Early Steps in The Processing of Oligosaccharide Chains of Rat α_1-AGP

by

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A Thesis submitted to the Faculty of Graduate Studies in partial fulfilment of the requirement for the Degree Masters of Science

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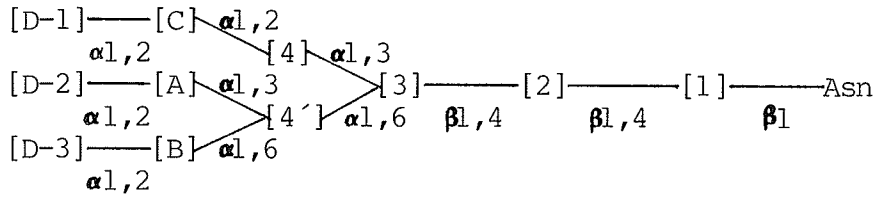
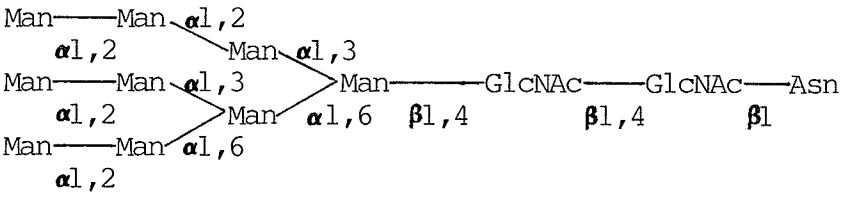
Abbreviations

α_1-AGP	Alpha$_1$-Acid Glycoprotein
Asn	Asparagine
CMP	Cytidine 5´-Monophosphate
D_2O	Deuterium Oxide
Endo-H	Endo Glucosaminidase-H
ER	Endoplasmic Reticulum
Fuc	Fucose
Gal	Galactose
GDP	Guanosine 5´-Diphosphate
Glc	Glucose
GlcNAc	N-acetylglucosamine
HPLC	High Performance Liquid Chromatograpy
(J)	Coupling Constant
(δ)	Chemical shift
Man	Mannose
MHz	Mega Hertz
mRNA	Messenger Ribonucleic Acid
mA	milliamperes
MW	Molecular weight
nm	nanometres
NMR	Nuclear Magnetic Resonance
OD_{280}	Optical Density at 280 nm
PAGE	Polyacrylamide Gel Electrophoresis
ppm	Parts per million
RNA	Ribonucleic Acid
RER	Rough Endoplasmic Reticulum
SA	Sialic Acid
SDS	Sodium Dodecyl Sulfate
SER	Smooth Endoplasmic Reticulum
TEMED	N,N,N´,N´-Tetraethylmethylenediamine
TRIS	[Tris(hydroxymethyl)aminomethane]
UDP	Uridine 5´-Monophosphate
V	Volts

Abbreviations

The following scheme has been employed throughout this thesis to denote specific mannose residues found in high mannose oligosaccharides and glycopeptides.





ABSTRACT

The early steps of oligosaccharide processing of rat α_1-AGP were studied by the characterization of processing intermediates from intracellular forms of α_1-AGP isolated from inflamed rat liver. Structures of glycopeptides and oligosaccharides released from RER and SER derived α_1-AGP were determined by proton NMR. These studies showed that within the RER, α_1-AGP is processed as far as a $Man_8GlcNAc_2$. At least 85% of these Man_8 structures were the "A" isomer. Following transport out of the RER, other mannose residues were removed from the "A" isomer in a specific order. The sequence of removal of mannose residues is believed to be controlled by conformational changes that occur within the oligosaccharide molecule upon the release of individual mannose residues. This view is supported by the finding that oligosaccharides other than the "A" isomer of Man_8 follow a different processing pathway outside of the RER.

Introduction

1.1 Historical Aspects of Glycoprotein Research

The term "protein" first appeared in chemical literature in 1838 in a paper by Gerardus Mulder. The term was used to describe the smallest fundamental unit of a variety of different substances of biological origin. The empirical formula of "protein" was $C_{40}H_{62}N_{10}O_{12}$ and when combined with one atom of sulphur and one atom of phosphorus, would produce fibrin (blood clot) and egg albumin (ovalbumin); with equal simplicity, it could produce blood serum albumin by the addition of a second sulphur atom. Although the discovery was greeted with great euphoria at first, it became increasingly obvious, that the structure of albumins, caseins and fibrins was indeed complex. All that remained of Mulder's theory by 1850, was the name protein.

With the rise of organic chemistry in the second half of the 19th century, great strides were taken in the elucidation of protein structure. Yet, the the first of these important steps had occurred prior to Mulder's theory of protein. In 1806, Louis Vauquelin and Jean Pierre Robiquet isolated a substance called asparagine from the juice of the asparagus plant. Heinrich Ritthausen and August Plisson in 1822 characterized the acidic component of vegetable protein hydrolysate, aspartic acid. Almost 50 years would pass before the proper structures would be assigned to these two amino acids. During this time it became more apparent that proteins were composed of a variety of amino acids, that were linked in what was first thought to be a ureido linkage

(NH-CO-NH). This was later corrected, by Franz Hoffmiester, to the peptide linkage.

Throughout this time and up until the 1940's, the major difficulty in the study of any protein or peptide was a suitable method for its purification. At best, crude purifications could be achieved by precipitation with alcohol, salt or heat. Yet, highly pure protein was close at hand. This was crystalline hemoglobin. As early as 1840, reports describing the appearance of "blood crystals" in the partially dried blood of a number of animals had been made. In 1853, Ludwig Teichmann showed that the treatment of dried blood with a sodium chloride solution, followed by hot glacial acetic acid yielded "hemin" (heme). By 1857, this reaction had become a standard forensic test for blood. Almost 15 years would pass before this technique was applied on a larger scale to blood crystals. Felix Höppe-Seyler was able to show that blood crystals treated in this way would produce in addition to the colored heme, a colorless "albuminoid" (having the chemical proporties of albumin) substance. It was at this time, that Höppe-Seyler introduced the term "prosthetic group", to describe the functional portions of proteins that did not have protein-like characteristics. In the ensuing years, some discussion followed as to whether hemoglobin should be classified as an "albuminoid" or a conjugated protein. By 1880, it was decided that it should fall into a category of conjugated proteins, to be known as the "chromo-proteins". At this time it was to join another group of conjugated proteins, the "glycoproteins". Through the application of color tests, specific for carbohydrate, it had been suggested that ovalbumin be placed in this

category. Similarly, studies by Oswald Schmiedeberg on chondroitin sulphate (from cartilage), and Johann Scherer and Einar Hammarsten on mucins (from ovarian fluids etc.), had indicated the widespread occurrence of carbohydrate-protein conjugate molecules in nature. The question of whether carbohydrate groups were significant in protein structure or function was greeted with some skepticism by the early 1900's.

In 1893 Pavy (1) published a paper describing the formation of a glucasone from the alkaline hydrolysate of coagulated egg white. From his results, he assumed that all proteins contained fairly large amounts of carbohydrate and that this "pre-formed" sugar was responsible for the conversion of protein into carbohydrate, which occurs in the bodies of animals. Because of the exotic nature of his physiological hypothesis, Pavy's correct chemical findings were ignored. In 1898, Eicholz (2) confirmed Pavy's findings by showing that ovalbumin hydrolysates were hexose positive. Later that year, Hoffmiester (3) crystallized ovalbumin and obtained the osazone from its strong base hydrolysate. In 1900, Seemann (4) isolated glucosamine hydrochloride from similar ovalbumin hydrolysates. The amount of saccharide isolated by both Seemann and Hoffmiester was very small and it was considered by them to be a contaminant. It was generally accepted by 1900 that proteins were composed of amino acids, with the occasional prosthetic group (to which sugars certainly did not belong).

In the years 1900 to 1919, the great sugar chemist Emil Fischer focused his attention on the structure and function of proteins, further weakening the cause of glycoproteins. He approached the problem

from two directions, the first being the isolation and characterization of components of protein hydrolysates (amino acids and di- and tripeptides) by fractional distillation of their corresponding methyl esters. His second approach was even more remarkable, namely the synthesis of polypeptides from optically active amino acids. By 1907, he had produced a 14 residue polypeptide which had all of the essential features of a protein. These were: precipitability, a strong Biuret reaction and susceptibility to digestion with "pancreatin". Later that year, he had produced an 18 residue polypeptide (3 leucines and 15 glycines) and embarked on the production of a more highly mixed hetropeptide. Through their work, Hoffmiester and Fischer were credited with having formulated the peptide hypothesis which further strengthened the conviction that proteins were composed strictly of amino acids. Ironically, the two chemists most likely to appreciate the importance of glycoproteins, Hoffmiester who crystallized ovalbumin and Fischer the sugar chemist, failed to recognize their existence.

By 1920 however, flaws began to appear in the peptide hypothesis. Most notable of these, and by Fischer´s own admission important, was the inability of pepsin to digest any of his synthetic peptides. This lack of digestibility led to a somewhat more relaxed stance regarding the structure and composition of proteins and renewed interest in glycoproteins.

In 1927 Fränkel and Jellinek (5) hydrolysed non-crystalline ovalbumin with barium hydroxide, precipitated the free amino acids with lead acetate and isolated an alcohol precipitable polysaccharide. When this polysaccharide was then hydrolysed with weak acid, mannose and

glucosamine hydrochloride were obtained. In 1929 Levine and Mori (6) conducted the same experiments as did Fränkel and Jellinek with several modifications. Crystalline ovalbumin was hydrolysed with a strong base resulting in the recovery of only minute quantities of hexose. Levine and Mori concluded that the crystallization procedure had freed the ovalbumin of saccharide and that the sugar which was found was due to ovomucoid. Two years later, Rimmington (7) repeated these experiments on horse serum proteins using the same protocol as Levine and Mori and came to a similar conclusion. In 1934 Sørensen, (8) using colorimetric techniques, detected the presence of carbohydrates in almost all proteins she studied and estimated ovalbumin to be 1.8% saccharide by weight.

The question as to whether proteins were glycosylated or not was finally resolved in 1938 by Neuberger (9). In an exhaustive series of experiments performed on ovalbumin, Neuberger showed that the carbohydrate content could not be revoved from the protein by denaturation, ultrafiltration or repeated crystallization. In addition, he determined that ovalbumin was glycosylated at a single location, and that the oligosaccharide had a molecular weight of 1200. The empirical formula that he tentatively assigned to this oligosaccharide was four mannose and two glucosamine residues linked covalantly to an unidentified nitrogenous component.

Almost 20 years would pass before any further work was done to identify this nitrogenous component. During this time, the introduction of a variety of chromatographic techniques made the resolution, characterization and quantitation of amino acids, peptides and

glycopeptides possible. In 1957, Cunningham et al. (10) isolated a glycopeptide from ovalbumin and determined the linkage between amino acid and saccharide to be through the β-carboxy terminal of aspartic acid. Nine months later, Neuburger's group reported that ovalbumin glycopeptides digested exhaustively with pepsin, followed by trypsin and chymotrypsin, yielded a complex consisting of 5 mannose, 3 glucosamine, 1 aspartic acid, 1 leucine and 0.5 mole equivalents of serine and threonine. Analysis of this compound showed it to have 7 mole equivalents of nitrogen, as opposed to the 6 that were expected. Neuburger was unable to account for this extra mole of nitrogen. Several years later two other groups, led by Yamashita (11) and by Bogdanov (12) had published similar findings, with respect to the glucosamine aspartic acid linkage. In 1963, Neuburger (13) was able to account for the unexpected nitrogen by comparing synthetic glycopeptides with those from ovalbumin. From this, it was determined that the linkage was between the C-1 of glucosamine and the β-nitrogen on asparagine.

1.2 Structural Features of Glycoprotein Oligosaccharides

Figure 1 shows the main structural features of the most important linkages that exist in nature between protein and carbohydrate. Figure 1A shows the type of linkage that is present in the N-linked glycoproteins, which are the main linkages of structures of interest for the purpose of this thesis. The linkage is an N-glycosylamine bond involving the amide group on asparagine and the hydroxyl on C-1 of GlcNAc. Other sugars are then attached by a glycosidic bond to the GlcNAc *via* the C-1 hydroxyl on the incoming sugar and the hydroxyl on C-4 or C-6 of the GlcNAc. As a result, the oligosaccharide will always remain non-reducing. This type of linkage is characteristic of serum glycoproteins, ovalbumin and many cell membrane glycoproteins. The other structures in Figure 1 (1B-1E) are characteristic of the O-linked glycoproteins.

N-Linked oligosaccharides are widely distributed throughout nature as they are found in virtually all eukaryotic cells. Through the examination of oligosaccharides isolated from a variety of glycoproteins, it is evident that they fall into one of three structural categories, the high mannose, the hybrid and the complex. All three types share a common core pentasaccharide to which differing outer branches are added. For the high mannose type, two to six additional mannose residues are added to the core. The hybrid structures have features common to both the complex and high mannose molecules. Also, in most situations the hybrid chains are "bisected" with a β1,4 linked GlcNAc linked to the β-linked mannose. The complex chains have from two

to four branches although in some instances, the avian mucoids, up to five have been found (14). The outer branches consist of a N-acetyllactosamine unit which is substituted with additional residues, Gal, Fuc, SA and GlcNAc (See Figure 2).

The two most frequently encountered outer branches have a fucose α1,3-liked to the lactosamine GlcNAc as in human lactotransferrin or SA α2,6 linked to the Gal as in human transferrin (15 & 16). Interestingly, oligosaccharides will not possess outer branch terminals of both Fuc and SA on the same molecule (17). Gal residues may also be substituted with α1,2 or α1,6 linked Fuc or an additional α1,3 linked Gal. Linear 2,8 linked polysialylated chains are found linked α2,3 to Gal in a number of neural glycoproteins (18) (See Figure 3). One unusual outer chain sequence consists of Galβ1,3GlcNAc that is di-substituted with SA 2,3 or α2,4 on Gal and α2,6 on GlcNAc (19). Other specialized outer branches have been found and they include the poly-N-acetyllactosaminoglycans, $(Gal\beta1,4GlcNAc\beta1,3)_n$, found on the erythroglycans (20) and the polymannosyl extensions, $(Man\alpha1,6Man\alpha1,6)_n$, found on yeast mannoproteins (21 & 22). With the variety of outer branches, linkages, bisection and fucosylation a vast number of different oligosaccharides are possible.

Figure 1. The five major carbohydrate-peptide linkages.

1A. β-N-Acetylglucosaminyl-asparagine or N-glycosidic

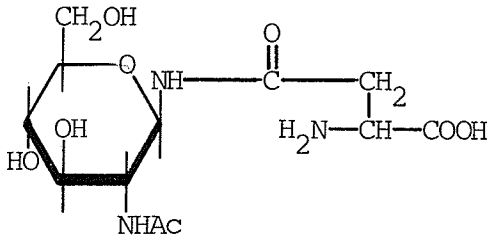
1B. α-N-Acetylgalactosaminyl-serine/threonine or O-glycosidic

1C. Xylosyl-serine

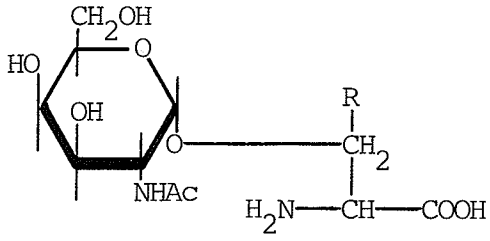
1D. Galactosyl-hydroxylysine

1E. L-Arabinosyl-hydroxyproline.

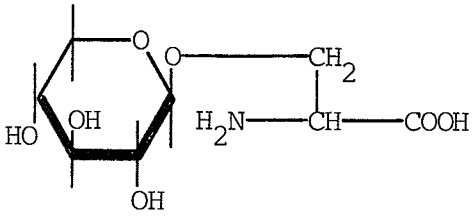
N-linked glycoproteins are widely distributed throughout nature in all eukaryotes. O-linked glycoproteins have thus far only been isolated from animals, reptiles and fish. The Xylosyl-serine linkage although found predominantly in proteoglycans, has also been characterized in thyroglobulin. Galactosyl-hydroxlysine linkages are found in collagens. Thus far only glycoproteins from plants and algae have had the L-Arabinosyl-hydroxyproline linkage.



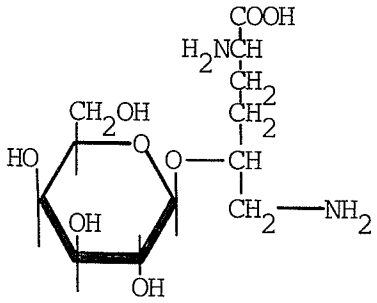
1A.



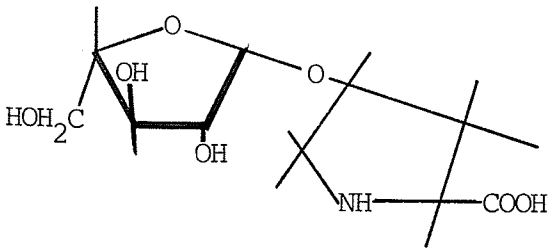
1B.



1C.



1D.



1E.

Figure 2. The three types of oligosaccharide structures.

The high mannose structure shown has been isolated from a variety of glycoproteins including: porcine thyroglubulin, porcine kidney acid mannosidase and soybean agglutinin (56, 71 & 72). The bisected hybrid structure depicted was isolated from hen ovalbumin (73). The complex bisected triantennary structure has been found on turkey ovomucoid (14).

The region enclosed in the box is the core pentasaccharide, common to all types of N-linked oligosaccharides.

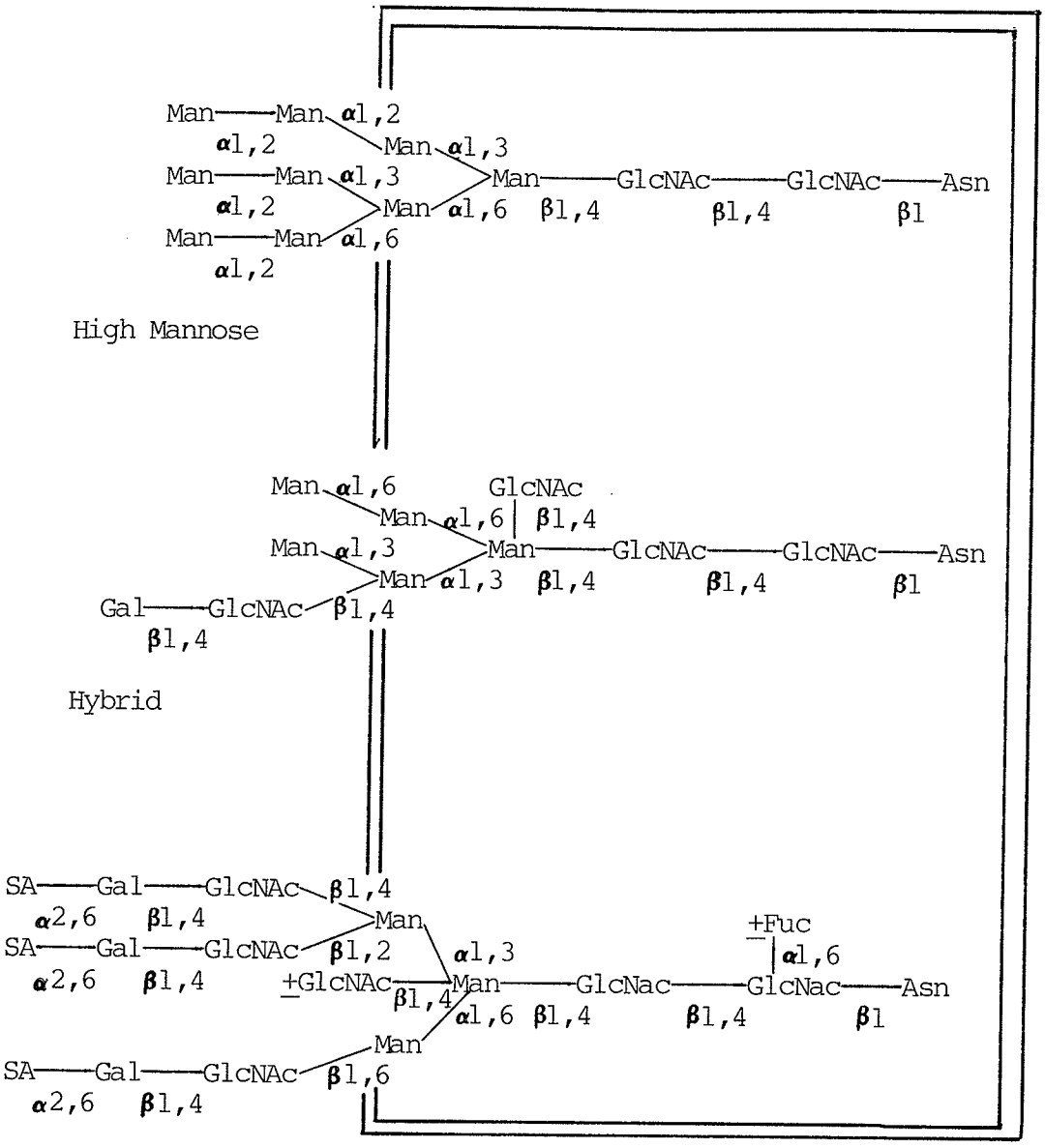
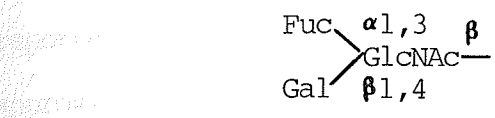
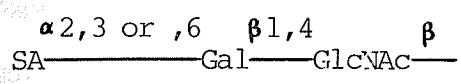
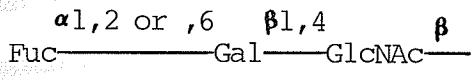

Man——Man α1,2
α1,2 Man α1,3
Man——Man α1,3 Man——GlcNAc——GlcNAc——Asn
α1,2 Man α1,6 β1,4 β1,4 β1
Man——Man α1,6
α1,2
High Mannose
Man α1,6 GlcNAc
Man α1,6 β1,4
Man α1,3 Man——GlcNAc——GlcNAc——Asn
Man α1,3 β1,4 β1,4 β1
Gal——GlcNAc β1,4
β1,4
Hybrid
SA——Gal——GlcNAc β1,4
α2,6 β1,4 Man
SA——Gal——GlcNAc β1,2 α1,3 +Fuc
α2,6 β1,4 ±GlcNAc β1,4 Man——GlcNac——GlcNac——Asn
α1,6 β1,4 β1,4 β1 α1,6
Man
SA——Gal——GlcNAc β1,6
α2,6 β1,4
Triantennary

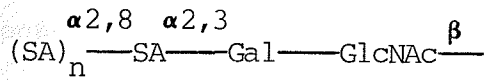
Figure 3. Various outer chain sequences found on complex oligosaccharides.

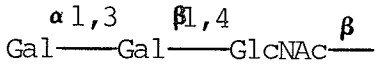
The six outer chain sequences are arranged in frequency of occurrence. Those occurring most frequently at the top, less frequently the bottom. Complex structures may have from two to four outer branches and in addition they may also be bisected with GlcNAc on the α-linked mannose and fucosylated on the Asparagine linked GlcNAc. The boxed in area is the core pentasaccharide.

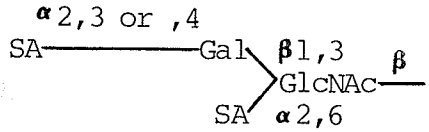

Fuc
α1,3
β
GlcNAc
Gal
β1,4

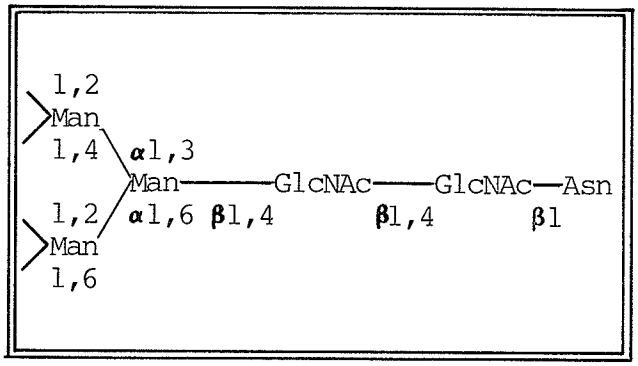

α2,3 or ,6
β1,4
β
SA
Gal
GlcNAc


α1,2 or ,6
β1,4
β
Fuc
Gal
GlcNAc


α2,8
α2,3
β
(SA)n
SA
Gal
GlcNAc


α1,3
β1,4
β
Gal
Gal
GlcNAc


α2,3 or ,4
SA
Gal
β1,3
β
GlcNAc
SA
α2,6


1,2
Man
1,4
α1,3
Man
GlcNAc
GlcNAc
Asn
1,2
α1,6
β1,4
β1,4
β1
Man
1,6

1.3 The Biosynthesis of N-linked Oligosaccharides

Prior to 1970, it was thought that N-linked oligosaccharides were constructed by the sequential addition of sugar residues to the non-reducing terminal of the growing oligosaccharide chain. While this is true, for the non-mannose residues of the complex and hybrid structures, the situation for the pentasaccharide core and the high mannose glycans is somewhat more complex. In 1972, Parodi et al. (23) reported that a glucose containing, lipid-linked oligosaccharide of approximately 20 residues could be synthesized and transferred to an acceptor protein in a cell free system prepared from rat liver. This result, along with others from subsequent experiments (24), indicated that these lipid linked oligosaccharides consisted of up to 3 glucose residues (of which, if any were present, at least one was at a non-reducing terminal), 4 to 10 mannose residues and a core Manβ1,4GlcNAcβ1,4GlcNAc which was joined via a pyrophosphate bond to an α-saturated, poly-isoprenol alcohol (dolichol) of 18-20 units. The synthesis of a glucosylated lipid-linked oligosaccharide and its transfer to an acceptor protein, is known as the dolichol cycle.

The dolichol cycle begins by the formation of a Dolichol Pyrophosphate-N-Acetylglucosamine (GlcNAc-P-P-Dol) complex in which GlcNAc-1-P is transferred from GDP-Man to Dol-P by a N-acetylglucosamyl transferase. Chain elongation is continued by the addition of the second GlcNAc and 5 mannose residues derived from their respective GDP-sugars. The remaining mannose and glucose residues to produce the $Glc_3Man_9GlcNAc_2$-P-P-Dol required for transfer to an acceptor protein

appear to be obtained from the respective Dol-P-sugars (25). What follows is the key step in the synthesis of glycoproteins, where oligosaccharides are transfered from the glucosylated lipid oligosaccharide donor en bloc to an asparagine sequon on an elongating (33 & 34) or newly synthesized protein (See Figure 4). The sequon is a tripeptide that has the sequence asparagine-X-serine/threonine where X may be any amino acid except proline or aspartic acid (25).

Glycoproteins that are produced by the dolichol cycle, are found in a variety of environments; enclosed in vesicles, bound in membranes and secreted extracellularly. With such a widespread occurrence, a number of questions regarding the physical processes that mediate translocation must be asked. The most pertinent of these is: how are glycoproteins that are synthesized on the cytoplasmic face of a lipid bilayer transported to the lumenal side of a solute impervious vesicle? The answer, to what might seem to be a physical impossibility, lies in the function of three highly specialized proteins that allow nascent, elongating proteins to be passed through a transient pore, as a single strand. This entire process, was first termed the signal hypothesis, when it was originally proposed by Blöbbel and Dobberstein in 1975 (26). Since then, it has been modified somewhat and been re-named more appropriatley, as the ER translocation system (27).

The most important of three proteins involved in protein translocation across the ER, is the Signal Recognition Particle (SRP), which is a complex protein, composed of six non-identical polypeptide chains of MW 72, 68, 54, 19, 14 and 9 Kd along with a single strand of RNA of approximately 300 bases. The SRP may exist in four states on the

cytoplasmic side of the ER, a free state in solution or three bound states. One bound to a SRP receptor protein and the other two to ribosomes. Upon translation of mRNA coding for the signal sequence (a hydrophobic N-terminal region of the peptide that is addressed to receptors in the ER translocation system), a high affinity complex forms between the SRP, the ribosome and the signal sequence. Concurrent with this complex formation is the arresting of further protein translation by some unknown mechanism. The SRP-arrested ribosomes are then targetted to the SRP-receptor. The steps following the formation of the arrested ribosome-SRP-SRP receptor complex are not clearly understood. It is believed that other integral membrane protein(s) now interact with the ribosome to anchor it and to create a pore-like structure. Once formed, the SRP is released from the ribosome into the cytoplasm and protein elongation is re-established. Under most circumstances, the signal region of the nascent protein is removed by a signal peptidase found within the vesicle. For those glycoproteins that are integral in the membrane, it is believed that some event triggers the release of the ribosomes from the pore forming protein prior to the completion of chain elongation thereby embedding the protein within the membrane (See Figure 5).

In light of the complexity of protein translocation, the only logical location for protein glycosylation would be on the lumenal side of the ER. The problem with this is that it requires the transport of sugars across a lipid barrier. To resolve this dilemma, the dolichol cycle is split into two distinct halves. Studies done to determine the orientation of lipid-linked oligosaccharides, indicate that species up

to $Man_5GlcNAc_2$ in size are found on the cytoplasmic face of the ER. Larger oligosaccharides, which have additional mannose and glucose residues, are found exclusively on the lumenal face. These results, taken along with the observed substrate requirements, suggest that lipid oligosaccharides up to the $Man_5GlcNAc_2$ compound are constructed on the cytoplasmic face with the GDP-sugars while the addition of further residues occurs in the lumen, with dolichol sugar phosphates as the donors (25). The mechanism by which the lipid sugar phosphates or the lipid oligosaccharides are transported across the ER is not understood (See Figure 4).

Following glycosylation, a series of processing reactions (also known as post translational modifications) will occur, that will ultimately shape the final oligosaccharide structure. The earliest processing reactions, occur in the RER and involve the removal of the three glucose residues along with one to three mannose residues. Glucosidase I releases the terminal α1,2 linked glucose while the two inner α1,3 linked residues are released by glucosidase II (28 & 29). The resultant $Man_9GlcNac_2$, may then be acted upon by at least three α-mannosidase activities. Two are located in the RER, one is specific for a single mannose residue (30) while the other is less specific and is capable of the release of three mannoses (31). The remaining mannosidase is located in the Golgi complex and is capable removing all four α1,2 linked mannoses (32 & 54). The precise timing of these early processing events is under debate as evidence exists to support both co- and post translational processing as far as the removal of the three glucose residues is concerned (33).

Once processed to the stage of a Man_8-$Man_6GlcNAc_2$ glycoprotein, these glycoproteins are packaged in vesicles and transported to the Golgi complex. In the cis/trans compartment of the Golgi, all secretory glycoproteins are processed to a $Man_5GlcNAc_2$, at which point, a single GlcNac is added to unbranched terminal α1,3 linked mannose. This reaction is catalysed by GlcNAc transferase I and is essential for further processing. The branched terminal α1,3 and α1,6 linked mannose residues are removed by a GlcNAc dependant mannosidase (Golgi mannosidase II) and a second GlcNAc is added to the nascent terminal mannose. Following this, the glycoproteins are moved to the trans Golgi, where the addition of other sugar residues occurs, to yield the various complex forms seen on mature glycoproteins (See Figure 6).

Other glycoproteins, notably those found in the RER and in the lysosomal compartments are also processed, but to a different extent. Glycoproteins which reside in the RER, are processed only to the extent of a Man_8-$Man_6GlcNAc_2$ (35 & 36). Those targetted to the lysosome are phosphorylated to produce a Man-6-phosphate residue which acts as a signal to transport the lysosomal enzyme from the Golgi to the lysosomal compartment of the cell (25).

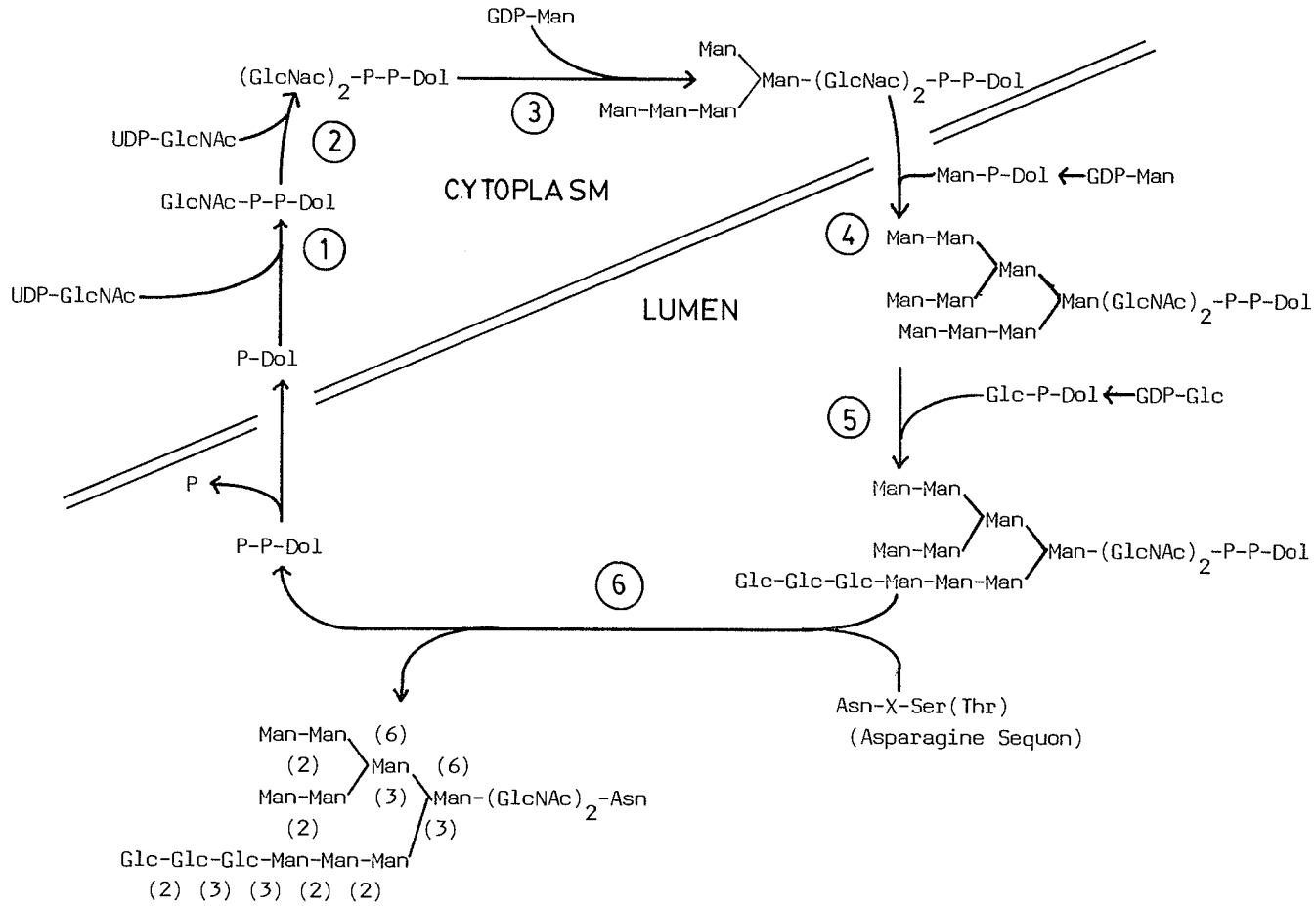


Figure 4. The dolichol cycle based on that proposed by Turco and Robbins (74) and Sharon and Lis (75). The circled numbers indicate six main sequential reactions in the cycle. The numbers in the parentheses indicate the type of linkage α1,( ).

Figure 5. The ER translocation system as proposed by Blobel (27). Upper case characters denote the ribosome cycle, lower case, the SRP cycle.

[A] Free ribosome and [a] SRP (Signal Recognition Particle) in the cytoplasm. [B,b] Binding of SRP to ribosome and commencement of translation. [C,c] Formation of high affinity SRP-ribosome complex, protein elongation and arrest of translation by signal peptide-SRP interaction. [D,d] Docking of SRP arrested ribosomes to SRP-receptor, migration of ribosome receptors and formation of transient pore. [E] Pore formation completed, resumption of protein elongation and release of SRP into cytoplasm [e] via the SRP-receptor. [F] Protein elongation and release of signal peptide with signal peptidase. [F´] Continued elongation and separation of ribosome receptors (membrane bound only). [F´´] Formation of trans-membrane segment and continued elongation (membrane bound only). [G] Completion of elongation and release of ribosome into cytoplasm.

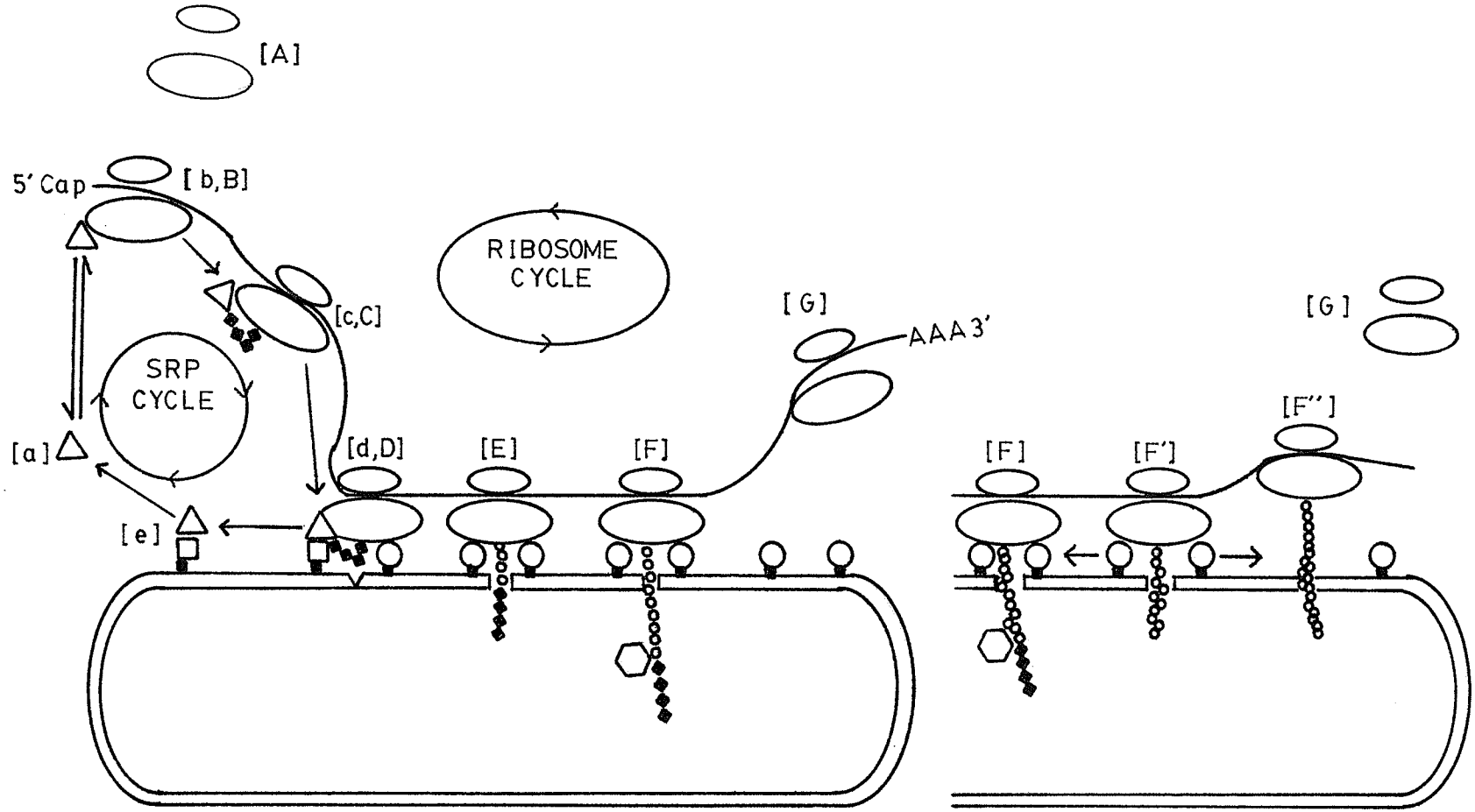

[A]
5' Cap
[b,B]
[c,C]
RIBOSOME
CYCLE
SRP
CYCLE
[G]
AAA 3'
[G]
[F'']
[a]
[d,D]
[E]
[F]
[F]
[F']
[e]

Figure 6. Schematic pathway of oligosaccharide processing (25).

The reactions are catalysed by the following enzymes:

A- Oligosaccharyltransferase,

B- α-Glucosidase I,

C- α-Glucosidase II,

D- ER α1,2 Mannosidase,

E- N-acetylglucosaminylphosphotransferase,

F- N-acetylglucosamine-1-phosphodiester α-N-acetylglucosaminidase,

G- Golgi mannosidase I,

H- N-acetylglucosaminyltransferase I,

I- Golgi α-mannosidase II,

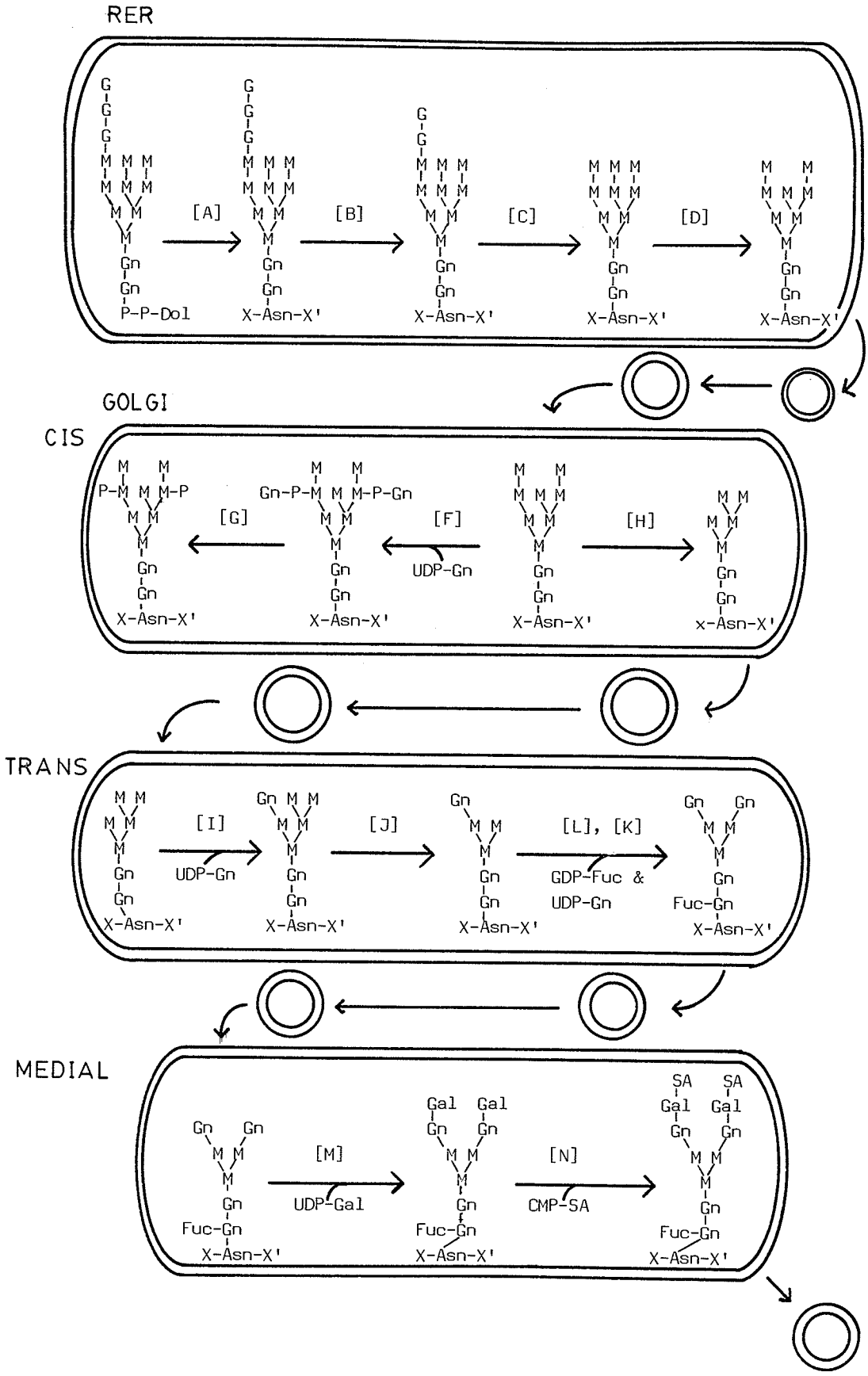
J- N-acetylglucosaminyltransferase II,

K- Fucosyltransferase,

L- Galactosyltransferase,

M- Sialyltransferase.

G	-Glucose
M	-Mannose
Gn	-N-acetylglucosamine
Fuc	-Fucose
Gal	-Galactose
SA	-Sialic Acid
Dol-P-P	-Dolichol Pyrophosphate


RER
[A]
[B]
[C]
[D]
P-P-Dol
X-Asn-X'
GOLGI
CIS
[G]
[F]
[H]
UDP-Gn
TRANS
[I]
[J]
[L], [K]
UDP-Gn
GDP-Fuc &
UDP-Gn
MEDIAL
[M]
[N]
UDP-Gal
CMP-SA

1.4 Methods of Structural Determination

The determination of oligosaccharide structure is not a task which is easily accomplished by the application of any one specific chemical or physical method. Rather, it is obtained by the correlation of information yielded by a variety of methods. The two aspects of oligosaccharide structure which necessitate this approach have their origins in the mechanism of biosynthesis and in the chemical properties of their constituent monosaccharides. As was noted earlier, proteins, DNA and RNA are either translated or transcribed from a highly invariant template. N-linked oligosaccharides are processed. Even though processing is remarkably conservative, it does result in structures that exhibit microheterogeneity. In addition to this, glycosidic bonds are optically active and N-linked structures are branched.

The oldest and now least used method for structural determination is permethylation analysis. First described in 1937 by Howarth for the determination of cellulose structure, it was not until 1964 that Hokamori devised methodology that made it applicable on a scale small enough that it could be applied to protein derived oligosaccharides (37). In the typical permethylation procedure, a small sample of oligosaccharide is dissolved in dimethyl sulfoxide under a nitrogen atmosphere. An ammount of methylsulfinyl carbanion equivalent to the hydroxyl content of the oligosaccharide is generated *in situ* by the addition of a measured quantity of sodium hydride. After a 10 min incubation, the reaction is stopped by the addition of excess methyl

iodide. Following work-up, the product, a per-O-methyl oligosaccharide (one in which all hydroxyls have been converted to methyl ethers) is hydrolysed and esterified with glacial acetic acid. The resultant mixture of alditol acetates is then resolved and quantitated, by either gas-liquid, or thin layer chromatography. Although the procedure is not very difficult, it does have three drawbacks: it is destructive, it requires several milligrams of oligosaccharide which may not always be available, and it may give more than one possible answer.

Currently, the most frequently employed techniques for determining oligosaccharide structure are affinity chromatography or other chromatographic techniques, alone or in combination with enzyme digestion and proton NMR. All are more sensitive and are able to yield more structural detail than methylation analysis. The use of chromatographic techniques and NMR offers an additional advantage, that they are non-destructive.

The most rapid and easily applied method for screening glycan structure, affinity chromatography, owes its utility to a family of molecules known as the lectins. Lectins are defined as proteins of a non-immune origin, which are multivalent for the binding of carbohydrates. To date, approximately 120 lectins have been isolated, from a wide variety of sources, mostly plant seeds (38). Lectins show their versatility, not only in the ability to bind specific monosaccharides, but also, in their ability to distinguish complex structural details. When used in affinity chromatography, the lectins are immobilized on an inert support such as Sepharose or agarose and placed in a column. A solution containing the oligosaccharide under

study is passed through the column and if the structural feature required for binding to the lectin is present, the oligosaccharide will be reversibly bound. Later, the bound oligosaccharide may be released by eluting the column with a buffer containing a molar excess of an appropriate glycoside.

There are six commonly used lectins for screening oligosaccharide structure that allow for the rapid determination of gross structural detail. The most widely used lectin, Concanavalin A (Con A), has its highest affinity for the trimannosyl portion of the common core pentasaccharide (39). This affinity however, is strongly moderated by the further addition of sugar residues. For this reason, Con A has no affinity for either tri or tetra-antennary complex structures and a progressively increasing affinity, for biantennary, hybrid and high mannose oligosaccharides. Wheat Germ Agglutinin (WGA) is able to bind complex and hybrid structures, having a bisecting GlcNAc. Pea and lentil lectins have affinities for two structural features, biantenary complex chains- that are fucosylated on the GlcNAc linked to asparagine, or similarly fucosylated, triantennary structures that have a 2,6 disubstituted, α-linked mannose. Erythroagglutinating Phytohaemaglutinin (E-PHA) is specific for biantennary complex chains, that have galactose at the non-reducing terminals and are bisected by a GlcNAc. Leukoagglutinating Phytohaemagglutinin (L-PHA) is specific for complex chains, having at least one 2,6 disubstituted α-linked mannose and non-reducing terminal galactose residues- hence, tri and tetra-antennary glycans (40).

Degradative sequencing of oligosaccharides, either through

chemical or enzymic digestion, has many similarities to the traditional sequencing methods employed for proteins and nucleic acids. In a generalized enzymic approach the glycan in question is incubated with a highly purified glycosidase. An alteration in the chromatographic behaviour, before and after digestion, indicates the presence of a specific residue or linkage. Due to the catalytic properties of enzymes, it is possible to sequence from the terminals of the glycan, using exo-glycosidases and from within the glycan, using endo-glycosidases.

Chemical hydrolysis of oligosaccharides is rather limited in its use due to the similar nature of all glycosidic bonds. A number of exceptions to this rule do exist. The sialic acids are very sensitive to mineral acid hydrolysis (0.02 M HCl is more than adequate) and therefore de-sialylation of complex structures is possible without damaging the rest of the chain. Acetolysis is a technique that allows one to hydrolyse α1,6 linked mannoses selectively. Recently, it has been successfully applied to the determination of high mannose oligosaccharide structure (41).

Although all of the methods described thus far are able to give us a great deal of insight into the structure of an oligosaccharide, our ultimate understanding of the mechanisms responsible for processing and for the relationship between structure and function are dependent upon a highly detailed picture at the molecular level. If sufficient oligosaccharide is available (approximately 200μg), simple proton NMR at 300 MHz or greater can give us some of the desired detail. Pioneering work in this field was done at 220 MHz in 1974 by Wolfe et

al., (42 & 43) on compounds isolated from the livers and urine of individuals having GM_1-gangliosidosis. However it was not until the late 1970's, when spectrometers of 360 and 500 MHz became available, and sophisticated computation systems that allowed for resolution enhancment, higher field homogeneity and Lorenzian to Gaussian transformation, that proton NMR became an invaluable tool. Since that time, a number of workers have expanded the catalogue of available spectra to include examples of all types of N-linked glycans. Most notable of these are the groups of Vliegenthart (44), Schachter (45), Carver (46), Ballou (47) and Montreuil (48).

To facilitate the interpretation of complex NMR spectra, Vliegenthart introduced the concept of "structural reporter groups" (49 & 50). Structural reporter groups embody three distinct but interrelated parameters of a proton signal that provide information essential to permit the assignment of primary structure. These characteristics are chemical shifts (δ) at clearly distinguishable positions of the spectra, together with the coupling constant (J) and the line width of the signal (See Table 1). In addition to being essential elements in the structural reporter groups, the spectral line widths are also indicative of the local mobility of protons.

Besides the aforementioned NMR parameters [chemical shift (δ), coupling constant (J) and spectral line width], spectral integration can give valuable information. The relative intensities of reporter group signals are directly proportional to the absolute numbers of that proton. Often, from integral information, it can be deduced whether or not a sample consists of one or more closely related isomers.

1.5 An Introduction to Present Work

Previous work done in the laboratory of J.C. Jamieson has shown that α_1-AGP is an acute phase reactant, which is elevated four to six fold in the serum and two to four fold intracellularly, within 24 hrs of tissue injury. Studies on the intracellular forms of α_1-AGP, from both control and inflamed rats indicated that they were forms with partially processed oligosaccharide chains. Affinity chromatography on Con-A and chemical analysis of acid hydrolysates indicate that the most likely oligosaccharides to be found associated with RER derived α_1-AGP would be a $Man_9GlcNAc_2$ structure (51). Similar studies suggest that the SER also contains high mannose forms of α_1-AGP, but the oligosaccharide chains contain fewer mannose residues. In these early studies no information could be obtained with respect to the precise structures associated with the intracellular forms of α_1-AGP.

Our current understanding of the early processing reactions that take place in the RER and Golgi have been obtained from three basic experimental approaches. The groups of Kornfeld et al. (30, 32 & 52), Bause et al. (28 & 31), Rothman et al. (53) and Touster et al. (54) have studied the effect of glycosidase activities isolated from microsomes on glucosylated and high mannose oligosaccharide substrates. Other groups, notably those of Herscovics et al. (55), Atkinson et al. (56) and Ballou et al. (57) have looked at the synthesis of manno-proteins in yeast, which require a partially processed, high mannose oligosaccharide substrate prior to their elongation with a polymannosyl branch. Lastly,

Liscum et al. (35), Rosenfeld et al. (36) and Kornfeld et al. (41) have characterized the oligosaccharides from glycoproteins that reside in the RER. All results from these workers point to the presence of two glucosidase activities and one or possibly two α-mannosidases in the RER.

The work described in this thesis seeks to determine pool sizes and the structures of oligosaccharides isolated from intracellular α_1-AGP. In doing this we can examine the early stages of glycoprotein processing without some of the limitations inherent in the experimental approaches of others. Therefore, rather than assuming that a certain step in glycoprotein processing occurs based on an enzyme activity isolated from a subcellular fraction and assayed with a synthetic substrate, the actual in vivo processing intermediates are characterized. Structural detail and pool sizes were determined by proton NMR of glycopeptides and oligosaccharides. As a further confirmation of structure, α_1-AGP oligosaccharides were labelled in vivo with [^{14}C]glucosamine and characterized by HPLC. The results obtained indicate that in rat liver α_1-AGP is exposed to only one α-mannosidase while in the RER. This RER α-mannosidase is capable of releasing a specific mannose residue and approximately 45% of the Man_9 chains on α_1-AGP residing in the RER were processed with respect to this mannose residue. Further processing occurring outside the RER is responsible for the sequential removal of the remaining mannose residues obligatory for complex oligosaccharide synthesis.

Table 1. Selected Structural Reporter Groups

Name	Shift ppm	Line Shape	Information
Anomeric Protons (H-1 Protons)	4.8-5.6	Man Broad Singlet Glc Sharp Doublet	The kind of sugar residue and the type and configuration of its anomeric linkage.
Mannose H-2 and H-3 Atoms	4.0-4.7	Broad Singlets	The pattern of their signals is, as a whole, indicative of the type of substitution of the common mannotriose branching core.
Sialic Acid H-3 Atoms	1.7-1.8 & 2.6-2.7	Triplet Quartet	Their chemical shifts are characeristic for the type and configuration of the glycosidic linkage of the sialic acid residue, and, in some cases, for the location of the residue in the chain.
Fucose H-5 and CH_3 Atoms	4.1-4.3 1.1-1.3	Broad singlet Doublet	The chemical shifts of these protons, together with that of H-1 of the residue, are indicative of the type and configuration of its glycosidic linkage, and of the structural environment, in particular of the residue to which fucose is attached.
Galactose H-3 and H-4 Atoms	3.9-4.2	Doublets or Quartets	Their chemical shifts are, in some cases, useful for characterizing the type and configuration of the Glycosidic linkage between galactose and its sustituent.
Amino Sugar N-Acetyl CH_3	1.95-2.10	Very sharp singlets	Their chemical shifts are sensitive to even small structural variations, making this region of the spectrum highly informative.

2.1 Materials

D-[1-^{14}C]-Glucosamine HCl (7.0 mCi/mMole) was purchased from New England Nuclear Corp., Montreal, Quebec; Lubrol-X was obtained from Imperial Chemical Industries, South Dorking, Manchester, England; Dextran T-70 and Sephadex G-100 and G-50 superfine,from Pharmacia, Dorval, Quebec; Pronase from Boehringer-Mannheim, Montreal, Quebec; Endo-H from Miles Laboratories, Naperville, Illinois; Bio-Gel P-4 from Bio-Rad Laboratories, Toronto, Ontario; Concanavalin-A Sepharose 4-B from Sigma, St Louis, Missouri; D_2O from Aldrich, Milwaukee, Wisconsin; NMR tubes from Wilmad, Buena Park, New Jersey; All other chemicals were obtained from local suppliers and were of the highest quality available.

2.2 Treatment of Experimental Animals

Male Long and Evans hooded rats of 200-300 g were obtained from a local supplier and were maintained on a diet of Purina Rat Chow and water ad libitum. Rats receiving injections were placed under general anesthasia by the inhalation of air saturated with diethyl ether at room temperature for approximatly 1 minute. Experimental inflammation was induced by the subcutaneous injection of turpentine oil in the dorsal lumbar region at the rate of 0.5 ml/100 grams body weight (51); control animals received injections of 0.15 M NaCl sterilized by filtration through Millipore Millex-GV filters. Rats were starved 24 hours prior to sacrifice which was between 10 AM and 12 PM to minimize nutritional and diurnal effects.

In all experiments, prior to sacrifice rats were heavily etherized.

After severing the jugular vein, blood was collected in a glass beaker, allowed to clot at room temperature for 1 hour and stored overnight at 4°C. Serum was prepared by centrifugation of non-clotted blood components at $5000g_{av}$ for 20 min. Livers were perfused *in situ* via the portal vein or hepatic artery with ice-cold 0.25 M sucrose, 100 mM α-D-Mannose, then excised and transferred to ice cold 0.25 M sucrose, 10 mM α-D-Mannose.

2.3 Chemical and Physical Measurements

Hexose was assayed using a resorcinol-sulphuric acid reagent, consisting of 1.5 g of resorcinol dissolved in 10 ml water and diluted to 100 ml with concentrated sulphuric acid. For glycopeptide samples, 200 µl of column eluate was added to 1.25 ml of the reagent and incubated for 30 min a boiling water bath. Samples were allowed to cool to 50°C and their optical densities were read at 428 nm. For samples containing smaller amounts of hexose, 50 µl of the test solution was added to 250 µl of the resorcinol reagent. Following incubation the samples were cooled, diluted to 1.25 ml with water and read at 428 nm. Tests showed the limit of sensitivity for this methodology to be 10 µg mannose per ml. Based on these results, the molar extinction coefficient for mannose was determined to be 2.66×10^6.

Protein was determined using the method of Bradford with bovine serum albumin as a standard (58). Radioactivity was measured on an LKB Rackbeta Liquid scintillation counter with preprogrammed internal quenching curves. For volumes of less than 0.4 ml samples were counted in 4 ml of scintillation cocktail and in plastic mini-vials; larger volumes were counted in 10 ml of cocktail and in glass vials.

2.4 Subcellular Fractionation

All subcellular fractionations were done in a Beckmann L5-50 ultracentrifuge. A Ti-60 fixed angle rotor was used for preparation of RER and SER; Golgi membranes were prepared in a SW-28 rotor. All centrifugations were performed at 4°C.

RER and SER were prepared on a discontinuous sucrose density gradient as descibed by Jamieson and Friesen (59). Perfused livers were homogenized in 3 volumes of 0.25 M sucrose containing 10 mM α-D-mannose in a Potter-Elvehjem homogenizer with 8 strokes of a motor driven Teflon pestle revolving at 2000 rpm. The homogenate was then centrifuged at 12,500 rpm for 20 min and the upper two thirds volume of the supernatant was collected, adjusted to 15 mM with 1.0 M $CsCl_2$ and layered over 10 ml of 1.3 M sucrose containing 15 mM $CsCl_2$ in a polycarbonate bottle. The gradient was then centrifuged at 40,000 rpm for 150 min. The RER collected as a pellet at the bottom of the tube, while the SER collected as a "fluffy layer" at the interface in the gradient. This material was aspirated, diluted 4 fold with 0.25 M sucrose, 100 mM TRIS pH 9.1, adjusted to 10 mM with 1.0 M $MgCl_2$ and pelleted at 40,000 rpm for 90 min.

Golgi membranes were prepared as described by Jamieson and Ashton (60) with only minor modifications. Livers were homogenized by 8 strokes of a Potter-Elvejhem homogenizer in 4 volumes 0.25 M sucrose-medium-A (medium-A: 100 mM TRIS-HCl pH 7.6, 10 mM $MgCl_2$, 10 mM α-D-mannose) and centrifuged for 5 min at 3000 rpm in a Ti-60 rotor. Supernatants were pooled and 12 ml were layered over a discontinuous

gradient consisting of 13 ml of 1.3 M and 12 ml of 0.7 M sucrose in medium-A. Following centrifugation for 45 min in a SW-28 rotor at 28,000 rpm, the "fluffy layer" which collected at the interface between the 0.7 and 1.3 M sucrose was pooled, diluted 4 fold with 0.25 M sucrose in 100 mM TRIS-HCl pH 9.1 and pelleted at 40,000 rpm for 90 min in the Ti-60 rotor.

2.5 Isolation of α_1-AGP From Rat Serum

The first step in the preparation of α_1-AGP involved the isolation of a glycoprotein rich fraction based on the procedure of Simkin et al. (61). One volume of rat serum was diluted 10 fold with 0.15 M NaCl at room temperature. This solution was slowly adjusted to 0.6 M in perchloric acid with rapid stirring by the addition of a 1.8 M perchloric acid solution and allowed to stand for 10 min. Following centrifugation at $600g_{av}$ for 20 min the supernatant was collected, adjusted to neutrality with 2.0 M NaOH and dialysed exhaustively against water. The dialysate was then concentrated in an Amicon Model 8400 ultrafiltration cell fitted with a UM10 filter and lyophilized. The resultant material which is rich in α_1-AGP was termed the perchloric acid (PCA) soluble fraction.

α_1-AGP was further purified from the PCA soluble fraction with CM-Cellulose chromatography based on the procedure of Jamieson et al. (62). The CM-Cellulose was prepared by washing twice with two bed volumes of 0.25 M NaOH, two volumes of 0.25 M NaCl and with water until neutral. The CM-Cellulose was then placed into a 2.5x50 cm column and equilibrated with a 50 mM sodium acetate buffer pH 4.9 at 4°C overnight. A sample of 250 mg of the PCA soluble fraction was dissolved in the

running buffer, and centrifuged for 2 min at 8000 g_{av} and the supernatant was loaded onto the column. The column was eluted with the running buffer at 50 ml/hr and 5 ml fractions were collected; those having an OD_{280} greater than 0.05 were pooled, dialysed, lyophilized and designated the CMC-1 fraction.

CMC-1 was further purified by isoelectric focusing on a pH 1-5 gradient. The dense electrode solution consisted of 0.15 ml concentrated sulfuric acid, 16 ml glycerol and 3.85 ml water. The dense gradient solution contained 0.1 g monochloroacetic acid, 0.1 g dichloroacetic acid, 0.1 g phosphoric acid, 35 ml glycerol and 20 ml water. The light gradient solution contained 0.1 g acetic acid, 0.1 g formic acid, 0.1 g citric acid, 0.1 g glutamic acid, 0.05 g aspartic acid, 0.30 ml pH 5-8 Ampholine Carrier Ampholytes (LKB 1809-126), 30 mg CMC-1 and 60 ml water. The gradient was generated using an LKB 8121 gradient mixer fitted with an LKB 8123-1 stirrer and was layered over dense gradient solution in an LKB 8100-1 isoelectric focusing column. Light electrode solution, 0.1 M NaOH, was added until the column was filled. Focusing involved the application of a constant current of 15 mA until the applied voltage reached 600 V this was followed by holding the potential constant at 600 V and allowing the current to drop below 1 mA. At this point 1 ml fractions were collected; those having an OD_{280} greater than 0.05 and a pH in the range of 2.85 to 3.30 were pooled, dialysed exhaustively and lyophilized. This material was deemed to be pure rat serum α_1-AGP as determined by SDS-PAGE and double diffusion analysis against both anti-whole rat serum and anti-rat α_1-AGP.

2.6 Immunological Methods

Antisera were prepared essentially as described by Simkin et al. (63) using full lop male albino rabbits. A mixture of 0.75 mg antigen, 0.20 ml 0.25 M NaCl and 0.40 ml Freund's complete adjuvent were emulsified by sonication with a 15 second burst at 50% power with an Artek Sonic Dismembranator fitted with a P200-1 probe. The emulsion was injected intramuscularly, one half of the dose into each thigh. Six days later, 1.25 mg antigen was prepared and injected as above. After a further 22 days, a series of intravenous injections of an antigen-aluminium hydroxide co-precipitate were given. The co-precipitate was prepared by adding sufficient 1.0 M $NaHCO_3$ to neutralize a mixture of 0.4 ml 10% potassium alum, 0.2 ml sterile water and 6.0 mg antigen. This neutralized mixture was allowed to stand over night at 4°C; the precipitate was then collected by centrifugation and suspended in 0.40 ml of 0.10 M potassium phosphate pH 7.4, containing 0.01% thiomersal. Volumes of 0.05, 0.10 and 0.25 ml of the suspension were injected on alternating days. Animals were bled twice, at 5 and 7 days following the last injection. Blood was collected directly into conical bottom centrifuge tubes, allowed to clot for 1 hr and stored overnight at 4°C. Non-clotted blood components were collected and centrifuged at $3000g_{av}$ for 20 min. Antisera collected was stored at -20°C until required.

Double diffusion analysis was performed as described by Ouchterlony (64). The medium consisted of 1.25% Nobel agar, 0.15 M NaCl and 0.01% NaN_3. Wells were cut, using a Bio-Rad universal template and punch. Following the loading of the sample wells, plates were sealed with

Parafilm and allowed to develop at room temperature for up to 24 hrs.

2.7 Electrophoretic Methods

SDS-PAGE was conducted using the discontinuous buffer system of Laemmli (65) in an LKB-2001 vertical slab gel electrophoresis unit. The stacking gel consisted of 3.0% acrylamide, 0.08% BIS-acrylamide, 0.1% SDS, 0.025% ammmonium persulfate, 0.0375% TEMED in 0.125 M TRIS, pH 8.8. The resolving gel consisted of 10% acrylamide, 0.26% BIS-acrylamide, 0,1% SDS, 0.25% ammonium persulfate, 0.025% TEMED in 0.375 M TRIS, pH 6.8. Samples of test proteins and MW standards were prepared in a buffer consisting of 0.0625 M TRIS pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.001% bromophenol blue by incubation in a boiling water bath for 2 min. Upon cooling, volumes of up to 100 ul containing up to 0.1 mg protein were loaded into the sample wells. Electrophoresis was conducted using a 0.2% SDS, 0.025 M TRIS-0.192 M glycine buffer, pH 8.3, at a constant current of 25 mA per gel at 15°C until the dye front migrated 95% the length of the gel.

Gels were stained using the procedure of Fairbanks (66) and were carried out at room temperature in a sealed Pyrex baking dish. Gels were first stained for 12-16 hrs in a solution of 0.05% Coomassie Brilliant Blue R-250, 10% acetic acid, 25% isopropanol. De-staining was then done using a two step procedure, first, in 0.003% Coomassie Brilliant Blue R-250 10% acetic acid, 60% isopropanol and then, repeated 3 to 5 times for 2 hrs in fresh 250 ml volumes of 10% acetic acid. Gels were photographed by supporting them on a glass plate and then placing them over a white backlit backround.

2.8 Labelling and Isolation of Oligosaccharides From α_1-Acid Glycoprotein

α_1-AGP was labeled in vivo as described by Jamieson and Friesen (59) by the intraperitoneal injection of 5 μCi of D-[1-^{14}C]-Glucosamine HCl dissolved in 0.5 ml of sterile normal saline into both control and 24 hr inflamed rats. After a 45 min labelling period, animals were sacrificed and the RER and SER were prepared as described. Pellets of RER and SER from 8-10 rats were extracted with 10 ml of 1% Lubrol-W by homogenization and followed by centrifugation for 1 hr at 250,000 g_{av}. The supernatant was collected and then concentrated 5 fold by pressure dialysis against 1.0% Lubrol-WX containing 0.15 M NaCl, 100 mM TRIS pH 7.4 and 0.05% NaN_3. Prior to the precipitation with anti-sera specific for rat α_1-AGP, a heterologous immune system was employed to remove any non-specific precipitating material (51). The Lubrol extract was adjusted to a final concentration of 100 mM TRIS pH 7.4, 4.7% Dextran T-70, 0.25 M α-D- Mannose and 0.05% NaN_3. Approximately 500 ug of human serum albumin was dissolved in 0.1 ml of normal saline and added along with 1.0 ml anti-human serum. The entire mixture was incubated for 45 min at 37°C and then refrigerated for 48 hrs at 4°C. Immune precipitates of human serum albumin were pelleted by centrifugation. To the supernatant 300 ug of human serum fraction IV and an additional 1 ml of anti-human sera was added and the incubation and centrifugation procedure was repeated. The incubation procedure was repeated once more to assure that no further precipitation occurred and precipitin curves were generated to determine the optimum anti rat α_1-AGP to Lubrol extract ratio. A quantitative precipitation of α_1-AGP was performed and the precipitates were then

analysed by SDS-PAGE and digested with Endo-H.

2.9 Large Scale Isolation of α_1-AGP

RER and SER were prepared as earlier described and pellets from 8-10 rats were homogenized in 50 ml of 100 mM TRIS pH 9.5 with 5 strokes of the pestle in a Potter-Elvejhem homogenizer. The homogenate was then sonicated for 2 min at full power with an Artek sonic dismembranator using a P-200 probe. The solution was then centrifuged at 250,000 g_{av} for 1 hr, the supernatant was collected, dialysed against 2 changes of 1 mM TRIS pH 10.0 and lyophilized. Sonic extracts from 80 rats were pooled and dissolved in 100 ml of 100 mM TRIS pH 9.1; Insoluble material was removed by centrifugation and filtration through Whatman GF/A glass fibre filters. Fifty ml aliquots of this material were then loaded onto a 3.5x250 cm column of Sephadex G-100 superfine and eluted with 50 mM TRIS pH 9.1. Fractions collected were monitored for protein, hexose and immunological activity towards anti-rat α_1-AGP. The α_1-AGP positive region (as judged by arc formation on double diffusion plates) was pooled, dialysed and lyophilized.

This α_1-AGP rich material was then dissolved in 3 ml Con-A binding buffer (100 mM acetate pH 6.0, 1.0 M NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 1 mM EDTA) and bound in a 1.0x15 cm column of Con-A Sepharose. The column was then eluted with Con-A binding buffer, first containing 10 mM α-methyl glucoside followed 500 mM α-methyl mannoside. The α-methyl mannoside eluate was dialysed and lyophilized prior to a final chromatographic separation on a 1.0x75 cm column of Sephadex G-50. The eluate was monitored for OD_{280} the peaks pooled and tested for α_1-AGP.

2.10 Preparation of Glycopeptides and Oligosaccharides

Oligosaccharides from immune precipitates of [^{14}C] glucosamine labelled α_1-AGP were prepared by a procedure similar to that of Trimble (67). The immune precipitates were solubilized and denatured by boiling in 1 ml of 1% SDS for 5 min followed by dialysis against 3 changes of 100 mM acetate buffer pH 5.8 at room temperature. Dialysates were then diluted 5 fold with acetate buffer and digested with 5-15 milli units of Endo-H for 48 hrs at 37°C under a toluene atmosphere. Following digestion any residual protein was precipitated with 2 volumes of methanol, the mixture was centrifuged and the supernatant lyophilized. Oligosaccharides were then de-salted on a 1x100 cm column of Bio-Gel P-4 (+400 mesh) eluted with distilled-deionized water. Fractions were collected and 100 ul aliquots were monitored for ^{14}C.

Unlabelled α_1-AGP was digested with Pronase as described by Mutsaers (68). Samples were dissolved in 5 ml of 100 mM TRIS pH 7.95 containing 2 mM $CaCl_2$ and incubated for a total of 96 hrs at 37°C in a toluene atmosphere. At 0, 24 and 48 hours Pronase amounting to 4% of the weight of the protein to be digested was added and the pH was re-adjusted to 7.95 if necessary. Following digestion the samples were incubated in a boiling water bath for 2 min, centrifuged and the supernatant was lyophilized. The freeze dried material was then dissolved in the minimum volume of water required and was resolved on a 1.5x200 cm column of Bio-Gel P-4 (200-400 mesh) with 100 mM pyridine-acetate buffer pH 5.1. Fractions were collected, assayed for hexose, pooled and lyophilized. Pools were then analysed by NMR to determine which fraction contained glycopeptide.

Glycopeptides were digested with Endo-H as described by Trimble (67). Samples were dissolved in 1.0 ml of 100 mM acetate pH 5.8 with 5-20 milli units of Endo-H. Incubations and de-salting were performed as described above for immune precipitates and the fractions were assayed for hexose.

2.11 Fractionation of Oligosaccharides by HPLC

Oligosaccharides were fractionated on a 0.46x25 cm resolving column and 0.46x5 cm guard column of 5μ Aminospherisorb on a Perkin-Elmer Series 4 Liquid Chromatography system. Samples were pre-filtered on 0.22 μ Millipore Millex-GV membrane, lyophilized and then dissolved in 100 ul of 60:40 acetonitrile-water (all ratios of solvents are V:V). Samples were then chromatographed essentially as described by Herscovics (55) with the exception that the solvents employed were unbuffered. The elution was performed isocratically at 1.5 ml per min with 60:40 acetonitrile-water for 14 min followed by a linear gradient to 42:58 acetonitrile-water over 64 min. Fractions from labelled samples were collected at 1 min intervals and counted. Non-labelled samples were monitored at 196 nm and peaks were collected and analysed by ^{1}H NMR. The column was pre-calibrated with ^{14}C labelled $Glc_3Man_9GlcNAc$-$Glc_1Man_9GlcNAc$ kindly provided by A. Herscovics and with $Man_9GlcNAc$-$Man_7GlcNAc$ oligosaccharides whose structures were determined by NMR.

2.12 NMR Analysis of Glycopeptides and oligosaccharides

Samples were dissolved in 0.5 ml of 99.8% atom percent D_2O, filtered through a Whatman GF/A filter and lyophilized. The solvation-lyophilization procedure was repeated twice and the sample was then stored over P_2O_5 for 48 hrs prior to solvation in 0.6 ml of 99.998 atom

percent D_2O under a nitrogen atmosphere. NMR spectra were obtained on a Bruker AM-300 spectrometer operating at 300 MHz in the FFT mode by the University of Manitoba NMR group. Chemical shifts (δ) are expressed in ppm down-field from an internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured by reference to internal acetone (δ 2.225 ppm in D_2O at 27 C), within an accuracy of 0.002 ppm.

Results

3.1 NMR Studies on Total Intracellular Glycoprotein Oligosaccharides

In order to determine the feasibility of characterizing glycopeptides and oligosaccharides isolated from intracellular α_1-AGP by proton NMR, a preliminary study was conducted on a total protein preparation derived from RER, SER and Golgi. Total proteins were isolated from RER, SER and Golgi prepared from 120 g of rat liver. Following exhaustive pronase digestion, the resultant glycopeptide mixture was chromatographed on Bio-Gel P-4 and the glycopeptide containing fractions were detected by assaying for total hexose. Based on the hexose elution profile, fractions were pooled as indicated in Figures 7-9 and prepared for NMR. The NMR spectrum for each pool was interpreted by comparison with the spectra of three standard glycopeptides (See appendix 1) and through structural reporter groups.

The RER and SER are known to contain glycoproteins that have either high mannose or partially completed complex oligosaccharides (25). While the complex structures have a number of excellent reporter groups (See table 1), the same may not be said of those of the high mannose variety. The only reporter group of any value for characterizing these structures is the anomeric or H-1 proton and in some instances the H-2 protons. To complicate matters further, the prediction of high mannose oligosaccharide structure by the shift values of anomeric proton signals and their integrals is very deceiving especially if the sample in question exhibits microheterogeneity. This

is because of the so called second order effects (also known as virtual couplings) in which the shifts of two or more different protons either overlap or are so close to one another that they are impossible to resolve. In such a situation, spectral integration of a signal thought to be due to a single proton may actually be that of a number of protons. This is most obvious for the terminal α1,2 linked mannose residues D-1, D-2 and D-3 on high mannose oligosaccharides. The H-1 protons for these three residues all resonate in the range of 5.04 to 5.07 ppm while the H-2 protons all fall at 4.07 ppm (See Fig 36). For a homogenous $Man_9GlcNAc_2$ this poses no problem as direct integration of the total D-1, D-2 and D-3 signal will suffice. In a mixture of Man_5-$Man_9GlcNAc_2$ structures, however, this simple integration is no longer applicable. The loss of D-2 substitution from residue A results in the shift of H-1 of A from the unique 5.40 ppm to 5.09. Similarly the loss of D-1 substitution of C causes the H-1 of C to shift from 5.30 to 5.05 ppm and the loss of D-3 substitution causes the H-1 of B to move from 5.15 to 4.90 ppm. While this may not seem that complicated it must be kept in mind that the unsubstituted H-1 resonance of residue A now overlaps with the H-1 of GlcNAc 1 and the H-1 of an unsubstituted residue C will overlap with the H-1 of D-1, D-2 and D-3. As a result determination of whether D-1 or D-2 is present or absent and its quantitation in a mixture of Man_6-$Man_9GlcNAc_2$ glycopeptides is only possible indirectly through the the H-1 resonances of the substituted C and A mannose residues and only in comparison with the total H-1 signal due to residue B (the sum of signals at 5.14 and 4.91 ppm) or that of residues 4 or 4´.

Of the five pools of hexose positive material from the RER protein digest, only R-3 had the essential spectral features indicative of an N-linked oligosaccharide. These were structural reporter groups for the acetates on GlcNAc, the anomeric protons, the mannose H-2 protons and the CH_2 protons on asparagine (See Figure 10). By comparing the published shift values for high mannose glycopeptides and spectral integration in the anomeric proton region, it was determined that glycopeptides isolated from the RER were most likely a mixture of Man_6-$Man_9GlcNAc_2$ structures (See Figure 11). The reasoning behind the presumed isomeric mixture is as follows: the integrals of the signals for the H-1 protons of substituted C, A and B residues are not equal to 1 when normalized against the signal of 4 or 4´, which indicates that not all A, B and C residues are substituted. The signal for residue 4 appears to be a poorly resolved doublet as a result of through space coupling which occurs between residue D-1 and 4. In the absence of D-1 and when residue 4 is substituted with C, the shift for H-1 of residue 4 is 5.347 ppm. When C is itself substituted with D-1, the shift of residue 4 will move downfield slightly to 5.336 ppm. Lastly, a split resonance exists for residue B in which the substituted B will resonate at 5.15 ppm and the unsubstituted at 4.90 ppm.

In addition to these high mannose structures, evidence exists to suggest the presence of a small amount of $Glc_1Man_9GlcNAc_2$. Although this structure has never been characterized by NMR, $Glc_3Man_9GlcNAc$ has been prepared from a mannoprotein from mutant yeast in sufficient quantity to allow for its characterization by NMR (57). The chemical shift of the H-1 reporter on the innermost glucose residue in the

$Glc_3Man_9GlcNac$ is 5.27 ppm. The effect of α1,3 substitution by the "middle" glucose residue is expected to be a downfield shift of the H-1 reporter of the innermost residue of 0.05-0.08 ppm. As a result, the H-1 signal of Glc in $Glc_1Man_9GlcNAc_2$ would be expected in the range of 5.19-5.22 ppm. The coupling constant for glucose in an α-linkage is 2-4 Hz. Therefore the doublet seen at 5.192 ppm may be the result of a glucose α1,3 linked to residue D-1.

As might be expected, the elution profile for the SER glycopeptides proved to be somewhat more complex than that seen for the RER (See Figure 8). Of the 8 pools of hexose positive material collected, only 4 contained N-linked oligosaccharides. Due to the small quantities of material isolated, characterization of the structures in pools S-3 and S-4 proved to be a difficult task. From the elution profile, it was estimated that S-3 contained 400 μg of glycopeptide and that this represents the limit of sensitivity at 300 MHz for the determination of high mannose glycopeptide structure. The only structural information that could be obtained from spectra of S-3 and S-4 was that they were high mannose glycopeptides which differed subtantially in their peptide moieties. Pools S-5 and S-6 proved to be far more interesting because they appeared to represent two distinct steps in glycoprotein processing. Integrals of the signals in the anomeric region of the spectra of S-5 showed that only 20% of the A residues were unsubstituted, compared to 50% of the B and C residues (See Fig 12). Similar integration of the spectra for S-6 showed that all C residues, 90% of B residues and 60% of A residues were unsubstituted (See Figure 13). In addition to high mannose structures,

The simple characterization of RER and SER α_1-AGP glycopeptides by NMR alone would not yield adequate information regarding structure due to the high probability of heterogeneity and second order effects. These two problems could be lessened somewhat by the digestion of any α_1-AGP glycopeptides with Endo-H which removes GlcNAc 1 and any amino acid residues. Although oligosaccharide spectra appear to be more complex than those of the glycopeptide from which they were derived (due to anomerization effects caused by the generation of a reducing terminal on GlcNAc 2), oligosaccharides do offer two advantages over glycopeptides. Oligosaccharide mixtures may be resolved into homologues by HPLC on amino-silica columns. As a result, instead of having 12 possible high mannose stuctures, HPLC resolved oligosaccharides are at worst a mixture of 4 isomers in the case of Man_7. The second advantage of oligosaccharides is that they lack the H-1 of GlcNac 1 and therefore allow the visualization of an unobscured H-1 signal from an unsubstituted C residue.

The final limitation to the results obtained by NMR are that they are of little quantitative value from sample to sample. As a result of this, a method independent of it should be used to quantitate the various oligosaccharide species found on α_1-AGP. The method to be employed involves labelling of oligosaccharides with [^{14}C]glucosamine followed by their digestion with Endo-H. The resultant oligosaccharide mixture is then resolved by HPLC and the oligosaccharides were quantitated by the determination of label associated with each fraction.

3.2 D-[1-^{14}C]-Glucosamine Labelling of Intracellular α_1-AGP Oligosaccharides

α_1-AGP was labelled *in vivo* with D-[1-^{14}C]-Glucosamine HCl administered intraperitoneally to 8 control and 8-24 hr inflamed rats. Following a 45 min incorporation period the animals were sacrificed and RER and SER were prepared from their livers. α_1-AGP was released from the membranes by homogenization in 1% Lubrol-X. Following concentration and centrifugation, the Lubrol extracts were precipitated with a heterologous immune system to remove any non-specific precipitating material as described by Friesen (51) prior to precipitation with anti rat α_1-AGP. Throughout the isolation procedure, steps were taken in an effort to minimize endogenous glycosidase activity which could alter oligosaccharide structure during the isolation procedure. This was accomplished by perfusing livers with sucrose containing 100 mM α-D-mannose. RER and SER were prepared in the presence of 10 mM α-D-mannose. Lubrol extracts were both dialysed and precipitated in a solution that contained 100 mM TRIS which has been shown to be an effective inhibitor of glucosidase I and II activity as well as that of Golgi mannosidase I (32 & 70). The pH chosen, 7.4, was well above the optimum for ER mannosidase (30), but low enough to not interfere with immunological reactions.

Once obtained, the α_1-AGP immune precipitates were solubilized with 1% SDS, dialysed and diluted 5 fold prior to digestion with Endo-H. Although SDS is a powerful denaturant at concentrations below 0.2%, it has no apparent effects on the enzymic properties of Endo-H (67). Following digestion oligosaccharides were recovered by alcohol

precipitation of residual protein and de-salting on Bio-Gel. The oligosaccharides were then resolved by HPLC.

The distribution of oligosaccharide species found in the RER was as expected. Only traces of oligosaccharides smaller than $Man_8GlcNAc$ were found in both the control and 24 hr inflamed rats (See Figures 15 & 16). For the most part the oligosaccharides found in the control were $Man_8GlcNAc$ and $Man_9GlcNAc$ at 32% and 59% respectively, along with a much smaller quantity (6%) of Glc_1 $Man_9GlcNAc$. In addition to having a 2.6 fold increase in total labelled oligosaccharides, the experimental animals also had a different oligosaccharide distribution. Glucosylated oligosaccharides jumped to 13% along with a modest increase in the Man_9 population (65%). The most significant change occurred in the Man_8 population which dropped to 19% of the total.

The most interesting aspect in the variation of oligosaccharide populations can be seen if we compare the ratio of incorporated ^{14}C (Experimental:Control) for a given oligosaccharide species to that of the total. As was mentioned previously, total incorporation of label increased 2.6 fold yet the label found in the $Man_8GlcNAc$ increased only 1.6 fold. The amount of label incorporated in other oligosaccharides was found to increase to a greater extent than in the $Man_8GlcNAc$ and these findings are summarized in Table 2.

The results from SER α_1-AGP oligosaccharides show that essentialy no label was associated with oligosaccharide structures that had retention times characteristic of high mannose oligosacchardes. In the control sample more than 90% of the label eluted with the column void volume and only one other significant peak was noted, that at 29 min

(See Figure 18). The structures of oligosaccharides in these peaks could not be positively determined. In the inflamed sample, only 75% of the label was associated with the void volume. The remainder of the label was associated with 3 peaks. One at approximately 29 min and the other two at 7 and 10 min (See Figure 18). Again, a positive identification of the peak eluting at 29 min was not possible. The two peaks at 7 and 10 min are probably the result of GlcNAc transferase I activity which would place label on the non-reducing end of unlabelled protein bound $Man_5GlcNAc_2$.

Figure 7. Bio-Gel P-4 chromatography of pronase digests of total RER proteins.

Lyophilized 96 hr pronase digests were dissolved in 1 ml of H_2O and eluted with 0.1 M pyridine-acetate pH 5.1 from a 1.5x175 cm column of Bio-Gel P-4 (200-400 mesh) at a constant pressure of 20 PSI. Fractions of 2.2 ml were collected and 200 µl aliquots were assayed for hexose. Fractions were pooled as indicated by the arrows and analysed by proton NMR.

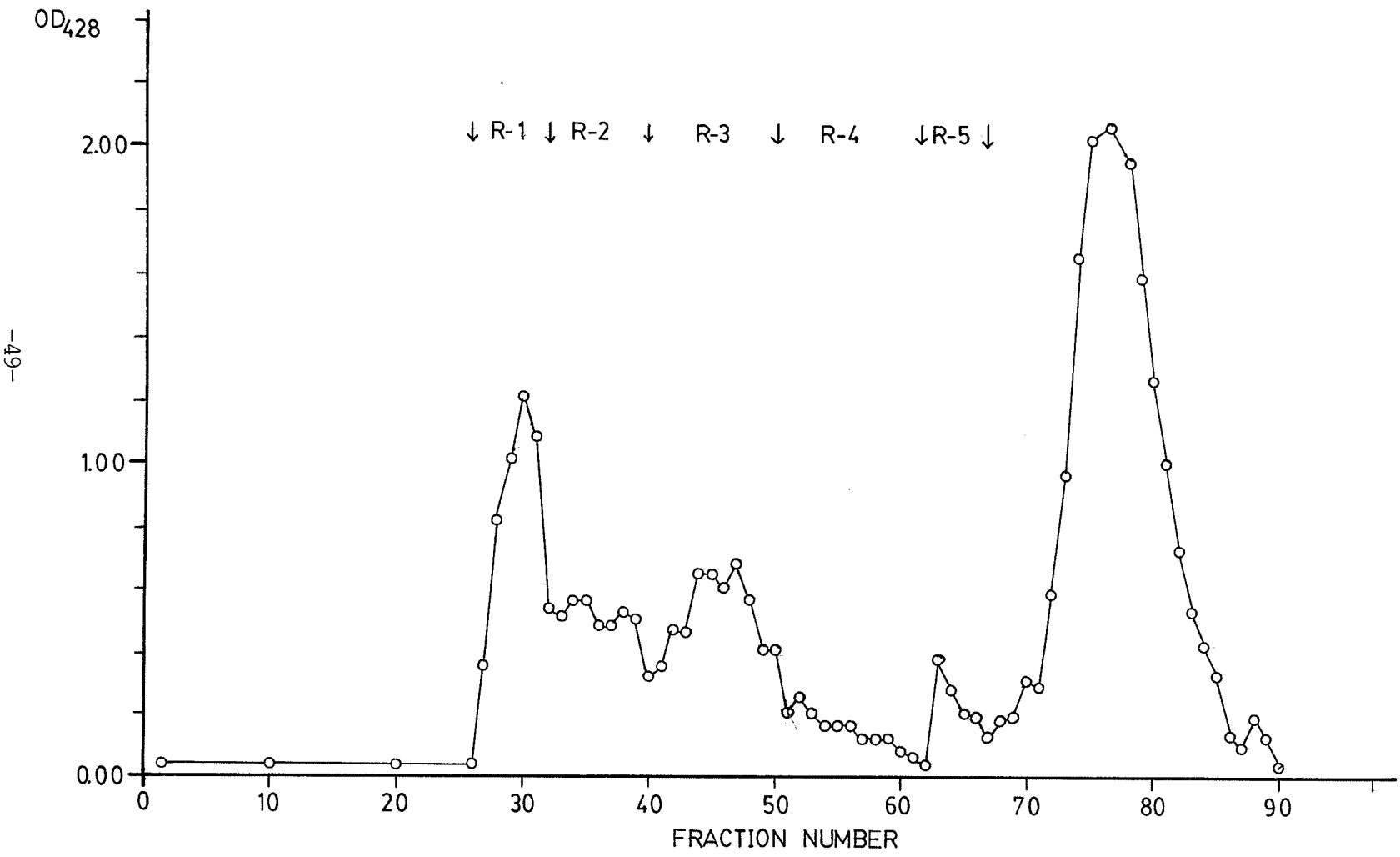

OD428
2.00
1.00
0.00
↓ R-1 ↓ R-2 ↓ R-3 ↓ R-4 ↓R-5 ↓
0
10
20
30
40
50
60
70
80
90
FRACTION NUMBER

Figure 8. Bio-Gel P-4 chromatography of pronase digests of total SER proteins.

Lyophilized 96 hr pronase digests were dissolved in 1 ml of H_2O and eluted with 0.1 M pyridine-acetate pH 5.1 from a 1.5x175 cm column of Bio-Gel P-4 (200-400 mesh) at a constant pressure of 20 PSI. Fractions of 2.2 ml were collected and 200 µl aliquots were assayed for hexose. Fractions were pooled as indicated by the arrows and analysed by proton NMR.

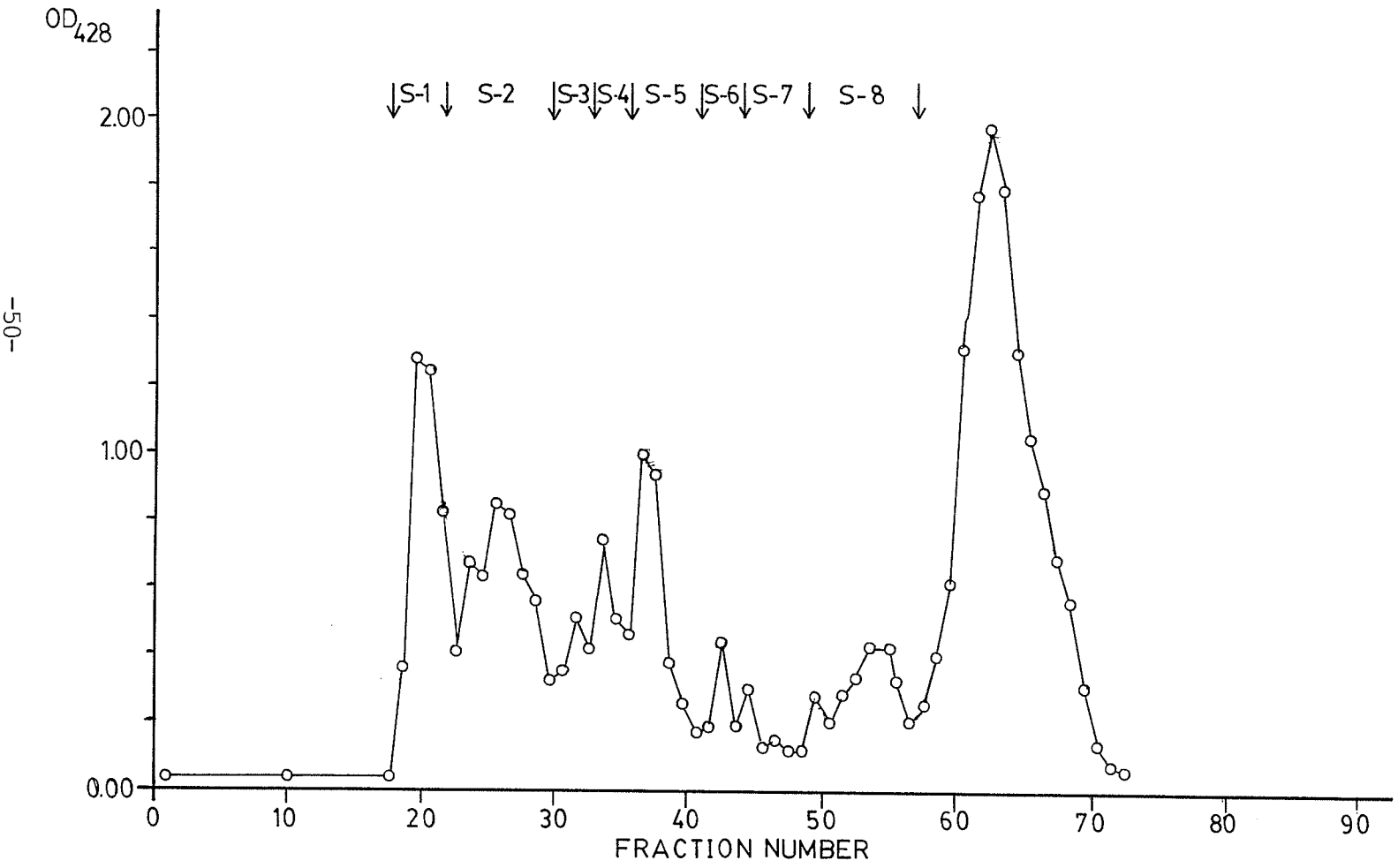

OD428
S-1
S-2
S-3
S-4
S-5
S-6
S-7
S-8
2.00
1.00
0.00
0
10
20
30
40
50
60
70
80
90
FRACTION NUMBER

Figure 9. Bio-Gel P-4 chromatography of pronase digests of total Golgi proteins.

Lyophilized 96 hr pronase digests were dissolved in 1 ml of H_2O and eluted with 0.1 M pyridine-acetate pH 5.1 from a 1.5x175 cm column of Bio-Gel P-4 (200-400 mesh) at a constant pressure of 20 PSI. Fractions of 2.2 ml were collected and 200 µl aliquots were assayed for hexose. Fractions were pooled as indicated by the arrows and analysed by proton NMR.

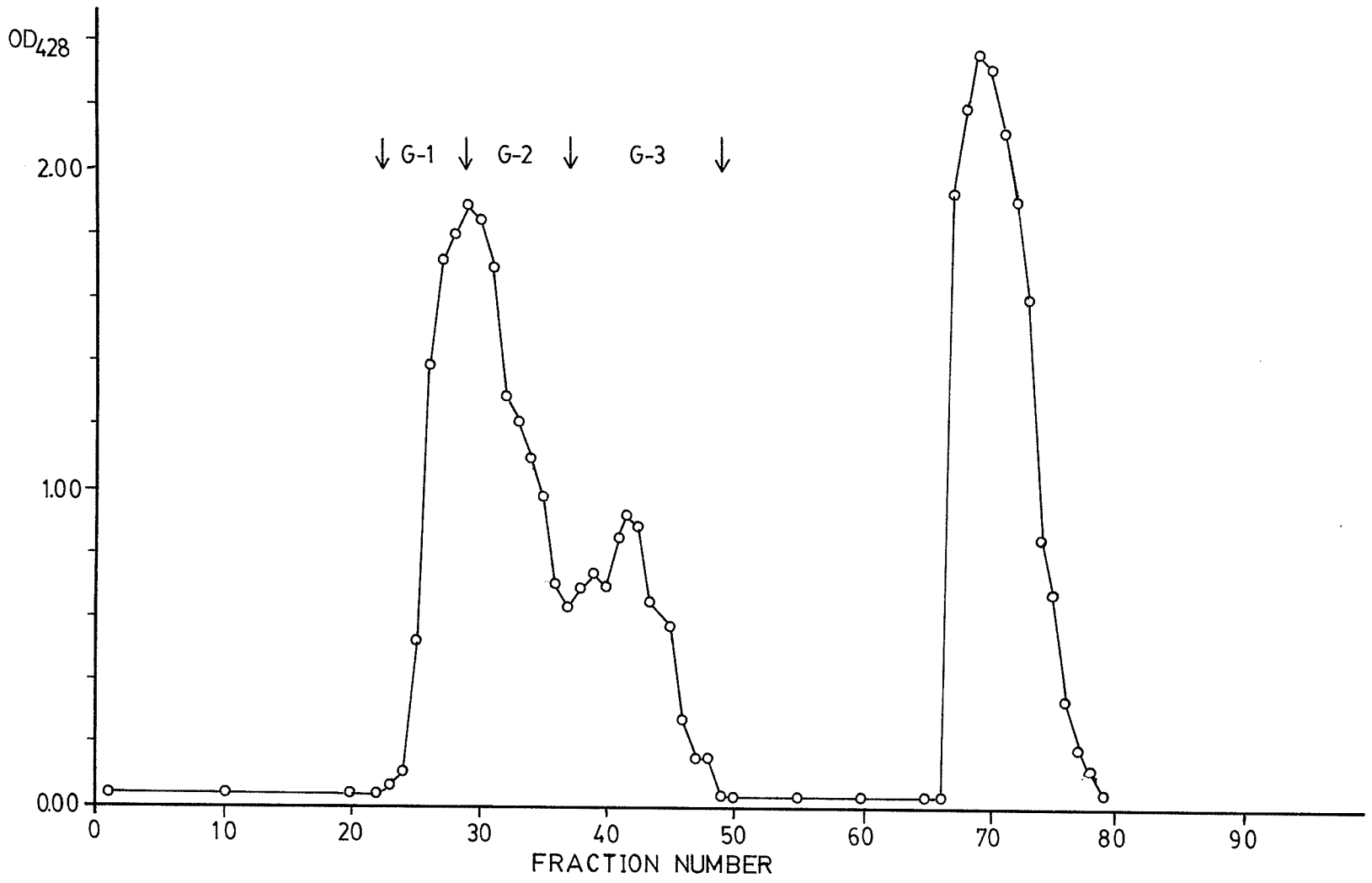

OD428
2.00
1.00
0.00
↓ G-1
↓ G-2
↓ G-3
↓
0
10
20
30
40
50
60
70
80
90
FRACTION NUMBER

Figure 10. NMR spectra of glycopeptide fraction R-3 [a].

Shift Range	Reporter Group [b]	Spectral Information
5.45-4.80 ppm	All mannose H-1 protons	Any shifts downfield of 5.30 ppm are due to the anomeric protons on an 1,2 substituted hexose. Therefore Man_{6-9} structures are present.
4.80-4.65	HOD	The normal isotopic impurity found in dueterium oxide.
4.65-4.55	GlcNAc 1 H-1 Protons	
4.65-3.95	All mannose H-2 protons	Sometimes useful for characterizing mixtures of isomers.
3.95-3.25	Sugar skeleton protons.	Of little or no use.
3.25-2.95	β-CH_2 on Asparagine	Positive conformation of N-linkage.
2.10-2.00	Acetate on GlcNAc	Highly sensitive to local environment. Will yield a complex, splitting pattern with heterogenous peptide moiety.

[a] For elution profile see figure 7. [b] See table 1 for description of structural reporter groups.

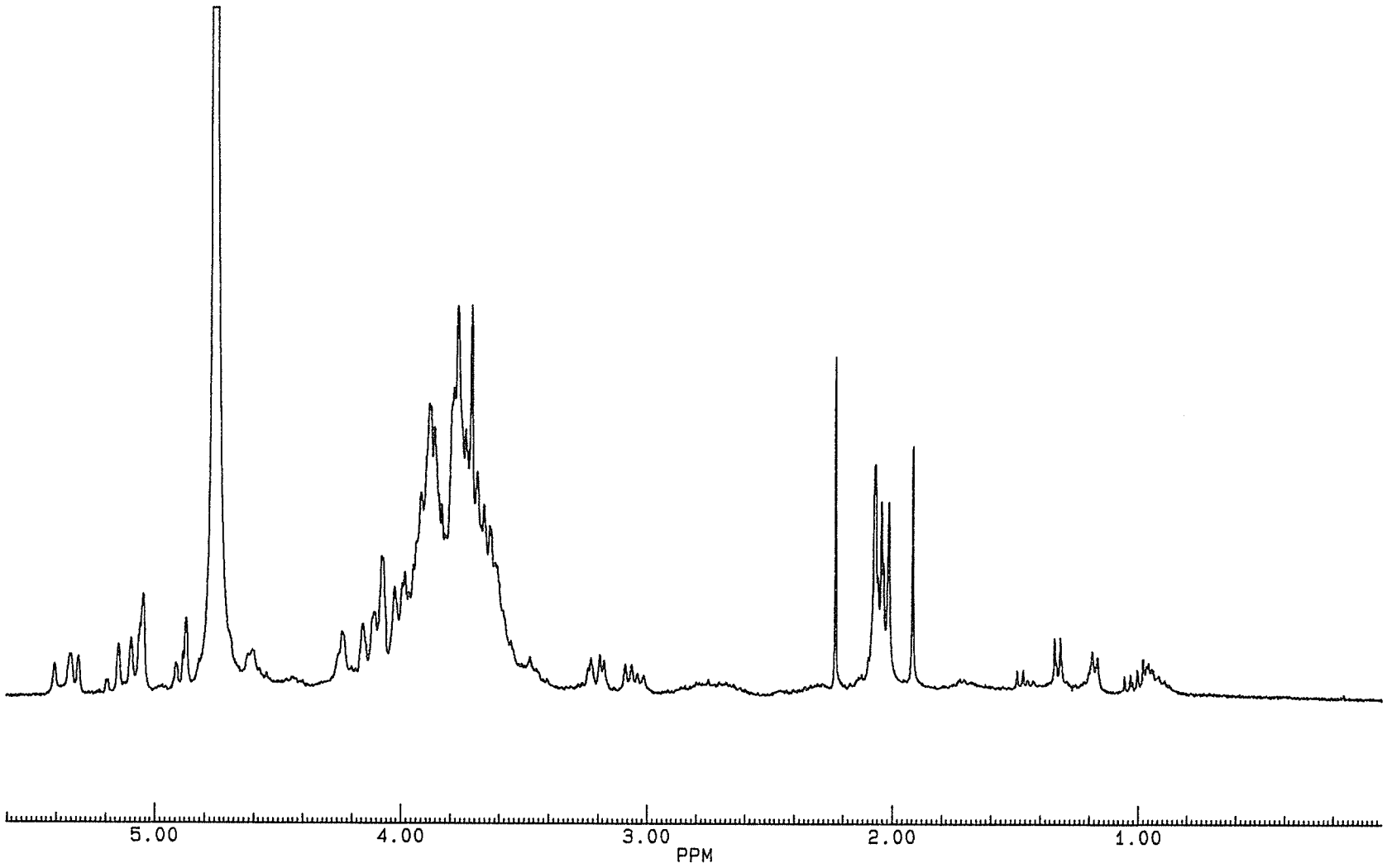
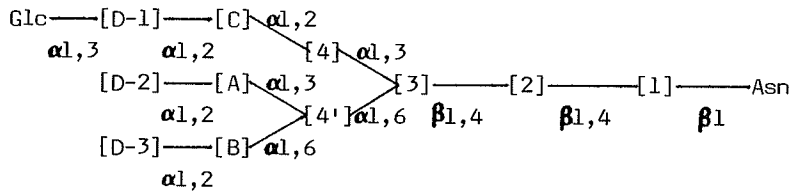

5.00
4.00
3.00
2.00
1.00
PPM

Figure 11. Anomeric region of glycopeptide fraction R-3 [a].

Shift	Reporter Group[b]	Spectral Information
5.403	H-1 of Man A	Substituted residue A
5.347	H-1 of Man 4	Substituted residue 4
5.336	H-1 of Man 4	Substituted residue 4 with long range coupling to D-1
5.306	H-1 of Man C	Substituted residue C
5.192	H-1 of Glc[c]	α1,3 Linked Glc residue
5.143	H-1 of Man B	Substituted residue B
5.093	H-1 of GlcNAc-1	GlcNac β1 linked to Asn
''	H-1 of Man A	Unsubstituted residue A
5.057	H-1 of Man D-2	
''	H-1 of Man C	Unsubstituted residue C
5.041	H-1 of Man D-1 and D-3	
4.911	H-1 of Man B	Unsubstituted residue B
4.870	H-1 of Man 4'	

[a] For elution profile see figure 7. [b] See (44) for shift assignment. [c] See section 3.1 for residue assignment.



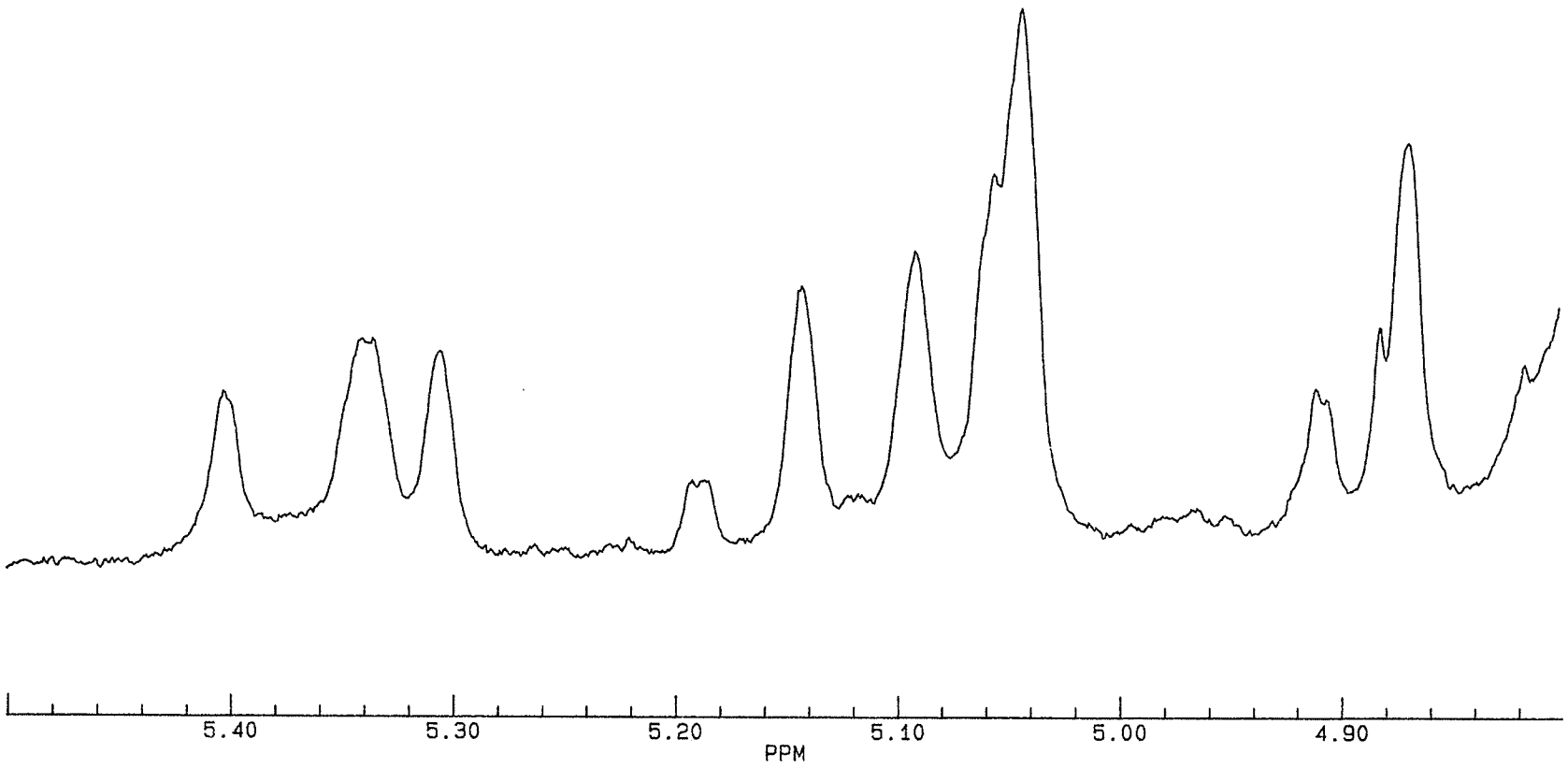
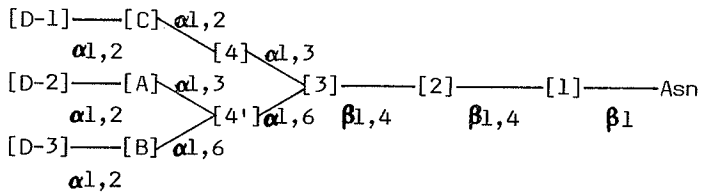

5.40
5.30
5.20
5.10
5.00
4.90
PPM

Figure 12. Selected regions of NMR spectrum of glycopeptide fraction S-5[a].

Shift/Integral[b]	Reporter Group[c]	Spectral Information
5.403/0.75	H-1 of Man A	Substituted residue A
5.347/1.00	H-1 of Man 4	Substituted residue 4
5.336/ --	H-1 of Man 4	Substituted residue 4 with through space coupling to D-1
5.306/0.50	H-1 of Man C	Substituted residue C
5.179/	?	?
5.143/0.50	H-1 of Man B	Substituted residue B
5.093/1.25	H-1 of GlcNAc-1	GlcNac β1 linked to Asn
'' / --	H-1 of Man A	Unsubstituted residue A
5.057/	H-1 of Man D-2	
'' /	H-1 of Man C	Unsubstituted residue C
5.041/	H-1 of Man D-1 and D-3	
4.911/	H-1 of Man B	Unsubstituted residue B
4.870/	H-1 of Man 4'	
4.55-4.35	H-1 of Gal	Structural reporters for Gal residues.
2.10-2.00	GlcNAc Acetates	Structural reporters for acetates on GlcNAc.

[a] For elution profile see figure 8. [b] Integral values are normalized for residue 4=1. [c] See (44) for shift assignment.



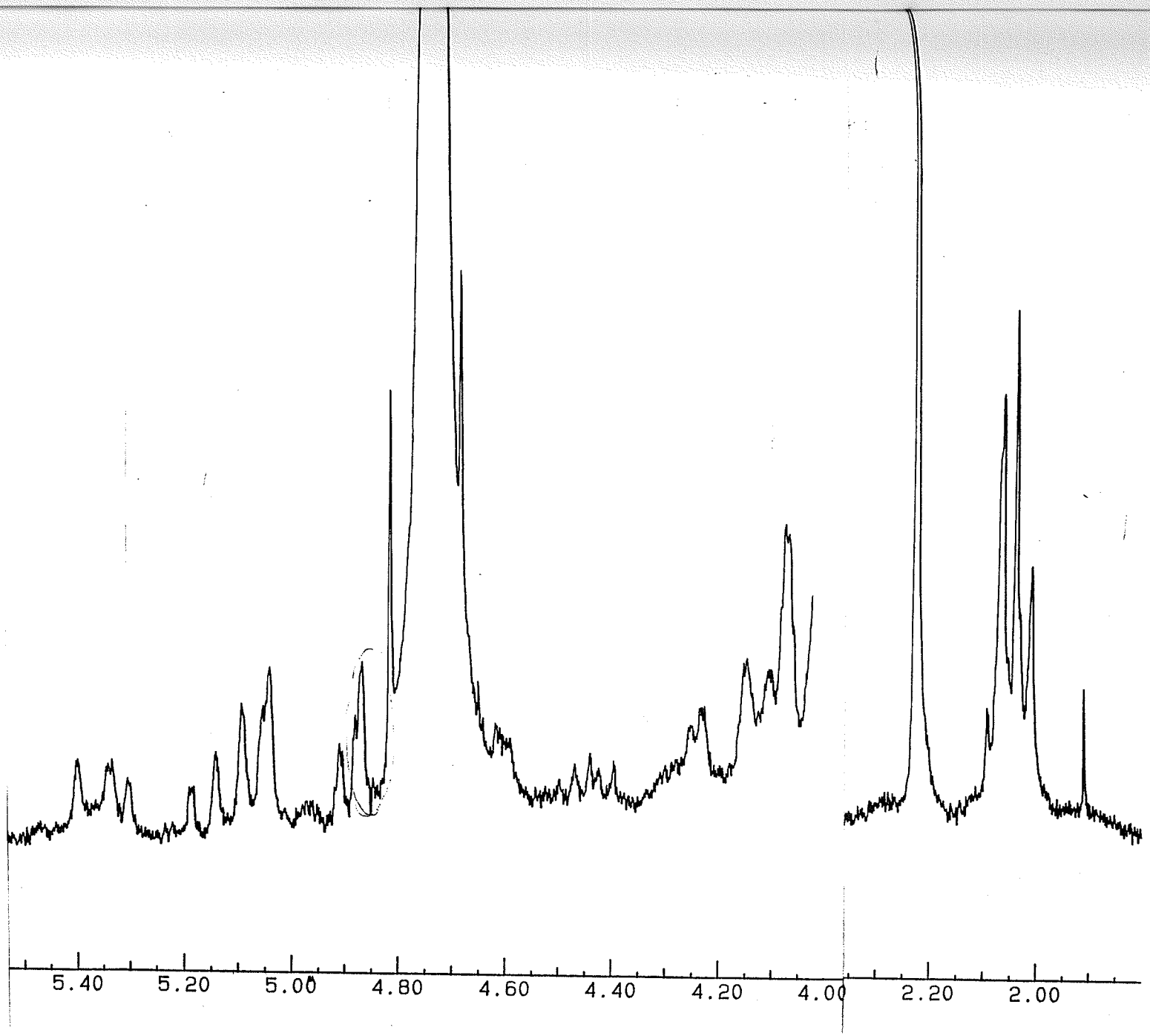
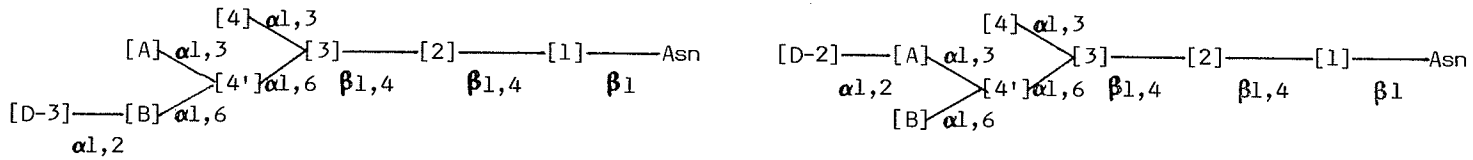

5.40
5.20
5.00
4.80
4.60
4.40
4.20
4.00
2.20
2.00

Figure 13. Selected regions of the NMR spectrum of glycopeptide fraction S-6[a].

Shift	Reporter Group[b]	Spectral Information
5.403	H-1 of Man A	Substituted residue A
5.347	H-1 of Man 4	Substituted residue 4
5.179	?	Contamination
5.143	H-1 of Man B	Substituted residue B
5.093	H-1 of GlcNAc 1	GlcNac β1 linked to Asn
''	H-1 of Man A	Unsubstituted residue A
''	H-1 of Man 4	Unsubstituted residue 4
5.057	H-1 of Man D-2	
5.050	H-1 of Man D-3	Unsubstituted residue C
4.911	H-1 of Man B	Unsubstituted residue B
4.870	H-1 of Man 4'	
4.55-4.35	H-1 of Gal	Structural reporters for Gal residues
2.10-2.00	GlcNAc Acetates	Structural reporters for GlcNAc residues

[a] For elution profile see figure 8. [b] See (44) for shift assignment.



Possible structures present in mixture.

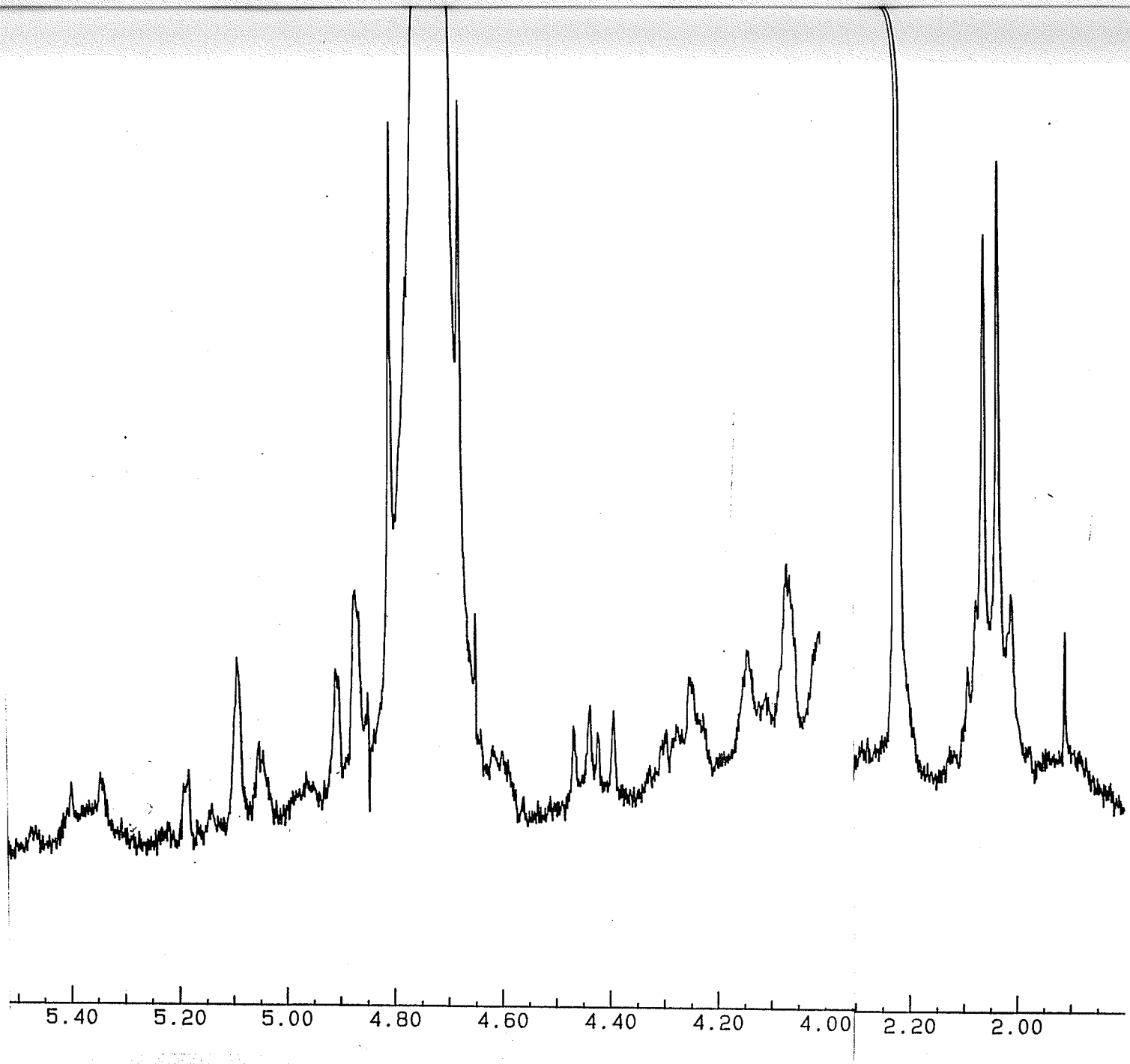

5.40
5.20
5.00
4.80
4.60
4.40
4.20
4.00
2.20
2.00

Figure 14. NMR spectra of glycopeptide fraction G-2[a].

Shift Range	Reporter Group[b]	Spectral Information
5.45-4.80 ppm	All mannose H-1 protons	Any shifts downfield of 5.30 ppm are due to the anomeric protons on an α1,2 substituted hexose. Therefore Man_6-Man_9 structures are present.
4.80-4.65	HOD	The normal isotopic impurity found in deuterium oxide.
4.65-4.55	GlcNAc 1 H-1 Protons	
4.55-4.35	Galactose H-1 protons	Indicative of complex oligosaccharide structure.
4.35-3.95	All mannose H-2 protons	Sometimes useful for characterizing mixtures of isomers.
3.95-3.25	Sugar skeleton protons.	Of little or no use.
2.10-2.00	Acetate on GlcNAc	Highly sensitive to local environment. Will yield a complex splitting pattern with heterogenous peptide moiety.
1.20-1.15	CH_3 of α1,3 linked fucose	The presence of complex oligosaccharides.

[a] For elution profile see figure 9. [b] See table 1 for description of structural reporter groups.

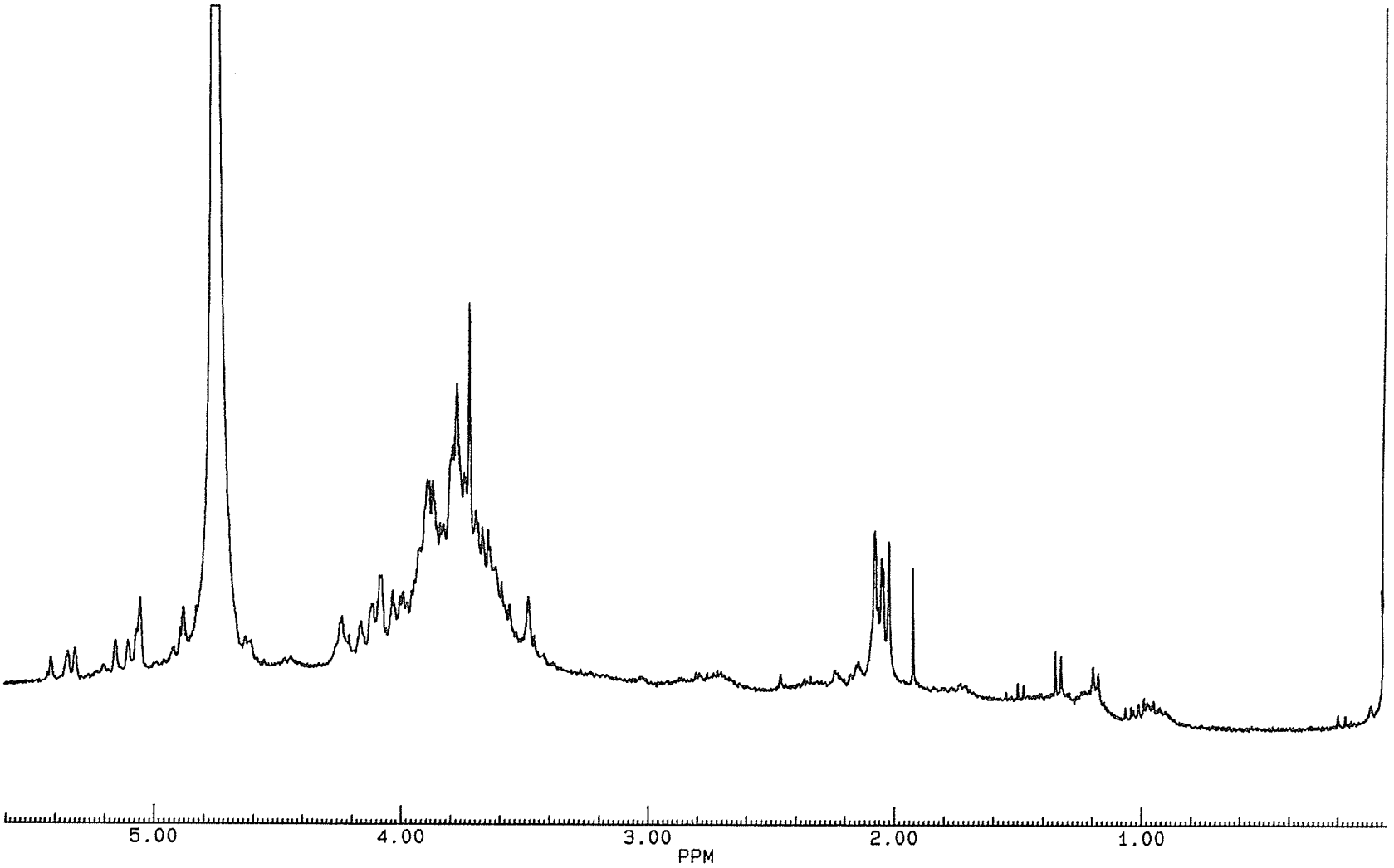
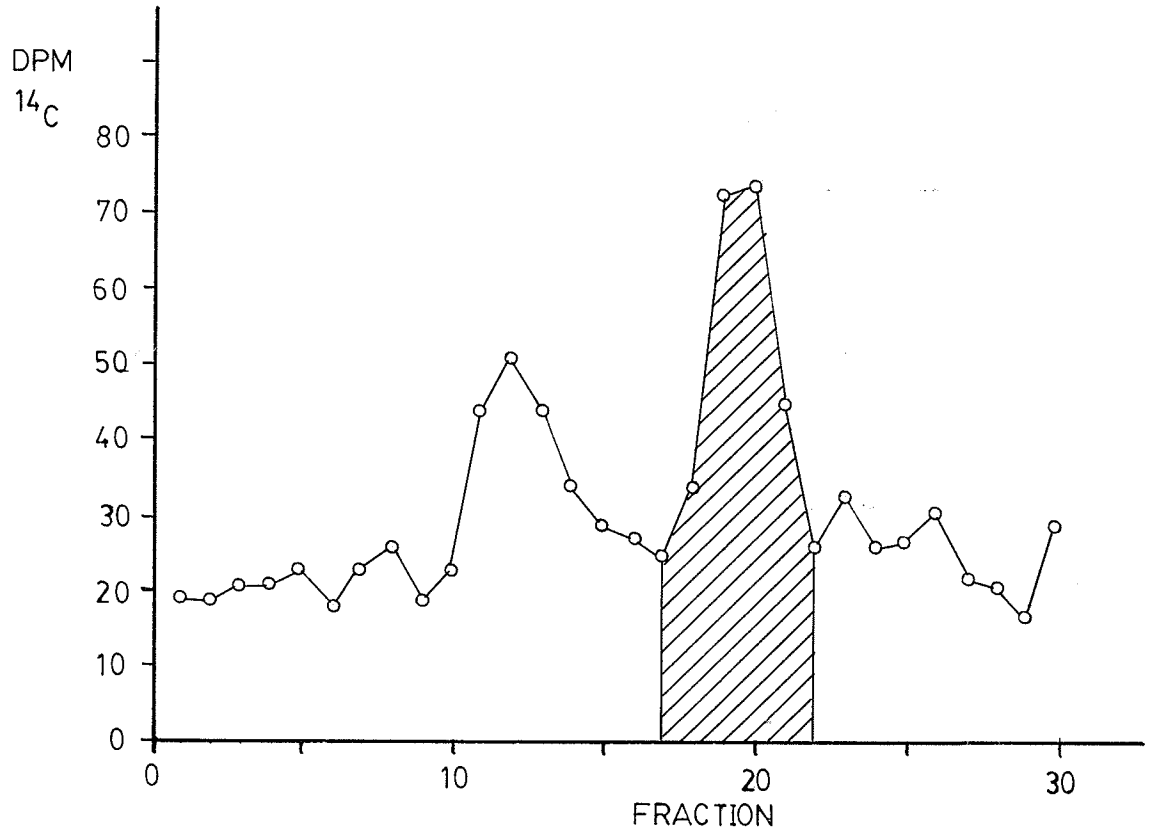

5.00
4.00
3.00
2.00
1.00
PPM

Figure 15. Isolation and resolution of [^{14}C]-glucosamine labelled oligosaccharides from inflamed rat RER α_1-AGP.

Endo-H digests of SDS solubilized α_1-AGP immune precipitates were de-salted on a 1x100 cm column of Bio-Gel P-4 (+400 mesh) with distilled de-ionized water, 2.2 ml fractions were collected and 200 µl aliquots were monitored for ^{14}C (upper panel). The shaded region which contained oligosaccharides was pooled, lyophilized and resolved by HPLC. Fractions were collected every 30 seconds (0.75 ml) and monitored for ^{14}C (lower panel). The arrows indicate elution times for high mannose oligosaccharides whose structures were unequivocally determined by NMR. Elution times for glucosylated oligosaccharides were determined by standards provided by A. Herscovics.


DPM
^{14}C
80
70
60
50
40
30
20
10
0
0
10
20
30
FRACTION

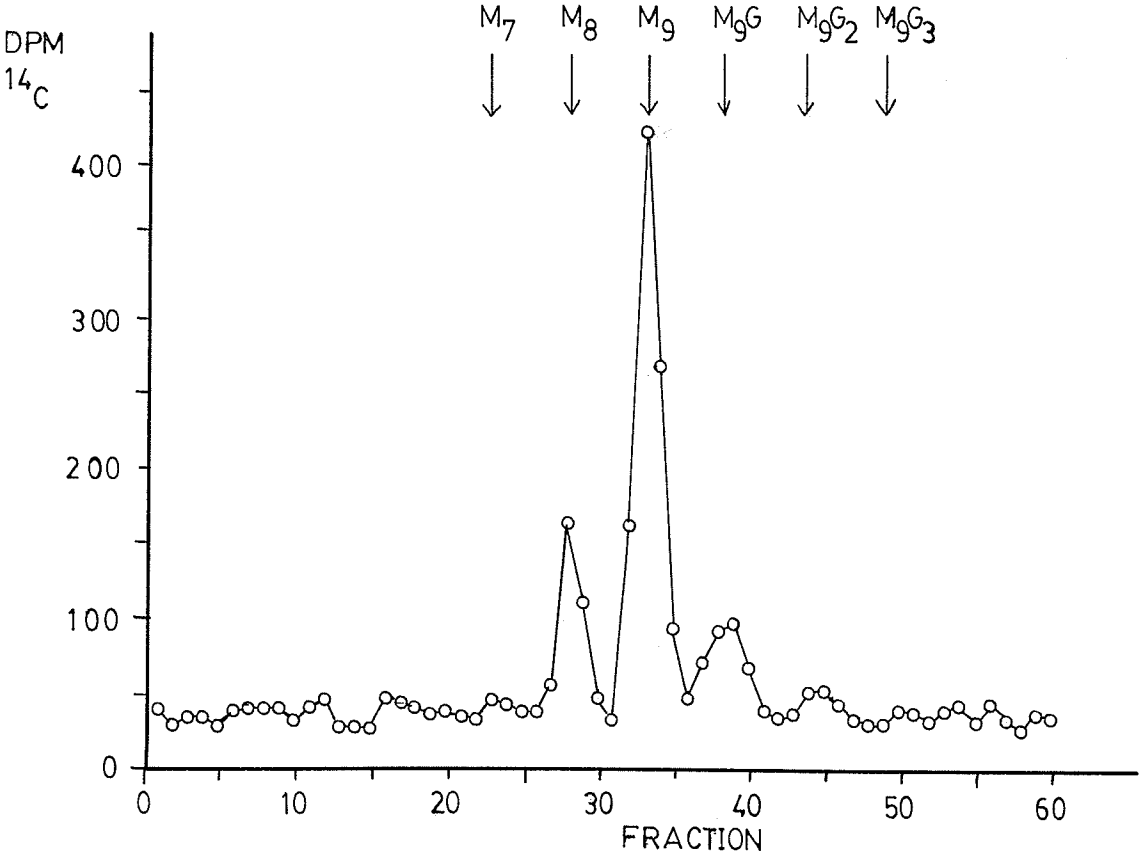
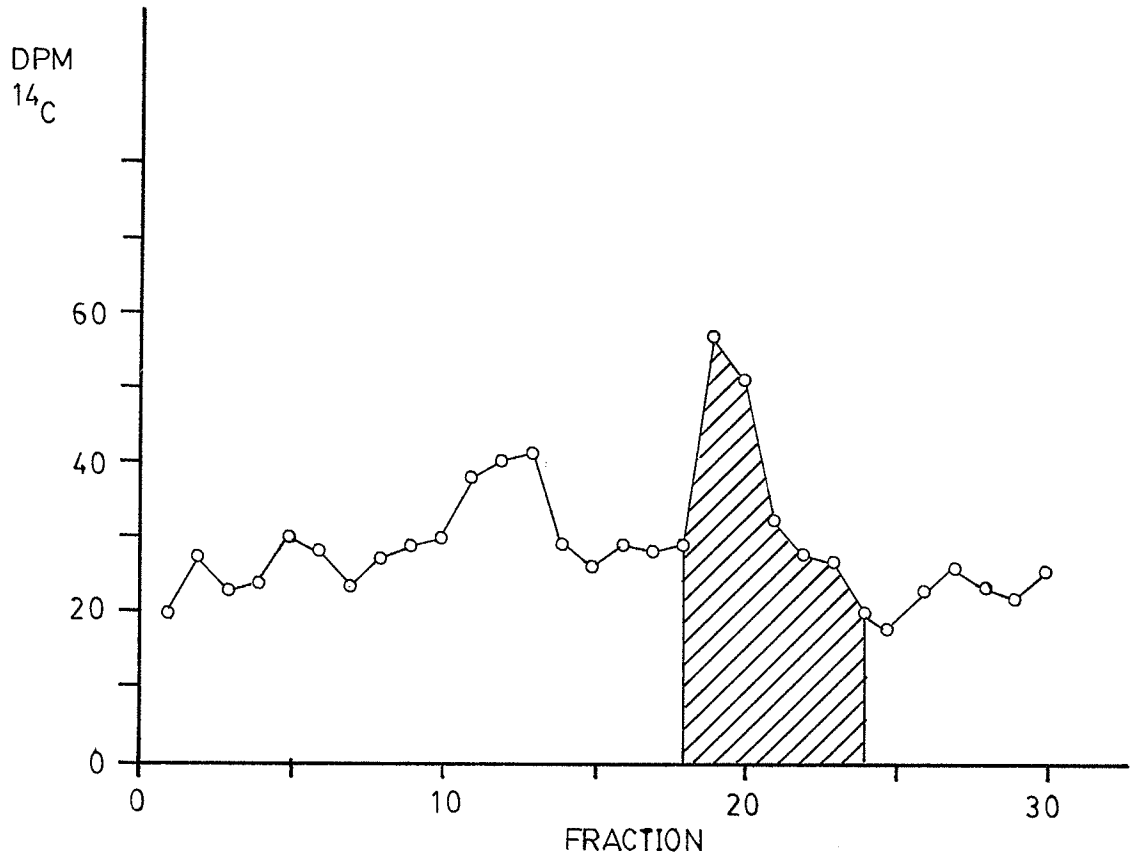

M_7
M_8
M_9
M_9G
M_9G_2
M_9G_3
DPM
^{14}C
400
300
200
100
0
0
10
20
30
40
50
60
FRACTION

Figure 16. Isolation and resolution of [^{14}C] glucosamine labelled oligosaccharides from control rat RER α_1-AGP.

Endo-H digests of SDS solubilized α_1-AGP immune precipitates were de-salted on a 1x100 cm column of Bio-Gel P-4 (+400 mesh) with distilled de-ionized water, 2.2 ml fractions were collected and 200 μl aliquots were monitored for ^{14}C (upper panel). The shaded region which contained oligosaccharides was pooled, lyophilized and resolved by HPLC. Fractions were collected every 30 seconds (0.75 ml) and monitored for ^{14}C (lower panel). The arrows indicate elution times for high mannose oligosaccharides whose structures were unequivocally determined by NMR. Elution times for glucosylated oligosaccharides were determined by standards kindly provided by A. Herscovics.


DPM
^{14}C
60
40
20
0
0
10
20
30
FRACTION

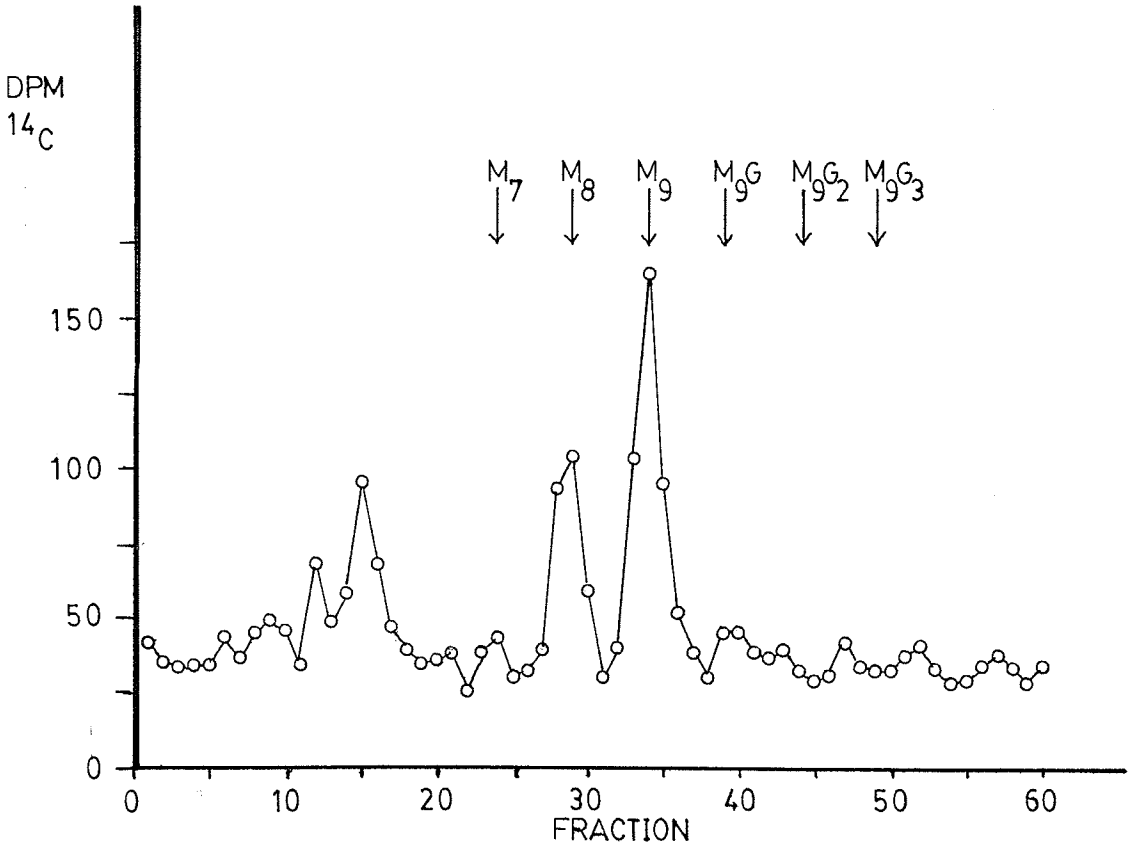

DPM
^{14}C
M_7
M_8
M_9
M_9G
M_9G_2
M_9G_3
150
100
50
0
0
10
20
30
40
50
60
FRACTION

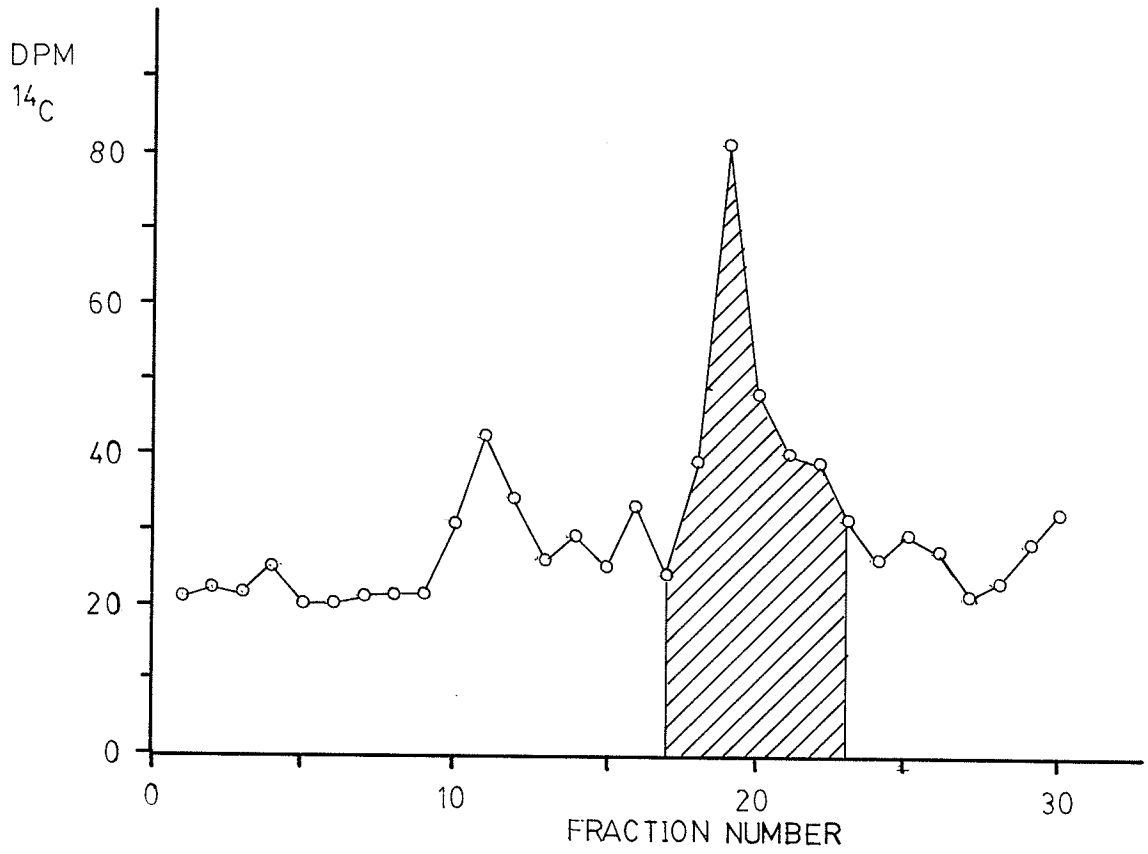
Table 2. Distribution of ^{14}C label in oligosaccharides from control and inflamed RER $_1$-AGP[a].

	Control		Inflamed			
Structure	DPM ^{14}C	% Total	DPM ^{14}C	% Total	DPM Inflamed / DPM Control	(Inf./Con.) / (Total Inf./Total Con.)
$Glc_2Man_9GlcNAc$	---	---	24	2.0	---	---
$Glc_1Man_9GlcNAc$	29	6.2	142	11.6	4.89	1.86
$Man_9GlcNAc$	275	59.1	800	65.3	2.90	1.11
$Man_8GlcNAc$	150	32.2	234	19.1	1.56	0.59
$Man_7GlcNAc$	11	2.4	25	2.0	---	---
Total	465	100	1225	100	2.65	---

[a] See figures 15 and 16 for elution profiles. DPM values reported are total peak areas for each oligosaccharide species less a backround of 35 DPM per fraction collected.

Figure 17. Isolation and resolution of [^{14}C] glucosamine labelled oligosaccharides from inflamed rat SER α_1-AGP.

Endo-H digests of SDS solubilized α_1-AGP immune precipitates were de-salted on a 1x100 column of Bio-Gel P-4 (+400 mesh) with distilled de-ionized water, 2.2 ml fractions were collected and 200 µl aliquots were monitored for ^{14}C (upper panel). The shaded region which contained oligosaccharides was pooled, lyophilized and resolved by HPLC. Fractions were collected every 30 seconds (0.75 ml) and monitored for ^{14}C (lower panel). The arrows indicate elution times for high mannose oligosaccharides whose structures were unequivocally determined by NMR. Elution times for glucosylated oligosaccharides were determined by standards provided by kindly provided by A. Herscovics.


DPM
^{14}C
80
60
40
20
0
0
10
20
30
FRACTION NUMBER

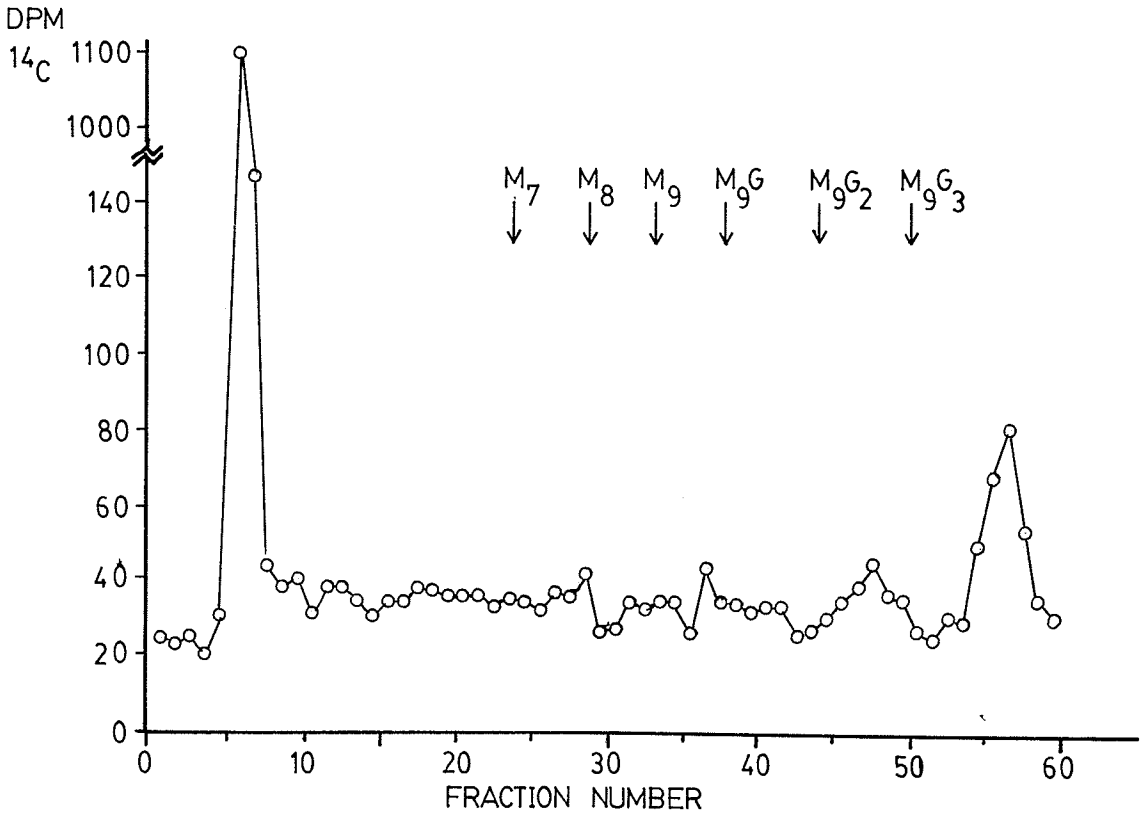
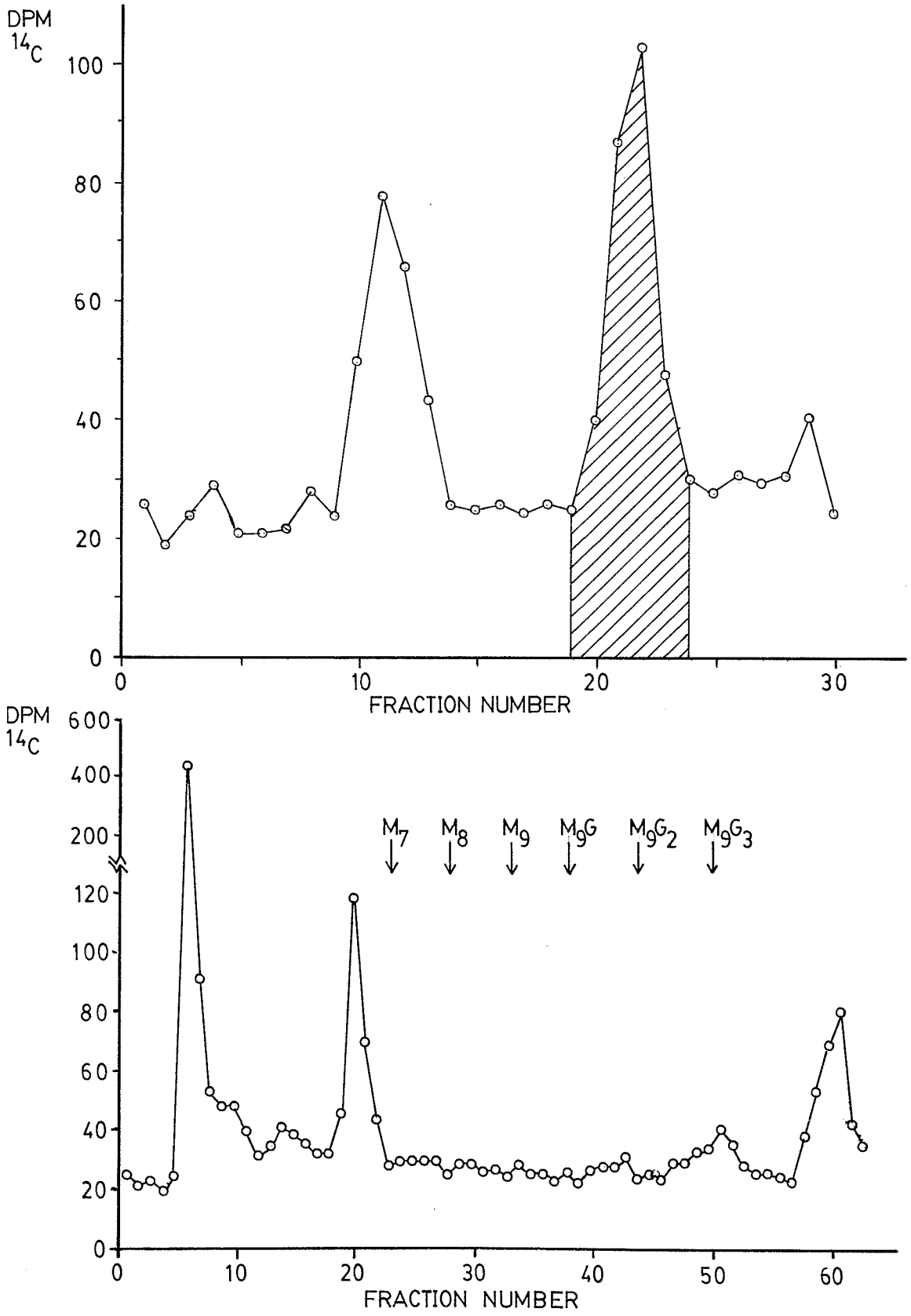

DPM
^{14}C
1100
1000
140
120
100
80
60
40
20
0
M_7
M_8
M_9
M_9G
M_9G_2
M_9G_3
0
10
20
30
40
50
60
FRACTION NUMBER

Figure 18. Isolation and resolution of [^{14}C] glucosamine labelled oligosaccharides from control rat SER α_1-AGP.

Endo-H digests of SDS solubilized α_1-AGP immune precipitates were de-salted on a 1x100 column of Bio-Gel P-4 (+400 mesh) with distilled de-ionized water, 2.2 ml fractions were collected and 200 μl aliquots were monitored for ^{14}C (upper panel). The shaded region which contained oligosaccharides was pooled, lyophilized and resolved by HPLC. Fractions were collected every 30 seconds (0.75 ml) and monitored for ^{14}C (lower panel). The arrows indicate elution times for high mannose oligosaccharides whose structures were unequivocally determined by NMR. Elution times for glucosylated oligosaccharides were determined by standards provided by kindly provided by A. Herscovics.


DPM
^{14}C
100
80
60
40
20
0
0
10
20
30
FRACTION NUMBER
DPM
^{14}C
600
400
200
120
100
80
60
40
20
0
M_7
M_8
M_9
M_9G
M_9G_2
M_9G_3
0
10
20
30
40
50
60
FRACTION NUMBER

3.3 Isolation of Intracellular α_1-AGP for Study by NMR

The previous studies using HPLC fractionation of [^{14}C]-glucosamine labelled oligosaccharides from intracellular α_1-AGP shows the presence of Man_9 and $Man_8GlcNAc$ oligosaccharides as the major structures associated with this protein from the RER. In order to characterize these oligosaccharides by NMR a larger quantity of intracellular α_1-AGP had to be prepared. The procedure that was employed differed from that used for the labelled oligosaccharides due to the limited availability of anti rat α_1-AGP. In addition to avoid any complications that might arise from the use of detergents, subcellular fractions were extracted by ultra sound.

Intracellular α_1-AGP was isolated from the sonic extracts of RER and SER prepared from 1.2 Kg of inflamed rat liver. The isolation followed a three step procedure, sonic extracts were first passed down a large column of Sephadex G-100 (See Figures 19 & 21). Those samples exhibiting the highest reactivity towards anti rat α_1-AGP were pooled, dialysed and freeze dried. This material was then chromatographed on Con-A Sepharose 4B to seperate high mannose α_1-AGP from albumin and other non glycosylated protein. Those protein containing fractions that eluted with 500 mM α-methyl mannoside were pooled, dialysed and lyophilized. Finally, this material was chromatographed on Sephadex G-50 and those fractions containing α_1-AGP were pooled, dialysed and lyophilized (See Figures 20 & 22). This methodology yielded approximately 10 mg of protein from both the RER and SER which was judged to be 90% α_1-AGP by SDS-PAGE (See Figure 23). Double diffusion analysis of this protein against anti whole

rat serum and anti rat α_1-AGP resulted in a diffuse precipitin line confirming the identity of the preparation (See fig 24).

Figure 19. Isolation of an intracellular α_1-AGP enriched fraction from sonic extracts of RER.

RER sonic extracts from 80 rats were dissolved in 100 ml of 100 mM TRIS-HCl pH 9.1. Following centrifugation and filtration a 50 ml aliquot of this solution was loaded onto a 3.5x250 cm column of Sephadex G-100 superfine and eluted with 50 mM TRIS-HCl pH 9.1, 2.2 ml fractions were collected and 100 µl aliquots were assayed for protein. Those fractions containing protein were then screened for α_1-AGP content by double diffusion analysis against anti rat α_1-AGP. Fractions containing α_1-AGP were pooled as indicated by shading.

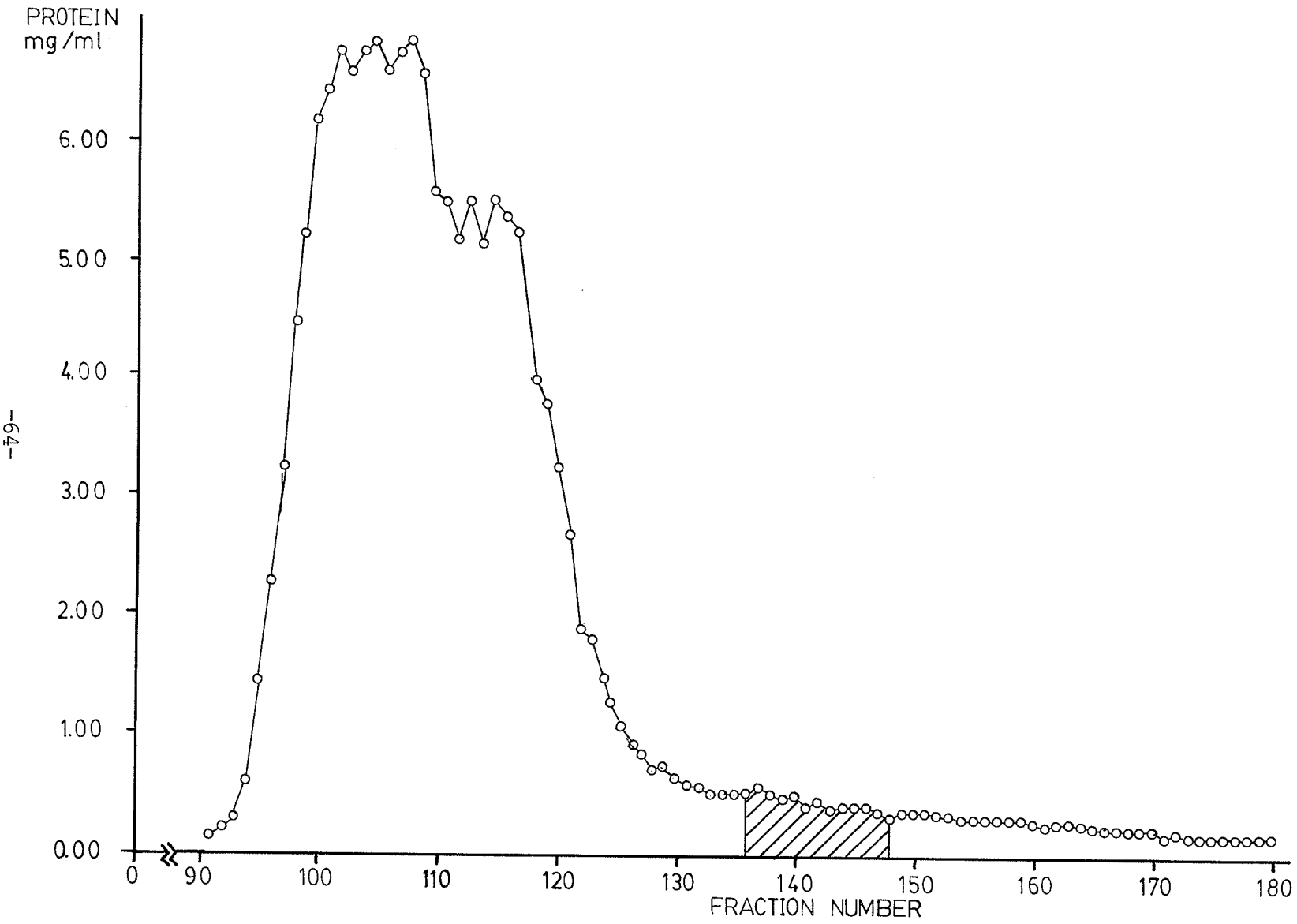
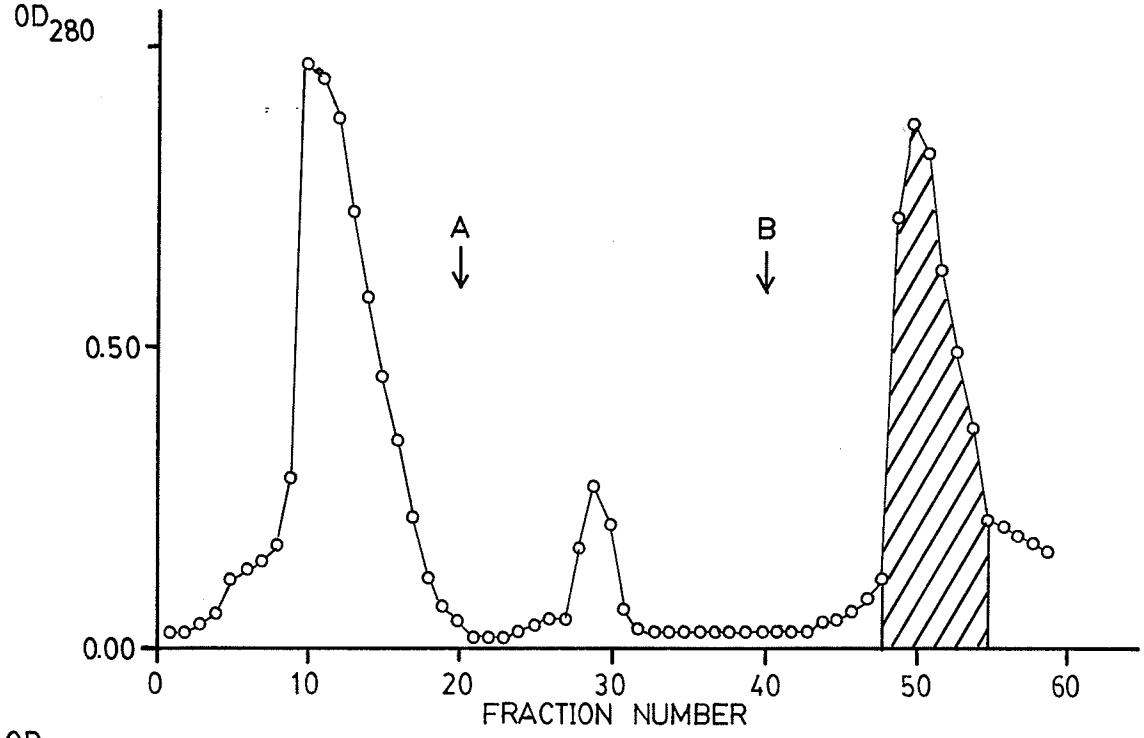

PROTEIN
mg/ml
6.00
5.00
4.00
3.00
2.00
1.00
0.00
0
90
100
110
120
130
140
150
160
170
180
FRACTION NUMBER

Figure 20. The purification of RER α_1-AGP.

Protein enriched in RER α_1-AGP (See Figure 19) was dissolved in 2 ml of Con-A binding buffer (0.1 M acetate, pH 6.0, 1.0 M NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 1 mM EDTA) and loaded onto a 1x15 cm column of Con-A Sepharose 4B. The column was then eluted in a stepwise manner. First with Con-A binding buffer, then with binding buffer containing 10 mM α-methyl-glucose (arrow A) and 500 mM α-methyl-mannose (arrow B). Fractions of 2.2 ml were collected and monitored for OD_{280}. Those protein containing fractions eluting with 500 mM α-methyl-mannose were pooled as indicated by shading, dialysed and lyophilized. This material was then dissolved in 50 mM TRIS, pH 9.1 and chromatographed on a 1x75 cm column of Sephadex G-50. Fractions of 2.2 ml were collected and monitored for OD_{280}. They were then pooled as indicated by shading, lyophilized and tested for α_1-AGP by double diffusion analysis and SDS-PAGE (See Figures 23 and 24).


OD280
0.50
0.00
A
B
0
10
20
30
40
50
60
FRACTION NUMBER

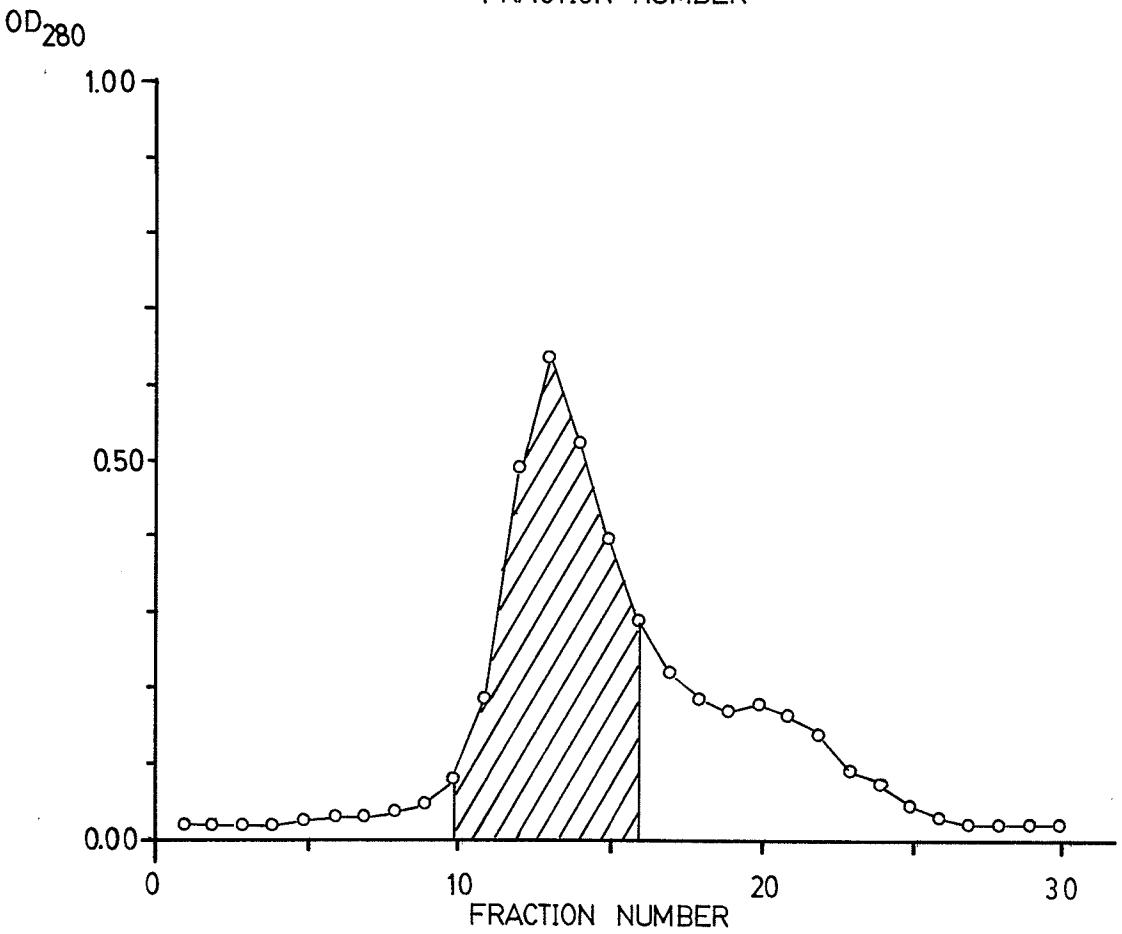

OD280
1.00
0.50
0.00
0
10
20
30
FRACTION NUMBER

Figure 21. Isolation of an intracellular α_1-AGP enriched fraction from sonic extracts of SER.

SER sonic extracts from 80 rats were dissolved in 100 ml of 100 mM TRIS-HCl pH 9.1. Following centrifugation and filtration a 50 ml aliquot of this solution was loaded onto a 3.5x250 cm column of Sephadex G-100 superfine and eluted with 50 mM TRIS-HCl pH 9.1, 2.2 ml fractions were collected and 100 ul aliquots were assayed for protein. Those fractions containing protein were then screened for α_1-AGP content by double diffusion analysis against anti rat α_1-AGP. Fractions containing α_1-AGP were pooled as indicated by shading.

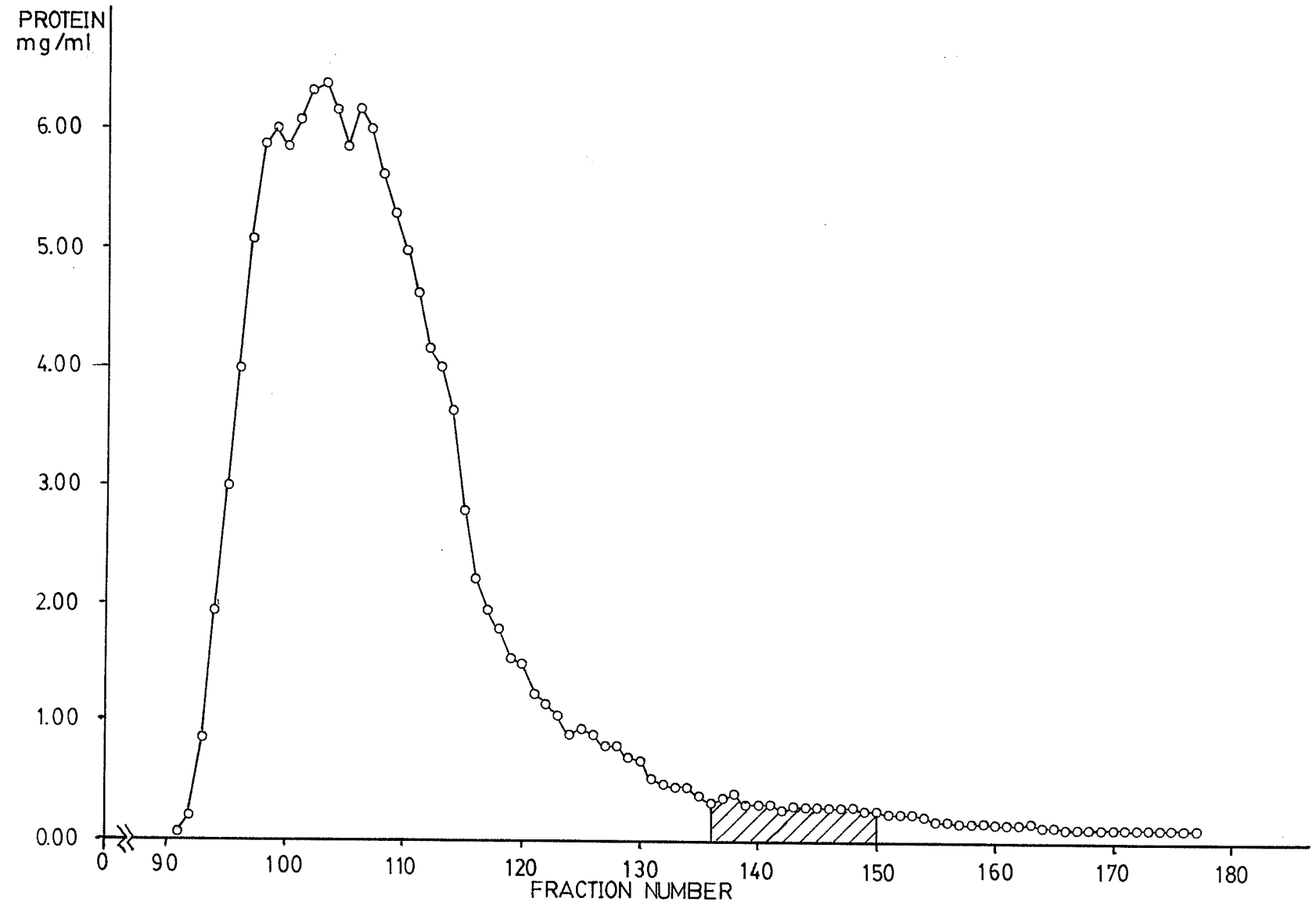
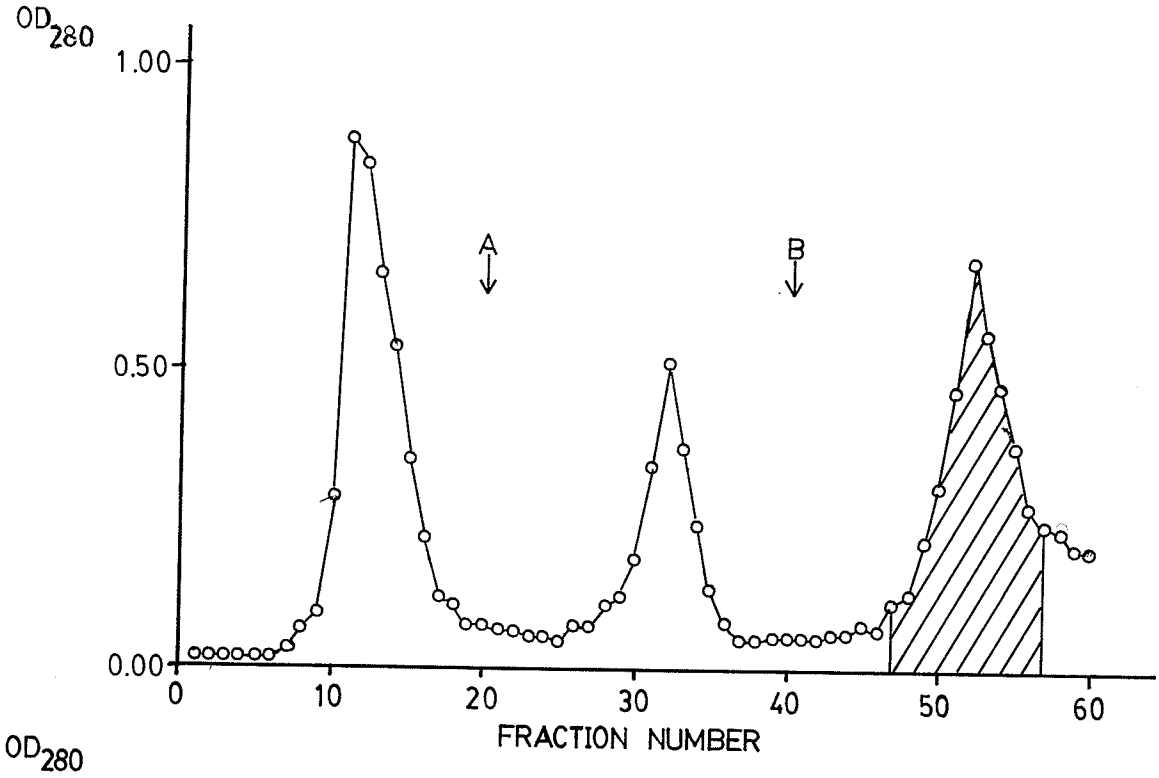

PROTEIN
mg/ml
6.00
5.00
4.00
3.00
2.00
1.00
0.00
0
90
100
110
120
130
140
150
160
170
180
FRACTION NUMBER

Figure 22. The purification of SER α_1-AGP.

Protein enriched in SER α_1-AGP (See Figure 21) was dissolved in 2 ml of Con-A binding buffer (0.1 M acetate, pH 6.0, 1.0 M NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 1 mM EDTA) and loaded onto a 1x15 cm column of Con-A Sepharose 4B. The column was then eluted in a stepwise manner. First with Con-A binding buffer, then with binding buffer containing 10 mM α-methyl-glucose (arrow A) and 500 mM α-methyl-mannose (arrow B). Fractions of 2.2 ml were collected and monitored for OD_{280}. Those protein containing fractions eluting with 500 mM α-methyl-mannose were pooled as indicated by shading, dialysed and lyophilized. This material was then dissolved in 50 mM TRIS, pH 9.1 and chromatographed on a 1x75 cm column of Sephadex G-50. Fractions of 2.2 ml were collected and monitored for OD_{280}. They were then pooled as indicated by shading, lyophilized and tested for α_1-AGP by double diffusion analysis and SDS-PAGE (See Figures 23 and 24).


OD280
1.00
0.50
0.00
A
B
0
10
20
30
40
50
60
FRACTION NUMBER

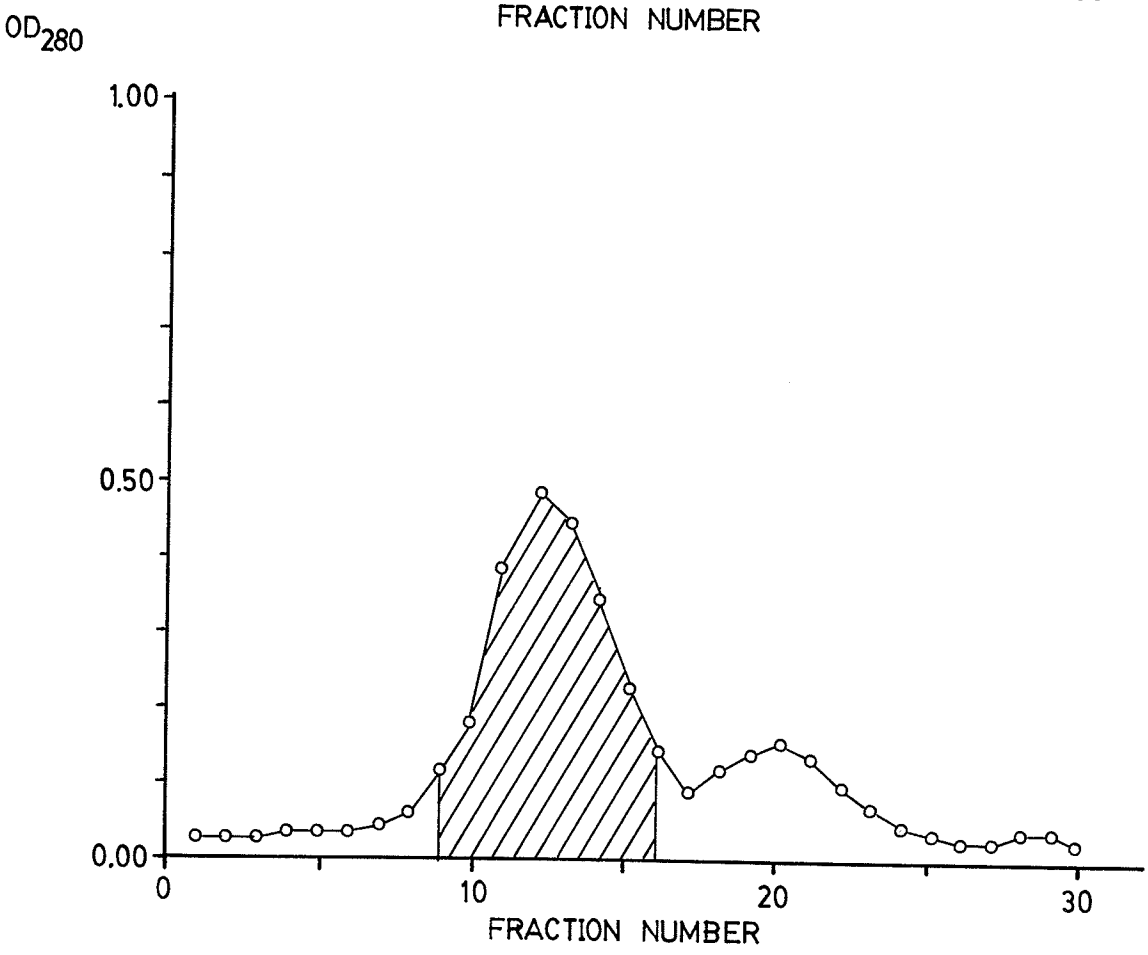

OD280
1.00
0.50
0.00
0
10
20
30
FRACTION NUMBER

Figure 23. SDS-PAGE analysis of RER and SER α_1-AGP.

Intracellular forms of α_1-AGP were run on a discontinuous gradient consisting of a 3% stacking gel and a 10% resolving gel. Lane 1 contains MW standards BSA (66 kD) and Ovalbumin (45 kD). Lane 2 contains RER α_1-AGP and lane 3 contains SER α_1-AGP. The appearence of two distinct bands at approximately 68 kD and 55 kD is typical of intracellular α_1-AGP on SDS-PAGE (51).

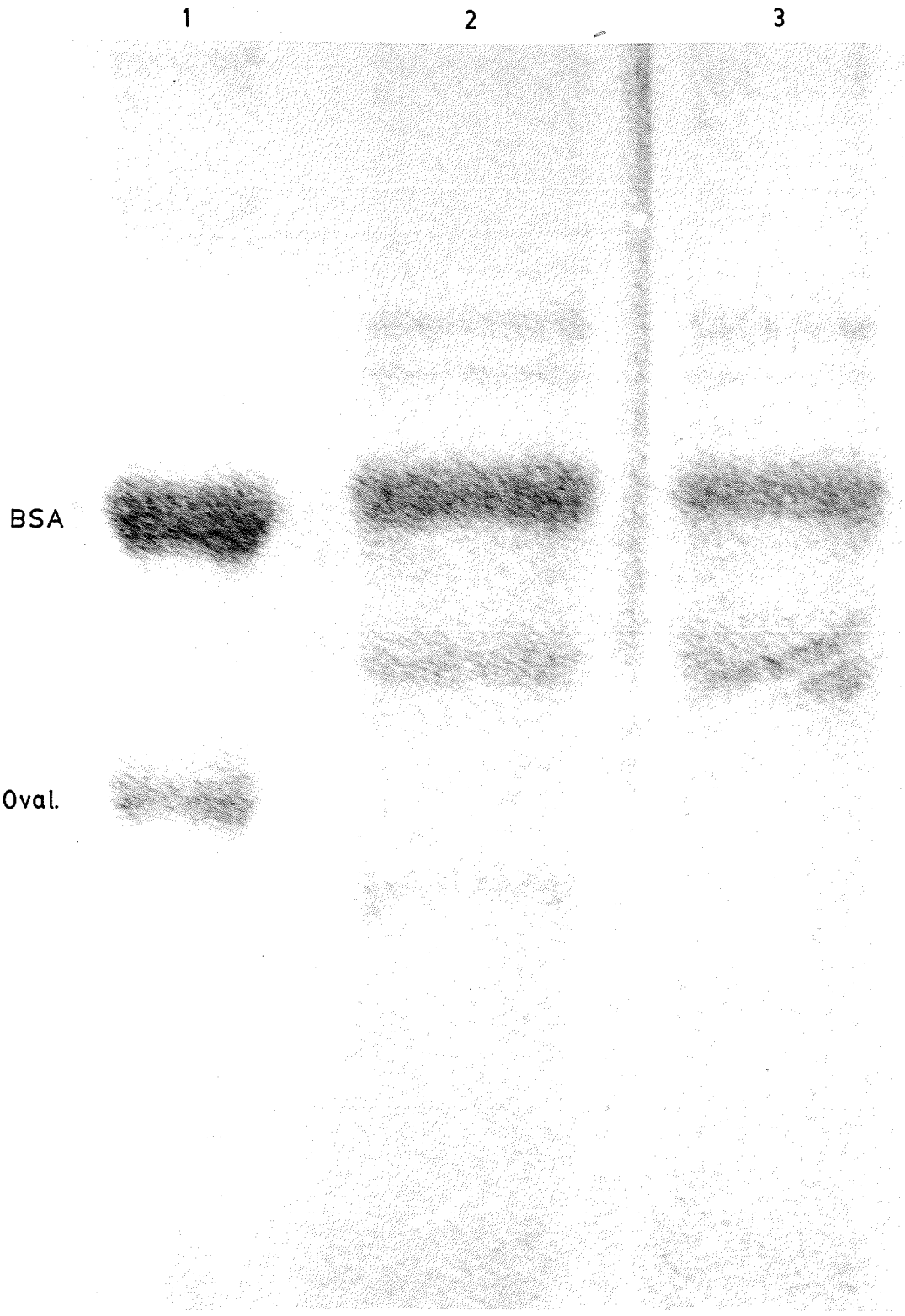
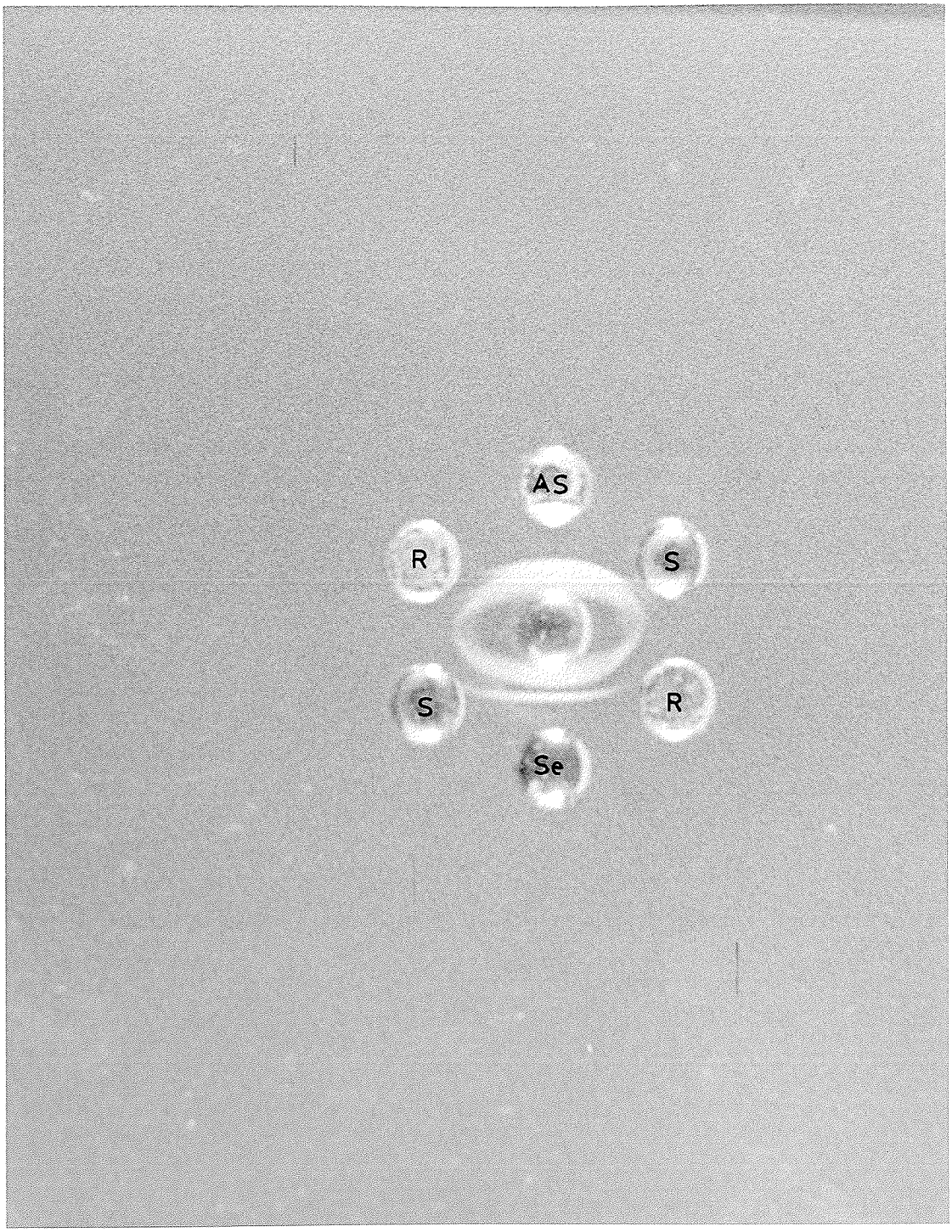

1
2
3
BSA
Oval.

Figure 24. Double diffusion analysis of RER and SER of rat α_1-AGP.

The center well contains anti rat α_1-AGP and the other wells contain RER α_1-AGP (R), SER α_1-AGP (S), Aisialo α_1-AGP (AS) and serum α_1-AGP (Se). Diffusion was at room temperature for 24 hrs.


AS
R
S
S
R
Se

3.4 NMR Studies of RER α_1-AGP Oligosaccharides

RER α_1-AGP glycopeptides were prepared by exhaustive pronase digestion and isolated by chromatography on Bio-Gel P-4 (See Figure 25). The elution profile of the RER α_1-AGP pronase digest was of some concern due to the exceptionally large peak of hexose positive material that eluted with the void volume. Although the approximate elution volumes for high mannose glycopeptides were known based on the previous total protein studies, four pools of hexose positive material were collected and analysed by NMR. The first two pools R-1 and R-2 (Figure 25) consisted of a mixed linkage glucose containing oligosaccharide that was believed to be due to "bleeding" of the Sephadex columns used for protein isolation. This conclusion was based on two pieces of data. The assayed amount of RER α_1-AGP was approximately 10 mg, yet the dry weight of material prior to digestion was 50 mg. The NMR spectra of this material showed it to have two signals in the anomeric region. A large doublet at 4.975 ppm having a coupling constant of 3.45 Hz (α1,6 linkage) and a smaller doublet at 5.33 ppm, J=3.30 Hz (α1,3 or α1,4 linkage).

As was expected, pool R-3 contained N-Linked oligosaccharides of the high mannose variety (See Figure 26). Integration of the H-1 structural reporter groups for residues 4 and 4´ yielded identical values which were normalized to 1 for comparison to other structural reporter groups. Integrals for the H-1 signals of substituted A, C and B residues were 0.50, 0.90 and 1.00 resepectively. This result taken together with results from the glucosamine labelling experiments

suggests a mixture of three glycopeptides, with the two predominant structures being a $Man_9GlcNAc_2$ and an isomer of $Man_8GlcNAc_2$ lacking residue D-2 (an unsubstituted residue A). Several other signals were also observed on this spectrum. A small doublet at 5.20 ppm was believed to be from a small quantity of glucosylated $Man_9GlcNAc_2$. This signal was previously observed in the total RER glycoprotein oligosaccharides. The doublet at 4.975 ppm was due to Sephadex oligosaccharide contamination. The most troubling of these signals however was what appeared to be a doublet at 4.910 ppm. This shift value corresponds to that seen for the structural reporter group for the H-1 of an unsubstituted residue B. While a structure lacking substitution on residue B cannot be ruled out, it was very likely this was not the situation as the normalized signal integral for a substituted B is equal to 1.

To help further characterize this mixed glycopeptide, it was digested with Endo-H and resultant mixed oligosaccharides were then resolved by HPLC. A survey spectra of the Bio-Gel P-4 de-salted oligosaccharide mixture revealed that substantial amounts of contaminant were removed from the sample. This was due to an alteration in chromatographic behavior caused by the digestion of the glycopeptide. While the molecular weight of the contaminants remained constant, that of the digested material decreased and therefore allowed for their separation on a short Bio-Gel column. While the resolution of sub-microgram quantities of radio labelled high mannose oligosaccharides is a routine procedure the same may not be said for unlabelled milligram quantities. Only one publication, that by Herscovics (55), makes a brief

reference to this type of procedure and although employed essentially as described, it was of limited success.

Prior to the resolution of the oligosaccharides, a suitable method for their detection in the column eluates had to be devised. A number of different methods were considered. A simple hexose assay of collected fractions, the addition of a labelled oligosaccharide and scintillation counting or oligosaccharide derivatization and UV-visible detection. However, each was deemed inappropriate. Hexose assay was ruled out due to the limited supply of oligosaccharide. Addition of a radio label was avoided because it too would require one to assay aliquots of the collected fractions resulting in the loss of oligosaccharide. In the event that an oligosaccharide of sufficient specific radioactivity could be prepared that losses would be negligible, one then had to consider the possibilty of contamination of the sample probe in the NMR spectrometer in the event of tube breakage. UV-visible detection of a derivatized oligosaccharide (assuming a good derivatizing agent could be found) was also unsatisfactory because it would effect both the chromatographic behavior and the NMR spectra in an unknown manner. The method which was ultimately chosen was UV detection of column eluates at 196 nm. This method which uses the -NH-CO- group in GlcNAc as a chromophore also has its problems. The -NH-CO- group has a low molar extinction coefficient, 30,000 at 196 nm. The UV cut off for one of the eluting solvents, acetonitrile is 192 nm; because oligosaccharide resolution required a gradient, one would have to contend with a constantly decreasing baseline. A second problem is that virtually all organic molecules have a strong absorbance band in this region of the

spectra.

The RER α_1-AGP oligosaccharide mixture was dissolved in 300 ul of 40:60 acetonitrile-water (V:V). A 100 ul aliquot was then chromatographed. Based on the elution profile described by Herscovics it was expected that the $Man_8GlcNAc$ would elute in the range of 40 to 44 min and the $Man_9GlcNAc$ between 45 and 49 min. The elution profile of this sample (See Figure 27) showed two peaks, one at 33 min and the other at 38 min somewhat earlier than expected. The remaining 200 ul of the oligosaccharide mixture were also chromatographed in 100 ul aliquots. The early and late eluting peaks for each of the three aliquots chromatographed were pooled and analysed by NMR. As was expected, the pool of late running peaks contained $Man_9GlcNAc$ oligosaccharide (See Figure 28). The assignment of $Man_9GlcNAc$ structure was straightforward, as the reporters for H-1 of residues A, B, C, 4 and 4´ were all equal to 1. Reporters for D-1, D-2 and D-3 yielded an integral of 3. The anomeric region also featured a new structural reporter group, a doublet at 5.23 ppm due to the H-1 of the anomer of GlcNac 2. The integral of this reporter group is 0.60 and indicates that the oligosaccharide exists in an anomeric ratio of 6:4 (α:β).

The early running pool contained a mixture of two isomers of $Man_8GlcNAc$ oligosaccharide (See Figure 29). Based on the integrals of the reporter groups for H-1 of substituted A, B and C residues it was determined that 85% of the isomers lacked residue D-2 (the "A" isomer) and 15% lacked residue D-1 (the "C" isomer). The spectrum also showed several other interesting characteristics of the $Man_8GlcNAc$

oligosaccharide. Integration of the H-1 for the α anomer of GlcNAc shows that it too exists in an anomeric ratio of 6:4 (α:β). The shift for this signal also is moved downfield 0.02 ppm in comparison to the $Man_9GlcNAc$ as the result of a through space coupling with residues A and D-2. Further evidence of this spatial relationship exists in the structural reporter group for the unsubstituted A residue. Through space coupling to the β-anomer results in a shift of 5.10 ppm while for the α-anomer this shift is 5.07 ppm [§]. One final interesting aspect of this spatial interaction can be seen if one compares the effects of α1,2 mannose substitution on various residues. When residues C, B or 4 are substituted the chemical shift of their H-1 protons moves +0.244 ppm. When residue A is α1,2 substituted its H-1 shifts +0.31 ppm.

The doublet at 4.975 ppm in the $Man_8GlcNAc$ (Figure 29) spectra is a curiosity. While it can't be positively identified, it is assumed to be traces of Sephadex. This is in itself not unexpected, however no such signal is seen on the $Man_9GlcNAc$ (Figure 28) spectrum. Why Sephadex would "bleed" fragments of a certain size is not understood.

[§] This signal assignment is based on signal integrals and differs from that proposed by Atkinson (56) who suggests that the shifts for the α and β anomers are 5.10 and 5.07 ppm respectively.

Figure 25. Bio-Gel P-4 chromatography of pronase digests of RER α_1-AGP.

Lyophilized 96 hr pronase digests of RER α_1-AGP were dissolved in 1 ml of H_2O and eluted with 0.1 M pyridine-acetate pH 5.1 from a 1.5x175 cm column of Bio-Gel P-4 (200-400) mesh at a constant pressure of 20 PSI. Fractions of 2.2 ml were collected and 200 µl aliquots were assayed for hexose. Fractions were pooled as indicated by the arrows and analysed by proton NMR. The shaded region was found to contain high mannose glycopeptides (See fig 26).

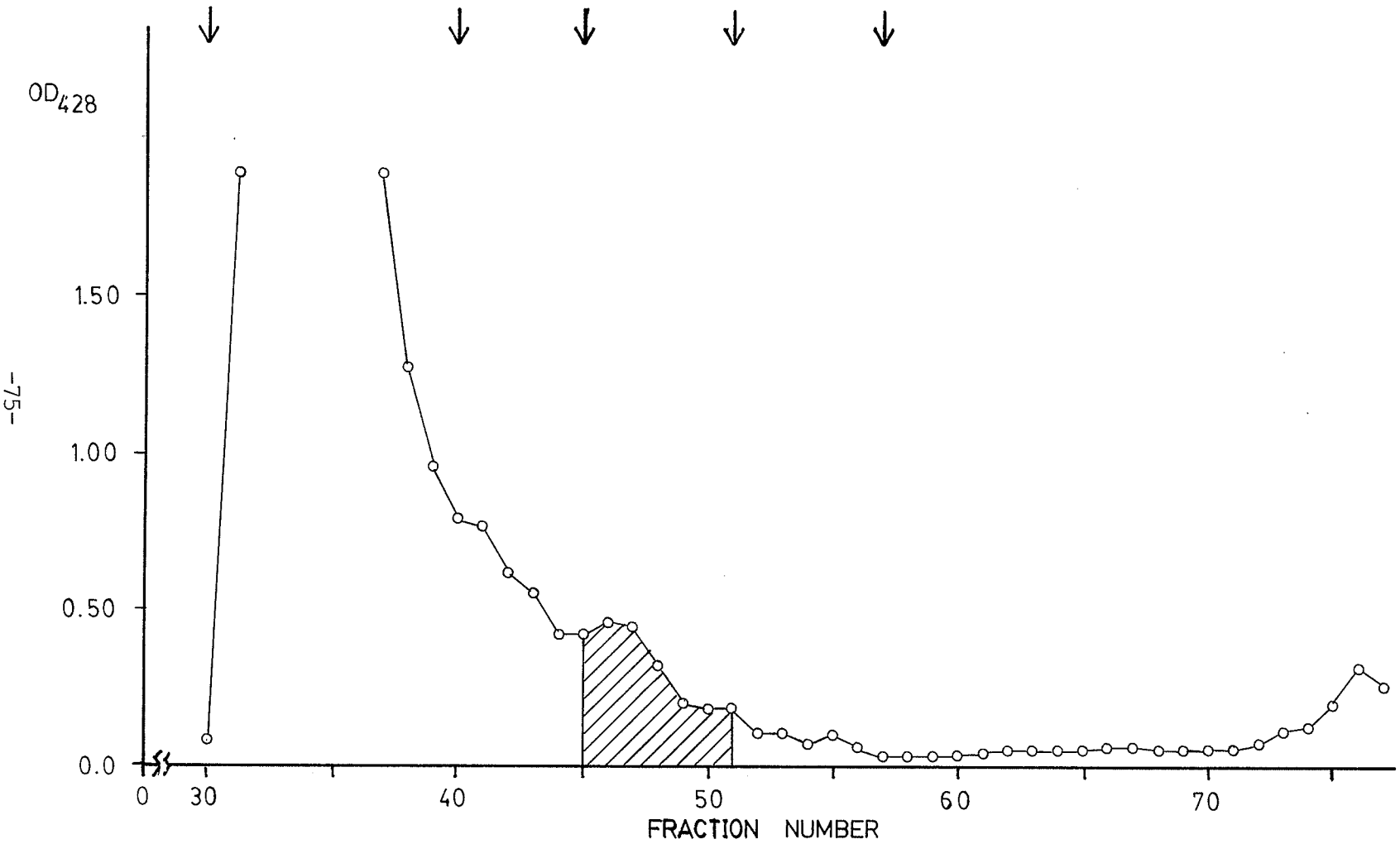
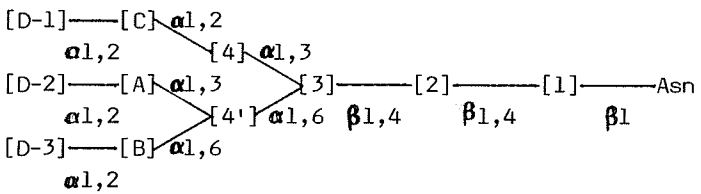

OD428
1.50
1.00
0.50
0.0
0
30
40
50
60
70
FRACTION NUMBER

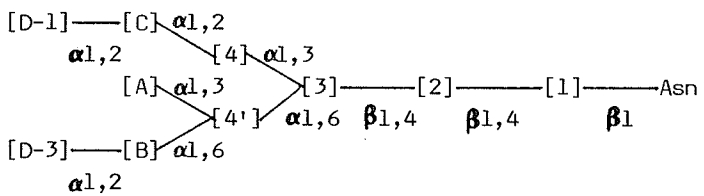
Figure 26. Anomeric region of glycopeptide mixture fraction R-3 from RER α_1-AGP [a].

Shift/Integral[b]	Reporter Group[c]	Spectral Information
5.403/0.50	H-1 of Man A	Substituted residue A
5.335/1.00	H-1 of Man 4	Substituted residue 4
5.306/0.90	H-1 of Man C	Substituted residue C
5.192/ --[d]	H-1 of Glc[e]	α1,3 linked glucose
5.142/1.00	H-1 of Man B	Substituted residue B
5.093/4.00	H-1 of GlcNAc 1	GlcNac β1 linked to Asn
'' / ''	H-1 of Man A	Unsubstituted residue A
5.057/ ''	H-1 of Man D-2	
5.050/ ''	H-1 of Man C	Unsubstituted residue C
5.043/ ''	H-1 of Man D-1 and D-3	
4.975/ --[d]	?	Contamination Sephadex bleed
4.910/0.10	H-1 of Man B	Contaminant of unknown origin
4.889/1.00	H-1 of Man 4'	

[a] For elution profile see figure 25. [b] Integral values are normalized for residue 4 or 4'=1. [c] See (44) for shift assignment. [d] Due to spin-spin splitting shift value reported as an average value of the two signals. [e] See section 3.1 for assignment.

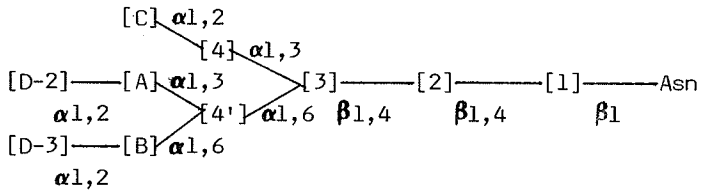


45%



50%

Structures of isomers present in mixture.



5%

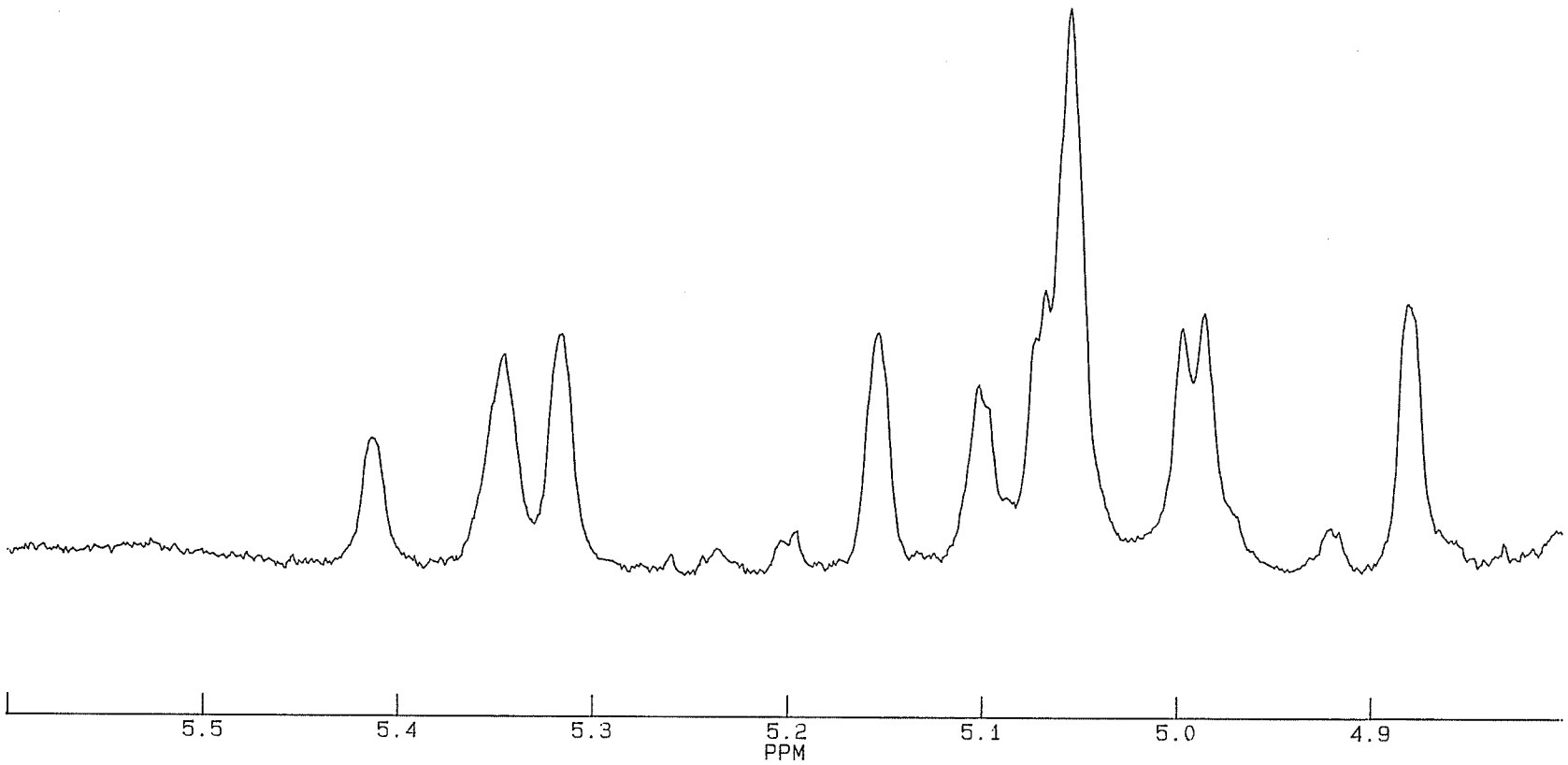
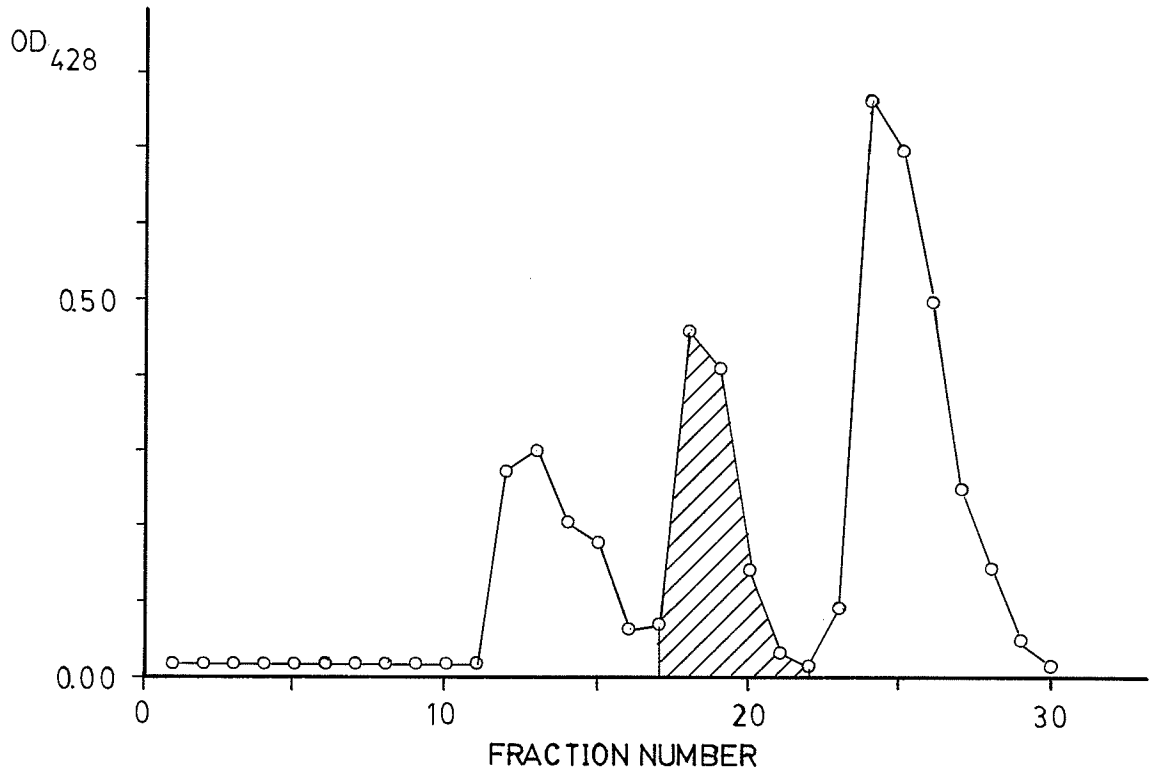

5.5
5.4
5.3
5.2
PPM
5.1
5.0
4.9

Figure 27. Isolation of oligosaccharides from RER α_1-AGP glycopeptides.

RER derived glycopeptides (See figs 21 and 22) were digested 48 hrs with 30 milli units of Endo-H and were de-salted on a 1x100 cm column of Bio-Gel P-4 (+400 mesh) with distilled deionized water (upper panel). Fractions of 2.2 ml were collected, 100µl aliquots were assayed for hexose and those in the shaded area were pooled and analysed by NMR. This material was then dissolved in 300 µl of 40:60 water-acetonitrile (V:V) and 100 ul aliquots were chromatographed on a 0.46x25 cm column of 5u Aminospherisorb. The column was eluted isocratically for 14 min with 40:60 water-acetonitrile and then by a linear gradient to 60:40 water-acetonitrile over 64 min. Eluate was monitored at 196 nm and peaks were collected as indicated by arrows. Peaks 1 and 2 were then prepared for and analysed by NMR (See figs 28 and 29).


OD428
0.50
0.00
0
10
20
30
FRACTION NUMBER

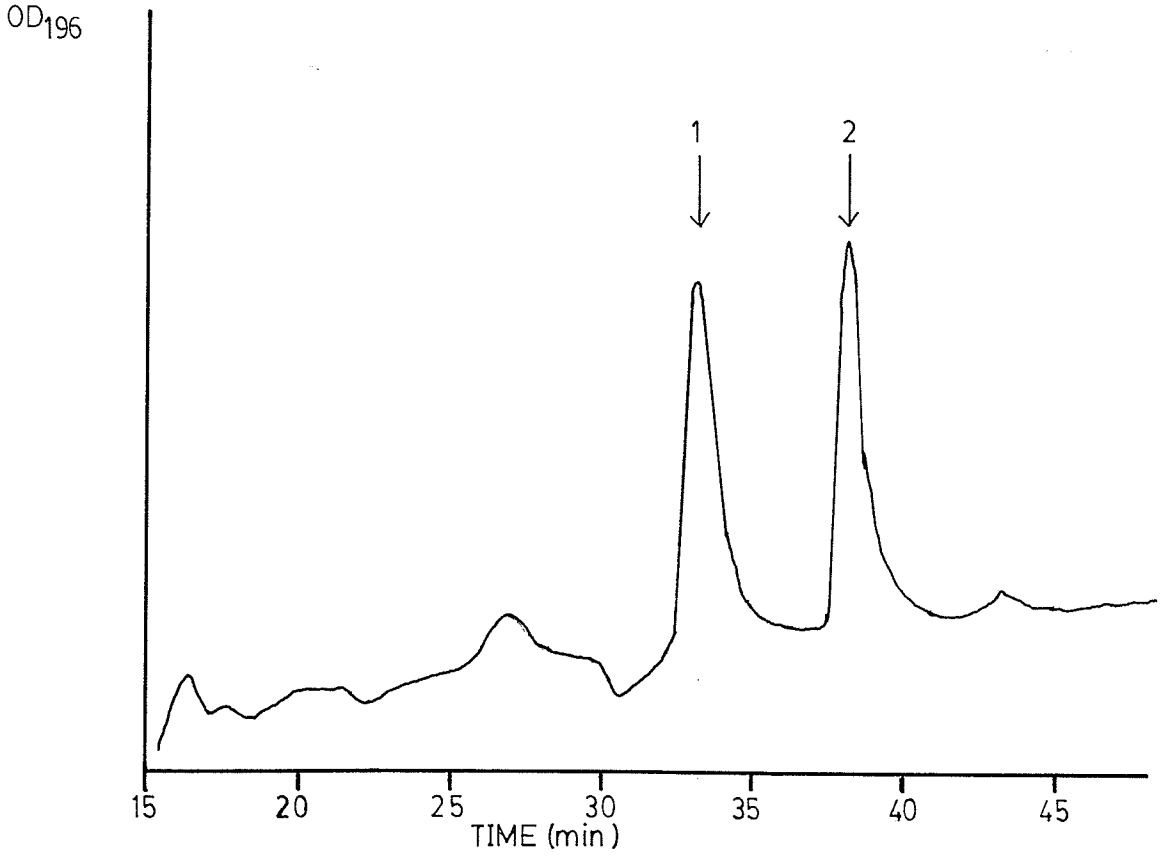
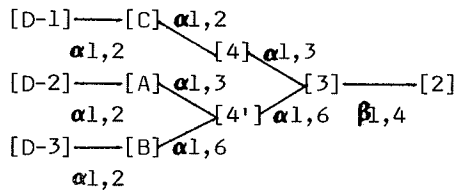

OD196
1
2
15
20
25
30
35
40
45
TIME (min)

Figure 28. Anomeric region of HPLC fraction 2 from RER α_1-AGP mixed oligosaccharides [a].

Shift/Integral[b]	Reporter Group[c]	Spectral Information
5.407/1.00	H-1 of Man A	Substituted residue A
5.337/1.00	H-1 of Man 4	Substituted residue 4
5.310/1.00	H-1 of Man C	Substituted residue 4
5.230/0.60[d]	H-1 of GlcNAc 2	α anomer of GlcNAc
5.148/1.00	H-1 of Man B	Substituted residue B
5.061/3.00	H-1 of Man D-2	
5.045/ --	H-1 of Man D-1	
5.039/ --	H-1 of Man D-3	
4.889/1.00	H-1 of Man 4'	

[a] For elution profile see figure 24. [b] Integral values are normalized for residue 4=1. [c] See (44 and 56) for shift assignment. [d] Due to spin-spin splitting shift value reported as average of two signals.



Oligosaccharide exists in a mixture of α and β anomers of residue 2 in the ratio 6:4 (α:β).

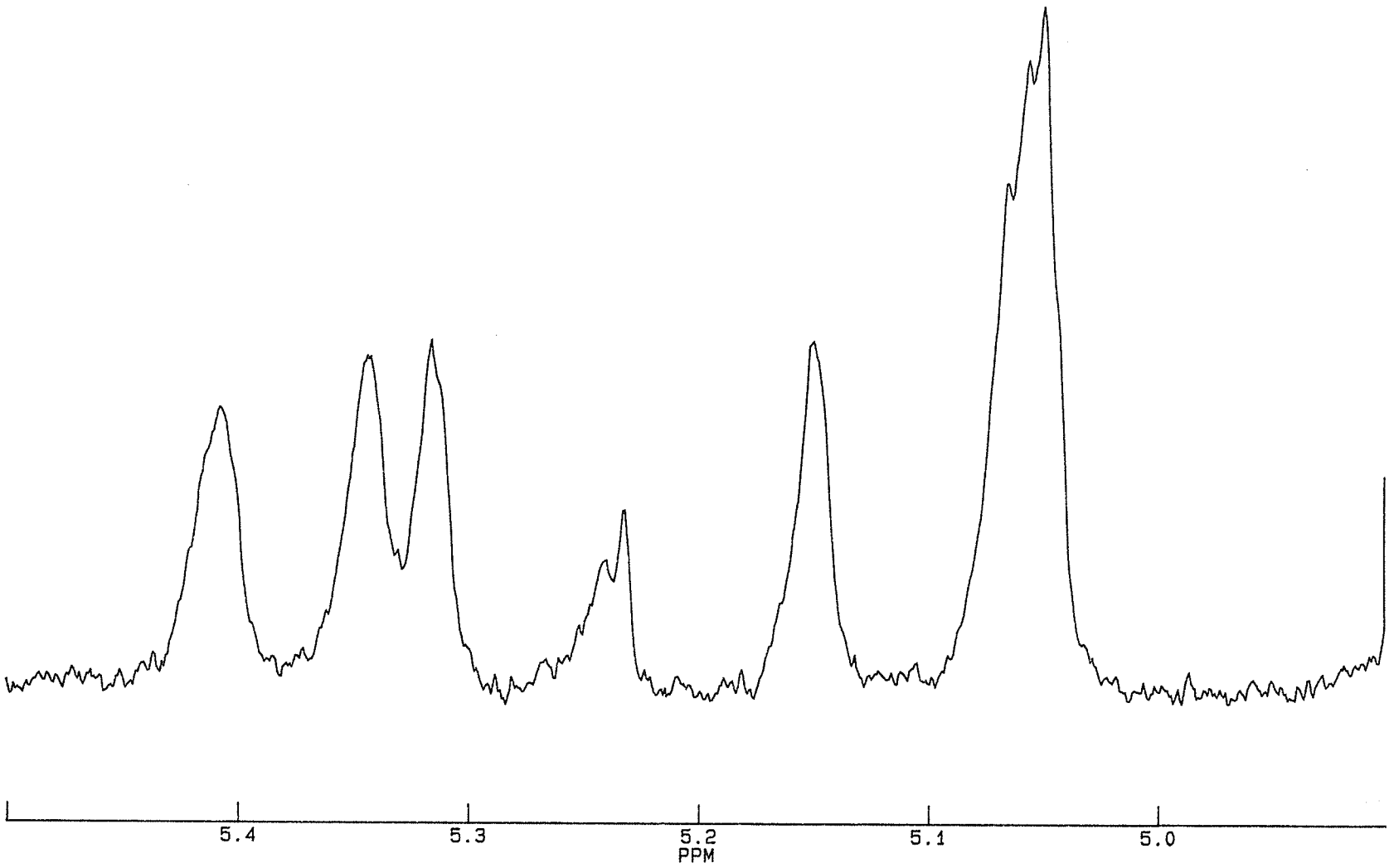
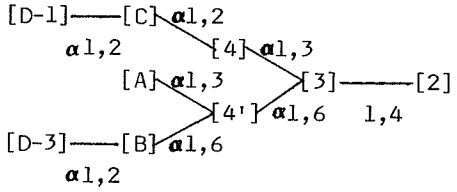

5.4
5.3
5.2
PPM
5.1
5.0

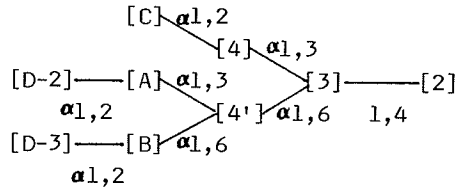
Figure 29. Anomeric region of HPLC fraction 1 from RER α_1-AGP mixed oligosaccharides [a].

Shift/Integral[b]	Reporter Group[c]	Spectral Information
5.397/0.15	H-1 of Man A	Substituted residue A
5.340/1.00	H-1 of Man 4	Substituted residue 4
5.306/0.90[d]	H-1 of Man C	Substituted residue C
5.244/0.60[d]	H-1 of GlcNAc 2	α anomer of GlcNAc
5.148/1.00	H-1 of Man B	Substituted residue B
5.100/0.35[d]	H-1 of Man A	Through space coupling of H-1 of unsubstituted A to the β anomer of GlcNAc
5.072/0.50[d]	H-1 of Man A	Through space coupling of H-1 of unsubstituted A to the α anomer of GlcNAc
5.053/2.10	H-1 of Man D-2	
'' / --	H-1 of Man C	Unsubstituted residue C
5.045/ --	H-1 of Man D-1	
5.038/ --	H-1 of Man D-3	
4.889/1.00	H-1 of Man 4'	

[a] For elution profile see figure 27. [b] Integral values are normalized for residue 4=1. [c] See (44 and 56) for shift assignment. [d] Due to spin-spin splitting shift value reported as average of two signals.

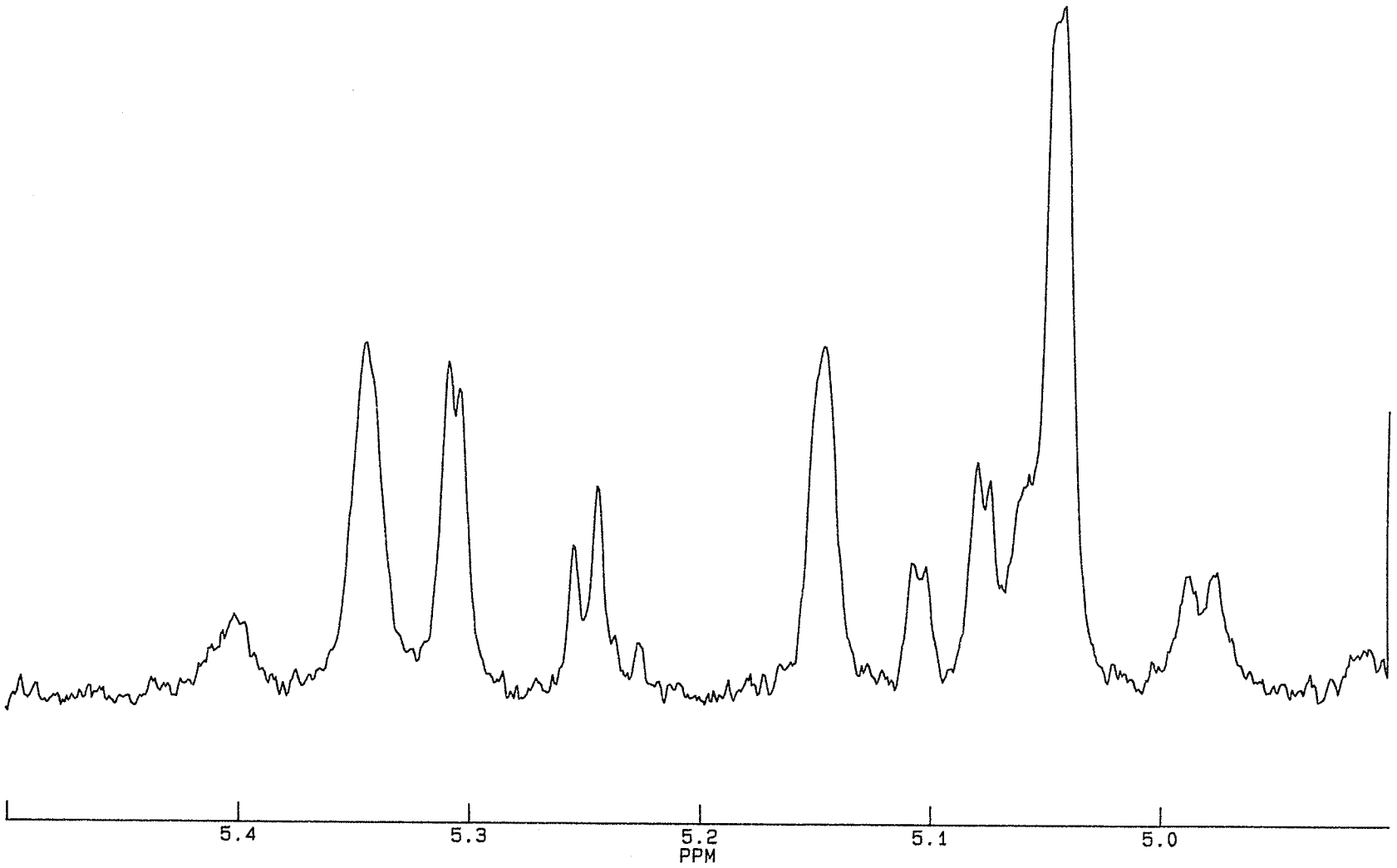


85%



15%

Both oligosaccharides exist in α and β anomers in the ratio of 6:4 (α:β).


5.4
5.3
5.2
PPM
5.1
5.0

3.5 NMR Studies of SER α_1-AGP Oligosaccharides

The analysis of SER α_1-AGP glycopeptides and oligosaccharides is an extension of those studies done on RER α_1-AGP. Therefore, the approach employed was the same. SER α_1-AGP was digested with pronase and glycopeptides were isolated by gel filtration chromatography on Bio-Gel P-4 (See Figure 30). NMR analysis indicated a complex mixture of high mannose structures. Integrals of the H-1 reporters for substituted A, C and B residues did not shed any light on the composition of the mixture other than indicating the presence of Man_6-$Man_9GlcNAc_2$ structures (See Figure 31). Following Endo-H digestion and de-salting the resultant oligosaccharides were resolved by HPLC (See Figure 32). Three pools of eluate were collected and examined by NMR. Pool 3 contained $Man_9GlcNAc$; pool 2 contained a mixture of the "A" and "C" isomers of $Man_8GlcNAc$ in a ratio of 3:1. Pool 1 contained a mixture of at least 3 isomers of Man_7. All lacked two of the three D residues. Integrals of reporters for substituted C, A and B residues indicate that 50% of the structures have residue D-3 as their most outer, branch terminal. The remaining 50% have D-1 and D-2 as the most outer branch in a 1:1 ratio. No evidence could be found that would suggest the presence of one other possible $Man_7GlcNAc$ structure, that which lacks residues D-1 and C. It should be noted however, that in this type of a mixture the only evidence for this structure would be obtained indirectly, by a difference in the integrals of signals for substituted residues 4 and 4´.

Figure 30. Bio-Gel P-4 chromatography of pronase digests of SER α_1-AGP.

Lyophilized 96 hr pronase digests of SER α_1-AGP were dissolved in 1 ml of H_2O and eluted with 0.1 M pyridine-acetate pH 5.1 from a 1.5x175 cm column of Bio-Gel P-4 (200-400) mesh at a constant pressure of 20 PSI. Fractions of 2.2 ml were collected and 50 µl aliquots were assayed for hexose. The shaded region was found to contain high mannose glycopeptides (See fig 31).

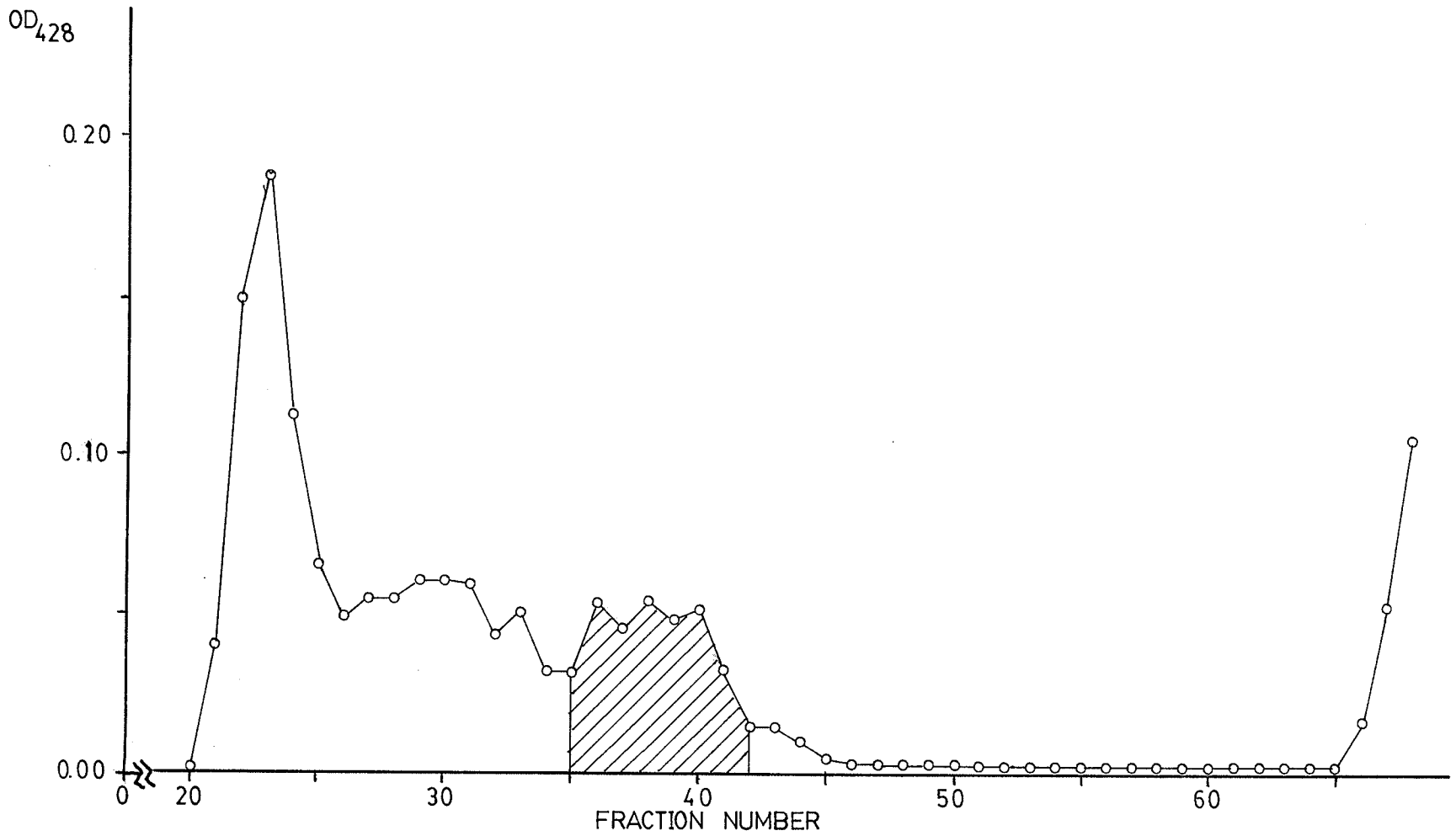
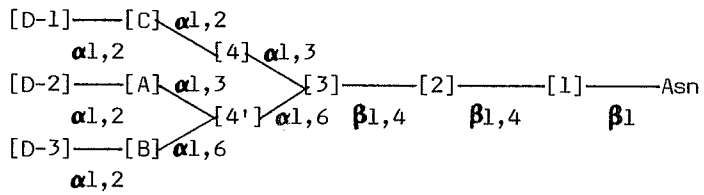

OD428
0.20
0.10
0.00
0
20
30
40
50
60
FRACTION NUMBER

Figure 31. Anomeric region of glycopeptide mixture from SER α_1-AGP[a]

Shift/Integral[b]	Reporter Group[c]	Spectral Information
5.403/0.40	H-1 of Man A	Substituted residue A
5.332/1.00	H-1 of Man 4	Substituted residue 4
5.307/0.75	H-1 of Man C	Substituted residue C
5.140/0.85	H-1 of Man B	Substituted residue B
5.090/1.30	H-1 of GlcNAc 1	GlcNac β1 linked to Asn
'' / ''	H-1 of Man A	Unsubstituted residue A
5.057/2.70	H-1 of Man D-2	
5.050/ ''	H-1 of Man C	Unsubstituted residue C
5.044/ ''	H-1 of Man D-1 and -3	
4.910/0.25	H-1 of man B	Unsubstituted residue B

[a] For glycopeptide preparation see figure 30. [b] Integral values are normalized for residues 4 and 4' =1.00 [c] See (44) for shift assignment.



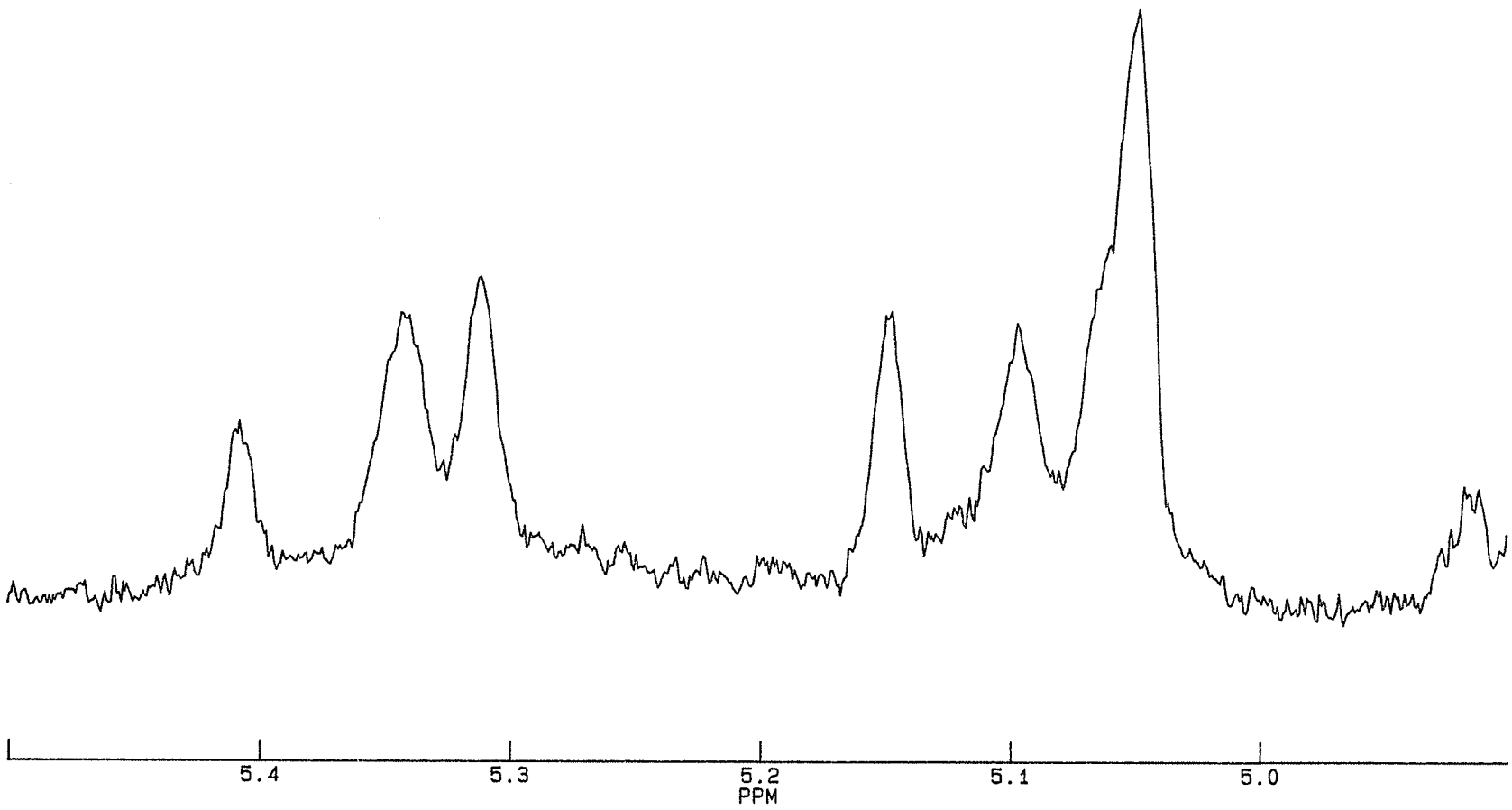
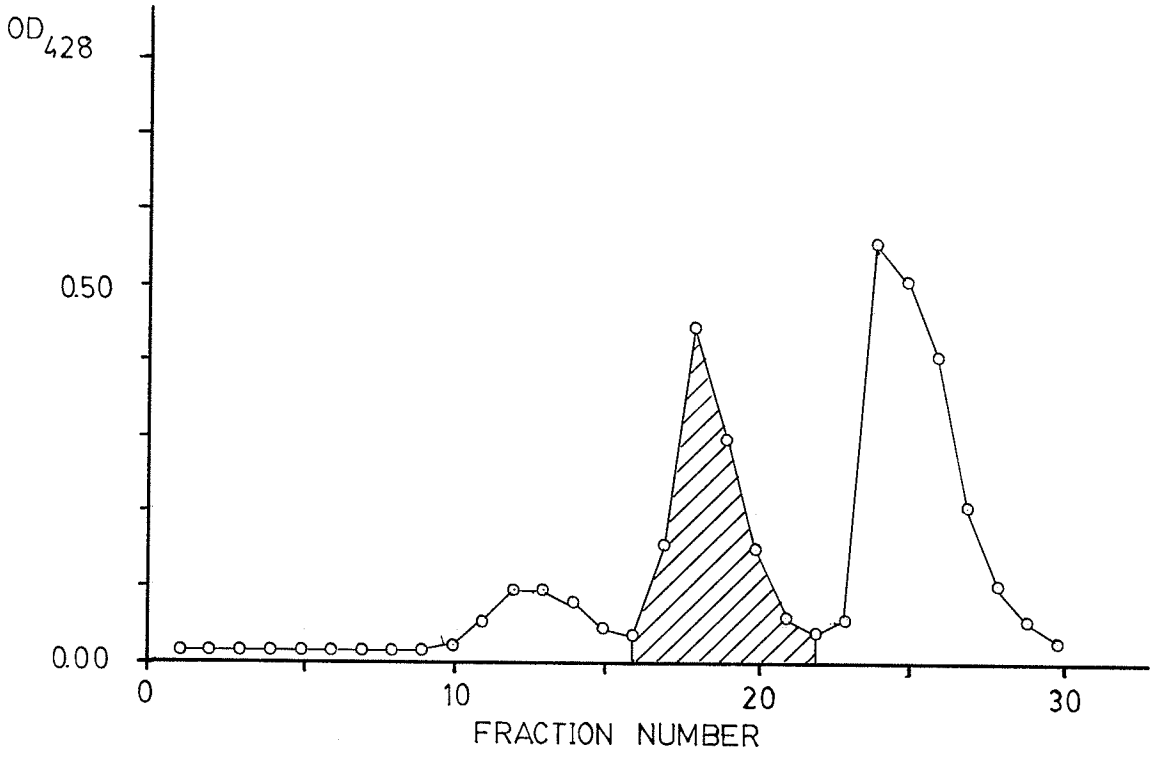

5.4
5.3
5.2
5.1
5.0
PPM

Figure 32. Isolation of oligosaccharides from SER α_1-AGP glycopeptides.

SER α_1-AGP glycopeptides (See figs 30 and 31) were digested 48 hrs with 30 milli units of Endo-H and were de-salted on a 1x100 cm column of Bio-Gel P-4 (+400 mesh) with distilled deionized water (upper panel). Fractions of 2.2 ml were collected and those in the shaded area were pooled and lyophilized. This material was then dissolved in 100 ul of 40:60 water-acetonitrile (V:V) and chromatographed on a 0.46x25 cm column of 5μ Aminospherisorb. The column was eluted isocratically for 14 min with 40:60 water-acetonitrile and then by a linear gradient to 60:40 water-acetonitrile over 64 min. Eluate was monitored at 196 nm and peaks were collected as indicated by arrows. Peaks were then prepared for and analysed by NMR. Peaks 2 and 3 were Man_8 and Man_9 oligosaccharides, respectively, as seen for the RER derived α_1-AGP (See figs 28 and 29). Peak 1 was found to be a mixture of Man_7 oligosaccharides (See fig 33).


OD428
0.50
0.00
0
10
20
30
FRACTION NUMBER

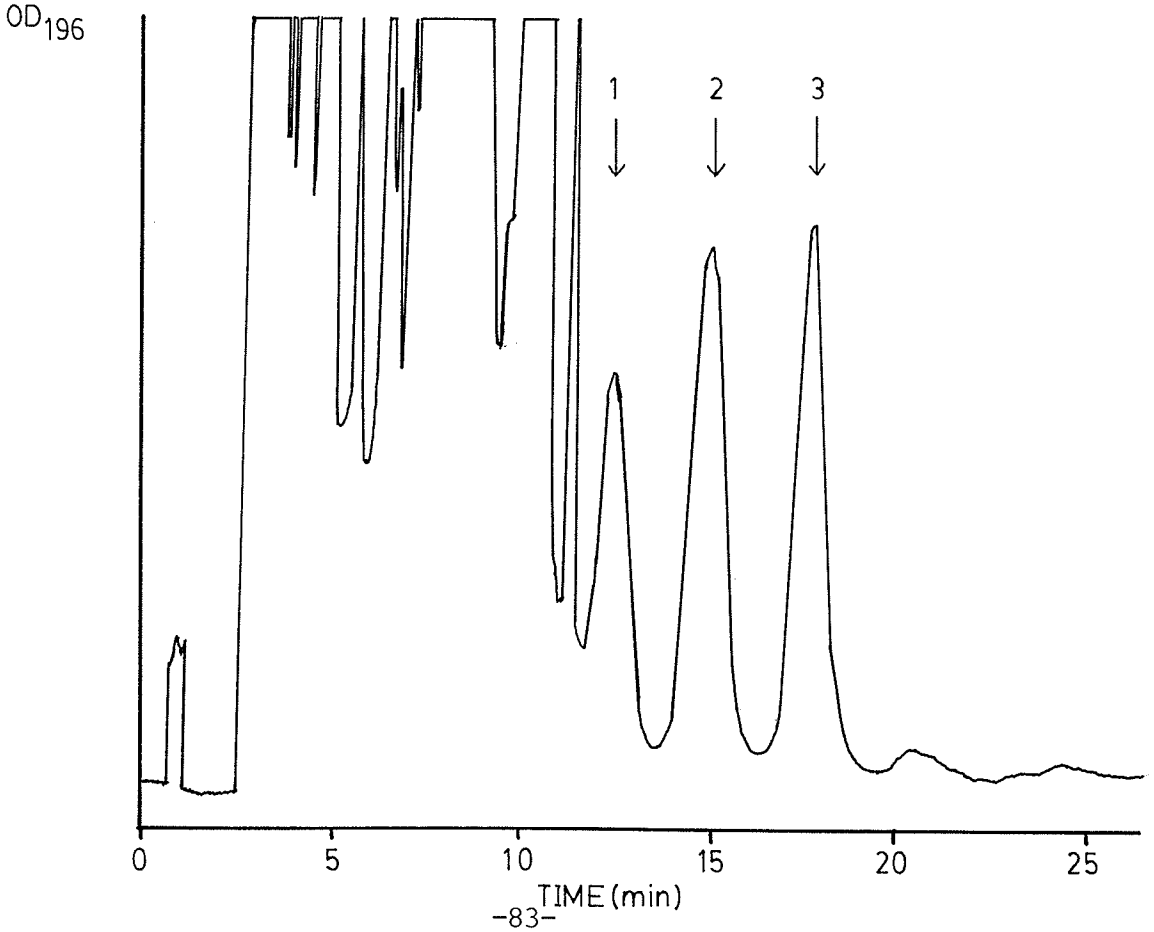
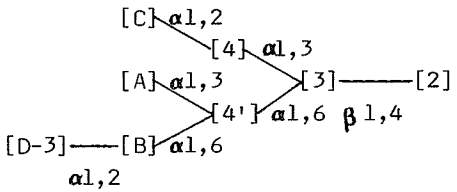

OD196
1
2
3
0
5
10
15
20
25
TIME (min)

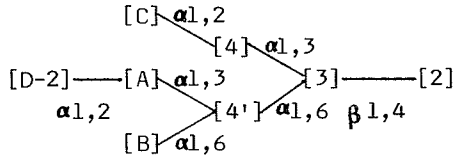
Figure 33. Anomeric region of HPLC fraction 1 from SER α_1-AGP mixed oligosaccharides [a].

Shift/Integral [b]	Reporter Group [c]	Spectral Information
5.398/0.25	H-1 of Man A	Substituted residue A
5.347/1.00	H-1 of Man 4	Substituted residue 4
5.304/0.25 [d]	H-1 of Man C	Substituted residue C
5.243/0.60 [d]	H-1 of GlcNAc 2	α anomer of GlcNAc
5.141/0.50	H-1 of Man B	Substituted residue B
5.101/0.55 [d]	H-1 of Man A	Through space coupling of H-1 of unsubstituted A to the β anomer of GlcNAc
5.076/0.60 [d]	H-1 of Man A	Through space coupling of H-1 of unsubstituted A to the α anomer of GlcNAc
5.053/1.51	H-1 of Man D-2	
'' / --	H-1 of Man C	Unsubstituted residue C
5.045/ --	H-1 of Man D-1	
5.040/ --	H-1 of Man D-3	
4.908/0.52	H-1 of Man B	Unsubstituted residue B
4.889/1.00	H-1 of Man 4'	

[a] For elution profile see figure 32. [b] Integral values are normalized for residue 4=1. [c] See (44 and 56) for shift assignment. [d] Due to spin-spin splitting shift value reported as average of two signals.

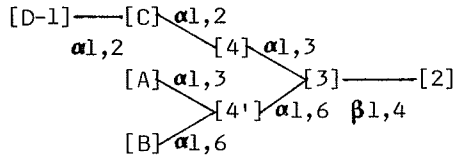


50%

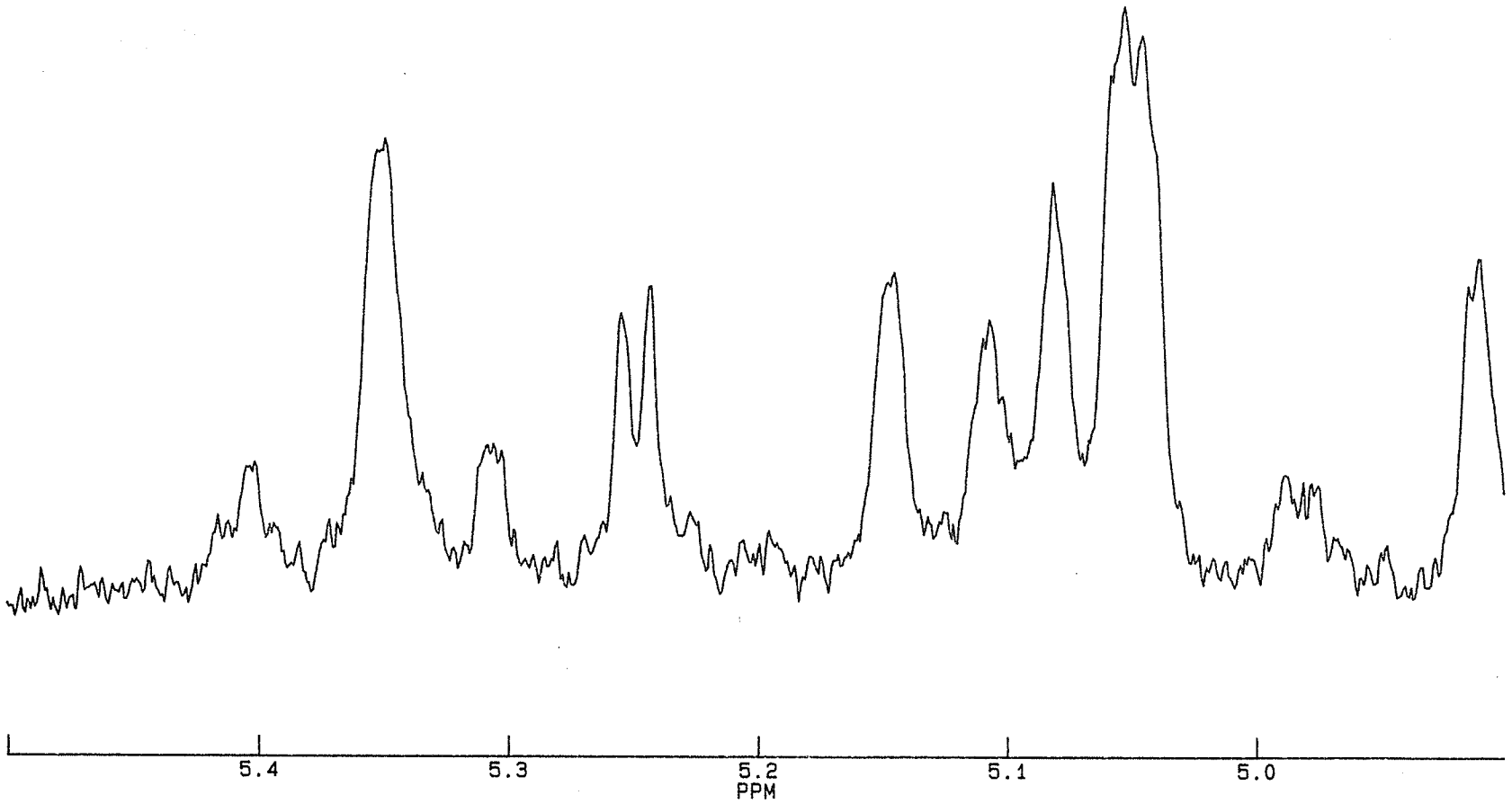


25%

All oligosaccharides exist in α and β anomers in the ratio of 6:4 (α:β).



25%


5.4
5.3
5.2
PPM
5.1
5.0

Discussion

4.1 Characterization of Total Glycoprotein Oligosaccharide Structures

The biosynthesis and secretion of glycoproteins by the liver is a remarkably complex pathway and is divided into a number of distinct phases that occur in a successive series of locales within the cell. Amino acids, ribosomes and the associated "machinery" required for protein synthesis are found on the cytoplasmic face of the ER. Through the ER translocation system and a peptide signal sequence found on secretory proteins, proteins are similtaneously synthesized and transported across the ER (27). Evidence exists to suggest that glycosylation may occur concurrent with peptide elongation (33 & 34), although other studies suggest glycosylation is also a post ribosomal event (25). These oligosaccharides are assembled on a lipid carrier on the cytoplasmic face of the ER, and then are internalized prior to glucosylation and transfer (25). Following this, a series of processing reactions occur that result in the removal of three glucose residues. At this point processing may cease or continue further and ultimately result in formation of high mannose, hybrid or complex structures (See section 1.4).

The currently accepted mechanism by which processing reactions occur has been elucidated using two experimental approaches. The more common is the assay of enzyme activities obtained from sub-cellular fractions. The other has been through *in vitro* labelling experiments done on sub-cellular fractions, tissue slices or mutant cell lines. These experiments take the form of pulse chase studies or the

characterization of labelled intermediates. Few, if any studies, have chosen to examine the oligosaccharide structures of glycoprotein processing intermediates isolated from intact organs removed from animals. The reason for this is that labelling studies in live animal's are difficult due to the rapid spread and dilution of the labelled precursor by endogenous pools thoughout the animals body. Therefore, very little is known about the dynamics of processing in vivo. The approach taken and described in this thesis avoids the limitations imposed by labelling or enzyme assays through the characterization of intermediates of the processing reactions by proton NMR.

Previous work done in this laboratory has shown that α_1-AGP is an acute phase reactant whose intracellular and serum level is substantially elevated by turpentine inflammation. Intracellular species that were isolated from the RER, SER and Golgi differed substantially in both hexose and amino acid content from the serum form. Results of hexose analysis indicated that the form isolated from the RER was most likely a high mannose form. Other species from the SER and Golgi were less easily characterized but did appear to represent the results of a continuation in processing reactions.

At the time of writing of this thesis, no published report exists that describes the characterization of any intermediate form of a glycoprotein by analysis of the oligosaccharide by NMR. For this reason a number of primary studies were performed on total protein preparations isolated from ultrasonic extracts of RER, SER and Golgi to determine if it was reasonable to proceed to study a specific protein such as α_1-AGP. This approach had been applied successfully by the

groups of Atkinson and Ballou in the study of mannoprotein oligosaccharides obtained from mutant yeast cultures (56 & 57).

The interpretation of NMR spectra from the RER derived glycopeptides indicated that all structures isolated were high mannose forms. The distribution of structures and the nature of any isomeric forms could not be determined. Spectral integration of H-1 reporters for substituted A, C and B residues showed that 80% of these were substituted. Whether this was due to a 4:1 mixture of $Man_9GlcNAc_2$:$Man_6GlcNAc_2$ or one which was more complicated, could not be positively determined. Not surprisingly, spectral evidence pointed to the existence of a small amount of glucosylated oligosaccharide. With this in mind, a heterogenous mixture seemed highly probable.

Both the elution profile and NMR spectra of SER glycopeptides proved to be far more complicated than was expected. A total of 4 glycopeptide containing pools were partially characterized. Two pools contained small amounts of what appeared to be $Man_9GlcNAc_2$ structures that differed in peptide composition. The remaining two pools had structures that had undergone considerable processing. One pool contained a complicated mixture which was impossible to delineate. The other, while being a mixture, contained a significant quantity of two $Man_6GlcNAc_2$ isomers (See Figure 13). At the time it was isolated, the uniqueness of these isomers was not fully appreciated (the characteristics of this oligosaccharide will be described in the next section). As a result, no special precautions were taken to preserve the sample or the spectral data. Later, when its significance was realized, the sample had degraded and no additional studies could be performed

on it. In addition to high mannose structures, the SER also showed spectral features that were indicative of complex oligosaccharides. These were only minor components and as a result no structural detail could be obtained.

The glycopeptides isolated from the Golgi were remarkably simple in comparison to those isolated from the SER. Once again, the dominant structure found was high mannose and even less complex structure was evident than was seen in the SER glycopeptides. This was an unexpected result, because all glycosyltransferase activities that have been characterized have been localized in the Golgi. This being the situation, one would expect large quantities of complex and comparatively little high mannose glycopeptide to be isolated from this fraction.

The results obtained from these experiments on total protein preparations were invaluable as they acted as a guide for all remaining studies to be done with intracellular α_1-AGP. A high degree of structural heterogeneity was observed in the glycopeptides isolated from total RER glycoproteins. As a result, any high mannose α_1-AGP glycopeptides that were isolated would have to be resolved into a homologous series. Members of this series could then be characterized by NMR. At the time this work was planned, only one method, that of Herscovics, described such a separation (55). The procedure involved digestion of glycopeptides with Endo-H and chromatography of the resultant oligosaccharides on an Aminospherisorb column. Although the method was employed essentially as described, it was not entirely successful. From the elution profile described by Herscovics, it was

expected that $Man_8GlcNAc$ oligosaccharides would elute from the column between 40 and 44 min and the $Man_9GlcNAc$ between 45 and 49 min. The elution profile of the first sample of RER α_1-AGP oligosaccharides was quite close to this with the $Man_8GlcNAc$ and $Man_9GlcNAc$ eluting at 33 and 38 min respectively (See Figure 27). Chromatography of subsequent aliquots of the same material, however, resulted in retention times for the $Man_8GlcNAc$ and $Man_9GlcNAc$ of 29 and 33 min for the second sample and 25 and 29 min for the third. This steady decrease in the retention time was ultimately traced to the destruction of the column packing material caused by the formation of a Schiff base between the aldehydic group on the oligosaccharide and amino groups of the column packing material (76). However, the column survived sufficiently long to allow for the completion of the study. The obvious remedy to this problem was to reduce the oligosaccharides to their corresponding N-acetylglucosaminitols prior to chromatography. Recently, a second procedure has been described by Vliegenthart that allows for the separation of homologous oligosaccharide structures as their corresponding dansylated glycopeptides (68). This procedure avoids some of the problems associated with detection and employs inexpensive reverse phase columns.

The second important result of the total protein studies was that it would be difficult if not impossible to characterize complex glycopeptides from α_1-AGP from Golgi. The reason for this was that it only small quantities of complex structure could be isolated from this fraction. Whether this was due to the method chosen for sub-cellular fractionation or to very rapid processing and hence small pool size is

not known. In the event the fractionation method chosen was at fault and large quantities of Golgi α_1-AGP could be isolated, one would have to contend with a prohibitively large number of potential processing intermediates.

As a result of these preliminary experiments, it was decided that any research to be done on intracellular α_1-AGP should focus on the characterization of the high mannose structures that could be isolated from the RER and SER.

4.2 Characterization of Oligosaccharides From Intracellular α_1-AGP

Recently, several workers have successfully determined the structures of high mannose oligosaccharide processing intermediates. These results came from characterization of the activities of mannosidases found in the RER, and labelled oligosaccharide intermediates generated *in vivo*.

The first pieces of evidence suggesting the existence of mannosidase activity in the RER began to accumulate in the early 1980's. Godelaine *et al.* (77) found bovine thyroglobulin containing $Man_8GlcNAc_2$ in the RER membrane fraction of thyroid slices and discovered that it accumulated when the slices were incubated with carbonyl cyanide **m**-chlorophenyl hydrazone, a compound that blocks intracellular transport from the RER to the Golgi. Later, Hercz and Harpaz (78) found that the Z variant of human plasma α_1 anti-trypsin that accumulates in the RER of liver contains oligosaccharides of the composition Man_8-$Man_6GlcNAc_2$.

In 1983 Kornfeld and Bischoff (30) presented evidence for the existence of an **α**-mannosidase in rat liver RER. The enzyme which they characterized differed substantially from the two previously described Golgi **α**-mannosidases. Physically, it was not bound by Con-A Sepharose. It was active against p-nitrophenyl-**α**-D-mannoside and it was not inhibited by swainsonine, a specific inhibitor of Golgi processing **α**-mannosidases. It had no activity towards the Golgi mannosidse II substrate, $GlcNAcMan_5GlcNAc_2$ and was most active against $Man_9GlcNAc_2$. Based on the substrate specificities, Kornfeld suggested that this

mannosidase was similar to one found in yeast by Atkinson et al. (56) which released a single mannose residue from $Man_9GlcNAc_2$ structures.

In 1985 Herscovics et al. (55) reported the isolation and characterization of an α-mannosidase from Sacchromyces cerevisiae. This mannosidase had similar characteristics to the RER mannosidase isolated from rat liver. It was unaffected by inhibitors of Golgi mannosidase I and II and it was active towards p-nitrophenyl-α-D-mannopyranoside. When incubated with a $Man_9GlcNAc$ substrate, it would remove only one mannose residue. This $Man_8GlcNac$ product was characterized by NMR and was shown to be a single isomer lacking residue D-2.

Later in 1985 Hickman and Theodorakis (79) proposed the sequence of removal of α1,2 linked mannose residues from IgA in MOPC 315 plasmacytoma cells. This sequence was D-2, D-1, D-3 and 4 and was determined by acetolysis of mannose labelled oligosaccharides from immune precipitates of intracellular IgA.

A further evalution of processing in the RER was published in two reports by Kornfeld et al. in 1986. In the first (41), it was found that UT-1 cells[§] grown in the presence of 1-deoxymannojirimycin and labelled mannose for 15 hr would only produce HMG-CoA reductase containing $Man_8GlcNAc_2$ and $Man_9GlcNAc_2$ oligosaccharides. When this experiment was repeated and cells were grown for an additional 3 hr in nonradioactive medium without inhibitor some of the oligosaccharides were processed to $Man_6GlcNAc_2$ and $Man_7GlcNAc_2$. These results

[§] A variant of Chinese Hamster Ovary Cells that overproduces HMG CoA Reductase (3-hydroxy-3-methylglutaryl-CoA reductase) due to gene amplification and stores it in a smooth membrane organelle known as the crystalloid ER.

could be explained in two ways. Prehaps a second α-mannosidase exists within the crystallioid ER that was sensitive to 1-deoxymannojirimycin. Alternatively, that small amounts of Golgi mannosidase I may be "misdirected" in transit from the RER to the Golgi.

In the second report Kornfeld's group (80) characterized a soluble mannosidase found in the RER (80). Originally termed the cytosolic α-mannosidase by Touster and Burns (81) it was found to have many properties similar to those of the RER mannosidase. Among these were the ability to hydrolyse p-nitrophenyl-α-methyl mannopyranoside and insensitivity towards Golgi mannosidase inhibitors. A polyclonal goat antibody raised against the purified soluble form of the enzyme specifically cross reacted with the membrane bound RER α-mannosidase. From these similarities between the two enzymes it was proposed that the soluble form was derived from the membrane bound RER α-mannosidase by proteolytic release. The only significant differences between the two forms of the enzyme were seen in long term incubations with a $Man_9GlcNAc$ substrate. The soluble form was capable of producing $Man_7GlcNAc$ along with smaller quantities of $Man_6GlcNAc$ and $Man_5GlcNAc$. This is in contrast to the bound form which only yielded a $Man_8GlcNAc$.

Approximately five months after Kornfeld proposed the existence of a second RER α-mannosidase, Bause (31) characterized such an enzyme in calf liver. Termed the Man_9 mannosidase, it had enzymic and physical properties that distinguished it from all previously described α-mannosidases. Unlike Golgi mannosidase I and II it would not bind to Con-A Sepharose. Unlike the RER α-mannosidase it was unable to hydrolyse p-nitrophenyl-α-methylmannopyranoside and was strongly

inhibited by 1-deoxymannojirimycin. The most unusual aspect of this enzyme was its residue specificity. Long term enzyme assays done with a $Man_9GlcNAc_2$ substrate resulted in the production of $Man_6GlcNAc_2$ which is believed to lack residues D-1, 4 and either D-2 or D-3.

In the work described in this thesis, the glycopeptides and oligosaccharides isolated from RER α_1-AGP have structures that are consistent with what is known regarding the processing of glycoproteins. The structures isolated and positively identified by NMR are $Glc_1Man_9GlcNAc_2$, $Man_9GlcNAc$ and the "A" and "C" isomers of $Man_8GlcNAc$.

The small quantities of $Glc_1Man_9GlcNAc_2$ recovered are not unexpected and may be explained in one of two ways. Assays done on purified glucosidase II with a $Glc_2Man_9GlcNAc_2$ substrate have shown that the rate of removal of the first Glc residue to be from 1.5 to 2 fold greater than that of the second (82). As a result, a buildup of α_1-AGP bound $Glc_1Man_9GlcNAc_2$ could be expected. A second possibility is transient reglucosylation of α_1-AGP bound $Man_9GlcNAc_2$. This type of a reaction has been shown to occur in mammalian cells (83).

The homogeneity of the $Man_9GlcNAc$ oligosaccharide isolated indicates that de-glucosylation is an essential step prior to processing by RER α-mannosidases. No evidence could be found for a $Glc_1Man_8GlcNAc$ lacking residues D-2 or D-3. The Man_8 "A" isomer is a result of RER α-mannosidase activity and appears to represent the major processing pathway for oligosaccharides on α_1-AGP. Approximately 85% of all oligosaccharides on α_1-AGP that are processed as far as a $Man_8GlcNAc_2$ in the RER are this isomer. The "C" isomer, while only representing a small percentage of all structures isolated, is a

curiosity. Two possible mechanisms could explain its existence. The first is that it is a result of soluble RER α-mannosidase activity as described by Kornfeld. The second is that it would involve Man_9 mannosidase activity as described by Bause. While both may give a superficial explanation of the origin of this structure it is not clear why the "B" isomer of $Man_8GlcNAc_2$ is also not produced as both of these enzymes are capable of producing this isomer under in vitro assay conditions.

In addition to the sequence information obtained from the NMR spectra, a small amount of spatial detail was also revealed. The most interesting aspect was that of the strong interaction between the D-2, "A" branch and GlcNAc residue 2. No doubt it is this spatial relationship that is responsible for the preferential removal of mannose residue D-2 from $Man_9GlcNAc_2$ by the RER α-mannosidase. Similarly, Kornfeld (25) has shown that the pattern of removal of mannose residues from a $Man_9GlcNAc$ substrate by Golgi mannosidase I results in isomers of Man_8-$Man_6GlcNAc$ that retain mannose residue D-2 (This type of structure is seen in Figures 13 and 22). Upon formation of the $Man_8GlcNAc_2$ "A" isomer the interaction between GlcNAc 2 and residue A is intensified. This manifests itself in the well resolved split resonance pattern seen at 5.10 and 5.07 ppm. Atkinson (56) has proposed that this is a stabilizing interaction that allows for an increased internal rotation about the α1,6 bond of residue B. In the production of yeast mannans, it is believed that this rotation exposes residue D-3 to the mannosyl transferase required for elongation into larger oligosaccharides.

Although the α_1-AGP oligosaccharides isolated from the SER are of uncertain subcellular origin (the SER is a mixture of membranes that includes Golgi), they do provide some valuable information. In addition to $Man_9GlcNAc_2$ and two isomers of $Man_8GlcNAc_2$ the SER α_1-AGP contained three isomers of $Man_7GlcNAc_2$. The major $Man_7GlcNAc$ oligosaccharide isolated lacked residues D-1 and D-2. This indicates that the next step in the processing of the "A" isomer of $Man_8GlcNAc_2$ is the removal of residue D-1. It therefore appears as if structural stabilization in the "A" isomer of $Man_8GlcNAc_2$ which results in rotation about the α1,6 bond of residue B prevents -mannosidase from acting on residue D-3. More simply stated, stabilization of the "A" isomer of $Man_8GlcNAc_2$ results in enhanced α-mannosidase susceptibility of residue D-1 in rat liver or enhanced mannosyl transferase susceptibility of residue D-3 in yeast. It is not clear which mannosidase is responsible for the release of residue D-1 from α_1-AGP. The most likely candidate would be Golgi mannosidase I.

The other two $Man_7GlcNAc$ isomers isolated, while not unexpected, are found in large enough amounts that an explanation of their origin is required. One isomer, that lacking residues D-1 and D-3 is probably a result of Golgi mannosidase I acting on $Man_9GlcNAc_2$ or the "C" isomer of $Man_8GlcNAc_2$ (which was shown to exist in the RER) that have been exported from the RER. As has been indicated earlier, if Golgi mannosidase I is presented a substrate containing residue D-2, it will preferentially release all other α1,2 linked mannose residues. The other isomer lacking residues D-2 and D-3 is more difficult to account for if we assume that it originated from the "A" isomer of $Man_8GlcNAc_2$.

If this were the situation, from previous results we would expect release of residue D-1 and not D-3. It would therefore appear that processing of the "A" isomer of $Man_8GlcNAc_2$ may proceed by the release of either D-1 or D-3, but that D-1 is favored.

Comparison of the relative ratios of oligosaccharides from labelled and unlabelled RER α_1-AGP also provides an interesting insight into glycoprotein processing. In yeast, Atkinson (56) has reported that the initial oligosaccharide transferred to protein is rapidly ($t_{1/2}$= 3 min) trimmed to $Man_8GlcNAc_2$ which slowly ($t_{1/2}$= 10-20 min) undergoes further processing. Studies done by Kornfeld (80) on RER mannosidase activity with high mannose substrates use incubation times up to two hours. Work done by Friesen (51 & 59) in which specific radioactivities of mannose labelled intracellular α_1-AGP from 24 hr inflamed rats were measured, showed that within 30 min of injection levels of label in the RER and SER forms began to drop. Based on these results, the labelling time chosen, 45 min, was thought to be a good compromise. It was believed that this incorporation time would allow for the proper distribution of label within the various oligosaccharide species that are found on intracellular α_1-AGP. In labelling studies it was found that almost 65% of all oligosaccharides isolated from inflamed rat liver RER α_1-AGP are $Man_9GlcNAc$ structures, another 20% are $Man_8GlcNAc$ and some 15% are glucosylated. Spectral integration of the glycopeptide mixture and peak integration of the HPLC elution profile indicates a different distribution. Almost 50% of structures are $Man_8GlcNAc$, 45% are $Man_9GlcNAc$ and 5% are glucosylated. These no doubt represent the true intracellular populations of oligosaccharide structures.

If processing occurred on the time scale as suggested by Atkinson then most of the label should have been found in $Man_8GlcNAc$ oligosaccharides. If processing to a $Man_8GlcNAc_2$ was on a longer time scale, such as the one chosen for the incorporation period, one would expect to find label distributed in oligosaccharides in roughly the same proportions as oligosaccharide species in the NMR spectra. It appears that the $t_{1/2}$ for processing of glucosylated structures to $Man_8GlcNAc_2$ is in the range of 60-120 min. Additional proof of this is seen if we look at the labelled oligosaccharides isolated from SER α_1-AGP. In these samples, no label was found associated with GlcNAc at the reducing terminal of high mannose oligosaccharides.

A comparison between control and inflamed labelled RER α_1-AGP oligosaccharide populations indicates that production of a $Man_8GlcNAc_2$ is a rate limiting step in inflamed rats. Although the recovered oligosaccharide increases approximately three fold between the control and inflamed animals this increase is not uniform among the various oligosaccharide species. In control rats all structures $Man_9GlcNAc$ and larger comprise 65% of oligosaccharides recovered while the remaining 35% are $Man_8GlcNAc$ and smaller. In the inflamed case, this shifts to 79% $Man_9GlcNAc_2$ and larger while the remaining 21% are $Man_8GlcNAc_2$ and smaller. If the conversion of $Man_9GlcNAc_2$ to $Man_8GlcNAc_2$ was not rate limiting, then one would not see a change in oligosaccharide distributions between inflamed and control animals.

The work described in this thesis has established that two major oligosaccharide species are found on RER derived α_1-AGP. These are the archetype $Man_9GlcNAc_2$ and the "A" isomer of $Man_8GlcNAc_2$. This finding

agrees with that of Kornfeld who has characterized an -mannosidase in the RER of rat liver which is capable of releasing a single specific mannose residue. The presence of small quantities of a second $Man_8GlcNAc_2$ isomer suggests the possible existence of a second α-mannosidase in the RER. Other oligosaccharides isolated from SER α_1-AGP indicate that terminal α1,2 linked mannose residues are removed in a sequential fashion consistent with that proposed by Hickmann and Theodorakis (79). In addition to a preferred pathway for the removal of terminal mannose residues a second less favoured pathway exists (See Figure 34). Whether both pathways lead to the same Man_5 structure required by GlcNAc Transferase I is not clear.

A second aspect of the analysis of intracellular oligosaccharides by NMR suggests that the sequential removal of mannose residues is result of changes that occur in oligosaccharide conformation as residues are removed. Therefore, following the release of glucose residues, the conformation of $Man_9GlcNAc_2$ is such that the D-2 residue is preferentially released. It could be argued that this release is catalysed by a highly specific enzyme, the RER mannosidase. However, in light of the observations made by Kornfeld regarding Golgi mannosidase I, it would appear that the RER mannosidase is a recognizes the unique spatial orientation of the D-2, A branch. Once formed, the "A" isomer of $Man_8GlcNAc_2$ assumes a conformation that enhances the rate of release of residue D-1. Similarly the remaining two mannose residues are removed. In the situation where mannose residue D-2 is not removed, it appears that processing proceeds more slowly.

4.3 Proposal for Future Work

Several questions regarding the processing of α_1-AGP remain unanswered. The most interesting question is whether the processing that occurs in the RER is random or domain specific. Rat α_1-AGP is glycosylated on five sequons and the experiments done here made no attempt to determine if a specific structure was associated with a given sequon. If processing is random and both $Man_9GlcNAc_2$ and $Man_8GlcNAc_2$ oligosaccharides are found on all sequons it may be assumed that no interaction of any sort exists between the protein moiety and the ER mannosidase. However, this seems highly unlikely as mature α_1-AGP does contain at least 6 different oligosaccharide structures. If a relationship between domain and structure can be found to exist in the RER then this may in some way dictate the ultimate complex oligosaccharide structure that is seen on the mature protein.

This experiment could be easily performed by isolating intracellular α_1-AGP and subjecting it to limited proteolysis. The peptide fragments could then be dansylated, resolved by reverse phase HPLC and screened for glycosylation on Con-A. Those peptide fragments that were glycosylated could be digested with Endo-H and the resultant oligosaccharides could be reduced with tritiated $NaBH_3$ prior to resolution by HPLC. The assignment of domain could be done by simple amino acid analysis or automated Edman degradation of the glycosylated fragments.

A second experiment is a continuation of the work done in this thesis and would involve the isolation of Man_6 structures from intracellular α_1-AGP. Two Man_6 isomers would be expected, one lacking residues D-1, D-2 and D-3. The other lacking residues D-1, C and D-3

(as seen in Figure 13). If this second isomer could be isolated it would be of interest to see if it was a suitable substrate for GlcNac transferase activities. If it was a substrate, the product of such a reaction, $GlcNAc_1Man_6GlcNAc_2$, may in some way act as a signal to further direct processing of the oligosaccharide chain.

Figure 34. The release of terminal α1,2 linked mannose residues from α_1-AGP.

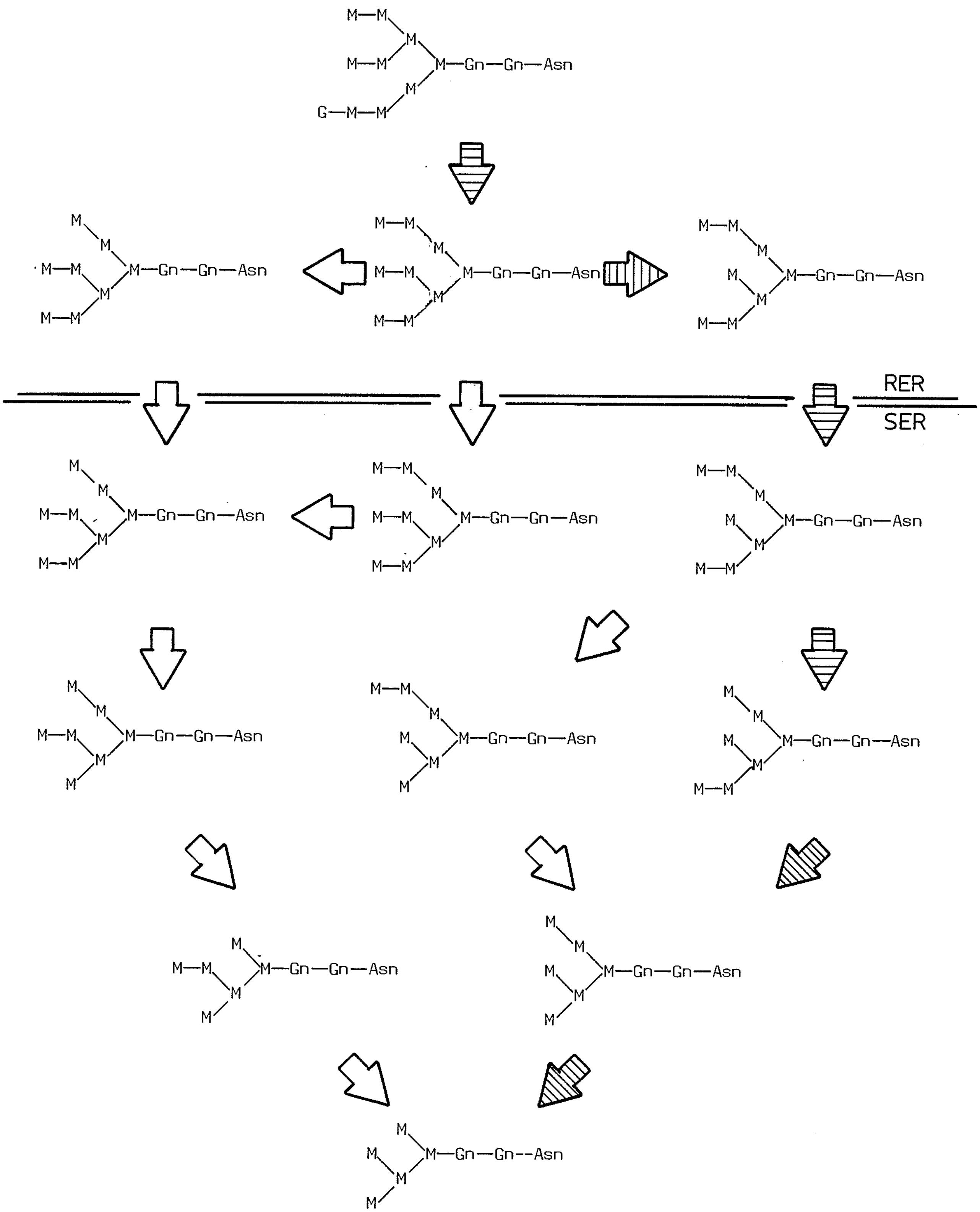
Proposed pathways for the release of terminal mannose residues from α_1-AGP. All structures depicted except Man_6 and Man_5 were isolated from intracellular α_1-AGP. Shaded arrows indicate the central pathway which is followed by at least 50% of all oligosaccharides. This pathway is based on the work on α_1-AGP descibed in this thesis and as a result of structural determinations of oligosaccharides. This same favoured pathway has been reported by one other group using a different experimental approach (79).

M - Mannose

G - Glucose

Gn - N-Acetyl Glucosamine

Asn - Asparagine

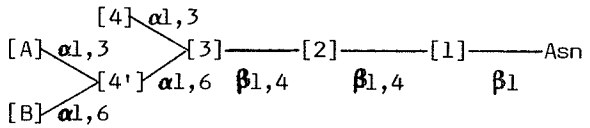

RER
SER

Appendix 1

Figure 35. NMR of $Man_5GlcNAc_2$-Asn glycopeptide standard.[a]

Shift/Integral[c]	Reporter Group[b]	Spectral Information
5.106/2.00	H-1 of Man 4	Unsubstituted residue 4
'' / --	H-1 of Man A	Unsubstituted residue A
5.071/1.00	H-1 of GlcNAc 1	
4.908/1.00	H-1 of Man B	Unsubstituted residue B
4.879/	H-1 of Man 4'	
4.606/	H-1 of Man 2	
4.251/	H-2 of Man 3	
4.140/	H-2 of Man 4'	
4.077/	H-2 of Man 4	
4.072/	H-2 of Man A	
3.984/	H-2 of Man B	
2.064/	Acetate of GlcNAc 2	
2.010/	Acetate of GlcNAc 1	

[a] Kindly provided by J. Carver. [b] See (44) for shift assignment. [c] Integral values are normalized for residue 4 or 4'=1



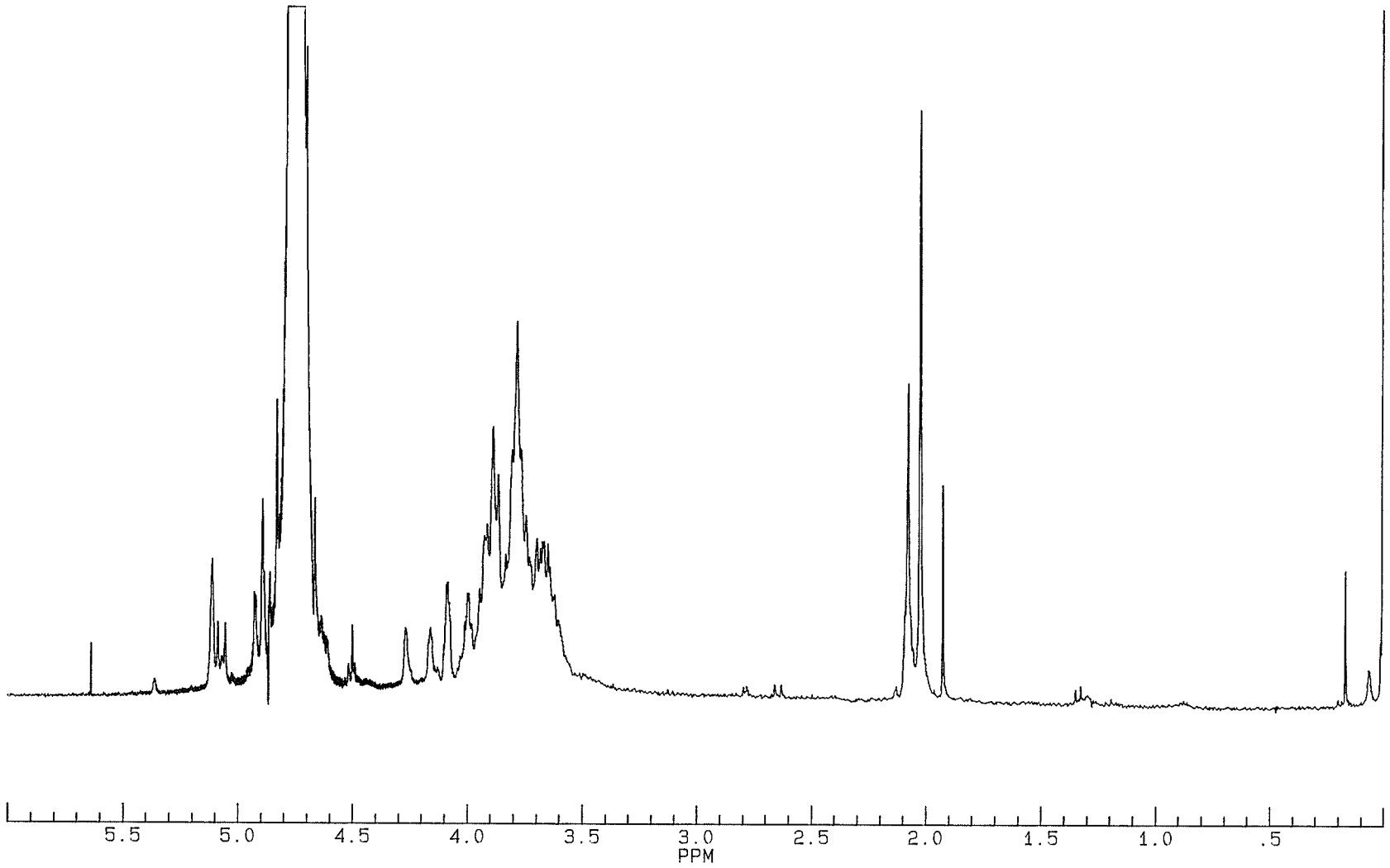
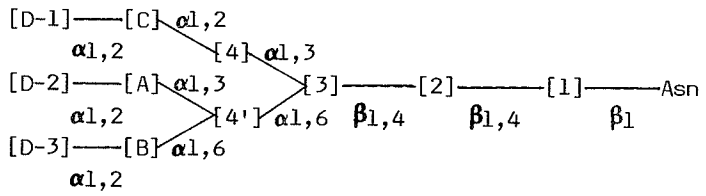

5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
.5
PPM

Figure 36. NMR of $Man_9GlcNAc_2$-Asn glycopeptide standard.[a]

Shift/Integral[b]	Reporter Group[c]	Spectral Information
5.404/1.00	H-1 of Man A	Substituted residue A
5.334/1.00	H-1 of Man 4	Substituted residue 4
5.306/1.00	H-1 of Man C	Substituted residue 4
5.144/1.00	H-1 of Man B	Substituted residue B
5.089/1.00	H-1 of GlcNAc 1	GlcNac β1 linked to Asn
5.063/3.00	H-1 of Man D-2	
5.058/ --	H-1 of Man D-1	
5.046/ --	H-1 of Man D-3	
4.867/	H-1 of Man 4'	
4.225/	H-2 of Man 3	
4.156/	H-2 of Man 4'	
4.102/	H-2 of Man A, C and 4	
4.072/	H-2 of Man D-1 and D-2	
4.067/	H-2 of Man D-3	
4.014/	H-2 of Man B	
2.063/	Acetate of GlcNAc 2	
2.014/	Acetate of GlcNAc 1	

[a] Kindly provided by J. Carver. [b] Integral values are normalized for residue 4 or 4'=1[c]. See (44) for shift assignment.



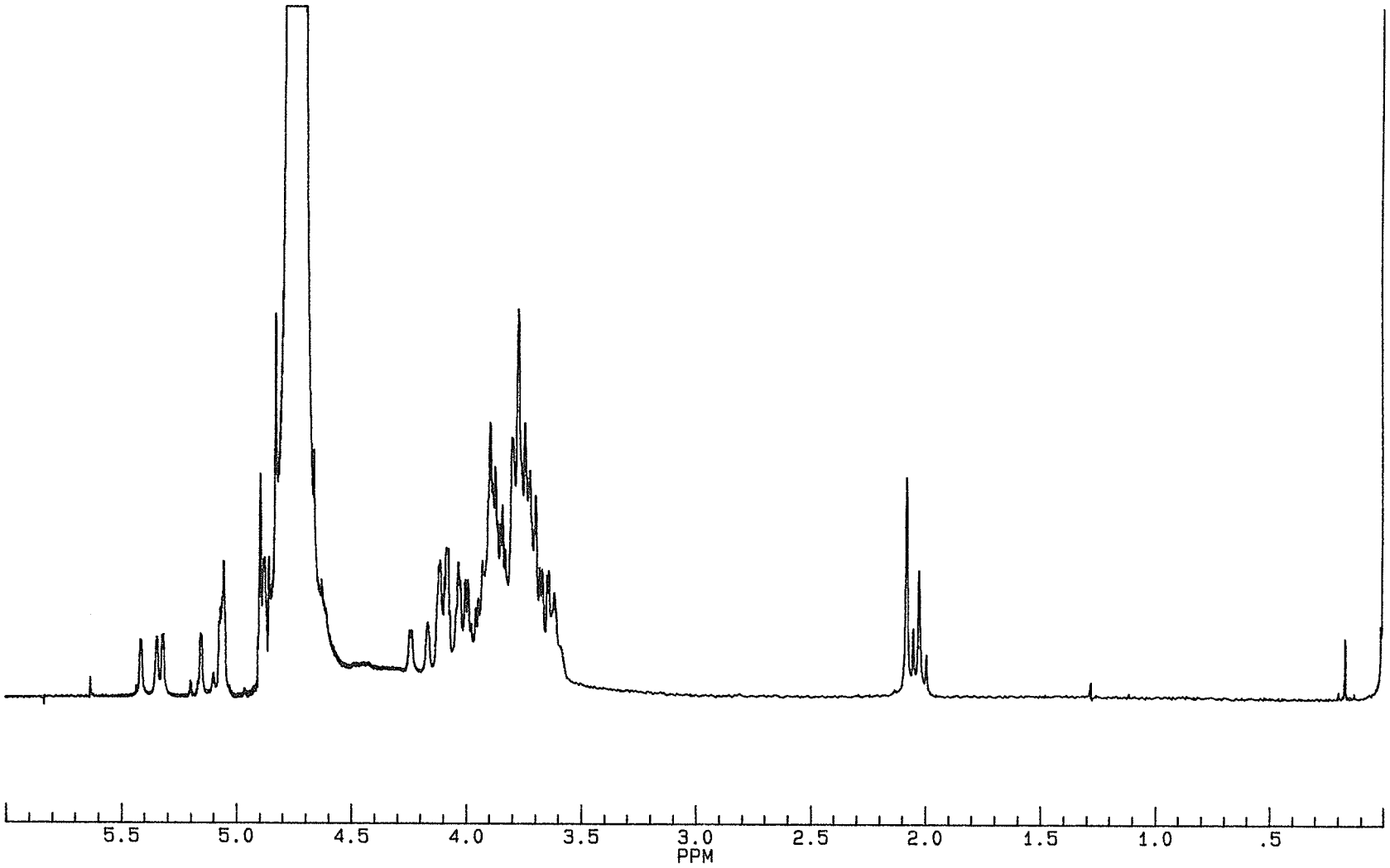
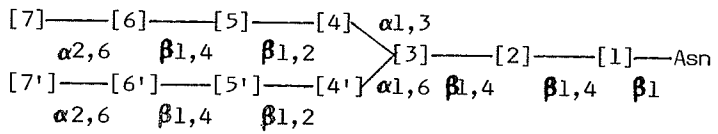
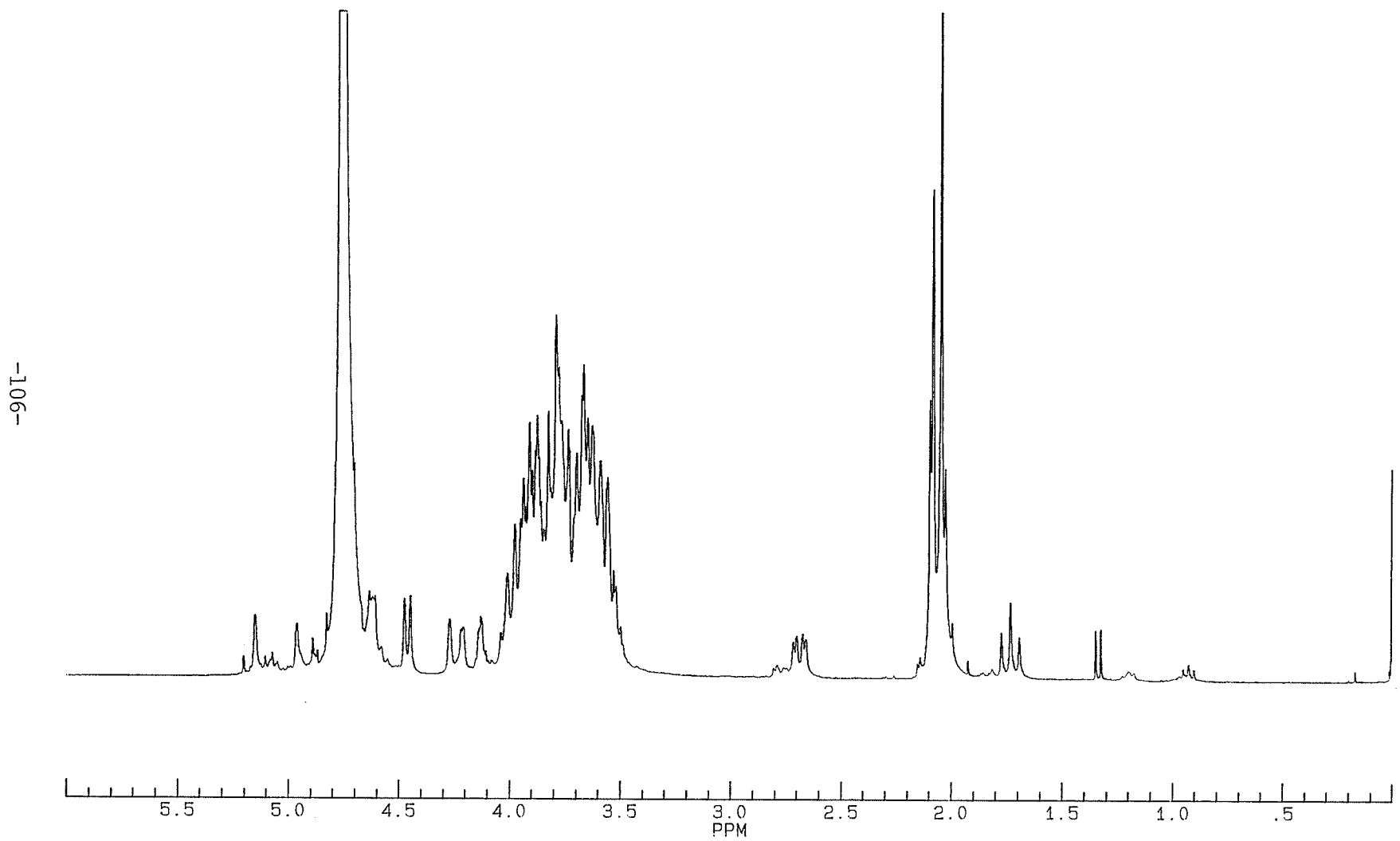

5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
.5
PPM

Figure 37. NMR of complex biantennary glycopeptide standard.[a]

Shift[b]	Reporter Group	Spectral Information
5.133	H-1 of Man 4	GlcNAc substituted mannose
5.088	H-1 of GlcNAc 1	
4.944	H-1 of Man 4'	GlcNAc substituted mannose
4.773[c]	H-1 of Man 3	
4.617	H-1 of GlcNAc 2	
4.597	H-1 of GlcNAc 5 and 5'	GlcNAc β1,2 linkage
4.457	H-1 of Gal 6'	α2,6 substituted galactose
4.431	H-1 of Gal 6	α2,6 substituted galactose
4.253	H-2 of Man 3	
4.200	H-2 of Man 4	
4.112	H-2 of Man 4'	
2.676[d]	H-3 equatorial of SA 7'	α2,6 linked sialic acid
2.661[d]	H-3 equatorial of SA 7	α2,6 linked sialic acid
2.079	Acetate of GlcNAc 2	
2.067	Acetate of GlcNAc 5	Branching terminal GlcNAc
2.065	Acetate of GlcNAc 5'	Branching terminal GlcNAc
2.029	Acetate of SA 7 and 7'	
2.011	Acetate of GlcNAc 1	
1.715[d]	H-3 axial of SA 7 and 7'	α2,6 linked sialic acid

[a] Kindly provided by J. Carver. [b] See reference (44) for shift assignment. [c] Obscured by HOD. [d] Due to spin-spin splitting, the shift value is reported as an average of signals.




-106-
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
.5
PPM

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