PHYSICOCHEMICAL STUDIES ON CELL SURFACE GLYCOCONJUGATES OF NEURONS FROM DEVELOPING HUMAN BRAINS

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by

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CERTIFICATE

I, Bobby Zachariah hereby certify that I had personally carried out the work depicted in the thesis entitled "PHYSICOCHEMICAL STUDIES ON CELL SURFACE GLYCOCONJUGATES OF NEURONS FROM DEVELOPING HUMAN BRAINS" except where external help sought and acknowledged.

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DECLARATION

This is to certify that Sri.Bobby Zachariah 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 his thesis entitled "PHYSICOCHEMICAL STUDIES ON CELL SURFACE GLYCOCONJUGATES OF NEURONS FROM DEVELOPING HUMAN BRAINS" was carried out under my direct supervision.

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ABBREVIATIONS

ANSA	1,2,4-aminonaphthol sulfonic acid
AHA	Artocarpus hirsuta agglutinin
BSA	Bovine serum albumin
Con A	Concanavalin A
DNs-Cl	Dansyl chloride (1-dimethylaminonaphthalene-5-sulfonyl chloride)
DABITC	4-NN-dimethylaminoazobenzene 4'-isothiocyanate
DABTH	4-NN-dimthylaminoazobenzene thiohydantoin
DTT	Dithiothreitol
DNase	Deoxyribonuclease
EDTA	Ethylenediamine tetraacetic acid
FDNB	1-fluoro 2,4-dinitrobenzene
FITC	Fluoresceine isothiocyanate
Fuc	Fucose
Glc	Glucose
Gal	Galactose
GlcNAc	N-aetylglucosamine
GalNAc	N-acetylgalactosamine
GPI	Glycosylphosphatidyl inositol
HRP	Horseradish peroxidase
JSA	Jack fruit seed agglutinin

Man	Mannose
α-MG	Methyl-α-D-glucopyranoside
NeuNAc	N-acetylneuraminic acid
PPO	2,5-diphenyloxazole
POPOP	1,4-bis-2-(5-phenyloxazolyl) benzene
PBS	20mM sodium phosphate buffer containing 150mM NaCl
PAGE	Polyacrylamide gel electorphoresis
PITC	Phenylisothiocyanate
PMSF	Phenyl methane sulfonyl fluoride
PNA	Peanut agglutinin
RCA	Ricinus communis agglutinin
SDS	Sodium dodecyl sulfate
SBA	Soybean agglutinin
TEMED	N,N,N',N'-tetramethylenediamine
TFMS	Trifluoromethanesulfonic acid
TFA	Trifluoroacetic acid
TBS	20mM Tris / HCl buffer containing 150mM NaCl
WGA	Wheat germ agglutinin

CHAPTER - I

GENERAL INTRODUCTION

INTRODUCTION

Development of the nervous system is one of the most complex examples of morphogenesis. It involves both the formation of intricate tissue structures and their precise interconnection. The brain tissue exhibits diversity in the types of cells with neurons and glia forming its major components. There are three main sources of variability in neural networks during development: somatic developmental sequence of network formation and neuroanatomy, the chemical variation related to neurotransmitters and the electrical variation. These processes emerge in a clear cut order of development. None of these processes is fully independent of others, although their relative contributions vary in time. The period in brain development following establishment of adult neuronal populations, when dendrite growth and increase in synaptic connectivity overlaps with development of glia is known as the period of the brain "growth spurt". Besides being an unique period of actual physiological importance, it is also recognized as a vulnerable period in brain development.

The formation of a complex nervous system from a simple neuroepithelium in the embryo involves many events, such as cell migration, differentiation, cell death and the formation of specific efferent and afferent connections. Of the many cellular events that contribute to the neural development, cell-cell

recognition has received particular attention. An example of such interaction is the axonal guiding system, which directs the correct axons to precise targets during development. It has been increasingly evident that the molecules of the cell surfaces and the extracellular matrix mediate such interactions and both genetic and epigenetic processes regulate such interactions during development. There are significant biochemical evidences to indicate that the cell surface carbohydrate residues present on glycoproteins and glycolipids play important roles during cell proliferation, differentiation and other events during development.

The role of oligosaccharide chains in brain development is emphasized by the facts that N-glycosylation is critical for neuronal differentiation and for the assembly and maintenance of functional voltage-sensitive sodium channels in neuroblastoma cells(126,295). Enzymological studies with microsomal preparations from developing brain have demonstrated increases in activities in the N-glycosylation pathway during active myelination and synaptogenesis(117). The close temporal relationship between the induction of N-glycosylation and glial differentiation suggests a possible role of N-linked glycoproteins in the differentiation process(19).

The development of neural structures proceeds in an orderly sequence as follows (a) neural induction, i,e formation of the primitive neural elements; (b) differentiation of the neural

plate; (c) formation of the neural tube which will give rise to the central nervous system: (d) formation of the neural crest dorsal to the neural tube, from which cells will migrate to form peripheral, sensory and autonomic ganglia and other derivatives; (e) formation of germinal zones which generate neuronal and glial precursors; (f) cell migration during which neuronal and glial elements will travel to their permanent locations; (g) cellular differentiation and maturation during which neurons in their final locations grow and establish connections; and (h) massive neuronal death and attrition.

The central nervous system develops from a thickened area of embryonic ectoderm, the neural plate, which appears by 18 day of gestation. The development of the nervous system is initiated by an interaction between dorsal ectoderm cells and underlying mesoderm cells. This process, termed neural induction, is poorly understood. The neural plate gives rise to the neural tube and the neural crest. The wall of the neural tube is initially composed of a thick neuroepithelium. The dividing cells of the neural tube move toward the lumen to form the ventricular germinal zone in which the cells proliferate. Mitosis takes place in the germinal zones and the neurons migrate only after the completion of mitosis. Some of the dividing neuroepithelial cells differentiate into immature neurons or neuroblasts. The glioblasts differentiate from neuroepithelial cells mainly after neuroblast formation has ceased. The production of neurons begins

at around six weeks of gestation. The stage of cell differentiation begins roughly during the 16th week and continues well into the postnatal period. During cell differentiation, neurons grow, develop their dendrites and axons and establish synaptic contacts. The advancing axon can select a specific path to the target from many of the possible choices. At the tip of the axon is the nerve growth cone. The growth cone, in particular its cell surface is the element of the advancing neurite to encounter a novel component. Myelinogenesis takes place in an orderly, regionalized fashion which begins before birth and continues well into adult life. The great post natal increase in brain weight is largely the result of accumulation of myelin. A crucial factor in cortical development, which helps to match the number of presynaptic neurons with available synaptic sites, is the process of cell death, which reduces the great excess of neurons produced in fetal life.

With few exceptions, neurons in the mammalian brain originate at a considerable distance from the place where they reside in the adult. The migration of neurons differs from the movement of cells from other organs. First the migration is initiated only after completion of the final cell division. Second, individual postmitotic cells move actively along preferred surface rather than being passively shifted as a group of cells. Third the final location of each individual neuron, which depends on both the form of its migratory pathway and the

speed of its migration, determines its synaptic relations and therefore specifies its function. Since the phenotype of an individual neuron has been proved in several regions of the brain, to be specified at the time of the last cell division, failure of the migrating cell to reach the correct destination leads to either neuronal death or inappropriate synaptic relationships, in either case producing functional deficits. Examination of migratory behaviour of postmitotic cells strongly suggests that the process depends on surface-mediated interactions. With regard to pathway selection, migrating neurons fall into three major categories (a) gliophilic cells that follow glial fibres and ignore neuronal surfaces (b) neurophilic cells that follow neuronal particularly axonal surfaces and ignore glial shafts; and (c) biphilic cells that display temporal or regional affinities towards either glial or neuronal surfaces. The gliophilic migration is the most prevalent type in mammalian brain(230). Selective displacement of migrating neurons along preferred surfaces can be explained by the presence of at least two types of bonds: one attaching neurons to neurons and the other binding neurons to glial cells.

The proper development of the nervous system depends upon a group of cell surface glycoconjugate molecules which are possibly involved in cell-cell interaction and recognition. The discovery of surface glycoproteins involved in cellular adhesion has opened the way for the analysis of the molecular events involved in this

important developmental process(66). These molecules have been functionally defined as cell adhesion molecules since they influence the specific adhesion of cells as judged by in vitro assays.

In the last few years several high molecular weight glycoproteins have been reported which play crucial roles in neural development. Surface membrane glycoproteins may be viewed as decoders of extracellular information. As such when a cell interacts with a substratum or with ligands on the surface of another cell, the information as to whether to form an adhesive complex or not must rest in the molecular architecture of these glycoproteins. If the correct combination of molecular signals is present, the cell will begin to organize an adhesive complex. Ultrastructural studies and a careful examination of the adhesion process in culture show that there are several types of adhesive interactions, and that adhesion is a stepwise process. According to this idea, the cell initially forms loose attachments to its substratum or to a neighbouring cell. This attachment triggers a series of molecular events in which more glycoproteins are recruited into the attachment areas, resulting in stabilization of adhesion and perhaps eventually in the formation of adhesive plaques or well-defined junctional complexes. This is a complicated series of events that could be disrupted (and perhaps controlled) at any point. It will be difficult both to prove whether a particular glycoprotein is involved directly or

indirectly with the adhesion process, and to establish precisely what role the glycoprotein plays in the series of events leading cellular adhesion. Such proof will require carefully correlated biochemical, immunological, biological and ultrastructural studies.

The process by which cells recognise and adhere each other is obviously one of the most crucial and complicated events in biology. When this process goes well as in normal embryonic development, the result is absolutely fascinating- the establishment of the intricate circuitry of the brain and peripheral nervous system. But when this process fails the results are disastrous: terribly malformed individuals on one extreme or rampant metastasis on the other. The information for establishing and maintaining the correct contacts must lie in the expression and organization of molecules on the cell surface. Two general approaches have been taken in identifying the molecules involved in cell-cell adhesion. One has been to establish in vitro systems of cell-cell adhesion involving dispersed embryonic tissue or even cultured cell lines and to identify factors that either promote or inhibit the particular cell-cell interaction being studied(88). A second approach to identify surface molecules involved in aggregation consisted of preparing a broad-spectrum antiserum capable of disrupting some of cell-cell interaction, and then attempting to identify the antigens involved(96).

The most thoroughly studied cell-cell adhesion glycoprotein to be discovered using antibodies is neural cell adhesion molecule (N-CAM)(67). The N-CAM migrated on SDS-PAGE as a broad and continuous band. The heterogeneity was due to the sialic acid content of the N-CAM. A comparison of N-CAM from adult and embryonic nervous system was made by Rothbard et al(239). The two N-CAM preparations cross reacted serologically and each was able to neutralize the adhesion-blocking activity of anti-N-CAM. The embryonic form of N-CAM appeared to have a higher molecular weight than the adult form. The adult form of N-CAM contained about one-third the amount of sialic acid present on the embryonic form. In both cases the sialic acid was present as a polymer. The role of N-CAM in histogenesis was explored in several ways(242,240). N-CAM was found on retinal optic nerve, spinal cord and to some extent on muscle and liver cells in developing embryos. Anti-N-CAM Fab was found to block neurite fasciculation of cultured chick dorsal root ganglia. This effect was blocked by purified N-CAM. The above made observations are all consistent with N-CAM being a molecule involved in cell-cell adhesion. The presence of N-CAM on muscle cells suggested that it might play a role in the neuromuscular interactions leading eventually to the formation of myoneural junctions(241). The binding of myoblasts to monolayers of neural retinal cells could be blocked by anti-N-CAM(110). The N-CAM exhibited homophilic binding properties. The N-CAM has also been detected in human

neural tissue(194). The change in sialic acid content from the embryonic to the adult form of N-CAM did not occur in the "staggerer" mouse mutant(63). Edelman has speculated that the failure of this embryonic to adult conversion might result in incorrect neuronal connections causing the neurological disorder seen in staggerer mice(64).

To understand N-CAM's role in development, it is necessary to consider morphogenetic mechanisms other than strict chemospecificity. These include the regulation of N-CAM expression, effects of the carbohydrate heterogeneity on binding function and the biological consequences of a homophilic adhesion mechanism. N-CAM seems to be continuously expressed during the formation of both nerve and striated muscle tissues. A striking aspect of N-CAM expression is the transient appearance or disappearance of the molecule at sites associated with dynamic reorganization of tissue structure. The heterogeneity in N-CAM's sialic acid content influences its binding properties, as removal of this negatively charged sugar increases the rate of binding of N-CAM to cells. The potential exists that the differences in both the amount and carbohydrate heterogeneity of N-CAM can produce a hierarchy of binding affinities.

The cell surface adhesion molecules can be divided into two groups defined by the calcium ion requirement of the reaction they mediate in a particular in vitro assay(277). The N-CAM participated in Ca^{++}-independent rather than Ca^{++}-dependent cell

aggregation. In contrast Grunwald et al found that in the neural retina, Ca^{++}-dependent receptors are maximally expressed during the height of morphogenetic movement(111). The neural cell surface molecules, Ng-CAM and L1 antigen have also been identified as components of calcium-independent adhesion system. In addition, there is evidence that N-CAM and L1 co-exist on the same cell and could act in combination to augment the specificity of neural cell-cell adhesion(232).

Several groups have made antisera to complex antigens in order to identify molecules involved in cell-substratum adhesion(32). These antibodies are of interest only if they perturb adhesion. They do so in vitro by causing the cells to completely detach from the substratum or by causing rounding or other morphological changes in cells previously stuck and spread on the substratum. Many of these antisera will also prevent or delay the initial attachment of cells to a substratum. Hsieh and Sueka prepared antiserum against whole rat neuronal cells that inhibited spreading of these cells(136). Using antibody absorption by different adherent and non-adherent cell lines and SDS-PAGE analysis, these workers showed that adherence correlated with the presence of 120 and 80 KDa glycoproteins.

It is possible to think the cell-cell and cell-substratum adhesion within the same conceptual framework. However each type of adhesion appears to require some specificity and there must be many molecules unique to each system. Studies on N-CAM indicated

that anti-N-CAM will block neurite fasciculation and the interaction of neurites with myoblasts, but has no effect on the interaction of neurites with the substratum.

The sequential addition and / deletion of monosaccharides is one mechanism for appearance, disappearance and reappearance of antigens during stages of embryogenesis, differentiation and also during oncogenesis and other insults to cells. The L1 antigen has been found in central nervous system on postmitotic neurons(133). It appears to be involved in migration of granule cells as well as in neuron-neuron adhesion and neurite outgrowth(85). An antibody prepared against L1 prevented the migration of granule cells across the molecular layer in cerebellar slices(176). L1 is involved in two types of binding mechanisms. In one type, L1 serves as its own receptor with slow binding kinetics. In the other, L1 is modulated in the presence of N-CAM on one cell (cis-binding) to form a more potent receptor complex for L1 on another cell(trans-binding)(150). To date all neural cell types in which L1 has been detected also express N-CAM. These neural cell types include subsets of postmitotic neurons and certain neural tumours(200). In contrast to N-CAM which is uniformly expressed on the cell surface of differentiated neurons, L1 is predominantly expressed on axons. The association between L1 and N-CAM appears to be based on the presence of properly biosynthesized complex or hybrid carbohydrate chains(159). The versatility of adhesion mechanisms would be of obvious benefit

for a complex tissue such as nervous system during its development. The adhesion molecules can function either individually or in conjunction, with a concomitant change in their adhesive affinities, allowing for an economic use of few adhesion molecules in their various combinations. It is likely that L1 and another cell surface glycoprotein Ng-CAM are similar. The glial cell surface glycoprotein J-1 and the extracellular glycoprotein cytotactin have similar molecular weights and antigenic properties.

The myelin associated glycoprotein is restricted to periaxonal loop of myelin in the central nervous system and is believed to be involved in axon-myelinating cell interactions in the central and peripheral nervous system(227). The myelin associated glycoprotein has been shown to mediate neuron-oligodendrocyte and oligodendrocyte-oligodendrocyte interactions(193).

The remarkable feature of the molecules N-CAM, L1, J1 and myelin associated glycoprotein was that they were all antigenically related as they share a common carbohydrate epitope as recognised by the L2 and HNK-1 antibodies(197,304). The exact nature and function of this carbohydrate epitope on these cell adhesion molecules are still unknown. The lipid extract from adult brains from various mammalian species did not contain the antigen for HNK-1 antibody. However when embryonic rat, pig, and human fetal brains were examined, HNK-1 reactive glycolipids were

observed easily(251). Gangliosides and neutral glycoshingolipids, considered as cell surface antigens, have been implicated in various cell surface phenomena such as the control of cellular growth, differentiation and receptor function. Several studies have noted that ganglioside GD3 constitutes a large proportion of total gangliosides in the immature vertebrate central nervous system, which then decreases with development and is replaced by more complex gangliosides(252). Gangliosides, such as GQ1b, when added to nerve cell culture medium induce neurite outgrowth and a number of chemical investigations which claim or disclaim the role of gangliosides in the therapy of a variety of nervous system disorders have appeared(173). Molecules that contain the L2/HNK-1 epitope make up the L2/HNK-1 family of cell-adhesion molecules. Further, since only some of the polypeptides of a particular adhesion molecule express the epitope, the expression of the carbohydrate structure seems to occur independently of the protein backbone(168). The importance of a "cell-adhesion family" lies in the finding that molecules expressing the epitope appear to have similar physiological roles and there are hints that the epitope itself may be involved in cell adhesion. A second cell-adhesion family is characterized by the L3 carbohydrate epitope(7). Members of this family include some members of the L2/HNK-1 family (i.e.L1 and MAG), as well as distinct molecules such as adhesion molecule on glia (AMOG) (7).

The D2-cell adhesion molecule was originally described as nervous system specific membrane protein enriched in fractions of synaptosomal plasma membranes(28). It has been shown that the antibodies against D2 can inhibit fasciculation of neurites from cultured rat sympathetic ganglia. The human D2-cell adhesion molecule exhibited a decrease in molecular weight with development(27). The decrease in molecular weight was probably due to the removal of sialic acids during development. Lectin positive and lectin negative forms of D2 were present in varying proportions during development, indicating a heterogeneity of the carbohydrate moiety of D2 at any stage of development(27). The concentration of D2 was higher at early stages of development than in adult tissues(30). The glycoprotein was involved in intercellular recognition processes during synaptogenesis(147).

A monoclonal antibody, anti-BSP-2, has been produced against glycoproteins extracted from neonatal mouse brain(130). Immunoprecipitates prepared with BSP-2 antibody contained a triplet of high molecular weight glycoproteins. In primary cultures of dissociated cerebellar cells, the antibody bound to neuronal cell types but not to astrocytes(130). Thy-1 was another cell surface glycoprotein purified from rat and mouse brain and lymphoid cells(195).

The adhesion molecule on glia (AMOG) is an integral membrane glycoprotein expressed by glia and was reported to be involved in neuron-glia interaction but not astrocyte-astrocyte adhesion(7).

AMOG is an adhesion molecule that mediates cerebellar granule cell migration by specifying the direct contact between the migrating neuron and the "contact-guiding" astroglial process, the Bergmann glial fibre. AMOG is detectable shortly before the onset of granule cell migration and unlike L1 and N-CAM that remain expressed also in adulthood, ceases to be detectable on Bergmann glial cells at the end of migratory period. Its developmental appearance and disappearance coincides temporally with granule cell migration.

Edmondson et al reported an antiserum raised against whole mouse cerebellar cells that blocks specific neuron-glia association, an activity which they named as anti-astrotactin(69). Astrotactin is a cell surface glycoprotein with an apparent molecular mass 100 KDa present on granule neurons, harvested from mice. Preliminary evidence indicates that the astrotactin activity is needed for both neuronal migration along astroglia and for neuronal regulation of astroglial cell growth. Further evidence suggests that the astrotactin activity is missing or defective on granule cells from the neurological mutant mouse weaver, an animal that suffers a failure of glial-guided neuronal migration. In cultures from normal cerebellum, granule neurons bind to and migrate along glial processes(121). Weaver neurons fail to bind or to migrate on either weaver or normal glia. In contrast normal neurons bind to and migrate on

both weaver and normal glia. The weaver mutation primarily affects granule neurons.

Ng-CAM is a cell surface glycoprotein reported to be present on neuronal cells of the chick embryo and is responsible for the interaction of neuronal cells with glial cells(109). The Ng-CAM is distinct from the previously described N-CAM. Moreover, since Ng-CAM is not detected on glial cells, neuron-glial interaction, unlike neuron-neuron adhesion, appears to be between different molecules, one of which is Ng-CAM. The adhesion between neurons and myotubes, for example appears to be mediated by a homophilic mechanism in which N-CAM on the neuron binds N-CAM on the myotube, whereas the interaction between neurons and glial cells appears to be heterophilic. In contrast to the N-CAM which is a primary cell adhesion molecule, Ng-CAM is has been termed as a secondary cell adhesion molecule. The Ng-CAM is not expressed on neurons until they become postmitotic and migrate from their proliferative zones.

A glycoprotein, G4 antigen, isolated from chick brain by monoclonal antibody and lectin affinity chromatography was related to mouse L1 antigen(233). A second glycoprotein, F11 antigen, was also isolated from adult chick brain using monoclonal antibody F11(233). Both G4 and F11 antigens share with N-CAM, the HNK-1/L2 carbohydrate epitope. The antibodies against the glycoproteins significantly reduced the average length of

chick sympathetic neurites growing on chick sympathetic axons(46).

The isolation and biochemical study of cell surface adhesion molecules have allowed an analysis of their spatial and temporal distribution and mechanism of action during various epochs of neural development. The accumulated data suggest that a small number of adhesive surface glycoproteins can mediate the neural development, provided that their binding activities are locally modulated by epigenetic means in dynamic fashion(65). Potential modulation mechanisms include differential chemical alteration, variation in temporal expression and variation in prevalence or surface density in different regions of a tissue or organism. By changing the binding behaviour of cell adhesion molecules, the various modulation events would directly or indirectly alter the dynamics and interactions of other primary processes of development such as cell movement, differentiation, division and death. Changes either in prevalence of cell adhesion molecules at the cell surface or in their individual binding strength through embryonic to adult conversion would be expected to lead to different interactions among the cells that were subject to other primary processes. Both the above mentioned modulatory changes were observed with N-CAM. When neural crest cells first appear they stain for N-CAM. As soon as they begin their migration, however they lose this staining. When the neural crest cells reach their destination, and just before they form ganglia,

N-CAM reappears on their surface. During brain development, while N-CAM continues to be used in later stages, at least one new specificity (Ng-CAM) is required for neuronal migration. Moreover, in later periods it was observed that not only changes in the prevalence of Ng-CAM and N-CAM but also embryonic to adult conversion are called into play, particularly at times at which various neuronal fibre tracts are being mapped. The rate of conversion, its time of initiation, or its degree in different cell types may differ in biologically different regions of the brain.

One of the main objectives of the study of neural development is to explore the role of cell adhesion molecules in disease. Primary genetic abnormalities involving the cell surface glycoproteins may either be lethal or difficult to detect, because the molecules play a fundamental role in very early development. Defects in processes mediated by N-CAM in specific subpopulations of cells might, however, lead to cytoarchitechtonic abnormalities such as defects of neural tube closure, microgyria, lissencephaly or any of the heterotopias(194). N-CAM might also be involved more indirectly in other diseases, perhaps as a target in various polyneuropathies or encephalomyelitis that may have autoimmune etiologies. The cell adhesion molecules have a role in the metastasis of particular kinds of cancer cells. Recent studies have shown, for example that transformation of embryonal neural

cells by tumour viruses such as Rous sarcoma virus leads to a loss of N-CAM mediated adhesivity(107).

In addition to their role in brain development, the surface glycoproteins also partake in the complex central nervous system cell sociology. Morphological studies of the central nervous system revealed an incredibly complex meshwork of neuronal and glial somata, axons, dendrites, glial processes, myelin and capillaries. These cellular entities differ on the basis of anatomical and neurophysiological properties. The human brain contains an estimated 10^{11} neurons, some of which may have up to 80,000 synaptic contacts on its surface(51). One of the goals of the neurobiologist is to acquire information on the structure and function of membrane components at a molecular level. Within this area of study, one of the major interests has focused on membrane glycoproteins. Studies during the past two decades have concentrated on the identification and characterization of membrane glycoproteins that may be useful in distinguishing or are unique to, specific classes of neurons, and different types of synapses. Cell-cell interactions in the nervous system are assumed to have developed to a high degree of precision because of the many cellular connections diverging and converging from one cell to another. The molecular mechanisms underlying these phenomena remain largely obscure, but it may be assumed that specific cell surface glycoproteins play an important role. The study of neural cell membrane markers is therefore expected to

improve our understanding of cellular communications. The extreme heterogeneity of the nervous system though a major problem in understanding the place of glycoconjugates in the functioning of the nervous system, is at the same time the essential basis of its function.

The nervous system is characterized by unusually high concentrations of cerebrosides, sulfatides and gangliosides. Gangliosides are overwhelmingly concentrated in neuronal plasma membranes(57,238). The glycosidases and other enzymes involved in the hydrolysis of glycoproteins and glycolipids do not appear to be markedly specific in vitro and the synaptosomal plasma membrane neuraminidase appears to hydrolyse both glycoproteins and glycolipids(95,143,226). Confirmation of this lack of specificity has been obtained in vivo, since defects in ganglioside degradation are accompanied by similar changes in glycoprotein degradation(37,38,36).

In all solid tissues, cells adhere to each other and to the substratum. The cell surface glycoproteins play an important role in the adhesive events which are instrumental for the maintenance of tissue architecture. Synaptic glycoproteins seems to be involved in the exocytosis-endocytosis cycle which takes place during transmitter release(129). The cell surface glycoproteins have been implicated in receptor-ligand interactions. Neurons especially rely upon plasma membrane glycoproteins for circuitry formation and other intracellular connections(50). Many

neurotransmitter receptors that have been identified thus far have been glycoproteins. These include the α-adrenergic and those for dopamine and muscarinic acetylcholine receptors(257,256,156). Of further importance are those studies that have demonstrated differences in the surface glycoconjugates between perikaryon and perikaryal projections and between different neuron types (122,221,60,59). The neuron type specificity of surface glycoproteins are consistent with the concept that surface carbohydrates are involved in neuronal recognition(222).

The cell to cell adhesion appears to be largely a mechanical phenomenon: that is, cell surface adhesion molecules physically hold two membranes together. However there is evidence that cell-cell adhesion can also generate biochemical signals which lead to alterations in gene expression and / functional properties of neural cells(1). The direct contact between neurons and glial cells in vitro promotes biochemical changes in several different types of glial cells. Such changes include the induction of cortisol-mediated glutamine synthetase in chick retinal and tectal glial cells, stimulation of cell division and morphological differentiation accompanied by cessation of proliferation(177,178,118,123). Neuron-neuron contact has also been shown to produce changes in cellular biochemistry in vitro. Levels of neurotransmitter biosynthetic enzymes and of neuropeptides are regulated by cell-cell contact in rat and chick sympathetic neurons(2,3,4). There are two general biochemical

mechanisms by which cell contact could cause alterations in cell biochemistry: (a) the cell adhesion glycoproteins could serve to hold the appropriate cells together, while different molecules actually generate signals between cells or (b) a single molecule or ligand-receptor complex could function simultaneously in both capacities. In E10 chick sympathetic neurons, there is a cell contact-dependent increase in the specific activity of choline acetyl transferase over the first several days in culture(1). The above mentioned effect was similar to that which has been previously described using rat sympathetic neurons, where contact between neurons resulted in an increase in both choline acetyl transferase activity and substance P concentration(3).

The significance of surface glycoconjugates in the central nervous system cell sociology was further illustrated by the identification of endogenous lectins in brain(258,316,40). One of them called cerebellar soluble lectin has been detected in oligodendrocytes and seems to be involved in myelin formation(317). The lectin was found to be present in compact myelin and in zones of contacts between different myelin sheaths or oligodendrocyte membranes. The cell contacts between different oligodendrocytes or between adjacent lamellae in myelin were mediated by lectin-glycoprotein interactions. This hypothesis was demonstrated by two effects of anti-lectin Fab fragments on oligodendrocyte cultures: (1) The almost complete detachment of the cell layer from the culture substratum, and (2) the loss of

myelin compaction by a separation of lamellae at the intraperiod line. The developmental regulation of the lectin in neonatal rat brain has been demonstrated(258).

Understanding the specific molecular interactions that govern the social behaviour of cells is of pivotal scientific and clinical importance. When considering the modulation of recognitive phenomena there is still a strong prejudice towards thinking in terms of proteins. It is now however beginning to be appreciated that the carbohydrate moieties of glycoproteins satisfy important prerequisites to take part in recognitive mechanisms. By their enormous potential to form structural diversity they can serve as ideal candidates to carry biological information. Consequently it is not surprising to unravel programmed and strictly regulated changes in carbohydrate composition and sequence in the expression of glycoconjugates within the course of complex processes such as development and differentiation. The difference in the structure and arrangement of oligosaccharide chains seems to be a crucial factor of cell individuality and contributes to specific cell contacts. If a cell looses its specific properties and / its contacts with the environment are changed, e.g. during growth, differentiation, ageing or malignant transformation, dramatic changes often occur in the glycoconjugate composition of cells(203).

The significance of cell surface glycoproteins has also been reflected in the field of oncology. The cell surface interactions

are important during tumorigenesis, tumour progression and metastasis. Cell surface glycoconjugates, particularly are known to be involved in the complex process of metastasis. Surface glycoconjugates of tumour cells play multiple roles in the interactions of malignant cells with host systems including the host immune system. The differences in surface molecule expression are related to the resistance of the metastatic tumour to killing by the host's immune mechanisms(269,196,104). High and low metastatic malignant lymphoma cells have differential cell surface glycoconjugate expression particularly of sialylated molecules(149,234). The metastatic lymphoma cells were resistant to natural killer mediated cytolysis in contrast to the parental cells which could be due to the differential expression of surface glycoconjugates(148). The cell surface carbohydrates mainly associated with glycolipids and integral membrane glycoproteins, can cover the entire cell surface. By consequence, they form the first layer of interactions between cells and contribute significantly to the chemical, immunological and "lectinological" identity of the cell(115,262).

Neoplastic cell surface glycoproteins showed aberrant glycosylation. It has been established that a structural change in fucose containing, N-glycosidically linked carbohydrates constitutes a discrete step in the progressive, multistep process of malignant transformation, being a specific and necessary determinant of the tumorigenic phenotype(297,261,298). The

increase in apparent molecular weight of the surface glycoproteins was due to an increase in terminal sialic acid residues in more highly branched structures(276,312). Terminal sialylation of cell surface glycoconjugates has also been correlated with increased metastatic potential in a number of murine tumour cell models(314). An increased level of sialylation of surface-exposed carbohydrates in tumour cells may be a necessary condition for reducing the tumour cell adhesion to extracellular matrix proteins and susceptibility to immune destruction, phenotypes essential for the manifestation of invasiveness and tumorigenicity(56). The invasion was directed by a gradient in the level of carbohydrate sialylation, irrespective of a normal or malignant origin of the confronting tissues(245). However the sialic acid has a known negative impact on the adhesive forces between cells which are mediated by cellular adhesion molecules(239). One of the most consistently observed alterations following neoplastic transformation is a shift toward the synthesis and expression of larger Asn-linked oligosaccharides(298,289). An increase in cell-surface Asn-linked complex type oligosaccharides that contain an N-acetylglucosamine residue linked β1-6 to mannose has been demonstrated following viral transformation(223). A shift in the glycoform population of glycoproteins on the tumour cell surface towards those with the branching oligosaccharides was associated with a corresponding increase in the metastatic potential of that cell. The expression

of polylactosamine sequences may contribute to the decreased tumour cell adhesion on extracellular matrix proteins. Presumably the actual metastasis of a particular tumour cell would occur once a critical threshold of the appropriate glycoform populations had been achieved. The cells expressing high densities of high mannose type, hybrid type, and incomplete complex type (i,e.exposed GlcNAc) N-linked oligosaccharides are more sensitive to natural killer cell lysis than tumour cells expressing completely processed sialylated complex type oligosaccharides which appear to escape natural killer surveillance(55).

Structural changes in surface carbohydrate expression may be the result of (a) blocked synthesis, sometimes accompanied by accumulation of precursor oligosaccharides; (b) loss of crypticity by membrane conformational change; (c) activation of glycosyltransferases in progenitor cells resulting in carbohydrate neosynthesis; (d) accelerated degradation of cell surface glycoconjugates; or (e) a switch in the qualitative expression pattern of certain glycoconjugates. As a result of these changes in carbohydrate content particularly glycolipids, the tumour cell membrane may result in an altered fluidity, which in turn may affect the function of receptors, membrane enzymes and cellular metabolism(115). The removal of cell surface fucose by enzyme treatment decreased the metastatic but not tumorigenic properties of malignant cells(307). A remarkable feature of the

tumour associated carbohydrate antigens is their close relationship with the classically known blood group antigens(213). Most types of tumour associated carbohydrate antigens defined by monoclonal antibodies have been found to be highly expressed at certain defined stages of embryogenesis and fetal development. One such structure SSEA-1, often found on a polylactosamine backbone was also expressed at the blastocyte stage in the mouse(23). The addition of multivalent SSEA-1 containing structures has been shown to decompact pre-implantation mouse embryos in tissue culture, suggesting that the structure is part of a stage-specific recognition system(23). In addition monoclonal antibodies that react with SSEA-1 structure have been shown to inhibit cell-substratum adhesion of F9 teratocarcinoma cells(212). Embryogenesis is an orderly process involving cell proliferation, cell invasion and cell migration over extracellular matrix, characteristics that appear to be similar to those required of metastatic tumour cells(179). Although the re-expression of embryonic carbohydrate structures by tumour cells may simply reflect the expression of earlier genetic programs, it is also possible that certain structures that are involved in embryonic cell-cell interactions may enhance the malignant potential of tumour cells.

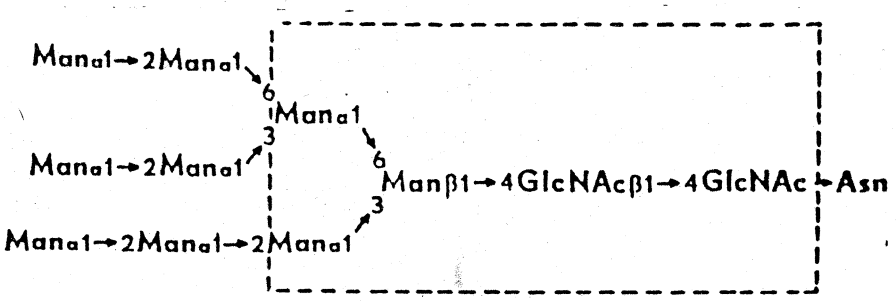
The functional significance of the above mentioned surface glycoconjugates could be attributed to their carbohydrate residues. Glycoconjugates found in the biological systems include

glycoproteins, glycolipids and the proteoglycans. Glycosphingolipids are widely distributed in animal tissues, particularly in synaptic membranes of brain and cell surfaces. The commonly occurring glycosphingolipids can be classified into two different families, the acidic gangliosides and the neutral glycosphingolipids. Long chain glycosphingolipids of blood group or globoside families are important constituents of the plasma membranes of normal and transformed cells. Very little is known about the function of these glycolipids on cell surfaces.

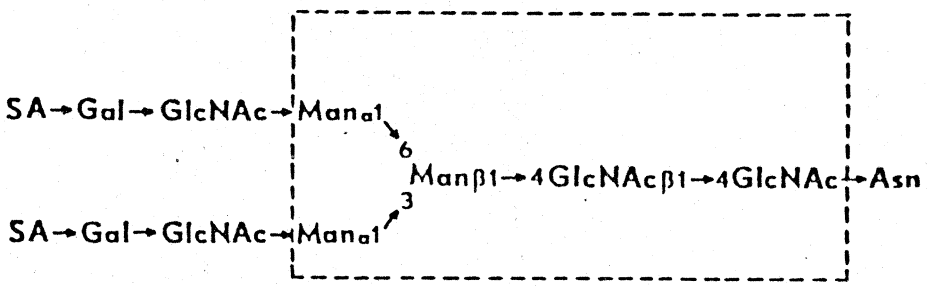
Glycoproteins are proteins with covalently linked sugars ubiquitous in nature and are found in all living organisms(215). Glycoproteins occur in cells, both in soluble and membrane-bound forms, as well as in the intercellular matrix and in extracellular fluids. This class includes enzymes, immunoglobulins, hormones, toxins, lectins, and structural proteins. Although 200 different monosaccharides are found in nature, only 11 are known to occur in glycoproteins(253). Most of these are hexoses or their simple derivatives, such as N-acetylhexosamines and uronic acids. L-fucose and two pentoses- L-arabinose and xylose are also glycoprotein constituents. More than 20 different sialic acids have been identified; the most common being N-acetylneuraminic acid and N-glycolyl-neuraminic acid. The sugars in glycoproteins also occur as sulfate or phosphate derivatives.

Glycosaminoglycans are high-molecular-weight linear carbohydrate polymers that are generally composed of disaccharide repeating units of an uronic acid and a hexosamine. Chondroitin sulfate and heparan sulfate occur as proteoglycans in which the polysaccharide chains are covalently linked through xylose residues to the hydroxyl groups of serine residues of a protein moiety. The glycoproteins differ from the glycosaminoglycans in the following major respects: (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 heterosaccharides, which are often branched; and (d) they contain several sugars that are not characteristic compounds of glycosaminoglycans.

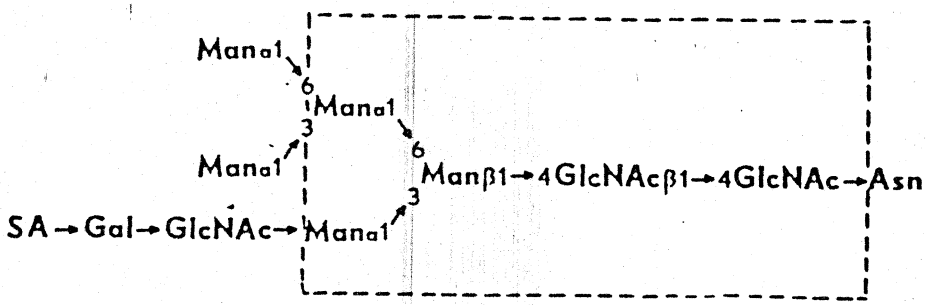
The most distinctive feature of glycoproteins is the carbohydrate-peptide linkage. The sugar chains of glycoproteins can be classified into two groups by the structure of their linkage regions to the polypeptide backbone. Sugar chains attached to the polypeptide by an O-glycosidic linkage and the second major class of N-glycosidically linked sugar chains where the linkage is between the N-acetylglucosamine to the amide nitrogen of asparagine(163). The structures of different oligosaccharide chains found in glycoproteins are given figure 1. Several types of O-glycosidic linkage occur between N-acetylgalactosamine, galactose and xylose on the one hand and the hydroxyl groups of serine, threonine, hydroxylysine, and



I. HIGH-MANNOSE TYPE



II. COMPLEX TYPE



III. HYBRID TYPE

Figure 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.

hydroxyproline on the other. Recently a novel type of glycosylation in which N-acetylglucosamine is O-glycosidically attached to protein was described and was found to be enriched on many proteins localized to the cytoplasmic and nucleoplasmic compartments of the cell(164,135).

Majority of the carbohydrate in brain glycoproteins is linked via N-acetylglucosamine to the amide nitrogen of asparagine residues in the protein moiety(189). The N-linked oligosaccharides contain a core pentasaccharide Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc(203). Structurally, the asparagine-linked sugar chains can be classified into three subgroups. Oligosaccharides which contain only mannose and N-acetylglucosamine residues, have been classified as high mannose type. The high mannose type oligosaccharides typically have two to six additional mannose residues linked to the pentasaccharide core. In complex type sugar chains structural variation arises by the addition of outer branches with lactosamine sequence to the α-mannose residues of the pentasaccharide core(219,310,220). The complex type structure can be further modified by the addition of extra sugar residues that elongate the outer chains. The hybrid chain structures are so named because they have features of both high mannose and complex type oligosaccharides(275,311). Most hybrid molecules contain a "bisecting" N-acetylglucosamine linked β1-4 to the β-linked mannose residue, although some exceptions exist(139,292,313). In the polypeptide chains, the amino acid

sequence next to the glycosylated asparagine was Asn-X-Ser/Thr where X can be any amino acid other than proline and aspartic acid(192).

There are large variations in the number and type of carbohydrate units attached to glycoproteins. At one extreme are glycoproteins such as ovalbumin and bovine ribonuclease B which contain a single oligosaccharide unit per molecule. At the other extreme is ovine submaxillary mucin which contains 205 disaccharide chains per molecule. A single glycoprotein may contain more than one type of carbohydrate-peptide linkage.

Sugar chains are formed by the sequential action of glycosyl transferases. The structures of the final sugar chains produced are determined by the specificity of each glycosyltransferase. A shortage in certain nucleotide sugars, changes in relative glycosyl transferase activities and other factors can theoretically induce changes in the major sugar chain structures. Such a mechanism has been proposed to explain the microheterogeneity of sugar chains.

Glycoproteins are important constituents of cell membranes. The membrane glycoproteins are amphipathic molecules- that is, in addition to the hydrophilic sequences usually found in soluble proteins, they contain a hydrophobic sequence which anchors them to the lipid bilayer. Because of this, glycoproteins can be extracted from membranes only by solvents that break hydrophobic bonds. They are therefore classified as integral or intrinsic

membrane proteins(253). The carbohydrate units are often clustered near one end of the molecule, and this hydrophilic portion is exposed on the external side of the plasma membrane. Carbohydrates are thus asymmetrically distributed across the membrane bilayer. At least some of the intrinsic glycoproteins pass all the way through the bilayer so that they are exposed to both the external and internal environment. The internal segments of such intrinsic glycoproteins may be closely associated with some of the proteins located at the cytoplasmic face of the membrane. In this way, the transmembrane glycoproteins may provide a means of communication across the bilayer, either for the transport of solutes and water or for the transmission of signals in response to external stimuli, such as hormones, antibodies or other cells.

The cell surface carbohydrates have an enormous potential for coding biological information. In peptides and oligonucleotides, the information content is based on the number of monomeric units and their sequence, whereas in carbohydrates, information is also encoded in the position and anomeric configuration (α or β) of the glycosidic units and in the occurrence of branch points. Further structural diversification may occur 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 leading to the hypothesis that "the specificity

of many natural polymers is written in terms of sugar residues and not of amino acids or nucleotides"(254). There are strong evidences that this diversity is biologically significant, since in many cases carbohydrates modify the activities of proteins to which they are attached and also serve as markers of cell differentiation, development and pathological states(114,208,229).

The carbohydrates of glycoproteins modify the physicochemical properties of proteins by changing their hydrophobicity, electrical charge, mass and size. Glycoproteins, especially if they are sugar rich are more resistant to proteolysis than the nonglycosylated ones. Nonglycosylated fibronectin synthesized in chick embryo fibroblasts in presence of tunicamycin was degraded intracellularly at a much faster rate than the glycosylated protein(216). Carbohydrates serve as important recognition markers on glycoproteins in solution as well as on cell surface. The classical work of Ashwell and his coworkers had demonstrated that removal of sialic acid from circulating glycoproteins leads to a dramatic enhancement in the rate of glycoprotein clearance from the circulatory system(206). The asialoglycoproteins are rapidly taken up and catabolized by the liver. The binding protein that was presumed to be responsible for this reaction was purified from rabbit liver(153). Clearance systems in which sugars other than galactose serve as determinants also have been identified, and in

some cases the specific carbohydrate-binding proteins have been isolated. For example, a system specific for clearance of glycoproteins terminating with mannose and N-acetylglucosamine is present in chicken, rat, and rabbit livers(186). A different system, in which carbohydrates on soluble glycoproteins serve as determinants of recognition is that responsible for the intercellular segregation and selective uptake of lysosomal glycosidases by fibroblasts. The major role of the phosphomannose residues on newly synthesized acid hydrolases and phosphomannose receptors on some membranes is to allow segregation in the Golgi apparatus. The receptor-ligand system thus prevents loss of the lysosomal enzymes from cells. In analogy to the role of carbohydrates in determining the survival time of glycoproteins in the circulation system, sugars on cell surfaces may be important in determining the life span to circulating cells and the distribution in specific organs in the body. Thus human erythrocytes which normally persist in the circulatory system for about 120 days are absorbed within hours and phagocytosed by liver after treatment with neuraminidase(278). The sugars serve as recognition markers in cell-cell, cell-virus and cell-bacteria interactions. Moreover, cell surface glycoproteins are the immunodeterminant structures of blood group A,B,H and M/N specificities or act as acceptors for a number of lectins(300). Evidence has been presented to indicate the fundamental importance of carbohydrate structure recognition in the immune

system. The cell surface glycoproteins presumably play a structural role in stabilizing the cell membrane. Oligosaccharide chains can influence the ability of protein to fold properly. Studies with the sialic acid rich glycoproteins of saliva and intestinal, tracheal or cervical mucus have led to the conclusion that the sialic acid is responsible for the high viscosity and the function of those mucins as lubricants(105). On the other hand, the available information shows clearly that the presence of carbohydrate is not always essential for the particular function of the glycoprotein in which it occurs.

The asparagine-linked oligosaccharide is assembled in endoplasmic reticulum on the lipid carrier dolichol phosphate. The lipid linked oligosaccharide is known as the "G-oligosaccharide". The sequence of reactions of "dolichol phosphate cycle" for the formation of G-oligosaccharide is given in figure 2. An examination of protein sequences has revealed that only about one third of the potential Asn-X-Ser/Thr sites in proteins are actually glycosylated(165). The glycosylation occurs co-translationally and the asparagine that is to be glycosylated is part of a growing peptide chain that is in the process of folding. Consequently the period of time during which glycosylation can occur may be quite brief. Once the protein has folded, potential glycosylation sites are no longer accessible to the oligosaccharyl transferases(224). The efficient glycosylation of proteins is dependent on a sufficient pool of completely

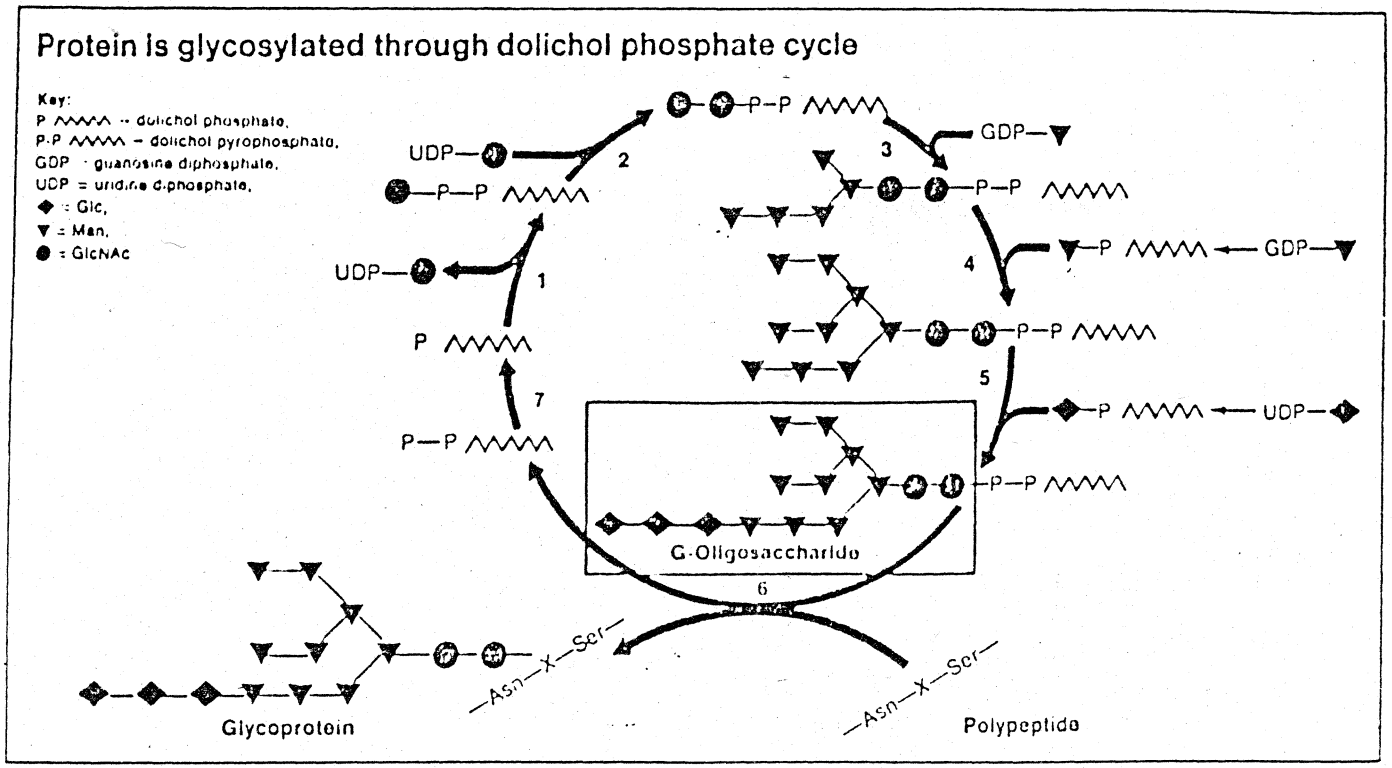


Figure 2. Dolichol phosphate cycle for the glycosylation of proteins in the endoplasmic reticulum.

Adapted from Sharon,N., and Lis,H.(1981) Chemical and Engineering News 59,21-44.

glycosylated lipid-linked oligosaccharide donor, an adequate activity of oligosaccharyl transferase and a properly oriented and accessible Asn-X-Ser/Thr sequence in the acceptor. The precursor $Glc_3Man_9GlcNAc_2$ is transferred from the lipid donor to an asparagine in a nascent polypeptide during its vectorial transport across the membrane of the rough endoplasmic reticulum. The processing of the oligosaccharide chains begins in the endoplasmic reticulum and have been shown to occur co-translationally(11). Both integral membrane and secreted glycoproteins undergo the same sequence of events in rough endoplasmic reticulum, but with various glycoproteins some or all of the steps, including the glycosylation event, may occur after the polypeptide chain is completely synthesized(137). With the exception of glycoproteins that are permanent residents of the endoplasmic reticulum membrane, the newly synthesized glycoproteins are next transported to the cis Golgi cisternae by means of vesicles which are believed to bud from the rough endoplasmic reticulum and then fuse with the Golgi membrane(144). When the glycoproteins arrive in the Golgi, they traverse the stack from the cis through medial to trans cisternae by vesicular transport(18). Further oligosaccharide processing takes place in the Golgi complex. The schematic pathway of oligosaccharide processing of glycoproteins is given in figure 3. The final oligosaccharide structure assembled on a glycoprotein is dictated to a large extent by the order in which that glycoprotein

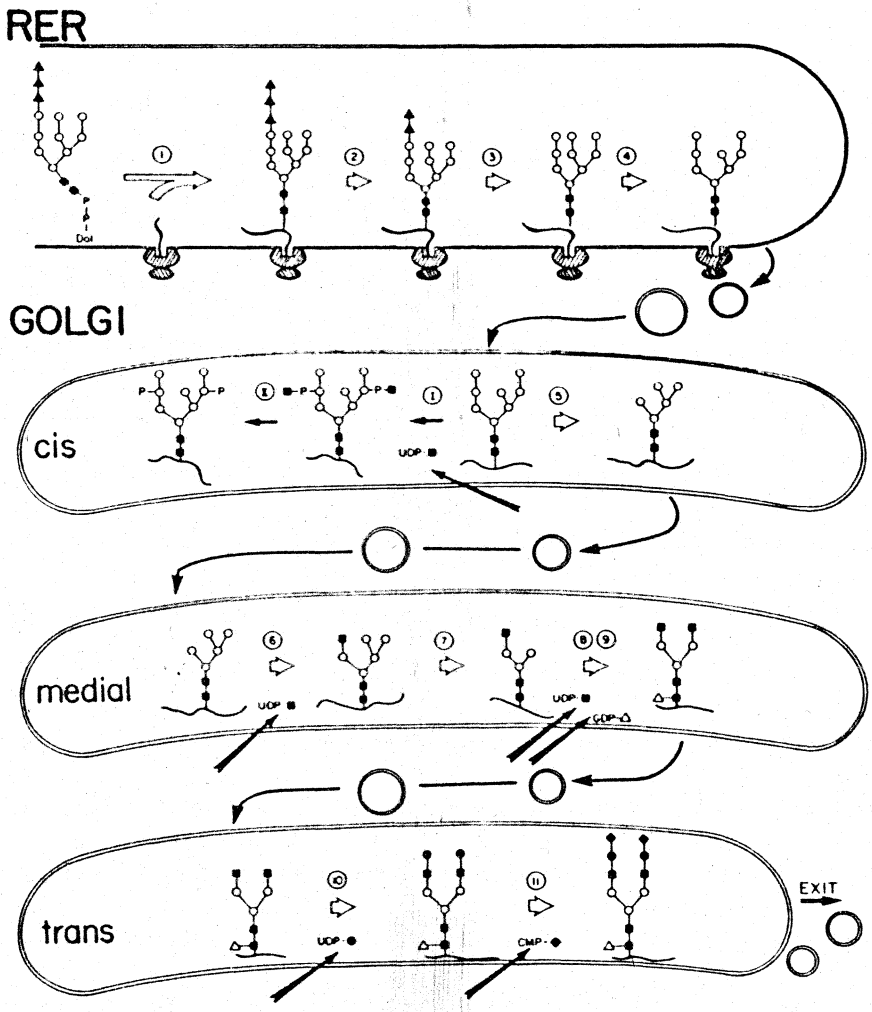


Figure 3. Schematic pathway of oligosaccharide processing on newly synthesized glycoproteins. The reactions are catalyzed by the following enzymes: (1) oligosaccharyl-transferase, (2) α-glucosidase I, (3) α-glucosidase II,(4) ER α 1,2-mannosidase, (I) N-acetylglucosaminylphospho-transferase, (II) N-acetylglucosamine-I-phosphodiester α-N-acetylglucosaminidase, (5) Golgi α-mannosidase I, (6) N-acetylglucosaminyltransferase I, (7) Golgi α-mannosidase II, (8) N-acetyl glucosaminyltransferase II, (9) fucosyl-transferase, (10) galactosyltransferase, (11) sialyl-trasferase. The symbols represent: ■,N-acetylglucosamine; ○,mannose; ▲,glucose; △,fucose; ●,galactose; ◆,sialic acid. Adapted from Kornfeld,R.,and Kornfeld,S.(1985) Ann.Rev. Biochem. 54,631-664.

encounters the processing glycosidases and glycosyl transferases and their specificity. It is apparent that many thousands of different oligosaccharide structures could be assembled from the combined and sequential action of the processing enzymes. Yet only a limited number of structures is observed. This is due to the rigid substrate specificity of the processing enzymes. The asparagine-linked oligosaccharides may undergo further post-translational modifications, including phosphorylation of mannose residues, sulfation of mannose and N-acetylhexosamine residues and O-acetylation of sialic acid residues.

The synthesis of O-linked oligosaccharides appears to occur almost entirely by sequential glycosylation where the product of one glycosyltransferase is utilized as an acceptor substrate by another glycosyl transferase. Synthesis begins with the transfer of N-acetylgalactosmine to the hydroxyl group of threonine or serine by the enzyme UDP-GalNAC: polypeptide transferase. The initial glycosylation event can occur on nascent chains as they are being synthesized on the rough endoplasmic reticulum(267). After the sequence GalNAc Thr/Ser is formed, either galactose or sialic acid can be transferred next. The addition of sialic acid before galactose would terminate the oligosaccharide as a disaccharide. Once the disaccharide Galβ1-3GalNAc Thr/Ser was formed, the preferred sequence of addition of further sugar residues is sialic acid, fucose and N-acetylgalactosmine respectively.

Even within a single glycoprotein, the oligosaccharide structures exhibit considerable microheterogeneity. On first principles, it would seem evident that oligosaccharide structure is under genetic control at the level of the expression of the number and type of glycosyltransferases which are produced in a cell. The relative amounts of the various glycosyltransferases produced by a cell may determine which of the several alternative biosynthetic pathways will predominate. The situation at branch points can simply be viewed as competition for a common acceptor substrate by two glycosyl transferases.

The presence of carbohydrates offers a convenient handle for the use of lectins in the glycoprotein isolation and characterization. Lectins are a group of sugar-binding and cell-agglutinating proteins of non-immune origin that are ubiquitous in plants, but are also found in microorganisms and animals. Glycoproteins bind noncovalently to lectins and the complexes thus formed can be dissociated by adding the respective hapten sugar for which the lectin is specific. Several different types of N-glycosidically linked carbohydrate units are known to occur in brain and can be partially fractionated by affinity chromatography on concanavalin A-Sepharose under standardized conditions. Most of the N-glycosidically linked carbohydrate units in nervous tissue glycoproteins are of the complex type(189).

In the complex oligosaccharides of brain glycoproteins, much of the fucose is linked to peripheral GlcNAc residues in an oligosaccharide with the structure NeuAc(α2-3)Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-(166). Over half of the N-acetylglucosamine residues are 4-O-substituted, whereas approximately one-quarter are terminal, and a small proportion are 3,4- and 4,6-di-O-substituted partially by fucose(167). Galactose apparently accounts for one of the substituents found at C-3 of GlcNAc residues, since significant amounts of Gal(β1-3)GlcNAcol could be detected after partial acid hydrolysis and reduction of a brain glycopeptide fraction(166).

Although most of the sialic acid in brain glycoproteins is in the expected nonreducing terminal position, in adult rat brain a small fraction has been shown to be substituted by another sialic acid residue(189). Such disialosyl groups had previously been known to occur in gangliosides, and they are present in particularly large amounts in glycoproteins of certain microsomal and plasma membrane preparations from brain(83). Recent studies have demonstrated that in developing brain there is a significant proportion of tri- and tetraantennary N-glycosidic glycopeptides that contain di- and polysialosyl carbohydrate units on their outer branches(80). Glycopeptides containing these highly anionic oligosaccharides, which also have ester sulfate residues, were precipitated by cetylpyridinium chloride(189).

The O-glycosidically linked oligosaccharides in brain consist of the typical core disaccharide galactosyl(β1-3)N-acetylgalactosamine, which may occur either as such or substituted with sialic acid residues at C-3 of galactose and/or C-6 of N-acetylgalactosamine. The tetrasaccharide accounts for the half of the total O-glycosidically linked oligosaccharides in rat brain glycoproteins(190). The α-anomer of the core disaccharide (which has been detected only in a nonsialylated form) appears to be present only in nervous tissue(82).

Majority of the brain glycoproteins are integral components of cell surface and internal membranes, whereas much of the glycosaminoglycan content of nervous tissue is either soluble or easily releasable from the particulate fraction(189). One or both of these classes of complex carbohydrates has been found in all of the cellular and subcellular fractions examined, including bulk-isolated neurons, axons, astrocytes, and oligodendroglia, as well as nuclei, mitochondria, cytoplasm, myelin, and synaptic membranes, junctions, and vesicles. Membrane-bound complex carbohydrates are known to be transported in the fast component of axonal flow(70,6).

AIMS AND OBJECTIVES

Cell surface glycoproteins play significant roles in central nervous system cell sociology, oncology and in its development. The morphological, physiological and pathological changes in the nervous system are accompanied by specific changes at the molecular level. Among the many factors which govern the brain function, cell surface glycoproteins are an important source of information. The physicochemical characterization of these surface glycoproteins is essential in understanding their crucial roles in normal brain function and pathology.

As evident from the earlier discussions, most of the information about of neural cell surface glycoproteins were obtained from non-human sources. The information related to neural surface glycoproteins of developing human brain is lacking. It is quite likely that cell adhesion during the process of stabilization of tissue form requires further components in addition to the presently known neuronal cell surface glycoproteins. Information on the cell surface glycoproteins from developing human brain would lead to a better understanding of its function and in the treatment of various neurological disorders. The present study was undertaken in an attempt to obtain information on the concanavalin A-binding neuronal surface

glycoproteins of developing human brains. Efforts to achieve this goal comprised of the following sections:

(1) Isolation of neurons and the major neuronal cell surface Con A-binding glycoproteins of developing human brains.

(2) Physicochemical characterization of Con A-binding neuronal cell surface glycoproteins.

(3) Comparative study of the major neuronal cell surface glycoproteins from second trimester and full term fetuses.

(4) Immunogenicity of the Con A-binding glycoproteins.

CHAPTER - II

MATERIALS AND GENERAL METHODS

MATERIALS AND GENERAL METHODS

Coomassie brilliant blue G, sodium dodecyl sulfate, galactose oxidase (from Dactylium dendroides), neuraminidase (from C.perfringens),horseradish peroxidase, trypsin (TPCK treated) from bovine pancreas, fetuin, bovine serum albumin, ovalbumin, wheat germ, DL-dithiothreitol, dithioerythritol, 5-hydroxytryptamine hydrochloride (serotonin), fluoresceine isothiocyanate, N-acetyl-D-glucosamine, N-acetyl neuraminic acid (sialic acid), iodoacetic acid sodium salt, dansyl chloride, standard dansyl amino acid kit, iodoacetamide, chitin, 4-NN-dimethylaminoazobenzene 4'-isothiocyanate (DABITC), N,N,N',N'-tetramethylenediamine (TEMED), 2-thiobarbituric acid, sodium cyanoborohydride, acrylamide, N,N'-methylene-bis-acrylamide, 1,4-butanediol diglycidyl ether and Tris were purchased from Sigma Chemical Co., St.Louis, USA. The standard amino acid kit was purchased from Serva Feinbiochemica, Heidelberg, New York. Human IgG was purchased from Behringwerke AG, Marburg, Federal Republic of Germany. Coomassie brilliant blue R-250 was purchased from Pierce Chemical Co.,Illinois, USA. Deoxyribonuclease II from bovine spleen, Triton X-100, Tween 20, trifluoromethanesulfonic acid (TFMS), phenyl isothiocyanate, guanidine hydrochloride, phenylmethane sulfonyl fluoride, benzamidine hydrochloride were the products of Fluka, Buchs, Switzerland. Ficoll 400, Sepharose 4B, Sepharose 6B, Sephadex G-50, Sephadex G-75 and Blue dextran

2000 were purchased from Pharmacia Fine Chemicals, Sweden. Bio-gel P-4, Bio-gel P-6DG and nitrocellulose membrane were purchased from Bio-Rad Laboratories, California, USA. Methyl-α-D-glucopyranoside, N-ethylmorpholine and pronase from Streptomyces griseus were purchased from Koch-Light Laboratories Ltd, Colnbrook, England. Trifluoroacetic acid and precoated silica gel plates were the products of E.Merck, Federal Republic of Germany. Polyamide sheets (5 X 5cm) were purchased from Schleicher and Schull, Federal Republic of Germany. Goat anti rabbit gamma globulin was purchased from Immunodiagnostics, New Delhi, India. [^{3}H]-sodium borohydride and [^{3}H]-acetic anhydride were the products of Amersham International plc, U.K. [^{14}C]-acetic anhydride was purchased from Bhabha Atomic research Centre, Bombay, India. Other chemicals used were of analytical grade and obtained from local sources. The seeds of Canavalia gladiata, Ricinus communis, Artocarpus integrifolia, Artocarpus hirsuta, Arachis hypogea and Pisum sativum were obtained locally. Soybean agglutinin was a kind gift from Prof. Nathan Sharon, Dept. of Biophysics, Weizmann Institute of Science, Rehovot, Israel.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

The molecular weight range of the neuronal glycoproteins was determined by SDS-PAGE on 8% and 10% slab gels according to the method of Laemmli(170).

Reagents:

(1) Gel solution: Fifteen grams of acrylamide and 0.4g of bis acrylamide were dissolved in distilled water and the volume made up to 50ml. The solution was filtered and stored at 4°C in amber coloured bottle.

(2) Buffer I: 0.614M Tris / HCl pH 8.8. For one hundred millilitre of the buffer, 164mg of SDS was dissolved.

(3) Buffer II: 0.147M Tris pH 6.8. For one hundred millilitre of the buffer, 108mg of SDS was dissolved.

(4) Chamber buffer: 0.25M Tris / 0.192M glycine pH 8.3 containing 0.1% SDS.

(5) Ammonium persulfate(15mg/ml).

For the preparation of 10% separating gel, 6ml of acrylamide, 11ml of buffer I, 0.9ml of ammonium persulfate and 20µl of TEMED were mixed together at the time of polymerization. For the preparation of spacer gel, 1ml of acrylamide, 8.5ml of buffer II, 0.5ml of ammonium persulfate and 10µl of TEMED were mixed together at the time of polymerization. Bovine serum albumin (67,000), IgG heavy chain (50,000), ovalbumin (45,000), Con A (27,000) and IgG light chain (23,500) were used as standard proteins. The small slab gel (7.3 X 8.3cm) was run at 10mA and the bigger slab gel (12 X 15cm) at 40mA till the bromophenol blue dye had reached the bottom of the gel. The gels were stained with Coomassie brilliant blue R-250 and destained with methanol: acetic acid: water (1: 1.5: 7.5,v/v)(301). The gels were also

stained with silver nitrate according to the method of Wray et al(306).

Gradient SDS-PAGE on 3-12% gels was performed by modifications of the method described by Hames as given below(116).

Solutions:

(1) 50% acrylamide / bis-acrylamide solution (37:1): 48.6g of acrylamide and 1.315g of N,N'-methylene bis acrylamide were dissolved in 100ml of distilled water and filtered.

(2) Separating gel buffer (3M Tris-HCl pH 8.85 containing 0.4% SDS): 36.3g of Tris was dissolved in distilled water and the pH was adjusted to 8.85 before making up the volume to 100ml. Then added 400mg of SDS.

(3) Running buffer (50mM Tris-192mM glycine-0.1% SDS, pH 8.3): 4.23g of Tris, 9.98g of glycine and 700mg of SDS were dissolved in distilled water and the volume made upto to 700ml.

(4) Ammonium persulfate: 25mg / 250µl of distilled water.

Three percent acrylamide solution was prepared by mixing 1.2ml of 50% acrylamide with 5ml separating gel buffer and 13.8ml of distilled water. For the preparation of 12% acrylamide solution, 4.8ml of 50% acrylamide was mixed with 5ml of separating gel buffer and 10.2ml of distilled water. The preparations were degassed. Prior to the gradient formation, 16ml of the 3% buffered acrylamide solution was mixed with 7µl of TEMED and 45µl of ammonium persulfate solution. Seventeen millilitres of 12%

buffered acrylamide solution was mixed with 7µl of TEMED and 35µl of ammonium persulfate solution and the gradient was formed in a gradient mixer. The electrophoresis was carried out with a current of 40mA till the bromophenol blue indicator had reached the bottom of slab gel.

Preparation of protein-conjugates

(1) Peroxidase-conjugates

Periodate oxidised horseradish peroxidase was coupled to the various lectins and goat anti rabbit gamma globulin by the method of Nakane et al(210,303). To one milligram of horseradish peroxidase in 0.2ml of 0.3M sodium bicarbonate, 10µl of 1% FDNB in absolute ethanol was added and incubated at 25°C for 1h with occasional mixing at intervals. The incubation was continued for another 30 min at 25°C after adding 0.2ml of 0.06M sodium periodate. The excess periodate was eliminated by the addition of 0.2ml, 0.32M ethylene glycol and incubation for 1h at 25°C. The activated peroxidase and the lectins / immunoglobulin to be conjugated were dialysed separately against 0.01M Na_2CO_3-$NaHCO_3$ pH 9.5 for 16h at 4°C. One milligram of lectin / immunoglobulin in 2ml of the buffer was mixed gently with the activated peroxidase and incubated for 4h at 4°C . Fifty microlitre of 1% potassium borohydride was added to block free aldehyde groups and incubated for 1h at 4°C. The peroxidase conjugate was dialysed against 10mM $NaHCO_3$ pH 8.3 followed by 20mM Tris-HCl pH 8.0 and finally against 20mM Tris-HCl pH 7.5-0.1M NaCl-0.05% Tween 20.

(2) Fluoresceine isothiocyanate-concanavalin A

The preparation of FITC-Con A was carried out according to the method described by Felt kamp et al(280). Fluoresceine isothiocyanate(0.5mg) was added to 10mg Con A dissolved in 1ml of 0.25M Na_2CO_3-$NaHCO_3$ pH 9.0 and mixed gently for 16h at 4°C. The free FITC was separated from the conjugate by gel filtration on Biogel P-4 (1.1 X 21cm) equilibrated with TBS pH 7.4 containing 1mM $CaCl_2$, 1mM $MgCl_2$ and 1mM $MnCl_2$.

Protein estimation

Protein was estimated by a modified Lowry's method in a total volume of 0.81ml in presence of SDS to eliminate the interference due to Triton X-100(185,62).

Reagents:

(A) 2% sodium potassium tartrate.

(B) 1% copper sulfate.

(C) 2% sodium carbonate solution in 0.1N sodium hydroxide.

(D) Alkaline copper reagent: One millilitre each of reagent A and B were mixed together at the time of experiment and made up to 100ml with reagent C.

(E) 1N Folin's reagent.

One hundred and fifty microlitre of the sample and 0.6ml of alkaline copper reagent containing 3% SDS were mixed together and incubated at 25°C for 10min. This was followed by the addition of 60µl, 1N Folin's reagent and incubated at 25°C for 30min.

Absorbance was measured at 660nm. Ovalbumin was used as a standard.

Protein was also estimated by the Bradford's method using bovine serum albumin as a standard(33).

Reagent: The coomassie brilliant blue G dye was prepared as a 0.06% solution in 0.3M perchloric acid. One hundred and fifty milligrams of the dye was dissolved in 250ml of distilled water containing 4.49ml perchloric acid. The preparation was filtered through Whatman No.1 filter paper before use.

The protein estimation was carried out by mixing 1.5ml of the sample with an equal volume of the reagent and absorbance was measured at 620nm.

Carbohydrate estimation

The total neutral sugar was estimated by phenol-sulfuric acid method of Dubois et al in a total volume of 1.1ml with mannose as standard(61).

Reagents:

(A) Sulfuric acid.

(B) 5% phenol.

One hundred microlitre of the sample was mixed with 0.2ml of 5% phenol, followed by 0.8ml of chilled sulfuric acid. After 15min incubation at 25°C, absorbance was taken 485nm.

Sialic acid estimation

The sialic acid content of the neuronal glycoproteins was estimated by the method of Warren(296).

Reagents:

(A) 0.2M sodium metaperiodate in 9M o-phosphoric acid.

(B) 10% sodium meta arsenite in 0.5M Na_2SO_4 in 0.1N H_2SO_4.

(C) 0.6% thiobarbituric acid in 0.5M Na_2SO_4.

Twenty five microlitre of the sample was mixed with an equal volume of reagent A and incubated at 25°C in the dark for 25min. Two hundred and fifty microlitre of reagent B was added to reduce excess periodate followed by the addition of 750µl of reagent C. The sample was heated at 100°C for 15min. Seven hundred and fifty microlitre of methyl cellosolve was added and the preparation was centrifuged. The absorbance of the supernatant was measured at 549nm.

Determination of N-terminal sequence

The N-terminal analysis of the neuronal glycoproteins was performed by the both direct an indirect Edman degradation procedures.

(1) Dansyl-Edman method

The sample was first dansylated according to the method of Gray(106). Two hundred microgram of the sample was boiled for 2 min in 100µl of 40mM Li_2CO_3 / HCl pH 9.5 containing 2% SDS. Five microlitre of dansyl chloride (2.5mg / 0.1ml acetonitrile) was added to give a final concentration of 5mM Dns-Cl. The contents were mixed well and the reaction was allowed to proceed for 1h at 37°C. After the dansylation, 20µl of 1N HCl was added followed by 0.4ml cold acetone. The precipitated proteins were washed twice

with cold acetone:200mM HCl (4:1), and dried. To the dried sample 200µl of 6N HCl was added and transferred to an ampule. The ampule was flushed with nitrogen, sealed and hydrolysed at 108°C for 18h. After the hydrolysis, the ampules were opened and the contents dried.

For the identification of N-1 and N-2 amino acids, 400µg of the sample was boiled in 100µl of 2% SDS for 2min. One hundred microlitre of dry pyridine was added followed by 5µl of phenylisothiocyanate. The tube was flushed with nitrogen, covered with parafilm and incubated for 1h at 37°C. After the reaction, another 10µl of PITC was added, flushed with nitrogen and incubated for 1h at 37°C. Three hundred microlitre of distilled water was added to the preparation and the mixture dried under vacuum. Two hundred microlitre of anhydrous trifluoroacetic acid was added to the dried sample and the tube flushed with nitrogen and sealed with parafilm. The reaction was allowed to proceed for 1h at 37°C and the sample was air dried. The nonvolatile by-products were removed by extraction with n-butyl acetate. The dried residue was suspended in 0.5ml of 0.01N HCl, followed by the addition of 1ml n-butyl acetate. After thorough mixing, the two phases were separated by centrifugation. The aqueous phase was extracted twice with 1ml aliquots of the organic phase. The aqueous phase was dried in vacuum over NaOH. The dried protein was suspended in 200µl of distilled water and divided into two equal aliquots. One of the aliquots was further dried and

subjected to the dansylation procedure to determine the N-1 amino acid. The second aliquot was subjected to another cycle of Edman degradation procedure. The dansyl amino acids were separated by HPLC using gradient elution on a 5µm Lichrosorb RP-18 Ultropac column (4 X 250mm) with a guard column of 7µm (4 X 30mm) according to the method of Marquez et al(191). The HPLC gradient was formed from solvent A (0.6% acetic acid-0.008% TFA) and solvent B (methanol) as illustrated in Table 1. Dansyl amino acids were monitored by their fluorescence (Ex max 333nm, Em max 510nm).

(2) DABITC / PITC double coupling method

In the direct DABTIC / PITC double coupling method, the detection of released DABTH-amino acid derivatives was carried out on TLC(45,44).

First coupling: To one hundred microgram of the sample suspended in 20µl of 50% pyridine, 10µl of the DABITC solution (2.82mg/ml) in pyridine was added and vortexed. The tube was flushed with nitrogen, sealed with parafilm and incubated for 50min at 70°C.

Second coupling: Two microlitre of PITC was added, flushed with nitrogen and incubated for 15min at 70°C.

First extraction: Two hundred microlitres of heptane: ethyl acetate (2:1) [saturated with 67% pyridine], vortexed thoroughly and centrifuged for 2min at 2000rpm. The organic phase was discarded. The process was repeated five times.

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First coupling: To one hundred microgram of the sample suspended in 20µl of 50% pyridine, 10µl of the DABITC solution (2.82mg/ml) in pyridine was added and vortexed. The tube was flushed with nitrogen, sealed with parafilm and incubated for 50min at 70°C.

Second coupling: Two microlitre of PITC was added, flushed with nitrogen and incubated for 15min at 70°C.

First extraction: Two hundred microlitres of heptane: ethyl acetate (2:1) [saturated with 67% pyridine], vortexed thoroughly and centrifuged for 2min at 2000rpm. The organic phase was discarded. The process was repeated five times.

TABLE.1. LICHROSORB RP-18 HPLC SOLVENT GRADIENT PROGRAM FOR SEPARATION OF DNS-AMINO ACIDS. Solvent A: 0.6% acetic acid and 0.008% TEA in 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		

Cleavage: The aqueous phase was air dried, followed by further drying over P_2O_5 and KOH in vacuum. Fifty microlitre of anhydrous TFA was added, vortexed, flushed with nitrogen and incubated for 10min at 55°C.

Second extraction: The preparation was evaporated to dryness and suspended in 50µl of distilled water. One hundred microlitre of butyl acetate was added, vortexed and centrifuged to separate the phases. The aqueous phase was dried over P_2O_5 and KOH in vacuum and used for the next cycle.

Conversion: The butyl acetate extracts were dried over P_2O_5 and KOH in vacuum. Fifty microlitre of 50% TFA was added, vortexed. The tube was flushed with nitrogen, sealed with parafilm and incubated for 10min at 80°C. The preparation was evaporated to dryness over P_2O_5 and KOH in vacuum. After dissolving in 10µl of 80% ethanol the liberated DABTH-amino acid derivatives were subjected to TLC.

Preparation of standard DABTH derivatives of amino acids

The DABTH-derivatives were prepared according to the method of Chang(43)

Reagents: (1) Triethylamine-acetic acid buffer. To fifty millilitre of distilled water, 50ml of acetone was added, followed by 0.5ml of triethylamine and 5ml of 0.2M acetic acid. The pH was adjusted to 10.65 with triethylamine and or 1N HCl. (2) DABITC solution. 1.13mg/ml in acetone.

One milligram of amino acid was dissolved in 200µl of triethyl amine-acetic acid buffer and the pH was adjusted to 10 in case of His, Asp and Glu with 1N NaOH. One hundred microlitre of DABITC solution was added and incubated for 1h at 54°C. The preparation was dried over P_2O_5 and KOH in vacuum. The dried residue was dissolved in 200µl of 50% TFA and incubated for 45min at 55°C. The contents were further dried over P_2O_5 and KOH in vacuum and dissolved in 200µl of 80% alcohol.

DABTH derivative of diethylamine was prepared by mixing 10µl of double distilled diethylamine with 100µl of distilled water. To sixty microlitre of the mixture, 60µl of DABITC solution (2.4mg/ml in ethanol) was added. Four hundred and forty microlitre of ethanol was added, mixed well and stored at -20°C.

Identification of DABTHs by TLC on polyamide sheets

Less than 0.5µl of DABTH sample was applied to the origin (about 6mm from the edges of two adjacent sites) of a 2.5 X 2.5 cm polyamide sheet. Internal DABTC-diethylamine was always co-chromatographed with unknown samples. The sheet was run two-dimensionally by ascending solvent flow; acetic acid-water (1:2,v/v); toluene- n-hexane-acetic acid (2:1:1,v/v/v). The sheet was dried after each run with a drier at low heat. The sheet was exposed to HCl vapours, when all yellow spots turned red or blue.

Acid-urea-poly acrylamide gel electrophoresis

The acid-urea-PAGE was carried out according to the method of Sherton et al(255).

Reagents:

(1) Gel solution: To a mixture of 1g acrylamide, 28mg N,N'-methylene bis acrylamide and 7.2g urea dissolved in distilled water, 116µl of TEMED and 1ml of glacial acetic acid were added. The total volume was made up to 19.0ml after adjusting the pH 4.2 with 3N NaOH.

(2) Chamber buffer: To glycine (2.8g) dissolved in distilled water, 0.3ml of galcial acetic acid was added. After adjusting the pH to 4.2 with 3N NaOH, the total volume was made upto 200ml.

(3) Sample buffer: Chamber buffer containing 8M urea, 10% glycerol and 5% β-mercaptoethanol.

For the preparation of gel, 14.5ml of the filtered gel solution was mixed with 0.5ml of 6% ammonium persulfate at the time of polymerization. The protein sample for the electrophoresis was precipitated by 8 volumes of methanol, dissolved in the sample buffer and boiled for 2 min before loading. The electrophoresis was carried out till the methylene blue dye had moved out of the gel.

Alkaline-urea-polyacrylamide gel electrophoresis

Alkaline-urea-PAGE was carried out as described by Sherton et al(255).

Reagents: (1) Gel solution. A mixture of 2.13g acrylamide, 0.079g of N,N'-methylene bis acrylamide, 1.39g of boric acid, 0.35g of EDTA, 1.62g of Tris and 0.13ml of TEMED were dissolved in

distilled water and the volume was made upto 10ml after adjusting the pH to 8.6.

(2) Chamber buffer: A mixture of 1.92g of boric acid, 2.24g Tris base and 0.48g of EDTA were dissolved in distilled water and the volume was made upto 200ml after adjusting the pH to 8.6 with NaOH.

For the preparation of gel, 18.75ml Of 8M urea, 5.75ml of the gel solution and 0.5ml of 1.5% ammonium persulfate were mixed together at the time of polymerisation.

Electroelution of protein bands from SDS-PAGE

The electroelution was carried out by a modification of the method described by Stralfors and Belfrage(266).

Reagents:

(A) Solubilization buffer: 25mM Tris / 75mM glycine pH 8.8-1% SDS(w/v)-5mM DTT-40% glycerol(v/v).

(B) Recovery solution: 25mM tris-75mM glycine pH 8.8-1mM DTT-40% glycerol(v/v).

(C) Chamber buffer: 50mM Tris-150mM glycine pH 8.8.

The protein bands were cut from SDS-PAGE gels and soaked in the solubilization buffer with 4 changes of 30 min each. The preparation was boiled during the last change. Two or three equilibrated gel slices were packed over a supporting gel (6% Laemmli's separating gel) cast in electrophoresis tubes (0.6 X 9cm). Recovery solution was added to the electrophoresis tube, so that the gel slices were completely immersed in the solution.

On top of the recovery solution, 2M NaCl was carefully layered to fill the tube. The electrophoresis was carried out with a current of 4mA/tube for 90min at 25°C. The eluted protein formed a precipitate at the interface between the recovery solution and 2M NaCl. The protein precipitate along with the recovery solution was collected and precipitated with 8 volumes of methanol.

[^{3}H]-acetic anhydride labelling

The neuronal glycoproteins were labelled with [^{3}H]-acetic anhydride according to the method of Finne and Krusius(81). To five hundred micrograms of delipidated neuronal glycoproteins suspended in 1ml of 0.5M $NaHCO_3$, [^{3}H]-acetic anhydride (5.3 X 10^4cpm / 300µl of acetone) was added and incubated for 30min at 25°C. The concentration of $NaHCO_3$ was made to 1M by the addition of solid crystals followed by the addition of 25µl of cold acetic anhydride. After 30min incubation at 25°C, the reaction was arrested by the slow addition of 0.25ml glacial acetic acid. The preparation was dialysed extensively against distilled water. There was an incorporation of 1.5 X 10^4 cpm to the total sample. For measuring the radioactivity, aliquots were mixed with 10 volumes of Bray's scintillation fluid and counted. The scintillation fluid consisted of 4g of PPO, 0.2g of POPOP and 60g of naphthalene dissolved in 20ml of ethylene glycol, 100ml of methanol and 880ml of dioxane(35).

Carboxymethylation of neuronal glycoproteins

Carboxymethylation of neuronal glycoproteins was carried out as described by Gurd(112). One hundred microgram of protein was dissolved in 200µl of 0.1M $NaHCO_3$-6M guanidine hydrochloride-20mM β-mercaptoethanol, flushed with nitrogen, sealed with parafilm and incubated for 1h at 37°C. Two hundred microlitre of 200mM iodoacetic acid in 0.1M $NaHCO_3$(pH adjusted to 8.5 with NaOH) was added to the protein solution, flushed with nitrogen, sealed with parafilm and incubated for 2h in the dark at 37°C. After the incubation, 200µl of 6N HCl was added and the proteins were precipitated with 8 volumes of methanol. The precipitated proteins were washed with methanol:water (8:1v/v).

Phosphorous estimation

Phosphorous was estimated according to the method of Bartlett(17).

Reagents:

(A) Ammonium molybdate reagent: Ammonium molybdate (4.4g) was dissolved in 200ml of distilled water. Fourteen millilitres of sulfuric acid was added and the volume made upto 1 litre.

(B) Reducing reagent (ANSA): Thirty grams of sodium bisulfite, 6g of sodium sulfite and 0.5g of 1,2,4-aminonaphthol sulfonic acid were ground in a mortar. The preparation was dissolved in distilled water and the volume made upto 250ml. The solution was allowed to stand for 3h in the dark and filtered.

(C) Standard 0.5mM KH_2PO_4: Five millilitres of 10mM KH_2PO_4 (1.361g made upto 1000ml) stock solution was diluted to 100ml. One hundred microlitre of this standard contained 0.05 micromoles of phosphate. A few drops of chloroform were added to the preparation and stored at 4°C. For the standardisation, phosphate was taken in the range of 10 to 50 nanomoles.

Fifty microlitre of the sample was mixed with 200µl of perchloric acid and refluxed for 2-4h in a sand bath at 240°C until the solution became clear. The process was accelerated by the addition of 25µl of 5% ammonium molybdate(58). To the digested sample 0.6 ml of ammonium molybdate reagent and 0.6ml of 1:12 diluted ANSA were added. The preparation was boiled for 10min at 100°C, cooled to room temperature and the absorbance was measured at 830nm.

Colour development for peroxidase

The substrate for HRP-conjugates was prepared just before use(97). To fifty millilitres of TBS pH 7.4, 10mg o-dianisidine in 1.5ml methanol was added followed by 15 l of 30% H_2O_2(w/v). For colour development, the nitrocellulose strips were incubated with the chromogenic substrate for 30min at 25°C. The intensity of colour developed was visually estimated.

Hemagglutination assay

Hemagglutination assay was carried out using human or rabbit erythrocytes in a total volume of 0.25ml(181). Blood collected in acid-citrate-dextrose anticoagulate was washed three times with

10 volumes of PBS pH 7.4. Five percent cell suspension was made in PBS pH 7.4 containing 0.1% trypsin and incubated for 1h at 37°C. The trypsinised cells were washed three times in PBS pH 7.4. Lectin stock solution in PBS pH 7.4 (1mg/ml) was serially diluted to a total volume of 0.2ml. Fifty microlitre of 5% cell suspension was added and agglutination was checked after 1h.

Preparation of lectins

Concanavalin A was purified from Canavalia gladiata using Sephadex G-50 as the affinity matrix according to the method of Surolia et al(273).Wheat germ agglutinin was purified on a chitin column according to Bloch and Burger(25). The WGA was also prepared by affinity chromatography on GlcNAc-Sepharose 4B(293). Wheat germ was extracted with 10 volumes of PBS pH 7.2. The preparation was subjected to 35% ammonium sulfate saturation and the precipitated proteins were extensively dialysed against PBS pH 7.2 at 4°C. The supernatant obtained by centrifugation at 10,000g X 20min was loaded on GlcNAc-Sepharose 4B. The column was equilibrated with PBS pH 7.2 and eluted with 0.1M acetic acid at 4°C. Ricinus communis agglutinin was purified on cross linked guar gum according to the method of Appukuttan et al(9). Jackfruit seed agglutinin was purified from Artocarpus integrifolia according to the method of Sureshkumar et al(272). Artocarpus hirsuta agglutinin was purified from the wild jack (Artocarpus hirsuta) seeds according to Antony et al(8). Cross linked guar gum was used as the affinity matrix for the

purification of JSA and AHA. Pea lectin was prepared from Pisum sativum using Sephadex G-50 as the affinity matrix according to Van Driessche et al(291).

Pea nut agglutinin was prepared from Arachis hypogea using asialo fetuin-Sepharose 4B as the affinity matrix. The extraction of the lectin was carried out according to Lotan et al(184). One hundred grams of seeds soaked overnight in 250ml of 0.15M NaCl was homogenised and centrifuged to get a supernatant. The pellet was reextracted with another 250ml of 0.15M NaCl and centrifuged. The combined supernatants were filtered through cheese cloth and subjected to 60% ammonium sulfate precipitation. The precipitate was dissolved in minimum volume of distilled water and dialysed against distilled water and finally against 0.15M NaCl. The preparation was loaded on asialo fetuin-Sepharose 4B (1.2 X 8.5cm) equilibrated with 0.15M NaCl. The bound lectin was eluted with 75mM galactose in 0.15M NaCl and dialysed against TBS pH 7.4.

Protein A was extracted from Staphylococcus aureus (Cowan strain)(131). Two grams of bacteria(wet weight), suspended in 20ml of 0.05M Tris-HCl pH 7.5-0.15M NaCl was mixed with 0.5mg lysostaphin and DNase. The preparation was incubated at 37°C for 2h. The suspension was then centrifuged at 4°C. The pellet obtained was washed with 15ml of distilled water and centrifuged. The pH of the pooled supernatants was adjusted to 3.5 with 5N HCl and centrifuged. The supernatant was neutralized to pH 7.0 with

6N NaOH and subjected to 80% ammonium sulfate saturation. The precipitated protein was dialysed against PBS pH 7.4 followed by 0.1M $NaHCO_3$ pH 8.5 at 4°C. The protein A was purified by affinity chromatography on immunoglobulin-Sepharose 4B column.

Preparation of affinity matrices

N-acetyl-D-glucosamine-Sepharose 4B was prepared by coupling the aminosugar to epoxy activated Sepharose(287,271). Sepharose 4B was washed with distilled water followed by 0.6N NaOH. To ten grams of washed and suction-drained gel, 10ml of 1,4-butanediol diglycidyl ether and 10ml of 0.6N NaOH containing 20mg $NaBH_4$ were added. The mixture was rotated mechanically for 10h at 25°C. The epoxy-activated gel was washed with excess of distilled water and 0.1M NaOH. Four hundred milligrams of N-acetyl glucosamine in 10ml of 0.1M NaOH was added to the suction-drained gel and incubated for 15h at 45°C with gentle mixing. The product was washed extensively with distilled water followed by 0.05M Tris / HCl pH 8.0-0.5M NaCl and 0.05M sodium formate buffer pH 4.0-0.5M NaCl. The gel was finally equilibrated in PBS pH 7.2.

Asialofetuin was prepared by chemical desialylation of fetuin by acid hydrolysis. The asialofetuin was coupled to CNBr activated Sepharose 4B according to the method of Axen et al(12). Twenty millilitre of thoroughly washed Sepharose 4B was suspended in 20ml of distilled water and 40ml of 2M sodium carbonate. The temperature of the slurry was maintained at 10°C and 0.6g of cyanogen bromide dissolved in minimum volume of dimethyl

formamide was added to the gel suspension with stirring. After 5min the activated gel was filtered through a sintered glass funnel and washed thoroughly with 0.1M sodium bicarbonate buffer pH 8.5. The gel was transferred to asialofetuin solution (200mg asialofetuin dissolved in 20ml of 0.1M sodium bicarbonate, pH 8.5) and stirred gently for 18h at 4°C. The unreacted active groups were blocked by treatment with ethanolamine (1ml/100ml gel). The gel was washed successively with 0.1M sodium bicarbonate pH 8.5, 0.1M NaCl, distilled water and 20mM Tris / HCl pH 7.4.

Concanavalin A was coupled to cyanogen bromide-activated Sepharose 4B according the method of Kohn and Wilchek(160). Forty grams of packed Sepharose 4B was washed extensively with distilled water in a sintered glass funnel, followed by 400ml of cold acetone: water (3:7) and finally with 400ml of cold acetone: water(6:4). The washed gel washed was transferred to a beaker containing 40ml of acetone: water (6:4), maintained at -10°C using ice and NaCl. Four hundred milligrams of CNBr (10mg/g of the gel) dissolved in 4ml of acetone was added. Six hundred and eight milligrams (0.84ml) of triethylamine (15.2mg/g gel) diluted in 4ml of acetone was added in drops with gentle stirring for 2 min. After the activation, the gel was transferred to a sintered glass funnel, washed extensively with distilled water followed by 500ml of 0.1M $NaHCO_3$ pH 8.3. Four hundred milligrams of Con A (10mg/g gel) dissolved in 40ml of 0.1M $NaHCO_3$ pH 8.3 was

TABLE 2. LICROSORB RP 8 HPLC SOLVENT GRADIENT PROGRAMS FOR THE SEPARATION OF PROTEIN DERIVATIVES.
Sol A:water-0.1% TFA, Sol B:isopropanol -0.1% TFA. Flow rate=0.5ml/min.

GRADIENT I		GRADIENT II		GRADIENT III	
Time (min)	% Sol B	Time (min)	% Sol B	Time (min)	% Sol B
0	0	0	0	0	0
3	0	5	30	5	30
20	30	65	60	50	60
40	60	80	0	70	100
60	100	85	0	80	100
70	100			85	0
75	0				
85	0				

added to the activated Sepharose and gently stirred for 18h at 4°C. The unreacted groups were blocked by the addition of 0.4ml of ethanolamine (1ml/100g gel) and further incubation for 2h at 4°C. The coupled gel was washed with 0.1M $NaHCO_3$ pH 8.3 followed by extensive washing with distilled water and finally equilibrated in TBS pH 7.4 containing 1mM $CaCl_2$, 1mM $MgCl_2$ and 1mM $MnCl_2$.

RP-HPLC of proteins

Derivatized protein samples were chromatographed on reverse phase Lichrosorb RP 8 column of 5µm (4 X 250mm) and guard column of 7µm (4 X 30mm) HPLC system. Three gradients designated I, II, III respectively, were formed between solvent A (water-0.1% TFA) and solvent B (isopropanol-0.1% TFA) as given in Table 2.

CHAPTER - III

RESULTS AND DISCUSSION

PART I -ISOLATION OF NEURONS FROM HUMAN FETAL BRAINS

Morphological studies of the central nervous system revealed an incredibly complex meshwork of neuronal and glial somata, axons, dendrites, glial processes, myelin and capillaries. It is apparent that biochemical properties of this tissue reflect the sum of the individual properties of each separate cell type. Yet we know that neurons and glia have quite different functions and different biochemical properties. The isolation of neurons from human fetal brains would facilitate the physicochemical and biochemical studies of its surface glycoproteins.

There have been several procedures developed for the bulk isolation of brain cells that show promise for a variety of investigations(209,47,270). Ideally one desires a procedure that can efficiently and benignly resolve the brain tissue into its component cells so that their individual properties can be determined. Some of the reported procedures seem to be reasonable approaches towards the isolation of pure neurons, but at present no one method could be described as being totally satisfactory for all purposes.

The problems of isolating pure neurons in bulk are mainly: (a) the selection of a medium which will maintain the integrity of the cells, (b) the disruption of tissue through sieves (with or without prior treatment) to dissociate it into single cells, and (c) selection of differential centrifugation procedures to

separate the various cell types. All media used so far incorporate a high molecular weight material; either Ficoll, polyvinylpyrrolidone or albumin. The mode of action of these polymers was not clear but they appear to lessen cell breakage. The sieving procedures were all similar and involve forcing the tissue through nylon or stainless steel sieves of successively smaller meshes. The centrifugation procedures developed varied considerably, involving step gradients of sucrose or sucrose and Ficoll or continuous gradients of zonal rotors. The g forces used in these procedures have also varied over a wide range.

The storage medium used for storing the brain tissue in the present study was fortified with dimethyl sulfoxide, a cryoprotective substance. Dimethyl sulfoxide has the advantage over other neutral solvents such as glycerol in that it is less viscous and penetrates rapidly. The prevention of ice crystal formation and local high concentration of electrolytes or other components is believed to be important in the recovery of intact cells after freezing and thawing. The human fetal brain tissues stored under the above medium retained cellular structures. Intact neurons could be isolated from brain tissues preserved in the storage medium for one year.

The isolation of neurons from human fetal brain was a modification of the procedures of Poduslo & Norton and Farooq & Norton(225,74).

Solutions:

(1) Tissue storage medium: Medium I containing 8% dextrose,5% fructose and 20%(v/v) dimethyl sulfoxide(86).

(2) Medium I: 10mM phosphate buffer pH 6.0 (PBS)-0.1mM PMSF-15mM NaN_3-1mM benzamidine hydrochloride.

(3) Tissue disruption medium: Medium I containing 0.32M sucrose and 2-4% Ficoll 400.

(4) Sucrose solutions for density gradients: 0.9M, 1.35M, 1.55M, 1.65M and 2.0M sucrose solutions were prepared in medium I and adjusted to pH 6.0 with 2N NaOH.

Developing human fetal specimens were collected from the Department of Obstetrics and Gynaecology, Medical College Hospital, Trivandrum. Utilisation of the tissues for the present study was permitted by Ethics Committee of the Trivandrum Medical College. The second trimester specimens were obtained from those cases after medical termination of pregnancy. The specimens were collected in ice and brought to the laboratory within 4-6h. Full term fetuses were collected from cases where the intrauterine death had occurred within 6h prior to parturition. The gestation ages were determined by measuring the crown to rump length of the fetuses. The brains were dissected out and stored at -20°C in the storage medium.

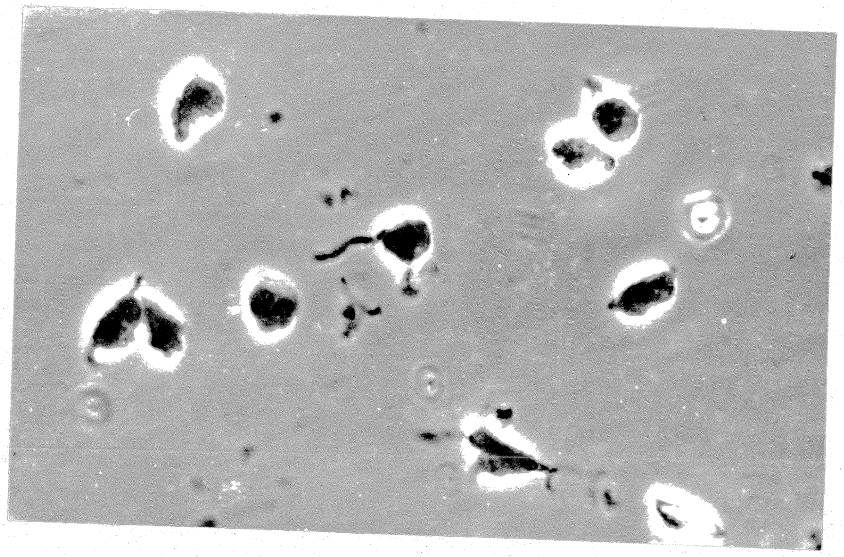
The tissue, preserved in the storage medium was thawed by suspending in PBS at 25°C and centrifuged at 2000g X 10 min, to remove the solution. All further steps were carried out at 0°-4°C.

The tissue was suspended in the tissue disruption medium (1g/2ml) and finely minced with a scalpel. The mechanical disruption was carried out by passing successively through 1mm pasteur pipette followed by 80 and 200 mesh nylon sieves under mild vacuum into Buchner flask. The cell suspension, thus obtained was diluted with the sucrose-Ficoll medium to a protein concentration of 5-10mg/ml. The cell suspension was further dispersed with a Down's homogenizer to obtain a homogenous suspension. Highly viscous cell suspensions sometimes obtained due to extensive cell lysis and release of nuclear materials into the medium was mixed with DNaseII(0.1-0.3% of DNase:protein) and incubated for 14-16h at 4°C(201). A discontinuous sucrose density gradient was carried out in HS-4 Sorvall-RC-5B centrifuge. The density gradient consisted of, from top to bottom, 9ml of 0.9M, 7ml of 1.35M, 7ml of 1.55M, and 5ml of 2.0M sucrose solutions. The cell suspension corresponding to a protein concentration of 50-100mg in 10ml was layered over the gradient and centrifuged at 3300g X 15 min. The centrifugation resulted in the distribution of cell homogenate into 4 layers. The two molar interface comprised of almost pure neuronal perikarya, which was collected and required no further purification. The neurons were also present in the 1.35M and 1.55M layers contaminated with glia, with more number of neurons in the 1.55M than in the 1.35M layer. The above two layers were pooled together(28ml) and layered over 1.65M sucrose (10ml) for centrifugation at 3300g X 45 min. Neuronal perikarya formed a

pellet which was pooled with the previous 2M neuronal fraction. The neurons still entrapped in the 1.65M layer was isolated by a third centrifugation process. The entrapped neurons in 1.65M layer was diluted with medium I and layered over another 1.65M sucrose layer. The neurons formed a pellet when centrifuged at 3300g X 45 min and the process was repeated 2-3 times till all the neurons could be isolated from the 1.65M layer. The neurons were washed with medium I containing 0.15M NaCl and stored in ice in the same medium. The neurons were characterised by light and phase contrast microscopy.

By a major modification of the above mentioned procedure, the isolation of neurons could be made easier. The first step density gradient centrifugation was avoided. The cell suspension obtained by mechanical disruption was mixed with an equal volume of 2M sucrose and layered over 1.65M sucrose. The preparation was centrifuged at 3300g X 45 min to obtain a neuronal pellet. Neurons still entrapped in the 1.65M layer was recovered by subjecting it to another centrifugation process over 1.65M layer. The process was repeated 2-3 times till all the neurons could be recovered from the 1.65M layer. In both methods, the 1.65M layer after the removal of neurons contained pure glia.

Almost pure and morphologically intact neurons and glia could be isolated by the two methods as revealed by light and phase contrast microscopy [Fig.4]. Most of the neurons were without axons and dendrites but could be identified on account of



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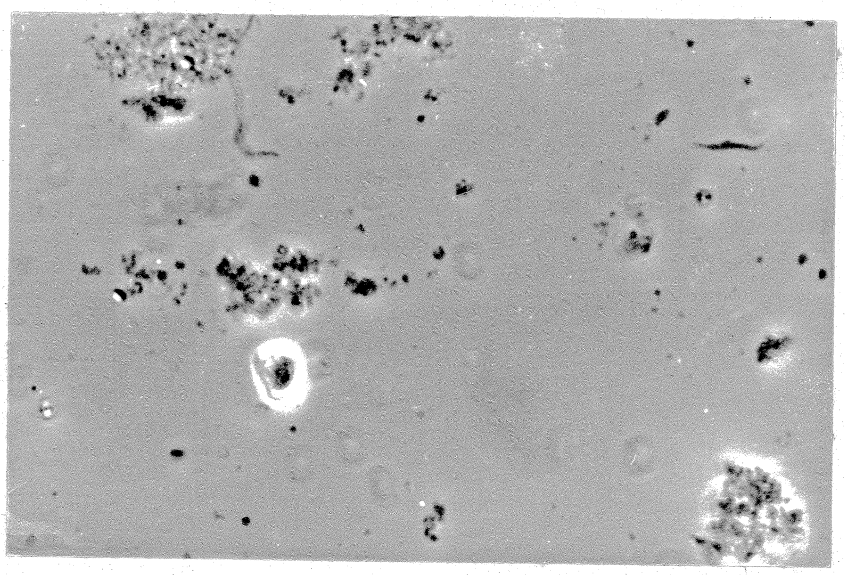


B

Figure 4. Phase contrast micrograph of isolated brain cells (X 300 magnification). A. Neurons, B. Glia.

their large size, large nucleus, single prominent nucleolus and abundant cytoplasm. Aniline dyes tolidine blue and methylene blue stained the ribonucleoproteins present in the nucleolus and the nissle granules of the cytoplasm. The glial layer contained both astrocytes and oligodendroglia.

Based on protein as determined by Bradford's method in the tissue homogenate, neuronal and glial preparations respectively, yields were calculated as 1-15% for neurons and 5-25% for glia. The yield of neurons decreased as the fetal age increased. A similar phenomenon was observed by Sellinger et al with rat cortices(145).

The age of human fetuses were calculated from their crown to rump(CR) length(205). The fetuses collected for the present study were classified into two groups based on their CR lengths. The crown to rump length of the first group ranged from 11-17 cm and the corresponding age was found to be in the range of 14-20 weeks (second trimester). For the second group of full term fetuses, CR length ranged from 30-33cm.

The time consuming and cumbersome density gradient preparation was avoided in the second method, enabling the isolation of neurons faster as compared to the first one. Moreover the exposure of neurons to hypertonic sucrose was minimised in the later procedure. In both methods, no attempt was made to reach density equilibrium. The large size of neurons

enabled them to be sedimented quickly at a comparatively low centrifugal force.

In agreement with Norton and Poduslo's observation, pH 6.0 was found to be the optimum for human fetal brain neurons. In the present method, tissue disruption was carried out by mechanical sieving alone and the conventional trypsinization process was avoided because of the extensive lysis of fetal brain cells.

Isolation of viable neurons was not a prerequisite for the present study. The morphologically intact neurons isolated by the two methods had been used satisfactorily for the present study.

PART II - PREPARATION OF NEURONAL PLASMA MEMBRANE ENRICHED FRACTION AND THE ISOLATION OF CONCANAVALIN A-BINDING CELL SURFACE GLYCOPROTEINS

The cell surface glycoproteins play an important role in mammalian development. Physicochemical studies on the neuronal surface glycoproteins would enable in understanding the nervous system development at the molecular level. This would require the isolation of neuronal surface glycoproteins of the developing brains. The present study was confined to those glycoproteins binding to concanavalin A only. Earlier workers have used the conventional methods, ion exchange chromatography, selective partitioning and gel filtration for the isolation of brain glycoproteins(228,288). The development of a combination of iterative immunisation, monoclonal antibody and affinity methods provided a powerful armamentarium for the isolation of neural glycoproteins from retina and brain of various species(32,132,99). Ninety percent of the brain glycoproteins were integral components of cell surface (189). With the exception of a few extrinsic proteins, the bulk of membrane proteins embedded in the lipid matrix are insoluble in aqueous medium as they are hydrophobic. Extraction of membrane lipids with organic solvents results in the irreversible denaturation of membrane proteins. Myelin proteolipid- apoprotein and basic

proteins however are the exceptions to this rule(279,73). A prerequisite for the isolation of membrane glycoproteins is the selection of a method for solubilization of these compounds. Different detergents have been employed for the solubilization of membrane compounds(127). Once solubilized the integral membrane glycoproteins can be purified by conventional methods followed by affinity chromatography on immobilized lectins or antibodies. The detergent solubilized integral membrane glycoproteins from brain cells have been purified by lectin affinity chromatography in presence of detergents(318,319,248). The lectins are made of subunits which, in many cases are held together by noncovalent forces. The detergents used for the solubilization of membrane may dissociate the native lectin molecules and or change their active conformation(182,180). In addition to denaturing the lectin, detergents can interfere with the lectin affinity chromatography by eluting the lectin from the matrix together with the material of interest(180,315,246). The great heterogeneity and complexity of brain in terms of its cell types and histological organization has hindered attempts to isolate and characterize specific intact glycoproteins, especially in view of their large numbers and the small amounts. Approximately 85-90% of the carbohydrate in brain glycoproteins is linked via N-acetylglucosamine to the amide nitrogen of asparagine residues in the protein moiety(189). Several different types of such N-glycosidically linked carbohydrate units are known to occur in

brain and can be partially purified by affinity chromatography on Con A-Sepharose under standardised conditions(81). The so called "neutral"or "high mannose" glycans are strongly bound to Con A. However most of the N-glycosidically linked carbohydrate units in nervous tissue glycoproteins are of the "complex" type(189). For the present study, Con A-Sepharose chromatography was employed for the isolation of neuronal surface glycoproteins.

Identification of Con A binding glycoproteins on the neuronal surface was a prerequisite before attempting to isolate them. A variety of reagents have been employed for the identification of cell surface glycoconjugates which include radioactive and fluorescent compounds as well as lectin derivatives. The following methods were carried out to identify the neuronal cell surface glycoproteins.

Tritium-labelling of intact neurons was attempted after periodate oxidation (93,290). Neurons(2mg protein) were washed twice in 10mM phosphate buffered saline pH 6.0 (PBS) and suspended in 1ml of 20mM $NaIO_4$ in the same buffer. The preparation was incubated in the dark for 20min at 4°C. After the incubation the neurons were pelleted at 15000g X 15 sec, washed 3 times in 10mM PBS pH 8.0 and suspended in 1ml of the same buffer. Ten microlitres of NaB^3H_4(10^5cpm) in 10mM NaOH was added and incubated at 25°C for 30 min. The neurons were then washed 3 times with 10mM PBS pH 6.0, followed by methanol and subjected to delipidation [chloroform: methanol (2:1,v/v)]. The dried

delipidated preparation was dissolved in 2ml of 2% SDS by boiling for one minute. Aliquots were counted for radioactivity. There was no radioactivity incorporation in the neuronal proteins. However the purified neuronal glycoproteins could be labelled with periodate-NaB^3H_4 as described later.

Tritium-labelling of intact neurons was attempted after treatment with galactose oxidase(92). The galactose oxidase selectively oxidises substrates on the cell surface. Treatment with neuraminidase prior to galactose oxidase has been used to remove the terminal sialic acid and achieve maximum labelling of the galactose / N-acetyl galactosamine residues. Neurons (15mg protein) were washed twice in 10mM phosphate buffered saline pH 6.0 (PBS) and suspended in 3ml of 40mM $NaBH_4$ in the same buffer. The preparation was incubated at 25°C for 30 min. Washed 5 times in PBS and divided into 3 aliquots; each sample was suspended in a final volume of 1ml PBS. To one set of cells, 0.6ml(60U) of galactose oxidase in PBS and 1mg (200mU) of neuraminidase were added; to another set galactose oxidase alone; the third set remained without enzymes. The cells were incubated at 37°C for 1h, washed 3 times with and suspended in 1ml of PBS. Five microlitres of NaB^3H_4(5 X 10^4cpm) in 10mM NaOH was added and the reduction was continued for 30 min at 25°C. The preparations were washed 3 times in PBS and aliquots were counted for radioactivity. There was no difference in the radioactivity incorporation into each of the three samples and the low

radioactivity incorporated could be due to nonspecific labelling. From each set of neurons a plasma membrane enriched fraction was prepared and subjected to Con A- Sepharose chromatography (0.9 X 7.5cm). The Con A eluates from each of the 3 samples did not have radioactivity.

In another experiment the binding of FITC-Con A to intact neurons was investigated(280). Neurons (1mg protein) were suspended in 0.2ml of buffer I (20mM Tris/Maleate pH 6.0-150mM NaCl-4% BSA). As a control neurons corresponding to the same amount of protein was suspended in 0.2ml of buffer II (buffer I containing 2M α-MG). To both test and control 0.6ml of FITC-Con A (10mg/ml in TBS pH 7.4-1mM $MgCl_2$-1mM $CaCl_2$-1mM $MnCl_2$) was added and incubated for 30 min at 4°C. The neurons were then pelleted at 5000g X 5 min and washed twice with 20mM Tris/Maleate pH 6.0-150mM NaCl (TMS) containing 1% BSA followed by two more washes in TMS alone. The BSA had a protective function on the stability of neurons. The preparations when observed under a fluorescence microscope, revealed the presence of intensely fluorescent neurons whereas the neurons in the control did not exhibit fluorescence [Fig.5].

The preparation of neuronal plasma membrane fraction would facilitate the isolation of its surface glycoproteins. Many workers have developed methods for the preparation of plasma membrane from neurons(284,305,41). Some of the methods involved $ZnCl_2$ or latex bead treatment prior to harvesting,

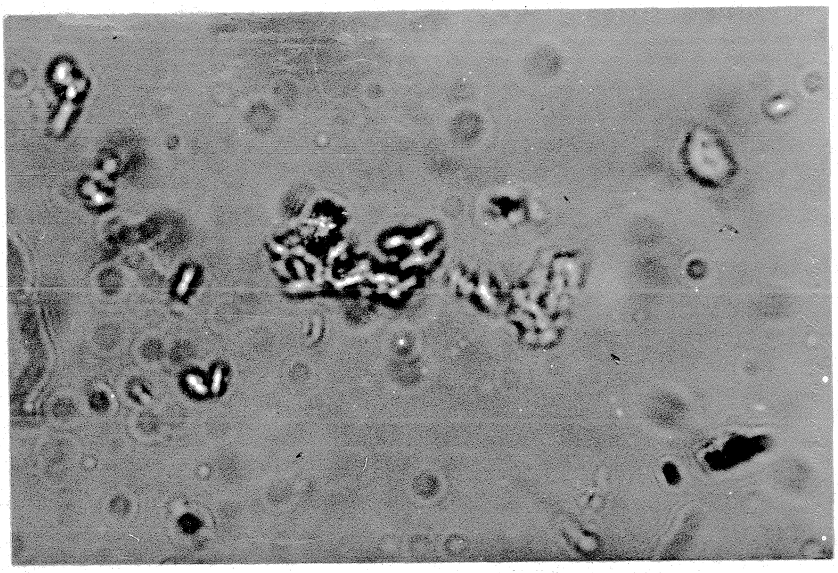


Figure 5. Fluorescence micrograph of neurons treated with FITC-Con A (X 300 magnification). Details of the experiment are described in the text.

homogenization, centrifugation and density gradient centrifugation. The preparation of plasma membrane from human fetal neurons was attempted by the following method(72).

A ten percent (v/v) suspension of neurons was made in 15 ml of the hypotonic medium (10mM Tris/HCl pH 7.4-0.1% NaN3-1mM PMSF-5mM β-mercaptoethanol) and homogenized in Potter Elvehjm homogenizer. The homogenate was centrifuged at 1000g X 10 min to get a pellet. The supernatant was further centrifuged at 1500g X 10 min to get a second pellet. The two pellets were together suspended in the hypotonic medium. Deoxyribonuclease II was added to 3%(w/w) of the total protein concentration. The crude plasma membrane suspension was layered over a 25-45% continuous sucrose density gradient and centrifuged at 90,000g X 180 min in SW.25-1 Spinco Beckman ultracentrifuge. After the centrifugation a plasma membrane band was visible on top of the gradient corresponding to an equilibrium sucrose density of 1.09. Truding et al has reported an equilibrium sucrose density of 1.14 for the plasma membranes isolated from cultured neuroblastoma cells(284). The plasma membrane isolated from the bovine system had an equilibrium density of 1.127. The yield of plasma membrane fraction isolated from human fetal neurons was in the range of 0.1-0.2% based on protein estimations. However the purity of the preparation could not be evaluated due to the absence of marker enzymes. While the activities of 5'nucleotidase(10), glutamine synthetase(198) and carbonic anhydrase(91) were found to be nil,

no recordable activity could be detected for acetyl cholinesterase(174). In the bovine system the various stages of plasma membrane isolation could be monitored by following the enrichment of 5'nucleotidase.

Attempting to prepare pure plasma membrane fractions in the absence of detectable markers could result in impure preparations and also lower percentages of yield. In order to minimise the possible loss of the plasma membrane glycoproteins of interest, the concept of pure plasma membrane preparation by the conventional density gradients was abandoned. Instead a plasma membrane enriched fraction was prepared as given below.

Preparation of plasma membrane enriched fraction

The plasma membrane fraction was prepared by lysing the cells with an aqueous solution of 10mM EDTA, 0.2% β-mercaptoethanol in Potter Elvehjm homogenizer. The neurons were homogenized by 10 strokes in the homogenizer followed by gentle stirring at 4°C for 1h before subjecting to 10 more strokes. The 8000g X 10 min pellet was washed 4 times with distilled water to obtain a plasma membrane enriched fraction.

Affinity chromatography on Con A-Sepharose 4B

The isolation of neuronal cell surface glycoproteins required the extraction of neuronal proteins in a suitable form compatible with lectin affinity chromatography. Initial attempts for the standardisation of the isolation procedure was carried out with intact neurons or delipidated cells. For the

solubilization of neuronal proteins only two detergents, Triton X-100 and SDS were employed. Each detergent presents some advantages over the other. The positive and negative aspects of these detergents should be considered relative to three parameters: nature of solubilization process, biological activity of the solubilized molecules and possibility of purification of individual solubilized molecules.

More than 80% of the total neuronal proteins could be solubilized with SDS whereas with Triton X-100 the solubility was comparatively low and required mild sonication. Higher solubilization of the neuronal proteins with Triton X-100 could be achieved at very low protein concentrations and high detergent / protein ratio. In addition only SDS was effective in solubilizing delipidated neuronal proteins. The term `solubilization' does not have the same meaning when used for membranes directly solubilized in Triton X-100 or SDS, when compared to the solubilization of delipidated membrane protein residues in SDS. However when compared to SDS, Triton X-100 has very low inhibitory effect on the binding efficiency of Con A. Lotan and coworkers have reported 2% for Triton X-100 and 0.2% for SDS as the highest concentrations which do not affect Con A mediated haemagglutination(182). The effects of detergents on soluble lectins may not possibly be directly extrapolated to the lectins immobilized on insoluble matrices. Covalent attachment to

the polymeric matrix stabilizes lectin molecules and decreases their susceptibility to the detergent inactivation(315).

Con A-Sepharose chromatography in presence of SDS

The extraction of neuronal proteins was carried out in presence of SDS according to the method of Gombos(103). The neurons were delipidated by two cycles of extraction with methanol-chloroform, (1:1,v/v) and (1:2,v/v). The lipid free pellet was left overnight in saturated EDTA containing 10%(v/v) β-mercaptoethanol. Divalent cations were eliminated by the EDTA which helped in the release of proteins from aggregates formed by interactions between cations and proteins. After centrifugation at 100,000g X 60 min, the pellet obtained was dissolved at room temperature in a minimum possible volume (1ml / 5mg proteins) of 20mM Tris / HCl pH 7.0 - 4% SDS - 2mM β-mercaptoethanol - 0.1 % NaN_3 - 0.1mM PMSF. The high SDS concentration was used for obtaining solubilization in a small volume. The pH of the solution was brought to 8.0 with 2 N NaOH and a 10 fold excess of iodoacetamide was added. Alkylation was allowed to take place in the dark for 12h, at 25°C, under nitrogen. The solution was dialysed for 48h against 49 volumes of 20mM Tris/HCl pH 7.0. The solution was centrifuged at 100,000g X 60 min. The dialysis steps eliminated excess iodoacetamide, and were calculated to decrease the SDS concentration to 0.08% while maintaining the protein in solution. The percentage of extraction for the neuronal proteins was 75-80% as estimated by Lowry's method. The protein solution

contained 3-4.5% bound carbohydrate as measured by phenol sulfuric acid method. Sodium dodecyl sulfate polyacrylamide gel electrophoresis in 10% gels of the solubilized proteins, revealed protein bands with molecular weight ranging from 12kDa to 100kDa [Fig.6].

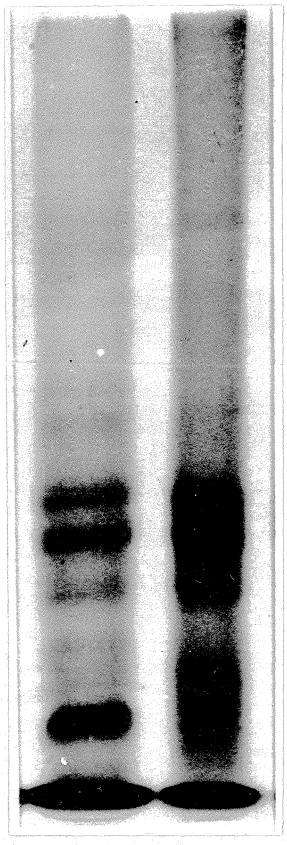
The Con A-Sepharose chromatography was carried out in presence of SDS according to the method of Zanetta et al(315). Freshly coupled Con A-Sepharose contains large amounts of free unbound Con A which must be eliminated before the chromatography. This was achieved by repeated washes at alkaline and acid pH. Con A-Sepharose filtered in a sintered glass funnel was washed with the following solutions.

(a) 100mM $NaHCO_3$ pH 8.3.

(b) 100mM sodium acetate-acetic acid pH 4.0, 500mM NaCl.

(c) 20mM Tris-HCl pH 7.4 (TBS) containing 0.08% SDS.

Between the successive solutions the gel was washed with distilled water. After the removal of noncovalently associated Con A, the Con A-Sepharose was regenerated by washing with TBS containing 1mM $CaCl_2$, 1mM $MgCl_2$,and 1mM $MnCl_2$. The gel was washed with TBS, resuspended and stored at 4°C in the same buffer. The SDS solubilized neuronal proteins were chromatographed on Con A-Sepharose 4B column at 25°C, equilibrated in buffer A (20mM Tris/HCl pH7.0-100mM NaCl-0.04% SDS-0.2mM PMSF-15mM NaN_3). The neuronal proteins (6mg) were loaded on the Con A-Sepharose column (0.9 X 6.0 cm) after the addition of NaCl to a concentration of



1 2

Figure 6. SDS-PAGE of total delipidated neuronal and glial proteins on 7.5% acrylamide gel stained with Coomassie blue. 1. Neuronal proteins, 2. Glial proteins.

100mM. The column was washed with 10 bed volumes of buffer A and eluted with 500mM α-MG in buffer A. The α-MG eluate on SDS-PAGE showed major bands corresponding to Con A itself. The Con A leaching phenomenon was further illustrated by the fact that protein content of the break through, washings and eluate put together was more than that of the loading protein. The Con A leaching was found to be more during the elution step when compared to the loading and washing processes as evident from the intensity of Con A bands on SDS-PAGE. In fact towards the end of washing, there was no Con A leaching as indicated by the absence of protein, checked by absorbance at 280 nm.

To minimise the leaching of Con A, affinity chromatography was carried out in presence of buffer B (buffer A containing 0.1% Triton X-100 instead of SDS) at 25°C. The SDS solubilized protein was dialysed against 0.1% Triton X-100 before loading. The Con A-Sepharose was washed with buffer B and eluted with 500mM α-MG in buffer B. The eluate was concentrated by ultrafiltration on Amicon YM10 membrane. The α-MG was removed either by gel filtration on Biogel P6DG or by dialysis against 0.08 % SDS and further concentrated by rotary evaporation. The Con A eluate on SDS-PAGE revealed Con A as the major component.

Con A leaching has been reported during the Con A-Sepharose chromatography of brain membranes solubilized in SDS(315). The size and capacity of the chromatography columns are critical in minimising this Con A leaching. Lotan and coworkers have observed

the release of Con A subunits from immobilized columns after the exposure to 0.05% SDS for 1h at room temperature(182). They have reported a higher release of Con A subunits after 16h incubation in 0.05% SDS. Analogous experiments carried out with 0.1% SDS for 1h, released twice as much Con A subunits as did the 16h incubation with 0.05% SDS. Prolonged incubation (16h) in 0.1% SDS released more lectin subunits and caused a dramatic reduction in its binding efficiency. The SDS released noncovalently bound Con A subunits rather than coupled or uncoupled lectin subunits. Lectin leaching has also been reported during the purification of glycoproteins from rat muscle cells in presence of SDS(246). According to Zanetta and Gombos, a certain amount of degradation products are formed during the chromatography which also contributes to the Con A leaching(315). In the present method this was minimised by reducing the column size. It is obvious that prewashing the Con A Sepharose at alkaline and acid pH was not sufficient in removing the noncovalently bound lectin subunits.

Extraction with Triton X-100.

Intact neurons were suspended in 20mM Tris/HCl pH 8.0-1% Triton X-100-1mM PMSF-4mM β-mercaptoethanol at a protein concentration 5mg/ml. The suspension was subjected to sonication in MSE Soniprep 150 at 20 microns amplitude for 4 X 1 min. The 15,600g X 5 min supernatant contained 75-80% of the total cell protein. Delipidated neurons required too drastic conditions of

sonication to enable the extraction of proteins. The Triton extracted neuronal proteins were subjected to Con A-Sepharose chromatography(1.2 X 8.0cm) after adjusting the pH to 7.4 with dilute acetic acid. The column was equilibrated and washed with 20mM Tris/HCl pH 7.4-0.2% Triton X-100. The 500mM α-MG eluate on SDS-PAGE revealed Con A as the major component.

Extraction without detergents.

To overcome the problem of Con A leaching, lectin affinity chromatography was carried out in the absence of detergents. Chloroform:methanol(2:1) delipidated neuronal proteins were suspended in 20mM Tris/HCl pH 8.0 at a protein concentration of 1mg/ml. The suspension was subjected to sonication at 15 microns amplitude for 2min. The carbohydrate percentage in the delipidated neuronal proteins was found to be in the range of 6±1. The turbid homogenate was passed through Con A-Sepharose(1.2 X 8.0cm) at 4°C after adjusting the pH to 7.4 with dilute acetic acid. The column was washed with 20mM Tris/HCl pH 7.4-150mM NaCl (TBS pH 7.4) and eluted with 500mM α-MG in the same buffer. The Con A breakthrough was chromatographed on RCA-Sepharose(1.2 X 8.0cm) equilibrated with TBS pH 7.4. The RCA binding proteins were eluted with 200mM lactose in TBS pH 7.4 at 25°C. Haemagglutination assay with trypsinized rabbit and human RBC, of respective lectin bound fractions were positive for RCA-Sepharose and Con A-Sepharose eluates (181). This correlated with the major RCA and Con A bands on SDS-PAGE of the respective eluates. The

haemagglutination titre of the Con A eluate was much lower than that of Con A. This could be due to the inhibition of Con A by the Con A binding glycoproteins or due to the dissociation of the lectin into its subunits during the process of leaching. This further illustrated the fact that Con A leaching was due to the dissociation of its noncovalently bound subunits. The Con A-Sepharose chromatography revealed a poor yield of Con A-binding glycoproteins, which could be due to the drastic sonication step where about 25% of the covalently bound carbohydrates in the neuronal proteins were lost. This was evident from the following experiment. Delipidated neuronal proteins were suspended in and dialysed against 20mM Tris/HCl pH 8.0. The protein suspension was subjected to sonication at 15 microns amplitude for 5 X 30sec. A fraction of the sonicate was kept aside for carbohydrate and protein estimations and the remaining sample was dialysed against 20mM Tris/HCl pH 7.0 overnight. The protein and carbohydrate estimations were carried out before and after dialysis. The results are given in Table 3.

There was a 50% decrease in the carbohydrate content of the Con A-Sepharose breakthrough and washings compared to that of the initial protein solution. Reducing the time or intensity of sonication resulted in non-homogenous protein solutions which caused column blocking.

The effect of ionic strength on the extraction of neuronal proteins was investigated. Delipidated neuronal proteins were

TABLE 3. EFFECT OF SONICATION ON THE CARBOHYDRATE CONTENT OF NEUROAL PROTEINS.

	Before dialysis	After dialysis
Protein (mg/ml)	1.0	1.0
Carbohydrate (µg/ml)	85	63

extracted by sonication with Tris/HCl pH 8.0 at 20mM and 100mM concentrations of the buffer. The preparations were sonicated at 15 microns amplitude for 5 X 30sec and centrifuged at 15000g X 5 min to obtain soluble supernatants. To asses the percentage of extraction, the protein content of the supernatants were estimated by Lowry's method. With the 100mM buffer, only 50% of the total proteins could be recovered in the supernatant as compared to the 90% recovery of proteins with the 20mM buffer. In general the solubility of hydrophobic proteins decreased with an increase in the ionic strength of the solubilization buffer. The above observations illustrated the hydrophobic nature of neuronal proteins.

The problem of extracting neuronal proteins in a suitable form compatible with lectin chromatography without drastic sonication has been circumvented with a western blot buffer used for hydrophobic proteins(260). A neuronal plasma membrane enriched fraction was homogenized in the modified western blot buffer 25mM Tris-700mM glycine pH 7.8-1mM PMSF-NaN_3 at a concentration of 0.2-0.5mg/ml. The homogenate was subjected to sonication at 1-2 microns amplitude for 4 X 15sec. The preparation was centrifuged at 150g X 5 min to remove large particles. The pH was adjusted to 7.4 with dilute acetic acid and 20ml of the supernatant was mixed with an equal volume of Con A-Sepharose 4B. After incubating for 2h at 4°C with occasional stirring, it was packed into a column(1.9 X 9.0cm) and washed at

25°C with one bed volume of 25mM Tris-700mM glycine pH 7.4 followed by 20mM Tris/HCl pH 7.4-150mM NaCl (TBS pH 7.4). The bound glycoproteins were eluted at 25°C with 500mM α-MG in TBS pH 7.4. The unbound fraction was repeatedly rechromatographed on washed and reequilibrated Con A-Sepharose, till the eluate contained no protein. The pooled Con A-binding fractions were concentrated by ultrafiltration and passed through Biogel P6DG to remove α-MG. The preparation was further concentrated by flash evaporation and dissolved in 2% SDS. The Con A eluate contained 180±60µg bound carbohydrate / mg protein. The SDS-PAGE of the preparation revealed the presence of Con A subunit as its major component, in addition to the faint high molecular weight bands when stained with Coomassie blue. These high molecular weight bands stained for carbohydrate with Thymol-H_2SO_4 (94). They were also labelled by FITC-Con A(90). The presence of Con A as the major contaminant was further confirmed by N-terminal analysis of the Con A eluate. Since the Con A leaching was observed even in the absence of detergents, the phenomenon could be due to an inherent property of the neuronal proteins. The above mentioned neuronal protein extract was subjected to WGA-Sepharose chromatography (0.9 X 7.5cm) equilibrated with TBS pH 7.4. The WGA-binding proteins were eluted with 100mM N-acetylglucosamine in TBS pH 7.4 at 25°C. The WGA eluate on SDS-PAGE revealed the presence of glycoprotein bands, which correlated with the Con A

binding high molecular weight bands. There was no protein band corresponding to WGA.

Pretreatment of Con A-Sepharose 4B with 8M urea

The problem of Con A leaching was overcome by pretreating the Con A-Sepharose 4B with 8M urea.

Reagents for urea pretreatment:

(1) 0.1M sodium acetate/acetic acid pH 4.0-1M NaCl-8M urea.

(2) 8M urea,1M NaCl.

(3) 0.1M Tris/HCl pH 8.5-1M NaCl-8M urea.

About 70ml of Con A-Sepharose was mixed with an equal volume of reagent 1 and stirred gently at 25°C for 30min. Filtered through a sintered glass funnel and the process was repeated 4 times. The gel thus obtained was washed with 5 bed volumes of reagent 2. Con A-Sepharose was further suspended in an equal volume of reagent 3 and stirred gently at 25°C for 30min. Filtered through sintered glass funnel and the process was repeated 4 times. After the urea treatment, the Con A-Sepharose was washed extensively with distilled water, equilibrated in TBS pH 7.4-1mM $CaCl_2$-1mM $MgCl_2$-1mM $MnCl_2$ and stored at 4°C in the same buffer.

There was a significant decrease in Con A leaching when the chromatography was carried out using the urea treated Con A-Sepharose as revealed by the decreased intensity of Con A bands on SDS-PAGE. However the Con A leaching could not be totally eliminated. The urea treatment resulted in the improved yield of neuronal glycoproteins.

The yield of Con A-binding glycoproteins was almost doubled when the extraction of neuronal proteins was carried out in presence of the western blot buffer containing 1% Triton X-100. However the carbohydrate content of the Con A eluate remained in the same range of 180+60µg/mg protein. The presence of Triton X-100 has enhanced Con A leaching as revealed by a faint Con A band on SDS-PAGE. When the chromatography was attempted in presence of 20% ethylene glycol instead of Triton X-100 the Con A leaching further decreased. However the chromatographic process was time consuming because of the very slow flow rate due to the presence of ethylene glycol.

The yield of neuronal Con A binding glycoproteins was compared under various sets of chromatographic conditions. Neuronal plasma membrane enriched preparation corresponding to a protein concentration of 14mg was extracted with the Triton-western blot buffer and divided into 4 equal aliquots. After adjusting the pH to 7.4 with dilute acetic acid, each aliquot was mixed with the urea treated Con A-Sepharose(35ml) and incubated at 4°C for 2h with occasional mixing. The samples were packed into columns(2.6 X 7.5cm). They were washed and eluted as shown in Table 4.

The Con A eluates were diluted with distilled water and concentrated by ultrafiltration. The proteins were precipitated with 80% methanol, after adding SDS to a concentration of 10%. The precipitated proteins were suspended in 0.5ml of 2% SDS and

TABLE 4. YIELD OF CONA-BINDING GLYCOPROTEINS UNDER VARIOUS CHROMATOGRAPHIC CONDITIONS.

Sample No.	1	2	3	4
Protein (µg).	150	210	100	80

Sample 1: Washed with 10 bed volumes of TBS pH 7.4-0.1% Triton X-100 at 25°C and eluted with 2 bed volumes of 500mM α-MG in the same buffer at 25°C.

Sample 2: Washed with 10 bed volumes of TBS pH 7.4-0.1% Triton X-100 at 4°C and eluted with 2 bed volumes of 500mM α-MG in the same buffer at 4°C.

Sample 3: Washed with 10 bed volumes of TBS pH 7.4-0.05% Triton X-100 at 4°C and eluted with 2 bed volumes of 500 mM α-MG in the same buffer at 4°C.

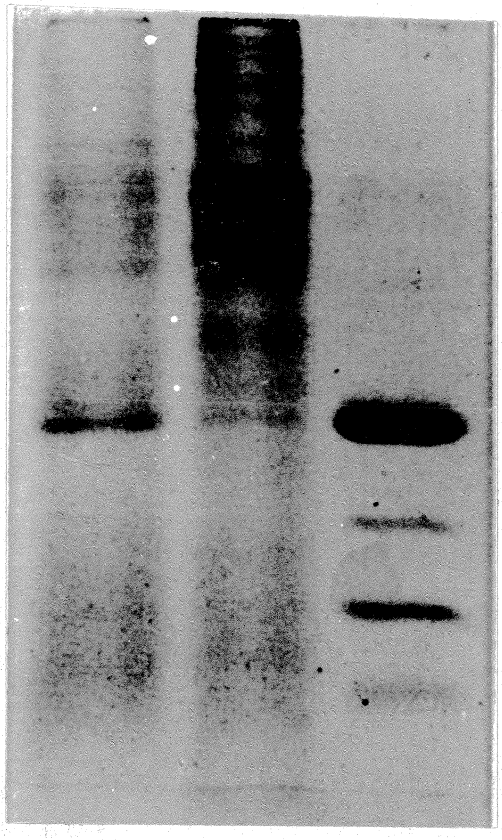
Sample 4: Washed with 10 bed volumes of TBS pH 7.4 at 4°C and eluted with 2 bed volumes of 500 mM α-MG in the same buffer at 4°C.

the protein estimations were carried out according to the modified Lowry's method.

It is evident from the above observations that the yield of neuronal glycoproteins was maximum when the Con A chromatography was performed in presence of 0.1% Triton X-100 containing buffer at 4°C. The Con A leaching was more when the washing and elution were carried out at 25°C instead of 4°C as evident from the increased intensity of Con A bands on SDS-PAGE [Fig.7]. The yield of neuronal glycoproteins was more at 4°C as compared to the chromatography at 25°C. This could be due to the extensive leaching of Con A at 25°C resulting in the loss of Con A bound glycoproteins. In successive chromatographies of the Con A breakthrough, the Con A leaching was found to be increasing as revealed by the intensity of Con A bands on SDS-PAGE. The procedure used in the present study for the isolation of neuronal surface glycoproteins is described below.

Isolation of Con A binding surface glycoproteins

The plasma membrane enriched preparation was suspended in 25mM Tris-700mM glycine pH 7.8-10mM β-mercaptoethanol-1mM PMSF-NaN_3 at a protein concentration of 0.2-0.5mg/ml. The suspension was homogenised by 10 strokes in the Potter Elvehjm homogenizer followed by a mild sonication of 1-2 microns amplitude for 4 X 15sec. After the addition of Triton X-100 (1%v/v), the homogenate was stirred at 4°C for 1h and centrifuged at 150g X 5min. After adjusting the pH to 7.4 with dilute acetic acid, 60ml of the



1 2 3

Figure 7. SDS-PAGE of Con A eluate obtained at different temperatures on 10% acrylamide gel stained with Coomassie blue. 1. Con A eluate obtained at 25°C, 2. Con A eluate obtained at 4°C, 3. Con A.

supernatant was mixed with 40ml of urea treated Con A-Sepharose 4B. Incubated at 4°C for 4h with occasional mixing and packed into a column (2.6 X 7.5cm). The column was washed with 10 bed volumes of TBS pH 7.4-0.1% Triton X-100 at 4°C and the bound glycoproteins were eluted with 2 bed volumes of 500mM α-MG in the same buffer at 4°C. The Con A-Sepharose column was washed with 1M NaCl between successive chromatographies. The Con A eluates from different chromatographies were pooled together, concentrated 10 times by ultrafiltration in Amicon YM-10 membrane. The concentrated sample was diluted 10 times with TBS pH 7.4 and concentrated again by ultrafiltration. The process was repeated 3 times to ensure the removal of excess Triton X-100. The Con A eluate was further concentrated to 1ml by lyophilization or by flash evaporation and precipitated with 8 volumes of methanol.

The neuronal glycoproteins eluted from Con A-Sepharose column were contaminated with noncovalently associated glycolipids. The amounts of contaminant Con A and glycolipids in the Con A eluate was more when the washing and elution were carried out at 25°C instead of 4°C. The yield of neuronal Con A binding glycoproteins was in the range of 1-4%.

The nonionic detergents are considered to be the most suitable for lectin affinity chromatography, because their effects on lectins are negligible. Triton X-100 has been found to bind hydrophobic membrane glycoproteins and to increase their tendency to aggregate (182). Based on the hydrophobic nature of

protein(s) chromatographed, the inhibitory effect of Triton X-100 on lectin binding can vary. Kahane et al have reported a 30-40% inhibition of glycophorin binding to WGA-Sepharose at 0.06% Triton X-100(182). The glycophorin is an amphiphilic glycoprotein containing a hydrophobic segment which can bind large amounts of detergents. This emphasises the fact that during the lectin chromatography, detergents interacted with lectin subunits which was governed to some extent by the hydrophobic nature of the protein(s) chromatographed. The higher local concentration of detergents can denature and / dissociate the lectin subunits during chromatography. The above mentioned observations can be extended to explain the increased Con A leaching phenomenon observed during the Con A chromatography of neuronal plasma membrane proteins. The neuronal plasma membrane proteins being hydrophobic were not sufficiently soluble in ordinary aqueous buffers and required Triton X-100 for solubilization. The enhanced interaction of Triton X-100 with Con A subunits during the chromatography due to the presence of hydrophobic neuronal glycoproteins might have resulted in the Con A leaching.

Removal of contaminant Con A and glycolipids from the Con A binding glycoproteins

Any possible contamination of Con A and glycolipids as and when occurred was eliminated by an electrophoretic method which was a modification of the electrophoretic elution of proteins from polyacrylamide gel(266). Electrophoretic tubes(0.5 X 9.0cm)

were filled with 12% laemmli's separating gel up to 3/4 of its length(170). About 100µl of the neuronal Con A eluate boiled in 2% SDS was loaded. In order to judge the movement of the contaminant Con A, a control tube was loaded with 100µl of 2% SDS treated Con A-FITC [Fig.8A]. A current of 4-5mA/tube was applied in the forward direction so that the proteins would migrate from the top of the gel to its bottom. The movement of Con- FITC was monitored from its fluorescence by making the room dark and focusing ultraviolet light on the gel. The electrophoresis was carried out till the major Con A-FITC band had moved out of the gel. After the electrophoresis the chamber buffers as well as the buffer inside the electrophoretic tubes were discarded. For the second electrophoresis 100µl of recovery solution [25mM Tris/75mM glycine pH 8.8-1mM dithioerythritol-40%(v/v) glycerol] was first loaded. On top of the recovery solution, 2M NaCl was carefully layered to fill the tube [Fig.8B]. The current was reversed, so that the proteins would migrate in the opposite direction. A current of 4-5mA / tube was applied for double the duration of time taken by Con A-FITC to migrate in the downward direction. The Con A free neuronal glycoproteins formed a precipitate at the interface between the recovery solution and 2M NaCl. The protein ppt. along with the recovery solution was collected and precipitated by 80% methanol. The glycoproteins obtained by this method was free of Con A and glycolipids. As an alternative the Con A free neuronal glycoproteins could be extracted by

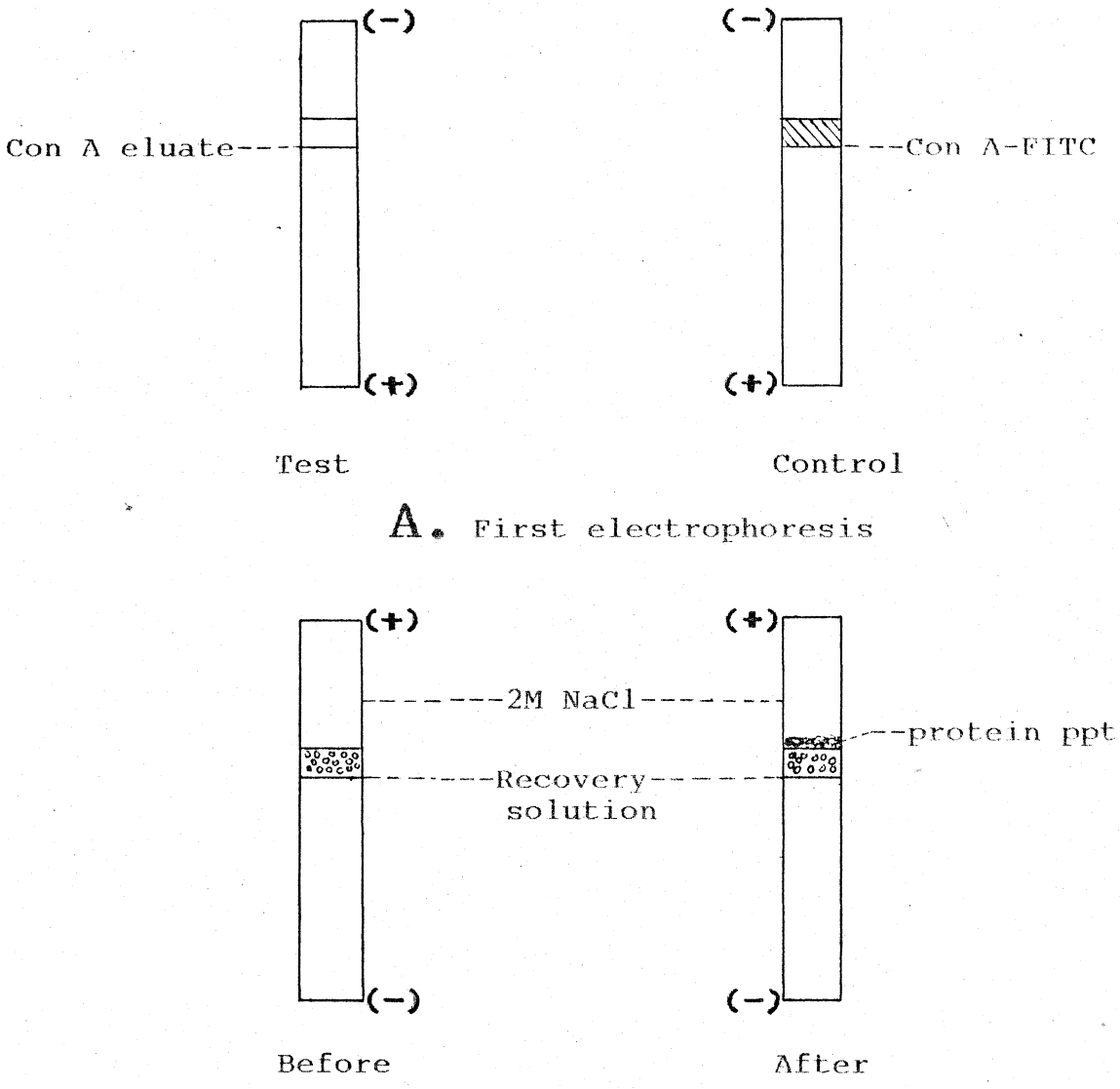


Figure 8. Electrophoretic removal of contaminant Con A from Con A eluate.

homogenizing the gel after the first electrophoresis with laemmli's chamber buffer containing 2% SDS.

In addition to the above mentioned electrophoretic method contaminant glycolipids could be removed by subjecting the neuronal Con A eluate to delipidation with water:methanol: chloroform[3:8:4] (274). The contaminant Con A was heat inactivated for the purpose of differential lectin binding studies.

PART III - CHARACTERIZATION OF NEURONAL CONCANAVALIN A-BINDING SURFACE GLYCOPROTEINS

The role of surface glycoproteins in cell sociology, oncology and brain development is well documented. The brain tissue exhibits diversity in the types of cells with neurons and glia forming its major components. The physicochemical studies on the neuronal surface glycoproteins would improve our knowledge about nervous system function. Advances in the areas of biochemistry and immunology have made possible new approaches that have led to a better understanding of the molecular and functional properties of membrane glycoproteins of the nervous system. The protein behaviour is hard to predict and varies considerably due to the large differences in size, net charge, amino acid composition, solubility and native secondary and tertiary structure. Generally applicable isolation and characterisation schemes cannot be implemented for every protein. This makes protein chemistry rather difficult, and much experience and technology are necessary to manipulate small amounts of protein optimally. On the other hand this makes dealing with peptides and proteins more challenging compared to other compounds. The hydrophobicity of membrane proteins and its insolubility in aqueous solutions lead to considerable difficulties in their characterization studies. The majority of

procedures available for characterization of a given protein require the protein to be present in pure form and in suitable amounts. Such conditions were difficult to fulfil for many proteins of the nervous system(27). To asses the purity, identity and quantity of the minute amounts of samples has been one of the most challenging tasks in protein chemistry. The simplest and most commonly used technique is SDS-gel electrophoresis with sensitive staining methods.

Determination of Molecular Weight:

Determination of molecular weight by SDS-PAGE gave values within 10% of the actual molecular weight, but anomalous results were often obtained with glycoproteins. Use of two or more sets of conditions, such as different concentrations of acrylamide might indicate the occurrence of anomalies. It was best to regard molecular weights derived from SDS-PAGE as apparent until confirmed by other evidences.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the neuronal Con A eluate in 3-12% linear gradient gels indicated 3 major groups of diffuse bands of apparent molecular weights 65-72 KDa; 52-63 KDa; and 43-48 KDa respectively in addition to the faint 27 KDa band of Con A when it was present [Fig.9A,9B]. On eight percent and ten percent SDS-PAGE the neuronal glycoproteins revealed 6-8 bands in the molecular weight range of 43-72 KDa, which differ from each other by 2-4 KDa [Fig.10]. In general the solubility of neuronal glycoproteins in 2% SDS was low and a

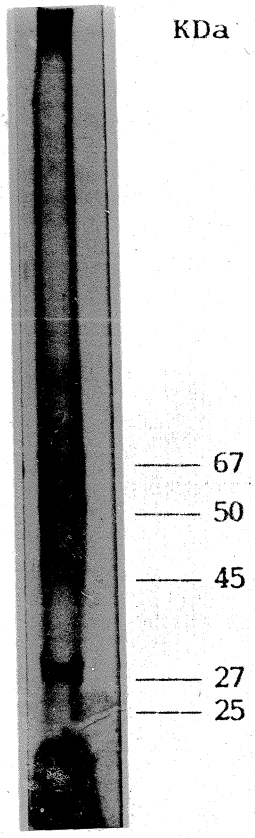


Figure 9A. Gradient SDS-PAGE of neuronal glycoproteins on 3-12% linear acrylamide gel stained with silver.

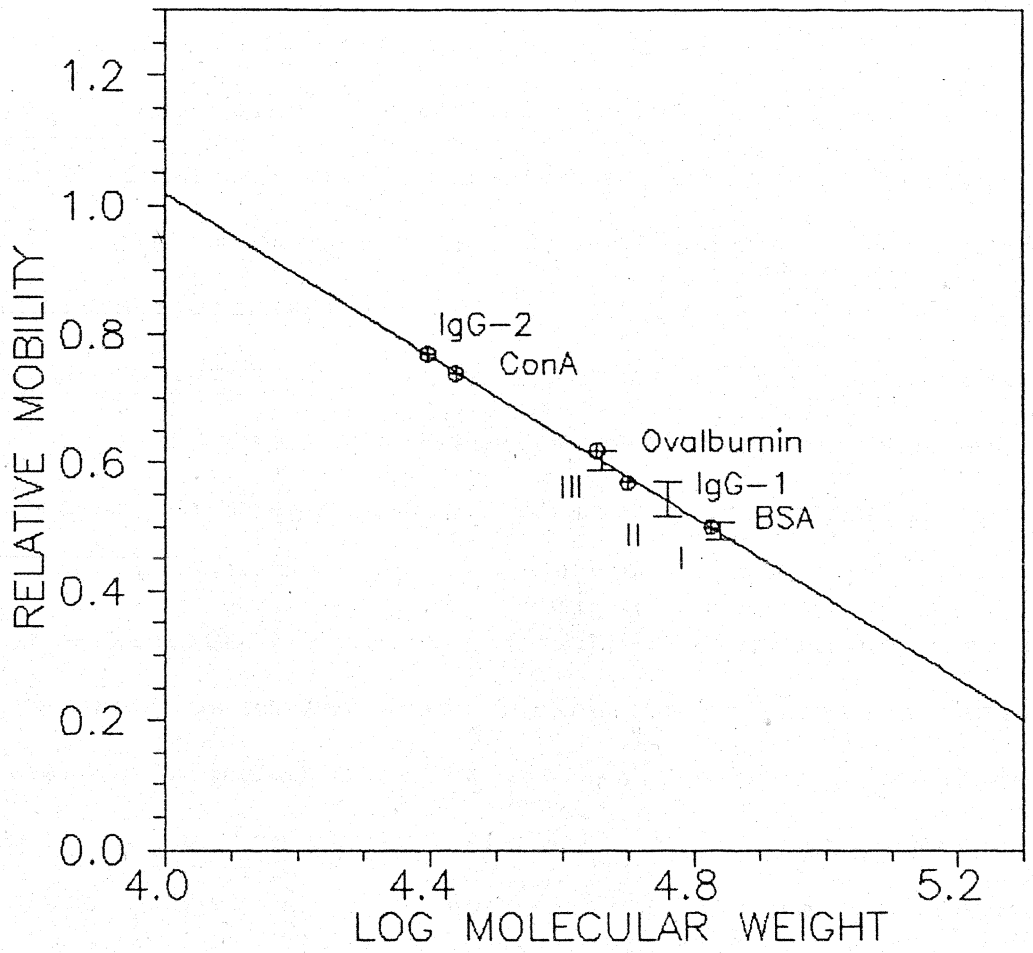


Figure 9B. Molecular weight range determination of neuronal glycoproteins by SDS-PAGE on 3-12% linear acrylamide gel. IgG-1 for IgG heavy chain and IgG-2 for IgG light chain.

soluble preparation of 1mg/ml was difficult to obtain. Only partially solubilized protein suspensions in 2% SDS could be used for SDS-PAGE which resulted in the accumulation of proteins as aggregates at the point of application. The accumulated proteins were removed from the gel by electroelution. They were further subjected to electrophoresis under similar experimental conditions. This resulted in the formation of all the 3 groups of bands of the original sample. A fraction of the protein still remained at the origin. Successive reelectrophoresis of the accumulated protein aggregates, at one stage resulted in its complete removal from the top of gels. In gradient SDS-PAGE gels there was extensive streaking of proteins throughout the gel. Drying the proteins at any stage during its preparation enhanced the process of aggregation resulting in a higher percentage of their retention at the origin. The above mentioned observations on SDS-PAGE indicated the hydrophobic nature of neuronal glycoproteins. The addition of 8M urea to the SDS solubilized neuronal glycoproteins did not improve their solubility as revealed by the same amount of protein aggregates remaining at the point of application in SDS-PAGE gels. Hydrophobic proteins when heated in presence of Laemmli's sample buffer exhibited an enhanced aggregation(128). The process of aggregation was directly proportional to the temperature and the period of exposure to the heat. However in case of the neuronal glycoproteins, boiling for 10 min in presence of 2% SDS did not

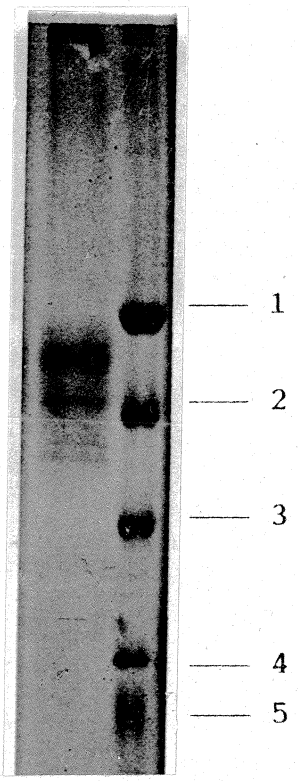


Figure 10. SDS-PAGE of neuronal glycoproteins on 8% acrylamide gel stained with Coomassie blue. Lane A - Neuronal glycoproteins, Lane B - Standards: 1. BSA (67,000); 2. IgG heavy chain (50,000); 3. Ovalbumin (45,000); 4. Con A (27,000); 5. IgG light chain (23,500).

enhance the aggregation process. The Con A-binding glycoproteins from both second trimester and full term fetal brain neurons exhibited similar multiple band pattern on SDS-PAGE.

Anomalous behaviour on SDS-PAGE

For the convenience of study the 6-8 protein bands on 8% and 10% SDS-PAGE were grouped into 3 major groups corresponding to that of gradient PAGE. Each of the 3 groups of bands obtained on 3-12% gradient-SDS-PAGE were cut, electroeluted and reloaded on 8% SDS-PAGE. Each group of gradient-PAGE band gave rise to the same profile of all the 3 groups of bands on 8% SDS-PAGE [Fig.11]. In another experiment each of the 3 gradient-PAGE protein bands were subjected to a second gradient-PAGE under similar experimental conditions. Each of the 3 gradient-PAGE bands upon reelectrophoresis gave rise to all the 3 groups of bands of the original sample. The results were the same when each of the 3 groups of 8% SDS-PAGE bands were subjected to electrophoresis on another 8% SDS-PAGE under similar experimental conditions.

The abnormal behaviour of neuronal glycoproteins on SDS-PAGE could be explained by an anomalous SDS binding to neuronal glycoproteins. The anomalous SDS binding may be attributed to the hydrophobicity of neuronal glycoproteins and / or contributed by their carbohydrate moieties. Hydrophobic membrane proteins have higher affinity for detergents. The erythrocyte membrane glycoprotein glycophorin binds SDS at ratios as high as 5-7mg/mg

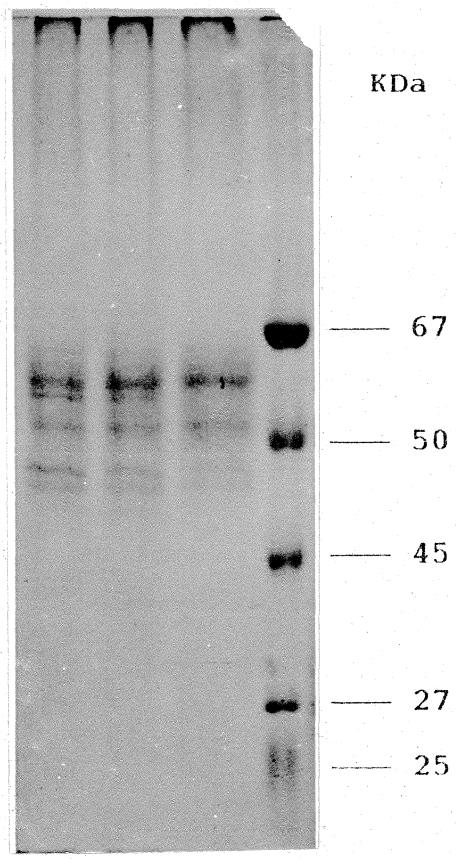


Figure 11. Re-electrophoresis of neuronal glycoprotein bands from gradient-PAGE on 8% SDS-PAGE. The protein bands were visualised by staining with Coomassie blue. A. Gradient-PAGE upper band. B. Gradient-PAGE middle band. C. Gradient-PAGE lower band. D. Standards.

protein which is much higher than the 1-2mg / mg protein ratios found with non-membrane proteins and glycoproteins(182). On the contrary the carbohydrate moieties in glycoproteins reduce SDS binding resulting in their higher apparent molecular weights on SDS-PAGE(172,243). The chances of hydrophobicity or the presence of carbohydrate residues by themselves individually contributing to the abnormal SDS-PAGE pattern of neuronal glycoproteins may be considered as remote and the phenomenon could be an additive effect of both factors.

The solubilization of membrane proteins by SDS is due to the formation of mixed micellae of SDS with the hydrophobic proteins. Complete solubilization results in the formation of homogenous micellae. Partial solubility can result in the formation of heterogenous micellae. The heterogeneity can be due to a fixed protein concentration with varying amounts of SDS or with a fixed amount of SDS and varying amounts of protein in the micellae or by a combination of both processes. The anomalous SDS-PAGE behaviour of neuronal glycoproteins could be explained by a partial solubility of these proteins in SDS, resulting in the formation of heterogenous micellae. The formation of mixed micellae with a fixed number of neuronal glycoproteins with varying amounts of SDS could account for the subtle differences in the charge of these molecules resulting in their differential migration on SDS-PAGE. The major bands on 8% SDS-PAGE differ from each other by 2-4 KDa. Formation of mixed micellae having a fixed

amount of SDS with varying amounts of neuronal glycoproteins could answer the SDS-PAGE anomalous behaviour only by the presence of a 2-4 KDa polypeptide in the neuronal Con A eluate as its major component. This probability should be considered as remote because majority of the integral membrane glycoproteins are of high molecular weight. The formation of heterogenous mixed micellae could be a dynamic equilibrium process, with micellae of one particular composition giving rise to others depending on their relative concentration. Under the conditions of SDS-PAGE, the dynamic equilibrium must be favouring the formation of micellae only in the molecular weight range of 43-72 KDa. During reelectrophoresis, micellae of one particular composition could be regenerating others represented by the various protein bands on SDS-PAGE. This explained the phenomenon of one protein band giving rise to other bands when the reelectrophoresis was carried out.

The term microheterogeneity was used to describe the structural differences in the carbohydrate chains of glycoproteins. In addition to the molecular weight differences, the finer variations in hydrophobicity imparted by microheterogeneity on the neuronal glycoproteins could further magnify their anomalous binding by SDS. The multiple band pattern of neuronal glycoproteins on SDS-PAGE could be attributed to an additive effect of anomalous SDS binding, protein aggregation,

heterogenous mixed protein-SDS micellae formation and microheterogeneity.

Effect of chemical deglycosylation on SDS-PAGE

The study of deglycosylated glycoproteins has yielded insight into the structure and function of glycoproteins. Specific applications have included the removal of glycoprotein microheterogeneity for molecular weight determination of the peptide portion(283) and the preparation of substrates for protein sequence determination(100). Deglycosylation of glycoproteins has been achieved enzymatically(87), biosynthetically using inhibitors of glycosylation(264) and chemically(14). Chemical deglycosylation by TFMS treatment has been employed to determine the molecular weight contributed by carbohydrate part of glycoproteins(99). The TFMS treatment destroys the carbohydrate residues retaining the intact polypeptide chain. The present deglycosylation studies were carried out to investigate the probable contribution of glycoprotein microheterogeneity to the anomalous SDS-PAGE pattern. Desialylation has been reported to decrease the molecular weight of glycoproteins on SDS-PAGE(235,239). Increase in apparent molecular mass on SDS-PAGE following desialylation is characteristic of glycoproteins bearing a high level of sialylated O-linked oligosaccharides such as leukosialin(39).

Trifluoromethanesulfonic acid(TFMS) treatment:

The TFMS treatment was carried out according to the method of Sojar and Bahl(265). The neuronal glycoproteins were alkylated with iodoacetic acid in presence of 4% SDS. The alkylated proteins were subjected to ion pair extraction with acetone:triethyl amine:acetic acid:water[85:5:5:5] to remove the SDS(161). Two aliquots of the alkylated glycoproteins(100µg) were dried under vacuum overnight at 25°C. Thirty microlitre of TFMS was added to one of the samples, flushed with nitrogen, sealed with parafilm and incubated at 0°C for 120min. The second sample was kept aside as control without the addition of TFMS. Subsequently, the reaction mixtures were cooled to -20°C and neutralized by the gradual addition of 150µl of 60% pyridine in water also previously cooled to -20°C. The neutralized samples were dialysed against distilled water and precipitated with 4 volumes of methanol.

Gradient SDS-PAGE (3-12%) revealed an increase in the apparent molecular weights for the TFMS treated sample [Fig.12]. In the control sample there were two bands of 55 KDa and 43 KDa in addition to the 27 KDa band of Con A subunit. The usual 65-72 KDa band was missing from the control. The TFMS treated sample on gradient PAGE indicated the presence of two bands with apparent molecular weights of 66 KDa and 55 KDa. The contaminant Con A band was absent in the deglycosylated preparation. The increase in apparent molecular weights could be due to an enhanced

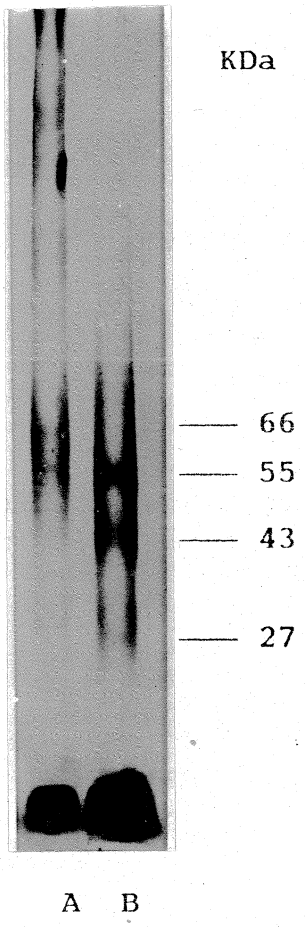


Figure 12. SDS-PAGE of TFMS treated neuronal glycoproteins on 3-12% linear gradient acrylamide gel stained with silver. A. Test; B. Control.

aggregation process. The destruction of oligosaccharide chains by TFMS treatment might have imparted more hydrophobicity on the protein which could have caused the enhanced aggregation. The absence of Con A subunit band in the deglycosylated preparation could be due to the fact that Con A also might have joined in the aggregation process on account of the hydrophobic patches present on the Con A molecule. In general TFMS causes a decrease in the molecular weight of glycoproteins as well as a reduction in their apparent heterogeneity, resulting in sharpening of the stained zones on SDS-PAGE(68). The probable decrease in the molecular weight of neuronal glycoproteins might be masked by an apparent increase due to their aggregation.

Chemical desialylation with 0.1N H_2SO_4 and nitrous acid deamination caused an enhanced aggregation of neuronal glycoproteins resulting in a higher percentage of their accumulation on top of gels(124,78). However these chemical modifications did not change the multiple band pattern of neuronal glycoproteins on SDS-PAGE.

Acid-Urea-Polyacrylamide Gel Electrophoresis

The neuronal glycoproteins on acid-urea-PAGE in 5% gels indicated 4 major bands [Fig.13]. Both second trimester and full term neuronal glycoproteins revealed the same electrophoretic profile. The solubility of neuronal glycoproteins in 8M urea was lower resulting in the accumulation of protein aggregates at the sample wells. The neuronal glycoproteins did not migrate in

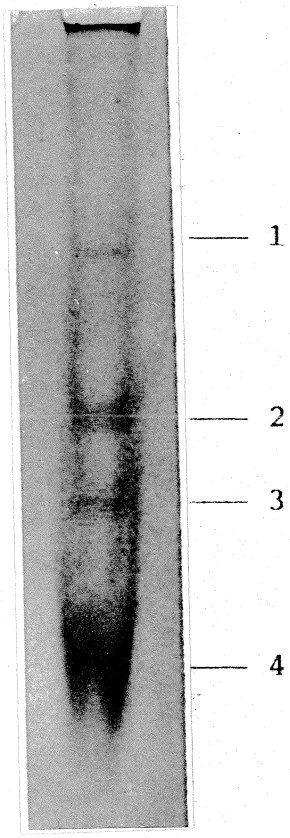


Figure 13. Urea-PAGE of neuronal glycoproteins at pH 4.2 on 5% acrylamide gel stained with coomassie blue.

alkaline- urea- PAGE at pH 8.6 in 5% gels which could be due to the absence or a lower percentage of acidic amino acids.

The urea-PAGE bands 2, 3 and 4 were cut from 50% methanol fixed and coomassie blue stained gels. The gel slices were soaked in the solubilization buffer (urea-PAGE chamber buffer pH 4.2 containing 8M urea, 40% glycerol and 5mM DTT) for 3h. The solubilization buffer was changed every hour, with boiling the sample after the second and third changes. For the second urea-PAGE, the gel slices were inserted inside the sample wells and a current of 15 mA was applied. The electrophoresis was carried out till the marker dye came out and was continued for 15min more. The gel was fixed in 50% methanol washed several times and finally boiled in distilled water before staining by silver. All the three bands moved to the same distance and their migration was only till band 1 of the native total Con A eluate [Fig.14]

The unusual migration of neuronal glycoproteins in the second urea-PAGE cannot be explained. However the possibility of the glycoproteins to form self micellae even in the presence of urea and the extent of solubilization achieved for the fixed proteins inside the gel slices should be taken into account while attempting to explain the unusual Urea-PAGE reelectrophoretic behaviour.

Carbohydrate content

The carbohydrate content of neuronal Con A-eluate varied depending on the temperature at which the chromatography was

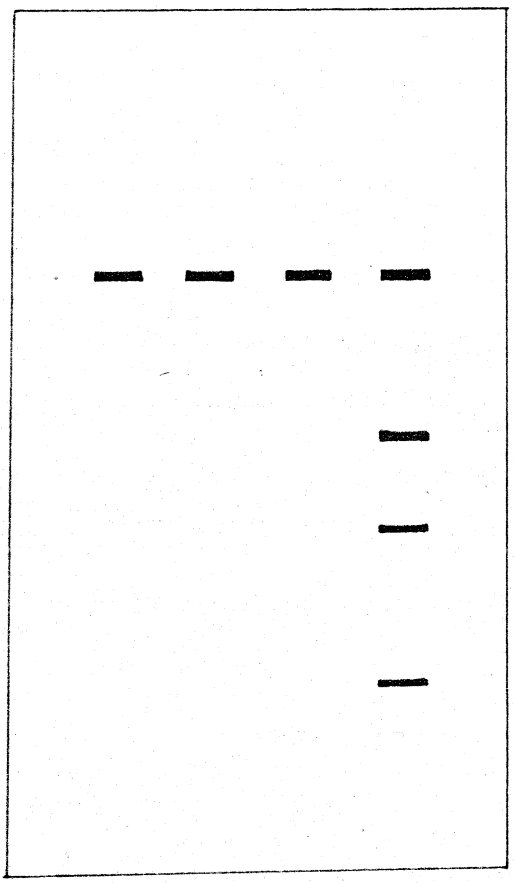


Figure 14. Diagramatic representation of the re-electrophoresis of neuronal glycoprotein bands from acid-urea-PAGE on second acid-urea-PAGE. A. Band 2; B. Band 3; C. Band 4; D. Neuronal glycoproteins.

carried out. The electroeluted SDS-PAGE protein bands as well as the neuronal glycoproteins eluted at 4°C, contained 180±60µg carbohydrate/mg protein. The carbohydrate content increased to 360±120µg/mg protein when the washing and elution of Con A-Sepharose column was performed at 25°C instead of 4°C. The difference in carbohydrate content was due to the presence of glycolipids in the Con A eluate obtained at 25°C. The higher carbohydrate content in the 25°C eluted glycoproteins was inspite of the presence of contaminant Con A which was absent in the cold Con A eluate as well as in the electroeluted protein bands. The carbohydrate content of neuronal glycoproteins from both the second trimester and full term brains were in the same range.

Sialic acid content

The sialic acid content of the cold eluted glycoproteins were in the range of 35±5 nmoles / mg protein. For the sample eluted at 25°C, sialic acid content was found to be 20±5 nmoles / mg protein. The higher sialic acid content / mg protein in cold eluted sample was due to the absence of contaminant Con A as compared to its presence in the room temperature eluted sample. The neuronal glycoproteins from both the second trimester and full term fetal brains had the same sialic acid content.

Presence of noncovalently associated glycolipids

The neuronal Con A eluate was subjected to delipidation according to the method of Svennerholm and Fredman(274). The present method of Svennerholm has the advantage that even the

highly glycosylated glycolipids (polyglycosyl ceramides) become solubilized and most glycoproteins remain insoluble(79). The lipid extract was flash evaporated and dissolved in a minimum volume of chloroform:methanol(2:1) and subjected to TLC on precoated silica plates (2.5 X 10 cm). Twenty microlitre of the sample was applied on the plate and developed with the solvent system, n-propanol: water [7:3](169).

The following observations revealed the presence of noncovalently associated glycolipids in the neuronal Con A eluate obtained at 25°C.

(a) Higher percentage of carbohydrate in the 25°C eluted glycoproteins as compared to the 4°C eluted preparation.

(b) Higher percentage of carbohydrate in the 25°C eluted glycoproteins as compared to the electroeluted SDS-PAGE protein bands.

(c) Decrease in carbohydrate content of the 25°C eluted proteins after delipidation and not in the 4°C eluted preparation.

(d) Decrease in sialic acid content after delipidation only in the 25°C eluted proteins but not in 4°C eluted sample.

(e) The lipid extract was positive for carbohydrate.

(f) The lipid extract on TLC rendered positive staining for general lipids, glycolipids and gangliosides(259). The above mentioned observations are summarised in Table 5.

The area stained by iodine vapour on the TLC plate was larger when compared to the other two methods, indicating the

TABLE 5. EFFECT OF TEMPERATURE ON THE THE COMPOSITION OF CON A ELUATE.

ELUTION TEMPERATURE	4°C	25°C
CARBOHYDRATE(µg/mg protein)	180±60	360±120
CARBOHYDRATE AFTER DELIPIDATION (µg/mg protein)	180±60	180±60
SIALIC ACID (nmoles/mg protein)	35±5	20±5
SIALIC ACID AFTER DELIPIDATION (nmoles/mg protein)	35±5	18±5
CON A BAND ON SDS-PAGE	+	++++
TLC OF THE LIPID EXTRACT		
(a) IODINE VAPOUR	+	++++
(b) ORCINOL-H_2SO_4	+	++++
(c) RESORCINOL-HCL	+	++++

(+) for weak and (++++) for very strong intensity of staining.

presence of lipids other than glycolipids. The area stained by resorcinol-HCl was much smaller than that of orcinol-H_2SO_4 revealing that the majority of glycolipids were non-sialylated with a very minor ganglioside component. The carbohydrate content of the 25°C Con A eluate decreased by 50% following delipidation as compared to the 10% decrease in sialic acid which confirmed the lower percentage of gangliosides. The lower percentage of gangliosides was further reflected by an apparent increase in the sialic acid content of 4°C eluted sample. The lower Con A contamination in the 4°C eluted preparation would explain its apparent higher sialic acid content. The position of resorcinol-HCl region near the solvent front indicated the possible presence of gangliosides belonging to the GM_3-GM_4 class. The absence or a lower percentage of non-covalently associated glycolipids in the 4°C eluted neuronal glycoproteins was further confirmed on TLC.

The lipid extract from both the second trimester and full term neuronal glycoproteins revealed similar profiles on TLC. The increase in glycolipid content at 25°C, correlated with the enhanced leaching of Con A observed at the same temperature. The α-anomeric glucose residues in glycolipids cannot interact with Con A. The glycolipids must have appeared in the Con A eluate in micellar association with Con A and the Con A binding glycoproteins.

Plasma membranes of animal cells are particularly rich in glycolipids as compared with intracellular membranes and has been

used as the plasma membrane marker(302,157,158). The association of glycolipids is an additional evidence for the fact that neuronal glycoproteins are from the cell surface.

Covalently linked lipids.

In recent years much attension has been focused on the post translational modification by long chain fatty acids to eukaryotic proteins. Three modes of fatty acid linkage to eukaryotic proteins have been described. These are: (a) attachment of fatty acid (primarily palmitic acid) in a thioester or ester bond to cysteine, serine or threonine residues(217), (b) cotranslational myristoylation of proteins on aminoterminal glycine residues via an amide linkage(250), (c) carboxyterminal addition of a phosphatidyl inositol(GPI)-containing glycan moiety to proteins(77). While some acylproteins clearly require their acyl chains for stable association with cellular membranes(151), others normally behave as soluble proteins(207). Several palmitoylated proteins are surface-oriented glycoproteins while all of the myristoylated proteins appear to be internal. Nervous system proteins with GPI-anchors include acetyl cholinesterase, Thy-1 and $N\text{-}CAM_{120}$. The release of radioactive fatty acid from metabolically labelled acylproteins with the nucleophilic hydroxylamine or by mild alkali has been used to identify ester linkages of acyl chains(247). These treatments did not cleave fatty acid amide bonds. Acid hydrolysis of acyl proteins released both ester-linked and amide-linked fatty acids in addition to

breaking the polypeptide backbone(5). In the present study, the presence of covalently linked lipids and its possible contribution to the anomalous SDS-PAGE pattern were investigated.

Nitrous acid deamination.

The cleavage of the GPI-anchor was carried out by the method of Ferguson et al(78). The neuronal glycoproteins precipitated with 8 volumes of methanol was subjected to delipidation by successive extractions with chloroform-methanol(1:2,v/v), (1:1,v/v), (2:1,v/v) and suspended in 1ml of distilled water. Three aliquots of the delipidated proteins(100µg) were dried under vacuum. To two of the aliquots(A and B), 450µl, freshly prepared 200mM $NaNO_2$ in 250mM sodium acetate/acetic acid buffer pH 3.5 and to the third sample(C) 450µl distilled water were added. The preparations were incubated at 25°C for 5h, followed by the addition of 45µl of 1N HCl, 1.2ml chloroform and 0.6ml methanol. The mixture was vortexed and the phases were separated by centrifugation. After the removal of lower organic phases, the upper aqueous phases were reextracted with preequilibrated lower phase. As reagent controls 450µl each of distilled water and $NaNO_2$ reagent were subjected to the same procedure. The lower phases were dried in a boiling water bath. The sample A lower phase was kept aside for phosphorous estimation. The dried lower phase from sample B was subjected to hydroxylamine treatment. The lower phase obtained after the treatment was washed twice with 2ml of toluene and dried under vacuum. Phosphorous estimations

were carried out as described in materials and methods. No recordable inorganic phosphorous could be detected in the sample A lower phase and in the lower phase obtained from sample B after hydroxylamine treatment indicating the possible absence of GPI-anchor in neuronal glycoproteins.

Methanolic potassium hydroxide treatment

Hydrolysis of the ester linked fatty acids by methanolic KOH was carried out by the method of Reindeau et al(236). Delipidated neuronal glycoproteins(100µg) were suspended in 1ml of 0.3M KOH in methanol and incubated for 30min at 56°C. It was acidified with glacial acetic acid and vortexed with 1ml of toluene. The phases were separated by centrifugation and the upper toluene phase was collected. As a blank 1ml of methanolic KOH reagent was subjected to phase separation and the upper phases were dried under vacuum for TLC analysis.

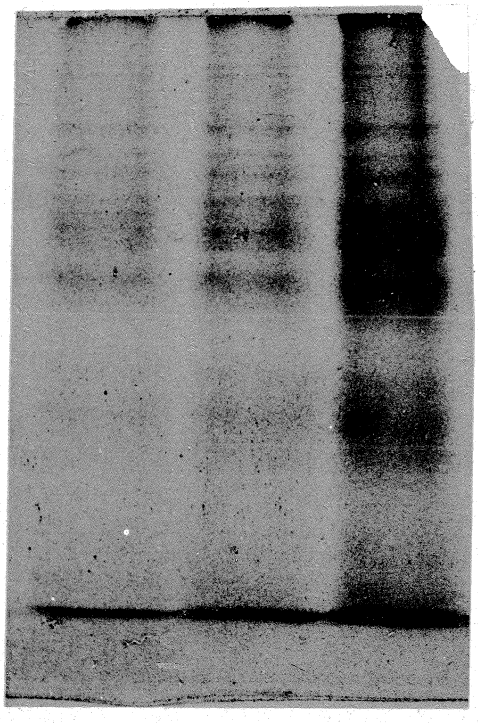
Hydroxylamine treatment

The neuronal glycoproteins were subjected to the hydroxylamine treatment according to the method of Reindeau et al(236). Delipidated neuronal glycoproteins(100µg) were suspended in 1ml of 1M hydroxyl ammonium chloride, adjusted to pH 7.0 and incubated at 25°C for 2h. Two millilitre of toluene was added and mixed thoroughly. The phases were separated by centrifugation. As a blank 1ml of hydroxylamine reagent was also subjected to phase separation with toluene. The upper phases were collected and dried under vacuum for TLC analysis.

The dried organic phases obtained after the methanolic KOH and hydroxylamine treatment were dissolved in 100µl of chloroform: methanol (2:1) and subjected to TLC on precoated silica gel G plates (5X25cm). The plates were prerun with chloroform: methanol(2:1) and activated overnight at 100°C. Twenty microlitre of the above samples were spotted on the plate. The plates were developed in the solvent system toluene: methanol: acetic acid [80:20:1] till the solvent front reached 1cm below the top of the plate. After the run the plates were dried in air. The plates were exposed to iodine vapour, subsequently sprayed with saturated potassium dichromate in 70%(v/v) aqueous H_2SO_4 and heated at 100°C for 1h. Both methanolic KOH and hydroxylamine treatments did not reveal the presence of fatty acids. After the deacylation treatments the proteins were dried under vacuum, dissolved in 2% SDS and subjected to 8% SDS-PAGE. The multiple band pattern of neuronal glycoproteins on SDS-PAGE remained unchanged after the deacylation treatments[Fig.15].

N-terminal analysis

The arrangement of integral membrane glycoproteins in the plasma membrane is determined by the topological distribution of their hydrophobic and hydrophilic domains. The structural organization of these molecules can therefore be inferred from an analysis of the position of their hydrophobic amino acid residues once the primary structure is determined. The elucidation of the amino acid sequence of cell surface glycoproteins proved to be a



1 2 3

Figure 15. SDS-PAGE of neuronal glycoproteins after chemical deacylation treatments on 8% acrylamide gel stained with Coomassie blue. 1. Nitrous acid deamination; 2. Hydroxylamine treatment; 3. Methanolic KOH treatment.

formidable task owing to their very low abundance and their complexity. Information about the secondary or tertiary structure of proteins could only be gained if the amino acid sequence is available. N-terminal analysis helps in assessing the purity, identity and quantity of minute amounts of protein samples. It can also be applied to compare the homology of structurally related proteins, to analyze the detailed pathway of limited proteolysis or to locate selectively modified Lys/Arg residues. The classical approach uses cleavage of the protein with trypsin to produce small peptides which could be sequenced. The order of the tryptic peptides was then found using overlapping peptides isolated from other digests of the protein.

N-terminal analysis of the neuronal glycoproteins was performed by both direct and indirect Edman degradation procedures. In the indirect Dansyl Edman procedure a portion of the sample after each cycle was dansylated and subjected to acid hydrolysis. The acid stable dansyl amino acid derivatives were detected on HPLC. In the direct DABITC / PITC double coupling method the detection of released DABTH-amino acid derivatives was carried out on TLC. N-terminal analysis of the electroeluted individual SDS-PAGE protein bands by the above two methods revealed a blocked N-terminal for the neuronal glycoproteins [Fig.16]. The probability of N-terminal blocking during the process of SDS-PAGE was investigated. The Con A eluate was first dansylated in presence of 2% SDS. The contaminant Con A was

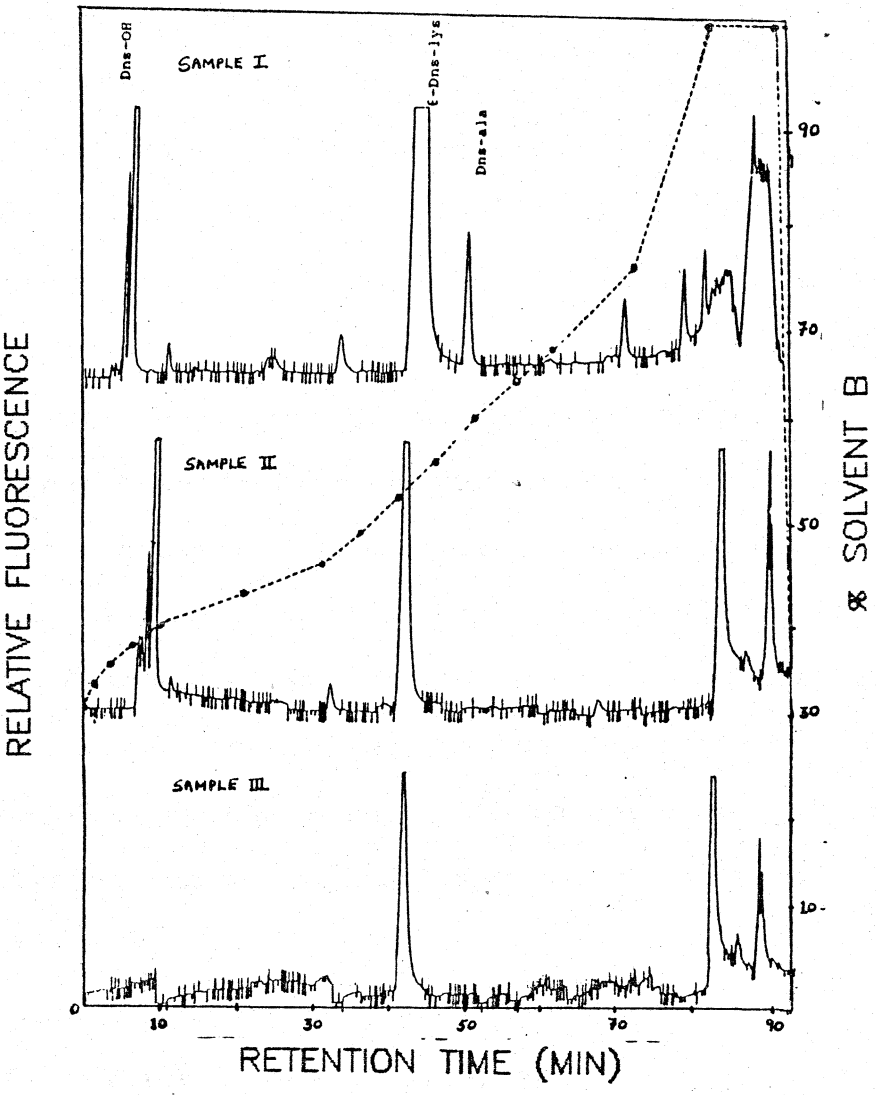


Figure 16. N-terminal analysis of neuronal glycoprotiens by Dansyl-Edman method. The experimental details are described in the text. Sample I. Total neuronal Con A eluate, Sample II. Electroeluted SDS-PAGE bands, Sample III. Neuronal glycoproteins after the electrophoretic removal of contaminant Con A.

eliminated from the dansylated preparation by the electrophoretic method described earlier using FITC-Con A as a marker. The electroeluted proteins were precipitated with 8 volumes of methanol and subjected to acid hydrolysis. High performance liquid chromatography of the hydrolysate revealed the presence of ε-group of lysine, confirming the blocked N-terminal of neuronal glycoproteins [Fig.16].

A number of cell surface receptors, especially those involved in signal transduction through interaction with guanine nucleotide binding proteins are blocked at the amino terminal and are not amenable to N-terminal analysis in the intact form of the receptor(231). Fragmentation of the protein by chemical or enzymatic methods and separation of the peptide fragments by reversed phase HPLC is the strategy that has generally proved useful in analyzing the structures of membrane proteins blocked at the amino terminal.

Gel filtration on Sepharose 6B

The gel filtration of neuronal glycoproteins was carried out on Sepharose 6B (100 X 1.4 cm) equilibrated with 25mM Tris-700mM glycine pH 7.8 containing 15mM NaN_3. The chromatography was carried out at 25°C at a flow rate of 6ml/h. The neuronal glycoproteins, labelled with (^{3}H)-acetic anhydride were extensively dialysed against distilled water and dissolved in the equilibration buffer at a concentration of 0.25 mg/ml. Centrifuged at 15,000g X 3 min to obtain a supernatant. One ml of

the supernatant($4X10^5$cpm) was loaded on the column. Two millilitre fractions were collected and 0.1ml aliquots from each fraction was counted. The neuronal glycoproteins resolved into two peaks [Fig.17]. The first peak (peak I) appeared at the void volume, and the second peak (peak II) appeared at the end of total bed volume. The fractions were pooled together and the peaks dialysed against distilled water. Aliquots were counted to estimate the recovery of total radioactivity. The peak I contained 10,000 cpm and the peak II contained 30,000 cpm. The gel filtration process was repeated with another batch of neuronal glycoproteins. The gelfiltered peaks from the two preparations were pooled together and concentrated to 1ml by lyophilization. The buffer composition of the preparation was made to 25mM Tris / 700mM glycine pH 7.8 by the addition of solid Tris and glycine. The concentrated peaks were further subjected to gel filtration on Sepharose 6B under similar conditions. Each of the gel filtration peaks on rechromatography eluted at the same volumes as that of earlier chromatography, indicating that the gelfiltration peaks were not interconvertible [Fig.17]. Aliquots were counted to asses the recovery of the total radioactivity after second gelfiltration. This time peak I contained 5000 cpm and the peak II contained 16000 cpm. The gel filtered peaks were further dialysed against distilled water, concentrated by lyophilization and subjected to dot-enzyme-linked-lectin assay. Both gel filtration peaks were positive for

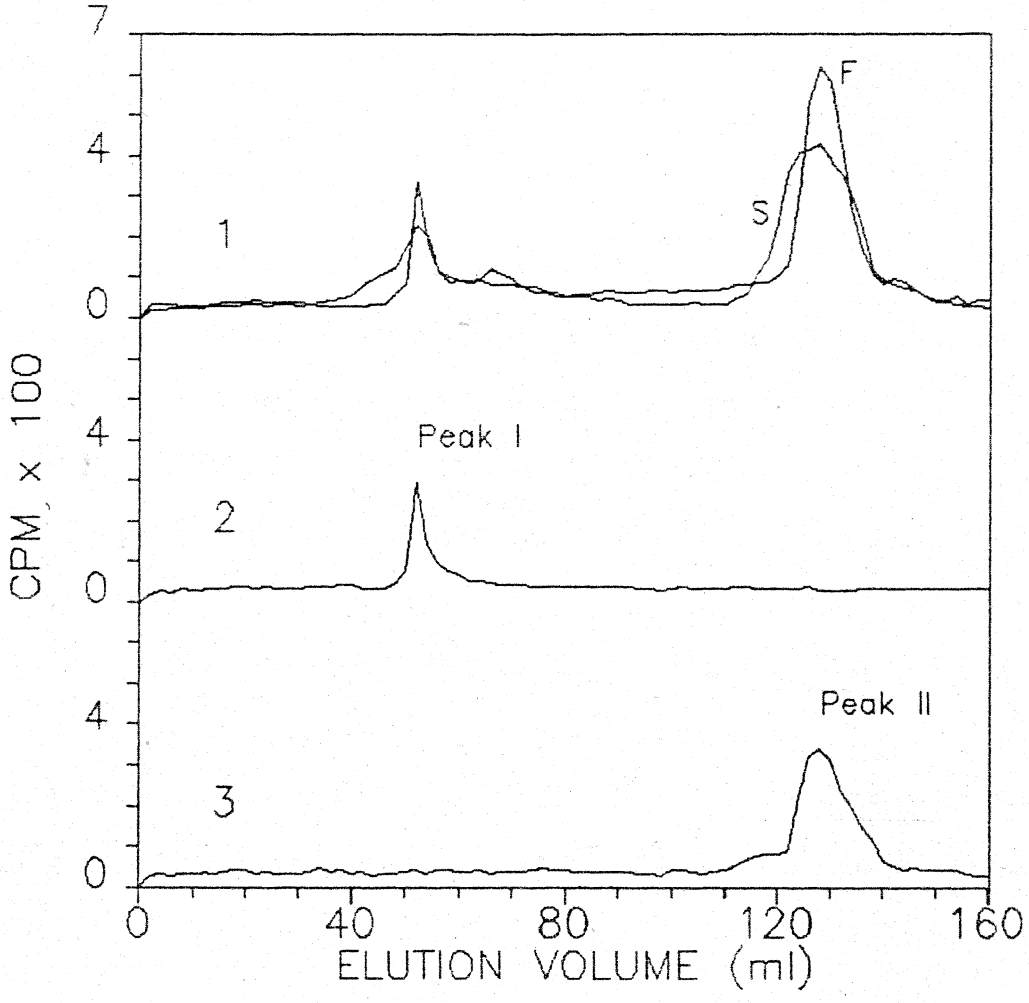
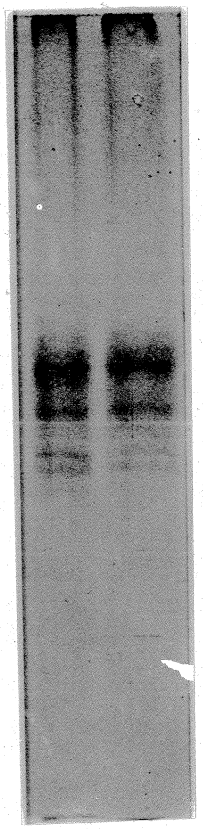


Figure 17. 1. Gel filtration of [^{3}H]-labelled neuronal glycoproteins on Sepharose 6B (100 X 1.4cm). S-Second trimester neruonal glycoproteins, F-Full term neuronal glycoproteins. 2. Re-gel filtration of Peak I. 3. Re-gel filtration of Peak II.

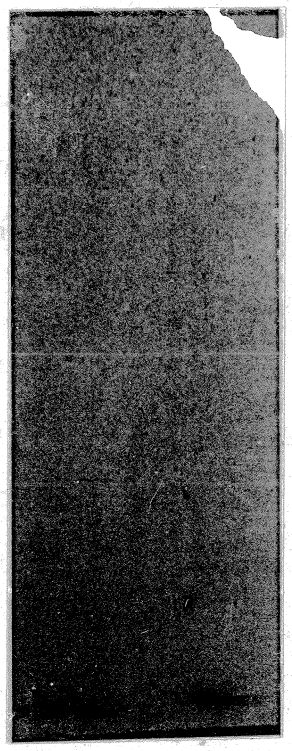
interaction with Con A, RCA and WGA. Both gel filtered peaks on 8% SDS-PAGE revealed the same multiple band pattern as that of the original sample[Fig.18]. The similar multiple band pattern for both gel filtered peaks could be due to the anomalous SDS binding to neuronal glycoproteins discussed earlier. On acid-urea-PAGE both gel filtered peaks gave a single fast moving band of equal mobility [Fig.19]. Mobility of this urea-PAGE band was higher than that of the native glycoprotein bands. To asses the possibility of glycoproteins forming insoluble aggregates, the gelfiltered peaks were centrifuged at 100,000g X 60 min. After the centrifugation, 100% of the total radioactivity could be recovered in the supernatants. The fact that even peakI proteins remained in the soluble supernatant illustrated the absence of protein aggregation. The Sepharose 6B chromatography of tritiated neuronal glycoproteins from full term fetal brains also resulted in two peaks, with similar retention times as mentioned earlier [Fig.17].

The gel filtered peak II was further subjected to gel filtration on Sephadex G-75 to obtain its molecular weight. The protein sample (8500cpm) was extensively dialysed against distilled water and concentrated to 0.3 ml by lyophilization. The buffer composition of the preparation was made to 25mM Tris/700mM glycine pH 7.8 by the addition of solid Tris and glycine. The preparation was loaded on the gel filtration column (63X0.5cm) equilibrated with 25mM Tris/ 700mM glycine pH 7.8 containing 15mM



A B

Figure 18. SDS-PAGE of Sepharose 6B gel filtered neuronal glycoprotein peaks on 8% acrylamide gel stained with coomassie blue. A. Peak I, B. Peak II.



A B

Figure 19. Acid-urea-PAGE of Sepharose 6B gel filtered neuronal glycoprotein peaks on 5% acrylamide gel stained with coomassie blue. A. Peak I, B. Peak II.

NaN_3. The chromatography was carried out at 25°C with a flow rate of 6ml/h. Fractions of 0.5ml were directly collected in scintillation vials and counted. The peak II eluted long after cytochrome c (12.5KDa) indicating a low molecular weight for the glycoprotein [Table 6]. However the exact molecular weight could not be estimated due to the anomalous migration of low molecular weight proteins on gel filtration.

The Sepharose 6B void volume peak might represent a large neuronal surface glycoprotein. When compared to the globular proteins, glycoproteins in general exhibit higher apparent molecular weight in gel filtration columns, due to its extended nature. The presence of high molecular weight glycoproteins on the neuronal surface could not be ruled out. The molecular mass of the cell surface glycoprotein epitectin as determined by gel filtration ranged from 1.0-1.5 X 10^4 daltons with two bands of apparent molecular weight 390KDa and 350KDa on SDS-PAGE (15).

The gel filtered peak II might represent the monomers of the peak I protein. The similar electrophoretic pattern for both peaks on urea-PAGE and SDS-PAGE substantiated this possibility. According this hypothesis, the components of neuronal Con A eluate include a high molecular weight glycoprotein excluded by Sepharose 6B and its dissociated monomers. The native unlabelled neuronal glycoproteins did not produce a fast moving urea-PAGE band corresponding to that of the tritiated gel filtered peaks. Treatment with urea might be insufficient in dissociating the

TABLE 6.

GEL-FILTRATION OF PEAK II FROM SEPHAROSE 6B ON SEPHADEX G-75.

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	_____	44

native neuronal glycoprotein to its monomers. This could be due to the formation of heterogenous micellar aggregates by the neuronal protein subunits. The four urea-PAGE bands of the native protein probably represented the different stages of micellar aggregation. Acetic anhydride labelling might have destabilized micellae formation enabling the complete dissociation of the protein to its monomers by urea. This explained the higher mobility of acetic anhydride labelled neuronal glycoproteins on urea-PAGE as compared to the native unlabelled glycoprotein.

Another possibility was the formation of a high molecular weight protein micellar aggregate represented by the void volume peak formed from a low molecular weight protein represented by the peak II. According this hypothesis, the major component of neuronal Con A eluate could be a low molecular weight glycoprotein with an inherent property to form micellar aggregates. Glycophorin is known to form aggregates in neutral aqueous solutions, even in presence of 6M guanidine hydrochloride(182). The phenomenon of self micellae formation could be related to the amphipathic nature of integral membrane glycoproteins. Both the hydrophilic carbohydrate moieties and their hydrophobic domains contribute to the amphipathic nature of surface glycoproteins. The formation of micellae is a critical process which depends not only on the amphipathic nature of the solute but also on the monomerisation power of solvents. Methanol is known to destabilize the micellae formation by gangliosides.

To some extent hydrogen bonds are essential for the formation of surface hydrophobic patches in protein molecules represented by their tertiary structure. Urea interferes with the hydrogen bond formation in protein molecules diminishing their hydrophobicity. The diminished hydrophobicity might destabilize the protein micellae formation converting them to smaller micellae or the complete dissociation of the protein to its monomers. Urea treatment could be insufficient in the complete dissociation of the neuronal protein micellae to its individual protein molecules. The incomplete dissociation was manifested by the formation of heterogenous smaller micellae represented by the 4 bands on urea-PAGE. Acetic anhydride labelling might have destabilized the micellae formation, enabling its total dissociation to individual protein molecules by urea. This explained the higher mobility of acetic anhydride labelled neuronal glycoproteins on urea-PAGE.

The acetic anhydride labels free amino groups in proteins, resulting in the reduction of their positive charge. Reduction in the total number of positive charges on neuronal glycoprotein(s) by acetic anhydride labelling was expected to decrease its mobility on acid-urea-PAGE. The electrophoretic mobility of a protein is governed by its charge/mass ratio. The decreased surface charge of neuronal glycoprotein(s) appeared to be compensated by the dissociation of micellar aggregates to its monomeric form causing a net increase in the charge/mass ratio.

The above mentioned observations indicated the presence of a low molecular weight protein in the neuronal Con A eluate, represented by the gelfiltration peak II. The probability of acetic anhydride labelled neuronal glycoproteins migrating in the opposite direction in acid-urea-PAGE was not investigated. As mentioned earlier the native neuronal glycoproteins did not migrate in alkaline-urea-PAGE. A change in the isoelectric point, due to the acetic anhydride labelling could alter the electrophoretic behaviour of proteins.

High Performance Liquid Chromatography

The tritiated Con A eluate and the gel filtered peaks were subjected to reverse phase HPLC. The HPLC procedure was carried out with a 10µm Lichrosorb RP 8 column(4 X 250 mm). The HPLC gradients were formed from solvents A (water, 0.1%TFA) and solvent B (isopropanol, 0.1%TFA). Tritiated second trimester neuronal glycoproteins were dissolved in 25mM Tris / 700mM glycine pH 7.8 at a concentration of 0.25 mg/ml and centrifuged at 15,000g X 3 min. The supernatant was extensively dialysed against distilled water. The pellet obtained was washed twice and suspended in distilled water. The aqueous suspensions(100µl) of supernatant (12,000cpm) and the pellet (27,000cpm) were mixed with equal volumes of TFA, vortexed and centrifuged at 15,000g X 3 min to obtain supernatants. One hundred microlitre of the supernatants were injected into HPLC. From the pellet only 50% of the total proteins could be solubilized by this procedure.

Fractions of 0.5ml, at 1 min intervals were directly collected in scintillation vials. The fractions were counted for radioactivity. In gradient I both supernatant and the pellet migrated as broad peaks with retention times of 54 min [Fig.20]. The HPLC analysis of neuronal glycoproteins from full term fetal brains with gradient I, also resulted in a broad peak with the similar retention time as mentioned above [Fig.21].

The gelfiltered peaks of second trimester neuronal glycoproteins were extensively dialysed against distilled water and concentrated by lyophilization. During the process, proteins precipitated from their solutions. The aqueous suspensions (75µl) of peak I (8,500cpm) and peak II (10,500cpm) were mixed with an equal volume of TFA, vortexed and centrifuged at 15,600g X 3 min to obtain supernatants. One hundred microlitre of the supernatants were injected into HPLC. Radioactivity measurements in the supernatants indicated 50% solubility for the peak I protein and 100% for the peak II protein. In gradient I, the gel filtered peaks migrated as single broad peaks with retention times of 58 min for peak I and 47 min for peak II [Fig.20]. The gel filtered peaks of neuronal glycoproteins from full term fetal brains also exhibited similar retention times as mentioned above when subjected to HPLC analysis under similar experimental conditions [Fig.21].

In gradient II, the (^{3}H)-acetic anhydride labelled neuronal glycoproteins migrated as a single broad peak with a retention

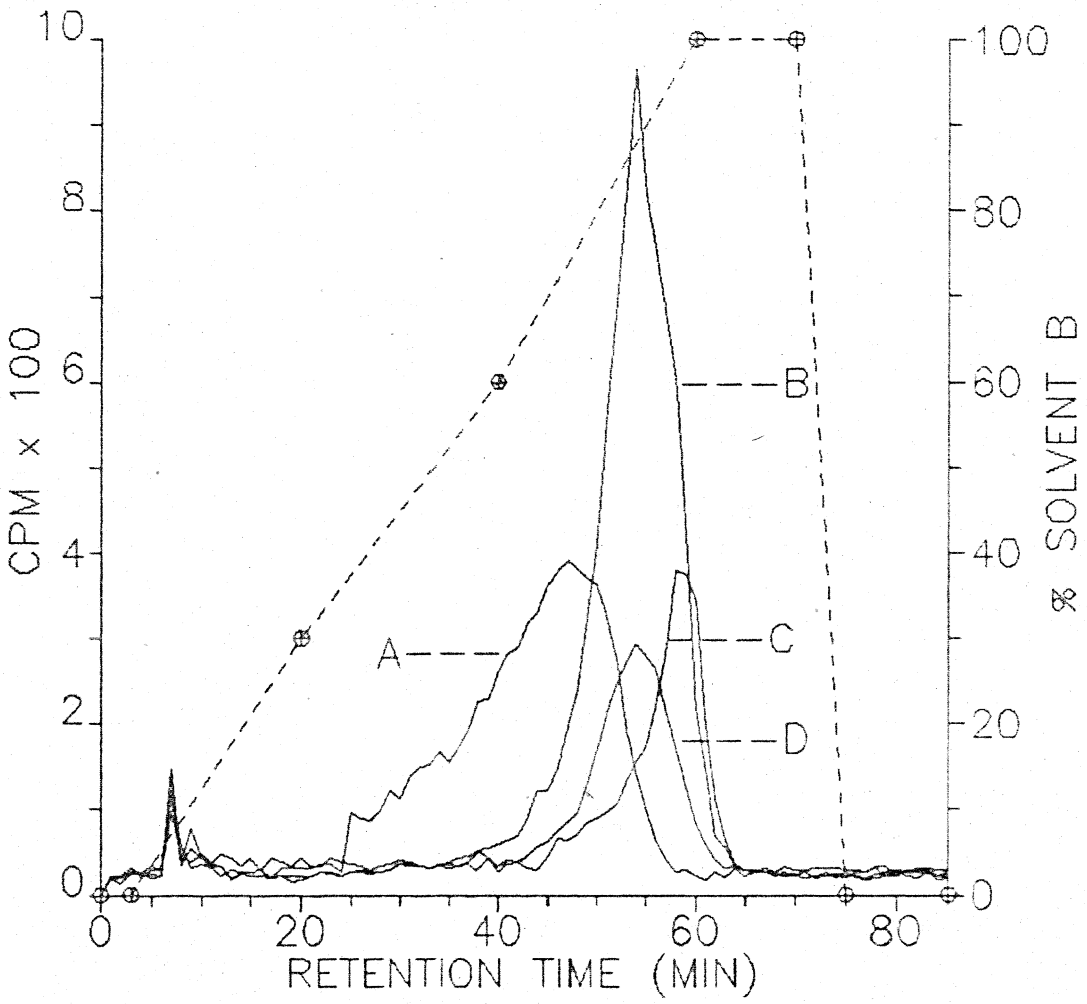


Figure 20. HPLC of [^{3}H]-labelled second trimester neuronal glycoproteins on RP-8 in gradient I (0---0). Flow rate = 0.5ml/ min; Solvent A = Water-0.1% TFA; Solvent B = Isopropanol-0.1% TFA; A. Gel filtered peak II, B. Pellet, C. Gel filtered peak I, D. Supernatant. For details see the text.

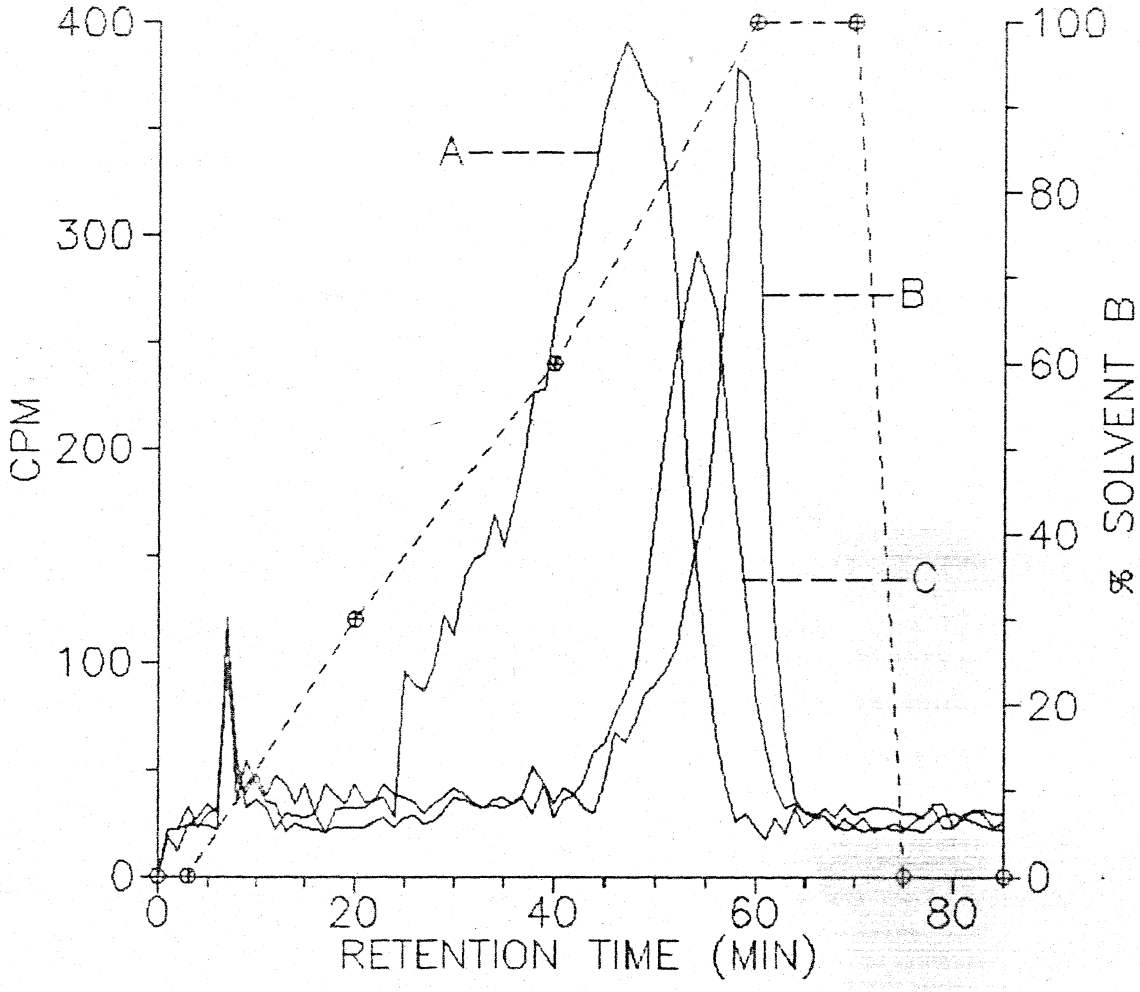


Figure 21. HPLC of [^{3}H]-labelled full term neuronal glycoproteins on RP-8 in gradient I (0---0). Flow rate = 0.5ml/min; Solvent A = Water-0.1% TFA; Solvent B = Isopropanol-0.1% TFA; A. gelfiltered peak II, B. total glycoproteins, C. gel filtered peak I. For details see the text.

time of 71 min [Fig.22]. The HPLC peaks were concentrated to dryness and checked for lectin interaction by dot enzyme linked lectin assay. All the above mentioned HPLC peaks were positive for their interaction with Con A, WGA and RCA. In both gradient I and II in addition to the major peaks described above, there was a minor peak at 7 min which was positive for lectin binding.

Carboxymethylated neuronal glycoproteins were subjected to reverse phase HPLC after dansylation. The carboxymethylated proteins(200µg) were dissolved in 200µl of 40mM Li_2CO_3 / HCl pH 9.5 containing 1% SDS. Twenty microlitre of dansyl chloride [2.5mg / 0.1ml acetonitrile] was added and incubated at 37°C for 1h. After dansylation the proteins were precipitated with 4 volumes of methanol and washed three times with 80% methanol. It was dried under vacuum and dissolved in 200µl of 50% TFA. One hundred microlitre of the 15,600g X 3 min supernatant was subjected to HPLC. One minute fractions of 0.5ml were collected. The fractions were diluted to 2.5 ml with distilled water and their fluorescence was measured. In gradient I, the dansylated neuronal glycoproteins migrated as a broad peak with a retention time of 61 min [Fig.23].

Digestion with Trypsin and Pronase

In the present study of proteolytic digestion, carboxymethylated glycoproteins were labelled with periodate-NaB^3H_4, at their carbohydrate moieties. The proteins were further dansylated in presence of SDS to block the free amino groups. The

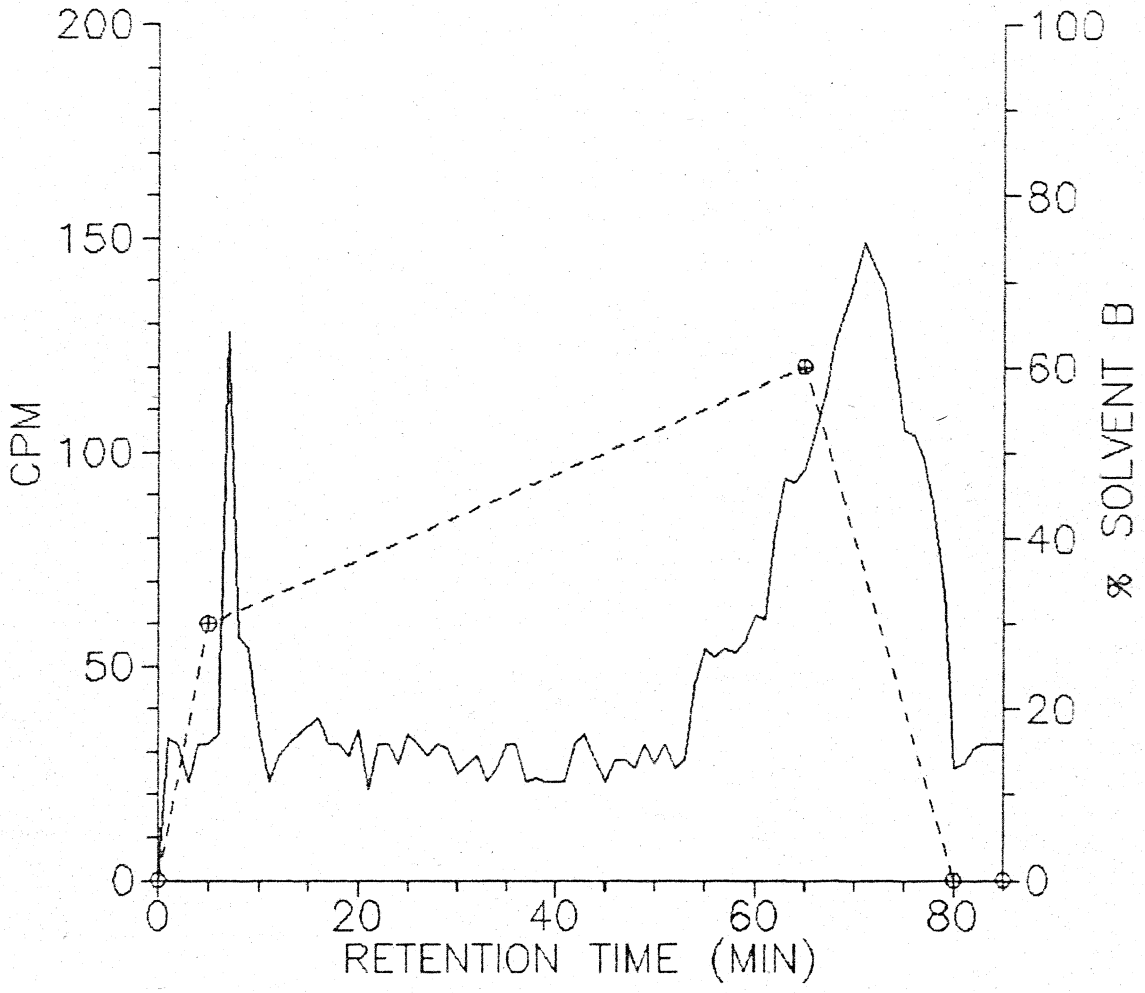


Figure 22. HPLC of [^{3}H]-labelled neuronal glycoproteins on RP-8 in gradient II (0---0). Flow rate = 0.5ml/min; Solvent A = Water-0.1% TFA; Solvent B = Isopropanol-0.1% TFA. For details see the text.

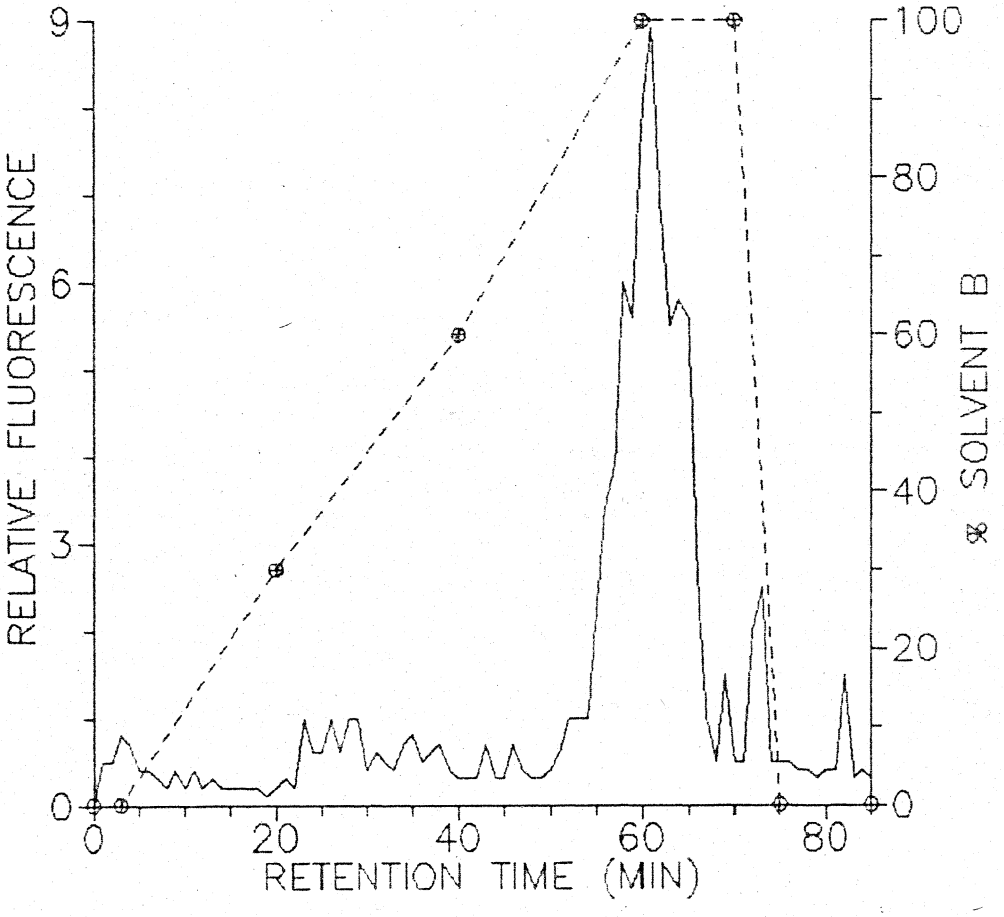


Figure 23. HPLC of dansylated and carboxymethylated neuronal glycoproteins on RP-8 in gradient I (0---0). Fluorescence was measured with Ex.Max = 333nm and Em.Max = 510 nm. Experimental details are described in the text.

proteolytic digestion would liberate new amino groups which could be labelled with (^{14}C)-acetic anhydride. The proteolytic cleavage was analyzed by HPLC with a RP 8 column.

Periodate-NaB^3H_4 labelling:

The periodate oxidised neuronal glycoproteins were reduced with NaB^3H_4 according to the method of Lenten and Ashwell(290). Carboxymethylated neuronal glycoproteins(1mg) were suspended in 0.1ml 100mM sodium acetate buffer pH 5.0 containing 8M urea and boiled for 2 min to denature the proteins. The preparation was diluted to 0.3ml with 100mM sodium acetate buffer pH 5.0 and 0.1ml of 40mM sodium meta periodate dissolved in the same buffer was added. The preparation was incubated at 4°C in the dark for 16h. Ethylene glycol(10µl) was added and further incubated at 25°C for 1h in the dark. Twenty microlitre of NaB^3H_4(8 X 10^6 cpm) in 10mM NaOH was added and incubated at 25°C for 90 min. The proteins were precipitated with 8 volumes of methanol and washed 5 times with methanol and dried under vacuum. It was suspended in 500µl of 40mM Li_2CO_3 / HCl pH 9.5 containing 2% SDS, aliquots were counted for radioactivity. There was a net incorporation of 45,000 cpm to the glycoproteins. The proteins were dansylated as described under materials and general methods.

Proteolytic Digestions:

The tritiated and dansylated neuronal glycoproteins were subjected to trypsinisation as given below (263). A suspension of the protein(500µg) in 500µl of 100mM phosphate buffer pH 7.6

containing 2M urea was prepared and divided into 2 aliquots. To the test 20µg TPCK-trypsin in 20µl of 0.001N HCl was added and incubated at 37°C for 5h. This was followed by the addition of another 20µg of trypsin and further incubation for 24h. After the digestion the trypsin activity was inactivated by boiling the preparation at 100°C for 5 min. In the control sample trypsin (40µg) was added just prior to the boiling.

The pronase digestion of tritiated and dansylated neuronal glycoproteins was carried out as given below(71,81). A suspension of the protein(500µg) in 500µl of pronase digestion buffer (50mM N-ethyl morpholine / HCl pH 8.0-10mM $CaCl_2$-2M urea) was prepared and divided into 2 aliquots. To the test 20µg pronase dissolved in 20µl of the pronase digestion buffer was added and incubated at 37°C for 24h. One drop of toluene was added as a bacteriostatic agent. Another 20µg pronase was added and further incubated for 24h, which was followed by the addition of a third aliquot of 10µg pronase and incubation for another 24h. Pronase activity was inactivated by boiling the preparation at 100°C for 5 min. In the control sample 50 µg pronase was added just prior to the boiling.

^{14}C-labelling of Glycoprotein:

The pH of the pronase treated preparations were adjusted to 8.5 by the addition of N-ethyl morpholine and for the trypsin treated samples the pH was adjusted to 8.5 by the addition of 1N NaOH. Fifty microlitre of (^{14}C)-acetic anhydride (2 X 10^4 cpm) in

acetone was added and incubated at 25°C for 45 min. Cold acetic anhydride(10µl) was added and the pH was adjusted to 8.5 before incubating for another 45 min. One hundred microlitres of glacial acetic acid was added to the preparation followed by the addition of 100µl of TFA. It was centrifuged at 2000 rpm for 5 min to remove the precipitated proteins. Supernatant was flash evaporated to dryness. Added 0.6 ml of 100mM pyridine/acetate pH 5.0 and further flash evaporated to dryness. The process was repeated 5 times to ensure the removal of free (^{14}C)-acetic anhydride. The ^{14}C- incorporation to the preparations were very low. To the dried preparations 100µl of 50% TFA was added, mixed and centrifuged at 15,600g X 3 min. Supernatants (100µl) were subjected to HPLC using a 10µm Lichrosorb RP 8 column (4 X 250 mm). The HPLC gradient III was formed from solvent A (water, 0.1% TFA) and solvent B (isopropanol,0.1%TFA). Fractions of 0.5 ml were collected at 1 min intervals directly into scintillation vials. The fractions were counted in LKB Liquid Scintillation Counter at ambient temperature.

The same protocol of proteolytic digestion was carried out for the neuronal glycoproteins from second trimester and full term fetal brains. The HPLC analysis revealed the absence of proteolytic cleavage by trypsin and pronase for the neuronal glycoproteins from both sources [Fig. 24,25]. The very low incorporation of ^{14}C in to the glycoproteins after the proteolytic digestion, itself was a manifestation for the absence of

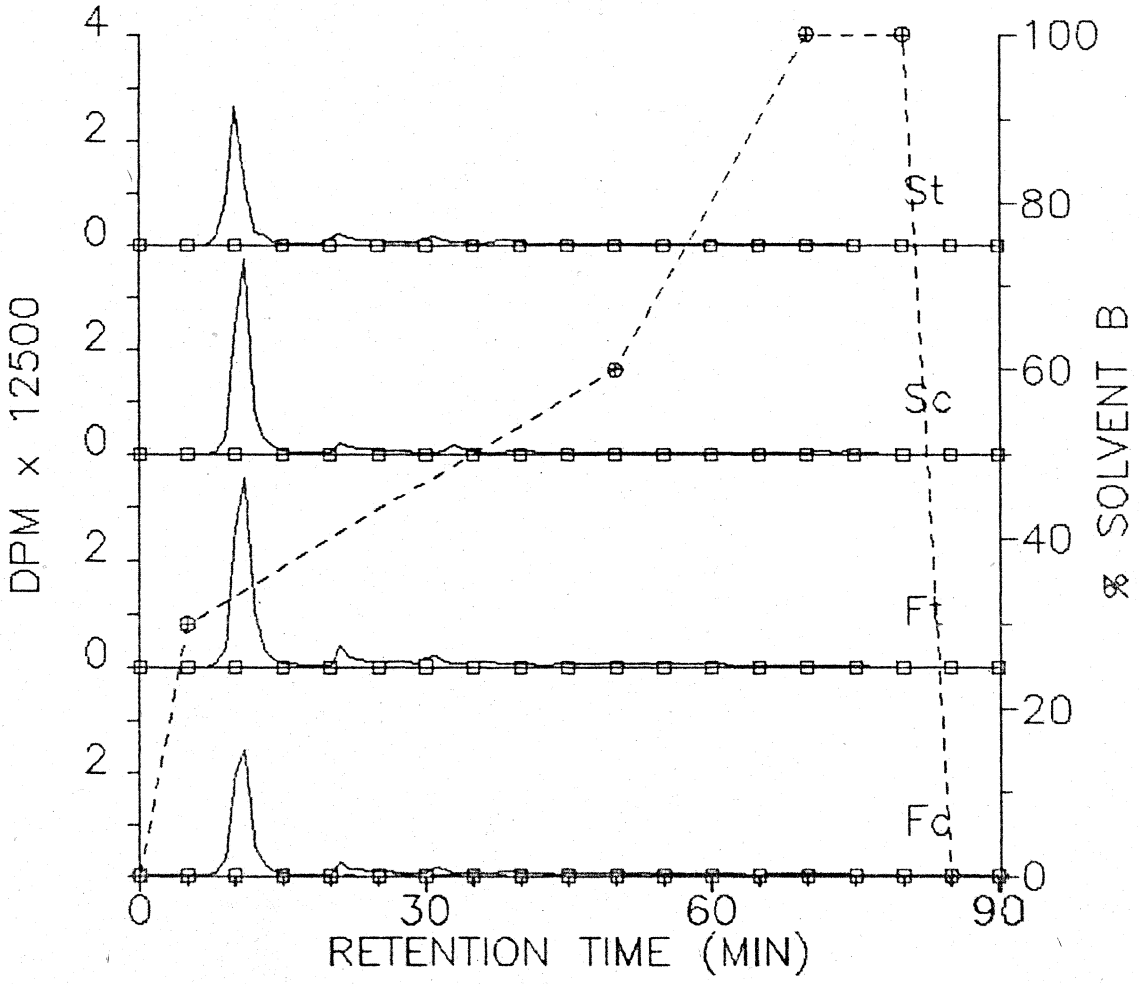


Figure 24. HPLC analysis of trypsin digested neuronal glycoproteins on RP-8 in gradient III (0---0). Flow rate = 0.5ml/ min. Experimental details are described in the text. St-Second trimester neuronal glycoproteins test, Sc-Second trimester neuronal glycoproteins control, Ft-Full term neuronal glycoproteins test, Fc-Full term neuronal glycoproteins control.

□ [^{3}H] — [^{14}C]

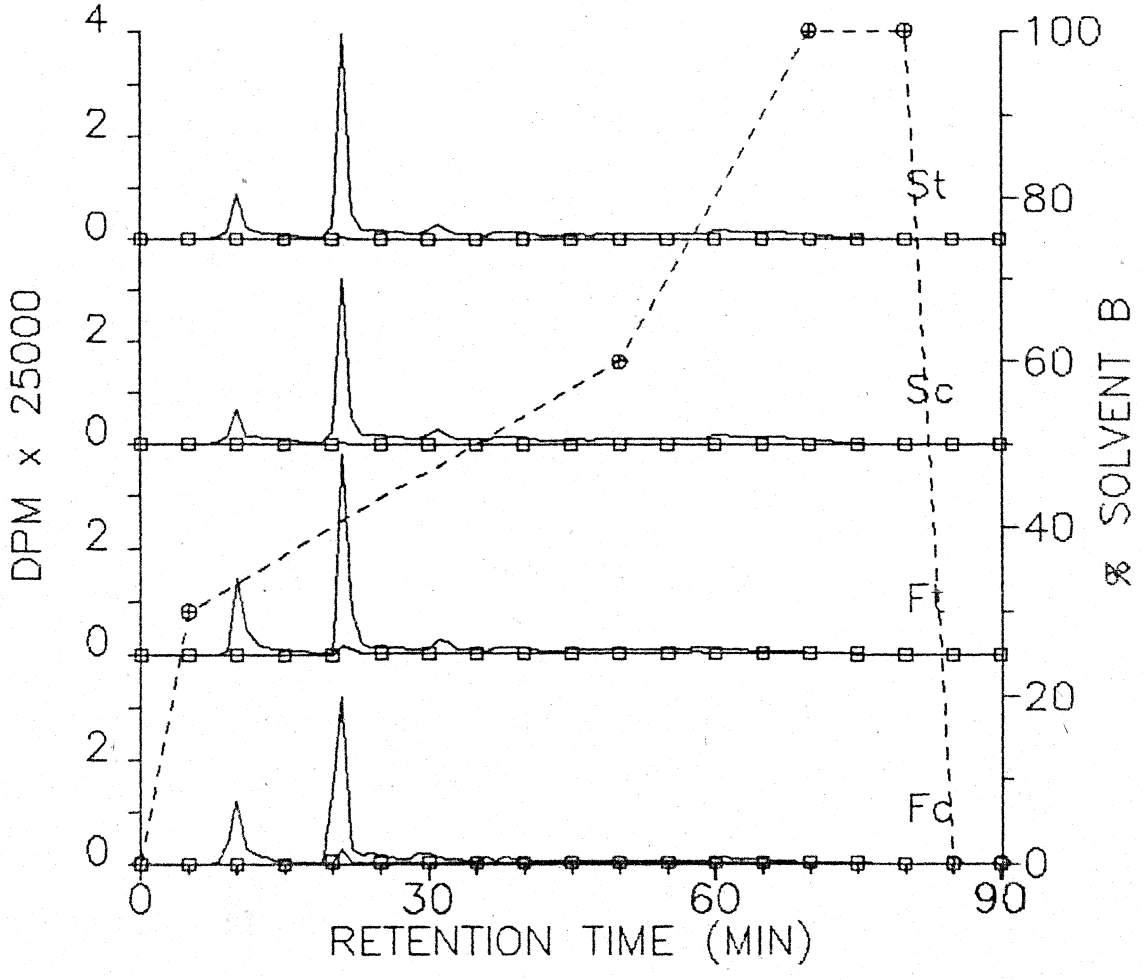


Figure 25. HPLC analysis of pronase digested neuronal glycoproteins on RP-8 in gradient III (0---0). Flow rate = 0.5ml/ min. Experimental details are described in the text. St-Second trimester neuronal glycoproteins test, Sc-Second trimester neuronal glycoproteins control, Ft-Full term neuronal glycoproteins test, Fc-Full term neuronal glycoproteins control.

□ [^{3}H] — [^{14}C]

proteolytic cleavage. The Dansyl-Edman procedure demonstrated the presence of lysine residues in neuronal glycoproteins, the potential sites of cleavage for trypsin. The resistance of neuronal glycoproteins against proteolytic digestion could be attributed to their carbohydrate chains having a protective function. The steric hindrance imparted by the oligosaccharide chains must be restricting the accessibility of pronase and trypsin for their cleavage sites. Deglycosylation or periodate oxidation of carbohydrates of peroxidases have been reported to increase their susceptibilities to proteolysis by trypsin(138). The hydrophobic nature of cell surface proteins renders them resistant to reduction and alkylation reducing the yield of peptide fragments generated by trypsin(231). The murein lipoprotein of the outer membrane of E.Coli was not completely digested by trypsin, chymotrypsin or papain(34).

Phase partitioning of neuronal glycoproteins

Neuronal glycoproteins labelled with (^{3}H)-acetic anhydride was extensively dialysed against distilled water to remove the free acetic anhydride. After dialysis 0.6ml of the preparation(26,000 cpm) was mixed with 1 ml of methanol and 2ml of chloroform. It was mixed and centrifuged to separate the phases. The separated phases were dried under vacuum and counted for radioactivity. The phase partitioning resulted in the recovery of 87% radioactivity in the lower organic phase, 2% in the upper aqueous phase and 11% in the protein interphase. The

upper and lower phases were negative for lectin binding whereas the interphase was positive. Phase partitioning of (^{3}H)-acetic anhydride was carried out by the same procedure described above, which resulted in the recovery of 11% radioactivity in the upper phase and 89% in the lower phase.

In another experiment the tritiated neuronal glycoproteins (26,000 cpm) were suspended in 0.1ml of distilled water and precipitated with 8 volumes of methanol and washed 4 times with methanol. The methanol washings were pooled together, dried under vacuum and counted for radioactivity. Of the total radioactivity recovered, 96% was present in the methanol washings and the remaining 4% in protein pellet. The methanol washings were negative for lectin binding whereas the protein pellet was positive.

PART IV - DIFFERENTIAL LECTIN BINDING STUDIES ON NEURONAL CONCANAVALIN A-BINDING SURFACE GLYCOPROTEINS

Elucidation of the structure of membrane glycoprotein sugar chains is indispensable in analyzing the molecular mechanisms of cell sociology and to study the cell surface changes during differentiation. In most cases membrane glycoconjugates are very difficult to isolate in sufficient quantities for structural studies. The glycoconjugates usually exhibit microheterogeneity in the structure of their carbohydrate moieties which further hinders the characterisation studies.

The structures of carbohydrate chains of glycoproteins have been analyzed by conventional methods such as gas-liquid chromatography and mass spectrometry. These analytical methods are often limited in application because they require relatively large amounts of sample and expensive instrumentation.

Lectins are sugar binding proteins of non-immune origin that agglutinate cells or precipitate glycoconjugates(102). Each lectin binds specifically to a certain sugar sequence in oligosaccharides and glycoconjugates(218,101). Lectins have been widely used for histochemical detection of sugar chains on the cell surface(249), staining and structural elucidation of electrophoretically separated membrane glycoproteins(141,142), separation of cells in different differentiation stages(31,299)

and isolation of membrane glycoconjugates on a preparative scale by affinity chromatography(183).

A combination of chemical and enzymatic treatments with lectin affinity studies have been used to establish the structures of oligosaccharide chains in glycoproteins(108,214). Different lectins are able to recognize different saccharide sequences on the same glycan structure. However in general, the broad specificity and cross-reactivity of lectins with different oligosaccharide structures make it impossible to elucidate their correct structure. Glycoprotein visualization with lectins after electrophoretic separation followed by transfer to nitrocellulose paper offers the advantage of greater accessibility of oligosaccharide side chains to the various ligands(214,49). Once a complex has been created and detected, it can further be analyzed by subjecting to solutions containing sugar haptens; thus the bound lectin can be specifically competed off the blot. Protein blotting has been used for the analysis of glycoproteins in three different ways. The first has been via probing with lectin (53,16,98). The second approach entails in situ enzyme modification of glycoproteins(237,155) and the last involves a direct sugar stain, for example enzyme-hydrazides(154). The procedure of in situ enzyme modification is based on the fact that glycoprotein is immobilized yet accessible to glycosidase treatment. By choosing various endo- or exoglycosidases and the right lectin, one can "sequence" an oligosaccharide side chain.

For the present study, lectins coupled to periodate oxidised horse radish peroxidase were used to carry out the differential lectin binding studies.

The neuronal glycoproteins separated on 8% SDS-PAGE was electrophoretically transferred to nitrocellulose for lectin affinoblotting. The transfer buffer consisted of 25mM Tris / 192mM glycine pH 8.3-20% (v/v) methanol(282). The gel was rinsed in distilled water after electrophoresis and soaked briefly in the transfer buffer along with the nitrocellulose membrane. They were placed one above the other and secured in the Electrophoretic Transfer Kit, between layers of Whatman No.1 filter paper(6 each), also soaked in the transfer buffer. A current of 1mA/cm^2 was applied for 2h with the nitrocellulose sheet facing the anode. The blot was stained with amidoblack (0.1% in 45% methanol-10% acetic acid) and destained with 90% methanol-2% acetic acid(244). The electrophoretic transfer of neuronal glycoproteins by the above method was complete as indicated by the absence of stainable protein bands in the gel after the transfer.

Lectin affinoblotting of neuronal glycoproteins after electrophoretic transfer to nitrocellulose

As described earlier the neuronal glycoproteins exhibited anomalous behaviour on SDS-PAGE. The lectin affinoblotting on nitrocellulose transfers of neuronal glycoproteins was carried out in order to further asses the above mentioned phenomenon. The

probable differential binding of the various SDS-PAGE protein bands by different lectins would give further informations on the possible anomalous SDS binding to neuronal glycoproteins. It was carried out by the same protocol as described later for the dot enzyme linked lectin assay. The HRP-conjugates of Con A, WGA and RCA stained all the bands of neuronal glycoproteins. The higher sensitivity of HRP-lectin conjugates detected even the minor bands between the major SDS-PAGE bands making their identification difficult. The optical density of the stained protein bands was measured by densitometry. The nitrocellulose was dried on Whatman No.1 filter paper and immersed in liquid paraffin to achieve translucency for scanning densitometry. The optical density of the stained protein bands were measured at 610 nm for amidoblack and 545 nm for HRP-lectin stain. The densitometry revealed the same profile of optical density for the protein bands with each of the 3 lectins [Fig.26]. The above mentioned observations further substantiated the anomalous SDS binding to neuronal glycoproteins. Different types of oligosaccharide chains could be present on the same polypeptide chain, which explained the positive interaction with all the lectins for each of the SDS-PAGE protein bands. However the optical density of the various stained bands could be different with the different lectin, depending on the distribution and number of oligosaccharide chains present on the polypeptide chains. In the present study, staining intensity for each of the

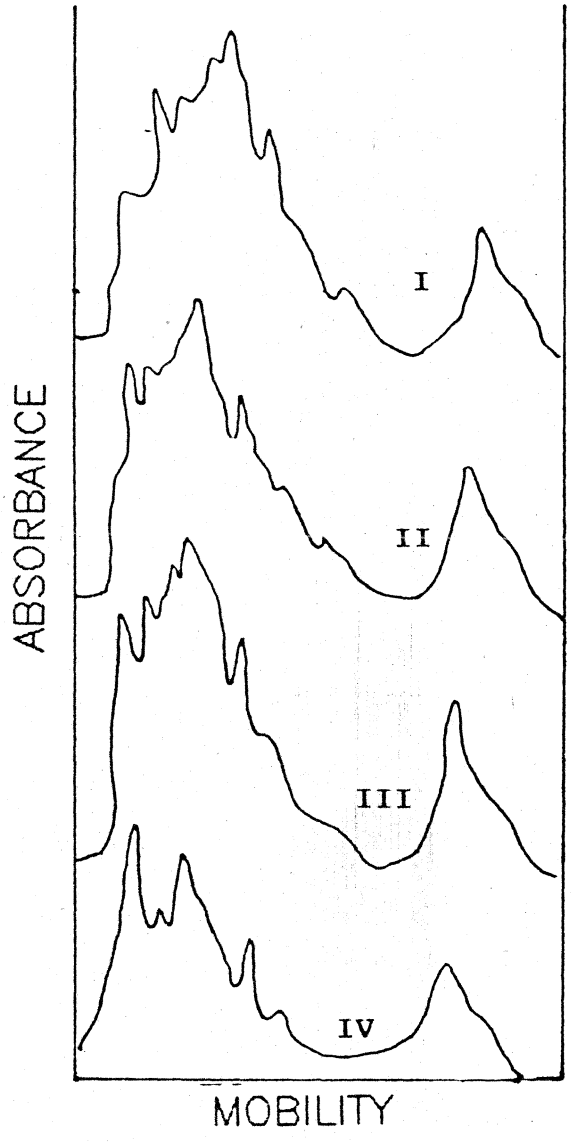


Figure 26. Scanning densitometry of the lectin affinoblott of neuronal glycoproteins. I. HRP-Con A, II. HRP-RCA, III. HRP-WGA, IV. Amidoblack. The optical density was measured at 610 nm for amidoblack and 545 nm for HRP-lectin stain. The experimental details are described in the text.

SDS-PAGE protein bands was the same with all three lectins, which could be due to the anomalous SDS-PAGE behaviour of neuronal glycoproteins.

Dot Enzyme Linked Lectin Assay

Differential lectin binding studies after chemical and enzymatic deglycosylation and the lectin binding in presence of their hapten sugars were performed by the dot enzyme linked lectin assay described below(97). The neuronal glycoproteins precipitated with 8 volumes of methanol was extracted twice with water: methanol: chloroform (3:8:4) to remove the noncovalently associated glycolipids. The possible contaminant Con A present in the glycoproteins would interfere with the differential lectin binding studies. The delipidated proteins were boiled at 100°C for 2min to inactivate the contaminant lectin. The proteins were dissolved in 40mM Li_2CO_3 pH 9.5-0.05% SDS at a concentration of 200µg/ml. The stock solution was serially diluted in the same buffer and 5µl aliquots were spotted on strips of nitrocellulose paper. They were soaked in the quenching buffer (20mM Tris/ HCl pH 7.4-150mM NaCl-0.05% Tween 20) and incubated at 25°C for 2h. The quenching buffer was replaced with HRP-lectin conjugates(10-20µg/ml) dissolved in the same buffer and further incubated at 4°C for 2h. The HRP-serotonin was used at a concentration of 100µg/ml. In case of Con A, the conjugate was dissolved in the quenching buffer containing 1mM $MnCl_2$, 1mM $MgCl_2$, and 1mM $CaCl_2$. After the incubation, the nitrocellulose strips were washed 3

times with the quenching buffer at 4°C followed by 20mM Tris/HCl pH 7.4-150mM NaCl at 25°C. The nitrocellulose strips were incubated with the chromogenic substrate for 30 min for colour development. The nitrocellulose strips were washed twice with distilled water. The colour intensity depends on the affinity between glycoproteins and lectin-peroxidase reagents and also on the specific activities of the lectin-peroxidase reagents. The lectin probes provided some information as to the nature and composition of oligosaccharide side chains in glycoproteins.

Lectin binding in presence of hapten sugars

The dot enzyme linked lectin assay was performed with HRP-conjugates of Con A, WGA, RCA, JSA, AHA, SBA, PNA and pea lectin. All the lectins except SBA and PNA interacted with the neuronal glycoproteins. For hapten sugar inhibition studies, the respective hapten sugars were also incorporated in the quenching buffer and in the HRP-lectin solutions according to the concentrations given in Table 7. For the hapten inhibition study with Con A, the starting concentration was 325µg/ml for the neuronal glycoproteins instead of 200µg/ml used with other lectins. The interactions of all the above mentioned lectins with the glycoproteins could be inhibited by their hapten sugars [Table 7]. When compared to the complete inhibition of Con A binding with 500mM α-MG, inhibition by 15mM α-MG was partial. The affinity of JSA, AHA and Con A for the neuronal glycoproteins was higher as compared to RCA.

TABLE 7. LECTINS BINDING TO NEURONAL GLYCOPROTEINS

HRP-LECTIN	HAPTEN SUGAR	TEST	CONTROL
CON A	(a) 15mM α-MG	+ + + + + +	+ + + + - -
	(b) 0.5M α-MG	+ + + + + +	- - - - - -
RCA	0.2M Lactose	+ + + - - -	- - - - - -
WGA	0.1M GlcNAc	+ + + + + +	- - - - - -
JSA	0.2M Galactose	+ + + + + +	- - - - - -
AHA	0.2M Galactose	+ + + + + +	- - - - - -
Pea Lectin	0.2M Glucose	+ + + + + +	- - - - - -
SBA		- - - - - -	
PNA		- - - - - -	

Lectin affino dot blotting was carried out for serially diluted glycoproteins with the concentration increasing from left to right. (+) for positive and (-) for negative lectin binding. For experimental details see the text.

Differential lectin binding studies after chemical and enzymatic deglycosylation

Deglycosylation of glycoproteins has been achieved enzymatically, biosynthetically using inhibitors of glycosylation such as tunicamycin, and chemically(171,14,265). The enzymatic approach has been found to be superior for structural studies because of its narrow substrate specificity.

Trifluoromethanesulfonic acid treatment

Deglycosylation by TFMS results in the destruction of oligosaccharide chains retaining the intact polypeptide chain. The extent of deglycosylation depends on the duration and the temperature. The neuronal glycoproteins were subjected to TFMS treatment according to the method of Sojar and Bahl(265). Forty microlitres of anhydrous TFMS was added to 100µg of dry protein taken in a test tube, flushed with nitrogen and sealed with parafilm. The preparation was incubated at 25°C for 3h. It was cooled to -10°C in ice and neutralized by the gradual addition of 200µl of 60% aqueous pyridine also previously cooled to -20°C. The proteins were precipitated with 4 volumes of methanol after 50µl of 10% SDS was added and further washed with 80% methanol. The glycoproteins were not susceptible to total deglycosylation under the experimental conditions as indicated by the residual binding of Con A, WGA, JSA and AHA [Table 8].

TABLE 8. LECTIN BINDING TO NEURONAL GLYCOPROTEINS AFTER TFMS TREATMENT AND PERIODATE OXIDATION

HRP-LECTIN	CONTROL	TFMS	$NaIO_4$
CON A	+ + + + + +	+ + - - - -	+ - - - - -
WGA	+ + + + + +	+ + + - - -	+ + + - - -
RCA	+ + + - - -	- - - - - -	- - - - - -
JSA	+ + + + + +	+ + + - - -	+ + - - - -
AHA	+ + + + + +	+ + + - - -	+ + - - - -
Pea Lectin	+ + + + + -	+ + - - - -	+ - - - - -

Lectin affino dot blotting was carried out for serially diluted glycoprotins with the concentration increasing from left to right. (+) for positive and (-) for negative lectin binding. For experimental details see the text.

Periodate oxidation

The susceptibility of carbohydrate moieties to periodate oxidation was examined. The oligosaccharide chains were modified by a mixture of 40mM $NaIO_4$ and 80mM $NaCNBH_4$ in 200mM sodium acetate buffer pH 3.5 mixed in equal volumes just before use(281). Two hundred microlitres of the reaction mixture was added to 50µg of the protein suspended in 200µl of 200mM sodium acetate buffer pH 3.5. The preparation was mixed well and incubated at 4°C for 24h in the dark. One hundred microlitres of 20%(v/v) glycerol was added to stop the reaction. The proteins were precipitated with 4 volumes of methanol after 80µl of 10% SDS was added. The glycoprotein oligosaccharide chains were not susceptible to total modification under the experimental conditions as indicated by the residual binding of Con A, WGA JSA and AHA [Table 8].

Nitrous acid deamination

The glycoproteins were subjected to nitrous acid deamination according to the method of Ferguson et al(78). One hundred micrograms of protein dissolved in 0.45 ml of 250mM sodium acetate buffer pH 3.5 containing 200mM $NaNO_2$ was incubated at 25°C for 5h. The proteins were precipitated with 4 volumes of methanol. The lectin binding to the glycoproteins was not sufficiently altered [Table 9]. Glucosaminyl linkages in oligosaccharides are cleaved by nitrous acid deamination. The removal of acetyl groups from the N-acetylglucosamine residues is

TABLE 9. LECTIN BINDING TO NEURONAL GLYCOPROTEINS AFTER NITROUS ACID DEAMINATION

HRP-LECTIN	CONTROL	TEST
CON A	+ + + + + +	+ + + + + −
WGA	+ + + + + +	+ + + + + −
RCA	+ + + − − −	+ + − − − −
JSA	+ + + + + +	+ + + + + +
AHA	+ + + + + +	+ + + + + −
Pea Lectin	+ + + + + +	+ + + + + −

Lectin affino dot blotting was carried out for serially diluted glycoproteins with the concentration increasing from left to right. (+) for positive and (-) for negative lectin binding. For experimental details see the text.

essential for the deamination to take place(119). The insufficient deamination observed in the present study indicated the fact that all glucosamine residues in neuronal glycoproteins were N-acetylated.

Chemical desialylation

Fifty micrograms of dry proteins were suspended in 200µl of 0.1N H_2SO_4 and incubated at 80°C for 1h. The proteins were precipitated with 4 volumes of methanol after the protein solution was made to 2% SDS. The chemical desialylation diminished the binding of serotonin and WGA, whereas the galactose binding lectins (RCA, JSA and AHA) did not exhibit enhanced affinity [Table 10].

Neuraminidase treatment

Neuraminidase treatment of neuronal glycoproteins immobilized on nitrocellulose paper was performed as given below(120). Aliquots (5µl) of the serially diluted glycoproteins were spotted on nitrocellulose, with a starting concentration of 200µg/ml. Soaked in the blocking buffer (100mM sodium acetate / acetic acid pH 5.6-0.05% Tween 20) and incubated for 2h at 25°C. The blocking buffer was drained off and incubated with neuraminidase (1U/20ml) in the same buffer for 18h at 37°C. As a control another nitrocellulose paper spotted with serially diluted protein was incubated in the blocking buffer alone under similar conditions. The nitrocellulose sheets were washed 4 times with 20mM Tris / HCl pH 7.4-0.05% Tween 20, followed by

TABLE 10. LECTIN BINDING TO NEURONAL GLYCOPROTEIN AFTER CHEMICAL DESIALYLATION

HRP-CONJUGATE	CONTROL	TEST
SEROTONIN	+ + + + - - - -	+ - - - - - - -
WGA	+ + + + + + -	+ + - - - - -
RCA	+ + + - - - - -	+ + + - - - - -
JSA	+ + + + + + + - -	+ + + + + + + - -
AHA	+ + + + + + + - -	+ + + + + + + - -
SBA	- - - - - - -	- - - - - - -
PNA	- - - - - - -	- - - - - - -

Lectin affino dot blotting was carried out for serially diluted glycoproteins with the concentration increasing from left to right. (+) for positive and (-) for negative lectin binding. For experimental details see the text.

incubation with the respective HRP-conjugates of lectins and serotonin for 16h at 4°C. The neuraminidase treatment resulted in decreased binding to WGA and serotonin [Table 11].

The effect of NaCl on serotonin binding was checked by incubating the protein spotted nitrocellulose paper with HRP-serotonin dissolved in 20mM Tris / HCl pH 7.4-1M NaCl-0.05% Tween 20. After 16h incubation at 4°C, the nitrocellulose sheet was washed 3 times with the same buffer before colour development. The serotonin binding to the glycoproteins decreased significantly in the presence of 1M NaCl as compared to the control.

The following factors should be taken into consideration while attempting to analyze the observations on differential lectin binding studies. The study was carried out with total Con A eluate which could be a mixture of glycoproteins. It is difficult to make conclusions when different oligosaccharide chains are present even on one polypeptide chain. The source of the glycoproteins was a developing tissue where the synthetic pathway is incomplete. As described earlier, the glycoproteins exhibited a tendency to form micellae. The formation of micellae in aqueous solutions might prevent the accessibility of oligosaccharide chains for chemical and enzymatic modifications. In general the hydrophobic membrane proteins tend to form aggregates diminishing their accessibility for chemical reagents.

The biantennary complex type oligosaccharide chains are

TABLE 11. EFFECT OF NEURAMINIDASE TREATMENT ON THE BINDING OF WGA AND SEROTONIN TO NEURONAL GLYCOPROTEINS

HRP-CONJUGATE	CONTROL	TEST
WGA	+ + + + + -	+ + - - - -
SEROTONIN	+ + - - - -	- - - - - -

Lectin affino dot blotting was carried out for serially diluted neuronal glycoproteins with the concentration increasing from left to right. (+) for positive and (-) for negative lectin binding. For experimental details see the text.

bound only weakly to Con A and can be eluted with 15mM α-MG. High mannose and hybrid type oligosaccharide chains establish strong interaction with immobilized Con A and required 500mM α-MG for elution(218). The neuronal glycoproteins required 500mM α-MG for the complete inhibition of Con A binding which indicated the presence of high mannose and hybrid or only one of the two types of oligosaccharide side chains. The partial inhibition of Con A binding by 15mM α-MG revealed the presence of biantennary complex type of oligosaccharide side chains.

According to the recent reports, the GlcNAcβ1-4Manβ1-4GlcNAc β1-4GlcNAc-Asn structure is essential for the tight binding of glycopeptides to WGA(218). Glycopeptides with hybrid and bisected biantennary sugar chains fulfilled this structural requirement and were reported to bind with WGA-Sepharose(308). High mannose and biantennary or triantennary complex type glycopeptides did not bind on WGA-Sepharose. The presence of sialic acid residues in glycopeptides has been implicated in their enhanced interaction with WGA as found in the present study. Residual binding of neuronal glycoproteins to WGA after enzymatic and chemical desialylation indicated either the presence of hybrid and / bisected biantennary types of oligosaccharide side chains. Several workers have shown that the binding of WGA to cells or glycopeptides was decreased after treatment with neuraminidase(21,202,42). However the inhibitory effect of N-acetyl neuraminic acid is weaker than that of

GlcNAc(20) and the presence of clustering sialyl residues may be necessary for the strong interaction of sialoglycoconjugates with WGA(22). The poly(N-acetyllactosamine)-type glycans found in mammalian cell surface glycoproteins has been reported as another major binding site for WGA (89,285).

Significant decrease in the binding of serotonin to the neuronal glycoproteins after the chemical and enzymatic desialylation further demonstrated the presence of sialic acid residues. The serotonin binding to sialic acid was an electrostatic interaction and was inhibited by 1M NaCl. Affinity chromatography utilising the interaction of serotonin with NeuAc and its derivatives have been employed for the isolation of sialoglycoproteins(268). The sialic acid residues of neuronal glycoproteins were involved in binding to serotonin at the cell surface. In general a higher percentage of sialic acid has been reported in the developing and oncological tissues(194,314). The comparatively lower sialic acid content of the Con A-binding neuronal glycoproteins could be attributed to the mild sonication step involved in their isolation procedure. As mentioned earlier, drastic sonication of delipidated neuronal proteins resulted in the loss of 25% bound carbohydrate. Sialic acids were the terminal and the most labile carbohydrate residues in glycoproteins.

Peanut agglutinin and soybean agglutinin have been demonstrated to bind preferentially to mucin type

glycopeptides(218). Peanut agglutinin binds preferentially to Galβ1-3GalNAcα1-Ser/Thr and SBA shows higher affinity for GalNAcα1-Ser/Thr. The meagre interaction of SBA and PNA with neuronal glycoproteins indicated limited number of O-linked oligosaccharide chains. These results are in agreement with the previous studies on whole brain, where the O-linked oligosaccharides was found to comprise only 10-20% of the glycoprotein-bound carbohydrates(189). The limited number of O-linked oligosaccharide chains present in the neuronal glycoproteins might not have been available for lectin binding. In glycoproteins the O-linked carbohydrate chains are comparatively short and buried inside masked by the longer and more branched N-linked oligosaccharide chains. The micellae formation of neuronal glycoproteins could have further occluded the O-linked sugar chains making it less accessible to the lectins. The steric hindrance due to the large size of PNA molecule might have prevented it from interacting with specific sugar chains of the glycoproteins. There have been reports that in addition to the carbohydrate chains, lectins recognise the peptide portion of glycoprotein structure(309). The number of O-linked carbohydrate chains in neuronal glycoproteins could be developmentally regulated. The incomplete synthetic pathway in developing brain might explain the lower number of O-linked oligosaccharide chains. In many cases desialylation exposed the PNA binding structures(53). In case of the neuronal glycoproteins

chemical desialylation did not expose PNA binding sites. The poor sensitivity of detection for PNA and SBA could be another factor which resulted in their almost negative interaction with the neuronal glycoproteins.

The affinity of Con A for the glycoproteins was more as compared to RCA, indicating a probable higher percentage of high mannose chains with less number of complex type oligosaccharide chains. Ricinus communis agglutinin primarily binds to the terminal Galβ1-4GlcNAc and much more weakly to the Galβ1-3GalNAc sugar sequence. The affinity of JSA and AHA for the neuronal glycoproteins was more as compared to RCA. The lower affinity for RCA interaction was further demonstrated by its total absence of binding after periodate oxidation and TFMS treatments. The jack fruit seed agglutinin and AHA have higher affinity for the α-galactose residues when compared to the β-galactose residues (272,8). The differential anomeric specification of these galactose binding lectins could explain their different binding affinities for the neuronal glycoproteins. The glycoproteins tend to form aggregates, diminishing the accessability of their oligosaccharide chains for lectin binding. Steric hindrance for the large RCA molecules to the galactose residues could be more as compared to JSA and AHA.

Terminal α-linked galactose units has been reported to occur in several cell membrane glycoconjugates(294). The possible presence of α-galactose residues in human brain might impart the

higher affinity of JSA and AHA for the neuronal glycoproteins compared to RCA. Alpha-galactose residues are considered to be either absent or cryptic in human system since serum antibody against α-galactose residues (anti-gal) was found to be present in humans. The brain cells function in an environment isolated from other organ systems in the body because of the blood brain barrier. Lacking both immunocompetent cells and antigenic stimulation, the normal brain does not synthesize antibody. Considering the above mentioned immunological background, the presence of α-galactose residues in human brain should not be considered a remote possibility. The immunological privilege of brain depends upon the integrity of the blood brain barrier. In pathological conditions the barrier breaks down and the inflammatory process allows lymphocytes and antibody to enter the nervous system. One of the causes for the central nervous system autoimmune disorders may be the immunological reactions against α-galactose residues. The blood brain barrier is incomplete in the human fetus. The permeability persists to the end of gestation and into the neonatal period. Antibodies of the IgG class from the mother pass across the placenta which include the anti-gal. The above mentioned observations questions the possible presence of α-galactose residues in fetal brain. However in the fetal brain, the α-galactose residues could remain cryptic during the period of brain development and its concentration may be developmentally regulated.

Investigations on the ligand specificity of jack seed agglutinin revealed its high affinity for the T-antigenic structure [1-β-D-galactopyranosyl-3-(α-2-acetamido-2-deoxygalactopyranoside)] in glycoproteins(48). This disaccharide forms the typical core structure of oligosaccharides linked to serine or threonine(13). The above mentioned data suggest the binding of JSA is specific for O-linked oligosaccharides and is not expected to bind any N-linked oligosaccharides(134). The neuronal glycoproteins exhibited strong interaction with JSA which indicated the possible presence of O-linked oligosaccharide chains furnishing the α-2-acetamido-2-deoxygalactopyranoside required for the lectin binding. When compared to PNA and SBA the sensitivity of JSA could be more in detecting the O-linked oligosaccharide chains. Hortin and coworkers have demonstrated the occurrence of a single O-linked oligosaccharide chain (even when sialylated) as adequate in mediating the binding of a glycoprotein to JSA(134). The higher sensitivity of JSA might have enabled it in detecting the limited number of O-linked oligosaccharide chains in the neuronal glycoproteins. The smaller size of JSA molecule further facilitated its interaction with the glycoproteins. The probable presence of α-galactopyranose residues in neuronal glycoproteins could explain their affinity for JSA. Most N-linked oligosaccharides do not contain any galactopyranose or 2-acetamido-2-deoxygalactopyranose units in α-anomeric linkages. The uncommon exceptions to this rule are N-

linked oligosaccharides bearing blood group antigens, which have been demonstrated to have low affinity for JSA(188). It is of further interest that the presence of a brain-specific disaccharide α-galactosyl-(1-3)-N-acetylgalactosamine has been demonstrated in rat, rabbit and chick brains(84).

The presence of a fucose residue attached to the innermost GlcNAc residue in the core of glycopeptides is essential for high affinity binding to pea lectin(162). The positive interaction with pea lectin demonstrated the presence of fucose residues in neuronal glycoproteins. Even an asparagine residue is required for high affinity binding of glycopeptides to pea lectin-agarose(309).

Ricinus communis agglutinin binds primarily to galactose moieties in complex or hybrid type sugar chains(218). Exposure of the underlying galactose residues by desialylation has been reported to render the protein positive or enhance the binding affinity for galactose binding lectins(53). The impairment of RCA interaction by sialylation is much greater when the galactose residues are substituted by sialic acid at C-3 rather than C-6(54). However desialylation did not enhance the affinity of galactose binding lectins (RCA, JSA and AHA) for neuronal glycoproteins. Desialylation of rat Transferrin with commercial neuraminidase or by acid hydrolysis did not result in its binding to RCA-Sepharose column whereas desialylation performed by incubation with rat liver endothelium caused its retention on

RCA-sepharose column(140). The galactosyl residues exposed by neuraminidase treatment or acid hydrolysis might have been modified by the desialylation process so as to make them unavailable for binding to RCA(140). The above mentioned observations can be extended to explain the similar results obtained with the neuronal glycoproteins. This was consistent with and supports the work of Hatten et al(125). They have reported the penultimate galactosyl residues exposed by neuraminidase treatment or acid hydrolysis were not susceptible to enzymatic cleavage by β-galactosidase. An alternative explanation would be that the penultimate galactosyl residues may be normally modified so that they would not bind to the galactose binding lectins after desialylation(140). Polysialosyl carbohydrate units have been reported to occur in developing rat brain(80). The probable presence of similar structures in developing human brain could be another explanation for the limited exposure of terminal galactose residues in the neuronal glycoproteins after desialylation.

The incomplete deglycosylation of neuronal glycoproteins by TFMS treatment and the partial periodate oxidation could be attributed to their tendency to form micellae making the oligosaccharide chains inaccessible to the reagents. The fixed duration for which these chemical reactions were carried out might not have been sufficient in unfolding the protein micellae and exposing their carbohydrate chains. In general the

hydrophobic membrane proteins tend to form aggregates diminishing the accessibility for chemical reagents.

The differential lectin binding studies were carried out with neuronal glycoproteins from second trimester and full term fetal brains. The glycoproteins did not appear to exhibit any prenatal developmental changes in the structure of their oligosaccharide chains under the limited conditions of study.

PART V - IMMUNOLOGICAL STUDIES

Immunochemical recognition of an antigen combined with characterization by physicochemical techniques allows the antigen to be characterized in molecular terms. In addition to immunodetection, the recognition of an antigen as specific to the nervous system would enable in characterizing the behaviour of the antigen during cell growth, differentiation and oncogenesis. This would lead to a better understanding about the molecular basis of neural development and in assessing the role of cell surface glycoproteins in cell sociology. Polyclonal and monoclonal antibodies have been used for the identification of carbohydrate structures on cell surfaces(75,76). The behaviour of oncofetal antigens which undergo changes with cell differentiation and oncogenesis have been characterized with antibodies against known, specific cell surface carbohydrate epitopes(152). From earlier reports 25% of the antibody produced against cell surfaces are directed towards carbohydrate residues present on the cell surface(187). Sensitivity and specificity of the immunological techniques makes them a valuable tool for the detection, localization and quantitation of antigens in complex tissues. The majority of procedures available for characterization of a protein require the protein to be present in pure form and in sufficient amounts. Such conditions are difficult to fulfil for many proteins of the nervous system (26).

However, immunochemical procedures are not limited by these requirements and have proven valuable tools in the analysis of neural markers. The introduction of monoclonal antibody technology was a major advance and has already led to the recognition of many new cell type-specific antigens. By this technology it is possible to obtain specific antibodies using preparation containing many antigens.

Immunization of rabbits and guinea pigs with whole neurons was attempted with the objective of raising polyclonal antibodies against neuronal cell surface. Production of polyclonal antibodies against neuronal cell surface Con A-binding glycoproteins would enable further characterisation these glycoproteins with respect to the following aspects.

(a) To ascertain the specificity of these glycoproteins with regard to cell type, tissue, species and subcellular location.

(b) To asses the changes in concentration and localization of these cell surface glycoproteins during the various stages of development.

The presence of antibody was checked by the following immunochemical techniques(199,24).

(a) Ouchterlony double diffusion: The gel plates were prepared with 1% agarose in phosphate buffered saline pH 7.4-0.1% NaN_3-0.1% Triton X-100. After loading the antigen and antibody the plates were incubated at 25°C in moist atmosphere for 3-4 days.

(b) Counter current electrophoresis: The gel plates were prepared in 1% agarose in 0.075M barbitone buffered saline pH 8.3-0.1% Triton X-100. Twenty microlitre of sample was electrophoresed at 5 mA/plate for 90 min. For a better visualization of precipitin lines, the gels were stained with coomassie brilliant blue.

(c) Immuno electrophoresis: Twelve millilitres of 1% agarose prepared in 50mM barbitone / acetate pH 8.6-0.2% Triton X-100-0.1% NaN_3 was layered on glass plates (84 X 94mm). The antigens (20µl) were layered in the wells and subjected to electrophoresis in the same buffer without Triton X-100, at 5-10 mA / plate for 90 min. Two hundred microlitre of antibody was loaded into slots after the electrophoresis and incubated for one week at 4°C. Slides were further incubated in the 0.9% NaCl at 4°C with 3 changes for 2 days. The gels were blotted dry with filter paper and dried at 50°C for 30 min. The dried gels were stained for 5 min with coomassie brilliant blue and destained.

(d) Dot enzyme linked Immunosorbent assay: Five microlitre of the antigen at a concentration of 0.5-1 mg/ml was spotted on nitrocellulose paper and incubated at 25°C for 2h in quenching buffer [20mm Tris/HCl pH 7.4-150mM NaCl(TBS) containing 2.5% gelatin, 0.05% Tween 20 and 0.1% NaN_3]. This was followed by incubation with rabbit antibody (6-10mg/ml) in the incubation buffer (TBS containing 0.25% gelatin and 0.05% Tween 20) overnight at 4°C. It was washed 3 times with the quenching buffer at 25°C at intervals of 10 min. It was then incubated at 4°C for

2h in goat anti rabbit Γ-globulin-HRP conjugate(200µg/ml) in the incubation buffer. The plates were washed 3 times at 25°C in the quenching buffer followed by TBS. The colour was developed by incubating in chromogenic substrate for HRP as described earlier.

Immunization of rabbits and guinea pigs with intact second trimester neurons was attempted. Neurons corresponding to a protein concentration of 0.5-1mg in 1ml saline was mixed with an equal volume of Fruend's complete adjuvant and injected intraperitonially in rabbits and at multiple intramuscular sites in guineapigs. Succeeding doses were given after every 10 days, intramuscularly or subcutaneously at multiple sites in complete or incomplete adjuvant for rabbits. Guinea pigs were administered every week at 4 intramuscular sites in Fruend's complete adjuvant. Preimmune sera was collected and sera was collected periodically after the immunization to check for the antibody production. Neurons were solubilized by sonication(20 X 2min) in PBS pH 7.4 containing 3% Triton X-100. The preparation was mixed with an equal volume of PBS, so that the concentration of Triton X-100 is lowered to 1.5%. The 100,000g X 60 min supernatant was used as antigen at a concentration of 5-10 mg protein/ml. The presence of antibody in immune serum was checked by immunodiffusion and counter current immunoelectrophoresis against the antigen. There was no antibody production against intact neurons after 6 months of immunisation in rabbits and 2 months in guinea pigs as detectable by the above two methods. However the

animals were sacrificed and sera collected for checking antibody response against neuronal Con A-binding glycoproteins. The preimmune serum and antisera were 50% saturated with ammonium sulfate at 4°C. The precipitated Γ-globulin fraction was collected by centrifugation at 10,000g for 15min and dialysed extensively against TBS pH 7.4-0.05% Tween 20 at 4°C. The presence of antibodies to neuronal Con A-binding glycoproteins in these fractions were checked by dot enzyme linked immunosorbent assay. The glycoproteins did not bind to the Γ-globulin fractions purified from neuron immunized rabbit and guinea pig sera.

In another attempt immunization of rabbit was carried out with neuronal Con A-binding glycoproteins. The second trimester neuronal Con A-binding glycoproteins(400µg) were resolved on 10% SDS-PAGE. The 50% methanol fixed, coomassie brilliant blue stained gel corresponding to the region of neuronal glycoprotein bands were cut out. The gel pieces were homogenised with Potter Elvehjm-homogenizer in 6ml of PBS pH 7.4. The preparation was further mixed with 200µg of delipidated neuronal glycoproteins and divided into 4 equal aliquots of 1.5ml each. Each of the 4 aliquots were injected into the rabbit at 10 day intervals. The aliquots were mixed with equal volumes of Fruend's complete adjuvant and injected intramuscularly to the 4 limbs of the rabbit. The preimmune serum was collected from the animal before immunization. Formation of cysts was observed at the sites of injection. The cyst formation could have been due to the presence

of acrylamide in the injected sample. The animal was bled 10 days after the last injection. Sera was collected randomly from 5 rabbits as control. Antibody production against the glycoproteins was checked using the dot enzyme linked immunosorbent assay. The neuronal glycoproteins cross reacted with its antiserum as well as with the antiserum raised against Con A-binding glycoproteins from second trimester human fetal glial cells. Moreover the preimmune serum and all the 5 control sera also cross reacted with the neuronal Con A-binding glycoproteins. The binding of the antisera to the neuronal glycoproteins was traced to the Γ-globulin fraction obtained by 50% ammonium sulfate saturation of the respective antiserum. This indicated an immunological cross reaction between the antigen and antisera.

To further asses the nature of the immunological cross reaction, the Γ-globulin fractions from control and immune sera were resolved by Protein A-Sepharose 4B chromatography. The column (1 X 7cm) was equilibrated in 20mM Tris buffered saline pH 7.4 (TBS) at 4°C and the sample was loaded in the same buffer. The column was washed with 10 bed volumes of TBS and eluted with 300mM glycine/HCl pH 3.0. The eluted fractions were immediately neutralized with 2M Tris base and precipitated with 50% ammonium sulfate saturation. The pellet obtained by centrifugation at 10,000g X 30 min was suspended in and dialysed against TBS containing 0.05% Tween 20. Protein A-binding IgG fractions from both the immune serum and the control sera cross reacted with the

neuronal glycoproteins. The possibility of specific antibody production against the glycoproteins in addition to the nonspecific cross reaction was investigated. Serially diluted antigen was spotted on two strips of nitrocellulose paper. One strip was incubated with the control serum and the other was incubated with immune serum. The protein content of the two sera was similar. Dot enzyme linked immunosorbent assay revealed no difference between the test and the control indicating the absence of specific antibodies against neuronal glycoproteins. In conclusion, the neuronal glycoproteins could not elicit an immune response. Immunoelectrophoresis and counter current electrophoresis did not reveal any difference between the immune and control sera further confirming the absence of specific antibody production against the neuronal glycoproteins.

The lack of antibody production could be due to the low antigenicity of neuronal glycoproteins. Immunosuppression due to the presence of similar antigenic determinants in the injected animals could be another factor for the lack of antibody response. The tendency of neuronal glycoproteins to form micellae might be preventing their accessibility to the immune system of the injected animals. Hydrophobic membrane proteins tend to form aggregates which can hinder the recognition of their antigenic determinants by the immune system.

In general brain proteins in pure form are of low immunogenicity. Proteins such as S-100 and protein 14-3-2

elicited antigenic response only when administered conjugated to methylated BSA(175,204). Synaptin and D1, D2, D3 brain specific membrane proteins were defined by means of a polyspecific polyclonal rabbit antiserum raised against fractions of rat brain synaptosomal plasma membranes (29,146). Myelin proteins and glial fibrillary acidic protein on the other hand were highly immunogenic(211,286). Purified brain glycolipids are potentially antigenic. They are haptens and can elicit an immune response only when covalently linked to carrier polymer, as micellar aggregates with a heterologous carrier polymer or as natural membrane fragments(113). It may be possible to elicit an antibody response against the neuronal glycoproteins by immunization after covalently linking it to another protein like bovine serum albumin or after incorporating the glycoproteins into liposomes.

CHAPTER - IV

GENERAL DISCUSSION

GENERAL DISCUSSION

Development of nervous system is one of the most complex examples of morphogenesis. It involves both the formation of intricate tissue structures and their precise interconnection. There are three main sources of variability in neural networks during development: somatic developmental sequence of network formation and neuroanatomy, the chemical variation related to neurotransmitters and the electrical variation. The developmental changes in the human central nervous system are accompanied by specific changes at the molecular level. The cell surface glycoproteins play an important role in brain development. These cell surface glycoproteins have been postulated to coordinate the morphogenetic steps: neural induction, cell proliferation, migration, aggregation, cytodifferentiation, synapse formation, cell death and synapse elimination, all of these together constitute the neural development.

The present study was carried out with the aim of obtaining information on the Con A-binding neuronal surface glycoproteins from developing human brains. As part of the study the prenatal developmental changes in the Con A-binding glycoproteins were also investigated.

In developing brain, the synthetic process is incomplete, which resulted in the problems encountered during the purification and characterization of the glycoproteins. During

the isolation of neurons, the conventional trypsinisation of brain tissue could not be employed due to the extensive lysis of cells. The intercellular cementing substances might not have fully formed in the developing brain, which resulted in the lysis of the cells. The preparation of pure plasma membrane fractions could not be monitored due to the absence of conventional plasma membrane markers. In developing brain the synthetic pathway for the marker enzymes might be incomplete. The processing of asparagine-linked oligosaccharide chains of glycoproteins is a post translational process which takes place in the Golgi complex. According to the earlier reports most of the N-glycosidically linked carbohydrate units in nervous tissue glycoproteins were of the complex type(189). However the higher affinity of Con A for the neuronal glycoproteins as compared to RCA indicated a predominant number of high mannose carbohydrate chains with less number of complex type oligosaccharide chains. During the post translational glycosylation of proteins, the high mannose oligosaccharide chains are formed first followed by their modification to complex type carbohydrate chains in the golgi complex. The incomplete synthetic pathway in developing brain would explain the predominance of high mannose oligosaccharide chains in the neuronal glycoproteins. The above mentioned observations reflected the developing stage of the brain tissue used in the present study.

The isolated glycoproteins were hydrophobic in nature which added to the difficulties encountered in their isolation and characterization studies. The glycoproteins were insoluble in ordinary aqueous buffers and required detergents for their solubilization. The hydrophobicity of neuronal glycoproteins was further demonstrated on SDS-PAGE. In general the problems encountered during SDS-PAGE of hydrophobic membrane proteins included: (a) the accumulation of silver- or coomassie blue-stainable material at the sample wells, (b) extensive streaking of stainable material occurring throughout most of the gel lanes and (c) the inability to detect much stainable material in the gels that were substantially overloaded. All these problems were encountered during the SDS-PAGE of neuronal glycoproteins. Deglycosylation by TFMS treatment caused an apparent increase in the molecular weight on SDS-PAGE due to aggregation. An increase in hydrophobicity due to the removal of hydrophilic carbohydrate chains would explain the enhanced aggregation of neuronal glycoproteins following TFMS treatment. The removal of Triton X-100 from the Con A-eluate by dilution with TBS pH 7.4 and ultrafiltration resulted in the precipitation of neuronal glycoproteins even at concentrations of 0.25mg/ml. The hydrophobic membrane proteins are associated with a phospholipid bilayer and in general tend to form insoluble aggregates in aqueous media when they are removed from the hydrophobic media. The hydrophobicity of membrane proteins could be due to their

higher content of hydrophobic amino acids and their hydrophobic tertiary structural domains.

The neuronal glycoproteins appeared to have an inherent capacity to form micellae. This was evident from a number of observations during the study. Carbohydrate and sialic acid estimations before and after delipidation of the total Con A eluate indicated the presence of glycolipids in the 25°C eluted preparation [Table 5]. At the higher temperature of elution there was also an increase in the percentage of contaminant Con A in the preparation. The temperature dependant contamination of Con A and glycolipids in Con A eluate could be explained on the basis of the inherent capacity of neuronal glycoproteins to form micellae.

The neuronal plasma membrane proteins being hydrophobic were not sufficiently soluble in ordinary aqueous buffers and required Triton X-100 for solubilization. During Con A-Sepharose chromatography the hydrophobic neuronal proteins might have enhanced the interaction of Triton X-100 with Con A subunits. Moreover previous reports indicated the occurrence of possible hydrophobic interactions between lectins immobilized on CNBr-activated Sepharose and detergent solubilized glycoproteins(52). The surface hydrophobic patches of Con A molecules might have further facilitated this interaction. During the process of chromatography the neuronal glycoproteins could be forming mixed micellae with Triton X-100, associated lipids and

the Con A subunits. It is probable that the 500mM α-MG elution could be resulting in eluting these mixed micellae rather than only the neuronal Con A-binding glycoproteins. The mixed micellae formation could explain the presence of Con A and associated glycolipids in the Con A eluate. The formation of mixed micellae might be a dynamic equilibrium process, in direct proportion to the temperature at which the washing and elution were carried out. The washing and elution at 25°C instead of 4°C must have enhanced the mixed micellae formation which explained the presence of higher amounts of Con A and associated glycolipids in the Con A eluate when chromatography was carried out at the higher temperature. The presence of Triton X-100 might have further enhanced the formation of mixed micellae. Polyacrylamide gel electrophoresis in presence of Triton X-100 of proteins derived from membranes directly solubilized in the same detergent showed protein bands of extremely high molecular weight which may be due to the formation of heterogenous micellae(182). Glycophorin has been reported to undergo aggregation in neutral aqueous solutions, even in the presence of 6M guanidine hydrochloride(182). The self micellae formation of neuronal glycoproteins could explain the Con A leaching observed even during the chromatography of delipidated proteins in the absence of detergents.

The abnormal leaching of Con A observed during the chromatography of neuronal proteins in presence and absence of

Triton X-100 is not an universal phenomenon. When the same Con A-Sepharose was used for the purification of glycoproteins from other sources the extensive Con A leaching was not observed. The process can be attributed to the amphipathic nature of neuronal glycoproteins, which enables them to form self micellae or mixed micellae.

The anomalous SDS-PAGE behaviour of neuronal glycoproteins could also be explained on the basis of their tendency to form mixed micellae with SDS. The heterogenous mixed micellae formation could be a concentration dependant dynamic equilibrium process which explained the phenomenon of one protein band giving rise to the other bands on SDS-PAGE. The micellae formation of the glycoproteins was further manifested by gel filtration on Sepharose 6B. The gel filtered peak I might represent a micellar aggregate formed from a low molecular weight protein represented by peak II protein. Sephadex G75 chromatography confirmed the low molecular weight for the peak II protein. Urea-polyacrylamide gel electrophoresis further substantiated the tendency of the glycoproteins to form micellae. Heterogenous micellar aggregates formed from a low molecular weight protein (Gel filtered peak II) might represent the 4 bands on Acid-Urea-PAGE. The acetic anhydride labelling might have destabilized the micellae formation, leading to its total dissociation into monomers in the presence of urea, represented by the fast moving Urea-PAGE band of the gel filtered peaks. Urea alone might be insufficient in

the complete dissociation of the protein micellae to its monomers. The incomplete dissociation was manifested by the formation of heterogenous smaller micellae represented by the 4 bands on urea-PAGE.

The micellae formation could be attributed to the amphipathic nature of protein molecules. The presence of a hydrophobic domain separated from another hydrophilic region, together forming a bipolar configuration is essential for the formation of an amphipathic molecule. Integral membrane glycoproteins are potential candidates that fulfill this structural requirement. In the integral membrane glycoproteins, carbohydrate units are often clustered near one end of the molecule, and this hydrophilic portion is exposed on the external side of the plasma membrane. The asymmetric distribution of carbohydrate chains in the membrane glycoproteins might contribute to the bipolar configuration required for an amphipathic molecule. The blocked N-terminal for the neuronal glycoproteins could be due to the clustering of carbohydrate chains at that end. Acetic anhydride labelling of the neuronal glycoproteins might have diminished the number of positive charges present in the protein molecule essential for the micellae formation in presence of urea. The presence of detergents like Triton X-100 in the medium might enhance the protein micellae formation. There was an enhanced Con A leaching when the Con A-Sepharose chromatography was carried out in

presence of Triton X-100 as compared to its absence. The presence of Triton X-100 might have enhanced the mixed micellae formation resulting in the increased Con A leaching.

The inherent capacity of neuronal glycoproteins to form micellae explained many of their physicochemical properties. The glycoproteins were resistant to proteolytic digestion by trypsin and pronase. They were not susceptible to total deglycosylation by TFMS and periodate oxidation under the experimental conditions. Soybean agglutinin and peanut agglutinin could not detect the limited number of oligosaccharide chains present in the glycoproteins. The above observations could be attributed to the tendency of neuronal glycoproteins to form micellae making the oligosaccharide chains less accessible to the reagents. One of the causes for the low immunogenicity of neuronal glycoproteins could be their tendency to form self micellae preventing the accessibility of antigenic determinants to the immune system.

The same protocol of physicochemical studies were carried out for the neuronal glycoproteins from second trimester and full term fetal brains. Based on the limited number of physicochemical studies carried out with the available technology, no structural differences were detected in the glycoproteins during the prenatal development. Carbohydrate and sialic acid contents of the glycoproteins were in the same range. Determination of molecular weights on SDS-PAGE gave similar results. Sepharose 6B

chromatography resolved both sets of glycoproteins into two peaks with similar elution volumes. Reverse phase HPLC retention times were same for the glycoproteins. Both sets of glycoproteins exhibited resistance for proteolytic digestion with pronase and trypsin. The N-terminal of the glycoproteins were blocked in either case. Differential lectin binding studies did not reveal any prenatal structural changes in their oligosaccharide chains. However by the combination of more sensitive technology and monoclonal antibody methods, it may be possible to elucidate the structural changes during brain development.

One of the important observations in the present study was an indication for the probable presence of α-linked galactose residues in human brain. The higher affinity of JSA as compared to RCA and the strong interaction of JSA as compared to the almost negative interaction with SBA and PNA suggested the probable location of the α-galactose residues in carbohydrate chains. Considering the immunological privilege of brain, the presence of α-galactose residues in human brain cannot be considered as a remote possibility.

SUMMARY

SUMMARY

The present study was undertaken in an attempt to obtain information on the concanavalin A-binding neuronal cell surface glycoproteins of developing human brains. The study was expected to improve our knowledge about nervous system function, development and oncology. The isolation of neurons was an adaptation of previous procedures involving mechanical disruption of tissue by sieving followed by discontinuous sucrose density gradient centrifugation. Almost pure and morphologically intact neurons could be isolated by the two methods described. Fluoresceine isothiocyanate-concanavalin A binding of the intact neurons indicated the presence of Con A-binding surface glycoproteins.

The concept of pure plasma membrane preparation was abandoned due to the absence of detectable plasma membrane markers. Instead a plasma membrane enriched fraction was prepared by lysing the cells with 10mM EDTA and centrifugation at 8000g X 10 min. Glycoproteins were isolated by affinity chromatography on Con A-Sepharose 4B. Initial procedures with whole cell or plasma membrane enriched preparations, with or without prior delipidation, in the presence or absence of detergents like SDS or triton X-100, resulted in extensive leaching of lectin from the affinity matrix. The Con A leaching was minimised by pretreatment of Con A-Sepharose with 8M urea. The western blot

buffer 25mM Tris-700mM glycine pH 7.8 enabled an improved extraction of neuronal proteins.

The glycoproteins contained 180±60µg carbohydrate / mg protein. Sialic acid content of the glycoproteins was in the range of 35±5nmoles / mg protein. The glycoproteins eluted from Con A-Sepharose column contaminated with noncovalently associated glycolipids. Majority of the glycolipids were non-sialylated with a minor ganglioside component. The association of glycolipids is an additional evidence for the fact that neuronal glycoproteins were from the cell surface.

The amounts of glycolipids and Con A in the Con A eluate was more when the elution was carried out at 25°C as compared to 4°C elution. Temperature dependent mixed micellae formation between the glycoproteins, Con A and glycolipids might account for the higher amounts of Con A and glycolipids present in the Con A eluate obtained at 25°C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated the presence of 3 major groups of diffuse bands of molecular weights 65-72KDa; 52-63KDa; and 43-48KDa respectively. Each group of bands upon reelectrophoresis gave rise to all the 3 groups of bands of the original sample. The SDS-PAGE multiple band pattern may be accounted for by an additive effect of anomalous SDS binding, protein aggregation, heterogenous mixed micellae formation and microheterogeneity.

Chemical deglycosylation and deacylation did not alter the multiple band pattern on SDS-PAGE. The trifluoromethanesulfonic acid treatment caused an increase in the apparent molecular weights. Acid-urea-PAGE revealed 4 bands for the glycoproteins. They did not migrate in alkaline-urea-PAGE at pH 8.6, indicating an absence or a lower percentage of acidic amino acids. The glycoproteins did not contain ester linked fatty acids and GPI-anchor. The N-terminal of the glycoproteins were blocked.

The glycoproteins interacted with Con A, WGA, RCA, JSA and pea lectin and were inhibited by their respective hapten sugars. The various SDS-PAGE bands were not differentiated by Con A, RCA and WGA as evident from lectin affinoblotting. High mannose, hybrid and biantennary complex type oligosaccharide chains were present in the glycoproteins. The higher affinity of Con A as compared to RCA indicated predominant number of high mannose sugar chains over the complex type oligosaccharide chains. The higher affinity of JSA and AHA as compared to RCA indicated the probable presence of α-galactose residues in the neuronal glycoproteins. The positive interaction with JSA demonstrated the presence of O-glycosidically linked oligosaccharide chains. However the almost negative interaction of SBA and PNA with the glycoproteins indicated a limited number of O-linked carbohydrate chains. The probable presence of α-galactose residues in the carbohydrate chains would further explain the strong interaction of JSA with the glycoproteins as compared to SBA and PNA. The

glycoproteins were not susceptible to total deglycosylation by TFMS and periodate oxidation under the experimental conditions. The presence of fucose was demonstrated by pea lectin binding. Desialylation diminished the binding of serotonin and WGA whereas the galactose binding lectins (RCA, JSA and AHA) did not exhibit enhanced binding. Nitrous acid deamination did not produce sufficient change in the lectin binding indicating the glucosamine residues in the neuronal glycoproteins were all N-acetylated.

Gel filtration on Sepharose 6B resolved the tritiated glycoproteins into two peaks which exhibited similar elution pattern upon rechromatography. On SDS-PAGE both gel filtered peaks revealed the same multiple band pattern as that of the original sample. On acid-urea-PAGE, both the gelfiltration peaks produced a single fast moving band of equal mobility. Mobility of this Urea-PAGE band was higher than that of the native Con A-binding glycoprotein bands. The second gel filtration peak may represent monomers of Peak I protein. Another possibility is the formation of a protein micellar aggregate represented by peak I formed from a low molecular weight protein represented by peak II. Heterogenous micellar aggregates formed from the low molecular weight protein might represent the 4 bands on urea-PAGE. Acetic anhydride labelling might have destabilized the micellae formation, leading to its total dissociation into

monomers in the presence of urea, represented by the fast moving Urea-PAGE band of the gel filtered peaks.

In reversed phase HPLC, the gel filtered peaks migrated with different retention times, whereas the total Con A eluate migrated as a single broad peak. The glycoproteins were not susceptible to proteolytic digestion by trypsin and pronase. Attempts to raise antibodies against intact second trimester neurons and glycoproteins in rabbit and guinea pigs were unsuccessful.

Based on the limited number of physicochemical studies carried out with the available technology, no structural differences were detected between the neuronal glycoproteins of second trimester and full term human fetal brains. In conclusion the neuronal glycoproteins appear to have an inherent capacity to form micellae which explained many of their physicochemical properties.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Acheson,A.,and Rutishauser,U.(1988) J.Cell Biol.106,479-486.
2. Acheson,A.L.,and Thoenon,H.(1983) J.Cell Biol.97,925-928.
3. Adler,J.E.,and Black,I.B.(1985) Proc.Natl.Acad. Sci.U.S.A.82,4296-4300.
4. Adler,J.E.,and Black,I.B.(1986) Dev.Brain Res.30,237-241.
5. Aitken,A.,Cohen,P.(1984) Methods Enzymol.106,205-210.
6. Amborn,R.T.,and Schwartz,J.H.(1979) Complex carbohydrates of Nervous Tissue.(Margolis,R.U.,and Margolis,R.K.,eds) Plenum Press, New York,pp.269-289.
7. Antonicek,H.,Persohn,E.,Schachner,M.(1987) J.Cell Biol.104,1587-1595.
8. Antony,L.,Basu,D.,and Appukuttan,P.S.(1989) Indian J.Biochem.Biophys.26,361-366.
9. Appukuttan,P.S.,Surolia,A.,and Bachhawat,B.K.(1977) Indian J.Biochem.Biophys.14,382-384.
10. Aronson,N.N.,Touster,O.(1974) Methods Enzymol.31,92-102.
11. Atminson,P.H.,and Lee,J.T.(1984) J.Cell Biol.98,2245-2249.
12. Axen,R.,Porath.,and Ernback,S.(1967) Nature 214,1302-1304.
13. Baenziger,J.U.(1984) in The Plasma Proteins (Putnam,F.W.,ed), Vol.4,pp.271-315,Academic Press,New York.

14. Bahl,O.P.,and Sojar,H.T.(1987) Methods Enzymol.138,341-350.

15. Bardales,R.,Bhavanandan,V.P.,Wiseman,G.,and Bramwell,M.E.(1989) J.Biol.Chem.264,1980-1987.

16. Bartles,J.R.,and Hubbard,A.L.(1984) Anal.Biochem.140,284-292.

17. Bartlett,G.R.(1959) J.Biol.Chem.234,446-468.

18. Bergmann,J.E.,and Singer,S.J.(1983) J.Cell Biol.97,1777-1787.

19. Bhat,N.R.,and Waechter,C.J.(1988) J.Neurochem.50,375-381.

20. Bhavanandan,V.P.,Katlic,A.W.(1979) J.Biol.Chem.254,4000-4008.

21. Bhavanandan,V.P.,Katlic,A.W.,Banks,J.,Kemper,J.C., Davidson,E.A.(1981) Biochemistry 20,5586-5594.

22. Bhavanandan,V.P.,Umemoto,J.,Banks,J.R.,Davidson,E.A.(1977) Biochemistry 16,4426-4437.

23. Bird,J.M.,and Kimber,S.J.(1984) Dev.Biol.104,449-460.

24. Bjerrum,O.J. (1983) electroimmunochemical analysis of Membrane Proteins (Bjerrum,O.D.,ed)pp.3-44, Elsevier Science Publishing Company,Inc.New York.

25. Bloch,R.,and Burger,M.M.(1974) Biochem.Biophys.Res. Commun.58,13-19.

26. Bock,E.(1978) J.Neurochem.30,7-14.

27. Bock,E.(1984) Handbook of Neurochemistry. 2nd edn. (Lajtha,A.,ed) Vol.7,pp.231-244. Plenum Press.New York.

28. Bock,E.,and Jorgenson,O.S.(1975) FEBS Lett.52,37-39.

29. Bock,E.,Jorgensen,O.S.,and Morris,S.J.(1974) J.Neurochem.22,1013-1017.

30. Bock,E.,Rasmussen,S.,Albeck,M.,Sensenbrenner,M., Petmann,B.,and Louis,J.C.(1983) Electroimmunochemical Analysis of Membrane Protiens (Bjerrum,O.J.,ed). Elsevier, Amsterdam.pp.275-286.

31. Brabec,R.K.,Peters,B.P.,Berbsteub,I.A.,Gray,R.H.,and Goldstein,I.J.(1980) Proc.Natl.Acad.Sci.U.S.A.77,477-479.

32. Brackenburry,R.,Thiery,J.P.,Rutishauser,U.,and Edelman,G.M.(1977)J.Biol.Chem.252,6835-6840.

33. Bradford,M.M.(1976) Anal.Biochem.72,248-254.

34. Braun,V.,and Bosch,V.(1972) Eur.J.Biochem.28,51-69.

35. Bray,W.R.(1960) Anal.Biochem.1,279-285.

36. Brunngraber,E.G.,Brown,B.G.,and Aro,A.(1974) J.Neurochem. 22,125-128.

37. Brunngraber,E.G.,Berra,B.,and Zambotti,U.(1973) Clin.Chem. Acta 48,173-181.

38. Brunngraber,E.G.,Berra,B.,and Zambotti,V.(1973) FEBS Lett.34,350-352.

39. Carlosson,S.R.,and Fukuda,M.(1986) J.BIol.Chem.261,12779-12786.

40. Caron,M.,Joubert,R.,Baldier,D.(1987) Biochim.Biophys. Acta 925,290-296.

41. Chakravarthy,B.R.,Spence,M.W.,Clarke,J.T.R.,andCook,H.W. (1985) Biochim.Biophys.Acta 812,223-233.

42. Chandrasekaran,E.V.,Davidson,E.A.(1979) Biochemistry 18,5615-5620.

43. Chang,J.Y.(1983) Methods Enzymol.91,455-466.

44. Chang,J.Y.(1988)Anal.Biochem.170,542-556.

45. Chang,J.Y.,Brauer,D.,and Wittmann-Liebold,B.(1978) FEBS Lett.93,205-214.

46. Chang,S.,Rathjen,F.G.,and Raper,J.A.(1987) J.Cell Biol.104,355-362.

47. Chao,S.W.,and Rumsby,M.G.(1977) Brain Res.124,347-351.

48. Chatterjee,B.P.,and Uhlenbruck,G.(1982) Experentia 38, 1225-1226.

49. Clegg,J.C.S.(1982) Anal.Biochem.127,389-394.

50. Cotman,C.W.,and Kelly,P.T.(1980) Macromolecular Architecture of CNS synapses.(Cotman,C.W., Nicolson,P.G.,eds) pp.505-533. Elsevier, Amsterdam.

51. Cotman,C.W.,and Kelly,P.T.(1980) The Cell Surface and Neuronal Function.(Cotman,C.W.,Poste,G.,and Nicolson,G.L.,eds) pp.503-533,Elsevier, Amsterdam.

52. Davey,M.W.,Huang,J.W.,Sulkowski.,and Carter,W.A.(1974) J.Biol.Chem.249,6354-6363.

53. De Maio,A.,Lis,H.,Gershoni,J.M.,and Sharon,N.(1986) FEBS Lett.194,28-32.

54. Debray,H.,Decout,D.,Strecker,G.,Spik,G.,and Montrenil,J.(1981) Eur.J.Biochem.117,41-45.

55. Dennis,J.W.,Laferte,S.,Fukuda,M.,and Dell,A.,and Carver,J.P.(1986) Eur.J.Biochem.161,359-373.

56. Dennis,J.W.,Waller,C.,Timpl,R.,and Schirrmaeher,V.(1982) Nature 300,274-276.

57. Derry,G.M.,and Wolfe,L.S.(1967) Science 158,1450-1452.

58. Dittmer,J.C.,and Wells,M.A.(1969) Methods Enzymol.14,482-487.

59. Dodd,J.,and Jessel,T.M.(1985) J.Neurosci.5,3278-3294.

60. Dodd,J.,Solter,D.,and Jessel,T.M.(1984) Nature 311,469-472.

61. Dubois,M.,Gilles,K.A.,Hamilton,T.K.,Rebers,P.A.,and Smith,F.(1965) Anal.Chem.28,350-356.

62. Dulley,J.R.,and Grieve,P.A.(1975) Anal.Biochem.64,136-141.

63. Edelman,G.,and Chuong,C.M.(1982) Proc.Natl.Acad.Sci. U.S.A.79,7036-7040.

64. Edelman,G.,Gallin,W.J.,Delouvee,A.,Cunningham,B.A.,and Thiery,J.P.(1983) Proc.Natl.Acad.Sci.U.S.A.80,4384-4388.

65. Edelman,G.M.(1976) Science 192,218-226.

66. Edelman,G.M.(1986) Ann.Rev.Cell.Biol.2,81-116.

67. Edelman,G.M.,Gallin,W.J.,Delouvee,A.,Cunningham,B.A.,and Thiery,J.P.(1983) Proc.Natl.Acad.Sci.U.S.A.80,4384-4388.

68. Edge,A.S.B.,Faltynek,C.R.,Holf,L.,Reichert,L.E.,and Weber,P.(1981) Biochemistry 118,131-137.

69. Edmondson,J.C.,Liem,R.K.H.,Kuster,J.E.,and Hatten,M.E.(1988) J.Cell Biol.106,505-517.

70. Elam,J.S.(1979) Complex Carbohydrates of Nervous Tissue (MargolisR.U.,and Margolis,R.K,eds) Plenum Press, New York,pp.235-267.

71. Entlicher,G.,Kocoured,J.(1976) Methods in Carbohydrate Chemistry.(Whisle,R.L.,and Bemille,J.N.,eds) 7,191-194.

72. Evans,W.H.(1979) in Laboratory Techniques in Biochemistry and Molecular Biology (Work.T.S.,and Work,E.,eds) 7 PartI,pp.11-266.

73. Eylar,E.H.,Salk,J.,Beveridge,G.(1969) Arch.Biochem. Biophys.132,34-38.

74. Farooq,M.,and Norton,W.T.(1978) J.Neurochem.31,887-894.

75. Feizi,T.(1985) Nature 314,53-57.

76. Feizi,T.,and Childs,R.A.(1985) Trends Biochem.Sci. 10,24-29.

77. Ferguson,M.A.J.,and Williams,A.F.(1988) Ann.Rev.Biochem. 57,285-320.

78. Ferguson,M.A.J.,Low,M.G.,and Cross,G.A.M.(1985) J.Biol.Chem.260,14547-14555.

79. Finne,J.(1980) Eur.J..Biochem.104,181-189.

80. Finne,J.(1982) J.Biol.Chem.257,11966-11970.

81. Finne,J.,and Krusius,T.(1982) Methods Enzymol.83,269-277.

82. Finne,J.,and Krusius,T.(1976)FEBS Lett.66,94-97.

83. Finne,J.,Krusius,T.,Rauval,H.,and Hemminki,K.(1977) Eur.J.Biochem.77,319-323.

84. Finne,J.,Krusius,T.(1977) FEBS Lett.66,94-98.

85. Fischer,I.,Shea,T.B.,Sapirstein,V.S.,Kosik,K.(1986) Dev.Brain.Res.25,99-109.

86. Fleischer,S.,and Kervina,M.(1974) Methods Enzmol.31,1-6.

87. Fukuda,M.N.(1985) Biochemistry24,2154-2163.

88. Frazer,W.,and Glaser,L.(1979) Annu.Rev.Biochem. 48,491-523.

89. Fukuda,M.,Fukuda,M.N.,Hakamori,S.(1979) J.Biol.Chem. 253,3700-3703.

90. Furlan,M.,Perret,B.A.,and Beck,E.A.(1979) Anal.Biochem. 96,208-214.

91. Gabriel,O.(1971) Methods Enzymol.22,578-604.

92. Gahmberg,C.G.(1978) Methods Enzymol.50,204-206.

93. Gahmberg,C.G.,Myllyla,G.,Leikola,J.,Pirkola,A.,and Nordling,S.(1976) J.Biol.Chem.251,6108-6116.

94. Gander,J.E.(1984) Methods Enzymol.104,447-451.

95. Gatt,S.(1969) Methods Enzymol.14,134-149.

96. Gerisch,G.(1977) Curr.Top.Dev.Biol.14,243-270.

97. Gershoni,J.M.(1988) Methods Biochem.Anal.33,1-58.

98. Gershoni,J.M.,and Palade,G.E.(1982) Anal.Biochem.124,396-405.

99. Girgah,N.,Cruz,T.F.,Letarte,M.,and Mooscarello,M.A.(1988) Biochem.J.256,351-356.

100. Glassman,J.H.S.,and Todd,C.N.,and Shively,J.E.(1978) Biochem.Biophys.Res. Comm.85,209-216.

101. Goldstein,I.J.,and Hayes,C.E.(1978) in Advances in Carbohydrate Chemistry and Biochemistry.Vol 35.[Tipson,R.S.,and Horton,D.,eds] pp.12-340. Academic Press,New York,San Francisco,London.

102. Goldstein,I.J.,Hughes,R.C.,Monsigny,M.,Osawa,T.,and Sharon,N.(1980) Nature 285,66.

103. Gombos,G.(1976) in Concanavalin A as a Tool. (Bittiger,H., and Schnebli,H.P.,eds.)pp.379-387. John Wiley and Sons Ltd,London.

104. Goroelik,E.,Fogel,M.,Segal,S.,and Feldman,M.(1979) J.Supra Molecular Struct.12,385-402.

105. Gottschalk,A.(1960) Nature 186,949-954.

106. Gray,W.R.(1972) Methods Enzymol.251,121-138.

107. Greenberg,M.E.,Brackenburry,R.,and Edelman,G.M.(1984) Pro.Natl.Acad.Sci.U.S.A.81,969-973.

108. Green,E.D.,and Baenzinger,J.U.(1988) J.Biol.Chem.263,25-29.

109. Grumet,M.,and Edelman,G.M.(1984) J.Cell Biol.98,1746-1756.

110. Grumet,M.,Rutishauser,U.,and Edelman,G.M.(1982) Nature 295,693-695.

111. Grunwald,G.B.,Geller,R.L.,and Lilien,J.(1980) J.Biol. Chem.85,766-776.

112. Gurd,F.R.N.(1972) Methods Enzymol.25,424-438.

113. Hakamori,S.(1972) Methods Enzymol.28,232-236.

114. Hakomori,S.(1984) Trends Biochem Sci.9,455-459.

115. Hakomori,S.(1984) Ann.Rev.Immunol.2,103-126.

116. Hames,B.D.(1981) Gel electrophoresis of protein- A practical approach (Hames,B.D.,and Rickwood,eds) IRL Press Ltd,P.O.Box I, Eyaham, Oxford OX81JJ, England, pp.71-76.

117. Harford,J.B.,and Waechter,C.J.(1980) Biochem.J.188,481-490.

118. Harrison,G.R.,and Partlow.(1980) Brain Res.192,371-381.

119. Hase,S.,and Ikenaka,T.,and Mataushima,Y.(1979) J.Biol.Chem.85,989-994.

120. Hansen,S.N.,Lihme,A.O.F.,Hansen,T.CB.,and Bjerrum,O.L.(1985) in Lectins Biology-Biochemistry Clinical Biochemistry (Hansen,E.,ed) Vol.4,pp 241-252, Walter de Gruyter.,Berlin.New York.

121. Hatten,M.E.,Leim,R.K.H.,and Mason,C.A.(1984) J.Cell Biol.98,193-204.

122. Hatten,M.E.,Schachner,M.,and Sidman,R.L.(1979) Neuroscience 4,921-935.

123. Hatton,G.E.(1987) J.Cell Biol.104,1353-1360.

124. Hatton,M.W.C.,Mariz,L.,Berry,L.R.,Debanne,M.T.,and Regoeczi,E.(1979) Biochem.J.181,663-638.

125. Hatton,M.W.C.,Regoeczi,E.,and Wong,K.L.(1974) Can.J.Biochem.52,845-853.

126. Heacock,A.M.(1982) Brain Res.241,307-315.

127. Helenius,A.,and Simons,K.(1975) Biochim.Biophys. Acta 415,29-79.

128. Hennessey,J.P.,and Scarborough,G.A.(1989) Anal.Biochem. 176,284-289.

129. Heuser,J.E.,and Reese,T.S.(1973) J.Cell.Biol.57,315-344.

130. Hirn,M.,Pierres,M.,Deagostini,-Bazin,H.,Hirsch,M.,and Goridis,C(1981) Brain Res.214,433-439.

131. Hjelm,H.,Hjelm,K.,and Sjoquist,J.(1972) FEBS Lett.28,73-75.

132. Hoffman,S.(1982) J.Biol.Chem.257,7720-7729.

133. Holley,J.,and Schachner,M.(1985) Nature 316,728-730.

134. Hortin,G.L., and Trimple,L.(1990) Anal.Biochem.188,271-277.

135. Hott,G.D.,and Hart,C.W.(1986) J.Biol.Chem.261,8049-8057.

136. Hsieh,P.,and Sueoka,N.(1980) J.Cell Biol.86,866-873.

137. Hubbard,S.C.,and Robbins,P.W.(1979) J.Biol.Chem.254,4568-4576.

138. Hu,C and van Huystee,R.B.(1989) Biochem.J.263,129-135.

139. Hunt,L.A.,Davidson,S.K.,Golemboski,D.B.(1983) Arch.Biochem.Biophys.226,347-356.

140. Irie,S.,Minguell,J.J.,and Tvassoli,M.(1989) Biochem.J.259,427-431.

141. Irimura,T.,Nicolson,G.L.(1984) Cancer Res.44,791-798.

142. Irimura,T.,Nicolson,G.L.(1983) Carbohydr.Res.120,187-195.

143. Irwin,L.N.,and Mancini,J.,and Hills,D.(1973) Brain Res.53,488-491.

144. Jamieson,J.D.,and Palade,G.E.(1968) J.Cell Biol.39,589-603.

145. Johnson,D.E.,and Sellinger,O.Z.(1971) J.Neurochem.18,1445-1448.

146. Jorgensen,D.S.,and Bock,E.(1974) J.Neurochem.23,879-880.

147. Jorgensen,O.S.(1977) FEBS Lett,79,42-44.

148. Joshi,S.S.,and Sharp,J.G.(1988) Lectins and Glycoconjugates in Oncology (Gabins,H.J.,Nagel,G.A.,eds) Springer-Verlag, Berlin.

149. Joshi,S.S.,Tilden,P.A.,Jackson,J.D.,Sharp,J.G.,and Brunson,K.W.(1987) Cancer Res.47,3551-3557.

150. Kadmon,G.,Kowitz,A.,Altevogt,P.,and Schachner,M.(1990) J.Cell Biol.110,193-208.

151. Kamps,M.P.,Buss,J.E.,Sefton,B.M.(1985) Proc.Natl.Acad. Sci.U.S.A.82,4625-4630.

152. Kapadia,A.,Feizi,T.(1981) Exp.Cell.Res.131,185-189.

153. Kavasaki,T.,and Ashwell,G.(1976) J.Biol.Chem.251,1296-1302.

154. Keren,Z.,Berke,G.,and Gershoni,J.M.(1986) Anal.Biochem. 155,182-187.

155. Kerjaschki,D.,Sharkey,D.J.,and Farquhar,M.G.(1984)J.Cell Biol.98,1591-1596.

156. Kilpatrick,G.J.,Jenner,P.,Marsden,C.D.(1985) J.Pharm. Pharmacol.37,320-328.

157. Klenk,H.D.,and Choppin,P.W.(1970) Proc.Natl. Acad.Sci.U.S.A.66,57-62.

158. Reukonen,O.,Gahmberg,C.G.,Simons,K.,and Kariainen,L. (1970) Acta.Chem.Scand.24,733-737.

159. Kodman,G.,Kowitz,A.,Alterogt,P.,and Schachner,M.(1990) J.Cell Biol.110,209-218.

160. Kohn,J.,and Wilchek,M.(1984) Appl.Biochem.Biotech.9, 285-305.

161. Konigsberg,W.H.,and Henderson,L.(1983) Mehods Enzymol. 91,254-259.

162. Kornfeld,K.,Reitman,M.L.,Kornfeld,R.(1981) J.Biol.Chem. 256,6633-6640.

163. Kornfeld,R.,and Kornfeld,S.(1976) Ann.Rev.Biochem.45,217-222.

164. Torres,C.R.,and Hart,G.W.(1984) J.Biol.Chem.259,3308-3317.

165. Kronquist,K.E.,Lennaz,W.J.(1978) J.Supramol.Struct.8, 51-65.

166. Krusius,T.,and Finne,J.(1978) Eur.J.Biochem.84,395-403.

167. Krusius,T.,and Finne,J.(1977) Eur.J.Biochem.78,369-379.

168. Kucherer,A.,Faissner,A.,Schachner,M.(1987) J.Cell Biol.104,1597-1602.

169. Kuhn,R.,and Wiegandt,W.,and Egge,H.(1961) Angw.Chem. 73,580-584.

170. Laemmli,U.K.(1970) Nature 227,680-685.

171. Le,A.V.,and Doyle,D.(1985) Bichemistry 24,6238-6245.

172. Leach,B.S.,and Collawn,J.F.,Jr.,and Fish,W.W.(1980) Biochemistry 19,5734-5741.

173. Ledeen,R.(1984) J.Neurosci.Res.12,147-159.

174. Leuzinger,W.(1971) Methods EnZymol.17,782-787.

175. Levine,L.,and Moore,B.W.,(1965) Neurosci.Res.Prog. Bull.3,18-21.

176. Lindner,J.,Rathjen,F.G.,and Schachner,M.(1983) Nature 305,427-430.

177. Linser,P.J.,and Moscona,A.A.(1983) Dev.Biol.96,529-534.

178. Linser,P.J.,and Perkins,M.(1987) Dev.Brain Res.31,277-290.

179. Liotta,L.A.,Rao,N.,and Wewer,U.M.(1986) Ann.Rev. Biochem.55,1037-1057.

180. Lis,H.,and Sharon,N.(1986) in The Lectins:Properties, Functions and Applications in Biology and Medicine (Liener,I.I.,Sharon,N.,and Goldstein,I.J.,eds.)pp.293-370, Academic Press, Orlando.

181. Lis,H.,and Sharon,N.(1972) Methods Enzymol.28,360-365.

182. Lotan,R.,Beattie,G.,Hubbell,W.,and Nicolson,G.L.(1977) Biochemistry 16,1787-1794.

183. Lotan,R.,Nicolson,G.L.(1979) Biochim.Biophys.Acta 559,329-376.

184. Lotan,R.,Skutelsky,E.,Danon,D.,and Sharon,N.(1975) J.Biol. Chem.250,8518-8523.

185. Lowry,O.H.,Rosenbrough,N.J.,Farr,A.L.,and Randell,R.L. (1951) J.Biol.Chem.193,265-275.

186. Lunney,J.,and Ashwell,G.(1976) Proc.Natl.Acad.Sci.U.S.A. 73,342-345.

187. Magnani,J.L.,and Ginsberg,V.(1987)Methods Enzymol.138,195-207.

188. Mahanta,S.K.,Sastry,M.V.K.,and Surolia,A.(1990) Biochem.J. 265,831-840.

189. Margolis,R.K.,and Margolis,R.U.(1983) Handbook of Neurochemistry (Lajtha,A.,ed.)Vol.5,pp.177-204,Plenum Press,New York.

190. Margolis,R.K.,and Margolis,R.U.(1979) Complex Carbohydrates of Nervous Tissue(Margolis,R.U.,and Margolis,R.K,eds) Plenum Press, New York,pp.45-73.

191. Marquez,F.J.,Queseda,A.R.,Jimenez,F.S.,and De Castro,I.N.(1986) J.Chromatogr.380,275-283.

192. Marshall,R.D.(1972) Ann.Rev.Biochem.41,673-702.

193. Martoni,R.,and Schachner,M.(1986) J.Cell Biol.103,2439-2448.

194. McClain,D.,and Edelman,G.(1982) Proc.Natl.Acad.Sci.U.S.A. 79,6380-6384.

195. McClain,L.D.,Tamana,M.,and Acton,R.T.(1978) Brain Res.159,161-171.

196. McDermott,R.P.,Kienker,L.J.,Bertovich,M.J.,and Muchmore,A.V.(1981)Immunol.44,143-152.

197. McGarry,R.C.,Helfand,S.L.,Quarles,R.H.,and Roder,J.C.(1983) Nature 306,376-378.

198. Meister,A.(1985) Methods EnZymol.113,185-199.

199. Milford-Ward,A.(1981) Techniques in Clinical Immunology (Thompson.,ed.) pp.1-27,Blackwell Scientific Publications.Oxford.London.

200. Miragall,F.,Kadman,G.,Husman,M.,and Schachner,M.(1988) Dev.Biol.129,516-531.

201. Monad,J.N.,and Kent,C.(1986) Anal.Biochem.159,157-162.

202. Monsigny,M.,Roche,A.C.,Sene,C.,Maget,D.R.,Delmotte,F. (1980) Eur.J.Biochem.104,147-153.

203. Montreuil,J.(1980) Adv.Carbohydr.Chem.Biochem.37,157-223.

204. Moore,B.W.,and Perez,V.J.(1968) in Physiolgical and Biochemical aspects of Nervous Integration. (Carlson,F.D.,ed) pp.353-360. Prentice-Hall, Englewood Chiffs,NJ.

205. Moore,K.L.(1973) In The Developing Human Clinically Oriented Embryology,pp.71-84. W.B.Saunders Company-Philadelphia.

206. Morell,A.G.,Irvine,R.A.,Sternlieb,I.,Scheinberg,I.H., and Ashwell,G.(1968) J.Biol.Chem.243,155-159.

207. McIlhinney,R.A.J.,Chadwick,J.K.,Pelly,S.J.(1987) Biochem.J.244,109-115.

208. Muramatsu,J.(1988) J.Cell Biochem.36,1-6.

209. Nagata,Y.,Mikoshiba,K.,and Tsukada,Y.(1974) J.Neurochem.22,493-503.

210. Nakane,P.K.,and Kawaio,A.(1974) J.Histochem. Cytochem.22,1084-1091.

211. Nassbaum,J.L.,Delaynoy.,and Mandel,P.(1977) J.Neurochem.28,183-190.

212. Nomoto,S.,Muramatsu,H.,Ozawa,M.(1986) Exp.Cell.Res.164,49-62.

213. Nudelman,E.,Fukushi,Y.,Levery,S.B.,Higuchi,T.,and Hakomori,S.(1986) J.Biol.Chem.261,5487-5495.

214. Ochai,K.,Katagiri,Y.V.,Hatae,T.,and Okiyama,H.(1989) Biocmem.J,257,43-49.

215. Olden,K.,Parent,J.B.,and White,S.L.(1982) Biochim.Biophys. Acta 650,209-232.

216. Olden,K.,Pratt,R.M., and Yamada,K.M.(1978) Cell 13,461-465.

217. Olson,E.N.,Spizz,G.(1986) J.Biol.Chem.261,2458-2466.

218. Osawa,T.,and Tsuji,T.(1987) Ann.Rev.Biochem.56,21-42.

219. PazParrente,J.,Weiruszeski,J.M.,Strecker,G.,Montreuil,J., Fournet,B.,etal(1982)J.Biol.Chem.257,13173-13176.

220. Paz-parente,J.,Strecker,G.,Leroy,Y.,Montreuil,J.,and Fournet,B.(1983) FEBS Lett.152,145-152.

221. Pfenninger,K.H.,and Maylie-Pfenninger,M.F.(1981) J.Cell Biol.89,536-546.

222. Pfenninger,K.H.,Myelie-Pfenninger,M.F.,and Friedman,L.B.(1984) Dev.Biol.106,97-108.

223. Pierce,M.,and Arango,J.(1986) J.Biol.Chem.261,10772-10777.

224. Pless,D.D.,and Lennarz,W.J.(1977) Proc.Natl.Acad.Sci. U.S.A.74,134-138.

225. Poduslo,S.E.,and Norton,W.T.(1975) Methods Enzymol.35,561-579.

226. Preti,A.,Lomnbardo,A.,Tettamanti,G.,and Zambotti,V.(1973) J.Neurochem.21,1559-1562.

227. Quarles,R.H.(1983) Dev.Neurosci.6,285-303.

228. Quarles,R.H.,and Pasnak,C.F.(1977) Biochem.J.163,635-637.

229. Rademacher,T.W.,Parekh,R.B.,and Dwek,R.A.(1988) Ann.Rev. Biochem.57,785-838.

230. Rakic,P.(1985) in The Cell in Contact.(Edelman,G.M.,and Thiery,J.P.,eds) pp.67-91, John Wiley and Sons.New York.

231. Ramachandran,J.,Rodriguez,H.,Henzel,W.,and Tsubokawa,M.(1987) Methods in Protein Sequence Analysis (Walsh,K.A.,ed) pp.157-169. Humana Press.Clifton. New Jersey.

232. Rathjen,F.G.,and Rutishauser,U.(1984) EMBO J.3,461-465.

233. Rathjen,F.G.,Wolf,J.M.,Frank,R.(1987) J.Cell Biol.104,343-353.

234. Reading,C.L.,Brunson,K.W.,Toranni,M.,and Nicolson,G.I. (1980) Proc.Natl.Acad.Sci.U.S.A.77,5943-5947.

235. Rehm,R.(1989) FEBS.Lett.247,28-32.

236. Reindueau,D.,and Guertin,D.(1986) J.Biol.Chem.261,976-981.

237. Rohringer,R.,and Holden,D.W(1985) Anal.Biochem.144,118-127.

238. Rosner,h.,Wiegandt,H.,and Rahman,H.(1973) J.Neurochem. 21,655-665.

239. Rothbard,J.B.,Brackenbury,R.,Cunningham,B.,and Edelman,G.M.(1982) J.Biol.Chem.257,11064-11069.

240. Rutishauser,U.,Gall,W.E.,and Edelman,G.M.(1978) J.Cell Biol.79,382-393.

241. Rutishauser,U.,Grumet,M.,and Edelman,G.(1983) J.Cell Biol.97,145-152.

242. Rutishauser,U.,Thiery,J.P.,Brackenbury,R.,and Edelman,G.M.(1978) J.Cell.Biol.79,371-381.

243. Sarris,A.H.,and Palade,G.E.(1982) J.Cell Biol.93,583-590.

244. Schaffner,W.,and Weissmann,C.(1973) Anal.Biochem.56,502-504.

245. Schallier,D.C.C.,Bolscher,J.G.M.,and Smets,L.H.(1988) In Lectins and Glycoconjugates in Oncology (Gabrius,H.J., and Nagel,G.A.,eds)pp.87-96. Springer-Verlay, Berlin.

246. Scher,M.G.,Resneck,W.G.,and Bloch,R.J.(1989) Anal.Biochem. 177,168-171.

247. Schimidst,M.F.G.,Brecha,M.,and Schlesinger,M.J(1979) Proc.Natl.Acad.Sci.U.S.A.76,1687-1691.

248. Schmelzer,C.H.,and Poduslo,S.E.(1987) Biochim.Biophys. Acta 903,103-111.

249. Schrevel,J.,Gros,D.,Monsigny,M.(1981) Prog.Histochem. Cytochem.14(2),1-269.

250. Schultz,A.M.,Henderson,L.E.,Oroszlan,SGarber,E.A., Hanafusa,H.(1985) Science 227,427-429.

251. Schwarting,G.,Jungalwala,F.B.,Chou,D.K.H.,Boyer,A.M.,and Yomamoto,M.(1987) Dev.Biol.120,65-76.

252. Seyfried,T.N.,Miyazawa,N.,and Yu,R.K.(1983) J.Neurochem.41,491-505.

253. Sharon,N.,and Lis,H.(1981) Chemical and Enginering News 59,21-44.

254. Sharon,N.,and Lis,H.(1989) Science 246,227-234.

255. Sherton,C.C.,and Wool,I.G.(1974) Methods Enzymol.30,506-526.

256. Shirakawa,O.,Kuno,T.,and Tanaka,C.(1983) Biochem.Biophys. Res.Comm.115,814-819.

257. Shreeve,S.M.,Fraser,C.M.,and Venter,J.C.(1985) Proc.Natl.Acd.Sci.U.S.A.82,4842-4846.

258. Simpson,D.L.,Thorne,D.R.,and Loh,H.H.(1977) Nature 266,367-369.

259. Skipski,V.P.(1975) Methods Enzymol.35,396-425.

260. Small.G.M.,Imanda,T.,and Lazarow,P.B.(1988) Anal.Biochem. 169,405-409.

261. Smets,L.A.(1980) Biochim.Biophys.Acta 605,93-111.

262. Smets,L.A.,and Van Beek,W.P.(1984) Biochim.Biophys.Acta 738,237-249

263. Smyth,D.G.(1967) Methods Enzymol.11,214-222.

264. Soderquist,A.M.,and Carpenter,G.(1984) J.Biol.Chem.259, 12586-12594.

265. Sojar,H.T.,and Bahl,O.P.(1987) Arch.Biochem. Biophys.259,52-57.

266. Stralfors,P.,and Belfrage,P.(1983) Anal.Biochem.128,7-10.

267. Strous,G.J.A.M.(1979) Proc.Natl.Acad.Sci.U.S.A.76,2694-2699.

268. Sturgeon,R.J.,and Sturgeon,C.M.(1982) Carbohydr. Res.103,213-219.

269. Stutman,O.D.,Wisun,R.E.,and Lattime,E.C.(1980) Proc.Natl.Acad.Sci.U.S.A.77,2895-2898.

270. Subbalakshmi,G.Y.C.V.,and Murthy,CH.R.K.(1985) Neurochemical Research 10,239-250.

271. Sundberg,L.,and Porath,J.(1974) J.Chromatogr.90,87-98.

272. Sureshkumar,G.,Appukuttan,P.S.,and Basu,D.(1982) J.Biosci.4,257-261.

273. Surolia,A.,Prakash,N.,Bishayee,S.,and Bachhawat,B.K.(1973) Indian J.Biochem.Biophy.10,145-150.

274. Svennerholm,L.,and Fredman,P.(1980) Biochim.Biophys.Acta 617,97-109.

275. Tai,T.,Yamashita,K.,Setsuko,I.,and Kobata,A.(1977) J.Biol. Chem.252,6687-6692.

276. Takasaki,S.,Ikehira,H.,Kobata,A.(1980) Biochim.Biophys. Res.Comm.92,735-742.

277. Takeichi,M.(1979) J.Cell Biol.75,464-474.

278. Tanick,J.,and Schauer,R.(1974) Hoppe-Seyler.Z.Physiol. Chem.355,395-399.

279. Tenenbaum,D.,and Folch-Pi,J.(1966) Biochim.Biophys. Acta 115,141-147.

280. The,T.H.,and Feltkamp,T.E.W.(1970) Immunology 18,865-875.

281. Thorpe,P.E.,Setre,S.I.,Foxwell,B.M.J.,Brown,A.N.F., Skiilleter,D.N.,Wilson,G.,Foresta,J.A.,and Thipe,F.(1985) Eu.J.Biochem.147,197-206.

282. Towbin,H.,Staehelin,T.,and Gordon,J.(1979) Proc.Natl. Acad.Sci.U.S.A.76,4350-4354.

283. Trimble,R.B.,and Maley,F.(1977) J.Biol.Chem.252,4409-4412.

284. Truding,R.,Shelanskii,M.L.,Daniels,M.P.,and Morell,P. (1974) J.Biol.Chem.249,3973-3982.

285. Tsuji,T.,Irimura,T.,Osawa,T.(1980) Biochem.J 187,677-686.

286. Uyeda,C.T.,Eng,L.F.,and Bignami,A.(1972) Brain.Res.37,81-89.

287. Uy,R.,and Wold,F.(1977) Anal.Biochem.81,98-107.

288. Van Nieuw Amerongen,A., Van Den Eijnder, D.H.,Heijlman,J., and Roukema,P.A.(1972) J.Neurochem.19,2195-2205.

289. Van Beek,W.P.,Smets,L.A.,and Emmelot,P.(1975) Nature 253, 457-460.

290. Van Lenten,L.,and Ashwell,G.(1972) Methods Enzymol.28,209-211.

291. Van Drissche,E.,Smets,G.,Dejaegere,R.,and Kanarek,L.(1982) Lectin Biology, Biochemistry, Clinical Biochemistry, (Hansen,E.,ed) 2,729-740. Walter de Gruyter.,Berlin.New York.

292. Varki,A.,Kornfeld,S.(1983) J.Biol.Chem.258,2808-2818.

293. Vertblad,P.(1976) Biochim.Biophys.Acta 434,169-176.

294. Viala,J.,and Jainefelt,J.(1985) Trends Biochem.Sci.10,392-395.

295. Waechter,C.J.,Schmidt,J.W.,and Catterall,W.A.(1983) J.Biol.Chem. 258,5117-5123.

296. Warren,L.(1959) J.Biol.Chem.234,1971-1975.

297. Warren,L.,and Buck,C.A.(1980) Clin.Biochem.13,191-197.

298. Warren,L.,Buck,C.A.,Tusgynski,,G.P.(1978) Biochim.Biophys. Acta 516,97-127.

299. Watanabe,M.,Muramatsu,T.,Shirane,H.,Vagai,K.(1981) J.Histochem.Cytochem.29,779-790.

300. Watkins,W.M.,and Morgan,W.T.J.(1952) Nature 169,825-829.

301. Weber,K.,and Osborn,M.(1969) J.Biol.Chem.244,4406-4412.

302. Weinstein,D.B.,Marsh,T.B.,Glick,M.C.,and Warren,L.(1970) J.Biol.Chem.295,3928-3933.

303. Weir,D.M.(1985) Handbook of Experimental Immunology VolIV,pp.129.6-129.7.

304. Wernecke,H.,Linder,J.,and Schachner,M.(1985) J.Neuroimmunol.9,115-130.

305. Will,H.,Benson,A.,Devilliers,G.,and Mandel,P.(1982) J.Neurochem.39,924-932.

306. Wray,W.,Boulikas,T.,Wray,V.P.,and Hancock,R.(1981) Anal. Biochem.118,197-203.

307. Wright,L.C.,May,G.L.,Gregory,P.,Dyne,M.,Holes,K.T., Williams,P.G.,and Moumntford,C.E.(1985) J.Cellular Biochemistry 37,49-59.

308. Yamamoto,K.,Tsuji,T.,Matsumoto,I.,and Osawa,T.(1981) Biochemistry 20,5894-5899.

309. Yamamoto,K.,Tsuji,T.,Osawa,T.(1982) Carbohydr.Res.110,283-289.

310. Yamashita,K.,Kammerling,J.P.,and Kobata,A.(1983) J.Biol. Chem.258,3099-3106.

311. Yamashita,K.,Tachiubana,Y.,and Kobata,A.(1978) J.Biol. Chem.253,3862-3868.

312. Yamashita,K.,Tachibona,Y.,Ohhura,T.,and Kobata,A.(1985) J.Biol.Chem.260,3963-3969.

313. Yamashita,K.,Veda,I.,Kobata,A.(1983) J.Biol.Chem.258,14144-14147.

314. Yogeswaran,G.,and Salk,P.L.(1981) Sience 212,1514-1516.

315. Zanetta,J.P.,and Gombos,G.(1976) in Concanavalin A as a Tool(Bittiger,H.,and Schnebli,H.P.,eds) pp.389-398. John Wiley and Sons Ltd, London.

316. Zanetta,J.P.,Dontenwill,M.,Meyer,A.,and Roussel,G.(1985) Dev.Brain.Res.17,233-243.

317. Zanetta,J.P.,Meyer,A.,Kuchler,S.,and Vincendon,G.(1987) J.Neurochem.49,1250-1257.

318. Zanetta,J.P.,Morgan,I.G.,and Gombos,G.(1975) Brain Res. 83,337-348.

319. Zanetta,J.P.,Reeber,A.,and Vincendon,G.(1981) Biochim. Biophys.Acta 670,393-400.

SYNOPSIS

PHYSICOCHEMICAL STUDIES ON CELL SURFACE GLYCOCONJUGATES OF NEURONS FROM DEVELOPING HUMAN BRAINS

SYNOPSIS

by

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for Ph.D Degree

of

BIOCHEMISTRY

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INTRODUCTION:

The central nervous system displays complex cell sociology. Surface glycoproteins of brain cells play an important role in the brain tissue architecture. Differences in the surface glycoconjugates have been demonstrated between different neuron types(18). Neurons depend on plasma membrane glycoproteins for circuitry formation and other intracellular connections. Many neurotransmitter receptors, like α-adrenergic, and those for dopamine and muscarinic acetylcholine are glycoproteins (25), (26),(14). The significance of membrane glycoproteins have been implicated in receptor-ligand interactions, neuronal recognition as well as in intercellular communications.The molecular mechanisms underlying these phenomena remain largely obscure.

Neoplastic cell surface glycoproteins showed aberrant glycosylation. One of the most constantly observed alternations following neoplasmic transformation is a shift towards the synthesis and expression of larger asparagine-linked oligosaccharide chains(22). Terminal sialylation of cell surface glycoconjugates has been correlated with increased metastatic potential in a number of murine tumor cell models (30).Although the reexpression of embryonic carbohydrate structures by tumor cells may simply reflect the expression of earlier genetic programs,it is also possible that certain structures that are involved in embryonic cell-cell interactions may enhance the malignancy potential of tumor cells.

Development of nervous system is one of the most complex examples of morphogenesis. It involves both the formation of intricate tissue structures and their precise interconnection. There are three main sources of variability in neural networks during development: somatic developmental sequence of network formation and neuroanatomy, the chemical variation related to neurotransmitters and the electrical variation. Morphological changes in the human central nervous system are accompanied by specific changes at the molecular level. Among the many factors which guide the axons of embryonic neurons to their proper destinations, neuronal cell surfaces may be an important source of information. There is substantial evidence that cell surface glycoproteins play pivotal roles in the cell to cell adhesion during neural development. The role of oligosaccharide side chains in brain development is emphasized by the facts that N-glycosylation is critical for neuronal differentiation(12). Mammalian brain is rich in N-linked oligosaccharides which comprise about 80-90% of the glycoprotein-bound carbohydrates (15). Increases in activities in the N-glycosylation pathway have been demonstrated during active myelination(11), synaptogenesis(13), and glial differentiation(1). Cell surface carbohydrates undergo remarkable alterations during differentiation and development (7). Appearance of novel glycoproteins during the process of neural development has been demonstrated with lectin binding(17).In the last few years several neural cell surface

glycoproteins have been identified which appear to be involved in adhesive interaction between neurons, glial cells and matrices. These cell surface glycoproteins have been postulated to coordinate the morphogenetic steps: neural induction, cell proliferation, migration, aggregation, cytodifferentiation, synapse formation,cell death and synapse elimination, all of these together constitute the neural development. Edelman and coworkers have isolated and characterized a neural cell adhesion molecule (N-CAM) from chicken retina cells(29). N-CAM analogues have been found in mouse, rat and human prenatal brains. An adult form of N-CAM contains less sialic acid and it has been suggested that the sialic acid content of N-CAM influences the adhesiveness of the molecule. Myelin glycoproteins of mouse brain control myelination by regulating the contact between oligodendrocytes and axon(3). Another cell adhesion molecule Ng-CAM has been reported from the neuronal cells of chick embryo(9).A monoclonal antibody was employed to detect human Thy-1 on the membranes of some neuronal cell bodies and their processes(2). Rathjen et al showed the presence of chick neural cell surface molecules(20) related to L1 antigen, a mouse neural glycoprotein implicated in cell-cell adhesion (23).

It is evident from the above discussions that most of the informations about neural cell surface glycoproteins were obtained from rat, mouse and chicken. The information related to neural surface glycoproteins of developing human brain is

lacking. Cell adhesion during the process of neural development is a multistep process, and the stabilization of tissue form may require further components in addition to the presently known neuronal cell surface glycoproteins. The present study was undertaken in an attempt to obtain information on the concanavalin A (Con A)-binding neuronal cell surface glycoproteins of developing human brain. In order to obtain this information the following parameters were carried out.

(1) Isolation and characterization of neurons and the major neuronal cell surface Con A-binding glycoproteins of developing human brains.

(2) Physicochemical characterization of the Con A-binding neuronal cell surface glycoproteins.

(3) Comparative study of the major neuronal cell surface glycoproteins from second trimester and full term fetuses.

(4) Immunogenicity of the Con A-binding glycoproteins.

Developing human fetal specimens were obtained from Department of Obstetrics and Gynaecology, Medical College, Trivandrum, after the medical termination of pregnancy. Utilisation of the tissues for the present study was permitted by Ethics Committee of the Trivandrum Medical College. The brains were stored at -20°C in a cryopreservative medium (10 mM phosphate buffer pH 6.0, 8% dextrose, 5% fructose, 20%(v/v) dimethyl sulfoxide, 0.1mM phenyl methane sulfonyl fluoride, 15mM NaN_3). The fetuses collected for the present study were

classified into two groups based on their crown to rump (CR) lengths. The CR length of the first group ranged from 11-17 cm and the corresponding age was found to be in the range of 14-20 weeks (second trimester). For the second group of full term fetuses, CR length ranged from 30-33 cm .

RESULTS AND DISCUSSION:

The isolation of neurons was a modification of the procedures of Poduslo & Norton(19) and Farooq & Norton(6). The mechanical disruption of the brain tissue was carried out in an isotonic medium by successively passing through 80 and 200 mesh nylon sieves in presence of 2-4% Ficoll 400. The cell suspension was treated with DNAase to degrade DNA released during the lysis of cells in the process. A discontinuous sucrose density gradient centrifugation consisting of 2M, 1.55M,1.35M and 0.9M sucrose was carried out at 3300 g X 15 min. The 2M interphase comprised of almost pure neurons which was collected. The 1.35M and 1.55M layers were pooled together, layered over 1.65M sucrose and centrifuged at 3300 g X 45 min to obtain a neuron-rich pellet.

A major modification of the above mentioned procedure made the isolation of neurons easier. The first step density gradient was avoided. The cell suspension was mixed with an equal volume of 2M sucrose. The diluted preparation was layered over 1.65M sucrose layer and centrifuged at 3300 g X 45 min to obtain a neuron-rich pellet.

Almost pure, morphologically intact neurons could be isolated by the two methods. The yield of neurons from 16-20 week fetal tissues was 1-15% based on protein as determined by Bradford's method(4). The yield of neurons decreased as the age of the fetus increased. In the present method, tissue disruption was carried out by mechanical sieving alone and the conventional trypsinization process was avoided because of the extensive lysis of fetal brain cells.

The isolated neurons could be labelled with Con A-FITC indicating the presence of Con A-binding glycoproteins(28). However attempts made to label the neuronal cell surface glycoproteins by periodate-NaB^3H_4 and by galactose oxidase-NaB^3H_4 methods were not successful.

The isolation of neuronal cell surface glycoproteins required the extraction of neuronal proteins in a suitable form compatible for Con A-Sepharose 4B chromatography. For the solubilization of neuronal proteins,two detergents ;Triton X-100 and SDS were employed. Delipidated neuronal proteins were solubilized in SDS as described by Gombos(31) resulting in 75-80% of the proteins in solution. The protein solution contained 3-4.5% bound carbohydrate as measured by phenol sulfuric acid method. The SDS solubilized neuronal proteins were subjected to Con A-Sepharose chromatography after bringing down the SDS concentration to 0.04% by dialysis against 20mM Tris-HCl pH 7.0. The 500mM methyl α-D glucopyranoside(α-MG) eluate on

SDS-PAGE revealed Con A as major component. Extraction of intact neurons with 1% Triton X-100 also resulted in leaching of Con A during the 0.5M α-MG elution. This problem of Con A leaching was minimized when affinity chromatography was carried out in the absence of detergents. Delipidated neuronal proteins were subjected to sonication (15 microns for 2 min). Con A-Sepharose chromatography revealed a poor yield of glycoproteins, which may be due to the loss of covalently bound carbohydrates. When the intensity of sonication was reduced, it resulted in the poor extraction of neuronal proteins. This problem has been minimized with a western blot buffer: 25 mM Tris, 0.7M glycine pH 7.8 (27). A neuronal plasma membrane enriched preparation was homogenized in the western blot buffer by subjecting to very mild sonication. Con A chromatography after adjusting the pH of the homogenate to 7.4, resulted in Con A leaching.

The problem of Con A leaching was ultimately overcome by pretreating the Con A-Sepharose 4B with 8M urea. In the finalized procedure, a neuronal plasma membrane enriched fraction was first prepared by lysing the cells with aqueous solutions of 10mM EDTA, 0.2% β-mercaptoethanol in Potter-Elvehjm homogeniser. The homogenate was centrifuged at 8000 g X 10 min to obtain a plasma membrane enriched pellet. The pellet was then homogenized in 25mM Tris,0.7 M Glycine pH 7.8 with 1% Triton X-100, followed by mild sonication. The 150 g X 5 min supernatant was adjusted to pH 7.4 and chromatographed on urea treated Con A-Sepharose 4B at 4°C.

The electro eluted SDS—PAGE protein bands as well as the neuronal glycoproteins eluted at 4°C, contained 180 ± 60 µg carbohydrate / mg protein. The sialic acid content of the cold eluted glycoproteins were in the range of 35 ± 5 nmoles / mg protein.

The neuronal glycoproteins eluted from Con A-Sepharose column were contaminated with noncovalently associated glycolipids. The lipid extract was positive for carbohydrate.Thin layer chromatography of the lipid extract in solvent system, n-propanol:water (7:3) further confirmed the presence of glycolipids. The amount of glycolipids was more when the elution was carried out at 25°C than at 4°C. The association of glycolipids is an additional evidence for the fact that the neuronal glycoproteins are from the cell surface.

SDS-PAGE of neuronal glycoproteins in 8% or 10% gels and 3-12% linear gradient gels indicated three major groups of diffuse bands of molecular weights 65-72 KDa; 52-63 KDa; and 43-48 KDa respectively. These groups of bands were cut and electroeluted. They were further subjected to electrophoresis under similar experimental conditions. Each group of bands upon reelectrophoresis gave rise to all the 3 groups of bands of the original sample. The multiple band pattern of neuronal glycoproteins on SDS-PAGE may be accounted for by an additive effect of anomalous SDS binding, protein aggregation, heterogenous mixed protein-SDS micellae formation and microheterogeneity. Five

percent urea-PAGE at pH 4.2 in presence of 6M urea revealed 4 bands for the neuronal glycoproteins(24). They did not migrate in alkaline-urea-PAGE at pH 8.6.

The probable contribution of glycoprotein microheterogeneity and the presence of covalently linked lipids to the anomalous SDS-PAGE pattern was investigated. The neuronal glycoproteins were subjected to chemical deglycosylation and deacylation. Deglycosylation by trifluoromethanesulfonic acid (TFMS) treatment at 0°C for 2 h ,desialylation by acid hydrolysis ,and nitrous acid deamination caused an enhanced aggregation of neuronal glycoproteins resulting in a higher percentage of their retention at the bottom of SDS-PAGE sample wells. In addition, the TFMS treatment caused an increase in the average molecular weights of neuronal glycoproteins on SDS-PAGE. However these chemical modifications did not change the multiple band pattern of neuronal glycoproteins on SDS-PAGE.

The protein pattern on SDS-PAGE remained unchanged after deacylation with methanolic KOH and hydroxylamine treatments (21). The nitrous acid deaminated samples indicated the absence of glycosyl phosphatidyl inositol membrane anchor in neuronal glycoproteins. The glycoproteins did not contain ester linked fatty acids.

N-terminal analysis of the total Con A eluate as well as the individual SDS-PAGE protein bands by Dansyl Edman procedure(8),

(16) and DABITC-PITC method of Chang et al(5), revealed a blocked N-terminal.

The neuronal glycoproteins separated on 8% SDS-PAGE were electrophoretically transferred to nitrocellulose for lectin affinoblotting. Horse radish peroxidase (HRP)-conjugated Con A, wheat germ agglutinin (WGA) and Ricinus communis agglutinin (RCA) stained all the bands of glycoproteins.

Differential lectin binding studies were carried out after chemically modifying the neuronal glycoproteins. Dot-enzyme linked lectin assay was performed with HRP-conjugates of Con A, WGA, RCA, jack fruit seed agglutinin (JSA), Artocarpus hirsuta agglutinin (AHA), soybean agglutinin (SBA), peanut agglutinin (PNA) and pea lectin . All the lectins except SBA and PNA interacted with the neuronal glycoproteins and were inhibited by their respective hapten sugars. When compared to the complete inhibition of Con A binding with 500mM α-MG, inhibition by 15mM α-MG was partial, indicating the presence of both high mannose and hybrid type oligosaccharide chains. The affinity of JSA and AHA for the neuronal glycoproteins was higher compared to RCA. This could be explained by the differential anomeric specification of these galactose binding lectins or by steric hindrance of the large RCA molecule for galactose residues. This was further demonstrated by the total absence of RCA binding to neuronal glycoproteins after periodate oxidation and TFMS treatments. Deglycosylation with TFMS at 25°C for 3h was

incomplete as indicated by the residual binding of Con A , WGA, JSA and AHA. Periodate oxidation (10mM) at pH 3.5 for 24 h at 4°C did not completely modify the oligosaccharide side chains. The lectin binding was not significantly altered by deamination. The presence of fucose was demonstrated by pea lectin binding. In addition to the lectins , HRP-serotonin conjugate also interacted with the neuronal glycoproteins. Desialylation by acid hydrolysis diminished the binding of serotonin and WGA, whereas the galactose binding lectins (RCA,JSA and AHA) did not exhibit enhanced affinity. Neuraminidase treatment of neuronal glycoproteins also resulted in decreased binding to WGA and serotonin(10).

The gel filtration of neuronal glycoproteins was carried out on Sepharose 6B (100cm X 1.4cm) equilibrated with 25mM Tris,0.7M glycine pH 7.8. Glycoproteins labelled with (^{3}H)-acetic anhydride,when chromatographed resolved into two peaks. The first peak appeared at the void volume ,but the second peak appeared at the end of total bed volume. The peaks were positive for lectin binding . Each of the gel filtration peaks upon rechromatography under similar conditions exhibited identical elution pattern. The proteins remained in soluble supernatant when centrifuged at 100,000 g X 60 min. Both gel filtration peaks on 8% SDS-PAGE gave the same multiple band pattern as that of the original sample. On acid-urea-PAGE both gel filtration peaks gave a single fast moving band of equal mobility. Mobility of this urea-PAGE band

was higher than that of the native Con A-binding glycoprotein bands. The second gel filtration peak may probably represent the monomers of peak I protein. Treatment with urea may be insufficient in dissociating the native neuronal glycoprotein to its monomers. This could be due to the formation of heterogenous micellar aggregates represented by the 4 bands on urea-PAGE. Acetic anhydride labelling might have destabilized the formation of protein micellae, leading to its dissociation into monomers in urea. This would explain the higher mobility of acetic anhydride labelled proteins on urea-PAGE as compared to the native glycoprotein.

Phase partitioning of (^{3}H)-acetic anhydride labelled neuronal glycoproteins with chloroform:methanol:water (2:1:0.6) resulted in the recovery of 87% radioactivity in the lower organic phase. The upper and lower phases were negative for lectin binding whereas the interphase was positive. Acetic anhydride can form hydrophobic interactions with the neuronal glycoproteins.

The above labelled glycoprotein as well as the gel filtered peaks were chromatographed on HPLC with Lichrosorb RP-8 column. HPLC gradients were formed from Solvent A (Water, 0.1% TFA) and Solvent B (Isopropanol,0.1% TFA).In gradient I (60% solvent B at 40 min),the total Con A eluate migrated as a single broad peak with a retention time of 54 min. The gel filtered peaks eluted out of the column as single peaks with retention times of 58 min

for peak I and 47 min for peak II. Dansylated and carboxymethylated glycoprotein migrated as a broad peak in gradient I with a retention time of 61 min . In gradient II (60 % solvent B at 65 min), the (^{3}H)-labelled neuronal glycoprotein moved as a single broad peak with retention time of 71 min . The neuronal glycoproteins were not susceptible to proteolytic digestion with trypsin and pronase.

Attempts to raise antibodies against intact second trimester neurons in rabbit and guinea pigs were unsuccessful. In another attempt second trimester neuronal glycoproteins resolved on 10% SDS-PAGE, was used for immunizing rabbits along with the homogenized gel pieces. There was no antibody production. The lack of antibody production could be due to the low immunogenicity of neuronal glycoproteins.

Based on the limited number of physicochemical studies carried out with the available technology, no structural differences were detected between the neuronal glycoproteins of second trimester and full term human fetal brains.

REFERENCES:

1. Bhat,N.R.,and Waechter,C.J.(1988) J.Neurochem.50,375-381.
2. Bock,E.(1984) Hand Book of Neurochemistry.2 Edn (Lajtha,A., ed.) Plenum Press,pp.231-244.
3. Brady,R.O.,and Quarles,R.H.(1973) Mol.Cell.Biochem.2,253-256.

4. Bradford,M.M.(1976) Anal.Biochem.72,248-254.

5. Chang,J.Y.,Brauer,D.,and Wittmann-Liebold,B.(1978) FEBS. Lett.93,205-214.

6. Farooq,M.,and Norton,W.T.(1978)J.Neurochem.31,887-894.

7. Feizi,T.(1985) Nature 314,53-57.

8. Gray,W.R.(1972) Methods Enzymol.251,121-138.

9. Grumet,M.,and Edelman,G.M.(1984) J.Cell Biol.98,1746-1756.

10. Hansen,S.N.,Lihme,A.O.F.,Hansen,T.C.B.,and Bjerrum,O.L. (1985) in Lectins Biology Biochemistry Clinical Biochemistry. (Hansen,E.,ed.) Vol.4,pp.241-252, Walter de Gruyter,Berlin.

11. Harford,J.B.,and Waechter,C.J.(1980) Biochem.J.188,481-490.

12. Heacock,A.M.(1982) Brain.Res.241,307-315.

13. Idoyaga-Vargas,V.,and Carminatti,H.(1982) Biochem.J.202, 87-95.

14. Kilpatrick,G.J.,Jenner,P.,and Marsden,C.D.(1985) J.Pharm. Pharmacol.37,320-328.

15. Krusius,T.,and Finne,J.(1977) Eur.J.Biochem.78,369-380.

16. Marquez,F.J.,Queseda,A.R.,Sanchez-Jimenez,F.,and De Castro, I.N.(1986) J.Chromatogr.380,275-283.

17. Mikol,D.D.,and Stefansson,K.(1988) J.Cell Biol.106,1273-1279.

18. Pfenninger,K.H.,and Maylie-Pfenninger,M.F.(1981) J.Cell. Biol.89,536-546.

19. Poduslo,S.E.,and Norton,W.T.(1975) Methods Enzymol.35,

561-579.

20. Rathjen,F.G.,Wolff,J.M.,Frank,R.,Bonhoeffer,F.,and Rutishauser,U.(1987) J.Cell Biol.104,343-353.

21. Reindeau,D.,and Guertin,D.(1986) J.Biol.Chem.261,976-981.

22. Sater,U.V.,and Glick,M.C.(1979) Biochemistry 18,2533-2540.

23. Schachner,M.,Faissner,A.,Fischer,G.,Keilhauer,G.,Kruse,J., Kunemund,V.,Lindner,J.,and Wernecke,H.(1985) in The Cell In Contact (Edelman,G.M.,ed.),pp.257-275.

24. Sherton,C.C.,and Wool.I.G.(1974) Methods Enzymol.30, 506-521.

25. Shreeve,S.M.,Fraser,C.M.,and Venter,J.C.(1985) Proc.Natl. Acad.Sci.USA 82,4842-4846.

26. Shivakawa,O.,Kuno,T.,and Tanaka,C.(1983) Biochem.Biophys. Res.Comm.115,814-819.

27. Small,G.M.,Imanda,T.,and Lazarow,P.B.(1988) Anal.Biochem. 169,405-409.

28. The,T.H.,and Feltkamp,T.E.W.(1970) Immunology 18,865-875.

29. Thiery,J.P.,Brackenburry,R.,Rutishauser,U.,and Edelman,G.M. (1977) J.Biol.Chem.252,6841-6845.

30. Yogeswaran,G.,and Salk,P.L.(1981) Science 212,1514-1516.

31. Zanetta,J.P.,Morgan,I.G.,and Gombos,G.(1975) Brain Res.83, 337-348.

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Dated 19th March 1991

Dr. G.N.A. Nayar
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Dear Dr. Nayar,

Please refer to your letter No.SCTIMST/Acad/250.1/91, dated 8th Feb. 1991, I am enclosing herewith the reports of the Thesis of Ms. Yasmin Marikar and Mr. Bobby Zacharia, entitled "Biochemical Studies on Cell Surface Glycoproteins of Glial Cells in Developing Human Brain" and "Physicochemical Studies on Cell Surface Glycoconjugates of Neurons from Developing Human Brains" respectively.

Please acknowledge the same.

Yours sincerely,

(B.K. BACHHAWAT)