides*

The heterogeneity present in the population of oligosaccharides isolated from human Po is considerable. This is in contrast to the proposal of Sakamoto *et al.* (1987) for bovine Po, where all the glycans were proposed to be hybrid type with the same basic structure. Methylation analysis or composition data of unfractionated glycans as investigated in this work would have generated data consistent with a single average structure as proposed for bovine Po.

Treatment of the sulphated oligosaccharides with endoglycosidase H did not result in the cleavage of the chitobiose core. The presence of core fucose or sulphate has been reported to have no affect on the action of this enzyme (Merkle *et al.* 1985, Schwartz and Elbein 1985), and so resistance to hydrolysis may be due to some aspect of the overall oligosaccharide structure. The minimum structural requirement for action of

this enzyme appears to be Manα1→3Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc (Green *et al.* 1988a) and therefore this sequence would be absent from the Po glycans, and the mannose arm and core of the hybrid glycans would have the sequence Manα1→6Manα1→6Manβ1→4GlcNAcβ1→4(±Fucα1→6)GlcNAc. However, although it has not been investigated, it is possible that the presence of sulphate esters on the arm residues, in particular the terminal mannose, could prevent the action of the enzyme. Bearing in mind the fact that the specificity of endo H is still debated, conclusions with regard to the mannose containing arms of the hybrid glycans are tentative at best. The structure proposed by Sakamoto *et al.* (1987) for the Po glycan would be endoglycosidase H sensitive.

Overall there is a high incidence of hybrid glycans and biantennary complex oligosaccharides. There is a high level of core fucosylation. The hybrid glycans identified in POSA and POS1 are very similar, and are also of a similar structure to the sulphated glycans found on ovalbumin (Yamashita *et al.* 1983a) which were sulphated on a terminal mannose residue.

The defined glycans are predominantly galactosylated (table 2.10), and on biosynthetic grounds the hybrids are probably bisected (chapter one). The 13.5 gu glycan from POS1 is unusual in this regard, as it carries no terminal galactose residues, but has two fucose termini. This oligosaccharide was not identified in other charge fractions, and may carry a sulphate on one of the terminal GlcNAc residues. The repeat lactosamine glycan from POS2 carries four Galβ1→4(3)GlcNAc repeat sequences on one arm, and only mannose on the other arm. This structure was also restricted to a single charge fraction. The fucosylated biantennary saccharide (14.5gu) was identified in both POSA and POS1, and the hybrid glycans in POSA and POS1

Table 2.10 **PROPOSED STRUCTURES OF THE DEFINED OLIGOSACCHARIDES FROM L2 REACTIVE Po**

P4 Elution Position (gu)	Structure
Fraction POSA	
14.5	Galβ1→4(3)GlcNAcβ1→2Manα1→6 Manβ1→4GlcNAcβ1→4GlcNAc Galβ1→4(3)GlcNAcβ1→2Manα1→3 6 ↑ Fucα1
12.9	GlcNAcβ1→2Manα1→6 Galβ1→4(3)[Manβ1→4GlcNAcβ1→4GlcNAc GlcNAcβ1→2Manα1→3 4 ↑ GlcNAcβ1
12.3	±[Manα1→3](6)Manα1→6 Manβ1→4GlcNAcβ1→4GlcNAc Galβ1→4(3)GlcNAcβ1→2Manα1→3 6 ↑ Fucα1
9.2	Manα1→6 Manα1→6 Manα1→3 Manβ1→4GlcNAcβ1→4GlcNAc Manα1→3
Fraction POS1	
14.5	Galβ1→4(3)GlcNAcβ1→2Manα1→6 Manβ1→4GlcNAcβ1→4GlcNAc Galβ1→4(3)GlcNAcβ1→2Manα1→3 6 ↑ Fucα1
13.5	GlcNAcβ1→2Manα1→6(3) Manβ1→4GlcNAcβ1→4GlcNAc GlcNAcβ1→2Manα1→3(6) 6 3(6) ↑ ↑ Fucα1 Fucα1
12.3	Manα1→6(Manα1→3)Manα1→6 Manβ1→4GlcNAcβ1→4GlcNAc Galβ1→4(3)GlcNAcβ1→2Manα1→3

Table 2.10 continued

P4 Elution Position (gu)	Structure
11.5	Manα1→6 Manβ1→4GlcNAcβ1→4GlcNAc Galβ1→4(3)GlcNAcβ1→2Manα1→3 6 ↑ Fucα1

Fraction POS2

20.5	Manα1→3(6)Manα1→6 Manβ1→4GlcNAcβ1→4GlcNAc [Galβ1→4(3)GlcNAcβ1→3]$_{3}$Galβ1→4(3)GlcNAcβ1→2Manα1→3

Proposed primary structures for Po glycans. Those saccharides for which there was clear data have been reconstructed from the information obtained by enzymatic sequencing. POS3 and the 10.0 to 15.5 gu envelope from POS2 are not represented here because the data did not allow the reliable reconstruction of primary structures. The data presented in this table represent 63.2% of the glycans recovered from the second preparation of L2 reactive Po. The structures proposed are based on enzymatic sequence analysis, and also rely on a consideration of the standard biosynthetic pathway. Because methylation analysis and composition data are not available, the HexNAc residues assigned by β-hexosaminidase digestion could also be GalNAc, but this is considered to be unlikely. Molar incidences are not given, because these were not accurately determined.

appear to be the same basic structures with variable mannose substitution and core fucosylation. Therefore a large number of distinct structures were identified, and as Po carries a single glycosylation site, each of these glycans represents a different glycoform. The number of Po glycoforms in the total population may be considerable, as the L2 reactive material may be a small fraction of the total Po population.

If the incidence of hybrid glycans does increase with increased sulphation (table 2.11, and see results), then perhaps the hybrid glycans are better substrates for a sulphotransferase. It is difficult to envisage how three sulphate esters are linked to a hybrid oligosaccharide in the POS3 fraction and the possibility that these glycans are also sialylated cannot be ruled out. The occurrence of only two sulphates on the lactosamine is interesting, as this glycan has several potential sulphation sites and therefore the addition of the second sulphate group may prevent further action of the sulphotransferases. The role of the lactosamine repeat containing oligosaccharide may be of interest, as these saccharides have been found to be important in cell-cell interactions (Rastan *et al.* 1985). The observation of the same glycan structures in POSA and POS1 indicates that these oligosaccharides can serve as substrates for both the Schwann cell sulphotransferase and sialyltransferase. At least one of the sulphated glycans, eluting at 13.5gu in the POS1 fraction, is not a substrate for a sialyltransferase (table 2.10). Because the position of the sulphate esters was not identified, it cannot be determined if the sialyl- and sulphotransferases compete for the same position on the glycan. But the observation that there are tri-sulphated glycans with similar molecular weights as the mono-sulphated glycans indicates that there are several possible sites for sulphation. The high level of specificity observed with

Table 2.11(a)

CLASSES OF N-LINKED OLIGOSACCHARIDES ON L2 PEACTIVE Po

Structure	Class	% Molar Incidence
Structures neutral post desialylation		
	Oligomannose	2.0
	Biantennary Complex	9.2
	Hybrid	7.7
Structures Bearing One Sulphate Ester		
	Biantennary Complex	16.7
	Hybrid	25.7
Structures Bearing Two Sulphate Esters		
	Biantennary Complex	4.8*
	Lactosamine Repeat Containing Hybrid	1.9
	Hybrid	11.2

Table 2.11(a) Continued

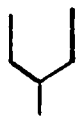
Structure	Class	% Molar Incidence
Structures Bearing Three Sulphate Esters		
	Complex	6.2*
	Hybrid	14.6

Table 2.11(b)

Summary of Incidences of Glycan Classes of the Oligosaccharides From L2 Reactive Po

Class	All Glycans	Unsulphated Glycans	Sulphated Glycans
Oligomannose	2.0	10.6	0.0
Hybrid	59.2	48.7	63.7
Lactosamine Repeat	1.9	0.0	2.3
Complex*	36.9	40.7	34.0
Total	100.0	100.0	100.0
Ratio Hybrid/Complex	1.6	1.2	1.9

The glycan classes were elucidated from the second Po preparation as detailed in the text. Icons represent the pentasaccharide core Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{\beta 1}$, with the α1→6 arm drawn on the right. The mannose containing arm (M) of the hybrid structures is proposed to be on the Manα1→6 arm on biosynthetic grounds. The complex glycans were all found to be biantennary type. Each icon represents class only which may be composed of more than one structure.

* The incidence of the hybrid glycans in the di- and tri-sulphated fractions is taken as 70% in each case, as argued in results.

The incidence of the classes presented is tabulated (b) for the whole glycan population and the unsulphated and sulphated glycans analysed in this study. It is clear that the majority of the saccharides are of the hybrid type.

N-glycan sulphotransferases so far studied (Smith and Baenziger 1988, Spiro and Bhoyroo 1988) may indicate that a battery of enzymes is responsible for sulphation of the Po oligosaccharides, rather than a single enzyme capable of catalysing sulphation at multiple sites.

It is interesting to note that the rat and bovine Po polypeptides are strongly conserved in evolution (Axel and Lemke 1980, Sakamoto *et al.* 1987), whilst the data for the oligosaccharide portion from lapine (Ishaque *et al.* 1980) and bovine Po (Sakamoto *et al.* 1987) suggests that the glycans are similar. The present study extends this to the human protein. For the glycans analysed here, many of the structures would be similar to those for the bovine and lapine Po. This is in contrast to examples of species specific glycosylation, e.g. α1-acid glycoprotein and γ-glutamyltranspeptidase (Kobata 1984), and may indicate that the precise structures of the glycans of Po play an essential role in the function of the glycoprotein, or that the glycosylation capability of Schwann cells has been conserved through mammalian evolution. Then conservation of the polypeptide sequence ensures that the same glycans are produced by biosynthesis.

The data presented here indicates that the microheterogeneity of the glycans on human Po is quite considerable and prompts the possibility that heterogeneity is also present amongst the Po glycans from different species. A reexamination of the oligosaccharides present on these molecules is required to establish if this is the case. Fuller studies of the POS2 and POS3 glycans are needed. Attempts made by the author were confounded by problems with the purity of donated samples. It would also be of interest to study the glycans of Po produced by Schwann cells in culture, as these cells can be maintained indefinitely and thus provide a ready source of

protein for study. Analysis of the Po glycans from cultured cells under different conditions, for example cell density, culture substrate or co-culture with neurones may allow further elucidation of the rôle that this glycoprotein plays in myelin formation.

2.5.6 *L2 Affinity Chromatography of Oligosaccharides*

The failure of the released human Po oligosaccharides to bind to immobilised L2 antibodies was unexpected, as the glycoprotein was isolated by affinity chromatography with an L2 antibody proposed to recognise a carbohydrate determinant. More in line with expectations was the binding by the column of some radioactivity from the murine brain oligosaccharides. In this case approximately 0.1% of the glycans were bound by the L2 antibodies. This would be reasonably expected for a single determinant with potential biological activity. Due to the small amount of bound material it was only possible to obtain a radioelectrophoretogram of these glycans (figure 2.11), and this was of poor quality. However, it is clear from figure 2.11 that the bound glycans are heterogenous. Chromatography with the Po glycans indicates that a significant population of oligosaccharides that are L2 positive when present on a polypeptide are not seen by the antibody when they are released from the protein. In addition, attempts to isolate glycopeptides that carry the L2 determinant from murine brain have also been unsuccessful (B. Schmitz, personal communication).

These data can be taken to indicate that released saccharides or glycopeptides do not bind very efficiently to L2 antibodies under affinity chromatography conditions. The glycans bound by the L2 antibodies from the whole brain oligosaccharides may represent exceptionally high affinity

ligands, and therefore do not reflect the total population of L2 reactive glycans in the CNS. Several possible reasons can be proposed to explain these observations. (1) Removal from the polypeptide and subsequent reduction of the glycans may alter the configuration of the glycan core. However, because the SGGL glycan is distinct from the N-linked saccharides of Po, and murine brain glycopeptides also fail to bind to L2 antibody columns this possibility can be excluded. (2) The conformation of the glycan may be influenced by the polypeptide so that none of the saccharides are available for interaction when in the released form. These possibilities can also be discounted because binding of L2 antibodies is observed with glycolipids which would not be expected to have the same conformational constraints on the glycans. (3) The glycolipid determinant is a high affinity ligand for L2, and does not need additional contributions in binding energy from a polypeptide, whilst the Po glycans and the majority of the CNS oligosaccharides are lower affinity ligands, so that additional binding energy is required from the polypeptide. This possibility cannot be discounted with the data availible here. (4) The concentration of the ligand may have been too low to allow efficient interaction between the glycan and the antibody. With the experiments here, as only one nanomole of Po saccharides were used, this may have been the case . The affinity of many anticarbohydrate antibodies can be low, thus high ligand concentrations may be required to produce effective binding. This problem can be overcome with blotting or histochemical staining techniques, where the ligand densities in a band or area of a section can be very high. It is also possible that coupling of the glycan to a hydrophobic moiety, i.e. a lipid in the case of the SGGLs, or the polypeptide of Po for the Po glycans, may produce a more favourable binding

energy for interaction between the saccharide and antibody, due to hydrophobic interactions. This is consistent with contempory views on antibody:antigen interactions (e.g. Roberts *et al.* 1987). This last possibility is consistent with proposal (3), in that a specific structure may not necessarily be required from the polypeptide, but a physicochemical contribution may an important factor in contributing binding energy. In ELISAs, a large amount of whole brain oligosaccharides (about 15µg sugar per microtitre well, i.e. millimolar concentrations, of which 20% are sulphated) have been found to be necessary to effect 50% inhibition of L2 binding to murine brain membrane proteins (B. Schmidt, personnal communication).

Therefore a combination of concentration, hydrophobicity, and a requirement for additional polypeptide determinants are candidate explanations for the observation that glycans cannot be efficiently bound back to an L2 column after removal from the polypeptide. None of these possibilities can be excluded with the information available at present. This problem might be circumvented by the application of large amounts of oligosaccharides to the L2 column, or derivatisation of the glycans (e.g. to neoglycolipids (Gooi *et al.* 1987)) to facilitate the exploitation of blotting methods.

2.5.6 *The HNK-1/L2 Epitope*

The data presented here formally demonstrate that glucuronic acid is not required for L2 binding to a glycoprotein, but that sulphation of the oligosaccharides is important. This is in agreement with the recent data of Gowda *et al.* (1989), who showed that sulphated lactosamine repeat glycans

were reactive with HNK-1. As argued above it is likely that the unsulphated glycans were derived from Po that had been retarded on the L2 column by aggregation with the L2 reactive Po, and therefore the non-sulphated glycans are not L2 reactive. The neutralised glycan structures have been encountered elsewhere (e.g. the glycohormones, ovalbumin, serum glycoproteins), and therefore it is unlikely that these structures are responsible for the HNK-1/L2 reactivity *per se*. Because of the purification strategy employed, it is highly improbable that HNK-1/L2 reactive contaminating material was responsible for the isolation of the Po in this study. Because the Po glycoprotein carries a single site, most of the oligosaccharides isolated should be capable of binding to HNK-1/L2, at least in the context of the glycoprotein (with the reservations discussed above). Therefore, the HNK-1/L2 epitope on glycoproteins is not the same as that found on the SGGL glycolipids. None of the structures of the human Po glycans was the same as the SGGL glycan.

The Bio-Gel P4 chromatograms of the L2 reactive and the total populations of Po sulphated oligosaccharides following methanolysis are remarkably similar (figure 2.12), and therefore L2 does not show specific binding to the underlying sulphated glycans from Po. As only some sulphated carbohydrates are HNK-1 reactive (e.g. chondroitin sulphate is not), there is some restriction on the nature of the sulphate-oligosaccharide moiety required for HNK-1 binding. Likely candidates are Gal-SO_4 and GlcNAc-SO_4. As sulphatide (Gal-3-SO_4-Cer) is active as an L2 saccharide analogue *in vitro* (Kunemund *et al.* 1988), and galactose-3-sulphate is a known component of some N-glycans (see Spiro and Bhoyroo 1988), and could also be present in the Po N-linked oligisaccharides, there is precident that galactose-3-sulphate is the HNK-1/L2 epitope. However, it has been stated that

sulphatide does not bind HNK-1 directly (Gowda *et al.* 1989), which may reflect differences between L2 and HNK-1. GlcNAc-SO_4 is also a possible candidate, and this moiety could be present in the Po glycans and the sulphated glyans reported as HNK-1 active (Gowda *et al.* 1989). It should be noted that free GlcA-3-SO_4 was not found to be active as an L2 saccharide analogue *in vitro* (Kunemund *et al.* 1988), and therefore for L2 binding there may be an additional requirement for the sulphated residue to be part of a larger structure and positioned at the non-reducing terminus.

In the neutralised glycans the presence of arm GlcNAc in all of the saccharides could be considered as a novel determinant suitable for discrimination by the L2 monoclonal if it is also 3'-sulphated. This is additional evidence for the HNK-1/L2 epitope not being a feature of the glycan core itself The 13.5 gu oligosaccharide from POS1 is the only glycan characterised in the sulphated fractions not bearing at least one terminal galactose residue following neutralisation. Therefore if this glycan is L2 reactive its determinant could be GlcNAc sulphate. From the present data it is not possible to conclude if Gal-SO_4 and/or GlcNAc-SO_4 are the HNK-1/L2 determinant. However it is clear that the assumption made by many authors that the SGGL sulphated glycan, or part of it, is present on HNK-1/L2 binding glycoproteins, is incorrect. This is the first report of a detailed structural analysis that disputes this dogma.

The demonstration of a high incidence of sulphated oligosaccharides amongst the N-linked oligosaccharides of CNS glycoproteins raises the question of the selectivity of HNK-1/L2 binding. HNK-1/L2 activity against glycoprotein determinants is restricted to certain anatomical regions, and within these to specific glycoproteins (Yamamoto *et al* 1988). Regulation of epitope expression may be achieved by controlling the activity of a

sulphotransferase. It will be of interest to establish if the observations made here for human Po can be extended to other species and other glycoproteins carrying the HNK-1/L2 epitope.

Of special interest is N-CAM. The activity of this CAM is thought to be modulated in the embryonic chick by the presence of polysialic acid (Edelman 1987) and control of the HNK-1/L2 saccharide in later life may be an example of the exploitation of carbohydrate structural diversity at different points in ontogeny to increase the scope of a single polypeptide to mediate adhesion events. Elucidation of the HNK-1/L2 reactive glycans from N-CAM would serve to strengthen these proposals. Glycan structure modulation coupled with the high number of mature mRNA species generated by alternate splicing of the N-CAM message (e.g. Santoniz *et al.* 1989) could generate an immense level of diversity in the N-CAM glycoproteins that are produced in the CNS, with a potential for very subtle regulation of function between different splicoforms and glycoforms.

2.6 *Conclusions*

The information presented in this chapter represents the first investigation of the detailed structure of the asparagine-linked oligosaccharides derived from a glycoprotein carrying the HNK-1/L2 epitope. In particular the glycans present on the fraction of Po glycoforms that are reactive with an L2 monoclonal antibody were investigated and the epitope shown to be distinct from that encountered in the previously defined SGGL glycan. The same may be true for other glycoproteins bearing this determinant. With regard to the HNK-1/L2 epitope on Po, it is highly probable that the important determinant is a small part of the total

glycan, with Gal- or GlcNAc-SO_4 as strong candidates.

Because the L2 saccharide is itself able to influence cell adhesion (Kunemund *et al.* 1988), a receptor for this glycan must exist *in vivo*. Nothing is known about the structure or biochemistry of this molecule. Because the expression of HNK-1 reactivity shifts from glycolipid to glycoprotein during development, the L2 receptor must either be able to recognise both, or else consist of a group of adhesion molecules recognising a common structural feature. This is reminiscent of the integrin adhesion molecule superfamily, which bind to the tripeptide sequence Arg-Gly-Asp (RGD) from extracellular matrix glycoproteins, but also discriminate between the molecules that carry the RGD peptide (Rhouslatti and Pierschbacher 1987). From the study here, it is valid to conclude that L2 binding is conferred not by a glycan of one structure, but a whole set, with a small common or equivalent determinant. Co-ordinate regulation of carbohydrate and the polypeptide that carries the glycan has enormous potential for highly sophisticated control of cell adhesion events.

Partial characterisation of the oligosaccharides derived from whole murine brains allowed some appreciation of the heterogeneity of these glycans to be made. In particular the range of anionic moieties expressed by this population of saccharides is very high. The ability to selectively remove the charged groups from the glycans will be of considerable use in the further characterisation of the HNK-1/L2 determinant, which is of great importance to increasing our understanding of the molecular basis of cell-adhesion and the role that carbohydrates play in this process.

CHAPTER THREE

AN INVESTIGATION OF THE N-GLYCOSYLATION OF SERUM AND CEREBROSPINAL FLUID GLYCOPROTEINS OF MULTIPLE SCLEROSIS PATIENTS

3.1 *Preamble*

In this chapter the immunology of multiple sclerosis (MS) is reviewed, with specific reference to oligoclonal banding of IgG in the cerebrospinal fluid (CSF), the most consistently observed clinical parameter in MS. An analysis of the N-linked oligosaccharides from total serum and CSF glycoproteins from MS patients is presented. Linkage analysis of the CSF N-glycans following fractionation on immobilised Concanavalin A (ConA) demonstrated that the CSF N-glycans are similar to serum glycoprotein N-glycans. A method for the isolation of CSF IgG and study of the N-glycans by exoglycosidases derived from this glycoprotein is detailed. The oligosaccharides of CSF IgG were found to be exclusively biantennary complex, and similar to those found on serum IgG.

From the data presented in this chapter the following conclusions can be drawn. (1) There is no alteration in the total N-linked oligosaccharides of total serum or CSF or serum IgG in MS. (2) There is a clear difference between the N-linked oligosaccharides from total serum and CSF glycoproteins, which is conserved in MS. (3) The N-glycans of intrathecal IgG are the same as those in serum IgG, but the relative incidence of core fucose is decreased to 60% of the serum IgG value in normal CSF IgG, but not in MS CSF IgG. It is concluded from these data that altered

glycosylation does not result when an individual contracts MS, and that abnormal glycosylation cannot account for this autoimmune disease. The alteration in N-glycans of CSF IgG in MS is argued to result from of an influx of B lymphocytes into the central nervous system (CNS).

3.2 Introduction

Multiple sclerosis (MS) is a neurodegenerative autoimmune disease of the human CNS of unknown aetiology. The causative agent of the disease has been variously proposed to be viral, bacterial or environmental. Epidemiology of the disease, reviewed by Kurtzke (1983), indicates that the susceptibility varies with geographical location. The incidence rate is high in Europe, across the central region of North America and in Southern Australasia. Within the high risk regions, pockets of extremely great risk can be defined. In Scandinavia the geography of MS has been mapped to a high resolution and the greatest risk areas appear to extend along lines of easier or more frequent communication and high population density. MS is slightly more common in females, with a mean onset age of about 30 and a death rate maximum at 60 years. Evidence from epidemiology in support of an infective cause has been cited (Kurtzke and Hyllested 1979, Kurtzke *et al.* 1980, Kurtzke 1978), but an agent that correlates with the disease has not been identified (Meulen and Stephenson 1983).

Much insight into MS has come from a study of an animal model for the disease, experimental autoimmune encephalomyelitis (EAE). EAE is initiated by immunisation of an animal with myelin extracts. Myelin basic protein (MBP) appears to be the most effective encephalytogenic component in the extracts. Cross reaction between this protein and measles virus antigens

has been proposed to initiate autoimmunity in the brain of MS patients, and sequence homology between MBP and measles virus surface antigens supports this. Unfortunately only about half of the MS brains analysed contain measles virus DNA (Haase *et al.* 1981), the same proportion as found in non-MS brains. A similar lack of a correlation has been observed for a large number of viruses which have been proposed as possible causative agents for MS (see Meulen and Stephenson 1983, Vandvik and Norrby 1989).

The disease course can take two paths. The chronic and progressive forms present a slow decine with accumulated loss of function, whilst the relapsing-remitting form is episodic with the patient remaining quiesent and even improving between relapse periods. During a relapse a significant loss of faculties can occur over a very short time. The episodic nature of relapsing-remitting MS tends to smooth out into the chronic form as the disease progresses.

MS is characterised by extensive demyelination of axon fibres resulting in plaques. These grow by immunological activity localised chiefly at the margins of the plaques. Perivascular cuffs, surrounding the capillaries, contain lymphocytes, plasma cells and monocytes. From the cuffs monocytes invade the parenchyma and are transformed into macrophages which actively phagocytose damaged myelin. The status of *multiple* sclerosis results from the impairment of more than one neural function that can be correlated to physically separate regions of the brain. A common defect occurs in processing of visual information, which is used as a clinical test for MS. Elevated immune function in the CNS can often be observed, i.e. the number of lymphocytes and the concentration of CSF immunoglobulins can be raised (reviewed in Walsh and Tortellotte 1983). Whilst the CNS has been considered as an organ of immunological privilege, this is clearly

incorrect as a considerable level of immunological activity is evident in CNS tissue (Merrill 1987).

3.2.1 *Immunology and Oligoclonal Banding*

Rapid migration of T cells into the CSF of progressive MS patients has been observed (Hafler and Weiner 1987). The persistent inflammation may require this continual replenishment. The CNS appears to sequester antigen-specific T cells during viral encephalomyelitis. The selective accumulation of some T and B cell clones in the CNS could be due to selective migration or proliferation and expansion of a restricted set of lymphocytes. T cells in the G1 phase of the cell cycle have been detected in MS CSF, and their activation appears more pronounced during exacerbations (Noronha *et al.* 1980). The mechanism of damage at the plaque is not known, but the presence of activated macrophages and natural killer (NK) cells suggests that the process is not antigen specific, although NK-like T cells expressing functional T cell receptor γ-chains have been observed in the CSF (Ang *et al.* 1987).

The immune dysfunction in MS may be due to a defect in T cell supression circuits. Abnormal T8 T cell function has been demonstrated in MS peripheral blood (Antel *et al.* 1986). Morimoto *et al.* (1987) extended this finding to show that the suppressor-inducer T cell subset (T4+, 2H4+) was defective in MS patients. The autologous mixed lymphocyte reaction is decreased in MS patients (Hafler *et al.* 1985). A recent study has demonstrated that the variable domains of T cell receptors expressed by encephalitogenic T cells in EAE belong to a very restricted subset and that monoclonal antibodies directed against these receptors can alleviate the

disease (Urban *et al.* 1988). A similar therapy for use in MS itself remains to be found. A cruder approach using anti-CD4 or anti-CD2 mouse monoclonal antibodies resulted in some immunosuppression in MS patients (Hafler *et al* 1988). Classical therapy has been administration of immunosuppressants like cyclosporin, and nonsteroidal anti-inflammatrants.

Susceptibility to MS has been mapped to the HLA-D gene locus, DW2 being an important predisposing allele (Waksman 1985) with less strong linkage to HLA A3 and B7 antigens (Paty *et al* 1977). The latter two haplotypes may correlate with the unresponsiveness of T cells to mitogens. There is also a tentative link to the IgG allotype genes. The overbearing observation is that no haplotype engenders a high risk factor, so that the contribution of the MHC to MS is probably minor in the majority of cases.

The most reliable clinical parameter is oligoclonal banding of IgG from the MS brain as visualised by isoelectric focusing. Laterne *et al.* (1970) demonstrated that oligoclonal bands of γ-globulin were specific for MS. Over 99% of clinically definite MS patients have these bands, which are absent in the serum (Tortellotte *et al.* 1983). The banding resembles that of serum IgG after repeated immunisation with a single antigen. The oligoclonal IgG has also been found in some other inflammatory CNS diseases, e.g. neurosyphillis. Tortellote *et al.* (1986) have proposed that oligoclonal bands result from the output of a small population of circulating plasma B-cells that are held within the CNS. The IgG is at least partially synthesised within the CNS because the blood brain barrier (BBB) is considered to be intact in MS (Tibbling *et al.* 1977, Link and Tibbling 1977).

The specificity of the oligoclonal IgG is not known. Minor components are directed against autoantigens and viral determinants, but the major

portion (>90%) is undefined. Clearly, autoimmune stimulation of a restricted set of B cells as found in the CNS would lead to production of oligoclonal IgG. Grimaldi *et al.* (1985) have demonstrated the presence of oligoclonal IgA in some MS CSF. Analysis of immune complexes from the CSF of MS patients (Coyle 1985) showed the presence of Herpes simplex virus type 1 antigens, myelin basic protein and antibodies directed against brain glycolipids in complexes from some individuals. Anti-idiotype analysis of the oligoclonal IgG indicates that there is a limited V gene usage so that the anti-IgG raised against one MS patient's oligoclonal immunoglobulins can cross react with some others, but not with CSF IgG from non-MS individuals (Tachovsky *et al.* 1982). Therefore the vast majority of oligoclonal IgG in MS would appear to be an immune response against an unknown antigen.

Hyperimmunisation of rabbits results in altered glycosylation of IgG (Murray and Brown 1988), and abnormal glycosylation may underlie the establishment of autoimmunity in some cases (Rademacher *et al.* 1988b). Therefore, it was of interest to determine if the IgG in the CNS of MS patients exhibited a change in glycosylation due to repeated antigen stimulation of the B cells (Tortellote *et al.* 1986). In addition, because inflammation can alter the glycosylation of some serum proteins (Bierhuizen *et al.* 1988), it was important to establish if changes in the overall population of N-linked oligosaccharides were associated with MS. Clearly, this latter could be due to changes in the levels of individual glycoproteins, their glycosylation or both of these factors.

3.2.2 *Glycosylation of CSF Proteins*

Few proteins that exist in both the CNS and systemic compartments have been studied with respect to their glycosylation. Ribonuclease (RNAse) represents a higher proportion of CSF protein than serum protein. Schieven *et al.* (1982) demonstrated that its glycosylation was altered in the CSF compared to serum, with some loss of sialic acid and galactose residues. These authors suggested that the alteration may be due to the transport of the RNAse into the CNS across the BBB. Alliquant *et al.* (1984) have shown that there is no correlation between serum and CSF RNAse activity and between CSF albumin and CSF RNAse activity, which would indicate that the RNAse is either synthesised intrathecally, or is specifically transported into the CSF.

It has been proposed that the N-linked glycans of neural glycoproteins display unique structural features (Parekh *et al.* 1987), which may act as an organ specific marker. Therefore the structure of the N-linked oligosaccharides of total CSF glycoproteins and IgG were studied to determine whether the CNS displays glycosylation patterns that can be considered as organ specific, or if the oligosaccharides were similar to those observed for systemic glycoproteins.

3.3 ***Methods and Materials***

Serum and CSF from patients with clinically definate MS used for the analysis of total serum and CSF N-glycans were provided by Prof. H. Weiner, Brigham and Women's Hospital, Boston, MA, USA (3M, 6F, ages 21-60, mean ages 36). CSF from normal humans for total N-glycan analysis (4M, 2F ages

39-49, mean age 44) and CSF from normal humans (sample#1; 2M, 1F, ages 19, 47 and unknown, sample#2; 1M, 3F, ages 19-74, mean age 62) possible MS (2M, 2F, ages 15-55, mean age 42), definate MS (4F, ages 23-38, mean age 31) and dementia (1M, age unknown), used in the isolation of intrathecal IgG, were kindly obtained by Dr. C.J. Edge of the Glycobiology Unit from local hospitals in the Oxford area.

3.3.1 *Purification of Proteins*

IgG was isolated from unpooled serum of MS patients by ammonium sulphate precipitation and DEAE ion exchange as described in appendix one. IgG was purified from pooled CSF samples (20ml) and from normal serum (150µl) by protein G HPLAC using the method given in appendix one. CSF samples were pooled due to the low level of material (25-30µg/ml γ-globulin). The protein G bound material (IgG) was pooled, concentrated and analysed by SDS-PAGE and immunoelectrophoresis (figures 3.1 and 3.2).

3.3.2 *Oligosaccharide Analysis*

N-linked oligosaccharides were released from protein, purified and radiolabelled as described in appendix one.

Oligosaccharides were separated on the basis of net charge by HVE in the presence of pyridine-acetate. The relative incidence of neutral and acidic glycans was assessed after elution of the origin region (neutral glycans) and the rest of the paper (acidic glycans) by liquid scintillation counting. The number of sialic acid residues on oligosaccharides obtained from purified IgG was determined by ion exchange chromatography on monoQ as

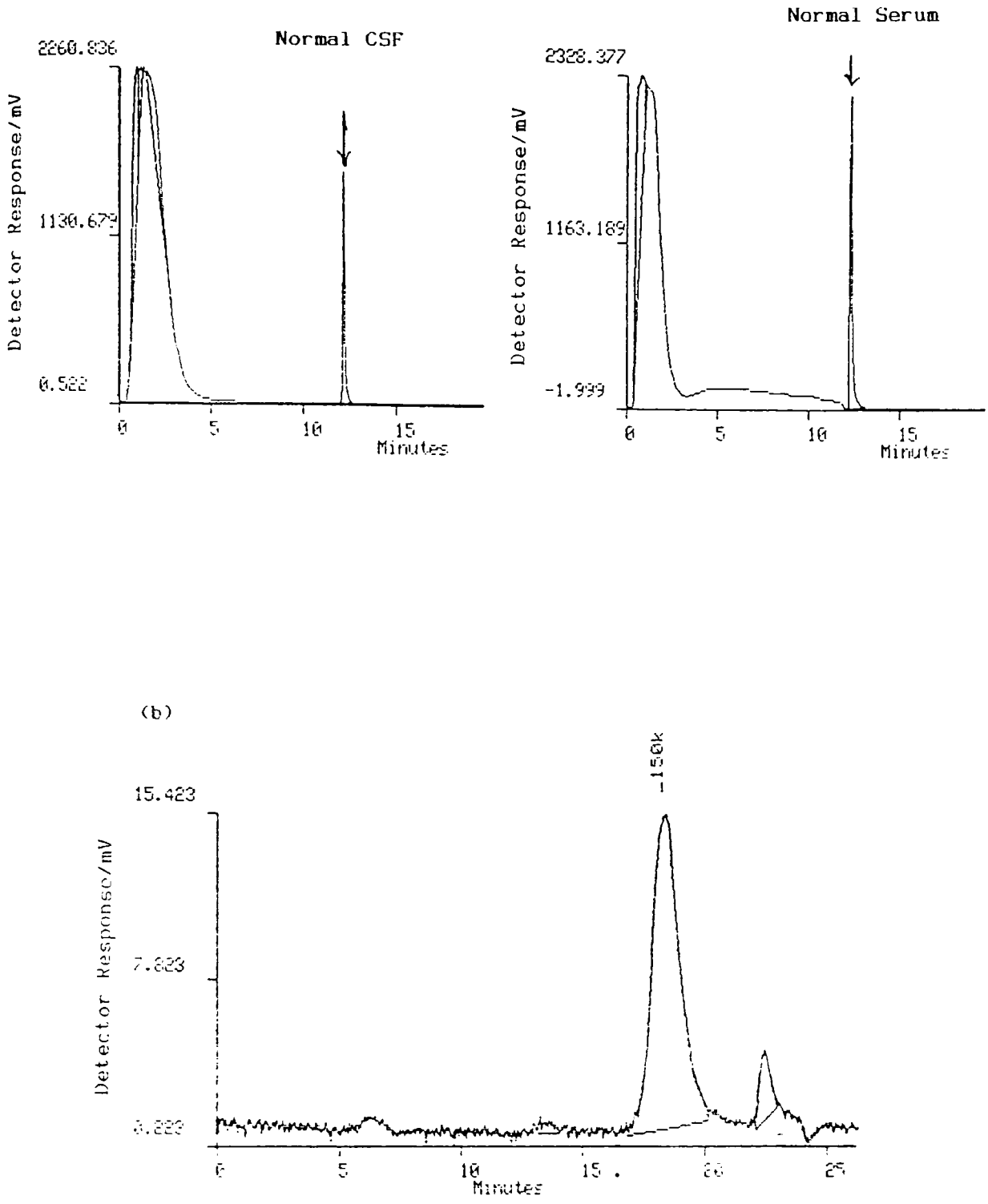


Figure 3.1 (a) Elution profiles at 280nm of human serum and CSF on protein G high performance liquid affinity chromatography (HPLAC). The conditions used are given in appendix one. Material eluting between 0.0-5.0 minutes and 12.0-13.0 minutes was pooled seperately and analysed by SDS-PAGE and immunoelectrophoresis. In addition the bound material was analysed by gel filtration on Zorbax 250/450 tandem column as described in chapter 5. (b) Zorbax 250/450 gel filtration elution profile at 280nm of protein G bound material from human CSF.

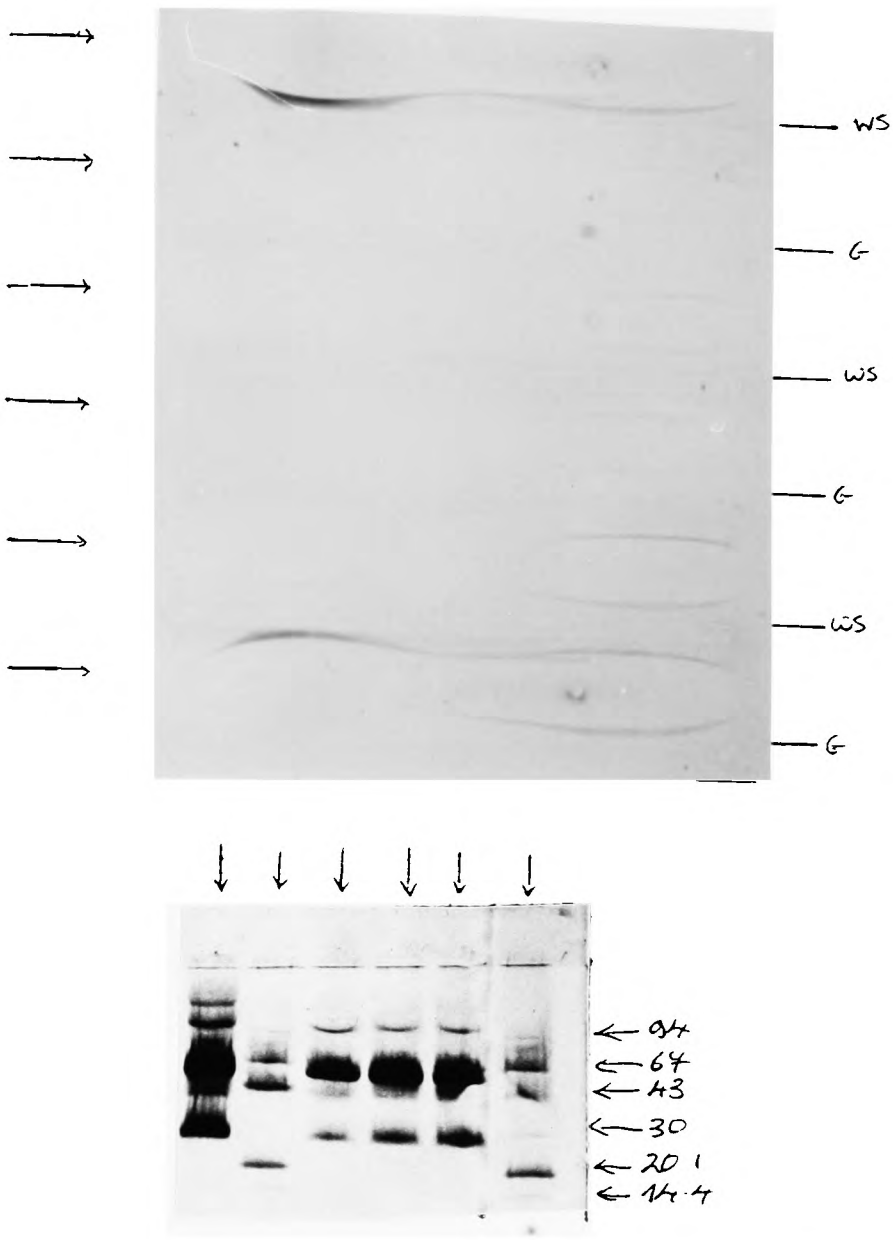


Figure 3.2 (a) Immunoelectrophoresis of material retained by protein G. Lanes one and six; unfractionated normal serum, lanes two to five; protein G bound material from normal CSF, possible MS CSF, dementia CSF and normal serum respectively. Lanes are indicated from top to bottom (left) and troughs at right. WS and G indicate the positions of troughs containing anti-whole human serum or anti-IgG γ-chain. The arc at the cathode (right) was identified as IgG and no other proteins were visualised except in the serum sample (lane five) where a faint precipitin arc corresponding to serum albumin can be seen.
(b) SDS-PAGE (8-25%), silver stained, of the protein G retained material. Lanes are indicated from left to right. Lane one; serum, lane two and six; standards, lane three; dementia CSF, lane four; possible CSF, lane five; normal CSF. The major two bands are at 50 and 25 kdal consistent with their assignment as heavy and light chains of IgG. Small amounts of high molecular weight proteins are due to under reduction (150kdal). In addition a small amount of albumin may be present in the serum IgG. Molecular weights of the standard protein mix are indicated at right.

described in appendix one. The pooled neutral and acidic glycans were treated with *Artherobacter ureafaciens* neuraminidase, and subjected to electrophoresis in the presence of pyridine-acetate as before. Neutral glycans were subjected to Bio-Gel P4 chromatography. To provide a semiquantitative analysis of the complex elution profile of body fluids, molar ratios of glycans eluting as peaks from Bio-Gel P4 were determined by liquid scintillation counting.

Fractionation of the desialylated CSF glycans using Con A was carried out as described in appendix one. The resulting pools were chromatographed on Bio-Gel P4. In the case of pools B and C (eluted with α-methylmannoside and hydrochloric acid repectively), methylation analysis was performed directly. For pool A (unbound by Con A), partial removal of the dextran hydrolysate internal standard was performed by borate high voltage electrophoresis, followed by digestion with yeast α-glucosidase as described in appendix one. This procedure removed about 60% of the dextran from the CSF oligosaccharyl alditols. Methylation analysis was performed as described in appendix one.

The nature of the anionic moiety of the N-linked glycans of CSF IgG was identified by incubation of the glycans with neuraminidase followed by analysis of the products by HVE in the presençe of pyridine-acetate. The relative incidence of the glycans on IgG from serum and CSF terminated with one, two or no β-galactose residues was determined with a mixture of exoglycosidases (see appendix one). The substituents on the pentasaccharide core Manα1→3(Manα1→6)Manβ1→4GlcNAcα1→4GlcNAc$_{\omega 1}$ (i.e. fucose and bisecting GlcNAc) were determined by first removing the nonreducing terminal β-galactose residues from the desialylated IgG glycans with bovine epididymal β-galactosidase or *Streptococcus pneumoniae* β-galactosidase, and

then digesting the degalactosylated cores with *S. pneumoniae* β-hexosaminidase under linkage-specific conditions (see appendix one).

3.4 *Results*

3.4.1 *Analysis of Total N-linked Oligosaccharides from Serum and CSF and Serum IgG*

A representative radioelectrophoretogram of total CSF N-linked oligosaccharides and the desialylated oligosaccharides is shown in figure 3.3. A peak at the origin (N) and two anionic peaks (A1 and A2) can be seen in the untreated glycan profile as well as some very highly mobile material. Treatment with neuraminidase resulted in a collapse of this profile to a single peak at the origin, with a smear of radioactivity over the remaining paper. From this it can be concluded that most, if not all, of the charged glycans were sialylated, with sialic acid in α2→3 and/or α2→6 linkage. The relative incidence of neutral and charged oligosacccharides before and after neuraminidase treatment is given in table 3.1. The incidence of anionic material following neuraminidase treatment was very variable (table 3.1), and was probably radiochemical contaminant.

Comparison of the Bio-Gel P4 and charge distribution data for the total N-linked oligosaccharides from serum and CSF shows no difference between the normal and MS groups (tables 3.1 to 3.4). A representative 'asialo' serum Bio-Gel P4 elution profile is shown in figure 3.3, with the pooling fractions indicated. The relative radioactivity of these pools for normal and MS individuals is given in table 3.3. Similarly an 'asialo'

CHARGE DISTRIBUTION OF N-LINKED OLIGOSACCHARIDES FROM CEREBROSPINAL FLUID OF MS AND NORMAL HUMANS

Patient	Percent		Percent (Post Neuraminidase)	
	N	A	N	A
MS Group (n=7)				
SN	30	70	53	47
EA	24	76	ND	ND
JO	23	77	60	40
JH	28	72	58	42
KM	32	68	68	32
PS	37	63	63	37
CA	50	50	ND	ND
Mean(±SD)	32(9)	68(9)	60(5)	40(5)
Control Group (n=7)				
DB	38	62	44	56
AS	23	77	57	43
WB	33	67	75	25
SC	33	67	57	43
MF	26	74	ND	ND
BB	26	74	ND	ND
HV	23	77	ND	ND
Mean(±SD)	29(5)	71(5)	58	42

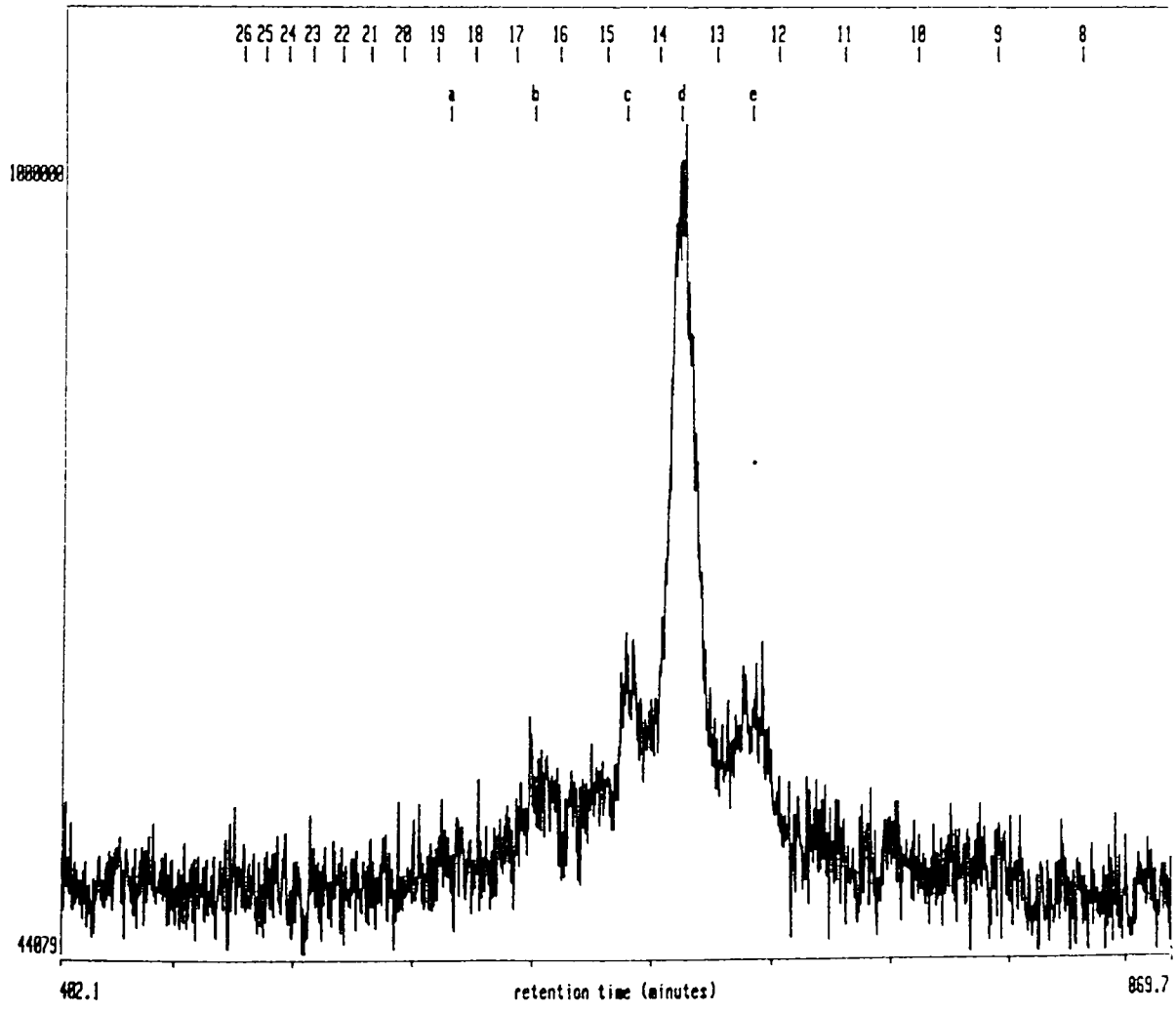
Table 3.1 The charge distribution for the CSF N-linked oligosaccharides. The neuraminidase resistant acidic material is probably radiochemical blank (see text). Values have been normalised to 100% in each case. ND; not determined. Means only are given for the control group following neuraminidase as there are too few points to calculate a standard deviation.

SIALYLATION OF TOTAL SERUM N-LINKED OLIGOSACCHARIDES FROM MS PATIENTS

Patient (n=8)	Percent	
	Neutral	Sialylated
SN	23	77
KM	33	67
JO	36	64
JH	19	81
PS	29	71
CA	32	68
HB	24	76
MG	24	76
Mean (± SD)	27(6)	73(6)

Table 3.2 The relative incidence of naturally neutral and sialylated N-linked oligosaccharides from the serum of multiple sclerosis patients. Values were obtained as described in the text, and have been normalised to 100% of the radioactivity returned after electrophoresis.

Figure 3.3 Representative Bio-Gel P4 gel filtration profile of total asialo serum N-linked oligosaccharides. The numbers across the top of the profile denote the elution positions of isomaltose oligomer internal standards. Letters a to e indicate the positions of the pools of oligosaccharides that were used for quantitation.



POPULATIONS OF N-LINKED GLYCANS FROM MS SERUM

Patient (n=8)	Percent				
	A	B	C	D	E
SN*	1.5	9.0	7.8	56.5	25.2
KM	3.0	14.0	16.0	47.0	20.0
JH	4.5	19.1	12.4	46.9	17.1
CA	5.2	10.0	17.3	45.8	21.8
HB	5.0	12.4	14.7	48.5	19.3
JO	5.2	9.5	17.6	49.1	18.6
MG	5.9	14.9	19.4	48.0	11.8
PS	5.5	9.5	21.6	49.1	14.3
Mean(±SD)	4.9(0.9)	12.8(3.3)	17.0(2.8)	47.8(1.2)	17.6(3.2)
Normal**(±SD)	5.9(2.9)	12.7(2.5)	19.5(2.8)	43.6(3.8)	17.6(4.0)

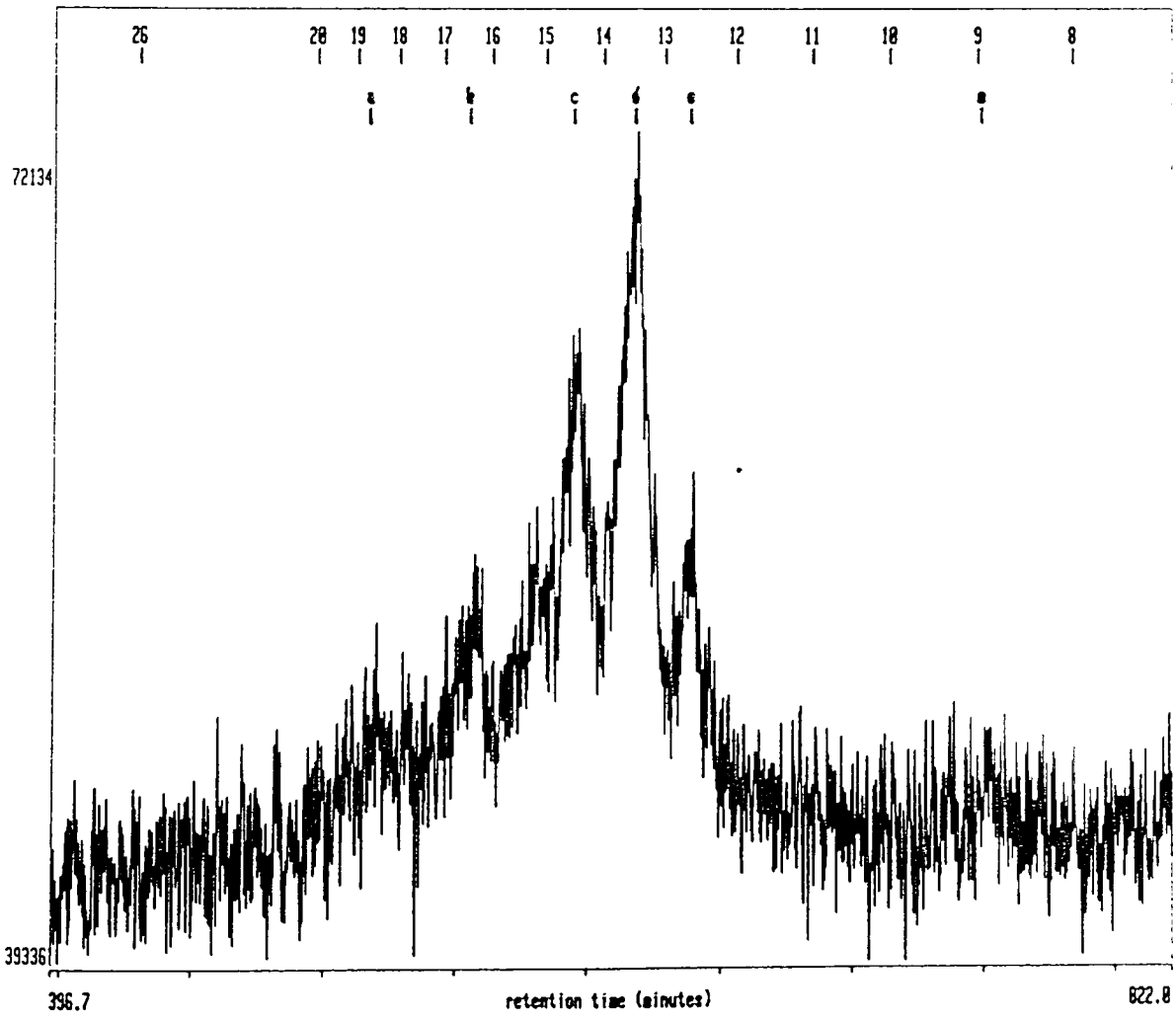
Table 3.3 Incidences of pools of N-glycans from the serum of patients with MS fractionated by gel permeation on Bio-Gel P4. Values are given for the arithmetic mean and the standard deviation.
* indicates not included in calculation of mean.
** Normal serum values (n=19). Normal serum values provided by S. Amatyakul, of the Glycobiology Unit.

POPULATIONS OF N-LINKED GLYCANS FROM HUMAN CSF

Patient	Percent					
	A	B	C	D	E	M
MS Group (n=7)						
EA	9.6	16.3	13.0	32.9	21.7	6.5
CA	12.3	12.9	20.3	23.8	15.2	15.5
JO	9.2	17.4	20.7	24.1	15.5	13.1
SN	13.1	23.1	21.4	19.6	13.3	9.4
JH	11.1	14.4	12.9	32.0	21.2	8.1
KM	2.0	17.5	16.1	29.5	21.6	13.3
PS	10.7	12.2	12.0	27.2	20.3	17.6
Mean(±SD)	9.7(3.4)	16.3(3.4)	16.6(3.8)	27.0(4.5)	18.4(3.3)	11.9(3.8)
Control Group (n=6)						
WB	10.8	13.9	11.3	37.9	21.8	4.3
AS	12.1	15.4	22.9	28.1	15.2	6.3
DB	11.8	20.6	8.4	33.0	22.6	3.5
HV	12.4	14.9	31.6	20.6	12.9	7.6
SC	10.4	16.2	26.9	27.3	15.1	4.1
BB	10.4	16.1	14.3	36.4	15.4	7.4
Mean(±SD)	11.3(0.8)	14.2(5.8)	19.2(8.5)	30.6(5.9)	17.2(3.6)	4.5(2.5)

Table 3.4 Pools of human CSF N-glycans fractionated by gel permeation on Bio-Gel P4. The arithmetic mean and the standard deviation are given for each pool.

Figure 3.4 Representative Bio-Gel P4 gel filtration profile of total asialo CSF N-linked oligosaccharides. The numbers across the top of the profile denote the elution positions of isomaltose oligomer internal standards. Letters a to m indicate the positions of the pools of oligosaccharides that were used for quantitation.



elution profile of total CSF N-linked oligosaccharides is shown in figure 3.4 and the relative incidence of each pool is given in table 3.4.

The gel filtration profiles of the serum and CSF are similar, but distinct. The major peak in both cases eluted at 13.5 glucose units (gu), and glycans with both higher and lower molecular weights can be seen. Each pool contains many glycans; pools A and B contain the tri- and tetraantennary complex oligosaccharides, pools C and D predominantly the biantennary glycans, pool E the hybrids and incomplete complex glycans and pool M (CSF only) the oligomannose saccharides. Some differences are evident between the CSF and serum N-linked oligosaccharide gel filtration profiles. The prevalance of the 13.5gu material is much less in the CSF than the serum. In both serum and CSF profiles this material composes pool D. The mean incidence for pool D in serum is 47.8% whilst the CSF value obtained by averaging the MS and control sets is 28.7%. The incidence of pools C and E, which contain a similar molecular weight fraction of the glycans in both cases, is about the same for serum and CSF, being approximately 17% in both cases (tables 3.3 and 3.4). There is a slight increase in the high molecular weight glycans (pools A and B) in CSF compared to serum (Pool A_{CSF} = ca. 10%, Pool A_{serum} = ca.5% and Pool B_{CSF} = ca. 15%, Pool B_{serum} = ca. 13%), and a concomitant decrease in the incidence of the low molecular weight oligosaccharides in the serum (due mainly to the absence of a pool M, the oligomannose, hybrid and incomplete complex oligosaccharides). Therefore, there are clear and reproducible differences in the gel filtration profiles of the asialo oligosaccharides from serum and CSF but not between the MS and control groups for the same body fluid. This provides strong evidence that the blood brain barrier is intact in MS as proposed by Link and Tibbling (1977). Any break in the BBB

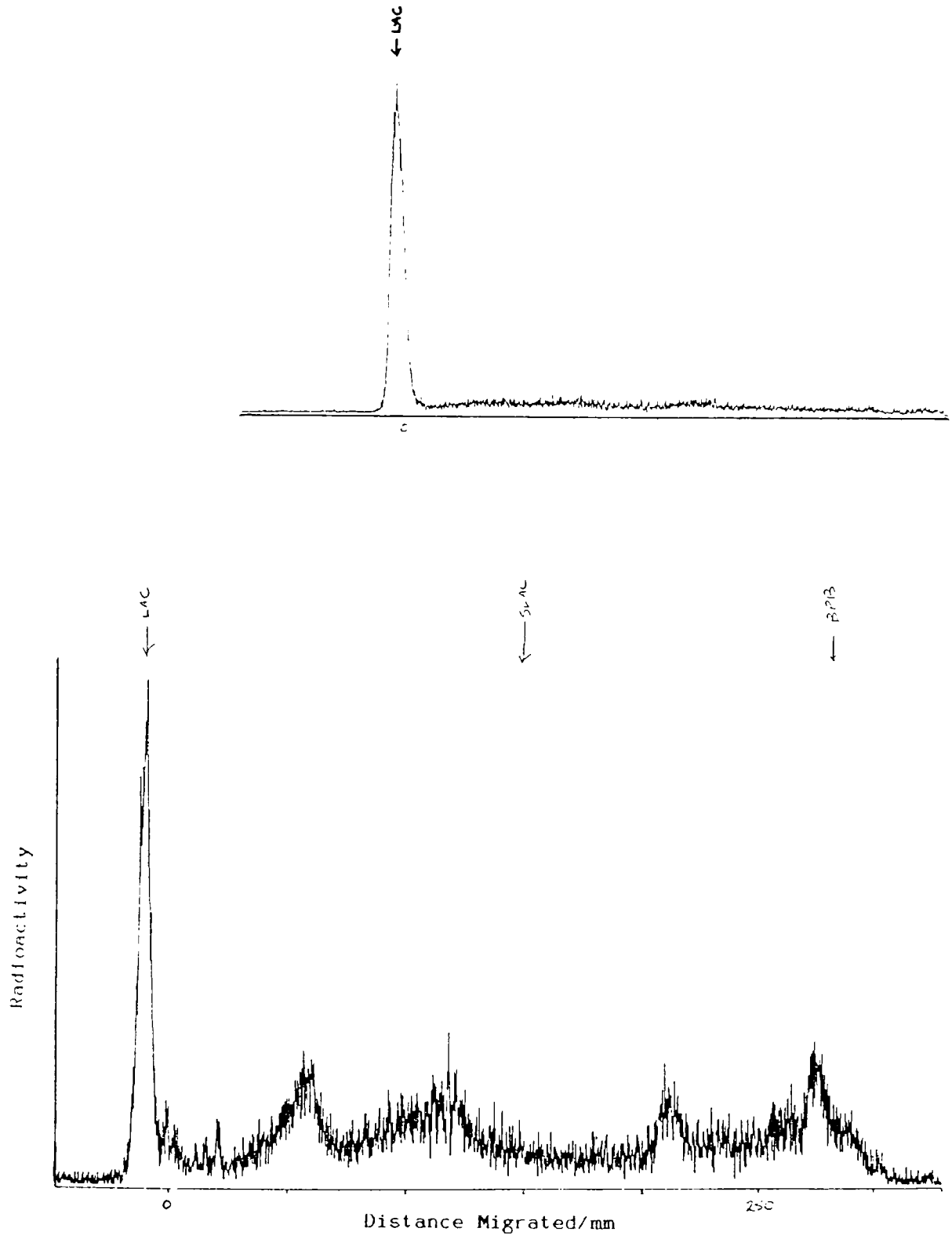


Figure 3.5 High voltage radioelectrophoretogram of total human CSF N-linked oligosaccharides (inset shows the resultant electrophoretogram following neuraminidase treatment of the oligosaccharides). The migration positions of standards (bromophenol blue, lactitol and sialyllactitol) are indicated.

that allowed the influx of serum macromolecules would be expected to result in a marked alteration in the CSF N-linked oligosaccharide pool, as serum is far more concentrated with respect to glycoproteins than CSF. Analysis of CSF oligosacccharides could be used as a method to assess the integrity of the blood brain barrier, in addition to analysis of albumin/IgG ratios (Link and Tibbling 1977).

No evidence of an alteration to the glycosylation of IgG in the serum is seen from the total serum profiles. This is true for the serum IgG as shown in figure 3.6, because apart from patient SN all the MS serum IgG galactosylation values fall within the normal range. The data are given in table 3.5. The incidence of neutral, mono- and di-sialyl glycans, and the levels of fucosylation and bisecting GlcNAc on the core were also determined for the serum IgG glycans (table 3.6). With the exception of the fucosylation in patient KM, these values are within the normal range (the mean values of the normal incidences of the four cores are given for comparison). Because there was no shift seen for all the patients for any of the parameters analysed here, a change in serum IgG glycosylation cannot be associated with MS *per se*.

3.4.2 *Methylation Analysis of CSF N-Glycans*

Fractionation of pooled desialylated CSF N-linked oligosaccharides from the normal and MS groups on ConA resulted in three pools, A, B and C. The Con A elution profile for the normal CSF glycans, is shown in figure 3.7 and the Bio-Gel P4 gel filtration profiles of the pools in figure 3.8. The relative incidences of these pools are given in table 3.7. Because of a lack of material pool C from the MS CSF glycans was not analysed further.

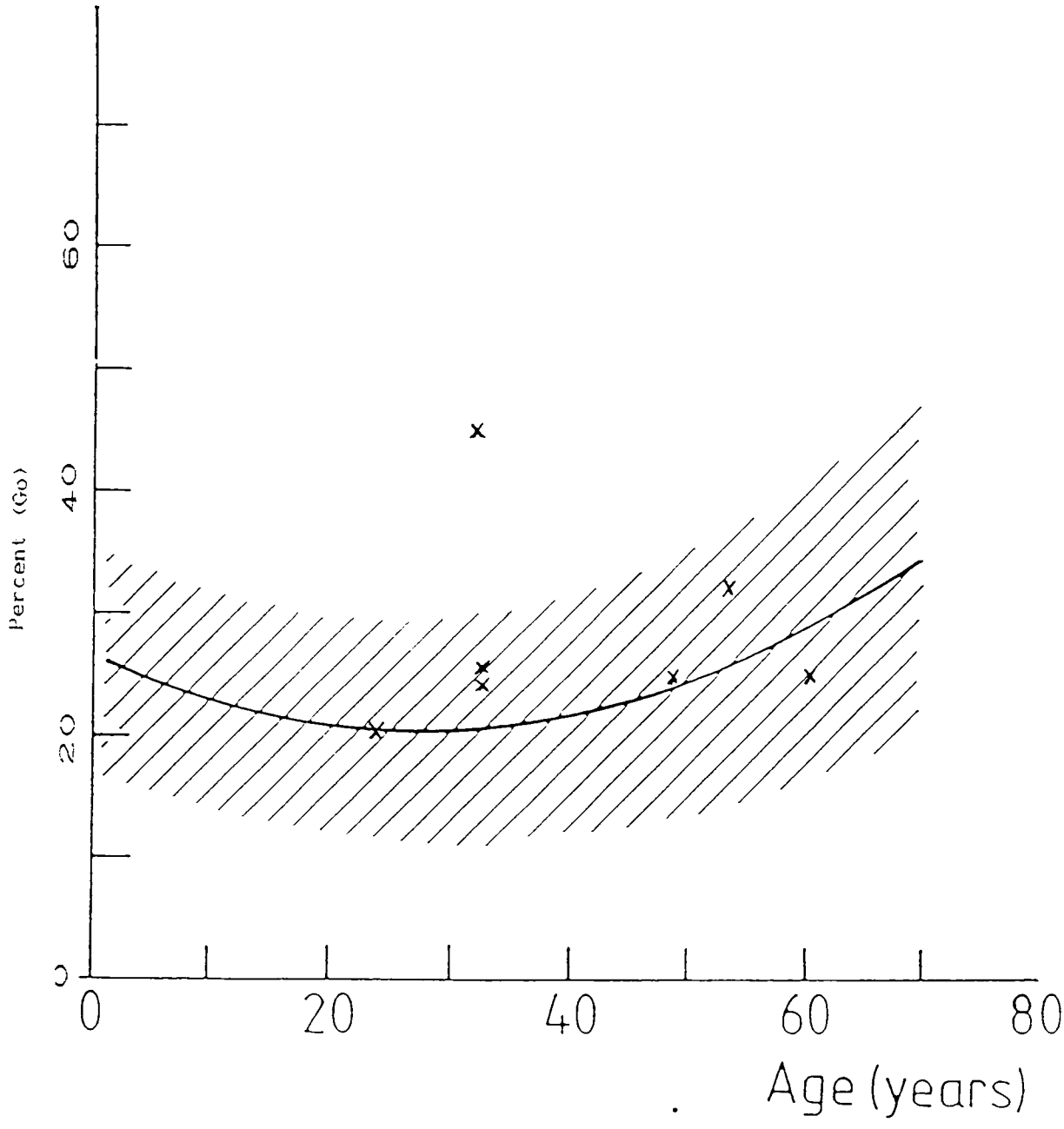


Figure 3.6 Plot of Go against age for MS IgG N-linked glycans. Go is the relative incidence of N-linked oligosaccharides devoid of terminal galactose. The solid black line is the mean for normal serum IgG Go against age (n = 111), and was determined by Parekh *et al.* (1988b).

GALACTOSYLATION OF SERUM IgG FROM MS PATIENTS

Patient	Age	Incidence(%) of terminal β-galactose residues		
		0	1	2
CA	23	21	37	42
MG	NA	19	43	38
SN	32	45	26	29
HB	53	32	41	27
KM	38	26	37	37
JO	60	25	44	31
PS	32	26	36	38
JH	32	24	44	32

Table 3.5 The incidence of serum IgG N-glycans with zero to two non-reducing terminal β-galactose residues for the patients with MS are given, together with the age of the patient (see figure 3.6).

CORE SUBSTITUTION AND SIALYLATION OF SERUM IgG FROM MS PATIENTS

Patient	Sialylation % Incidence		
	N	A1	A2
CA	74	20	6
MG	76	18	8
SN	79	16	5
HB	78	15	7
KM	78	17	5
JO	71	22	7
JH	76	18	6
PS	78	16	6
Std(±SD)*	75.7(5.0)	20.5(5.0)	3.8(1.6)
Mean(±SD)	76.3(2.5)	17.1(1.6)	6.3(1.0)

Patient	Core substituents					
	None	+B	+F	+B+F	ΣB	ΣF
CA	30	8	52	10	18	62
HB	27	8	50	15	23	65
KM	53	17	22	8	25	30
JO	24	7	58	11	18	69
JH	33	5	53	14	19	67
Std(±SD)*	18.5(6.5)	6.6(2.5)	61.8(10.6)	13.2(3.1)		
Mean	29.25	7.0	53.25	12.5	19.5	65.75

Table 3.6 Sialylation and core substituents of MS serum IgG N-glycans. It is not possible to derive standard deviations for the core data because only four samples are in the normal range. The values for patient KM were not included in calculating the mean. +B, +F, +B+F represent oligosaccharides with bisecting GlcNAc, with fucose and with both, respectively. ΣB, ΣF are values for total bisect and total fucose. All data are expressed as percent incidence. N is neutral glycans and A1, A2 with one or two sialic acids respectively.

* Data taken from Parekh *et al.* (1985).

Table 3.7

CSF	Percent Incidence of Pool From Con A		
	A (Unbound)	B (α-methylmannoside)	C (HCl)
Control (n=7)	38.3	52.4	9.3
MS (n=7)	45.8	53.6	0.6

The methylation analysis data are shown in table 3.8. As expected no difference in the gel filtration chromatograms for the MS and control groups could be seen, and the methylation analysis detected the same partially methylated alditol acetates for both groups in the corresponding fractions, with the exception that terminal and 3-linked mannose were detected in the MS CSF glycan pool A. Pool A eluted from Bio-Gel P4 at a position consistent with multiantennary complex glycans, and this was confirmed by the methylation analysis. The reason for the detection of additional PMAAs in the MS pool A is unknown, and is not consistent with the Con A fractionation or the gel filtration analysis, and may be artifactual. Pool B eluted from Bio-Gel P4 in a position consistent with biantennary oligosaccharides with a peak maximum at 13.5gu. The methylation analysis confirms this, except 2-linked mannose was not detected. Considering the gel filtration (figure 3.9), methylation analysis data (table 3.8), and the specificity of the lectin, it appears that pool B contains biantennary complex and hybrid oligosaccharides. Core fucose and bisecting GlcNAc were detected in pools A and B, on the basis of the presence of terminal fucose and 4,6-linked $GlcNAc_{bi}$, and terminal GlcNAc and 3,4,6-linked mannose respectively. Evidence for hybrid type glycans in

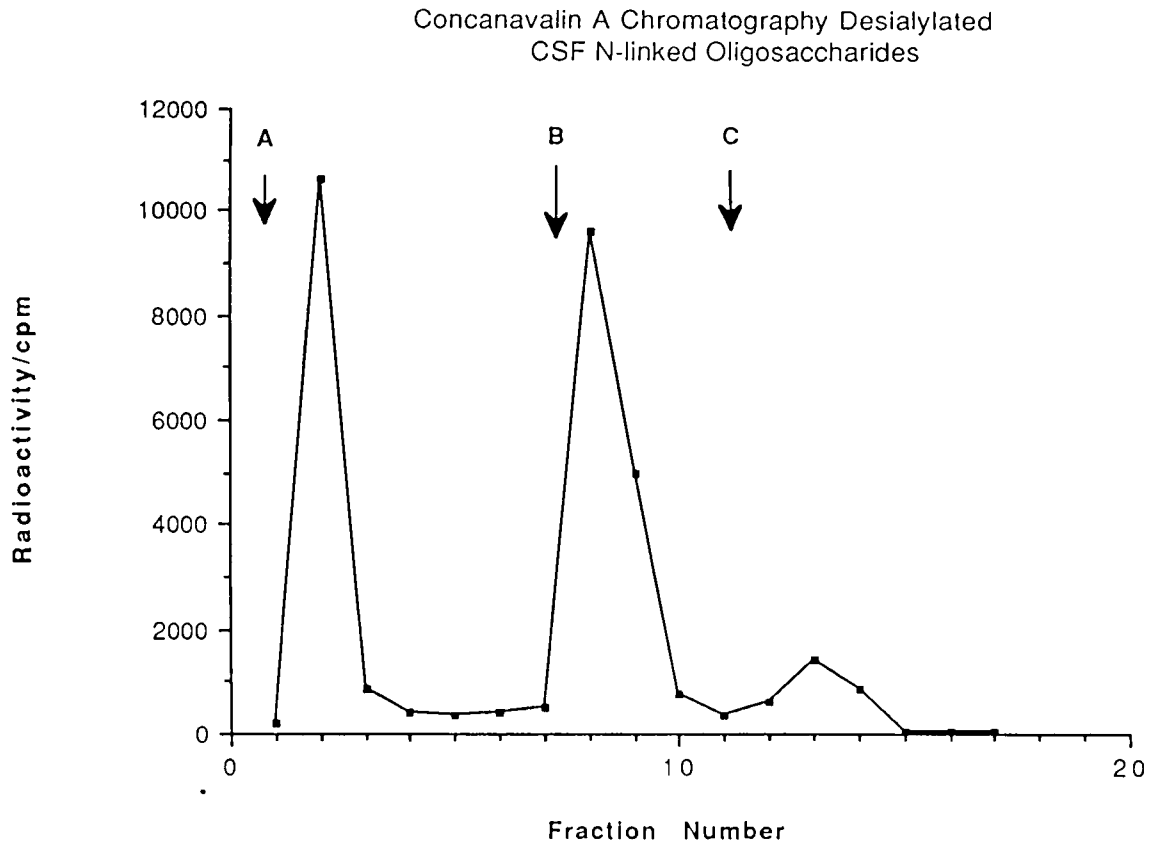
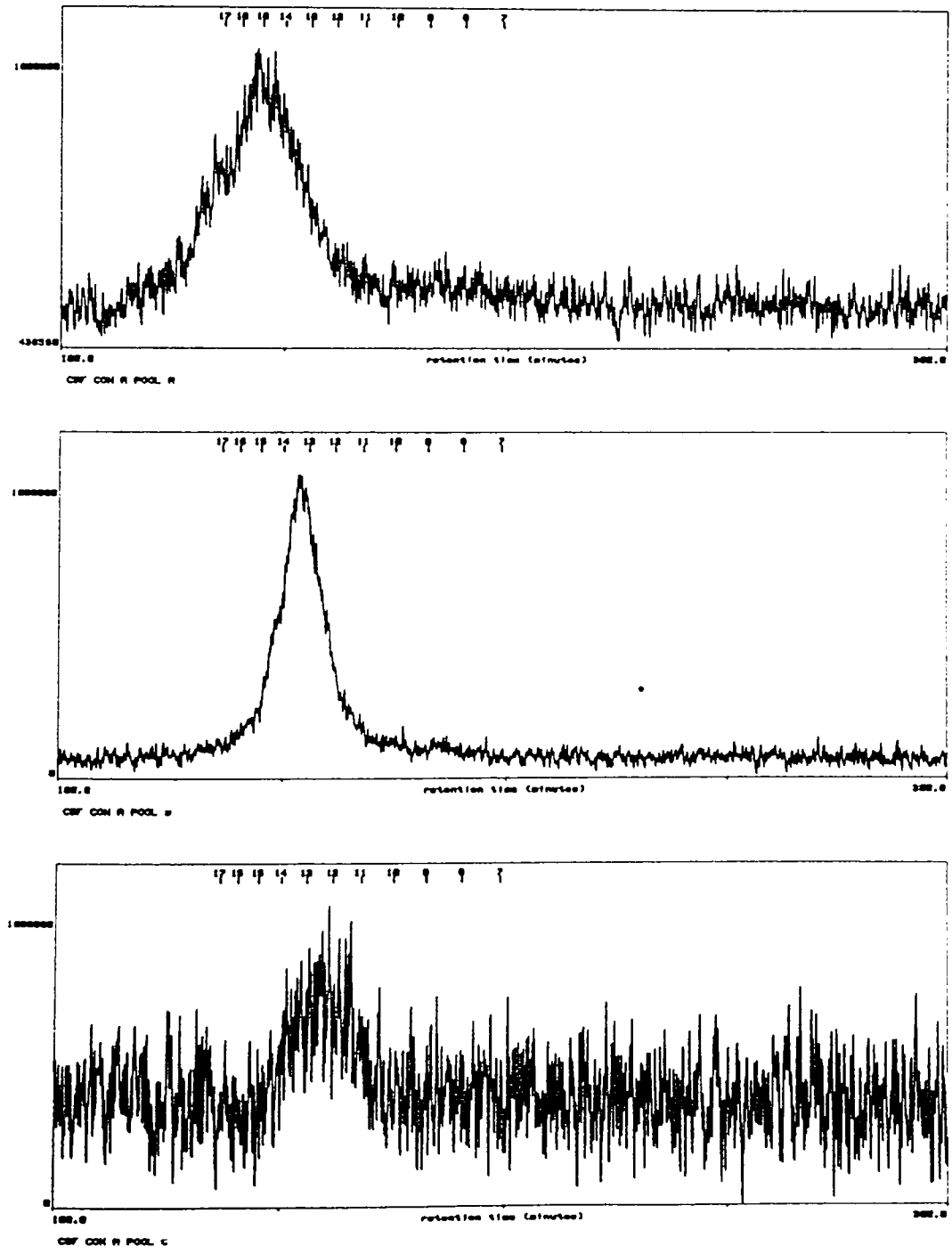


Figure 3.7 Chromatogram of total desialylated CSF N-linked oligosaccharides from normal humans on Concanavalin A Sepharose. The positions of the application of the buffers to elute the pools are indicated by A, B and C.

Figure 3.8 Bio-Gel P4 chromatograms of Con A pools of total desialylated CSF N-linked oligosaccharides, (a, top) pool A, (b, middle) pool B, (c, bottom) pool C. The positions of isomaltose oligomer internal standards are indicated.



METHYLATION ANALYSIS DATA FOR DESIALYLATED N-LINKED OLIGOSACCHARIDES OF HUMAN CEREBROSPINAL FLUID

Residue	Control		ConA Fraction	MS	
	A	B	C	A	B
T-Fuc	+	+	-	+	+
T-Gal	+	+	-	+	+
T-GlcNAc	+	+	+	+	+
4-GlcNAc	+	+	+	+	+
4-GlcNAc$_{o1}$	+	+	+	+	+
4,6-GlcNAc$_{o1}$	+	+	-	-	+
T-Man	-	+	+	+	+
2-Man	+	-	+	+	-
2,4-Man	+	+	+	+	+
3,6-Man	+	+	+	+	+
2,6-Man	+	-	+	+	-
3,4,6-Man	+	+	-	+	+
3-Man	-	+	+	+	+

Table 3.8 The partially methylated alditol acetates (PMAAs) are reported as the residues from which they originate. No quantitation was possible due to the low amount of material, which made integration of the flame ionisation detector response unreliable. The conA fraction refers to the pools obtained from conA chromatography as described in the text. + indicates that the residue was positively identified from the mass spectrum and the retention time by the GC column, and - indicates that a residue could not be unambiguously identified. Neutral and amino sugar derivatives were analysed by gas-chromatography mass-spectrometry with a CP-Sil-8 column and the neutral sugars further analysed with a SP2860 60m GC column. The failure to detect 4,6-GlcNAc$_{o1}$ in the MS pool B was probably due to the poor recovery of this residue by the analysis method used here. In addition the failure to identify 2-linked mannose in the pool B fractions is considered equivocal as discussed in the text. The PMAA that results from this residue elutes very closely to the PMAA derivative of 3-linked mannose on the SP2860, and close to 4-linked glucose on the CP-Sil-8 column, so that the spectrum could be obscured by the ions derived from other PMAAs.

pool B stems from the observation of terminal and 3-linked mannose in the methylation analysis. Pool C eluted from Bio-Gel P4 at a position consistent with oligomannose and hybrid oligosaccharides, and the methylation analysis is consistent with this assignment. The detection of terminal GlcNAc in pool C indicates that some hybrid glycans devoid of galactose are present. The methylation analysis data indicates that the glycans are, in the main, similar to those encountered in serum glycoproteins.

3.4.3 *CSF IgG Isolation*

Elution profiles of normal CSF and normal serum on protein G HPLAC are shown in figure 3.1. The profiles were identical, except that some material eluted between 3.5 and 12.0 minutes. This quantitatively insignificant material was not analysed. The results of SDS-PAGE and IEP of the purified IgGs are shown in figure 3.2 and indicate that IgG and serum albumin are present in the protein G bound fractions. Analysis by IEP of the material in serum and CSF not bound by protein G showed that no IgG was present. A preparation of CSF isolated by the protein G method was analysed by HPLC gel filtration; all protein eluted with a retention time consistent with a molecular weight of 150kdal (figure 3.1b). Serum albumin is not glycosylated therefore it did not affect the subsequent oligosaccharide analysis. From the A_{280nm} a yield of 25µg of IgG per millilitre of CSF was calculated. This compares well with a level of 25-30µg γ-globulin/ml of CSF.

3.4.4 *The N-linked Oligosaccharides of Human CSF IgG.*

The relative incidences of neutral and acidic glycans from the CSF and serum IgG purified by the same method are shown in table 3.9. These values are consistent with those observed in normal serum IgG. Neuraminidase treatment resulted in the neutralisation of all acidic glycans. Therefore all sialic acid was in α2→3 or α2→6 linkage, and similar levels of sialylation are seen in CSF and serum IgG N-glycans.

Gel filtration of the normal serum and the CSF IgG glycans on Bio-Gel P4 produced the elution profiles shown in figure 3.9. The serum and MS CSF IgG profiles are very similar, whilst the normal CSF IgG profile is somewhat different, with the lower molecular weight peaks being more prevalant. The profile of possible MS IgG glycans is intermediate between these two extremes. The dementia CSF IgG gave an unusual profile but was not analysed further because insufficient material was recovered following gel filtration.

The data for the incidence of terminal β-galactose substitution, core fucosylation and bisecting GlcNAc, as determined by exoglycosidase digestion, are given in table 3.9. The overall heterogeneity of the CSF IgG is similar to serum IgG. Treatment of the oligosaccharides with a mixture of exoglycosidases containing jack bean β-hexosaminidase and β-galactosidase and bovine epididymal α-fucosidase resulted in the conversion of all oligosaccharides to the pentasaccharide core, eluting from Bio-Gel P4 at 7.5gu. The sensitivity of the oligosaccharides to the various exoglycosidases indicates that the linkages present are the same for serum and CSF IgG glycans. In addition, the specificity of the *S. pneumoniae* β-galactosidase, used to remove galactose from some of the IgG

Table 3.9 ANALYSIS OF HUMAN CSF IgG N-LINKED GLYCANS

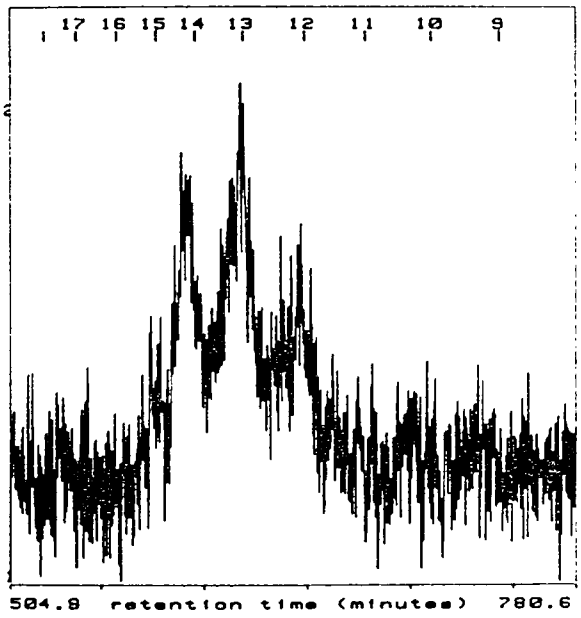
	Sialylation(%)		Galactosylation(%) (Number of β-galactose termini)		
IgG[fn]	Neutral	Sialylated	0	1	2
MS	69.6	30.4	27.0	39.0	34.0
Poss. MS	78.5	21.3	33.5	33.8	32.7
Normal#1	84.5	15.5	28.0	41.0	31.0
Normal#2	67.1	32.9	39.4	31.7	28.9
Dementia	84.5	15.5	-	-	-
Serum#1	75.0	25.0	19.5	36.7	43.8
Serum#2	78.8	21.2	20.5	39.8	39.7
Std(±SD)	75.7(5.0)	24.3(5.0)			

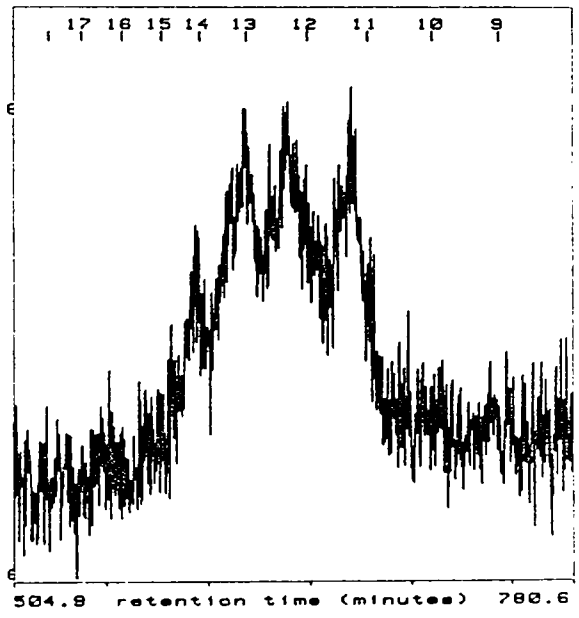
	Core substituents					
	None	+B	+F	+B+F	ΣB	ΣF
MS	13.8	4.4	62.2	19.6	24.0	81.8
Poss. MS	32.0	13.2	47.8	7.0	20.2	54.8
Normal#1	49.8	13.1	27.9	9.2	22.3	37.1
Normal#2	29.1	7.2	46.6	17.1	24.3	63.7
Mean Norm. CSF	*39.5*	*10.1*	*37.3*	*13.1*	*23.3*	*50.4*
Serum#1	11.2	5.9	67.1	15.8	21.7	82.9
Serum#2	15.7	4.4	67.9	12.0	16.4	79.9
Mean Serum	*13.4*	*5.2*	*67.5*	*13.9*	*19.1*	*81.4*
Std(±SD)*	18.5(6.5)	6.6(2.5)	61.8(10.6)	13.2(3.1)	19.8	75.0

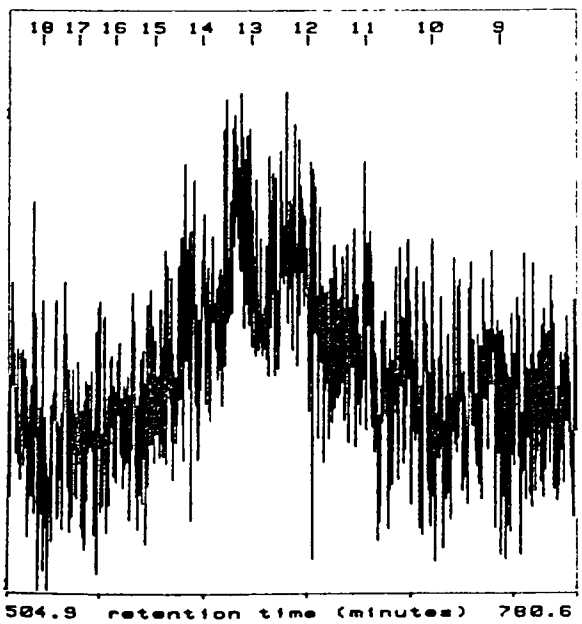
Table 3.9 The N-linked oligosaccharides of human CSF IgG. Abbreviations given in table 3.6.

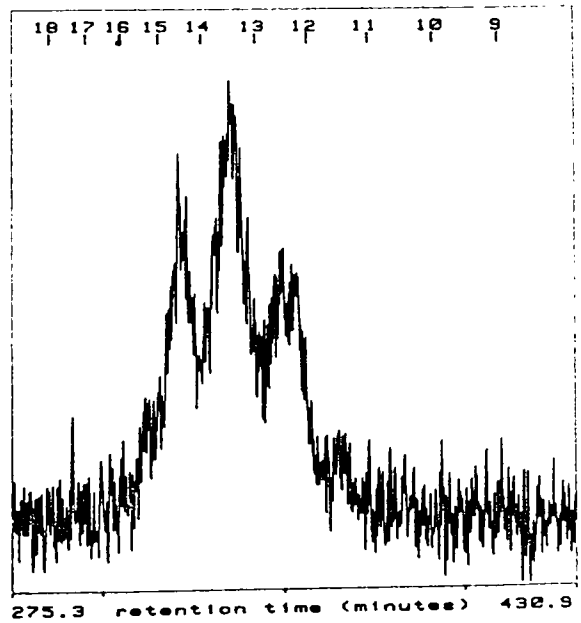
Nomenclature: MS; IgG from pooled CSF of patients with clinically definite MS, Poss MS; IgG from pooled CSF from patients diagnosed as possible MS, Normal; IgG from pooled CSF from patients without MS, and Serum; IgG from pooled normal human serum. #1 and #2 refers to two seperate samples prepared from different pools of material (CSF) or two preparations from the same pool of material (serum). * Data for normal human serum IgG is included for comparison (Parekh *et al.* 1985).

Figure 3.9 Gel permeation Bio-Gel P4 chromatograms for total asialo IgG N-linked oligosaccharides prepared by protein G HPLAC. The numbers across the top of the profile denote the elution positions of isomaltose oligomers. (a, top left) Human serum IgG, (b, top right) normal CSF IgG, (c, bottom left) possible MS IgG, (d, bottom right) definite MS. Note the similarity of the profiles in panels a and d.









glycan samples in the core analysis, determined that all galactose was in β1→4 linkage, the same as serum IgG. The incidence of glycans terminating in two, one or zero β-galactose residues is within the normal range for serum IgG.

However the fucosylation of the normal CSF IgG glycans is low. This effect is probably significant because the IgG was isolated from a pool of CSF samples, and a decrease in fucosylation of IgG from one individual would have been balanced by the others if they were at normal levels. Note that the fucosylation of normal #2 is higher than normal #1, but is still below that for the serum IgG. The reason for the variation between the two groups is unknown, but may relate to the age of the patients, which was greater for normal #2, and also to the fact that the normal CSFs were obtained from patients with lumbar disc problems, so that heterogeneity in pathlogy may be evident. However, calculation of the mean value clearly indicates that there is a significant decrease in the level of fucose in the normal CSF IgG compared to serum or MS CSF IgG. The differences in the incidence of core fucose accounts for the observations made from the 'asialo' Bio-Gel P4 profiles.

3.5 Discussion

It is clear from the data presented in this chapter that a glycosylation defect is not involved in the pathology of MS, and additionally abnormal glycosylation is not a feature of the oligoclonal IgG observed in MS.

In the unfractionated CSF N-linked oligosaccharides the sialylation level does not alter in the MS brain and neither does the overall

distribution of structures. This is interesting in that the large amount of inflammation that occurs in the CNS of MS patients is the result of considerable immunologic activity. The absence of a significant shift in the structures and sialylation of the CSF N-glycans implies that the blood brain barrier remains essentially intact, because the oligosaccharides are different from those in serum. Any leakage of serum glycoproteins into the CSF would be expected to result in an alteration of the oligosaccharides so that the CSF glycans come to be more like those in serum. In fact the only pool where there is significant deviation between the two study groups is in pool M (table 3.4). This region of the chromatogram corresponds to 8-10gu, which would contain either oligomannose, hybrid or incomplete biantennary glycans. However, with regard to the Con A fractionation, the relative incidence of pool C, which contains the oligomannose glycans, is decreased in MS, casting doubt on the significance of this observation. Overall, the glycans in the MS CSF appear to become less reactive towards the lectin, as evidenced by an increase in the incidence of pool A in the MS sample, and therefore a greater proportion of multiantennary complex oligosaccharides was found. A loss of Con A binding activity has been observed for serum α-1 acid glycoprotein in inflammatory conditions, and is caused by an increased prevalence of triantennary glycans on this protein (Bierhuizen *et al.* 1988). A similar cause may be underlying the observation made here.

Methylation analysis of the Con A pools shows that the component residues of the glycans are similar to those expected for serum glycoprotein structures, and taken together with the elution positions of these pools on Bio-Gel P4, are consistent with the presence of oligomannose, hybrid and lactosamine complex oligosaccharides, with

bisecting GlcNAc and core fucose. This contrasts with evidence that CNS N-glycans are different from those in other tissues (Krusius and Finne 1977, Schieven *et al.* 1982). In particular no evidence for a high incidence of fucose residues on arm GlcNAc residues as reported in rat brain (Krusius and Finne 1977) was obtained by the analysis here. The difference may be due to the fact that in this study secreted glycoprotein oligosaccharides were analysed, and CNS specific glycosylation may be more a feature of membrane bound glycoproteins than secreted glycoproteins. It was determined that neuraminidase sensitive sialic acid was the only anionic moiety present on the CSF glycans, which is in contrast to the high incidence of sulphated glycans encountered for cell associated glycoprotein oliogosaccharides from murine brain as discussed in chapter two, impling that the pathways of biosynthesis of saccharides on glycoproteins destined to become secreted or membrane bound are distinct (see chapter one).

The technique of pooling regions of Bio-Gel P4 chromatograms of complex mixtures of oligosaccharides has been used in this laboratory for some time in the analysis of serum glycans. The extension of the method to CSF allowed quantitative information on the distribution of N-linked oligosaccharides to be obtained and the direct comparison of the glycans from two body fluids, without the need for full structural characterisation of the component oligosaccharides. It would not be possible to obtain comparable data by other methods because of the low amounts of material that are available. Typically about four nanomoles of oligosaccharides were obtained from 1ml of CSF.

The glycosylation of serum IgG from MS patients was shown to be normal. This is not unexpected, because, despite some abnormalities, the systemic immune system is not considered to play a major role in MS. The

observation of elevated Go in IgG from patient SN and decreased fucosylation of IgG from patient KM cannot be considered significant as the alterations were not present in all of the IgGs analysed.

From figure 3.9 it was immediately apparent that the serum IgG prepared on protein G has an identical N-glycan gel filtration profile to that obtained by ion exchange purification, which is the standard method used for serum IgG isolation in this thesis. Therefore the glycans analysed from the protein G purified IgGs can be compared directly to those obtained by ion exchange. The normal CSF IgG glycans were somewhat different to those obtained from serum IgG in that the same set of peaks were present, but with an increase in the incidence of the lower molecular weight peaks, due to a lower level of core fucose in comparison to serum IgG. Therefore, the CNS B cells are not different from systemic B cells in terms of their capacity to glycosylate IgG, except that the incidence of core fucose residues is decreased by 40%. The definate MS IgG glycans are very similar to the normal serum IgG. All linkages in the CSF IgG glycans, where determined, were the same as found in serum IgG oligosaccharides. It is felt that a larger number of patients need to be studied to back up the observations made here.

The data from the total CSF N-linked oligosaccharide pools indicates that the BBB is intact (above), but analysis of the CSF IgG indicates that the MS CSF IgG is similar to serum IgG in terms of carbohydrate, whereas normal CSF IgG is hypofucosylated compared to serum IgG. Possible explanations for this observation are; (1) some of the IgG is derived from a serum source, i.e. transported across the BBB as a free glycoprotein (2) that the inflammation observed in the MS brain alters the glycosylation of the the IgG made by the resident B cells, or (3) some of the IgG is

produced by B cells recruited from systemic sources, which make IgG with the same carbohydrate as serum IgG. It is unlikely that the first proposal is valid because it has been shown that much of the IgG in the CSF of MS patients is made within the CNS (Walsh and Tortellote 1983). Because only a small alteration in the CSF total N-linked carbohydrates is seen between the MS and control groups (pool M), it is also unlikely that the second possibility is correct, as it would be expected that an inflammatory state would lead to significantly altered glycosylation of other CSF glycoproteins as IgG. Such a severe change would not be expected to affect only one glycoprotein. The final proposal, of increased numbers of systemic B cells in the MS CSF, is the strongest hypothesis. It is known that large numbers of lymphocytes circulate through the CNS of MS patients (Hafler and Weiner 1987), and that the number of lymphocytes within the CNS is increased in MS (Walsh and Tortellotte 1983), and therefore a posssible mechanism for the recruitment of systemic B cells into the CNS exists. These cells may become activated on entering the CNS and account for both elevated immunoglobulin levels and the oligoclonal IgG seen in the CNS of MS patients (Tortellotte 1986).

To the author's knowledge this is the first time that the glycans of an immunoglobulin isolated from the CNS have been analysed. It has been proposed that neural glycosylation may be different to the systemic compartment, acting as a tissue marker, or that specific carbohydrates may act as recognition molecules for BBB lectin receptors that then carry captured glycoproteins across the epithelium and release them into the CSF (Rademacher *et al.* 1988b). This hypothesis is not borne out by the observation that no novel glycans exist on CSF IgG, and that the only difference between the glycans for normal and MS CSF IgG is a decreased

incidence of core fucose. If the alteration of the CSF IgG glycans in MS represents the influx of systemic lymphocytes, then the interaction underlying the change in the CSF IgG glycosylation must be governed by B cell homing to the inflammed CNS. In addition, the glycosylation of the IgG secreted by the B cells in the MS brain does not alter that seen for normal CSF IgG, and therefore if these B cells are recruited from the systemic compartment they do not alter their IgG glycosylation on entering a new environment, i.e. the CNS.

In conclusion the following points can be made. (1) There is no evidence for an alteration in total or IgG N-linked oligosaccharides from serum of MS patients compared to normal individuals. (2) Only small differences between the normal and MS CSF N-linked glycans were detected on the basis of Con A fractionation. (3) The N-glycans in the CSF appear to be similar to serum glycoprotein N-linked oligosaccharides on the basis of carrying sialic acid as the sole anionic moiety and linkage analysis. (4) CSF IgG N-glycans are the same as those encountered on serum IgG, except that there is a decrease in the incidence of core fucose. In MS the CSF IgG is glycosylated in an identical manner to serum IgG, which is taken as evidence that the increased level of IgG in the MS brain is produced by B cells recruited from the systemic compartment. (5) Conserved differences in the CSF and serum total N-glycans between normal individuals and MS patients indicates that the BBB remains intact in MS.

An inability to provide consistent and convincing biochemical data on alterations in MS has been a common feature of studies into this disease. Whilst the information presented in this chapter indicates that alterations in glycosylation are not significant features of MS, the search for further

biochemical and immunological data on this disease remains a pressing question in contempory clinical neurology.

CHAPTER FOUR

AN ANALYSIS OF GLOMERULONEPHRITIS-ASSOCIATED CHANGES IN THE GALACTOSYLATION OF SERUM IMMUNOGLOBULIN G

4.1 *Preamble*

Patients with a number of diseases have been found to exhibit abnormalities in the glycosylation of serum IgG when compared with age-matched controls. Specifically there is an increased prevelance of N-linked oligosaccharides from serum IgG that lack galactose (Go) and terminate in N-acetylglucosamine (Parekh *et al* 1989). Amongst the group of diseases studied to date the elevation in Go is confined to patients with rheumatoid arthritis (RA), tuberculosis, Cröhn's disease and patients with both systemic lupus erythematosis (SLE) and Sjörgren's syndrome (SS). An elevated Go does not correlate with increased immunoglobulin synthesis. A reduction in the activity of B-lymphocyte β-galactosyltransferase may account for the increased Go in RA (Axford *et al.* 1987).

The aetiology of glomerulonephritis is poorly understood, but one important factor leading to initial damage of the glomerulus is increased immune complex (IC) deposition in the subepithelial space of the glomerulus or recognition of self-antigens by autoantibodies (e.g. in Goodpasture's syndrome). A predisposition to formation of immune complexes could lead to increased levels of glomerular IC deposition. It has been proposed that hypogalactosylated IgG has a greater propensity to aggregate (Rademacher *et*

al. 1988b). A possible predisposing factor for glomerulonephritis, therefore, may be an increase in levels of serum IgG with high Go.

The incidences of Go were determined for serum IgG isolated from a group of patients with glomerulonephritis. These data indicate that in at least some of these patients the serum IgG is hypogalactosylated when compared to age-matched controls, and that glomerulonephritis patients with highly elevated Go values also show evidence of severely impared glomerular function.

4.2 *Introduction*

It has been difficult to elucidate the mechanisms of renal disease for two main reasons. Firstly, the glomerulus and interstitium appear to have a limited response to injury so that most insults result in a similar structural alteration, leading to sclerosis and loss of function, and secondly, even if the source of injury is removed the kidney tends to deteriorate futher (Klahr *et al.* 1988). The end stage kidney is reduced in mass and has a decrease in the number of glomerular capillaries. There is localised cell proliferation and an eventual collapse of the capillary bed. Fibrosis is characteristically observed, and atrophy with an influx of inflammatory cells is usually encountered. The concomitant loss of function parallels these structural alterations. Proteinuria is the most common symptom at presentation.

Glomerular injury can arise from a number of sources. These factors include the deposition of immunoglobulin, circulating immune complexes (CICs) or complement proteins, intra and extracapillary coagulation, and the local elevation of cytokine and other factor activities. All of these

events have at some time or other been proposed as causative or sustaining agents of renal diseases (Klahr *et al.* 1988). The resulting sclerosis can be focal, i.e. restricted to small regions of the kidney, segmental, where only segments of the glomeruli are involved, or diffuse and involving all the glomeruli. Diffuse glomerulonephritis arises from a number of systemic diseases, e.g. SLE, Goodpasture's syndrome and diabetes. In fact, in aging normal humans a slow progressive loss of glomerular function of this type is observed.

Attempts to define immunological abnormalities in glomerulonephritis have, in general, met with little success. Cagnoli *et al.* (1982) have shown that the T4/T8 T cell ratio is increased in membranous glomerulonephritis, due to a loss of CD8 positive cells. The same alteration was found in IgA nephropathy (Bannister *et al.* 1983b). Therefore, some loss of regulation of the immune response may be present in these diseases, and erroneous activation of lymphocytes may occur. The observation that lupus nephritis in autoimmune mice can be suppressed by treatment with monoclonal antibodies directed against the interleukin 2 receptor indicates that a generalised increase in immunological activity can give rise to nephritis (Kelley *et al.* 1988).

4.2.1 *Immune Complexes In Glomerulonephritis*

The ability of the immune response to damage the glomerulus has been demonstrated experimentally by the immunisation of rats with homologous renal tubular epithelial antigen, which results in a glomerulopathy termed Heymann neuritis (Keraschki and Farquar 1983). The binding of antibodies to the glomerular epithelium promotes proteinuria. Recently it has been found

that the Heymann antigen, gp330, acts as a receptor to a serum protein (Kanalas and Makker 1988). Renal disease in Goodpasture's syndrome is probably derived from autoimmune recognition of a collagen component of the glomerular besement membrane (Saus *et al.* 1988). Immune complex disease due to serum sickness also results in glomerular damage. Immune complexes can trigger complement and cause the release of vasoactive amines from mast cells and basophils, leading to an increase in vascular permiability, which favours further IC deposition. ICs can promote aggregation of platelets by binding to platelet Fc receptors, and produce a microthrombus, which can also cause release of histamine. Attempts by macrophages to ingest deposited ICs can result in inadvertant tissue damage.

For membranous glomerulonephritis (MGN), the subepithelial deposition of ICs in the glomerulus appears to be of utmost importance for development of the disease. This event can be triggered by chronic infection, with e.g. hepatitis B. There is some association with MHC antigens (HLA 8, 18 and DR-3 being more highly represented in MGN patients than in the general population), but the relative risk is not high (Mallick *et al.* 1983). The disease is more common in males. Interestingly, less than 25% of patients diagnosed as MGN progress to end stage renal failure, and so any therapeutic measures have to take into account this high level of spontaneous remission (Cattran *et al.* 1989). It is not known how initially antigen(s) becomes deposited in the glomerulus and go on to form ICs *in situ*. Preformed circulating ICs can also become localised at the glomerulus. A number of disease states are associated with MGN. As already mentioned, chronic infection may supply an exogenous antigen, whilst endogenous antigens could be shed by tumours (e.g. carcinoembryonic antigen (Costanza *et al.* (1973)), and become deposited in the glomerulus.

Immunological abnormalities are also associated with MGN, e.g. lymphoma (Hodgkin's disease), autoimmunity (SLE, SS) or chemotherapy, e.g. penicillamine and captopril. In the case of SLE, the deposition of DNA at the glomerulus could allow the localisation of anti-DNA autoantibodies. In addition, MGN is not observed as a complication of RA in tha absence of chemotherapy (Samuels *et al.* 1978), and therefore any immunological abnormalities in common between these two diseases must be accompanied by other factors that lead to the establishment of either RA or MGN. A recurrence of MGN following renal transplantation could reflect either a systemic immunological defect (responsible for the initial disease), or the immunological abnormalities that arise from immunosupression chemotherapy. Therefore in MGN there is clear evidence for the importance of ICs in the establishment of the disease.

In membranoproliferative glomerulonephritis (MPGN) the deposition of ICs is observed to occur in the mesangium and along the capillary walls, which is in contrast to the subepithelial IC deposition observed in MGN. Additional factors may be important in this disease, as cellular proliferation is observed in the mesangium and in the capillary walls. The disease associations for MPGN are similar to MGN (Donadio 1988). In addition, MPGN patients have an increase in the levels of an autoantibody to the complement C3 convertase, called nephritic factor. This antibody is able to stabilise the C3 convertase and so prolong its activation of C3.

Damage to the glomerulus is associated with the infiltration of macrophages and lymphocytes into the mesangium. The primary damage does not appear to result from the direct destructive potential of the macrophage *per se*, but rather these cells play a role in the regulation of other cells, e.g. stimulation of fibroblasts, release of interleukin 1 (IL-1) and

tumour necrosis factor α (TNFα). Stimulation of rat mesangial cells with interferon-γ *in vitro* leads to their expression of MHC class II antigens, and therefore when the glomerulus becomes inflammed the mesangium may present antigen direct to T lymphocytes (Martin *et al.* 1989). In addition the macrophage can stimulate endothelial cells to increase their ability to activate platelets (endothelia are usually antithrombogenic). Platelets in turn can release a number of factors that can affect glomerular structure and function, for example thromboxane A2 and heparinase. Platelet activation may be particularly important in chronic renal disease e.g. MPGN (Klahr 1988). The effects of these factors on haemodynamics may also be important (Pusey *et al.* 1988).

Therefore, the initial formation of immune complexes in the glomerulus, or the deposition of circulating complexes probably provides the basis for the initial insult to the kidney in many cases. Subsequent damage due to the presence of macrophages or the activation of complement further complicate the lesion. The release of immunogenic material from the glomerulus into the circulation may provide positive feedback for continued assault against the kidney.

4.1.2 *Immune Complex Deposition and Clearance*

It is hard to define the relative importance of recognition of autoantigens or the trapping of circulating immune complexes in the glomerulus of patients with nephritis (Pusey *et al.* 1988). Several factors are important in removal or deposition of immune complexes. Two distinct steps may be important in the establishment of deposited immune complexes in the glomerulus; (1) the planting of antigens (e.g. DNA) and (2)

subsequent antibody adhesion to the antigen. The incidence of rheumatoid factor (RF) and antiidiotypic antibodies in the circulation can influence the subsequent build-up of a larger IC following the formation of an initial antibody-antigen complex at the glomerulus. RF can inhibit the binding of aggregated human γ-globulins to glomeruli *in vitro* (Boulton *et al.* 1982), and therefore the raised levels of RF in RA sera may, in part, explain the absence of glomerulonephritis in RA.

The molecular configuration of ICs as well as the absolute levels in the circulation can influence clearance. Large complexes are removed by the liver faster than smaller ones, so that if there is a genetic element controlling IC size then this may influence the subsequent disease course. The size of an immune complex also affects the site of deposition. Small complexes can become localised in the subepithelial region, whilst large complexes are unable to cross the glomerular basement membrane. The removal of particulate ICs requires both C3 and IgG, but soluble ICs have no complement requirement. Phagocytosis of ICs is enhanced by complement. Adler has shown that proteinuria resulting from circulating IC (CIC) deposition is complement mediated (Adler *et al.* 1983). Therefore, paradoxically, a complement deficiency (e.g. in MPGN) can result in excessive deposition of CICs, whilst the damage to the glomerular basement membrane may be mediated by complement proteins.

The dynamics of IC formation and deposition may be regulated by a number of factors including the type of IgG present. IgG3, which can activate complement and bind to macrophages, is preferentially deposited in the glomerulus (Bannister *et al.* 1983a). The deposition of CICs appears to be strongly influenced by vascular permeability. An increase in permeability results in greater deposition, and in the NZB/NZW autoimmune

mouse treatment with histamine antagonists results in less renal pathology. The glomerular site is particularly important in this regard because the blood pressure in glomerular capillaries is very high and this favours deposition. In addition, activation of macrophages at distant sites by systemic inflammation (e.g. SLE) may result in an altered response to ICs deposited on the glomerulus, i.e. such macrophages may cause greater damage to the glomerulus than would otherwise be expected.

Therefore, a number of factors influence the formation and clearance of ICs at the glomerulus. In the majority of glomerulonephritis cases, a cause for IC formation, by infection or disease is not discernable, and the nephritis is considered idiopathic. Clearly, if an alteration to the glycosylation of serum IgG does lead to increased self-aggregation, then this phenomenon may explain some of the idiopathic disease that occurs, or may underlie susceptibility to glomerulonephritis in some individuals. Evidence for a role of IgG glycosylation in spontaneous aggregation of IgG is discussed fully in chapter 5 (section 5.2.6).

4.3 *Methods and Materials*

Human serum samples from patients with diagnosed glomerulopathies were obtained from hospitals in the Oxford area by Dr C.J. Edge of the Glycobiology Unit. Additional information on serum creatinine levels, creatinine clearance and urinary proteinuria at the time of taking the blood sample were also obtained for these patients wherever possible. Individuals with rheumatoid arthritis were not included in this study. Isolation of serum IgG, release, purification and radiolabelling of the N-linked oligosaccharides, the incidence of neutral, mono-and di-sialylated

oligosaccharides and the relative incidence of glycans bearing zero, one or two non-reducing terminal β-galactose residues were determined as described in Appendix 1.

4.4 *Results*

The relative incidence of serum IgG N-linked glycans terminating in zero, one and two β-galactose residues are given in table 4.1, and the incidences of sialylated glycans are given in table 4.2. Table 4.3 shows the renal function parameters together with the Go values. Sialylation levels were not determined for all samples. The incidence of N-linked glycans devoid of terminal galactose are plotted against age in figure 4.1. Patients were grouped into five sets, membranous, membranoproliferative and chronic glomerulopathies, minimal change syndrome, and other nephropathies. Residual values, i.e. the difference between the Go for an individual determined here, and the mean value for age-matched controls are also given in table 4.3. The level of serum creatinine is plotted against Go for the individuals where both of these parameters are known in figure 4.2. A two order polynomial curve was fitted to the data for serum creatinine versus incidence of Go using a Macintosh SE/30 computer with the Cricket Graph programme.

4.5 *Discussion*

The incidence of the neutral and sialylated IgG glycans in the glomerulonephritis patients does not show a deviation from normal IgG N-linked oligosaccharide sialylation. Three patients, MB, JD23 and JM do

GALACTOSYLATION OF SERUM IgG FROM PATIENTS WITH GLOMERULONEPHRITIS

Patient	Age (years)	Sex	Relative Incidence of terminal βgalactose residues (Percent)		
			0	1	2
Membranous Glomerulonephritis (n=9)					
MC5	35	M	33.6	34.9	31.5
EM3	67	M	42.1	32.7	25.2
AM	68	M	58.1	28.9	15.0
MP	25	M	48.1	32.0	19.9
WW	80	M	42.4	35.1	22.5
AB	76	M	37.8	32.2	30.0
JM	39	M	31.8	42.5	25.7
NK2	42	M	50.2	31.4	18.4
MB	21	F	28.0	38.6	33.4
Chronic/Membranous Glomerulonephritis (n=4)					
JL	49	M	29.9	35.5	34.4
GP	74	F	44.5	40.0	15.5
WB3	76	F	50.6	34.2	15.2
PU	41	M	22.0	40.2	37.8
Membranoproliferative Glomerulonephritis (n=2)					
FD	68	F	53.0	31.8	15.2
FW	52	M	42.3	36.8	20.9
Minimal Change Syndrome (n=4)					
MS6	40	M	26.5	46.0	27.5
AK7	16	M	24.7	41.7	33.6
LM11	32	F	34.3	48.9	16.8
IJ1*	32	M	33.2	37.1	29.7
Others (n=5)					
SB9	30	F	42.4	37.0	20.6
CS	67	M	50.1	33.9	16.0
BO	79	F	59.1	26.5	14.4
JD23	57	F	37.3	33.9	28.8
BT	72	F	41.0	36.4	22.6

Table 4.1 Incidences of N-linked glycans terminating in zero, one and two β-galactose residues from serum IgG of patients with renal disease. * patient also has ankylosing spondylitis. Key to other diseases: SB9 Focal segmental glomerulonephritis, CS glomerulonephritis and polyarthritis, BO Goodpasture's syndrome, JD23 IgA nephropathy, BT proliferative glomerulonephritis. Patient sex denoted by F, female, M, male.

SIALYLATION OF SERUM IgG FROM
PATIENTS WITH GLOMERULONEPHRITIS

Patient	Incidence		
	N	A1	A2
Membranous glomerulonephritis			
MC5	77.1	20.6	2.3
EM3	80.2	17.6	2.2
AM	87.0	9.2	3.8
MP	62.2	28.1	9.7
AB	77.8	19.4	2.8
JM	65.7	28.5	5.8
NK2	77.4	14.3	8.3
MB	66.0	28.0	6.0
Chronic/membranous glomerulonephritis			
JL	81.8	16.3	1.9
PU	73.0	19.9	7.1
Other Nephropathies			
CS	71.0	21.1	7.9
BO	84.1	10.7	5.2
JD23	57.7	37.0	5.3
IJ1	79.7	18.0	2.3
Mean(±SD) (n=14)	74.3(8.4)	20.6(7.3)	5.1(2.6)
Std values(±SD)*	75.7(5.0)	20.5(5.0)	3.8(1.8)

Table 4.2 Sialylation of the IgG glycans from patients with glomerulonephritis. Key N neutral, A1 monosialyl, A2 disialyl. All patient designations as for table 4.1 except that IJ1 is grouped with other nephropathies in this table.
* Standard values for IgG sialylation taken from Parekh *et al.* (1985).

RENAL FUNCTION PARAMETERS FOR THE PATIENTS INCLUDED IN THIS STUDY

Patient	Age	Go	Residual*	SCr[$]	CrC[#]	Urinary Protein[@]
Membranous Glomerulonephritis (n=9)						
MC5	35	33.6	+12	121	71	15.6
EM3	67	42.1	+12		69	6.5
AM	68	58.1	+27	987	3	
MP	25	48.1	+27	982	7	
WW	80	42.4		276	40	
AB	76	37.8		194	12	
JM	39	31.8	+9	99	34	4.9
NK2	42	50.2	+27			
MB	21	28.0	+7	607	2	
Chronic/Membranous Glomerulonephritis (n=4)						
JL	49	29.9	+5	154	63	6.5
GP	74	44.5		541		
WB3	76	50.6		804		
PU	41	22.0	-1	80	99	1.3
Membranoproliferative Glomerulonephritis (n=2)						
FD	68	53.0	+21	534		
FW	52	42.3	+17	101	68	17
Minimal Change Syndrome (n=4)						
MS6	40	26.5	+4	79		
AK7	16	24.7	+4	63	78	23
LM11	32	34.3	+13	120		55.7
IJ1	32	33.2	+12			7.2
Others (n=5)						
SB9	30	42.4	+21	106	68	0.26
CS	67	50.1	+20		•	
BO	79	59.1				
JD23	53	37.3	+19	94	6.3	
BT	72	41.0		507		

Table 4.3

* The residual Go value was calculated as described in methods and materials. [$] Serum creatinine given in μmoles/litre, [#] creatinine clearance rate given in ml/min, and [@] urinary protein given in g/24hours.

Mean residuals are; Membranous glomerulonephritis +13
Chronic glomerulonephritis +2
Membranoproliferative glomerulonephritis +19
Minimal Change Syndrome +8

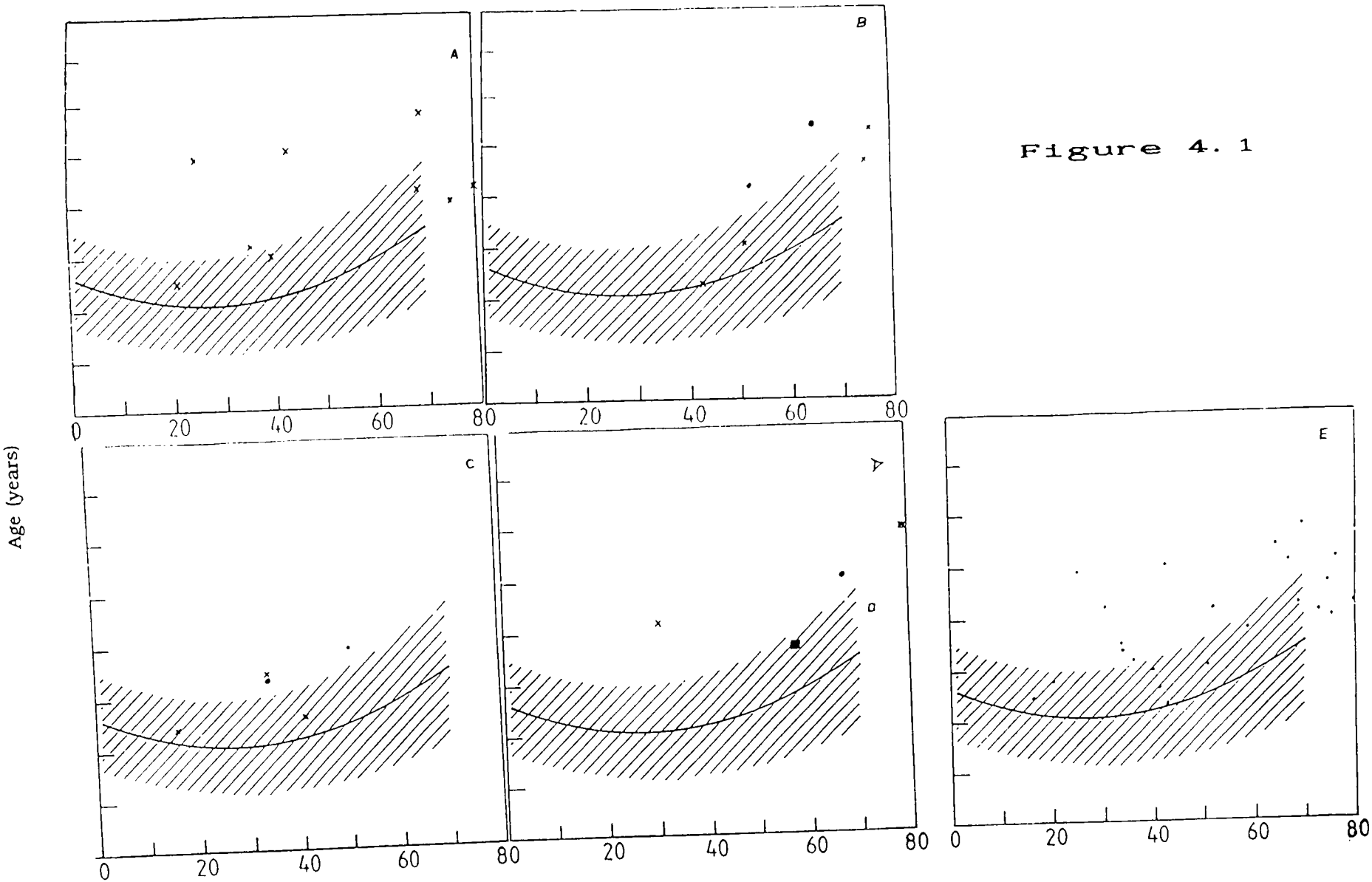


Figure 4. 1

Figure 4.1

Panel A; Percentage incidence of agalactosyl oligosaccharide sequences, Go, from the IgG of patients with membranous glomerulonephritis related to age (n = 9).

Panel B; Percentage incidence of agalactosyl oligosaccharide sequences, Go, from the IgG of patients with chronic membranous glomerulonephritis (n = 4) and membranoproliferative glomerulonephritis (n = 2) related to age. x Chronic membranous glomerulonephritis, ● Membranoproliferative glomerulonephritis.

Panel C; Percentage incidence of agalactosyl oligosaccharide sequences, Go, from the IgG of patients with minimal change syndrome (n = 3) and minimal change with ankylosing spondylitis (n = 1) related to age. x Minimal change syndrome, ● Minimal change with ankylosing spondylitis.

Panel D; Percentage incidence of agalactosyl oligosaccharide sequences, Go, from the IgG of patients with nephropathies related to age (n = 5).
x Focal segmental glomerulonephritis, ● Glomerulonephritis and polyarthritis, ☒ Goodpasteur's syndrome, ■ IgA nephropathy, □ Cresenteric glomerulonephritis.

Panel E; Percentage incidence of agalactosyl oligosaccharide sequences, Go, from the IgG of all patients with glomerulonephritis related to age (n = 23).

Solid line and shading indicates the regression line and the region ±two standard deviations from the line, respectively, for normal serum IgG Go incidence related to age (n=111) (Parekh *et al.* 1989)

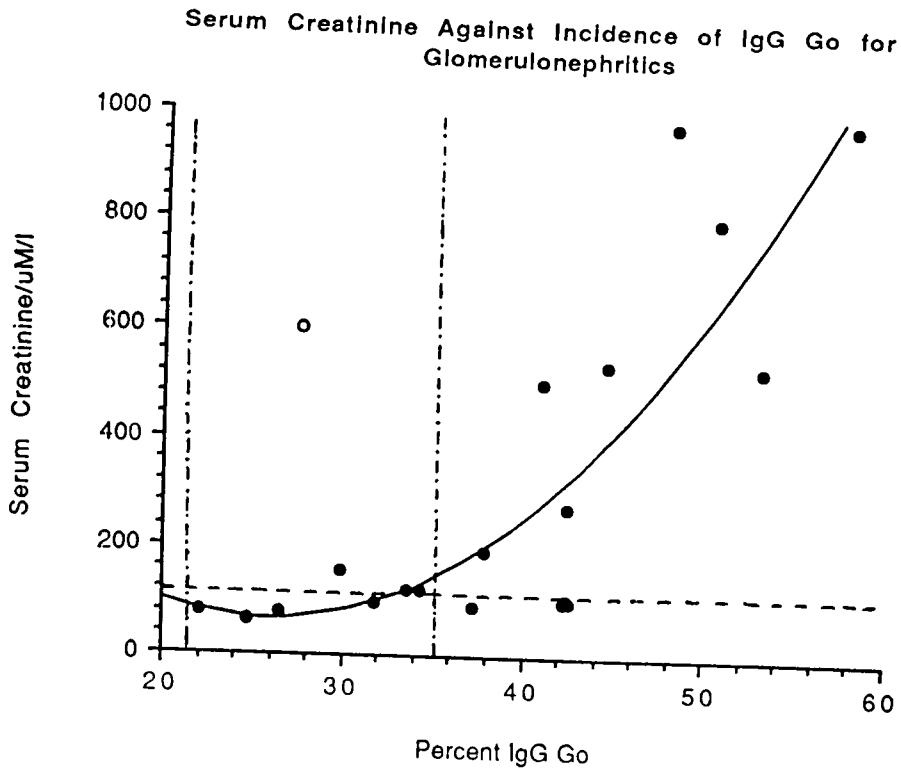


Figure 4.2 Plot of incidence of Go against serum creatinine for patients with glomerulonephritis in this study (n = 20). The curve represents a two order polynomial fit to the raw data ($y = 708.19 - 49.564x + 0.953x^2$). The open circle represents patient MB, not included in the calculation of the best fit curve. The dashed line represents the mean normal level of serum creatinine at 109µM/litre (Source; Documetia Geigy), and the dotted and dashed lines represent the normal range of serum IgG Go for age 0 to 70 years (21-35%)

show a decrease in neutral glycans, but as all three were studied as part of the same batch of samples from hydrazinolysis, this may reflect small variability in the loss of sialic acid resulting from this procedure and may be artefactual.

Figure 4.1 and table 4.3 clearly show that the terminal galactosylation of this set of serum IgGs is lower than for a normal set. All the Go values for the glomerulonephritis patients are above the regression line of the normal set and therefore are not coincident with the normal Go values. Comparison of this to figure 4.3 for IgG from rheumatoid arthritis patients clearly shows that the glomerulonephritis patients as a group not exhibit as severe a hypogalactosylation as the RA group. Analysis of the residual difference between the Go values for the MPGN and MGN groups does indicate that these patients have elevated Go when compared to normals (table 4.3), which are the two forms of glomerulonephritis where IC deposition is probably most important for the establishment of disease. It should be noted in this regard that the individual data for the RA patients less than 60 years old often fall close to the +2SD line but are always above it. This is not the case with the glomerulonephritis patients, where a high proportion of the Go values fall within the ±2SD area. The ±2SD region is extremely broad and this reflects the natural variability in the Go parameter in the general population. Also RA patients older than 60 years can fall into the 2SD area of the graph, and because of the spread of the data in this region extrapolation beyond 70 years is not possible. Because of the sample size, and the natural spread of the data, statistical analysis was not performed.

Separation of the patients into the various nephropathies indicates that the elevation of Go for only some of the glomerulonephritis patients

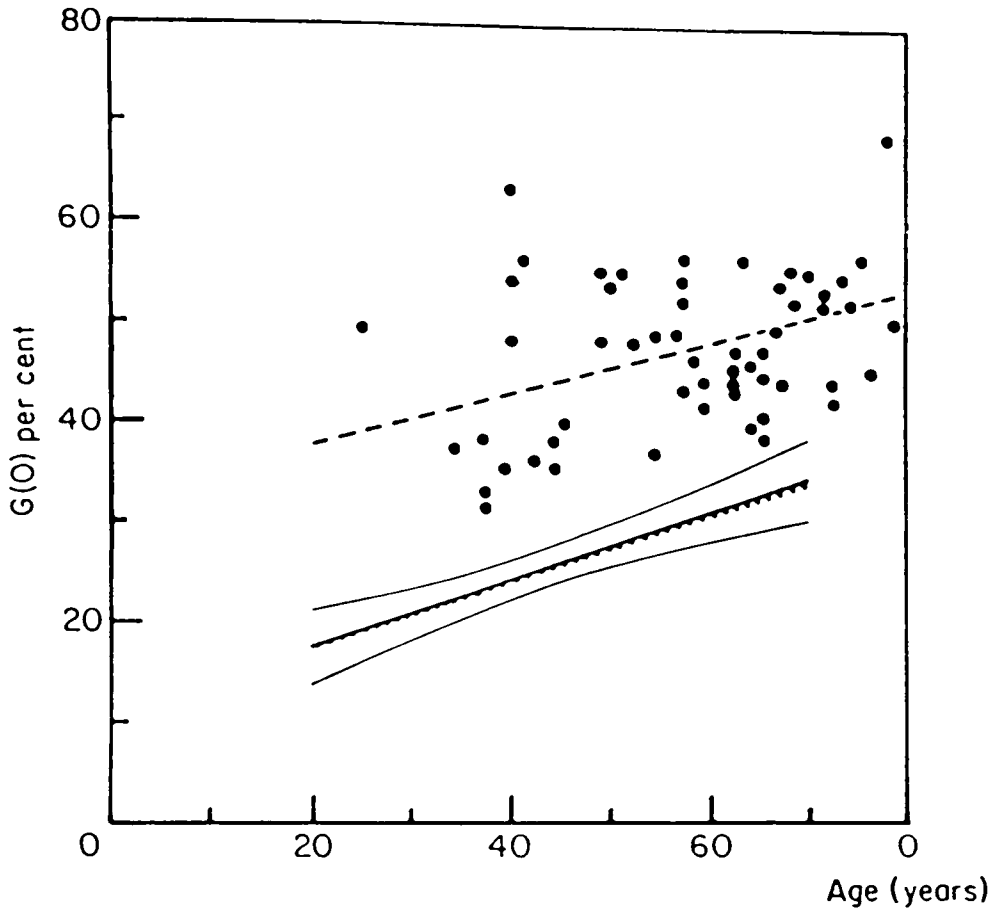


Figure 4.3 Percent incidence of agalactosyl oligosaccharide glycans from IgG of patients with rheumatoid arthritis plotted against age. The figure is reproduced from Parekh *et al.* (1989).

is observed in the individual disease types. For the MGN patients four of the individuals are clearly outside the 2SD range. One patient, MP, was known to have very active disease when the blood sample was taken and the Go value for this IgG is very high (48% incidence with an age of 25 years, over double the mean for the control data). The MPGN set are elevated whilst the chronic membranous glomerulonephritis patients are not elevated. Again, because of the sample size statistical analysis was not performed on these data. The minimal change group also do not show a great increase in Go in comparison to the MGN and MPGN groups. Interestingly, one patient had ankylosing spondylitis as well as minimal change syndrome renal disease but this did not alter the Go for the IgG glycans (ankylosing spondylitis is not associated with hypogalctosylation of IgG glycans (Parekh *et al.* 1989)). It is difficult to draw strong conclusions from the other diseases because of too few points, but the data here does indicate that the patient SB9 (focal segmental glomerulonephritis) has an increased Go, and patient CS (polyarthritis and glomerulonephritis) is also elevated. Therefore it can be concluded that some MGN and MPGN patients show hypogalactosylation of their serum IgG. Whilst statistical analysis was not performed, inspection of the data (figure 4.1e) indicates that the correlation between increased Go and glomerulonephritis is less strong than is observed for Go and RA.

With regard to the association of Go with renal function (figure 4.2), it is of interest to note that the individuals with the highest serum creatinine levels (indicative of severe imparement of glomerular function) have the most elevated Go. In figure 4.2 the ranges for serum creatinine and IgG Go for healthy individuals are shown. The glomerulonephritis patients clearly comprise two groups, the first with serum creatinine in

the normal range and with normal or slightly raised Go, and a second group comprised of patients with elevated serum creatinine and highly elevated Go. The curve fitted to the data is presented purely to show that the two groups present a contiguous group, from normal parameters to highly abnormal ones. The single exception to the trend (patient MB) had a near normal Go but high serum creatinine. This patient was known to have very poor renal function, and the reason for this individual not conforming to the overall trend is not known. The patients with high levels of serum creatinine also show a very low creatinine clearance rate (where these data are available, table 4.3). Therefore in the majority of cases highly impared renal function correlates with an increase in Go. Obviously there is a question of cause or effect in this instance, and it will be of importance to establish if an increase in Go precedes or postdates severe loss of renal function.

The involvement of a glycosylation alteration in self aggregation of IgG (Middaugh and Litman 1987, Hymes and Millinax 1979, Rademacher *et al.* 1988b), IC formation and hence complement activation may contribute to the immunopathology of glomerulonephritis by increasing the propensity of IgG to aggregate and become deposited in the glomerulus. Potential macrophage activation by the recognition of exposed GlcNAc terminating glycans (Rademacher *et al.* 1988b) could also increase the level of damage to the mesangial membrane. Therefore in some cases elevated Go may be a contributary factor to the establishment of glomerulonephritis.

Because glomerulonephritis is distinct from RA it implies that the hypogalactosylation may be necessary or contributory, but not sufficient, for the establishment of both of these diseases (section 4.1.1). In this regard it should be noted that SLE is not associated with an elevated

incidence of Go oligosaccharides and SLE in humans is associated with renal disease. In SLE, planting of an autoantigen in the glomerulus is probably an important initial event in the development of nephritis, and therefore provides the primary mechanism whereby IC formation can occur in the kidney. A multifactoral cause for glomerulonephritis may explain the observation that only some of the patients in this study display hypogalactosylation. However, the observation that the MGN and MPGN groups are elevated, and the fact that in these diseases IC deposition is considered to be a primary lesion does indicate that hypogalactosylation of serum IgG may be a predisposing factor in some instances.

The limited response of the kidney to damage from a wide range of sources adds a level of difficulty in the interpretation of the results of any study into nephritis. The incomplete correlation of highly elevated Go with disease may reflect this. Obviously, in patients where antigen planting or autoantibody recognition of a glomerular basement membrane antigen provides the underlying cause for initiation of renal disease, there is no requirement *per se* for a factor that increases the spontaneous rate of IC formation. However, in other individuals where such mechanisms may not be responsible for the establishment of glomerulonephritis, aggregation of IgG may be the primary cause for deposition of Ig in the glomerulus. It is of interest that the presence of high levels of RF in the serum may offset this, so that RA does not lead to renal failure, whereas the increase in the level of CICs as seen in RA might be expected to lead to kidney disease in the absence of RF.

In both RA and glomerulonephritis immune complex formation is an important event in disease progression. The deposition and accumulation of ICs at different sites (the glomerulus in nephritis and the synovium in RA)

are clearly mediators of tissue damage, which may subsequently become self-sustaining as antigenic material is released into the circulation. An increased propensity for aggregation of serum IgG in these individuals may underlie one route by which disease can become established, but clearly other factors are also of importance, for example infection and pre-exsiting immunological abnormalities. The deposition of antigens (either exogenous or endogenous), for which there are circulating antibodies may be of importance to the establishment of glomerulonephritis, but if, in addition, some individuals are producing IgG that is more capable of forming complexes, then these individuals may be at greater risk than the rest of the population for the development of renal disease.

Clearly a larger number of patients need to be studied to confirm the observations made here. The question of cause or effect for glycosylation abnormalities in serum IgG and renal disease needs to be addressed in order to rationalise more fully both the possible role of hypogalactosylated IgG in glomerulonephritis and to determine if levels of galactosylation are an important factor in determining the ability of IgG to self-associate *in vivo*.

Studies on glomerulonephritis have shown that this group of diseases are caused by a large number of factors e.g. infection, systemic autoimmunity, chemotherapy The establishment of a clearcut aetiology remains an important question. Indeed, it has been commented that

'It appears that there may be genetic markers for the suseptibility of membranous glomerulonephritis, but the resulting immunological abnormalities, if any, have not been delineated clearly.' (Coggins 1988)

A possible role for alterations in IgG glycosylation may go some way to answering this problem.

CHAPTER FIVE

THE GLYCOSYLATION OF HUMAN SERUM IMMUNOGLOBULIN A1

5.1 *Preamble*

In this chapter the structure and function of IgA are discussed, and a description of aspects of rheumatoid arthritis (RA) relevant to a role for IgA in this disease is given. The glycosylation of IgA1, as determined here, is compared to the analysis of previous workers. Alterations of human IgG N-linked carbohydrate that are found in certain diseases, especially RA are discussed. The N-glycosylation of IgA1 from normal and RA patients was compared in order to determine if RA associated alterations in carbohydrate also occur in another glycoprotein.

Monomeric serum IgA1 was chosen for study for the following reasons. (1) IgA is a B cell product, similar to IgG in terms of synthesis, secretion, and polypeptide secondary structure. (2) The N-linked glycosylation of IgA has been reported as being of the complex type, and as such is similar to that of IgG and, therefore, carbohydrate biosynthesis is expected to involve common enzymes. (3) IgA is predominantly a monomer in serum, similar to IgG. (4) Evidence has been presented for a role of IgA in RA pathogenesis. (5) IgA1 could be isolated in sufficient quantity and purity for analysis from small volumes of serum obtained from individual patients. (6) The glycosylation of IgA1 has only been studied in detail for a myeloma protein, and therefore normal serum IgA1 has not been analysed. Points (1) to (5) make IgA1 a candidate for RA-associated alterations in

glycosylation.

Based on a study of various published methods, a two step purification scheme for human serum IgA1 was used. A full characterisation of the N- and O-linked glycans from human serum IgA1 by the use of NMR, gas-chromatography mass-spectrometry (GCMS) and exoglycosidase degradation analysis is presented. The terminal substitution, i.e. galactosylation and sialylation, of the N-linked glycans derived from serum IgA1 from healthy individuals and patients with rheumatoid arthritis was also investigated.

From these data it is concluded that: (1) the N-glycosylation of serum IgA1 in the human is predominantly of the biantennary complex type, with full substitution of the glycan termini with galactose and sialic acid, which is in contrast to that observed in IgG, (2) the O-linked glycans of IgA1 are heavily sialylated, (3) an alteration in the N-glycosylation of serum IgA1 in RA patients, of equivalent severity to that seen for IgG, was not observed. Finally these findings are discussed with reference to the differences in glycosylation between IgA1 and IgG and the possible importance of N- and O-glycans in modulating the function and stability of IgA1.

5.2 *Introduction*

5.2.1 *The Molecular Structure of IgA*

IgA in many species is synthesised in amounts greater than any other immunoglobulin isotype. Due to the unique body fluid distribution of IgA the serum level is quite low (Mestecky and McGhee 1987). Large amounts of IgA are found in the external secretions e.g. saliva, tears, milk, and

mucus of the respiratory, urinogenital and intestinal tracts (Heremans 1974). In some species, including the human, the serum and secretory compartments are quite distinct. IgA in the secretions is mostly produced locally, by plasma cells residing in the Peyer's patches or equivalent organs associated with the mucosal surfaces. Serum IgA is produced primarily in the bone marrow (Heremans 1974). A total rate of synthesis in the human has been calculated as 66mg/kg/day (Delacroix 1985).

An unusual feature of the structure of human IgA is the large number of possible molecular configurations that it can present (figure 5.1), which appear to be regulated for specific compartments. There are two subclasses, A1 and A2, and also additional allotypic variants. The major structural difference between the subclasses is that IgA1 possesses a 20 amino acid hinge region C-terminal to Cα1 and N-terminal to Cα2, whereas there is no comparable structure in IgA2. The hinge polypeptide is composed exclusively of proline, serine and threonine residues, adopts an extended conformation and carries the O-linked glycans (Baenziger and Kornfeld 1974b).

Monomeric IgA has the typical immunoglobulin tetrameric polypeptide structure (two light chains and two α-chains), with a molecular weight of 160kdal. 80-90% of serum IgA exists in this form. In the secretions IgA is dimeric and tetrameric and contains additional polypeptides; the J chain and the secretory component (SC). A very small amount of monomeric IgA is also found in the secretions (Heremans 1974). Subclass distribution is dissimilar between the serum and secretory forms; in serum 80-90% of IgA is IgA1, whilst in secretions IgA1 and IgA2 are about equally represented.

The three constant domains of the α-chains of both subclasses are similar in sequence. The C-terminus is similar to the μ-chain C-terminus. The penultimate cysteine residue is highly reactive, and in the polymeric

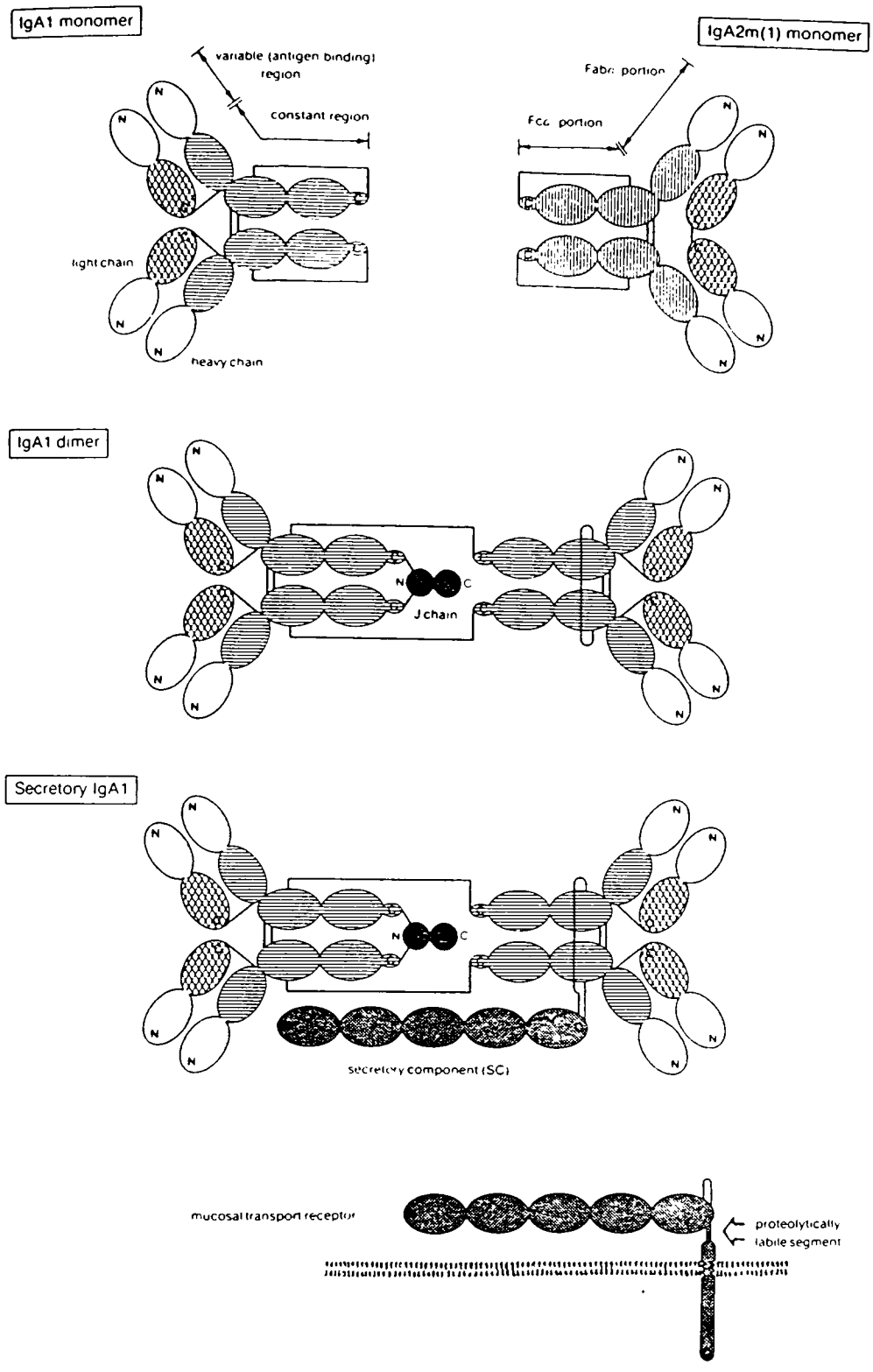


Figure 5.1 Schematic diagram of the covalent structure of human IgA. Each elliptical section represents an Ig domain. Disulphide bridges are indicated by solid lines. The positions of the J chain and the secretory component are not fully defined by present data, and their positions are hypothetical. The figure is taken from Underdown and Schiff (1986).

form this amino acid forms a disulphide bridge to the J chain. In monomers other proteins can be disulphide-linked, e.g. serum albumin (Mannik 1967) and α1-antitrypsin (Tomasi and Hauptmer 1974). Whilst other polypeptides have been demonstrated to be covalently bound to monomeric IgA, e.g. protein HC (Grubb *et al* 1986) and α1-microglobulin, (Vincent *et al* 1985), the protein-protein linkage has not been defined in these cases.

The precise three-dimensional structure of the polymeric IgA complex is not known. The presence of the J chain would appear to be of great importance to the assembly of the polymeric IgA molecule. Neither the precise configuration of the J chain in relation to the α-chain, nor if the J chain connects two monomers or is associated with only one of them is known. The number of J chains per dimer may vary (Brandtzaeg 1976). It is probable that a single J-chain is associated with five monomer IgM molecules to form a pentamer, so the precise stoichiometry of the J-chain to heavy chain may be independant of the number of constant regions in the final complex. A correlation between the amount of J chain present in polymeric IgA and the ability of the molecule to bind SC has been suggested, but other experiments have not been able to confirm this (Schiff *et al.* 1986, Brandtzaeg and Prydz 1984). Polymeric IgM has a higher affinity for SC than IgA.

The SC is composed of five homologous Ig domains most homologous to light chain variable domains (Eiffert *et al.* 1984). The fifth domain is less homologous and has a ragged C-terminus, due to irregular proteolysis when cleaved from the trans-membrane domain (see below). The J chain has low homology with Ig domains, but circular dichroism measurements indicate that it also assumes the Ig fold (Heremans 1974). Therefore the polymeric IgA molecule is made entirely of polypeptides belonging to the Ig

superfamily.

The positions of the N- and O-linked glycans have been determined. This information is summarised in figure 5.2. The same sites are probably used in the serum and secretory forms of the molecule. None of the IgA glycosylation sites are in homologous positions to the sites on IgG.

5.2.2 *Function and Biology of IgA in Normal Humans*

Most B cells at the mucosal surface are IgA producers (Bienenstock and Befus 1980). The IgA producing B cells are enriched at the Peyer's patches in the small intestine. Antigens in the intestinal lumen are sampled by specialised 'M' cells located over the Peyer's patches and bordering the lumen. M cells appear competent to transfer antigens into the patches so that they are exposed to the resident B cells.

Following antigenic stimulation IgA plasma cell precursors migrate to the blood via the lymphatic system and home back to the same mucosal site or a distant one (Czerinsky *et al.* 1987). The T cell control of these cells begins by the stimulation of IgM producing B cells which later differentiate to become IgA producers. Some T cells bear Fcα receptors, and this subset regulate the IgA positive B cells. A number of soluble IgA binding factors are also important in regulation of the IgA producing cells (Kiyono *et al.* 1985).

IgA is transported from the mucosal tissues to the gut lumen by binding to the poly Ig receptor (SC membrane form) at the basolateral membrane of the epithelial cells facing the synthesis site. IgA remains bound to this receptor and is transported through the cell and released at the mucosal surface by proteolytic cleavage of the receptor to generate the SC fragment

GLYCOSYLATION SITES ON HUMAN IMMUNOGLOBULIN A

Domain	Position[a]	Subclass		
		A1[b]	A2 Allotype	
			m(1)[c]	m(2)[d]
V	(Generation of Asn-X-Ser/Thr by variation)			
Cα1	116		N[e]	N
	211			N
Hinge	224	O		
	230	O		
	232	O		
	238	O		
	240	O		
Cα2	263	N	N	N
	337		N	N
Cα3	459	N	N	N

Figure 5.2 The N and O-glycosylation sites of human immunoglobulin A
[a] Amino acid numbering as for IgA1 Bur. Sequon location data from Putnam F., *et al.* (1979) ([b], IgA1 Bur), Tsuzukida Y., *et al.* (1979) ([c], IgA2 Lan) and Torano A., and Putnam F., (1978) ([d], IgA2 But).

(e) N = Asn linked, O = Ser linked

Adapted from Mestecky J. and Kilian M., (1985).

which remains in association with the IgA dimer. In man, as in most species, the secretion occurs along the length of the gut. The SC transport system functions exclusively for polymeric IgA and IgM. The presence of IgA at the mucosal surfaces is thought to cause the aggregation of microorganisms, decreasing their ability to adhere to and penetrate the mucosal epithelium. Such interference gives greater opportunity for lysozyme, peroxidase and other nonspecific agents to act (Tenuovo *et al.* 1982).

The recent identification of a large number of IgA1 specific proteases secreted by pathogenic micro-organisms suggests that IgA1 has an important role in mucosal defense. These proteases all cleave the hinge region at a specific site, but the precise position depends on the individual enzyme. All three principle causes of bacterial meningitis, *Haemophillus influenzae, Neisseria meningitidis* and *Streptococcus pneumoniae* produce IgA1 specific proteases (Kilian *et al.* 1988). Neutralising antibodies to the protease have been detected in the secretions of patients infected with IgA1 protease producing bacteria. Cleavage of IgA1 may destroy function and allow the bacteria to colonise and penetrate the mucosa. The monovalent Fab fragments produced may even protect the bacterium by blocking access of intact antibodies to the cell surface, and thus prevent their aggregation. Kilian *et al.* (1988) proposed that natural infection with *H. influenzae, N. meningiditis* or *S. pneumoniae*, which occurs in the majority of infants, leads to immunisation against the IgA1 protease so that the enzyme is neutralised and the pathogen cannot mask itself with Fabs and thereby escape immunosurveillance.

Circulating serum IgA plays a role in antigen clearance. Some serum IgA is mucosal derived, possibly from active IgA producing plasma cells in the

gut-associated lymphoid tissue (GALT). These antibodies are useful for the removal of dietary antigens, which may be considerable in gut inflammatory diseases. Immune complexes containing IgA appear to be taken up by the hepatic Kuppfer cells. In the mouse, α1 heavy chains, but not α2 heavy chains, have been shown to bind to the murine hepatic asialoglycoprotein receptor. In addition, Hiemstra *et al.* (1987) recently demonstrated that both IgA subclasses can efficiently activate complement by the alternative pathway, and that the presence of the $(Fab')_2$ fragment is essential for this activity. Conversely IgA may inhibit the binding of IgG with C1q, by acting as an IgG antagonist, attenuating the classical complement pathway (Stockert *et al.* 1982).

Serum IgA binds Fcα-receptors on a wide variety of cells. Of particular importance is binding to macrophages which may have a role in antigen opsonisation. In the rat the interaction with IgA appears to be mediated by the O-linked glycans at the hinge region (Gorter *et al.* 1988). Fcα-receptors are important mediators of antibody-dependant cellular cytotoxicity (ADCC). Suitable effector cells for ADCC e.g. NK cells, may be present at the mucosal surface and in the circulation.

The detection of mucosally produced IgA in serum supports the possibility that oral injestion of antigen may modify subsequent systemic immune responses, and prevent hypersensitivity to food antigens. A single oral dose of several miligrams can totally suppress the systemic response to subsequent challenge (oral tolerance) (Mowat 1987). Sub-milligram doses can prime, acting as active immunogens. Antigens that result in tolerance are usually thymus-dependant, i.e. requiring T cells to mediate the effect. The mechanism of tolerance appears to be due to T cell anergy. Depletion of suppressor T cells (Ts) or pretreatment of mice with anti I-J antiserum can

prevent oral tolerance to ovalbumin being established. It is possible that the intestinal epithelium presents antigen direct to supressor T cells, and this mediates tolerance (Bland and Warren 1986).

5.2.3 *Rheumatoid Arthritis and the Role of IgA*

5.2.3.1 Clinical Features

Rheumatoid arthritis (RA) is a disease that affects 3% of the population, being more common in women than men. It is considered a modern disease, with little evidence of existence before the 15th century. Clinically, RA is characterised by inflammation at synovial joints and eventual erosion of the tissues, although the disease is not restricted to these areas. The most notable immunologic abnormality is the presence of antibodies that bind to immunoglobulin (Rheumatoid factor, RF). The spectrum of symptoms is very diverse. RA is essentially a multi-system disorder, with many organs becoming involved, but with autoimmune attack focused on the synovial tissue. Non-specific symptoms (e.g. lethergy, anorexia, weakness and stiffness) often antedate overt joint symptoms. Pyrexia and lymphadenopathy may occur. Extra-articular manifestations of RA include development of subcutaneous nodules, which contain lymphocytes and plasma cells. The symptoms and natural history of RA have been recently reviewed by Morrow and Isenberg (1987).

5.2.3.2 Aetiology

The aetiology of RA is unknown and is probably multifactoral. Arthropathies have been associated with infection by bacterial (e.g. *Yersinia spp*), viral and fungal agents. There is evidence that mycobacteria can lead to arthritis in experimental animals, but there is no proof of a correlation with RA. Enteric bacteria, e.g. *Clostridium perfringens*, can cause an acute arthritis in swine. In human studies it has been difficult to isolate potential arthritogenic agents. Peptidoglycans derived from bacteria have both adjuvant and arthritogenic properties, which lends weight to the theory that bacteria are the root cause of some cases of RA. Immunisation of mice with RF has shown that the idiotypes can cross react with peptidoglycan derived from *Streptococcus pyrogenes*. There is little evidence in support of viruses as an aetiological agent in RA, although rubella can lead to transient arthritis in humans.

5.2.3.3 Immunopathology

The HLA locus probably plays some role in predisposition to RA. About 70% of RA cases are DR4 positive. DR4 confers a relative risk of 6.0, which is not excessively high. Todd *et al.* (1988) have observed an increased prevalence of DR1 and DRw10 in RA patients. There is also a negative correlation for DR2 and the DR4 Dw10 haplotype, perhaps suggesting that Dw10 is protective in the DR4 background. Inspection of the DRβ1/111 alleles that have an increased prevalence in RA patients indicates that the third hypervariable regions (amino acids 65-75) are similar amongst these alleles. This region is a known T cell recognition site and therefore these

gene products may contribute directly to the disease, perhaps by allowing the presentation of self-antigens, or by being defective in presentation to Ts cells.

All RA patients' sera contain antibody that react with autologous or heterologous immunoglobulin. The contribution of IgM RF to the disease progression is not well defined, and a strong correlation between IgM and IgG RF levels and prognosis has not been observed. The specificity of RF is broad, and most react with Ig from a number of species, preferentially binding to aggregated Ig. The RF binding site is usually located on the Fc, but sites on the Fab have been documented.

Autosensitisation, possibly due to an altered molecular structure, has been proposed as a primary immunological lesion in RA. Parekh *et al* (1985) have shown that the IgG in RA has decreased galactose substitution on the Fc glycans. The effect of this may be to lead to exposure of protein determinants masked by the oligosaccharide, or that the new termini of the glycans may themselves be immunogenic (Rademacher *et al.* 1988b).

Idiotype analysis and Southern blotting of RNA for RF autoantibodies in mice has demonstrated that the V domain genes utilised in these antibodies is restricted. Most of the V domains arise from a restricted set of gene families towards the 3' end of the V gene cluster. Preferential expression of the 3' genes is known to occur early in development (Manheimer-Lory *et al.* 1986). The sequences of autoreactive antibodies seen in several diseases are commonly derived from genomic sequences with minimal somatic mutation of the germ line sequence, and are also biased towards a restricted set of V region families (Bona 1988).

Autoantibodies against other determinants such as collagen types I-V have been observed. The antibodies appear to be directed against denatured

or degraded collagen which occurs in synovial joint damage. Anti-keratin antibodies have been found in the sera of about 50% of patients. Many cytoskeleton components are also targets for autoantibodies. Anti-nuclear antibodies have been detected, but unlike systemic lupus erythematosus (SLE), these antibodies do not bind to DNA. The contribution that this diverse group of antibodies may make towards disease progression is not known, and is probably secondary to the lesion causing the disease.

Elevated levels of immune complexes are present in the sera of RA patients. The immune complexes (ICs) are composed of Ig and complement components, and no common antigen has been identified. Elevation of IC levels is more marked in the synovium where most of these complexes are produced. Immune complexes can fix complement by both the classical and alternative pathways. This in turn leads to inflammation and the release of hydrolases and proteases due to tissue dammage. Release of material from such sites could result in immunisation and/or further immune complex generation, and the complexes may themselves stimulate RF B cells.

The serum levels of complement components are little altered in RA, although C3d is increased, and C3 may show some elevation. In the synovium there appears to be increased breakdown of complement, as expected. The acute-phase proteins serum amyloid A and C-reactive protein (CRP) are elevated in RA patients.

5.2.3.4 Lymphocyte Abnormalities

In human RA, no clear alteration to the numbers of T cells has been found, although a decrease in the numbers of T cells rossetting sheep erythrocytes has been documented. These cells are probably an immature

subpopulation, and their titre correlates inversely with disease activity (Youinov *et al.* 1983). Monoclonal antibody/fluorescent activated cell sorter (FACS) analysis has not been able to reveal any major changes in the incidence of T cell subsets. Cross and Hazelton (1985) have reported that the autologous mixed lymphocyte reaction (AMLR), which measures the effector T cell activity, is suppressed and is influenced by disease activity and chemotherapy. Cytotoxic T cell activity is reduced and there may be an increase in the contrasuppressor T cell (T_{cs}) activity (see below). Adoptive transfer experiments demonstrated that in experimental allergic arthritis a T cell line reactive with *M. tuberculosis* and rat type II collagen can transfer arthritis to an irradiated rat (Holoshitz *et al.* 1983). Paradoxically this same T cell line was protective for rats challenged with complete Freund's adjuvant, which normally results in an acute arthritis.

Several reports indicated that the control of B cell activity is awry in RA, with overproduction of immunoglobulin. No phenotypic alterations are seen in the B cell population, but Irving *et al.* (1981) observed decreased IgM production by RA B cells stimulated *in vitro* by Epstein-Barr virus. The activities of interleukin (IL) 2 in the serum and synovium and γ-interferon (γ-IFN) in the synovium are reduced, and there may also be an IL-1 dysfunction. The cytokines responsible for control of IgA and some subclasses of IgG are different to those governing production of IgM and other IgG subclasses (Beagley *et al.* 1988), but it is not known how the levels of these isotype-specific lymphokines alter in RA, if indeed they do.

Therefore, several lines of evidence indicate that the immune system in RA is abnormal, but the spectrum of alterations seen in this disease are

not indicative of a primary lesion, and may be secondary to it. This is clearly compounded by the fact that the establishment of RA may be multifactoral, and the clinical picture can be very diverse even amongst those patients with definite RA. Recently there has been some evidence for the involvement of γδ T cells in synovial immunity (Holoshitz *et al.* 1989) and in the immune response to mycobacteria and heat shock proteins (see Raulet 1989), but this remains to be investigated further.

5.2.3.5 IgA in Rheumatoid Arthritis

As most individuals possess circulating autoantibodies in low levels (including rheumatoid factor), these antibodies cannot be considered abnormal *per se*, and may be an important element in normal immunoregulation (Cohen and Cooke 1987). The levels of different isotype RFs do not necessarily correlate (Cowling *et al.* 1980). A high titre of IgA RF is indicative of a poor prognosis in RA, whilst the levels of IgM RF are not informative (Teitsson *et al.* 1984).

A characterisation of IgA RF in Sicca syndrome patients showed that the autoantibody was enriched in the dimeric form compared to the normal serum distribution (Elkon *et al.* 1982). Some workers have taken this finding to imply that the IgA RF is of mucosal origin and that the trigger for some rheumatic disease could be gut associated. However, Schohenloher *et al.* (1985) demonstrated that the quantity of monomeric IgA RF was underestimated, and the ratio of monomeric to polymeric IgA RF varied greatly in different patients (Schohenloher *et al.* 1986). They also observed polymeric IgA RF in the synovium, suggesting that not all the polymeric IgA was of GALT origin. This conclusion is supported by the

observation that polymeric IgA RF is produced in IgA nephropathy which does not involve the GALT (Czerkinsky *et al.* 1986).

Treatment of RA patients with D-penicillamine and gold (sodium aurothiomalate) does not always result in a decrease in the level of IgA RF, but when it does the patient goes into remission (Stanworth 1985). In addition, elevated levels of an IgA-α1-antitrypsin (α1AT) covalent complex (α1AT is an acute phase protein) in RA serum are reduced by D-penicillamine treatment. Both isotypes of IgA can form this complex. Administration of D-penicillamine does not alter the serum concentration of either free α1AT or IgA. A fall in the complex concentration does correlate with clinical improvement. The IgA-α1AT complex appears to be formed in the synovium and has never been found in healthy individuals, but does also occur in ankylosing spondylitis (Stanworth 1985). The efficacy of thiol containing reagents, like D-penicillamine, and heavy metal treatment (e.g. gold and copper) may be in blocking the reactive cysteine on the IgA (Stanworth 1985).

In presensitised rabbits that are given an articular injection of antigen resulting in experimental arthritis, the serum IgA concentration rises rapidly, and IgA plasma cells are seen in the synovial space. This rise can be prevented by giving D-penicillamine, which also alleviates the arthritis (Stanworth 1984). Raised IgA levels in these cases could lead to an increase in the formation of the α1AT complex, although the precise role of this complex in pathology is unclear. α1AT has been proposed to inactivate cell surface enzymes on lymphocytes preventing their proliferation. The sequestration of the α1AT could prevent its action as an inhibitor of degradative enzymes, which is presumably important because, as an acute phase protein, it would be raised under inflammatory conditions.

In addition the α1AT-IgA complex may activate macrophages, mediating joint damage.

5.2.4 *The Glycosylation of Immunoglobulins*

The carbohydrate moities of human immunoglobulins have been extensively studied (see references in figure 5.3). IgG is the best characterised, the other immunoglobulins substantially less so. This is, in part, due to the difficulty in isolation of sufficient quantities of IgA, IgD, IgE and IgM, and most studies on these immunoglobulins have focused on myeloma proteins.

Data from analysis of different paraproteins indicates that the glycans may vary from one myeloma to another, especially in the relative abundance of various oligosaccharides, for example IgD (see figure 5.3). As discussed in chapter one, the cell phenotype can alter the glycosylation markedly, and therefore the glycans on the myeloma immunoglobulins may be a poor reflection of those found on the normal population of Igs *in vivo.* Indeed, Takahashi *et al.* (1987) have observed that the spectrum of glycans on serum IgG is different for that found on myeloma IgG. Mizuochi *et al.* (1982) have also observed this, and found that the glycans expressed on the IgG from normal individuals were much less variable. Both of these studies showed that the glycans of myeloma IgGs tended to be less substituted than the normal serum IgG glycans.

Most of the Ig glycans occur in the Fc. The specific site varies with isotype, subclass and allotype. It is not considered that the positions of sites show significant homology from one Ig to another. The amount of carbohydrate varies greatly from one isotype to another (e.g. about 3% for IgG and 12% for IgM). IgA2 is an example of conserved Fab glycosylation,

Figure 5.3 Summary of The N-linked Glycans of Human Serum Immunoglobulins

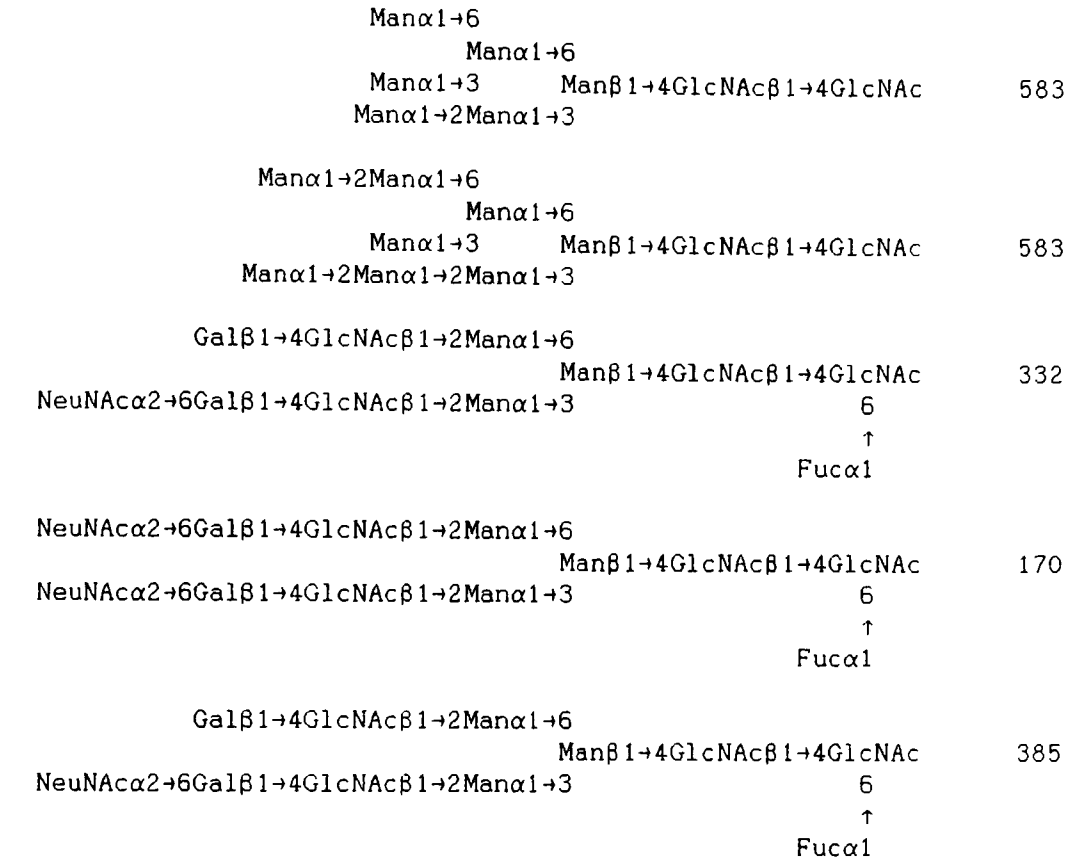
(a) Immunoglobulin E: The oligomannose structure illustrated is in fact a series from Man9 to Man5. Only the Man9 glycan is drawn here. Data from Baenziger *et al.* (1974) and Rearick *et al.* (1983). Studies performed with myeloma proteins. The sites of the sequons are not known.

Structure	Number of chains
± NeuNAcα2→6Galβ1→4GlcNAcβ1→2Manα1→6 Manβ1→4GlcNAcβ1→4GlcNAc NeuNAcα2→6Galβ1→4GlcNAcβ1→2Manα1→3 6 ↑ Fucα1	2
NeuNAcα2→6Galβ1→4GlcNAcβ1→2Manα1→6 Manβ1→4GlcNAcβ1→4GlcNAc Galβ1→4GlcNAcβ1→2Manα1→3 6 ↑ Fucα1	1
Manα1→2Manα1→6 Manα1→6 Manα1→2Manα1→3 Manβ1→4GlcNAcβ1→4GlcNAc Manα1→2Manα1→2Manα1→3 (Man9 to Man5)	1

(b) Immunoglobulin M: The studies were performed with myeloma proteins. The oligomannose structures were determined by Chapman and Kornfeld (1979a, b). Small amounts of Man 9 to Man 7 were also found. Cohen and Ballou (1980) detected additional isomers on a different IgM myeloma by NMR. Baenziger and Kornfeld (1974b) reported the complex structures, and the cores were confirmed by Tarentino *et al.* (1975). In addition Cahour *et al.* (1984) reported the presence of disialyl fucosylated triantennary glycans (with both disubstituted mannose isomers present, not shown).

Structure	Site
Manα1→6 Manα1→6 Manα1→2Manα1→3 Manβ1→4GlcNAcβ1→4GlcNAc Manα1→3	402
Manα1→6 Manα1→6 Manα1→3 Manβ1→4GlcNAcβ1→4GlcNAc Manα1→3	402

Figure 5.3 continued



(c) Immunoglobulin D (NIG 65): Positions of sialic acid residues are not known. Data taken from Ishihara *et al.* (1983).

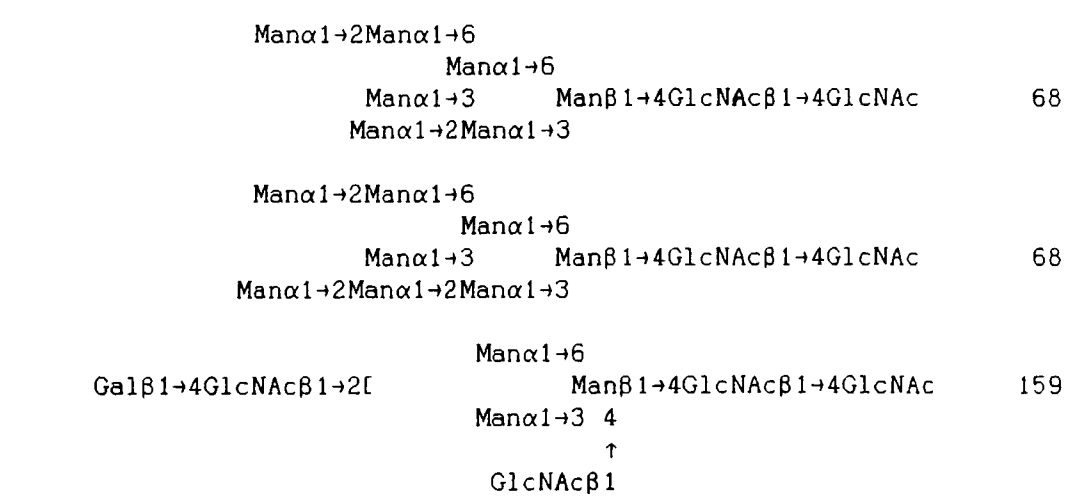
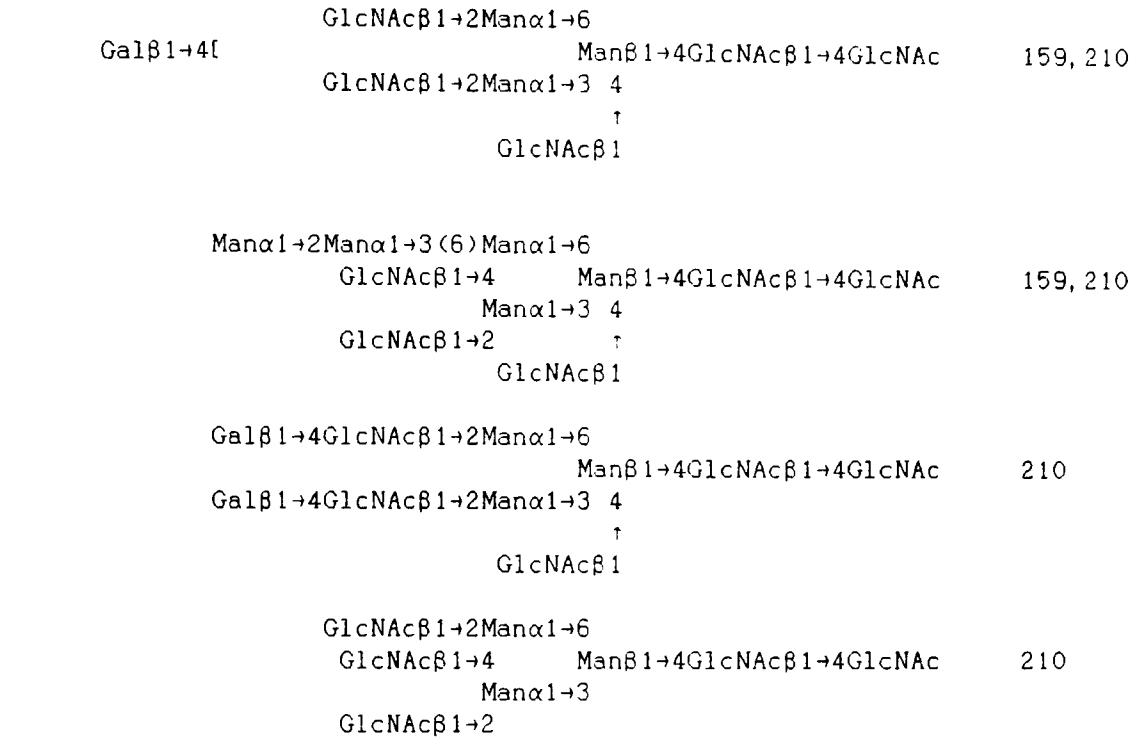
Structure	Site
	68 68 159, 210

Figure 5.3 continued



(d) Immunoglobulin D (WAH): Data from Mellis and Baenziger (1983).

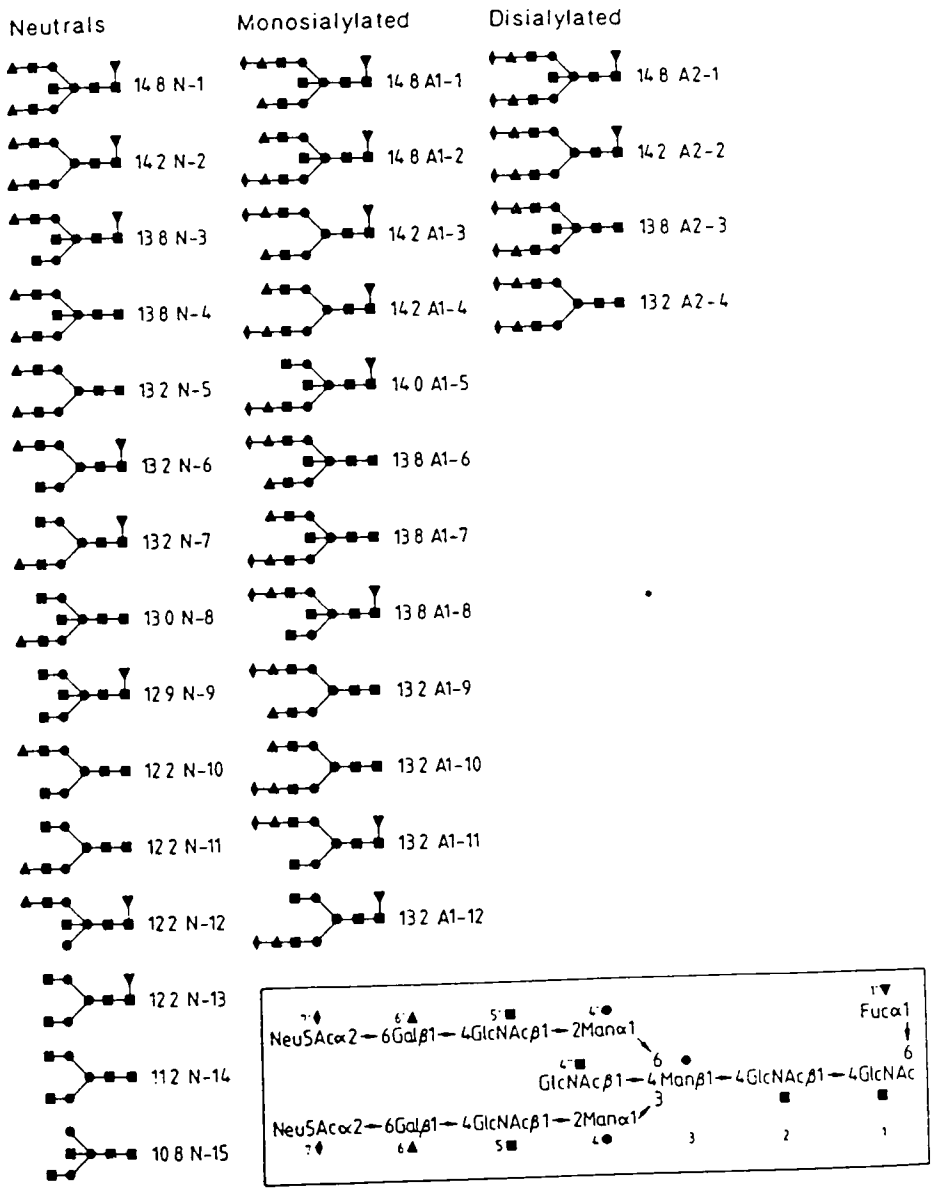
Structure	Molar % (at site)	Site
Man9 to Man 5	78	354
Glucosylated Man 9-7	22	354
Galβ1→4GlcNAcβ1→2Manα1→6 Manβ1→4GlcNAcβ1→4GlcNAc Galβ1→4GlcNAcβ1→2Manα1→3	36	496
Incomplete biantennary	27	496
Bisected biantennary	16	496
Bisected incomplete biantennary	21	496

Figure 5.3 continued

±50% NeuNAcα2→6Galβ1→4GlcNAcβ1→2Manα1→6
Manβ1→4GlcNAcβ1→4GlcNAc
Galβ1→4GlcNAcβ1→2Manα1→3 6
↑
±40%Fucα1

	29	445
Incomplete	44	445
Bisected	7	445
Bisected incomplete	21	44

(e) Immunoglobulin G: The asparagine linked glycans of IgG. The figure is taken from Parekh *et al.* (1985).



whilst fortuitous sites are generated in the V domain by recombination and somatic mutation. This latter, as well as alterations to carbohydrate biosynthesis associated with transformation (see chapter one) makes the analysis of monoclonal immunoglobulins an inaccurate route for the estimation of polyclonal Ig glycosylation.

Figures 5.3 and 5.4 summarise what is currently known about the structures of the oligosaccharides of human serum immunoglobulins.

5.2.5 *Glycosylation of Serum IgA*

The first study of the glycans of human IgA1 was performed by Dawson and Clamp (1968) who reported the carbohydrate composition of glycopeptides derived from an IgA1 myeoloma protein, and noted that the glycopeptides exhibited considerable heterogeneity in their sugar components. They documented the existence of two different carbohydrate protein linkages (N- and O-linked), but were unable to propose any structures that are consistent with contempory views of glycan structure.

Baenziger and Kornfeld studied the N and O-glycans from an IgA1 myeloma. Glycopeptide fragments were analysed by methylation and composition analysis and enzyme digestion. The structures that they proposed for the glycans are shown in figure 5.4. The carbohydrate composition of IgA is distinct for the two subclasses (Tomana *et al.* 1976). IgA2 has greater overall carbohydrate content than IgA1, with more fucose, mannose and glucosamine. IgA1 also contains galactosamine. No difference was observed in the levels of galactose and sialic acid between the subclasses (Tomana *et al.* 1976). As shown in figure 5.2, IgA2 has four conserved N-glycosylation sites per heavy chain whereas IgA1 has two, hence

Structure	Approximate number per heavy chain
(a) N-linked Oligosaccharides	
Galβ1→4GlcNAcβ1→2Manα1→6 Manβ1→4GlcNAcβ1→4GlcNAc NeuNAcα2→6Galβ1→4GlcNAcβ1→2Manα1→3 4 ↑ GlcNAcβ1	1
NeuNAcα2→6Galβ1→4GlcNAcβ1→2Manα1→6 Manβ1→4GlcNAcβ1→4GlcNAc NeuNAcα2→6Galβ1→4GlcNAcβ1→2Manα1→3 6 ↑ Fucα1	1
(b) O-linked Oligosaccharides	
GalNAc	1
Galβ1→3GalNAc	4

Figure 5.4 The glycans of human serum IgA1 as determined by Baenziger and Kornfeld (1974a, b) for a myeloma protein. See text for further discussion. The glycans of milk secretory IgA have been analysed by Pierce-Cretel *et al.* (1982, 1984), and are more complex. Some of the glycans bear outerarm fucose residues and repeat lactosamine sequences. This is typical of glycosylation of milk glycoproteins.

the greater level of carbohydrate in IgA2. The saccharide composition of IgA2 is consistent with the glycans being primarily of the complex class. It is surprising that the levels of sialic acid and galactose were found to be the same for both subclasses in view of the lower number of N-glycans being present on IgA1, and the fact that Baenziger and Kornfeld reported that the O-linked chains were not sialylated and could not account for the extra sialic acid on the IgA1 (if the N-glycan structures are essentially the same). Some variation between the composition of IgA isolated from different individuals was also observed (Tomana *et al.* 1976). Differential glycosylation has been reported for polymeric IgM compared to the monomer (Davies *et al.* 1989), and thus the molecular configuration of the isolate may affect the glycosylation. In addition, disease state or age may influence the glycosylation of IgA as is seen in IgG.

Tunicamycin treatment has been reported to decrease the level of secretion of an IgA myeloma (MOPC 315) by 85% (Hickman *et al.* 1977), and therefore correct glycosylation may be important for efficient secretion of IgA.

In milk, the glycosylation of IgA is very different from serum IgA. The oligosaccharides are much longer, containing repeat lactosamines and peripheral arm fucose residues (Pierce-Cretel *et al.* 1984). This type of glycosylation reflects that found in mucosal proteins (e.g. Finne *et al.* 1989), and is clearly under different functional and biosynthetic constraints to serum glycosylation. To the authors knowledge the glycosylation of IgA in the gut lumen has not been assessed.

5.2.6 *Alteration of IgG Glycosylation with Disease*

Alterations observed in glycan structure in myeloma IgG have been discussed (section 5.2.4). The first observation that IgG oligosaccharide structure variation could correlate with diseases other than malignancy was provided by Margolies and Boat (1983) who determined that serum IgG in cystic fibrosis patients was hypogalactosylated and hyposialylated, and speculated that this could play a role in immune complex formation.

Parekh *et al.* (1985) demonstrated a severe deficiency in the galactosylation of serum IgG in patients with rheumatoid arthritis when compared to normal individuals. The number of N-glycans lacking galactose (Go) increased from 25% in normal serum IgG to 50% in RA. There was no significant alteration in the levels of sialylation or in the core structures (i.e. fucose and bisecting GlcNAc residues), and no novel structures were encountered. It was proposed that this defect could underlie the observation that most RF activity is directed against the Cγ2 domain of the Fc, where the glycans are attached. The defect in galactosylation is highly specific, with similar alterations only being observed in juvenile RA, Crohn's disease, tuberculosis and individuals with both systemic lupus erythematosis and Sjörgren's syndrome (Parekh *et al.* 1989b). The level of galactosylation has been shown to correlate with disease activity so that the galactose level returns to the normal range for patients with inactive disease, or in remission (Parekh *et al.* 1988b). The galactosylation of the N-linked glycans of IgG from normal individuals changes as a parallel function of age. This may reflect the immunological competence of the individual (Parekh *et al.* 1988c, and see figure 5.5). The majority of the sialic acid is present on the Fab oligosaccharides, with a

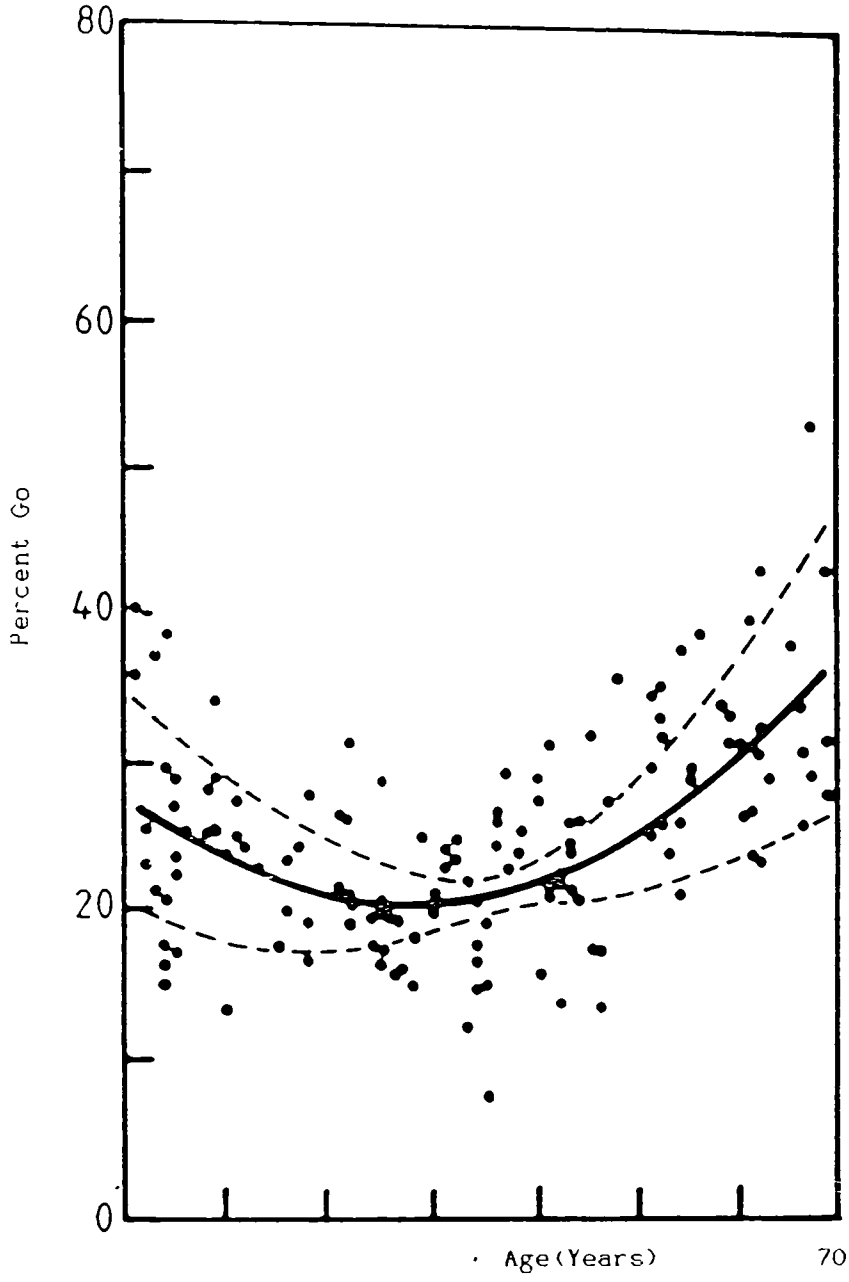


Figure 5.5 The variation of the relative incidence of N-linked oligosaccharides devoid of terminal galactose for human serum IgG with age. The solid line depicts the regression curve for the data (n=111) and the dotted lines the 95% confdidence limit of the regression analysis. The figure is taken from Parekh *et al.* (1988c).

total absence of disialylated glycans from the Fc (Rademacher *et al.* 1988b).

The carbohydrate composition of IgG in complexes isolated from the serum of RA patients invariably shows an increase in the level of Fab associated sialic acid as well as decreased galactose in the Fc glycans (Rademacher *et al.* 1988b). Removal of Fab associated sialic acid or carbohydrate prevents the formation of IgG aggregates (Middaugh and Litman 1987, Hymes and Mullinax 1979). It is therefore possible that the formation of complexes in RA serum is due to both Fab glycosylation and Fc hypogalactosylation. The alteration of the Fc surface topology by loss of a terminal galactose could allow aggregation by binding of an Fab oligosaccharide in a lectin-like fashion to a newly exposed pocket in the Fc, or an autoantibody may recognise carbohydrate or protein determinants that were previously masked (Rademacher *et al.* 1988b).

The exposure of a terminal GlcNAc residue could contribute to pathogenesis by eliciting an autoantibody response or interacting with various cell surface receptors (Rademacher *et al.* 1988b). Cross reaction with adjuvant bacterial components (peptidoglycans) containing GlcNAc could explain the arthritogenic activity of these compounds. A mouse monoclonal recognising agalactosyl human IgG Fc was produced by immunisation with group A *Streptococcus* peptidoglycan (Rook *et al.* 1988). Anti-GlcNAc antibodies directed against the Fc of IgG may stimulate an oligoclonal B cell population that produces IgG with a low galactose level. Possible mechanisms by which this may be mediated are illustrated in figure 5.6.

Based on reactivity against a panel of monoclonal antibodies, rabbits subjected to hyperimmunisation show an alteration in the antigenicity of IgG Fc, which is centralised at the Cγ2 domain. Partial deglycosylation of

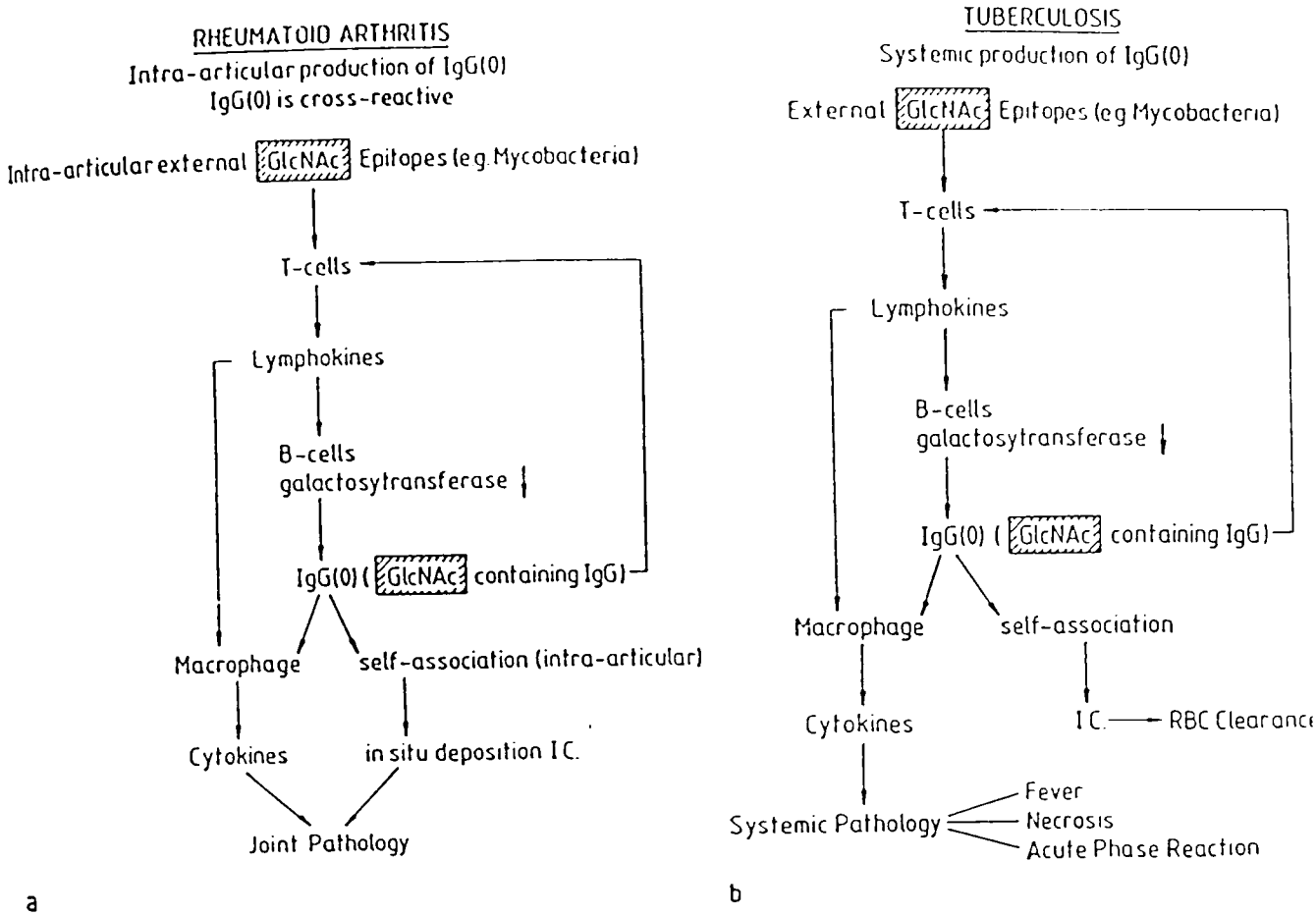


Figure 5.6 Proposed mechanisms for the production of IgG with agalactosyl Fc N-linked oligosaccharides in response to an external GlcNAc epitope in rheumatoid arthritis (a) and tuberculosis (b). It has been proposed that a reduction in B cell β-galactosyltransferase could be a response to T cell cytokines. The anatomical site of the production of such hypogalactosylated IgG may account for the differences in pathology of these two diseases. IL-1, interleukin 1, TNF, tumour necrosis factor, I.C. immune complexes. The figure is taken from Rademacher *et al.* (1988b).

pre-immune rabbit IgG led to equivalent antigenicity as the hyper-immune IgG (Murray and Brown 1988). Therefore, repeated immunisation can affect glycosylation of immunoglobulins. Based on the observation that the hyperimmune IgG could be specifically recognised by monoclonal antibodies, the glycosylation alterations can be detected by the immune system.

Investigation of the primary lesion that gives rise to hypogalactosyl-IgG in rheumatoid arthritics has shown that in the rheumatoid B cell there is a decrease in the activity of β-galactosyltransferase, and it has been proposed that the altered activity is specific for the γ-chain (Axford *et al.* 1987). Glycan alterations in certain diseases may therefore be the result of changes in the activity of substrate-specific transferases, and in this particular system it is possible that IgG is the only protein affected. Alternatively, other immunoglobulins may be altered as well as IgG. At present it is not known if the decrease in B cell immunoglobulin galactosyltransferase is restricted to a subset of lymphocytes or if all are affected. There is a precedent for the occurrence of highly specific glycosyltransferases, as these enzymes have been documented in other systems, e.g. N-acetylgalactosamine transferase in the pituitary will only add GalNAc to LH and FSH glycans (Smith and Baenziger 1988) and not to the same saccharide on a different protein.

Therefore it is important to determine if the glycans of IgA show a comparable alteration in structure to those of IgG in RA. In addition, the structures of the glycans themselves need to be established from normal human serum IgA, as this is not known. This knowledge will be important for future investigations of disease-associated glycosylation alterations of

immunoglobulins, as well as defining more accurately the structure of IgA as a glycoprotein.

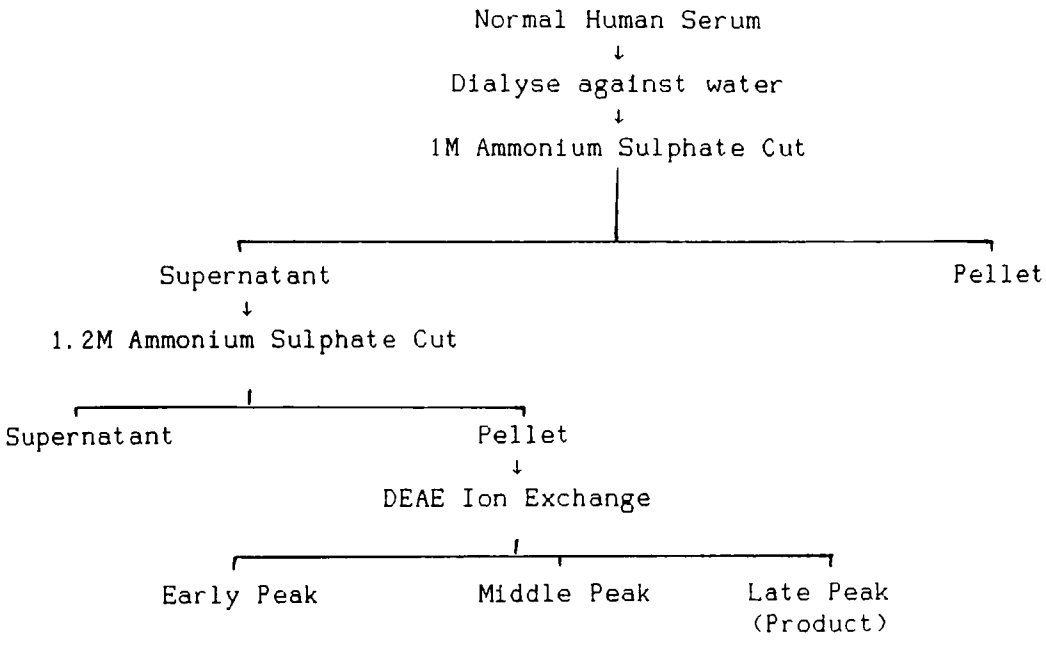
5.3 *Methods and Materials*

The methods that were used for the isolation of human serum IgA are given below. Each method illustrates a distinct approach, and does not involve procedures that would be expected to damage the glycans. Pooled normal human serum (NHS) obtained from the blood bank at the John Radcliff Hospital, Oxford, was used throughout this study.

5.3.1 *Ion Exchange Chromatography Isolation of IgA*

The method of Dawson and Clamp (1968) was followed (scheme 5.1). Twenty millilitres of NHS was dialysed against distilled water at 4°C. The retentate was then aliquoted into polycarbonate centrifuge tubes and an equal volume of 2M ammonium sulphate solution added. After 18 hours at 4°C the material was centrifuged at 10 000g for 30 minutes at 4°C. Pellets were discarded and solid ammonium sulphate added to the supernatant to a final concentration of 1.2 Molar. The material was incubated for a further 4 hours at 4°C and centrifuged as before. The supernatants were discarded and the pellets taken up in 3mls of 20mM Tris-HCl, pH7.4, and exhaustively dialysed against this buffer at 4°C. The retentate was then applied to a column of DEAE-Sephacel (Sigma) (1.5cm x 20cm) equilibrated in 20mM Tris-HCl, pH7.4, with a flow rate of 15ml/hour. The column was then washed with 90ml of Tris buffer and then a linear gradient was applied to 0.5M NaCl in 20mM Tris-HCl, pH7.4 over 150ml. The gradient was applied using an FPLC

Human Serum IgA Purification by Ion Exchange



Scheme 5.1 Purification of IgA from human serum by the method of Dawson and Clamp (1968), section 5.3.1. The product was taken for analysis (see results).

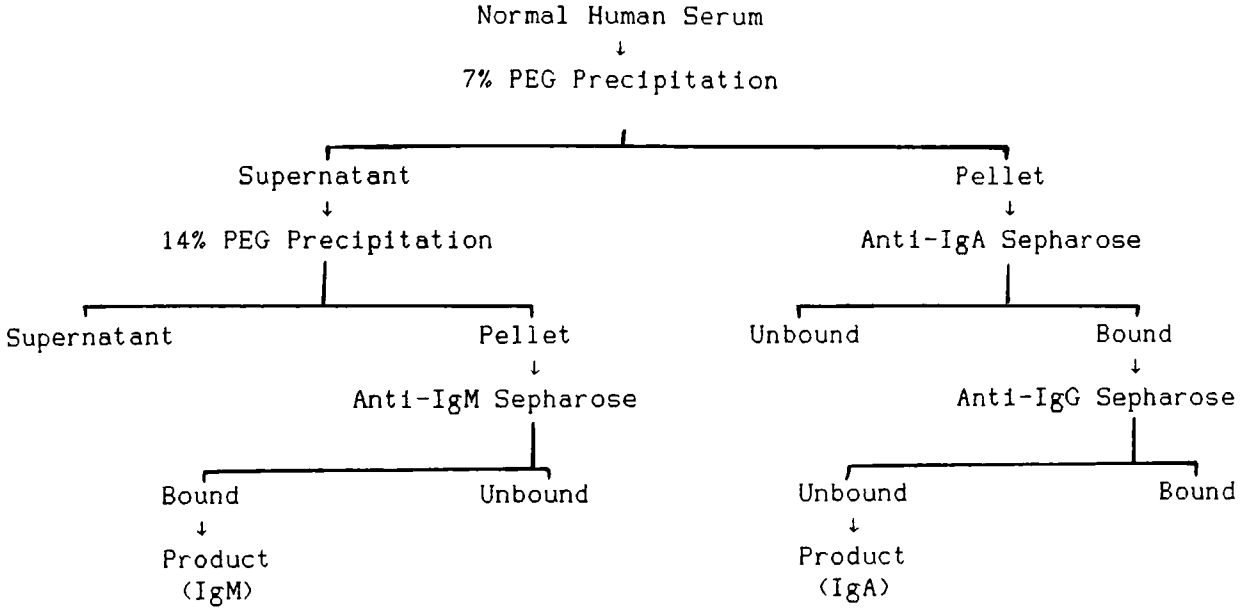
system and the eluate monitored at 280nm. Three peaks were observed to elute from the DEAE column, and these were pooled and concentrated in an amicon ultrafiltration cell using a YM10 membrane (figure 5.7). IgA was detected by double diffusion only in the last peak to have eluted from the column. The late peak pool was dialysed aginst water at 4°C, lyophilised and taken up in 1ml of water and injected onto a TSK SWG3000 HPLC column running in 100mM potassium phosphate buffer pH7.2 (KPi). The eluate was monitored at 280nm, and 0.5 ml fractions were collected. The material indicated by the bar (figure 5.7) was analysed.

5.3.2 *Isolation of IgA by PEG Precipitation and Anti-Immunoglobulin Antibody Affinity Chromatography*

The method of Cripps *et al.* (1983) was followed (scheme 5.2). Ten milliliters of NHS was mixed in a 50ml sterile plastic centrifuge tube with an equal volume of 14% polyethylene glycol (PEG) 6000 (Sigma) in 10mM Tris-HCl pH 8.0, and placed on a tube roller for 18 hours at 4°C. The precipitate was harvested by centrifugation at 1600g in a TJ-10 bench centrifuge (Beckman) (7% pellet). The supernatant was removed and mixed with an equal volume of 21% PEG 6000 in 10mM Tris-HCl pH 8.0, and rolled for a further 18 hours at 4°C and the precipitate harvested as before (14% pellet). Both precipitates were resuspended seperately in 10mM Tris-HCl pH 8.0 and centrifuged at 10 000g for 30 minutes at 4°C. Both solutions were then filtered by passage through 0.45µm PTFE filters (Millipore).

Immobilised antibody columns were made by coupling immunopurified anti-heavy chain antibodies to CNBr-activated sepharose as described in appendix one. An anti-IgA column, 2ml bed volume, was made with 4mg of goat anti-

Human Serum IgA Purification by Polyethylene Glycol Precipitation



Scheme 5.2 Human Serum IgA and IgM isolation by the method of Cripps *et al.* (1983), section 5.3.2. The products were taken for analysis (see results).

human α-chain (Calbiochem). An anti-IgM column, 2ml bed volume, was made with 4mg goat anti-human μ-chain (Calbiochem). Goat anti-human γ-chain $F(ab')_2$ antibodies (5mg, Sigma) were immobilised onto 1ml of CNBr sepharose.

All columns were equilibrated in 10mM Tris-HCl pH 8.0 containing 150mM NaCl (TBS) and bound proteins were eluted with 3M NaSCN in TBS. The flow rate was maintained at 5ml/hour and 1ml fractions were collected. Elution was monitored by measuring the A_{280nm} of the fractions in a spectrophotometer. The 7% pellet was applied to the anti IgA column, bound proteins eluted, dialysed into TBS, and then applied to the anti-IgG column. The unbound protein was collected. The 14% pellet was applied to the anti IgM column and bound proteins eluted and dialysed into TBS.

Both of the final pools from the columns were concentrated by ultrafiltration (Centricon 30, Amicon) and then subjected to HPLC gel filtration on a Zorbax 250XL preparative column (Hichrom), flowing at 0.5ml per minute with 100mM KPi as the mobile phase. The eluant was monitored at 280nm.

The Zorbax 250XL eluate from the 7% pellet preparation after affinity chromatography was analysed for IgA by double diffusion and the IgA containing fractions were pooled and analysed by IEP and HPLC gel filtration on a tandem column consisting of one Zorbax GF250 and one Zorbax GF450 in series, eluted with 50mM KPi at 1ml/min. The material eluting at the void of the Zorbax 250XL column from the 14% pellet following affinity chromatography was pooled and analysed by IEP.

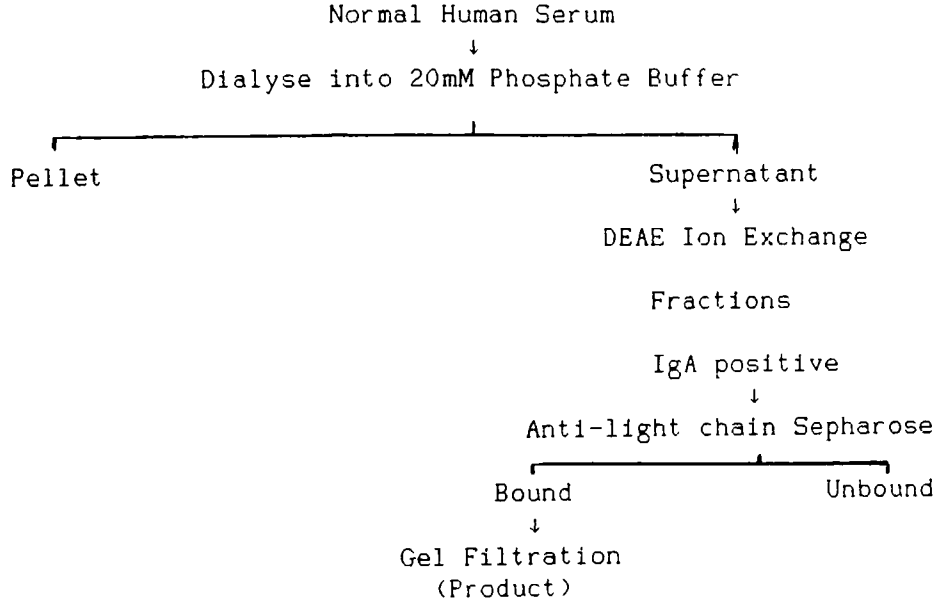
5.3.3 *Ion Exchange and Affinity Chromatography*

The Method of Litman and Good (1972) was used (scheme 5.3). Ten millilitres of NHS were dialysed against 20mM KPi at pH6.3, 4°C. The retentate was then spun in an MSE clinical centrifuge at 3000rpm to remove the precipitate and the supernatant was applied to a DEAE Sephacel column (1.5cm x 20cm) equilibrated in 20mM KPi, pH6.3. The column was pumped at 12ml/hour and 4ml fractions were collected. The eluate was monitored by measuring the A_{280nm} of the fractions. When the absorbance had dropped to 0.05 AU the column was eluted with 50mM NaCl in the 20mM KPi and fractions collected as before. IgA was detected in the eluate by double diffusion, and those fractions that contained IgA were pooled and dialysed into TBS. This material was not processed further (see results).

5.3.4 *Jacalin-Affinity Chromatography*

The Method of Roque-Barreira and Campos-Neto (1985) was followed with minor modifications (scheme 5.4). Ten millilitres of NHS were spun in 1ml aliquots in a microfuge for 5 minutes and the supernatants were removed and pooled. This material was then applied to a 0.6cm.x 20cm column of jacalin agarose (Pierce) equilibrated in PBS. The column was pumped at 20ml/hour and 2 ml fractions were collected. The eluate was monitored by taking A_{280nm} measurements. When the absorbance had dropped to less than 0.05 AU the bound proteins were eluted with 0.8M D-galactose (Sigma) in PBS. The elution profile was subsequently monitored with the BioRad protein assay reagent (figure 5.9). IgA was detected in the eluate by double diffusion. The bound protein was pooled and concentrated to 3ml in an Amicon

Human Serum IgA Isolation By Ion Exchange and Light Chain Affinity Chromatography



Scheme 5.3 Purification of human serum IgA by the method of Litman and Good (1972), section 5.3.3. The purificatio nwas not taken beyond the ion exchange step in this study.

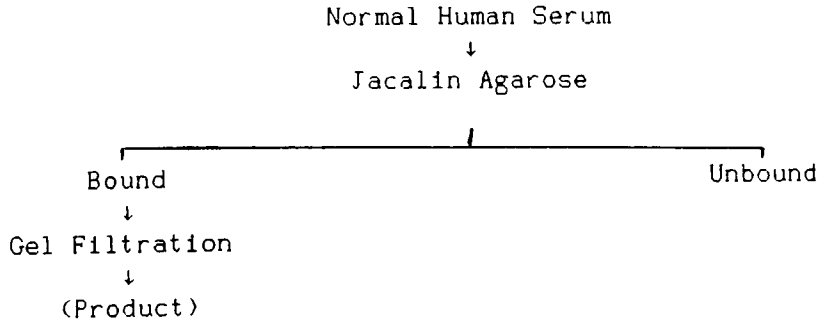
ultrafiltration cell against a YM10 membrane. The material was injected onto a TSK SWG 3000 column and eluted as described above (section 5.3.3). The fractions were analysed by double diffusion and the IgA containing fractions were pooled and analysed by IEP and gel filtration (figure 5.9) on tandem Zorbax 250/450 columns as described above (section 5.3.2).

5.3.5 *Hydrophobic Interaction Chromatography*

The Method of Dolgaust and Plaut (1976) was followed (scheme 5.5). Twenty five millilitres of NHS were mixed with 16.7 ml of 2.5M ammonium sulphate in 100mM Tris-acetate buffer pH8.0 and the solution incubated at 4°C for 18 hours. The solution was clarified by centrifugation at 10 000g for 30 minutes at 4°C and the supernatant was loaded onto a 0.6cm x 20cm column of phenylalanine-agarose (Sigma) equilibrated in 1M ammonium sulphate in 40mM Tris acetate at pH 7.6 at a flow rate of 20ml/hour. The column was then eluted sequentially with 100ml of each of the following buffers; 0.8M ammonium sulphate in 32mM Tris acetate at pH 7.6, 0.6M ammonium sulphate in 24mM Tris-acetate at pH 7.6, 0.4M ammonium sulphate in 16mM Tris-acetate at pH 7.6, 250mM NaCl in 50mM Tris-acetate pH8.0 and finally 250mM Tris-HCl pH10.5. 10ml fractions were collected and IgA detected by double diffusion. It was confirmed that IgA eluted with the second elution buffer. In addition the breakthrough contained a significant quantity of IgA, which was due to overload, and small amounts of IgA were detected in the later eluting peaks, by double diffusion.

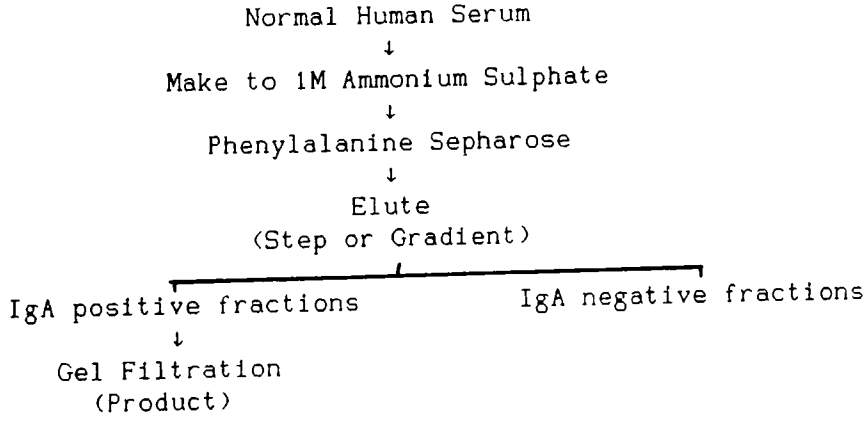
Because of the poor resolution obtained with this method a modification was made. The serum was prepared and loaded onto the phenylalanine column as before, but a linear gradient was applied from the starting buffer down

Isolation of IgA From Normal Human Serum by Jacalin Affinity Chromatography



Scheme 5.4 Purification of human serum IgA by the method of Roque-Barriera and Campos-Neto (1985), section 5.3.4. The product from the gel filtration was taken for analysis (see results).

Isolation of IgA From Normal Human Serum by Hydrophobic Interaction Chromatography



Scheme 5.5 Purification of human serum IgA by the method of Dolgaust and Plaut (1976).

to 0.4M ammonium sulphate in 16mM Tris acetate at pH 7.6 over 6 column volumes. This modification resulted in the elution of the IgA in the middle of the gradient as a broad peak. The earlier fractions from this peak contained more IgA as the precipitin reaction was more intense in the double diffusion assay. The material at the leading edge of the peak was pooled seperately and concentrated down to 3ml in an Amicon ultrafiltration cell with a YM10 membrane, and injected onto a TSK SWG 3000 HPLC column as described above (section 5.3.2). IgA was again detected by double diffusion and the positive fractions pooled and analysed by IEP.

5.3.6 *Purification of Immunoglobulins A and G From Individual Human Serum Samples Used for Oligosaccharide Studies*

The method of Roque-Barreira and Campos-Neto (1985) was used subsequently for the isolation of IgA1 for carbohydrate analysis. Serum for the control set was obtained from volunteers in the Glycobiology Unit, and for the RA group from clinically definite RA patients from Dr D. Isenberg at the Middlesex Hospital, London. IgG was isolated from aliquots of the same individual sera used to isolate the IgA as described in appendix one (A1.2.2.2). In one case, to ensure that the presence of undetected contaminants did not affect the oligosaccharide analysis, IgA was purified from pooled serum by the Roque-Barreira and Campos-Neto method, and a second aliquot of the serum was fractionated before jacalin agarose chromatography on Affigel blue DEAE (BioRad) as follows. Ten millilitres of serum were dialysed against 20mM sodium phosphate buffer pH 7.0 (NPi). The retentate was then applied to a column of Affigel blue DEAE (0.9cm x 25cm) at a flow rate of 12ml/hour and 1ml fractions were collected. The column

was washed with 20ml of NPi and the bound proteins eluted with a linear gradient of 20mM to 300mM NPi over 100mls. The IgA containing fractions were detected by double diffusion, pooled and dialysed into PBS before jacalin agarose and gel filtration chromatography. This procedure will remove most of the IgG and serum albumin from the IgA and substantially enrich the IgA over other serum proteins.

For analysis of the sialic acid linkage of serum IgA N-linked oligosaccharides, and isolation of IgA O-linked glycans, pooled normal human serum was used as a starting material, and processed in an identical manner as described (section 5.3.4).

For the analysis of subclass glycosylation, 250µg of affinity purified human serum IgA1 and IgA2 were purchased from Calbiochem. The proteins were analysed by SDS-PAGE as described (appendix one section A1.2.2.3.1).

5.3.7 *Isolation of N- and O-linked Glycans*

N-linked oligosaccharides were isolated from the immunoglobulins, purified and radiolabelled as described in appendix one (A1.2.1.1.1 and following sections). O-linked glycans were liberated by alkali treatment and purified as described (section A1.2.1.1.6).

5.3.8 *Carbohydrate Analysis*

5.3.8.1 Assessment of Galactosylation of Complex Glycans

The degree of substitution of the N-linked oligosaccharides with terminal β-galactosyl residues was determined by incubating the purified

oligosaccharides with a mixture of exoglycosidases as described in appendix one (section A1.2.1.3.6), and analysis on Bio-Gel P4 columns. The IgG glycans needed no further purification, but the IgA1 oligosaccharides were observed to be contaminated with low molecular weight material that had been labelled by the reduction procedure, which was removed by treatment with neuraminidase and passage through Bio-Gel P4 prior to incubation with the exoglycosidase mix. This contaminant was probably derived from the O-linked chains, which are released by hydrazinolysis under the conditions used.

5.3.8.2 Determination of the Levels of Sialylation of The Glycans From IgA

The total oligosaccharides of IgA1 were analysed by anion exchange chromatography on Mono Q (appendix one (section A1.2.1.2.3)). The relative incidence of the neutral and charged species was assessed by quantitation of the radioactivity in the eluate. IgA1 oligosaccharides of the RA and control groups fractionated by anion exchange were pooled separately and digested with neuraminidase before analysis on Bio-Gel P4 columns. Some low molecular weight contamination was observed, and the raw data obtained from the analysis of the individual IgA glycans were corrected to allow for this (table 5.5). The nature of the anionic moiety of the N-linked glycans of human serum IgA1 was identified by incubation of the glycans with *Arthrobacter ureafaciens* neuraminidase followed by analysis of the products by HVE in the presence of pyridine-acetate.

The linkage position of the sialic acids were determined as follows. Radiolabelled human serum IgA1 glycans from pooled normal serum were isolated and separated from low molecular weight material by gel filtration

on a Bio-Gel P4 (200-400 mesh) column (1.5 x 30cm) with 50mM pyridine acetate pH 5.5 as the eluant. The column was run at room temperature with a flow rate of 1ml/min. The column was calibrated with a set of standard glycans. Material eluting with a hydrodynamic volume greater than 10.5 glucose units was pooled seperately from the remaining material. These two fractions were then analysed by one-dimensional NMR at 600MHz (appendix one, section A1.2.1.3.5).

5.3.8.3 Elucidation of the Primary Structure of the N-linked Glycans from IgA1

N-linked glycans from the control individuals IgA1s were pooled and analysed by one dimensional NMR following neuraminidase treatment to determine the anomericity of the monosaccharide substituents and to provide some information on the primary sequences of the glycan mixture. The oligosaccharides were then fractionated on aqueous Bio-Gel P4 columns and individual peaks were pooled. Aliquots were taken from the P4 pools for methylation analysis by GCMS. Further aliquots were subjected to sequential digestion by exoglycosidases.

5.3.8.4 Elucidation of the Primary Sequence of the O-linked Glycans

The O-linked oligosaccharides were analysed and fractionated by Mono Q, and the neutral and charged glycan fractions pooled seperately. Each fraction was treated with neuraminidase and analysed by Bio-Gel P4 gel filtration chromatography. An aliquot of the total glycans was desialylated and analysed by Bio-Gel P4 chromatography. The anomericity of the bonds in

the oligosaccharides was determined by neuraminidase and bovine β-galactosidase digestion, and the identity of the saccharide eluting at 2.5gu on the Bio-Gel P4 chromatograph was determined by borate paper electrophoresis. The linkage positions of the monosaccharides were determined by methylation analysis before and after neuraminidase treatment. In the case of the oligosaccharide that eluted from Mono Q with a retention time equivalent to a disialylated glycan selective ion monitoring (section A1.2.1.3.1) was performed to identify the reducing terminal residue, which was recovered at a very low level. The sialic acid present was identified by GCMS of the per-TMS glycoside (appendix one, section A1.2.1.3.4).

5.4 *Results*

5.4.1 *Protein Purification: Selection of a Suitable Method for IgA Isolation*

In table 5.1 the results of the study of the methods for isolation of serum IgA are summarised. Yields have been calculated only where a product was obtained in sufficient purity to make this possible.

The ammonium sulphate anion exchange method (Dawson and Clamp 1968) was not suitable. Levels of protein eluting from the DEAE column were low. Both the 1.0M ammonium sulphate pellet and 1.2M ammonium sulphate supernatant contained IgA (scheme 5.1). The breakthrough peak (peak E) from the DEAE column was found to be pure IgG by IEP, whilst the second peak (peak M) was predominantly γ-globulin of the A and G class. The high molecular weight material on the TSK profile of peak L was also γ-globulin, probably IgM

Table 5.1

SUMMARY OF IgA PURIFICATION METHODS

Method	Yield (g/ml serum)	Purity	Technical Considerations	Comments
Ion Exchange (Section 5.3.1)	60µg	Not pure	Easy	Suitable for large scale purifications.
PEG Precipitation (Section 5.3.2)	14µg IgA 100µg IgM	Pure Pure	Easy	Suitable for large scale purification. Yields both IgA and IgM.
Ion Exchange and Affinity Chromatography (Section 5.3.3)	NK	NK	Easy	Method unsuitable, and not pursued (see text).
HIC (Section 5.3.4)	NK	Not pure	Difficult	Product not obtained pure. Very difficult for routine use.
Jacalin Affinity (Section 5.3.5)	1.5mg	Pure	Easy	High yield and purity. Suitable for small scale purifications

Table 5.1 Yields are given where a product was obtained in sufficient purity for this to be calculated. Purity was assessed by gel filtration and immunoelectrophoresis, except in the case of the jacalin method where SDS-PAGE was also performed. Details of the protocols can be found in the indicated text sections. Comments are made on the method in light of the aim of the investigation, which was to be able to routinely isolate IgA from small serum samples.
Yield is given as mass per ml of serum. 'Pure' indicates that the product was judged to be >95% pure. NK indicates not known. HIC indicates hydrophobic interaction chromatography. 'Easy' denotes that a method was found to be straightfoward and did not require complex manipulations that could not be reproduced reliably when used for a large number of purifications.

from the molecular weight (figure 5.7). The final product (indicated by a bar in the gel filtration profile in figure 5.7) was predominantly IgA, but was contaminated with at least one other protein as identified by IEP. This method was rejected on the grounds that the product was obtained at 60µg/ml of serum, an unacceptibly low yield. This method was devised for isolation of a myeloma IgA present at high concentration in serum, and this may explain the disappointing yield that was obtained here.

The PEG precipitation and affinity chromatography method (Cripps *et al.* 1973) was easy to perform and yielded pure immunoglobulins (figure 5.8). However the yield was 100µg and 14µg for IgM and IgA respectively per millilitre of serum. Cripps *et al.* used 100ml of serum, whereas only 10ml was used in this study. IgA and IgM were detected in the unbound eluates from the respective antibody columns, so that the capacity of these columns was clearly too low to isolate all the immunoglobulin in a single passage. This method was rejected on the following grounds; (1) the possibility of needing to repass the material through the antibody columns several times, and (2) risk of cross contamination from one sample to another by incomplete desorption of bound immunoglobulin from the columns.

The ion exchange and light chain affinity chromatography method (Litman and Good 1972) was rejected at an early point of assessment. A large amount of protein was eluted from the ion-exchange column with the salt step, indicating that several proteins co-eluted. Previous work by the author of this thesis showed that ion exchange of human serum under the conditions used here would not completely resolve IgA from IgG and IgM, and therefore affinity chromatography on immobilised anti-light chain antibodies would not accomplish complete purification of IgA. The method was rejected for the following reasons; (1) additional steps would have to be incorperated

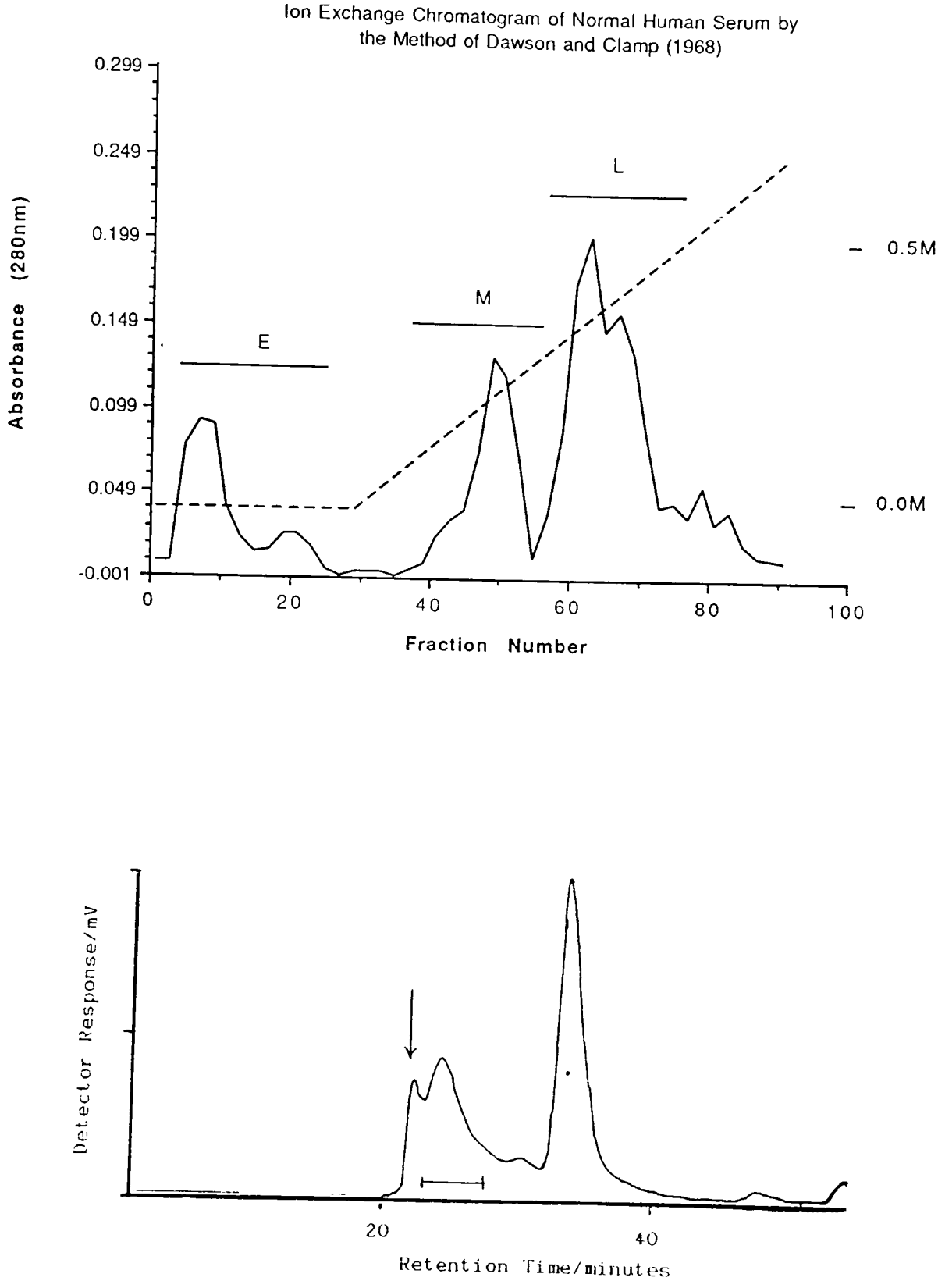


Figure 5.7 (a) DEAE ion exchange chromatogram of material produced from human serum by ammonium sulphate fractionation by the method of Dawson and Clamp (1968). The concentration of NaCl is indicated by the dashed line. A_{280nm} measurements were made on the individual fractions.
(b) Gel filtration A_{280nm} profile of the late peak (L) from the ion exchange column. IgA elutes at about 25 minutes under these conditions.

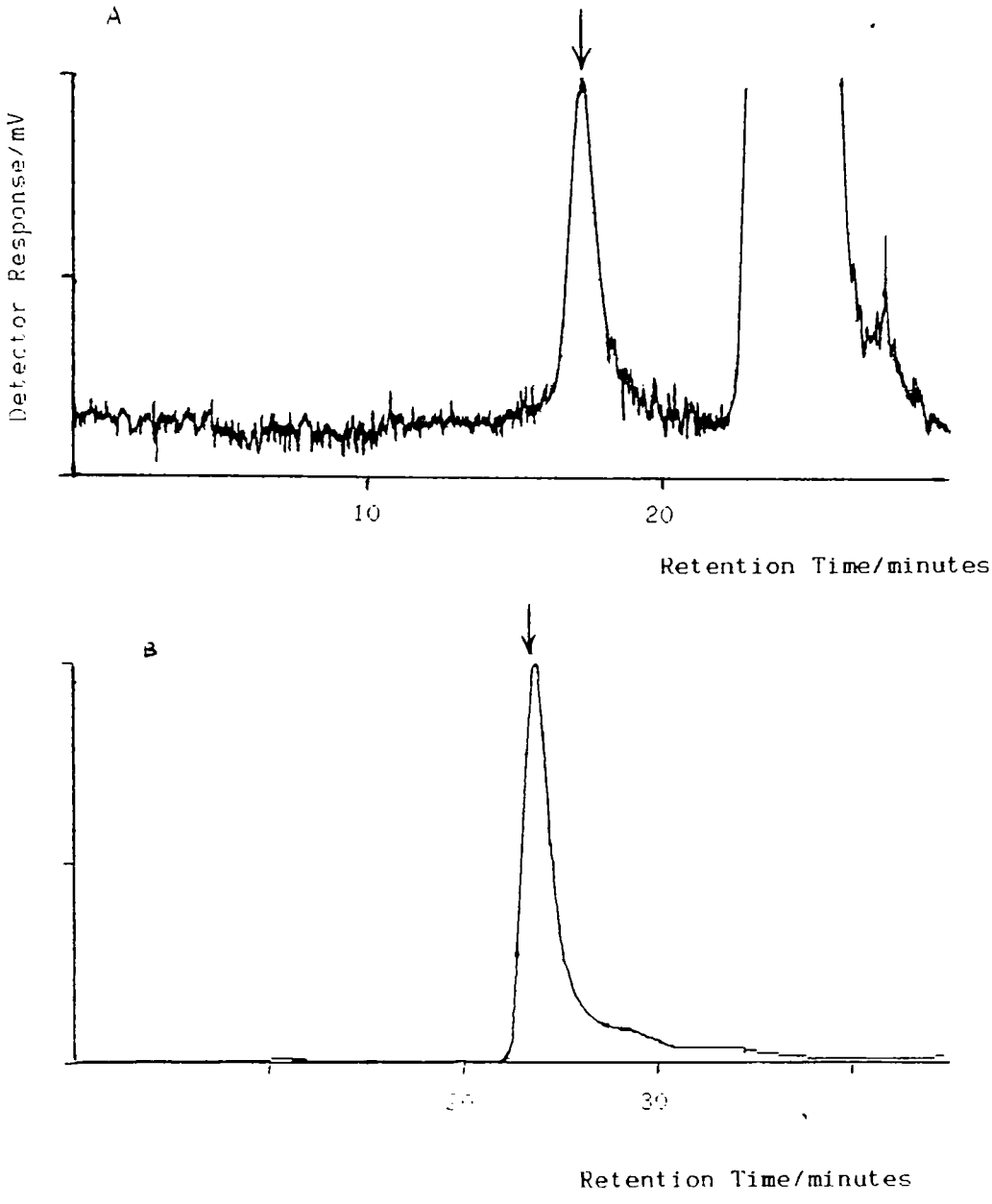


Figure 5.8 Gel filtration A_{280} profiles of IgA (a) and IgM (b) isolated by the method of Cripps *et al.* (1983). The arrows indicate the elution positions of IgA and IgM respectively, which were taken for analysis. The large peak at Vt in panel A is due to salt from the sample.

into the method, making it cumbersome for repeated purifications, (2) because several steps would be needed, the yield would be low, and (3) the same criticisms as detailed above for antibody columns apply to the affinity chromatography step used in this method.

Using hydrophobic interaction chromatography (HIC) (Dolgaust and Plaut 1976) was found to be technically difficult. The capacity of the phenylalanine-Sepharose column was much lower than expected, so that substantial breakthrough occured. The bound IgA did elute as predicted with the second buffer, but some IgA was detectable in other fractions eluted later from the column. Therefore the published method was rejected for the following reasons; (1) the step elution of the phenylalanine agarose column was complex, and not easy to accurately reproduce, (2) a large phenylalanine column would have to be used on account of the low calacity of the gel, which is undesirable for small samples, and (3) IgA would be eluted by several of the elution buffers. As the authors reported that IgA eluted with two of their buffers, but only discussed the complete purification of IgA from one of them, they did not address this problem. In addition the ammonium sulphate pellet was IgA positive by IEP.

An attempt to simplify this method by gradient elution across the region where IgA elutes was also unsuccessful because a large number of proteins were co-eluted, which were not resolved. Gel filtration of the proteins eluted at the leading edge of this peak on the TSK SWG 3000 column showed that there were several proteins present, and IgG had co-eluted with IgA as detected by IEP. Therefore this methoc was also rejected.

A substantially different approach was utilised by the jacalin-agarose protocol (Roque-Barreira and Campos-Neto 1985). The jackfruit lectin (which binds to the T antigen) was used to isolate O-glycosylated glycoproteins.

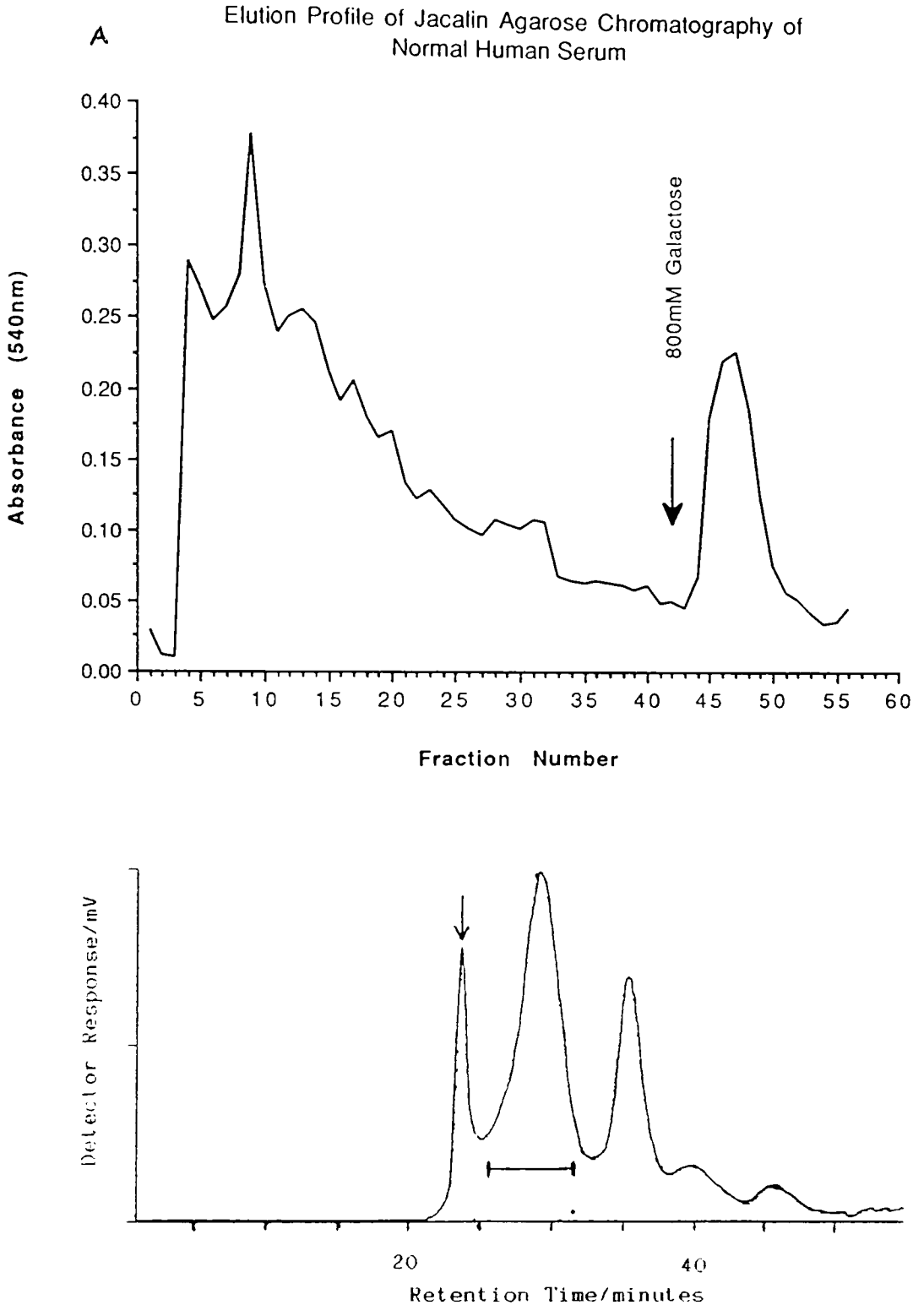
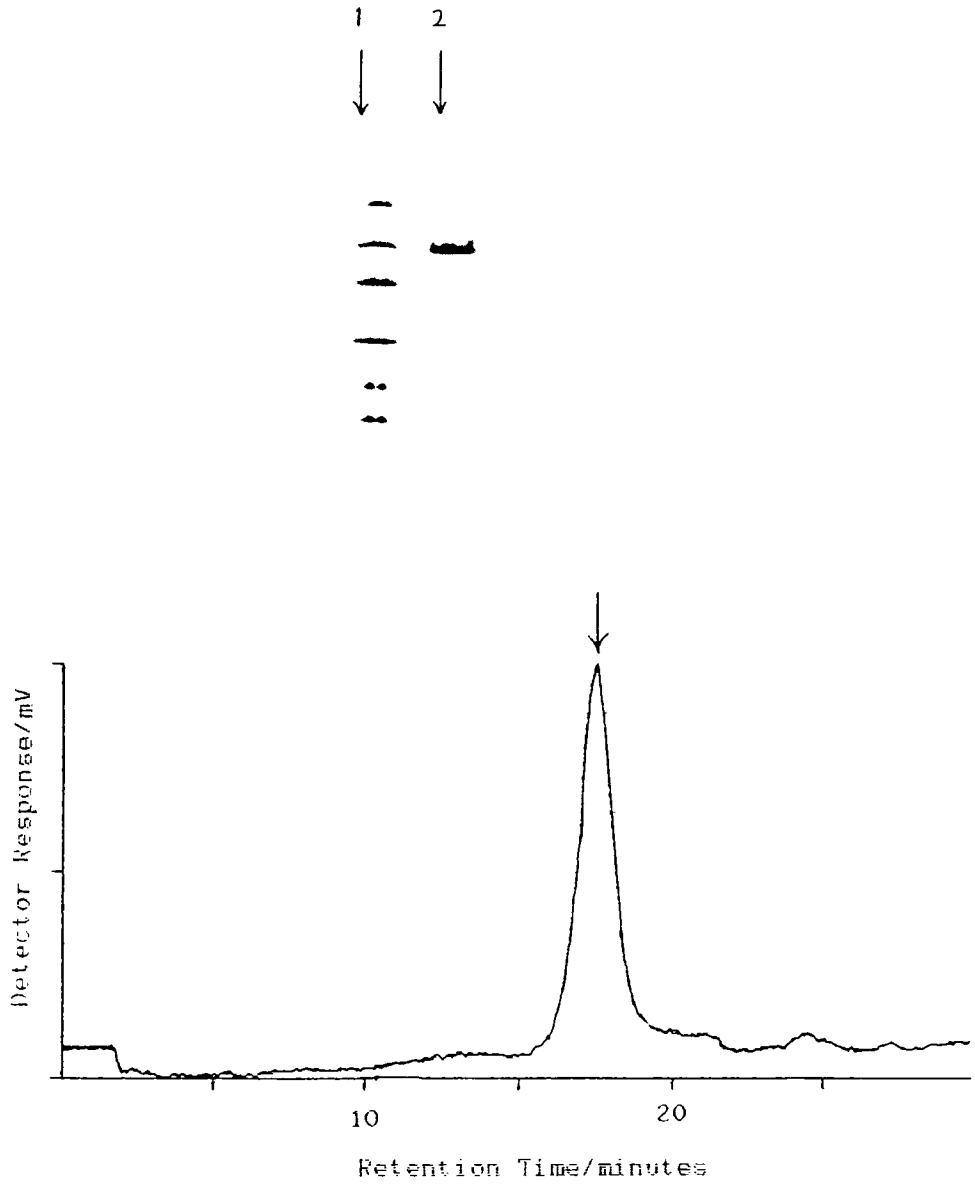


Figure 5.9 (a) Upper panel; Elution profile for human serum on immobilised jacalin. The point of application of 800mM galactose is indicated. Lower panel; A_{280} profile of gel filtration on a TSK SWG 3000 column of proteins bound by immobilized jacalin and eluted with 800mM galactose. The bar indicates the IgA immunoreactive fractions, and the arrow the void volume of the column.
(b) Upper panel; Coumassie stained SDS polyacrylamide gel of purified IgA run under reducing conditions (lanes 1 and 3), and molecular weight markers (lanes 2 and 4). Markers included rabbit phosphorylase (97.4kdal), bovine serum albumin (66.2kdal), hen ovalbumin (42.7kdal), bovine carbonic anhydrase (31.0kdal), soybean trypsin inhibitor (21.5kdal), and hen egg lysozyme (14.4kdal). The additional high molecular weight band observed in the IgA1 lane is due to under reduction, and was also observed in commercial IgA samples analysed by the same method. Lower panel; Gel filtration of purified human serum IgA1 on a tandem Zorbax 250-450 analytical column. The arrow indicates the elution position of IgA1.


1
2
Detector Response/mV
10
20
Retention Time/minutes

This method worked very well, and was easy to perform. The gel filtration step was replaced by HPLC, which increased the speed and yield of the procedure. In some cases a single repassage of unbound protein over the jacalin column was necessary to remove all IgA. The lectin has recently been shown to bind IgA1 and not IgA2 (Biewenga *et al.* 1989). Jacalin clearly does not bind to N-glycosylated glycoproteins as IgG, transferrin and a number of other N-glycosylated proteins are not retained by the column. The IgA made by this method was > 96% pure by IEP, HPLC gel filtration and reducing SDS-PAGE. A yield of 1.5mg/ml of serum was obtained.

Therefore, this last method was chosen as the most suitable for the following reasons; (1) the product could be obtained in greater yield than the other methods, and at high purity, (2) the method was easy to perform on a routine basis, and was much faster than any other method, (3) bound material could be easily desorbed completely from the lectin, therefore preventing cross contamination by retention of material on the affinity matrix. The need for a high yield was particularly important in this study as only small volumes of serum from individuals, i.e. ca. 3-5ml, were availible. The caveat of using a lectin, which could result in the purification of specific glycoforms was later dispelled (see discussion).

5.4.2 *Purification of Immunoglobulin A1 for Comparative Oligosaccharide Analysis*

A panel of IgA1s were purified from a set of individual normal and RA sera using the method of Roque-Barreira and Campos-Neto (1985) as described above (section 5.3.4). The IgA peak eluted from the TSK gel fitration

column with a retention time indicating a molecular weight of 220kdal. This is clearly smaller than a dimer, which would elute at over 300kdal. Re-chromatography of the purified IgA on a tanden Zorbax column resulted in the elution of the glycoprotein with a molecular weight of approximately 160kdal. Therefore, in consideration of the subclass specificity of the jacalin lectin used in the initial fractionation, and the molecular weight of the final product, it is concluded that the purification protocol yields monomeric IgA1.

5.4.3 *Analysis of the Galactosylation of the N-linked Glycans on IgA1 and IgG in Normal Humans and RA Patients*

The 'asialo' profiles of human serum IgA1 oligosaccharides are shown in figure 5.10. Most of the saccharides elute at 13.5gu or larger, which indicates that the glycans are predominantly complex type. The envelope at 13.5gu to 15.2gu accounts for about 80% of the N-glycans, and elution in this region of the chromatogram suggests a biantennary structure. The oligosaccharides with a hydrodynamic volume greater than 15.2gu would be expected to be tri- and tetraantennary complex oligosaccharides. The 'asialo' Bio-Gel P4 chromatograms did not show any variation between the normal and RA IgA1 N-glycans (figure 5.10). The low molecular weight material, eluting at 3.5 and 2.5gu was probably derived from O-linked glycans, and was not analysed further.

Treatment of the whole pool of oligosaccharides with the mixture of exoglycosidases (section A1.2.1.3.6) altered the gel filtration profile. An example is shown in figure 5.11. Peaks resulting from the digestion of complex glycans bearing zero to three terminal β-galactosyl residues can be

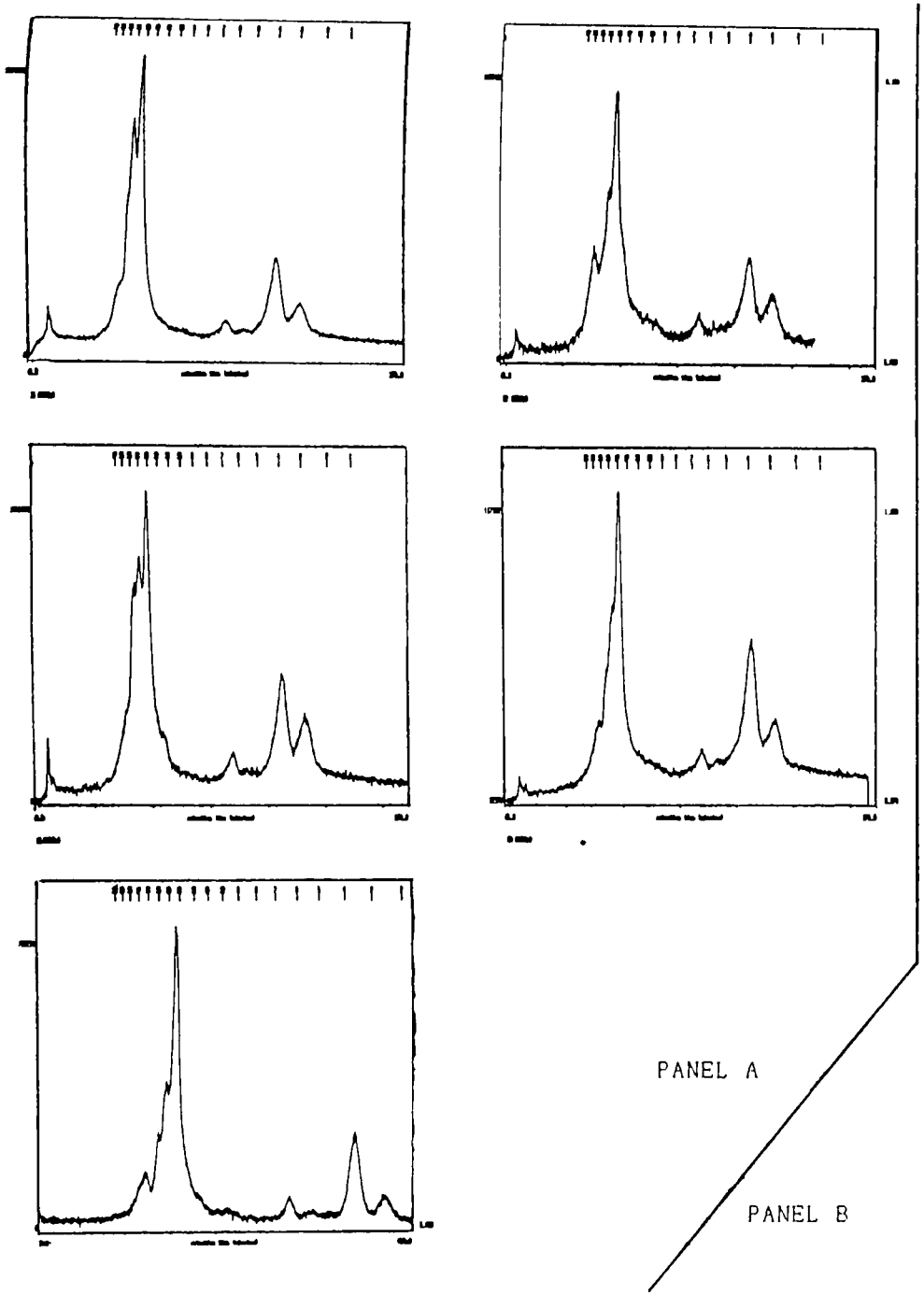
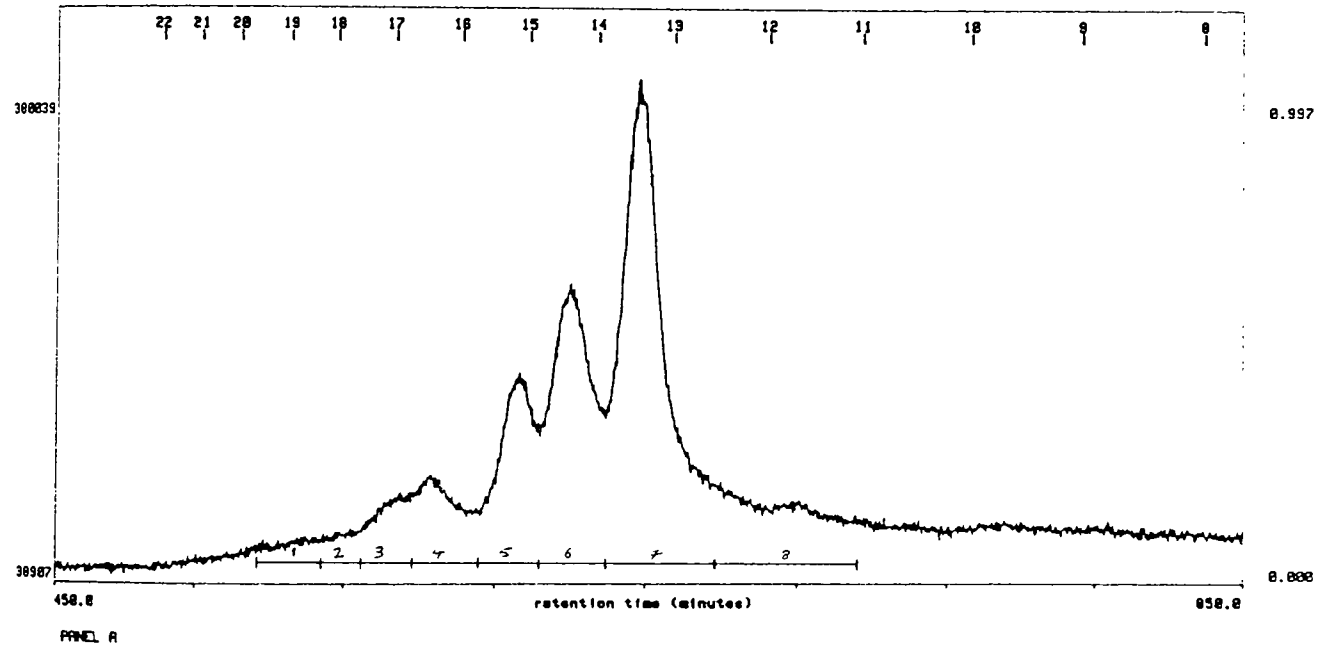
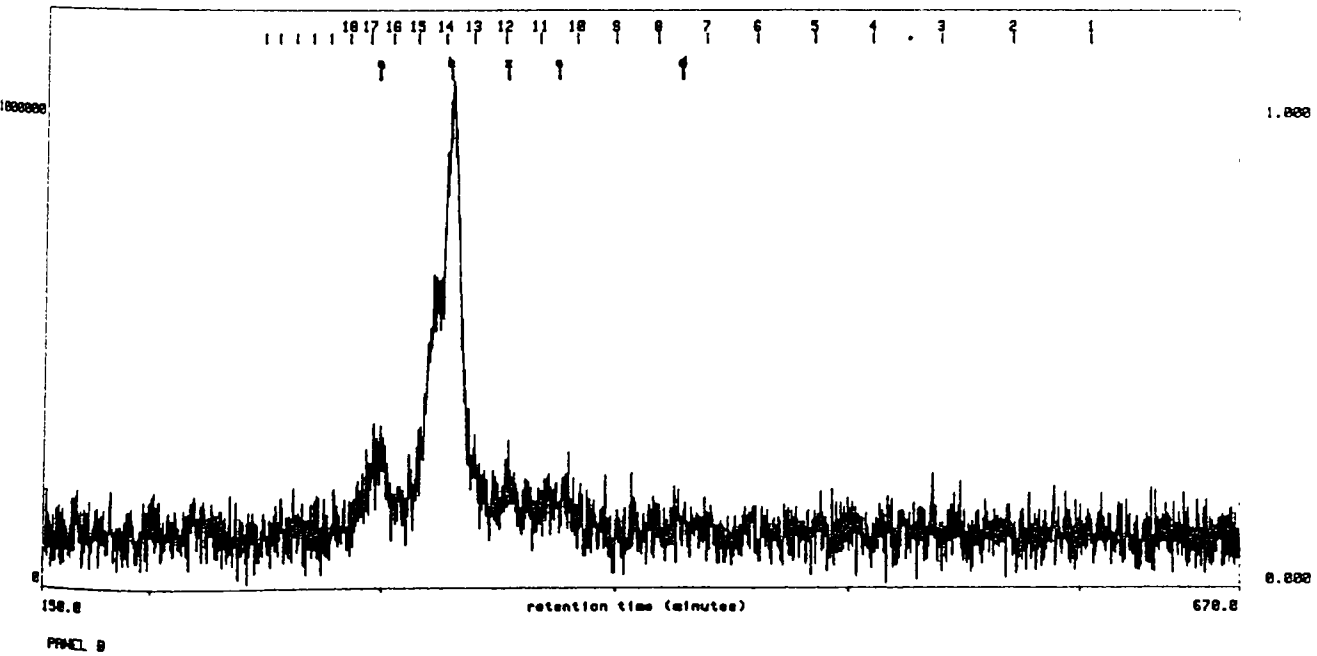


Figure 5.10 Bio-Gel P4 chromatograms of IgA1 N-linked oligosaccharides following desialylation. The numbers across the top of the profiles indicate the elution positions of isomaltose oligomers co-chromatographed with the sample. Panel A; control set (MC not shown), panel B; RA set.

Figure 5.11 (a) Bio-Gel P4 chromatogram of IgA1 oligosaccharides following desialylation. The pools used in the oligosaccharide analysis are indicated beneath the profile (1-8). Isomaltose oligomer internal standards are indicated as in figure 5.10.
(b) Bio-Gel P4 chromatogram of IgA N-linked oligosaccharides following digestion with the outerarm galactose exoglycosidase mix (see text). The positions of the resultant glycans bearing three to zero terminal galactose residues are indicated (e, d, b, a respectively), and the 11.8gu peak is indicated by c. Isomaltose oligomer elution positions are indicated as in (a).





seen at 7.5, 10.5, 13.5 and 16.5 gu (see table 5.2). The identity of these peaks was confirmed by digestion with jack bean β-galactosidase and α-mannosidase, which resulted in the movement of the peaks by the expected number of glucose units, i.e. three, two or one galactose residues were removed from the 16.5, 13.5 and 10.5gu glycans respectively, and the 7.5gu glycan lost two mannose residues on digestion with the mannosidase. The small peak that elutes at approximately 11.8gu was later characterised as a mixture of an oligomannose and a hybrid glycan (section 5.4.5) and would not be expected to move with the enzyme mixture. In addition the sensitivity of the glycans to jack bean β-galactosiadase determined that all galactose was in β1→4 linkage.

The prevalence of the glycans terminating with none to three β-galactosyl residues on serum IgA and IgG was determined (table 5.3). The IgG Go values are plotted against age in figures 5.12, and the IgA1 Go-G_3 values for the RA and control group are compared in figure 5.13. The incidence of Go of IgG and IgA1 from the same individual are plotted against each other in figure 5.14. At this resolution no difference could be seen between the RA and control set for the galactosylation (table 5.3). In addition no evidence was found for an age related alteration in the prevalence of galactose termini (not shown).

Comparison of the galactosylation of IgA1 glycans isolated from pooled serum by jacalin affinity chromatography and initial fractionation by ion exchange showed that the additional purification step did not affect the glycans recovered from the glycoprotein (table 5.4). This confirms that the method used here resulted in a product that was of sufficient purity for oligosaccharide analysis.

Table 5.2

Structures Generated From Complex Type Oligosaccharides With The Exoglycosidase Mix

Number of Galactose Residues	Designation	Structure	Hydrodynamic Volume/gu
0	G0	M_3N_2	7.5
1	G1	$(GN)_1M_3N_2$	10.5
2	G2	$(GN)_2M_3N_2$	13.5
3	G3	$(GN)_3M_3N_2$	16.5

Abbreviations; G = galactose, N= N-acetylglucosamine and M= mannose.

Galactosylation of Serum IgA1 and IgG in the Same Individual

		Serum IgA1					Serum IgG				
Control Set (n=5)	Age/Yrs	G_0	G_1	G_2	G_3	H*	G_0	G_1	G_3	Mean IgG Go For Age	Difference
Pool		2.4	8.6	71.8	10.1	7.1	26.2	37.4	36.4		
MC	25	1.9	8.9	71.3	12.7	5.2	27.4	41.7	30.9	21.6	+5.8
JA	35	1.3	9.2	73.2	11.2	5.1	21.4	40.1	38.5	17.8	+3.6
AD	40	2.2	12.5	66.7	13.0	5.6	26.4	37.8	35.8	24.7	+1.7
GT	25	2.9	13.3	55.7	21.4	6.7	19.1	40.9	40.0	21.6	-2.5
AM	21	3.3	11.8	68.2	11.4	5.3	28.0	38.6	33.4	21.6	+6.4
Mean (±SD)	29.2(7.9)	2.3(0.8)	11.1(2.0)	67.0(6.8)	13.9(4.2)	5.6(0.7)	21.0(11.3)	39.8(1.6)	35.7(3.7)	18.9	+3.0(3.6)
Rheumatoid Set (n=5)											
PC	45	4.0	6.6	68.5	12.6	8.3	31.4	40.0	29.6	22.5	+8.9
DB	60	3.7	9.6	61.9	17.0	7.8	37.5	37.6	24.9	32.2	+5.3
MS	28	2.7	7.7	69.0	12.3	8.3	59.6	25.0	15.4	18.9	+40.7
CF	66	4.2	11.5	64.6	11.1	8.6	38.6	31.8	29.6	34.0	+4.0
RS	56	3.5	11.0	68.0	11.1	6.3	45.5	33.1	21.3	32.2	+13.3
Mean (±SD)	51.0(15.0)	3.6(0.6)	9.3(2.1)	66.4(3.1)	12.8(2.4)	7.9(0.9)	42.5(10.8)	33.5(6.0)	24.2(6.0)	28.9(6.8)	+14.4

Table 5.3 The relative incidence of glycans terminating in zero to three galactose residues are shown for IgA1 and zero to two galactose residues for IgG isolated from the same individual, and the same serum sample. Values are given as percent of total for each immunoglobulin. The age of the individual at the time of bleed is also shown, as well as the mean and standard deviation for each group. Mean and standard deviations have been calculated for the data from the individuals only. The mean age-corrected IgG Go has been calculated from the data obtained by a study of 174 normal individuals (Parekh *et al.* 1988c). The values are given for the mean and standard deviation for an age range of five years, and the difference between this value and that for the IgG glycans studied here are given. The mean Go difference for the control and RA groups is also given.

* H denotes the material that elutes at 11.8gu from Bio-Gel P4 after mixed exoglycosidase treatment. See text and figures 5.12, 5.13 and 5.14 for further presentation of this data.

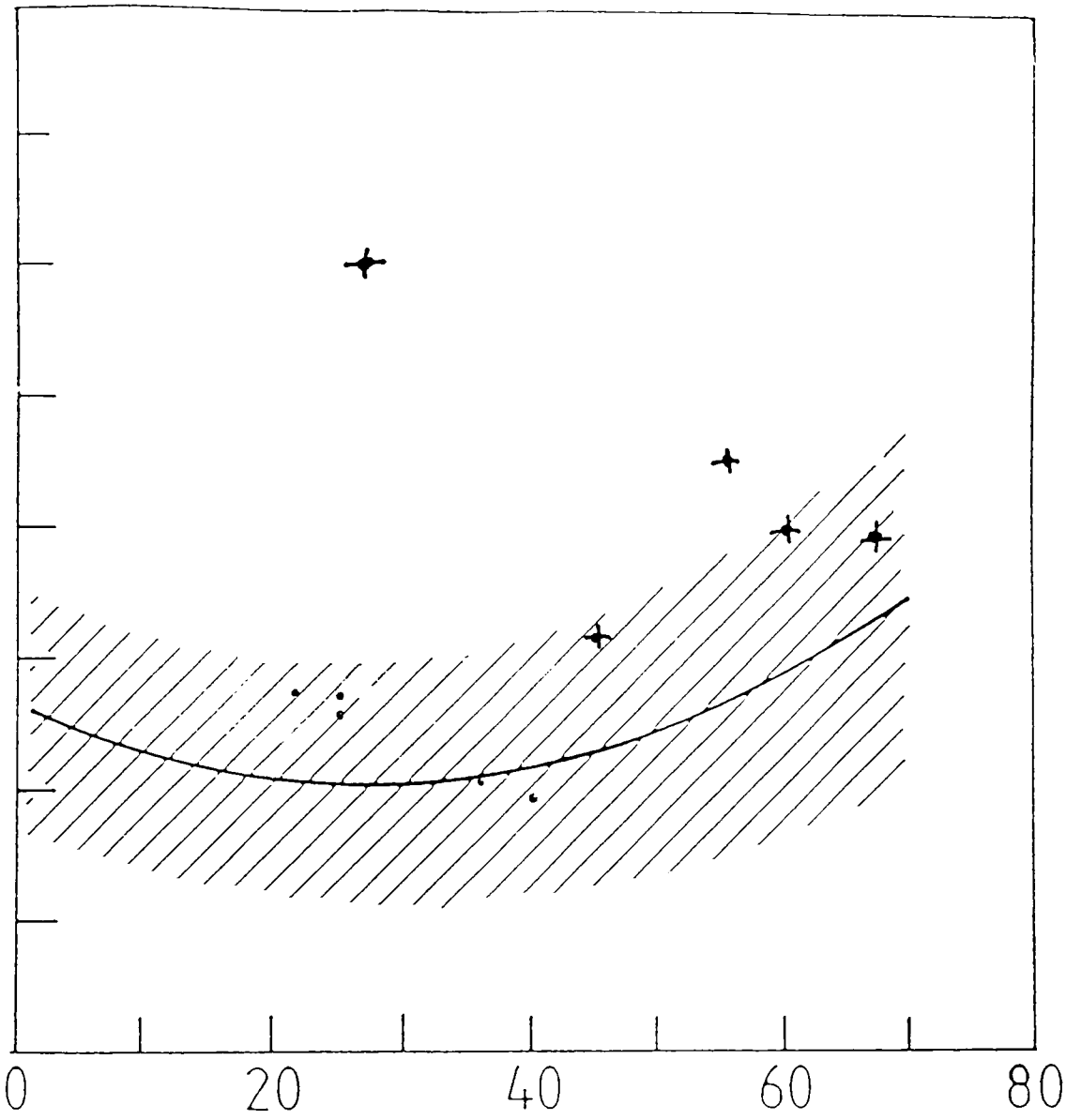


Figure 5.12 Plot of relative incidence of the N-linked oligosaccharides devoid of terminal galactose from human serum IgG against age for the individuals included in this study. The control set (n=6) are plotted as •, and the rheumatoid set (n=5) as +. Note that the control set fall within the normal area, except for one sample, and that the rheumatoid set are elevated from the controls. The solid line is the regression line for IgG from normal individuals, and the shaded area indicates the are ±2 standard deviations from the regression line (taken from Parekh *et al.* 1988a).

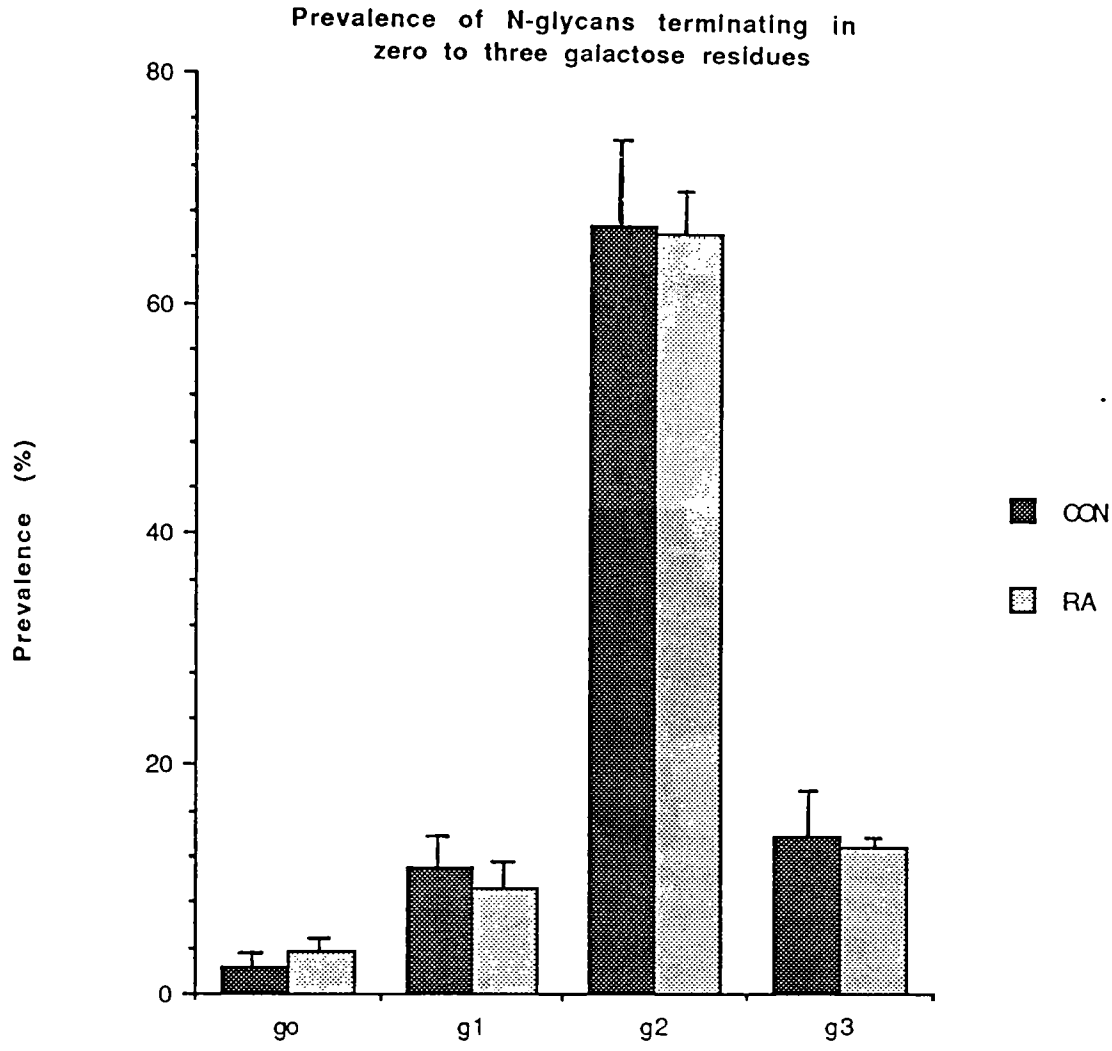


Figure 5.13 Prevalence of structures terminating in zero (go), one (g1), two (g2) and three (g3) β-galactose residues from human serum IgA1. The data from table 5.3 is presented as a histogram. It is clear from these data that there is no significant difference in the galactosylation of the glycans from the control and RA sets. Only the data from the individual RA patients (n=5) and the control group (n=5) are included in this figure.

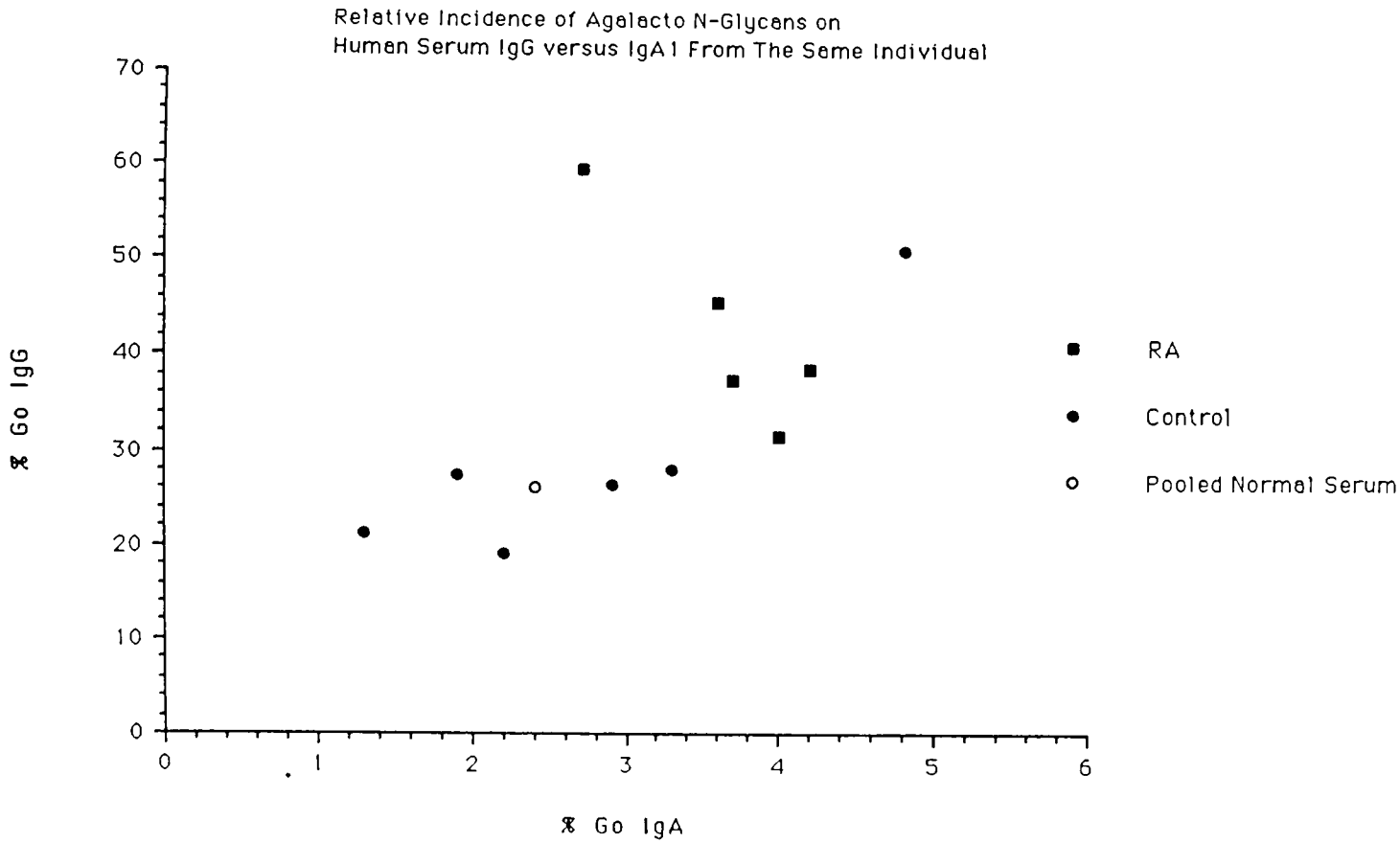


Figure 5.14 Plot of Go values for IgA1 versus IgG N-linked glycans isolated from the same individual. Note that the horizontal axis has been expanded greatly with respect to the vertical axis.

Table 5.4

Galactosylation of Oligosaccharides From IgA Purified By Two Different Routes

Method	% Galactosylation*			
	G0	G1	G2	G3
1	2.6	9.3	77.2	10.9
2	3.4	10.3	76.0	10.3
Mean	3.0	9.8	76.6	10.6

Method 1; IgA1 was purified by the method described (section 5.4.2). Method 2 was performed by initial fractionation of serum by ion exchange on Affigel-blue DEAE prior to jacalin affinity chromatography (see text). A variation of between 2-5% in the incidence of the products is encountered when the same sample is subjected to digestion with an exoglycosidase mix.
* Corrected to ignore 11.8 glucose unit saccharides.

5.4.4 *Analysis of the Sialylation of the IgA1 N-linked Oligosaccharides.*

The sole anionic moiety of the glycans was found to be sialic acid, based on complete neutralisation of the IgA1 glycans with neuraminidase. The hydrazinolysis procedure removes all N substituents, therefore this information is lost. As N-acetylneuraminic acid is the only derivative reported for human serum glycoproteins (Corfeld 1982) it is probable that this residue is the sialic acid present on serum IgA1. The anion exchange chromatograms for the IgA1 glycans are shown in figure 5.15. The incidences of the neutral and acidic N-glycans, corrected to ignore low molecular weight material present in some of the fractions (see below) are given in table 5.5, and are compared for the RA and control set in figure 5.17. It was immediately apparent that the sialylation of IgA1 is much more extensive than that observed for IgG (Parekh *et al.* 1985).

When the pooled serum IgA1 glycans, following fractionation on buffered Bio-Gel P4, were analysed by one dimensional NMR spectroscopy, it was determined that the sialic acid on the N-linked glycans was linked α2→6 (figure 5.18b). Greater than 90% of the N-acetylneuraminic acid in the high molecular weight Bio-Gel P4 fraction (greater than 10.5gu) was judged to be in α2→6 linkage by the intensities of resonances in the spectral region at δ of 1.719 and 1.800 for the axial H3 protons and at 2.668 and 2.756 for the equatorial protons (Vliegendthart *et al.* 1983). This is in agreement with the report of Baenziger and Kornfeld (1974). A small amount of α2→3 linked sialic acid was detected in this material. Desialylation of both the fractions from the buffered Bio-Gel P4 separation with neuraminidase, and analysis on Bio-Gel P4 confirmed that [illegible] amount of low molecular weight material was present in the N-linked glycan fraction (>10.5gu).

Panel A

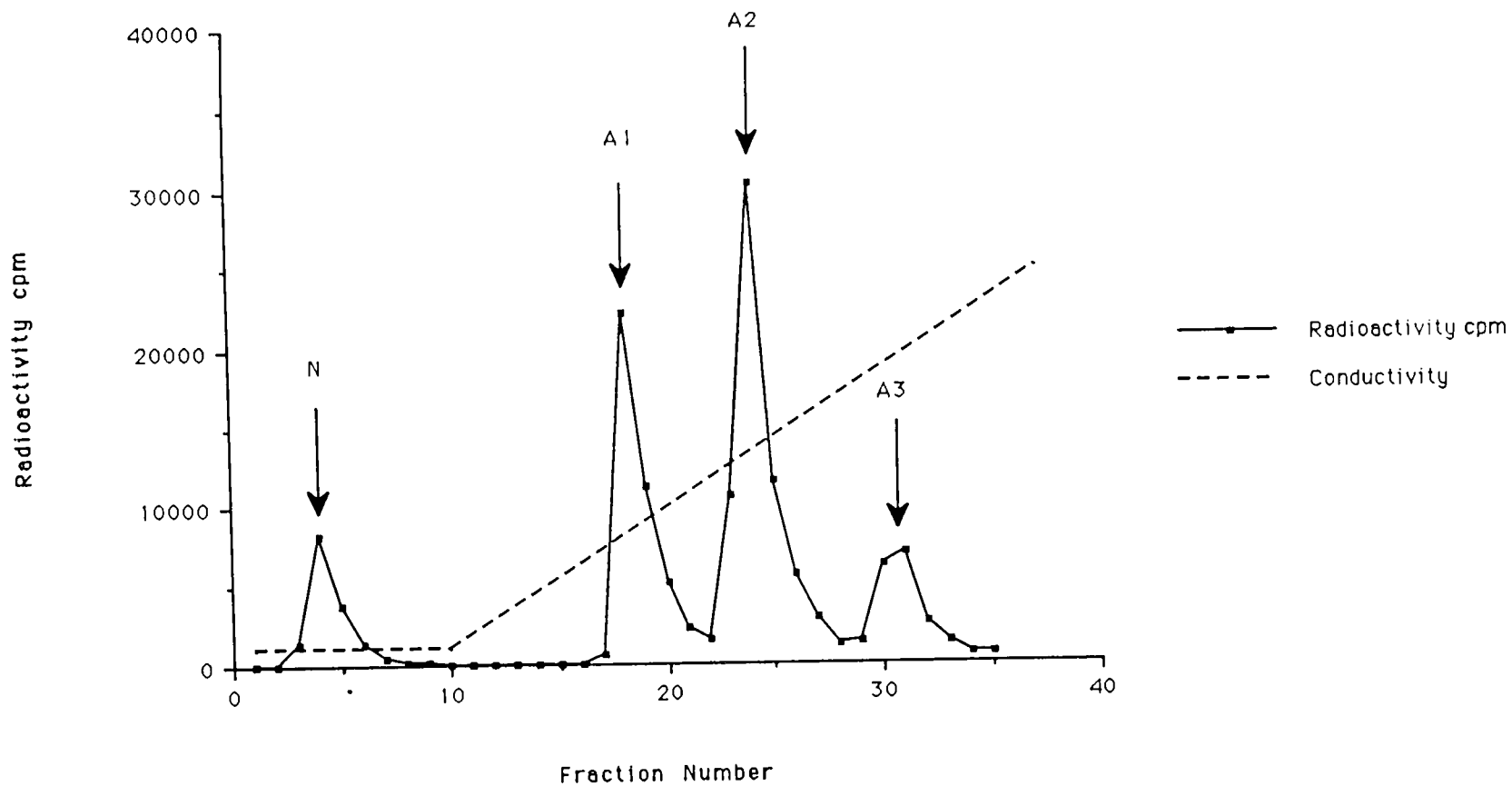
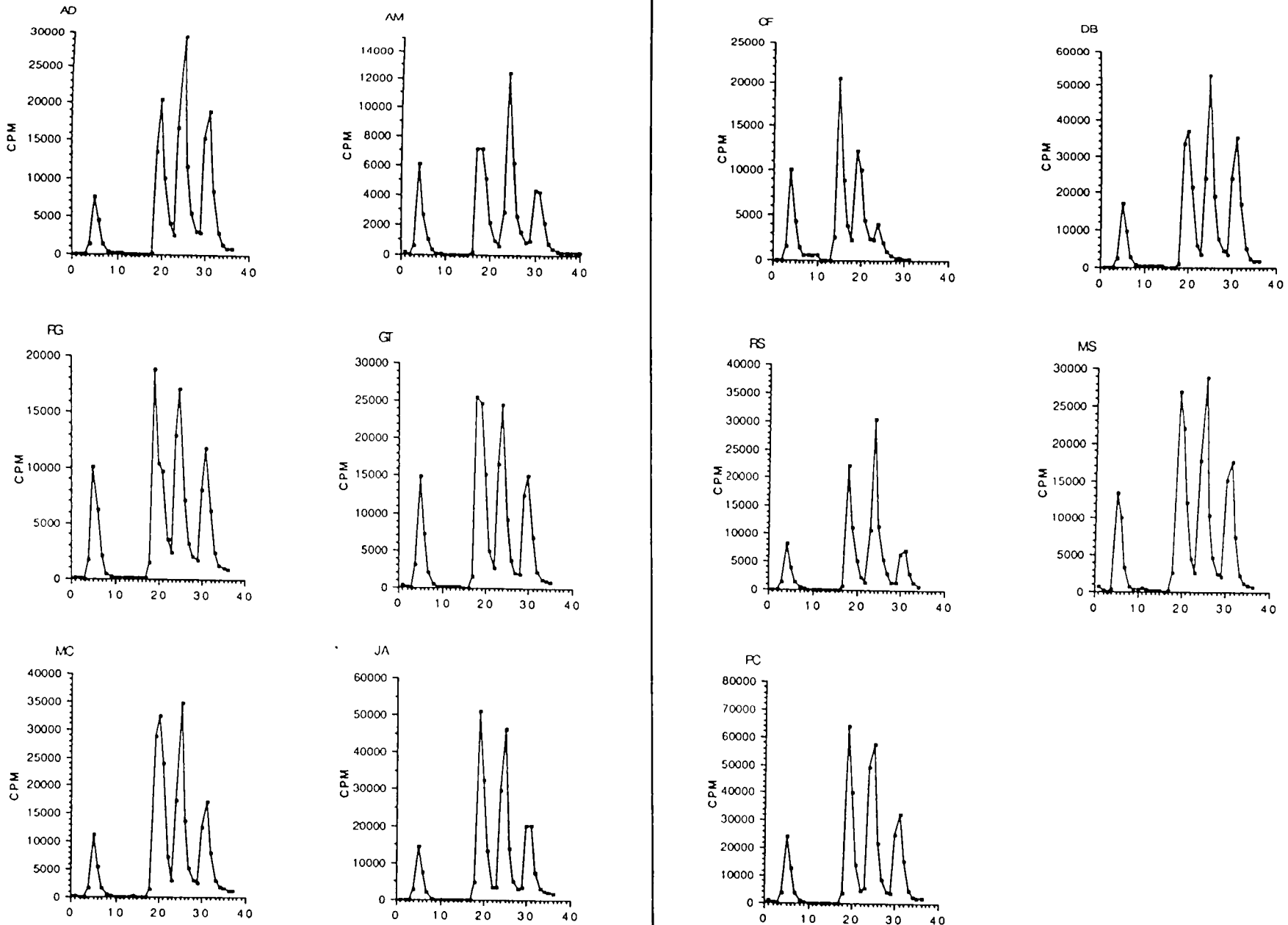


Figure 5.15 (a) Representative Mono-Q ion exchange chromatogram of the oligosaccharides isolated from normal human serum IgA1 by hydrazinolysis. The neutral and acidic fractions are indicated by N and A1 to A3 respectively. The ammonium acetate gradient is shown by a dashed line. Mono-Q ion exchange chromatograms are shown for all IgA1 glycan samples in panel b (control set) and panel C (rheumatoid set).



PANEL B PANEL C

Table 5.5

Sialylation of Human Serum IgA N-linked Oligosaccharides

Control Set (n=6)	**N**	**A1**	**A2**	**A3**
MC	10.7	44.1	39.6	5.6
JA	11.4	40.5	41.7	6.4
AD	10.5	34.3	47.0	8.2
GT	17.1	40.8	36.0	6.1
RG	15.6	44.8	33.5	6.1
AM	18.6	30.9	44.9	5.6
Mean (± SD)	14.0 (3.6)	39.2 (5.5)	40.5 (5.2)	6.3 (1.0)
Rheumatoid Set (n=5)				
PC	14.0	34.0	45.8	6.2
DB	13.4	34.1	43.9	8.6
MS	17.3	37.0	39.3	6.4
CF	22.1	39.1	36.4	2.4
RS	13.0	31.3	51.8	3.9
Mean (± SD)	16.0 (3.8)	35.1 (3.0)	43.4 (6.0)	5.5 (2.4)

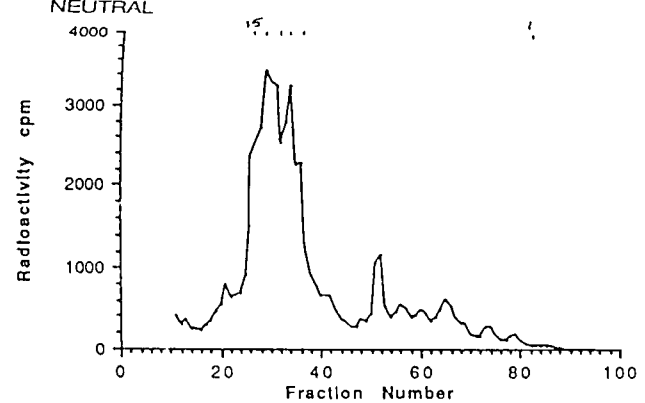
The data were obtained by anion exchange of the IgA1 glycans. The figures have been corrected for the presence of low molecular weight radiochemical material in some of the fractions and normalised to 100% for each sample (see text).

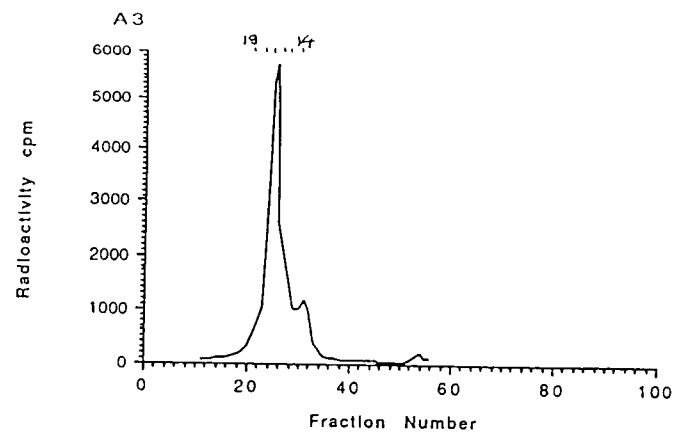
whilst the low molecular weight fraction all eluted from Bio-Gel P4 with a hydrodynamic volume equal to or less than 3.5gu. Therefore it is proposed that the small amount of α2→3 sialic acid detected in the high molecular weight glycans from IgA1 was due to the presence of some O-linked oligosaccharide, and therefore the sialic acid on the N-linked glycans is exclusively in α2→6 linkage.

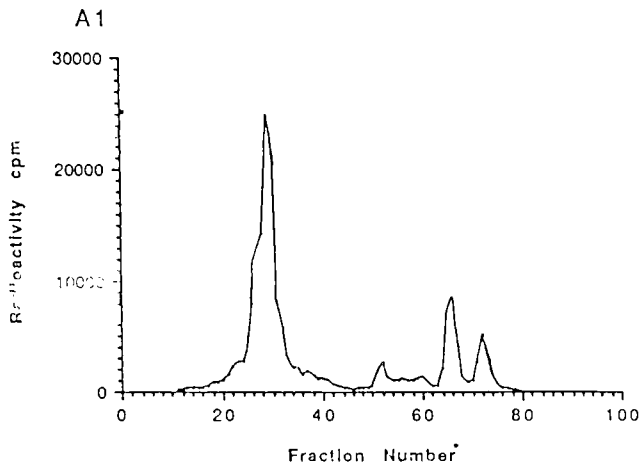
The Bio-Gel P4 profiles of the pooled neutral and acidic glycans from the control and RA set of IgA1s after fractionation by anion exchange and treatment with neuraminidase are shown in figure 5.16. No difference between the profiles obtained with the pooled RA and control sets was discernable. Therefore, based on the similarities of the Bio-Gel P4 (figure 5.16) and anion exchange (figure 5.15) chromatograms it is concluded that there is no significant difference in the sialylation of serum IgA1 between normal individuals and RA patients. In addition, no age-related alteration in sialylation was observed (not shown).

5.4.5 *The Primary Structure of the N-linked Glycans from Human Serum IgA*

Fraction 8 - Radioactive fraction 8 migrated on Bio-Gel P4 at 11.8gu (figure 5.11, scheme 5.6). Treatment of 8 with *S. pneumoniae* β-hexosaminidase under linkage sensitive conditions resulted in the movement of 72% (8C) to 10.2gu suggesting the loss of one GlcNAc. Digestion of F8C with jack bean β-hexosaminidase resulted in the oligosaccharide eluting from Bio-Gel P4 at 7.5gu, indicating removal of two GlcNAc residues. Treatment with jack bean α-mannosidase resulted in the loss of two mannose residues. Therefore F8C is proposed to be a bisected biantennary oligosaccharide with no terminal galactose.







PANEL A

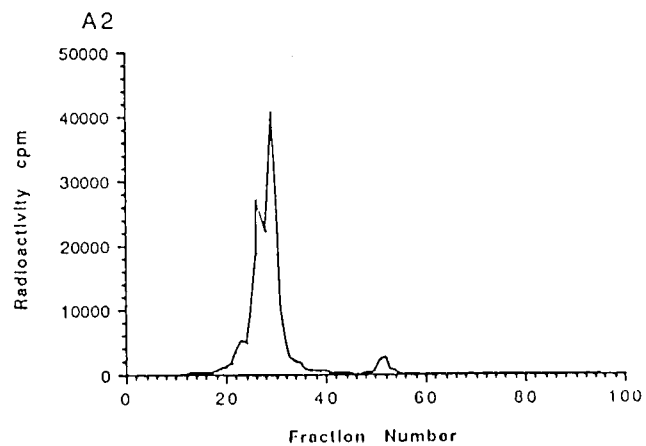
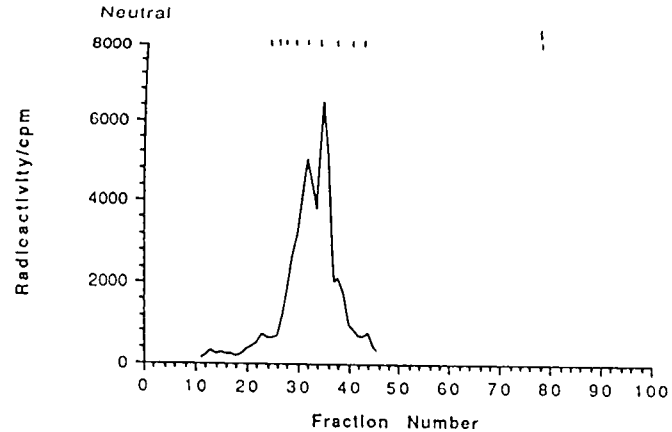
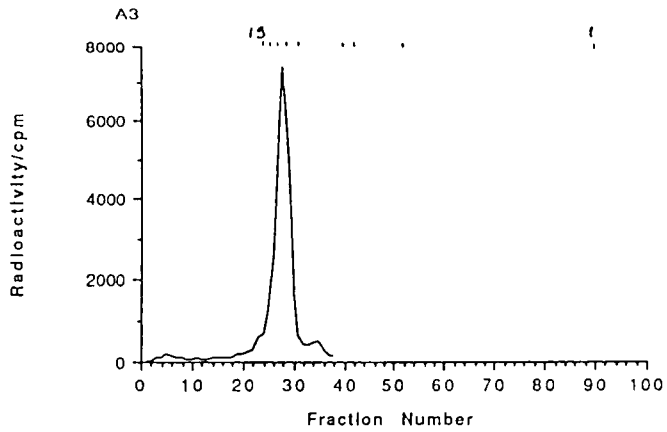
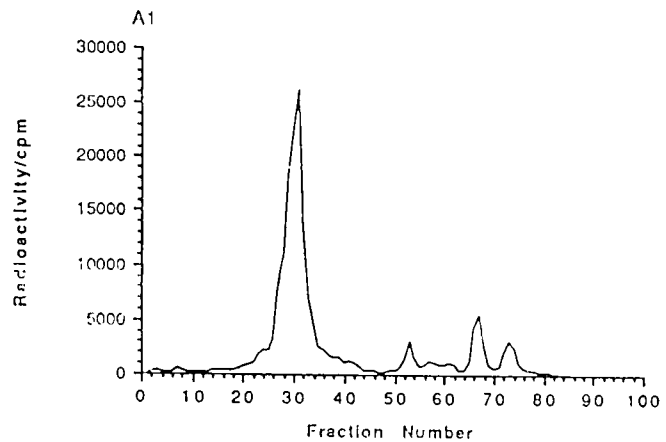


Figure 5.16 Bio-Gel P4 chromatograms of the pooled control group (panel A) and rheumatoid set (panel B) neutral, mono, di and trisialylated oligosaccharides derived from human serum IgA1 following fractionation on Mono-Q and desialylation (N to A3 respectively). Isomaltose oligomers included as an internal standard are indicated by numbers across the top of the profiles.







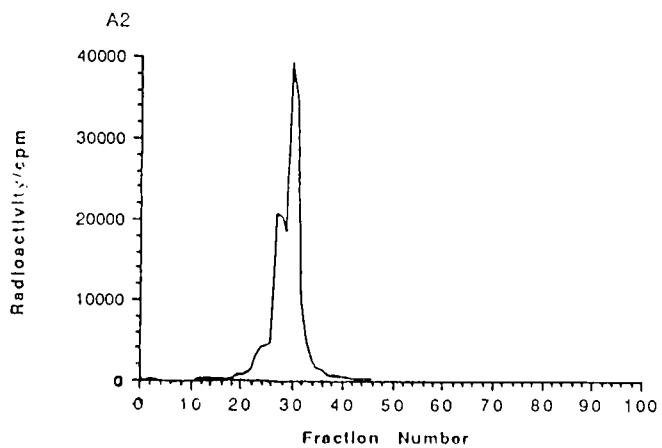


Figure 5.16 Panel B

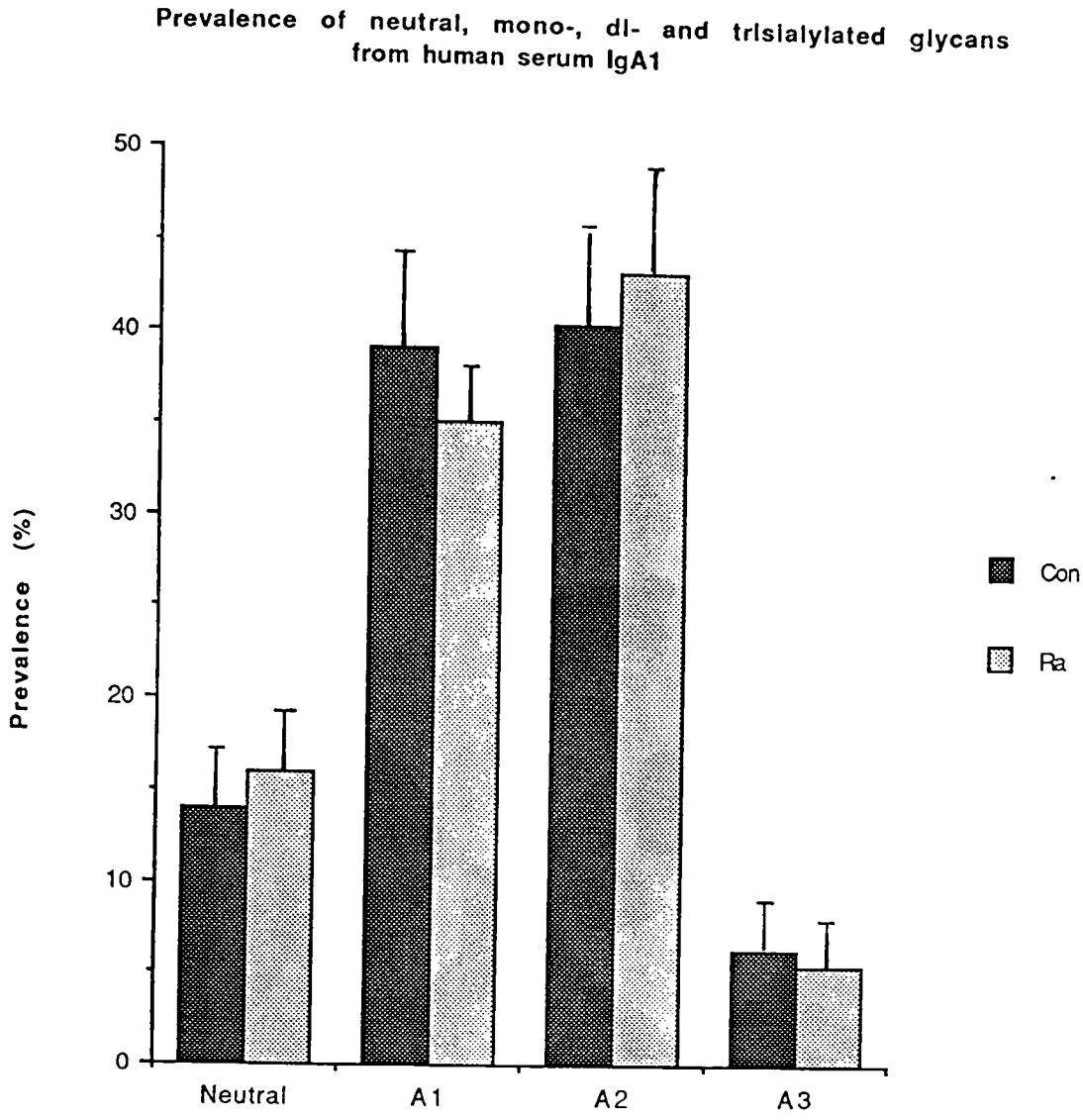


Figure 5.17 Prevalence of neutral, mono-, di- and trisialylated glycans from human serum IgA1. Data taken from table 5.5. From these data it is clear that there is no significant difference in the sialylation of the glycans from the RA and control groups.

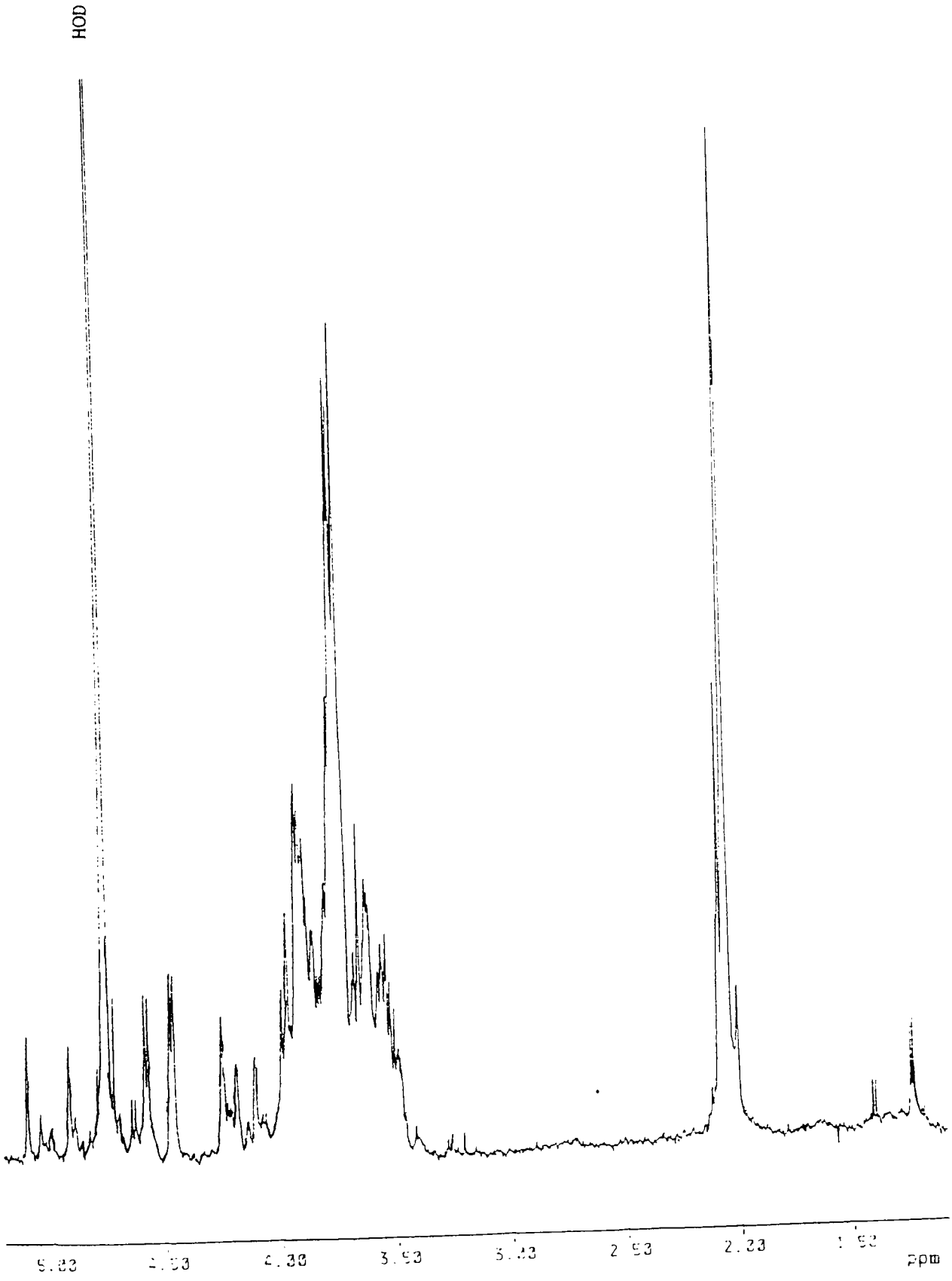


Figure 5.18 One dimensional 500MHz proton nuclear magnetic resonance spectrum at 300K of desialylated human serum IgA N-linked oligosaccharides in D_2O. The sugars analysed here were pooled from the control set of IgAs. Details of the spectrum are discussed in the text.

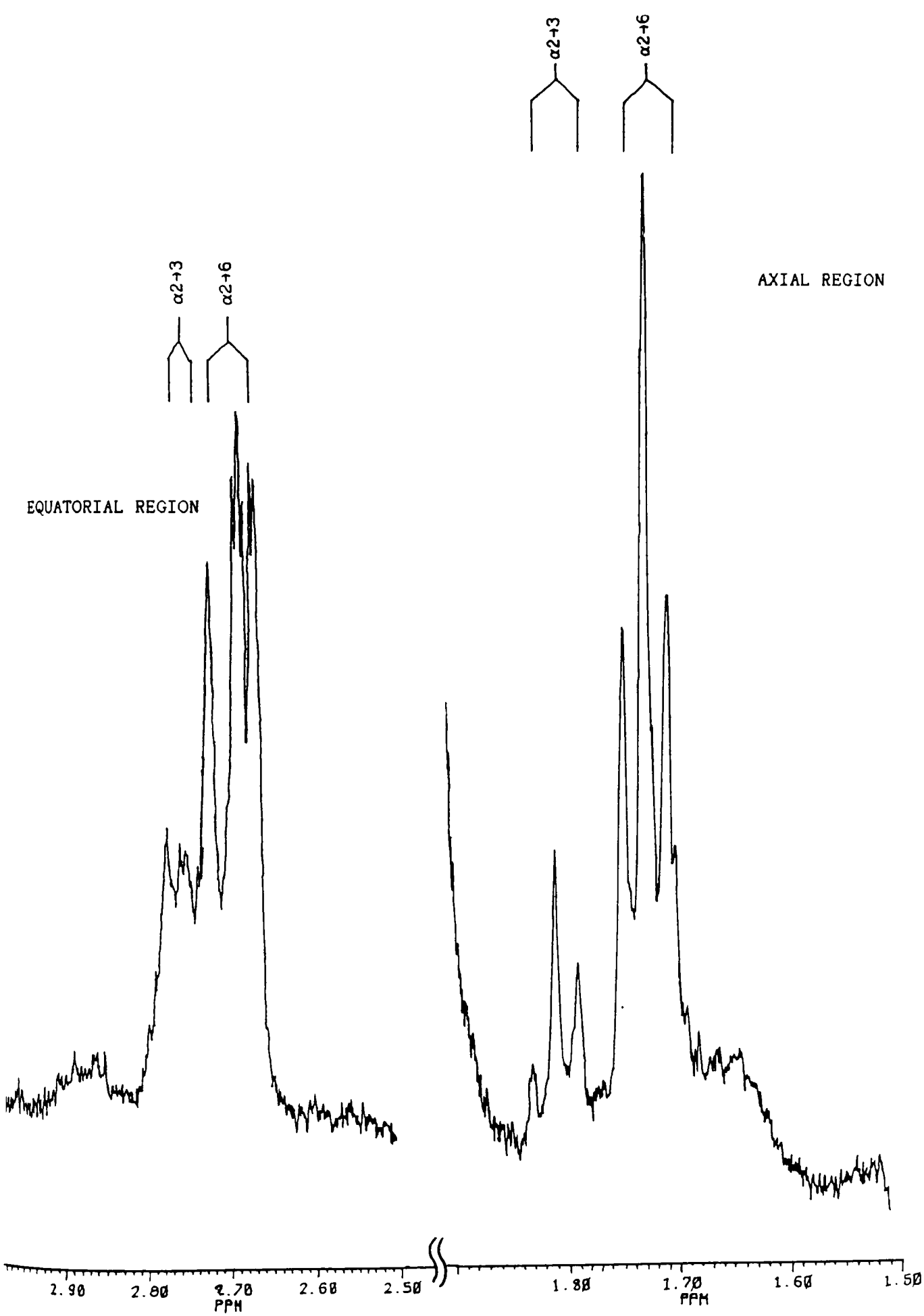
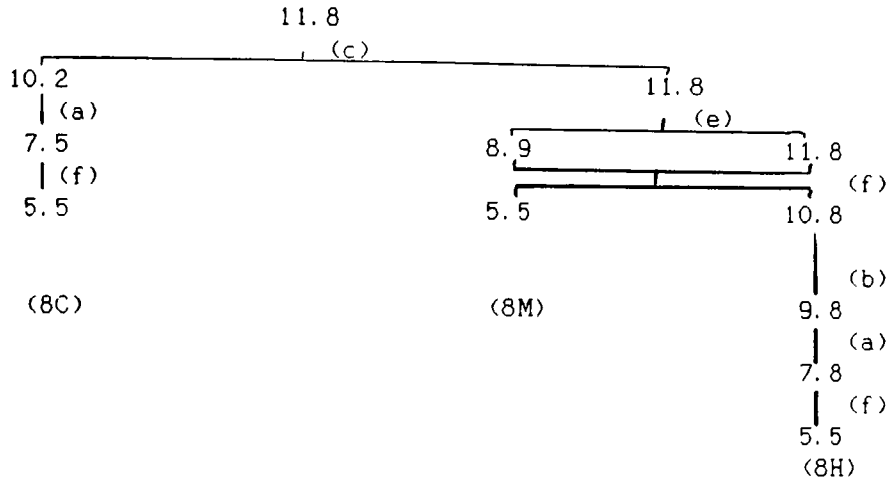


Figure 5.18b One dimensional proton nuclear magnetic resonance spectrum at 600MHz of the N-linked oligosaccharides derived from pooled human serum IgA1 following fractionation on Bio-Gel P4. The regions of the spectrum that contain the resonances for the axial and equatorial protons from the sialic acid substituents are shown. The resonances that originate from N-acetylneuraminic acid in α2→3 and α2→6 linkage are indicated.

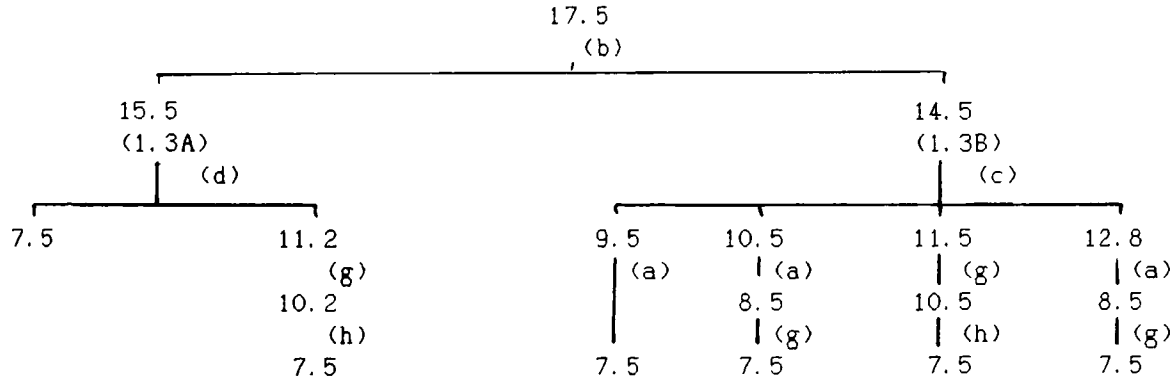
The part of fraction 8 that was resistant to *S. pneumoniae* β-hexosaminidase was digested with *A. saitoi* α-mannosidase which resulted in the removal of three mannose residues from 30% of this material. Repooling all the hexosaminidase resistant oligosaccharides and digestion with jack bean α-mannosidase resulted in the movement of the *A. saitoi* sensitive material (8M) to 5.5gu, i.e. removing four α-mannose residues, and the remainder to 10.8gu, i.e. removal of one α-mannose residue. The material now at 10.8gu, designated 8H, was treated with bovine epidydimal β-galactosidase, which removed one galactose residue, and subsequent digestion with jack bean β-hexosaminidase removed a GlcNAc. Finally, digestion of this glycan with jack bean α-mannosidase resulted in the radioactivity eluting at 5.5gu. These data indicate that glycan 8M is an oligomannose with eight mannose residues, whilst 8H is a hybrid oligosaccharide with one terminal mannose and one terminal galactose. Methylation analysis of fraction 8 (table 5.6) detected terminal galactose, terminal mannose, terminal GlcNAc, 3,6-disubstituted mannose, 3,4,6-trisubstituted mannose, a high amount of 2-linked mannose and 4-linked GlcNAc. This is consistent with the glycan structures proposed for fraction 8, and indicates that the galactose is 4-linked to the GlcNAc.

Fraction 7 - Treatment of fraction 7, which migrated on Bio-Gel P4 at 13.5gu, with bovine epididymal β-galactosidase removed two galactose residues. Subsequent digestion with *S. pneumoniae* β-hexosaminidase under linkage specific conditions resulted in the removal of two GlcNAc residues. Methylation analysis detected terminal galactose, 2-linked and 3,6-linked mannose, 4-linked GlcNAc and reducing terminal 4-linked GlcNAc, consistent with the structure of the glycan being biantennary complex, with galactose in 4-linkage.

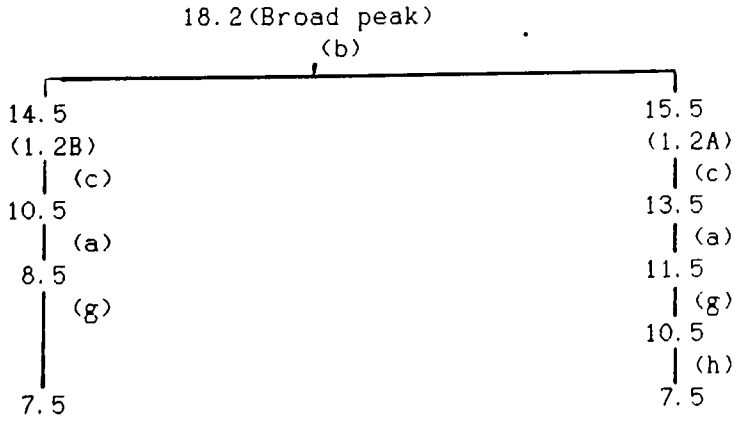
Scheme 5.6 **Exoglycosidase Analysis of Pool 8**



Scheme 5.7 **Exoglycosidase Analysis of Pool 3**



Scheme 5.8 **Exoglycosidase Analysis of Pool 2**



Methylation Analysis of Human Serum Immunoglobulin A1 N-linked Oligosaccharides

Methylated Alditol Acetate	Fraction[a] 1a	1b	2	3	4	5	6	7	8	8b	Deduced position in oligosaccharide
Galactitol											
2,3,4,6-tetra-O-Me	1.18	2.06	3.08	0.87	2.56	1.28	2.45	1.89	0.82	1.40	non-reducing terminal
Mannitol											
2,3,4,6-tetra-O-Me	-	-	0.22	-	-	-	-	-	0.37	0.34	non-reducing terminal
3,4,6-tri-O-Me	2.57	2.16	3.42	1.42	1.06	1.30	3.18	1.48	1.95	3.11	internal: 2-substituted
2,4-di-O-Me	1.00[b]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	internal: 3,6-disubstituted
2,6-di-O-Me	0.17	0.23	-	-	-	-	-	-	-	-	internal: 3,4-disubstituted
3,4-di-O-Me	0.18	0.21	0.12	0.34	-	-	-	-	-	-	internal: 2,6-disubstituted
3,6-di-O-Me	0.20	0.19	0.48	0.47	0.78	-	-	-	-	-	internal: 2,4-disubstituted
2-O-Me	-	0.39	0.15	-	-	0.39	0.32	-	0.29	0.25	internal: 3,4,6-trisubstituted
N-Acetylglucosaminitol											
1,3,5,6-tetra-O-Me	-	-	0.43	0.19	Trace	-	Trace	0.66	-	1.04	reducing terminal: 4-sub.[c]
1,3,5-tri-O-Me	-	-	0.55	-	-	0.82	-	-	-	-	reducing terminal: 4,6-disub.
3,4,6-tri-O-Me	1.23	-	-	-	-	0.58	0.96	-	0.47	-	non-reducing terminal
3,6-di-O-Me	-	1.39	5.80	2.21	4.22	1.87	2.40	2.69	3.57	-	internal: 4-substituted
Fucitol											
2,3,4-tri-O-Me	-	-	0.22	-	-	0.59	0.82	-	-	-	non-reducing terminal
Molar Incidence (%)	0.9		2.0	3.3	6.7	9.4	22.8	49.1	5.8		

Table 5.6 Methylation analysis data for the fractionated N-linked oligosaccharides from normal human serum IgA1.
a. = fraction designation. The nomemclature is the same as used in figure 5.11. For fraction 1, the sample was further subdivided into a high (a) and a low (b) molecular weight component in the molar ratio 2:3 (a:b). Also, 8b denotes a low molecular weight component from fraction 8 which was methylated and analysed separately
b. = incidence of residues expressed relative to 1.00 for 2,4-di-O-Me mannitol (3,6-linked mannose).
Trace indicates that a residue was seen, but could not be quantified. - indicates that a residue was not observed.
c. sub = substituted

Fraction 6 - Fraction 6 eluted at 14.3gu from Bio-Gel P4. This material was resistant to jack bean β-hexosaminidase digestion. Treatment with bovine epididymal β-galactosidase resulted in the removal of two galactose residues. Treatment of this material with *S. pneumoniae* β-hexosaminidase under linkage specific conditions generated three peaks, a major one at 8.2 gu (82%) corresponding to a fucosylated core, and two minor peaks at 11.0 (6%) and 10.2gu (12%) corresponding to bisected cores (i.e. containing GlcNAcβ1→4 substituents on the core Manβ1→4 residue), with and without fucose, respectively. Methylation analysis of F6 indicated the presence of terminal galactose, 2-, 3,6- and 3,4,6- linked mannose, terminal GlcNAc, 4-linked GlcNAc and terminal fucose. These data indicate that the structure of the major species in fraction 6 is a fucosylated biantennary complex oligosaccharide, whilst some minor species, which are bisected biantennary glycans are also present (structures 5 and 7B).

Fraction 5 - Fraction 5 eluted at 15.2gu from Bio-Gel P4. This material was resistant to digestion with jack bean β-hexosaminidase. Digestion of fraction 5 with bovine epididymal β-galactosidase removed two galactose residues. This peak appeared assymetric, indicating that fraction 5 was heterogeneous. These were resolved, after digestion with *S. pneumoniae* β-hexosaminidase under linkage sensitive conditions, into three species. 82% of the glycans eluted at 11.0gu, consistent with a bisected and fucosylated pentasaccharide core, whilst 15% of the oligosaccharides eluted at 8.2gu indicating the presence of a fucose substitution only. The remaining 3% of the sugars eluted at 10.2gu corresponding to a bisected core. Methylation analysis of fraction 5 indicated the presence of terminal galactose, GlcNAc and fucose, 2-, 3,6- and 3,4,6-linked mannose, 4-linked GlcNAc and 3,6-linked reducing terminal GlcNAcol. These data are consistent

with the assignment of the major species in fraction 5 as a fucosylated and bisected biantennary, with the minor species being a fucosylated biantennary glycan and a bisected biantennary glycan (structures 6 and 7B).

Fraction 4 - This material eluted from Bio-Gel P4 at 16.5gu. Digestion with bovine epididymal β-galactosidase resulted in the loss of three galactose residues. Subsequent digestion with jack bean β-hexosaminidase resulted in the glycan eluting at 7.5gu, i.e. three GlcNAc residues were removed. Methylation analysis of fraction 4 indicated the presence of terminal galactose, 2-, 2,4- and 3,6-linked mannose and 4-linked GlcNAc. These data are consistent with the proposal that fraction 4 contains a tri-antennary glycan, with 3 terminal galac tose residues and no core substitution. The methylation also indicates that one mannose is disubstituted at the 2 and 4 positions. Therefore structure 4 in table 5.7 is proposed for this glycan.

Fraction 3 - This material initially eluted from Bio-Gel P4 at 17.5gu (figure 5.11, scheme 5.7). Treatment with bovine epididymal β-galactosidase followed by *S. pneumoniae* β-hexosaminidase and jack bean β-hexosaminidase resulted in the conversion of most of this material to 8.5 and 7.5gu. A small amount of material could not be digested beyond 11.5gu with these enzymes (see scheme 5.7). All the 8.5gu cores could be converted to 7.5gu by digestion with bovine epididymal α-fucosidase indicating the presence of core fucose on these glycans, and the 11.5gu material was converted to 10.5gu, indicating the removal of an α-fucose residue. Methylation analysis of fraction 3 indicated the presence of terminal galactose, , 2-, 3,6-, 2,6 and 2,4-linked mannose, 4-linked GlcNAc and 4-linked GlcNAcol. Neither terminal GlcNAc nor terminal fucose were seen by the methylation analysis. The sensitivity of some of the fraction 3 glycans to fucosidase, and the

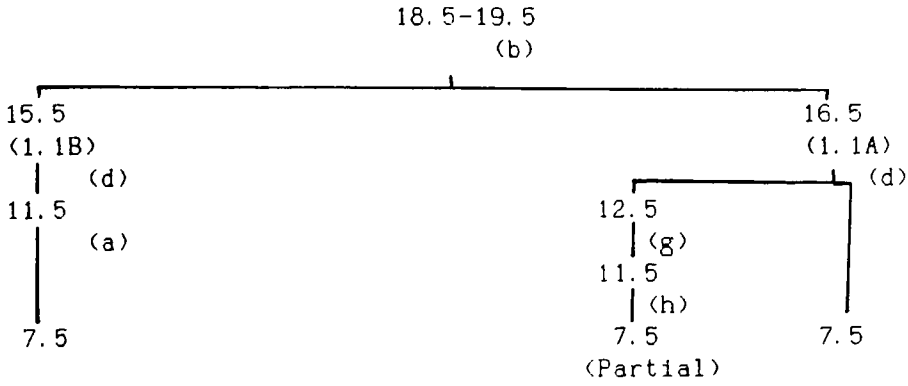
observation that some of fraction 3 only lost two galactose residues on digestion with galactosidase indicates that both of these residues were in fact present (scheme 5.7).

From these data it was clear that fraction 3 contained several structures, and it was not possible to reliably reconstruct the primary structures of all of these glycans. However, it can be confidently concluded that fraction 3 contains predominantly triantennary glycans containing core fucose, and a small amount of tetraantennary oligosaccharides.

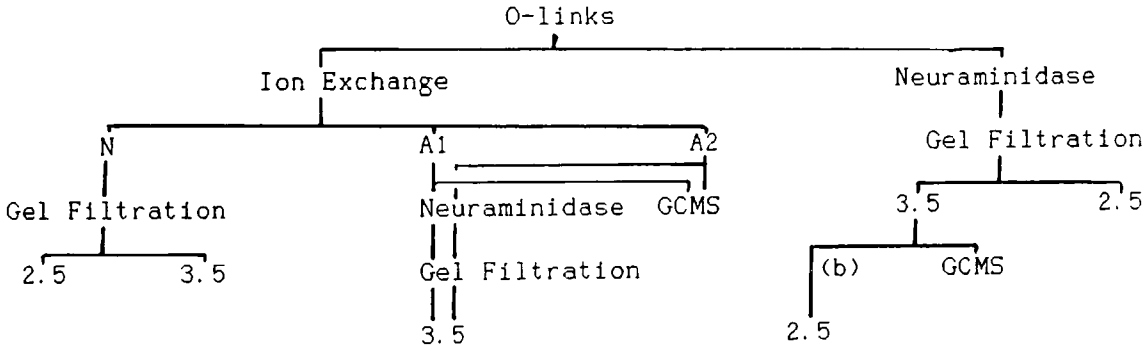
Fractions 2 and 1 - These fractions accounted for only 2.9% of the glycans (figure 5.10), and eluted from Bio-Gel P4 at 18.2 (fraction 2) and 19.0gu (fraction 1). Methylation analysis indicated that these oligosaccharides were multi-antennary complex glycans (table 5.6). A small amount of terminal fucose was detected in fraction 2, and some terminal GlcNAc in the high molecular weight component of fraction 1.

Digestion of fractions 1 and 2 with bovine epididymal β-galactosidase, *S. pneumoniae* β-hexosaminidase and jack bean β-hexosaminidase also indicated that these fractions contained multi-antennary complex oligosaccharides (schemes 5.8 and 5.9). Based on the observation that all of the glycans from fraction 1 were digested to 7.5gu and some of the glycans from fraction 2 were digested to 8.5gu with the galactosidase and hexosaminidase, and then to 7.5gu with bovine epididymal α-fucosidase it is concluded that some of the glycans in fraction 2 contain core fucose residues, but the fraction 1 glycans were not fucosylated, which is in agreement with the methylation analysis. From the initial elution positions on Bio-Gel P4 and the behaviour of the oligosaccharides when subjected to the various enzymes it is concluded that fraction 2 contained core

Scheme 5.9 **Exoglycosidase Analysis of Pool 1**



Scheme 5.10 **Structural Analysis of IgA1 O-linked Oligosaccharides**



The schemes 5.6 to 5.10 summarise the more complex exoglycosidase sequence analysis and the O-link structure elucidation. Figures refer to the elution position of a glycan on Bio-Gel P4 in glucose units. The following single letter code is used to designate the enzymes used; (a) jack bean β-hexosaminidase, (b) bovine epididymal β-galactosidase, (c) *Streptococcus pneumoniae* β-hexosaminidase under linkage specific conditions, (d) *Streptococcus pneumoniae* β-hexosaminidase, (e) *Aspergillus saitoi* α-mannosidase, (f) jack bean α-mannosidase, (g) bovine epididymal α-fucosidase, (h) mild acid hydrolysis followed by jack bean β-hexosaminidase. Refer to text for further details.

fucosylated triantennary glycans and fraction 1 contained tetraantennary oligosaccharides without core fucose. From this analysis it was clear that fractions 1 and 2 were heterogenous, and that the level of any one oligosaccharide was vanishingly small compared to the major structures from fractions 4 to 7.

One dimensional proton NMR (figure 5.18) of the pooled desialylated N-linked oligosaccharides derived from the normal set of purified serum IgA1 allowed the anomericity of the residues present in the mixture of glycans to be assigned from the chemical shifts and the $J_{1,2}$ couplings observed. These were; exclusively β for galactose (with H1 chemical shift (δ) at 4.452 and 4.463) and arm N-acetylglucosamine (H1δ at 4.588 and 4.572, with resonances from the core GlcNAc residues at 4.635), exclusively α for fucose (H1δ at 4.893), and both α and β for mannose (α with H1δ at 5.150 and 4.921, and β with H2δ at 4.252). The spectrum was consistent with assignment of the vast majority of the glycans to the biantennary complex type (Vliegenthart *et al.* 1983). The incidence of core α-fucosylation, from the intensity of the resonance of the three methyl-group protons at 1.209ppm, was estimated at approximately 30%. Similarly, the ratio of the H1 resonances of the core α-mannose residue, in the presence (δ=5.053) and absence (δ=5.150) of a bisecting GlcNAc allowed the incidence of the bisect to be estimated at approximately 30%. The spectrum was consistent with all core structures being of the pentasaccharide Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{\beta 1}$ sequence, which is also in agreement with the methylation analysis data.

An aliquot of the total asialo N-linked oligosaccharides was digested with endoglycosidase H. A very small amount of radioactivity (ca.2%) was recovered at 2.5gu, indicating that essentially all the N-glycans of IgA1

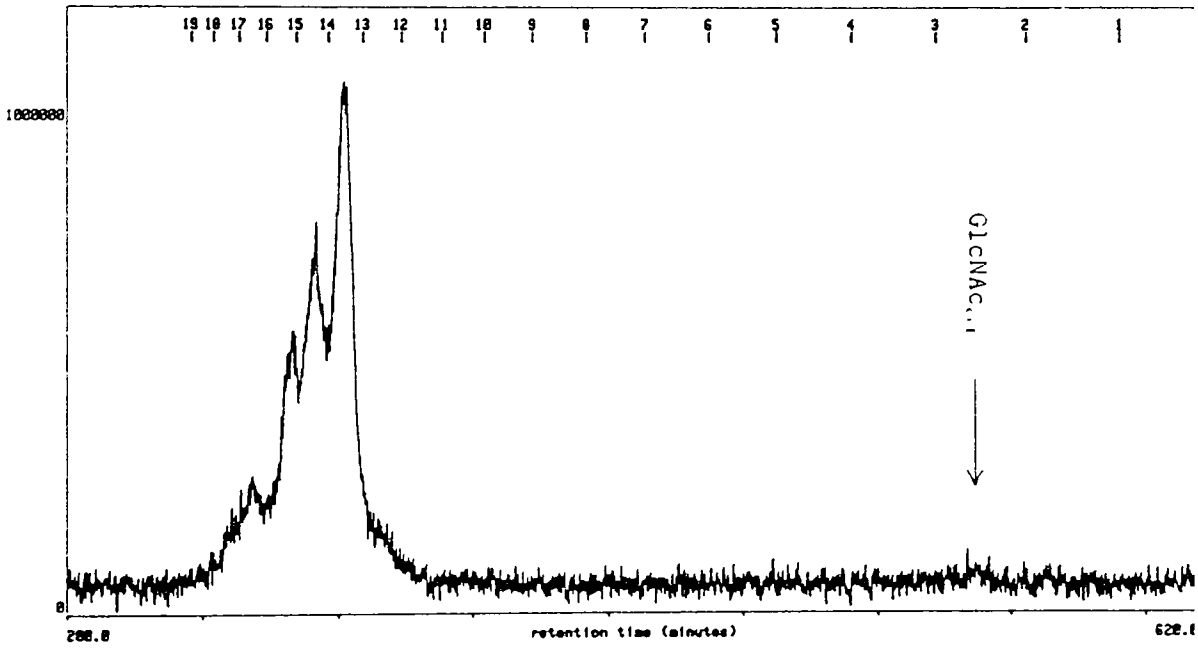


Figure 5.19 Bio-Gel P4 chromatogram of total IgA1 H-linked oligosaccharides digested with Endoglycosidase H

were of the complex type (figure 5.19). The small amount of endo H sensitivity is consistent with the observation of glycans 8H and 8M. The reducing terminus of all the IgA1 N-linked glycans was shown to be GlcNAc$_{1,1}$ by borate high voltage electrophoresis following complete hydrolysis.

The proposed primary structures of the major N-linked oligosaccharides of normal human serum IgA1 are shown in table 5.7, together with the incidence of the tri- and tetraantennary glycans determined to be present in fractions 1, 2 and 3.

5.4.6 *Primary Structural Elucidation of the O-linked Glycans From Human Serum Immunoglobulin A*

Fractionation of the O-linked oligosaccharides from IgA1 by anion exchange yielded a neutral component (N, 36.0%) and two distinct charged glycans (A1, 54.5% and A2, 9.5%) which eluted from Mono Q with retention times similar to authentic mono- and disialylated oligosaccharides (scheme 5.10, figure 5.20). Methylation analysis (table 5.8) of A1 and A2 identified 3-linked galactose and 3-linked N-acetylgalactosaminitol (A1), and 3-linked galactose and 3,6-linked N-acetylgalactosaminitol (A2). These data indicate that the sialic acid on A1 is linked $\alpha 2 \rightarrow 3$ to the galactose, whilst A2 contains two moles of sialic acid, one linked $\alpha 2 \rightarrow 3$ to the galactose, and the other linked either$\alpha 2 \rightarrow 6$ or $\alpha 2 \rightarrow 3$ to the GalNAc. Analysis of per-TMS-glycosides of A1 and A2 by GCMS following mild acid hydrolysis determined that N-acetylneuraminic acid was the only sialic acid present.

Digestion of an aliquot of all of the glycans with *A. ureafaciens* neuraminidase converted the oligosaccharides to neutral. Gel filtration of these glycans eluting at 3.5 and 2.5gu (figure 5.21). Bio-Gel P4

Table 5.7

PROPOSED PRIMARY STRUCTURES OF THE N-LINKED OLIGOSACCHARIDES OF HUMAN IMMUNOGLOBULIN A1

Designation	Structure	Molar Incidence (%)	HDV* (gu)
8C	GlcNAcβ1→2Manα1→6 Manβ1→4GlcNAcβ1→4GlcNAc GlcNAcβ1→2Manα1→3 4 ↑ GlcNAcβ1	4.0	11.8
8H+	Manα1→3(6)Manα1→6 Manβ1→4GlcNAcβ1→4GlcNAc Galβ1→4GlcNAcβ1→2Manα1→3	1.2	11.8
8M+	Manα1→2Manα1→6 Manα1→6 Manα1→3 Manβ1→4GlcNAcβ1→4GlcNAc Manα1→2Manα1→2Manα1→3	0.5	11.8
7	Galβ1→4GlcNAcβ1→2Manα1→6 Manβ1→4GlcNAcβ1→4GlcNAc Galβ1→4GlcNAcβ1→2Manα1→3	46.8	13.5
7B	Galβ1→4GlcNAcβ1→2Manα1→6 Manβ1→4GlcNAcβ1→4GlcNAc Galβ1→4GlcNAcβ1→2Manα1→3 4 ↑ GlcNAcβ1	5.3	14.0
6	Galβ1→4GlcNAcβ1→2Manα1→6 Manβ1→4GlcNAcβ1→4GlcNAc Galβ1→4GlcNAcβ1→2Manα1→3 6 ↑ Fucα1	20.3	14.4
5	Galβ1→4GlcNAcβ1→2Manα1→6 Manβ1→4GlcNAcβ1→4GlcNAc Galβ1→4GlcNAcβ1→2Manα1→3 4 6 ↑ ↑ GlcNAcβ1 Fucα1	8.9	15.4

Table 5.7 Continued

Designation	Structure	Molar Incidence (%)	HDV (gu)
4	Galβ1→4GlcNAcβ1→2Manα1→6(3) Galβ1→4GlcNAcβ1→4 Manβ1→4GlcNAcβ1→4GlcNAc Manα1→3(6) Galβ1→4GlcNAcβ1→2	7.1	16.5
Triantennary glycans from fractions 2 and 3		4.5	17.5-18.5
Tetraantennary glycans from fractions 1 and 3		1.4	18.5-19.5

Oligosaccharides are drawn with the reducing terminus to the right of the figure. *; HDV = hydrodynamic volume in glucose units determined from the initial position of the glycan on Bio-Gel P4.
† These glycans account for the small incidence of endoglycosidase H material detected by digestion of an aliquot of the whole mixture of N-linked oligosaccharides (figure 5.19).

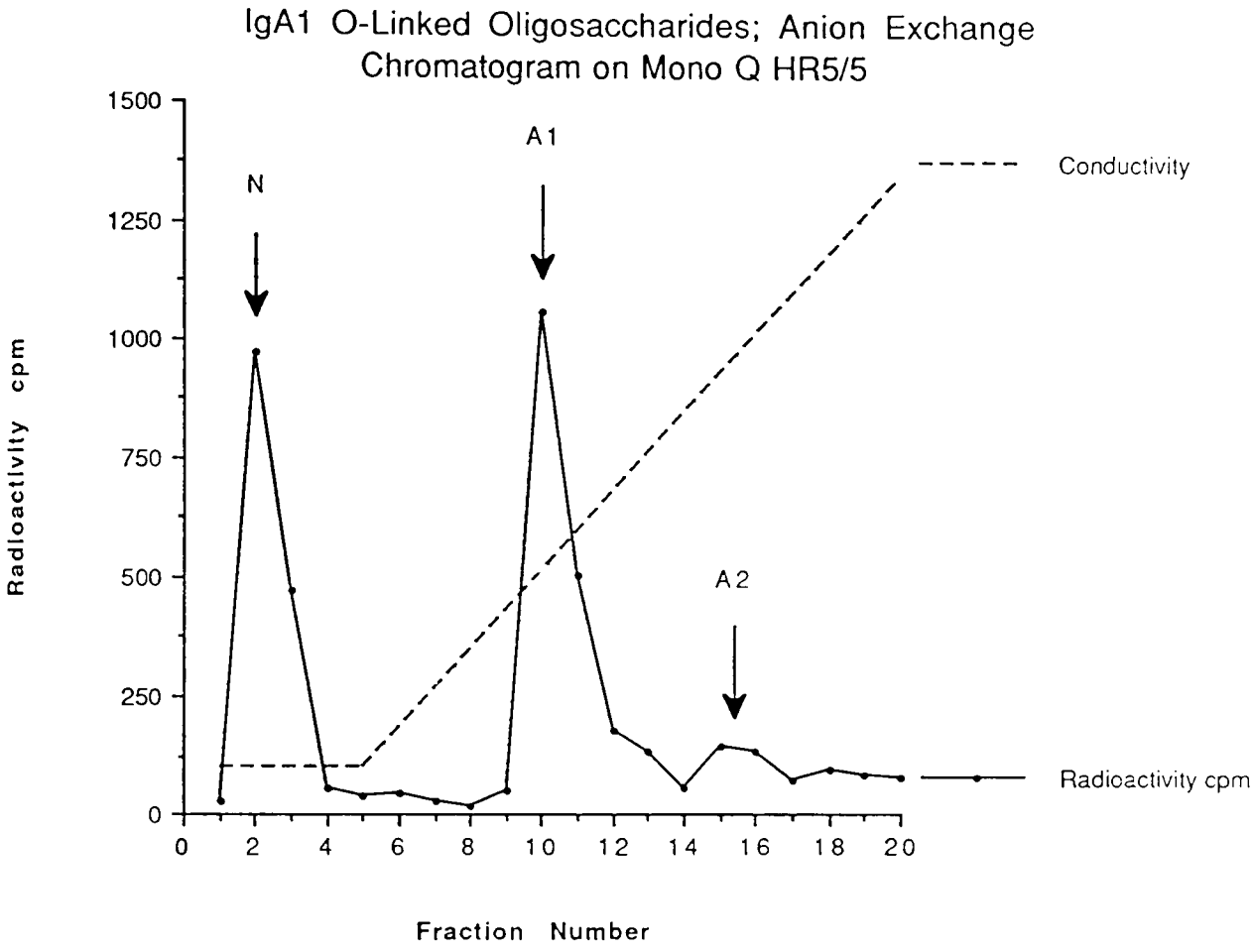


Figure 5.20 Mono-Q ion exchange chromatogram of O-linked oligosaccharides from human serum IgA run in a gradient of ammonium acetate (indicated by the dashed line). The neutral and acidic fractions are indicated by N, and A1 and A2 respectively. The relative incidence of the neutral, monosialylated and disialylated glycans was determined from the radioactivity in the fractions as N 36.0%, A1 54.5% and A2 9.5%.

Table 5.8

Methylation Analysis Of IgA O-Linked Glycans

	Residue			
	T-Gal	3-Gal	3-GalNAc$_{o-1}$	3,6-GalNAc$_{o-1}$
3.5gu post BioGel P4	+	-	+	-
Untreated A1 post ion exchange	-	+	+	-
Untreated A2 post ion exchange	-	+	-	+

Monosaccharides were detected by GCMS as their PMAA derivatives. For each fraction no other PMAA could be detected. The 3.5gu material post P4 chromatography represents the total disaccharides isolated post neuraminidase, and therefore defines the neutral and aminosugar composition and the galactose to N-acetyl galactosamine glycosidic linkage position for the neutral 3.5gu glycan and the A1 and A2 oligosaccharides. For the A2 fraction the levels of the PMAAs were very low and could only be detected by selective ion monitoring. A peak at 10.4 minutes was detected on the total ion current (TIC) and the spectrum was consistent with 3-linked galactose, and a peak at 14.8 minutes had ions of m/z 89, 131, 161, 247, 318 and 219, which is consistent with a 3,6-linked HexNAcol. From the data for the total 3.5gu material, the only HexNAc present is GalNAc, therefore the A2 contains 3,6-GalNAcol. Two further peaks were seen on the TIC, one close to 3-Gal was 2-linked glucose, and the second at 11.4 minutes was not identifiable as a PMAA. In addition the position of the second sialic acid on the A2 sugar must be at the six hydroxyl because only the 4 and 6 positions are vacant, and the specificity of the *A. ureafaciens* neuraminidase precludes the presence of 4-linked neuraminic acid.

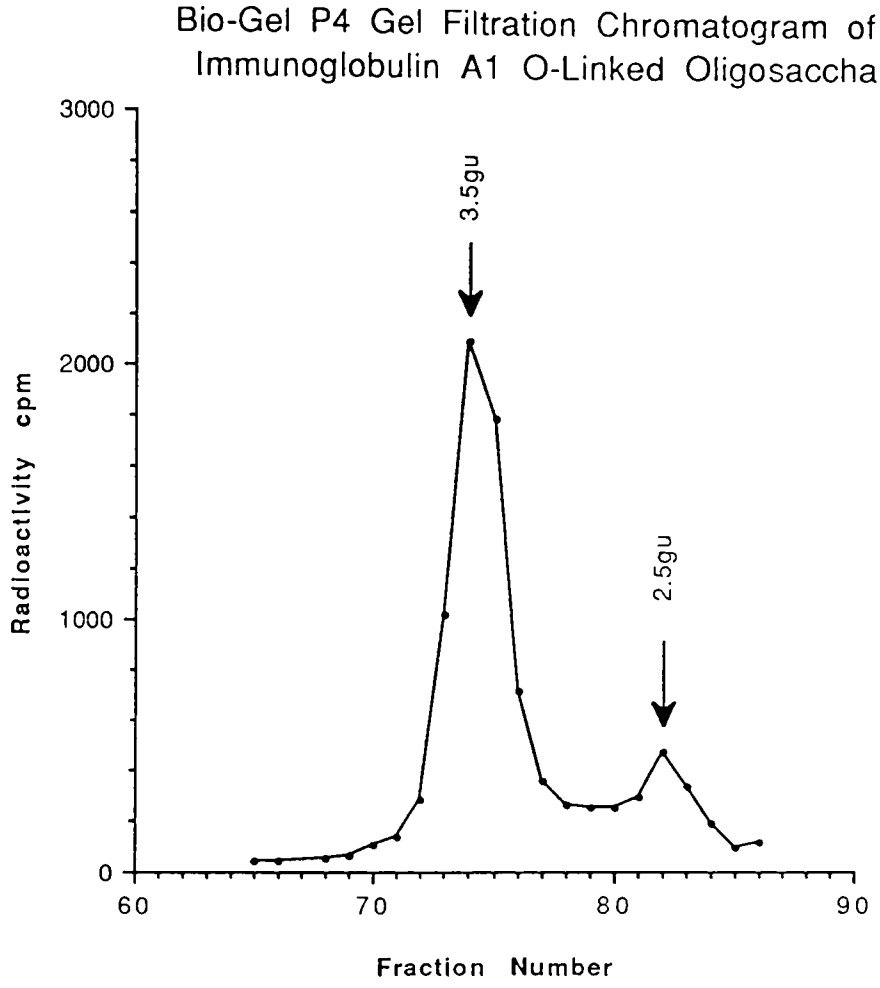


Figure 5.21 Bio-Gel P4 gel filtration profile of the total O-linked glycans from human serum IgA post desialylation. Two peaks at 3.5 and 2.5gu are indicated, with a relative incidence of 81.1% and 18.9% respectively. Some material eluted at 15-12 gu, which was not radiolabelled but was seen by the refractive index detector. GCMS composition analysis of this material did not detect any carbohydrate, so it is concluded that this material is not oligosaccharide, and may be residual peptide that co-purified with the O-linked glycans.

chromatography identified two components in the naturally neutral fraction (N), eluting at 3.5gu (N2, 48.7%) and 2.5gu (N1, 51.3%), whilst in the acidic fractions following desialylation only a 3.5gu glycan was observed. All the 3.5gu glycans were found to be composed of terminal galactose and 3-linked N-acetylgalactosaminitol by methylation analysis. The 3.5gu oligosaccharides eluted at 2.5gu from Bio-Gel P4 following digestion with bovine epididymal β-galactosidase, demonstrating that the 3.5gu glycan had the structure Galβ1→3GalNAc, and that in A2 one of the sialic acids was linked to the six position of the GalNAc. The naturally occuring 2.5gu saccharide migrated on borate high voltage electrophoresis to a position consistent with N-acetylgalactosaminitol.

Based on these data the structures in table 5.9 are proposed for the O-linked oligosaccharides of human IgA1.

From the specific acitivity of the [^{3}H]-sodium borohydride it can be calculated that a theoretical incorperation of 1μCi per 160nM of oligosaccharide would be obtained under conditions where complete reduction was obtained. Following purification of the saccharides it was determined that 1.27μCi had been incorperated, from 3mg of protein. Therefore there are approximately 200nM of O-linked carbohydrate per 20nM of glycoprotein (assuming a molecular weight of 160kdal), and thus about 10 O-glycans per molecule. This is in agreement with the previously reported value of five O-links per α-chain hinge region, but because an internal standard was not included the figure of 10 chains per IgA1 molecule provides only a lower limit estimate.

Table 5.9

Primary Structures of The O-Linked Oligosaccharides of Human Serum IgA

Designation	Structure	Molar Percent	Number per αchain
N1	$GalNAc_{OL}$	18.9	1.0
N2	$Gal\beta1\rightarrow3GalNAc_{OL}$	17.1	1.0
A1	$NeuNAc\alpha2\rightarrow3Gal\beta1\rightarrow3GalNAc_{OL}$	54.5	2.5
A2	$NeuNAc\alpha2\rightarrow3Gal\beta1\rightarrow3GalNAc_{OL}$ (NeuNAcα2 ↓ 6)	9.5	0.5
	Total	100.0	5.0

The relative incidence of the saccharides was calculated following fractionation by Mono-Q ion exchange and gel filtration. A hypothetical incidence is presented at right, assuming five O-linked oligosaccharides are present on each heavy chain polypeptide, and that all O-linked oligosaccharides were associated with the α-chain (Baenziger and Kornfeld 1974b).

5.4.7 *N-Glycosylation of the Subclasses of Human IgA*

The analysis of the human serum IgA subclasses from a commercial supplier was restricted to Bio-Gel P4 gel filtration following treatment with *A. ureafaciens* neuraminidase, as there was very little material availible (figure 5.22). Radiochemical incorperation into the total glycans following purification was 0.7 x 10^5 d.p.m. for IgA1, and 2.2 x 10^5 d.p.m. for IgA2. Assuming an equal efficiency of reduction for these two samples, there are approximately three times as many N-linked glycans on IgA2 as IgA1. These data are in agreement with the known number of used sequons on the two subclasses, i.e. two per α-chain for IgA1 and four or five per α-chain for IgA2.

The Bio-Gel P4 profiles were very similar, with a range of structures eluting between 18gu and 13gu, peaking at 13.5gu. Therefore, from this data at the gross level the glycosylation of the subclasses of human serum IgA is very similar.

5.4.8 Jacalin-Agarose Affinity Chromatography Of Released N-linked Oligosaccharides

An aliquot of untreated N-linked oligosaccharides from IgA1, isolated from patient CF (2.5x10^7dpm), and 4x10^5dpm of a biantennary oligosaccharide isolated from human IgG;

Galβ1→4GlcNAcβ1→2Manα1→6[Galβ1→4GlcNAcβ1→2Manα1→3]Manβ1→4GlcNAcβ1→4GlcNAcol

were fractionated on jacalin agarose (section A1.2.1.2.5). 0.5% of the IgA1 oligosaccharides were retained by the column and eluted with galactose, whilst all of the IgG biantennary saccharide was recovered in the unbound

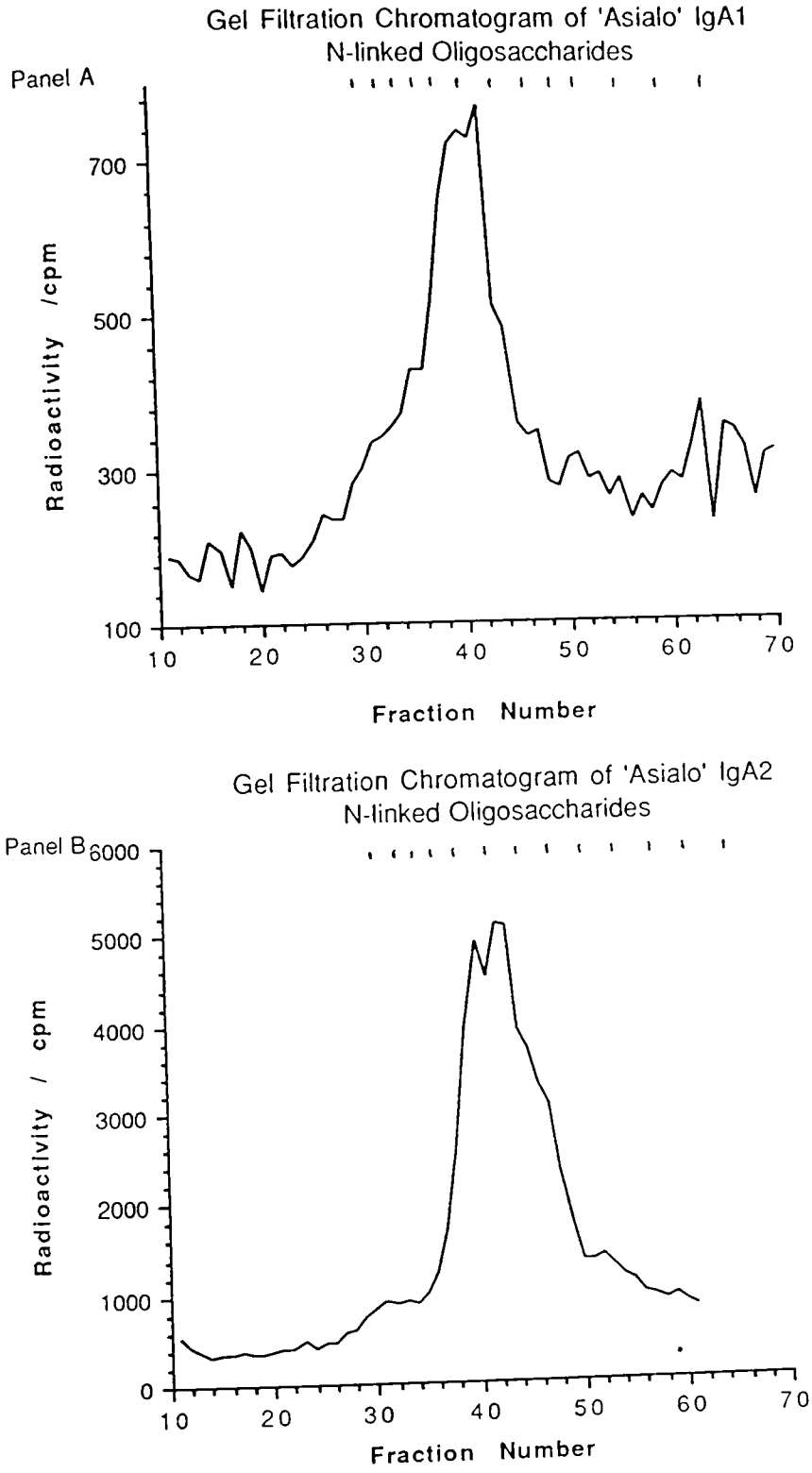


Figure 5.22 Bio-Gel P4 gel filtration chromatograms of normal human serum IgA1 N-linked oligosaccharides (panel A), and normal human serum IgA2 N-linked oligosaccharides (panel B. Both proteins were obtained from Calbiochem. The elution positions of isomaltose oligosaccharides (dp 6-18) run as an internal standard as indicated by vertical lines above the profile.

fractions. When the IgA1 oligosaccharides that had been unbound and retained by the column were seperately desialylated and chromatographed on Bio-Gel P4 it was found that the profiles were identical, and therefore it is concluded that jacalin agarose does not interact specifically with released N-linked oligosaccharides from human serum IgA1.

5.5 *Discussion*

5.5.1 *Protein Purification Study*

Purification of normal serum IgA1 by jacalin affinity chromatography from small amounts of serum enabled analysis of the N-linked glycans of IgA1 from individual humans to be performed, providing a first detailed insight into the possible variability that may be encountered in the carbohydrates present on this immunoglobulin in different individuals.

The use of a lectin to isolate a glycoprotein was initially a cause for concern because only some of the IgA molecules may have been isolated. However, the jacalin column was found to be able to remove all α-chain immunoreactivity from the unbound fraction, indicating that the vast majority of the IgA was bound by the column. Although jacalin bound the intact IgA1 molecule, the lectin had no binding activity against the released N-linked oligosaccharides (section 5.4.9), free lactosamine (Sastry *et al.* 1986), nor N-glycosylated proteins like transferrin, IgG and IgM(Roque-Barriera and Campos-Neto 1983). As the vast majority of the IgA was bound by the lectin it is unlikely that a selective purification was obtained and therefore the N-glycans are representative of the total population.

One possible caveat is that the lectin isolated O-linked glycoforms because sialylated versions of the T-antigen (Galβ1→3GalNAc) are present on IgA1 (table 5.9). It is not known how these glycans are distributed amongst the population of IgA1 or if the sialylated T-antigen oligosaccharides can be bound by jacalin (Sastry *et al.* 1986). If the sialylated glycans are not ligands for jacalin and if the unsubstituted T-antigen is not present on all molecules, then some IgA1 would not have be isolated. If there is some coupling between the O-linked and N-linked structures present on a given IgA1 molecule, then the population of N-linked oligosaccharides studied would not be representative. As discussed above, the majority of the IgA1 molecules were bound by jacalin, and therefore a hypothetical unbound population would be very small. This observation is in agreement with the studies of other workers where all the IgA1 molecules appear to be bound by the lectin (e.g. Biewenga *et al.* 1989, Kobayashi *et al.* 1988).

Analysis of the galactosylation of the N-linked glycans of an IgA1 purified with jacalin following an initial fractionation of pooled serum by ion exchange on Affigel-blue DEAE indicated that the N-linked glycan population was essentially the same as when this step was omitted (table 5.4). The Bio-Gel P4 chromatogram of commercially available IgA1 was consistent with the N-glycans being the same as observed for the jacalin purified IgA1 (figure 5.22). All these data, together with the evidence that the IgA1 was of high purity (>96%, section 5.3.4, figure 5.9), makes it very unlikely that either selective purification or impurity had a significant affect on the results obtained in the glycan analysis. Clearly, to further increase the purity of the IgA would require additional steps and hence decreased yield. As one of the aims of this project was to investigate the glycans from IgA isolated from individuals this was not

attempted routinely.

There has been some controversy in the literature about the precise specificity of jacalin with respect to IgA subclass, and reports have been conflicting (Aucouturier *et al.* 1988, Kondoh *et al.* 1987). Recently analysis of the binding of proteolytic fragments of IgA has conclusively show that IgA1, and not IgA2, binds to the lectin (Biewenga *et al.* 1989). The failure to detect residual IgA2 in the unbound material from the jacalin column elutions in this work was probably due to the low levels of this immunoglobulin in these eluates (IgA2 accounts for ca. 10% of serum IgA), and therefore it is concluded that the purification of IgA used here results in the isolation of IgA1 only. Therefore any conclusions that can be drawn for IgA1 from the data in this chapter cannot be extended to IgA2.

At the practical level the jacalin method was far superior in terms of yield, purity and technical ease. The other methods for purification that were tested were either much more complex, requiring a greater number of manipulations to be performed, or were unsuitable because of the small-scale isolation that was required. These considerations, together with the arguments above for non-selective isolation and purity of the IgA, made jacalin affinity chromatography the method of choice.

5.5.2 *N-linked Oligosaccharides*

The results presented above define the N-linked oligosaccharides of human serum IgA1. The vast majority of the oligosaccharides are complex biantennary type (table 5.10). The results are in general agreement with the earlier study of Baenziger and Kornfeld (1974).

The differences between this and the earlier study are probably due to

Table 5.10

Classes of Pooled IgA1 Glycans from Human Serum as Determined in This Thesis (1989) and by Baenziger and Kornfeld (1974)

Class	Molar Incidence (%)	
	1989	1974
Oligomannose	0.5	
Hybrid	1.2	
Total Complex	98.3	100.0
Biantennary	85.3	100.0
Triantennary	11.6	
Tetraantennary	1.4	

The incidence of classes of the glycans from IgA1 as determined in this thesis and in an earlier study are shown. The complex class is subdivided into bi-, tri- and tetraantennary.

the following. The earlier study was performed on a myeloma protein, which may have had altered N-glycans from those for polyclonal serum IgA1 because it was a paraprotein and a monoclonal. In this study the total IgA1 glycan heterogeneity from normal individuals was sampled, and therefore included IgAs with both different polypeptide sequences and secreted from cells at different points in ontogeny. With the technology available at the time of the earlier work, it would have been impossible to detect the less abundant oligosaccharides that were identified in this study. In fact, the two oligosaccharides that were identified in 1974 are identical to the two most abundant N-linked glycans present on IgA1 as determined here, i.e. 7 and 6. These two sugars alone account for 67.1% of the N-linked oligosaccharides found in this work, and are much more abundant than any of the other oligosaccharides present. From the present study the molar incidence of these glycans was determined which was not known previously. Hydrazinolysis is particularly powerful for the elucidation of the heterogeneity of N-linked oligosaccharides because non-selective and quantitative release is achieved. This does create a problem as it is difficult to determine at what incidence an oligosaccharide is no longer significant. Even though the purity of the IgA1 studied here was high, the minor components identified (specifically the oligomannose and tetra-antennary glycans) cannot be considered unequivocally to be derived from IgA1.

By far the largest group of structures (85.3%) are the biantennary complex oligosaccharides (table 5.10). All four possible cores were detected, with an incidence of core fucose of 29.2% and bisecting GlcNAc of 14.2%. In comparison to IgG the level of core fucose is much lower, being 29.2% for IgA1, and 75% for IgG (see table 3.9). The incidence of bisecting

GlcNAc is quite simliar for the two Igs, 14.2% for A1 and 19.8% for G. This low incidence of bisect may account for the absence of high levels of hybrid glycans on both proteins, whilst the lower level of core fucose in IgA1 indicates that the activity of the fucosyltransferase for the IgA1 glycans is decreased with respect to IgG glycans. It is not known if this reflects either a difference in the B cell glycosylation pathway for α+ and γ+ cells or a difference in the ability of the fucosyltransferase to act on these two proteins. All of these glycans are fully substituted with galactose.

About 10% of the glycans recovered are triantennary, and 1.5% are tetraantennary. Some of the triantennary oligosaccharides in fraction 1-3, accounting for less than 3% of the total, were resistant to enzymatic degradation beyond a core at 10.5gu. The reason for this is not known, but may be due to either a steric block, due to the linkage of the monosaccharides remaining in the glycans, or a problem with low substrate concentration. All of the multi-antennary complex oligosaccharides are highly galactosylated. The glycans found in fractions 1 to 3 were very heterogenous, and therefore the level of the individual components in these fractions was very low, and therefore it is felt that these oligosaccharides are quantitively minor.

A single oligomannose glycan, (8M) was detected, which was a Man-8 oligosaccharide. The isomer was not identified, but based on biosynthetic considerations it is probable that the Manα1$\rightarrow$2 from the Manα1$\rightarrow$3Manα1$\rightarrow$6 arm is lost. A single hybrid glycan (8H) was also present but based on the observation that the oligomannose accounts for only 0.7% of the oligosaccharides, the hybrid is probably also sensitive to endoglycosidase H, which cleaved about 2% of the glycans, suggesting that the terminal

mannose of 8H was linked to the core Manα1→6 at the 3 position (Hubbard 1988). However, the prevalence of 8H and 8M indicates that these oligosaccharides are also very minor components.

Therefore the overall impression of the glycans from normal serum IgA1 is that they are highly processed and that the chains are highly substituted with galactose, and almost exclusively biantennary. Because of this, the heterogeneity amongst the major components is much lower than seen in IgG, where incomplete galactosylation generates a large number of structures (Figure 5.3).

5.5.3 *Sialylation and Galactosylation of the N-linked Glycans*

The glycans are clearly highly charged. The sole anionic moiety was identified as N-acetylneuraminic acid, predominantly in α2→6 linkage. This observation is the same as that for IgG. This linkage is common amongst human serum glycoprotein N-glycans (Corfeld 1982). However, a small amount of sialic acid in α2→3 linkage cannot be excluded, but this would have to account for less than 5% of the total sialic acid present on these oligosaccharides.

Analysis of the neutral and sialylated glycans on Bio-Gel P4 allowed the identification of the underlying structures to be made (Figure 5.16). As expected the neutral fraction contained the oligomannose, hybrid and nongalactosylated biantennary oligosaccharides as well as some of the galactose substituted biantennary glycans. The mono- and disialo- fractions are almost exclusively biantennary, with only a small amount of higher molecular weight material. The trisialo- fraction contained the triantennary oligosaccharides. The sialylation of the glycan classes, i.e.

biantennary, triantennary and the low molecular weight saccharides (fraction 8) can be calculated from this data (table 5.11). Overall about 70% of the β-galactosyl residues are substitited with sialic acid. This is in marked contrast to the glycans of IgG where only about 20% of the galactose residues are sialylated (Parekh *et al.* 1985). The biantennary oligosaccharides carry 1.3 sialic acids on average. The incidence of the mono- and disialylated biantennary saccharides is equal. The triantennary oligosaccharides carry 2.3 sialic acid residues per glycan. There is an increased prevelance of the triantennary oligosaccharides amongst the more highly charged fractions. Therefore, the complex saccharides have a small but significant level of terminal galactose.

Comparison of the pattern of the sialylation of the IgA1 oligosaccharides from the RA and control sets indicated that there was no difference between these two groups.

With regard to galactose substitution, it is clear that IgA1 is highly galactosylated. This is again in marked contrast to IgG, and therefore the addition of both sialic acid and galactose is much more efficient for IgA compared to IgG. The variance in the relative incidence of the IgA1 N-glycans terminating in zero to three β-galactose residues was small between the different individuals as assayed by exoglycosidase digestion, despite a wide variance in the galactosylation of the IgG glycans (table 5.2, figures 5.12-5.14). Residual analysis (table 5.3) of the difference in the Go values for the IgGs from the age-matched mean allows the calculation of a mean difference of +14.4% (n=5) for the RA patients, compared to +3.0% (n=5) for the control samples. Therefore the RA Go values are elevated as a group, whereas the control set are not elevated.

The actual percent incidence of Go of the IgA glycans is very low (ca.

Sialylation of the N-linked Glycan Classes of Human Serum IgA1

Charge	Non-Complex*	Biantennary	Multiantennary*
Neutral	100.0	10.2	0.0
Monosialo	0.0	44.9	20.8
Disialo	0.0	44.9	30.8
Trisialo	0.0	0.0	48.4

Table 5.11. The percent incidence of each glycan class in the neutral and sialylated fractions is presented. The total incidence for each class has been normalised to 100 for clarity (therefore the sum of the figures across the table has no significance).
The data for this table were generated by considering the sialylation of the N-linked glycans as detailed in table 5.5, the asialo structures in table 5.7 and the relative incidences of the glycans seen by gel filtration following fractionation by ion exchange (figure 5.16).
* For clarity the oligosaccharides 8H, M and C have been grouped as a class, and * the tri and tetraantennary glycans have been grouped as one class.

3-4%), and thus it is difficult to evaluate the significance of a shift in the value as determined experimentally. That a Go glycan is present can be concluded from the occurence of glycan 8C. However, a doubling of the incidence of this oligosaccharide would only increase the incidence to about 6-8%, in contrast to a doubling of IgG Go incidence, which could increase values from about 30% to 60%. A shift in this latter case can be clearly observed. Therefore, it is concluded that from this analysis there is no evidence for an RA-associated shift in the incidence of Go in IgA1. In addition, the small normal level of IgA1 Go, and the inability to see a marked increase in Go even in an individual with a very high IgG Go (patient MS, table 5.3, but see below) makes it unlikely that IgA1 hypogalactosylation has a significant role in the pathology of rheumatoid arthritis. This conservation of galactosylation of the IgA1 glycans is completely different to that encountered for IgG glycans. In particular, an inverse relationship between the incidence of Go and G2 (i.e. glycans bearing two galactose residues) as occurs in IgG (Parekh *et al.* 1988b) is clearly not occuring in the IgA1 oligosaccharides.

Even though the IgA1 Go does not vary greatly, when the values of Go for IgG and IgA1 from the same individual are compared, it appears that IgA1 Go correlates with IgG Go (figure 5.14) but because of the low absolute incidence of the Go oligosaccharides many more data points would be needed to confirm these observations, and therefore this hypothesis is unproven at present. In any case, a small fluctuation in the incidence of Go superimposed on the very high level of galactosylation of the IgA1 glycans indicates that any variation is very minor, and that the overriding feature of IgA1 glycosylation is constancy amongst the group of individuals studied in this work. This is the case for both galactose and sialic acid

substitution. Therefore the alteration to β-galactosyltransferase activity observed in rheumatoid B lymphocytes (Axford *et al.* 1987) does not appear to affect the α-chain of IgA1, and therefore the hypogalactosylation in RA may be restricted to the γ-chain of IgG.

Overall the glycosylation of IgG and IgA1 is very different. The glycans of IgA1 are more similar to other serum glycoproteins, and several structures are in common with them, e.g. transferrin, α1-acid glycoprotein, C1q (Kobata 1984). In this light IgG must therefore be considered an unusual glycoprotein even amongst the immunoglobulins.

It cannot be assumed that all immunoglobulin secreting B cells are similar with respect to their glycosylation phenotype and very obvious differences in the α-positive and γ-positive subsets are evident from an analysis of their products, i.e. IgG and IgA1. In the mouse control of IgA production appears to be regulated by interleukin 5 (IL 5), and IgG1 production is under control of IL 4 (Beagley *et al.* 1988). Therefore differences in the glycosylation of IgA and IgG could be due to modulation of the cellular glycosylation apparatus by differentiation as well as the effects of differences in the structure of the immunoglobulin polypeptide itself. However the information presented in this thesis indicates that any alterations in the cytokines that govern B cell Ig production that may occur in RA do not affect the glycosylation of IgA1 to any significant degree. This is of interest, as it has been found that the level of Go on IgG does not correlate with the level of circulating IgG in the serum (Parekh *et al.* 1989), so that altered galactosylation does not simply reflect a shift in the rate of biosynthesis of IgG.

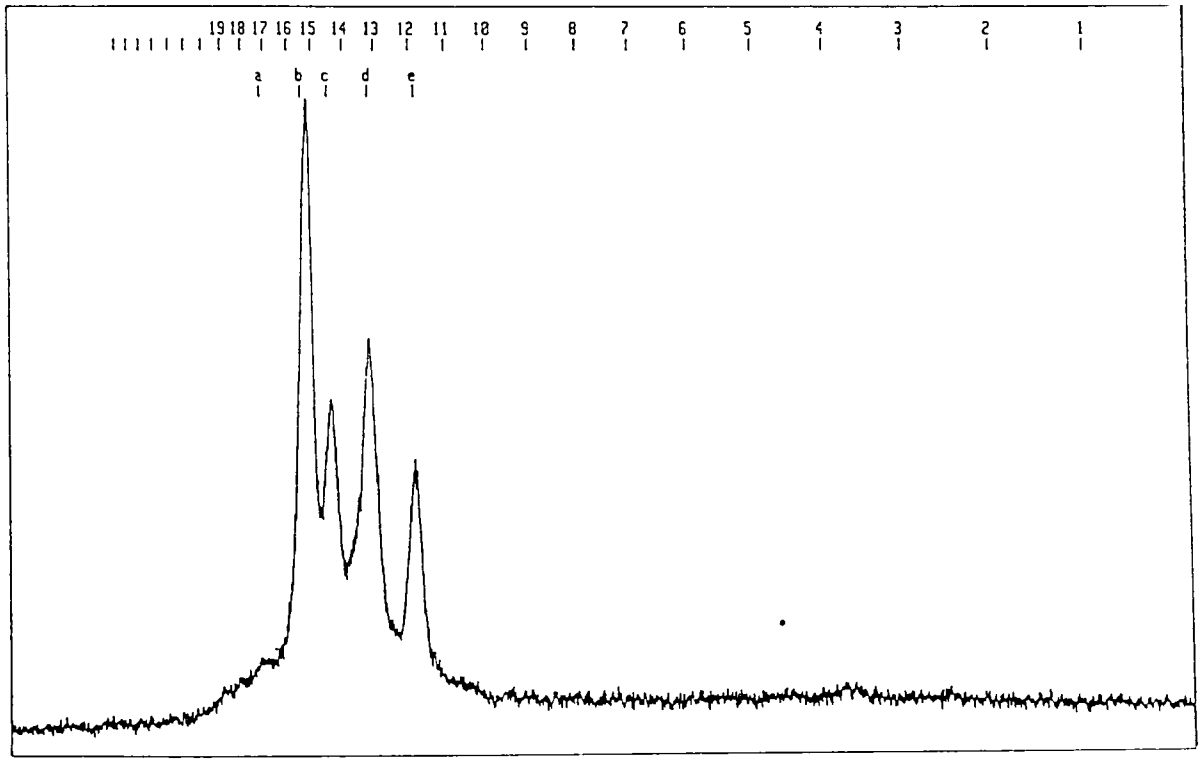
As discussed in chapter one (section 1.3.2) it has been proposed that the conformation of the Fc polypeptide of IgG influences glycan processing

(Schachter 1986). In addition it is thought that the saccharides pair, so that one of the sugars must carry only one galactose, so that both glycans can fit into the pocket formed between Cγ2 and Cγ3 of the heavy chain (Rademacher *et al.* 1986). In the case of IgA1, neither of the sites on the α-chain are in a homologous position. One of the sites is on Cα3 and the second site, which is on Cα2, is not in an equivalent position to the IgG sequon (figure 5.2). Therefore constraints on IgG glycosylation, allowing a maximum of 50% digalacto- oligosaccharide to be present on the Fc, do not apply to IgA1, and indeed the majority of the oligosaccharides derived from IgA1 are of the digalactobiantennary type. If the glycans on the Fc of IgA1 are exposed to the solvent, which is almost certainly the case, then the influence of terminal galactose on serum clearance or terminal GlcNAc on possible autoimmune mechanisms would be far greater than for IgG where the determinants are, to some extent, shielded from interaction by the polypeptide. Therefore, in the case of IgA1, full substitution of the glycans with galactose and sialic acid would be not only possible, but necessary, to ensure that neither a low serum half-life nor the activation of an autoimmune response is engendered by incomplete synthesis of the Fc oligosaccharides. It should be noted that the similarity of the level of bisecting GlcNAc for the IgA1 and IgG glycans is particularly interesting. It has been found that the majority of the bisecting residues are present on the Fab of IgG, as it has been considered that the steric constraints on biosynthesis of the Fc glycans precludes the addition of the GlcNAc (chapter one). As the incidence of bisect is not greater for IgA1, it may be that the steric influences on the addition of this residue are not as great as proposed. Alternatively some other factor could be modulating the addition of GlcNAcβ1→4 to the core of the IgA1 oligosaccharides.

The question of site specific glycosylation is raised by the fact that there are two sequons on the α1 chain. Isolation of the oligosaccharides from the two subclasses of human serum IgA obtained from a commercial source and chromatography on Bio-Gel P4 demonstrated that the glycans were similar, and of the complex type. The levels of radiochemical incorperation were consistent with the observation that IgA2 possesses greater total N-linked carbohydrate than IgA1 (Tomana *et al.* 1976). The presence of a higher number of N-linked sites on IgA2 did not significantly alter the glycan profile compared to IgA1 (figure 5.21) indicating that the extra glycans do not affect the processing of the oligosaccharides at common sites, whilst glycans at sites unique to IgA2 are similar to those on the common sites. This suggests that major differences in the glycans at different sites are are not present.

Finally, it is of interest to compare the Bio-Gel P4 profiles for desialylated N-linked oligosaccharides from normal human serum IgA1 and the Fc of a human myeloma IgA1 (SCH) as obtained by hydrazinolysis (figures 5.11 and 5.22, data shown courtesy of S. Amatyakul of the Glycobiology Unit). In both cases, the major peaks elute with the same hydrodynamic volumes (i.e. 11.8, 13.5, 14.3 and 15.2gu, with an envelope from 16.5-20.5gu), but the intensities of the peaks are clearly different. Whilst the glycans for protein SCH have not been analysed, it is probable that the difference in the two profiles is due to a shift in the incidence of the glycans rather than a change in the glycan structures expressed. The differences could be accounted for by an increased incidence of core fucosylation and a greater prevalence of the oligosaccharides identified in pool 8 for the serum IgA1. Therefore a clear shift in the glycosylation of IgA1 is seen in malignancy, compared to no shift in an autoimmune disease.

Figure 5.23 Bio-Gel P4 gel filtration chromatograph of the oligosaccharides from human myeloma IgA1 (SCH) post neuraminidase. The elution positions of isomaltose oligomer oligosaccharide internal standards are indicated.



Similarly, greater alterations in the glycans of IgG have been observed in myeloma proteins compared to IgG in other disease states (Mizuochi *et al.* 1982).

5.5.4 *O-Linked Glycan Sequences*

The structures of the O-linked glycans of IgA1 have been encountered in other glycoproteins e.g. glycophorin (Krotkiewski 1988). In an earlier study one GalNAc was reported for every four Galβ1→3GalNAc disaccharides (Baenziger and Kornfeld 1974b). In the present work the molar incidence of GalNAc was found to be 18.9% of the total O-linked glycans, and the remainder had asialo cores of $Gal\beta1\rightarrow3GalNAc_{>1}$, which is the same as the previous study within the accuracy of the analysis. The T antigen Galβ1→3GalNAc has been found to be the most avidly bound ligand by jacalin (Sastry *et al.* 1986). Two sialylated O-linked glycans were found. The reason for the fact that the structures reported by Baenziger and Kornfeld (1974b) did not include these structures could be due to the myeloma IgA1 investigated being derived from cells with defective sialyltransferase, or removal the sialic acid during the isolation of the hinge peptide. The conditions used make the latter unlikely.

The structures of the O-linked oligosaccharides from IgA1 determined in this study (table 5.7) account for the conserved levels of sialic acid and galactose in IgA1 compared to N-acetylglucosamine, mannose and fucose which are greater for IgA2 (Tomana *et al.* 1976).

The presence of sialoglycans in the hinge region of IgA1 could alter the physicochemical properties of that part of the molecule as the peptide portion is composed of uncharged amino acids (Putnam *et al.* 1979). On

average 2.5 monosialyl- and 0.5 disialyl-chains would be present per heavy chain if all the O-linked glycans were associated with the α-chain, i.e. seven moles of sialic acid per mole of IgA1 would be contributed by these glycans. If the distribution is nonuniform, considerable diversity in this region could result by the generation of glycoforms based on differential O-glycosylation. It has recently been shown that the rat peritoneal macrophage IgA receptor binds to the hinge of human IgA1 in a manner that involves the O-linked oligosaccharides (Gorter *et al.* 1988). These workers showed that the binding of IgA1 could not be inhibited by IgA2, but that bound IgA1 could be competed out by IgA1. Free galactose was able to attenuate the binding of the Fcα receptor for human IgA1. Therefore the structural definition of the hinge region may have important biological implications. If there exist O-linked glycoforms then individual IgA1 molecules may show differential binding to Fcα receptors and therefore control of O-glycosylation may influence the function of the molecule.

A model of the hinge region of IgA1 incorperating the glycan structures elucidated in this work was prepared using molecular graphics software (figure 5.24, Field *et al.* 1989). From this it can be seen that the glycans contribute a significant amount of the surface area of this region of the molecule, and therefore may affect the topology of the hinge greatly. The influence of these charged glycans on the conformation of the peptide is not known, but may greatly alter its flexibility, and thus modulate the area of space that the Fab combining site can sample. This may, in turn, have important consequences for the function of IgA1 in antigen recognition and complement activation.

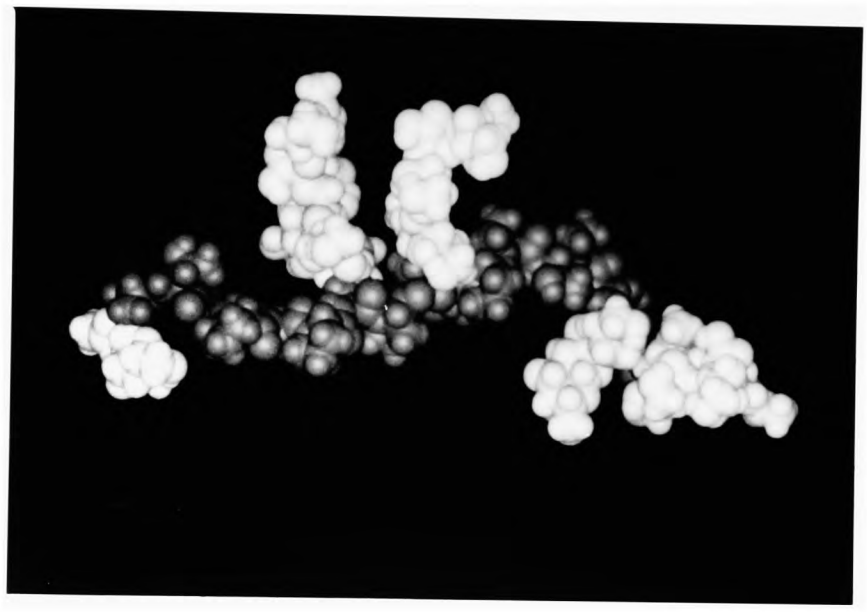


Figure 5.24 Computer model of the hinge region of IgA1. Peptide atoms are shown in dark, whilst the glycans are shown in light. The peptide sequence was minimised independantly, using BIOSYM software, and the carbohydrates then attached to appropriate serines (assuming the location of neutral structures is as determined by Baenziger and Kornfeld (1974b)) in resonable solution conformations as determined by NMR of similar compounds. The peptide sequence and the linkage positions of the glycans used for the model were;

NH_2-S[N1]TPPTPS[A2]PS[A1]TPPTPS[N2]PS[A1]-COOH

with the glycans indicated by [] <u>following</u> the residue to which they are attached. Glycan designations are as given in table 5.9. The distribution of the sialic acids is arbitrary.

5.5.5 *Implications and Future Directions*

The application of a number of independant techniques in the analysis of the oligosaccharides is very powerful as cross checks of the assignments are inherent in the strategy. Information gained from methylation analysis simplified subsequent exoglycosidase sequencing. For example all the GlcNAc was found to be 4 linked or terminal and therefore all galactose was in Galβ1→4 linkage, and this information did not need to be determined by exoglycosidase analysis. Insufficient material prevented NMR analysis of the fractionated oligosaccharides, but NMR spectroscopy of the total oligosaccharides allowed the assignment of some structural features, in particular the anomericity of the monosaccharides and the incidence of the core substituents. Subsequent use of exoglycosidases allowed the connectivity of the residues to be determined once the linkage positions had been provided from methylation analysis. With plentiful samples NMR and methylation analysis of purified components is possible, but for the quantities availible here the use of the enzymatic method is very powerful. Indeed, in the case of pool 1 less than one nanomole of sugar was analysed.

It would be of interest to examine the O-linked glycans from IgA1 isolated from RA patients to determine if O-glycosylation has a role in this disease. Regulation of macrophage activity in the synovium of the RA joint is an important component in disease pathlogy, and therefore potential mechanisms whereby this may be altered could be important. It would also be of interest to assess the glycosylation of IgA in other diseases where IgA cointaining immune complexes or deposition of IgA is an important mediator of disease, e.g. IgA nephropathy.

CHAPTER SIX

SUMMARY

The glycosylation of the glycoproteins studied in this thesis, IgA1, IgG and Po are considered with respect to possible constraints on biosynthesis that may be present. The results from the investigation of the whole murine brain glycans and the Po oligosaccharides are considered, with reference to the HNK-1/L2 epitope and CNS glycosylation. The implications of the data obtained from the studies of MS and glomerulonephritis are discussed.

6.1 *Glycosylation of IgA1, IgG and Po*

Po and immunoglobulins A1 and G are all members of the immunoglobulin superfamily. As such, they are all expected to adopt similar secondary structural conformations, i.e. the Ig fold (Barclay and Williams 1987). When comparing the glycosylation of these three proteins, despite proposed similarities in polypeptide structure, the glycosylation is clearly different.

With regard to IgA1 (chapter five), the N- and O-linked glycans are highly processed. The vast majority of the N-glycans are complex type with a high incidence of sialic acid and fully substituted with galactose, whilst the O-linked glycans also carry high levels of sialic acid. This contrasts with IgG, which has been shown (Mizuochi *et al.* 1982, Parekh *et al.* 1985) to carry glycans that are incomplete. The reason for this is

considered to be due to the unique position of the Fc sequon on IgG and the conformation of the Fc polypeptide which confers a steric constraint on the construction of the glycans (chapter one). It is of interest that the oligosaccharides on the Fab of IgG are much more heavily sialylated and galactosylated than the Fc glycans (Rademacher *et al.* 1988b) so that steric control of construction of the Fab chains must be absent (chapter one). Because the sequon positions of the IgA1 Fc are not in analogous positions to those of the IgG Fc, the IgA1 oligosaccharides are accessible to the processing enzymes in the Golgi, and consequently become fully substituted with galactose and sialic acid. This results in low heterogeneity of N-glycans on IgA1. Indeed, only five of the desialylated glycans that were identified can be considered as major components (table 5.5).

The accessibility of the IgA1 glycans is important for determination of the final spectrum of glycan structures seen. Because the oligosaccharides are exposed, any incomplete chains could act as ligands for the asialoglycoprotein receptor, or as potential triggers for autoimmunity as suggsted in RA (Rademacher *et al.* 1988a). Sampling of serum immunoglobulin glycans, as performed in this study, analyses the steady state, so that if IgA1 glycoforms having high levels of terminal galactose are produced, then the contribution of these glycoforms to the circulating population would be minor because of rapid clearance by the liver. However, IgG, with a low level of sialic acid, may accumulate because the Fc oligosaccharides may be prevented from interaction with the asialoglycoprotein receptor because of steric considerations, which do not appear to apply in the case of IgA1. It is interesting that the glycans of IgA1 and IgA2 appear similar. Because of the different number of glycans present on these two subclasses, this evidence suggests that a high level of site-specific glycosylation is not

occurring, and is further evidence that IgA glycans are not susceptible to steric control and become fully processed.

Human Po glycoprotein (chapter two) was observed to have a high incidence of hybrid glycans, estimated at approximately 60%. The prevalence of hybrid oligosaccharides found on Po appears to be conserved in lapine, bovine and the human proteins (chapter two), although detailed structural data on the glycans from the former two proteins are not availible. As the polypeptide primary structures of Po in rat, cow and humans are also conserved, this may reflect conservation of the Schwann cell glycan biosynthetic apparatus and hence suggests an important functional requirement for the Po glycoprotein, and perhaps also Po glycoforms, in cell-cell adhesion.

The incidence of hybrid oligosaccharides appears to be governed by the relative activity of GlcNAc-transferase III and Golgi mannosidase II. Therefore in the Schwann cell the activity of the GlcNAc-transferase compared to the mannosidase must be higher than in e.g. the B cell, to produce the spectrum of structures seen. Because Po is a membrane protein, this may influence the processing of the glycans (Fukuda *et al.* 1988).

6.2 *Disease Studies*

The fuller processing of the IgA1 oligosaccharides made it difficult to assess the occurrence of RA-associated alterations to the N-glycans (chapter five), as the normal level of galactosylation was very high. Glycosylation defects observed in disease may be consequences of one or more of at least three factors: (1) an altered biosynthetic enzyme, e.g. β-galactosyltransferase (Axford *et al.* 1987), (2) the presence of an affected

glycoprotein as a significant proportion of the steady state population, and (3) the position of the sequon, which allows the glycoprotein to be sensitive to an alteration in the biosynthetic pathway. The evidence presented in this thesis indicates that significant changes in IgA1 glycosylation in RA do not occur, and this may be due to any or all of the factors mentioned above.

Myeloma IgA1 (protein SCH) also possesses complex type glycans like normal serum IgA1, but from the Bio-Gel P4 analysis it is clear that the incidence of structures is different. It may be noted that the myeloma IgA1 glycans are still mostly fully processed in contrast to observations for IgG myelomas (Mizuochi *et al.* 1982), where incomplete glycans become more prevalent.

The data presented for multiple sclerosis (chapter three) indicated that a major glycosylation disorder such as seen in RA does not occur in the glycoproteins studied, despite severe inflammation in the brains of MS patients. An important conclusion of this study was the substantiation of the hypothesis that the blood brain barrier remains intact in MS patients. The glycosylation of IgG found in the CSF showed little evidence of alteration: data suggests a possible increase in the incidence of core fucose in the MS CSF IgG when compared to the normal CSF IgG. This may be evidence for an influx of serum B cells into the brains of MS patients, which is also in agreement with proposals for MS (Tortellotte *et al.* 1984).

By contrast, in some glomerulonephritis patients, evidence was found for a decrease in the levels of galactose on the glycans of serum IgG, particularly in those with the severest disease, as assessed by serum creatinine levels (chapter four). Whilst the increase in Go was not complete, as observed for RA (which may be unique in this regard), these

data suggests that an alteration to the molecular structure of IgG may underlie the pathogenesis of renal disease in some cases.

6.3 *The HNK-1/L2 Epitope*

Evidence is presented that HNK-1/L2 reactivity can be confered to glycoproteins by sulphated glycans (chafter two). No evidence was obtained for a novel structure on Po containing a glucuronic acid residue, the site of addition of sulphate in the SGGL glycans. This is of considerable importance in neural cell adhesion, as the HNK-1/L2 epitope forms the basis of one of the major experimental systyems currently under study. The results presented here highlight a severe problem in the use of monoclonal antibodies as the sole reagent for structural assignments, and clearly fuller investigations into this system are required to clarify the issues raised here. Experiments attempting to bind released glycans to L2 antibodies suggest that in this case antibody binding is a complex phenomenon, requiring contributions from both the glycan and protein moieties (chapter two).

6.4 *Whole Brain Glycans*

The studies into the murine brain oligosaccharides (chapter two) brought to light a number of points: a high incidence of charged glycans was observed, with a significant incidence of sulphated oligosaccharides. This contrasts with the exclusive presence of sialic acid as the anionic moiety in human CSF N-glycans. Whilst this may reflect a species difference, it also suggests that the membrane bound glycoproteins, such as

Po, express different oligosaccharides to those encountered on soluble glycoproteins. This may reflect a difference in the processing of glycans close to the Golgi membrane (Po) and those free in the lumen, i.e. soluble proteins like IgA. For human serum N-linked glycans all the anionic moieties are sialic acids, and this may be a general feature of body fluid protein glycosylation. Metabolic labelling of murine brain glycans with $^{35}SO_4$ demonstrated the presence of two classes of sulphated glycan in the CNS, a population of oligosaccharides with a small number of sulphates and a second class consisting of polysulphated glycans. This second population may be glycosaminoglycan-type saccharides.

Methods for the removal of sulphate and phosphate esters were derived and characterised for their use on N-linked oligosaccharides (chapter two). These two procedures, methanolysis, and hydrogen fluoride treatment, were shown to be non-destructive when applied to N-linked oligosaccharides, and therefore may be of general applicability in the future.

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APPENDIX ONE

METHODS AND MATERIALS

*

APPENDIX TWO

AN OVERVIEW OF METHODOLOGIES EMPLOYED FOR STRUCTURAL STUDIES OF OLIGOSACCHARIDES DERIVED FROM GLYCOPROTEINS

*

APPENDIX THREE

THE BIOSYNTHESIS OF O-LINKED OLIGOSACCHARIDES

*

APPENDIX ONE

GENERAL METHODS AND MATERIALS

Methods and materials that are considered to be of general application throughout the work described in this thesis are described in this appendix. Other methods are given in the individual chapters.

A1.1 *Materials*

Bio-Gel P4 (200-400mesh) and (-400mesh), Dowex AG-50 X12, AG-3 X4A and Chelex-100 were obtained from Bio-Rad Laboratories Ltd, (Watford, Hertfordshire, UK), QAE-Sephadex A-25 was obtained from Pharmacia-LKB (UK) Ltd (Milton Keynes, Buckinghamshire, UK). Chromatography paper was obtained from Whatman, Maidstone, Kent, UK.

Enzymes: *Canavalia ensiformis* (jack bean) β-hexosaminidase, β-galactosidase and α-mannosidase, coffee bean α-galactosidase and *Aspergillus saitoi* α-mannosidase were purified in the Glycobiology Unit, *Streptococcus pneumoniae* β-hexosaminidase and β-galactosidase, bovine epidydimal β-galactosidase, *Bacteriodes fragillis* endo-β-galactosidase and *Streptomyces plicatus* endoglycosidase H were obtained from Boehringer Mannheim, (Lewis, East Sussex, UK). *Artherobacter ureafaciens* neuraminidase was purchased from Calbiochem (Cambridge Bioscience, Cambridge, UK). bovine epididymal α-fucosidase and bovine intestinal alkaline phosphatase were obtained from Sigma (Poole, Dorset, UK). Attenuated Newcastle disease virus was obtained from Gist-Brocades Animal Health, UK. In addition the jack

bean β-hexosaminidase used in the mixed glycosidase reaction to determine terminal β-galactose substitution was obtained from Sigma (UK).

Bovine intestinal alkaline phosphatase was purchased from Sigma.

Lectins: Concanavalin A agarose was obtained from Pharmacia-LKB. Jacalin agarose was purchased from Pierce (Chester, UK).

Proteins: Purified IgA1 and IgA2 from human serum were purchased from Calbiochem. Bovine siderophillin was obtained from Sigma.

Antisera: All antisera were purchased from Sigma. Anti-immunoglobulin antisera were heavy-chain specific. Antisera to whole human serum, immunoglobulin M and immunoglobulin G were raised in rabbit, and the antisera against human serum albumin and immunoglobulin A were raised in goat. Standard human serum as an immunoelectrophoresis marker was obtained from Sigma.

Carbohydrates: Transferrin oligosaccharides were obtained by hydrazinolysis of bovine siderophillin (Sigma) and purification of the oligosaccharides as described below. Bovine kidney heparan sulphate, glucose-6-sulphate (potassium salt), N-acetylglucosamine-3 and 6-sulphates (sodium salts) and colominic acid (from *Escherichia coli*) were purchased from Sigma. Isomaltose oligosaccharide standards for gel filtration were obtained by partial hydrolysis of dextran (Sigma) in 0.1M HCl at 100°C for 4 hours. Lactone glycosidase inhibitors were purchased from Sigma. Scylloinositol was purchased from Calbiochem. Lactose and bovine sialyllactose were obtained from Sigma. Radiolabelling of sugars by reduction was performed by the author.

Radioactivity: Tritiated sodium borohydride (10Ci/mmol) was obtained from New England Nuclear, USA.

Suppliers of other specific reagents used in particular pieces of work

can be found in the relevant chapters or are given below.

All general reagents used, unless otherwise stated, were of analytical grade or higher. For mass spectrometry Aristar reagents were used throughout.

A1.2 *Methods*

A1.2.1 *Oligosaccharide Methodology*

A1.2.1.1 Preparations

A1.2.1.1.1 Hydrazinolysis

Purified glycoproteins or body fluids were exhaustively dialysed against double distilled water in prepared dialysis tubing or in a flow dialyser (BRL, Bethesda, USA) at 4°C with a 12 kdal cut off membrane, lyophilised in virgin plastic tubes or acid washed glass vessels and then placed in acid washed hydrazinolysis tubes and again freeze dried. The hydrazinolysis tubes were then equipped with a valve and the samples were cryogenically dried by placing the tubes over activated charcoal in one arm of an inverted U-shaped vessel. The vessel was then evacuated and the pressure brought down to <0.1 Pa by placing the second arm in liquid nitrogen (-196°C). The samples were dried in this manner for at least 48 hours. The vessel was repressurised with dry argon and 200-500µl of fresh double-vacuum-distilled anhydrous hydrazine (25°C, 1.3kPa) (Aldrich, Gillingham, Dorset, UK) was immediately added with a glass syringe and the tubes flame sealed. Hydrazinolysis was carried out by heating the tubes in

an oven with a temperature ramp of 10°/hr from 30°C to 85°C followed by 12hrs at a constant temperature of 85°C. The tubes were then removed from the oven and allowed to cool to room temperature.

A1.2.1.1.2 Purification of N-linked Oligosaccharides

The hydrazine was removed by cracking open the hydrazinolysis tube with a glass knife and a 'hot finger' followed by reduced pressure evaporation at 25°C. Residual hydrazine was removed by five co-evaporations of 200µl each of freshly distilled anhydrous toluene at reduced pressure (BDH, Bristol, UK).

The tube was kept on solid CO_2 until re-N-acetylation was performed. Re-N-acetylation was performed within one hour to minimise degradation of the released oligosaccharides and was achieved by the addition of cold saturated sodium bicarbonate (Fluka, Glossop, Derbyshire, UK) immediately followed by an aliquot of acetic anhydride (Fluka), and incubated for ten minutes on wet ice. The tubes were then allowed to warm to ambient and a second aliquot of acetic anhydride was added, and the incubation continued for a further 50 minutes. A total of a ten fold molar excess of acetic anhydride over amino acids was used, added as the two aliqouts. Sufficient sodium bicarbonate was used so that at the end of the reaction the acetic anhydride had hydrolysed to give a one molar solution of acetic acid in sodium bicarbonate.

Following the second incubation the sample was applied to a column of Dowex AG50 (H^+ form) with a five fold excess of the resin over sodium ions and the column washed with five column volumes of water and dried by rotary evaporation at 27°C. The material was then spotted onto water-washed

3 x 50cm 3MM paper strips. The paper was subjected to descending paper chromatography in butan-1-ol/ethanol/water (4/1/1, v/v/v) (Solvent I) for 48hrs at 30°C to remove remaining proteinaceous material. The strips were then dried in air and the origin inspected for fluoresence under long wavelength ultraviolet light. Peptidic material is highly fluorescent at this wavelength and can be easily visualised. Oligosaccharides are less fluorescent than the chromatography paper, and thus appear as a dark area on the paper background. After 48hrs of chromatography the peptidic material can be seen as a smear towards the bottom of the paper strip, and the oligosaccharides can usually be seen at the origin. The origin of the chromatogram (-1 to +3cm, or -1 to +1cm for total serum samples) was eluted with water and then dried by rotary evaporation at 27°C.

Residual hydrazones were removed by treatment with 1mM copper (II) acetate, at a five fold molar excess of copper over the calculated amount of oligosaccharide, in a stoppered glass tube at 30°C for 45 minutes. The solution was then desalted on a double tandem column of Chelex 100 (Na^+ form) and 50µl of AG50 (H^+ form). A ten fold excess of Chelex over copper ions was used. The column was washed with five column volumes of water and the sample filtered through a 0.45µm PTFE Millex SV filter (Millipore, Harrow, UK). The sample was then dried by rotary evaporation at 27°C and the residue taken up in 100µl of water. This solution was then transfered to a small glass tear-drop vial, dried in a Speedvac by reduced pressure and rehydrated with 20µl of water. Samples were stored, capped with PTFE tape, at -20°C until reduction.

A1.2.1.1.3 Radiochemical Reduction

Immediately prior to reduction the samples were dried in a Speedvac as above. The samples were radiolabelled with tritiated sodium borohydride at 6mM in 50mM sodium hydroxide adjusted to pH 11.0 with saturated boric acid in a reaction volume calculated to give 1mM oligosaccharide. The reductant reagent must be made up immediately prior to use to ensure maximal incorporation of radiolabel because at this pH the borohydride will hydrolyse at a slow but appreciable rate.

The vial was then sealed with PTFE tape and incubated for 4hrs at 30°C after which an equal volume of 1M sodium borodeuteride (Fluka) in sodium hydroxide/boric acid buffer pH 11.0 was added to complete the reduction. The incubation was continued for a further 2hrs. The pH of the reaction is critical for efficiency of incorporation and has been determined to be most efficient at pH 11.0 as well as reducing the possibility of C-2 epimerisation and 'peeling' of the glycan from the reducing terminus (Ashford *et al* 1987). The use of at least a fivefold molar excess of reductant over sugar results in the cleanest incorporation of radiolabel, i.e. these conditions do not completely maximise the specific activity of the oligosaccharide, but result in the greatest incorporation relative to background.

The reaction was stopped by the addition of aliquots of 1M acetic acid until effervescence had ceased and the sample was desalted with AG50 (H^+ form) at a fivefold excess over sodium ions. The sample was dried by rotary evaporation at reduced pressure at 27°C. Boric acid was removed by repeated evaporation (5 times) with methanol acidified with 1% acetic acid (v/v) at

27°C and the residue spotted on 3 x 50cm 3MM paper strips and developed in solvent I for 48hrs. The strips were dried and the radioactivity was detected with a Packard model 7220/21 radioactivity scanner (Canberra-Packard, Pangbourne, Berkshire, UK) and the reduced glycans that remain at the origin were eluted with water, rotary evaporated to dryness at 27°C and dissolved in 1ml of water. An aliquot was withdrawn for scintillation counting.

A1.2.1.1.4 Removal of Acidic Radiochemical Contaminants

This proceedure was only used where it was known that all glycans in the mixture were either neutral or sialylated. The oligosaccharides were dried by rotary evaporation at 27°C and then dissolved in 1ml of 1M acetic acid and the solution was passed through 500µl of Dowex AG3 4A resin (OH^- form). The column was washed with 3ml of water and the eluate rotary evaporated to dryness at 27°C and taken up in 1ml of water. An aliquot was removed for scintillation counting.

A1.2.1.1.5 Large Scale Hydrazinolysis

Large scale hydrazinolysis was performed on whole murine brains as follows. Approximately six grams of lyophilized whole mouse brains were homogenised in 150ml of cold acetone (-10°C) for 3 minutes with a mechanical homogeniser. The homogenate was filtered through Whatman 50 paper in a glass scintered buchner and the residue resuspended in a second 150ml of cold acetone and homogenised and filtered as before. The final residue was dried under reduced pressure with a water pump and then a

vacuum pump. The dried material was made into a slurry with 80ml of water and exhaustively dialysed against water at 4°C. The retentate was then placed in a large scale hydrazinolysis tube and frozen. The material was then dried by lyophilization for three days and by cryogenic drying for at least one week.

To the dried residue was added 40ml of freshly distilled anhydrous hydrazine under argon and the tube sealed, incubated and freed from hydrazine as described above for small scale hydrazinolysis. The dried residue was then re-N-acetylated by the addition of 200ml of cold saturated sodium bicarbonate and 15ml of acetic anhydride. The mixture was immediately transferred to a round bottom flask and incubated with gentle stiring at 0°C for 10 minutes. The flask was then allowed to warm to ambient temperature, a second 15ml aliquot of acetic anhydride was added and the mixture incubated for a further 60 minutes at room temperature. The contents of the flask were then passed through 750ml of AG50 resin (H^+ form) and the column washed with five column volumes of water. The eluate was collected and reduced in volume in a rotary evaporator at 30°C, and the material was then freeze dried.

The material, about 200mg, was loaded onto a fined microcrystalline cellulose column (60ml, Whatman) equilibrated in solvent I. The column was eluted first with 5 column volumes of solvent I, 1 column volume of methanol and finally 5 column volumes of water. The water eluate was collected seperately, and rotary evaporated and lyophilised to dryness as before.

Residual hydrazones were removed by incubation with copper (II) acetate as described above. Treatment of an aliquot of this material with AG3 (OH^- form) as described above was performed (see chapter two) but was

discontinued in later experiments. In addition some material was taken for radiochemical reduction as described above.

A1.2.1.1.6 Release of O-Linked Oligosaccharides

A modification of the method of Amato *et al* (1988) was used. Three milligrams of purified glycoprotein were transfered to a glass hydrazinolysis tube equipped with a reactivalve. The oligosaccharides were released by the addition of 2ml 50mM sodium hydroxide containing 1M sodium borodeuteride (Fluka) and 10mCi [^{3}H] sodium borohydride (NEN) and incubation for 18hrs at 50°C.

The reaction was stopped by the addition of 4M acetic acid until effervescence had ceased. Sodium ions were removed by desalting on a 1ml column of Dowex AG50 (H^{+}) in water. Boric acid was removed by repeated evaporation with acidified methanol as above and the residue applied to a 50 x 5cm strip of 3MM paper (Whatman), and developed by descending paper chromatography at 30°C in solvent I for 24hrs. A parallel track containing tritium-reduced N-acetylglucosaminitol, lactitol and bovine sialyllactitol was also run on the same paper strip to ensure correct localisation of the released oligosaccharidi tols. After 24hrs the strip was dried and radioactivity detected with a Berthold model LB2432 linear analyser (Lab Impex, Twickenham, Middlesex, UK) and data accumulated on a 9386 Hewlett Packard (Winersh, Buckinghamshire, UK) computer. The region of the chromatogram -2 to +8 cm from the origin, containing all the radioactivity remaining on the paper, was eluted with water and rotary evaporated to dryness at 27°C. The residue was taken up in 1ml of water, and applied to a C18 SepPac cartridge (Millipore) washed with methanol and equilibrated in

water. The column was then eluted with 5ml of water and the eluate dried by rotary evaporation at 27°C and taken up in 1ml of water. An aliquot was removed for scintillation counting and the remainder was stored at -20°C.

A1.2.1.2 Fractionation

A1.2.1.2.1 Bio-Gel P4 Gel Permiation Chromatography

Before chromatography the glycans were passed through a layered column containing 100µl bed volume each of Chelex 100, Dowex AG-50(H^+ form), Dowex AG-3 X4A(OH^- form) and QAE-Sephadex A-25 (Pharmacia-LKB). The eluate was filtered through a 0.45µm teflon filter (Millex SR) and dried by rotary evaporation at 40°C. The oligosaccharides were taken up in water and 25µl of isomalto-oligosaccharides added (20mg/ml). This addition was omitted from samples that were intended for methylation or NMR analysis. This solution was applied to a high resolution gel filtration system comprising either one Bio-Gel P4 (-400 mesh) column (1.5cm x 100cm) or two of these columns in series. In addition, for fractionation of small glycans, purification of immunoglobulin A N-linked oligosaccharides, desalting purposes, or analysis of immunoglobulin G oligosaccharides following digestion with mixed exoglycosidases, a 1.5cm x 50cm column was used. The columns were maintained at 55°C and eluted with water at a flow rate of 0.2ml/min. The effluent was monitored by a Berthold HPLC radioactivity flow monitor (model LB503) and an Erma refractive index monitor (model ERC7510, HPLC Technology Ltd, UK) prior to collection by a fraction collector. Typically 1ml fractions were collected. Analog signals from the monitors were digitized using a Nelson Analytical ADC interface (Anachem, Luton,

Bedfordshire, UK) and the digital values were collected and analysed by model 9836C computer. Additionally the analog output was plotted directly on a multi-channel chart recorder, which also received event marks from the fraction collector. In cases where insufficient radioactivity was applied to the column to be detected by the flow monitor, aliquots were withdrawn from the fractions for determination of radioactivity by scintillation counting. Fractions containing radiolabelled oligosaccharides were pooled following the chromatography and concentrated by rotary evaporation at 40°C.

A1.2.1.2.2 Diaminobutane Silica HPLC.

Neutral oligosaccharides were subjected to fractionation on a Lichosorb silica SI 60 (5µm) HPLC column, 5mm x 25cm ID (Hichrom, Reading, UK) run under the conditions of Turco (1981). The column was equilibrated in acetonitrile-water (65:35 v/v) containing 0.05% diaminobutane (DAB, Aldrich) (Solvent II) at a flow rate of 1ml/min. Oligosaccharides were injected onto the column using a WISP 710B sample processor (Waters, Harrow, Middlesex, UK).

Solvent mixtures were generated by proportionating solvent II with acetonitrile-water (40:60 v/v) containing 0.05% DAB (Solvent III) using two Waters model 510 HPLC pumps controlled by a DEC PC-380 computer (Waters). The gradient elution was programmed at 100% solvent II for five minutes followed by a linear gradient to 80% solvent III over 45 minutes. The effluent was monitored with a Berthold HPLC radioactivity flow monitor , before collection. The analog signal from the monitor was collected and analysed using a DEC PC-380 computer. Fraction size was 330µl.

Radioactivity was determined in the fractions by scintillation counting. Oligosaccharides were pooled following elution and passaged through 2ml of Dowex AG50 (H^+ form) to remove DAB and rotary evaporated to dryness and taken up in water.

A1.2.1.2.3 Anion Exchange Chromatography

Anion exchange on Mono Q was performed as follows. Oligosaccharides in 200µl water were loaded onto a Mono Q HR5/5 column (Pharmacia-LKB) equilibrated in water. Fractions of 0.5ml were collected and a flow rate of 1ml/min was maintained throughout the experiment. The column was eluted for five minutes with water and then a linear gradient was applied from zero to 200mM ammonium acetate pH5.5 over 60 minutes by proportionating the water with 0.5M ammonium acetate pH5.5. The chromatography was carried out using an FPLC gradient system (Pharmacia-LKB). Oligosaccharides were detected by scintillation counting of aliquots of the fractions. In cases when oligosaccharides were recovered and pooled, the fractions were first passed through AG50 (H^+ form) resin to remove ammonium ions and then the eluate was concentrated by rotary evaporation. Residual acetic acid was removed by co-evaporation with toluene by rotary evaporation and finally by high vacuum drying with a two stage vacuum pump equipped with a liquid nitrogen trap. The oligosaccharides were then taken up in water and stored at -20°C.

Anion exchange on MicroPac AX5 was performed essentially as described by Green and Baenziger (1986). The precise conditions of the elution were optimised by the author. Oligosaccharides in water were injected onto a MicroPac AX-5 (5µm 25cm x 5mm ID) column (Jones Scientific, Hengoed, Mid Glamorganshire, UK) equilibrated in water using a WISP 710B sample

processor. Flow rate was maintained at 1ml/min. Elution with phosphate buffer at pH4.0 as described by Green and Baenziger was performed with a two segment, linear gradient of zero to 125mM sodium phosphate buffer from zero to 15 minutes, and up to 500mM buffer from 15 to 40 minutes. Solvent mixtures were generated by proportionating the flow from two Waters model 510 HPLC pumps controlled by a PC-380 computer. The effluent was monitored with a Berthold HPLC radioactivity flow monitor, before collection. The analog signal from the monitor was collected and analysed using a DEC PC-380 computer. Latterly a modification to this method was devised by the author using sodium acetate as the eluant. The sample was loaded in water onto the column which was equilibrated in 2mM sodium acetate buffer pH 4.0. Uncharged material was eluted with this buffer and then the charged oligosaccharides were eluted with a two segment linear gradient to 0.5M sodium acetate at 25 minutes and then to 1.0M sodium acetate at 80 minutes. Eluant flow rate was maintained at 1ml/min throughout the experiment.

A1.2.1.2.4 Concanavalin A Affinity Chromatography

Affinity chromatography of mixtures of neutral oligosaccharides on Concanavalin A, the lectin from *Canavalia ensiformis*, was performed as described below. A glass column 0.6cm x 20cm (Altex) was packed with 10ml of Con A Sepharose (Pharmacia-LKB) in 100mM sodium acetate buffer, pH5.0, allowed to settle and pumped with a peristaltic pump at 10ml/hr. The column was washed with 50ml of 0.1M HCl to convert the lectin to the monomer form, followed by 50ml of 10mM sodium acetate buffer pH 5.0 containing 5mM methyl α-D-mannopyranoside (Sigma), 5mM $MnCl_2$ and 5mM $CaCl_2$. All subsequent steps were performed at 4°C. The column was then equilibrated in 0.1M sodium

acetate pH5.0 with 5mM $MnCl_2$ and $CaCl_2$ to remove the methylmannoside. The sample, in 200µl of water, was loaded on to the column by the peristaltic pump and allowed to remain within the gel volume for at least 30 minutes. The column was then sequentially eluted with 20ml of the following solutions at 10ml/hr; 10mM sodium acetate pH5.0 containing 100mM NaCl and 1mM $MnCl_2/CaCl_2$ (Buffer A), 10mM sodium acetate pH5.0 containing 20mM methylmannoside and 1mM $MnCl_2/CaCl_2$ (Buffer B), and finally 100mM HCl (Buffer C). The eluate was collected as 0.5ml fractions and radioactivity was detected by scintillation counting. Tri- or tetraantennary N-linked glycans as well as those biantennary glycans with a bisected core are eluted with buffer A. Buffer B elutes the non-bisected biantennary glycans and some of the hybrid structures, whilst buffer C elutes the oligomannose oligosaccharides as well as those hybrid glycans that carry a large number of mannosyl residues on the manα1→6 arm. The oligosaccharides were pooled and metal ions removed with Dowex AG50 (H^+ form) and the chloride ions with AG3 (OH^- form). Further purification of the oligosaccharides was performed by gel filtration on Bio-Gel P4 columns eluted with water.

A1.2.1.2.5 Jack Fruit Lectin Affinity Chromatography

Affinity chromatography with the lectin from *Arthocarpus integrifolia* lectin (jacalin) was performed using lectin immobilised on agarose (Pierce). The gel was packed into a glass column to a 1ml bed volume. All operations were performed at room temperature. The column was pumped at 20ml/hr and equilibrated in PBS (8g/l NaCl, 0.2g/l KCl, 1.45g/l Na_2PO_4 and 0.2g/l KH_2PO_4, pH 7.2). Oligosaccharides were pumped onto the column in 100µl of water and allowed to remain within the bed of the column for at

least 30 minutes by stopping the flow. The unbound oligosaccharides were eluted with PBS at 20ml/hr and 10ml fractions were collected. Bound oligosaccharides were eluted from the lectin with 800mM galactose (Sigma) in PBS with the same flow rate and fraction size. For analysis of the oligosaccharides on analytical Bio-Gel P4 columns the oligosaccharides were first desalted by passage through a 1.5cm x 30cm Bio-Gel P4 (200-400 mesh) column equilibrated in water, with a flow rate of 1ml/min delivered from a P500 pump (Pharmacia-LKB). Fractions of 1ml were collected. The eluate was monitored with a conductivity flow cell (BioRad), and the radiolabelled oligosaccharides were located by scintillation counting of aliquots of the fractions. The oligosaccharides were pooled and concentrated by rotary evaporation before processing further.

A1.2.1.2.6 High Voltage Paper Electrophoresis

High voltage electrophoresis (HVE) of reduced glycans in pyridine/acetic acid/water (3:1:387 v/v/v) pH5.4 was performed at 80V/cm on 20cm x 43cm 3MM paper sheets (Whatman) in a Camag electrophoresis cell (Geneva, Switzerland). [^{3}H]-reduced lactitol and bovine sialyllactitol were run in a seperate track as standards and migration distance was monitored visually with bromophenol blue (Sigma). The paper was wetted with the buffer prior to electrophoresis. Electrophoresis was continued for 45 to 90 miniutes depending on the experiment being performed. The cell platen was maintained at 15°C by a circulating water bath. After completion of the experiment the paper was dried and scanned using a Berthold linear analyser and data were collected and analysed on a Hewlett Packard 9836C computer.

High voltage electrophoresis in borate buffer: reduced saccharides were

applied to a 12.5cm x 120cm piece of Whatman 1 paper and the paper wetted with 60mM sodium tetraborate buffer pH 9.5 and then electrophoresed using the same buffer for 5 hours at 8.5kV/m in a custom built 1.2m flat-bed high-voltage electrophoresis unit (Locarte, London, UK). The platen was maintained at 20°C throughout the experiment. A set of standard ^{3}H-monosaccharidi tols were run on a parallel track when the technique was used for monosaccharide analysis, and bromophenol blue was used as a migration marker in all cases. When monosaccharides were being separated electrophoresis was carried out for five hours. For complex oligosaccharides the experiment was continued for 18 hours, and a second drop of bromophenol blue was placed at the origin after 8 hours. Following electrophoresis the paper was dried and the radioactivity localised with a linear scanner as described above. Identification of monosaccharides was made by comparison of the positions of the unknown and the standard sugar alditols, using the internal standard [^{3}H]-deoxyribose as a calibration point. Oligosaccharides were recovered from the paper by eluting with water. The eluate was concentrated to 1ml by rotary evaporation at 40°C, and the solution was passed through 500µl of Dowex AG50(H^{+}). The column was washed with five column volumes of water, and the combined eluate was taken to dryness by rotary evaporation. Boric acid was removed by five co-evaporations from acidified methanol (methanol/acetic acid 100/1 (v/v)).

A1.2.1.2.7 Descending Paper Chromatography Following High Voltage Electrophoresis in Pyridine Acetate

In order to remove an electrolysis product that arises from the pyridine in pyridine/acetate paper electrophoresis, and which is a potent

inhibitor of sialidases, oligosaccharides were applied to a 3cm x 50cm 3MM paper strip (Whatman) following HVE, and subjected to descending chromatography in pyridine/ethyl acetate/acetic acid/water (5:5:1:3 v/v/v/v) for 4hrs at 30°C. The paper was then dried and scanned as described above and the oligosaccharides eluted with water. The inhibitory compound chromatographs at the solvent front whilst the oligosaccharides migrate a shorter distance (R_f <0.5).

A1.2.1.3 Analysis

A1.2.1.3.1 Gas-Chromatography Mass-Spectrometry (GCMS)

All GCMS analysis was performed in the electron impact mode using a Hewlett Packard 5996 instrument with a CP-SIL-8 30m capillary column (Supelco, Saffron Walden, Essex, UK), I.D. 0.25mm or a SP2860 60m capillary column (Jones Scientific, ID 0.25mm). Quantitation was achieved by flame ionisation detection. Gas-chromatography (GC) data were recorded on a Hewlett Packard integrator (model 3393A).

For analysis of partially methylated alditol acetates (section A1.2.1.3.2) the GC oven had the following programme; 90°C to 140°C at 30°/minute, 140°C to 250°C at 5°/minute. In some cases the following programme was used, 90°C to 140°C at 30°/minute, 140°C to 250°C at 5°C/minute with selective ion monitoring (SIM) of the following m/z 319, 318, 291, 277, 259, 247, 245, 234, 217, 185, 161, 143, 131, 129, 118, 89, with a dwell time on each ion of 20mS. In addition the N-glycans from human cerebrospinal fluid were analysed by SIM on the CP-SIL-8 column and the SP6320 column. The GC oven conditions for the SP2860 were 90°C isothermal

for 1 minute, 90°C to 170°C at 30° per minute, 170°C to 230°C at 5° per minute, and 230°C to 231°C at 5° per minute, and isothermal at 251°C. Mass-Spectral (MS) data were collected by SIM of ions at the following m/z; 101, 102, 118, 129, 130, 145, 149, 161, 162, 173, 189, 190, 205, 233, 234, and 333, with a dwell time of 20mS.

For analysis of trimetylsilane methylglycosides (section A1.2.1.3.3) the GC oven had the following programme; 90°C to 140°C at 30°/minute, 140°C to 180°C at 4°/minute, 180°C to 240°C at 10°/minute.

Analysis of per-TMS derivatives of sialic acids (section A1.2.1.3.4) was performed by SIM with a dwell time of 20mS, and the following ions were monitored; m/z = 814, 726, 712, 624, 536, 444, 375, 356, 261, 205 and 173. The ions at m/z 814, 712, 444 and 261 are diagnostic for N-glycoyl-neuraminic acid, those at m/z 726, 536, 356, and 173 are diagnostic for N-acetyl-neuraminic acid and the remainder are common to both the sialic acid derivatives. The GC oven had the following programme; 90°C to 140°C at 30°/minute, 140°C to 180°C at 4°/minute, 180°C to 240°C at 10°/minute.

A1.2.1.3.2 Methylation Analysis

Purified oligosaccharides were permethylated as described by Ciucani and Kerek (1984), and converted to their partially methylated alditol acetates (PMAAs) by the method of Ferguson *et al* (1988).

The oligosaccharide sample was dried in a Speedvac in a reactivial (Pierce) with a wedge shaped PTFE stir bar. 50µl of dry DMSO (Fluka) was added and the sample sonicated for 20 minutes. 50µl of DMSO containing 120mg/ml sodium hydroxide, ground in a glass mortar and pestle, was added and the mixture stirred for 30 minutes. Three 10µl aliquots of methyliodide

(Fluka) were added at 10 minute intervals and the reaction stirred throughout. 300µl of chloroform and 1ml of sodium thiosulphate (100mg/ml) was then added and the vial mixed and centrifuged. The aqueous phase was removed and the organic phase washed four times with 1ml of water, and then dried in a Speedvac.

Hydrolysis of the glycosidic linkages was performed by the addition of 100µl of 0.25M sulphuric acid in 93% aqueous acetic acid (v/v) to the dried oligosaccharides and incubation for 150 minutes at 80°C. The hydrolysate was desalted by passage through a column of AG3 (acetate form, 0.5ml) equilibrated and eluted with 50% methanol (aq). The sample was dried by rotary evaporation and residual acetic acid removed by co-evaporation with toluene. The monosaccharides were reduced with 200µl of sodium borodeuteride (10mg/ml, Fluka), in water, at 30°C, for 150 minutes, and neutralised by two additions of 100µl glacial acetic acid. Boric acid was removed by five evaporations from acidified methanol and the excess acetic acid drawn off by high vacuum.

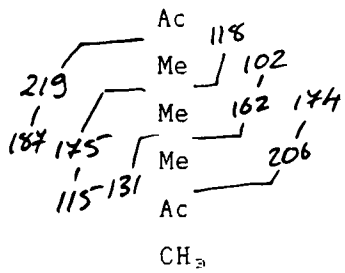
Per-acetylation was then achieved by the addition of 250µl of acetic anhydride to the material, followed by incubation at 100°C for 120 minutes, after which the anhydride was removed with reduced pressure. The PMAAs were dissolved in 500µl of dichloromethane and the organic phase washed with 1ml of water to remove residual sodium acetate and other contaminants. The organic phase was recovered, dried and taken up in a small volume (20µl) of dichloromethane and an aliquot used for GCMS. A transferrin biantennary oligosaccharide was included amongst each batch of methylation samples to monitor the quality of the procedure.

Table A1.1

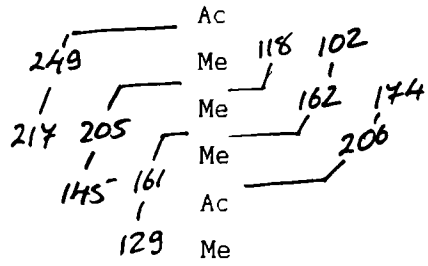
FRAGMENTATION PATTERNS OF PARTIALLY METHYLATED ALDITOL ACETATE MONOSACCHARIDE DERIVATIVES PRODUCED BY MASS SPECTROMETRIC ANALYSIS IN ELECTRON IMPACT MODE

Neutral Sugars

Terminal Residues

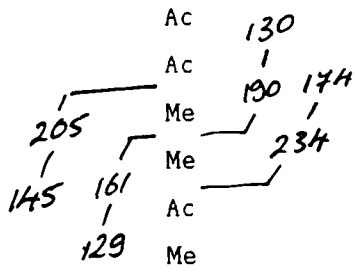


T-6-deoxyhexose-*p*

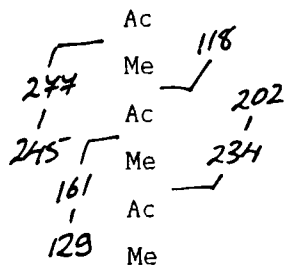


T-hexose-*p*

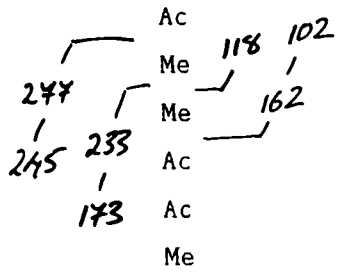
Monosubstituted



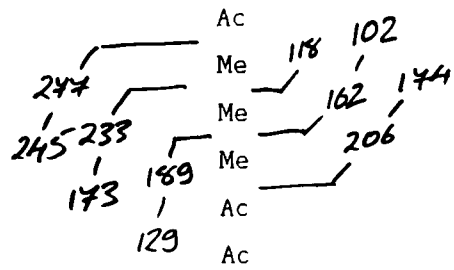
2-hexose-*p*



3-hexose-*p*

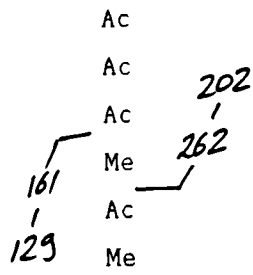


4-hexose-*p*

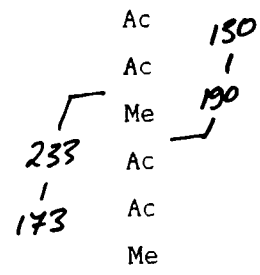


6-hexose-*p*

Disubstituted



2, 3-hexose-*p*



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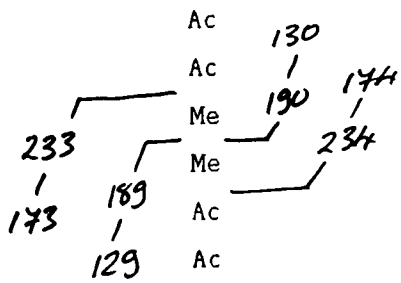

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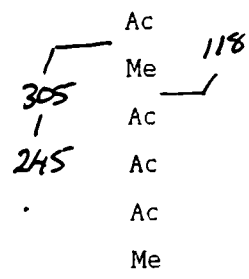
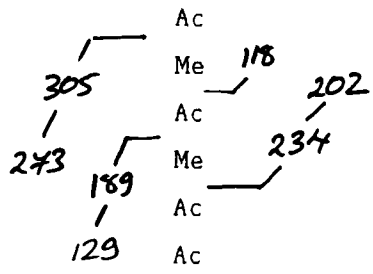


3, 4-hexose-*p*



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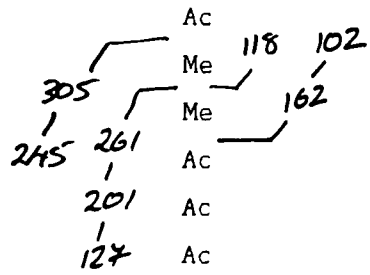
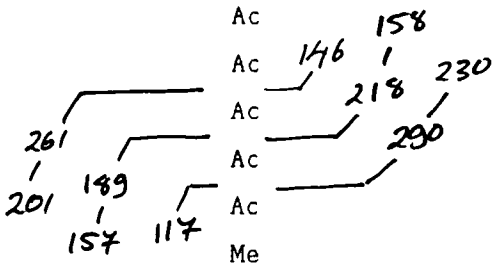
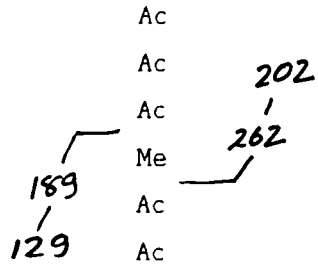


4, 6-hexose-*p*

Trisubstituted



2, 3, 4-hexose-*p*



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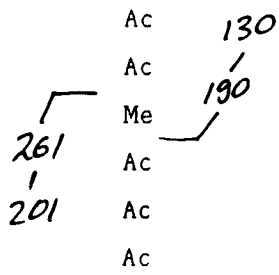

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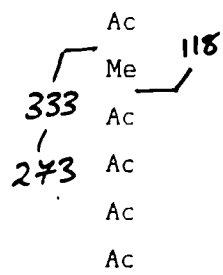
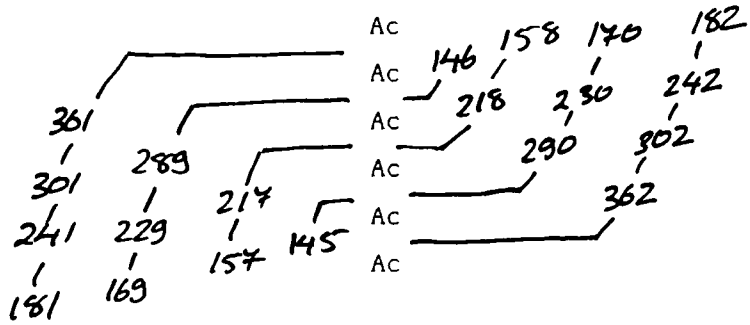


3, 4, 6-hexose-*p*

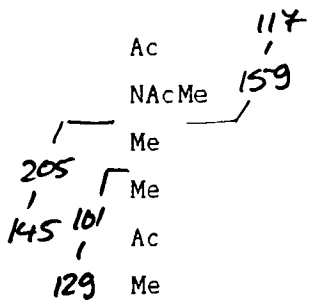
Tetrasubstituted



2, 3, 4, 6-hexose-*p*

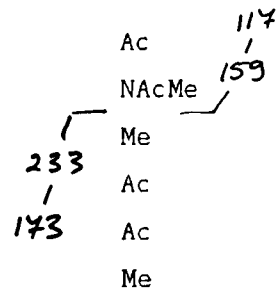
Amino Sugars

Terminal



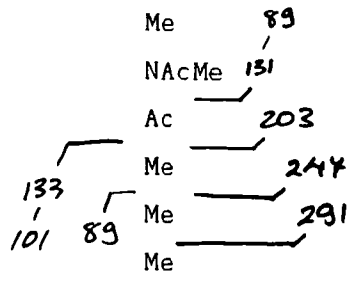
T-hexosamine-*p*

Monosubstituted

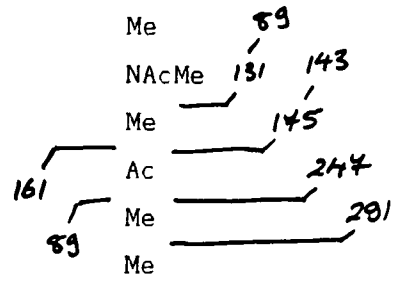


4-hexosamine-*p*

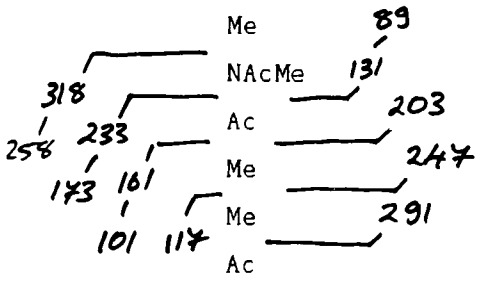
Reducing Terminal Sugars



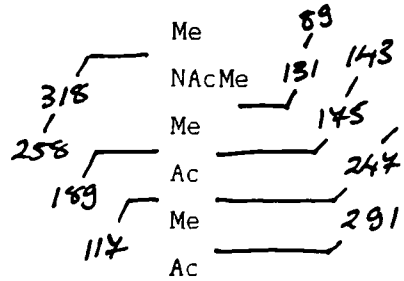
3-hexosaminitol



4-hexosaminitol



3, 6-hexosaminitol



4, 6-hexosaminitol

Notes for table A1.1; All mass fragments quoted are for the deuterium reduced residue.
The diagrams are arranged so that the anomeric carbon (C1) is upper-most. Carbons that carry methyl groups are indicated by Me, and those that are acetylated are indicated by Ac. NAcMe indicates the amine function in the amino sugars, which is monomethylated by this proceedure. Only the diagnostic and strong ions are given in this table. .
Each derivative is named as the residue from which it was derived, and is not given the full systematic name, for reasons of clarity of presentation. A full treatment of this method of analysis is availible in Biermann and McGinnis (1989). Fragmentation for per-O-TMS esters of neuraminic acids can be found in Schauer (1987).

A1.2.1.3.3 Composition Analysis

Composition of oligosaccharides was performed by conversion of the glycans to their trimethylsilane-methylglycosides. This was achieved as follows; 10nm of scylloinositol was added as an internal standard to the oligosaccharides which were then dried down in a glass capillary tube that had been flame sealed at one end. 50µl of dry 0.5M methanolic-HCl (Supelco) and 10µl of methyl acetate (Aldrich) were then added. The vial was then flame sealed and incubated at 80°C for 16hrs. Following the methanolysis the vial was cracked open and the oligosaccharides re-N-acetylated by the addition of 10µl of pyridine followed by 5µl of acetic anhydride. After 30 minutes incubation at room temperature the sample was dried in a speedvac and further dried by flash evaporation with 100µl of dry methanol. Then 15µl of a proprietry TMS reagent, Sigma-sil A, (Sigma) (trimethylchlorosilane: hexamethyldisilazane: pyridine 1:3:9 v/v/v) was added and the methylglycosides incubated for two hours by placing the tube in a sealed plastic vessel at room temperature containing dessicant before analysis by GCMS.

A1.2.1.3.4 Sialic Acid Analysis

Analysis was performed according to the method of Schauer (1987). The sialic acid glycosidic bonds were hydrolysed with 100µl 0.1M HCl (aq) (Pierce, sequenal grade) for 2 hours at 85°C in sealed capillary tubes, which does not remove N-substituents. The sugars were dried in a Speedvac and flashed once with methanol, and converted to the per-O-TMS glycosides by the addition of 15µl Sigma-sil A. Aliquots were taken for analysis by

GCMS. The standard sugars, N-glycoyl-neuraminic acid and bovine sialyllactose were obtained from Sigma. Using a CP-SIL-8 capillary GC column, run under the conditions described in section A.1.2.1.3.1, it was determined that the N-glycoyl derivative eluted with a R_T of 1.1 compared to N-acetyl-neuraminic acid.

A1.2.1.3.5 Nuclear Magnetic Resonance Spectroscopy (NMR)

Neutral oligosaccharides for NMR analysis were prepared by passage through a layered column containing 100µl each of Chelex 100 (Na^+ form), AG50 (H^+ form), AG3 (OH^- form) and QAE-Sephadex, followed by filtration through a 0.45µm PTFE filter. Charged oligosaccharides were prepared for NMR by passage through a layered column of 100µl Chelex 100 (Na^+ form) and 100µl AG50 (H^+ form) and filtered through a 0.45µm PTFE filter. The oligosaccharides were deuterated by repetitive dissolution in 99.96% D_2O (Aldrich) with intermediate evaporation. Samples were finally dissolved in 400µl 99.996% D_2O (Aldrich) and analysed in precision bore glass NMR tubes (Aldrich).

Fourier transform NMR spectra of neutral oligosaccharides were recorded at 500MHz on a Bruker AM500 NMR spectrometer, driven by an Aspect 3000 computer at a probe temperature of 300K. A total of 8192 real data points were collected in the free induction decay (FID) mode, with a sweep width of 3496Hz, and the data was zero filled to 16k points for fourier transform analysis. Charged oligosaccharides were analysed by fourier transform NMR at 600MHz with a Bruker AM600 spectrometer at a probe temperature of 300K. A total of 250 scans were collected with a sweep width of 3496Hz. A total

of 8192 real data points collected in the FID mode, and the data zero filled to 16K for fourier transform analysis.

A1.2.1.3.6 Enzymatic Treatments

The activities of the exoglycosidases were determined by assay with paranitrophenol-glycosides (PNP-glycosides) or by other personel in this laboratory against oligosaccharide substrates. For PNP-glycoside determinations a reaction mixture containing the relevant PNP-glycoside at 10mM, with 1mg/ml BSA, in a buffer of the same composition as used for the oligosaccharide analysis. Aliquots of the reaction mixture, typically 100µl, were added to 1.5ml eppendorf tubes and the tube warmed to 37°C. A small volume of enzyme, typically 5µl was added and the solution incubated for 15 or 30 minutes. The reaction was stopped and the free PNP visualised by the addition of 1ml of 0.5M sodium carbonate. The A_{400nm} was determined. A reagent blank must be included. The activity can be calculated with the following formula;

$$\text{Activity (Units)} = \frac{(A_{400nm[sample]} - A_{400nm[blank]}) \times 200}{17700 \times \text{time of incubation in minutes}}$$

One unit is defined as that amount of enzyme which hydrolyses 1µmol of substrate in one minute. It is important to realise that the activity determined against a PNP-glycoside does not necessarily relate to the activity against a complex oligosaccharide.

For the determination of the level of non-reducing terminal

β-galactosyl residues in a mixture of complex glycans the oligosaccharides were incubated with the following mixture of exoglycosidases; 100U/ml jack bean β-hexosaminidase, 4U/ml bovine epididymal α-fucosidase, and 5U/ml *A. ureafaciens* neuraminidase in 0.1M citrate-phosphate buffer, pH5.5, with 0.1mM sodium azide. The mixture also contained γ-galactonolactone (Sigma) at 10mg/ml to inhibit galactosidase activity.

Enzyme digestion conditions are given in table A1.2. In cases where a low substrate concentration was present (>10pmoles), the volume of the digest was decreased to 50% of the standard reaction volume to increase the substrate concentration. After incubation at 37°C for 18 hours the cap of the microcentrifuge tube was removed, and the digestion continued for a further 90 minutes, after which the sample was rehydrated in 50μl of water and stored at -20°C. Relevant enzyme cleavage specificities are shown in table A1.3.

Analysis of exoglycosidase digestions by Bio-Gel P4 chromatography depends on the ability to obtain structural data from the hydrodynamic volume of an oligosaccharide (Kobata 1984). Because the radiolabelled glycan is co-chromatographed with isomaltose oligosaccharides accurate hydrodynamic volume in glucose unit (gu) equivalents to ±0.2 glucose units (gu) can be obtained. The monosaccharide components of oligosaccharides contribute various volumes to the overall elution position of a glycan (Kobata 1987). For most purposes the following values are used; galactose 1.0gu, mannose 0.9gu, fucose 0.8gu, N-acetylaminosugars 2.0gu, and bisecting GlcNAc contributes 0.5gu, whilst reduction of the reducing terminal residue adds a further 0.5gu to the overall hydrodynamic volume.

Table A1.2

GLYCOSIDASE DIGESTION CONDITIONS

Enzyme	Reaction Volume (µl)	[Enzyme]/Uml^{-1}	Buffer
Jack Bean β-Galactosidase	30	10	A
Streptococcus pneumoniae β-Galactosidase	25	0.2	D
Bovine epididymal β-Galactosidase	25	2	A
Coffee Bean α-Galactosidase	15	5	E
Jack Bean β-Hexosaminidase	20	10	B
Streptococcus pneumoniae β-Hexosaminidase	20	0.3	D
Streptococcus pneumoniae β-Hexosaminidase (Bisect sensitive)	30	0.008	D*
Jack Bean α-Mannosidase	30	50	C
Aspergillus saitoi α-Mannosidase	20	2µg/ml	F
Bovine epididymal α-Fucosidase	15	2	D
Yeast α-Glucosidase	50	50	K*
Bacteriodes fragillis Endo-β-Galactosidase	20	0.5	E
Streptomyces plicatus Endoglycosidase H	50	0.01	D
Flavobacterium meningosepticum Peptide-N-Glycanase	100	0.05	H
Artherobacter ureafaciens Neuraminidase	60	20	G

Table A1.2 (continued)

Enzyme	Reaction Volume (µl)	[Enzyme]/Uml^{-1}	Buffer
Newcastle disease virus Neuraminidase	50	*	G*
Outerarm Galactose Exoglycosidase Mix	15	See text	J
Bovine Intestinal Alkaline Phosphatase	50†	60	I

Conditions: all digestions were performed at 37°C for 18 hours in 1.5ml microcentrifuge tubes unless otherwise indicated.

Buffer compositions;

A 0.1M citrate-phosphate pH 3.5
B 0.1M citrate-phosphate pH 4.0
C 0.1M citrate-phosphate pH 4.5
D 0.1M citrate-phosphate pH 6.0
E 0.1M sodium acetate pH 5.8
F 0.1M sodium acetate pH 6.0
G 0.1M sodium acetate pH 5.0
H 50mM Tris-HCl pH 7.0
I 0.1M ammonium bicarbonate
J 0.1M citrate-phosphate pH 5.5
K 0.1M Potassium phosphate pH 7.2

§ Bovine serum albumin added at 1mg/ml as stabiliser.
† Digestion continued for 44 hours.
* Digestion continued for 30 hours.
* For Newcastle disease virus neuraminidase digestions a suspension of attenuated virions was used, containing 1 x 10^9 EID_{50}/ml.
ᵛ The buffer also contained a cocktail of lactones (galactonolactone, mannonolactone, fuctonolactone, glucuronic acid lactone, and N-acetylgalactosaminyllactone) at 2mg/ml each.

Table A1.3

GLYCOSIDASE SPECIFICITIES

Enzyme	Specificity
Jack Bean β-Galactosidase	Galβ1→6>4>3
Strepococcus pneumoniae β-Galactosidase	Galβ1→4
Bovine epididymal β-Galactosidase	Galβ1→3/4 (1→6 slowly)
Coffee Bean α-Galactosidase	Pan
Jack Bean β-Hexosaminidase	HexNAcβ1→2/3/6
Streptococcus pneumoniae β-Hexosaminidase	Pan
Streptococcus pneumoniae β-Hexosaminidase (Bisect sensitive)	HexNAcβ1→2 (1→6 slowly, and steric considerations[#])
Jack Bean α-Mannosidase	Manα1→2=6>3
Aspergillus saitoi α-Mannosidase I	Manα1→2
Bovine epididymal α-Fucosidase	Fucα1→6 (residual α1→3 activity in commercial preparation[*])
Yeast α-Glucosidase	Pan[*] (Glcα1→6 slowly)
Bacteriodes fragillis Endo-β-Galactosidase	GlcNAcβ1→3Galβ1→↓→4(3)GlcNAc[¶]
Streptomyces plicatus Endoglycosidase H	Complex (see text)
Artherobacter ureafaciens Neuraminidase	NeuNAcα2→6/3
Newcastle disease virus Neuraminidase	NeuNAcα2→3

Table A1.3 continued

Notes:

- * Fucα1→3 activity detected by the author.
- ** Branched, fucosylated and sulphated lactosamine structures are not split.
- *** This enzyme has complex properties. At low concentrations the enzyme will cleave HexNAcβ1→2 linkages only. In addition, the presence of a bisecting GlcNAcβ1→4 on the core β-mannose residue of a biantennary oligosaccharide inhibits the cleavage of the sequence GlcNAcβ1→2Manα1→6(GlcNAcβ1→4)Manβ1→4-R, but GlcNAcβ1→2Manα1→3(GlcNAcβ1→4)Manβ1→4-R is cleaved at the β1→2 linkage. At high concentrations the enzyme appears to be pan specific, and allows quantitative removal of all proximal arm GlcNAc residues
- ** Yeast α-Glucosidase used in this thesis for the partial removal of dextran from radiolabelled oligosaccharide alditols.

Many of the enzymes show differential activity depending on the size of the substrate. The conditions given in table A1.2 have been determined to be effective on oligosaccharide alditols as studied in this thesis, for a substrate at 1-20nMoles per reaction.

Sources of information: Kobata (1984), Beeley (1985), Keesley (1987).

A1.2.1.3.7 Hydrogen Fluoride Treatment

Complete removal of mono or diphosphate esters was achieved by incubating the glycans with 50µl of 50% aqueous HF (Pierce) at 0°C for 48 hours in a microcentrifuge tube. The sample was then added to 250-300µl of saturated frozen LiOH on dry ice in a microcentrifuge tube. The precise volume of LiOH required to bring the sample to about pH6.0 was determined with 50µl blank HF aliquots incubated along with the sample and titrated with saturated LiOH. pH was measured with pH papers (Sigma). The LiF precipitate was removed by centrifugation and the supernatant removed. The pellet was washed once with 100µl of water and the two supernatants combined. An equal volume of saturated sodium bicarbonate was added to the sample to raise the pH and two additions of 10µl acetic anhydride were made over a 30 minute period to re-N-acetylate the glycans. For this step it is only necessary to raise the pH to >8.0. The sample was then desalted by passage through a 1ml column of Dowex AG50 (H^+ form) and dried by rotary evaporation.

A1.2.1.3.8 Chemical Desialylation

The conditions described by Green and Baenziger (1988a) were used. The oligosaccharide was incubated in 500µl of 2M acetic acid at 100°C for 15 to 30 minutes in a reactivial. The acetic acid was removed by speedvac evaporation, followed by evaporation with 5µl of toluene, and the residue re-N-acetylated and desalted as described (section A1.2.1.3.7). The product was then analysed by paper electrophoresis or MonoQ anion exchange.

A1.2.1.3.9 Chemical Removal of Fucose

The oligosaccharide was incubated in 200µl of 0.1M HCl at 100°C for 1 hour. Chloride ions were removed by passage through AG3 (OH^-), and the column washed with five column volumes of water. The eluate was then dried by rotary evaporation and the glycans re-N-acetylated (A1.2.1.1.2).

A1.2.1.3.10 Removal of Sulphate Esters

Sulphate esters were removed from glycans by incubation with methanolic HCl. The oligosaccharides were freeze dried overnight in a hydrazinolysis tube equipped with a reactivalve. In early experiments the vessel was repressurised with dry argon to exclude moisture, but this was found to be unneccessary and in later experiments the vessel was simply opened to the atmosphere. 50mM methanolic-HCl was made by diluting one part 0.5M methanolic HCl (Supelco) with nine parts of methanol dried by storing over molecular sieves. Five hundred microlitres of the dilute methanolic-HCl were added to the oligosaccharides through the reactivalve which was then closed. The methanolysis was performed by incubating the oligosaccharides at room temperature for 24 hours. The reaction was terminated by removing the solution from the hydrazinolysis tube and rotary evaporating to dryness. Oligosaccharides were re-N-acetylated (section A1.2.1.1.2) and analysed by high voltage electrophoresis. This method is discussed in detail in chapter two.

A1.2.1.3.11 Identification of the Reducing-Terminal Monosaccharide

The identity of the reducing terminus monosaccharide of tritium-reduced glycans was determined by complete hydrolysis of the glycan followed by high voltage electrophoresis in borate buffer (section A1.2.1.2.6). The glycan was hydrolysed by the following procedure. The oligosaccharide was dried down in a glass vial and incubated at 100°C in 1M HCl for 3 hours. The material was transfered to a rotary evaporator tube and repeatedly (five times) dried from 500µl water to remove HCl, and re-N-acetylated as described (section A1.2.1.1.2). Sodium ions were removed by passage through Dowex AG50 (H^+ form) and the residue dried down. The residue was then subjected to a second round of hydrolysis, re-N-acetylation and finally mixed with tritium-reduced 2-deoxyribitol as an internal standard.

A1.2.2 *Protein Methodology*

A1.2.2.1 Liquid Chromatography

A1.2.2.1.1 HPLC

Normal phase HPLC was performed using a Waters system with two model 510 pumps and an in-line solvent degasser. Depending on sample size or the system configuration, a U6K injector or a WISP 710B sample processor was used to inject the sample. The eluate was monitored at 280nm and 214nm with a Waters 490 programmable multi-wavelength detector. The system was controlled and data were collected with a DEC PC-380 computer. Flow was directed to waste or to a fraction collector using a PSV-1 solenoid

valve (Pharmacia-LKB). Column details and chromatography conditions are given in the relevant chapters/sections.

A1.2.2.1.2 Low pressure liquid chromatography

All chromatography was performed in acid cleaned glass columns (Altex) with PTFE tubing (0.1cm ID, Anachem) and flanged connectors, except where otherwise stated. Solvent was delivered with a Minipuls 2 peristaltic pump (Gilson) and fractions were collected with a fraction collector. Gradients were generated with a Pharmacia gradient maker.

A1.2.2.2 Purification of Immunoglobulin G

IgG was purified from human serum by the following method. Aliquots of serum, typically 1ml, were precipitated with ammonium sulphate (33% saturation) on ice for 2hrs. The precipitate was separated by centrifugation at 5000g for 20mins at 4°C and the pellet washed with 40% saturated ammonium sulphate and then dissolved in 1ml of 20mM potassium phosphate buffer pH 7.2 (20mM KPi). This fraction was then dialysed exhaustively against 20mM KPi in a flow dialyser with a 12kdal cut off membrane (BRL) at 4°C overnight. The retentate was applied to a 4ml DEAE-Sephacryl (Sigma) column equilibrated in 20mM KPi and flowing under gravity, and eluted by the sequential addition of 1ml aliquots of 20mM KPi and collecting the eluate until all protein had been eluted, typically 15ml. IgG is the only protein not retained by the anion exchanger under these conditions. The A_{280nm} of the individual fractions was measured with a spectrophotometer and the protein containing fractions pooled. Purity was

assessed by gel filtration on a Zorbax 250-450 (Hichrom) tandem HPLC column, flowing at 1ml/min in 50mM KPi.

Isolation of IgG from pooled human cerebrospinal fluid (CSF) was performed using protein G affinity chromatography. The CSF, typically a pool of 20ml total volume, was lyophilised and reconstituted with 2ml of water. The material was then dialysed in a BRL flow dialyser with a 12kdal cutoff membrane against 100mM potassium phosphate buffer pH 7.5. The retentate was then injected onto a Protein G Spherisorb (Persorb, Uppsala, Sweden) high performance liquid affinity chromatography column (HPLAC) equilibrated in the same buffer and flowing at 1ml/min. The eluant was monitored at 280nm with a Waters model 490 multi-wavelength detector. Unbound protein was washed from the column, and after ten minutes the flow rate was increased to 3ml/min and the bound protein eluted with 50mM glycine-HCl buffer pH 2.5. Fractions of 1 min were collected throughout. The fractions eluted with the acidic buffer were neutralised immediately by the addition of 150µl 1M Tris base. The fractions containing bound material were pooled and dialysed against water and then lyophilised.

Analysis of this material was performed by both SDS-polyacrylamide gel electrophoresis and immunoelectrophoresis (see below), and demonstrated that the bound protein was composed of IgG and albumin. All subclasses of human IgG have been shown to be retained by immobilised protein G (Björck and Kronval 1984).

A1.2.2.3 Gel Electrophoresis

A1.2.2.3.1 Polyacrylamide Gel Electrophoresis (SDS-PAGE)

All analytical SDS-PAGE was performed using the Phast System (Pharmacia-LKB). Protein solutions for analysis were added to an equal volume of Laemlli sample buffer (Laemlli 1970) containing 0.1M Tris-HCl pH6.8, 12% (w/v) sucrose, 4% (w/v) SDS, 0.005% (w/v) bromphenol blue and 0.1% (w/v) β-mercaptoethanol (Sigma), and incubated at 100°C for 3 minutes. A 1µl sample was loaded onto an 8-25% gradient precast thin-film gel using a sample applicator and electrophoresis performed at 60V/cm for 200V minutes. The cell plated was maintained at 15°C throughout the experiment. Bio Rad low molecular weight markers were run as standards. Silver staining was performed as directed in the Phast System manual, except that after the addition of the silver nitrate and the following water washes the gel was removed from the development unit and placed in a plastic petri dish. Developer was added and the appearance ofbands monitored visually. The reaction was stopped by the addition of 5% acetic acid at the optimum point. Coomassie blue staining of the gels was performed in petri dishes. Gels were stained and fixed with a solution containing 1% (w/v) Brilliant blue R (Sigma), 10% (v/v) acetic acid and 50% (v/v) methanol. and destained with 4.1.3 (v/v/v) methanol/acetic acid/water.

A1.2.2.3.2 Immunoelectrophoresis

Agarose gel immunoelectrophoresis was performed essentially as described by Grabar and Williams (1953). Gels were cast between two warmed

grease free glass plates, with the lower sheet covered with gelbond. Melted 1% agarose (no residual electroendosmosis, Sigma) in barbitone buffer (1.84g/l barbitoic acid (Sigma), 10.31g/l sodium barbitone (Sigma), and 3% (w/v) PEG 3000 (Sigma), pH 8.2.) was poured between the plates and the gel allowed to set. Wells and troughs were then cut into the gel. Later experiments were carried out using dried immunoelectrophoresis films (Calbiochem), that were precast and rehydrated in barbitone buffer. The gel was placed on the platen of a flat bed electrophoresis apparatus (Pharmacia-LKB). The platen was cooled to 10°C with a circulating water bath. Samples, typically 10µl, were pipetted into the wells. Normal human serum, containing bromophenol blue was run on the gel as a standard. Paper wicks of Whatman 3MM were used, and the electrode buffer was barbitone buffer. Electrophoresis was performed at 10V/cm and typical run times of six hours were used. Antisera were diluted with barbitone buffer and pipetted into the troughs. The gels were incubated overnight at room temperature. Free protein was washed out with phosphate buffered saline (PBS), and the gel was fixed and stained with coomassie blue as described for SDS-PAGE. Anti-immunoglobulin antisera were affinity purified and specific for heavy chain determinants. Specific antisera were diluted 1 in 25, and the anti-whole human serum antiserum was diluted 1 in 10. Antisera were stored at -20°C, undiluted and were thawed just before use.

A1.2.2.4 Immobilisation of Protein on Cyanogen Bromide-Activated Sepharose.

Proteins were immobilised on cyanogen bromide-activated Sepharose (CNBr-Sepherose) (Pharmacia-LKB). The gel was rehydrated with 1mM HCl on a Nalgene filter unit. About 200ml of HCl per gram of gel was used. The gel

was then resuspended in coupling buffer (500mM NaCl in 100mM $NaHCO_3$, pH 8.0) and washed five times. An equal volume of protein in coupling buffer (not greater than 2mg/ml) was added and the reaction allowed to proceed overnight at 4°C. A small aliquot of the supernatant was removed and assayed for protein to determine the coupling efficiency. The residual reactive groups were blocked by incubating the gel in 200mM glycine buffer pH 7.0 for 4 hours and the gel was then washed alternately with coupling buffer and 0.5M sodium chloride in 0.1M sodium acetate, pH 4.0 three times. Gels were stored at 4°C in PBS until required.

A1.2.2.5 Protein Estimation

Protein was estimated with the BCA reagent (Pierce) or the Bio Rad Bradford system kit. Bovine serum albumin was used as a standard. For both assays the reaction volume was decreased to 250µl to facilitated the use of a microtitre plate.

The BCA reagent was made up following the manufacturers instructions (50:1 reagent A to reagent B, v/v). 200µl of the working reagent were added to 50µl of a protein solution in a microtitre plate well and the plate incubated at room temperature for 30 minutes. The plate was read on a Flowlabs Multiskan plate reader (MC340) at 600nm.

The BioRad reagent was diluted with four parts of water and the assay performed in an identical manner to the BCA assay, except that the plate was read immediately, at 600nm.

Purified IgG concentrations were estimated by A_{280nm}, assuming an absorbance of 1.4AU/mg/cm.

A1.2.2.6 Dialysis of Proteins

Small volumes of protein were dialysed in a BRL flow dialyser using a 12kdal cutoff membrane or a Pierce microdialyser with a 6kdal cutoff membrane. Larger volumes were dialysed in dialysis tubing that had been cleaned as follows; the tubing was boiled for 5 minutes in 5% (w/v) sodium carbonate and then washed with water. The tubing was then boiled in 50mM EDTA pH8.0, washed and stored in 20% (v/v) ethanol at 4°C until use. Immediately before addition of the protein the tubing was extensively washed with deionised water. All dialysis was performed at 4°C.

A1.2.2.7 Double Diffusion

Immunochemical detection of protein in column eluates was performed by fused double diffusion in 1% agarose in barbitone buffer. The gel was cast in a plastic petri dish, and holes punched into the gel. Troughs were cut close to the wells (3mm distance). Aliquots of the fractions collected from the column elution were added to the wells and antisera diluted in barbitone buffer (1:25 v/v) added to the trough. The gels were incubated at 4°C overnight. Staining was performed as described for immunoelectrophoresis (section A1.2.2.3.2). Simple double diffusion was performed in identical gels but with holes cut in hexagonal arrays.

A1.2.3 *Miscellaneous methods.*

A1.2.3.1 Absorbance Measurements

Absorbances were determined with a single beam ultraviolet/visible variable wavelength spectrophotometer (Perkin Elmer, Beaconsfield, Buckinghamshire, UK) in quartz or UV grade plastic cuvettes with a path length of 1cm.

A1.2.3.2 Conductivity and pH Measurement

Conductivity was measured with a Radiometer conductivity probe (model CDC114) and meter (model CDM83). pH was determined with a Radiometer silver electrode and pH meter (model PHM84).

A1.2.3.3 Determination of Radioactivity

Radioactivity was estimated by scintillation counting, using a propriatry scintillation cocktail (Readysolv, Beckman, High Wickam, UK). Samples were counted in plastic vials in an LC-501 scintillation counter (Beckman).

For counts per minute determinations, typically 10 to 100µl of sample was added to 2ml of scintillation cocktail in a vial, and the contents mixed before counting. With large volume samples (>100µl) large vials were used, and 10ml of scintillation fluid was added. When fractions from chromatographic separations were analysed, a constant sample volume was counted for all the fractions obtained from that run.

For determination of disintigrations per minute, a constant volume of 100µl of sample in 2ml of scintillation fluid was used to standardise the quenching of the sample. The LC-501 was calibrated with standard activity vials supplied by the manufacturer.

APPENDIX TWO

AN OVERVIEW OF THE METHODOLOGIES EMPLOYED FOR STRUCTURAL STUDIES OF OLIGOSACCHARIDES DERIVED FROM GLYCOPROTEINS

The strategy for determination of the primary sequence of any glycan falls into four steps; (1) the glycan is released from the protein, either chemically or enzymatically, (2) the sugars are purified away from the deglycosylated protein or peptide, (3) the glycans are fractionated, and (4) analysis itself is performed. It is usually desirable to use more than one independant method to unequivocally define a structure. At some point in the procedure a label, either radiochemical or otherwise, can be introduced to aid detection and/or quantitation. This latter point is particularly important for microscale sequence determination as performed in this thesis as chemical detection is insufficiently sensitive to permit accurate quantitation or detection at low levels. Several workers rely on NMR spectroscopy alone, but usually reference to spectroscopic data from previously characterised glycans is used to confirm the assignments (Vliegenthart *et al.* 1983).

Table A2.1 **ELUCIDATION OF GLYCAN STRUCTURE**

(a) Information required to define the primary structure of a glycan

(1) Composition, i.e. the monosaccharides
(2) Linkage position, i.e. the hydroxyls involved in the glycosidic bond
(3) Anomeric configuration of the glycosidic bonds
(4) Order of the residues (sequence)

(b) Methods used in sequence determination

(1) Liquid chromatography, GCMS
(2) GCMS (methylation analysis), enzymatic degradation
(3) and (4) FAB-MS, NMR, antibodies, lectins, chemistry, and enzymatic degradation

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To completely define an oligosaccharide, all the information in (a) must be obtained. Common methods for obtaining this are listed in (b). In some cases, a single method can provide all the information reguired, e.g. NMR and enzymatic degradation, but it is more usual for a variety of methods to be applied to determine a structure. With more material, a greater number of options are availible. For very small levels of oligosaccharide, analysis is usually restricted to GCMS and enzymatic methods.

A2.1 *Release*

A2.1.1 *Chemical Release*

Asparagine linked glycans can be released by hydrazinolysis. The procedure, when performed correctly, results in the complete cleavage of all amide bonds, i.e. that linking the reducing terminal GlcNAc to the asparagine, all the peptide bonds in the protein or peptide, and the removal of the N-acyl substituents from hexosaminyl and neuraminic acid residues (Takasaki *et al* 1982). Information on these substituants is therefore lost. Figure A2.1 summarises the reactions that ensue following hydrazinolysis. Degradation is a large problem in this procedure. The hydrazine must be strictly anhydrous (less than 0.05% water), necessitating fresh distillation with toluene as an entrainer (Schmidt 1984 and references therein) as close to the time of use as possible. If the hydrazine becomes wet the alkaline conditions that are produced lead to a peeling reaction by β-elimination of residues from the reducing terminus. Further rearrangements of the reducing terminus can result from the production of a free amine on the glucosamine which stabilizes the glycosylamine (Product 1 in fig A2.1). This can be destroyed by the re-N-acetylation procedure by substitution of the amine function. The glycosylamine becomes labile and deaminates to yield the free sugar. The formation of hydrazones has been observed by the reaction of hydrazine with the glycosylamine. These derivatives are quite stable and are alkali resistant. They cannot be reduced or derivatized in a quantitative manner, and though they have not been studied it is to be expected that they will

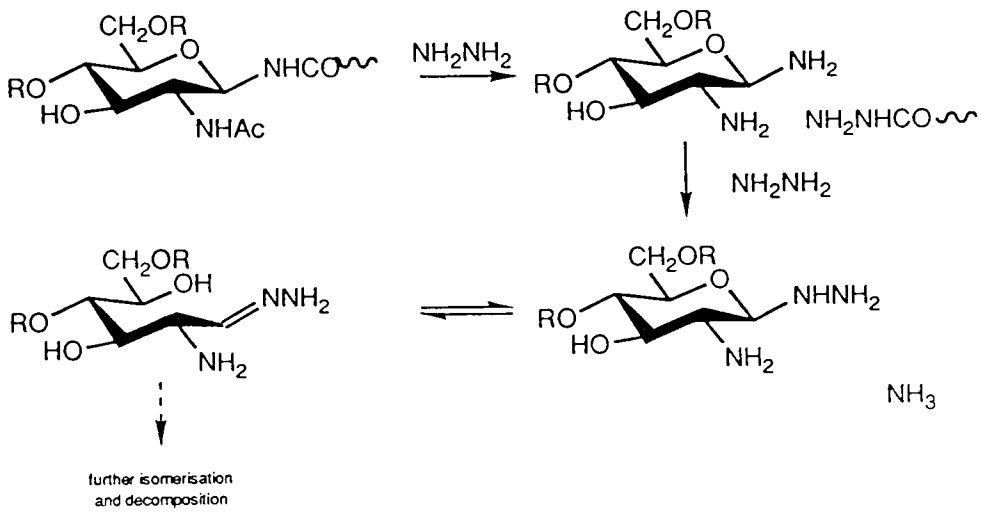

Figure A2.1 Diagrammatic representation of the reaction that occurs at the oligosaccharyl-protein linkage under hydrazinolysis. This reaction is discussed in more detail in the text.

not behave identically to the homologous free sugar, making analysis difficult. Removal of the hydrazone has been achieved by Manger (unpublished), who used a Lewis acid, the Cu^{++} ion, to hydrolyse residual hydrazones after the re-N-acetylation procedure. The behaviour of the reducing terminus GlcNAc during hydrazinolysis and subsequent work-up has been studied by the use of model compounds (Michalski *et al* 1984). This group observed the production of eight byproducts which they propose were derived from the hydrazone.

The great advantage of hydrazinolysis over other methods for N-link release is that it is not selective for specific structures and the protein under study has little or no influence on the reaction. Problems may be encountered when dealing with complex mixtures, like whole organ extracts (chapter two).

The O-linked glycans may be released by alkali treatment. This is the case for both the mucin chains and GAGs. Alkaline β-elimination of the O-serine bond must be rapidly followed by reduction of the free oligosaccharide to prevent the peeling reaction from taking place (Carlson 1968, Kobata 1984).

A2.1.2 *Enzymatic Release*

An increasing use of enzymes for the release of glycans from proteins is taking place. There are several valid reasons for this. As the glycoprotein is left intact sensitivity to various deglycosidases can be used to probe the gross glycan structure of a glycoprotein which is afterwards analysed by SDS-PAGE or other methods. This approach is very powerful in studies of biosynthesis or for assessing the influence of

carbohydrate on function. In addition, partial digestions can provide an estimate of the number of sites present (Sundblad *et al* 1988). Secondly, the rearangements due to the de-N-acetylation of the hexosamines or generation of the hydrazones as encountered with hydrazinolysis do not occur with enzymatic release. Thirdly information about the N-acyl groups is not lost, which is a severe limitation for hydrazinolysis if these substituents are of interest. For example some glycoconjugates have been shown to contain nonacetylated glucosamine (Ferguson *et al* 1988) whilst sialic acids can contain various moieties substituted on the amine.

The endoglycosidase that are useful reagents for studies of oligosaccharides fall into three categories; (1) the endo N-acetyl β-D glucosaminidases, D from *Streptococcus pneumoniae* (Koide and Muramatsu 1974), H from *Streptomyces plicatus* (Tarentino and Maley 1974) and F from *Flavobacterium meningosepticum* (Plummer *et al* 1984), (2) the endo β-galactosidases of *S. pneumoniae* and *Baccilus fragillis* (Fukuda 1985), and (3) the endoglycopeptidases peptide N-glycosidases (PNGases) (Tarentino *et al* 1985) and peptide O-glycosidases (Umemato *et al* 1977). The PNGases are the most useful if complete release of the oligosaccharides is required, and have been isolated from *S. pneumoniae, F. meningiosepticum* and almond emulsin. The minimum saccharide that can be cleaved appears to be $GlcNAc_2$-Asn (Chu 1986). Greater activity is observed if the substrate is denatured and high activities are required to achieve complete deglycosylation. The PNGases have not been found to exhibit preferential cleavage towards particular glycan classes, but have decreased activity against saccharides present at the N or C terminus (Thotakura and Bahl 1987). The endo N-acetyl β-D glucosaminidases all cleave between the two GlcNAc residues of the chitobiose core. Endo H and F have preference for oligomannose, but in

addition endo F can remove biantennary complex structures unless they are bisected. No tri or tetra antennary glycans are hydrolysed and F will not act on the agalacto ovomucoid structures (Tarentino and Plummer 1987). There is also some debate about the effect on endo H of a Fucα1→6 substituent or sulphate esters on the core as well as the precise action on some hybrid glycans. Endo D acts on biantennary glycans if the manα1→6 arm is substituted (Kobata 1979). The O-glycopeptidase only removes O-linked Galβ1→3GalNAc, and is thus of little value as a method of release (Umento *et al.* 1977)

The major problems with the use of these reagents are twofold; (1) differential activity of some endoglycosidases against certain structures is encountered, and in some cases, e.g. endo H, there is still disagreement about the precise specificity, and (2) the protein may play a role in selectivity of glycan release. For N-glycanase it has been observed that the efficiency of release of glycans alters with different substrates (Hirani *et al* 1987). Green *et al* have used this enzyme in their studies of the glycohormones and bovine fetuin (Green *et al.* 1988a, b, d), and obtain total release of the glycans by extensive incubation. Heterogenous mixtures of proteins, e.g. cell membranes or body fluids present a particularly difficult problem, which hydrazinolysis can deal with easily. Digestion of the protein matrix may facilitate the more general use of N-glycanases in the future.

The analysis of limit glycopeptides has been a common strategy in the past, because the problems of glycan release could be circumvented. However the fractionation of the peptides has proved to be difficult because of incomplete digestion of the protein (Finne and Krusius 1982). The

availibility of broad specificity peptide-N-glycosidases has reduced the use of this method.

A2.2 *Purification*

There are several methods that can be employed for the isolation of the glycan fraction from the source protein. Gel filtration and reverse-phase HPLC have been used. Alternatively the material can be chromatographed on paper (Takasaki 1982) or through microcrystalline cellulose by partitioning of the components. Further steps may also be necessary.

A2.3 *Fractionation*

Oligosaccharides can be fractionated either underivatised or derivatised. One of the commonest methods is gel permiation chromatography on Bio-Gel, usually P4 or P6, which separates by hydrodynamic volume (Kobata 1984 and references therein). As this can be rationalised to take account of the primary structure of the glycan, this method is extremely powerful when coupled to exoglycosidase degradation for determination of sequence. A number of high performance liquid chromatography (HPLC) methods have been described. Amine bonded phase silica columns are the basis of many methods (Blanken *et al* 1985, Mellis and Baenziger 1981, Turco 1981). This methodology was extended to fractionate charged oligosaccharides by ion-suppresion amine adsorption HPLC (Green and Baenziger 1986). Affinity chromatography can be performed with released oligosaccharides. Monoclonal antibody columns are especially useful in defining epitopes detected by histochemistry (Zopf *et al* 1987). The use of lectins to fractionate or

characterise oligosaccharides is a powerful approach (Osawa and Tsuji 1987), but has the problem of low reproducibility when using soft bed columns. As a result many workers are switching to HPLC affinity chromatography (HPLAC). Harada *et al* (1987) fractionated human IgG oligosaccharides by serial chromatography. Green has systematically redefined a number of lectin binding requirements using HPLAC, which was only possible because the controlled chromatography allowed the chromatogram to be reliably divided into several diagnostic regions (Green *et al* 1987 a, b). Townsend and coworkers (1988) have used a novel method developed by the Dionex Corperation that involves ion exchange at high pH where the hydroxyl groups become ionised, with pulsed ampermetric detection.

A new approach, which utilises pyridylamino derivatives of released oligosaccharides has the advantage of allowing the oligosaccharides to be detected fluorescently and to be chromatographed on reverse phase HPLC (Yamamoto *et al.* 1989). This method may become an important technique in the future.

Anion exchange has been performed using strong anion exchangers, classically QAE, and now Mono Q and Micropac AX5 (Hallenbeck *et al* 1987, Green *et al.* 1988b). Soft exchangers, e.g. DEAE are still commonly used for GAG fractionation. GAGs have also been fractionated by polyacrylamide gel electrophoresis (Hampson and Gallager 1984) and on ion exchange resins.

A huge array of methods for fractionating derivatised oligosaccharides have been reported, but few are in general use (Honda 1984). Post column derivatisation is common. Precolumn methods have the advantage of rendering sugars hydrophobic and thus allowing their application to reverse phase columns (RPHPLC). Precolumn derivatization is usually a reductive amination

to the reducing terminus, which is easy to perform. Detection can then be performed by fluorometry. *Para*-amino benzoic ethyl ester and the azo dye 4'-*N*,*N*-dimethylamino-4-azobenzene have been used succesfully (Muramoto *et al* 1987, Wang *et al* 1984). Acetylated and methylated oligosaccharides have also been successfully fractionated. Derivatisation can preclude the subsequent use of the oligosaccharides in bioactive systems or for lectin or immunochemical studies.

A2.4 *Analysis*

There are several approaches that can be used to analyse oligosaccharides and to gain structural information. Affinity chromatography on lectins has already been mentioned. Enzymatic degradation with exoglycosidases of defined specificity is a powerful method, which has been fully exploited by Kobata and others. The use of high specific activity radiolabelled alditols in enzymatic sequencing provides the most sensitive method availible for detailed sequence determination to date, although fluorescent derivatives are coming into use, which have the potential of even greater sensitivity. A compilation of the specificities of the panel of enzymes that are commonly used can be found in appendix one. This method has the advantage that both linkage and anomericity are determined, but clearly relies on the availibility of suitable enzymes. This is an obvious major drawback. A less apparent problem is the determination of the arm that a particular residue resides upon. A simple method for circumventing this is not availible, although partial acetolysis to cleave $\alpha 1 \rightarrow 6$ linkages can resolve some ambiguities (Kocourek and Ballou 1969).

The use of gas chromatography (GC) mass spectrometry (MS) of derivatised oligosaccharides or monosaccharides has allowed detailed linkage information and composition data to be obtained relatively easily. Specifically the methylation analysis of Hakamori (1964) and subsequent modifications to this procedure is very powerful. Anomeric information cannot be obtained but butanolysis can give the absolute configuration of the composite monosaccharides. Retention times and fragmentation patterns for methylation analysis of relevant monosaccharides can be found in appendix one. Methylation analysis is performed using electron impact ionisation to produce small fragment ions. A second MS method that is becoming used more frequently is fast atom bombardment MS (FAB-MS). The sample in a matrix is ionised by the impact of a beam of highly energetic ions such as argon. It has the advantage that nonvolatile polar substances give a strong molecular ion and cleavage is preferential across the glycosidic bond, with a series of ions arising from cleavage at both ends of the molecule. Limit glycopeptides can also be analysed. The great advantage of this method is that connectivity data are obtained, which in combination with methylation analysis can give most of the information required to elucidate a structure fully. FAB-MS of derivatised oligosaccharides, usually methylated or acetylated, is often used to improve the data that can be obtained by this method. Mass spectrometric methods have been compiled recently (Biermann and McGinnis 1989).

Finally, NMR has been an extremely informative method for the sequence determination of oligosaccharides, and under some circumstances can deal with mixtures. In addition, nuclear overhauser effect experiments can provide conformational information. Two dimensional NMR is usually required for oligosaccharide analysis. Proton NMR is the most common method although

^{13}C NMR has been used. Over recent years Vliegenthart, Carver and others have built up a large database of coupling constant and chemical shift assignments for anomeric protons and structural reporter groups, which allows the rapid assignment of one dimensional spectral data. The major drawback of this approach is in the study of unknown compounds, but this is also true for many of the methodologies employed. A detailed review of this topic can be found in Vliegenthart *et al.* (1983) and Sweely and Nunez (1985).

A substantial battery of techniques are now availible for the isolation and characterisation of oligosaccharides. The pace at which new structures are being identified has intensified over the last five years or so, and whilst carbohydrate sequence analysis still represents a substantial time and financial commitment, the proliferation in the number of laboratories that are addressing the roles of oligosaccharides in biology signifies that this field of biology is entering a new an exciting era.

APPENDIX THREE

THE BIOSYNTHESIS OF O-LINKED GLYCANS

A brief overview of the biosynthesis of O-linked glycans is given, with some examples of the functions of these moities.

A3.1 *Biosynthesis of Glycosaminoglycans*

Glycosaminoglycans (GAG) chains occur as substituents of a large number of proteins and also as free polysaccharides. Proteins that carry GAGs are termed proteoglycans (PGs). The linkage to protein is almost invariably at a Ser-Gly sequence which becomes xylosylated on the serine hydroxyl and then substituted by two galactosyl residues and one glucuronosyl residue The construction of the Ser-Xyl-Gal-Gal-GlcA core takes place in the RER. Subsequent elongation occurs in the Golgi by the addition of uronic acids and N-acetyhexosamines from the corresponding nucleotide sugars (Rodin 1980). The steps are shown schematically in figure A3.1 Two types of chain can be constructed by the alternating addition of one hexosamine and one uronic acid. Type I chains usually contain GalNAc and GlcA (chondroitin and dermatan) whilst the type II chains are elongated with GlcNAc and GlcA (heparin, heparan sulphate). There is also heterogeneity in the linkages between consecutive monosaccharides. After chain elongation the chains are modified by uronosyl epimerases, which convert GlcA to iduronic acid and sulphotransferases which add sulphate esters to the hydroxyl groups. Chondroitin is directly sulphated but in dermatan sulphate epimerisation of

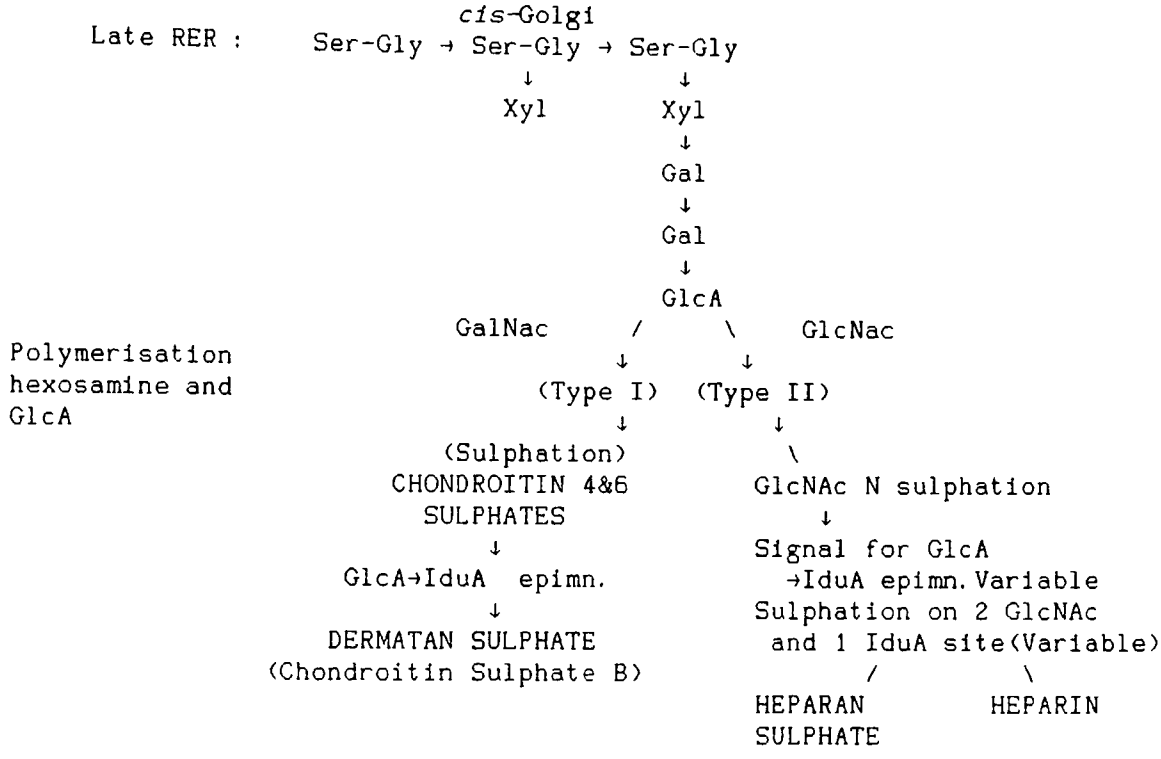

Figure A3.1 Schematic representation of the biosynthesis of Type I and Type II glycosaminoglycans. The relevant monosaccharides are added by the action of transferase enzymes using UDP-sugar as the low molecular weight substrate. Adenosine 3'-phosphate 5'-phosphosulphate (PAPS) is the donor of sulphate in this pathway, as well as for all other glycan biosynthetic sulphation so far characterised.

GlcA takes place prior to the addition of the sulphate esters. Type II chains are also N-sulphated following de-N-acetylation of the GlcNAc, which acts as a signal for epimerisation of the GlcA (Lindahl *et al* 1986). The variation possible due to these processes generates great heterogeneity in the primary sequences of the GAGs.

Proteoglycans are a major component of the extracellular matrix (ECM), and act as both structural components and as regulators of cellular responses to other factors. Free GAG chains are also found in the ECM and in the circulation. For example heparin can inhibit vascular smooth muscle proliferation and affect interactions of these cells with EGF (Scott-Burden and Bühler 1988) as well as exhibiting antithrombolitic properties. PG molecules have been identified that carry from one GAG chain to twenty or more. The properties of these PGs are diverse, reflecting the differences in their molecular structures. PGs are able to bind to cell surfaces by a variety of mechanisms, either directly by a membrane spanning polypeptide domain of the core protein, and thus are potentially able to interact wuth the cytoskeleton, or by a glycosylphosphatidylinositol membrane anchor (Ishihara *et al* 1987). In addition they may bind non-covalently to receptors (Höök *et al* 1984). Therefore the distribution of GAG chains and PGs is extremely widespread.

Recently it has become clear that different cells secrete PGs of distinct sizes and that the core proteins can also be N-glycosylated or carry mucin type O-glycans. A recent report (Spiro *et al.* 1989) has demonstrated that blockage of N-linked glycan maturation by inhibitors of glucosidase I prevents addition of GAG chains to a myeloma PG and to the MHC γ-chain. Therefore it is possible that the different modes of glycosylation show some coordination in their control mechanisms. It has

also been shown that GAG chains can be initiated on N-linked glycan cores in some cell lines (Sundblad *et al.* 1988).

Cell surface PGs have been reviewed by Fransson (1987). Most proteins that carry GAGs appear to always carry them, but some proteins are expressed both as PGs and non-GAG substituted glycoproteins. The hepatocyte transferrin receptor and the γ-chain of Ia major histocompatibility antigens (MHC) are examples of such proteins. The hepatocyte heparan sulphate PG (HSPG) has a low molecular weight of 35kdal, whilst that present on fibroblasts is a dimer of 90kdal subunits. The protein core of this molecule appears to be identical to the hepatocyte transferrin receptor. Human colon carcinoma cells express an HSPG of 240-400kdal which carries over 10 heparan sulphate (HS) chains and 100 O-linked glycans (Iozzo 1988). Mixed PGs, carrying chondroitin sulphate and HS have been detected (Rapraeger *et al 1985). Krusius et al* (1986) have detected keratin sulphate containing PGs from rat brain that are linked to the core protein by a mannose residue. The variety of combinations of GAG-chain that can reside on a given core is very large, and therefore the potential for sophisticated modulation of function is very great.

Glycosaminoglycans also respond to alterations of cell phenotype and have been demonstrated to be sensitive to various growth factors (Bassols and Massagué 1988, Elias *et al* 1988). Transformation of 3T3 cells with SV40 leads to the production of undersulphated HS chains (Underhill and Keller 1975). This change appears to be widespread amongst many cell types but is specific for HS, and is not observed on chondroitin or dermatan sulphate (Nakamura and Kojima 1981). Functionally, this change may result in loss of ECM adherence, especially towards fibronectin,, and in addition could alter growth factor responses, as HS is known to bind GM-CSF and interleukin 3

(Roberts *et al* 1988). Rider and Hart (1987) have demonstrated specific differences in the sulphation position of chondroitin from T and B cells. Murine splenic B-cells exhibit more abundant 6-O-sulphation than 4-O-sulphation, but in T-cells the reverse is true. Hart has also shown that resting thymocytes only secrete chondroitin 4-sulphate (Hart 1982). Thus, in a similar way to N-linked oligosaccharide biosynthesis, the synthesis of GAGs is sensitive to the cell state, and therefore can provide a means by which that state is expressed.

A3.2 *Mucin-Type Glycan Biosynthesis and Function*

The synthesis of mucin-type O-linked glycans occurs in the Golgi, although the addition of the reducing terminal residue has been reported to take place in a compartment that lies between the RER and the cis-Golgi cisternae (Tooze *et al.* 1988). Subsequent chain elongation is similar in broad terms to the biosynthesis of the GAGs in that the chain is built up on the protein by the sequential action of Golgi monosaccharide transferases.

The most common O-linkage to protein is GalNAc-Ser/Thr, and is exemplified by the mucins and most of the mammalian serum O-glycoproteins. However, other linkages to the peptide do occur (Kornfeld and Kornfeld 1980). For example in the yeast *S. cerevisiae* and a number of other fungi the reducing terminus of the glycan is mannose. In the earthworm *Lubricus terrestris* Gal-Ser linkages have been found. Fucose linked directly to Ser or Thr has been identified in human or rat tissues. Krusius *et al.* (1985) also identified reducing terminal mannose in rat brain glycoproteins. The linkage of O-glycans to hydroxylysine (Hyl), as found in collagen, is also

Structure	Reference
GlcNAc	1
NeuNAcα2→3Galβ1→4GlcNAcβ1→6 GalNAc Galβ1→3	
NeuNAcα2→3Galβ1→4(3) GlcNAcβ1→6 Fucα1→3(4) GalNAc Galβ1→3	2
NeuNAcα2→8NeuAcα2→6 GalNAc NeuNAcα2→3Galβ1→3	
GlcNAcβ1→6 GalNAc NeuNAcα2→3Galβ1→3	3
6'SO_4Galβ1→4GlcNAcβ1→3Galβ1→3GalNAc	4
Galβ1→4GlcNAcβ1→3Man 3 • ↑ Fucα1	
NeuNAcα2→3Galβ1→4GlcNAcβ1→3Man	5

Figure A3.2 Examples of O-Glycan structures. References and sources (1) Hart 1988, nuclear cpore proteins (2) Yurwicz *et al* 1987, cervical mucus glycoproteins (3) Krotkiewski 1988, glycophorin(4) Mawhinney *et al* 1987, mucus glycoproteins (5) Krusius *et al* 1986, rat brain proteoglycan.

unusual, in that the sequence Glcβ1→3Gal→Hyl has been identified in a glycopeptide from the glomerular basement membrane collagen of guinea pigs. This sequence appears to be highly antigenic, with important caveats for glomerular disease. In addition, O-linked GlcNAc has been detected on cytoplasmic and nuclear pore proteins, and on proteins involved in transcription control (Hart *et al.* 1988, Jackson and Thian 1988). The pathway for this synthesis is not yet defined. The discussion of O-linked glycan biosynthesis given below will be restricted to the mucin-type (GalNAc terminating) oligosaccharides.

Abeijon and Hirschberg (1987) have located the rat liver polypeptide:N-acetylgalactosaminyltransferase to the Golgi and were unable to detect activity in the RER. As mentioned above, it is possible that the addition actually occurs post-RER, in vesicles between the RER and the Golgi stack (Tooze *et al.* 1988). However, addition of chain synthesis certainly occurs after N-linked glycan addition. Therefore the O-glycans are exposed to the same battery of Golgi transferases and exoglycosidases as the N-linked glycans.

UDP-GalNAc acts as the donor nucleotide sugar for the reducing terminus. The chain is elongated by the sequential addition of monosaccharide residues, and typically galactose and GlcNAc contribute the bulk of the residues that are added. Further GalNAc residues and fucose can also be added (Kornfeld and Kornfeld 1980). Both sialic acid and sulphate esters have been found as substituents of the O-linked glycans. Polysialic acids, with the linkage SAα2→8SA are common in mucin glycoproteins.

In the mucus glycoproteins a high density of oligosaccharide chains substituted onto the core protein is typically encountered, and this property allows such glycoproteins to form a gel. Overall the proteins

appear as long flexible rods (Sheenan and Carlstedt 1987). The mucins probably play a key role in first line defence against invading pathogens. The cervical mucus glycoproteins of the higher primates have been studied in great detail, and the glycans appear to alter with the ovulatory cycle. In the monkey a shift from sialic acid in α2→6 linkage to α2→3 is observed in the premenstrual mucus glycans, and a similar alteration is found in the human (Yurewicz *et al.* 1987). This alteration in linkage of the sialic acid may allow the addition of GlcNAc to the C6 hydroxyl of the reducing terminal GalNAc, and hence chain elongation, and thus alter the properties of the mucus profoundly. It may be, in this case, that the biosynthetic pathway is under hormonal control, as the quality of cervical mucus is a critical determinant of fertility.

The O-linked glycans from the serum glycoproteins are usually smaller and less charged that those encountered on mucus glycoproteins, but many structures are found in common between the two groups of proteins. Amano *et al.* (1988) have recently shown that the population of O-linked glycans on hCG alters in malignant disorders so that larger glycans come to dominate. This increase in complexity parallels that found in the N-linked oligosaccharides discussed above, and thus the biosynthesis of these glycans is also sensitive to the cellular state.

A3.3 *Conclusions*

In a manner similar to N-linked oligosaccharides, mucin type and GAG chains are important components of glycoproteins. Biosynthesis of these moieties is sensitive to the cell state, so that the control of O-link

glycan production provides a means by which the cell can express it's phenotype, and modulate the properties of the core proteins.