

**BIOCHEMICAL STUDIES ON CELL SURFACE
GLYCOPROTEINS OF GLIAL CELLS IN
DEVELOPING HUMAN BRAIN**

A THESIS PRESENTED

by

YASMIN MARIKAR

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CERTIFICATE

I, YASMIN MARIKAR hereby certify that I had personally carried out the work depicted in the thesis entitled "BIOCHEMICAL STUDIES ON CELL SURFACE GLYCOPROTEINS OF GLIAL CELLS IN DEVELOPING HUMAN BRAIN", except where external help sought and acknowledged.

Signature:

YASMIN MARIKAR

Date: 2nd Jan 1991.

Prof. Debkumar Basu

Neurochemistry Division

DECLARATION

This is to certify that Ms. Yasmin Marikar in the division of Neurochemistry this Institute, has fulfilled the requirements of the regulations relating to the nature and prescribed period of research for the Ph.D degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum. The work relating to her thesis entitled "BIOCHEMICAL STUDIES ON CELL SURFACE GLYCOPROTEINS OF GLIAL CELLS IN DEVELOPING HUMAN BRAIN", was carried out under my direct supervision.

Signature

Debkumar Basu

Prof. Debkumar Basu

(Guide)

The thesis
entitled

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IN DEVELOPING HUMAN BRAIN**

Submitted
by

YASHIN HARIKAR

for

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in

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of

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FOR
MEDICAL SCIENCES AND TECHNOLOGY
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evaluated and approved
by

Name of the
(Guide)

(Names of thesis examiners)

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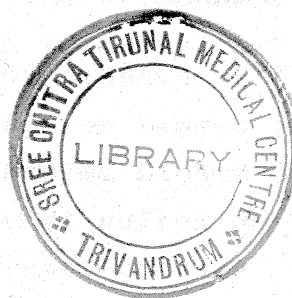
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YASMIN MARIKAR

ABBREVIATIONS

1. AHA	-	<i>Artocarpus hirsuta</i> agglutinin
2. ANSA	-	1,2,4-Amino naphthol sulfonic acid
3. BSA	-	Bovine serum albumin
4. C:M:W	-	Chloroform:Methanol:Water
5. CBB	-	Coomassie Brilliant Blue
6. CNS	-	Central nervous system
7. Con A	-	Concanavalin A
8. CSF	-	Cerebrospinal fluid
9. CTP	-	Cytidine triphosphate
10. DABITC	-	4-N,N'-Dimethyl aminoazobenzene-4'-isothiocyanate
11. DELISA	-	Dot Enzyme Linked Immunosorbant Assay
12. DELLA	-	Dot Enzyme Linked Lectin Analysis
13. DMSO	-	Dimethyl sulfoxide
14. DNAase	-	Deoxyribonuclease
15. Dns-Cl	-	Dansyl chloride (1-dimethylamino-naphthalene-5-sulfonyl chloride)
16. DTT	-	Dithiothreitol
17. EDTA	-	Ethylenediamine tetraacetic acid
18. FITC	-	Fluorescein isothiocyanate
19. Fuc	-	L-Fucose
20. Gal	-	D-Galactose
21. GalNAc	-	N-Acetyl-D-galactosamine
22. GDP	-	Guanosine diphosphate
23. Glc	-	D-Glucose
24. GlcNAc	-	N-Acetyl-D-glucosamine
25. Gn.HCl	-	Guanidine hydrochloride
26. GPI	-	Glycophosphatidyl inositol
27. HRP	-	Horseradish peroxidase
28. IgG	-	Immunoglobulin G
29. JSA	-	Jack fruit seed agglutinin
30. Man	-	D-Mannose

31.	Man-6-P	-	Mannose-6-phosphate
32.	α -MG	-	1-O-methyl α -D-glucopyranoside
33.	2-ME	-	2-mercaptoethanol
34.	M _r	-	Molecular weight
35.	NeuAc	-	N-Acetylneuraminic acid
36.	PBS	-	0.02M Phosphate buffered saline
37.	PITC	-	Phenyl isothiocyanate
38.	PMSF	-	Phenyl methane sulfonyl fluoride
39.	PNA	-	Peanut agglutinin
40.	POPOP	-	1,4-Bis-2-(5-phenyloxazolyl) benzene
41.	PPO	-	2,5-Diphenyloxazole
42.	PSA	-	<u>Pisum sativum</u> agglutinin
43.	RCA	-	<u>Ricinus cummunis</u> agglutinin
44.	RER	-	Rough endoplasmic reticulum
45.	SBA	-	Soybean agglutinin
46.	SDS	-	Sodium dodecyl (lauryl) sulfate
47.	TBS	-	0.02M Tris buffered saline
48.	TEA	-	Triethylamine
49.	TEMED	-	N,N,N',N'-Tetramethylenediamine
50.	TFA	-	Trifluoroacetic acid
51.	TFMS	-	Trifluoromethane sulfonic acid
52.	TMS	-	0.02M Tris maleate buffered saline
53.	UDP	-	Uridine diphosphate
54.	WELLA	-	Western Enzyme Linked Lectin Analysis
55.	WGA	-	Wheat germ agglutinin



CHAPTER - I

GENERAL INTRODUCTION

INTRODUCTION

Tissue morphogenesis is governed by a series of epigenetic sequences acting synergistically and comprised of the primary processes - cell division, cell movement, cell adhesion, cell interaction, cell differentiation and cell death (79,68). These phenomena contained within the broad term 'cell-sociology' are requisites for the normal development, growth and maintenance of cells. The molecular basis of various aspects of cell-sociology is still largely obscure. Analyses at the molecular level of biological phenomena such as recognition, adhesion and interaction between cells as well as cells and extracellular matrix components is imperative towards understanding how organised tissues arise from a mass of undifferentiated cells. Besides, some insight would be gained about the causes and factors determining cell pathology, which basically is a manifestation of a deranged cell-sociology.

The nervous system by virtue of its unique anatomical diversity, architecture and functional complexity exemplifies cell-sociology in its broadest most evolved form. Development of the mammalian nervous system would therefore be one of the most sophisticated examples of tissue morphogenesis in that it involves both the formation of intricate tissue structures and the precise establishment of neuronal pathways and interconnections. All neural tissue, primarily, comprise of two types of cells - the neurons, involved with the generation and conduction

of electrical impulses, synaptic transmission and the establishment of specific connections; and glial cells, which form the supportive connective tissue network to maintain the neurons. As our objective was to study the molecular basis of glial cell development and function in human central nervous system, the following discussion, though applicable in a general context, is of particular relevance to the central nervous system and more specifically, the neuroglia.

Cellular membranes in general and the surface membrane in particular, which serves as a link between the cell and its microenvironment are critical components in the development and maintenance of the structural and functional integrity of eukaryotic systems (253). According to the fluid mosaic model proposed by Singer and Nicolson, membranes are comprised of phospholipids and glycolipids arranged asymmetrically to form a bilayer configuration, with proteins and glycoproteins intercalated into this lipid bilayer (308). The distinct topological orientation of carbohydrates, facing the inside of intracellular membranes/organelles but placed on the outer leaflet of plasma membranes facing the external environment, is a manifestation of the biosynthetic, sorting and transport mechanisms determining the final destination of these components.

Glycoconjugates are quantitatively major components of plasma and internal membranes, where they undoubtedly contribute to the structural properties of such membranes. In addition,

these biomers have been implicated in many cellular responses and as receptors for a multitude of signal and regulatory molecules. Glycoconjugates may be glycolipids, proteoglycans or glycoproteins.

Glycolipids contain carbohydrates conjugated to ceramide, the lipid component. Addition of a single sugar to ceramide render glucocerebrosides and galactocerebrosides. Myelin is comprised of predominantly galactocerebrosides and their sulfate esters, termed sulfatides. The commonly occurring glycosphingolipids are classified into (a) acidic or neuraminic acid containing gangliosides and (b) neutral glycosphingolipids containing mono-, di-, or oligoglycosyl chains attached to ceramide. Oligoglycosyl ceramides are further divided into the blood group glycolipids containing N-acetylated lactosyl ceramide core structure and the globoside series containing no amino sugars. Much of the glycolipid carbohydrate, at least in normal cell surfaces, appeared unavailable for reaction with antisera, enzymes such as sialidase and lectins. It was thus inferred that these glycolipids may be cryptic, possibly concealed by the ramified glycoprotein molecules (112). Gangliosides are normal components of mammalian cell plasma membranes and are particularly abundant in nervous tissue. Developmental studies in humans have revealed that the prenatal period of 'growth spurt' occurring between 15 and 30 weeks of gestation is paralleled by

an increase in the content of gangliosides and a change in their composition (184).

Proteoglycans are a diverse group of proteins containing glycosaminoglycan side chains. Glycosaminoglycans are high molecular weight linear polysaccharides, generally composed of disaccharide repeating units of a uronic acid [D-glucuronic acid or L-iduronic acid] and a hexosamine [N-acetylglucosamine or N-acetylgalactosamine]. The heterogeneity of these molecules is due to different types of repeating disaccharide units, their modification by sulfation and epimerisation of specific sugar residues, and their occurrence on a variety of different core proteins. Normally very little glycosaminoglycan is present in the brain [0.02% of brain wet weight in humans]. Most of the chondroitin sulfate and hyaluronic acid in brain is present in a soluble form, whereas heparan sulfate largely appears to be an integral component of plasma membranes. Glycosaminoglycans, in particular, hyaluronic acid, are probably important constituents of the extracellular matrix in nervous tissue, where they could be expected to play a role in the regulation of brain cell microenvironment (216).

Existence of clinically and biochemically well characterised lysosomal storage disorders, sphingolipidoses and mucopolysaccharidoses and their related neurological manifestations have boosted glycolipid and glycosaminoglycan research to a great extent. Glycoproteins, on the other hand, have been least studied

amongst the nervous system glycoconjugates. Approximately 90% of brain glycoproteins are integral components of the cell surface and internal membranes. Purification of this species to homogeneity, a prelude to biochemical characterisation has proven difficult. However, in the past few years, it has become increasingly apparent that glycoproteins are involved in various aspects of cell sociology, as well as in a multitude of disease processes, the most notable being malignant transformation.

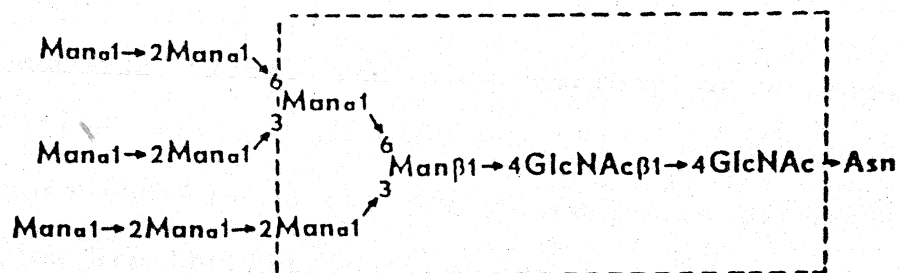
Structure of Glycoproteins

Glycoproteins differ from glycosaminoglycans in that (a) they do not usually contain uronic acid (b) they lack a serially repeating unit (c) they contain a relatively low number of sugar residues in the heterosaccharide, which is often branched and (d) they contain several sugars that are not characteristic components of glycosaminoglycans, e.g. fucose, sialic acid, galactose and mannose. Depending on the mode of linkage of the glycan side chain to the polypeptide backbone, glycoproteins may be O-linked or N-linked.

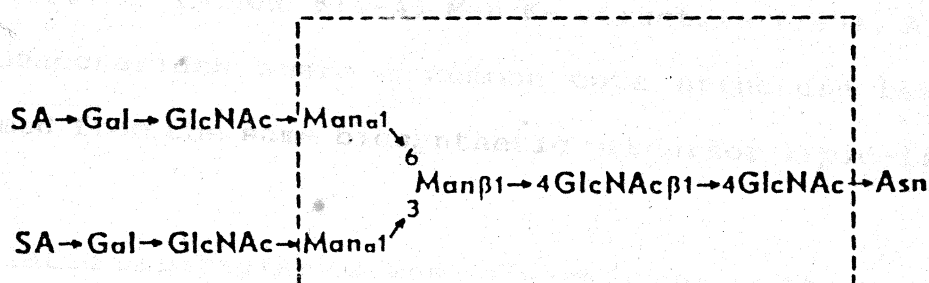
Mucins, blood group glycoproteins and certain serum and membrane glycoproteins contain oligosaccharide units α -O-linked via GalNAc to the hydroxyl groups of serine and/or threonine of the protein moiety. The GalNAc is directly substituted at C-6 by sialic acid in submaxillary mucins or it may be substituted at C-3 or C-4 by a β -galactosyl residue which serves as the core region of a variety of larger oligosaccharide chains. Collagens

and basement membranes contain another type of glycoprotein linkage structure consisting of Gal and Glc β (1-2)Gal disaccharides linked O-glycosidically to the hydroxyl group of hydroxylysine in the peptide (180). Glycosaminoglycans are linked to protein in proteoglycans through the typical Xyl-serine linkage (277). A novel GlcNAc-Ser/Thr linkage has recently been identified on many proteins localized in the nucleoplasmic and to a lesser extent, the cytoplasmic compartments of the cell (136).

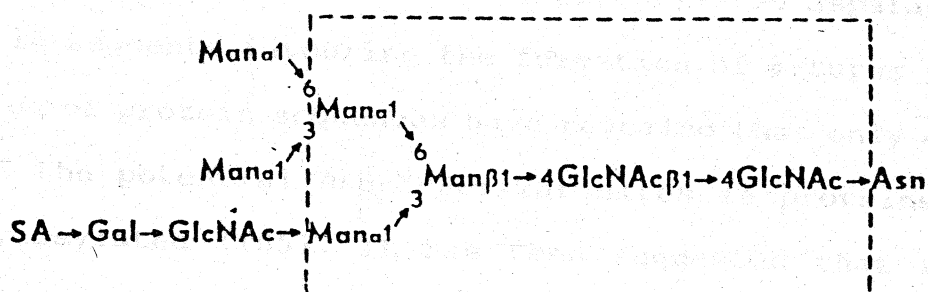
Carbohydrate chains may be β -N-linked through GlcNAc to the amide nitrogen of asparagine residues in the protein moiety. All Asn-linked oligosaccharides share a common pentasaccharide core structure enclosed within the boxed area in Fig.1. Based on the structures of their outer branches, N-linked glycans are categorised as high mannose, complex and hybrid types. The high mannose-type oligosaccharides have 2-6 additional mannose residues linked to the (Man)₃(GlcNAc)₂ core. Complex-type structure contains the sialyl lactosamine sequence and presents two other commonly found substituents, namely, a fucose α (1-6) linkage to the innermost GlcNAc residue in the core and a 'bisecting' GlcNAc linked β (1-4) to the β -linked mannose residue. This complex-type structure may be modified both by the addition of extra branches on the α -mannose residues or by the addition of extra sugar residues that elongate the outer chains. The majority of complex-type chains contain two, three or four outer branches or antennae, but units with five outer branches have been found



I. HIGH-MANNOSE TYPE



II. COMPLEX TYPE



III. HYBRID TYPE

Fig.1.

Structures of the major classes of asparagine-linked oligosaccharides. The box delineated by the broken line indicates the common pentasaccharide core structure.

Adapted from Elbein, A.D. (1984) CRC Crit. Rev. Biochem. 16, 21-49.

on avian ovomucoids (256). The wide variety of complex-type glycans identified, contain GlcNAc, Man, Gal, NeuAc and Fuc as the basic monosaccharide units. The third class of Asn-linked oligosaccharides are the hybrid-types which specify features of both high-mannose and complex-type glycans. Most hybrid molecules have the 'bisecting' GlcNAc $\beta(1-4)$ Man β - structure (345). All N-linked oligosaccharides share a common core structure because they are formed from the same biosynthetic precursor lipid-linked glycan (181).

Glycosylated asparagines almost always occur in the sequence Asn-X-Ser/Thr where X could be any amino acid except possibly proline and aspartic acid (219). Most N-glycosylated asparagines are located in segments favouring the formation of β -turns (23). An examination of protein sequences have revealed that only about one third of the potential Asn-X-Ser/Thr sites in proteins are actually glycosylated (185). It has been suggested that rapid folding of the nascent polypeptide is responsible for the lack of glycan chains at potential glycosylation sites. Since glycosylation occurs co-translationally, once the protein has folded, potential glycosylation sites are no longer accessible to the oligosaccharyl transferases (259). Amino acid sequences on proteins serving as potential sites for O-glycosylation have not been identified.

Glycoprotein Biosynthesis

The polypeptide backbones of all glycoproteins, lysosomal, secretory or plasma membrane, are synthesized on membrane bound polyribosomes of the rough endoplasmic reticulum [RER] by the same machinery that assembles non-glycosylated proteins on free ribosomes. Each of these proteins contain a hydrophobic amino-terminal signal peptide that interacts with the signal recognition particle, an 11S ribonucleoprotein, thereby initiating vectorial transport of the protein across the RER membrane into the lumen of the organelle (348). During synthesis the proteins are extruded into the lumen of the RER where they remain membrane bound [membrane glycoproteins] or become soluble [secreted glycoproteins] depending on their ultimate destination. The attachment of the carbohydrate side chain commences during translation, although some residues are incorporated post-translationally. The glycoproteins are glycosylated and processed as they traverse various functional regions of the endoplasmic reticulum, the cis, medial and trans Golgi system to the last Golgi compartment, the trans Golgi network. Movement of glycoproteins through the endomembrane chain is a dissociative process most probably mediated by vesicles budding from the rims of the donor ER or Golgi membranes and moving to and fusing with the target cisternae. Stepwise synthesis of both N-linked and O-linked oligosaccharides are carried out by different mechanisms on the luminal side of the endomembrane system by specific

glycosyltransferases. Each of these enzymes catalyses the precise and sequential transfer of a sugar residue from an activated donor substrate to the hydroxyl group of an acceptor substrate on the growing oligosaccharide chain (26). Donor substrates are usually nucleotide sugar derivatives, although lipid-linked sugars dolicholphosphoryl mannose [Dol-P-Man] and dolicholphosphoryl glucose [Dol-P-Glc] also serve as donor substrates for several enzymes. Glycosyltransferases are conveniently classified according to the sugar transferred.

The biosynthesis of Asn-linked glycans is a multistep process, involving (a) assembly of a lipid-linked oligosaccharide intermediate (b) transfer of the oligosaccharide from the lipid carrier to the growing peptide chain and (c) processing of the oligosaccharide bound to protein.

Synthesis of the lipid-linked oligosaccharide entails sequential addition of sugars to the lipid membrane anchor, dolichol phosphate [Dol-P], a phosphorylated polyprenol containing 16-22 isoprene units, with a saturated α -isoprenoid unit, by membrane bound glycosyltransferases of the RER (24). The dolichol phosphate cycle results in the formation of an oligosaccharide consisting of $\text{Glc}_3\text{Man}_5\text{GlcNAc}_2$ linked to dolichol via a pyrophosphate as illustrated in Fig.2.

Monophosphoryl dolichol is derived from dolichol pyrophosphate by hydrolysis [Step 7] or from phosphorylation of dolichol by CTP [Step 8]. Synthesis begins with the reversible

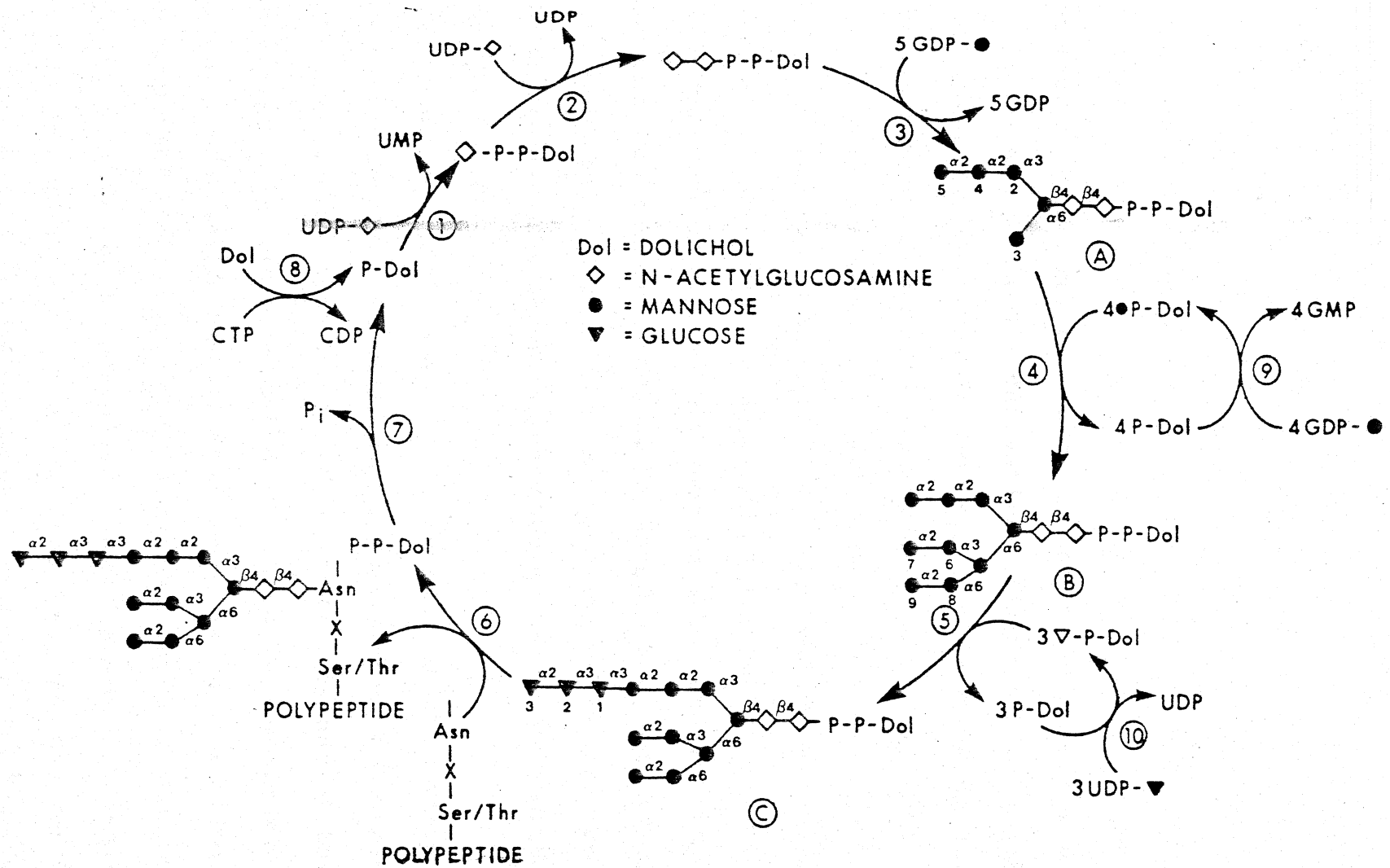


Fig.2. The dolichol phosphate [Dol-P] cycle.

transfer of a GlcNAc moiety from UDP-GlcNAc to Dol-P [Step 1] followed by the irreversible transfer of a second GlcNAc residue from UDP-GlcNAc to give GlcNAc₂-P-P-Dol [Step 2]. The first five Man residues are added directly from GDP-Man [Step 3]. Addition of the last four Man residues and all three Glc residues proceed via the corresponding Dol-P derivatives [Step 4 & 5], formed from GDP-Man [Step 9] and UDP-Glc [Step 10] respectively. The Man residues are added sequentially in order 1 to 5 [Step 3] and 6 to 9 [Step 4] as indicated. Similarly the Glc moieties are added sequentially in the order 1 to 3. The precursor Glc₃Man₉GlcNAc₂ is transferred en bloc from the lipid donor to available asparagine sites on the nascent polypeptide during its vectorial transport across the membrane of the rough endoplasmic reticulum. Co-translational glycosylation, however, is not obligatory. Presence of Glc in the lipid-linked oligosaccharide serves as a signal for its en-bloc transfer to protein.

Processing is initiated by the removal of the terminal Glc residue by a specific glucosidase I. The two inner Glc residues are then removed by glucosidase II to render glycans of the high mannose-type. The glucosidases are located in the membranes of the rough endoplasmic reticulum as is a specific α -mannosidase which catalyses the removal of at least one $\alpha(1-2)$ linked mannose residue. It has been postulated that glucose trimming is necessary for efficient movement from the RER to the Golgi, possibly because the deglycosylated oligosaccharides form part of

a recognition site for a transport receptor for certain secretory proteins (205). An alternative explanation for these findings was that glucose removal is necessary for the maturation of glycoproteins to a correct functional conformation (297). When the glycoproteins arrive at the Golgi, they traverse the stack from the cis through medial to trans cisternae by vesicular transport (25,282,281). Lysosomal enzymes undergo a highly specific mannose phosphorylation in the cis Golgi. The high mannose oligosaccharides on non-lysosomal glycoproteins are further trimmed by mannosidase I to yield $\text{Man}_5\text{GlcNAc}_2$ structure in either the cis or medial Golgi cisternae. Those oligosaccharide chains destined to become complex-type structures are further processed by addition of a GlcNAc residue catalysed by GlcNAc transferase I. The resulting intermediate forms the basis of the hybrid structure types which are stabilized by addition of another GlcNAc moiety to the core β -linked Man to give a bisected structure. Further processing involves removal of two mannose residues by Golgi α -mannosidase II, the subsequent addition of another outer chain GlcNAc residue by GlcNAc transferase II and the possible transfer of a fucose residue to the innermost GlcNAc in the core. The final steps of complex oligosaccharide synthesis take place in the trans Golgi cisternae and consist of addition of outer chain galactose, sialic acid and other terminal sugar residues catalysed by their respective transferases. The newly synthesised glycoproteins are sorted and packed into secretory,

lysosomal or membrane bound vesicles in the trans Golgi network and transported to their respective final destinations where they eventually transfer their contents to the appropriate preexisting cellular organelle by membrane fusion or exocytosis.

Exactly how these membrane vesicles recognize the appropriate membrane partner is enigmatic. It has been proposed that carbohydrate acts as a chemical 'tag' which upon interaction with a specific intracellular membrane receptor direct glycoproteins to specific cellular organelles following synthesis on the rough endoplasmic membrane. Final targeting is lectin mediated at least for lysosomal enzymes, but a similar mechanism has been suggested for other glycoproteins as well (182). During processing of lysosomal enzymes in the cis Golgi membrane one or more mannose residues in the high mannose chains are phosphorylated to generate the Man-6-P recognition marker. These markers are recognised by Man-6-P specific lectins localised on lysosomal membranes. Removal of phosphate in the highly acidic prelysosomal compartments may be a mechanism for permanently restricting the enzymes within the lysosome (166). Vesicles containing membrane bound glycoproteins fuse with the inner surface of the plasma membrane transferring the structural protein into the cell surface. Carbohydrate residues originally intra cisternal in the Golgi complex are thus externalised. Secretory vesicles are believed to migrate from the Golgi to the plasma membrane to

not been fully understood. The specific localization of the

disgorge their contents by reverse pinocytosis after fusion with the plasma membrane (320).

The biosynthesis of O-linked oligosaccharides does not involve lipid intermediates and occurs almost entirely by sequential glycosylation, where the product of one glycosyl transferase is utilised as an acceptor substrate by another glycosyltransferase. Initiation of O-glycosylation is catalysed by the enzyme UDP-GalNAc:polypeptide transferase localised predominantly in the Golgi membranes. Endogenous peptide acceptors for this transferase are essentially confined to the RER signifying that initiation of O-glycosylation is primarily post-translational. Synthesis commences with the transfer of GalNAc to the hydroxyl group of serine or threonine on the polypeptide acceptor. Transfer of Gal to form Gal $\beta(1-3)$ GalNAc directs the pathway towards the synthesis of a wide variety of chains containing Fuc, NeuAc, GlcNAc or Gal as the constituent monomers. Chain termination occurs in the Golgi by addition of fucose or sialic acid prior to packing for either secretion or incorporation into the plasma membrane. The diversity of O-linked glycans though impressive, is not disorganised suggesting the existence of a mechanism regulating the synthesis and destination of specific carbohydrate structures (284).

Biosynthetic controls that determine branching and microheterogeneity of protein bound oligosaccharides has still not been fully understood. The specific localisation of the

processing enzymes to the RER or the cis, medial or trans Golgi cisternae and their rigid substrate specificities, provides a mechanism for controlling their sequential action on newly synthesised glycoproteins in both space and time. The large number of interacting metabolic pathways traversed by a sugar residue en route to the glycosylation of a protein also furnish many potential sites for regulation. Factors that could affect glycosylation include the rate of synthesis of nucleotide sugar donors, the intracellular transport of donor substrates, the accessibility and structure of acceptor substrates, the concentration of inhibitors such as the nucleotide products of glycosyltransferase reactions, competition with other glycosyl transferases of overlapping specificities and depletion of substrates by degradative enzymes such as pyrophosphorylases or glycosidases. Genetic factors such as species and tissue specific differences, genetic polymorphism within a species, differential gene expression within an individual and endomembrane factors due to transport through the complex endomembrane assembly line complicate the issue further (294). Finally, signals transmitted by cell surface macromolecules may trigger glycoprotein biosynthesis as is obvious during development and differentiation (293).

Elucidation of the glycoprotein biosynthetic pathway has been accomplished primarily with the help of glycosylation deficient mutants and the use of inhibitors that block these

pathways at specific steps. The resulting accumulation of intermediates prior to the block and/or the formation of altered or unusual glycoproteins are in addition, useful tools to investigate the biological functions of the carbohydrate side chains of glycoproteins. Inhibitors of cholesterol biosynthesis such as compactin can also block the formation of dolichol, thereby limiting the levels of carrier lipid. The nucleoside antibiotic, Tunicamycin has been shown to inhibit the first reaction in the lipid-linked saccharide pathway, namely, the transfer of GlcNAc-1-P from UDP-GlcNAc to Dol-1-P to form GlcNAc-P-P-Dol, and is widely used to block N-glycosylation in vivo and in vitro. Inhibitors of glycan processing, such as Swainsonine, a potent inhibitor of α -mannosidase II and the plant alkaloid Castanospermine, which inhibits glucosidase I, diminished considerably the synthesis of complex type chains in various mammalian cell lines (73).

Post-translational modifications on oligosaccharide chains

Asn-linked oligosaccharides may undergo further post-translational modifications including phosphorylation of mannose residues, sulfation of mannose and N-acetyl hexosamine residues and O-acetylation of sialic acid residues. Lysosomal hydrolases acquire phosphomannosyl moieties which serve as the essential component of a recognition marker that allows binding to a specific Man-6-P receptor in the Golgi and subsequent translocation to lysosomes (50). Different mannose residues on

the glycan chain may be phosphorylated and individual molecules can contain one or more phosphates, however, oligosaccharides with phosphomonoesters are the physiologic ligands for binding to the receptor (345). Fibroblasts from patients with lysosomal storage diseases, I-cell disease [mucopolidosis II] and pseudo Hurler polydystrophy [mucopolidosis III] are severely deficient in N-acetyl glucosaminyl phosphotransferase activity (50,114).

Another recognition system involving Man-6-P has been detected in embryonic chick neural retina, in a family of glycoproteins that bind to the cell surface baseplate protein, ligatin (159). These glycoproteins contain high mannose oligosaccharides with the Man-6-P residues in diester linkage to the 1-carbon of terminal glucose residues (213). The binding of ligatin is mediated by the diester rather than the monoester as in the case of the lysosomal Man-6-P receptor. This diester is synthesized in a fashion analogous to the transfer of GlcNAc-P to mannose residues of lysosomal enzymes (183).

A variety of secretory and membrane glycoproteins are known to contain sulfate residues on their N-linked oligosaccharides. Several pituitary glycoproteins including bovine lutropin [LH] and thyroid stimulating hormone [TSH] specify, on their α -subunits, biantennary complex-type oligosaccharides with 4-O-sulfated GlcNAc and GalNAc residues at the two nonreducing termini (254,22,8). By contrast, placental human chorionic gonadotropin [hCG] which shares the common α -subunit, lacks

sulfate and has terminal sialic acid residues, indicating that the sulfation reaction can be tissue specific (226,170).

Anionic modification of N-linked oligosaccharides, such as sialylation, phosphorylation and sulfation are of interest because it is likely that these signals, and not the underlying oligosaccharides, confer biological specificity or function to some N-linked carbohydrates.

Nervous system specific carbohydrate structures

Approximately 85-90% of the carbohydrate in brain glycoproteins are N-glycosidically linked, the remainder being found as O-linked glycans. Most of the N-linked oligosaccharides in nervous tissue glycoproteins are of the complex-type (216). An unusual polysialosyl sequence $[\text{NeuAc } \alpha(2-8)]_n \text{ NeuAc } \alpha(2-3) \text{ Gal}$ has been found in the complex glycopeptides of developing rat brain by Finne (82). These unique structures were absent in adult brain. The number of sialic acid residues varied from 8-12 in glycopeptides having 3-4 outer branches. Polysialosyl glycopeptides have also been isolated from a neural tumour cell line (217). The polysialosyl chains have been identified during the period characterised by the outgrowth of axons and dendrites and the formation of synapses. Recent observations have implied the occurrence of similar developmentally regulated polysialosyl multiantennary complex-type glycoproteins in human brain too.

The O-linked glycans of neural tissue comprise of the core disaccharide $\text{Gal } \beta(1-3) \text{ GalNAc-}$, which occurs as such or is

substituted with sialic acid at C-3 of Gal and/or C-6 of GalNAc. The α -anomer of the core disaccharide has also been detected in rat brain O-glycans and appears to be specific to nervous tissue (84).

Chondroitin sulfate proteoglycan purified from rat brain contains sulfated N- and O-glycosidically linked glycoprotein oligosaccharides. In addition, this proteoglycan specifies a series of novel O-linked mannose oligosaccharides (83). This type of carbohydrate-protein linkage has subsequently been described for keratan sulfate side chains present as minor components of the predominantly chondroitin sulfate proteoglycan of brain (187).

Microheterogeneity

Different molecules of the same glycoprotein may differ in the size and composition of their carbohydrate units, most commonly in sialic acid or fucose distribution. Microheterogeneity represents that variation in carbohydrate groups of glycoproteins produced by partial substitution of sugar residues on a basically similar core structure. For example, the single carbohydrate unit of ovalbumin is not identical in all the molecules of a single preparation (227). Microheterogeneity is believed to be an outcome of the mechanism of biosynthesis of the carbohydrate units, which are secondary gene products and are attached to the nascent polypeptide chain by a co-translational modification. This is a non-template mechanism of information

transfer and hence is subject to a higher incidence of error than a template mechanism. It is evident that the factors regulating glycoprotein biosynthesis play key roles in determining the range and extent of heterogeneity encountered, and the variations in oligosaccharide structure that occur. The chain terminating mechanism may also play a role in the phenomena of heterogeneity, albeit the lack of knowledge of this mechanism. The paucidisperse homologues and microheterogeneity observed in many glycoproteins may represent oligosaccharides in varying stages of completion or resulting from transglycosylations that lack an absolute specificity. Although most cells have the capacity to process protein bound structures ranging from simple high mannose units to extended complex-type units and can produce such an array at any particular asparagine that is glycosylated, this kind of gross heterogeneity is not common. Rather, individual glycosylation sites tend to have characteristic oligosaccharides and when heterogeneity is encountered, it usually consists of a family of closely related glycans which differ from the oligosaccharides at another site on the same protein (4).

As a result of microheterogeneity, it is very often difficult to elucidate the structure of carbohydrate chains on glycoproteins. The use of immobilized lectins have facilitated the isolation and purification of such molecules and helped in coping to a great extent with the problem of microheterogeneity.

Lectins are a group of di- or polyvalent proteins of nonimmune origin that bind carbohydrate specifically and non-covalently, and cause the agglutination of cells or precipitation of glycoconjugates. Lectins are widely distributed in plant and animal systems, as soluble and membrane bound forms. Although lectin binding can be reversed by simple sugar inhibitors specific for each lectin, it has been evidenced that lectin interaction is governed by a more complex sequence of sugars as well as the conformation of sugars in the carbohydrate side chain. This aspect of lectin-carbohydrate interaction forms the basis of the use of lectins as tools in biochemical and clinical studies (192).

Catabolism of glycoproteins

Lysosomes are principal sites for the digestion of biosynthetic material. The numerous hydrolytic enzymes present in these organelles usually exert their actions within the cell, in the vacuolar system of the cytoplasm, either on exogenous material engulfed by endocytosis or on endogenous cell constituents segregated by autophagy. Nevertheless, lysosomal enzymes can also be excreted by exocytosis to be active outside the cell in the degradation of some extracellular components. The lysosomes contain a highly acidic environment, optimal for the activities of the numerous acid hydrolases associated with this organelle. Integrity of the membrane shield surrounding the lysosomal particles is essential for the latency of the enzymes.

An injury to the membrane by known abnormal situations can liberate the contained enzymes and lead to the digestion of the cell itself (56).

The products of intralysosomal digestion, if sufficiently small, either diffuse or are transported across the lysosomal membrane to enter cellular metabolism. The non-digestible products accumulate in the lysosome, and cause pathological overloading of these organelles, since most cells appear unable to discharge the residue of their lysosomal digestion. This phenomena is accelerated in various storage disorders characterised by the congenital lack of one or more of the lysosomal hydrolases. The observation that lysosomal storage diseases are characterised by the accumulation of abnormal glycolipids, glycosaminoglycans and glycoproteins presenting a common terminal residue whose catabolic pathway is blocked, indicates that glycoconjugates share common enzymes in their respective degradative pathways (341).

Lysosomes carry out digestion of biological material through a large collection of hydrolytic enzymes acting on most of the bonds found in proteins, nucleic acids, lipids, oligosaccharides and polysaccharides. The stepwise hydrolysis of glycoproteins by lysosomal hydrolases is schematically represented in Fig.3. Catabolism of glycoproteins is initiated by the action of either proteolytic enzymes or glycosidases. Proteins are digested through the synergistic action of a series of exo and endo

peptidases that release peptides and amino acids. The cathepsins A, B, C, and D are important amongst the peptidases of lysosomes. The cathepsins carry out extensive hydrolysis of proteins at acid pH, including the peptide backbone of glycoproteins (120).

Glycosidases, implicated in the catabolism of oligosaccharides and the carbohydrate chains of glycoconjugates are of two types - endoglycosidases acting within the main chain, and exoglycosidases mediating the stepwise attack at the nonreducing end groups of the polymorphic carbohydrate structures. Complete degradation of glycoprotein requires as many glycosidases as there are distinct glycosidic bonds in the molecule, an unknown number of proteolytic enzymes and a specific enzyme cleaving the carbohydrate to protein linkage. Lysosomes contain enzymes that cleave practically every type of glycosidic linkage found in glycoproteins. The carbohydrate portion of the glycoprotein is split off sequentially from the nonreducing end by exoglycosidases, the absence of a particular exoglycosidase preventing the action of the enzyme next in line. The first step in the degradation of the glycan side chains involves the removal of terminal sialic acid by lysosomal neuraminidase. Desialylated glycoproteins are then sequentially acted upon by β -galactosidases, N-acetyl- β -hexosaminidases, α -mannosidases and α -L-fucosidases.

The carbohydrate-peptide linkage in mucin-type glycans are cleaved by the lysosomal N-acetyl- α -galactosaminidase (321).

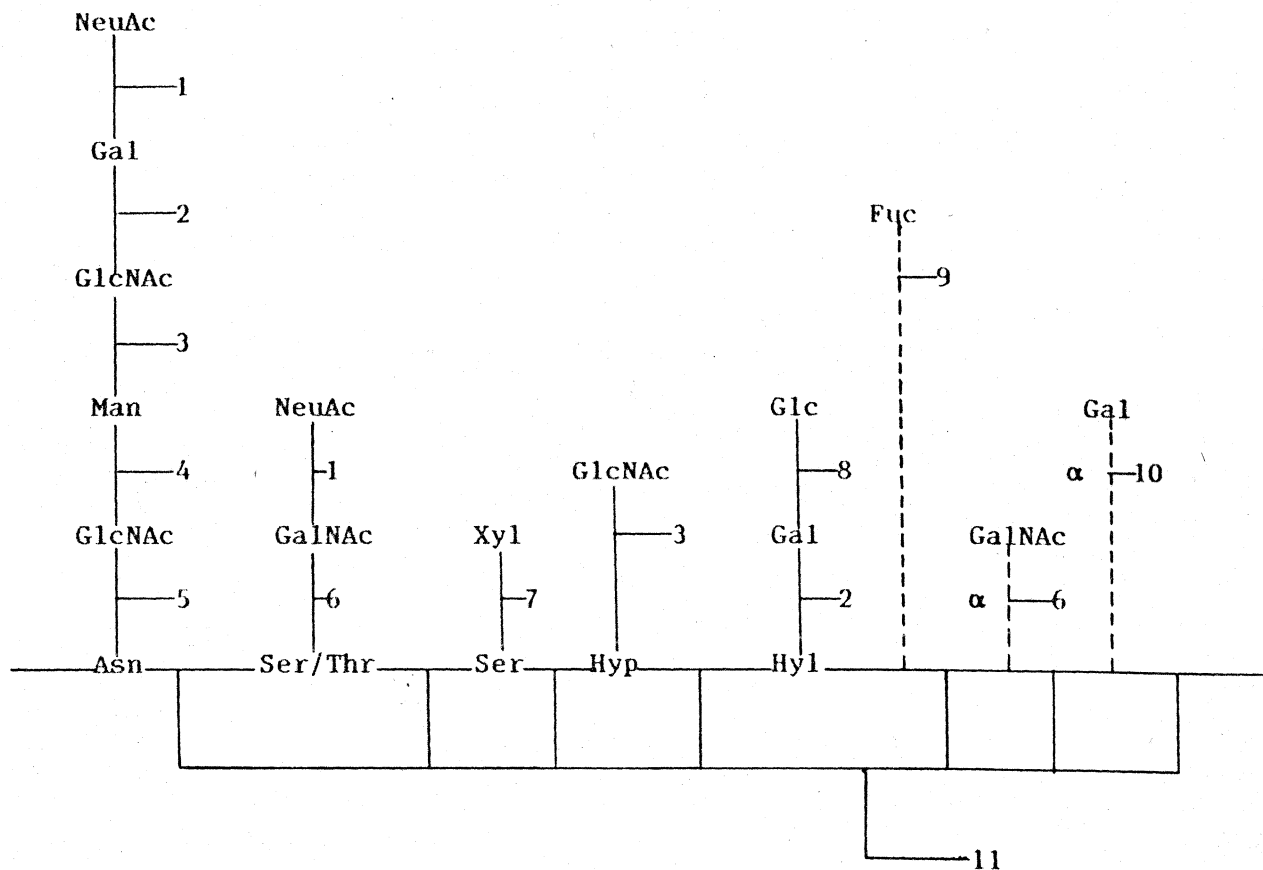


Fig.3. Catabolism of glycoproteins by lysosomal hydrolases.

- 1...Neuraminidase
- 2... β -D-Galactosidase
- 3...N-Acetyl- β -D-glucosaminidase
- 4... α -D-Mannosidase
- 5...4'-L-Aspartylglycosylamine amido hydrolase
- 6...N-Acetyl- α -D-galactosaminidase
- 7... β -D-Xylosidase
- 8... β -D-Glucosidase
- 9... α -L-Fucosidase
- 10... α -D-Galactosidase
- 11...Cathepsins

Adapted from Gregoriadis, G. (1975) *Lysosomes in Biology and Pathology* (Dingle, J.T. and Dean, R.T., eds) 4, pp. 264-294, North-Holland Publishing Company, Amsterdam.

Beta-Aspartyl acetyl glucosaminidase that splits the GlcNAc-Asn linkage has also been demonstrated (343). Certain endo-glucosaminidases are present in the cytoplasm and have a higher pH optima of pH 7.5 approximately, than the usual lysosomal acid hydrolases (258).

Functions of carbohydrate chains in glycoproteins

Cell surface glycoproteins play important roles in pinocytosis, differentiation, tumorigenesis, intercellular recognition and adhesion, as receptors for hormones, lectins, biogenic molecules, toxins and viruses, and as mediators of immunological specificity. Soluble glycoproteins function as enzymes, hormones, immunoglobulins, lectins, serum transfer and membrane transport proteins. The exact contribution of carbohydrate to biological activity of the protein is not always clear, apart from the observation that glycoconjugates are invariably present at sites of functional importance. Carbohydrate is considered to act as a chemical 'tag' labelling proteins destined for secretion from the cell. Glycosylation, however, is not obligatory for protein export or translocation, neither is it a prerequisite for biological activity of glycoproteins (243).

Nonetheless, the glycan chains can exert a modulatory control by altering the physicochemical properties of the protein such as hydrophobicity, electrical charge, molecular mass and size, in addition to acting indirectly by influencing protein

folding and conformation. Carbohydrate confer a resistance to cellular proteolysis, thereby increasing the life and stability of the newly synthesized glycoproteins. The protection against proteolytic degradation is attributed to steric hinderance by the carbohydrate as well as the more stable conformation adorned by the glycosylated protein (48). Glycoproteins are major constituents of plasma membranes where they contribute to the structural integrity of the membrane bilayer. Insertion, proper orientation and conformational stabilisation of integral membrane proteins is effected by the oligosaccharide side chains contained in them (264,156). Carbohydrate moieties of glycoproteins have also been implicated in the transport of metabolites across cell membranes (244). The high viscosity of mucus glycoproteins have been attributed to their saccharide chains (116). Antifreeze glycoproteins of Antarctic fish are dependant on the integrity of their disaccharide units for biological activity (57).

Carbohydrate moieties of glycoproteins provide important prerequisites for recognitive mechanisms by virtue of their enormous potential for structural diversity and thereby as potential carriers of biological information. In proteins and oligonucleotides, information content is based only on the number of monomeric units and their sequence, whereas in carbohydrates information can also be encoded in the position and anomeric configuration [α or β] of the glycosidic units and in the occurrence of branch points. Further structural diversification

is effected by covalent attachment of sulfate, phosphate and acetyl groups to the sugars. Thus, in theory, an enormous number of compounds can be derived from a relatively limited number of monosaccharides, augmenting that carbohydrates have an enormous potential for encoding biological information. The high degree of selectivity and specificity characteristic of biological processes is generally provided by a specific stereochemical fit between complementary molecules, one a carrier of biological information and the other capable of decoding such information, in other words the 'lock and key' hypothesis (305).

The immune response is eminently involved in recognition phenomena, best characterised being the interaction between antibodies and blood group substances. The blood group I/i, ABH and Lewis [Le] determinants on the surface of erythrocytes are carbohydrates. The fetal i antigen presents a linear repeating poly-N-acetyllactosamine structure, while the adult I determinant contains a branched structure. The ABH and Le structures are formed on I/i structures. I/i determinants appear on oligosaccharides, glycolipids and glycoproteins. Glycoproteins carry both O-linked and N-linked I/i antigens. Blood group I/i antibodies are less common than ABO antibodies and are often associated with disease states. Although the exact functions of blood group antigens are poorly understood, there are evidences that expression of these antigens are related to cell cycle and differentiation events.

Immune recognition is mediated through several types of carbohydrate-binding proteins. The glycosylation pattern of immunoglobulins can affect their binding specificities. More recently, evidence has accumulated to implicate recognition of carbohydrate structures in the activation of complement, namely by carbohydrate-binding C' components. Glycan structures are also recognised by soluble immune mediators such as lymphokines and monokines, surface lectins, cell surface enzymes including glycosyltransferases and glycosidases and carbohydrate-mediated uptake systems (273).

The ubiquitous presence of lectins in plant and animal systems focus on the possibility that carbohydrate-lectin interactions may be yet another facet of the lock and key complementarity mediating cell-sociology. Several membrane-bound lectins have been shown to participate in selective binding, uptake and clearance of circulating glycoproteins and cells in higher animals. The Gal and GalNAc specific lectins in rabbit hepatocytes are involved in clearance of glycoproteins from the circulatory system (11). Rat peritoneal macrophages express a galactose specific cell surface lectin that mediates binding to desialylated erythrocytes and glycoproteins as a prelude to phagocytosis and lysis. Similar mechanisms have been attributed to clearance of aged blood cells by Kupffer cells of the reticuloendothelial system. The intracellular trafficking of

lysosomal hydrolases already discussed, is a well characterised illustration of lectin-carbohydrate interaction.

Characteristics of polypeptide glycosylation include the following: (a) Different glycoproteins from the same cell contain different oligosaccharide structures (b) Individual polypeptides usually carry several different structures (c) Many structures are found at the same glycosylation site [site heterogeneity] (d) The pattern of oligosaccharide heterogeneity at a single glycosylation site during a constant physiological condition is reproducible and not random, and (e) The cell-type plays a role in oligosaccharide processing. This form of post-translational modification of a polypeptide serves to create discrete subsets or glycoforms of a glycoprotein that have different physical and biochemical properties leading to functional diversity. Glycosylation of a polypeptide creates a set of glycoforms all of which share an identical polypeptide but differ with respect to glycosylation. Hence, a glycoprotein has composite activity, which a cell can control by varying the relative incidence of each glycoform. Displaying the entire spectrum of oligosaccharides simultaneously may be necessary to avoid immunogenicity at later developmental stages.

Recent data suggest that oligosaccharide biosynthesis at a given glycosylation site is not independent of the biosynthetic events occurring at other glycosylation sites, thus giving rise to certain allowed glycoforms. Such an interaction would be

necessary if the oligosaccharides of cell surface glycoproteins are recognised in clusters and not individually. This may be one reason why the majority of glycoproteins contain multiple glycosylation sites. The set of glycoforms of a given polypeptide expressed by an organism is dependent on its physiological state. Examples include the control variations in the N-glycosylation of human serum transcortin and thyroxine-binding globulin during pregnancy (318), changes in hepatic N-glycosylation of α_1 -antitrypsin and α_1 -acid glycoprotein during acute inflammation (238) and changes in outer-arm galactosylation of human serum IgG N-linked oligosaccharides during aging (267). These variations may significantly affect the bioactivity of the glycoprotein in question. For example the increased specific bioactivity of TSH secreted after exposure of the pituitary to TRH [Thyroid Releasing Hormone] as compared with basal TSH has been attributed to a change in the oligosaccharides of TSH (228). The up and down regulation by glycosylation of the bioactivity of glycoproteins that interact with cell-bound receptors has become a recurring theme in the analysis of oligosaccharide function. Collagenous proteins such as gelatin are involved with fibronectin in forming a fibrillar network on the surface of various cells including connective tissue cells. The presence of poly lactosamine-containing oligosaccharides on the gelatin-binding domain of fibronectin resulted in weaker binding to the gelatin, compared with fibronectin which carried smaller complex-type glycans. The

differential expression of these glycoforms can result in fine control of the fibrillar network as required, for instance, at different stages of development (366). The differential binding activities of the A and E forms of N-CAM is another illustration.

The extent of glycosylation can control the biological activity of a polypeptide. The relative occupancy of glycosylation sequons on the regulatory IgE-binding protein is crucial to the activity of this protein in potentiating or suppressing IgE biosynthesis (155). Extrinsic tissue plasminogen activator [tPA] is a serine protease that converts plasminogen into plasmin and thereby induce clot lysis. Glycosylation of tPA is itself physiologically significant in that it prevents the protein moiety from converting plasminogen to plasmin in the absence of a fibrin clot. In addition, the two variant forms I and II differ in the number of attached oligosaccharide chains and exhibit different binding kinetics, thereby conferring greater control and sensitivity to the tPA mediated thrombolysis. The occupancy of N-linked glycosylation sites on tPA is also associated with the efficiency of secretion (262,60).

Species specificity of N-glycosylation is evident in some glycoproteins. Rat α_1 -acid glycoprotein is N-glycosylated in a quite different way from human α_1 -acid glycoprotein, the major difference relating to the degree of branching, fucosylation, and outer-arm decoration of the complex oligosaccharides (361). An inverse relationship between species-specific glycosylation

characteristics and the presence of naturally occurring antibodies against carbohydrate epitopes present in other species has been demonstrated with respect to terminal Gal $\alpha(1-3)$ Gal structure in new and old world primates and humans (104). Similarly tissue specific differences in oligosaccharide structures have been evidenced in the glycoforms of Thy-1 from rat brain and thymus (17). These differences may be a unique cellular means of defining its specific glycotype.

The location and nature of oligosaccharides on viral surface glycoproteins, in some cases, determine the efficiency and range of cellular infection. The expression of viral glycans are dependent and consistent with the biosynthetic pathways of the host, the latter therefore can play an important role in defining the infective range of a virus. Further, viral surface oligosaccharides may be ligands for host cell-surface lectins, and in this way exert a considerable effect on the spread of viruses between different hosts. It has also been proposed that the carbohydrate side chains on the surface of viruses might provide them with significant protection against the host's immune response during a primary infection.

Lymphocytes exhibit a variety of migratory patterns encompassing all or some of the organs of the body. These patterns can be altered during certain infections or disease states, either by change in the cell surface properties of lymphocytes or the target tissues. Lectin-carbohydrate

interactions have been suggested as a general mechanism for lymphocyte migration. Any change in the glycosylation of lymphocytes that render them ligands for any lectin of a tissue can lead to retention of those lymphocytes within that tissue with the consequent possibility of an 'autoimmune' reaction. Since lymphocytes also express a variety of lectins, tissue specific recognition of lymphocytes by endothelial cells may arise from cell-specific glycosylation characteristics of these latter cells with concomitant lymphocyte extravasation (267).

Development of the Nervous System

The nervous system develops from a thickened area of embryonic ectoderm known as the neural plate. This plate appears by day 18 of gestation and is infolded to form a neural groove and neural folds. When the neural folds fuse to form the neural tube, some neuroectodermal cells are not included but remain between the neural tube and the surface ectoderm as the neural crest. Development of the neural plate and neural tube is believed to be induced by the notochordal process and paraxial mesoderm. The cranial end of the neural tube forms the brain, consisting of the forebrain, the midbrain and the hindbrain. The forebrain gives rise to the cerebrum; the midbrain forms the adult midbrain; and the hindbrain differentiates into the pons, cerebellum and the medulla oblongata. The remainder and the longest part of the neural tube forms the spinal cord. The lumen of the neural tube gives rise to the ventricles of the brain and

the central canal of the spinal cord. The walls of the neural tube are thickened by proliferation of neuroepithelial cells, the source of all nerve and the macroglial cells, comprising of astrocytes and oligodendrocytes, in the central nervous system. When neuroepithelial cells cease the production of precursor neuroblasts and glioblasts, they differentiate into ependymal cells which gave rise to the ependymal epithelium lining the central canal of the spinal cord and ventricles. Microglia are believed to differentiate from mesenchymal cells which enter the CNS with the blood vessels. The neural crest gives rise to most of the peripheral nervous system consisting of cells in the cranial, spinal and autonomic ganglia, Schwann or neurilemmal cells, most of the autonomic nervous system and all chromaffin tissue, including the adrenal medulla (231).

The development of the nervous system is initiated between dorsal ectoderm cells and underlying mesoderm cells. This process termed neural induction is very poorly understood. Experiments relating to the early development of the nervous system in amphibian embryos suggest that there are two signals involved in the process of neural induction. The inductive signal had three consequences: morphogenetic movements leading to the formation of the neural tube began, the future anatomical organisation of the nervous system was laid out on the neural plate, and the physiological properties of neural plate cells altered which were manifested as the activation of additional sodium pumps within

the neuroectoderm, essential for subsequent expression of the neuronal phenotype (350). Remarkable findings were that the destiny of neural cells were already programmed at the earliest stages of neural tube formation and that the cells continued to differentiate sequentially and temporally in tissue culture as they did in vivo. Thus a unique model system was made available for the study of cell lineages of neural cells under controlled environmental conditions. The application of tissue culture methods, neuroimmunocytochemical techniques and the continuous addition of new cell markers have made possible the identification of specific cell populations. Studies on neural cells have established that astroglial and oligodendroglial cells begin to develop early in neurogenesis, rather than after neurons have completed their development, and that the development of various neural lineages are interdependent. Neurons depend on radial glia for guidance, and some early neural and oligodendroglial precursor cells depend on astroglial precursor cells as a substratum, at least in culture. Cell differentiation in the astroglial cell lineage occurs throughout embryogenesis and in postnatal life. The earliest cells from the astroglial lineage identified so far are the glioblasts (Fig.4.). These give rise to proastroblasts which contain vimentin intermediate filaments in their cytoplasm and express the Ran-2 antigen. Proastroblasts form astroblasts, identifiable by the presence of two types of intermediate filaments containing vimentin and glial fibrillary

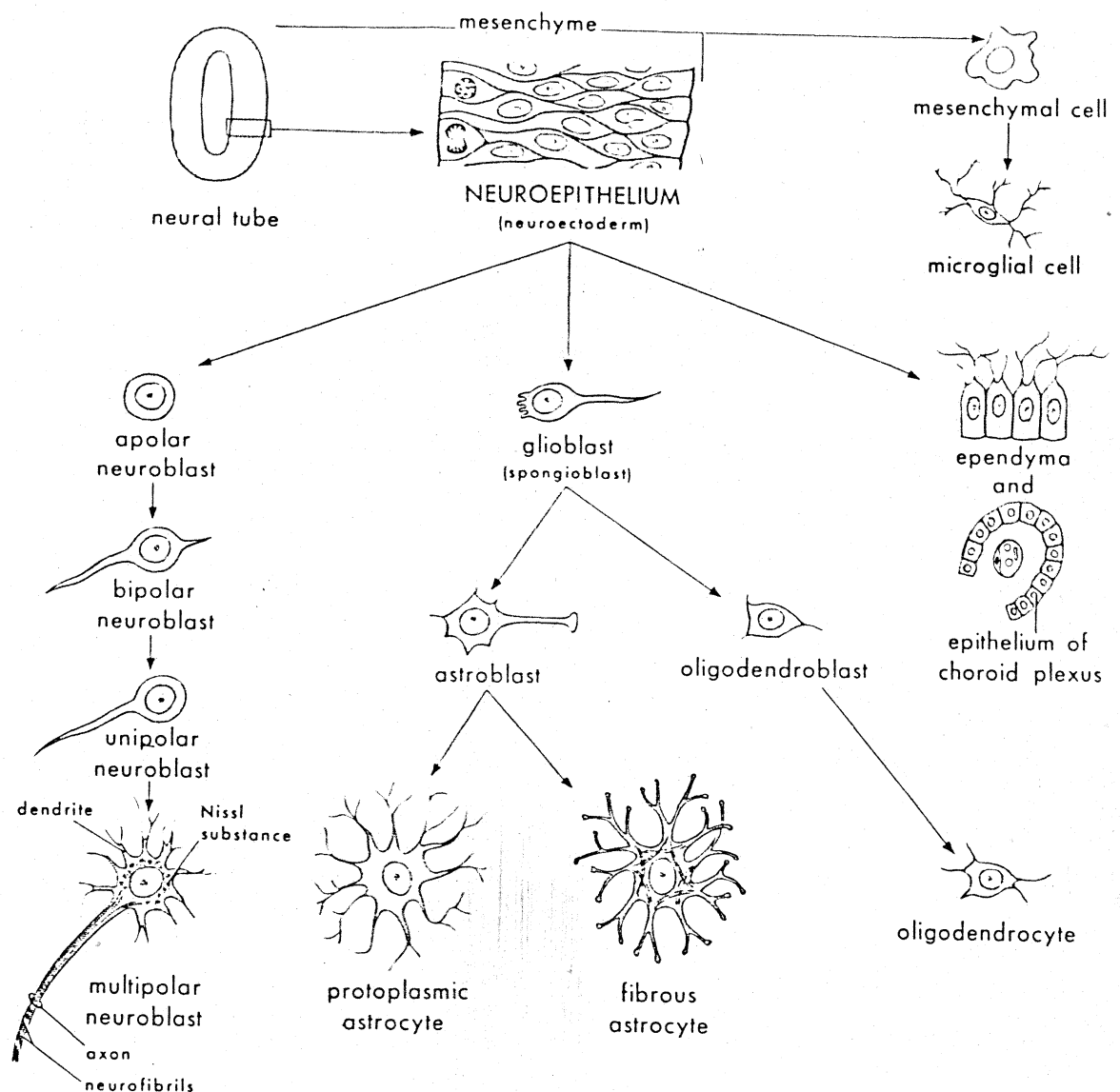


Fig.4.

Schematic diagram illustrating histogenesis of cells in the CNS. Neuroepithelial cells give rise to all neurons and macroglial cells. Multipolar neuroblasts develop into nerve cells or neurons.

Adapted from Moore, K.L. (1973) *The Developing Human – Clinically Oriented Embryology*, pp. 306–334, W.B.Saunders Company, USA.

acidic protein as well as expression of Ran-2 antigen. Astroblasts differentiate into astrocytes having a distinctive stellate shape. Oligodendroglial precursor cells give rise to oligodendroblasts containing galactocerebroside. Oligodendroblasts form oligodendrocytes, which are identified by the presence of galactocerebroside and myelin basic protein. Post-mitotic oligodendrocytes undergo three distinct stages of maturation forming light, medium and finally dark oligodendrocytes. The oligodendroglial lineage differs from the astroglial lineage in being biphasic. There appears to be a production of oligodendrocytes in the early stages of embryogenesis and then a period of quiescence, followed in postnatal life by another peak of oligodendrocyte production and maturation. Oligodendrocytes also have regional sequences of development which are related to the sequence of myelination. Intermediate forms between astrocytes and oligodendroglia have been observed, indicating that the lineages may have more plasticity than classical hierarchical concepts of lineages encompassed (77).

Nerve fibres which remain with the brain and spinal cord receive support from, and are ensheathed by neuroglial cells. The axons are enclosed in a sheath of myelin which facilitates saltatory conduction of impulses. In the peripheral nervous system these myelin sheaths are derived from the plasma membranes of Schwann/neurolemmal cells. The myelin sheaths of axons in the

CNS are formed in a similar manner by oligodendrocytes. Nerve fibres in different parts of the brain and spinal are myelinated at different stages of development that correlates with the time they become fully functional. In humans, myelin formation begins in the spinal cord during mid-fetal life [4th month] and is completed only by the second to third year of postnatal life. Fibres in the brain are largely unmyelinated at birth, myelination continues until adolescence. Myelination has been associated with the milestones of human development.

The nature of cell interactions and the signals inducing phenomena leading to the next stage of differentiation is still only the conceptual stages of exploration. Nevertheless, the involvement of cell surface carbohydrates are emphasized by reports that a marked induction of N-linked glycoprotein synthesis correlates temporally with the onset of neuronal and glial differentiation (27). Enzymological studies with microsomal preparations from developing brain also demonstrate increase in activities in the N-glycosylation pathway during active myelination (134) and synaptogenesis (157). The identification of several marker antigens specific to neural tissue have contributed valuable information with respect to nervous system morphogenesis, function and pathology. Histogenesis during embryonic development is often initiated by increased adhesiveness between cells. The available data suggest that basement membrane proteins and cell adhesion molecules [CAM] are

involved in these histogenetic events. All cell adhesion molecules are integral membrane glycoproteins synthesized by the cells on which they function.

Neural cell adhesion molecule [N-CAM] is first expressed in the chick embryonic blastoderm and is found in conjunction with liver cell adhesion molecule [L-CAM], another primary CAM probably expressed as early as the 2-cell stage. At the time of neural induction a dramatic change occurs in N-CAM and L-CAM distribution, N-CAM levels are markedly increased in the region of the neural plate while L-CAM rapidly disappears, whereas in the region of the somatic ectoderm, L-CAM is enhanced as N-CAM slowly diminishes. Placodes destined to build neural structures express both primary cell adhesion molecules but eventually lose L-CAM. After an initial differentiation event, N-CAM and L-CAM diverge in cellular distribution and are modulated in prevalence within various regions of inductions, or actually decrease greatly when mesenchyme appear or cell migration occur. N-CAM disappears from somatic ectoderm and from endoderm, except for a population of cells in the lung. L-CAM is expressed on all ectodermal and endodermal epithelia but remains restricted in the mesoderm to epithelial derivatives of the urogenital system (51).

N-CAM has been identified in chicken (151), rodent (49) and human neural tissues (221). This developmentally regulated glycoprotein manifests as a polysialylated embryonic [E] form containing 30% sialic acid by weight and a less sialylated adult

[A] form containing 10% sialic acid by weight. The molecule mediates homotypic binding of neuron to neuron and heterotypic binding of neuron to striated muscle, in both cases, by a calcium independent homophilic binding mechanism. Conversion from embryonic to adult forms occur during the perinatal period, with a concurrent increase in the efficacy of binding. Although sialic acid is not directly involved in binding, it modulates binding presumably through its high negative charge. N-CAM chains consist of three domains, an amino-terminal domain containing a region mediating homophilic binding, a middle domain containing three attachment sites for the bulk of carbohydrate, and a carboxy-terminal domain, part of which spans the plasma membrane (67). The glycoprotein is conserved structurally and functionally in widely differing species and is implicated in neurite fasciculation (283), retinal layering (42), formation of retinotectal maps (90) and in neuron-myotube formation (121). N-CAM is present on neurons, and on predominantly immature astrocytes, oligodendroglia and Schwann cells.

Just prior to the appearance of glia, a secondary adhesion molecule termed neuron-glia cell adhesion molecule [Ng-CAM] is expressed in chick embryo. These glycoproteins are present only on the post-mitotic neurons of the CNS and on neurons and Schwann cells of the periphery (168,330). The adhesion molecule binds homophilically to Ng-CAM on neurons and heterophilically to different molecules on astroglial cells (123). Ng-CAM is

structurally and immunologically similar to the mouse L1 antigen (272,291) and has been shown to be identical to the nerve growth factor inducible large external glycoprotein [NILE] in rat PC12 cells (91). These integral membrane proteins are involved in the formation and bundling of axons (271), migration of granular cells along radial glial cells (198,199), in neuron-neuron adhesion and neurite outgrowth (94,86). In developing cerebellum, granule cells continually express N-CAM but after they become post-mitotic in the superficial layers they express Ng-CAM, emit parallel fibres in the granule cell layer, and then migrate inward along Bergmann glial cells.

Cytotactin is an extracellular matrix glycoprotein of neural and non neural tissues that mediates glia-neuron interaction and is synthesized by glia but not neurons of the central and peripheral nervous system. Cells isolated from smooth muscle, kidney and lung also synthesize this protein (122). Immunochemical methods demonstrate that N-CAM, Ng-CAM and cytotactin are highly concentrated at the nodes of Ranvier of adult chicken and mouse. In contrast, unmyelinated axonal fibres are uniformly stained by specific antibodies to both CAMs but not by antibodies to cytotactin. Adhesive interactions between neurons and glia mediated by the CAMs and cytotactin may thus be a requirement for establishing and maintaining the nodes of Ranvier (275). Cytotactin has been found to share similarities in molecular

weight and antigenic properties with the antigen J1, another neuron-glia adhesion molecule (186).

Myelin associated glycoprotein [MAG] restricted to the periaxonal loop of myelin, is now believed to be involved in neuron to oligodendrocyte or oligodendrocyte to oligodendrocyte interactions and myelination (265,354).

All the above adhesion molecules are antigenically related and share common carbohydrate epitopes consisting of a sulfated glucuronic acid recognised by the monoclonal antibodies L2 and HNK-1 (162).

The novel adhesion molecule on glia [AMOG] does not belong to the L2/HNK-1 family. This calcium independent adhesion molecule is an integral membrane glycoprotein expressed by Bergmann glial cells in the cerebellum at critical developmental stages of neuron-glia interaction and granule neuron migration preceding synaptogenesis. Astrocytes synthesize AMOG, which mediates neuron to astrocyte but not astrocyte to astrocyte adhesion. It is also expressed by oligodendrocytes and possibly subserve an adhesive function on these glial cells too. AMOG is detectable shortly before the onset of granule cell migration and unlike L1 and N-CAM that persists also in adulthood, ceases to be expressed on Bergmann glia at the end of the migratory period. The adhesion molecule contains a carbohydrate epitope recognized by the monoclonal antibody L3, also present on L1 and MAG but not on N-CAM nor J1. The functional significance of these cross

reactivities and of the L3 epitope has not been established as yet (6).

Recent reports have demonstrated that AMOG is a homologue of the β -subunit of Na,K-ATPase suggesting that these molecules or variants of the β -subunit of the Na,K-ATPase, tightly associated with an α -subunit, are recognition elements for adhesion that subsequently link cell adhesion with ion transport (113).

Another cell surface glycoprotein distinct from N-CAM and NILE and termed Astrotactin, has been identified on mouse granule neuron cells. This antigen appears to play an important role in the formation of neuron-glia cell contacts and is required for neuronal migration along astroglia and for neuronal regulation of astroglial cell growth (70). Studies with normal and weaver mice strains have established that astroglial differentiation into forms that support migration, appear to rely on proper interactions with normal granule neurons (141). Thus it is likely that astrotactin is important for a variety of contact mediated relationships between neurons and astroglia.

Histogenesis is not limited to cell-cell interactions. The extracellular matrix components provide attachment sites for cells and thus mediate cell-matrix interactions necessary for the maintenance of a proper tissue histoarchitecture. All matrices contain glycoproteins, collagens and proteoglycans as major structural elements. The basement membrane is a special type of matrix that is deposited in a polar fashion around epithelial and

endothelial cells. Around fat cells, muscle and Schwann cells it is seen on all sides of the cell. Experimental studies on tissue repair have shown that basement membranes are important for the maintenance of tissue structure, and embryological studies suggest a role for these membranes in histogenesis (333).

The adhesive properties of basement membranes have been ascribed to glycoproteins. These substrate adhesion molecules [SAM] mediate cell-substrate and substrate-substrate interactions which can alter the biological properties of the individual basement membrane components crucial to morphogenesis (72). The extracellular matrix glycoproteins laminin and fibronectin are known to stimulate rapid and profuse neurite elongation from a wide variety of central and peripheral neurons. This fact, coupled with the presence of laminin and fibronectin immunoreactivity in embryonic tissues through which axons grow, suggest the involvement of these SAMs in nerve growth, *in vivo* (278). The increased expression of SAMs during embryogenesis corroborate its participation in the adhesion and migration of cells. The effect of matrix proteins would be mediated by binding to cell surface receptors. An illustration of CAM-SAM interaction is the conjugate relation between N-CAM expression and fibronectin appearance in the development of neural crest cells (331,62). Fibronectin has been detected in embryonic and early postnatal mouse brain restricted to a region along the route of cerebellar

granule cell migration. However, this glycoprotein does not appear to be present in adult brain (142).

The matrix proteins too demonstrate specific binding sites for cell surfaces and other matrix proteins. It is evidenced for laminin that a heparin binding domain appears to promote neurite outgrowth, another domain interacts with Type IV collagen while the central core region of the molecule is involved with cell binding. Fibronectin also has separate domains that bind respectively to collagen, heparin or cell surfaces (72).

The presence of L1, N-CAM, MAG and L2/HNK-1 epitopes have been demonstrated on basement membranes and collagen fibrils. Similarly, the adhesion molecule J1 has been observed on collagen fibrils in the proximity of denervated but not innervated neuromuscular junctions. It is suggested that these CAMs are secreted by unevenly localized cellular sources and are retained by the extracellular matrix, where they subserve yet unknown functions. Nevertheless, a salient inference is that a rigorous classification of adhesion molecules into those that mediate interactions with other cells and those mediating interactions with the extracellular matrix is futile, as adhesion molecules have now been found both as integral membrane constituents as well as part of the extracellular matrix (220).

It is evident that development of the nervous system specify distinct, yet coordinated morphogenetic steps : neural induction, cell proliferation, migration, aggregation, cytodifferentiation,

synapse formation, cell death and synapse elimination. Three separate kinds of supramolecular systems are involved in the generation and maintenance of these phenomena, viz. cell-cell adhesions mediated by the CAMs, cell-substrate adhesions mediated by the SAMs and cell contacts via intercellular junctions such as gap and tight junctions and desmosomes. Each system contain different gene products, but certain portions of each of these systems interact heterarchically with the others and mutually modulate their functions. It has been proposed that cell-cell recognition occurs by means of local cell surface modulation of a small number of proteins rather than by expression of large numbers of different cell surface markers.

Perturbation studies with antibodies against specific molecules indicate that migration of granule cells involve Ng-CAM, L1 antigen, cytotactin, a glial-derived neurite promoting factor [GdNPF] which in addition is a potent serine protease inhibitor (263), AMOG and possibly N-CAM.

Studies on mouse sciatic nerve have revealed that axon-Schwann cell interactions are characterised by the sequential appearance of cell adhesion molecules and myelin basic protein apparently coordinated in time and space. From this sequence it has been deduced that L1 and N-CAM are involved in fasciculation, initial axon-Schwann cell interaction, and onset of myelination, with MAG to follow and myelin basic protein to appear only in compacted myelin. The formation of compact myelin is the basis

for rapid propagation of action potentials through saltatory conduction in the axons. In contrast to L1, N-CAM is probably involved in the maintenance of compact myelin and axon-myelin apposition of larger diameter axons (286).

Another recent observation was that the cell adhesion molecule L1 interacted with itself, the avidity of which was enhanced by specific association of L1 and N-CAM on the cell surface of one of the interacting partner cells (163). This functional cooperativity between N-CAM and L1 was carbohydrate dependant, based on the presence of particular complex and hybrid glycan structures on these molecules (164).

It is thus probable that adhesion molecules do not act independently of each other, but cooperate synergistically exerting thereby an additional modulatory control to the overall morphogenetic processes. The modulatory effects of one adhesion molecule on the other would endow a particular cell with combinatorial possibilities in regulating the quality and strength of cell surface contacts.

At least three kinds of regulatory signals responsible for modulation have been suggested - (a) Prevalence modulation or signals for cell adhesion molecule gene expression, (b) polarity modulation or signals causing alterations in distribution at different locations on an individual cell, viz. mechanisms governing the transport of cell adhesion molecules to the cell surface, and their metabolic turnover, and (c) signals inducing

chemical modification of the cell adhesion molecules such as the E to A conversion of N-CAM. At present, very little is known regarding the nature of the signals responsible for activation of the various CAM genes in the expression sequence. One potential factor is the release of morphogens following cell contact, which serve as signals at the sites of embryonic induction as illustrated by the observation that nerve growth factor could enhance the expression of neuron-glia adhesion molecule in PC12 cells (91). There are evidences that physiological alterations can induce altered CAM expression as illustrated in the event of peripheral nerve injury and repair (54). The proliferation of neuroglial cells is important in the early development of the CNS. After the cessation of development in the mature CNS, nearly all of the neuroglial cells withdraw from the cell cycle and enter a quiescent state, or have a very low rate of turnover. It has been evidenced however, that certain of the glial cell types could respond to CNS trauma in adult animals by an increase in proliferative activity which contributed to the formation of a glial scar (173). These observations are indicative of a complex signalling system relating morphology to CAM expression. The possible role of neurotropic agents, hormones, neuropeptides and other environmental factors in the induction and coordination of morphogenetic events should therefore be a major consideration in the overall analysis of the modulatory and regulatory mechanisms governing the formation and maintenance of nervous tissue.

A second type of signal generating mechanism for CAM gene expression would be the asymmetric interaction at the cell surface of CAMs themselves, leading to global cell surface modulation that would directly effect induction through cytoskeletal changes. This would in turn alter CAM gene expression and CAM surface transport by propagation through the cycle. Another alternative, not yet demonstrated, is a cellular interaction with SAMs such as laminin, fibronectin and collagens, followed by global surface modulations and alterations in CAM expression. Cyclic appearances of N-CAM and L-CAM do occur in embryogenesis whatever the nature of the signals.

In vitro experiments have demonstrated that N-CAM turned over at the cell surface and that the E-form was replaced at least in part by the A-form. When combined with the structural work showing that sialic acid is modulated, and not the carbohydrate attachment sites, these observations suggest that an intracellular sialyl transferase or sialidase is being regulated along with the transport of the molecule to the cell surface.

Thus it is a combination of dynamics of expression, the regulatory loops, and various modulation mechanisms acting on a relatively few cell adhesion molecules of different specificity that epigenetically yield the differentiation patterns in neural histogenesis.

Adhesion is not a purely physical phenomena. Cell-cell adhesion can trigger biochemical signals leading to alterations

in gene expression and/or functional properties of neural cells. Direct contact between glial cells and neurons in vitro promote biochemical changes in several different types of glial cells. Such changes include the ability to express mature biochemical properties, such as cortisol-mediated glutamine synthetase induction in chick retinal and tectal glia (200,201), stimulation of rat Schwann cell division (133) and morphological differentiation accompanied by cessation of proliferation in mouse cerebellar astrocytes (140). Neuron-neuron contact has led to changes in cellular levels of the neurotransmitter biosynthetic enzymes tyrosine hydroxylase, choline acetyltransferase and of the neuropeptide, Substance P, in chick sympathetic neurons and rat PC12 cells (2,210,367).

Besides the comparatively well characterised adhesion molecules several neural marker protein antigens have been identified. These antigens have been mainly used to date as immunochemical tools to distinguish various neural cells and cell structures. Many of these proteins have been demonstrated in a wide variety of species. The conservation during evolution further supports the assumption that a protein not required by another tissue, has a potentially important function possibly specific to nervous system. A few relevant neuroglial markers have been enumerated.

Glial fibrillary acidic protein [GFA] was first isolated from tissues rich in fibrous astrocytes and from human brains

with a pathology leading to fibrous gliosis, e.g. multiple sclerosis plaques. Subsequently, this protein was isolated from normal brain tissue and from a wide range of vertebrate species. GFA is absent in peripheral nerve and other organs, is localized in glia, predominantly in astrocytes, and hence is used as an astroglial marker. Intermediate astroglial filaments distinct from actin or tubulin contain GFA as the major constituent. Proteins immunochemically related to GFA have been demonstrated recently in non-glial cells such as human fibroblasts (32).

A soluble brain specific acidic glycoprotein, α_2 -glycoprotein, has been reported to be specifically localized on astrocytes. This marker exhibits differential distribution in neural tumours and is always present in astrocytomas but absent in glioblastoma multiforme. However, the function is not known (349).

Nervous system antigen-1 [NS-1] is found only in brain and neural tumours. Several mammalian species manifest this antigen which appears before onset of myelination in oligodendroglial cell surfaces but not on astroglia or neurons (33).

Alterations in Glycosylation - Relationships to Disease

A comparative analysis of the N-glycosylation of particular glycoproteins provides an excellent probe for acquired or inherited cell-type-specific or tissue-specific dysfunction. Several inherited diseases categorized as lysosomal storage disorders are known to be associated with abnormalities in the

genes for glycosidases. Comparatively, only a limited number of genetic diseases caused by defective oligosaccharide biosynthesis are known, for e.g. I-cell disease and pseudo-Hurler polydystrophy in which a deficient phospho-N-acetylglucosaminyl transferase activity is manifested as defective transport and targeting of lysosomal enzymes.

Immune complex formation in rheumatoid arthritis has been attributed to an altered N-glycosylation in the Fab domain as well as the presence of agalactosyl structures in the Fc domains of these patients (342).

The presence and integrity of N-linked oligosaccharides of the α -subunit gonadotropic hormones are essential for the activation of adenyl cyclase in the target tissue but not for binding to the hormone receptor. Deglycosylation is associated with increased affinity of binding to the receptor (285).

Cancer is a disease in which abnormalities of both cell growth and cell development are found. Transformed cells have altered surface membranes as demonstrated by the occurrence of tumour-specific surface antigens, decreased cellular adhesiveness and cohesiveness, increased agglutinability by plant lectins, altered patterns in glycolipids and glycoproteins, loss of contact inhibition and metastatic spreading. Such alterations in the surface membranes are possibly related and could be manifestations of an overall process in which the regulation of internal metabolic events by cell-surface macromolecules is

altered by transformation (12). Transformed cells are capable of indefinite proliferation and are able to overcome some of the physiological and mechanical constraints that restrict the population growth and territorial expansion of normal cells. In addition, tumour cells are frequently immature and lack the functional attributes of normal cell counterparts.

Tumorigenesis, growth control and metastatic spread involve recognitive phenomena and are mediated through cell-surface biomolecules. Changes detected in structure and composition of carbohydrate moieties of glycoproteins in oncogenically transformed cells serve as a basis to propose functional involvement of the phenomenologically described alterations.

No causally related alterations in amino acid sequence have been found in tumour membrane glycoproteins, and subunit interactions of these components seem, in general, to conform to normal patterns. Changes in the glycan structure was observed with consistency but many of these were not associated exclusively with the malignant phenotype.

Appearance of an abundance of high molecular weight sialylated glycopeptides on tumour cells that promote premature migration of immature malignant variants have been evidenced. Malignant cells expressed more highly branched structures, containing an increased degree of sialylation. The high levels of sialic acid in the surface exposed carbohydrate of tumour cells is a necessary condition for the manifestation of invasiveness

and tumorigenicity and correlates directly with metastatic potential (360,296).

Comparison of fucose labelled glycopeptides derived from the surface of malignant and corresponding normal cells established that structural changes in fucose containing N-glycosidically linked carbohydrates, constituted a discrete step in the progressive multistep process of malignant transformation, and was a specific and necessary determinant of the tumorigenic phenotype. Increased binding of fucose specific lectins to more highly metastatic variants have also been evidenced. Transformation of a number of cell-types resulted in an increase in the size of fucose containing cell surface glycopeptides (312).

According to the lock and key complementarity hypothesis, proteins capable of specifically recognising these sugar determinants such as the glycosyltransferases, sugar specific antibodies or endogenous lectins should also reflect variations in expression and composition. Differentiation-dependant changes in the pattern of carbohydrate-binding proteins in the intracytoplasmic and intranuclear compartments of brain tumour cells, have been demonstrated suggesting the probable involvement of the 'glycoconjugate-endogenous receptor' system in malignant cell transformation (19). The endogenous lectins are assumed to participate in regulatory processes of tumour growth (96).

Tumour immunology has uncovered many tumour distinctive antigens defined by monoclonal antibodies selected by

preferential reactivity of tumour cells over normal cells and tissues. Many of these antigens are directed to carbohydrate antigens in glycolipids, glycoproteins or proteoglycans. The identified tumour associated glycan structures are closely related to the classically known blood group antigens and the major human alloantigens highly expressed in epithelial tissues (127,78). They are fucosylated, sialylated multiantennary chains. Most types of tumour associated carbohydrate antigens have been found to be highly expressed at certain defined stages of embryogenesis and fetal development, while others were found at the stem cells of a certain cell lineage during differentiation and maturation (107). In an attempt to explain human cancer the concept of a transforming gene or oncogene was introduced, however, the majority of human cancers did not express any oncogene whatsoever (189). In striking contrast, aberrant glycosylation could be found not only in experimental cases but essentially in all types of naturally occurring human cancers. Thus carcinogenesis presumably results from a combination of genetic and epigenetic alterations and involves aberrant glycosylation of glycoconjugates but not the amplification or expression of oncogenes (16).

The tumour antigens are expressed in the majority of human cancers, and combinations of these antigens seem to cover the expression of tumour-associated carbohydrate antigens in essentially all human cancers. In spontaneous human cancers,

tumour development is a multistep process from in situ cancer at its earliest stage to a highly metastatic tumour that becomes lethal to the host. Glycosylation patterns have been found to reflect the stage of tumour development.

Information with regard to (a) the mechanism of regulation of carbohydrate expression at the transcriptional and post-translational levels and the organisation of glycosyltransferases in membranes (b) the functional concept of carbohydrate expression and developmental changes associated with oncogenesis, and (c) the mechanism of immune recognition of carbohydrate chains, is fragmented. Currently, monoclonal antibodies and anti-idiotypes directed to carbohydrate antigens on oncogenically transformed cells, used alone, or as conjugates in cancer therapy, in tumour imaging, and in the targeting and delivery of drugs is under investigation. Active immunization with carbohydrate antigens is another probable line of approach to cancer therapy. Since cancer is essentially a disease of phenotype regardless of its genetic mechanism, diagnosis and treatment should be focused on phenotype. Of the many phenotypic characteristics expressed by tumour cells, aberrant glycosylation is the most obvious with chemically well defined parameters. It therefore appears to be the best target for diagnosis and therapy (129).

It is indisputable that glycoproteins and their oligosaccharides are important participants in cell-sociology and

physiology. Many glycoproteins have been identified in nervous system tissues, however, a detailed understanding of the composition and structure of complex oligosaccharides associated with only a few glycoproteins have been determined. In spite of the progress made in this area, the function of the majority of brain glycoproteins remains unknown. Major obstacles in the study of brain glycoproteins are due to the vast cellular heterogeneity of nervous system tissues as well as difficulties in obtaining large and homogeneous quantities of individual glycoproteins. The role of cell surface glycoproteins in adhesion phenomena has been best studied. Nonetheless, the described functions for such cell surface, nervous system-specific glycoproteins have in large part been elucidated or partially understood using in vitro systems. Their specific roles within the nervous system, either in a developmental or differentiation setting, await future investigation. In this context, an important goal in understanding nervous system development would be the identification of cell surface glycocomponents involved in neural recognition, guidance, migration and in the formation of synaptic contacts. Progress in technological areas as well as the use of monoclonal antibodies and other specific molecular probes should provide answers to some of these elusive questions, and the molecules involved, in the future.

AIMS AND OBJECTIVES

It is evident that cell surface glycoproteins are implicated in a multitude of cellular processes involving recognition phenomena, such as, morphogenesis, oncogenesis as well as receptor-ligand interactions. Cell-sociology has not yet been fully defined at the biochemical/molecular levels. This is especially so with respect to the mammalian nervous system due to its diverse and complex nature. Fetal tissue is a differentiating and developing system and would therefore manifest best the entire spectrum of events constituting cell-sociology. The molecular characterisation of a developing system is expedient to understanding the basis and mechanisms of cellular development and differentiation to form functionally mature cells, as also the kind of structural elements necessary for functional maturation. In addition, the studies would enable one to determine how the control of differentiation of tumour cells differed from that of normal cells. It has been established that a close parallel exists between normal immature cells and oncogenically transformed cells. Antigens claimed to be tumour-specific have been subsequently demonstrated as the adhesion-proliferation and differentiation antigens in fetal tissues or immature cells in the adult. The identification of oncofetal proteins and the expression of proliferation-differentiation antigens in transformed cells suggest that tumour cells are

probably arrested at, and representative of, a certain stage of maturation along the normal developmental pathway. The application of such differentiation markers in the classification of tumour cells as well as in the diagnosis, therapy and prognosis of cancer has in fact become a feasible possibility in recent years.

A number of nervous system specific, cell membrane glycoproteins mediating crucial cellular events in CNS development and sociology have been described in the preceding section. However, it is worth noting that most of the characterisation work on neural tissue are limited to non-human, mainly avian and rodent systems. Most of the information available on human CNS sociology has been obtained through indirect, extrapolatory protocols. Besides, in comparison with neuronal cell types very little information is available on the molecular aspects of glial cell development and function. Hence the characterisation of a few glial cell surface-specific glycoproteins from developing human brain would contribute towards a better understanding of glial cell-sociology and functions in general, and have more pertinence to its role in human development.

The objective of the present study is to isolate, identify and characterize glial cell surface-specific glycoproteins from developing human brains. The study involves, (a) Separation of glial cells and neurons from IInd [CR length = 9-20 cm] and IIIrd [CR length = 24-36 cm] trimester human foetal brains. (b)

Isolation of cell surface glycoproteins from glial cells of different gestational age groups. (c) Physicochemical/biochemical characterisation of the isolated glial cell surface glycoproteins. (d) Age related comparison of identified glycoproteins from IInd and IIIrd trimester groups to ascertain whether these glycoproteins are subject to developmental regulation, and (e) Immunological characterisation in order to verify if the isolated glial cell surface glycoproteins are cell, tissue or species specific.



CHAPTER - II

MATERIALS AND GENERAL METHODS

MATERIALS

D-glucose, D-galactose, D-mannose, N-acetyl glucosamine, N-acetyl galactosamine, N-acetylneuraminic acid, serotonin, bovine serum albumin, ovalbumin, ferritin, soybean trypsin inhibitor, fetuin, dansyl chloride, dansyl derivatives of amino acids, 4-N,N'-dimethyl aminoazobenzene-4'-isothiocyanate, 3,3'-diaminobenzidine, Coomassie Brilliant Blue G-250, Sudan Black B, Amido Black, fluorescein isothiocyanate, Tris, sodium lauryl sulfate, dithiothreitol, acrylamide, N,N'-methylene-bis-acrylamide, N,N,N',N'-tetramethylenediamine, iodoacetamide, iodoacetic acid, guar gum, thiobarbituric acid, 1,4-butanediol diglycidyl ether, NaCNBH_4 , Neuraminidase [*Clostridium perfringens*], Lysostaphin, Horseradish peroxidase, Trypsin, Trypsin [TPCK treated], Galactose oxidase [*Dactylium dendroides*], and Freund's adjuvant were purchased from Sigma Chemical Company, St. Louis, U.S.A. Coomassie Brilliant Blue R-250 was a product of Pierce Chemical Company, Rockford, U.S.A. Triton X-100, Tween 20, KBH_4 , phenyl isothiocyanate, phenyl methane sulfonyl fluoride, benzamidine hydrochloride, guanidine hydrochloride, 2-amino pyridine, amino naphthol sulfonic acid, trifluoromethane sulfonic acid, trifluoroacetic acid and Deoxyribonuclease II [from bovine spleen] were obtained from Fluka, Buchs, Switzerland. Sodium periodate, NaAsO_3 , NaBH_4 , 1-O-methyl- α -D-glucopyranoside, and N-ethyl morpholine were procured from Koch-Light Laboratories Ltd., Colnbrook, England. Silica gel TLC plates were obtained

from E. Merck, Darmstadt, Federal Republic of Germany. Soybean agglutinin was a gift from Dr. Nathan Sharon, Dept. of Biophysics, Weizmann Institute of Science, Rehovot, Israel. Barbituric acid and barbitone were obtained from BDH Chemicals, Ltd., England. Nitrocellulose paper and Biogel P-4 were products of BioRad Laboratories, Richmond, U.S.A. Ficoll 400, Sephadex G-75, Sepharose 4B, Sepharose 6B and Sephacryl S-300 were purchased from Pharmacia LKB Biotechnology, Uppsala, Sweden. DOWEX 50x8 and amino acid standards were from Serva Feinbiochemica, Heidelberg, Germany. Human IgG was purchased from Behringwerke, AG, Marburg, Federal Republic of Germany. Goat anti-rabbit Γ -globulin was procured from Immunodiagnostics, New Delhi, India. $\text{NaB}[\text{}^3\text{H}]\text{}_4$ and ^3H -acetic anhydride were supplied by Amersham International, plc, England. ^{14}C -acetic anhydride was a product of Bhaba Atomic Research Centre, Bombay, India. All other reagents were of analytical grade and were obtained from local sources.

GENERAL METHODS

Protein was estimated by an adaptation of Bradford's protein dye-binding method (37) or a modified Lowry method (64).

Reagents

- i) Coomassie Brilliant Blue G-250 dye solution - 0.06% in 0.3M perchloric acid.
- ii) Solution A - 2% Na-K tartrate in water.
- iii) Solution B - 1% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in water.

iv) Solution C - 2% Na_2CO_3 in 0.1N NaOH containing 3% SDS.

v) Alkaline tartrate reagent - the solutions A,B and C were mixed in the ratio A:B:C,1:1:98 (v/v/v) just prior to use.

vi) Folin reagent - 1N solution.

In the dye-binding assay, 1.5ml of protein solution was mixed with an equal volume [1.5ml] of Coomassie Blue dye solution and the resulting colour was read immediately at 620nm. Crystalline bovine serum albumin was used as the reference standard.

The modified Lowry estimation was carried out in a total volume of 0.81ml. To 150 μ l protein solution was added 600 μ l of freshly prepared alkaline tartrate solution. After 10 min, 60 μ l of 1N Folin reagent was added and mixed. The blue colour was read after 30 min at 660nm against the reagent blank. Crystalline ovalbumin was the standard protein employed.

Total carbohydrate was measured by the phenol-sulfuric acid method of Dubois et al in a total volume of 1.1ml (63). D-mannose was used as the standard. The assay system comprised of 100 μ l of carbohydrate solution to which was added 200 μ l of a 5% phenol solution and 800 μ l of ice-cold concentrated H_2SO_4 . The optical density of the developed colour was read after 15 min at 485nm.

Sialic acid was estimated by the thiobarbituric acid protocol of Warren (351).

Reagents

i) Periodate solution - 0.2M NaIO_4 in 9M H_3PO_4 .

ii) Arsenite reagent - 10% NaAsO_3 in 0.1N H_2SO_4 containing 0.5M Na_2SO_4 .

iii) TBA reagent - 0.6% thiobarbituric acid in 0.5M Na_2SO_4 .

Glycoconjugates were desialylated in 0.1N H_2SO_4 at 80°C for 1 h. The hydrolysate [25 μ l] was mixed with 25 μ l of periodate solution and kept in the dark at 25°C for 20 min. To this mixture was added 250 μ l of arsenite reagent and vortexed until colourless. The solution was then mixed with 750 μ l of TBA reagent and heated for 15 min at 100°C in a boiling water bath. The cooled solution was vortexed with 750 μ l of methyl cellosolve, centrifuged, and the supernatant filtered through a Whatmann No.1 microfilter apparatus. The absorbance of the clear filtrate was measured at 549nm against the standard, N-acetylneuraminic acid. Fetuin was used as a reference sialylated glycoprotein.

Phosphorus estimation was adapted from the procedure of Bartlett (21).

Reagents

i) Ammonium molybdate solution - 0.44g ammonium molybdate in 100ml water containing 1.4ml H_2SO_4 .

ii) ANSA reagent - 3g sodium metabisulfite, 0.6g Na_2SO_3 and 0.05g of 1,2,4-amino naphthol sulfonic acid was dissolved and made upto 25ml in water. The solution was kept in the dark for 12-16 h at 4°C and filtered. Working reagent was prepared by diluting the stock solution 1:12, ANSA:water (v/v).

The sample [50 μ l] was refluxed with 0.2ml perchloric acid and 25 μ l of ammonium molybdate solution for 2-4 h at 240°C in a sand bath to oxidise all organic material and quantitatively convert the organic phosphorus into inorganic phosphate. To the colourless solution was added 0.6ml of ammonium molybdate solution and 0.6ml of ANSA reagent. The mixture was boiled at 100°C in a boiling water bath for 10 min and cooled. The blue colour was measured at 830nm. Potassium dihydrogen orthophosphate was used as the standard.

Polyacrylamide Gel Electrophoresis

I. Sodium dodecyl sulfate - polyacrylamide gels of 7%, 8%, 10% and 12% (w/v) concentrations were respectively prepared as per the procedure of Laemmli (190). All preparative and analytical SDS-PAGE were carried out on vertical slab gels of 1.5mm thickness in a minigel unit [8.3 x 7.3cm] or in the Broviga vertical slab gel unit [12 x 15cm]. A 3% spacer gel was layered over the separating gels.

Preparation of 3-12% gradient SDS-polyacrylamide slab gels were adapted from procedures reviewed by Hames (131).

Reagents

- i) Acrylamide solution [50%, w/v] - 48.6g of acrylamide and 1.315g of methylene bisacrylamide was dissolved, made upto 100ml in water and filtered.
- ii) Separating gel buffer - 3M Tris-HCl, pH 8.85, containing 0.4% SDS.

- iii) Ammonium persulfate solution [100mg/ml] was prepared fresh.
- iv) 3% gel solution was made up of 5ml separating gel buffer, 1.2ml acrylamide solution and 13.8ml of water.
- v) 12% gel solution was made up of 5ml separating gel buffer, 4.8ml acrylamide solution and 10.2ml of water.

Similarly, for a 5-25% gradient gel the respective 5% and 25% gel solutions were prepared in a total volume of 20ml. All gel solutions were degassed before delivering into the gradient mixer apparatus.

A 3-12% gel gradient was formed with a gradient mixer apparatus. To the reservoir chamber of the apparatus transferred 17ml of 3% gel solution mixed with 40 μ l of ammonium persulfate solution and 7 μ l of TEMED. The mixing chamber contained 16ml of 12% gel solution to which was added 32 μ l of ammonium persulfate solution and 6 μ l of TEMED. The gel gradient was delivered from the mixing chamber via a peristaltic pump to the slab gel unit at a flow rate of 3-4ml/min to complete gradient formation within 15 min. Gradient gels were utilized directly without the 3% spacer gel.

Electrophoresis was carried out in 0.05M Tris-0.192M glycine -0.1% SDS, pH 8.3 chamber buffer, at a constant current of 15mA for minigels and 40mA for standard slab gels. The gels were fixed in 50% methanol containing 0.05% formaldehyde to remove SDS, replacing the solution thrice over a period of 1 h.

The gels were stained for protein with Coomassie Brilliant Blue-R250 and destained according to the method of Weber and Osborn (353).

Silver staining was carried out directly after electrophoresis or on CBB stained gels by the procedure of Wray et al (358). Gels destined for silver staining were first made completely free of SDS by several changes in fixing solution with boiling, followed by soaking for a minimum of 24 h in 50% methanol. This treatment abolished background colour during silver staining.

Carbohydrate in glycoproteins were specifically detected after SDS-PAGE by the Thymol-sulfuric acid staining protocol of Gander (108).

Identification of Con A-binding glycoproteins on SDS-PAGE gels was effected with FITC-Con A according to the method of Furlan et al (93).

Sudan Black B staining for lipids was carried out on 50% methanol fixed SDS-PAGE gels as per the procedure of Fringes et al (92).

The standard molecular weight marker proteins used were bovine serum albumin [67 KDa], immunoglobulin G light chain [25 KDa], immunoglobulin G heavy chain [50 KDa], ovalbumin [45 KDa] and Con A [27 KDa]. All the protein samples in chamber buffer containing 2% SDS, 10% 2-ME and 20% glycerol were heated for 3 min in a boiling water bath at 100°C prior to application on

the gel. Standards were loaded 5-15 μ g per well while glial proteins were applied at higher concentrations of 25-100 μ g to enable their detection.

II. Acid urea-PAGE was carried out at pH 4.2 in 5% polyacrylamide gels containing 6M urea by a modified procedure (307).

Reagents

i) Acrylamide-urea solution - 1g acrylamide, 27.7mg methylene bisacrylamide, 7.2g urea, 1.046ml glacial acetic acid and 116 μ l TEMED were dissolved in water and adjusted with KOH to pH 4.2. The solution was made upto a total volume of 19.33ml and filtered through Whatmann No.1 paper.

ii) Ammonium persulfate solution - 6% in water.

iii) Chamber buffer - 14g glycine and 1.5ml acetic acid was made upto 1L in water to give a pH 4.2.

The gel polymerisation reaction was commenced by mixing 14.5ml of acrylamide-urea solution with 0.5ml of ammonium persulfate. The mixture was quickly transferred to the slab gel apparatus and allowed to set at 25°C for 3 h. The gel was run in chamber buffer, pH 4.2 at ambient temperature in reverse mode at a constant current of 12mA with methylene blue as the tracking dye. Samples were prepared in chamber buffer containing 8M urea, 10% 2-ME and 20% glycerol, adjusted to pH 4.2, and heated for 3 min at 100°C before loading 50-100 μ g protein per well. The gels were stained with CBB and/or silver as described earlier.

Electroelution of proteins

The protein bands were excised from fixed CBB stained SDS-PAGE gels. During large scale preparation of protein a single well was stained on the methanol fixed gel and corresponding bands were then cut out from the rest of the wells. The protein was recovered from the gel slices by an electrophoretic elution procedure adapted from methods employing a discontinuous conductivity gradient (251,317).

Reagents

- i) Solubilization buffer, pH 8.8 - 0.025M Tris-0.075M glycine-1% SDS (w/v)-0.005M DTT-40% glycerol (v/v).
- ii) Recovery buffer, pH 8.8 - 0.025M Tris-0.075M glycine-0.001M DTT-40% glycerol (v/v).
- iii) Chamber buffer, pH 8.8 - 0.050M Tris-0.15M glycine.

The fixed protein in the gel was resolubilized within the gel by incubating in solubilization buffer, changing the buffer every 15-30 min three times at 25°C and finally boiling at 100°C for 3 min during the fourth change. The equilibrated gel slices were arranged over a 10% supporting tube gel of length 5cm, 3 pieces per tube, then immersed in and covered over with 250µl of recovery buffer, taking care to remove any trapped air bubbles. Above this, was carefully layered 2M NaCl, the high conductivity zone that served to retain eluted protein within the recovery buffer. Both chambers of the Broviga tube gel apparatus were filled with chamber buffer, taking care not to disturb the 2M

NaCl layer in the tubes. Electroelution was conducted in the reverse mode at 4mA/tube for 1.5 h. The eluted protein was distributed in the recovery solution and as a precipitate of SDS-protein complex at the NaCl interphase. The recovery buffer containing protein was collected, made 1% SDS (w/v), heated at 100°C for 3 min and filtered through Whatmann No.1 microfilter to remove contaminant gel pieces. The protein was first precipitated with 5 volumes of cold methanol at -20°C, then washed thrice in ice-cold 1:5, water:methanol (v/v) and stored at -20°C in minimal volume of distilled water.

Preparation of Lectins

Concanavalin A [Con A] was isolated from *Canavalia gladiata* according to the method of Surolia et al (323).

Wheat Germ Agglutinin [WGA] was purified from wheat germ on chitin as described by Bloch and Burger (30). This lectin was also prepared by affinity chromatography on GlcNAc-Sepharose 4B matrix. Wheat germ was extracted with 10 volumes of 0.02M phosphate buffered saline, pH 7.2. The protein precipitated at 35% ammonium sulfate saturation was dialysed extensively at 4°C against the same buffer and centrifuged at 10,000 x g for 20 min. The supernatant [20ml] was loaded on a GlcNAc-Sepharose 4B column [1 x 5cm] at 4°C. The column was washed with phosphate buffered saline, pH 7.2 and eluted at 4°C with 0.1M acetic acid. The eluted protein was dialysed against phosphate buffered saline,

pH 7.2 and checked for hemagglutination with trypsinized rabbit RBCs (203).

Castor Bean or Ricinus communis was the source for Ricinus Communis Agglutinin [RCA]. The purification of RCA was carried out by affinity chromatography on cross-linked guar gum according to Appukuttan et al (9).

Jack Fruit Seed Agglutinin [JSA] was prepared from Jack fruit, [Artocarpus integrifolia] seeds by the protocol of Suresh et al (322).

Artocarpus hirsuta Agglutinin [AHA] was isolated by the method of Antony et al from wild jack fruit seeds (7).

Preparation of Pea seed lectin [PSA] from Pisum sativum was according to the procedure of Driessche et al (61).

Arachis hypogaea was the source of Peanut Agglutinin [PNA] which was purified by a modified protocol. Peanut seed extract was prepared in 0.15M NaCl, saturated to 60% with ammonium sulfate and the resulting protein precipitate dialysed against saline at 4°C as per the procedure of Lotan et al (206). The protein was then chromatographed at 4°C on an asialofetuin-Sepharose 4B affinity column. The matrix was washed with saline and eluted with 0.05M galactose in saline. The lectin was dialysed to remove sugar inhibitor and checked for haemagglutinating activity (203).

Haemagglutination Assay (203)

Human or rabbit blood collected in acid citrate dextrose anticoagulant solution, was washed thrice with 10 volumes of cold 0.02M phosphate buffered saline [PBS], pH 7.4. A 5% cell suspension was prepared in PBS containing 0.1% trypsin and incubated at 37°C for 1 h. The trypsinized cells were washed three times in PBS, pH 7.4 and suspended in the same buffer. The agglutinin to be checked was diluted in PBS to a starting concentration of 1mg/ml. The haemagglutination assay was carried out on agglutinin, serially diluted with PBS in a total volume of 0.2ml. To these solutions were added 50 μ l of the 5% trypsinized RBC cell suspension. The agglutination was read after 30 min at 25°C.

Preparation of Affinity Matrices

Con A-Sepharose 4B was prepared by the procedure of Kohn and Wilchek (175). Sepharose 4B [10g] was washed respectively with 5 volumes of water, acetone:water, 3:7 (v/v) and acetone:water, 6:4 (v/v) at 0-4°C. The gel was prechilled in 10ml of acetone:water, 6:4 (v/v) to -15°C in a salt-ice bath with gentle stirring. To the cooled gel, 100mg CNBr in 1ml acetone was poured in one lot followed by dropwise addition of triethyl amine [152mg in 1ml acetone] over the 2 min reaction period, taking care to maintain the temperature between -15 to -10°C. The gel was quickly transferred to a sintered glass funnel and washed respectively with 10 volumes of ice-cold distilled water and 250ml of 0.1M

NaHCO_3 , pH 8.5, coupling buffer. Coupling of the CNBr activated Sepharose 4B to Con A was carried out by mixing the gel with 100mg of lectin in 10ml of coupling buffer, pH 8.5, and stirring slowly at 4°C for 18 h. The unreacted active groups on the matrix were then blocked by addition of 100 μ l of ethanol amine to the gel mixture and continuing with stirring for 3 h at 4°C. The gel was washed successively with 10-25 volumes each of ice-cold 0.1M NaHCO_3 buffer, pH 8.5, water, 0.05M sodium formate-acetic acid-1M NaCl, pH 4 buffer, water, 0.05M Tris-HCl-1M NaCl, pH 8 buffer, water, 2M sucrose, pH 7, water and finally equilibrated in 0.005M Tris-HCl, pH 7.4 containing 0.001M each of Ca^{2+} , Mg^{2+} and Mn^{2+} at 4°C. Con A-Sepharose 4B was stored at 4°C in the same equilibration buffer in presence of sodium azide.

Preparation of WGA-Sepharose and RCA-Sepharose affinity matrices were also carried out by similar protocols. The coupled gels were washed in 1M NaCl containing buffers at pH 4 and pH 8 respectively, and stored in the equilibration buffer comprised of 0.02M Tris-HCl-0.15M NaCl, pH 7.4 in presence of sodium azide at 4°C.

GlcNAc-Sepharose 6B was prepared by coupling the amino sugar (347) to epoxy activated Sepharose (339). Sepharose 6B was washed with water and 0.6N NaOH. To 10g of the washed and suction dried gel added 10ml of 1,4-butanediol diglycidyl ether, 10ml of 0.6N NaOH containing 20mg of NaBH_4 and stirred at 25°C for 10 h. The gel was subsequently washed in excess of distilled water and then

with 0.1M NaOH. Epoxy activated gel was coupled to GlcNAc by addition of 400mg of the sugar in 10ml of 0.1M NaOH followed by incubation with stirring at 45°C for 15 h. The product was washed extensively with water, 0.05M Tris-HCl-0.5M NaCl, pH 8 and 0.05M sodium formate-acetic acid-0.5M NaCl, pH 4 buffers respectively before equilibration in 0.02M phosphate buffered saline, pH 7.2 at 4°C.

Asialofetuin was prepared by desialylation of 200mg fetuin in 0.1N sulfuric acid at 80°C for 1 h (58). The desialylated protein was dialysed against 0.1M NaHCO₃, pH 8.5, with three changes, at 4°C for 18 h. Asialofetuin was coupled to 20ml of packed CNBr activated Sepharose 4B by the method of Axen et al (14). The washed gel was suspended in 20ml water and 40ml of 2M Na₂CO₃. When the temperature of the slurry was maintained at 10°C added 0.6g of CNBr dissolved in minimal volume of dimethyl formamide and stirred for 5 min. The activated gel was washed with 0.1M NaHCO₃, pH 8.5 and coupled to asialofetuin as described for Con A-Sepharose.

Protein A was extracted from Staphylococcus aureus [Cowan strain 11] by digestion with lysostaphin according to the procedure of Sjoquist et al (309) and immobilized on CNBr activated Sepharose 4B as per the method of Axen et al (14).

Preparation of Protein-conjugates

A procedure adapted from The and Feltkamp was employed for the preparation of FITC-Con A (329). Con A [20mg] was dissolved

in 2ml of cold 0.25M Na_2CO_3 - NaHCO_3 , pH 9.0 buffer. To this solution was added 0.05mg FITC/mg protein and stirred gently at 4°C for 12-16 h. The uncoupled FITC was removed by extensive dialysis at 4°C against 1L x 3 changes of 0.02M Tris buffered saline, pH 7.4 containing 0.001M each of Ca^{2+} , Mg^{2+} and Mn^{2+} followed by dialysis in the same buffer in absence of metals. Alternatively, the free FITC could be separated from the FITC-protein conjugate by gel-filtration on a BioGel P4 column [1 x 32cm] in Tris buffered saline containing metals at 4°C to recover FITC-Con A in the void volume.

Conjugation of horseradish peroxidase [HRP] with lectins, immunoglobulins and serotonin was carried out according to the method of Nakane and Kawaio (236). Horseradish peroxidase [1-10mg] dissolved in 1ml of 0.3M NaHCO_3 , pH 8.5, was mixed with 50 μ l of 1% 1-fluoro 2,4-dinitrobenzene solution in absolute ethanol and incubated with gentle mixing at 25°C for 1 h, in order to block the free amino groups on the protein. To this solution added 1ml of 0.06M NaIO_4 and continued incubation in the dark for 30 min at 25°C to generate reactive aldehyde groups on peroxidase. The excess periodate was neutralized with 1ml of 0.32M ethylene glycol, keeping at 25°C in the dark for 1 h. The activated enzyme was dialysed overnight at 4°C against 500ml x 3 changes of 0.01M Na_2CO_3 - NaHCO_3 , pH 9.5 buffer. Similarly, dialysed the lectin, immunoglobulin or serotonin to be conjugated to peroxidase against the same buffer. Equal proportions (1:1, w/w) of

peroxidase and protein/serotonin were conjugated by mixing at 4°C for 4 h. Free aldehyde groups were subsequently blocked with 1% KBH_4 , adding 100 μl /ml of protein solution and then incubating at 4°C for 1 h. The HRP-conjugate was successively dialysed at 4°C against 1L each of 0.01M NaHCO_3 , pH 8.3, 0.02M Tris-HCl-0.05% Tween 20, pH 8.0, and 0.02M Tris buffered saline-0.05% Tween 20, pH 7.4, to minimize precipitation of the conjugate. The supernatant obtained on centrifugation at 5000 x g for 10 min was used for analytical studies.

Radiolabelling of Glycoproteins

The amino groups in the protein were N-acetylated with ^3H -acetic anhydride by the method of Finne and Krusius (85). The protein [500 μg] in one volume of aqueous medium was extracted twice with four volumes of chloroform:methanol [C:M], 1:2 (v/v) (325). To the delipidated protein pellet suspended in 1ml of 0.5M NaHCO_3 , pH 8.5, was added ^3H -acetic anhydride [$12 \times 10^6 \text{cpm}/0.2\text{ml}$ acetone] and incubated at 25°C for 30 min with periodic shaking. At the end of this interval added 42mg of solid NaHCO_3 to maintain pH at 8.5, followed by 10 μl of cold acetic anhydride and incubated further for 15 min at 25°C. The mixture was acidified with 100 μl of glacial acetic acid and dialysed extensively against 1L x 6 changes of distilled water at 4°C.

Glycoproteins were labelled at the carbohydrate side chains by the periodate oxidation- ^3H -borohydride reduction procedure of Spiro (315). The protein [100 μg] in 200 μl of 0.2M sodium acetate-

acetic acid, pH 5.6 buffer was treated with an equal volume of 0.02M NaIO₄ solution in water and incubated at 4°C in the dark for 16 h. The reaction was arrested by addition of 10µl of ethylene glycol and keeping in the dark at 25°C for 30 min. The solution was adjusted with 1M NaOH [25µl] to pH 8.0-8.5 and reduction reaction carried out by addition of NaB[³H]₄ [5 x 10⁶ cpm/20µl 0.01N NaOH], incubating at 25°C for 1 h. The solution was cooled in ice, acidified with 25µl of 6N HCl and dialysed at 4°C against 1L x 6 changes of distilled water.

The excess free radioactivity was better removed by precipitation of the protein with 8 volumes of ice-cold methanol and repeated washing of the pellet in 1:8, water:methanol, (v/v) at 0-4°C.

Radioisotope measurement

Samples labelled with ³H or ¹⁴C were counted in Bray's liquid scintillation fluid (38). The scintillant contained 60g naphthalene, 4g PPO, 0.2g POPOP, 100ml methanol and 20ml ethylene glycol made upto 1L in 1,4 dioxane.

Radioactivity of proteins fixed in gels after SDS-PAGE was determined by the protocol of Bonner and Laskey (35). The lanes on the slab gel were transversally cut into 0.5cm segments. Each polyacrylamide gel piece [0.5 x 1 x 0.15cm] was incubated at 37°C for 24 h in 1ml of H₂O₂:30% ammonia solution, 19:1 (v/v) to degrade the gel matrix. The resulting solution was counted in 4ml of Bray's scintillant against a background gel blank. The recorded

cpm values were subjected to quench correction. The counting efficiency in presence of 1ml of the H_2O_2 reagent was calculated to be 8.5% for tritium from the prepared quench calibration plots.

Amino-terminal Analysis

All solvents were redistilled under anhydrous, non-oxidising and aldehyde free conditions before use. Glass double distilled deionized water was used. Solvents and standard samples prepared for HPLC were filtered through 0.45 μm microfilters [Millipore]. All solvents employed in HPLC were degassed prior to introduction into the system.

I. Dansyl-Edman procedure: The Edman degradation protocol was modified from Gray (117). Glial protein [150 μg] or Con A [6 n moles] in 150 μl of 2% SDS in water was boiled at 100°C for 5 min. Kept aside a 50 μl aliquot for dansylation. To 100 μl of sample added 100 μl of 5% PITC in anhydrous pyridine, flushed the tube with nitrogen, sealed with parafilm and kept for 1 h at 37°C. To this solution added 10 μl of PITC directly, flushed with nitrogen, sealed and incubated further for 1 h at 37°C. The contents were diluted with 300 μl water, air dried and then dried overnight in vacuo over NaOH. The residue was treated with 200 μl of anhydrous TFA, kept for 1 h at 37°C and air-dried. This sample was partitioned by addition of 1ml 0.01N HCl and 2ml of n-butyl acetate, vortexed, centrifuged, and the upper organic phase containing the cleaved off N-terminal amino acid as the phenyl-

thiohydantoin derivative was aspirated off. The lower phase was extracted thrice with 2ml each of n-butyl acetate, air-dried and then dried in vacuo over NaOH for 30 min. The residue was suspended in 20 μ l of 10% SDS and 80 μ l of water and boiled for 5 min at 100°C to solubilize. Another 50 μ l aliquot was kept aside for dansylation. The remaining protein solution was made up to 100 μ l with water and subjected to the next cycle of Edman degradation.

Due to the insoluble nature of the protein, dansyl derivatization was carried out in presence of SDS according to method of Tapuhi et al (327). To the air-dried protein aliquot added 20 μ l of 10% SDS and 100 μ l of 0.04M LiCO₃, pH 9.5 buffer, and boiled for 5 min at 100°C. The solution was mixed with 5 μ l of dansyl chloride solution [2.5mg Dns-Cl/0.1ml acetonitrile] and incubated in the dark at 37°C for 1 h. The protein was precipitated with 5 volumes of ice-cold acetone and washed twice in acetone:0.1N HCl, 5:1 (v/v) to remove SDS. The dansylated protein pellet was air-dried and subjected to acid hydrolysis. Digestion of the protein was carried out with 200 μ l of aziotropic 6N HCl in nitrogen flushed and sealed ampules at 110°C for 18 h. The hydrolysate was dried, dissolved in 50 μ l of LiCO₃ buffer and centrifuged at 15,500 x g for 1 min in an Eppendorf microcentrifuge. The supernatant [20 μ l] was injected into the RP-HPLC system.

TABLE.1.

LICHROSORB RP-18 HPLC SOLVENT GRADIENT PROGRAM FOR
SEPARATION OF DNS-AMINO ACIDS.

Solvent A: 0.6% acetic acid-0.008% TEA-water,

Solvent B: methanol. Flow rate = 0.5 ml/min.

Time (min)	% Sol B	Time (min)	% Sol B
0	30	45	56
1	32	50	60
3	34	55	64
5	36	60	67
10	39	70	75
20	42	80	100
30	45	90	100
35	48	92	30
40	52		

Separation and identification of Dns-amino acids by RP-HPLC was standardized employing a Lichrosorb RP-18 column [5 μ m, 4 x 250 mm] and guard column [7 μ m, 4 x 30mm] connected to the HPLC system [LKB-Bromma, Sweden]. The procedure was adapted from Marquez et al (218) and utilized a modified gradient programmed between the solvents, Sol A: 0.6% acetic acid and 0.008% triethyl amine in water, and Sol B: methanol, at a flow rate of 0.5ml/min. The gradient system has been tabulated in Table.1. Dansyl-amino acids were detected by their fluorescence at excitation maxima = 333nm and emission maxima = 510nm, using a Shimadzu Spectrofluorophotometer RF-540 and identified by their respective retention times obtained with standard Dns-amino acids.

II. DABITC-PITC procedure: The DABITC-PITC manual micro-sequencing method of Chang et al employed a double coupling technique wherein a first coupling with the coloured Edman reagent, 4-N,N'-dimethyl aminoazobenzene 4'-isothiocyanate was followed by coupling with phenyl isothiocyanate to achieve a quantitative coupling reaction (45,44). After acid cleavage and conversion, the red coloured DAB-thiohydantoin [DABTH] amino acids were easily distinguished from the by products which were coloured blue. As ensuing DABITC-PITC cycles were impeded by a progressive protein insolubility, only the first N-terminal amino acid was identified by this method.

Glial protein [100 μ g] or Con A [2-4 n moles] was washed free of salt and other contaminants with water:methanol, 1:5 (v/v)

followed by acetone:0.1N HCl,5:1 (v/v) and dried in vacuo. To the protein suspended in 20 μ l of 50% pyridine added 10 μ l of DABITC solution [2.82mg/ml pyridine], vortexed, flushed with nitrogen, sealed with parafilm and incubated at 70°C for 50 min. The second coupling was commenced with the addition of 3 μ l of PITC. The mixture was vortexed, flushed with nitrogen, sealed and incubated at 70°C for 15 min. By products like diphenylurea and diphenylthiourea were extracted out by successive partitioning [4-6 times] with 200 μ l aliquots of heptane:ethyl acetate:67% pyridine,4:2:3, upper phase (v/v/v). The yellow coloured upper organic phase containing the by products was discarded while the lower aqueous phase was dried in vacuo over P₂O₅ and KOH, adding small aliquots of ethanol periodically to ensure complete drying of the sample. The residue was treated with 50 μ l of anhydrous TFA, vortexed, flushed with nitrogen, sealed and incubated at 55°C for 15 min. The solution was evaporated to dryness in vacuo over KOH. To the dried sample added 50 μ l of water and extracted thrice the liberated N-terminal DAB-4'-thiazolinone amino acid derivative with 100 μ l each of n-butyl acetate. The pooled butyl acetate extract was evaporated in vacuo over P₂O₅ and KOH. Conversion to red coloured DABTH was effected in 50 μ l of 50% TFA, sealing under nitrogen and incubation at 80°C for 10 min. The DABTH-amino acid solution was dried completely over P₂O₅ and KOH in vacuo, dissolved in 5-10 μ l of 80% ethanol and identified by TLC.

Standard DABTH-amino acids were prepared by a procedure adapted from Chang (46). To 1mg amino acid in 200 μ l of TEA-acetic acid buffer [50ml water, 50ml acetone, 0.5ml triethyl amine and 5ml of 0.2M acetic acid, adjusted to pH 10.65] 100 μ l of DABITC solution [1.13mg/ml acetone] was added, mixed and incubated at 54°C for 1 h. The contents were dried in vacuo over P₂O₅ and KOH, redissolved in 200 μ l of 50% TFA and incubated at 54°C for 45 min. The solution was evaporated in vacuo and the residue was dissolved in 200 μ l of 80% ethanol. DABTH-diethylamine was co-chromatographed with every sample as an internal reference standard. This derivative was prepared by mixing together 5 μ l of diethylamine, 58.8 μ l of DABITC solution [2.4 mg/ml ethanol] with 441 μ l of ethanol and was used directly. All DABTH-derivatives were stored at -20°C.

Two dimensional thin layer chromatography was carried out on polyamide sheets [2.5x2.5cm, Schleicher and Schull, Germany]. The solvent system employed in TLC were,

1st dimension - acetic acid:water, 1:2 (v/v)

2nd dimension - toluene:n-hexane:acetic acid, 2:1:1 (v/v)

The spots were visualized after the run by exposing the dry plates to HCL vapours.

RP-HPLC of Protein

Derivatized protein samples were chromatographed on reverse phase Lichrosorb RP-8 column [5 μ m, 4 x 250mm] and guard column [7 μ m, 4 x 30mm] HPLC system. Two gradients, I and II run at the

same flow rate of 0.5ml/min but of differing shapes, formed between the solvents, Sol A: water-0.1% TFA, and Sol B: isopropanol-0.1% TFA were employed. The respective programs for Gradient I and Gradient II are tabulated in Table.2.

Separation and identification on HPLC of carbohydrate derivatives

Fluorescent labelling of carbohydrates at their reducing termini with 2-aminopyridine was attempted by the method of Hase et al (137,139). The potential aldehyde groups of carbohydrate were coupled to 2-aminopyridine by reductive amination with NaCNBH_4 . The product was isolated by adsorption on DOWEX 50 [H⁺] followed by washing and elution with 0.7M aqueous ammonia. The 2-aminopyridyl derivatives were separated by HPLC on LKB-Ultropac TSK-NH₂-60 column [5 μ m, 4.6 x 250mm] with LKB Ultropac Lichrosorb RP-8 [7 μ m, 4 x 30mm] precolumn. A gradient elution system between the solvents, Sol A: 0.015M KH_2PO_4 -KOH, pH 5.2 and Sol B: Acetonitrile, was employed at a flow rate of 1ml/min. The gradient program constituted an isocratic elution at 80% B for 25 min, a linear gradient from 80-35% B for 45 min followed by isocratic elution in 35% B for 10 min. The peaks were detected in a Shimadzu Spectrofluorophotometer RF-540 at an excitation maxima = 320nm and emission maxima = 400nm. Standard mono and oligosaccharide derivatives were quite well resolved under the given conditions. Characterisation of glycan side chains was however limited severely by the inability to release sufficient quantities of oligosaccharides from the prepared glial glyco-

TABLE.2.

LICHROSORB RP-8 HPLC SOLVENT GRADIENT PROGRAMS FOR
THE SEPARATION OF PROTEIN DERIVATIVES.

Solvent A: water-0.1% TFA, Solvent B: isopropanol-
0.1% TFA. Flow rate = 0.5ml/min.

GRADIENT I		GRADIENT II	
Time(min)	% Sol B	Time(min)	% Sol B
0	0	0	0
3	0	5	30
20	30	50	60
40	60	70	100
60	100	80	100
70	100	85	0
75	0	90	0
80	0		

proteins to enable their detection as fluorescent derivatives on HPLC.



CHAPTER - III

RESULTS AND DISCUSSION

PART I: ISOLATION OF NEURONS AND GLIAL CELLS FROM HUMAN FOETAL BRAIN

Morphological studies of the central nervous system have revealed an incredibly complex network of neuronal and glial somata, axons, dendrites and glial processes, myelin and capillaries. The biochemical characterisation of this tissue was a reflection of the sum of the individual properties of each separate cell type. It was apparent that neurons, astrocytes, oligodendroglia, microglia and the other cells composing the brain or even specialized regions of a single cell, such as, the dendritic tree, neuronal pericarya and axon, presented their own particular ultrastructure, function and complement of subcellular organelles. Therefore most probably they also specified an unique composition and biochemistry. Over the years many efforts have been made to resolve this complex tissue into individual cellular components and determine their respective contributions to the total biochemistry of the brain. A logical approach to the elucidation of any complex structure was to separate the individual components and then to study them in isolation. Microdissection techniques pioneered by Deiters, Lowry, Giacobini, Hyden and others (279) were restricted to certain large and possibly highly specialised or atypical cells, could not furnish pure glial cells, and of course, limited studies to very small samples.

The complexity of the nervous system, with its intertwined processes and minimal extracellular space, presumably inhibited attempts to extend the normally applied cell separation techniques to brain. The first major effort to prepare a cell suspension and CNS cells in bulk was credited to Korey and co-workers (176). They attempted to isolate glia from white matter. Although the cell separation was crude, the basic techniques used by these investigators have been continued in most of the subsequent investigations.

Several elegant procedures are now available for the bulk isolation of neurons and glial rich fractions from rat brain (280,147,75), rabbit brain (31), bovine brain (261), embryonic chick ganglia (257), and postmortem human brain (158). All these procedures were based on the following guidelines.

- i) Preparation of a suspension of free cells by disruption of intercellular adhesion without destruction of the cells. The extracellular matrix was disrupted mechanically by consecutive sieving through nylon or steel meshes (176,280,158), preincubation with or without hydrolytic enzymes such as trypsin (261), hyaluronidase and collagenase (148) or by employing organic media (288). Most popular methodologies combined a preincubation step with disruption through sieving.
- ii) Separation of different cell types by centrifugation. Most procedures utilized discontinuous density gradients of sucrose and/or Ficoll or continuous gradients of sucrose, Ficoll or

Percoll. A major consideration during cell isolation was that cellular integrity had to be maintained through the various steps involved. Temperature, pH, mechanical stress during disruption and centrifugation, hypertonicity of the gradient during cell separation, exogenous and endogenous proteolytic activity were factors affecting cell integrity. Hence the selection of a suitable medium which could maintain cell integrity was obligatory.

All media used so far incorporated a high molecular weight material, either Ficoll, polyvinylpyrrolidone, gangliosides or albumin. The mode of action of these polymers was not clear but they appeared to have a protective action on cell membranes, stabilising them and thereby reducing cell breakage. Low temperatures [0-4°C] were ideal as they reduced exogenous and endogenous hydrolytic activity. An optimum of pH 6 was favoured by many workers though there have been reports of cell isolation carried out at physiological pH 7.4. Processing freshly obtained brain tissue within minimal period of time obtained better yields of intact cells. However frozen tissue have also been used. The cellular integrity was better preserved when freezing was carried out in the presence of cryoprotective agents like glycerol or DMSO. These agents prevented the formation of ice crystals, the main factor causing tissue damage during the process of freezing and thawing (87). Protease inhibitors, bacteriostatic and

fungicidal agents have also been employed to minimize proteolytic and other degradative activities.

The objective as already stated was to study cell surface glycoproteins in glial cells of developing human brain. A preliminary aim to achieve this goal would naturally entail the isolation of human foetal glial cells. The methodology optimised was a modification of previously published procedures (75,261,158).

Collection of human foetal brain tissue

The collection and study of developing human foetal brain was approved by the ethics committee, Department of Obstetrics and Gynaecology, Medical College Hospital, Trivandrum - 695 011. Human foetal specimens were obtained from the Department of Obstetrics and Gynaecology, Medical College Hospital, Trivandrum - 695 011. The procured second trimester human fetuses were expelled through spontaneous or Ethacridine induced abortions. Third trimester and still born fetuses were mostly intra uterine deaths due to unknown causes. The crown-rump [CR] lengths measured in foetal position, correlated with age as calculated from the last menstrual period. The relationship between CR length and foetal age given by Hamilton et al (132) was used as a general guideline. The foetal specimens studied were categorized on the basis of CR lengths into IInd trimester [CR = 9-20cms] and IIIrd trimester [CR = 24-36cms] groups.

The foetal specimens were collected in ice, brought to the laboratory and processed within 12 h of abortion. The brain was dissected out, the meninges were removed and the tissue stored at -20°C in a cryopreservative Medium I made up in Buffer A [0.01M phosphate or Tris-maleate-0.1% NaN_3 -0.0001M PMSF-0.002M benzamidine.HCl, pH 6.0] containing 20% DMSO, 5% Fructose and 8% Dextrose. Brains have been stored in this medium for 1 year without appreciable loss in integrity. Dimethylsulfoxide served a cryoprotective role while dextrose, fructose, protease inhibitors and azide contributed to the preservative function of the medium. It has been documented that brain cells were better preserved at pH 6.0.

Isolation of Neurons and Glial cells from human foetal brain tissue

Brain tissue stored at -20°C was minced and brought to 25°C . The cryopreservative medium was removed by centrifugation at 4°C at $2000 \times g$ for 10 min. All further steps were carried out between $0-4^{\circ}\text{C}$ unless specifically mentioned. The tissue was suspended in 2 volumes (w/v) of Medium II [0.32M sucrose-2-4% Ficoll 400 in Buffer A, pH 6.0] and mechanically disrupted by forcing successively through 1mm Pasteur pipette, 80 and then 200 mesh nylon sieves under mild pressure into a Buchner flask. The resulting cell homogenate comprising of a suspension of free cells was diluted further in Medium II to obtain a protein concentration of 5-20mg/ml (37). Extensive cell lysis rendered

highly viscous cell homogenates. In such situations, the cell homogenate was mixed with 0.1-3% DNAase II [w/w protein:DNAase III] and incubated for a period of 1-10 h, to digest the released nuclear material. The homogenate was suspended with 12 strokes in a loose fitting Dounce homogeniser just prior to loading on a discontinuous sucrose density gradient. The discontinuous density gradient was prepared by layering 5ml of 2M, 7ml of 1.55M, 7ml of 1.35M and 9ml of 0.9M sucrose in Buffer A. The cell homogenate [10ml] was carefully loaded over this gradient and centrifuged [HS-4 rotor in Sorvall RC-5B or Spinco SW.25.1 rotor in Beckman L5-50 centrifuge] at 3300 x g for 15 min. Pure neuronal pericarya banded at the 2M interface and was collected with a syringe. Myelin and cell debris were found in the 0.9M layer while large particulate material, undisrupted tissue and capillaries pelleted at the bottom of the centrifuge tube. The 1.35M and 1.55M interfaces contained a crude glial fraction viz. glial cells contaminated by neurons. These fractions were collected, pooled, resuspended as before, layered over 8ml of 1.65M sucrose and centrifuged at 3300 x g for 45 min. The resulting neuronal pellet was pooled with the neurons obtained at the 2M sucrose interface. Relatively pure glial were collected from the 1.65M interface. The cells in hypertonic sucrose medium were diluted slowly with gentle stirring in 2 volumes of ice-cold Buffer A and pelleted at 3300 x g for 10 min. The cells were washed thrice with Buffer A-

0.15M NaCl, pH 6.0 [Medium III] and stored in the same medium in ice until further use.

Another more convenient procedure has also been employed for the separation of neurons and glial cells. The cell homogenate in Medium II containing 5-20mg protein/ml was diluted 1:1 with 2M sucrose in Buffer A, suspended by 12 strokes with Dounce homogeniser, layered [30ml] over 8ml of 1.65M sucrose and centrifuged at $3300 \times g$ for 45 min. Pure neurons pelleted and were collected. The 1.65M interface was resuspended and centrifuged over 8ml of 1.65M sucrose for 45 min as before. Relatively pure glia banded at 1.65M interface at the end of the 2nd or 3rd centrifugation steps. The neuronal and glial cell fractions were washed thrice in Medium III and stored in ice.

Based on protein estimated in the total cell homogenate, neuronal and glial cell fractions respectively, yields were calculated to be 5-20% for glial cells and 1-15% for neurons. Yields were found to be higher in gestation ages 16-24 weeks for neurons and 20-36 weeks for glial cells.

The cells were identified by light and phase contrast microscopy of wet preparations. Neurons appeared as elongated cells shorn of processes with prominent nuclei and nucleoli (Fig.5.). Glial cells were mostly astrocytes with small cell bodies with a radiating mesh of elongated processes. A few spherical oligodendroglia could be observed (Fig.6.). Morphologically the cells from foetal brain appeared similar to

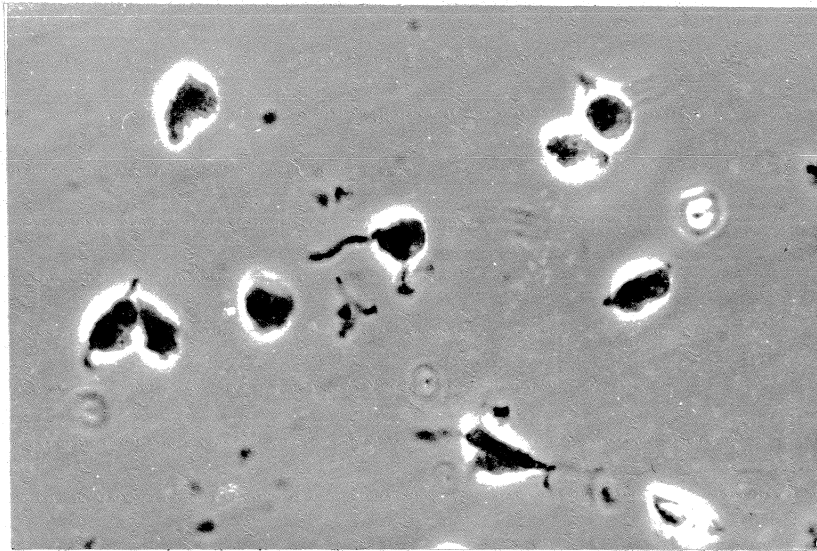


Fig.5. Human Foetal Neurons. Phase Contrast Micrograph (pH x 300).

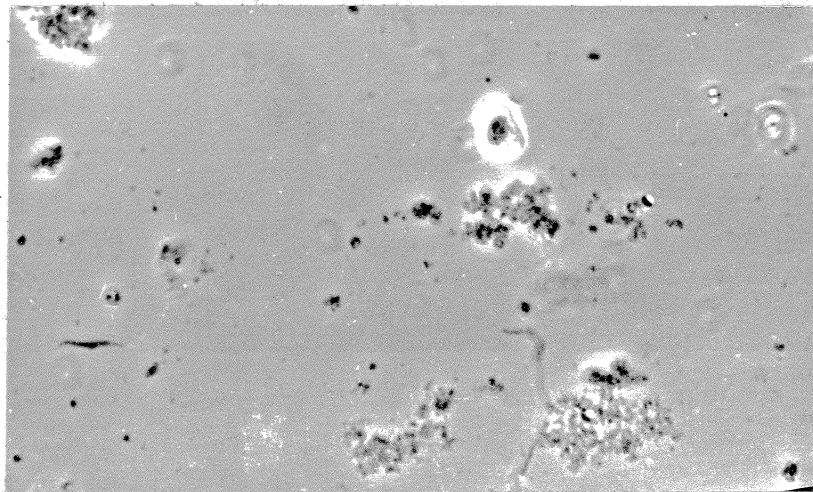
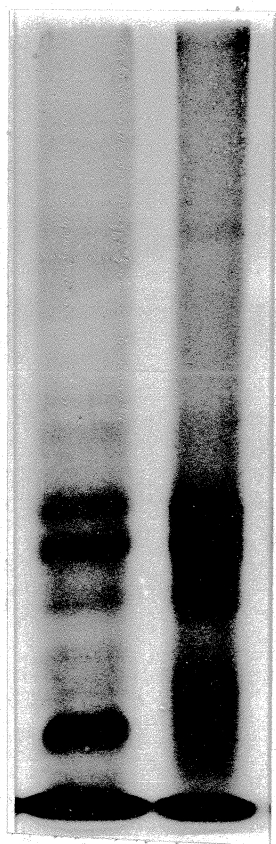


Fig.6. Human Foetal Glial Cells - Astrocytes and Oligodendroglia. Phase Contrast Micrograph (pH x 300).



1 2

Fig.7. 7% SDS-PAGE of delipidated neurons and glial cells. Coomassie Blue stain. Experimental details are given under General Methods. Lane 1: neurons, Lane 2: glial cells.

those isolated from other species and correlated with the descriptions already published in literature. Nevertheless, our criteria for ascertaining purity of glial fractions was to observe the complete removal of neuronal cells from the glial preparations. Recentrifugation over 1.65M sucrose was repeated until glial fractions were free of neurons. Generally this was achieved by the end of the second density gradient centrifugation step. Neurons were separated on the basis of rate zonal migration of these cells in the centrifugal field, a time dependant factor, whereas glial cells banded isopycnically on the 1.65M density layer.

The brain cell types isolated by the present procedure appeared morphologically intact and distinct from one another on microscopy. Delipidated and SDS solubilised glial and neuronal cells rendered dissimilar profiles on SDS-PAGE (Fig.7.). Further studies were pursued with the above mentioned glial cell rich fractions.

PART II: ISOLATION OF CONCAVALIN A BINDING GLYCO- PROTEINS FROM GLIAL CELL SURFACES

The well documented role of cell surface glycoproteins in cell-sociology has been described in the introductory chapters. Physicochemical characterisation of a few of these surface glycoproteins from developing human glial cells, would contribute further towards understanding the molecular basis of glial cell development and interaction.

Most procedures available for characterisation of a given protein, required the species to be present in pure form and in sufficient quantity. The priority therefore entailed the selection of a methodology suitable for the isolation of a homogeneous preparation of glial cell surface glycoproteins in sufficient yield. This was a complicated endeavour considering the heterogeneity of nervous tissue; largely circumvented by the development of procedures for isolation of specific cell types and highly enriched subcellular fractions; and because in the cell membranes that have been well characterised, glycoproteins were classified as integral membrane components (308).

Membrane proteins have been roughly classified into two categories depending on the conditions required to extract them from the membranes. Peripheral or extrinsic proteins were associated with one or other of the membrane surfaces by electrostatic and dipolar interactions and could be solubilized by chelating agents or by varying the pH/ionic strength of the

medium. Integral or Structural proteins contained regions partly embedded in the membrane matrix or lipid bilayer, that is, they were presumed to be stabilized by hydrophobic forces and were not easily solubilised and separated from membrane lipids into aqueous, low ionic strength, neutral solutions, compatible with the procedures used for further purification of the glycoprotein. Their solubilization required disruption of the bilayer by detergents, chaotropic agents or organic solvents, liable to cause denaturation and loss of biological activity. Another limitation for many extracted integral membrane glycoproteins was their tendency to aggregate. One of the causes of such aggregation was interaction between the hydrophobic segments of the peptide chain, normally buried within the lipid bilayer of the membrane. Protein denaturation during extraction could increase aggregation by exposing further, the hydrophobic sections of the peptide chain.

Some workers have avoided these solubilization related problems by using glycopeptides, released from cells or membrane preparations by proteolytic digestion, as their starting material for characterisation studies (179,85). This strategy was convenient if the objective was to characterise the carbohydrate moieties of glycoproteins. However, only a generalized picture of glycan composition and structure was obtained with little indication of in-situ localization of the glycopeptides of interest. A study of intact glycoproteins, though problematic

with regard to solubilization and handling, yielded more specific information.

Approximately, 90% of the brain glycoproteins were integral components of cell surface and internal membranes (216,269). Plasma membranes isolated from immature neurons (146), glial cells (289,290,260), synaptosomes (125) neuroblastoma and tumoral glial cells (126) have been shown to contain glycoproteins. More than 80% of the carbohydrate in nervous system glycoproteins were linked via N-acetyl glucosamine to amide nitrogen of asparagine residues of the protein moiety. Most of the N-glycosidically linked oligosaccharides of brain were of complex-type and were characterised as (a) containing galactose, fucose, and sialic acid in addition to mannose and glucosamine, and (b) being more extensively branched when compared to the neutral high-mannose glycopeptides (255).

Brain glycoproteins have been isolated using conventional procedures (229,266,340) or by affinity chromatography of detergent solubilised integral membrane glycoproteins on immobilized lectins (363,298,224,306) or specific antibodies (49,111,52).

The presence of cell surface glycoproteins on human foetal glia were confirmed as follows:

a) Radiolabelling of glial cells by periodate oxidation-tritiated borohydride reduction

This method introduced a tritium label specifically into carbohydrate units. The technique involved an initial oxidation of monosaccharide moieties with periodate to render aldehydes which were then labelled by reduction with tritiated borohydride. The procedure developed by Lee Van Lenten & Ashwell (344) for soluble glycoproteins was adapted for application to membrane bound glycoconjugates by many workers. Our method was a modification from those of Gahmberg et al (99,101) for labelling of cell surface glycoconjugates.

Glial cells [5mg] were washed thrice in 1ml ice cold 0.02M phosphate-0.15M NaCl-0.1% NaN_3 [PBS] pH 6.0 at 15,500 x g for 20 sec, and suspended in 1ml of the same buffer. The test sample was made 0.005M NaIO_4 by adding the weighed amount of solid periodate and incubated in the dark in ice [0°C] for 10-15 min. The cells were washed three times with cold PBS pH 8.0 at 15,500 x g for 20 sec and suspended in 1ml PBS pH 8.0. To this was added $\text{NaB}^{[3]\text{H}}$, [1-2 x 10^5 cpm/10-100 μ l 0.01N NaOH] and kept for 30 min at 25°C. The pelleted cells were washed 8 times with cold PBS pH 6.0 to remove free radioactivity. A control was run simultaneously wherein no periodate oxidation was carried out to correct for non specific labelling. About 5-10% of the total radioactivity was incorporated into glycoconjugates.

The labelled cells were delipidated by washing first with methanol and then extracting respectively with 3ml each 2:1,C:M (v/v), 1:1,C:M (v/v) and 1:2,C:M (v/v), centrifuging at 2000 x g for 15 min at 25°C. The pellet was dried in vacuo to remove organic solvents and then solubilized in 2% SDS by boiling for 5 min at 100°C. The organic phases were pooled and evaporated off. The delipidated pellet and lipid phase were estimated for radioactivity as given under Materials and General Methods. The incorporated tritium was distributed equally between lipid and protein indicating the presence of glycolipid and glycoprotein on glial cell surfaces.

b) Con A-FITC labelling of glial cell membranes

Freshly isolated glial cells [2mg] were washed thrice with 0.02M Tris-Maleate-0.15M NaCl-0.1% NaN₃ pH 6.0 [TMS] at 4°C at 3000 x g for 5 mins. To one aliquot of the packed cells was added 0.2ml TMS pH 6.0-4% BSA and 0.8ml Con A-FITC [5mg/ml in 0.02M Tris-HCl-0.15M NaCl-0.1% NaN₃ [TBS], pH 7.4]. The other aliquot was run as a control and contained cells in 0.2ml TMS, pH 6.0-4% BSA-2M α -methyl glucoside [α -MG] and 0.8ml Con A-FITC. Both samples were incubated in ice for 30 min. The cells were washed thrice with TMS, pH 6.0, containing 1% BSA at 4°C and observed under fluorescence microscope (Fig.8.). Glial cell surfaces were labelled by FITC-Con A indicating presence of Con A-binding cell surface glycoproteins. The controls were not labelled confirming the specificity of Con A-binding which was reversed in presence

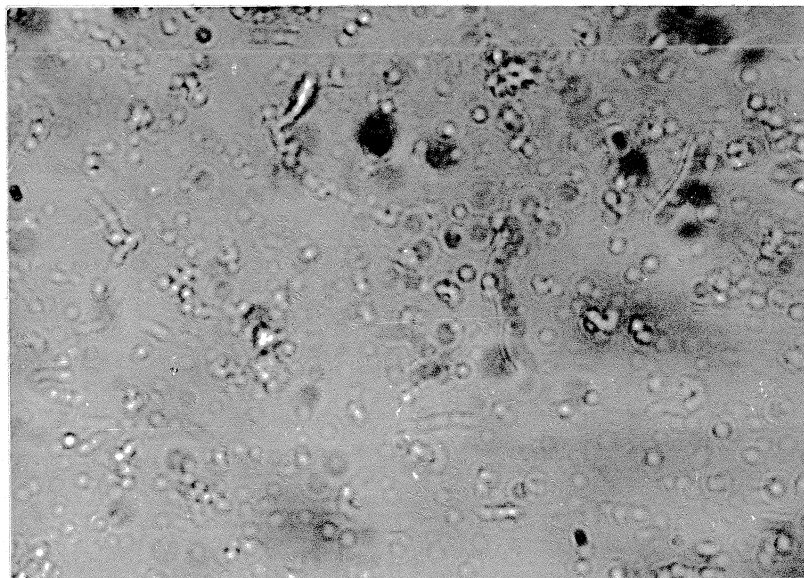


Fig.8. Fluorescence photomicrograph of FITC-Con A
labelled human foetal glial cells (300x).

of its specific hapten inhibitor α -MG. BSA prevented cell lysis in presence of Con A-FITC and thereby served a protective function.

c) Tritium labelling of cell surface glycoconjugates using Galactose oxidase (100)

Three samples containing 5mg each of glial cells were washed thrice with cold PBS, pH 6.0 at 15,500 x g for 15 sec. To these samples were added 0.95ml PBS, pH 6.0 and 50 μ l 0.2M cold sodium borohydride in 0.01 N NaOH and incubated at 25°C for 30 min with occasional stirring. This was to prevent non-specific labelling. The cells were washed 4 times in cold PBS, pH 6.0. To one aliquot [test] added 200mU Neuraminidase and 2.5U Galactose oxidase. To the second aliquot added Galactose oxidase [2.5U] alone. The third sample served as a control and contained no enzymes. All the samples were incubated at 37°C for one hour with occasional shaking. The cells were washed thrice with cold PBS, pH 6.0, suspended in 1ml buffer, treated with NaB[³H], 11 x 10⁵ cpm/50-100 μ l 0.01 N NaOH], and kept at 25°C for 30 min. The cells were washed 8 times in cold PBS, pH 6.0 until the washings were free of radiolabel. The pellets were suspended in 0.2ml buffer and counted for tritium. No label was incorporated on the cells over the control. Thus glial cells were not labelled by this technique.

Gahmberg and Hakomori (102) studied labelling of neutral glycosphingolipids in normal NIL cells and transformed NIL_{py} cells and observed that the specific activity of the labelled glyco-

lipids was 10 to 20 times higher in the transformed cells. These glycolipids were assumed to be 'cryptic' or less accessible in normal cells. This property of crypticity may be involved in many of the observations of changes in cell surface antigens with development and oncogenesis.

Our initial rationale for the isolation of some of these Con A-binding glycoproteins from human foetal glial cell surfaces was to prepare glial plasma membranes which could subsequently be used as a source for the glycoproteins of interest. A procedure modified from previously published methodologies (160,154,153) was adopted. Glial cells [100-200mgm protein] were suspended in 10-20ml of cold hypotonic Buffer B [0.01M Tris-HCl-0.0001M PMSF-0.1% sodium azide-0.005M 2-ME, pH 7.4] and stirred at 0-4°C for 1 h. The cells were lysed in Potter Elvehjm homogeniser with 45 strokes and centrifuged at 1500 x g for 15 min. The supernatant was recentrifuged at 1500 x g for 15 min. The pooled pellets were suspended in Buffer B containing 2% sucrose (w/v) and 3% DNase II (w/w protein) with Dounce homogeniser, loaded over 28ml of a 25-40% (w/v) continuous sucrose density gradient in Buffer B at 0-4°C, and centrifuged in a Spinco type SW.25.1 rotor at 100,000 x g for 180 min. Plasma membranes banding near the top of the gradient were collected, washed at 40,000 x g for 15 min in Buffer B and stored at -20°C.

Plasma membranes were characterised on the following criteria.

- a) Microscopy - observed under the limited magnification of 40-100x available with Light and Phase contrast microscopes.
- b) Isopycnic density - Plasma membranes banded at a sucrose concentration of 34% (w/v) corresponding to a density of 1.12.
- c) Estimation of plasma membrane enzyme markers - Glial cells and their plasma membrane fractions were assayed for the membrane markers, (i) 5' Nucleotidase (10), (ii) Acetyl choline esterase (195,150), (iii) Glutamine synthetase (222), (iv) Carbonic anhydrase (97,71) and (v) Sialic acid (351) respectively.

Bovine brain glial cells and plasma membrane fractions contained detectable 5'Nucleotidase and Acetyl choline esterase activities. Human foetal glial cells were positive for Acetyl choline esterase and sialic acid only. However, none of the specified membrane markers could be detected in the glial plasma membrane fractions from human foetal brain. The limitations of extremely low yields as well as improper characterisation of the plasma membrane preparations obtained, precluded our pursuing this route further.

The procedure of Zanetta et al for the isolation of synaptosomal plasma membrane glycoproteins by affinity chromatography on Con A (364) was extended to glial cells. Human foetal glia [50mg protein] stored in PBS, pH 6.0 at 0-4°C were washed once in distilled water and once in methanol at 2000 x g for 15 min. The cell pellet was delipidated by sequential extractions with 6ml each of 1:2,C:M (v/v), 1:1,C:M (v/v) and

2:1,C:M (v/v), stirring for 2 min and pelleting down at 2000 x g for 15 min at 25°C. The delipidated protein was washed with methanol, dried in vacuo and suspended in 7ml of 0.02M Tris-HCl, pH 7.0 containing 10% 2-ME saturated with EDTA and kept at 4°C overnight. This step released peripheral membrane proteins, broke down aggregates possibly formed by interactions between cations and proteins, and also caused breakage of disulfide bonds. The protein was centrifuged at 100,000 x g for 60 min and the ensuing pellet solubilised in 5ml of 0.02M Tris-HCl-0.1M NaCl-4% SDS-0.002M 2-ME, pH 7.0, following which the pH was adjusted to 8.0 with 1M NaOH. Alkylation of sulfhydryl groups was effected by adding 10 fold excess the protein concentration of iodoacetamide, flushing and sealing in nitrogen, and incubating for 12 h in dark at 25°C. The alkylated protein was dialysed against 49 volumes of distilled water at room temperature for 48 h to bring down SDS concentration to 0.08%. The 100,000 x g for 60 min supernatant containing about 10-15mg protein/ml (64) was loaded at 25°C on Con A-Sepharose 4B [6ml gel/ml sample] equilibrated in 0.02M Tris-HCl-0.1M NaCl-0.0002M PMSF-0.1% NaN₃, pH 7.0 [Buffer C] containing 0.04% SDS. The affinity column was washed with the same buffer and eluted at 25°C with 0.5M α -MG in Buffer C-0.04% SDS. The Con A eluate was precipitated with 4 volumes methanol and 1.5 volumes ethyl acetate and washed with 80% methanol to remove the hapten sugar.

On SDS-PAGE it was observed that the major protein band corresponded to Con A in molecular weight, suggesting the leaching of Con A from the matrix. Zanetta et al (365) have reported similar situations which has been attributed to two factors mainly.

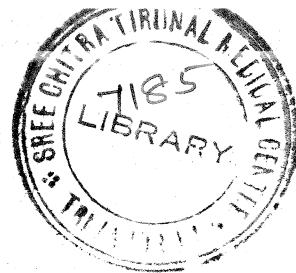
a) Presence of noncovalently bound Con A on the matrix. This was removed by prolonged and repeated washings of coupled gel at alkaline pH 9 and acid pH 4 in 0.1M buffers in presence of 1M NaCl, followed by washing in 0.5M sugar inhibitor and finally in buffer containing 0.08 % SDS. The gel was then equilibrated with 0.01M metals [Ca^{2+} , Mn^{2+} and Mg^{2+}] before bringing it to Tris buffer-0.04% SDS.

b) Formation of degradation products during chromatography. This effect was more significant at ambient temperatures and could be reduced by using concentrated protein solutions and minimum gel necessary for binding of specific glycoproteins.

Con A is made up of 4 subunits held together by noncovalent, hydrophobic forces. Detergents and chaotropic agents used for the solubilization of membrane glycoproteins could dissociate the native lectin molecules. Previous reports have indicated the occurrence of possible hydrophobic interactions between lectins immobilized on CNBr-activated Sepharose and water soluble and detergent solubilised glycoproteins (55,234). The effect of detergents on the activities and stability of immobilized lectins have been studied by Lotan et al (207). According to them anionic

detergents caused the most pronounced deleterious effects on the glycoprotein binding efficiency of Con A-Sepharose than cationic or zwitterionic detergents at concentrations of 1-2.5% .Con A was sensitive to ionic detergents even at 0.1% concentration. Sodium lauryl sulfate, a potent protein denaturant, under mild conditions [0.05% SDS for 1 h at 23°C] decreased the binding efficiency of Con A by 30% which correlated with the release of noncovalently bound lectin subunits from Con A-Sepharose. Even longer exposure [0.05% SDS for 16 h at 23°C] or more drastic conditions [1% SDS for 1 h at 23°C] did not dramatically decrease the activity of immobilized Con A. The binding efficiencies however, were markedly diminished due to extensive leaching of lectin subunits from the matrix. A higher SDS concentration [0.1%] was more harmful, especially after prolonged incubation. Nonionic detergents such as Nonidet P-40 or Triton X-100 at concentrations of upto 2.5% did not decrease the binding of glycoproteins to Con A-Sepharose by more than 25% . Helenius & Simons (145) had observed that Triton X-100 was very inefficient in breaking noncovalent bonds which were responsible for the quaternary structure of multimeric proteins. These observations suggested that nonionic detergents were most suitable for lectin affinity chromatography because their effects on lectins were minimal.

Considering these above factors lectin affinity chromatography was attempted, replacing SDS with the nonionic detergent Triton X-100. Chromatography was carried out at 4°C in TBS, pH



7.4-0.1% Triton X-100-0.0002M PMSF [Buffer D]. Glial proteins solubilized by Zanetta's procedure were dialysed against 0.1% Triton X-100 at 4°C before loading on the gel. The column was washed with Buffer D and eluted at 25°C with 0.5M α -MG in Buffer D. The eluted fractions were concentrated by ultrafiltration through YM-10 membrane and dialysed against cold water to remove α -MG. The leaching of Con A as revealed by SDS-PAGE, prevailed undiminished in spite of the presence of nonionic detergent and lower operating temperatures.

To verify whether this excessive leaching of Con A was caused by the presence of detergent, isolation of glycoproteins was carried out in the total absence of detergents. Glia were delipidated in chloroform-methanol and suspended in 0.02M Tris-HCl, pH 7.0-1% 2-ME-saturated with EDTA and kept at 4°C for 2 h. All further steps were carried out at 0-4°C. The suspension was centrifuged at 3000 x g for 20 min, the pellet washed thrice in water to remove soluble proteins, suspended in TBS-0.001M PMSF, pH 8.0 and sonicated at a frequency of 22 μ for 30 sec x 4 times. The pH was adjusted to 7.4 with dilute acetic acid and the turbid protein homogenate [0.2-0.5mg protein/ml by Bradford assay] was filtered through Whatmann No:1 filter paper to remove large particulate material. About 50% of the total protein was in the filtrate which was loaded on an equal volume of Con A-Sepharose 4B. The column was washed at 4°C with TBS, pH 7.4, and eluted at 25°C with two bed volumes of 0.5M α -MG in the same buffer. The

Con A eluate was concentrated by ultrafiltration through YM-10 to 5ml and dialysed extensively at 4°C against 3-4 changes x 1L of distilled water. Further concentration if required was achieved by flash evaporation or by precipitation in presence of 1% SDS with 4-5 volumes of cold methanol or acetone. This protocol also yielded an eluate containing Con A as the major protein component illustrating that detergent was not the only factor responsible for Con A leaching.

Prior delipidation of glial samples before the affinity chromatography step was carried out for basically two reasons - (a) an increase in the binding efficiencies of the glycoproteins of interest was imperative in absence of interfering glycolipid, and (b) presence of hydrophobic lipid could augment an already prevalent problem of leaching of Con A subunits from the matrix. Solubilization of the protein in the absence of detergents was difficult to achieve. Sonication was therefore used as a means to render homogenous cell suspensions in the absence of detergents. The suspensions prepared from delipidated glial cells, though filtered, caused considerable column blockage indicating ineffectiveness of the applied conditions to break down particulate aggregates. Increasing the frequency and/or time of sonication, mandatory to achieving better homogenisation, resulted in loss of carbohydrate. The loss was upto 25% as determined from carbohydrate estimation of samples dialysed extensively before and after sonication at 22 μ frequency for

30 sec x 4-8 times (63). Glial cells could be homogenised more easily without preliminary delipidation to render solutions more compatible with affinity chromatography.

Solubilisation was finally effected in a buffer consisting of 0.025M Tris-0.7M glycine, pH 7.8, used for transblotting of hydrophobic integral membrane proteins (311). Glial cells [30mg] were washed thrice at 5000 x g for 10 min [4°C] with distilled water. The cells were homogenised in 50ml of 0.025M Tris-0.7M glycine-0.1% NaN₃ [Buffer E] pH 7.8, with 10 strokes in Potter Elvehjem homogeniser followed by stirring for 1 h at 4°C. The pH was adjusted to 7.4 with dilute acetic acid and centrifuged at 150 x g for 1 min to settle large particulate material. The supernatant was chromatographed batchwise by mixing with an equal volume of Con A-Sepharose 4B, keeping for 2 h with occasional stirring at 4°C. The gel-protein mixture was packed into a glass column, washed with one bed volume of Buffer E, pH 7.4, and then in cold TBS, pH 7.4. Elution was carried out at 25°C with 0.5M α -MG in TBS pH 7.4. Significant leaching of lectin was observed in this procedure too (Fig.9.). Con A-Sepharose 4B affinity chromatography has been used routinely in our lab to isolate a wide variety of glycoproteins, however this extensive leaching of Con A to constitute the majority of the eluted protein has only been demonstrated in the present case.

Haemagglutination studies with trypsinized human RBC were carried out on the unbound and hapten eluted fractions derived

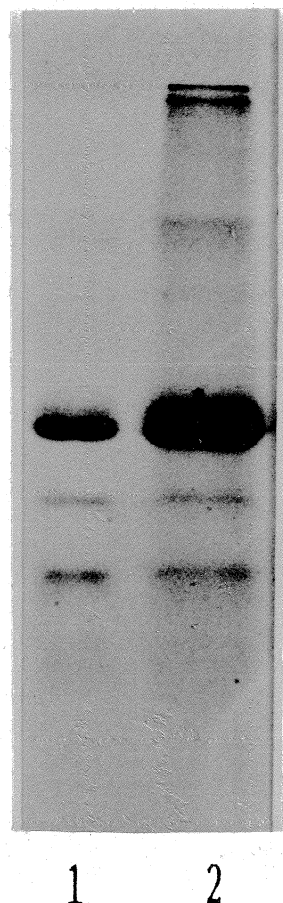


Fig.9.

Illustration of Con A leaching on 12% SDS-PAGE.
Coomassie Blue stain. Details are given in
text. Lane 1: Con A, Lane 2: Con A eluate.

from the above procedure. The fraction of glial homogenate that did not bind to Con A-Sepharose and was collected as breakthrough was diluted 1:1 (v/v) with 0.005M Tris-HCl, pH 7.0, and used directly for the haemagglutination assay at a protein concentration of 100 μ g/ml. The 0.5M α -MG eluted fraction was dialysed extensively against distilled water [1L x 4 changes] and concentrated by ultrafiltration through YM-10 membrane at 4°C. The concentrate was made 0.025M Tris-0.7M glycine by addition of solid salt, pH was adjusted to 7.4 with acetic acid and the solution diluted 1:1 with 0.005M Tris-HCl, pH 7.0 to render a protein concentration of 100 μ g/ml. Haemagglutination assay system comprised of 0.2ml protein solution and 50 μ l of a 5% suspension of trypsinized human RBC's in TBS, pH 7.4, at 25°C. The agglutination was monitored after 2 h.

The protein fraction collected in the breakthrough agglutinated human RBC's. Since the proteins did not exhibit this property before application on the lectin matrix it was concluded that agglutination was caused by Con A released from the matrix during loading of protein sample. This initial loss of lectin from the column during chromatography of glial proteins was a transient occurrence as no protein was detectable in the effluent at the end of the washing step. Con A leaching was again observed during elution of the bound glycoproteins with specific sugar. Although Con A constituted the major protein in the eluted fraction, a noteworthy observation was that the 0.5M α -MG eluted

fraction [Con A eluate] did not agglutinate RBC's under the conditions of the haemagglutination assay. The evidence suggested that Con A present in the Con A eluate was not in a native conformation favouring formation of crosslinkages between RBC cell surfaces. It was possible that noncovalently associated Con A subunits leaching from the gel during elution existed as monovalent forms incapable of haemagglutinating activity. Inhibition of the lectin in haemagglutination, by the co-existing Con A-specific glycoproteins eluted from the column, could not, however, be ruled out. Obviously prewashing the gel with buffers of high ionic strength and extremes of pH were ineffective towards removing Con A subunits noncovalently associated with the Sepharose matrix.

The problem has now been circumvented by introducing a pretreatment step for the immobilised lectin with guanidine hydrochloride [Gn.HCl]. Freshly prepared Con A-Sepharose 4B was washed respectively in 0.05M formate, pH 4.0 and 0.05M Tris-HCl, pH 8.0 buffers in presence of 1M NaCl, 0.3M sucrose, and finally in water. The gel was then treated at ambient temperature with 10 volumes 6M Gn.HCl, pH 7.0, over a period of 3 h. This step removed almost all the noncovalently associated Con A from the gel matrix. After the pretreatment step, Con A-Sepharose 4B was thoroughly washed with water and equilibrated overnight at 4°C in 0.01M Tris-HCl, pH 7.0, containing 0.001M each of Ca^{2+} , Mn^{2+} , and Mg^{2+} . This gel was used for affinity chromatography. Rechromato-

graphy was carried out on the same gel after washing with water, 1M NaCl and equilibration in buffer containing metals.

The binding capacity of this gel though diminished yielded glycoprotein fractions relatively free of contaminant Con A. The final procedure for the isolation of Con A-binding glycoproteins from glial cell surfaces has been elaborated below.

Preparation of plasma membrane enriched fraction from human foetal glial cells

About 50mg glial cells were suspended in 15ml ice-cold distilled water containing 0.01M EDTA-0.25% 2-ME, lysed by 20 strokes in Potter Elvehjm homogeniser and pelleted at 8000 x g for 10 min at 4°C. The pellet was rehomogenised as above by 20 strokes in same medium. The 8000 x g for 10 min pellet was suspended with 10 strokes in cold 0.25% 2-ME to ensure maximal cell lysis, which could be monitored by light or phase contrast microscopy. The plasma membrane enriched pellet was washed once more with 0.25% 2-ME and then twice in cold distilled water at 8000 x g for 10 min to remove adhering cytoplasmic constituents. This fraction was stored in ice in distilled water-0.1% NaN₃ until further use.

Isolation of Con A-binding glycoproteins from plasma membrane enriched fraction

The plasma membrane enriched pellet was suspended at 0-4°C by 10 strokes in Potter Elvehjm in Buffer E-0.001M PMSF-1% Triton X-100, pH 7.8, diluted in the same buffer to get a protein

concentration of 0.2-0.5mg/ml (64) and sonicated at 2-4 μ frequency for 15 sec x 4 times. The pH was adjusted to 7.4 with dilute acetic acid and the homogenate centrifuged at 150 x g for 2 min to pellet particulate material. The pellet, if any present, was resuspended in the above buffer, sonicated and centrifuged. The pooled supernatants [30-40ml aliquots] were mixed with equal volumes of the pretreated and equilibrated Con A-Sepharose 4B in a beaker and kept for a minimum of 2 h with occasionally stirring, at 4°C. The mixture was packed into a column, washed at 4°C first with one bed volume of Buffer E-0.1% Triton X-100, pH 7.4 and then in TBS-0.1% Triton X-100, pH 7.4 [Buffer F]. The column was eluted in bulk with two bed volumes of Buffer F containing 0.5M α -MG. The eluate was either filtered through Whatmann No.1 or centrifuged at 5000 x g for 10 min to remove gel particles if present, diluted 1:20 with 0.01M TBS, pH 7.4, and concentrated at 4°C by ultrafiltration through YM-10 or PM-10 membrane. The turbid concentrate [5ml] was dialysed extensively in the cold against 4 x 1L distilled water to remove hapten sugar and detergent. The dialysed protein was stored at -20°C.

The samples eluting at 25°C presented a faint band corresponding to Con A in molecular weight on SDS-PAGE (Fig.10.). That the 27 KDa protein was indeed Con A was confirmed by N-terminal analysis of this band cut out from SDS-PAGE gels. Electroelution of this protein from the gel slices and N-terminal sequencing by Dansyl-Edman and DABITC-PITC methods as described under Materials

& General Methods revealed that the N-terminal sequence for the 27 KDa band was Ala-Asp-The- which corresponded to that of Con A. The persistence of the Con A band in 25°C eluted samples, although considerably reduced after pretreating the gel matrix, could not be explained as an effect caused by detergent. Eliminating Triton X-100 in the above isolation procedure did not abolish this Con A band. Contrarily, Triton X-100 was found to increase the yield of eluted glycoprotein at least 8 fold, probably by enhancing the solubilisation process and hence was maintained in all the buffers.

Finally, it was established that carrying out the entire procedure of Con A chromatography and elution at 4°C rendered a glycoprotein fraction free of Con A. This fraction did not show any Con A band on SDS-PAGE (Fig.10.).

Leaching was apparently caused by dissociation of Con A subunits noncovalently but hydrophobically associated with the Sepharose matrix and/or with covalently linked Con A subunits. This dissociation was aided by the presence of hydrophobic glycoproteins, lipid or detergent molecules in the mobile phase. As Con A leaching was maximal during elution with 0.5M α -MG, it was likely that the glial Con A-binding glycoproteins furnished the hydrophobic environment suitable for the dissociation of Con A subunits from the gel.

The appearance of Con A even after drastic pretreatment of Con A-Sepharose in 6M Gn.HCl corroborated with the variation

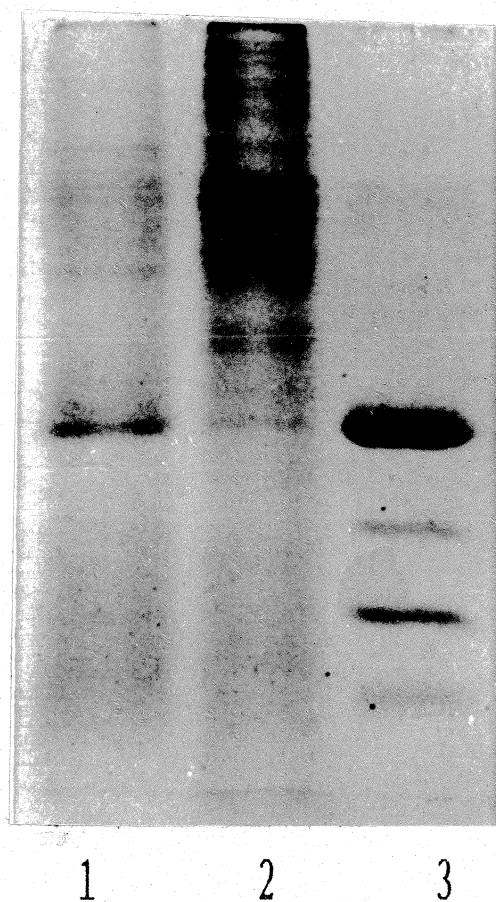
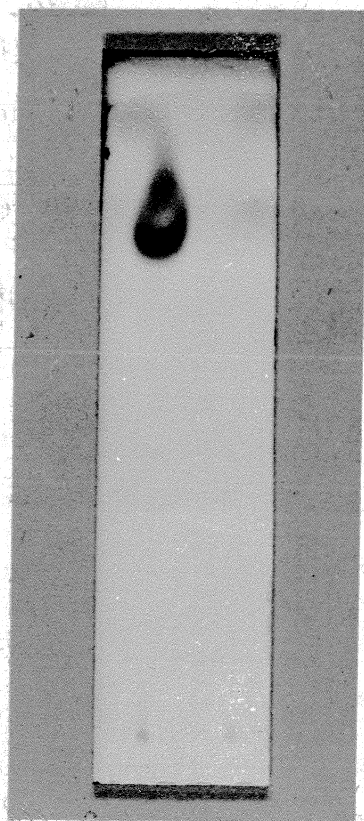


Fig.10. 10% SDS-PAGE of fractions eluted from Con A-
Sepharose 4B at 4°C and 25°C. Coomassie Blue
stain. Details are given in text.
Lane 1: 25°C eluate, Lane 2: 4°C eluate,
Lane 3: Con A.



A B

Fig.11. Orcinol-sulfuric acid stained thin layer chromatogram of lipid extracted from 400µg protein eluted respectively at 25°C [A] and 4°C [B]. TLC was carried out in 7:3, n-propanol:water (v/v) solvent system at 25°C. See text for details.

observed between the 25°C and 4°C eluted samples. This temperature dependent leaching of Con A from pretreated gels has now been ascribed to the presence of noncovalently associated glycolipid coeluting with the glycoprotein. Identification of this lipid fraction and characterisation of the Con A eluate has been dealt with in the succeeding chapters. Therefore only the role played by lipid in relation to lectin affinity chromatography has been discussed here. It was observed that during elution, the glycolipid profile closely paralleled that of Con A and that cold eluted samples contained negligible lipid as compared to the fractions eluted at 25°C (Fig.11.).

Considering the occurrence of hydrophobic patches on Con A, substantiated by reports on the possible occurrence of hydrophobic interactions between lectins immobilized on CNBr activated Sepharose and water or detergent solubilised glycoproteins (55,234), it was possible to hypothesize that hydrophobic membrane glycoproteins, detergent and lipid stabilized in the form of mixed micelles offered an environment conducive for solubilisation of Con A subunits. Con A subunits associated through hydrophobic interactions with covalently coupled Con A had a higher affinity towards these micelles, probably by virtue of the presence of a highly nonpolar micellar core, and thus eluted in the mobile phase. Low temperatures did not favour micelle formation restricting mobility of lipid due to phase transition, thereby decreased the hydrophobicity offered to

lectin subunits and prevented leaching of Con A. This could explain the relative absence of glycolipid and Con A evidenced when elution was carried out at 4°C.

One fact to be kept in mind however, was that Con A leaching was observed in the absence of detergent and/or lipid. A possible explanation for this phenomenon could be that the glycoprotein binding to Con A was itself amphipathic and could form self micelles of sufficient hydrophobicity to include Con A. Triton X-100 and glycolipid enhanced and further stabilized micelle formation in the form of mixed micelles of protein, lipid and detergent.

Con A-binding glycoproteins were prepared from glial cells isolated from IInd [CR=9-20cms] and IIIrd [CR=24-36cms] trimester brain tissue respectively. Glycoprotein yields were calculated as between 2-4% of the total protein loaded on the affinity matrix. Removal of contaminant lipid and Con A from the prepared glycoproteins

Lipid was extracted by the method of Svennerholm & Fredman (325). One volume of an aqueous suspension of glycoprotein was treated with 4 volumes of 1:2,C:M (v/v), to obtain a 4:8:3,C:M:W (v/v/v) mixture. The sample was vortexed for 2 min at room temperature and centrifuged at 3000 x g for 15 min. The pellet was suspended in water and extracted once again as above. The supernatant contained all the solubilized glycolipid and could be used for analysis of glycolipids. Lipid also separated from the

glycoproteins during SDS-PAGE and migrated as a diffuse band close to the dye front.

Con A was removed electrophoretically by SDS-PAGE in tube gels. Electroelution of glycoproteins directly from the gel or from cut gel slices containing individual glycoprotein bands was employed to recover samples absolutely free of Con A. The Con A eluate was subjected to SDS-PAGE in 12% tube gels of 5.5cms length according to the method of Laemmli (190) in 0.05M Tris-0.192M glycine-0.2% SDS, pH 8.3, chamber buffer. Con A-FITC was run simultaneously as a marker until the visible Con A-FITC band moved out of the gel. The proteins freed of Con A were electroeluted out directly from the tube gel. The lower anodic chamber was filled with fresh Laemmli buffer. The solution over the gel was blotted out and the gel layered with 150 μ l recovery solution consisting of 0.025M Tris-0.075M glycine-40% glycerol-0.001M DTT, pH 8.8 (317). Over this, 2M NaCl, a high conductivity zone, was carefully layered till the top of the tube. The upper cathodic chamber was filled with Laemmli buffer free of SDS, the current was reversed so that the upper chamber was the anode and electrophoresis carried out for twice the initial running time at 4mA/tube. Protein eluted into the recovery solution and was prevented from migrating further by the 2M NaCl high conductivity zone. After the run, protein was collected in the recovery solution, precipitated in 5 volumes ice cold methanol, washed 2-3 times with 1:8, 0.1N HCl:acetone (v/v) to remove SDS, freed of

purity. The acid was washed with water to remove the detergent
contaminant, and the glycerol was obtained after

PART III:

CHARACTERISATION OF CONCAVALIN A-

BINDING GLYCOPROTEINS

Glycoproteins were prepared from glial plasma membrane enriched fractions as described in the preceding section. Extensive dialysis against water did not remove the detergent completely, hence the glycoprotein fraction was obtained after Con A chromatography as a dispersion in water-Triton X-100 medium. This sample has been referred to as Con A eluate.

Most standard methodologies for protein characterisation were hindered appreciably by the poor to limited solubility of the isolated protein. Organic solvent precipitation of the glycoprotein aggravated the existing problem as expected. Exercising drastic conditions for solubilisation such as boiling for 5 min at 100°C in presence of denaturants like 2-4% SDS, 8M urea, or 6M Gn.HCl could only achieve solutions of protein concentration not greater than 0.5mg/ml. Characterisation studies were therefore carried out on homogeneous protein suspensions. Wherein protein solubilisation was imperative, highly sensitive techniques were employed to enable detection.

The protein was concentrated further by flash evaporation at 37°C, lyophilization or by methanol precipitation. The recovery was less when employing drying procedures as a means of protein concentration due to sticking of sample to the container walls. The entire protein could be precipitated with 4-5 volumes of ice-cold methanol in presence of 1% SDS. In the absence of SDS only

70% of the total protein precipitated in 80% methanol. Efficient recovery of protein in absence of SDS was possible by precipitation with 8 volumes of cold methanol. Organic solvent precipitation, in addition removed all the residual Triton X-100 carried over from the glycoprotein isolation step. The prior removal of the nonionic detergent by organic solvent precipitation was essential to procedures such as SDS-PAGE, because Triton X-100 was found to interfere with protein migration during electrophoresis and caused smearing of bands.

Protein concentrations were determined by the modified micro Lowry method (64). The percent carbohydrate/weight protein for the room temperature eluted Con A-binding fraction was 45 ± 20 % irrespective of whether the estimation was done directly on the Con A eluate or on methanol precipitated glycoproteins (63). The poor solubility in spite of such a high carbohydrate content could not be explained.

A complex banding pattern was observed on SDS-PAGE in 8% and 10% gels as well as 3-12% gradient slab gels for the Con A eluate as illustrated in Fig.12. and Fig.13.(190). An appreciable amount of protein did not migrate into the gel and remained at the bottom of the sample well. In addition were present faintly stained multiple bands of M_r ranging from 35 KDa to above 100 KDa. A faint but distinct band corresponding to Con A in M [27 KDa] was obtained for the Con A-binding fraction eluted at 25°C but was absent in samples eluted at 4°C (Fig.10.). Finally,

a broad diffuse band staining with silver or CBB was observed near the dye front at the bottom of the gel. For ease of study the glycoprotein bands were divided into five M_r groups, viz. G_0 [protein retained at the point of application], G_I [75-130 KDa], G_{II} [65-72.5 KDa], G_{III} [52-63 KDa] and G_{IV} [43.5-48.5 KDa] respectively (Fig.14.). The band corresponding in M_r to Con A was designated as Con A-band. Solubilizing the protein sample in larger volumes abolished the G_0 band, however protein concentration within the gel was too low for detection. A protein concentration of 0.5-1mg/ml was maintained optimally for samples loaded on SDS-PAGE.

Glial plasma membrane fraction and the Con A eluate at a concentration of 100µg protein/25µl/well were applied on a 7% mini slab gel and subjected to SDS-PAGE at a constant current of 10mA. The gel was stained for carbohydrate with thymol-sulfuric acid (108). Diffuse carbohydrate staining in the upper half of the gel indicated the presence of high molecular weight glycoproteins. The well containing Con A eluate however stained negative except at the point of application where the concentration of the insoluble glycoprotein was higher. Considering the low solubility of the sample it was probable that glycoprotein concentration within the gel was too low to be detected with the staining procedure used. A similar picture was seen on Con A-FITC staining of the Con A eluate after SDS-PAGE in 10% slab gels

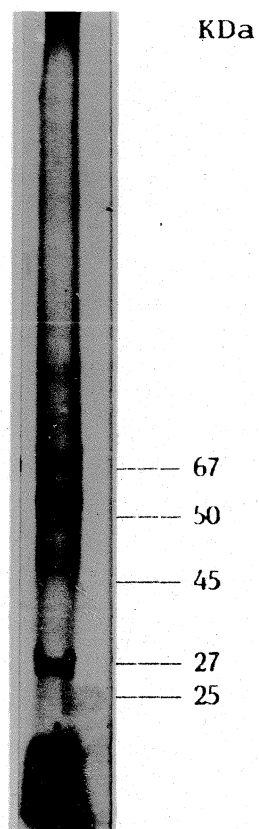


Fig.12. 3-12% Gradient SDS-PAGE of Con A eluate.
Silver stain. See text for details.

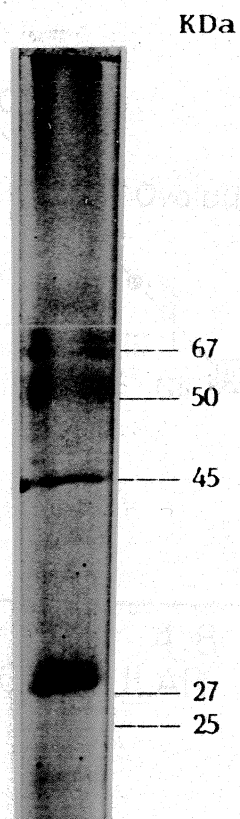


Fig.13. 8% SDS-PAGE of delipidated Con A eluate.
Coomassie Blue stain. See text for details.

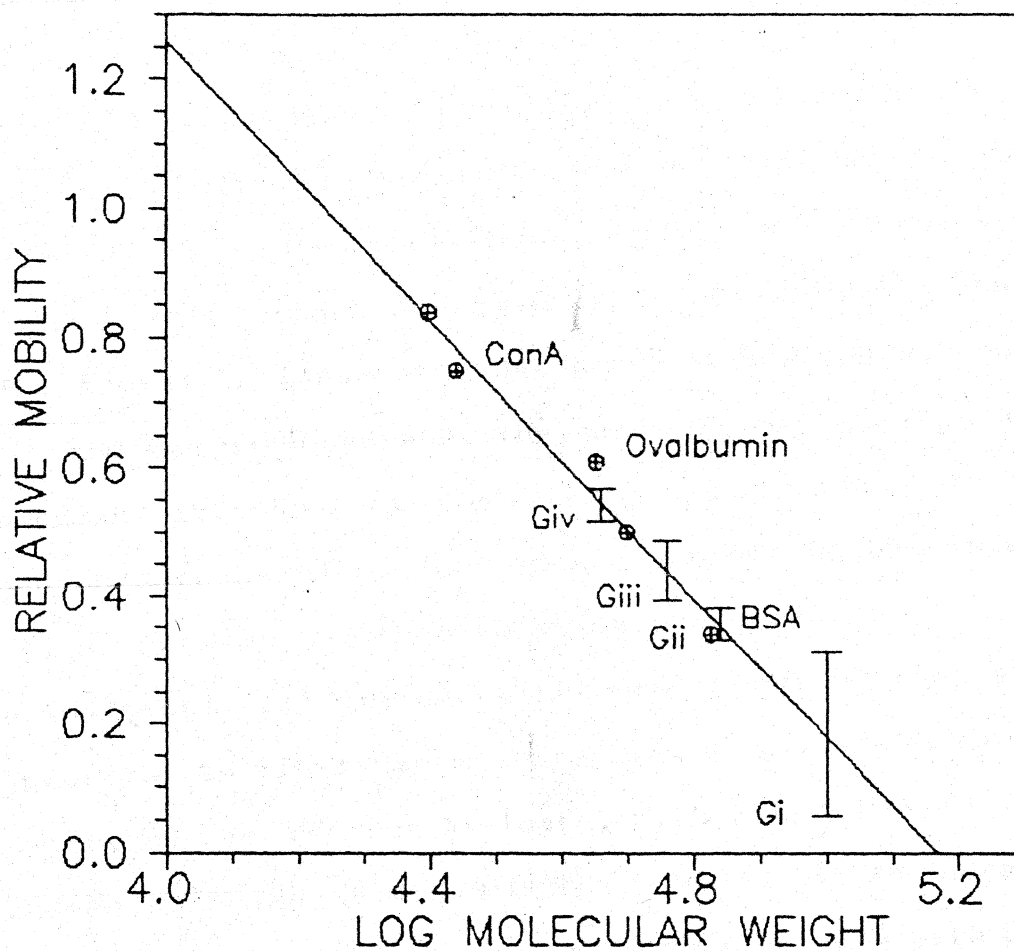


Fig.14.

Molecular weight determination of glial Con A-binding glycoproteins on 8% SDS-PAGE.

(93). Con A-FITC binding could be visualized only for protein at and close to the point of application on the gel.

To ensure that the CBB stained bands obtained after SDS-PAGE on 8% and 3-12% gradient polyacrylamide gels, respectively, were glycoproteins, individual bands were cut out and the protein was electroeluted. The eluted protein-SDS complex was washed 4 times in 80% methanol to remove contaminants, redissolved by boiling [100°C for 5 min] in 100 μ l of 0.04M LiCO₃-2% SDS, pH 9.5 buffer, and estimated for carbohydrate. The bands G₀, G_I, G_{II}, G_{III}, and G_{IV} each contained 160 $\pm$ 90 μ g carbohydrate/mg protein. The Con A-band contained no carbohydrate, an evidence supporting the existence of Con A, an artifact of the isolation protocol.

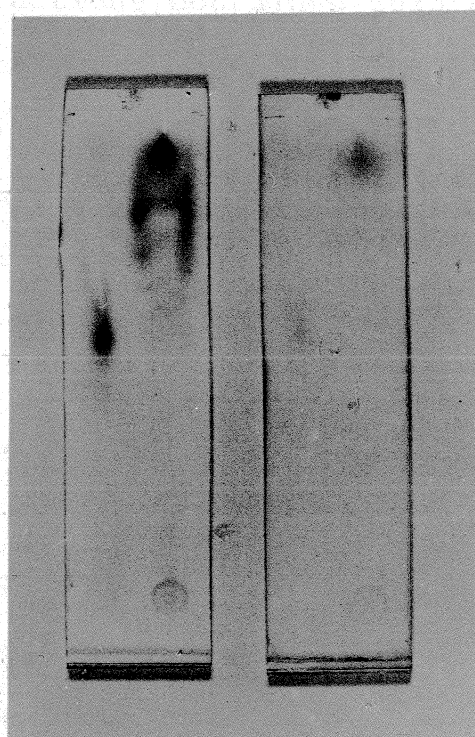
An analogous decrease in carbohydrate content from 450 $\pm$ 200 μ g/mg protein to 160 $\pm$ 90 μ g/mg protein was also noticed on delipidation of the 25°C Con A eluate, with 2:1,C:M (v/v) (88) or with Fredman's solvent, 8:4:3,M:C:W (v/v/v) (325). The evaporated organic phase was carbohydrate positive when reacted with phenol-sulfuric acid reagent. This discrepancy in carbohydrate content after SDS-PAGE or delipidation, supported by the presence of carbohydrate positive material extractable into organic solvents suggested the presence of glycolipid in the Con A eluate. The diffuse silver/CBB staining observed near the dye fronts after SDS-PAGE of the Con A eluate was attributed to this lipid (Fig.12.) component, since delipidated samples did not exhibit

this feature (Fig.13.). The presence of glycolipid was confirmed and identified by TLC.

Lipid analysis by TLC

Commercial Silica gel plates (2.5 x 10cms, E.Merck, Germany) were activated for 1 h at 110°C. The lipid, extracted from the Con A eluate in 8:4:3,M:C:W (v/v/v) was concentrated by evaporation in vacuo and 20 μ l spotted on TLC plates. Ascending chromatography in 7:3,n-propanol:water (v/v) solvent system was carried out at 25°C. The air dried plates were stained respectively with iodine vapour, orcinol-sulfuric acid, and resorcinol-HCl (188). All the lipid stained positive with iodine vapour were also stained with orcinol-sulfuric acid indicating a predominance of glycolipids. Resorcinol-HCl only stained a small area near the solvent front corresponding most likely to GM₁-GM₃ gangliosides (Fig.15.). The presence of noncovalently associated lipid component in the Con A eluate was thus evidenced. This lipid was obviously glycolipid in nature, the majority of which were nonsialylated, possibly as ceramide oligosaccharides. Ganglioside was present only as a minor component. It was apparent that these glycolipids closely associated with the Con A-binding glycoproteins were carried through the isolation procedures. The lipid contaminant was separated from the glycoprotein by SDS-PAGE or by delipidation with Fredman's solvent.

The glycoprotein fractions eluted from Con A-Sepharose at 4°C were found to contain 190 $\pm$ 60 μ g carbohydrate/mg protein which



A B

1

A B

2

Fig.15.

Thin Layer Chromatogram of the lipid fraction
 from II'' [A] and III'' [B] trimester glial
 Con A eluate. TLC was carried out in 7:3,
 n-propanol:water (v/v) solvent system at 25°C.
 Plate 1: orcinol-H₂SO₄ staining,
 Plate 2: resorcinol-HCl staining.

paralleled the observation that the glycolipid content was considerably reduced for these samples. A ganglioside component could barely be detected in these eluates. This aspect was verified further by sialic acid estimations of the Con A eluate. The 25°C eluted fractions contained 20 ± 5 n moles sialic acid/mg protein (351). A slight decrease to 18 ± 5 n moles/mg protein was obtained after delipidation of the above sample. The sialic acid content was 25 ± 5 n moles/mg protein for the 4°C eluted fractions and remained unchanged even after delipidation. Contamination of the cold eluted glycoprotein with Con A was negligible as visualised by the disappearance of the Con A-band on SDS-PAGE of these samples. The higher sialic acid content for the 4°C eluted fractions was therefore in concurrence with the increased purity of the isolated species.

It has been reported that plasma membranes were particularly rich in glycosphingolipids as compared with intracellular membranes (59,355,172,274). The presence of closely associated glycolipids could serve as an evidence in favour of the plasma membrane locale of the isolated glycoproteins.

Con A eluate in 0.6ml water, freshly labelled on carbohydrate residues by periodate oxidation-tritiated borohydride reduction (315) was subjected to Folch partitioning in 3ml of 2:1,C:M (v/v) (88). The lower phase and upper phases were respectively collected, evaporated to dryness and counted for tritium in 1ml of Bray's scintillant [refer Materials and General Methods]. The

protein interface was washed twice in 80% ice-cold methanol and counted as above. Only 30-40% of the total cpm was recovered collectively in the three phases. 80-85% of this fraction was protein label. The remaining label was in the Folch upper phase, most probably in the soluble glycolipid fraction. The organic phase was free of radiolabel. Con A eluate delipidated with Fredman's solvent also recovered in the supernatant and pellet together, only 30-40% of the initial radioactive label. Of this, more than 90% of the radiolabel was contained in the protein pellet. The observed loss in radioactivity was not delved into further.

The distribution of glycoprotein during organic phase partitioning was traced by DELLA [refer Part IV]. The separated organic, aqueous and interphase obtained on Folch partitioning of the Con A eluate were respectively evaporated, the residues were suspended in 0.04M LiCO_3 -0.05% SDS buffer, pH 9.5 and spotted on nitrocellulose sheets. The glycoproteins were detected by their binding to each of the HRP coupled lectins, Con A, WGA and JSA. The Folch upper phase and interphase bound the three lectins respectively, indicating that the glycoprotein was distributed in these two phases. This accounted for the small amount of radioactivity persisting in the Folch upper phase of samples delipidated prior to partitioning. The label separating into the Folch aqueous phase was most likely contributed to by glycolipid in conjunction with minor amounts of glycoproteins.

A relevant observation made in the course of radiolabelling studies was that the excess free radioactivity was not efficiently removed by extensive dialysis. More than 70% of this label was retained with the protein inside the dialysis bag. Washing the protein 6-8 times with 8 volumes of cold methanol was obligatory for complete removal of free radioactivity. Nonspecific sticking of label to hydrophobic proteins may have been a reason for this phenomena. However, considering the amount of free label retained in the present case, it was doubtful whether this was the only factor. The inherent property of the isolated glial glycoproteins to form micelles had already been hinted at in relation to Con A leaching. Entrapment of free radiolabel within glycoprotein micelles, thereby hindering their removal by dialysis could be yet another contributing factor. Methanol being an agent which destabilized micelle formation aided in the release of free radioactivity.

The above presumption was best illustrated during ^3H -acetic anhydride labelling of the glycoprotein fractions. Samples dialysed extensively against water to remove excess acetic anhydride, on Folch partitioning, gave up 80-90% of the total radiolabel into the lower organic phase. A control consisting of an aqueous solution of ^3H -acetic anhydride on phase partitioning rendered an identical amount [80-90%] of label into the Folch lower phase confirming that free acetic anhydride exhibited a higher affinity for the nonpolar phase. The preference shown by

acetic anhydride for the non polar phase was possibly satisfied within the hydrophobic core of glycoprotein micelles resulting in its retention along with protein under the conditions of dialysis.

The amphipathicity exhibited by glial glycoproteins was a characteristic of integral membrane glycoproteins in accordance with the fluid mosaic model of Singer and Nicolson (308). Several well studied integral cell membrane glycoproteins such as human RBC glycophorin (214,334), rhodopsin from mammalian retinal rods (250,135), lipophilin from myelin (98) etc were asymmetrically oriented across the membrane bilayer. These glycoproteins consisted of a hydrophilic carbohydrate rich N-terminal sequence exposed at the outer surface of the membrane and a hydrophobic section which interacted with the membrane bilayer.

N-Terminal Analysis

The determination of N-terminal sequence was based on the degradation scheme developed by Edman (69). Phenylisothiocyanate reacted with the amino-terminal amino acid of a polypeptide at basic pH to form phenylthiocarbamyl derivative. On acidification with trifluoroacetic acid, the first amino acid was cleaved off as its 2-anilino-5-thiazolinone derivative, exposing the amino group of the second amino acid. The thiazolinone derivative was converted to the more stable thiohydantoin in dilute acid. The amino acid derivative was removed by extraction, the remaining polypeptide was dried and the next Edman cycle repeated.

Amino-terminal analysis was carried out by two independent techniques. In the indirect Edman procedure, an aliquot of the protein was removed at the beginning and after each cycle of Edman degradation. The N-terminal of the aliquot was determined by dansylation of the protein followed by hydrolysis of the protein and analysis of the liberated dansyl amino acids by RP-HPLC. Intensely fluorescent derivatives resulted from the reaction of Dns-Cl with free amino groups. The dansyl amino acids were stable to acid hydrolysis and exhibited yellow fluorescence (118). The second protocol involved direct Edman degradation by DABITC-PITC followed by the isolation and identification of the cleaved off N-terminal DABTH-amino acid by TLC on polyamide sheets.

Amino-terminal sequence of the Con A eluate as determined by Dansyl-Edman procedure was Ala-Asp-Thr-. This was identical to the published N-terminal sequence of Con A (28), also confirmed by us. Although Con A eluate prepared at 4°C did not exhibit the Con A-band on SDS-PAGE, DABITC-PITC method revealed Ala- as N-terminal of these samples. The N-terminal was therefore determined in samples completely freed of Con A as follows.

Con A eluate was subjected to preparative 3-12% gradient slab gel electrophoresis, loading the sample in all the wells. Individual bands were cut out and the protein electroeluted out as described under general methods. The eluted proteins were washed thrice in 80% methanol and then with 80% acetone in 0.1N

HCl to remove SDS, dried in vacuo and sequenced. To ensure that artifactual N-terminal blocking by unpolymerised acrylamide or by other reactive agents during electrophoresis and electroelution did not occur (109), certain preventive measures were taken as suggested by Moos et al (232). All slab and tube gels used with proteins destined for sequencing were cast a day in advance so that polymerisation was complete. Pre-electrophoresis of the polymerised gels were carried out for 14-16 h at 40mA/slab or 4mA/tube in separating gel buffer [0.75M Tris-HCl-0.1% SDS, pH 8.85] containing 0.01M reduced glutathione in the cathodic chamber buffer. Glutathione removed charged impurities, reduced peroxides and residual radicals and served as a scavenger for uncharged reactive species like acrylamide monomer, free or incorporated acrolein or other reactive carbonyl compounds. The buffers were discarded after the run, the chambers were filled with the running chamber buffer and electrophoresis or electroelution was carried out with the sample.

The N-terminal appeared to be blocked for each of the glycoprotein bands G_0 to G_{IV} . DABITC-PITC procedure did not indicate any DABTH-amino acid on polyamide TLC. Identification of Dns-amino acids by reverse phase HPLC on Lichrosorb C_{18} column of hydrolysed dansylated proteins detected only ϵ -Dns-lys, confirming a blocked N-terminal for the glial glycoproteins (Fig.16.). The Con A-band however presented Ala as N-terminal amino acid in both the protocols.

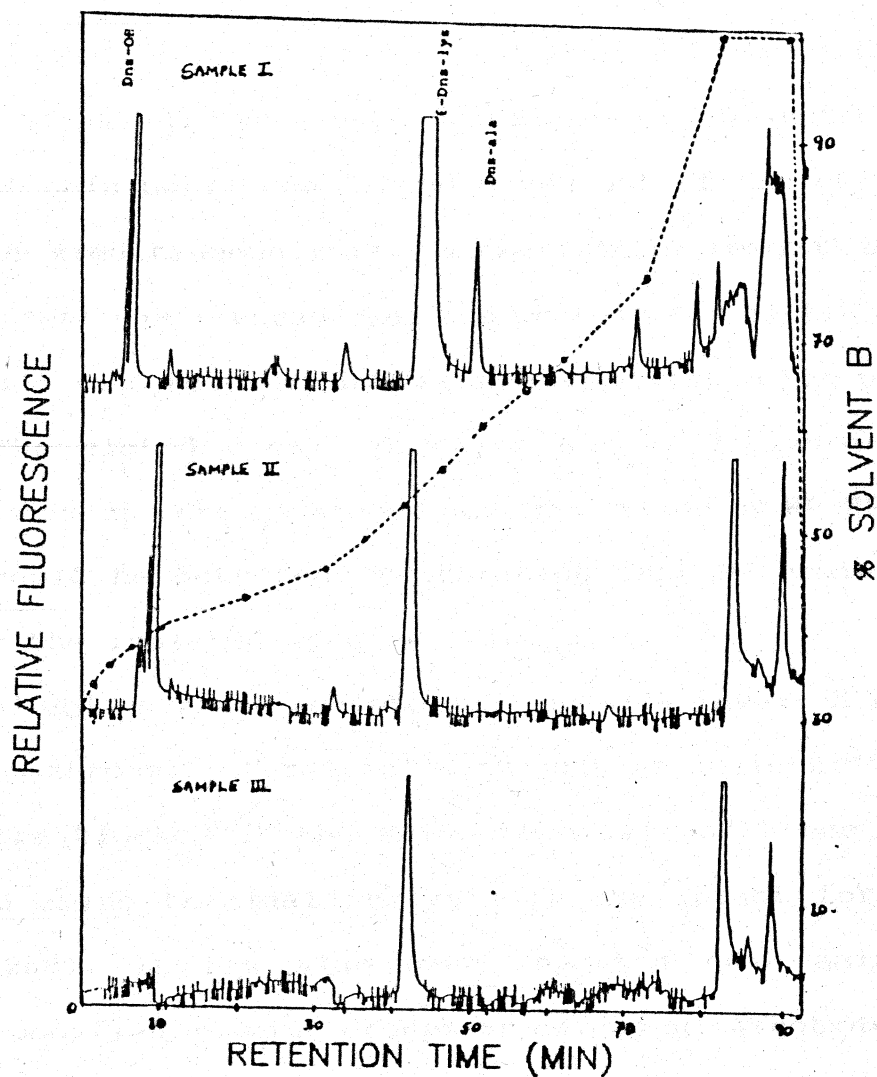


Fig.16.

RP-HPLC of dansyl amino acids for N-terminal Analysis by Dansyl-Edman procedure. Flow rate=0.5 ml/min.

Excitation=333nm, Emission=510nm, Sol A:0.6% acetic acid-0.008% TEA-water, Sol B:methanol. Gradient=30-100% B in 80 min. Sample I-Con A eluate (direct), Sample II-Bands G₁ - G₁₁ from SDS-PAGE, Sample III-Dansylated Con A eluate after removal of Con A. Refer text for details.

The validity of these observations were checked as follows. Con A eluate was first dansylated to rule out any artifactual amino group blocking. The dansyl proteins were subjected to SDS-PAGE in tube gels until Con A-band moved out of the gels. FITC-Con A was run simultaneously as a marker. After removal of Con A-band, the current was reversed and the protein was eluted directly out of the gel into recovery buffer as described in the preceding sections. The eluted dansyl proteins were hydrolysed and the dansyl-amino acids were separated and identified by RP-HPLC. Only α -dns-lys could be detected, reiterating that N-terminals were blocked for the isolated species (Fig.16.).

Several cell surface receptors especially involved in signal transduction through interaction with guanine nucleotide binding proteins were blocked at the amino terminal and hence were not amenable to amino terminal analysis in the intact form of the receptor (268). It has also been reported that amino acids containing modified α -amino groups or carrying carbohydrate side chains were resistant to Edman degradation (328,356). Presence of sites of glycosylation at or near the vicinity of the amino-terminal, commonly encountered in integral membrane glycoproteins, may account for the blocked N-terminal indicated by the sequencing procedures.

Re-electrophoresis on SDS-PAGE

The excised protein bands G_0 to G_{IV} obtained on SDS-PAGE in 10% and 3-12% gradient gels respectively, were subjected to a

second SDS-PAGE under identical conditions as the first run (Fig.17.). Each protein group generated the same multiband profile spanning the M_r range observed during the first SDS-PAGE. Protein concentrated predominantly in the M_r ranges corresponding to the bands G_{II} , G_{III} and G_{IV} . The Con A-band was absent illustrating that it was distinct from the other proteins. This evidence supported the established stature of the Con A-band as an artifact of the isolation procedure.

The obvious conclusion from the above experiment was that under the conditions of SDS-PAGE glial glycoproteins formed aggregates over a wide molecular weight range. These aggregates were interconvertible and exhibited a dynamic equilibrium between three major states corresponding to G_{II} , G_{III} , and G_{IV} . The high M_r bands G_o and G_I were converted to the lower M_r forms by a shift in this dynamic equilibrium towards the favoured and possibly more stable states. The tendency of hydrophobic membrane proteins to form irreversible aggregates in SDS (326,41) and the ensuing problems during SDS-PAGE (149) have been reported. Though these workers have recommended that SDS treatment of protein be carried out at lower temperatures [0-37°C] as boiling increased the proclivity for aggregation, we found no difference in the case of our samples. Higher loading protein concentrations could only be achieved after boiling in 2-4% SDS at 100°C for 3-5 min. Samples which proved particularly resistant to SDS solubilisation were

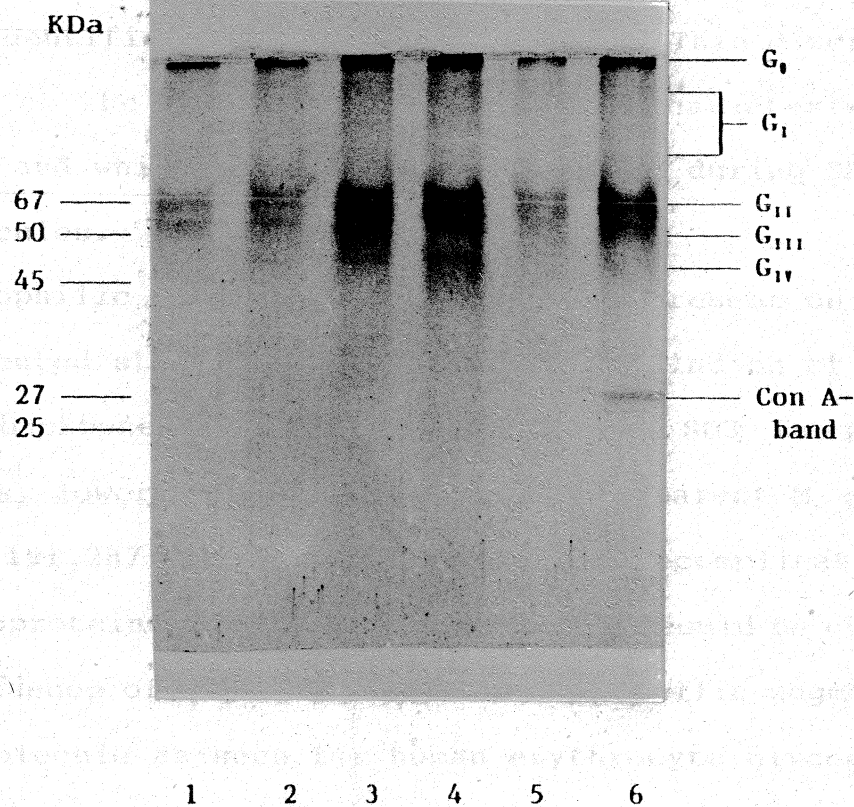


Fig.17. Second 10% SDS-PAGE of glial protein bands G₀-G_{IV}. Coomassie Blue stain. Lane 1: G₀, Lane 2: G_I, Lane 3: G_{II}, Lane 4: G_{III}, Lane 5: G_{IV}, Lane 6: Con A eluate. See text for details.

clarified to a limited extent by boiling with SDS in presence of urea (197,204). Urea had no effect on SDS-PAGE otherwise.

A notable observation was that contiguous protein bands visualised after SDS-PAGE differed in M_r by 2-4 KDa only. This signified a basic monomeric unit of very low molecular weight, if aggregation was the prime factor responsible for the complex multiband profile on SDS-PAGE. However such a species, if present, could not be identified on the gel or dye front. This discrepancy was explained on the basis of two properties characteristic of glycoproteins and which contributed appreciably during SDS-PAGE of these molecules.

The hydrophilic carbohydrate side chains present on glycoproteins prevented stoichiometric hydrophobic binding of SDS to the protein backbone resulting in decreased SDS to protein binding ratios, lower mobilities and higher apparent M_r on SDS-PAGE (302,40,191,287). The problem was more complicated for membrane glycoproteins where higher SDS binding could be obtained due to the presence of highly hydrophobic lipophilic segments on the protein molecule as seen for human erythrocyte glycophorin. This glycoprotein bound 5-7g SDS/g protein in contrast to 1-2g SDS normally bound per gram protein (119).

The anomalous SDS-protein binding ratios led to variations in surface charge and hence altered mobilities on SDS-PAGE. The apparent M_r therefore differed from the actual M_r for these glycoproteins. Another cause for the observed complexity of SDS-

PAGE profiles could be microheterogeneity of the oligosaccharide side chains in the glycoproteins. Fine differences in carbohydrate structure and sequence could manifest as minor differences in M on SDS-PAGE (328,227).

It was conceivable that all these factors viz. aggregation, anomalous SDS binding and microheterogeneity were collectively responsible for the glycoprotein profile on SDS-PAGE. A more committed suggestion would be that these aggregates were a heterogeneous mixture of protein-SDS micelles formed under the prevalent conditions of partial solubilisation of membrane proteins with SDS (39). It was clear that glial glycoproteins could not be totally solubilised in SDS. Mixed micelles containing varying amounts of protein and/or SDS and differing slightly in molecular weights were formed, thus giving rise to the complex multiband profile on SDS-PAGE. The slight differences in M_r of adjacent bands could be credited to variations in SDS concentration and to some extent microheterogeneity. The M_r of the basic protein monomer was irrelevant here. Micelle formation was also governed by a dynamic equilibrium dependent on solubilisation criteria. More conclusive evidence for this hypothesis would certainly contribute towards evolving a definite theory on the physicochemical nature of glial cell surface glycoproteins.

The factors responsible for such anomalous behaviour on SDS-PAGE were delved into. The first aspect to be verified was whether specific structural components in the glycoproteins influenced

their mobilities on SDS-PAGE. The presence and effect of structural elements demonstrating non-stoichiometric binding to SDS, such as highly hydrophilic or hydrophobic domains, and/or capable of imparting an inherent amphipathicity to the molecule thereby aiding in micelle formation were looked into. The extent of contribution of structural modifications characteristic of the glycoproteins towards their anomalous behaviour on SDS-PAGE were studied.

An increasingly large number of membrane associated proteins in eukaryotic cells are now known to contain covalently bound lipid. The hydrophobicity imparted by lipid helped in protein interaction with the membrane lipid bilayer and in anchoring of membrane proteins (301,337,81). Three modes of fatty acid linkage to eukaryotic proteins have been described (Fig.18.). These are (a) post-translational attachment of fatty acid [primarily palmitate though C_{12} to C_{18} acyl chain lengths were also found] in a thioester or ester bond to cysteine, serine or threonine residues. Myelin proteolipid proteins (316,98,89), mammalian transferrin receptor (248), rhodopsin (240) and HLA glycoprotein (167) were some examples of ester linked protein fatty acid acylation. The covalently bound fatty acid was readily released by hydroxylamine treatment (211,310) or mild alkaline methanolysis (247). (b) Co-translational myristoylation of proteins on amino terminal glycine residues via an amide linkage. The majority of these proteins were soluble, nonglycosylated

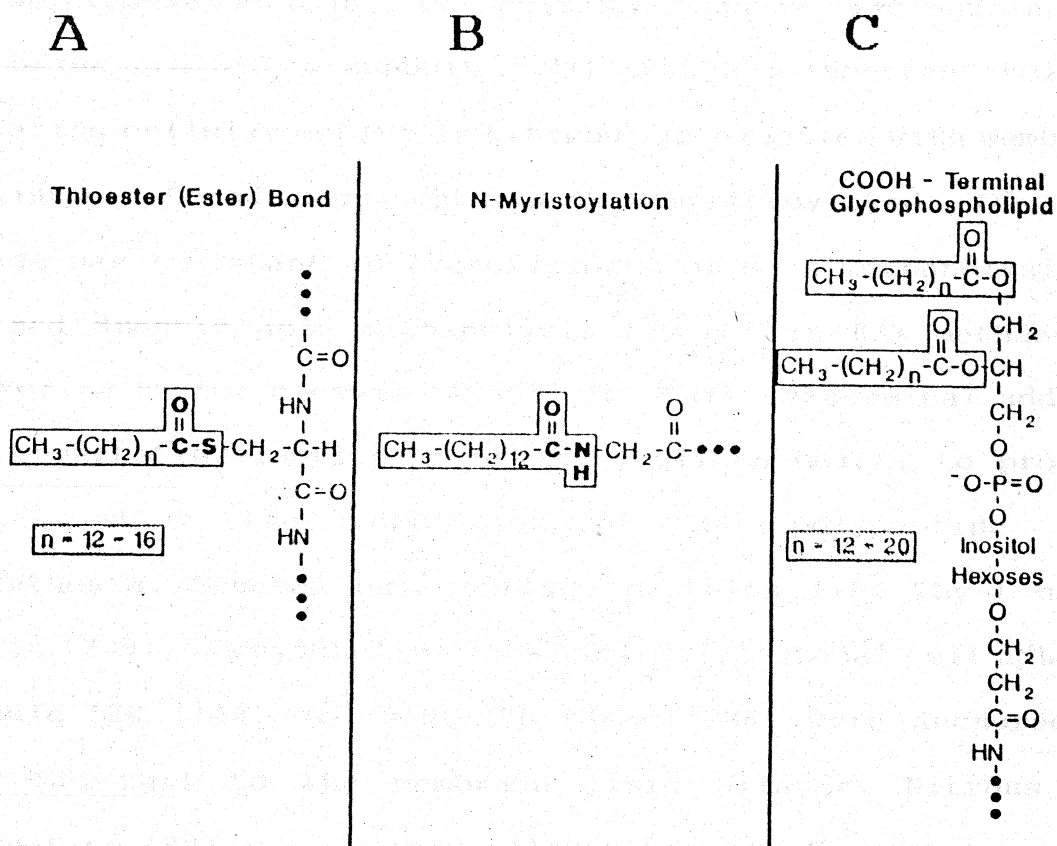


Fig.18. Types of fatty acid-protein linkages in eukaryotes.

A - Thioester/ester linkage, B - amide linkage and
C - glycosylphosphatidylinositol linkage.

Adapted from Towler, D.A., Gordon, J.I., Adams, S.P. and
Glaser, L. (1988) *Ann. Rev. Biochem.* 57, 69-99.

proteins, transiently associated with membranes. Protein kinases such as cAMP dependant kinase (43), tyrosine kinase p60^{src} of Rous Sarcoma Virus (300), phosphatases such as calcineurin B (3) and bovine brain G_o α -subunit (299) that had important roles in modulating cellular metabolism through interaction with membranes or membrane bound components were N-myristoylated. The amide linkage was resistant to hydroxylamine or mild methanolysis and required drastic acid methanolysis [2N HCl in 83% methanol at 95°C for 60 h] for cleavage (246). (c) Carboxy-terminal addition of a phosphatidylinositol containing glycan moiety to proteins shortly after the completion of the polypeptide chain biosynthesis. Several cell surface proteins like Thy-1 glycoprotein (209), acetylcholine esterase (95), neuronal cell adhesion molecule-120 (144) and 5'Nucleotidase (208) were anchored via their GPI-tail to the membrane lipid bilayer. Nitrous acid deamination (80) and cleavage with phosphatidylinositol specific phospholipases A₂, C or D (130,215) have been employed in cleavage of lipid from protein moiety.

Carbohydrate side chains added considerably to the hydrophilic nature of glycoproteins. Their effect on glycoprotein behaviour and mobility on SDS-PAGE was discussed above. In addition to inherent structural features certain external factors may have induced protein aggregation under the experimental conditions thereby giving rise to the complex interconvertible

multiband protein pattern on SDS-PAGE. This was the second aspect investigated.

At this point we sought answers to the following questions, (a) was covalently bound lipid the cause for aggregation? (b) did the glycan side chains influence protein behaviour on SDS-PAGE? and (c) could SDS itself be the cause for anomaly?

Glial cell glycoproteins were analyzed for the presence of covalently linked fatty acids (276). The glycoprotein [150µg] was extracted with 3ml aliquots of 8:4:3,M:C:W (v/v/v) twice and then with 2:1,C:M (v/v) once to eliminate all noncovalently associated lipid. The delipidated samples were used for lipid estimation studies.

Hydroxylamine hydrolysis of ester linked fatty acids

The protein [150µg] was treated with 400µl of 1M $\text{NH}_2\text{OH}\cdot\text{HCl}$ adjusted to pH 7.0 at 25°C for 120 min. The suspension was extracted three times with 1ml n-hexane. The organic phase was evaporated in vacuo and the residue analyzed for fatty acids by TLC.

Alkaline methanolysis cleavage of ester linked fatty acids

The protein [150µg] was digested at 56°C for 30 min with 400µl of 0.3M methanolic KOH and neutralized with 6N HCl. The liberated fatty acids were extracted into n-hexane as above and analyzed by TLC.

TLC of fatty acids

Fatty acids were separated on commercial Silica gel plates [20 x 20cm, E.Merck, Germany]. The solvent system employed was petroleum ether:diethyl ether:acetic acid,80:20:1 (v/v/v) (80). Myristic acid, stearic acid, palmitic acid and their corresponding methyl esters were used as standards, spotting 25 μ l sample per lane. Reagent blanks were run simultaneously along with test samples.

The chromatogram was visualized with iodine vapour and then stained for lipid with saturated $K_2Cr_2O_7$ in 70% sulfuric acid, followed by heating at 150°C for 15-20 mins. Lipids were perceived as charred spots (188).

Detection of GPI-Tail

Cleavage of phosphatidylinositol from glycan component of the GPI tail was effected by deamination with nitrous acid as per the protocol of Ferguson et al (80). The liberated phospholipid was then identified by estimation of its phosphorous content. The delipidated glycoprotein [300 μ g] was treated with 450 μ l of 0.25M $NaNO_2$ in 0.25M sodium acetate-acetic acid buffer, pH 3.5, at 25°C for 16 h. To this was added 45 μ l 1N HCl, 1.2ml chloroform and 0.6ml methanol, and the suspension was vortexed and centrifuged. The lipid partitioning into the organic phase was collected and evaporated to dryness. The residue was refluxed in 0.2ml perchloric acid in a sand bath [240°C] for 4-6 h in order to oxidise all organic material and convert ethereal phosphate to

inorganic phosphorus. The clear solution was assayed for phosphate by Bartlett's method (21). A protein sample run identically except for the deamination step which was excluded, served as control for nonspecifically liberated phosphate.

No identifiable fatty acid was detected by TLC. The phosphate determined for test and control protein samples were identical signifying absence of GPI-tail. The proteins after treatment with lipid cleavage reagents did not behave with any disparity on 10% SDS-PAGE when compared with untreated samples. Corroborative evidence was also obtained independently by staining glycoproteins separated on 10% SDS-PAGE for lipid with Sudan Black B (92). The proteins were not stained by Sudan Black B indicating the absence of covalently bound lipid components. Although the collective data suggested absence of covalently associated lipid, it was possible that the sensitivity of the protocols applied were not enough in the present situation. Nevertheless it was apparent that lipid was not the factor responsible for anomalous behaviour of the species on SDS-PAGE.

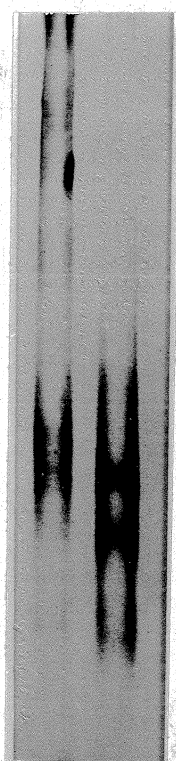
To assess whether carbohydrate side chains contributed to the abnormal behaviour of glial glycoproteins, SDS-PAGE was carried out on deglycosylated and desialylated samples. Vacuum dried delipidated glycoprotein [150 μ g] was subjected to chemical deglycosylation by treating with 50-100 μ l of TFMS at 0°C for 3 h according to Sojar and Bahl (314). The protein solution was cooled to -20°C and slowly neutralised by addition of 150-300 μ l

prechilled 60% pyridine. The deglycosylated sample was then precipitated with 8 volumes of cold methanol.

The glycoprotein [150 μ g] was desialylated in 300 μ l of 0.1N sulfuric acid at 80°C for 1 h and precipitated with 8 volumes of cold methanol (143).

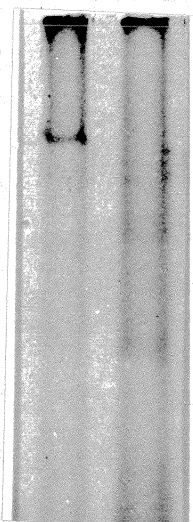
The above modified proteins were subjected to SDS-PAGE. An increase in M_r by 4 KDa was apparent for TFMS treated samples over the untreated controls (Fig.19.). Desialylation rendered a single band of M_r around 80 KDa (Fig.20.). The amount of protein retained at the point of application was considerably higher in both cases. The Con A-band characteristic of untreated samples was missing for TFMS and acid digested protein samples. It was likely that increased hydrophobicity imparted by removal of carbohydrate residues, resulted in the formation of larger aggregates. Apparently, a major portion of the protein did not enter the gel. The disappearance of Con A-band could probably be attributed to the association of Con A within these hydrophobic aggregates. However, whether this observed effect was itself a manifestation of the drastic chemical treatment rendered, was not studied further.

The role of SDS in glycoprotein aggregation and its consequential effects were then examined. Resolution of the Con A eluate was attempted in absence of SDS. Glycoproteins were solubilised in 8M urea buffers at pH 4.2 [100 μ g protein/well] and subjected to 6M urea-PAGE in 5% mini slab gels at pH 4.2 (307) as



1 2

Fig.19. 3-12% Gradient SDS-PAGE of TFMS treated glycoproteins. Silver stain. Lane 1: TFMS deglycosylated protein, Lane 2: native protein. Details are given in text.



1 2

Fig.20.

10% SDS-PAGE of desialylated glial Con A-binding glycoproteins. Coomassie Blue stain.

Lane 1: desialylated protein, Lane 2: native protein. See text for details.



described under Materials and General Methods. The glycoproteins were resolved into three components, a densely stained band at the point of application U_0 , a slow moving component U_I which actually comprised of two close bands in juxtaposition, and a moderately mobile band U_{II} (Fig.21.). The protein bands were cut out and resolubilized by incubation for 15-30 min in three changes of glycine-acetic acid chamber buffer, pH 4.2 containing 8M urea-40% glycerol-0.005M 2-ME, heating for 5 min at 100°C during the last change. The gel pieces containing the solubilised protein was placed directly in the sample wells of a 5% slab gel and a second acid urea-PAGE was carried out in conditions identical to the first run. It was found on re-electrophoresis that, U_0 resolved into U_I and U_{II} indicating the former to be the insoluble protein fraction. The band U_I remained unaltered in mobility while U_{II} manifested a portion of its protein as the slower moving forms, suggesting a possible conversion of U_{II} to the U_I form (Fig.22.). On interpretation there appeared to be two basic forms for the native glycoproteins, a slow moving component and a faster moving component which was inclined towards conversion into the former presumably higher M_r aggregated forms. This propensity for aggregation independent of SDS was studied in detail.

Gel-filtration studies on acetylated glycoproteins

The Con A eluate was delipidated in Fredman's solvent and radiolabelled with tritiated acetic anhydride (85). After

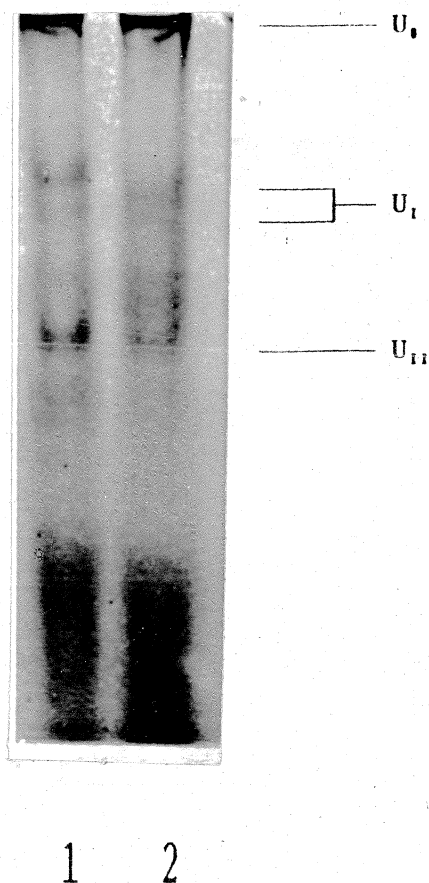


Fig.21. Acid urea-PAGE of Con A eluate. Silver stain. Lane 1: II' trimester glycoproteins, Lane 2: III' trimester glycoproteins. Samples were not delipidated. See text for details.

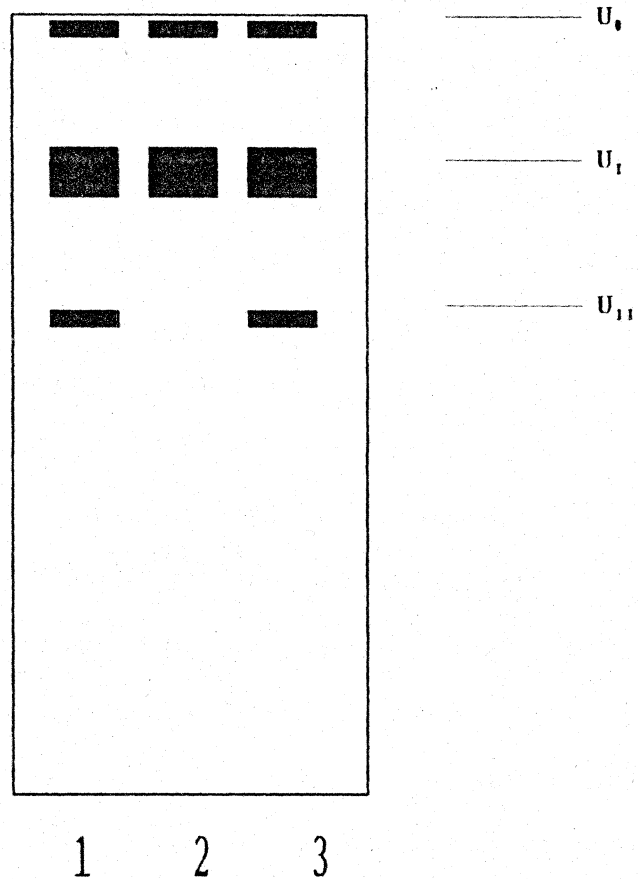


Fig.22. Second acid urea-PAGE of protein bands U_1 - U_{II} .
 Lane 1: U_{II} , Lane 2: U_I , Lane 3: U_1 . See text
 for details.

extensive dialysis against distilled water at 4°C, the aqueous protein suspension was made 0.025M Tris-0.7M glycine-0.1% NaN₃, pH 7.8 [Buffer E] and centrifuged at 15,500 x g for 5 min. The supernatant [S_{cf}] containing 80-85% of label was resolved by gel-filtration (5). The pellet [P_{cf}] was retained for further analysis by HPLC (Fig.23.). Initially, gel-filtration was attempted on Sephacryl S-300 [1.4 x 100cm] at 4°C in Buffer E, pH 7.8, loading 1ml of the supernatant [10⁵cpm]. A flow rate of 8-12 ml/h was maintained and 1ml fractions were collected. The radioactivity was measured in 200µl aliquots. All the label eluted within the void volume of indicating that the species was excluded on Sephacryl S-300 gel-filtration.

Gel-filtration was carried out at ambient temperature on Sepharose 6B column [1.4 x 100cm] in Buffer E, pH 7.8, at a flow rate of 8-12ml/h. The labelled sample was applied in 1ml and fractions were collected and counted as above. Two peaks were identified, Peak I eluted in the void volume and Peak II at 2.7 times the void volume. Peaks I and II were collected, dialysed against water and concentrated by lyophilization.

The two fractions were characterised on the following criteria.

a) Second gel-filtration of the respective peaks on Sepharose 6B under identical conditions as the first run. Reloading of each peak individually on the gel-filtration column did not alter their respective elution volumes. Peak I eluted in the void

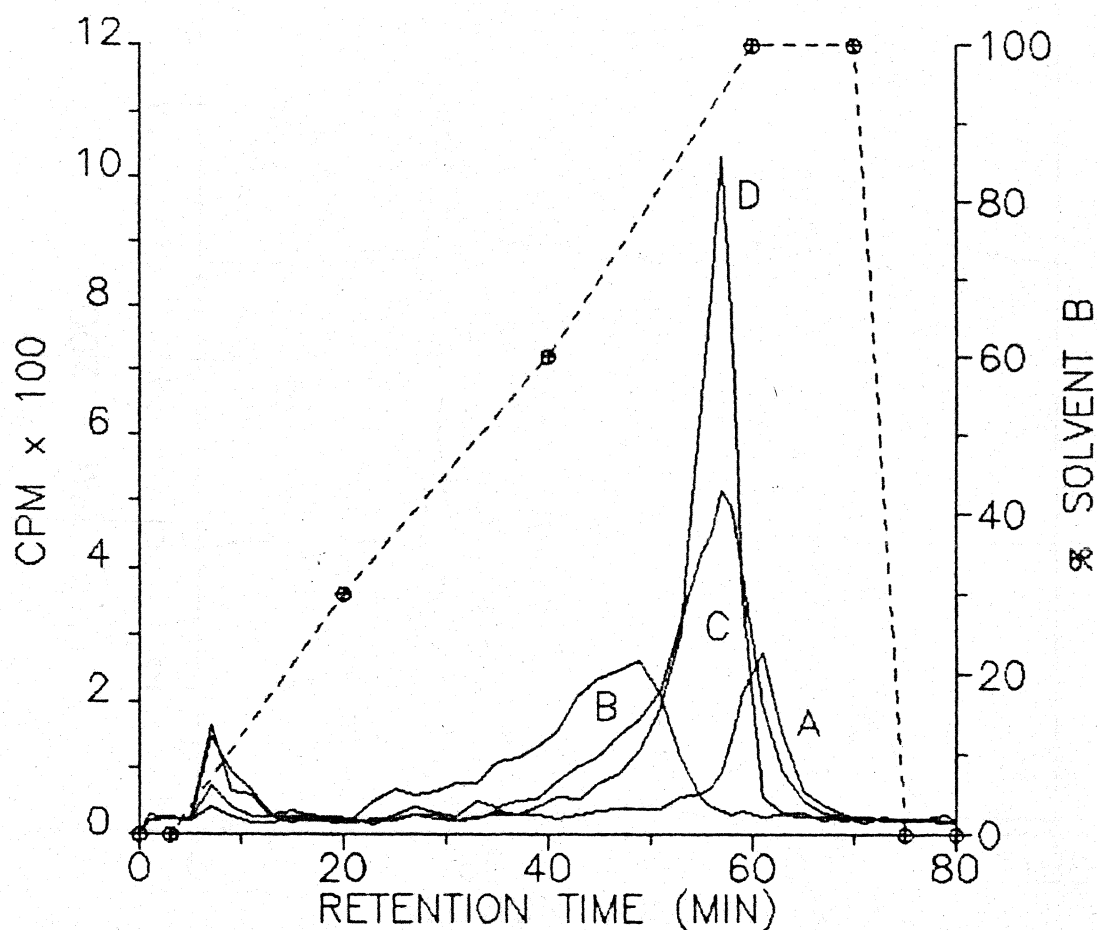


Fig.23.

RP-HPLC of [^3H] labelled glycoproteins. HPLC was carried out on Lichrosorb RP-8 column in Gradient I [0---100] between the Solvents, A:water-0.1% TFA and B:isopropanol-0.1% TFA at a flow rate=0.5ml/min for 80 min. Profiles A, B, C, D, correspond to Sepharose 6B gel-filtration Peak I, Peak II, samples S_i , and P_i , respectively. Refer text for details.

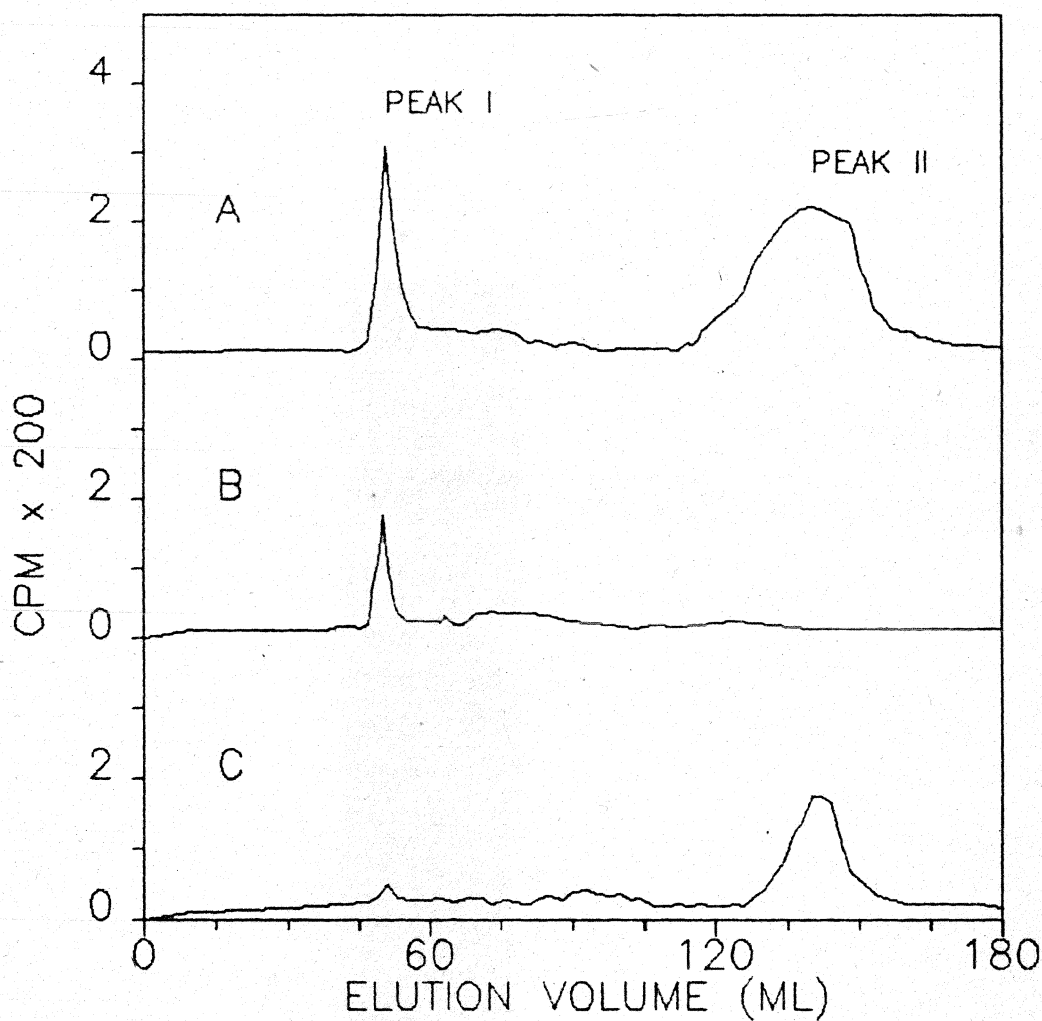


Fig.24.

Gel-filtration of [^3H] labelled glycoproteins on Sepharose 6B [1.4 x 100cm]. Eluant: 0.025M Tris-0.7M glycine-0.1% NaN_3 , pH 7.8. Flow rate=8-12ml/h. See text for details. A - first gel-filtration of Con A-binding glycoproteins, B - second gel-filtration of Peak I, C - second gel-filtration of Peak II.

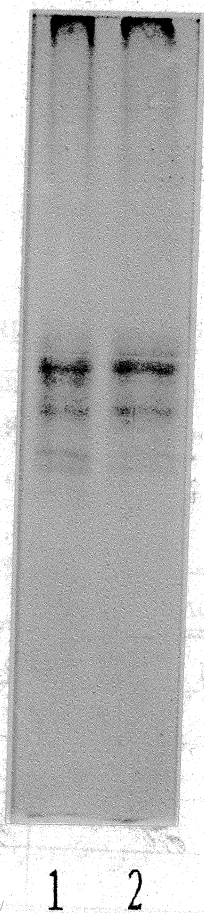


Fig.25. 8% SDS-PAGE of gel-filtration Peak I [Lane 1]
and Peak II [Lane 2]. Coomassie Blue stain.
Details are given in text.

TABLE.3.

RADIOACTIVITY INCORPORATED IN PROTEIN BANDS RESOLVED
ON 8% SDS-PAGE OF SEPHAROSE 6B GEL-FILTRATION PEAKS
I AND II. Corresponding bands were pooled. Background
 and Quench corrections have been applied.

SAMPLE	RADIOACTIVITY (cpm)
Total protein	100,000
Band G _i	660
Band G _{ii}	670
Band G _{iii}	506
Band G _{iv}	447

volume and Peak II at 2.7 times the void volume as before (Fig.24.). The observation suggested that the two peaks were distinct and non-interconvertible in the detergent free Tris-glycine buffer system used for the study. Peak I was excluded on Sepharose 6B indicating that it was of very high M_r . The centrifugation of this fraction at $100,000 \times g$ for 60 min recovered all the radioactivity in the supernatant thereby confirming that Peak I was a soluble very high molecular weight micellar aggregate.

b) Both Peaks exhibited analogous lectin binding properties on DELLA verifying that these fractions were glycoproteins with similarities in carbohydrate structure. The question of whether Peak I was a micellar form of Peak II arose at this juncture.

c) Peaks I and II appeared identical on 8% SDS-PAGE, furnishing multiband protein profiles comparable to those obtained for glial glycoproteins and concentrated in the regions corresponding to G_{II} , G_{III} and G_{IV} (Fig.25.). This observation established that the characteristic aggregation pattern on SDS-PAGE was in all probability an artifact of protein-SDS interactions. To ensure that the CBB stained bands coincided with the radiolabelled species, individual bands G_o , G_{II} , G_{III} and G_{IV} were excised and counted for radioactivity after digesting the gel matrix (35). A suitable piece of gel was counted for background (Table.3.).

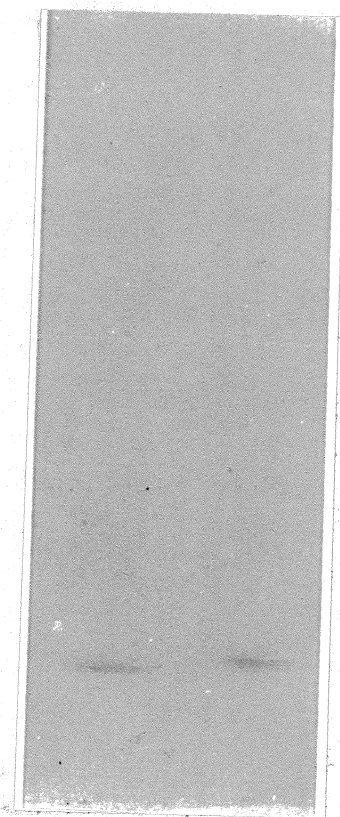
It was obvious that only 2% of the total radiolabel loaded on SDS-PAGE was recovered as protein label. An explanation for this

occurrence was the inefficient removal of free label by dialysis as discussed earlier. Methanol precipitation as a preliminary step although effective in eliminating the free radioactivity, reduced considerably the solubility of the protein in the detergent free Buffer E used in subsequent procedures, and hence was avoided.

d) The gel-filtration peaks were analyzed by RP-HPLC on Lichrosorb RP-8 column (5 μ m, 4 x 250mm) fitted with a guard column (7 μ m, 4 x 30mm). A gradient elution system (Gradient I) between the solvents, Sol A: water-0.1% TFA and Sol B: isopropanol-0.1% TFA was applied at a flow rate of 0.5ml/min at 25°C (refer Materials and General Methods). The gel-filtration samples S_{gr} , Peak I and Peak II in Buffer E, pH 7.8, was acidified with TFA to 10% concentration (v/v), while the undissolved pellet P_{gr} was dissolved in 50% TFA. The samples were centrifuged at 15,500 x g for 3 mins and 100 μ l of the supernatant (5000cpm) was injected on the RP-HPLC system. Fractions were collected every minute for 80 minutes and counted directly in 1ml scintillation fluid. The respective retention times for the four labelled samples were Peak I=61 min, Peak II=49 min, S_{gr} =57 min and P_{gr} =57 min (Fig.23.). The results suggested that Peaks I and II were distinct and non-interconvertible even in the non polar environment prevalent in RP-HPLC. Peak I eluting later, was more hydrophobic than Peak II. The micellar structure of the high M_r Peak I appeared to be retained under the conditions of RP-HPLC. Another surprising

observation was that S_{6r} which gave rise to Peaks I and II on Sepharose 6B gel-filtration, did not resolve correspondingly on RP-HPLC. This protein eluted as a single entity separate from Peak I and Peak II. The retention time of S_{6r} averaged those of Peaks I and II, possibly a cumulative effect of these two fractions. An identical profile obtained for the pellet P_{6r} only emphasized the limited solubility of the glycoprotein to optimal concentrations. The pellet comprised of the insoluble residual glycoprotein.

d) Peak I and Peak II on 6M urea-PAGE at pH 4.2, rendered a single fast moving band close to the dye front verifying that Peak I was a micellar form of a basic unit eluting as Peak II (Fig.26.). Protein migration in acid PAGE was dependant on two factors, charge contributed by basic amino acids present in the protein, and the mass of the protein. The fact that the isolated glial cell glycoproteins moved preferentially on acid PAGE was itself indicative of a higher content of basic amino acids in its polypeptide backbone. N-acetylation of the proteins should have decreased mobility on acid urea-PAGE. The opposite was perceived in this case. The acetylated gel-filtration peaks moved almost twice as fast as the band U_{11} obtained for non-derivatised Con A eluate on acid urea-PAGE in 5% gels. It was thus concluded that the decreased surface charge was presumably compensated by low molecular size of the acetylated species. A low M_r component eluting as Peak II, may be the basic monomeric unit constituting



1

2

Fig.26. Acid urea-PAGE of gel-filtration Peak I [Lane 1] and Peak II [Lane 2]. Silver stain. See text for details.

the micellar form [Peak I] of the isolated glycoprotein could be a possibility. It was noticed on comparison of gel-filtration peaks with non-acetylated glycoprotein on urea-PAGE that the latter resolved into at least three bands whereas the former rendered a single fast moving band. Mobility of the non-acetylated protein would be governed by both charge and mass. Since these proteins were comparatively slow moving it was inferred that the contribution of charge was negated by high molecular weight of the protein species, thereby resulting in retarded migration during electrophoresis. Alternatively, these bands could be disparately charged forms of similar molecular weight species which on acetylation rendered a single band. But as such a fast moving species was totally absent in non-acetylated protein, the first argument had greater credibility. The non-acetylated native protein probably existed under the conditions of urea-PAGE as soluble heterogenous micellar aggregates resistant to cleavage by urea into monomers. This incapability of urea to break up protein micelles was evident during resolubilization of fixed protein as a preliminary to the second urea-PAGE.

Micelles formed with N-acetylated protein, on the other hand appeared vulnerable to cleavage by urea and rendered low molecular weight monomeric units. Whether N-acetylation served to generate micelles of decreased stability by virtue of altered charge properties and thereby affected amphipathicity of the

glycoprotein was debatable. Experimentally however, N-acetylation appeared to be a key factor influencing micellar integrity in 8M urea.

Attempts were made to determine the M_r of Peak II proteins by gel-filtration. The M_r standards used in Sepharose 6B gel-filtration experiments were bovine serum albumin [67 KDa], ovalbumin [45 KDa], immunoglobulin G [150 KDa] and Ferritin [540 KDa] at a protein concentration of 10mg/ml in Buffer E, pH 7.8. The standard graph on extrapolation obtained an approximate value between 9-20 KDa for Peak II. On this premise, molecular weight determination was attempted by Sephadex G-75 gel-filtration. The Peak II protein was loaded at 25°C on Sephadex G-75 [1 x 63cm] equilibrated in Buffer E. The sample was loaded in 0.3ml and a flow rate of 6-8ml/h was maintained. Standard M_r proteins co-chromatographed were ovalbumin, bovine serum albumin, cytochrome c [12.5 KDa], soybean trypsin inhibitor [20.1 KDa] and horseradish peroxidase [40 KDa]. A linear standard graph could not be obtained especially with respect to low M_r standards, a likely possibility considering the limitations in techniques such as gel-filtration and SDS-PAGE in determining M_r of small molecules. Nevertheless, it was quite evident that Peak II eluted after cytochrome c, the protein having the smallest M_r amongst the standards loaded. Although an accurate estimation of the M_r was not possible, it was apparent that Peak II had a M_r below 12.5 KDa, most probably less than 10 KDa (Table.4.). Such a species

TABLE.4.

GEL-FILTRATION OF PEAK II FROM SEPHAROSE 6B ON
SEPHADEX G-75. See text for details.

PROTEIN	MOLECULAR WEIGHT	ELUTION VOLUME (ml)
BSA	67,000	21
Ovalbumin	45,000	24
Peroxidase	40,000	25
STI	20,100	27
Cytochrome C	12,500	30
Peak II	—	42

would be difficult to visualise on SDS-PAGE. The hypothesis that a multiband pattern on SDS-PAGE consisting of a series of bands differing only slightly in M_r may quite possibly be the result of aggregation of such low M_r monomers could not therefore be ruled out completely.

Carboxymethylation of Glial proteins

The procedure was adapted from Suryanarayana et al (324). The protein [100 μ g] was delipidated in Fredman's solvent and incubated in 50 μ l 0.1M NaHCO₃, pH 8.5 buffer containing 6M Gn.HCl and 0.02M DTT in a non-oxidising atmosphere of nitrogen at 37°C for 1 h. To the solution was added 50 μ l of 0.2M iodoacetic acid in the same buffer and flushed with nitrogen. The tube was sealed and incubated in the dark for 2 h at 37°C. The protein was precipitated and washed thrice in 8 volumes cold methanol.

Proteolytic digestion of the glycoprotein with trypsin (313)

The carboxymethylated protein was suspended in 400 μ l of 0.1M sodium phosphate buffer, pH 7.6 containing 2M urea and digested with 10 μ l TPCK-Trypsin [1mg/ml 0.01 N HCl] at 25°C for 24 h. The reaction was arrested by boiling at 100°C for 5 min and adjusting to pH 9.5 with NaOH. Protein controls were run simultaneously without enzyme. The free amino groups in the tryptic digests were dansylated by addition of 50 μ l Dns-Cl [2.8mg/0.1ml acetonitrile] and incubation at 37°C for 1 h in the dark. The solution was acidified with 50 μ l of 50% TFA and centrifuged at 15,500 x g for 3 min. The supernatant containing the peptide fragments was

collected and concentrated in vacuo. The samples were centrifuged to remove particulate material and 100 μ l was injected into Lichrosorb RP-8 column of the HPLC system. Enzyme blanks consisting of Trypsin treated identically with Dns-Cl were also run.

The dansyl peptides were separated and identified by RP-HPLC applying the same Gradient I at a flow rate of 0.5ml/min between Sol A:water-0.1% TFA and Sol B:isopropanol-0.1% TFA as mentioned earlier. However in this case peptides were identified by fluorescence of the coupled dansyl groups. The fractions were collected every minute for 80 min, made upto 2.5ml with water and the fluorescence was measured. The HPLC profiles for the proteins were obtained by plotting fluorescence against time. The peaks positive for fluorescence were collected, lyophilized to dryness, redissolved in 100 μ l of 0.04M LiCO₃, pH 9.5 buffer and subjected to differential lectin binding studies by DELLA as described in the succeeding section.

The HPLC profiles obtained were rather complex with fluorescent peaks distributed over the entire gradient (Fig.27.). All these peaks bound the different lectins in an identical manner and hence were indistinguishable on the basis of carbohydrate composition (Table.5.). The enzyme blank with Trypsin gave a small peak which did not bind lectins. However in presence of glycoprotein, as was the case for the test samples, this peak was overlapped by a lectin positive peak characteristic of the glycoprotein. The profiles obtained with the protein controls run

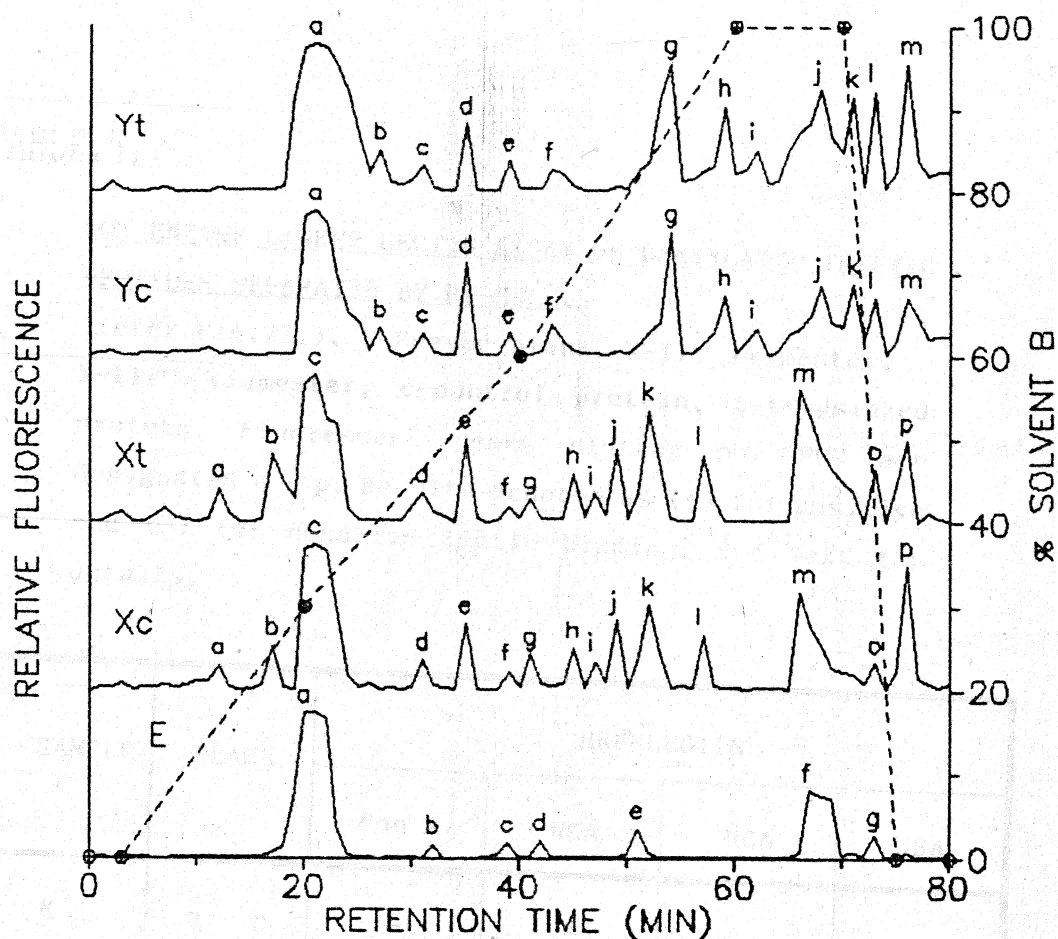


Fig.27.

RP-HPLC of Dansylated Trypsin digested glycoproteins.

HPLC was carried out on Lichrosorb RP-8 column in Gradient I [0—0] between the Solvents, A:water-0.1% TFA and B:isopropanol-0.1% TFA at a flow rate = 0.5ml/min for 80 min. Proteins were detected by fluorescence measured at Excitation = 333nm, Emission = 510nm [—]. E-enzyme blank, X-II'' trimer, Y-III'' trimer, c-control protein, t-trypsin digest. See text for details. Refer Table.5.

TABLE.5.

**DOT ENZYME LINKED LECTIN ASSAY OF DANSYLATED TRYPTIC
PEPTIDES SEPARATED BY RP-HPLC.**

(refer Fig.27.). E-Enzyme blank, X-IInd trimester,
Y-IIIrd trimester, c-control protein, t-trypsinized
protein. Fluorescent peaks eluting on HPLC are
designated a - p. Results denoted as (+) for positive
and (-) for negative lectin binding. See text for
details.

SAMPLE	PEAKS	HRP-LECTIN			
		Con A	WGA	RCA	JSA
E	a - g	-	-	-	-
Xc	a - p	+	+	+	+
Xt	a - p	+	+	+	+
Yc	a - m	+	+	+	+
Yt	a - m	+	+	+	+

in absence of enzyme, were similar to those observed for the trypsin digested test samples and indicated no peaks specific for the trypsinized protein. In addition, the trypsinized test samples and their corresponding protein controls on 5-25% gradient SDS-PAGE appeared identical (Fig.28.). It was possible that the multiple peaks on RP-HPLC were a result of glycoprotein aggregation enhanced by the increased hydrophobicity imparted through dansylation.

The refractory nature of the glycoprotein to cleavage by trypsin was verified by a different, more sensitive protocol. Protein was radiolabelled on carbohydrate residues to facilitate detection. All available amino groups were blocked and the so modified species was subjected to digestion by proteases. Proteolysis would generate free amino groups at the sites of cleavage which could be identified by labelling these groups with an isotope distinct from the label on carbohydrate. Acceptance of the second isotope was itself indicative of proteolytic cleavage. In addition, glycopeptides could be specifically identified in the protease digest.

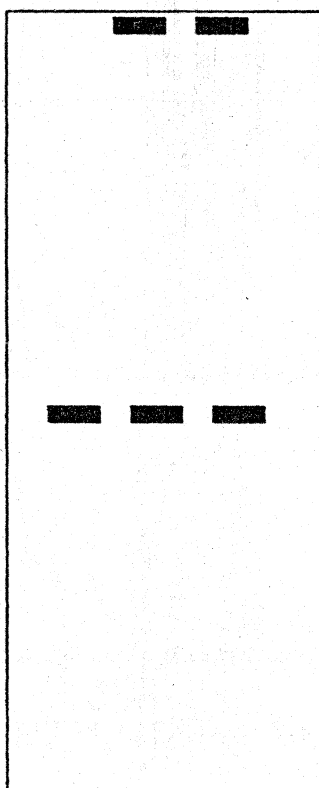
Carboxymethylated protein [400µg] was dansylated to block all free primary amino groups. The protein in 200µl of 0.04M LiCO_3 -2% SDS, pH 9.5 buffer was boiled at 100°C for 5 min. To this was added 30µl Dns-Cl [2.8mg/0.1ml acetonitrile] and incubated at 37°C for 1 h in the dark. The solution was acidified with 50µl of 6N HCl and the protein was precipitated by 8 volumes of ice-cold

acetone. The pellet was washed once with 1:8, 1N HCl:acetone (v/v) to remove SDS, suspended in 100 μ l of 0.4M sodium acetate buffer, pH 5.6 containing 8M urea and boiled for 5 min at 100°C to remove residual organic solvent. The carbohydrate residues of the glycoprotein were then labelled with tritium. Periodate oxidation was carried out with 0.01M NaIO₄ in 0.1M sodium acetate-2M urea, pH 5.6 buffer in a total volume of 400 μ l at 4°C in the dark for 16 h. Excess periodate was neutralised by addition of 10 μ l ethylene glycol followed by further incubation in the dark at room temperature for 30 min. The pH was adjusted to $\geq$ 8 with 25 μ l 1M NaOH, added NaB[³H], [15 x 10⁵ cpm/25 μ l 0.01N NaOH] and kept for 1 h at 25°C. The protein was precipitated in 8 volumes of cold methanol. The pellet was washed 4-5 times with 1:8, W:M (v/v) till the washings were free of radioactivity. The tritiated protein was divided into two aliquots. A portion was digested with TPCK-Trypsin while the remainder served as a protein control. Trypsinization was carried out in 400 μ l of 0.1M sodium phosphate buffer, pH 7.6 in presence of 2M urea. To this solution was added 20 μ l TPCK-Trypsin [1mg/ml 0.01N HCl] and incubated at 37°C for 5 h. A further aliquot of 20 μ l trypsin was added and the incubation continued for a total period of 24 h. The enzyme was inactivated by boiling at 100°C for 5 min. The trypsinized tests and control protein samples were N-acetylated with ¹⁴C-acetic anhydride. The samples were made to pH $\geq$ 8 by addition of 15 μ l 1M NaOH and incubated at 25°C for 30 min with ¹⁴C-acetic anhydride [4 x

10^6 cpm/30 μ l acetone]. Subsequently, 30 μ l of cold acetic anhydride was added, the pH adjusted to ≥ 8 with NaOH and incubation was continued for another 15 min. The samples were cooled in ice, acidified with 100 μ l glacial acetic acid and centrifuged to settle undigested protein. The supernatant was flash evaporated 4-6 times from 0.1M pyridine-acetic acid buffer, pH 5 to remove remainder radioactive acetic anhydride as acetic acid. The dried residue was redissolved in 50% TFA, centrifuged at 15,000 x g for 3 min and resolved by RP-HPLC. An enzyme blank was also run simultaneously.

Pronase digestion of glial glycoproteins

Pronase digestion was carried out on 400 μ g carboxymethylated dansylated glycoprotein whose carbohydrate side-chains were labelled with tritium (74,85). The protein was boiled at 100°C for 5 min in 800 μ l of 0.05M N-ethyl morpholine buffer pH 8.0, containing 0.01M CaCl₂ and 2M urea. Pronase [1mg/ml] was pre-incubated in the above buffer at 37°C for 30 min to destroy interfering activities. The enzyme was added at equal intervals in three lots [50 μ l totally] over an incubation period of 72 h at 37°C. The pH was maintained at 8.0 by addition of N-ethyl morpholine, if required. The reaction was arrested by boiling for 5 min at 100°C. The undigested protein was centrifuged and the supernatant was N-acetylated with ¹⁴C-acetic anhydride. The radiolabelled samples were resolved by RP-HPLC against a protein control and enzyme blank run under identical conditions.



1 2 3

Fig.28. 5-25% Gradient SDS-PAGE of Trypsin digested glycoproteins. Lane 1: Trypsin, Lane 2: II' trimester trypsin digest, Lane 3: III' trimester trypsin digest. See text for details.

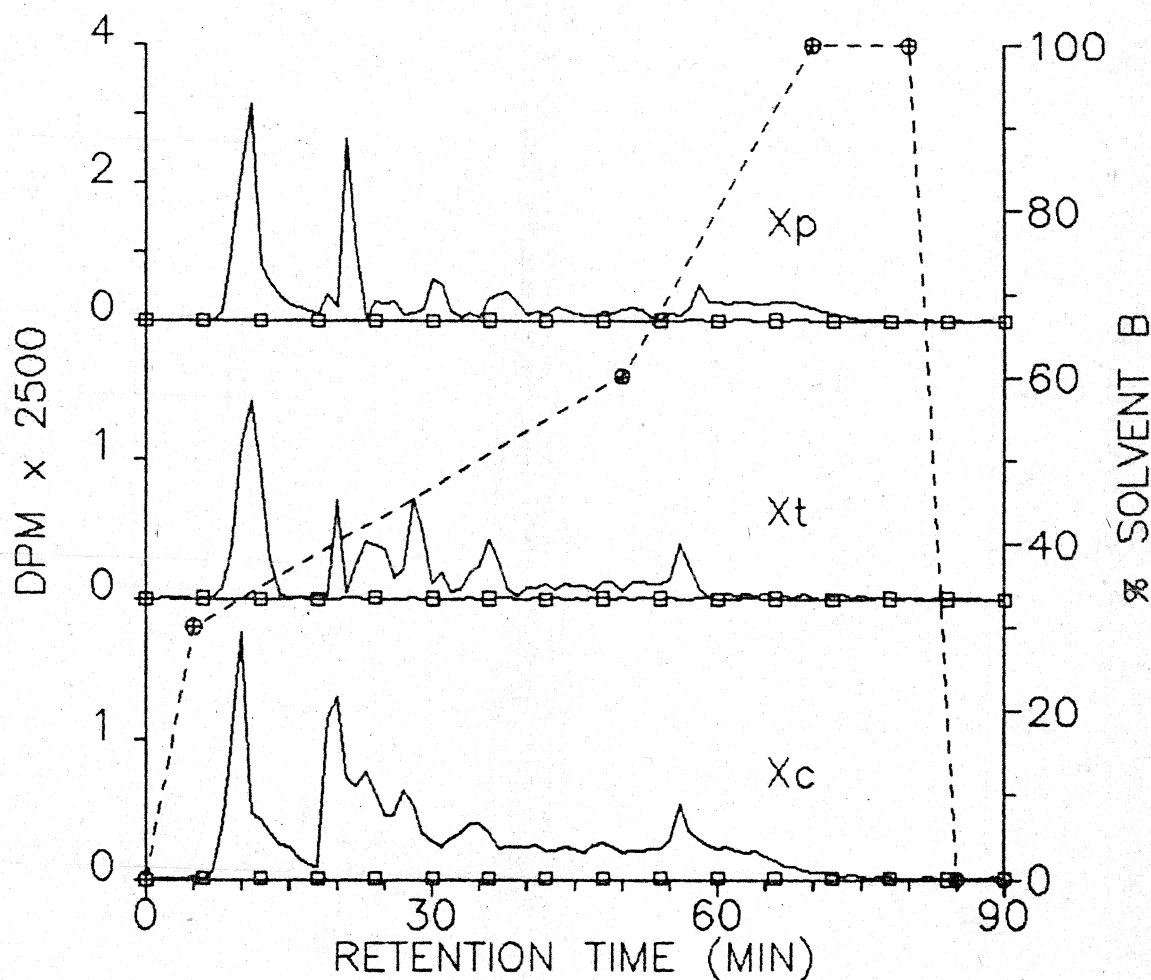


Fig.29.

RP-HPLC of trypsin and pronase digested II¹⁴ [X] trimeric glial glycoproteins labelled with [³H] and [¹⁴C]. HPLC was carried out on Lichrosorb RP-8 column in Gradient II [0---0] between the Solvents, A:water-0.1% TFA and B:isopropanol-0.1% TFA at a flow rate= 0.5ml/min for 90 min. ³H-dpm: [—], ¹⁴C-dpm: [□—□], c-control protein, t-trypsin digest, p-pronase digest. Refer text for details.

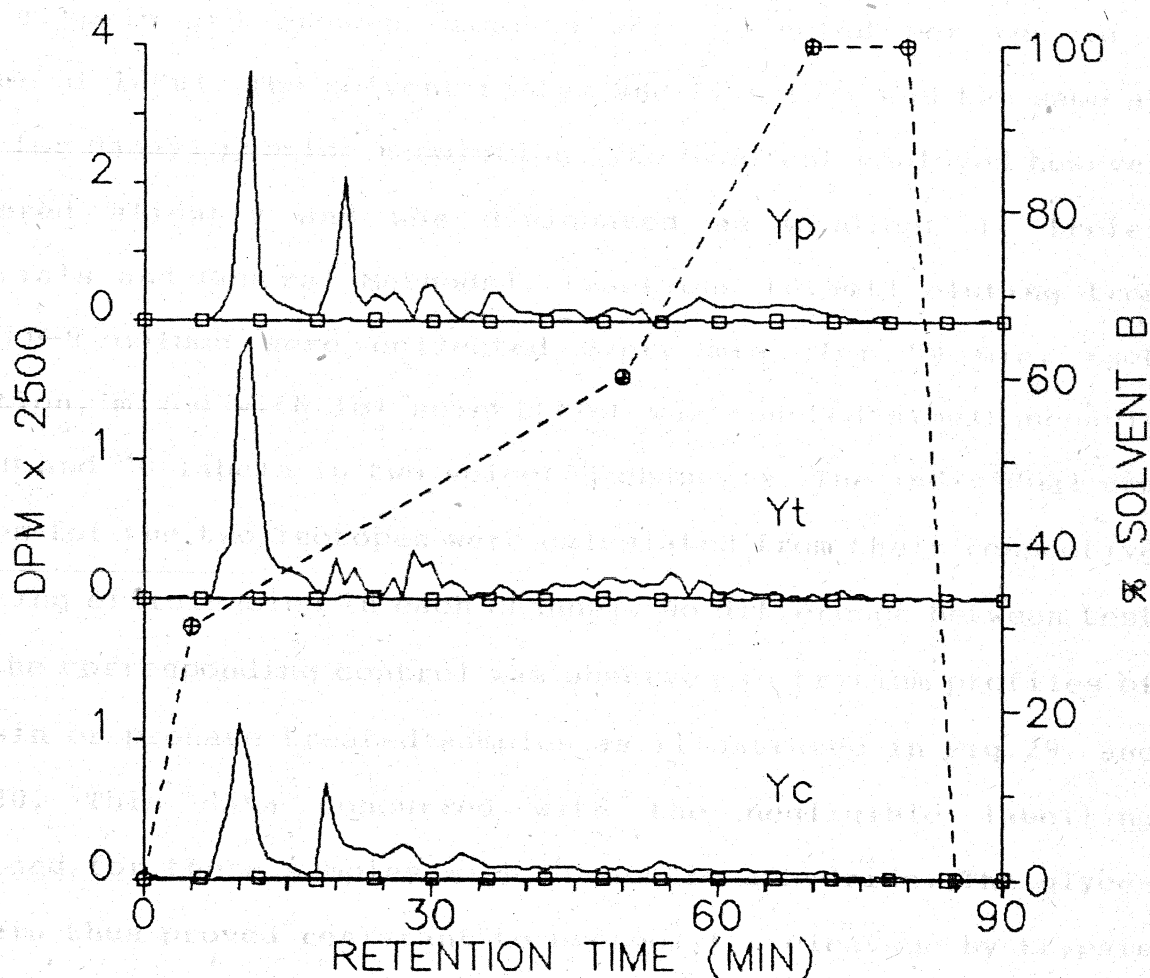


Fig.30.

RP-HPLC of trypsin and pronase digested III' [Y] trimester glial glycoproteins labelled with [^1H] and [^{14}C]. HPLC was carried out on Lichrosorb RP-8 column in Gradient II [0—0] between the Solvents, A:water-0.1% TFA and B:isopropanol-0.1% TFA at a flow rate=0.5ml/min for 90 min. ^1H -dpm: [—], ^{14}C -dpm: [□—□], c-control protein, t-trypsin digest, p-pronase digest. Refer text for details.

Separation of peptides by HPLC on Lichrosorb RP-8 column

Trypsin and pronase digests were injected per run in a volume of 100 μ l. The solvent system and flow rate was the same as used for dansyl peptide resolution. The Gradient employed however differed slightly and was designated as Gradient II [refer Materials and General Methods]. Fractions [0.5ml] eluting from the RP-8 column were collected every min, for 90 min. Each fraction, mixed with 1ml scintillant was counted simultaneously for ^3H and ^{14}C labels in two selected channels. The individual dpm values for the two isotopes were calculated from their respective counting efficiencies in each channel. No difference between test and the corresponding control was observed in tritium profiles of trypsin or pronase treated samples as illustrated in Fig.29. and Fig.30. This data concurred with the negligible labelling obtained for these samples with ^{14}C -acetic anhydride. The glycoprotein thus proved resistant to proteolytic cleavage by trypsin or pronase. Resistance to proteolysis has been reported for many glycoproteins (346). Carbohydrates on glycoproteins were known to serve a protective function during exposure to proteases, probably through steric hinderance (328).

The characterisation studies were carried out on glial cell glycoproteins prepared from IInd and IIIrd trimester brains respectively. No developmentally related variations could be identified under the present experimental conditions.

PART IV: STUDIES ON CARBOHYDRATE SIDE CHAINS OF GLIAL CONCAVALIN A-BINDING GLYCOPROTEINS

Partial structural characterisation of glycan side chains of the isolated glycoproteins was attempted. Elucidation of the carbohydrate structure of membrane glycoconjugates in their native forms posed many problems, such as, difficulty in isolating sufficient quantities required for structural studies, poor solubility and microheterogeneity of the oligosaccharide side chains.

Many workers have conveniently studied carbohydrate structure on glycopeptide or oligosaccharide fragments prepared from the parent glycoproteins (53,223). This approach yielded precise information with respect to carbohydrate content and structure, for individual glycopeptides or oligosaccharides. However due to the heterogeneity of fragments obtained from the parent glycoproteins the overall results were of a generalized nature. We therefore chose to study glycan structure on intact glycoproteins.

Lectins have been widely used to determine carbohydrate structure. Lectin binding was governed not only by the specific sugar but a more complex sequence of sugars in the oligosaccharide (178). By using a variety of lectins of different sugar binding specificities which were inhibited by simple sugar haptens, fine variations in oligosaccharide structure could be conveniently elucidated.

The high degree of sensitivity required to monitor lectin binding was achieved by enzyme linked lectin assays on nitrocellulose, of the Western or dot blotted glycoproteins of interest. Western Enzyme Linked Lectin Analysis [WELLA] or Dot Enzyme Linked Lectin Analysis [DELLA] was carried out by a protocol adapted from published methodologies (241,76,110).

Preparation of Western blots

Glial proteins [250µg] were loaded on a 10% gel and SDS-PAGE was carried out. The proteins were transferred electrophoretically onto a nitrocellulose sheet according to the method of Towbin et al (335). The nitrocellulose sheet was cut into strips corresponding to the lanes on the gel. One strip, which served as control, was stained for 5 min in Amido black to visualize the blotted proteins (295). The remaining strips were subjected to differential lectin binding analysis.

Dot blotting of glycoproteins

The sample was boiled in blotting buffer [0.04M LiCO_3 -0.05% SDS, pH 9.5] at 100°C for 5 min. The suspension was diluted to a stock protein concentration of 10-30µg/0.1 ml. This stock solution was serially diluted 0-512 times in the same buffer. Nitrocellulose strips were spotted 0.5-1cm apart with 5µl each of the serially diluted samples and allowed to dry at 25°C.

Differential HRP-lectin and HRP-serotonin binding assay

Nitrocellulose strips containing the blotted samples were blocked with TBS pH 7.4-0.05% Tween 20 quenching buffer at 25°C

for 2 h or at 4°C overnight. Each strip was then incubated in the respective HRP-conjugate solution at 4°C for 2 h. The HRP-lectins were solubilised in quenching buffer at a concentration of 10-15 µg/ml (64). These conjugates could be reused several times until loss in activity was observed. The conjugated lectins used in this assay were Con A, WGA, RCA, JSA, AHA, SBA, PNA and PSA. Con A binding required 0.001M Ca^{2+} , Mn^{2+} , and Mg^{2+} in the buffer. After incubation with HRP-lectin, the conjugate was poured off and the nitrocellulose strip was washed thrice at 10 min intervals with cold quenching buffer. Finally it was washed at 25°C with two changes of TBS pH 7.4 before incubating with freshly prepared chromogenic substrate. Two HRP substrates differing in the colour of their respective oxidised forms have been used, o-dianisidine which gave rise to a brown colour (13), and 3,3'-diaminobenzidine which gave a reddish colour (357). The substrate solution was prepared by mixing 50ml TBS, pH 7.4, at 25°C with 15µl H_2O_2 and 10mg respective substrate dissolved in 1.5ml methanol. The colour attributed to lectin/serotonin binding was read visually after 30 min incubation in the chromogen.

To ensure the specificity of lectin binding, blanks were run simultaneously in presence of the corresponding sugar inhibitors. Hapten sugars employed were 0.015M and 0.5M α -MG for Con A, 0.2M GlcNAc for WGA, 0.2M Gal for JSA, AHA and PNA, 0.2M GalNAc for SBA, 0.2M lactose for RCA and 0.2M Glc for PSA. The respective

sugar inhibitors were maintained in the quenching buffer through all the steps of the lectin binding protocol.

In addition to lectin binding, presence of sialic acid was detected by its binding to serotonin. The binding was ionic in nature and could be reversed with 1M NaCl. Nevertheless, it showed a high degree of specificity for N-acetylneuraminic acid (319). Serotonin-HRP was used at a concentration of 30-50 $\mu\text{g/ml}$ (64).

The Nitrocellulose strips containing Western blotted glycoproteins after differential lectin binding analysis were dried on filter papers and rendered transparent by immersing in liquid paraffin. Densitometric scanning was carried out at 610nm for Amido black and 540nm for o-dianisidine respectively (235,270).

The western blots stained identically with all the lectins indicating that the glycoprotein bands were not resolved on SDS-PAGE on the basis of carbohydrate structure (Fig.31.). Individual bands G_I to G_{IV} eluted from SDS-PAGE also exhibited lectin binding properties identical to each other and to the delipidated Con A eluate. Henceforth, all binding studies were executed on dot blotted delipidated Con A eluate, boiled in blotting buffer to inactivate native Con A, if any present.

The glial glycoproteins were found to bind Con A, WGA, RCA, JSA, AHA, and PSA (Table.6.). No binding was observed with PNA and SBA. Lectin binding was completely inhibited in presence of

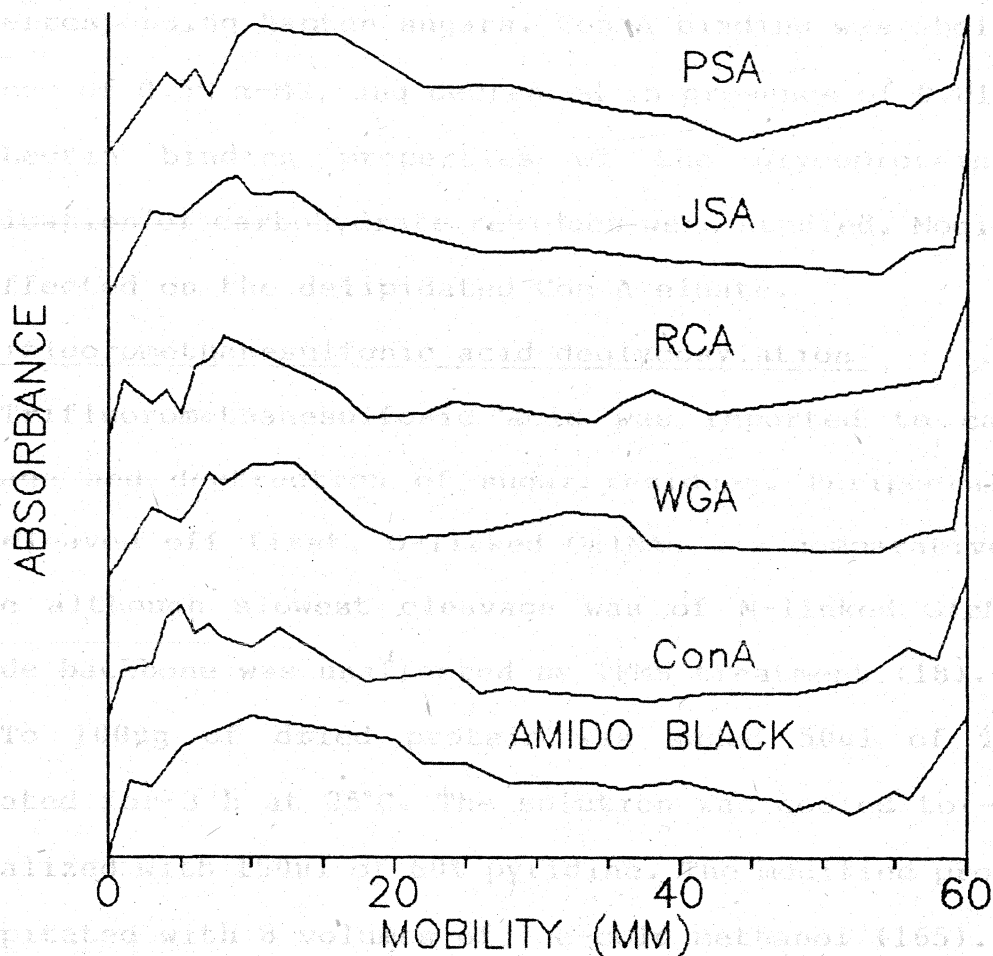


Fig.31.

Western enzyme linked lectin assay of glial Con A-binding glycoproteins - Densitometric Scan. Absorbance was read at 610nm for Amido black and 540nm for HRP-lectins. Refer text for details.

the corresponding hapten sugars. Con A binding was abolished in presence of 0.5M α -MG, and decreased in presence of 0.015M α -MG.

Lectin binding properties of the glycoproteins after modification of carbohydrate residues were studied. Modification was effected on the delipidated Con A eluate.

a) Trifluoromethanesulfonic acid deglycosylation

Trifluoromethanesulfonic acid was reported to cause the cleavage and destruction of sugar residues. Peripheral sugars were cleaved off first. O-linked GalNAc was comparatively more stable although slowest cleavage was of N-linked GlcNAc. The peptide backbone was unaffected by TFMS treatment (18).

To 100 μ g of dried protein was added 50 μ l of TFMS and incubated for 3 h at 25°C. The solution was cooled to -20°C and neutralized with 150 μ l of 60% pyridine. The modified protein was precipitated with 8 volumes of ice-cold methanol (165).

b) Nitrous acid deamination

Nitrous deamination has been employed in the cleavage of glucosaminyl linkages of oligosaccharide chains (138).

The protein [100 μ g] in 450 μ l of 0.25M sodium acetate buffer, pH 3.5, containing 0.25M NaNO₂ was incubated at 25°C for 5 h and 16 h respectively, and precipitated with 8 volumes of methanol (80).

c) Periodate oxidation of carbohydrate residues

Treatment with periodate resulted in the oxidative cleavage of vicinal diols in the oligosaccharide chains and the formation

of reactive dialdehydes (65). Subsequent reduction with borohydride minimized Schiff's base formation between the generated aldehyde groups and amino groups in the protein as well as nonspecific oxidation of amino acids. In contrast to borohydride, cyanoborohydride did not reduce the free carbonyl form of the sugar at $\text{pH} > 5$ (36). Thus the alkaline pH required for efficient borohydride reduction also favoured nonspecific side reactions, could be avoided with CNBH_4 as reducing agent.

The protein [100 μg] in 0.2ml of 0.2M sodium acetate, pH 3.5 buffer, was treated with 0.2ml of 0.04M NaCNBH_4 -0.02M NaIO_4 in acetate buffer and kept at 4°C for 24 h in the dark. The reaction was arrested with 100 μl of ethylene glycol and the protein precipitated with 8 volumes of methanol (332).

d) Chemical desialylation

The protein [100 μg] was digested at 80°C for 1 h in 0.1N H_2SO_4 and precipitated with 8 volumes of methanol (143).

e) Desialylation with Neuraminidase

Serially diluted glycoproteins were dot blotted on nitrocellulose strips, quenched at 25°C in 0.1M sodium acetate buffer pH 5.6-0.05% Tween 20 for 4 h and incubated with neuraminidase [50mu/ml] in the same buffer at 37°C for 18 h. The blots were washed with cold TBS pH 7.4-0.05% Tween 20 and incubated in the respective HRP-conjugate solutions. Controls run simultaneously through the entire sequence were not digested with the enzyme (171,233).

The results of differential lectin binding studies have been tabulated (Tables.6-9.). The characteristics and binding specificities of plant lectins have been reviewed by many groups of workers (115,249). Con A-binding glial glycoproteins were partially eluted with 0.015M α -MG indicating the presence of biantennary complex chains in addition to the high mannose and hybrid oligosaccharide side chains eluting with 0.5M α -MG (242,237). Chemical modification with TFMS and periodate resulted in an appreciable decrease in Con A binding.

The presence of bisected biantennary chains, also a characteristic of hybrid N-linked oligosaccharides was indicated by binding to WGA (359). Binding was obviously enhanced by the presence of sialic acid, since desialylation was found to decrease WGA binding. The relevance of sialic acid as a cell surface binding site for WGA has been reported (106). Significant residual WGA binding was observed after TFMS deglycosylation, NaNO_2 and periodate treatments, respectively.

Carbohydrates, containing penultimate galactose residues were found to interact with RCA (245). The comparatively limited RCA binding to glial glycoproteins was suggestive of a smaller proportion of tri- and tetra antennary complex N-linked glycans. These chains appeared to be most vulnerable to modifications by periodate, TFMS and NaNO_2 . Desialylation augmented RCA binding, probably by exposing more galactose residues.

The two lectins from the genre Artocarpus viz. JSA from Artocarpus integrifolia (322) and AHA from Artocarpus hirsuta (7) were also galactose specific. However, unlike RCA both these species were known to show a marked specificity towards α -linked galactopyranoside units and could not be inhibited by lactose. Both lectins were found to bind human serum IgA and IgG but not to IgM. Glial glycoproteins demonstrated a high affinity towards JSA and AHA. Binding was abolished in presence of 0.2M galactose. Surprisingly, JSA and ASA binding remained unaffected even after NaNO_2 or periodate modification. ASA binding activity appeared more refractory to chemical modification. Treatment with TFMS could not completely obliterate binding to these lectins. Desialylation evidenced a marginal increase in JSA and AHA binding.

The glycoproteins bound to Pea lectin signifying the presence of $\alpha(1-6)$ linked fucose in the innermost N-linked GlcNAc residue of the hexasaccharide core (177). Only TFMS deglycosylation affected this binding to a considerable extent.

The anti-T lectin, PNA, which was highly specific for the β -D-Gal(1-3)-D-GalNAc- α structure, did not bind to the isolated glycoproteins (206). This result was further supported by negative binding obtained with SBA, another lectin specific for GalNAc (202,304). Chemical modification of the N-linked glycan moieties did not aid PNA or SBA binding. The obvious inference was indicative of an absence of O-glycosidically linked side

chains with the characteristic GalNAc residues, however, considering the aggregated nature of the glycoprotein samples it was also probable that the relevant saccharide residues were inaccessible or masked by N-glycans and hence escaped detection by SBA and PNA.

From the lectin binding studies it could be concluded that the glial glycoproteins contained N-linked glycans of which, the biantennary, high mannose and hybrid types appeared the predominant species. Chemical modification affected binding to RCA the most. Cleavage by nitrous acid was rather ineffective, irrespective of whether nitrous deamination was carried out for 5 h or 16 h. Periodate oxidation of the oligosaccharide chains affected lectin binding to a moderate extent. Deglycosylation with TFMS was incomplete even after drastic treatment at 25°C, a condition reported to bring about complete deglycosylation of most glycoproteins. In this case, residual binding activity was observed.

Serotonin binding was employed as an additional parameter to monitor desialylation. Although serotonin binding to sialic acid was purely ionic in nature and could be inhibited with 1M NaCl, it was apparent that serotonin bound quite specifically to sialylated glycoproteins only, e.g. fetuin. Native glycoprotein bound serotonin-HRP appreciably (Table.9.). Chemical or enzymatic desialylation abolished this binding completely. This was used as

TABLE.6.

SPECIFICITY OF LECTIN BINDING TO GLIAL GLYCOPROTEINS.

Serial dilution of dot blotted glycoproteins increased from left to right. Lectin binding was carried out in presence [test] and in absence [control] of the specific hapten sugar. Results are denoted as (+) for positive and (-) for negative lectin binding. See text for details.

HRP-CONJUGATE	HAPTEN SUGAR	CONTROL	TEST
1. Con A	0.015M α -MG	+ + + + + +	+ + + + - -
	0.5M α -MG		- - - - - -
2. WGA	0.2M GlcNAc	+ + + + + +	- - - - - -
3. RCA	0.2M Lac	+ + + - - -	- - - - - -
4. JSA	0.2M Gal	+ + + + + +	- - - - - -
5. AHA	0.2M Gal	+ + + + + -	- - - - - -
6. PSA	0.2M Glc	+ + + + + +	- - - - - -
7. PNA		- - - - - -	
8. SBA		- - - - - -	

TABLE.7.

LECTIN BINDING TO CHEMICALLY MODIFIED GLIAL GLYCOPROTEINS. Serial dilution of dot blotted glycoproteins increased from left to right. Glycoproteins treated respectively with TFMS, NaIO₄, and NaNO₂, were compared with unmodified protein controls. Results are denoted as (+) for positive and (-) for negative lectin binding. Refer text for details.

HRP- CONJUGATE	CONTROL	TFMS	NaIO ₄	NaNO ₂
1. Con A	++++++	+-----	+++---	+++++-
2. WGA	++++++	+++---	++++---	+++++-
3. RCA	+++---	-----	-----	++-----
4. JSA	++++++	++-----	++++---	+++++-
5. AHA	+++++-	++++---	++++---	+++++-
6. PSA	++++++	+++---	++++---	++++++
7. PNA	-----	-----	-----	-----
8. SBA	-----	-----	-----	-----

TABLE.8.

LECTIN BINDING TO GLIAL GLYCOPROTEINS AFTERDESIALYLATION. Serial dilution of dot blotted

glycoproteins increased from left to right.

Chemically and enzymatically desialylated

glycoproteins were compared with unmodified protein

controls. Results are denoted as (+) for positive

and (-) for negative lectin binding. Refer text for details.

HRP-CONJUGATE	CONTROL	0.1N H ₂ SO ₄	NEURAMINIDASE
1. Con A	++++++	++++++	++++++
2. WGA	++++++	+++---	+++---
3. RCA	++++--	+++++-	+++++-
4. JSA	++++++--	++++++--	
5. AHA	++++++--	++++++--	
6. PSA	++++++	+++++-	
7. PNA	-----	-----	
8. SBA	-----	-----	

TABLE.9.

HRP-SEROTONIN BINDING TO GLIAL GLYCOPROTEINS.

Serial dilution of dot blotted glycoproteins

increased from left to right. Results are denoted

as (+) for positive and (-) for negative lectin

binding. See text for details.

SAMPLE	TREATMENT	SEROTONIN BINDING
CONTROL		+ + + + + -
TEST I	1M NaCl	- - - - -
TEST II	0.1N H ₂ SO ₄	+ + - - -
TEST III	NEURAMINIDASE	- - - - -

an index to monitor WGA binding and thereby correlate the difference in WGA binding of native and desialylated samples.

A noteworthy observation was the difference in binding affinities for the three galactose specific lectins RCA, JSA and AHA. It was surprising to evince residual binding to JSA and AHA even after drastic chemical modification. The sugars governing RCA binding were on the other hand most vulnerable to modification. One possible explanation for this behaviour may be the relative difference in the sizes of the lectin molecules. Larger molecules such as RCA would be less accessible to the binding sites on the glycoprotein aggregates as compared to JSA or AHA of lower molecular size. Another reason could be that the galactose residues interacting with RCA may differ from those specifically binding JSA and AHA. Since the WGA binding pattern paralleled that of JSA and ASA to a great extent, it was worth querying whether the galactose situated on WGA specific bisected biantennary and/or hybrid side chains were sites of JSA and AHA binding. These chains would be less accessible to RCA and to chemical modification as compared with the tri- or tetra antennary glycan side chains. The viewpoint however required further investigation. The third aspect to be examined was the higher specificity towards α -Gal residues exhibited by the artocarpus lectins. (352), human peripheral nerve (124), parietal. Recent reports (362) have revealed that jacalin [JSA] had a very high specificity for α -anomers of Gal and GalNAc. Further-

more, this lectin exhibited a particularly high binding affinity for the T-antigenic structure (47), the typical core structure of O-glycans (15). Hortin and Trimpe (152) have confirmed that the occurrence of a single O-linked oligosaccharide was adequate to mediate binding of a glycoprotein to jacalin-agarose. The lectin was not expected to bind N-linked oligosaccharides since most N-glycans lacked the α -anomers of Gal and GalNac. The exceptions to this were the N-linked blood group antigens, however these ligands were found to have low affinity for jacalin (212).

The fact that glial glycoproteins did not bind SBA and PNA was itself contradictory to its high binding affinity for JSA, taking into account the above mentioned criteria for JSA binding. It was conceivable that JSA binding was more sensitive and/or that of SBA and PNA were more susceptible to masking by other sugar residues. In addition, recent evidences have established that besides carbohydrate-binding sites, lectins may contain sites specific for noncarbohydrate ligands, and that these sites too, maybe critical for the recognition functions of the lectin (20,305). Finally, the presence of α -linked galactose in brain cannot be irrevocably rules out. A natural human IgG antibody with anti- α -galactosyl specificity has been reported (105). The presence of $\alpha(1-3)$ galactose moiety in human tissues such as blood group B substance (352), human peripheral nerve (124), parietal cells of gastric mucosa and fibroblasts (169), several cancer cells (194) and laminin (336) demonstrate the subtlety of

immune tolerance in man (103). Finne and Krusius (84) in the course of their studies on O-glycosidic carbohydrate units of glycoproteins of different tissues, identified a brain specific disaccharide, α -Gal-(1-3)-N GalNAc and elaborated that this unique linkage may occur on further glycoconjugates, notably in nervous tissue. The occurrence of such α -anomeric moieties could explain the higher affinity of the isolated glial glycoprotein towards JSA and AHA. These residues may be less vulnerable to modifying agents probably by virtue of their cryptic nature.

Two questions remained unanswered at this point. Was the predominance of high mannose and hybrid chains a result of the isolation technique, viz. the selective isolation of glycoproteins having a high affinity for Con A? Or was it a reflection of the plasma membrane glycoproteins in developing foetal glial cells? Intra uterine development of brain was a relatively slower process in humans as compared with other mammalian systems. The observed carbohydrate composition may be an indication of incomplete processing of the glycoproteins in the golgi-ER apparatus during their biosynthesis, before incorporation into the cell membranes.

Interpretation of carbohydrate structure from differential lectin binding studies presented certain limitations. As the isolated glial glycoproteins existed in the form of micellar aggregates, the demonstrated lectin binding properties were most likely a resultant effect pertaining to the glycoprotein

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aggregate as a whole and not a reflection of the individual glycan structures of the constituent glycoproteins. Another factor to be kept in mind was that detection of lectin binding by the technique of DELLA employed a chromogenic reaction and hence was subject to variation depending on the HRP-conjugate in use. Although satisfactory results were obtained with soluble standard glycoproteins such as fetuin, the possibility of an erroneous inference from the apparent binding intensities could not be ruled out.

Characterisation of glycan side chains by HPLC of 2-amino pyridine labelled oligosaccharides prepared from the glial glycoproteins was attempted. The procedure was limited severely by the inability to release sufficient quantities of oligosaccharides from the glycoproteins to enable their detection. This alternate approach to the elucidation of carbohydrate structure was thus abandoned.

Differential lectin binding studies were carried out on both IInd and IIIrd trimester glial glycoproteins. Both the samples demonstrated identical carbohydrate structure and lectin binding properties, signifying the absence of any developmentally related changes under the conditions of the study.

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Cell surface glycoproteins are unique functionally by virtue of their involvement in various aspects of cell-sociology within and outside the cell. Physicochemical characterisation per se cannot define these membrane components completely. Some information on the immunological behaviour of these molecules in combination with physicochemical techniques would furnish a more comprehensive molecular picture.

Immunochemical assays have been used widely to detect, localize and quantify minute amounts of antigen in complex tissues. Besides, immune recognition of an antigen specific to the nervous system would contribute tremendously towards a better understanding of brain development, differentiation, molecular biology and sociology, function and pathology. The study of such marker antigens was based on the rationale that a protein which was not required by another tissue, had an important function probably specific to nervous system.

It has been established that normal brain tissue to a great extent was immunologically inert. There were virtually no immunocompetent cells nor the presence of organized lymphoid tissue reflecting that the brain was not normally exposed to significant levels of antigenic stimulation. Immunological reactions in neural tissue were often muted and frequently delayed. This unique milieu was maintained by a series of

functional and anatomical barriers collectively known as the blood brain barrier. The brain was thus inaccessible, shielded from attack by invading organisms, and relatively impermeable to antigens reaching it from the blood stream. The immunological privilege of the brain was not absolute but relative and depended upon the integrity of the barriers separating it from the circulating blood. In disease, these barriers were breached and the inflammatory process allowed lymphocytes and antibodies to enter the nervous system. At this point, the brain developed into an organ capable of generating an immune response. The blood brain barrier was incomplete in the fetus, and the level of protein in the CSF was raised compared with that in the adult. This increase in permeability persisted to the end of gestation and into the neonatal period. Elevated levels of total protein and albumin were apparent at birth, decreased during early months of life and attained adult values at 8-12 weeks. Correspondingly, the levels of immunoglobulin were also raised 4-8 times the adult value at 22 weeks of gestation, and declined to 2-3 fold the normal adult values at birth. This level persisted upto 3 months after birth (1).

The central nervous system, therefore displayed a wide spectrum of immunological behaviour, ranging from relative unresponsiveness at one end, to the most florid manifestation of hypersensitivity and immunity at the other. Diseases such as multiple sclerosis and subacute sclerosing panencephalitis were

associated with active antibody synthesis in the CNS, while experimental allergic encephalomyelitis in animals provided a striking illustration of the fact that hypersensitivity could produce devastating lesions in the brain (193).

In general, brain proteins in the pure form were found to be of low immunogenicity. Proteins such as S-100 (196) and 14-3-2 (230) could elicit an antigenic response only when administered conjugated to methylated bovine serum albumin. Synaptin (34), D1, D2, and D3 brain specific proteins (161) were defined by means of a polyspecific polyclonal rabbit antiserum raised against fractions of rat brain synaptosomal plasma membranes. Proteins such as nervous system antigen 1 [NS-1] were identified by means of antisera raised against methyl cholanthrene-induced glioma, G26 in mice (292). Myelin proteins (239,303) and glial fibrillary acidic protein (338) on the other hand were highly immunogenic. Purified brain glycolipids were potentially antigenic and elicited an immune response only when linked to a heterologous carrier polymer, as micellar aggregates or as natural membrane fragments (128).

The use of conventional monospecific antisera, however, posed certain limitations such as (a) low titres (b) antibody heterogeneity in specificity and affinity (c) inability to reproduce the same pattern of antibody reactivity in another animal and (d) a finite supply of reagent. The advent of hybridoma technology (174) concomitant with the production of

specific monoclonal antibodies has facilitated the identification of several marker antigens of functional importance to the nervous system such as neural cell adhesion molecule (66) and adhesion molecule on glia (6).

Immunochemical characterisation of cell surface Con A-binding glycoproteins from human foetal glia were attempted with three objectives in mind. They were (a) to determine the immunogenicity of the antigenic species (b) to assess the nature of antibody produced in response to the immune stimuli and (c) to ascertain with the generated antibodies whether the antigen could serve as a marker glycoprotein, and if so, to what extent. Specificity with regard to species, tissue, cell type, subcellular location, developmental stage and finally cell pathology would be targeted. Immune reactivity was checked by the following immunochemical techniques (29,252,225).

a) Ouchterlony double diffusion

The gel plates were prepared with 1% agar in 0.02M phosphate buffer-0.15M NaCl-0.1% NaN_3 -0.1% Triton X-100, pH 7.4, and loaded with 20 μ l sample per well, replenishing the sample 4-5 times. Immunodiffusion was carried out at 25°C in a moist atmosphere for 4-5 days.

b) Countercurrent electrophoresis

Samples [20 μ l] were loaded into wells of the gel plate prepared with 1% agar in 0.075M barbitone-0.15M NaCl-0.1% Triton X-100, pH 8.3, and electrophoresed at 5mA/slide for 90 min. The

gels were stained with CBB for better visualization of the immune complex.

c) Immuno-electrophoresis

Glass plates [84 x 94mm] were layered with 12ml each of 1% agar in 0.05M barbitone-acetate, pH 8.6, buffer containing 0.2% Triton X-100 and 0.1% NaN_3 . The antigen was loaded in the wells and electrophoresed in the same buffer without Triton X-100 at 5-10mA/plate for 90 min. After the run 200 μ l of antibody was loaded into slots cut in the gel and incubated for one week at 4°C in a moist atmosphere. The precipitin arcs were stained with CBB. The slides were incubated at 4°C with 3 changes in 0.9% NaCl over two days. The gel was blotted dry with filter paper under pressure and dried at 50°C for 30 min. The dried gel was stained with CBB in methanol-water-acetic acid for 5 min and destained according to Weber and Osborn (353).

d) Dot Enzyme Linked Immunosorbant Assay [DELISA]

The antigen [2-5 μ g/5 μ l] was spotted on nitrocellulose and the strip incubated for 2 h at 25°C in the blocking buffer consisting of 2.5% gelatin-0.05% Tween 20 and 0.1% NaN_3 in TBS, pH 7.4. The antigen was then incubated with rabbit antibody [6-10mg/ml] in the above buffer, overnight at 4°C, followed by washing three times with blocking buffer at 10 min intervals. The bound antibody was detected by incubation in HRP-goat anti-rabbit Γ -globulin conjugate [200 μ g/ml] for 2 h at 4°C, washing thrice in blocking buffer at ambient temperature and subsequent visual-

ization in chromogenic substrate for HRP as described in the preceding section.

Immunization of rabbits and guinea pigs with intact IInd trimester glial cell preparations were attempted. Glial cells [0.5-1mg protein/dose] in 1ml saline was mixed with complete Freund's adjuvant and administered intraperitoneally for rabbits and at multiple intramuscular sites for guinea pigs. Succeeding doses were given every 10th day both intramuscularly and subcutaneously at multiple sites in incomplete adjuvant for rabbits. Guinea pigs were administered every week at four intramuscular sites in Freund's incomplete adjuvant. Sera were collected before commencing the study and then periodically. The sera were directly checked for antibody by immunodiffusion and counter current immunoelectrophoresis against antigen. Glial cells were solubilised by sonication [20 μ frequency for 15 sec x 8 times] in PBS, pH 7.4, containing 3% Triton X-100, and diluted 1:1 (v/v) in PBS to bring the detergent concentration to 1.5% . The 100,100 x g for 60 min supernatant was used as antigen at a concentration of 5-10mg protein/ml.

A very low antibody titre was first noticed in rabbit after the third month of immunization. Administration of antigen was continued for a total period of six months before sacrificing the animal. Guinea pigs were immunised for 2 months injecting 8 doses in all, and sacrificed. Both species elicited an immune response, however the delay period was longer in the case of rabbit. The

immunogenicity of the antigens appeared to be rather low since antibody titres were quite low. Immunodiffusion studies with serially diluted antisera gave a positive precipitin reaction only for the antiserum used directly. Another limitation was that the triton solubilised glial extracts diffused very slowly in agar and hence required longer incubation periods to enable immune complex formation. Control and antisera were saturated to 50% ammonium sulfate at 4°C, the precipitated Γ -globulin fraction was collected by centrifugation at 10,000 x g for 15 min and dialysed extensively [1L x 4 changes] against TBS-0.05% Tween 20, pH 7.4, at 4°C. The presence of antibodies to glial Con A-binding glycoproteins in these fractions were checked by DELISA. The glycoproteins did not bind the Γ -globulin fractions purified from glial cell immunized rabbit and guinea pig sera. This was an indication that immunization with intact glia could not serve as a route to generate antibodies specific to the cell surface glycoproteins of interest.

A more direct approach was tried out. Second trimester glial Con A eluate was resolved on 10% SDS-PAGE. The protein bands above the Con A band were cut out from the fixed and CBB stained gels and homogenised with Potter-Elvehjem homogeniser in 4ml PBS, pH 7.4. The protein-gel dispersion [0.1mg protein/ml] was mixed with an equal volume of Freund's complete adjuvant and administered at four intramuscular sites in rabbit. Three booster doses were given at 10 day intervals and the animal subsequently bled.

Formation of cysts were observed at the sites of injection, the animal otherwise appeared normal. Cyst formation may have been caused by acrylamide in the injected samples. Sera was also collected at random from 5 rabbit controls.

The Con A eluate delipidated in Fredman's solvent and suspended in 0.04M LiCO_3 -0.05% SDS, pH 9.5, was used as the antigenic component in the DELISA protocol used to monitor the immune response.

Glial glycoproteins cross reacted with its corresponding antisera as well as antisera raised against Con A-binding glycoproteins from human foetal neurons. Five out of the six control sera was found to cross react with the glial antigen. Considering the high degree of sensitivity of the DELISA technique, false positive results due to non-specific interaction was a tangible threat. The binding of the control pre-immunization sera to glial antigen was traced entirely to the Γ -globulin fraction precipitated at 50% ammonium sulfate concentration. Thus the positive DELISA obtained for the control sera against glial glycoproteins was attributed to the formation of an immune complex.

The next step was to check whether immunization increased the titres of the cross reacting antibody already prevalent in the non immunized rabbit controls. The serially diluted antigen was incubated with equivalent concentrations of control and antisera respectively by the DELISA technique. No difference was

observed between the control and antisera indicating that immunization did not elicit an immune response in rabbit. However it was apparent that some cross reacting species was already present in rabbit blood. Some preliminary investigations regarding the nature of this antibody in this species and whether there existed any disparity between the control and antisera, were carried out.

The Γ -globulin fraction was resolved by affinity chromatography on Protein A-Sepharose 4B [1 x 7cm] equilibrated in TBS, pH 7.4, at 4°C. The 50% ammonium sulfate fraction in 5ml TBS, pH 7.4, was loaded on the affinity matrix, washed with 10 bed volumes of the same buffer and eluted with 0.3M glycine-HCl, pH 3.0 buffer at 4°C. The breakthrough fraction containing IgA, IgM and possibly unbound IgG was collected. The eluted fractions containing the bound IgG was immediately adjusted to pH 7-8 with 2M Tris, pooled and precipitated by 50% ammonium sulfate saturation. The pellet obtained at 10,000 x g for 30 min was suspended in minimal volume of TBS, pH 7.4, and dialysed extensively at 4°C against the same buffer containing 0.05% Tween 20. The fractions resolved by affinity chromatography on Protein A-Sepharose were subjected to DELISA against the glial antigen. The control and the sera obtained after immunization with antigen were resolved on Protein A-Sepharose 4B. The breakthrough and eluted fractions from both these samples bound the glial

glycoprotein antigen suggesting that the cross reacting species most probably belonged to the IgG subclass.

The specificity of this antibody was examined. Tissue extracts of adult human brain, IInd trimester human foetal brain, liver, heart, spleen and placenta, IIIrd trimester human foetal brain and bovine brain were respectively prepared in 0.025M Tris-0.7M glycine-0.001M PMSF-1% Triton X-100-0.1% NaN₃, pH 7.8 buffer. The supernatant obtained after centrifugation at 15,500 x g for 3 min was used as antigen in immunological characterisation studies. The ensuing pellets were delipidated in C:M,2:1 (v/v), suspended in 0.04M LiCO₃-0.05% SDS, pH 9.5 buffer and spotted on nitrocellulose for DELISA.

The antibody present in the control and immune sera did not cross react with any of the tissue extracts on immunodiffusion or immunoelectrophoresis. However all the extracts appeared to bind antibody from control and immune sera respectively on DELISA. This observation was attributed to the high sensitivity of the DELISA method over the immunodiffusion and electrophoretic procedures. It was also likely that diffusion through agar gels was inhibited by the insoluble nature of the antigens under study limiting the latter procedures further. The DELISA results nevertheless suggested that the immunoreactive antibody present was not specific to brain tissue.

In conclusion, it was obvious that the isolated glial glycoproteins from human foetal brain were of low immunogenicity

and failed to elicit an immune response in rabbit and guinea pig. The tendency of these amphipathic glycoproteins to form self micelles was a possible reason for the low antigenic nature of the species. It was conceivable that potentially immunogenic regions on the polypeptide were buried within the micellar core. The protective masking effect due to carbohydrate would be an important consideration with regard to the refractory nature exhibited by such molecules. Finally, immune tolerance in the species selected for the study was a factor that could not be ruled out.

CHAPTER - IV

GENERAL DISCUSSION

CHAPTER - IV

GENERAL DISCUSSION

DISCUSSION

Glial cell development, sociology and function has been the least studied component of central nervous system biochemistry. An intimate relationship exists between neurons, the principal functional units of neural tissues, and neuroglia which supports and ensheathes these neuronal cells. Brain development is characterised by somatic differentiation, chemical variations as due to neurotransmitters and neuropeptides and electrical fluxes caused by the generation and conduction of action potentials. Glial cells are associated with all these events, such as in directing neuronal migration during establishment of neuronal pathways, in uptake mechanisms for neurotransmitters and in the propagation of electrical impulses by saltatory conduction through myelinated nerve fibres. Nevertheless, detailed knowledge of glial cell structure-function relationships is fragmented. The present study was targeted towards gaining some information on the molecular basis of glial cell-sociology with direct relevance to the developing human brain.

Human foetal brain tissue used for the study was a developing system wherein anabolic pathways were still operative and possibly dominated cellular metabolism. The presence of partially synthesized glycoproteins and other biomolecules was plausible and introduced a high degree of variability in the study. Another common problem with postmortem tissue was the extent of degradation undergone before collection and processing.

In addition, no information was available on the health or nutritional status of the mother. These factors could complicate the analysis further.

The above limitations were probably contributory factors which precluded the application of conventional methodologies for the isolation and characterisation of glial cell surface glycoproteins from developing human brains. For example, digestion of the extracellular matrix with proteases as a prelude to the separation of glial cells, was bypassed as trypsin digestion resulted in extensive lysis of foetal brain cells. Existence of an incompletely formed extracellular matrix in post-mortem foetal brain tissue which might have undergone partial decomposition, may have increased the fragility of the brain cells in the proteolytic milieu. Brain tissue disruption was therefore effected by mechanical sieving procedures under gentle suction in presence of cell stabilising polymers like Ficoll 400. Similarly, the preparation of pure plasma membrane subfractions was limited by the lack of detectable plasma membrane markers. It was possible that the marker enzymes may not have been fully functional as yet, or they had lost activity during storage.

The glial cell surface glycoproteins were hydrophobic and could not be solubilised in aqueous media even in presence of strong detergents like 4% SDS or in the presence of denaturants such as 8M urea or 6M Gn.HCl, at protein concentrations exceeding 0.5mg/ml. This property was particularly apparent on SDS-PAGE or

urea-PAGE of the Con A-binding glycoproteins, wherein a considerable portion of the applied protein was retained at the point of application. Another illustration of the limited solubility, was the precipitation of glial cell surface glycoproteins through aggregation when concentrated by ultrafiltration, even in presence of Triton X-100. Glycoprotein solutions, especially in the absence of detergents or denaturants, were too dilute to estimate protein or carbohydrate by the standard chemical micro-estimation methods. Hence protocols specifying solubilised protein components were carried out with radiolabelled samples. In general, suspensions of glycoproteins in the appropriate buffers were employed for characterisation studies.

Membrane proteins were commonly associated with hydrophobicity which enabled their interaction with the lipid components of the membrane bilayer (308). Hydrophobicity was imparted to proteins by the nonpolar amino acids in their polypeptide backbones and their hydrophobic tertiary structural domains. Since membrane proteins were stabilised by the membrane phospholipid bilayer, they tended to form insoluble aggregates in aqueous media when removed from this hydrophobic environment. Surfactants aided the solubilisation of membrane proteins in the form of protein-detergent micelles (145). Organic solvent precipitation removed the associated water of hydration and exposed additional hydrophobic domains in the protein,

consequently augmenting the formation of irreversible aggregates that resisted resolubilization in aqueous buffers.

Glial cell surface glycoproteins appeared to have an inherent tendency to form micellar aggregates. This property governed all its interactions and was manifested under different experimental criteria. Preparation of these glycoproteins by affinity chromatography on Con A-Sepharose 4B was a problematic endeavour due to excessive leaching of Con A from the matrix. This phenomena was abnormal and was apparently related to the nature of the protein under study. Con A-chromatography has been employed successfully for the isolation of a wide variety of glycoproteins and did not present the complication of lectin contamination in the eluted fractions. Dissociation of Con A from the matrix took place irrespective of the presence of lipid and/or detergent and the buffer composition during chromatography. Moreover, the lectin co-eluting with the glial glycoprotein fractions did not agglutinate trypsinized RBC, indicating that leaching was possibly due to the release of non-covalently associated Con A subunits from the matrix and not through cleavage of covalent bonds linking the lectin to the matrix. Pretreatment of Con A-Sepharose with 6M Gn.HCl effected the removal of non-covalently but hydrophobically bound Con A subunits from the immobilized Con A-gel matrix. Chaotropic agents disrupted hydrophobic domains by breaking the hydrogen bonds stabilising these tertiary structures in proteins.

The isolation of glial plasma membrane glycoproteins by RCA or WGA-Sepharose chromatography also presented similar problems of lectin leaching although WGA contamination in the eluted fractions were comparatively less than that of RCA. It was reasoned that since Con A and RCA were tetrameric proteins, covalent linkage of all the subunits to the matrix was less probable than for a dimeric protein such as WGA. The non-covalently immobilised subunits were associated mainly through hydrophobic interactions to the matrix bonded subunits, by virtue of hydrophobic patches on the lectin molecules. The existence of sufficiently nonpolar components in the eluting mobile phase caused the release of these subunits from the matrix.

The persistence of Con A subunits in the eluted fractions even after pretreatment of the gel suggested that 6M Gn.HCl was unable to remove completely all the non-covalently bound Con A from the affinity matrix. Obviously, the 0.5M α -MG eluate contained elements more competent than Gn.HCl in effecting the dissociation of Con A sub-units. The relative compositions and characteristics of the glycoprotein fractions eluted respectively at 25°C and 4°C have been summarised in Table.10. It was evident that Con A leaching was influenced by two factors, viz. the glycolipid content in the eluted fraction and the temperature at which elution was carried out. Elution at 25°C rendered glycoproteins contaminated with appreciable amounts of non-covalently associated glycolipid as well as Con A, whereas the

TABLE.10.

EFFECT OF TEMPERATURE ON THE COMPOSITION OF CON A ELUATE.

ELUTION TEMPERATURE	25°C	4°C
Carbohydrate [$\mu\text{g}/\text{mg}$ protein]	450 ± 200	160 ± 90
Carbohydrate after delipidation [$\mu\text{g}/\text{mg}$ protein]	160 ± 90	160 ± 90
Sialic acid [n moles/mg protein]	20 ± 5	25 ± 5
Sialic acid after delipidation [n moles/mg protein]	18 ± 5	25 ± 5
Con A-band on SDS-PAGE	++++	++
TLC of lipid extract:		
1. I_2 Vapour	++++	+
2. Orcinol - H_2SO_4	++++	++
3. Resorcinol - HCl	++++	++

4°C eluted samples were comparatively free of both glycolipid and Con A contaminants. Clearly, the Con A elution profile closely paralleled that of glycolipid and was temperature dependant. The leaching of Con A was inferred to be a result of temperature dependant phase transitional changes in the lipid component in the Con A eluate. At higher temperatures the mobile lipid constituents were included in the eluted mobile phase in the form of mixed micelles of glycoprotein, glycolipid and Triton X-100, containing a highly hydrophobic core. The greater affinity of Con A subunits for the hydrophobic environment contained within these mixed micelles facilitated their dissociation from the matrix and into the eluted fraction as glycoprotein-glycolipid-detergent-lectin micelles. At 4°C the comparatively immobile lipid was excluded from the eluting phase containing the less hydrophobic detergent-protein micelles. These were incapable of pulling out non-covalently bound Con A subunits from the matrix.

Prior to introduction of the Gn.HCl pretreatment step, it was established that Con A leaching took place even in the total absence of lipid and/or detergent. These observations hinted that glial glycoproteins could on their own cause the dissociation of Con A from the matrix of untreated affinity gels. Nevertheless, this phenomena was amplified in the presence of amphipathic molecules like glycolipids and detergents. Presumably these compounds stabilized micelle formation and increased the solubility of the glycoproteins in aqueous buffers. By and large

the best solubilisation medium for delipidated glycoproteins in the absence of detergents was 0.025M Tris-0.7M glycine, pH 7.8, (311).

The proclivity of glial glycoproteins to form micellar aggregates interfered with their resolution by SDS-PAGE. Non-stoichiometric protein-SDS binding, microheterogeneity and formation of micellar aggregates were responsible for the complex multiband patterns visualized on SDS-PAGE. This anomalous behaviour on SDS-PAGE favoured the existence of a gradation of mutually interconvertible micellar aggregates of SDS and protein in dynamic equilibrium with one another, in the molecular weight range 43-75 KDa.

Gel-Filtration studies verified that the glycoproteins formed self micelles in the absence of lipid and detergent. Sepharose 6B resolved the N-acetylated glycoproteins into an excluded Peak I which was soluble as confirmed by ultracentrifugation at 100,000 x g for 60 min, and an included Peak II, consequently suspected to be of low molecular weight, possibly less than 12.5 KDa. Both peaks rendered the same single fast moving band on acid urea-PAGE suggesting that Peak I was probably a micellar aggregate of the monomeric species eluting as Peak II. It also appeared that once the distribution into micellar and monomeric forms had occurred, the two species were non-interconvertible. This conclusion was arrived at after attempting resolution of individual peaks I and II by a second gel-

filtration on Sepharose 6B and also by RP-HPLC in an isopropanol-water gradient. Peaks I and II had their respective elution profiles and were not subject to interconversion nor further resolution by both the above protocols. The micellar peak I, as expected, had a higher retention time in the RP-HPLC system in agreement with its more hydrophobic nature. The observation that organic solvents employed in RP-HPLC were unable to break Peak I micellar aggregates was suggestive of a high degree of stability for these glycoprotein self micelles.

N-acetylated glycoproteins were resolved by acid urea-PAGE to low molecular weight, presumably monomeric forms. Urea-PAGE of the native protein however revealed comparatively slow moving bands. Mobility on acid urea-PAGE of native protein molecules was aided by an additional charge effect which was absent for N-acetylated proteins. The logical assumption was that the native glycoproteins rendered slow moving high molecular weight micellar forms, whereas N-acetylated glycoproteins were disrupted to their monomeric forms under the conditions of urea-PAGE. The decreased surface charge on the protein as a result of N-acetylation supposedly reduced the bipolarity of the glycoprotein and rendered them more vulnerable to H-bond cleavage by urea.

Additional evidence supporting the micellar structure of the isolated glycoproteins was gained in the course of radiolabelling procedures. It was observed that after N-acetylation of the glycoproteins with tritiated acetic anhydride, the excess free

acetic anhydride could not be removed by dialysis. Methanol or organic solvent precipitation and extensive washing in organic solvent media had to be employed to remove this excess free radioactivity. As acetic anhydride partitioned preferentially into a non-polar phase during phase partitioning, it was inferred that the free acetic anhydride molecules were held within the hydrophobic micellar core of the glycoproteins and thus were unable to be removed by dialysis against water. Methanol altered the dielectric constant of aqueous media destabilising micellar aggregates and hence served to dislodge the trapped acetic anhydride molecules.

Amphipathicity, a requisite for micelle formation was an inherent property of most integral membrane glycoproteins (320). With reference to the already characterised cell membrane glycoprotein structures, it was possible to postulate a similar amphipathic nature for the isolated glial glycoproteins. The postulated conformation correlated with all the identified characteristics of these glycoproteins. The bipolar glycoproteins specified distinct hydrophilic and hydrophobic domains consisting of hydrophilic carbohydrate chains and possibly the polar amino acids localized close to the N-terminal region, while the hydrophobic amino acids were situated in the vicinity of the carboxy-terminal. Micellar aggregates of these glycoproteins therefore comprised of a hydrophobic core made up of C-terminal peptides and the externally oriented N-terminal regions with

their associated carbohydrate, presumably in the form of a hydrophilic glycan coat. The exposed glycan coat on these micelles were probably responsible for the refractory nature of the glycoproteins.

The glycoproteins moved preferentially on acid urea-PAGE indicating a relatively higher proportion of basic amino acids on the polypeptide backbone. However, these glycoproteins were not susceptible to proteolytic cleavage by trypsin nor pronase. The glycan coat most likely served a protective role here through steric hinderance and masking of potential protease vulnerable amino acid sequences in the protein. Proteolytic digestion was carried out in partially denaturing conditions compatible with enzyme activity. The presence of 2M urea in the digestion medium did not however, render the glycoprotein more accessible to the proteases.

Amino-terminal analysis revealed a blocked N-terminal for the glycoproteins. Although many plasma membrane glycoproteins specified blocked amino terminals, the presence of glycan chains in the N-terminal region could also indicate an erroneous blocked amino group in the procedures used for determining N-terminal sequences, even if in actuality the amino terminal was free (328,356).

The glycoproteins were incompletely deglycosylated even under the drastic conditions of TFMS treatment. Deglycosylation resulted in the formation of a high proportion of insoluble

aggregates supposedly by partial destruction of the hydrophilic component of these amphipathic molecules. Desialylation imparted a similar but milder effect to render high molecular weight aggregates on SDS-PAGE.

The nature of carbohydrate side chains as analyzed by DELLA could only be subject to a general interpretation, as the results were a reflection of an aggregated entity and not of the individual component glycoprotein molecules. The glycoproteins appeared to contain a relatively higher proportion of Con A-binding glycans than tri- and tetraantennary complex-type chains binding to RCA. It has been reported that in brain membranes, N-linked glycans were predominantly of the complex-type (216). High mannose and hybrid-type chains were intermediates in the post-translational processing of newly synthesized glycoproteins to complex-type glycans (181). A higher contribution of high mannose and hybrid-type oligosaccharide chains on the isolated glycoproteins could be an illustration of incompletely processed glycan chains reflecting the immaturity of the tissue under study. Alternatively, this may be an artifact of the selective isolation of Con A-binding glycoproteins from glial plasma membrane enriched fractions.

In addition to high mannose and hybrid-type carbohydrate structures, the glycoproteins contained biantennary complex chains that were eluted with 0.015M α -MG. Sialic acid was detected by its interaction with serotonin and contributed to

25±5 n moles/mg protein. Sialylation augmented WGA binding to the glycoproteins. The presence of fucose linked $\alpha(1-6)$ to the innermost core GlcNAc residue was confirmed by the high affinity of pea lectin for the sample. The absence of SBA and PNA interaction suggested that O-linked glycans constituted a relatively minor share of the oligosaccharide side chains, or if present, they were masked and unavailable for interaction with these lectins. The *Artocarpus* lectins JSA and AHA, however, exhibited a very high affinity for the glycoproteins. Since JSA could detect O-linked oligosaccharides with a high degree of sensitivity, it was possible that JSA binding was an indication of mucin-type chains. Further, the *Artocarpus* lectins were also known to possess a high specificity for α -linked galactose and probably implied the presence of α -Gal containing glycans in developing human brain.

In order to determine whether the isolated glial glycoproteins were subject to developmentally related variations in structure and composition, the entire characterisation protocol was respectively carried out on glycoproteins from IInd and IIIrd trimester foetal brains. On comparison, no structural or physico-chemical differences were detected in the two groups with the limited available technology. Glycoproteins from both age groups specified similar carbohydrate and sialic acid contents and identical differential lectin binding characteristics. Behaviour on SDS-PAGE, acid-urea PAGE, gel-filtration and RP-HPLC were

similar. Both sets of glycoproteins were refractory to proteolytic digestion by trypsin and pronase. The amino-terminal was blocked for the IInd and IIIrd trimester glycoproteins. Although no prenatal changes could be detected under the present condition of study, with the help of more sensitive procedures and monoclonal antibody technology, it may be possible to pick up developmentally significant variations in structure and composition.

Glial glycoproteins exhibited low antigenicity which may also be a manifestation of its micellar structure. Potential immunogenic sites on the polypeptide were presumably made unavailable to the animal's immune system as a result of steric masking by the external glycan coat of the micellar aggregate. Immune tolerance in the species selected for study or the inherently low antigenic nature of brain proteins could not be ruled out. The failure to elicit an immune response with the isolated glial Con A-binding glycoproteins severely limited the present study. Questions such as whether the isolated glycoproteins were species, tissue or cell type specific, whether these glycoproteins could serve as cell membrane markers and what were their roles in neural development and function were unanswered. It was also not clear if the characterised physicochemical behaviour referred to a single glycoprotein monomer or a group of glycoproteins. Resolution of the Con A-binding glycoprotein aggregates to their component monomers could

shed some light on the structure-function relationships in these species. Monoclonal antibody technology would also aid in tagging these molecules in relation to already identified neural membrane markers, besides delving into the potential role of these glycoproteins as probes in brain pathology.

Summary

Development of the nervous system is a highly regulated process under genetic and environmental influences. The nervous system is a complex system that is highly organized and specialized. It is the central control system of the body, responsible for coordinating and integrating all aspects of behavior and thought. The development of the nervous system is a highly regulated process, involving the differentiation of neural stem cells into various cell types, including neurons, glia, and neuroendocrine cells. The process is controlled by a complex interplay of genetic and environmental factors, including growth factors, signaling molecules, and transcription factors. The development of the nervous system is a highly regulated process, involving the differentiation of neural stem cells into various cell types, including neurons, glia, and neuroendocrine cells. The process is controlled by a complex interplay of genetic and environmental factors, including growth factors, signaling molecules, and transcription factors.

SUMMARY

Several cell surface glycoproteins have been recognized as receptors for neurotransmitters and growth factors. These receptors are involved in the regulation of neuronal development and function. The development of the nervous system is a highly regulated process, involving the differentiation of neural stem cells into various cell types, including neurons, glia, and neuroendocrine cells. The process is controlled by a complex interplay of genetic and environmental factors, including growth factors, signaling molecules, and transcription factors. The development of the nervous system is a highly regulated process, involving the differentiation of neural stem cells into various cell types, including neurons, glia, and neuroendocrine cells. The process is controlled by a complex interplay of genetic and environmental factors, including growth factors, signaling molecules, and transcription factors.

SUMMARY

Cellular development, growth, maturation and maintenance under normal and pathological conditions are defined by a series of synergistically acting morphogenetic events involving cell-sociology. It has been documented that cell surface glycoproteins are implicated in processes involving recognition and adhesion phenomena as well as in receptor ligand interactions. Nervous system by virtue of its unique anatomical diversity, architecture and functional complexity exemplify cell sociology in its broadest most evolved form. Development of the nervous system is thus one of the most sophisticated examples of morphogenesis, in that, it involves both the formation of intricate tissue structures and the precise establishment of neuronal pathways and interconnections. Although considerable work has been carried out on nervous system cell-sociology, at the molecular level the picture is still largely obscure.

Several cell surface glycoproteins have been recognized to mediate adhesion among different cell types in the central nervous system at different developmental stages. Best characterized among them were the neural cell adhesion molecules N-CAM, L1, Ng-CAM, J1, and AMOG. The adhesion molecules did not act independently of each other but cooperated synergistically, thereby modulating the effect of one adhesion molecule on the other. It has also been evidenced that there was a marked induction of N-linked glycoprotein synthesis correlating

temporally with the onset of neuronal and glial differentiation in embryonic rat brain cells. Oncogenically transformed cells have been shown to express glycans altered in structure and composition. Malignant cells were found to synthesize preferentially, fucosylated and heavily sialylated multiantennary oligosaccharide side chains. These observations were illustrations of the role of cell surface glycoproteins in central nervous system sociology.

A noteworthy fact was that, with the exception of N-CAM, most of the characterization work on nervous tissue has been limited to avian or rodent systems. Also, in comparison with neurons, very little information was available on the molecular basis of glial development and function. Characterization of a few glial cell surface-specific glycoproteins from developing human brain would contribute towards understanding glial cell functions and sociology in general, and have more pertinence to its role in human development.

The objective of this study was to isolate, identify and characterize glial cell surface-specific glycoproteins of developing human brains of different gestational age groups. Selected proteins for study were physicochemically and biochemically characterized. An attempt was made to correlate the above properties with the developmental age. Immunochemical techniques were used to ascertain whether the isolated glycoproteins were cell, tissue or species specific.

Foetal brains, collected within hours of abortion, were categorized according to foetal crown-rump lengths as IInd [9-20 cms] and IIIrd [24-36cms] trimester groups and stored at -20°C in a cryopreservative medium containing 8% dextrose-5% fructose-20% DMSO in Buffer A [0.01M phosphate-0.0001M PMSF-0.002M benzamidine hydrochloride-0.1% sodium azide, pH 6.0].

Glial cell enriched fraction was prepared by a modified procedure involving mechanical disruption of tissue through sieving followed by isolation of glia by discontinuous sucrose density gradient centrifugation at 3300 x g for 45 min. Glial cells were collected from the 1.65M sucrose interface and consisted mostly of astrocytes and a few distinguishable oligodendroglia.

Glycoproteins were isolated from purified glia by affinity chromatography on Con A-Sepharose 4B. Initial procedures with whole cell or plasma membrane extracts, with or without prior delipidation, in the presence or absence of detergents like SDS and/or Triton X-100, resulted in leaching of lectin from the affinity matrix. This constituted a major portion of the eluted protein. Pretreatment of Con A-Sepharose with 6M Gn.HCl at 30°C for 3 h, minimized this problem. Cell lysis was effected with 0.01M EDTA-0.25% 2-ME in Potter Elvehjrn homogenizer. The plasma membrane fraction pelleting at 8000 x g for 10 min, was washed with water to remove adhering cytoplasmic constituents at 4°C. The glial plasma membrane enriched pellet thus obtained, was

homogenized in 0.025M Tris-0.7M glycine-1% Triton X-100, pH 7.8, buffer by sonication [frequency of 2-4 μ for 1 min] and chromatographed at pH 7.4, 4°C, on pretreated Con A-Sepharose 4B. The affinity column was washed with 0.02M Tris buffered saline-0.1% Triton X-100, pH 7.4, and eluted in the same buffer containing 0.5M α -methyl glucoside at 4°C.

The Con A eluate contained non-covalently associated glycolipid which was extracted into 8:4:3, methanol:chloroform:water (v/v/v), a probable affirmation favouring cell surface locale of the eluted glycoproteins. Thin layer chromatography revealed that the majority of glycolipids were non-sialylated with a very minor ganglioside component. The glycolipid profile closely paralleled that of Con A in the eluted fractions and was temperature dependent. Elution at 25°C rendered fractions containing a higher degree of Con A and glycolipid contamination as compared to the negligible contamination by these two components when elution was carried out at 4°C. This phenomena was attributed to the formation of heterogenous mixed micelles of glycoprotein, Con A, lipid and detergent which was enhanced at higher temperatures as a result of phase transitional changes in the lipid components.

Purified glycoprotein contained $160 \pm 90 \mu\text{g}$ carbohydrate/mg protein, and a sialic acid content of 25 ± 5 n moles/mg protein.

The N-terminal as determined by Dansyl-Edman procedure and the DABITC-PITC double coupling method revealed a blocked amino-terminal for the glycoproteins.

SDS-PAGE on 8% and 10% slab gels as well as on 3-12% gradient slab gels rendered multiple bands which were grouped as G_0 [protein retained at the point of application], G_I [75-130 KDa], G_{II} [65-72.5 KDa], G_{III} [52-63 KDa] and G_{IV} [43.5-48.5 KDa], respectively. Re-electrophoresis of groups G_0 - G_{IV} individually, under identical conditions as the first run on SDS-PAGE, generated the same multiband pattern for all samples, corresponding predominantly to G_{II} - G_{IV} in molecular weight. This observation was indicative of the existence of a dynamic equilibrium between these major interconvertible states. Solubilization of the glycoprotein at concentrations exceeding 0.5mg/ml even in presence of SDS could not be achieved. The insoluble G_0 band was an illustration of this limited solubility. The complex banding pattern on SDS-PAGE could be a resultant of many factors such as anomalous SDS binding, microheterogeneity and aggregation, probably as a heterogeneous mixture of protein-SDS micellar aggregates. The fact that contiguous protein bands differed in molecular weight by 2-4 KDa only, would favour micelle formation as the likely cause; considering that no low molecular weight monomer was observed on the gel or dye front.

The factors responsible for such anomalous behaviour on SDS-PAGE were delved into. Glycoprotein digested with methanolic potassium hydroxide or hydroxylamine to cleave ester linked lipid and with nitrous acid for removal of glycoposphotidylinositol tail, if any present, was subjected to 10% SDS-PAGE. No change

was observed indicating that covalently linked lipid, if any, was not the cause for anomalous behaviour on SDS-PAGE. No covalently bound lipid was detected by the techniques employed.

Chemical deglycosylation with trifluoromethane sulfonic acid or desialylation in 0.1N sulfuric acid, resulted in the formation of still larger hydrophobic aggregates on 3-12% gradient SDS-PAGE, most of which were unable to enter the gel.

The glycoproteins moved preferentially on 6M urea-PAGE (5% gel) at pH 4.2, suggesting a higher amount of basic amino acids in its polypeptide backbone.

The glycoproteins were N-acetylated with [^3H]-acetic anhydride. Gel-filtration on Sepharose 6B of the tritiated sample in 0.025M Tris-0.7M glycine, pH 7.8 buffer in absence of detergents, gave two distinct peaks - a very high molecular weight fraction, eluted at void volume [Peak I], and a low molecular weight [less than 12.5 KDa] glycoprotein [Peak II]. Rechromatography of Peaks I & II on the same column under identical conditions showed that the peaks were non-interconvertible. This was further substantiated by HPLC on a Lichrosorb RP-8 column [gradient elution system of 0-100% B at a flow rate of 0.5ml/min for 80 min between the solvents, Sol A:water-0.1% TFA, and Sol B:isopropanol-0.1% TFA]. Peak I did not pellet even at 100,000 x g for 60 min and remained in the supernatant. Urea-PAGE at pH 4.2, perceived a single fast moving protein band near the dye front for both the peaks, attesting the possibility of

Peak I being a micellar aggregate of Peak II. Both peaks rendered identical multiband profiles [G_0 - G_{IV}] on 10% SDS-PAGE verifying that the characteristic aggregation pattern observed could be an artifact of protein-SDS interactions. Peak II eluted long after cytochrome c [12.5 KDa] on Sephadex G-75 gel-filtration, corroborating the potential existence of low molecular weight species.

Acid urea-PAGE resolved the native protein into a slow moving band U_I and a moderately mobile band U_{II} . N-acetylated proteins on the other hand rendered a single fast moving band near the dye front. The decreased surface charge effected by N-acetylation appeared to be compensated by the low molecular size of the monomeric form. Apparently the acetylated protein was more vulnerable to disruption by urea than native protein which remained as slower moving micellar forms.

The glycoprotein was resistant to proteolytic digestion by both trypsin and pronase.

Western blotted glial glycoproteins rendered identical lectin binding patterns on treatment with different HRP-linked lectins. Carbohydrate structure was hence analyzed by Dot Enzyme Linked Lectin Assay. Dot blotted glycoproteins were assayed by differential binding to eight lectins of varying specificities. Binding studies detected primarily N-Linked glycans, an indication that the proteins contained mostly Asn-linked side chains. Of these, high mannose and hybrid-types were apparently predominant as indicated by limited binding to RCA. The presence

of a Con A-binding activity that was abolished with 0.015M α -MG was attributed to biantennary glycan chains on the proteins. The glycoproteins interacted with JSA but did not bind SBA or PNA, suggesting the possible masking of O-linked glycans in the micellar aggregates. The appreciable binding of glial glycoproteins to JSA and AHA may also be an indication of novel α -linked galactose residues in developing human brain. Presence of α (1-6) linked fucose in the core was demonstrated by binding to pea lectin. Chemical modification of carbohydrate with TFMS, and periodate could not obliterate lectin binding completely. Nitrous acid deamination did not alter binding characteristics of the glycoproteins. Desialylation achieved by chemical and enzymatic procedures diminished binding to serotonin and WGA and enhanced RCA binding.

Finally, the glial glycoproteins exhibited a low antigenicity and failed to elicit an immune response in rabbit and guinea pig. Immune tolerance in the species selected for study could not, however, be ruled out.

In summary, the isolated glycoproteins appeared to be amphipathic molecules with an inherent tendency to exist as self micelles. The molecules were of low molecular mass and in all probability had the carbohydrate chains located at or around the N-terminal region, with predominantly hydrophobic amino acids situated towards the C-terminal, thus contributing to a bipolar configuration characteristic of integral membrane glycoproteins.

The study was carried out on glial cell surface glycoproteins from IInd and IIIrd trimester human foetal brains respectively. The isolated glycoproteins did not seem to exhibit any prenatal developmental changes under the limited conditions of study and behaved identically in the protocols tried out so far.

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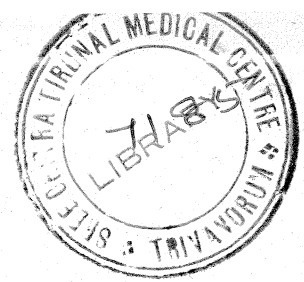
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SYNOPSIS

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**BIOCHEMICAL STUDIES ON CELL SURFACE
GLYCOPROTEINS OF GLIAL CELLS IN
DEVELOPING HUMAN BRAIN**

SYNOPSIS

by

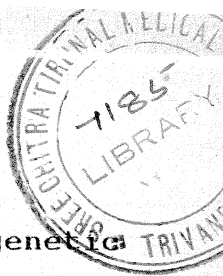
YASMIN MARIKAR

for Ph.D Degree

of

BIOCHEMISTRY

**SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL
SCIENCES AND TECHNOLOGY
TRIVANDRUM**



INTRODUCTION:

Tissue morphogenesis is governed by a series of epigenetic sequences. These synergistic events can be grouped into primary processes - cell division, cell movement, cell adhesion, cell differentiation and cell death. Cellular membranes are critical components in the development and maintenance of structural and functional integrity in eukaryotic systems (39). There is substantial evidence that cell surface glycoconjugates are implicated in processes involving recognition phenomena such as morphogenesis and oncogenesis (12,9,19) as well as receptor ligand interactions (2). Nervous system by virtue of its unique anatomical diversity, architecture and functional complexity exemplifies cell sociology in its broadest most evolved form.

Development of a nervous system is one of the most complex examples of morphogenesis, in that, it involves both the formation of intricate tissue structures and the precise establishment of neuronal pathways and interconnections. Analyses at the molecular level of phenomena such as recognition, adhesion and interaction between cells as well as cell surfaces and extracellular matrix components should provide major clues towards understanding how organised tissues arise from a mass of undifferentiated cells. The brain and other neural tissues are composed basically of two types of cells - neurons, involved with the generation and conduction of electrical impulses, and glial cells, which serve as connective, supportive tissue to maintain the neurons.

Several cell surface glycoproteins have now been recognised to mediate adhesion among different neural cell types at different developmental stages. Best characterised among them are the neural cell adhesion molecules [N-CAM] (10), from chicken, rodent and human neural tissues. The developmentally regulated glycoprotein mediates homotypic binding between neurons (7) and has a role in neurite fasciculation (44), retinal layering (5), formation of retinotectal maps (16) and in neuron-myotube formation (22). This structurally and functionally conserved molecule is present on neurons, predominantly immature astrocytes, oligodendroglia and Schwann cells, and manifests as a polysialylated embryonic form [30% sialic acid by weight] and a less sialylated adult form [10% sialic acid by weight] (8,38).

Adhesion molecule L1 (45) is immunochemically identical to the nerve growth factor-inducible large external glycoprotein [NILE] of PC12 cells (4), which in turn has been shown to be the mouse equivalent of chicken neuron-glia cell adhesion molecule [Ng-CAM] (17). These secondary adhesion molecules are expressed only in post mitotic neurons (29) and mediate heterotypic binding between neurons and glia. They appear to be involved in the formation and bundling of axons (42), migration of granular cells along radial glial cells (33,34) and in neuron-neuron adhesion and neurite outgrowth (18,15).

The antigen J1 (31) is another neuron-glia adhesion molecule sharing similarities in molecular weight and antigenic properties

with cytotactin (23), an extracellular matrix glycoprotein that mediates neuron-glia adhesion in vitro.

Myelin associated glycoprotein, is restricted to periaxonal loop of myelin, and is believed to be involved in glial axonal interaction (41,48,52).

All the above molecules are antigenically related, sharing common carbohydrate epitopes recognized by the monoclonal antibodies L2 and HNK-1 (31,30).

The adhesion molecule on glia [AMOG] does not belong to the L2/HNK-1 family of adhesion molecules. This novel glycoprotein is expressed by Bergmann glial cells in the cerebellum at critical developmental stages of neuron-glia interaction and granule neuron migration preceding synaptogenesis (1).

The adhesion molecules do not act independently of each other but cooperate synergistically, thereby modulating the effect of one adhesion molecule on the other. This would endow a particular cell with combinatorial possibilities in regulating the quality and strength of cell surface contacts.

Adhesion is not a purely physical phenomenon. There is evidence that cell-cell adhesion can trigger biochemical signals leading to alterations in gene expression and/or functional properties of neural cells (35,25,26). Bhat & Waechter (3) have demonstrated that there is a marked induction of N-linked glycoprotein synthesis correlating temporally with the onset of glial differentiation in embryonic rat brain cells. It has been

established that approximately 90% of the brain glycoproteins are integral membrane components, 85-90% of the carbohydrate of which are N-linked (36).

A noteworthy fact is, with the exception of N-CAM, most of the characterisation work on nervous tissue has been limited to avian or rodent systems. Also, in comparison with neurons, very little information is available on the molecular basis of glial development and function. Characterisation of a few glial cell surface-specific glycoproteins from developing human brain would contribute towards understanding glial cell functions and sociology in general, and have more pertinence to its role in human development.

The objective of this study was to isolate, identify and characterize glial cell surface-specific glycoproteins of developing human brains. Glial cell surface glycoproteins from human foetal brains of different gestational age groups were isolated. Selected proteins for study were physicochemically and biochemically characterized. An attempt was made to correlate the above properties with the developmental age. Immunochemical techniques were used to ascertain whether the isolated glycoprotein(s) was cell, tissue or species specific.

RESULTS AND DISCUSSION:

The collection and study of developing human foetal brain was approved by the ethics committee, Department of Obstetrics & Gynaecology, Medical College Trivandrum - 695 011. Foetal brains,

collected within hours of abortion, were categorized according to foetal crown-rump lengths as IInd [9-20cm] and IIIrd [24-36cm] trimester groups and stored at -20°C in a cryopreservative medium containing 8% dextrose-5% fructose-20% dimethyl sulfoxide in Buffer A [0.01M phosphate-0.0001M phenyl methane sulfonyl fluoride-0.002M benzamidine hydrochloride-0.1% sodium azide, pH 6].

Glial cell enriched fraction was isolated by an adaptation of previous procedures (11,40,28) involving mechanical disruption of tissue by sieving followed by isolation of glia by discontinuous sucrose density gradient centrifugation at 3300 x g for 45 min. Glial cells were collected from the 1.65M sucrose interface and consisted mostly of astrocytes and a few distinguishable oligodendroglia.

Glycoproteins were isolated from purified glia by affinity chromatography on Con A-Sepharose 4B. Initial procedures with whole cell or plasma membrane extracts, with or without prior delipidation, in the presence or absence of detergents like SDS and/or Triton X-100, resulted in leaching of lectin from the affinity matrix. This constituted a major portion of the eluted protein. Pretreatment of Con A-Sepharose with 6M guanidine hydrochloride at 30°C for 3 h, minimized this problem. Cell lysis was effected with 0.01M EDTA-0.25% 2-mercaptoethanol in Potter Elvehjem homogeniser. The plasma membrane fraction pelleting at 8000 x g for 10 min, was washed with water to remove adhering

cytoplasmic constituents at 4°C. The glial plasma membrane enriched pellet thus obtained, was homogenised in 0.025M Tris-0.7M glycine-1% Triton X-100, pH 7.8 buffer by sonication [frequency of 2-4 μ for 1 min] and chromatographed at pH 7.4, 4°C, on pre-treated Con A-Sepharose 4B. The affinity column was washed with 0.02M Tris buffered saline-0.1% Triton X-100, pH 7.4 and eluted in the same buffer containing 0.5M α -methyl glucoside at 4°C.

The Con A eluate contained non-covalently associated glycolipid which was extracted into 8:4:3, methanol:chloroform:water (v/v/v) (14), a probable affirmation favouring cell surface locale of the eluted glycoproteins. Thin layer chromatography revealed that the majority glycolipids were non-sialylated with a very minor ganglioside component.

Purified glycoprotein contained 160 ± 90 μ g carbohydrate/mg protein, and a sialic acid content of 25 ± 5 n moles/mg protein (50).

The N-terminal was determined by Dansyl-Edman procedure (21,37) and the DABITC-PITC double coupling method of Chang et al (6). The glycoprotein(s) revealed a blocked amino-terminal by these protocols.

SDS-PAGE (32,51) on 8% and 10% slab gels as well as on 3-12% gradient slab gels rendered multiple bands which were grouped as G_0 [protein retained at the point of application], G_I [75-130 KDa], G_{II} [65-72.5 KDa], G_{III} [52-63 KDa] and G_{IV} [43.5-48.5

KDa), respectively. Re-electrophoresis of groups G_0 - G_{IV} individually, under identical conditions as the first run on SDS-PAGE, generated the same multiband pattern for all samples, corresponding predominantly to G_{II} - G_{IV} in molecular weight. This observation was indicative of protein aggregation and the existence of a dynamic equilibrium between three major interconvertible states. Possibly, G_0 and G_I were converted to the lower molecular weight forms by a shift in this dynamic equilibrium. Solubilisation of the glycoprotein at concentrations exceeding 0.5 mg/ml even in presence of SDS could not be achieved. The insoluble G_0 band was an illustration of this limited solubility. The complex banding pattern on SDS-PAGE could be a resultant of many factors such as anomalous SDS binding, microheterogeneity and aggregation, probably as a heterogeneous mixture of protein-SDS micellar aggregates. The fact that contiguous protein bands differed in molecular weight by 2-4 KDa only, would favour micelle formation as the likely cause; considering that no low molecular weight monomer was observed on the gel or dye front.

The factors responsible for such anomalous behaviour on SDS-PAGE were delved into. At this point, we asked the questions, a) was covalently bound lipid the cause for aggregation? b) did the glycan side chains influence protein behaviour on SDS-PAGE? and c) could SDS itself be the cause for anomaly?

Glycoprotein digested with methanolic potassium hydroxide or hydroxylamine to cleave ester linked lipid (43) and with nitrous

acid for removal of glycoposphotidylinositol tail, if any present (13), was subjected to 10% SDS-PAGE. No change was observed indicating that covalently linked lipid, if any, was not the cause for anomalous behaviour on SDS-PAGE. No covalently bound lipid was detected by the techniques employed.

Chemical deglycosylation with trifluoromethane sulfonic acid (47) or desialylation in 0.1N sulfuric acid (27), resulted in the formation of still larger hydrophobic aggregates on 3-12% gradient SDS-PAGE, most of which were unable to enter the gel.

The glycoprotein(s) moved preferentially on 6M urea PAGE [5% gell at pH 4.2 (46), suggesting a higher amount of basic amino acids in its polypeptide backbone.

The glycoprotein(s) was N-acetylated with [^3H]-acetic anhydride (14). Gel-filtration on Sepharose 6B of the tritiated sample in 0.025M Tris-0.7M glycine, pH 7.8 buffer in absence of detergents, gave two distinct peaks - a very high molecular weight fraction, eluted at void volume [Peak I], and a low molecular weight [less than 12.5 KDa] glycoprotein [Peak II]. Rechromatography of Peaks I & II on the same column under identical conditions showed that the peaks were non-interconvertible. This was further substantiated by HPLC on a Lichrosorb RP-C8 column [gradient elution system of 0-100% B at a flow rate of 0.5ml/min for 80 min between the solvents, (A) water-0.1% trifluoroacetic acid, and (B) isopropanol-0.1% trifluoroacetic acid]. Peak I did not pellet even at 100,000 x g for 60 min and

remained in the supernatant. 6M Urea PAGE [5% gell] at pH 4.2, perceived a single fast moving protein band near the dye front for both the peaks, attesting the possibility of Peak I being a micellar aggregate of Peak II. The decreased surface charge effected by N-acetylation appeared to be compensated by the low molecular size of the monomeric form [Peak III]. Both peaks rendered identical multiband profiles [G_0 - G_{IV}] on 10% SDS-PAGE verifying that the characteristic aggregation pattern observed could be an artifact of protein-SDS interactions. Peak II eluted long after cytochrome c [12.5 KDa] on Sephadex G-75 gel-filtration, corroborating the potential existence of low molecular weight species.

Urea PAGE of native protein surprisingly resolved into a slow moving band U_I and a moderately mobile band U_{II} . The acetylated protein appeared more vulnerable to urea than native protein which remained as slower moving micellar forms.

The glycoprotein was resistant to proteolytic digestion by both trypsin and pronase.

Carbohydrate structure was analyzed by Dot Enzyme Linked Lectin Assay (20). Dot blotted glycoprotein was assayed by differential binding to eight lectins of varying specificities. Binding studies detected only N-Linked glycans, an indication that the protein contained mostly Asn-linked side chains. Of these, high mannose and hybrid types were predominant. Chemical modification of carbohydrate with TFMS (47), periodate (49) and

nitrous acid (13) was carried out. Desialylation was achieved by chemical (27) and enzymatic (24) procedures. TFMS deglycosylation abolished lectin binding almost entirely. Periodate oxidation, deamination and desialylation affected mainly the nonreducing terminal sugars. Complex chains were most vulnerable to modification .

In summary, the isolated glycoprotein(s) appeared to be amphipathic molecules with an inherent tendency to exist as self micelles. The molecule was of low molecular mass and in all probability had its carbohydrate chains located at or around the N-terminal region, with predominantly hydrophobic amino acids situated towards the C-terminal, thus contributing to a bipolar configuration characteristic of integral membrane glycoproteins.

This study was carried out on glial cell surface glycoproteins from IInd and IIIrd Trimester human foetal brains respectively. The isolated glycoprotein(s) did not seem to exhibit any prenatal developmental changes under the limited conditions of study and behaved identically in the protocols tried out so far.

Attempts to immunologically characterize this glycoprotein proved unsuccessful. Immunization of rabbits and guinea pigs with intact glial cell preparations resulted in a delayed but positive response against whole cell extracts. The antisera did not cross react with the isolated glycoprotein fraction. Attempts to prepare antibodies in rabbit against glycoprotein purified on

SDS-PAGE were unsuccessful. Low immunogenicity of the antigen and/or immune tolerance in rabbit were the probable causes of failure to elicit an immune response.

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