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MYELIN MEMBRANE PROTEIN BIOSYNTHESIS: AN *IN VITRO* STUDY

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BY

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DEDICATION

This thesis is dedicated to my Mother and Father.

ABBREVIATIONS USED IN THIS THESIS

ATP	-	Adenosine 5'-triphosphate
cAMP	-	Adenosine 3',5'-cyclic monophosphate
CNPase	-	2',3'-Cyclic nucleotide 3'-phosphodiesterase
CNS	-	Central nervous system
cpm	-	Counts per minute
CSK	-	Cytoskeleton
DAB	-	3,3'-Diaminobenzidine tetrahydrochloride
DPM	-	Dog pancreatic microsomes
DTT	-	Dithiothreitol
EDTA	-	Ethylenediaminetetra-acetic acid
EGTA	-	Ethyleneglycolbis(aminoethylether)tetra-acetic acid
EM	-	Electron microscope
GTP	-	Guanosine 5'-triphosphate
HEPES	-	N-2-Hydroxyethylpiperazine-N'-2'-ethanesulphonic acid
MAG	-	Myelin-Associated glycoprotein
MBP	-	Myelin basic protein
OD	-	Optical density
OLIGO(dt)	-	Oligodeoxythymidylate
PBS	-	Phosphate buffered saline
PIPES	-	Piperazine-NN'-bis-2-ethanesulphonic acid
PLP	-	Proteolipid protein
PNS	-	Peripheral nervous system
POLY (A)	-	Poly(adenylate)
POLY(U)	-	Poly(uridylate)
PTFE	-	Poly(tetrafluoroethylene)
SDS	-	Sodium dodecyl sulphate
SRP	-	Signal recognition particle
TCA	-	Trichloroacetic acid
TLCK	-	Nα-p-Tosyl-L-lysine chloromethyl ketone
TRIS	-	Tris(hydroxymethyl)aminomethane
WGE	-	Wheatgerm extract

ABSTRACT

The sites of biosynthesis and incorporation of the abundant CNS myelin proteins 2',3'-cyclic nucleotide-3'-phosphodiesterase (CNPase) and P2 protein into the growing myelin membrane were investigated.

Cell-free translation systems programmed with mRNA from rat brain, rabbit spinal cord, free and bound polysomes and purified myelin demonstrated conclusively that both CNPase and P2 are synthesized on free polysomes like the myelin basic proteins (MBPs) but unlike the proteolipid protein (PLP), the major intrinsic membrane protein of CNS myelin, which is known to be synthesized at the oligodendrocyte endoplasmic reticulum on bound polysomes (Colman et al., 1982).

These observations were supported by labelling studies on rats in vivo during the period of maximal myelin deposition. Newly synthesized CNPase associated with the myelin membrane very rapidly after labelling (~2 minutes) and this is consistent with the view that there is only a brief delay between synthesis and incorporation into their target membrane for extrinsic-type plasma membrane proteins. An RNA fraction isolated from purified CNS myelin was not enriched in mRNAs coding for CNPase and P2 but a considerable enrichment of mRNAs coding for MBPs was observed. This phenomenon has important implications for the cell biology of myelination since it suggests that although MBPs, CNPase and P2 are all basic extrinsic membrane proteins, and synthesized on free polysomes, different mechanisms for their transport to the myelin membrane exist.

The/

The addition of dog pancreatic microsomes (DPM) during translation showed no membrane association for CNPase however, at least 50% of MBPs were observed to non-specifically associate with these membranes. When newly synthesized MBP and P2 were incubated post-translationally with DPM or rabbit spinal cord myelin P2 only associated with myelin whereas MBP showed an equal affinity for both types of membranes. The segregation of MBP free polysomes at the myelin membrane during synthesis ensures that the nascent MBP polypeptides associate with the correct membrane.

Recent evidence has shown that the free polysome-mRNA complex is bound to the cytoskeleton during protein synthesis. After extensive characterization of the purified rat brain oligodendrocyte and myelin-associated cytoskeletons it was shown that the synthesis of MBPs and CNPase only occurs from mRNA that is associated with the cytoskeleton and not when it is part of the cytoplasmic mRNA pool. Lipid analysis of the purified rat brain myelin-associated cytoskeleton revealed the presence of tightly bound lipid with a considerable enrichment of cerebroside and sphingomyelin (the latter at the expense of phosphatidylethanolamine).

These studies on the cytoskeletal involvement in myelinogenesis suggest that extrinsic CNS myelin proteins are synthesized on the cytoskeleton and that post-translational cytoskeletal transport of these proteins to the growing myelin membrane may take place.

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Introduction

1.1 HISTORICAL ASPECTS OF MYELIN

The function of myelin is to insulate nerve fibres and thereby allow a considerable increase in the rate of nerve impulse conduction by comparison with unmyelinated fibres of similar diameter.

The presence of sheaths surrounding nerve axons was first reported by Virchow (1854) in the mid-19th century in the first study to establish myelin as a structural entity. Due to the lack of suitable techniques at that time it was some years before histologists, using newly developed stains and polarization microscopy, could visualize the structural relationships between the axon and its myelin sheath. Using these techniques it was established that the myelin sheath was not continuous over the entire axon but was segmented (Ranvier, 1871). Ranvier was later to investigate the involvement of the segmented junctions (Nodes of Ranvier) in saltatory conduction, the specialized form of impulse conduction found in myelinated axons.

The early work of Schmitt and his colleagues (Schmitt et al., 1935; Schmitt and Bear, 1939) established that myelin was a concentric, layered structure and that it consisted of a repeating unit. It was possible for Schmitt to write in 1939 that, "The proteins occur as thin sheets wrapped concentrically about the axons with two bimolecular layers of lipoids interspersed between adjacent protein layers".

The advent of the electron microscope and its application to neurobiology led to a burst of activity during which the major morphological features of myelin were elucidated.

Sjostrand/

Sjöstrand (1949) and Fernandez-Moran (1950) confirmed that the structure was multi-lamellar in nature and Geren and Schmitt (1954) showed that myelin formation was due to a spiralling of the membrane around the nerve axon to form the tightly packed myelin sheath. It must be said that most of the early work on the structural elucidation of myelin was carried out on the peripheral nervous system (PNS) and this was due to the accessibility of the tissue and the ease of fixation. Studies on the central nervous system (CNS) comprising the brain and spinal cord lagged behind the PNS and most of the structural foundation work was initiated in the early 1960's. Maturana (1960) and Peters (1960) confirmed that the CNS possessed a spiral configuration analogous to that of the PNS. CNS myelinogenesis, however, was the subject of much debate at that time, even the suggestion that the myelin sheath was a product of the axon itself (Hild, 1957). The origin of CNS myelin was finally resolved by Bunge et al. (1962) who showed that the cell responsible for myelination was the oligodendrocyte and that processes sent out by this cell contacted nerve axons and wrapped round them. Further work since that time has supported and refined Bunge's initial observations (Peters, 1964,; Okada, 1982).

1.2 STRUCTURE OF CNS MYELIN

i. Morphology

CNS myelin is a highly specialized biological membrane that has evolved to provide an environment of high resistance and low capacitance allowing a nerve impulse to be propagated along the axon with greater velocity and much less energy expenditure in doing so than/

than in unmyelinated axons (Waxman and Ritchie, 1985). What are the special features of this biological membrane that make it so unique and allow it to perform this function? Under the electron microscope the various stages of myelination can be observed (Figure 1) and certain features are immediately evident. In the early stages of CNS myelination (Figure 1a) a loose wrapping of an oligodendrocyte process around the nerve axon is seen. Myelination in the CNS is initiated when the axons to be myelinated reach a diameter of approximately lum, usually a few days after myelination has commenced in the PNS (Raine, 1984a). The oligodendrocyte process forms a loose cup around a segment of the axon and a further extension of the process results in one lip of the cup, the future "inner tongue" of the mature sheath, working it's way beneath the other. The multilamellar structure is formed by the rotation of this inner tongue around the axon. While the first few spiral turns are forming (Figure 1b) cytoplasm between layers and on the outer and inner surfaces disappears rapidly and the process of compaction has begun. Compaction of the membrane layers occurs very soon after the initial spiralling (Raine et al., 1968) and the oligodendrocyte cytoplasm is reduced to a thin, continuous strip (Schmidt-Lanterman Incisures) located at the outer, inner and paranodal edges of the mature sheath (Hirano and Dembitzer, 1967). This process continues until the layers have fused together (Figure 1c) forming a single major dense line between the inner surfaces of the unit membrane and an intraperiod line resulting from the close apposition of the outer leaflets of the membrane.

The ability of oligodendrocytes to synthesize myelin membrane

is/

Fig. 1a. EM photograph from a 4 day old kitten spinal cord. An oligodendrocyte process (✱) is seen loosely wrapped round an axon (A). The future inner loop (*) is also seen. Scale bar: 0.25μm x 48,000.

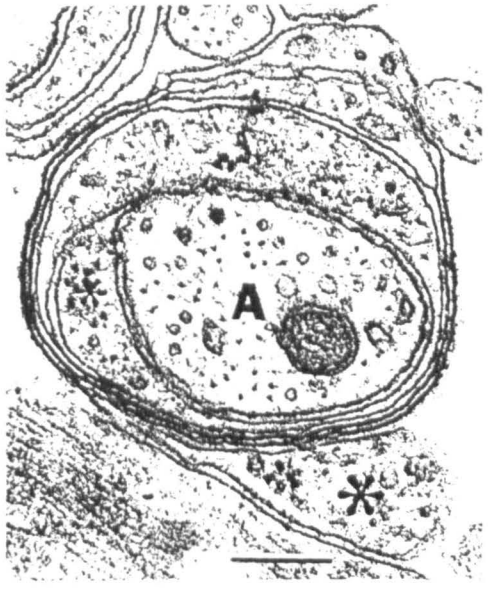


Fig. 1b. A later stage in CNS myelination than that in Fig. 1a. showing partial compaction of the sheath around it's axon (A). The outer (✱) and inner (*) loops are also observed. Scale bar: 0.5μm x 27,000

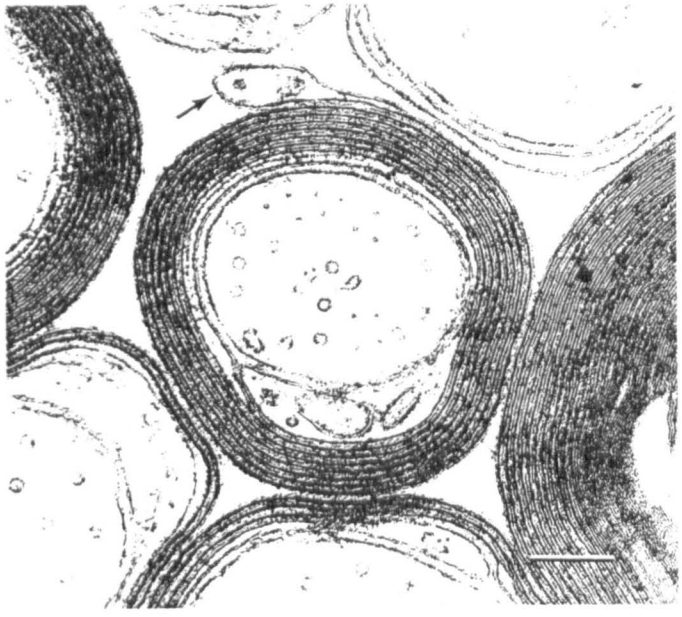


Fig. 1c. Transverse section of a mature CNS myelin sheath from canine spinal cord showing the outer tongue (→) and spiral nature of the sheath. Scale bar: 0.1μm x 150,000.

From Raine (1984a)

is prodigious; one oligodendrocyte can myelinate up to fifty axons, even those that are in nerve tracts distant from its cell body; it has been estimated that oligodendrocytes can produce three times their own weight in myelin every day (Crang and Rumsby, 1978; Norton and Poduslo, 1973b).

The presence of transverse bands between the lateral loop and the axolemma was reported by Hirano and Dembitzer (1982). These junctional complexes (zonulae occludentes) have also been observed by freeze-fracture techniques (Schnapp and Mugnaini, 1975) and are thought to be of functional significance since they develop after the sheath matures. The Node of Ranvier also contains desmosomes between adjacent lateral loops (Metuzals, 1965) however the functional significance of this is not known.

The classic work of Bunge et al.(1961) on the structural analysis of myelination showed that the oligodendrocyte process that myelinates an axon is shovel-shaped (Figure 2) and our understanding of the morphology of myelination has changed little since then.

ii. Molecular Organization

Myelination in the CNS is a complex process and must involve the involvement of many biochemical processes proceeding in a concerted manner. The functions of myelin lipids and proteins in myelinogenesis are unknown. However, we can speculate about certain steps which must be key stages in myelinogenesis.

1. The oligodendrocyte must reach a stage at which it is able to synthesize the specific components of the myelin sheath.

2. The axon must reach a stage at which it can be recognized as a myelinat able structure.

3./

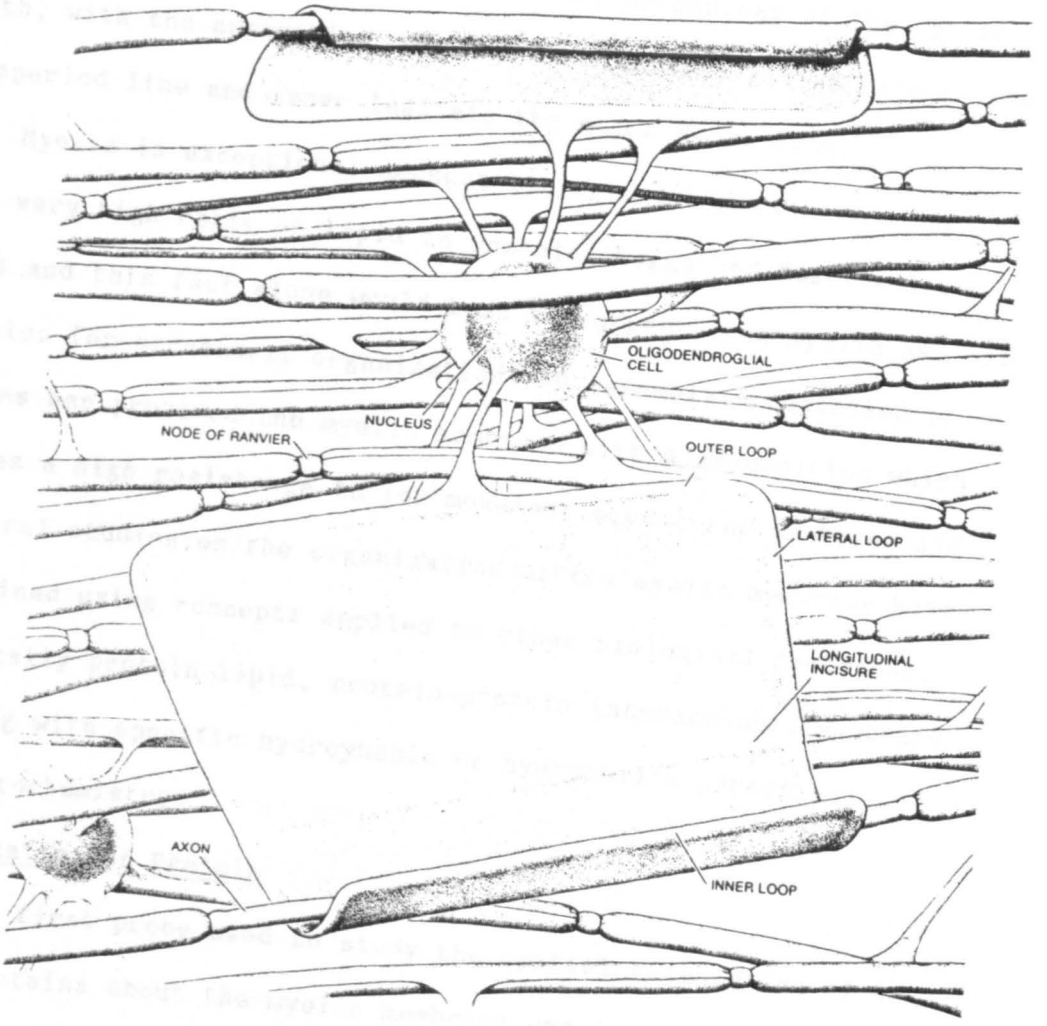


Fig. 2. Oligodendrocyte soma attached to numerous myelin sheaths unfolded to varying degrees. From Bunge et al. (1961).

3. The myelinating cell must recognize the axon to be myelinated as a specific target.

4. The wrapping of the myelinating cell processes must proceed in such a way as to build the characteristic morphology of the myelin sheath, with the apposition of the outer leaflets giving rise to the intraperiod line and inner leaflets the major dense line.

Myelin is exceptional amongst biological membranes in that it has a very high ratio of lipid to protein (Boggs and Moscarello, 1978a) and this fact alone would tend to suggest that myelin has its own rules for structural organization and assembly. Depletion of proteins has provided the myelin membrane with a composition which provides a high resistance to ion movement across the membrane and structural studies on the organization of the myelin membrane have been gained using concepts applied to other biological membranes, specifically protein-lipid, protein-protein interactions, membrane labelling with specific hydrophobic or hydrophilic probes and immunocytochemistry.

(a) Location of Protein

The first probe used to study the spatial orientation of CNS myelin proteins about the myelin membrane was lacto-peroxidase iodination (Poduslo and Braun, 1975) which showed that only PLP and some high molecular weight proteins were radio-iodinated. The results were regarded with caution however, since only the outer lamellae were seen to be labelled. A more permeable probe, salicylaldehyde, was used by Golds and Braun (1976) but resulted in all myelin proteins being labelled in both intact and fragmented myelin. A more definitive answer was obtained by using the hydrophobic/

hydrophobic probe 4, 4'-diisothiocyano -2, 2'- ditritiostilbene (Wood et al., 1977). Using CNS myelin fragments they showed that, on the basis of reactivity to the probe, MBP was much less accessible implying that it may reside in the major period zone rather than the intraperiod zone. Recent studies by Stevens et al. (1986) using adamantane diazirine (hydrophobic) and iodosulphanilic acid (hydrophilic) probes have indicated that PLP is an integral membrane protein whereas MBP is found at a site inaccessible to the hydrophobic probe ie. the cytoplasmic face of the membrane. Immunocytochemistry at the EM level has mapped MBP to the cytoplasmic faces of compact myelin and has confirmed the earlier observations using probes (Omlin et al., 1982).

In contrast to the two major CNS myelin proteins, PLP and MBP, less is known about the spatial disposition of MAG and CNPase in the membrane. Evidence to date has suggested that MAG is localized to the periaxonal region of the sheath and not in compact myelin. This has been linked to the idea that this protein is involved in the apposition of the myelin sheath to the axon (Quarles, 1984; Martini and Schachner, 1986). MAG has an extensive extracellular exposure (Poduslo et al., 1976) and proteolysis experiments have shown that a substantial part of the protein is firmly embedded in the lipid matrix (Sato et al., 1982).

CNPase has been mapped, by immunocytochemical techniques, to the major dense line of compact myelin analogous to the distribution of MBP (Roussel et al., 1978). Although this study observed that staining was evident only on the outer lamellae of the myelin sheath it was proposed that this was probably due to the inability of the antibodies/

antibodies to penetrate the dense wrappings of the sheath, a result that was confirmed by Nishizawa et al. (1981). Supporting evidence for the localization of CNPase in myelin sheaths has come from Sprinkle et al. (1983) and Nishizawa et al. (1985) who have clearly shown immunocytochemical staining of CNPase in myelin, oligodendrocytes and oligodendrocyte processes. No staining was observed in astrocytes or neurons.

(b) Biophysical Studies

Biophysical studies carried out to shed light on protein-lipid interactions have by and large been mainly of the reassociative type. That is, the reconstitution of purified proteins into or onto purified lipid vesicles, the so called model membrane systems. By the use of these techniques, particularly electron spin resonance (ESR), nuclear magnetic resonance (NMR) and liquid diffraction X-ray analysis the possible orientation of proteins with respect to the lipid bilayer and the changes in secondary structure of these proteins on binding can be detected.

Boggs et al. (1977) provided the earliest evidence that PLP preferentially associates with negatively charged lipids and that MBP also has a high affinity for this class of lipids. Since it has been shown that MBP is synthesized on free polysomes, is a peripheral type protein (Colman et al., 1982) and does not penetrate the lipid bilayer the question of the importance of electrostatic interactions in their relationship to the myelin membrane requires attention (Boggs et al., 1981). However the importance of the involvement of electrostatic forces in the structural association of MBP with the myelin membrane has been questioned by Braun (1984) since/

since the anionic lipid asymmetry of the myelin bilayer is unknown. He further argues that since only about 10 mole % of myelin lipids are anionic other myelin proteins (PLP is cationic at physiological pH for example) and cytoskeletal elements would also have access to some of these negatively charged residues.

Spin-label ESR (Brophy et al., 1984) and deuterium and phosphorus NMR (Sixl et al., 1984; Meier et al., 1987) studies on reconstituted PLP in model membranes have suggested that the protein has a hexameric arrangement in the membrane, a fact that was initially observed by Smith et al. (1983) using conditions that produced fewer structural perturbations to the protein than had previously been used. PLP with its potential surface membrane domains may have a role to play in anchoring the apposing lamellae of the compact myelin sheath (Laursen et al., 1984; Stoffel et al., 1984).

The ability of MBP to self-associate has also been observed by a number of workers (Chapman and Moore, 1976; Smith, 1982). The protein has a strong tendency to dimerize in low concentrations of SDS (Smith and McDonald, 1979) and the presence of covalently cross-linked dimers, but not higher oligomers, has been demonstrated in the intact myelin sheath (Golds and Braun, 1978a). An important discovery was made in 1977 when it was established that MBP could induce cross-linking of lipid bilayers <u>in vitro</u> (Smith, 1977) and a hypothesis that MBP played an important role in the interfacing of the cytoplasmic bilayer surfaces of CNS myelin was put forward by Braun (1977). Despite all the information gathered from studies such as X-ray diffraction, immunocytochemical localization of specific/

specific myelin proteins, freeze-fracture electron microscopy and molecular interactions in well-defined model membrane systems (ESR and NMR) we have only a tentative concept of molecular organization within the myelin membrane. Several models have been published (Rumsby and Crang, 1977; Boggs et al., 1982) and in the latest model (Braun, 1984) (Figure 3) the PLP is depicted as an integral membrane protein in both monomeric and oligomeric forms. Heterodimers of MBP and PLP (Golds and Braun, 1978b) are shown as is the peripheral nature of MBP. Also shown is MBP with one or more domains in very limited contact with the hydrophobic interior of the bilayer (Boggs and Moscarello, 1978b) together with the presence of MBP dimers. Braun stresses that although structural information obtained to date has been on PLP and MBP(s) and that together they comprise at least 60% of the total CNS myelin protein this does not mean that they are solely responsible for holding the membrane together. However from the work of Kirschner et al. (1979) and Hollingshead et al. (1981) he proposes that once the ordering of the lamellae has been initiated the major proteins play a role in the maintenance or stabilization of the interlamellar spacing.

iii. Origins of CNS Myelin - The Oligodendrocyte

(a) Differentiation In Vivo

It is generally accepted that the first description of the oligodendrocyte was given by Robertson (1899) when, experimenting with metal impregnation of CNS tissue, he noticed small branching cells of "very characteristic aspect" found throughout the white matter of the brain. The cell's name and detailed description was to appear several decades later in extensive work by the Spanish histologist/

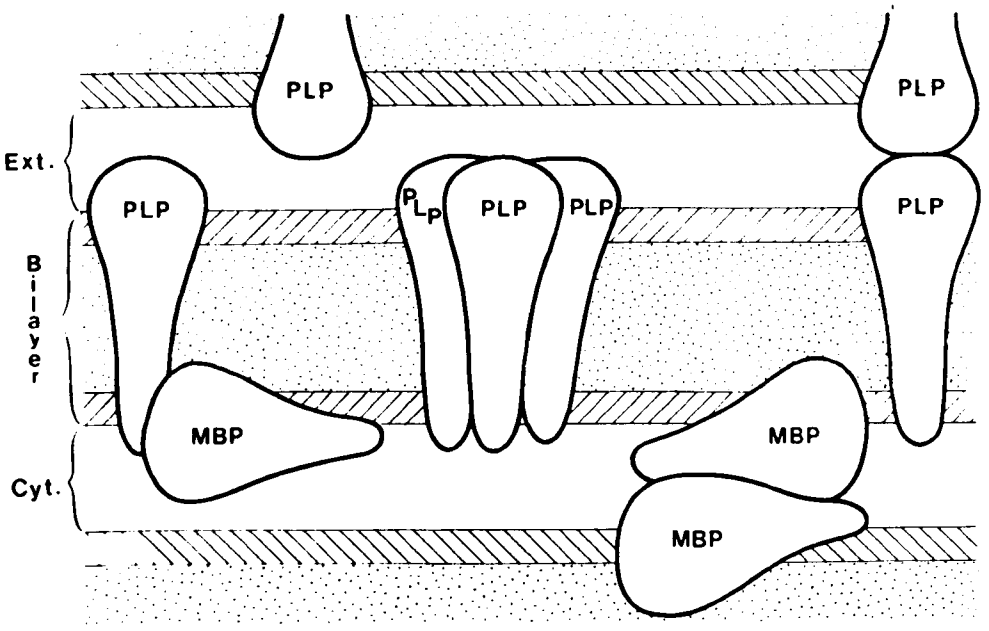


Fig. 3. Diagrammatic representation of the molecular organization in CNS myelin illustrating several possible arrangements of PLP and MBP. (Ext.) Extracellular apposition (intraperiod line); (Cyt.) cytoplasmic apposition (major dense line). After Braun (1984).

histologist (del Rio Hortega, 1919) who demonstrated that Ramon y Cajal's (1913) so called third element (ie. third brain cell type in addition to astrocytes and neurons) was composed of two types of cells: oligodendrocytes ("cells with few branches") derived from neuroectodermal cells, and microglial cells, originating from mesodermal cells. The general features of oligodendroglial ontogeny emerged from these early studies and a number of important questions still await answer. Present studies of oligodendroglial genesis in vivo are based on morphological studies using electron microscopy combined with labelling studies of cells with [^{3}H] thymidine and subsequent autoradiography (reviewed by Polak et al., 1982).

The major development of oligodendroglia occurs during postnatal life and is related to myelination in the various parts of the CNS. A layer of immature cells (the subventricular layer) persists into the postnatal period and it is from this layer that cells migrate to various parts of the brain. Differentiation into oligodendrocytes then commences (Sturrock, 1982; Paterson, 1983) with the sequence as follows: subventricular cells, glioblasts, oligodendroblasts, light oligodendrocytes, medium oligodendrocytes and finally, dark oligodendrocytes (Figure 4). Postmitotic maturation gives rise to three morphologically distinct oligodendrocytes. The light oligodendrocytes are the largest cells in the series and have a cytoplasm rich in organelles and microtubules suggesting high metabolic activity (Federoff, 1985). Federoff also points out that the formation of light oligodendrocytes corresponds roughly to the time of rapid myelination. After 4-7 days the light oligodendrocytes are transformed/

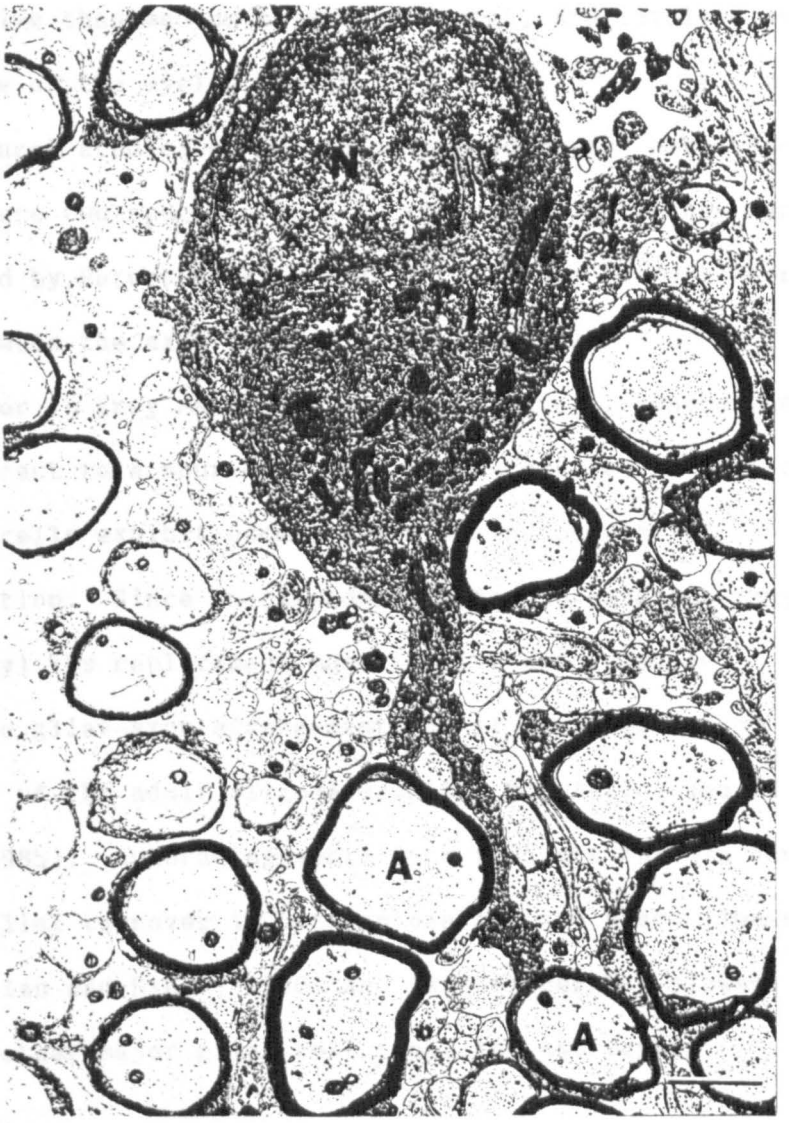


Fig. 4. EM picture of an oligodendrocyte from kitten spinal cord extending a process that branches to myelinate two axons (A). (N) Nucleus. Scale bar: 1μm x 15,000. From Raine (1984a).

transformed into medium oligodendrocytes and these cells appear to be less active metabolically. Around two weeks later maturation into the dark oligodendrocyte occurs. These cells have dense nuclei and cytoplasm and microtubules are difficult to see. Imamoto et al. (1978) have proposed that these cells, appearing even less metabolically active than medium oligodendrocytes, are involved only in the maintenance of the myelin sheath.

Ultrastructural studies of mature oligodendrocytes have also shown that there are two specific loci in the brain where they can be found (reviewed by Peters et al., 1976). They are found either lying in rows between the axon bundles (interfascicular oligodendroglia) or in grey matter as perineuronal cells where the cell body is adjacent to a neuronal soma. These studies have also shown that these cells exhibit common features independent of sub-type and location. Since an oligodendrocyte can ensheath many axons (up to fifty) its replacement must involve considerable disturbance to the glial-axon arrangement and it could be argued that any turnover of the adult oligodendroglial population should be slow (Sturrock, 1985). Autoradiographic data indicate that in adult mice oligodendroglial turnover is between one and two years (Imamoto et al., 1978; Kaplan and Hinds, 1980) and is considerably slower than that of the other major glial cell type, the astrocyte (Paterson, 1983) .

(b) Differentiation In Vitro

Although in vivo studies of oligodendrocytes have furnished us with knowledge of the general timetable and descriptive aspects of oligodendrocyte development and function relatively little information/

information has appeared about what factors influence oligodendrocyte differentiation. In recent years the development of cell culture techniques using enriched cultures of oligodendrocytes (McCarthy and de Vellis, 1980; Lisak et al., 1981) have yielded new information on oligodendrocyte development.

Using monolayers of optic nerves, a culture system that contains no neurons, Miller et al. (1985) have shown that the rat optic nerve contains three types of macroglial cells which develop in a strict sequence: Type 1 astrocytes appear first at embryonic day 16, oligodendrocytes at birth and Type 2 astrocytes between postnatal days 7 and 10. They have also demonstrated that the three cell types originate from two distinct precursor cells, one giving rise to Type 1 astrocytes and the other, oligodendrocytes and Type 2 astrocytes which they have called the O-2A progenitor cell. The presence or absence of foetal calf serum in the medium has a direct effect on the O-2A developmental pathway.

In the absence of foetal calf serum most cells develop into oligodendrocytes (Raff et al., 1983). O-2A progenitor cell proliferation in vitro is affected by a growth factor produced by Type 1 astrocytes (Noble and Murray, 1984) and further work by Raff et al. (1985) has suggested the existence of an intrinsic clock in the O-2A progenitor cell that counts cell divisions that are driven by the Type 1 astrocyte growth factor. In vitro the O-2A progenitor is a bipolar migratory cell which expresses gangliosides on its surface and the intermediate filament protein vimentin in its cytoplasm (Raff et al., 1984).

The Type 2 astrocytes derived from this lineage contribute to the/

the Nodes of Ranvier (ffrench-Constant et al., 1986; ffrench-Constant and Raff, 1986). The pattern of incorporation of lipid precursors into myelin-related lipids mimicks the time scale of development (Ayliffe et al., 1984) as does the expression of the myelin-related lipid and protein markers galactocerebroside (GC) (Hirayama et al., 1984) and CNPase (Roussel et al., 1983). The expression of MBP, usually in cells with more elaborate processes, is generally considered a further step in maturation (Roussel et al., 1981). This view has been supported by the fact that, in culture, all GC^+ cells can incorporate [^{3}H] thymidine whereas only a few GC^+/MBP^+ can (Bologa et al., 1983; Roussel et al., 1983). Furthermore, cells which were strongly positive for MBP did not incorporate [^{3}H] thymidine at all.

The exact mechanisms which underlie oligodendrocyte differentiation are still unclear. Temple and Raff (1986) have proposed that O-2A progenitor cells can only undergo a limited number of divisions (influenced by the Type 1 astrocyte growth factor) before they differentiate into oligodendrocytes. This hypothesis is supported by evidence that a single optic nerve progenitor cell divides a finite number of times on a Type 1 astrocyte monolayer before its progeny become multipolar cells most of which start to express GC after 10-12 days (Temple and Raff, 1986).

By studying the development of single progenitor cells for three weeks Dubois-Dalcq (1987) has shown that two antigenically different populations of cells emerge: non-proliferating GC^+ oligodendrocytes and slow proliferating GC^- multipolar cells which express/

express a cell surface protein, O_4, shortly before GC. This cell population requires specific signals provided by insulin and/or neurons for entry into final differentiation.

Tissue culture studies of myelination by mature oligodendrocytes are discussed in Section 1.4 ii (c).

1.3 BIOCHEMISTRY OF CNS MYELIN

i. CNS Myelin Lipids

(a) Composition

The dominant chemical feature of CNS myelin is the characteristically high lipid to protein ratio (Table 1). In the rat, some 25% of the dry weight of brain is accounted for by myelin, and because of the relative excess of lipids in this membrane compared to other subcellular fractions of the brain, 40% of the total lipid of brain is accounted for by myelin. In humans, where rather more of the dry weight of brain is due to myelin (35%) more than half of brain lipid is derived from this membrane (Norton, 1981). The most quantitatively significant lipids found in myelin include cholesterol, cerebroside and the plasmalogen form of ethanolamine. Of note is the presence of sulphatide (cerebroside-3-sulphate), a galactolipid, whilst not being entirely specific for the myelin membrane is considerably enriched with respect to other cellular membranes. Cerebroside and sulphatide are characterized by very long chain saturated and monoenoic fatty acids and Vandenheuval (1963) has suggested that this gives the myelin membrane increased stability. The phospholipids phosphatidylserine, phosphatidylcholine, sphingomyelin and phosphatidylethanolamine are less/

TABLE 1 COMPOSITION OF MATURE CNS MYELIN FROM SEVERAL SPECIES (Norton, 1981)

Components	Human	Bovine	Rat
		(% of dry weight)	
Protein	30.0	24.7	29.5
Lipid	70.0	75.3	70.5
		(% of lipid weight)	
Cholesterol	27.7	28.1	27.3
Total Galactolipid	27.5	29.3	31.5
Cerebroside	22.7	24.0	23.7
Sulphatide	3.8	3.6	7.1
Total Phospholipid	43.1	43.0	44.0
Ethanolamine[a]	15.6	17.4	16.7
Choline	11.2	10.9	11.3
Serine	4.8	6.5	7.0
Inositol	0.6	0.8	1.2
Sphingomyelin	7.9	7.1	3.2

[a] Primarily ethanolamine plasmalogen

less prominent in myelin than other membranes. Several minor lipid constituents found in myelin (particularly gangliosides) have attracted much attention since it is thought that they may have some dynamic function as opposed to the purely structural roles attributed to the major myelin-containing lipids. Around 0.2% of myelin lipid is composed of gangliosides (sialic acid - containing glycosphingolipids) primarily the monosialoganglioside G_{M1} (Ledeen et al., 1980). Cochran et al. (1982), amongst others, have found that in addition to G_{M1}, higher primates and birds also contain sialosylgalactosylceramide (G_{M4} or G_7); this lipid appears to be specific for myelin and oligodendrocytes. Sialic acid is thought to play a role in cell-cell recognition and communication and it has been suggested that these gangliosides many serve some similar function during development of the myelin sheath (Wiegandt, 1982).

(b) Metabolism

All the available evidence to date has shown that the biosynthetic pathways for CNS myelin lipids do not differ significantly from those found in other tissues, however these pathways must be extraordinarily active (Morrell and Toews, 1984).

It is generally assumed that the accumulation of "myelin specific" lipids (cerebroside and sulphatide are the most specific markers used) in the brain correlates with the time when oligodendrocytes are most active in synthesizing myelin. Studies in vivo using radioactive precursors of cerebroside and sulphatide have shown that incorporation of label increases as myelination begins, remains high during the period of active myelination and decreases as the rate of myelination decreases (Kishimoto et al., 1965; McKhann and/

and Ho, 1967). Galactosylation of ceramide and sulphation of cerebroside *in vitro* correlate well with the *in vivo* studies, the peak of enzyme activity corresponding to the peak rate of myelin accumulation (Constantino-Ceccarini and Morell, 1972).

The presence of various lipid-synthesizing enzymes in CNS myelin has received much attention recently and confounds the view that the myelin sheath is essentially an inert structure (reviewed by Ledeen, 1984). A high ethanolamine kinase activity has been found in purified myelin (Kunishita et al., 1987) and has completed the list of phospholipid synthesizing enzymes needed to synthesize phosphatidylethanolamine from diacylglycerol within the myelin membrane, CDP-ethanolamine:1, 2-diacyl-sn-glycerol ethanolaminephosphotransferase and CTP:phosphoethanolamine cytidylyltransferase having already been discovered (Wu and Ledeen, 1980; Kunishita and Ledeen, 1984). High levels of long-chain acyl-CoA synthetase, an enzyme that is involved in fatty acid incorporation into phospholipids and other lipids, has also been detected in purified myelin by Vaswani and Ledeen, (1987) and their results suggest the presence of different synthetases for arachidonate and oleate, analogous to the phenomenon reported in other tissues (Laposata et al., 1985).

In turnover studies on myelin lipids it has been observed that immediately following the period of maximal incorporation of a precursor in a given lipid there is a relatively fast metabolic decay, the half-life increasing as the time after injection increases. This observation has led to two hypotheses. One interpretation is that newly synthesized lipids not yet buried deep within/

within the myelin sheath are more accessible to catabolic enzymes involved in lipid turnover. This view has been supported by Miller and Morell (1978) using older animals where myelin accumulation is much slower. In this experiment only the rapid turnover phase was detected. The hypothesis of Freysz and Mandel (1980) argues that the rapidly turning over pool represents lipids at the major dense line (cytoplasmic face) and are more likely to come into contact with degradation enzymes in the cytoplasm. The stable lipid pool consists of lipid at the intraperiod line separated from the cytoplasmic face by the energy barrier of flipping from one bilayer leaflet to the other (flip-flop).

In young animals both lipid pools would be labelled since myelin is accumulating at a rapid rate, in older animals the cytoplasmic-face lipids would be preferentially labelled but would also be degraded rapidly.

For myelin lipids to be turned over presents a unique problem. Lateral diffusion to a specialized region of the sheath, for example the Schmidt-Lanterman Incisures, must take place since radial diffusion through consecutive layers of membrane seems unlikely on thermodynamic grounds. Once lipids have reached the cytoplasmic-containing areas of the sheath there is no evidence to suggest that significant catabolism takes place there. Therefore it is thought that the lipids have to travel all the way back to the oligodendrocyte perikaryon (an area midway between the cell body and the compact sheath) for degradation to take place. It is perhaps not surprising then, that lipid turnover is somewhat slower in myelin than in other biological membranes (Morell and Toews, 1984).

ii./

ii. Major Proteins of CNS Myelin

CNS myelin is unique not only because of the high lipid to protein ratio by comparison with other cell membranes but also because of its relatively simple protein composition when analysed by SDS-PAGE (see Results Section). Historically the protein composition has been divided into a low molecular weight group which includes MBP and PLP and a high molecular weight (>40kDa) region containing, amongst many minor protein bands, CNPase and MAG. Indeed, it has been calculated that these four protein species alone account for around 80% of total myelin protein (Eylar, 1972). However, despite a wealth of biochemical knowledge that has developed over the years, no single function has been attributed to any of the proteins and their individual role in myelin formation and stabilization remains obscure.

(a) Proteolipid Protein (PLP)

PLP, also known as lipophilin (Boggs et al., 1976) is the major integral membrane protein in the myelin sheath, in some species accounting for as much as 50% of total myelin protein. The term proteolipid was coined by Folch and Lees (1951) to describe a class of myelin proteins that were soluble in organic solvents and insoluble in aqueous media even when devoid of associated complex lipids. Subsequently it has been shown that the lipid-free apoprotein can be converted to a water soluble form (Lees et al., 1979), however classical methods of purification still involve extraction into chloroform-methanol. Cambi et al. (1983) have shown that the only protein found in chloroform-methanol extracts of bovine brain white matter was PLP. The protein has an apparent molecular/

molecular weight of 24-26kDa by SDS-PAGE although protein sequencing data of bovine PLP (Lees et al., 1983; Stoffel et al., 1983) and the amino acid sequence derived from rat brain cDNA clones (Milner et al., 1985; Dautigny et al., 1985; Naismith et al., 1985) shows that the protein has a true molecular weight of almost exactly 30kDa. PLP and its proteolytic fragments have a strong tendency to aggregate and precipitate in aqueous solutions due to their hydrophobic properties and this hampered protein sequencing studies. Examination of the amino-acid sequence (Figure 5) shows a sharp segregation into hydrophobic and hydrophilic domains. Sequence homology between the bovine and rat protein is very high, between 97-99%, and such an extreme conservation suggests a very strong structure/function relationship for PLP. Both bovine and rat PLP contain 14 cysteine/half-cysteine residues but only 25-30% react with sulphydryl reagents (Cockle et al., 1980) suggesting that the remainder form five disulphide bonds. From the sequence data a model can be constructed for the conformation of the protein within the lipid bilayer (Figure 6). The essential features of the model are 1) 3 transmembrane segments traversing the lipid bilayer, 2) 2 cis-membrane domains, extracellularly orientated and 3) 1 highly charged domain on the cytoplasmic side. The cis-membrane domains could promote, via hydrophobic interactions with the bilayer across the extracellular space, the formation and stabilization of the multilamellar myelin structure (Laursen et al., 1984).

PLP is not a glycoprotein (unlike the major integral membrane protein of PNS myelin, Po) but it is acylated, containing two molecules of palmitic acid per polypeptide chain (Stoffyn and Folch-Pi, 1971). Indeed, PLP was the first protein reported to contain/

MLLECLARCIVGAPFASLVATGLCVFGVAL
FLGLGHEALTGTEKLIETYFSKNYQDYEYL
INVIHAFQYVIYGTASFFFLYGALLLAEGF
YFIGAVRQIFGDYKTTICGKGLSATVTGGQ
KGRGSRGQHQAHSLERVCHCLGKWLGHPDK
FVGITYALTVVWLLVFACSAVPVYIYFNTW
TTCQSIAFPSKTSASIGSLCADARMYGVLP
WNAFPGKVCGSNLLSICKTAEFQMTFHLFI
AAFVGAAATLVSLLTFMIAATYNFAVLKLM
GRGTKF

Fig. 5. Amino acid sequence of rat brain PLP. Putative membrane-spanning regions are shown in bold type.

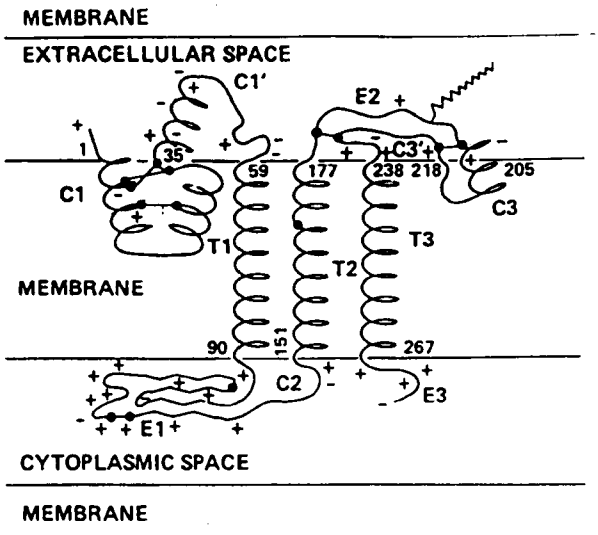


Fig. 6. Model of PLP in a membrane lipid bilayer. T1, T2 and T3 are possible trans-membrane segments; C1 and C3 are possible cis-membrane segments; and E1, E2, E3, C1' and C3' may be located outside of the bilayer. After Laursen et al. (1984).

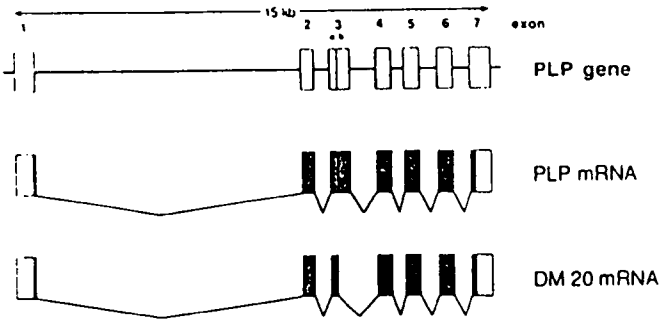


Fig. 7. PLP gene and transcript structures. Protein coding regions of spliced exons are shaded.

contain covalently bound fatty acids, a post-translational modification now recognized as widespread amongst membrane proteins (Schmidt, 1983). Bizzozero and Lees (1986) have identified palmitoyl-CoA as the lipid donor in purified myelin. The biological role of the esterified palmitic acid is unknown; since this confers increased hydrophobicity to PLP it may be important in maintaining lipid-protein interactions necessary for myelin stability.

The clone isolated by Milner et al. (1985) recognized two mRNAs of 3.2 and 1.6kb that encode PLP. Both mRNAs are the product of one PLP gene and arise from alternative recognition of two polyadenylation sites. Milner and colleagues also find no evidence of a cleavable amino-terminal signal sequence for PLP. This is somewhat unusual for an integral membrane protein and suggests that the insertion of the protein into the membrane during biosynthesis involves either a non-cleaved N-terminal signal or an internal signal sequence (Section 1.4 i).

A closely related protein which crossreacts with PLP antiserum is also present in CNS myelin. This protein (DM20) is much less abundant than PLP and has an apparent molecular weight of 20kDa as judged by SDS-PAGE. DM20 has been shown to share most structural and chemical properties with PLP, including amino-terminal sequences, carboxyl-terminal residue and amino-acid composition (Lees and Brostoff, 1984) and was thought, at that time, to be a deletion product of PLP. This has been verified by Nave et al. (1987) who have shown that DM20 is translated from an alternatively spliced mRNA lacking the coding region for residues 116-151 in the rat protein (Figure 7). Interestingly, the intron-exon structure of the/

the PLP gene (Diehl et al., 1986) shows that this arises by the selection of 5' splice donor sites rather than by the much more common process of independent exon splicing. Thus the PLP transcript utilizes alternative splicing to generate internal variants with altered coding capacities.

(b) Myelin Basic Proteins (MBPs)

MBPs are a family of highly charged peripheral or extrinsic membrane proteins and together constitute about 30% of total CNS myelin protein although this figure has been challanged recently. Careful dye-binding experiments using purified MBP have set the percentage at around 10 (Brophy et al., unpublished results). The proteins are easily extracted by acid and four MBPs can be recognised in rodents having molecular weights of 21.5kDa (pre-large), 18.5kDa (large), 17kDa (pre-small) and 14kDa (small) (Barbarese et al., 1977) with the two pre-species being present in smaller amounts than either the large or small MBP. Interestingly, the relative ratios of these proteins changes through development. In the young animal the ratio is 1:5:2:10 and in an adult 1:10:3.5:35 (Barbarese et al., 1978). Newman et al. (1987) have recently identified a second 17kDa protein by cDNA cloning which appears to be expressed at a lower level than the more widely characterized varient.

In humans, the presence of at least three MBP varients with molecular weights of 21.5, 18.5 and 17.3kDa have been reported (Deibler et al., 1986; Roth et al., 1986). To complicate the issue even further, a fourth human MBP of 20.2kDa has recently been discovered (Roth et al., 1987). All the MBPs are structurally and antigenically/

antigenically related and are the product of only one gene (de Ferra et al., 1985; Takahashi et al., 1985). In rodents the 32kb MBP gene is composed of seven exons and alternative splicing gives rise to each individual protein species (Figure 8). Identical MBPs are found in the PNS and Mentaberry et al. (1986) have shown that the same gene is responsible for the expression of these proteins. A 23kDa MBP which appears to be translated from a mRNA that is not a precursor to the other forms of MBP has recently been discovered in rat brain (Agrawal et al., 1986). The 21.5kDa MBP contains all the sequences found in the other basic proteins and the latter can be generated by splicing out exons 2 (18.5kDa), 6 (17kDa) or both (14kDa). Sequence homology between species is less than that of PLP, with rat and bovine MBP having 90% conservation. Several cDNA and genomic clones of MBP exist (Takahashi et al., 1985; de Ferra et al., 1985) and examination of the sequence (Figure 9) reveals no cysteinyl residues present therefore there is no capacity for intramolecular disulphide bridge formation. Although the basic protein is essentially a random coil in solution it is not completely without organization and there exist definite regions of localised structure (Mendz et al., 1982; Stoner, 1984).

The proposed folding of the protein is thought to arise from a hairpin structure (Stoner, 1984) and most of the ordered conformation is in the form of β-turns which overlap extensively resulting in a flat, pleated sheet-like structure (Martenson, 1981). The cationic residues in this configuration are ideally orientated for interaction with the phosphate groups of lipids at the cytoplasmic surface. Recent evidence has suggested, however, that/

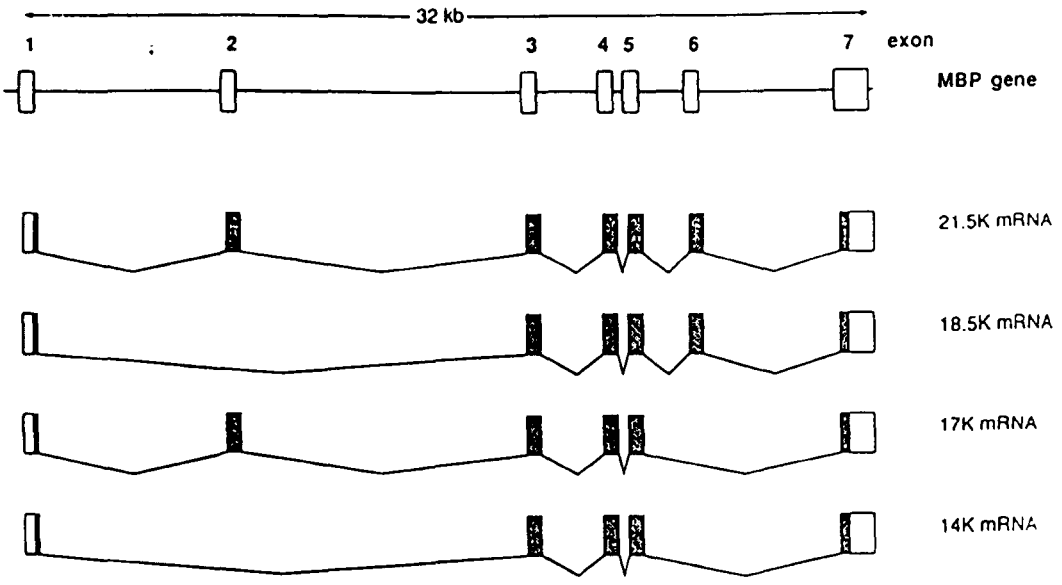


Fig. 8. MBP gene and transcript structures. Protein coding regions of spliced exons are shaded.

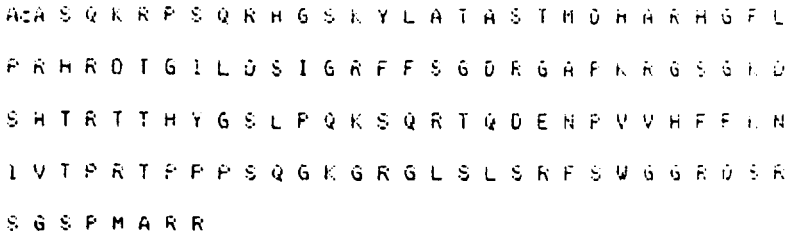


Fig. 9. Amino acid sequence of rat brain small MBP.

that MBP-lipid interactions may not be restricted to only electrostatic forces since it is thought a covalent linkage of polyphosphoinositide to Ser-54 of bovine MBP exists (Chang et al., 1986). This has been disputed recently by Smith et al. (1987) who have shown that MBP contains less than 4mol % of inositol and that the stoichiometry rules out a general mechanism for attachment of this cytoplasmically-orientated protein to its membrane. The amino acid sequence also reveals several other interesting features. There is an unusually high percentage (24%) of basic residues and apart from Lys and Arg the His content is much higher than in most proteins. Furthermore, the basic residues appear to be randomly distributed along the polypeptide chain (Lees and Brostoff, 1984). Most of the glutamic acid residues are amidated giving rise to the very high isoelectric point of $\geq$ 10. There is a single Trp present and it is thought that the sequences around it serve as a focal point for the known encephalitogenic properties of the protein (Eylar et al., 1970).

MBPs are not glycoproteins <u>in vivo</u> although the proteins have been shown to be acceptors of N-acetylgalactosamine when incubated with the appropriate galactosyl transferase <u>in vitro</u> (Cruz and Moscarello, 1983). Cruz et al. (1984) have identified the glycosylation site in human MBP to be Thr-95.

Three different post-translational modifications of MBPs have been discovered; methylation, acetylation and phosphorylation. In the course of determining the sequence of CNS MBP from bovine and human sources it soon became apparent that Arg-106 was methylated (Eylar et al., 1971). Further biochemical analysis has shown that

both/

both monomethyl and dimethylarginine are present and that the process is enzymatic and occurs at only one position in the molecule (Baldwin and Carnegie, 1971).

The role of methylation is unknown, however Crang and Jacobsen (1982) have demonstrated that methylation increases during myelination suggesting it is important in myelin formation. Labelling studies have recently shown a high rate of MBP methylation during synthesis in the brains of young mice, decreasing as the animals matured (Chandekar et al., 1986). Acetylation, the second post-translational modification, is confined to the N-terminal Ala in all species so far examined.

Phosphorylation of MBPs has been shown both in vivo and in vitro (Martenson et al., 1983; Kobayashi, 1984). Although its significance remains unclear it has been speculated that MBP phosphorylation might destabilize the compact myelin lamellae structure by causing a reversible separation of apposed cytoplasmic faces (the major dense line) at discrete areas of the sheath (Turner et al., 1984). If this is the case then the regulation of MBP phosphorylation and dephosphorylation would clearly play a pivotal role in the stabilization of the myelin structure. Phosphorylation of MBP in vivo is rapid with a turnover rate in the order of minutes (Des Jardins and Morell, 1983) however, only a small percentage of MBPs are in the phosphorylated form in vivo (Martenson et al., 1983). These observations also suggest a role for phosphatases in the regulation of myelin functions and the presence of an endogenous CNS myelin protein phosphatase (LP2) has recently been discovered (Yang et al., 1987).

Two/

Two reports have been published which show that MBP can interact *in vitro* with elements of the cellular cytoskeleton. In their study, Barylko and Dobrowolski (1984) found that when purified MBP was added to filamentous actin (F-actin) a complex of both proteins was precipitated. These authors suggest that either the contractile proteins are involved in transporting the MBPs to the membrane (Section 1.4 ib) or the MBP-associated microfilaments have a direct role in oligodendrocyte process formation itself, particularly since the microfilament-disrupting drug cytochalasin has been shown to cause unusual morphological changes in cultured oligodendrocytes (Kumagai et al., 1986). That MBP interacts with tubulin has been shown by Modesti and Barra (1986) and may be of significance *in vivo* since it is known that tubulin is a component of CNS myelin (de Néchaud et al., 1983).

(c) 2', 3'-Cyclic Nucleotide 3'-Phosphodiesterase (CNPase)

This enzyme (EC 3.1.4.37), which appears as a doublet on SDS-PAGE, was first demonstrated in bovine spleen (Whitfeld et al., 1955) and pancreas (Davis and Allen, 1956) but was later found at a much higher specific activity in the brain (Drummond et al., 1962) where it is primarily associated with myelin (Kurihara and Tsukada, 1967). CNPase activity is highest in the Corpus Callosum, an area of high activity and extensive myelin deposition (Kurihara and Takahashi, 1973). CNPase has recently been shown to be present at low activity in bovine adrenal medulla (Tirrell and Coffee, 1986), rat lymphoid tissue (Sprinkle at al., 1985), rat thymus and circulating blood lymphocytes (Bernier et al., 1987).

The CNPases were formerly called the Wolfgram proteins (W1 and W2)/

W2) after Wolfgram and Kotorii (1968) who concluded on the basis of differential amino acid analyses that a third major protein fraction, rich in dicarboxylic acids, exists in CNS myelin besides PLP and MBP. For many years the exact number and molecular weights of bands corresponding to CNPase were debated culminating in an extensive electrophoretic study by Waehneldt and Malotka (1980) who conclusively showed that CNPase (still called Wolfgram protein) consisted of a doublet in all mammalian species studies. The relative amounts of CNPase I and CNPase II vary with species as does the difference in molecular weight as established by SDS-PAGE. The rat enzymes have the highest apparent molecular weight (47kDa and 49kDa) for CNPase I and II respectively with the lowest molecular weight species present in human myelin (42kDa and 45kDa). The proteins are thought to be subunits of the native enzyme since electrophoresis in non-denaturing conditions puts the molecular weight at around 100kDa (Sprinkle et al., 1980). These workers and others (Drummond and Dean, 1980) have clearly shown by immunological, electrophoretic and amino acid compositional analysis that Wolfgram protein W1 and CNPase I are identical as are W2 and CNPase II.

Although the enzyme's existence in CNS myelin has been known for some time (Kurihara and Tsukada, 1967) it is only within the last 10 years that CNPase has been purified. This is, in part, due to the enzyme's strong binding to the membrane and the requirement for rather drastic solubilization procedures, for example detergent or guanidine as a pre-purification step (Drummond, 1979; Suda and Tsukada, 1980; Wells and Sprinkle, 1981). CNPase hydrolyzes, in vitro,/

vitro, 2' 3'-cyclic nucleotides to form 2'-nucleotides (Figure 10) and thin-layer chromatography has shown that only the 2'-isomer is produced (Sheedlo et al., 1984). It has also been shown that CNPase does not require divalent cations for activity and that the purified enzyme from human and bovine myelin has the very high specific activity of 1000-4400 μmoles of 2' 3'-cyclic AMP/min/mg protein.

The enzymatic activity for CNPase is unusual for two reasons. Firstly, 2' 3'-cyclic nucleotides are known to be enzymatic breakdown products of RNA and may be involved in RNA processing and splicing reactions, however 2', 3'-cyclic nucleotides are not known to accumulate in cells to any great degree (Olafson et al., 1969). Indeed, hydrolysis of these substrates is not confined exclusively to CNPase although CNPase is the only enzyme which converts them to 2'-nucleotides. Whether 2', 3'-cyclic nucleotides are the natural substrate is unknown as is the physiological role of CNPase. Two groups (Nakamura et al., 1979; Vogel and Thompson, 1986) have partially purified a nucleotide phosphomonoesterase from brain which is specific for 2'-nucleotides and this has led to the proposal of a pathway whereby the 2'-nucleotidase hydrolyzes the products of the CNPase reaction (Takahashi, 1981) (Figure 11). On a different line of investigation Sprinkle et al. (1987) have recently found a 5'-polynucleotide kinase activity associated with bovine and human CNPase. Purified wheatgerm and yeast RNA ligases have been shown to exhibit 2', 3'-cyclic nucleotide phosphosphodiesterase, ligase and 5'-polynucleotide kinase activities all residing in a single polypeptide chain (Phizicky et al., 1986; Pick and Hurwitz, 1986). The findings of Sprinkle et al. (1987) have led them to suggest that,/

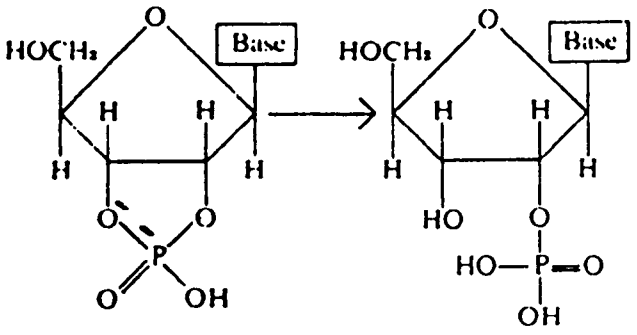


Fig. 10. Enzyme reaction catalysed by CNPase.

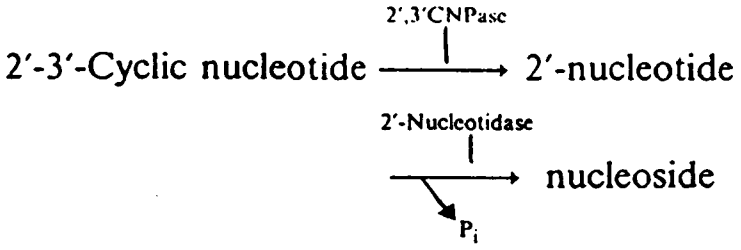


Fig. 11. Proposed pathway for the hydrolysis of the products of the CNPase reaction. After Takahashi (1981).

S S S G A K D K P E L Q F P F L Q D E D T V A T L H E C K T
L F I L R G L P G S G K S T L A R L N P W R S T T T A P R W
C L L M L T R S **F L A L G Q T S P R E Y K R L D E D L A G I**
L P R D I R V L V L D D T N H E R E R L D Q F F E N A D Q Y
Q Y Q V V L V E P K T A W R L D C A Q L K E K N Q W Q L L A
R I D D L K K L K P G L E K D F L P L Y F G W F L T K K S S
E T L R K D S R P G S F Q W K L G N H K A L R K S F D T S L
Y M G M N P R R S L P V S Y F G K R P P G V L H C T T K F C
D Y G K A T G A E E Y A Q Q D V V R R S Y G K A F K L S I S
A L F V T P K T A G A Q V V L N E Q E L Q L W P S D L D K P
S S S E S L P P G S R A H V T L G C A A D V Q P V Q T G L D
L L E I L Q Q V K G G S Q G E E V G E L P R G K L Y S L G K
G R W M L S L A K K M E V K A I F T G T M G R A N L Y R T R
Q P K G V A M Q I C T I I

Fig. 12. Amino acid sequence of rat brain CNPase I. The putative cyclic nucleotide binding sequence is shown in bold type. After Bernier et al. (1987).

that, since CNPase displays two out of the three enzyme activities associated with RNA ligases, the natural substrate for CNPase may be RNA and that the enzyme is involved in RNA metabolism. CNPase has recently been cloned from bovine (Vogel and Thompson, 1987; Kurihara et al., 1987) and rat brain (Bernier et al., 1987). The bovine clone codes for CNPase II and contains 400 amino acids, MW 44875Da, while that of rat brain is for CNPase I (404 amino acids, MW 45592Da). Interestingly both clones code for the CNPase species that is most abundant in the respective doublets, bovine being the only mammalian species so far studied that has a greater amount of CNPase II than I (Waehneldt and Malotka, 1980). Examination of the sequence from the rat brain clone (Figure 12) reveals that many of the acidic amino acids are amidated giving rise to the published isoelectric point of 8.5-9.0 for the purified rat enzyme (Wells and Sprinkle, 1981).

CNPase II has been shown to be phosphorylated *in vitro* in a cyclic-AMP and Ca^{2+} dependent manner by an endogenous myelin kinase (Bradbury and Thompson, 1984). As with MBP the turnover rate of this phosphorylation *in vivo* is rapid suggesting a physiological role for this post-translational modification. Vogel and Thompson (1987) have tentatively identified Ser-74, Ser-177 and Ser-305 as possible phosphorylation sites. In their rat brain clone Bernier et al. (1987) have identified a sequence (amino acids 69-81) that is homologous to known sites for cyclic nucleotide binding in other proteins. Northern blot analysis has failed to demonstrate whether there are two distinct mRNAs for each CNPase polypeptide, however an apparently single mRNA species from rat thymus has been shown to code/

code for both proteins (Bernier et al., 1987). These workers also provide evidence for a single CNPase gene and the idea that the proteins differ only be some form of post-translational modification (other than phosphorylation) is gaining ground.

(d) Myelin-Associated Glycoprotein (MAG)

The fourth most abundant protein of CNS myelin, MAG, accounts for about 1% of total myelin protein (Quarles et al., 1972). Several glycoproteins have been identified in CNS myelin on the basis of lectin-binding, fucose incorporation and periodic acid-Schiff staining but MAG is by far the most prominent (Poduslo and Braun, 1975). MAG has been purified from the chloroform-methanol insoluble residue of myelin by solubilization with the surface-active agent lithium diiodosalicylate, followed by phenol extraction (Quarles and Pasnak, 1977; Quarles et al., 1983). It is an integral or intrinsic membrane protein with an apparent molecular weight of 100kDa as judged by SDS-PAGE. The protein is almost one-third carbohydrate by weight and has a sugar composition similar to other glycoproteins with N-linked oligosaccharides. The sugars present are fucose (5%), mannose (23%), galactose (20%), N-acetylglucosamine (34%) and N-acetylneuraminic acid (sialic acid) (18%) (Quarles et al., 1983). MAG is also sulphated, probably on the sugar residues, as demonstrated by the incorporation of [^{35}S] sulphate *in vivo* (Matthieu et al., 1975). The protein has not been shown to be phosphorylated either *in vivo* or *in vitro* although a putative site for phosphorylation by a tyrosine kinase has been identified (Lai et al., 1987). Two MAG polypeptides are detectable in *in vitro* translation systems (Frail and Braun, 1984). The proteins/

proteins (72kDa and 67kDa) once glycosylated are presumed to co-migrate on SDS-PAGE as the characteristically broad band at 100kDa. The precise structural differences between the proteins have recently been elucidated (Lai et al., 1987). Earlier work had shown that peptide maps of both proteins were nearly identical (Frail and Braun, 1984). These workers have also shown that the mRNA for each MAG protein is developmentally regulated. 72kDa MAG (or L-MAG) mRNA appears at an earlier age than 67kDa MAG (S-MAG) and remains the dominant MAG mRNA species throughout the period of rapid myelination. As the rate of myelination decreases S-MAG mRNA becomes the dominant MAG mRNA species. This phenomenon was first observed by Quarles et al. (1973) where they measured the rate of incorporation of radiolabelled fucose into rat brain of various ages. MAG has recently been cloned (Lai et al., 1987; Salzer et al., 1987; Arquint et al., 1987) and all the clones isolated hydridize to two distinct mRNAs supporting the earlier in vitro translation results. The two MAGs share a common 23 amino acid membrane spanning domain but differ in the structure of their C-termini. Of particular interest is the tripeptide Arg-Gly-Asp (residues 118-120, Figure 13), located at the potentially extracellular disposed N terminus, which has been shown to be a crucial element in the interaction of a growing number of cell surface receptors, such as the fibronectin receptor (Tamkun et al., 1986) and the receptor for vitronectin (Pytela et al., 1985), with extracellular proteins. Of additional interest since MAG is thought to participate in myelin-axon recognition (Section 1.2 ii) is the finding that there is very strong homology between MAG and N-CAM, a well/

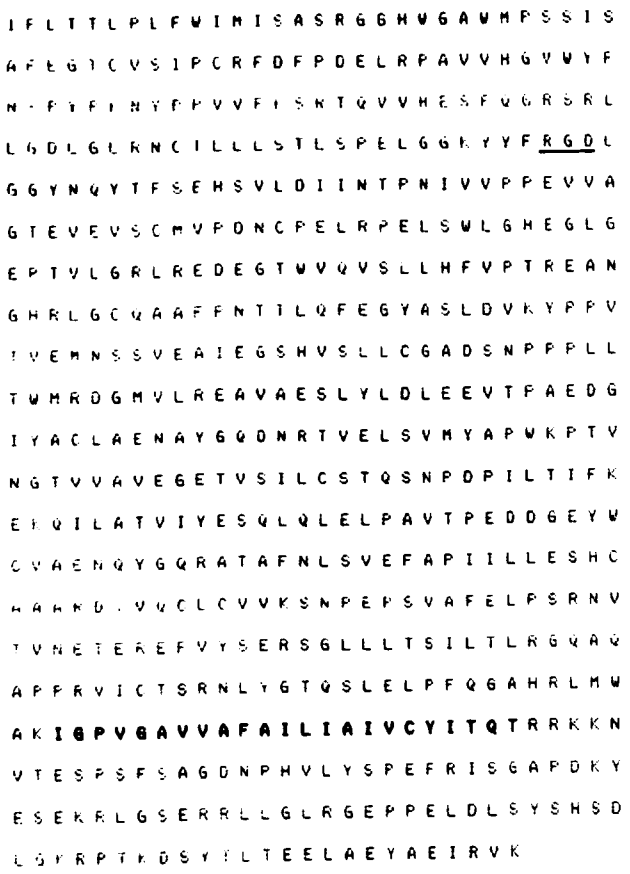


Fig. 13. Amino acid sequence of L-MAG. The tripeptide RGD is underlined and the potential transmembrane segment is shown in bold type. After Salzer et al. (1987).

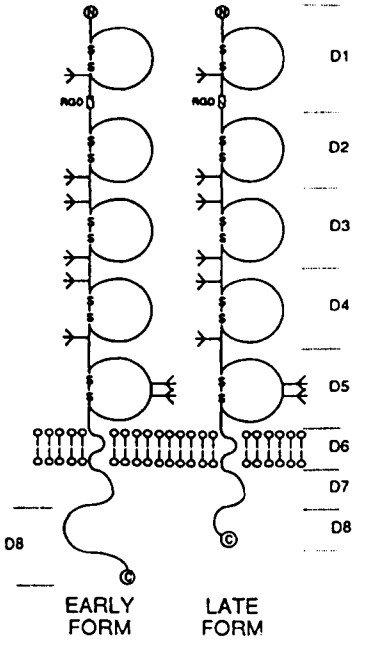


Fig. 14. Model of MAG showing the multiple domain structure. After Salzer et al. (1987).

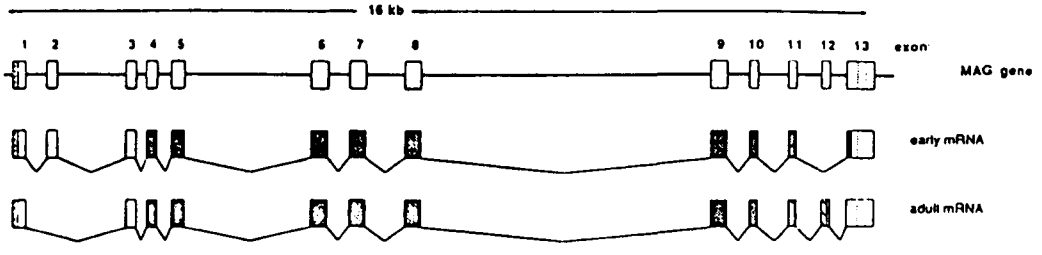


Fig. 15. MAG gene and transcript structure. Protein coding regions of the spliced exons are shaded.

well characterized cell adhesion molecule in the vertebrate nervous system which is thought to mediate neuron-neuron interactions (Edelman, 1984). Salzer et al. (1987) have concluded from their deduced amino acid sequence of L-MAG that there are five segments of internal homology each of which contain sequences that resemble those of immunoglobulin domains and they have classified MAG as a member of the immunoglobulin gene superfamily. These proteins all share a common extracellular subunit structure termed the immunoglobulin homology unit (Hunkapillar and Hood, 1986). The significance of this shared structure is not understood. A proposed model for MAG is shown in Figure 14. Both Salzer et al. (1987) and Lai et al. (1987) have provided evidence that a single gene for MAG exists and that, like PLP, DM20 and MBPs, the different forms of MAG arise from alternative splicing of the primary transcript (Figure 15). Of considerable interest has been the discovery that there is a shared antigenic determinant between human MAG and Leu-7 (HNK-1), a surface marker of human natural killer (NK) cells (McGarry et al., 1983; Murray and Steck, 1984). It is thought that this may be of significance with regard to autoimmune demyelination diseases since Tanaka et al. (1985) have shown that NK activity is reduced by the treatment of anti-MAG antibodies in vitro.

(e) P2 Protein

The P2 protein is a basic protein of apparent molecular weight 14kDa. The protein is most abundant in the PNS and was once thought to be a PNS-specific protein however immunological techniques have shown that it is present at decreased amounts in bovine, human and rabbit spinal cord (Uyemura et al., 1977), rabbit oligodendrocytes (Trapp et al., 1983) and areas of bovine brain associated with motor functions./

functions. P2 is not found in the higher areas of the CNS, optic nerve or non-neural tissue (Eaton et al., 1984). The amount of P2 found in CNS tissue is curiously variable from species to species and it has not been detected in the CNS of rodents (Kadlubowski et al., 1984). The variable amounts of P2 may be related to the observation that it is not a component of all nerve fibres but instead is present in some fibres and absent from others, also the proportion of fibres containing P2 tends to be larger in more caudal parts of the CNS (Trapp et al., 1983). The significance of this variation in distribution is not known however it raises the interesting question of variations in structure and even functions of myelin from different regions of the CNS.

The complete amino acid sequence of bovine (Kitamura et al., 1980), rabbit (Ishaque et al., 1982) (Figure 16) and human (Suzuki et al., 1982) P2 is known and each protein contains 131 amino acids differing by substitutions at nine locations. It has a lower percentage of basic amino acids (19%) than MBP but contains a higher percentage of hydrophobic amino acids leading to the suggestion that its interaction with the myelin membrane involve fewer electrostatic but more hydrophobic forces than for MBP (Lees and Brostoff, 1984). The membrane interaction is weak however since P2 can be isolated from non-delipidated myelin using extremely mild salt or acid conditions (Uyemura et al., 1977). P2 has an extensive β-sheet secondary structure and a virtual absence of α-helix and three possible structural models have been put forward by Martenson (1983) all essentially consisting of eight antiparallel β-strands with a two-stranded ribbon of antiparallel β-structure emerging from one/

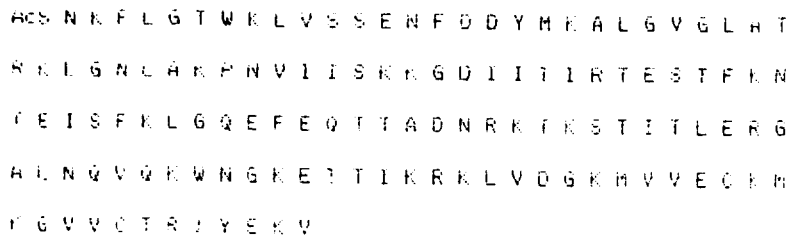


Fig. 16. Amino acid sequence of rabbit P2 protein.

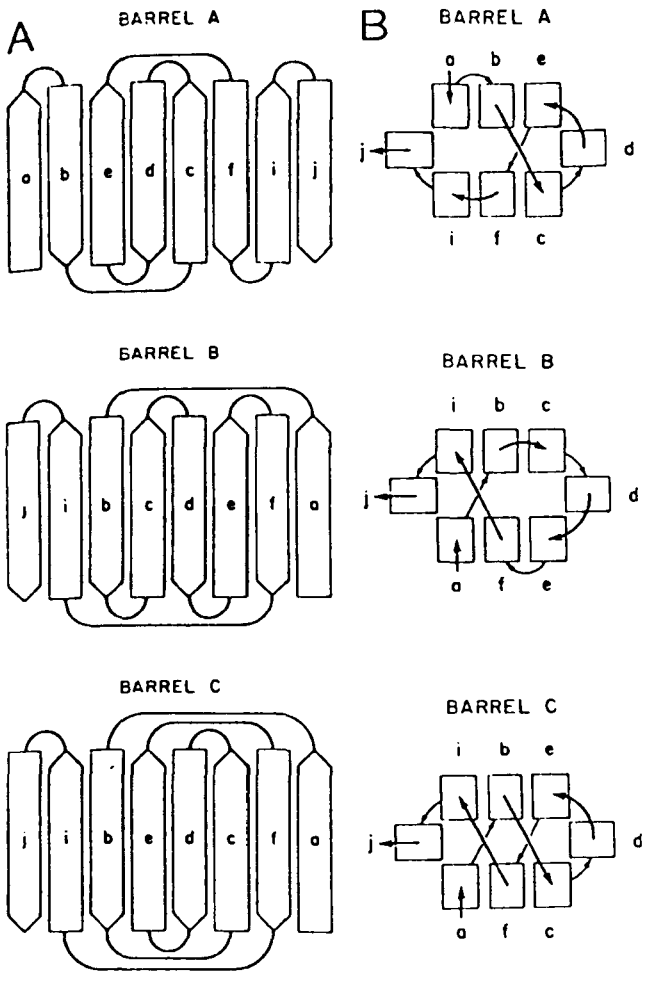


Fig. 17. Three possible models for the structure of P2 protein. After Martenson (1983).

one end (Figure 17). A striking sequence homology has been noticed between P2 and lipid-binding proteins from rat heart (Sacchettini et al., 1986), murine adipocytes (Bernlohr et al., 1984) and the cellular retinoid binding proteins (Takahashi et al., 1982). These proteins all have similar molecular weights and amino acid compositions and this has led to the suggestion that P2 may be a member of a group of intracellular proteins with a high affinity for lipids and a similar function (Lees and Brostoff, 1984). Since P2 is an extrinsic-type membrane protein as judged by the ease of extraction from the myelin membrane, it is conceivable that P2 has a role as a lipid carrier.

An important discovery was made in 1979 when Kadlubowsky and Hughes were able to induce experimental allergic neuritis (EAN) in Lewis rats by the administration of P2. Following on from this initial discovery Ishaque et al. (1981) were able to show that P2 was much more immunogenic when complexed with phospholipids indicating that the antigenic domain requires these lipids in order to attain the most favourable conformation for inducing a cell-mediated response that leads to disease. EAN is important because it is thought to be very similar to the human Guillain-Barre syndrome, an autoimmune demyelinating disease that specifically affects the PNS (Raine, 1984b).

1.4 CELL BIOLOGY OF MYELINATION IN THE CNS

i. Current Theories of Membrane Protein Biosynthesis

(a) Intrinsic Membrane Proteins

The unique orientation of intrinsic plasma membrane proteins within/

within the lipid bilayer with apolar residues interacting with lipid in the core of the bilayer and polar amino acids on both sides of the membrane presents an interesting problem for the cell's protein synthesis machinery. The apparatus for protein synthesis resides exclusively in the cytoplasm of the cell so this must mean that polar residues have to cross the lipid bilayer either during or after synthesis, yet it is their inability to do this that maintains the asymmetry of proteins within the plasma membrane. This complex process was an enigma until cell-free translation systems, assembled from heterologous components, were found to be able to reconstitute the early events in translocation of the nascent polypeptide through the membrane at its site of synthesis. The classic work of Palade and his colleagues (Palade, 1975) had shown that newly synthesised secretory proteins were initially found within the lumen of the rough endoplasmic reticulum (RER) and since then it has been conclusively shown that both intrinsic membrane (Lingappa et al., 1978) and lysosomal proteins (Erickson et al., 1981) both utilize the translocation machinery inherent in the RER.

Early studies by Milstein et al. (1972) had shown that larger forms of immunoglobulin light chains were synthesized than those eventually secreted from the cell and they postulated that the extra piece was at the NH_2-terminal and that this might serve as a signal for insertion into the ER membrane.

These studies also showed that in the presence of microsomes mature-length protein was produced thereby demonstrating that the signal sequence was cleaved during translocation into the RER. Further evidence for the existence of a 'signal sequence' in nascent polypeptides/

polypeptides began to appear (Kemper et al., 1974, Blobel and Dobberstein, 1975a; Blobel and Dobberstein, 1975b) and led to the signal hypothesis of Blobel and Dobberstein (1975b), an idea that had originally been put forward by Blobel and Sabatini in 1971. The essence of the signal hypothesis was that the information determining the specific association of polysomes with the RER membrane is contained in the amino-terminal portion of the nascent polypeptide and that this segment directs polysome-membrane attachment and assists in the cotranslational insertion of the nascent polypeptide through the microsomal membrane. Folding of the polypeptide chain would then serve to draw the remainder of the nascent chain through the postulated proteinaceous tunnel in the RER membrane.

Three components of the microsomal translocation apparatus have been purified from canine pancreas, the signal recognition particle (SRP) (Walter and Blobel, 1980), the SRP receptor also known as the docking protein (Gilmore et al., 1982; Meyer et al., 1982) and the signal peptidase (Evans et al., 1986). A fourth component of the microsomal translocation machinery has recently been discovered. Although the protein has not been purified it is known to be an intrinsic membrane glycoprotein of molecular weight 35kDa and has been named the signal-sequence receptor (SSR) (Wiedmann et al., 1987).

SRP is a complex of six polypeptides and a 7S RNA whose function is to bind to the signal sequence as it emerges from the ribosome during synthesis. A chain length of approximately 70 amino acids seems to be required before the interaction of SRP and the nascent/

nascent polypeptide chain can occur (Meyer et al., 1982) and it is known that after binding a temporary translation arrest occurs (Walter and Blobel, 1981; Meyer et al., 1982) until the SRP-ribosome complex binds to the docking protein located in the RER. The resultant attachment of the ribosome to the membrane is accompanied by SRP displacement from the ribosome by the docking protein (Gilmore and Blobel, 1983) and by a direct interaction between the signal-sequence and the SSR (Wiedmann et al., 1987). The ribosome is then free to continue chain elongation. The growing polypeptide chain passes through the ER membrane into the lumen where the cytoplasmically-situated signal peptidase removes the signal sequence. These events are depicted in Figure 18a. The mechanism of translocation remains unclear at the present time and it is not known whether it proceeds through a proteinaceous tunnel or directly through lipid (Wickner, 1979).

Once the polypeptide chain has entered the lumen of the RER core glycosylation and disulphide bridge formation can occur via enzymes located on the luminal side of the RER and once protein synthesis has terminated the ribosome is free to join the soluble pool of ribosomes and enter into a further round of protein synthesis. The exact route taken by the protein from the lumen of the RER to the plasma membrane is still under debate although it is presumed to enter the Golgi where trimming and modification of core oligosaccharides take place and is then finally transported via Golgi-derived vesicles to its target membrane (Palade, 1975). It is thought that the smooth areas of the RER, termed transitional zones, are/

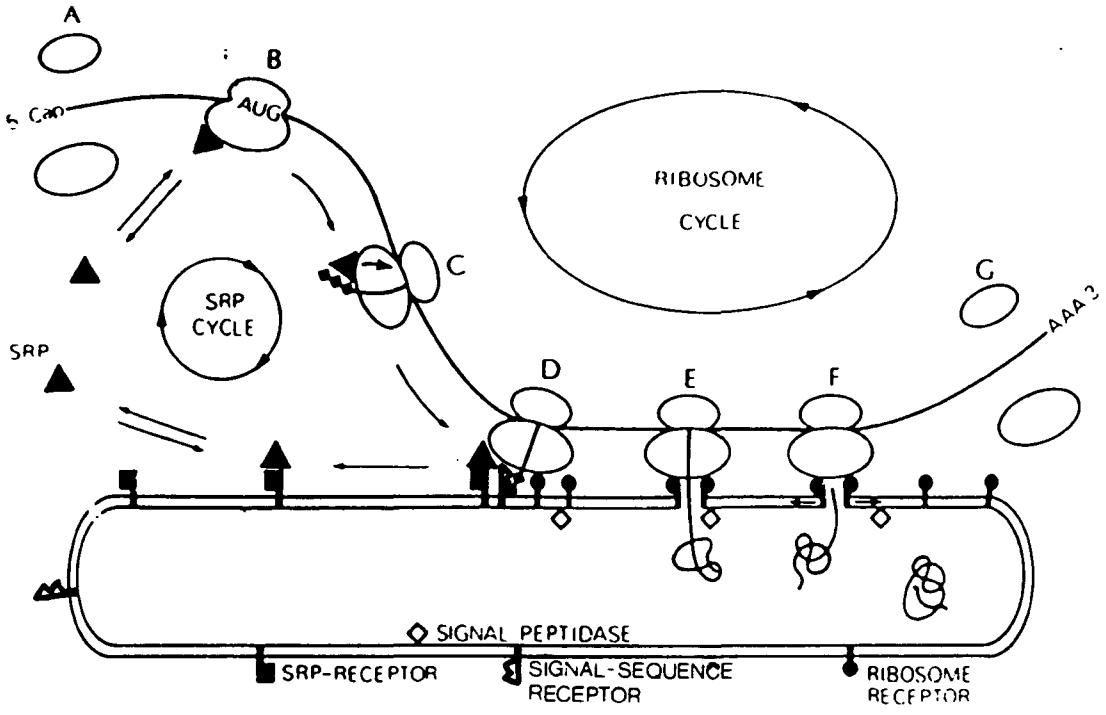


Fig. 18a. Diagrammatical view of protein biosynthesis on RER-bound polysomes showing co-translational insertion of the nascent polypeptide (A-G).

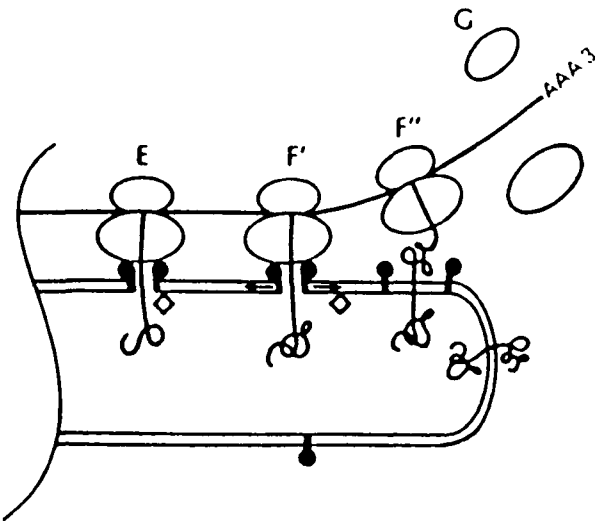


Fig. 18b. Biosynthesis of an intrinsic membrane protein showing a halt-transfer sequence anchoring the partially inserted polypeptide chain to the membrane.

are the sites for budding of protein- containing RER vesicles and Lodish et al. (1987) have succeeded in identifying a vesicle fraction that is enroute from the RER to the cis face of the Golgi. Furthermore, the exit of proteins from the RER has been shown to be the step at which maturation of secretory and intrinsic membrane proteins is regulated (Fries et al., 1984; Williams et al., 1985).

Recently it has been shown that translocation-competent binding of the ribosome to the ER membrane requires the participation of a GTP-binding protein in addition to the SRP and the docking protein (Connolly and Gilmore, 1986). These authors were also able to show that once the ribosome-membrane complex had formed, translocation of the nascent polypeptide through the ER membrane did not require GTP hydrolysis. One of the most striking features to emerge from these experiments and of central importance to the signal hypothesis is the tight coupling of synthesis of a polypeptide chain and its translocation across the ER membrane. Indeed, it is this phenomenon which distinguishes protein import into the ER lumen from the import of proteins into mitochondria and chloroplasts in which translocation of nascent polypeptides has been shown to be a post-translational event (Dobberstein et al., 1977; Maccechini et al., 1979). Subsequently it has been shown that in bacteria (Koshland and Botstein, 1982) and for some proteins of yeast (Hansen et al., 1986) translocation is not coupled to translation. Recently, Perara et al. (1986) have succeeded in uncoupling translocation from translation by using genetically engineered mRNAs devoid of termination codons. They have found that translocation does not occur spontaneously, a result also observed

by/

by Connolly and Gilmore (1986), and that translocation may require energy expenditure independent of protein synthesis.

The general mechanism described above can be modified to take into account the known complex topologies of different types of intrinsic membrane proteins (reviewed by Sabatini et al., 1982). Here, the concept of halt or stop-transfer signals is introduced. Proteins that span the lipid bilayer several times, for example PLP, would have several of these internal halt signals located within the polypeptide chain. Normal translocation would progress until a halt transfer signal was encountered. This hydrophobic sequence would anchor itself to the membrane while translation is continuing. When a polypeptide segment longer than the approximately 40 amino acids that are contained within the ribosome emerges it causes the ribosome to dislodge from the ER membrane. Translation would continue on the free ribosome until an internal signal sequence was synthesized then cotranslational insertion of this portion of the polypeptide would occur. By a combination of internal uncleaved signal sequences and hydrophobic halt-transfer signals the complex topology or disposition of the protein about the membrane would be constructed. Uncleaved signal sequences have been found in a number of intrinsic membrane proteins among them PLP (Colman et al., 1982) and the asialo-glycoprotein receptor (Holland et al., 1984). An examination of several putative halt-transfer signals contained within intrinsic membrane proteins has shown that, in addition to hydrophobic segments (which presumably span the bilayer), an adjacent group of highly charged residues is almost invariably present/

present on the carboxyl end of the hydrophobic sequence (Sabatini et al., 1982). The charged portion would not penetrate the bilayer and so would remain on the cytoplasmic side of the ER membrane. Whether ribosome dislodgement would be caused by the high concentration of charged amino acids at the site of ribosome-membrane binding or simply by steric hindrance due to the accumulation of nascent polypeptide under the ribosome is not known.

The generation of an intrinsic membrane protein with a membrane-spanning domain is shown in Figure 18b and much more has to be discovered about this process, particularly the crucial translocation step.

(b) Extrinsic Membrane Proteins

In contrast to intrinsic membrane proteins it had long been understood that membrane proteins of the extrinsic or peripheral type were synthesized on free polysomes that were distributed randomly about the cell cytoplasm. However, our understanding of how mRNA is transported within the cell and translated at discrete sites has undergone marked changes and considerable evidence has now accumulated showing that free polysomes are not simply 'floating' around in the cytoplasm but may in fact be intimately associated with the cellular cytoskeleton (Ben Ze'ev et al., 1981; Cervera et al., 1981; Ornelles et al., 1986). The cytoskeleton is an intracellular structure comprising three major filamentous systems, namely microfilaments (Lazarides, 1976), intermediate filaments (Lazarides, 1980) and microtubules (Tucker, 1979). In addition to this there are many filament-associated proteins and an irregular cytoskeletal/

cytoskeletal structure termed the microtrabecular lattice (Wolosewick and Porter, 1979).

Lenk et al. (1977) developed a fractionation procedure using non-ionic detergents whereby the non-extractable residue contained the cytoskeletal elements and the soluble fraction consisted of soluble proteins, tRNA, monosomes and other material. When examined under the EM it was observed that polysomes were invariably found associated with the cytoskeleton fraction. EM studies by Wolosewick and Porter (1976) had previously shown polysome clustering in the vicinity of the cytoskeleton in intact human diploid cells. Whilst the existence of polysome-cytoskeleton associations is unequivocal numerous reports have been published which suggest that the association is non-random. Polysomes in 3T3 cells are preferentially located in perinuclear regions (Fulton et al., 1980) and a discrete distribution of actin, tubulin and vimentin mRNA has been described in fibroblasts and myoblasts (Singer and Ward, 1982; Lawrence and Singer, 1986). In Xenopus oocytes a differential localization for actin, histone (Jeffrey, 1984) and Vg1 mRNA (Melton, 1987) has been observed. This spatial segregation of mRNAs indirectly suggests that polysome attachment to the cytoskeleton is via mRNA binding to the cytoskeleton. Direct evidence for this has come from Lenk et al. (1977) who have shown that ribonuclease treatment of the cytoskeleton results in the release of ribosomes and mRNA whereas treatment of the cytoskeleton with fluoride (Lenk et al., 1977) and pactamycin (Lenk and Penman, 1979), agents which cause ribosome-mRNA dissociation, results in the loss of ribosomes but/

but not mRNA from the cytoskeletal fraction.

Evidence that polysomes associated with the cytoskeleton are actively involved in protein synthesis has come from several studies. The initiation factors eIF-2, eIF-3, eIF-4A and eIF-4B (Howe and Hershey, 1984) and an aminoacyl-tRNA synthetase complex (Mirande et al., 1985) are preferentially associated with the cytoskeleton and it has also been shown that translation only functions when mRNA is bound to the cytoskeleton (Cervera et al., 1981; Moon et al., 1983; Ornelles et al., 1986). When the cytoskeleton is disrupted by the microfilament-specific drug cytochalasin D and mRNA is released into the soluble fraction protein synthesis in the cell was inhibited in direct proportion to the amount of mRNA released. In vitro studies have shown that the translational capability of mRNA released into the cytoplasm from cytochalasin treatment of HeLa cells is unaltered but the same mRNA is not translated in cytochalasin-treated intact HeLa cells (Ornelles et al., 1986).

In a study using several eukaryotic viral and cellular mRNAs Bonneau et al. (1985) were able to demonstrate that all the mRNAs associated with the cytoskeleton during protein synthesis irrespective of their 5' and 3' termini structure. The same report also argues that the association of mRNA with the cytoskeleton is required but is not sufficient for eukaryotic protein synthesis since infection of African green monkey cells by vesicular stomatitis virus inhibited actin synthesis but did not cause the release of actin mRNA from the cytoskeleton. In addition, the same group have found that upon suspension of anchorage-dependent cells

mRNAs are not released from the cytoskeleton although protein synthesis is greatly reduced. Brodeur and Jeffrey (1987) have provided recent evidence to support this view. On comparing the distribution of cycloheximide-induced polysome-mRNA accumulation in the cytoskeletal and soluble fractions of translationally active plasmodia and translationally dormant sclerotia of the slime mould Physarium polycephalum they found that translational suppression in the sclerotia was not caused by alterations in the sclerotial cytoskeleton during starvation since most of the polysomes were recovered with the cytoskeleton. In contrast, most of the plasmodial cyclohexamide-induced polysomes accumulated in the soluble fraction indicating that polysome formation is not dependent on a prior association of mRNA or ribosomes with the cytoskeleton and also that the cytoskeleton has a limited capacity for binding translational components. Taken together, these results indicate that protein synthesis does not require an association of polysomes with the cytoskeleton and is hence not involved in translational control.

The situation regarding the involvement of the cytoskeleton in eukaryotic protein synthesis will not be resolved fully until characterisation of the elements of the cytoskeleton involved in mRNA binding is attained. The development of in vitro systems in which the interaction between cytoskeletal and translational components can be studied will be of paramount importance in this area. Nevertheless, in terms of membrane protein biosynthesis, an involvement of the cytoskeleton in ensuring the synthesis of specific proteins at discreet sites within the cell's cytoplasm close/

close to their ultimate destination appears to be a rational hypothesis.

ii. Myelin Protein Synthesis

(a) In vitro

Studies involving in vitro methods have been carried out over the past ten years to determine the sites of synthesis of some of the major CNS myelin proteins. Most of the research to date has concentrated on the MBPs and all the evidence shows that these proteins are synthesized on free polysomes.

Early work by Campagnoni et al. (1978) had demonstrated the synthesis of MBPs by brain homogenates. Subsequent studies by the same group (Campagnoni et al., 1980; Mathees and Campagnoni, 1980) examined the ability of several subcellular fractions from mouse brain to synthesize MBP. Only the whole homogenate, postnuclear, postmitochondrial and postmicrosomal supernatants and free polysomes were able to synthesize MBPs. These results tended to rule out the possibility that MBPs were synthesized on membrane-bound polysomes. In these experiments the newly synthesized MBPs were identified by their comigration with authentic MBPs on carboxy-methyl cellulose, acid urea gels and SDS-PAGE. A major criticism of these detection methods is that they were unable to identify both the pre-large and pre-small MBPs, essential if a precursor-product relationship existed between the four MBPs of rodents. A much clearer picture emerged when Yu and Campagnoni (1982), using a similar experimental approach as before, identified newly synthesized MBPs with an antibody which recognized all four forms of mouse MBPs and concluded that there was no evidence for a precursor-product relationship between/

between the four protein species. The definitive work on the site of synthesis of MBPs has come from Colman et al. (1982). Here the investigators isolated rat brain mRNA from free and bound polysomes and used it to programme a wheatgerm translation system. Immuneprecipitation of MBPs clearly showed that MBPs were synthesized on free polysomes and were not synthesized as precursors. Like Yu and Campagnoni (1982) they also could find no evidence for a precursor-product relationship. Of considerable interest was the finding by Colman et al. (1982) that a mRNA fraction isolated from myelin-associated polysomes is considerably enriched in mRNAs coding for MBPs. This led the authors to suggest that the segregation of free polysomes engaged in the synthesis of MBPs within myelinating processes serves to ensure that these proteins specifically associate with their target membrane ie. myelin rather than with other intracellular membranes.

Two groups (Townsend and Benjamins, 1979; Colman et al., 1982), utilizing <u>in vitro</u> translation methods, have shown that PLP is synthesized on bound polysomes as might be expected since it is an intrinsic membrane protein (Section 1.4 ia). PLP is not synthesized as a precursor since the primary translation product is indistinguishable in electrophoretic mobility from the mature protein (Colman et al., 1982); these authors also showed a requirement for SRP in order to ensure efficient insertion into the RER membrane during synthesis.

The types of study described above not only determine the site of synthesis of a particular protein but are of use in elucidating precursor-product relationships and possible developmental controls of/

of transcription and/or translation.

(b) In vivo

The kinetics of incorporation of newly synthesized myelin proteins into the growing myelin membrane has provided information on the routes of protein traffic in the oligodendrocyte. Two of the earliest studies to examine the entry of newly synthesized proteins into myelin were those of D'Monte et al. (1971) and Smith and Hasinoff (1971). Both groups concluded that the high molecular weight proteins entered myelin first followed by MBPs and finally PLP. However, these experiments were carried out on rats of 40-50 days old and the rate of myelination is greatly decreased at this age. A subsequent study using intracranial injection of a radiolabelled amino acid into young rats (Benjamins et al., 1975) showed that newly synthesized CNPase and MBPs reached maximal specific radioactivity after 2 hours and remained constant for 24 hours. PLP, on the other hand, was observed to accumulate radioactivity for 6 hours then leveled off. This indicated a delay in the appearance of PLP in myelin relative to CNPase and MBPs. Short time point experiments have shown conclusively that newly synthesized MBPs (Colman et al., 1982) and CNPase (Benjamins et al., 1978) were incorporated rapidly into the growing myelin membrane indicating little delay between their synthesis and appearance in myelin. Both groups also observed a delay of at least 30 minutes until newly synthesized PLP was incorporated into myelin and Colman et al. (1982) detected labelled PLP in brain microsomal fractions as little as 2 minutes after injection. These results, particularly those of Colman et al . (1982) are consistent with a mechanism whereby/

whereby PLP is synthesized at the RER and is subsequently transported through the Golgi apparatus and from there to the plasma membrane. This has been supported by Townsend and Benjamins (1983) who used monensin to disrupt Golgi-derived vesicle transport. Under these conditions the entry of PLP into myelin was inhibited by 40% but little effect on the entry of the MBPs was observed.

Karin and Waehneldt (1985) have suggested that CNPase is synthesized on free polysomes since the labelled protein was found to accumulate in rat optic myelin rapidly after synthesis and monensin had no effect on the appearance of the protein. Of interest was the finding that labelled CNPase was also found in a microsomal fraction with the same kinetics of incorporation as that for myelin. The identity of the membrane(s) involved is unknown however the authors suggest that this may serve as a membrane intermediate in myelin formation.

Differences in the kinetics of entry of the major proteins into the growing myelin membrane suggests that the formation of the membrane is a multistep continuous process rather than a one-step discontinuous one. The intrinsic membrane proteins are synthesized at the RER and once they become incorporated into the oligodendrocyte plasma membrane, at the cell body or in the process itself, lateral diffusion may be responsible for delivery of the protein to the myelin membrane. Conversely, extrinsic proteins may be synthesized throughout the cytoplasm of the oligodendrocyte or in discrete areas close to the growing membrane (Colman et al., 1982) although the mechanism responsible for their transport to the myelin membrane may be the same as for intrinsic proteins (Benjamins, 1984)./

1984). Many investigators have tried to isolate membrane fractions that may correspond to stages in the transport of myelin proteins from their proposed sites of insertion to their ultimate destination, the compact myelin sheath.

Two main approaches have been taken: (1) the isolation of myelin-associated membranes in the hope of identifying specific membrane populations that may serve as intermediates or precursors to the mature membrane; (2) the fractionation, by sucrose gradients, of purified CNS myelin. These studies are aimed at identifying possible transition membranes, the differences in density reflecting different stages of maturation of the myelin sheath.

Using the first approach Agrawal et al. (1974) found that the membranes released from myelin during osmotic shock treatment contain myelin constituents and had metabolic properties that suggested they were myelin precursors. Further biochemical analysis has shown that this membrane fraction is really a heterogenous mixture of endoplasmic reticulum (Waehneldt and Mandel, 1972), axonal (Haley et al., 1981) and Golgi membranes (Benjamins et al., 1982).

More information about possible transition of precursor membranes to mature compact myelin has come from fractionation of the purified myelin membrane itself. In a double isotope labelling study, Benjamins et al. (1976) found that PLP appeared first in the denser subfractions (composed mainly of vesicular single-membrane structures) then later in lighter subfractions corresponding to typical multilamellar myelin. In contract, MBPs were observed to enter all membrane subfractions simultaneously. The results

suggested/

suggested that the denser subfractions contain pools of PLP and may serve as precursors to the lighter subfractions and that MBPs do genuinely enter all the membrane fractions simultaneously or move very rapidly from the lower 'precursor' subfraction to the lighter ones.

Three studies using young and adult rats (Waehneldt, 1978; Waehneldt and Lane, 1980; Lane and Fagg, 1980) have shown that the peak position of brain myelin shifts to a denser region of the sucrose gradient as the animal matures. Furthermore, several developmental trends regarding the abundance of myelin proteins in different membrane subfractions were apparent. The lighter membrane fractions contained a lower proportion of high molecular weight proteins than the heavy fractions, a higher level of MBPs and a lower level of CNPase and PLP. Lane and Fagg (1980) also observed that the ratio of PLP to MBPs decreased in the heavy myelin subfractions of young animals although in older animals where the overall rate of myelination has slowed considerably no clear trends were apparent and these observations suggest that MBPs are deposited in the myelin sheath slightly later than PLP. There is still considerable debate about this matter since during myelin development this ratio has been shown to increase (Morell et al., 1972), decrease (Magno-Sumbilla and Campagnoni, 1977), or remain unaltered (Adams and Osborne, 1973).

(c) <u>In Tissue Culture</u>

Several different culture systems exist for the study of myelination. These are explant cultures, dissociated-reaggregated cell cultures, dissociated-attached cell cultures, isolated oligodendrocytes/

oligodendrocytes and clonal lines (reviewed by Pfeiffer, 1984). The first three culture systems consist of a mixture of cell types the proportions of each depending on the particular experimental approach taken. Clonal lines of oligodendrocytes would appear to satisfy all the requirements for studying myelination in an environment which is not influenced by other cell types and providing optimum conditions for external manipulation. However the clonal lines that exist have not as yet realized their full potential and while they exhibit some myelin characteristics, for example CNPase activity (Sundarraj et al., 1975) difficulties in expressing appreciable levels of MBPs have been encountered. The development of methods for the selective enrichment of oligodendrocytes (McCarthy and DeVellis, 1980) and their ability to produce a myelin-like membrane in culture (Rome et al., 1986) has enabled investigators to study the metabolism of these cells in the virtual absence of astrocytes and neurons. The developmental expression of myelin proteins has been studied in cultured oligodendrocytes using two different techniques, immunocytochemistry using antibodies directed against the proteins themselves and *in situ* hybridization where, by the use of cDNA and/or oligonucleotide probes, the location and abundance of a particular mRNA can be established.

Zeller et al. (1985) found that the percentage of MBP mRNA and MBP-positive cells peaks at around 15 days postnatal in rat oligodendrocytes and decreases thereafter. Furthermore they reported that the time of emergence of the MBPs appeared to be determined at a very early stage in their development and also bore

a/

a striking correspondence to the time of MBPs appearance in the intact animal. MBP expression was observed in oligodendrocytes derived from spinal cord before those from mid-brain or hemispheres, a situation that is seen *in vivo*. Thus it appears that the developmental expression of the MBP gene occurs in a similar time frame *in vitro* and *in vivo*.

In a detailed study involving MBP, PLP and MAG, Dubois-Dalcq et al. (1986) have shown that both MBP and MAG first emerge between 5 and 7 days postnatal followed by PLP 1 to 2 days later. MBP was seen to be distributed throughout the cytoplasm whereas MAG was observed at perinuclear locations and PLP exhibited a granular staining pattern. These workers also found that MAG was detected in oligodendrocyte processes much earlier than MBP and PLP after their emergence in the oligodendrocyte cytoplasm suggesting that MAG may be transported to the periphery of the cell where it may be needed for specific interactions with axons as proposed earlier (Trapp et al., 1984). Both Zeller et al. (1985) and Dubois-Dalcq et al. (1986) have shown that these events take place in the absence of neurons and hence are not dependent on neuronal factors for their expression. Bologa et al. (1986) however have provided evidence that the production of MBP in cultured oligodendrocytes is regulated by a soluble neuronal factor. Support for this observation has come from Macklin et al. (1986) who have shown that MBP and PLP RNA levels increased fourfold when oligodendrocytes were co-cultured with chick spinal cord neurons. Thyroid hormone (T_3) has recently been shown to increase MBP mRNA levels (Shanker et al., 1987) indicating that MBP synthesis regulation by T_3 is a pre-translational/

pre-translational event. Regulation of mRNA levels by T_3 has been observed in other systems (Dozin et al., 1985; Gustafson et al., 1985) however the exact mechanism by which it does this is unknown.

Levels of CNPase activity have been shown to increase in the presence of cAMP (McMorris, 1983) however it remains to be established whether this is due to a general increase in CNPase concentration in the oligodendrocytes or an increased amount of a specialized CNPase-rich cellular compartment such as a myelin precursor membrane. cAMP induction of CNPase activity has also been shown in C_6 glioma cells. In this case the increase in CNPase activity is due to an increased number of newly synthesized CNPase molecules and not an increase in the catalytic activity of each CNPase molecule (McMorris and Sprinkle, 1982).

In summary, the use of tissue and cell culture to study the very early events of myelinogenesis is still in its infancy. However as these culture systems are becoming more established substantial gaps in our knowledge of this area will gradually be filled.

1.5 DEMYELINATION - MULTIPLE SCLEROSIS

Multiple sclerosis (MS), first described by Charcot in 1877, is by far the commonest of the demyelinating diseases in the Western world though its cause remains unknown. From a gross anatomical point of view the disease manifests itself as pale areas (plaques) of demyelination within the white matter of the brain and spinal cord. Functional damage is primarily in the spinal cord and results in the dysfunction of neurons concerned mainly with limbic function and/

and bladder control. Coronal sections through the brains of MS victims taken at autopsy reveal multiple disseminated plaques that range in size from about 1mm to several centimetres (Figure 19). Clinical diagnosis of MS is not easy since no specific test for the disease exists and diagnosis is usually based on detailed clinical history and extensive neurological examination (Traugott and Raine, 1984). The disease strikes mainly at people in early adulthood, predominantly female, and due to the variability of the duration and intensity of the disease it is almost impossible to give a prognosis regarding individual patients although the mean life expectancy has increased to about 35 years due to comprehensive care and the availability of antibiotics (Poser et al., 1982).

MS is classed as an "acquired autoimmune (inflammatory) and infectious disease of myelin" and the degeneration of the myelin sheath resulting in naked axons (Figure 20) is considered secondary to the putative infection. It is hypothesized that an autoimmune reaction to myelin antigens develops (Raine, 1984b).

Epidemiological data would seem to support the widely held view that the causative agent is viral in nature since high instances of the disease are found in temperate latitudes. In contrast, MS is almost unheard of in Africa and many parts of Asia. In addition to geographic factors, the presence of histocompatability (HCA) antigens A3, B7, DW2 and DRW2 appear to be significant (Oger et al., 1980) and might explain the increased incidence of MS within the families of diseased patients (Sadovnick and MacLeod, 1981).

Throughout the years many claims of having detected the "MS virus"/

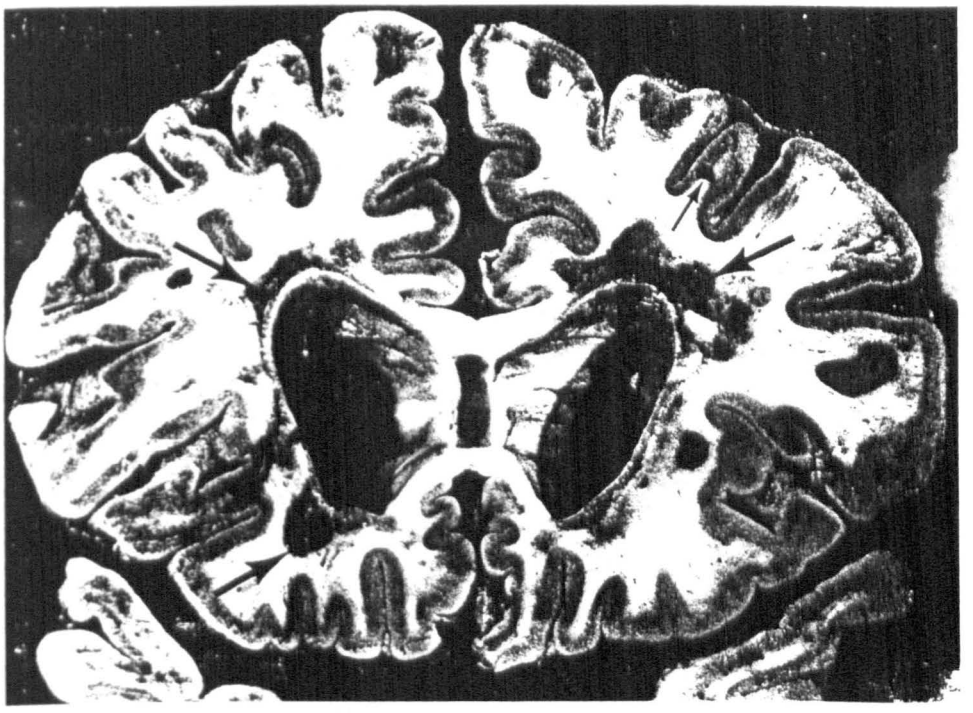


Fig. 19. Coronal slice from a chronic multiple sclerosis brain. The demyelinated plaques are clearly visible (arrows). From Raine (1984b).

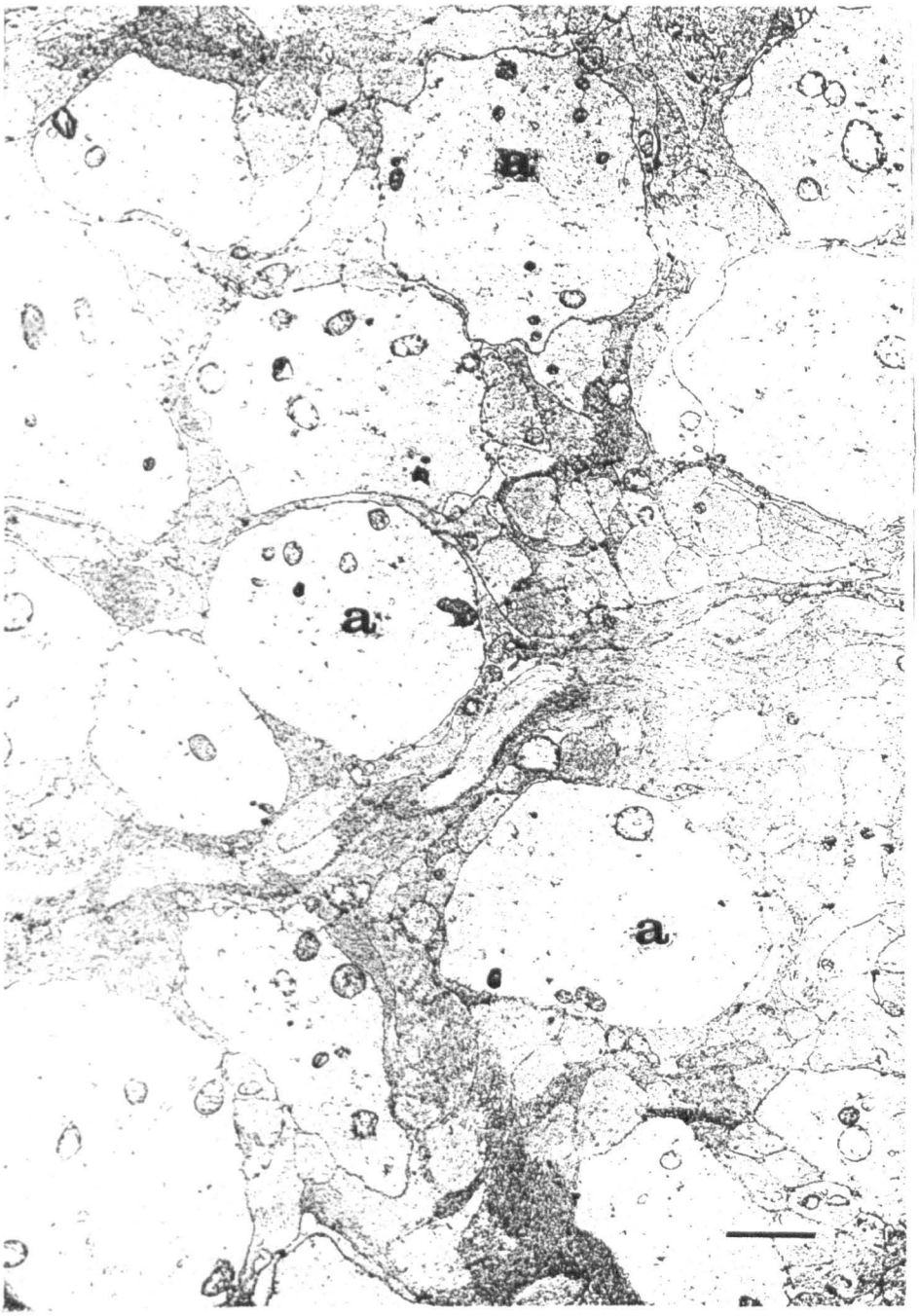


Fig. 20. EM picture of demyelinated axons (a). Scale bar: 2μm x 6000. From Raine (1984b).

virus" have been made, amongst them measles virus (reviewed by Iivanainen, 1981), and a 'paramyxoviruslike material' (Prineas, 1972). Other reports have also suggested that a coronavirus or distemper virus might be involved. However to date, no hard evidence to back up the virus theory has emerged although some circumstantial evidence from a number of conditions related to MS suggest that a virus is the likely candidate.

Immunological evidence that would indicate abnormality of immune regulation at the cellular and humoral level is equally inconclusive. Many workers have shown sensitization of blood lymphocytes against myelin components in MS (Lisak, 1980; Traugott and Raine, 1981) indicating involvement of the cell-mediated immune system. At the humoral level antibodies have been detected, in the cerebrospinal fluid (CSF) of MS patients, against MBP (Panitch et al., 1980) and oligodendrocytes (Traugott and Raine, 1981) and in general the IgG level in the CSF of the majority, but not all, of MS patients is elevated. This issue is further complicated by the presence of antibodies against myelin components in the sera of MS patients and sufferers of other neurological diseases totally unconnected with MS, for example peripheral neuropathy (Brophy, P. J., personal communication). During demyelination a rapid proliferation of astrocytes is observed (gliosis) and Bologa et al. (1985) have shown that MBP can stimulate their proliferation in vitro. These authors speculate that early attempts to immunosuppress MS patients to MBP (Romine and Salk, 1983) were unsuccessful due to increased astrocyte proliferation causing mechanical obstruction to remyelination.

Hope/

Hope for a cure for MS in the near future appears bleak. Charcot (1877) wrote at the time "It is not rare to meet with remissions and even with complete intermissions, which give rise to hope of a decided cure" and it is the limited ability of the CNS to remyelinate axons which is thought to be responsible for the variability in the prognosis of the disease. Remyelination of the CNS by injecting Schwann cells into spinal cord lesions of experimental animals has been demonstrated by Blakemore (1977) and Itoyama et al., (1983) and Harrison and Pollard (1984) have shown that these PNS myelin-forming cells accomplish remyelination in the CNS much more frequently than oligodendrocytes. The reasons for this are not clear.

More recently Kohsaka et al. (1986) have transplanted purified oligodendrocytes into the corpus striatum of Shiverer mice, a dysmyelinating mutant lacking MBP. Electron microscopy revealed that many axons became myelinated and stained heavily with antiserum against MBP. This study showed that mature oligodendrocytes are able to survive and myelinate host axons even in adult brains.

The administration of purified myelin-forming cells into CNS lesions, whilst not curing the problem, may arrest further degeneration of the patients facilities and must, therefore, be explored further.

1.6 AIMS OF THIS STUDY

A major goal of investigators engaged in membrane biosynthesis and assembly is to understand the mechanisms whereby proteins are synthesized, sorted and transported to specific sites on their target/

target membranes. The myelin membrane provides an excellent model for studying these problems due to its relatively simple protein composition and the high degree of purity with which it can be obtained in abundance.

The aim of the project was to establish the sites of biosynthesis of major CNS myelin proteins and to follow their incorporation into the growing myelin membrane. Furthermore, it was of considerable interest to determine if myelin-associated RNAs were enriched in messages encoding these proteins, as is the case for the MBPs (Colman et al., 1982). The involvement, if any, of the cytoskeleton in myelination was also to be investigated with a view to establishing a potential role for this structure in the assembly process.

Materials and Methods

2.1 BULK MYELIN ISOLATION

Advantage is taken of myelin's characteristically low buoyant density in order to isolate myelin essentially free of contaminating membranes.

The procedure is a modification of Norton and Poduslo (1973a). Wistar rats (4-6 weeks old) of both sexes from our own breeding colony were killed by CO_2 inhalation and the brains quickly removed and frozen in liquid N_2 or solid CO_2. A 5%(w/v) homogenate was prepared by homogenizing the brains in ice-cold 0.85M sucrose containing 10mM Hepes, pH7.4, 3mM DTT and 1mM TLCK using a Potter-Elvehjem glass homogenizer (Orme Scientific, Manchester, UK). The homogenate (40ml) was overlayed with 25ml of 0.25M sucrose/10mM Hepes, pH7.4/3mM DTT/1mM TLCK in polycarbonate centrifugation tubes and centrifuged at 70,000g for 90 min at 4°C in a MSE 3 x 70ml swing out rotor. After centrifugation, crude myelin was removed from the 0.25 - 0.85M sucrose interface and subjected to osmotic shocking by resuspending the myelin in 10 vol. of ice-cold double distilled H_2O containing 1mM TLCK. After centrifugation (23,000g for 30 min at 4°C, Sorvall SA600 rotor) the supernatant, containing contaminating microsomes, was discarded and the myelin pellet subjected to a further 2 rounds of osmotic shocking centrifuging at 17,000g on the final shock.

The purified myelin pellet was resuspended in a small volume of double distilled H_2O, aliquoted and stored at -20°C.

Myelin was also isolated from other tissue sources using this same general method.

2.2/

2.2 SUBCELLULAR FRACTIONATION AND RNA EXTRACTION

In all experiments involving RNA it is extremely important to exclude ribonuclease activity and sterile conditions must be maintained throughout.

i. Subcellular Fractionation

The method used was a modification of Colman et al. (1982). The concentration of all sucrose solutions were checked with a refractometer.

Sprague Dawley or Wistar rats of both sexes aged between 13-32 days old, a time when the rate of CNS myelination is maximal (Banik and Smith, 1977), were used in all experiments.

After decapitation, brains (including brainstem) were removed and immediately placed in ice-cold 0.25M sucrose/10mM Hepes, pH7.4/3mM DTT. Each brain was homogenized in this solution (3.5ml) using a Potter-Elvehjem glass homogenizer fitted with a PTFE pestle and centrifuged at 4,000g for 15 min at 4^{o}C to remove cell debris and nuclei. The supernatant was removed and centrifuged again. The post-nuclear supernatant was adjusted to 1.4M sucrose and this solution (9ml) was overlayed onto 1.9M sucrose (1.5ml). The discontinuous gradient was completed by layering 0.25M sucrose over the 1.4M sucrose solution. All sucrose solutions contained 10mM Hepes, pH7.4 and 3mM DTT. After centrifugation at 105,000g for 30h at 4^{o}C (MSE 6 x 16.5 swing out rotor) the rough microsomal fraction was removed from the 1.4 - 1.9M sucrose interface, diluted (1:1) with double distilled H_2O and centrifuged at 105,000g for 2h at 4^{o}C. Free polysomes were recovered in the pellet from the sucrose gradient. Myelin for these experiments was obtained by scaling/

scaling down the procedure in Section 2.1.

ii. Electron Microscopy

Electron micrographs of free polysomes and rough microsomes were obtained by a method similar to that of Hubbard et al. (1983).

Samples were fixed in glutaraldehyde (3%,v/v) in 0.5M $Na_2 HPO_4/NaH_2PO_4$, for 1h and centrifuged at 200,000g for 30 min at 20°C in a MSE 3 x 6.5ml swing out rotor. The pellets were washed in the phosphate buffer, post-fixed in 2%(w/v) OsO_4 and stained en bloc with 2%(w/v) uranyl acetate for 1h. After staining, the samples were dehydrated stepwise in solutions of ethanol, centrifuging briefly between steps, until finally they were suspended in anhydrous ethanol. The pellets were embedded in EMIX resin (Emscope, London, UK) in propylene oxide and ultra-thin sections obtained using a LKB Ultratome III microtome. The sections were stained with 0.2%(w/v) lead citrate in 0.1M NaOH and 8%(w/v) uranyl acetate for 20 min each at room temperature and electron micrographs obtained with a JEOL 100C electron microscope at 80V.

Electron micrographs of the Triton X-100 (cytoskeletal) pellet (Section 2.6 ii) were also obtained using this method.

iii. RNA Extraction

In recent years the use of guanidinium salts as potent chaotropic agents to dissociate ribonucleo-protein complexes and inactivate ribonucleases has proved helpful in the isolation of intact, biologically active RNA.

Depending on the initial sample volume two methods were used to isolate total RNA from brain or subcellular fractions.

(a)/

(a) Guanidine/Caesium Chloride Method (for large tissue samples and volumes > 3ml)

The method takes advantage of the fact that the buoyant density of RNA in CsCl is much greater than that of other cellular macromolecules.

The procedure used was a modification of Liu et al. (1979).

Cellular fractions and brains were homogenized (5%,w/v) at room temperature in 6M guanidine HCl (Grade 1, Sigma) containing 0.4%(v/v) Triton X-100 (BioRad Laboratories, Watford, UK), 25mM sodium acetate, pH5.5 and 10mM β-mercaptoethanol using a Potter-Elvehjem homogenizer. The homogenate (50ml) was layered over a 10ml cushion of 5.7M CsCl/100mM EDTA, pH6.1, and centrifuged at 70,000g for 22h at 22°C. The supernatant was discarded and the wall of the tube dried carefully while inverted. The crude RNA pellet was dissolved in 2ml of double distilled H_2O and heated gently if necessary. Phenol/chloroform (2ml) (pre-equilibriated with 0.1M Tris HCl, pH7.4) was added, the sample vortexed and centrifuged at 2,000g for 15 min at room temperature. The upper (aqueous) phase was then washed twice with chloroform.

Sodium acetate, pH5.5 was added to 0·2M and the RNA precipitated overnight at -20°C with 2.5 vol. of ethanol. Precipitated RNA was recovered by centrifugation at 14,000g for 45 min at 0°C (Sorvall, SA600 rotor) and the ethanol precipitation repeated. Purified RNA was washed with pre-cooled 80%(v/v) ethanol, centrifuged and dried under N_2.

The RNA pellet was dissolved in a small volume of double distilled H_2O and stored at -20°C.

For/

For quantitating the amount of RNA the optical density at 260nm and 280nm was measured. An OD_{260} of 1 corresponds to a concentration of 30µg/ml for RNA. The OD_{260}/OD_{280} ratio provides an estimate of the purity of the RNA, a pure preparation having a ratio of 2.0.

(b) Guanidine/Lithium Chloride Method (for small volume samples up to 3ml)

The combined use of guanidine and LiCl results in the precipitation of RNA species which are largely free of DNA fragments, polysaccharides and protein. The method used was that of Cathala et al. (1983) with modifications.

Lysis buffer (5M guanidine HCl/10mM EDTA/50mM Tris HCl, pH7.4/8%(v/v) β-mercaptoethanol) was added to samples at a ratio of 7ml buffer per ml or gram of sample, homogenized as for the CsCl method and 7 vol. of 4M LiCl added. The sample was well mixed and the RNA allowed to precipitate overnight at 4°C. After centrifugation at 14,000g for 90 min at 4°C (Sorvall, SA600 rotor) the supernatant was discarded and the pellet resuspended in 1ml of 2M LiCl/4M urea. The sample was re-centrifuged as before but for 1h and the crude RNA pellet dissolved by gentle heating and sonication (2 x 10s) in 1ml of 0.1%(w/v) SDS/1mM EDTA/10mM Tris HCl, pH7.4. Purification of RNA by phenol/chloroform and ethanol precipitation was carried out exactly as described in Section 2.2 iii (a).

iv. Poly(A)$^+$ RNA Isolation

The existence of poly(A) tails at the 3'-end of most eukaryotic mRNAs (Lim and Canellakis, 1970; Darnell et al., 1971) enables one to purify this RNA species via hybridization to oligo (dT) or poly(U) supports.

(a)/

(a) Oligo (dT)-Cellulose Chromatography

The poly(A)$^+$RNA fraction from total brain RNA was prepared by the method of Aviv and Leder (1972). Oligo (dT)-cellulose Type 7 (0.5g, equivalent to 20mg oligo (dT), Pharmacia) was washed successively with 10 vol. each of 0.1M NaOH, double distilled H_2O and 0.5M NaCl/0.1%(w/v) SDS/10mM Tris HCl, pH7.4 (NaCl buffer). Rat brain total RNA (1mg in 1ml NaCl buffer) was heated at 65^{o}C for 5 min, cooled on ice and applied to the column followed by 3 x 1ml of NaCl buffer. The run-through was re-applied to the column three times and poly(A)$^+$RNA eluted with 4 x 1ml of double distilled H_2O. The fractions were pooled and ethanol precipitated as described in Section 2.2 iii (a). The column was washed with 10 vol. of 1M NaCl containing 0.05%(w/v) NaN_3 and stored at 4^{o}C in this solution.

The average yield of poly(A)$^+$RNA was 12% of the total RNA.

(b) Poly(U) Affinity Paper

Rat brain total RNA (200µg in a volume not greater than 100µl) was heated at 65^{o}C for 5 min, chilled on ice and NaCl added to 0.5M. A 2cm^2 piece of Hybond mAP (Amersham) was cut out using a sterile scalpel blade, placed on several layers of 3MM paper (Whatman) and 40µl of 0.5M NaCl/1mM EDTA/20mM Tris HCl, pH7.4 spotted on carefully. The paper was allowed to dry in air then transferred to a sterile petri dish and the RNA sample spotted on slowly and left for 5 min. The paper was washed twice for 5 min each in 5ml of 0.5M NaCl/1mM EDTA/20mM Tris HCl, pH7.4 then twice for 2 min each in 10ml of 70%(v/v) ethanol and allowed to dry in air. The paper was sliced into 4 pieces, transferred to a microfuge tube/

tube and 100µl double distilled H_2O added. The sample was heated at 70°C for 5 min, vortexed and the strips removed from the liquid and trapped by the tube cap. Excess liquid was spun out of the paper (3,000g for 5 min, MSE microcentaur) and the poly(A)$^+$RNA ethanol precipitated (Section 2.2 iii (a)). The average yield of poly(A)$^+$RNA using this method was 10% of the total RNA.

2.3 IN VIVO LABELLING

The method used was similar to that of Colman et al. (1982).

Sprague Dawley or Wistar rats aged between 18-20 days old were anaesthetised under ether and injected, via the cerebellum, with 2.4mCi of [^{35}S] methionine (specific radioactivity 1,000 Ci/mmol, New England Nuclear), allowed to recover, then killed by decapitation at various time intervals. Subcellular fractionation was carried out (Section 2.2 i) except that 0.85M sucrose/10mM Hepes, pH7.4/3mM DTT (1ml) was included in the discontinuous gradient. All sucrose solutions contained 100 units/ml of Aprotinin (Sigma) to inhibit proteolysis. Myelin was collected from the 0.85 - 0.25M sucrose interface, subjected to osmotic shock and further purified on a 0.85M - 0.25M sucrose discontinuous gradient. All fractions were stored at -20°C.

2.4 POLYACRYLAMIDE GEL ELECTROPHORESIS AND WESTERN BLOTTING

i. Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples were routinely analysed in 5-15% gradient slab gels using the discontinuous system of Maizel (1971) as modified by Kreibich and Sabatini (1974). Samples (30µg protein per lane) were prepared/

prepared by boiling for 2.5 min in a sample buffer containing 2.5%(w/v) SDS and 125mM DTT. Electrophoresis was carried out at 4mA until the sample had entered the stacking gel then increased to 30mA. Protein bands were visualized by staining for at least 30 min in 0.2%(w/v) Coomassie Blue (BDH) in 50%(v/v) methanol and destaining in 25%(v/v) methanol/10%(v/v) glacial acetic acid.

ii. Two-dimensional PAGE

The method used was a modification (Althaus et al., 1983) of O'Farrell (1975).

Myelin proteins (50µg) were resolved by non-equilibrium isoelectric focusing in the first dimension in gels of 11cm x 4mm (internal diameter) containing 2%(v/v) ampholines of pH 2-11 (LKB) for 3h and separated in the second dimension by SDS-PAGE in 5-15% gradient slab gels.

iii. Silver Staining

Polyacrylamide gels were silver stained for protein in clean glass containers by the method of Ansorge (1985) as detailed below.

1.	50%(v/v) methanol/20%(w/v) TCA/2%(w/v) $CuCl_2$	(20 min)
2.	10%(v/v) ethanol/5%(v/v) acetic acid	(10 min)
3.	0.01%(w/v) $KMnO_4$	(10 min)
4.	10%(v/v) ethanol/5%(v/v) acetic acid	(10 min)
5.	10%(v/v) ethanol	(10 min)
6.	Double distilled H_2O	(10 min)
7.	0.1%(w/v) $AgNO_3$	(10 min in the dark)
8.	10%(w/v) K_2CO_3	(1 min in the dark)

Gels were rinsed briefly in double distilled H_2O between steps.

The/

The silver stain was developed in 0.01% (v/v) formaldehyde/ 2%(w/v) K_2CO_3 in the dark (usually 5-10 min) and stopped by briefly washing in 10%(v/v) ethanol/5%(v/v) acetic acid. Stained gels were stored in double distilled H_2O.

iv. Western Blotting

(a) Electrophoretic Transfer

After SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose paper (0.45μm, (Schleicher and Schuell, Anderman Ltd) at 0.25A for 6h in a TransBlot apparatus (BioRad Laboratories) by the method of Towbin et al. (1979). After transfer, strips were sliced off and stained in 0.1%(w/v) Amido black, destained using PAGE destain and finally immersed in double distilled H_2O. The remainder of the blot was blocked overnight in PBS containing 0.2%(w/v) gelatin and 0.1%(v/v) Triton X-100 (Buffer I) to quench non-specific binding of protein. Stained and blocked blots were dried on 3MM paper and stored at room temperature.

(b) Immunoblotting

Nitrocellulose blots were incubated in Buffer I containing the appropriate dilution of primary antibody for 1h at room temperature. Blots were washed four times for 5 min each in Buffer I minus antibody then incubated in Buffer I containing either goat anti-rabbit IgG (Northeast Biomedical Laboratories) (1:1000 or 1:2000 dilution) or sheep anti-mouse IgG (Scottish Antibody Production Unit) at 1:500. Both second antibodies were conjugated to horseradish peroxidase. After 1h incubation at room temperature in the dark, the blots were washed three times for 5 min each in Buffer II (PBS/0.1%(v/v) Triton X-100) and finally in PBS.

Immunostaining/

Immunostaining was visualized by the addition of DAB (Sigma) (50mg/100ml) in 50mM Tris HCl, pH7.4 followed by 30%(v/v) hydrogen peroxide (10µl per 10ml of DAB solution). The reaction was stopped by rinsing the blots in 2%(w/v) SDS and immunoblots were stored at room temperature in the dark.

(c) CNPase Activity Staining

Nitrocellulose blots of CNS myelin proteins were stained for CNPase activity exactly as described by Bradbury and Thompson (1984).

2.5 AFFINITY PURIFICATION OF CNPase AND PLP ANTIBODIES

i. Purification and Electroelution of CNPase

Rat brain CNPase was resolved by SDS-PAGE and the protein eluted from the gels pieces as described by Hunkapiller et al. (1984). Eluted material was precipitated by adding 10 vol. of acidified acetone.

CNPase antiserum was obtained from adult New Zealand White rabbits by the method of Colman et al. (1982). Blood taken from the ear vein was allowed to clot overnight at $4^{o}C$ and, once the clot had retracted, it was centrifuged at 14,000g for 20 min at $4^{o}C$ (Sorvall, SA600 rotor). The serum was decanted and serial dilutions tested in immunoblots of rat CNS myelin proteins.

ii. Affinity Chromatography

Sepharose 4B (5ml, Pharmacia) was thoroughly washed with 10 vol. of 0.5M NaCl and 10 vol. of double distilled H_2O. The resin was activated using cyanogen bromide according to the procedure of March et al. (1974). Excess liquid was removed by filtration and the activated resin washed with 10 vol. each of 0.1M $NaHCO_3$, pH9.5,/

pH9.5, double distilled H_2O and 0.1M Na_2CO_3/0.5M NaCl, pH8.35 (coupling buffer) and finally transferred to a small glass column (BioRad). Triton X-100 (2%,v/v) was added to the purified CNPase solution (3.2mg protein) and coupling was carried out using an end over end mixer for 12h at 4°C in the presence of 2 vol. of coupling buffer. After coupling, the column was washed with 10 vol. of coupling buffer and excess reactive sites blocked by incubating the resin in 2 vol. of 0.1M glycine, pH8.0 for 2h at room temperature. The column was washed successively with 10 vol. each of 0.1M sodium acetate/0.5M NaCl, pH4.5, 0.1M Tris borate/0.5 NaCl/0.5%(v/v) Triton X-100, pH8.5, PBS/0.1%(v/v) Triton X-100/2mM EDTA, pH7.4 and finally PBS, pH7.4. CNPase antiserum (4ml) containing 0.5M NaCl, 0.1%(v/v) Triton X-100, 2mM EDTA and 10mM Hepes, pH7.4 was centrifuged at 14,000g for 30 min at 4°C and the supernatant injected onto the column through a 0.22µm sterile filter (Flow Laboratories). Incubation was carried out for 2h at room temperature and the column was washed with PBS, pH7.4 until the optical density at 280nm of the eluent was less than 0.03. CNPase antibodies were eluted with 0.1M glycine/0.15M NaCl, pH3.0 and 1ml fractions were collected into 0.5ml of 0.5M NaH_2PO_4, pH8.0. An OD_{280} elution profile was obtained and relevant fractions pooled, dialysed against 5l of PBS, pH7.4 for 3h at 4°C, concentrated in a freeze dryer, aliquoted and stored at -70°C.

iii. PLP

PLP antiserum (330µl) (from Dr E Blair, Department of Biochemistry, University of Leeds) was affinity purified using a 1ml (approximately 1mg of PLP) affinity column scaling down the procedure/

procedure already described. This antiserum also contained antibodies versus MBP. These were removed using an appropriate affinity column.

2.6 CYTOSKELETAL EXTRACTIONS

i. Single-cell Suspensions

Wistar rats of both sexes aged between 15-19 days old were killed by CO_2 inhalation and the brains quickly removed and placed in Hanks Balanced Salt Solution (with $NaHCO_3$ and without Ca^{2+} and Mg^{2+}, Flow Laboratories) at room temperature. A single-cell suspension was prepared by coarsely chopping the tissue and forcing it through one layer of 136μm Nybolt (Plastok) then twice through one layer of 30μm Nybolt. The cells were harvested by centrifugation at 1,000g for 3 min at room temperature (MSE Multex) and gently resuspended in CSK buffer minus Triton X-100 (6ml per brain; see Table 2 for the buffer composition). After centrifugation as before, the cells were gently resuspended in CSK buffer (6ml per brain), lysed at 30°C for 1 min with gentle shaking and centrifuged at 14,000g for 5 min at 30°C (Sorvall, SA600 rotor). The supernatant (soluble fraction) was carefully removed and the pellet washed in an equivalent volume of CSK buffer (without Triton X-100) and centrifuged as before.

The supernatant was retained (wash fraction) and the cytoskeletal pellet resuspended in a volume of CSK buffer equal to that of the soluble fraction. This fraction was designated the cytoskeleton fraction. For protein distribution analysis, aliquots of each fraction were electrophoresed and immunoblotted (Section 2.4).

For/

TABLE 2 CONSTITUENTS OF THE CSK BUFFER

Stock Solution	Final Concentration
100mM Pipes, pH6.8	10mM
50%(v/v) glycerol	20%(v/v)
10%(v/v) Triton X-100	0.5%(v/v)
300mM $MgCl_2$	1mM
100mM EGTA	2mM
1M KCl	100mM
10mM Taxol	50μM
50mM TLCK	1mM
1mg/ml Leupeptin	10μg/ml
1mg/ml Antipain	10μg/ml
2mM Pepstatin A	15μM
1M Benzamidine	10mM

The pH was adjusted to 6.8 and the solution filtered through a 0.2μm sterile filter.

Leupeptin, Antipain and Pepstatin A were obtained from Peptide Institute, Osaka, Japan and Taxol from NIH, Bethesda, USA.

For RNA extraction the cytoskeletal pellet was further extracted by a modification of Bird and Sells (1986). The pellet was homogenized at 4°C using a Potter-Elvehjem homogenizer in 6ml CSK buffer containing 0.25M $(NH_4)_2SO_4$ but without KCl and incubated on ice for 10 min. After centrifugation at 10,000g for 20 min at 4°C (Sorvall, SA600 rotor) the supernatant was retained as the purified cytoskeleton. The pellet contained mainly nuclei, insoluble cell debris and heterogenous nuclear RNA. RNA was extracted from both soluble and purified cytoskeletal fractions (Section 2.2 iii) and the CSK buffer contained 5 units/ml Human Placental Ribonuclease Inhibitor (HPRI) (Amersham).

ii. Myelin-associated Cytoskeleton

Rat brain myelin was resuspended in CSK buffer (1mg/ml) and incubated for 10 min at 30°C. After centrifugation (13,400g for 5 min at room temperature, MSE microcentaur) the supernatant was retained (soluble fraction) and the pellet extracted again. The final pellet was resuspended in CSK buffer to a volume equivalent to that of the soluble fraction.

iii. Lipid Analysis

Rat brain myelin (2mg) and the cytoskeletal pellet from 10mg of rat brain myelin (Section 2.6 ii) were made to 1ml total volume and centrifuged (50,000g for 20h at 4°C, MSE 6 x 16.5 swing out rotor) through a continuous 0.3-1.2M sucrose gradient. Fractions (0.5ml) were collected and 50µl of each were solubilized in 2%(w/v) SDS at 50°C for 15 min, cooled and the OD_{280} obtained. A further 50µl from each fraction was analysed by SDS-PAGE (Section 2.4 i).

Lipid/

Lipid was extracted from the remainder of each fraction by the method of Bligh and Dyer (1959). Dried samples were dissolved in 20µl of chloroform/methanol (1:1,v/v) and spotted onto activated (100°C for 1h) Kieselgel 60 (Merck) TLC plates. After resolving the lipids in methyl acetate/isopropanol/chloroform/methanol/ 0.25%(w/v) KCl (25:25:25:10:9 by volume; Bürgisser et al., 1986) the plates were dried in air and the lipids stained lightly with iodine vapour.

For quantitative purposes total lipid extracts from either whole myelin or the cytoskeletal pellet were analysed for phospholipid (Eng and Noble, 1968), sulphatides (Kean, 1968), cerebrosides (Macala et al., 1983) and cholesterol (Sperry and Brand, 1943). Individual phospholipids were quantitated by scraping the relevant spots off the TLC plate and subjecting them to phosphate analysis as for the total phospholipids.

2.7 CELL-FREE TRANSLATIONS

i. Wheatgerm Extract (WGE) Preparation

It is extremely important to maintain sterile conditions throughout the entire preparation.

G25 (medium) Sephadex (Pharmacia) was pre-swollen in 20mM Hepes, pH7.4/50mM KCl/1mM $MgCl_2$ and autoclaved. DTT was added to 2mM and a glass column packed to a bed volume of 160ml. Wheatgerm (3.9g) (Robert Hutcheson's Mills, Kirkcaldy) was ground in liquid N_2 using a pre-cooled mortar and pestle. After the N_2 had evaporated the ground wheatgerm was added to 10ml of extraction medium (1mM Mg acetate/90mM KCl/2mM DTT) and centrifuged at 12,000g for/

for 12 min at 4°C (Sorvall, SA600 rotor). The supernatant was decanted from under the lipid layer using a pasteur pipette and made to 20mM Hepes, pH7.4 and 2mM Mg acetate. The sample was centrifuged as before and the supernatant layered onto the Sephadex column. The flow rate was adjusted to 75ml/l and, after discarding the front (1-1.5ml), 10ml of the turbid fraction of the void volume were collected, aliquoted, frozen in liquid N_2 and stored at -70°C.

ii. Optimization of the System

The ionic optima for translation in the WGE system must be determined for the particular RNA added. Depending on the set of experiments the K^+ and Mg^{2+} optima were determined using either total RNA or poly(A)$^+$RNA (4 OD Units/ml) in a translation mix that contained 20-120mM K acetate, 0.6-4mM Mg acetate, 4µl of Mastermix (see Table 3), 30-60µCi [^{35}S] methionine (specific radioactivity 1000 Ci/mmol, Amersham), 1mM TLCK, 200 units/ml HPRI and 10µl WGE in a total volume of 25µl (including RNA). Translation was initiated by the addition of RNA and incubation carried out for 90 min at 26°C. The amount of RNA used in each translation was also optimized using optimal K^+ and Mg^{2+} concentrations.
Incorporation of radioactivity was determined by spotting 2.5µl aliquots from each translation onto 3MM paper filter discs (2.1cm diameter, Whatman) and precipitation by TCA as outlined below:

Ice-cold 10%(w/v) TCA	(10 min)
Ice-cold 5%(w/v) TCA	(5 min)
Boiling 5%(w/v) TCA	(15 min)
5%(w/v) TCA	(5 min)
Ethanol	(5 min)
1:1(v/v)/	

TABLE 3 CONSTITUENTS OF THE WGE TRANSLATION MASTERMIX

Solution	Volume (µl)	Concentration in Translation
Double distilled H_2O	150	-
1mM amino acids (-met)	40	20µM
1M Hepes, pH7.6	40	20mM
100mM DTT	4	0.2mM
10mM Spermine	16	80µM
100mM ATP	20	1mM
12mM GTP	20	120µM
800mM Creatine phosphate	20	8mM
30mg/ml Creatine phosphokinase	8	0.12mg/ml

All additions were made on ice and after mixing, the Mastermix was aliquoted and stored at -70°C. Creatine phosphokinase from rabbit muscle (EC 2.7.3.2) and creatine phosphate were obtained from Boehringer Mannheim. All other reagents were from Sigma and of the highest purity available.

1:1(v/v) Ethanol/ether (5 min)

Ether (5 min)

Discs were dried under a heat lamp (5 min), transferred to scintillation vials and 5ml Fisofluor 3 (Fisons) added. Samples were counted for 1 min in a United Technologies Packard 200 CA Liquid Scintillation Counter.

iii. Membrane-binding Experiments

(a) Membrane Preparation

TLCK-treated rat brain myelin (5.4mg/ml) was sonicated heavily (3 x 5s) and centrifuged at 13,400g for 5 min at 4°C (MSE microcentaur). A 10 OD_{550}/ml suspension was prepared by diluting with double distilled H_2O. Salt extracted membranes (DPM) from dog pancreas and signal recognition particle (SRP) were prepared exactly as described by Shields and Blobel, (1978).

(b) Incubations

Rabbit spinal cord poly(A)$^+$RNA translations of equal TCA-precipitable cpm (60µl) containing 1µg/ml leupeptin, antipain and pepstatin A were incubated in the presence of H_2O (control), myelin or DPM (2µl of each) for 1h at 26°C with occasional shaking. Samples were centrifuged at 13,400g for 20min at 4°C (MSE microcentaur) and the supernatants carefully removed and 1µg/ml of proteinase inhibitors added to each. Additionally, 2µl of myelin were added to the supernatants from the myelin incubations. Membrane pellets were resuspended in 50µl of ice-cold PBS, pH7.4 containing 2µg/ml of proteinase inhibitors, sonicated lightly (2s) and re-centrifuged. The supernatants were discarded and 50µl of WGE (diluted 1:1.5 in double distilled H_2O) added to each. All samples were/

were sonicated (2s) and stored at -70°C.

For membrane-binding experiments involving MBPs, CNPase and PLP the same general method was used except that rat brain poly(A)$^+$RNA was used to programme the translations and DPM and SRP (1µl) were added at the start of the translations.

2.8 IMMUNOPRECIPITATION

The method used was a modification of Colman et al. (1982).

In vitro translations (200µl) and in vivo labelled samples (23.7µg protein in 200µl) were boiled for 2.5 min in 2%(w/v) SDS and diluted four-fold with Solution A (2.5%(v/v) Triton X-100/1mM EDTA/100 Units/ml Aprotinin/50mM Tris HCl, pH7.4, Goldman and Blobel, 1978). After centrifugation (13,400g for 5 min at room temperature, MSE microcentaur) to remove insoluble material, the supernatants were incubated overnight with constant shaking at 4°C with affinity purified antibodies (30µl) or antiserum (15µl) for translations and 100µl of each for samples labelled in vivo. Protein A-agarose (Sigma) suspension (30µl of a 33%(w/v) suspension in solution A) was added to each sample and, after 2h constant shaking at room temperature, the immune complexes were recovered by centrifugation (13,400g for 5 min at 4°C, MSE microcentaur) and washed three times with Solution A containing 0.1%(w/v) SDS and finally once with Solution A minus detergents. Immunoprecipitates were prepared for SDS-PAGE and eletrophoresed as described (Section 2.4 i). After staining and destaining, the gels were impregnated either with En^{3}Hance (New England Nuclear) or Amplify (Amersham) fluorographic reagents according to the manufacturers instructions.

Gels/

Gels were dried under vacuum (3h), placed under Kodak X-Omat XAR-5 or Konica A2 X-ray film in Harmer (London) cassettes containing Cronex intensifier screens and exposed at -70°C. Films were developed using Kodak LX-24 developer diluted 1:18(v/v) and Kodak FX-40 fixer at 1:4(v/v).

2.9 PHOSPHORYLATION OF CNPase II

Phosphorylation of CNPase II was carried out by a modification of the method of Bradbury et al. (1984). Rat brain myelin (1mg of protein) was suspended in 1mM $MgCl_2$/1mM TLCK/0.05%(v/v) Triton X-100/50mM Tris HCl, pH7.4 with or without 5μM cyclic AMP (Sigma). The reaction was started by the addition of 50μM ATP and 80μCi of [γ-^{32}P] ATP (specific radioactivity 0.5-3Ci/mmol, Amersham) and incubation carried out for 8 min at 37°C. Labelled myelin was harvested by centrifugation (14,000g for 15min at 4°C, Sorvall, SA600 rotor) and aliquots (20μg protein) processed for SDS-PAGE and antoradiography.

2.10 IODINATION OF MYELIN

Rat CNS myelin (15μg) was radio-iodinated using 250μCi of Bolton and Hunter reagent (N-Succinimidyl 3- (4-hydroxy-5-[^{125}I] iodophenyl) propionate) (specific radio-activity ~2000 Ci/mmol, Amersham) according to the method of Bolton and Hunter (1973).

2.11 PROTEIN DETERMINATION

Protein content was routinely carried out by a modification of Lowry et al. (1951) and included SDS to solubilize membrane proteins.

Samples/

Samples (200µl total volume) and 10-120µg bovine serum albumin (Fraction V, Sigma) were prepared in duplicate. 250µl of 1M NaOH/0.25%(w/v) SDS was added and the samples incubated at 60°C for 15 min. 2.5ml of Solution B (2%(w/v) Na,K,Tartrate (0.5ml)/1% (w/v)$CuSO_4$ (0.5ml) in 50ml of 2%(w/v) Na_2 CO_3) was added to each tube, mixed and incubated for 15 min at room temperature. Finally, 250µl of Folin-Ciocalteu reagent (BDH) freshly diluted (1:1) with double distilled H_2O was added and the optical density at 750nm measured after 45 min. A protein standard curve was constructed and the protein content of the samples determined from it.

Results

3.1 IDENTIFICATION OF CNPase IN RAT CNS MYELIN

i. The Protein Profile of Rat CNS Myelin

Figure 21, lane 1 shows the presence of the four abundant proteins MBPs, PLP, CNPase and MAG in purified rat brain myelin. The apparent molecular weight of CNPase I was calculated to be 48kDa and that of CNPase II 50kDa. These are similar to those reported previously (Drummond and Dean, 1980; Wells and Sprinkle, 1981). Also present are the cytoskeletal proteins actin and tubulin which are always observed to copurify with rat CNS myelin during isolation. For comparison purposes purified myelin from rat sciatic nerve is shown in lane 2 and shows an even simpler protein profile than that of CNS myelin, the major PNS integral membrane protein, Po, accounting for over 50% of the total protein composition.

ii. Characterization of CNPase Antiserum

The immunoblot of the two-dimensional gel shown in Figure 22b shows that both polypeptides are recognized by the affinity-purified CNPase antibodies. Furthermore, the immunoblot also reveals that no other polypeptides in rat CNS myelin cross react with the antibodies. Figure 23 (lanes 4 and 5) shows that both polypeptides that are recognized by the CNPase antiserum have CNPase activity.

iii. Phosphorylation of CNPase II

The pattern of phosphorylation of rat CNS myelin proteins is shown in Figure 23. Lanes 2 and 3 show that only two proteins were labelled to any great extent. MBPs were phosphorylated in a cyclic AMP-independent manner, while inclusion of 5μM cyclic AMP resulted in the phosphorylation of a protein of apparent molecular weight ~50kDa. Superimposition of the autoradiogram on to the stained and dried/

Fig. 21. Electrophoretic mobilities of rat brain (CNS) and sciatic (PNS) myelin proteins. Samples (30μg protein; lanes 1 and 2) were analyzed by SDS-PAGE in a 5-15% slab gel and stained with Coomassie blue. Lane 3 shows Coomassie blue stained molecular weight markers (myosin, 205kDa; B-galactosidase, 116kDa; bovine serum albumin, 67kDa; ovalbumin, 43kDa; glyceraldehyde-3-phosphate dehydrogenase, 36kDa; carbonic anhydrase, 30kDa; trypsinogen, 24kDa; soybean trypsin inhibitor, 20kDa; α-lactalbumin, 14kDa).

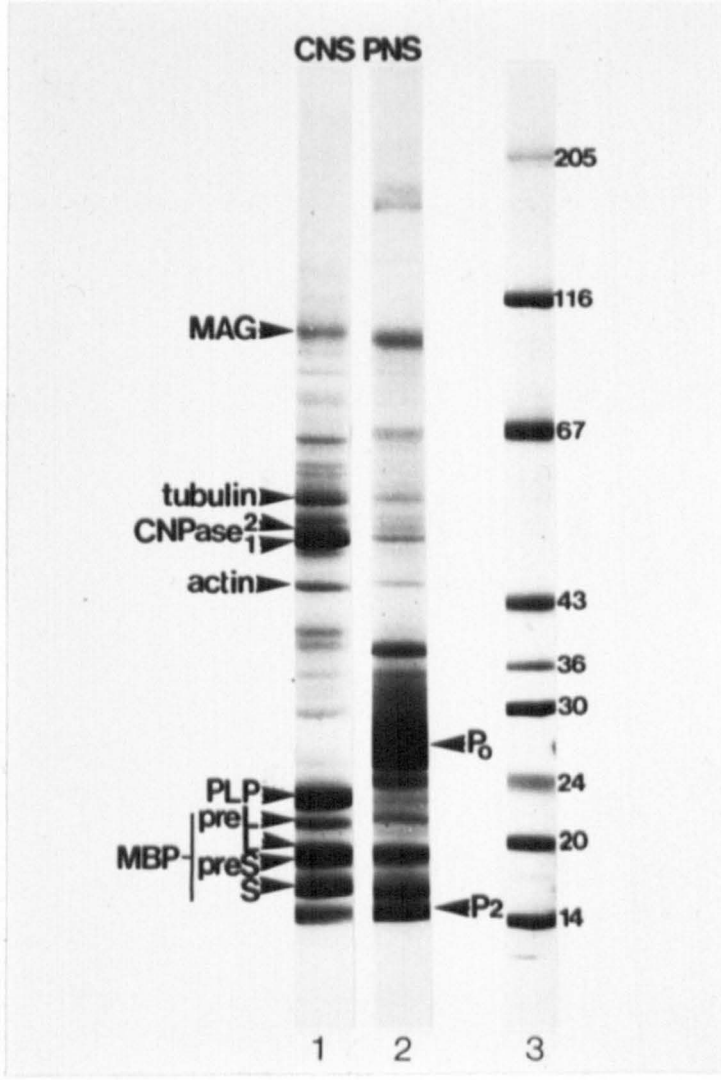

CNS PNS
205
116
MAG
67
tubulin
CNPase 2
1
actin
43
36
30
Po
PLP
24
preL
L
MBP
preS
S
20
P2
14
1
2
3

Fig. 22a. Two-dimensional analysis of rat brain myelin. Purified rat brain myelin (50μg protein) was subjected to isoelectric focusing in the first dimension followed, in the second dimension, by SDS-PAGE. The proteins were silver stained as described in the Materials and Methods Section. CNPase I is indicated by the arrow and CNPase II by the arrowhead.

Fig. 22b. Two-dimensional immunoblot of CNPase I and II. Purified rat brain myelin (50μg protein) was subjected to two-dimensional gel electrophoresis and the proteins transferred to nitrocellulose paper. CNPase I and II were detected using affinity purified rabbit anti-rat CNPase antibodies at 1:500 dilution and goat anti-rabbit IgG (peroxidase conjugated) at 1:1000. The proteins were visualized using DAB as the chromophore.


Base
Acid

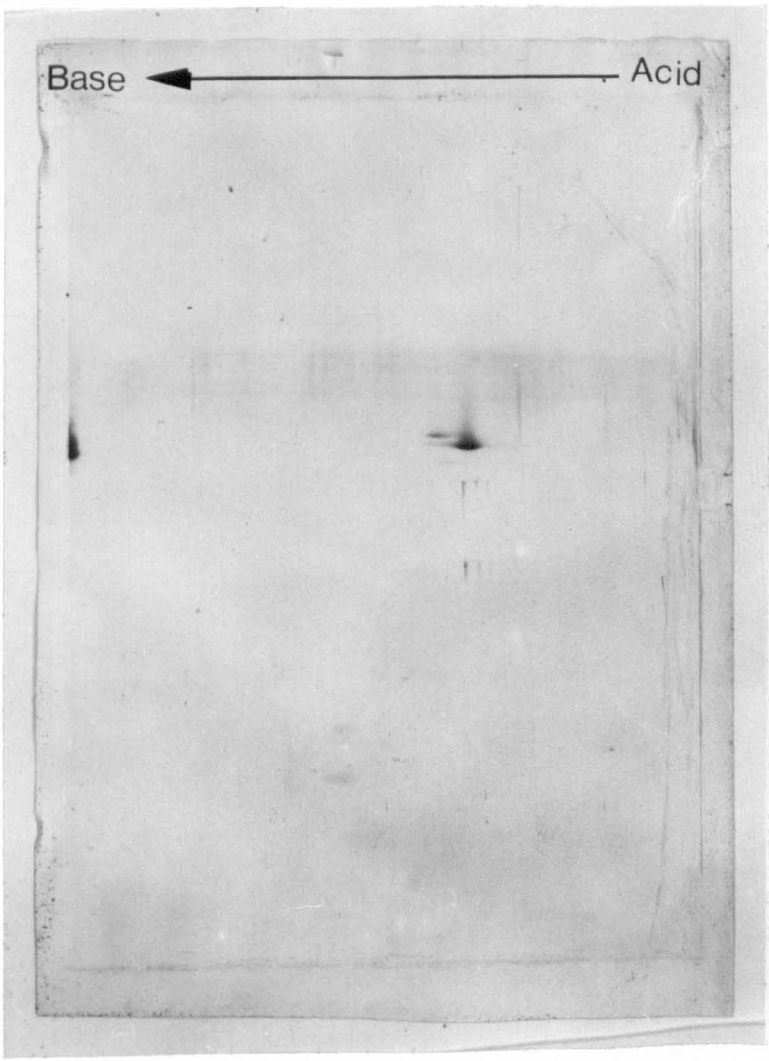
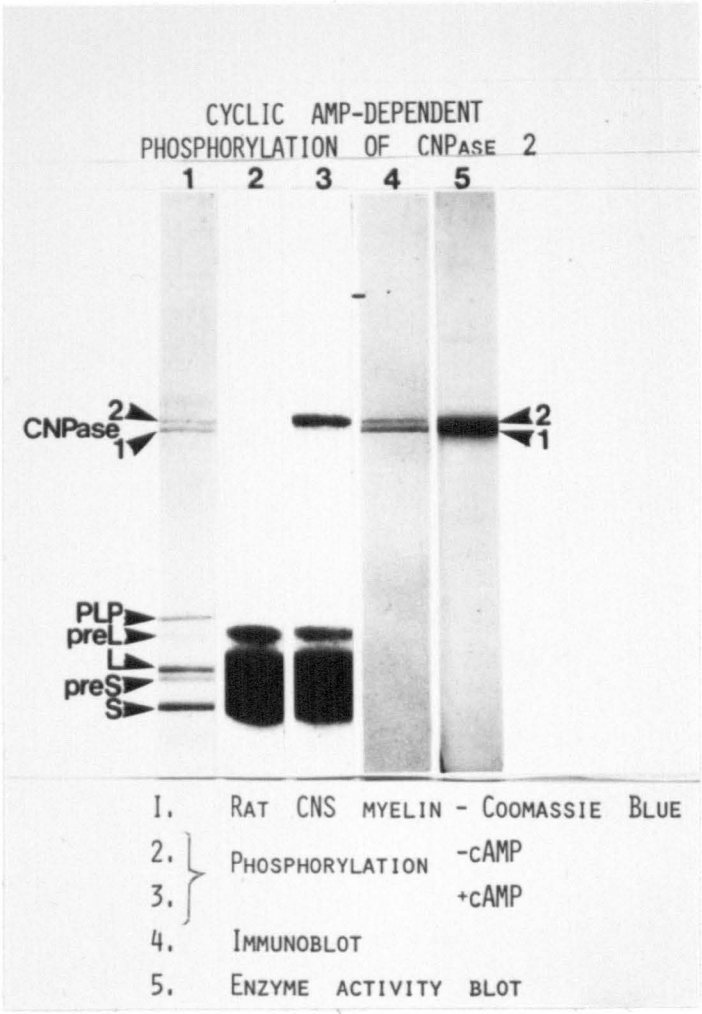

Base
Acid

Fig. 23. Cyclic AMP-dependent phosphorylation of CNPase II.

Rat brain myelin (20μg protein) was phosphorylated and subjected to SDS-PAGE. Lane 1 shows rat brain myelin stained with Coomassie blue and lanes 2 and 3 are autoradiograms of myelin phosphorylated in the absence or presence of 5μM-cyclic AMP respectively. Lane 4 is an immunoblot of CNPase I and II obtained using the same dilutions of primary antiserum and second antibody conjugate as described in Figure 22b. Lane 5 shows rat brain myelin stained for CNPase activity.


CYCLIC AMP-DEPENDENT
PHOSPHORYLATION OF CNPase 2
1 2 3 4 5
CNPase 2
1
2
1
PLP
preL
L
preS
S
1. Rat CNS myelin - Coomassie Blue
2. Phosphorylation -cAMP
3. +cAMP
4. Immunoblot
5. Enzyme activity blot

dried gel showed that the phosphorylated 50kDa protein co-electrophoresed with the upper component of the CNPase doublet. In this respect rat brain myelin CNPase II is similar to the equivalent rabbit enzyme (Bradbury and Thompson, 1984).

3.2 BIOSYNTHESIS OF CNPase

i. Subcellular Fractionation of Rat Brain

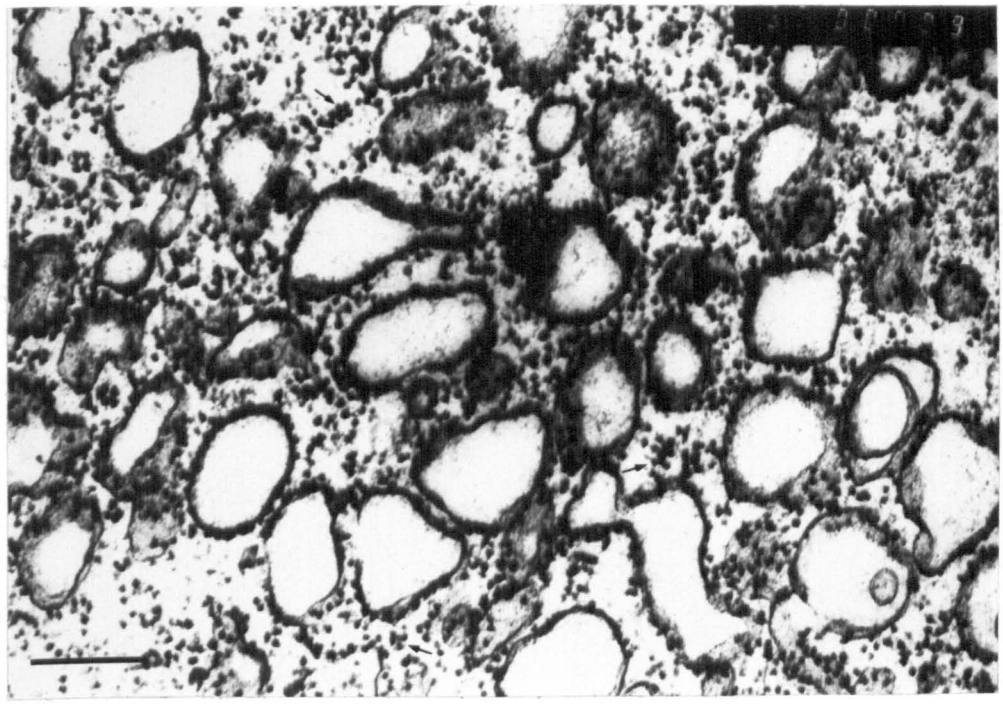
Rat brain was subjected to discontinuous sucrose gradient centrifugation and fractions corresponding to the rough microsomes (Figure 24) and free polysomes (Figure 25) were collected and a small portion of each checked by EM. The rough microsomal fraction contains the characteristic ribosome-studded membrane vesicles derived from the RER while the free polysomes (recovered in the pellet) show no contamination by membranes. The appearance of both fractions is similar to that reported by Colman et al. (1982).

ii. Site of Synthesis of CNPase

Prior to carrying out actual experiments the wheatgerm translation system was routinely optimized for potassium, magnesium and RNA concentrations for each source of RNA isolated. The results of a typical optimization for total brain poly(A)$^+$RNA are shown in Figure 26. For this particular RNA the optimal potassium and magnesium concentrations were found to be 60mM and 1.6mM respectively while the optimal RNA concentration was observed to be 4 OD U/ml (equivalent to 120µg RNA/ml of translation mixture). It is important to carry out these titration experiments to ensure that incorporation of the labelled amino acid is maximal, particularly so when dealing with proteins whose relative abundancy is low as is the case/

Fig. 24. Rough microsomes from myelinating rat brain. The rough microsomal fraction obtained from the 1.9/1.4M sucrose interface contained ribosome-studded membrane vesicles derived from the RER. Free polysomes that contaminate this fraction are indicated by arrows. x30,000 magnification.
Scale bar: 0.5 µm x 30,000

Fig. 25. Free polysomes from myelinating rat brain. Subcellular fractions were prepared from the brains of 15 day old Wistar rats by the procedure described in Materials and Methods. The free polysomes, not contaminated by membranes, were recovered in the pellet. x30,000 magnification.
Scale bar: 0.5 µm x 30,000



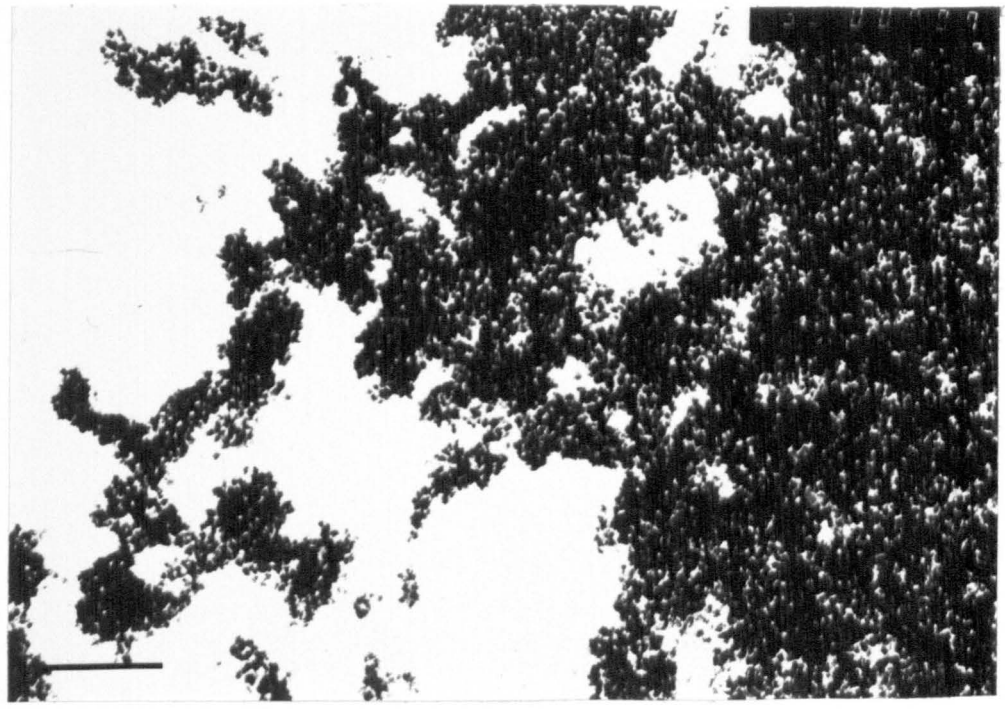
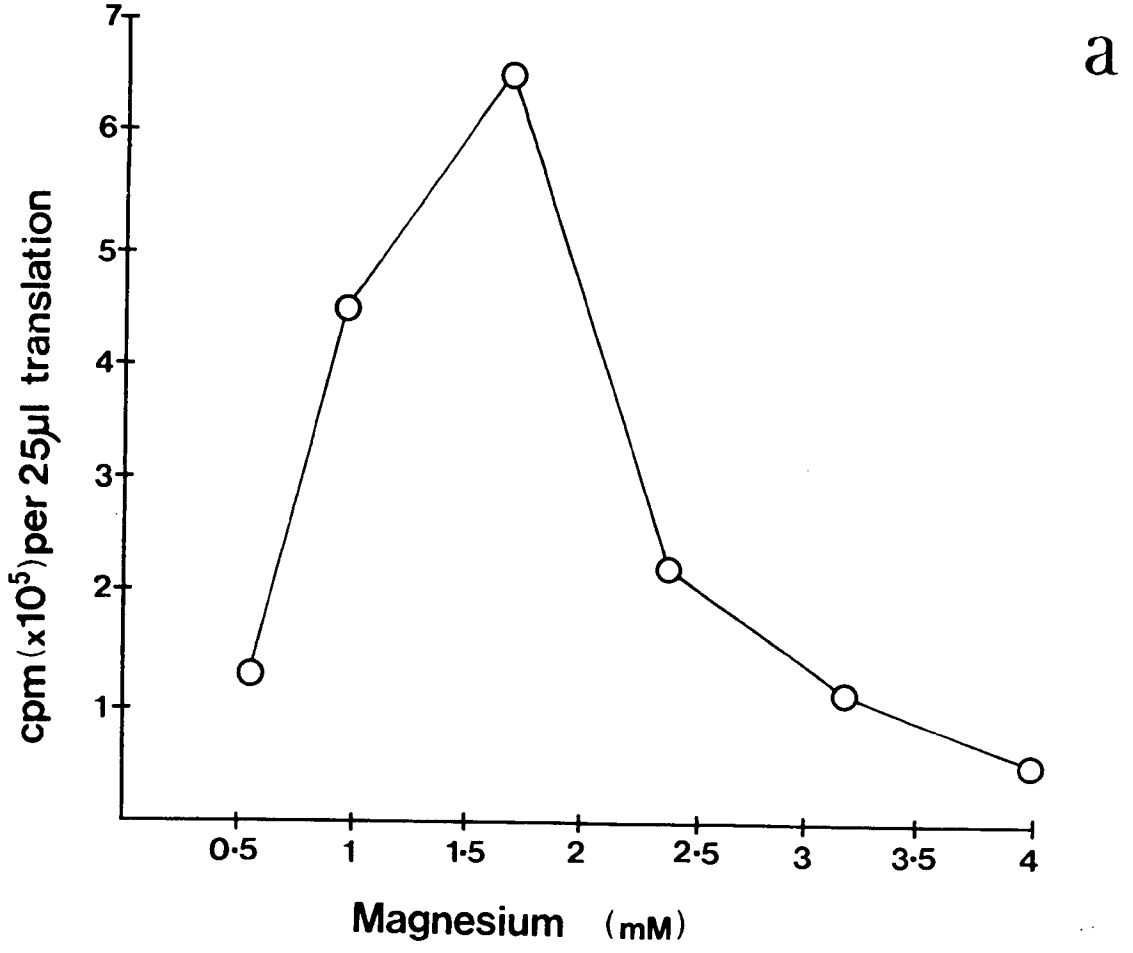
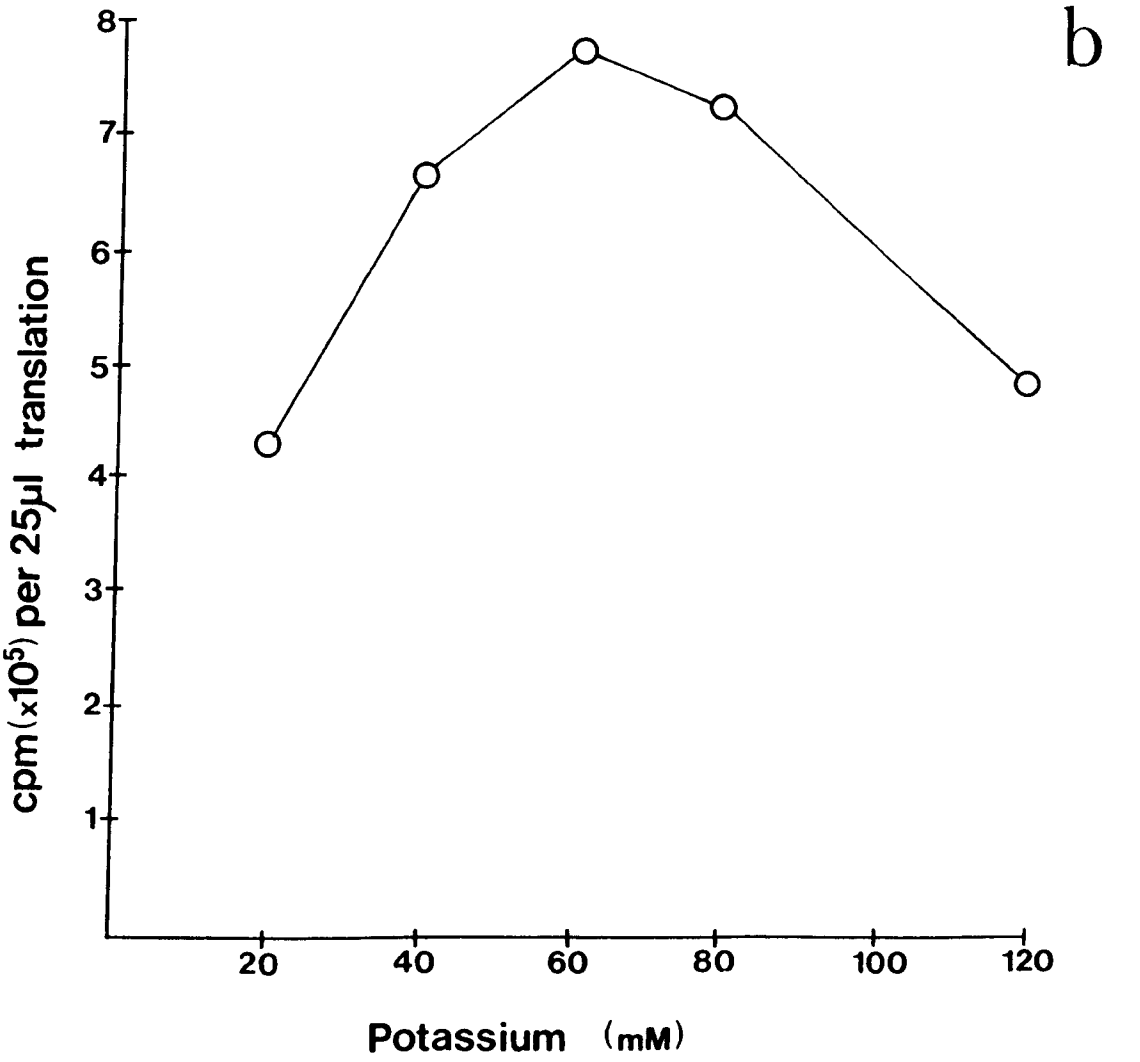


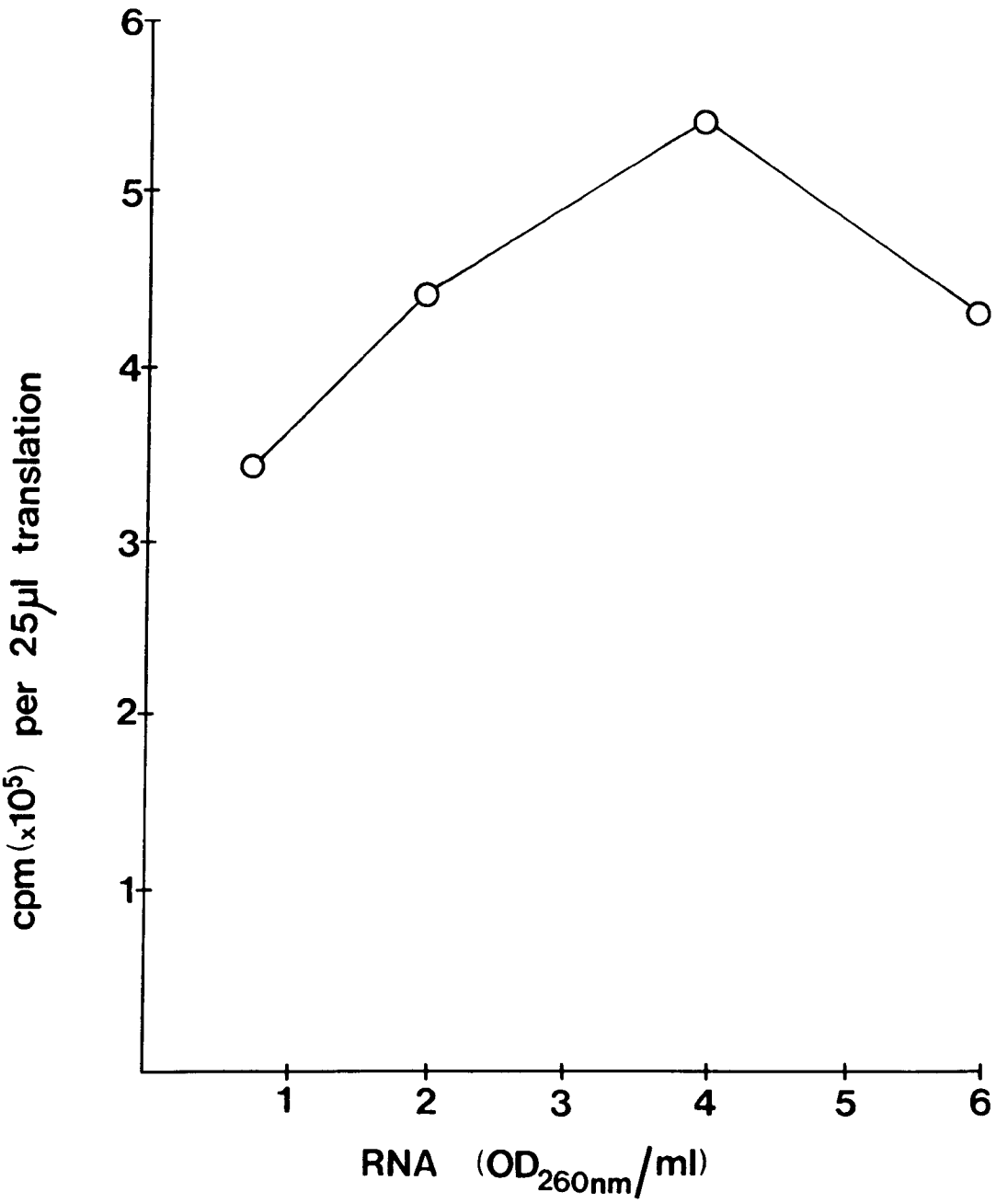
Fig. 26. Typical optimization of the wheatgerm translation system.

Translations were carried out in the presence of 4 OD U/ml rat brain RNA and varying concentrations of a) magnesium acetate (0.6-4mM) or b) potassium acetate (20-120mM) and RNA optimization was determined by translating varying amounts of c) rat brain RNA (0.7-6 OD U/ml) at optimal magnesium and potassium concentrations.


a
cpm (x10⁵) per 25µl translation
7
6
5
4
3
2
1
0·5
1
1·5
2
2·5
3
3·5
4
Magnesium (mM)


b
cpm (x10⁵) per 25µl translation
8
7
6
5
4
3
2
1
20
40
60
80
100
120
Potassium (mM)

C



case with CNPase.

RNA was extracted from both rat brain and rat brain subcellular fractions and translated at optimal efficiency in the wheatgerm translation system. CNPase and MBPs were immune-precipitated from translations containing identical numbers of TCA-precipitable cpm and the relative amounts of the polypeptides in translations programmed with total rat brain poly(A)$^+$RNA, free polysomal RNA and rough microsomal RNA were compared. CNPase I and CNPase II were shown to be synthesized on free polysomes (Figure 27, lane 3) and to share the same site of synthesis as MBPs (Figure 28). Small amounts of CNPases I and II and MBPs were detected in the RER lanes (Figure 27, lane 4 and Figure 28, lane 3 respectively) which was due to some free polysomal contamination in the rough microsomal fraction. Comparison of the size of the primary translation products produced by <u>in vitro</u> translation of total rat brain poly(A)$^+$RNA and those of the mature CNPase polypeptides found in purified ^{125}I-labelled rat brain myelin shows that CNPases I and II are not synthesized as precursors which are cleaved post-translationally (Figure 27, lanes 1 and 2). This is further support for the hypothesis that these polypeptides are synthesized on free polysomes.

iii. <u>Non-Enrichment of CNPase mRNA in Myelin-Derived RNA</u>

Colman et al. (1982) found that an RNA fraction could be extracted from purified rat brain myelin and that this RNA was enriched approximately 10-fold in MBP mRNA, relative to total rat brain mRNA. It was therefore of great interest to find out whether the message for another myelin protein which is also synthesized on free/

Fig. 27. Site of syntehsis of rat CNS CNPase. Poly $(A)^+$RNA from total brain (4 OD U/ml of translation mixture; lane 2) and total RNA from rat brain free polysomes (4 OD U/ml; lane 3), rough microsomes (2.7 OD U/ml; lane 4) and myelin (5.4 OD U/ml; lane 5) were used to programme a wheatgerm translation system. After translation, the samples were immune-precipitated (2×10^6 cpm per sample) with affinity purified anti-CNPase antibodies, electrophoresed and subjected to fluorography as described in Materials and Methods. The dried gel was exposed to X-ray film for 10 days at $-70^{\circ}C$. Lane 1 shows an autoradiogram of rat brain myelin (5μg) that had been iodinated using ^{125}I.

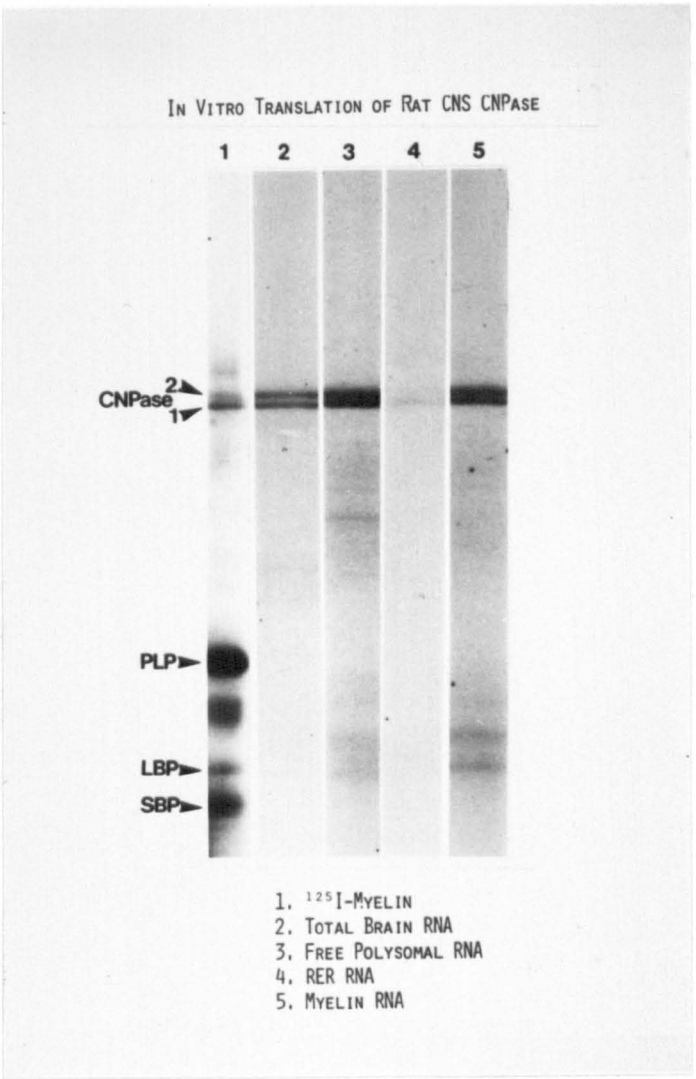
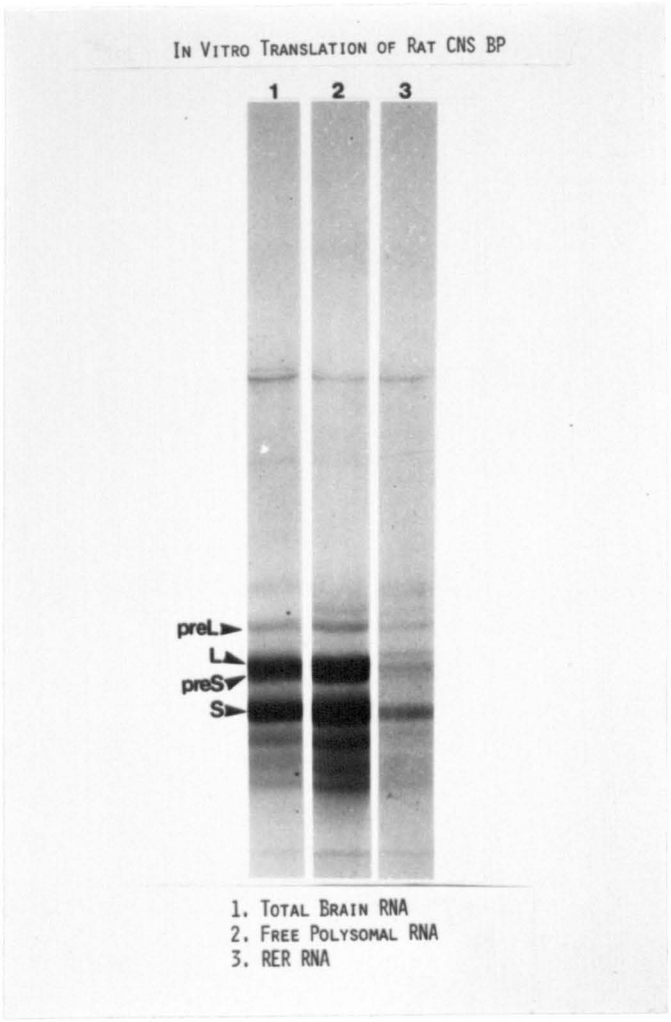
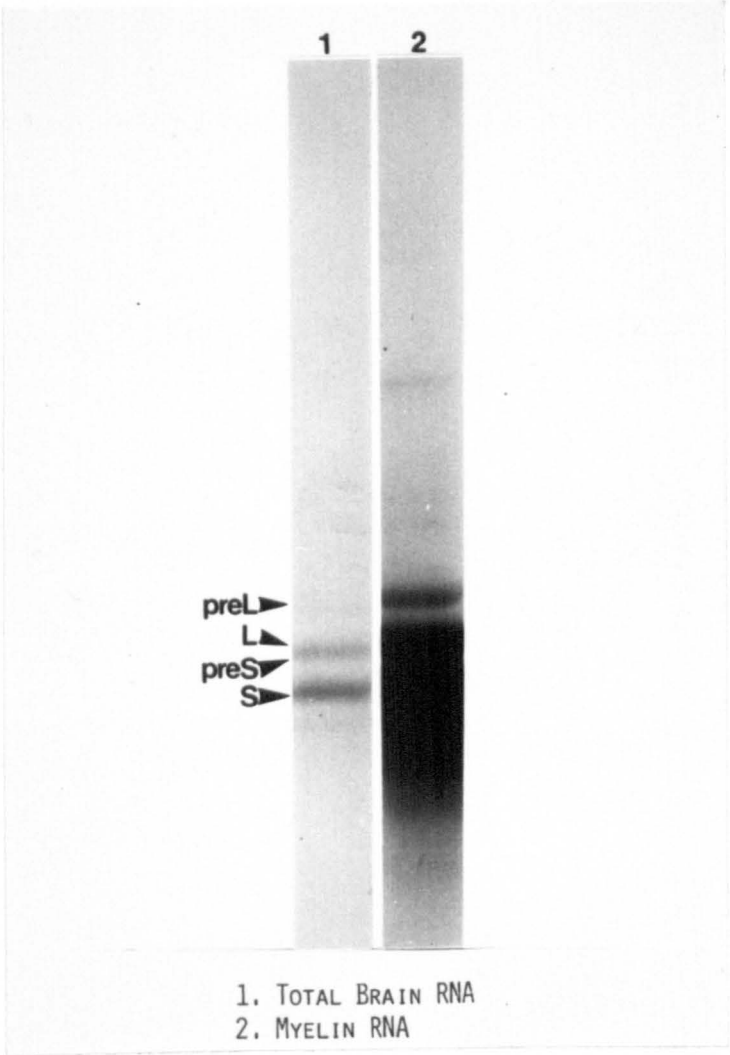

In Vitro Translation of Rat CNS CNPase
1
2
3
4
5
CNPase
2
1
PLP
LBP
SBP
1. 125I-Myelin
2. Total Brain RNA
3. Free Polysomal RNA
4. RER RNA
5. Myelin RNA

Fig. 28. Site of synthesis of rat CNS MBPs.
MBPs were translated, immune-precipitated with anti-MBP antibodies and detected by autoradiography as described in Figure 27. MBPs were translated using poly(A)$^+$RNA from total brain (lane 1), total free polysomal (lane 2) and rough microsomal (lane 3) RNA.

Fig. 29. The enrichment of MBP mRNAs associated with rat brain myelin.
Poly (A)$^+$RNA from total brain (4 OD U/ml; lane 1) and myelin-associated RNA (5.4 OD U/ml; lane 2) were translated and analysed by immunoprecipitation (2.5 x 10^6 cpm per sample) and autoradiography as described in Materials and Methods. After a 7 day exposure the fluorogram was placed over the dried gel to locate the relevant bands, which were excised and counted to determine the relative ratios of radioactivity in the total brain and myelin RNA MBP immunoprecipitates.


In Vitro Translation of Rat CNS BP
1
2
3
preL
L
preS
S
1. Total Brain RNA
2. Free Polysomal RNA
3. RER RNA


1
2
preL
L
preS
S
1. Total Brain RNA
2. Myelin RNA

free polysomes was also enriched in the myelin RNA fraction. Figure 27 (lanes 2 and 5) shows that when the CNPase polypeptides were immune-precipitated from translations containing the same amounts of TCA-precipitable radioactivity, there was no evidence of enrichment for CNPase message in the myelin RNA relative to total rat brain RNA whereas there was a pronounced enrichment of MBP message (Figure 29). The ratio of labelled CNPase synthesized by total brain mRNA to that produced from myelin-associated RNA was 1:1.3 as determined from the radioactivity contained in each CNPase lane (1832cpm and 2346cpm respectively). Myelin-associated mRNA coding for MBPs was enriched 13-fold over that for total brain mRNA (93536cpm and 7276cpm respectively).

iv. Kinetics of Incorporation of Newly-Synthesized CNPase into CNS myelin

A crude PLP antiserum was purified and samples from various stages of the purification were tested in immunoblots of rat brain myelin (Figure 30). The crude antiserum (lane 1) whilst containing PLP antibodies also contained antibodies that recognized a series of higher molecular weight proteins (~60-90kDa) and also several lower molecular weight proteins thought to be MBPs. Initial purification was carried out by affinity chromatography using column to which purified PLP had been covalently bound. This purification step removed antibodies recognizing the higher molecular weight proteins however, contamination by the presumed MBP antibodies was still apparent (lane 2). The fact that these antibodies had bound to the PLP column suggested that there was some MBPs that had been purified along with PLP, a likely explanation since the pre-large MBP runs very/

Fig. 30. Purification of PLP antiserum.

The crude PLP antiserum was purified as outlined in Materials and Methods. Rat brain myelin immunoblots were obtained using the PLP antiserum at various stages in the purification. Lane 1 shows the crude PLP antiserum (diluted 1:200); the same antiserum after purification via either a PLP (1:25; lane 2) or a MBP (1:5; lane 3) affinity column. Lane 4 shows the positions of the MBPs when the same blot was immunostained using MBP antiserum (1:1000).

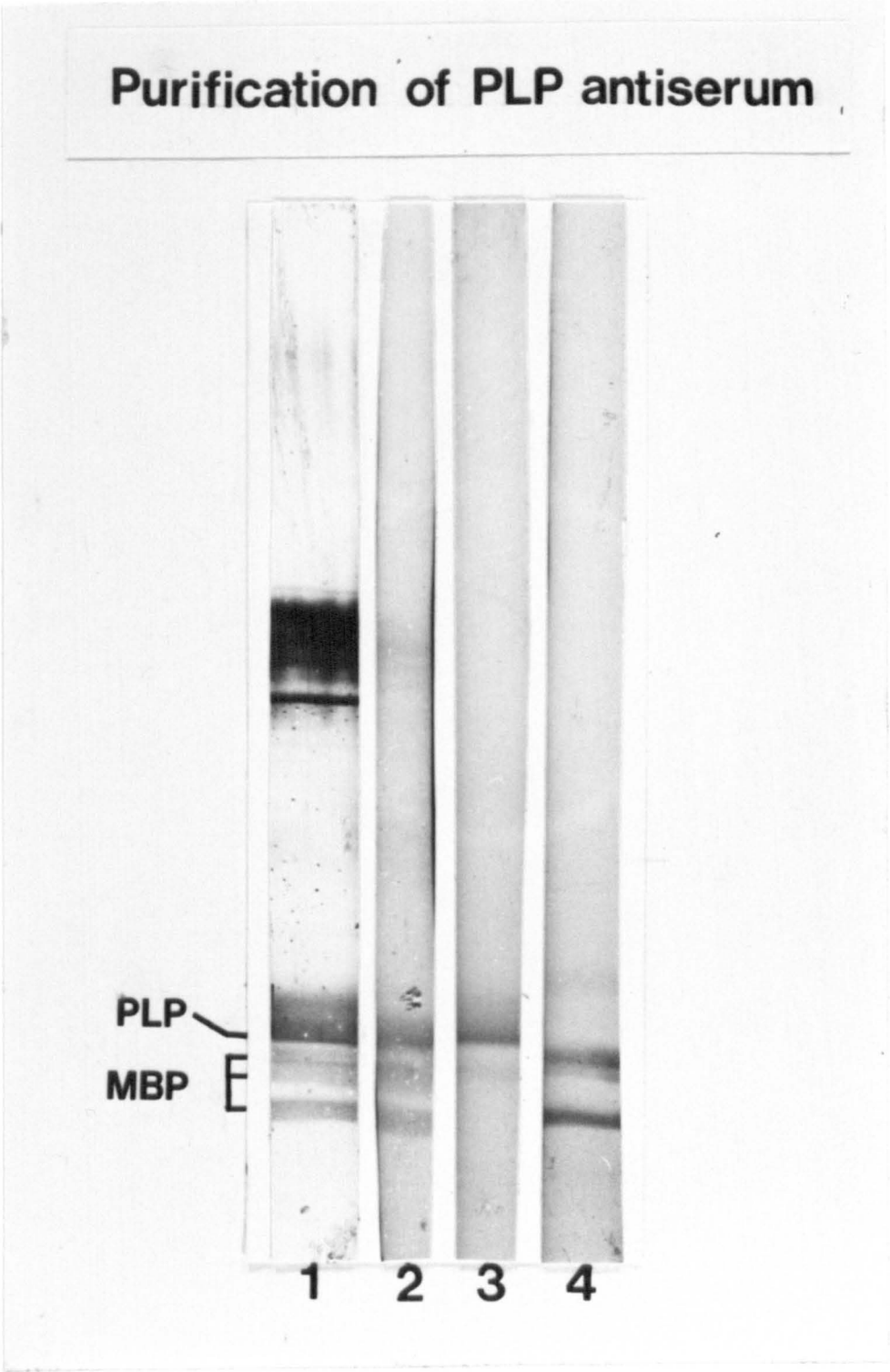

Purification of PLP antiserum
PLP
MBP
1
2
3
4

Fig. 31. Kinetics of incorporation of newly synthesized MBP, CNP and PLP into myelin. CNS myelin was isolated from rat pups that had received intracranial injections of ^{35}S-methionine (see Materials and Methods) and were allowed to survive for the designated times. Immunoprecipitates were obtained with anti-MBP antibodies (lanes a-c), anti-CNPase antibodies (lanes d-f) and anti-PLP antibodies (lanes g-i). The rapid appearance of MBPs and CNPase is in marked contrast to the delayed accumulation of labelled PLP, which is first detectable in myelin at 30 min post-injection.


MBP
CNP
PLP
2' 5' 30'
2' 5' 30'
2' 5' 30'
a b c d e f g h i

very close to PLP on SDS-PAGE. Lane 3 shows the results of passing the partly purified PLP antiserum through a column which had MBPs covalently bound to it. The contaminating MBP antibodies had been removed and the resulting affinity purified antiserum recognized only PLP. As a guide to the positions of the MBPs rat brain myelin was also immunostained with a polyclonal anti-MBP serum (lane 4).

The kinetics of incorporation of CNPase into myelin were determined after intracranial injection of ^{35}S-methionine and newly-synthesized CNPase was immune-precipitated from purified rat brain myelin. Newly-synthesized MBPs and PLP were also immune-precipitated from purified rat brain myelin. Figure 31 shows that newly-synthesized CNPase (lanes d-f) is incorporated into myelin with a time course similar to that of the MBPs (lanes a-c). This is in marked contrast to the delayed accumulation of PLP, which is first detectable in myelin at 30min post-injection. PLP has been shown to be synthesized on bound polysomes (Colman et al., 1982) and the kinetics of incorporation into myelin are consistent with a mechanism whereby the nascent protein is transported from its site of synthesis to the growing myelin membrane via the Golgi apparatus.

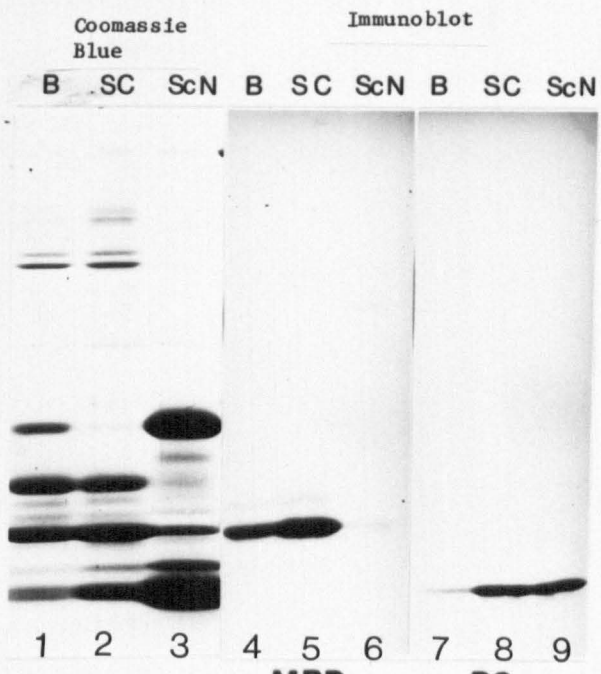
3.3 BIOSYNTHESIS OF P2

i. Immunological Identification of P2 in Rabbit CNS Myelin

Purified rabbit brain, spinal cord and sciatic myelin samples of equal protein content were electrophoresed and stained with Coomassie blue. Figure 32, lanes 1-3 shows an intensely staining band in the region corresponding to P2 in both spinal cord and sciatic myelin. Western blotting of identical samples using a polyclonal/

Fig. 32. Presence of P2 in rabbit spinal cord myelin. Rabbit brain, spinal cord and sciatic myelin was prepared according to the general method (see Materials and Methods) and samples of each (30μg protein for Coomassie blue staining and 2μg protein for immunoblots) were anlysed by SDS-PAGE in 5-15% gradient gels (lanes 1-3) or immunostained with anti-MBP antibodies (lanes 4-6) and anti-P2 antibodies (lanes 7-9).

P2 protein is a component of rabbit CNS myelin.



B - Brain
SC - Spinal cord
ScN - Sciatic nerve

polyclonal antibody raised against bovine P2 demonstrated conclusively that P2 is present in large amounts in both rabbit spinal cord and sciatic myelin. In contrast, the P2 antiserum revealed only a trace amount of P2 in rabbit brain (Figure 32, lanes 7-9). From the Coomassie blue stained gel the apparent molecular weight of P2 was calculated to be 14kDa which is in agreement with a previously published report (Kitamura et al., 1980).

Western blots using an antiserum directed against rat MBPs were also performed (Figure 32, lanes 4-6) to determine if the antiserum cross-reacted with the rabbit proteins and also as a positive control for the blots since it is known that MBPs, while components of the PNS myelin of all species studied so far, are present in much reduced amounts (Matthieu et al., 1980).

Only a very slight trace of MBP was observed in lane 6 and this is due to the reduction in the amount of antigen electro-transferred, necessary to avoid overstaining of the brain and spinal cord samples (lanes 4 and 5). Positive identification of P2 in abundant amounts in rabbit spinal cord myelin relative to the spinal cord myelin of bovine and rat (data not shown) led to the decision to use this source of P2 for all future experiments involving this protein.

ii. Site of Synthesis of P2

RNA was extracted from rabbit spinal cord and rabbit spinal cord free and bound polysomes and used to programme a wheatgerm translation system optimized for rabbit spinal cord total RNA. P2 was immune-precipitated from translations containing equal amounts of TCA-precipitable cpm and the relative amounts of the polypeptide in/

in each sample compared. Figure 33 clearly demonstrates that the P2 protein is synthesized on free polysomes, a situation akin to that previously found for MBPs and CNPase. The small amount of P2 observable in the RER lane (lane 3) is almost certainly due to free polysomal contamination in this fraction.

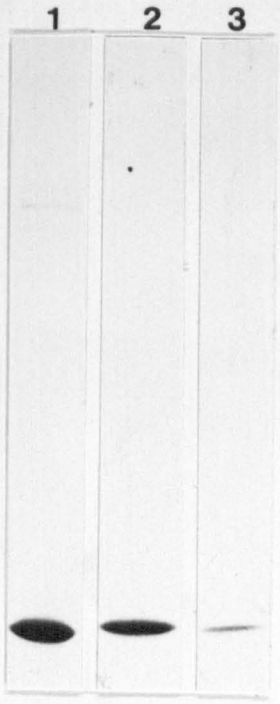
iii. Non-Enrichment of Myelin-Derived P2 mRNA

Like the MBPs, P2 is a highly basic protein of relatively low molecular weight and it was therefore decided to establish whether free polysomes synthesizing P2 were segregated in the myelin sheath (as is the case for MBPs) or whether their site of synthesis and incorporation into the growing myelin membrane followed more closely that of CNPase. Figure 34 demonstrates that when P2 was immune-precipitated from translations containing equivalent amounts of TCA-precipitable cpm there was no enrichment of P2 mRNA in the myelin-associated free polysomes. The ratio of labelled P2 translated from total spinal cord mRNA to that from myelin-associated mRNA was 1:1.1 as calculated from the radioactivity associated with each band (3600cpm and 4119cpm respectively; lanes 3 and 4). In contrast, a 7-fold enrichment of MBP mRNA was observed from RNA isolated from myelin-associated polysomes (28300cpm as against 4232cpm in the total spinal cord mRNA P2 band; lanes 1 and 2). Thus enrichment of mRNA synthesizing MBPs would appear to be a general phenomenon since it has been observed in myelin isolated from different parts of the CNS using two different species of animals.

Fig. 33. Site of synthesis of rabbit spinal cord P2. Poly(A)$^+$RNA from rabbit spinal cord (2.7 OD U/ml of translation mixture; lane 1) and RNA from rabbit spinal cord free polysomes (2.7 OD U/ml; lane 2) and rough microsomes (5.3 OD U/ml; lane 3) were used to programme a wheatgerm translation system. After translation, the samples were immune-precipitated (3 x 10^5cpm per sample) with anti-P2 antibodies, electrophoresed, fluorographed and the dried gel exposed to X-ray film for 7 days at -70^{o}C.

Fig. 34. The non-enrichment of P2 mRNA associated with rabbit spinal cord myelin.
Rabbit spinal cord poly(A)$^+$RNA (2.7 OD U/ml) and rabbit spinal cord myelin-associated RNA (2.7 OD U/ml) were translated and immune-precipitates were prepared (3 x 10^5cpm per sample) with anti-MBP antibodies (lanes 1 and 2) and anti-P2 antibodies (lanes 3 and 4) and analysed by autoradiography. After a 7 day exposure the fluorogram was placed over the dried gel to locate the relevant bands, which were excised and counted to determine the relative ratios of radioactivity in the spinal cord total and myelin RNA MBP and P2 immunoprecipitates.

In Vitro Translation Of Rabbit CNS P2



1. Total Sp.Cord RNA
2. Free Polysomal RNA
3. RER RNA

mRNA associated with rabbit CNS myelin is not enriched in P2 mRNA.

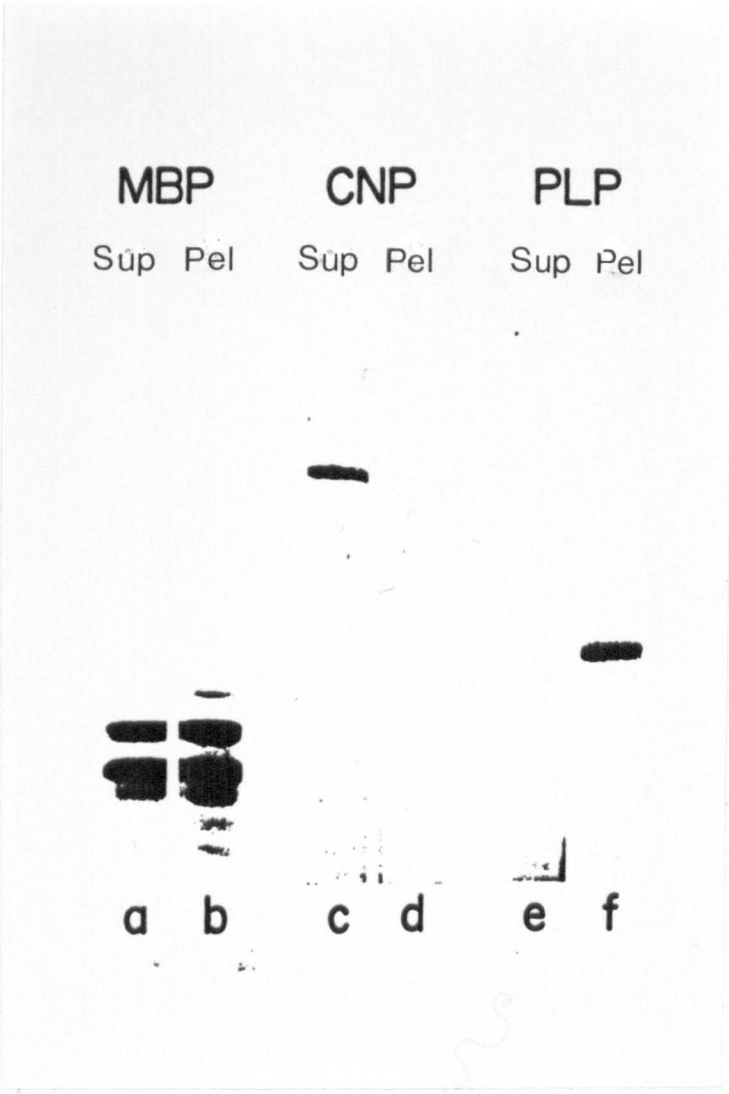
3.4 MEMBRANE ASSOCIATION OF NEWLY-SYNTHESIZED MYELIN PROTEINS

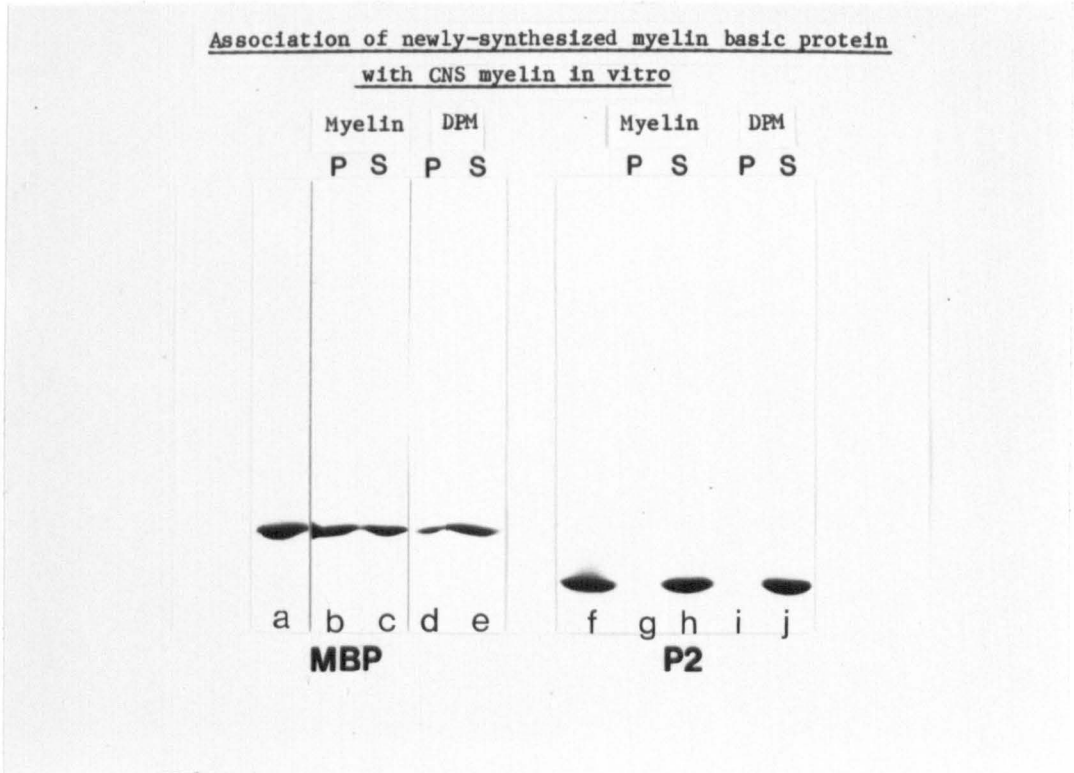
The interaction of *in vitro* synthesized MBPs, CNPase and PLP with microsomal membranes was examined in wheatgerm translations that had been programmed with rat brain poly(A)$^+$RNA, and to which dog pancreatic microsomes and signal recognition particle (SRP) had been added at the start of the translations (Figure 35). When MBPs, CNPase and PLP were immune-precipitated from the supernatant (lanes a, c and e) and pellet (lanes b, d and f) fractions it was observed that all of the newly synthesized PLP could be recovered with the sedimentable microsomes (lanes e and f) as predicted if this major intrinsic protein was undergoing cotranslational insertion during its synthesis. The reverse was found for the extrinsic protein CNPase where all of the newly synthesized product was found in the supernatants (lanes c and d) indicating that this protein has a specific affinity for the myelin membrane, its natural target membrane *in vivo*. The situation for MBPs was found to be somewhat different to that of CNPase however. Even though MBPs, like CNPase, are extrinsic membrane proteins synthesized on free polysomes, a large proportion (~50%) of the *in vitro* synthesized MBPs were recovered with the microsomes (lanes a and b). These data demonstrate striking differences in the behaviour of each of the *in vitro* synthesized polypeptides with the added membranes. The binding of the MBPs to microsomes derived from non-brain tissue suggests that these highly charged polypeptides will readily and non-specifically associate with acidic lipids or phospholipid vesicles irrespective of their origin.

In order to further test this hypothesis MBPs and P2 (like

Fig. 35. The association of newly synthesized MBP, CNPase and PLP with membranes in vitro. Wheatgerm translations were programmed with rat brain poly(A)$^+$RNA (4 OD U/ml) and were incubated at the start of the translations with dog pancreatic microsomes (DPM) and signal recognition particle (SRP). Supernatants (lanes a, c and e) and pellets (lanes b, d and f) were prepared and immunoprecipitated using MBP, CNPase and PLP antisera.

Fig. 36. Membrane affinity of newly synthesized MBP and P2.
Wheatgerm translations were programmed with rabbit spinal cord poly(A)$^+$RNA (2.7 OD U/ml) and were incubated post-translationally with either rabbit spinal cord myelin or DPM. Myelin (lanes b and g) and DPM (lanes d and i) pellets and myelin (lanes c and h) and DPM (lanes e and j) supernatants were prepared and immunoprecipitated using MBP and P2 antisera. Control immunoprecipitates that had been incubated in the absence of added membranes are also shown (lanes a and f).


MBP
CNP
PLP
Sup Pel
Sup Pel
Sup Pel
a b c d e f


Association of newly-synthesized myelin basic protein with CNS myelin in vitro
Myelin
DPM
Myelin
DPM
P S P S
P S P S
a b c d e
f g h i j
MBP
P2

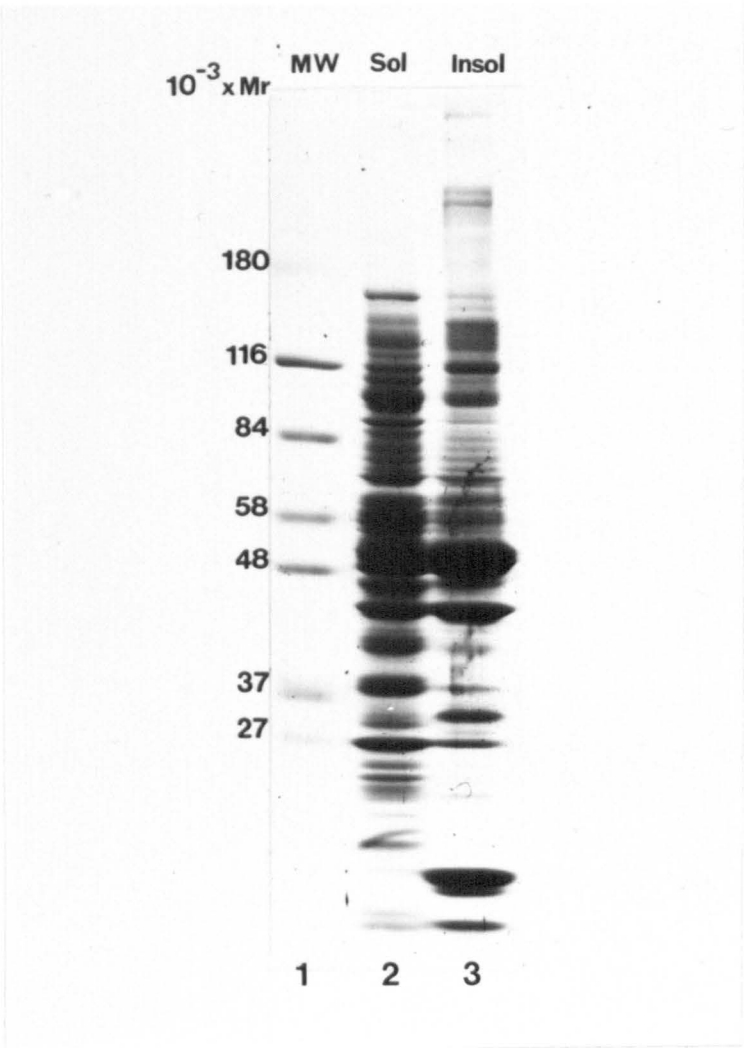
MBPs a highly charged protein of low molecular weight) were immune-precipitated from translations that had been programmed with rabbit spinal cord RNA and incubated post-translationally with either purified rabbit spinal cord myelin or DPM (Figure 36). The non-specific association of MBP with microsomes is seen in lanes d and e where a substantial amount of the polypeptides (~20%) are found associated with the microsomes. The distribution of MBPs observed when myelin was included in the incubation (lanes b and c) suggests that these proteins have a greater affinity for their natural membrane since approximately 50% was found associated with the myelin pellet. P2 on the other hand was always recovered in the supernatant irrespective of the source of the membranes (lanes g-j) and suggests that even though it is a myelin-specific protein its association with the myelin membrane is very weak. Lanes a and f are immune-precipitated MBPs and P2 from translations that had no membranes added and were included as positive controls.

3.5 CYTOSKELETAL CHARACTERIZATION

i. The Oligodendrocyte Cytoskeleton

Single-cell suspensions were prepared from freshly dissected rat brain and Triton X-100-soluble and -insoluble (cytoskeletal) fractions isolated as described in the Materials and Methods section. Equal volumes from each fraction were electrophoresed and either stained with Coomassie blue or transferred to nitrocellulose and immunoblotted. Figure 37 shows Coomassie blue staining of the fractions where it is apparent that the bulk of the proteins are isolated with the detergent-soluble fraction (lane 1). Most of the total/

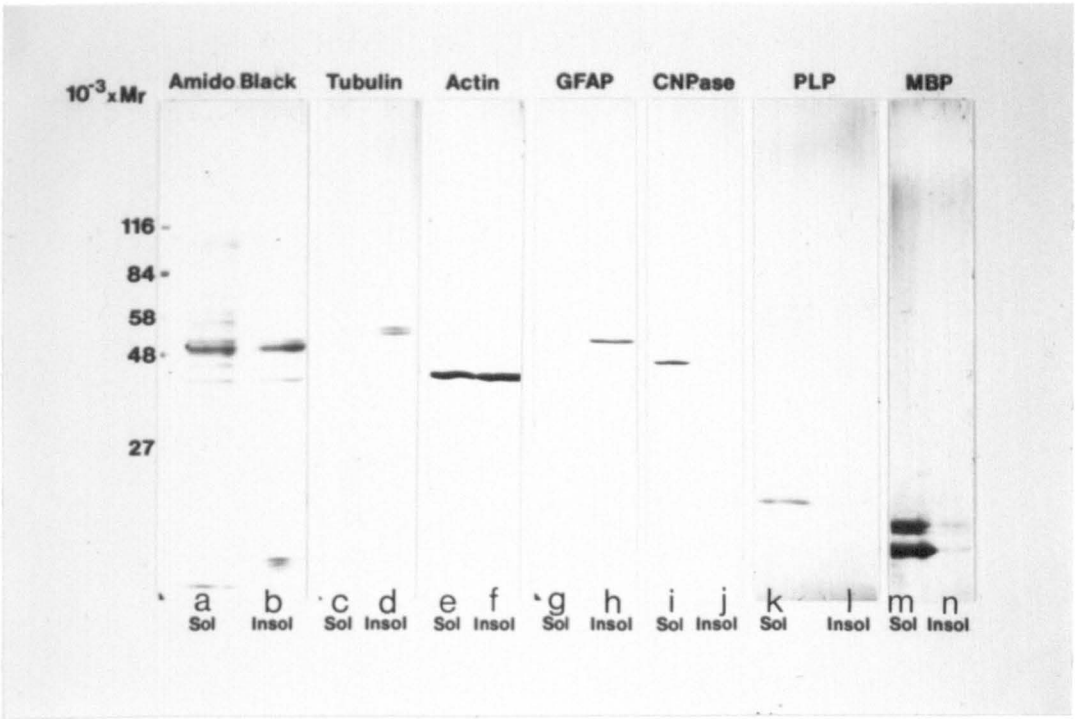
Fig. 37. The protein composition of rat brain detergent-soluble and -insoluble fractions. Single-cell suspensions were prepared from rat brain and the Triton X-100-soluble and -insoluble fractions isolated as described in Materials and Methods. Equal volumes from each fraction were analysed by SDS-PAGE in a 5-15% gradient gel and lanes 2 and 3 show the Coomassie blue stained pattern of proteins in the Triton X-100 -soluble and -insoluble fractions respectively. Lane 1 consists of Coomassie blue stained molecular weight markers (α_2-macroglobulin, 180kDa; B-galactosidase, 116kDa; fructose-6-phosphate kinase, 84kDa; pyruvate kinase, 58kDa; fumarase, 48kDa; lactate dehydrogenase, 37kDa; triosephosphate isomerase, 27kDa).


MW
Sol
Insol
10^{-3} x Mr
180
116
84
58
48
37
27
1
2
3

total protein (70%) was recovered in this fraction, the remaining 30% being associated with the cytoskeletal fraction. Prolonged extraction (up to 5 min) with the CSK buffer did not alter the protein distribution (data not shown). This protein distribution is in agreement with previous reports (Howe and Hershey, 1984; Fey et al., 1986) although a thorough characterization of the individual proteins contained within the fractions was not carried out by these workers. Of interest in Figure 37 is the enrichment of proteins in the tubulin region of the gel contained within the cytoskeletal fraction (lane 2). These preliminary results involving Coomassie blue staining were encouraging since it is known that microtubules are extremely susceptable to depolymerisation during isolation (Vallee and Collins, 1986).

A thorough characterization of the individual protein distribution within the soluble and cytoskeletal fractions was carried out by Western blotting of equal proportions of each sample (Figure 38). Proteins from the three types of cytoskeletal elements are represented in lanes c-h comprising tubulin (microtubules), actin (microfilaments) and glial fibrillary acidic protein (GFAP), an astrocytic intermediate filament marker. That the detergent-insoluble residue represents an authentic cytoskeletal fraction is shown by the enrichment of tubulin (100%), actin (approx. 60%) and GFAP (100%) found within this fraction. CNPase (lanes i and j) was found predominantly in the detergent-soluble fraction (approx. 90%) as was PLP (100%, lanes k and l) and all the four forms of MBPs (approx. 90%, lanes m and n). There was no evidence of a selective enrichment of a particular MBP species associated with the cytoskeleton. This was also true for both forms of/

Fig. 38. Protein distribution in detergent-soluble and -insoluble fractions derived from rat brain. Triton X-100-soluble and -insoluble fractions were prepared from single-cell suspensions of rat brain and equal volumes of each fraction electrophoresed in 5-15% gradient gels and the proteins electro-transferred to nitrocellulose paper. Immunoblots were obtained (lanes c-n) using a monoclonal anti-tubulin antibody (1/100 dilution; Serotec), anti-γ-actin (1:2000; Dr C Bulinski, UCLA), anti-GFAP (1:2000; Dr E Bock, University of Copenhagen), affinity purified anti-CNPase (1:500), anti-MBP (1:1000) and anti-PLP (1:4) polyclonal antibodies. Lanes a and b show a blot that had been stained with Amido black and contains molecular weight markers as described in the legend to Figure 37.


10-3 x Mr
Amido Black
Tubulin
Actin
GFAP
CNPase
PLP
MBP
116
84
58
48
27
a Sol
b Insol
c Sol
d Insol
e Sol
f Insol
g Sol
h Insol
i Sol
j Insol
k Sol
l Insol
m Sol
n Insol

of CNPase.

ii. The Myelin-Associated Cytoskeleton

Triton X-100-soluble and -insoluble (cytoskeletal) fractions were prepared from purified rat brain myelin and subjected to protein distribution analysis as for intact cells. Once the optimum extraction conditions for the myelin-associated cytoskeleton had been established (Figure 39) the pellet was viewed under the EM and the protein distribution assessed. The electron micrograph (Figure 40) shows the presence of filamentous material along with small amorphous particles. Thus the detergent-insoluble pellet consists of material with a definite macromolecular structure and is not simply the result of the sedimentation of protein aggregates. Figure 41 (lanes a-d) shows that all of the tubulin and approximately 40% of actin was found in the cytoskeletal fraction. Since the tubulin was all in the cytoskeleton it was decided that the detergent-insoluble residue was a true reflection of the cytoskeleton. The fact that a larger amount of actin was found in the detergent-soluble fraction was deemed unimportant since in all experiments involving cytoskeletal purifications a substantial amount of unpolymerised soluble actin is always observed.

The distribution of the myelin-specific proteins is interesting. Most of the CNPase I was found in the detergent-soluble fraction (approx. 70%) however a considerable enrichment (approaching 100%) of CNPase II was observed to be associated with the cytoskeleton. This appears to be peculiar to the myelin-associated cytoskeleton since it is not seen in the cytoskeletal extracts from oligodendrocytes (Figure 38). PLP, by contrast,/

Fig. 39. Protein composition of rat brain myelin and detergent-soluble and-insoluble fractions. Rat brain myelin was extracted with Triton X-100 as described in Materials and Methods. Rat brain myelin (30μg protein; lane 1) and equal volumes of the detergent-soluble and -insoluble fractions (lanes 2 and 3 respectively) were analysed by SDS-PAGE in 5-15% gradient gels and the proteins stained with Coomassie blue.

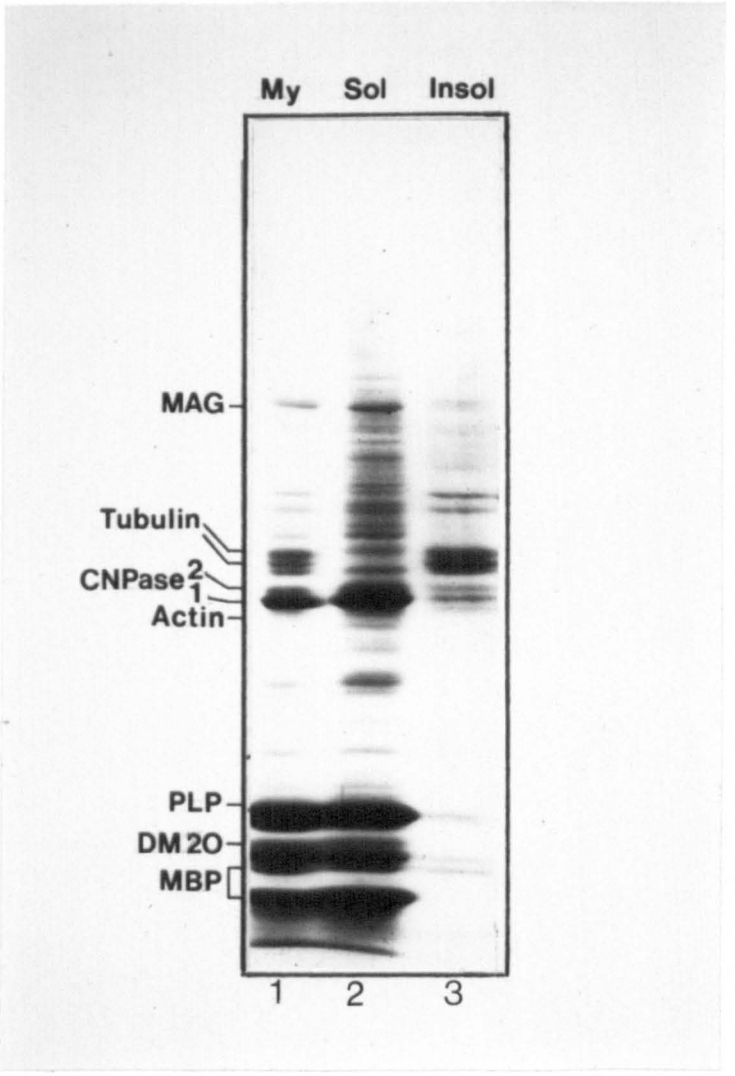

My
Sol
Insol
MAG
Tubulin
CNPase 2
1
Actin
PLP
DM 20
MBP
1
2
3

Fig. 40. Electron micrograph of the detergent-insoluble pellet derived from purified rat brain myelin.

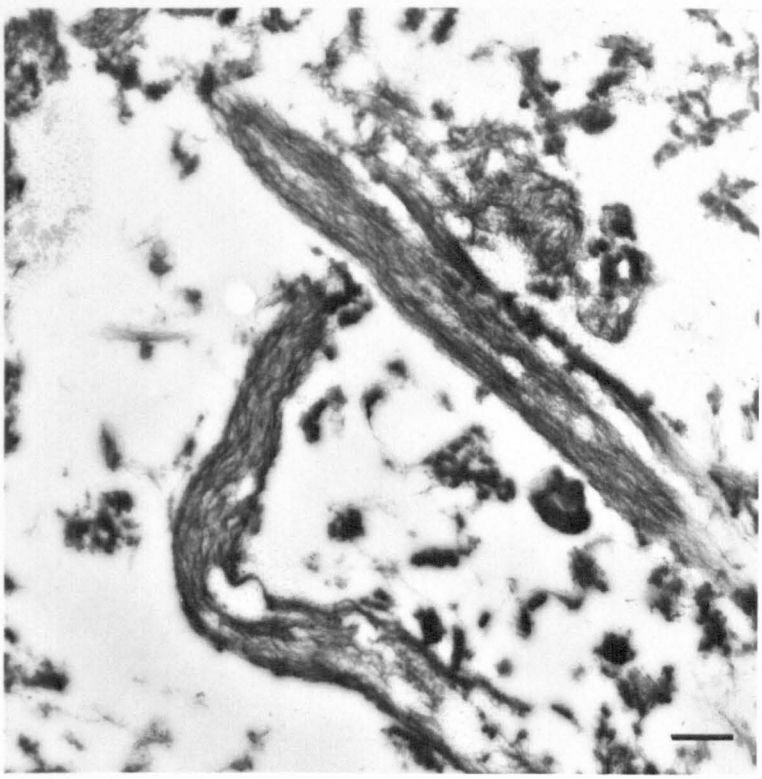
Rat brain myelin was extracted with Triton X-100 and the detergent-soluble and -insoluble fractions isolated. Electron micrographs were obtained from ultra-thin sections of the detergent-insoluble pellet as described in Materials and Methods.

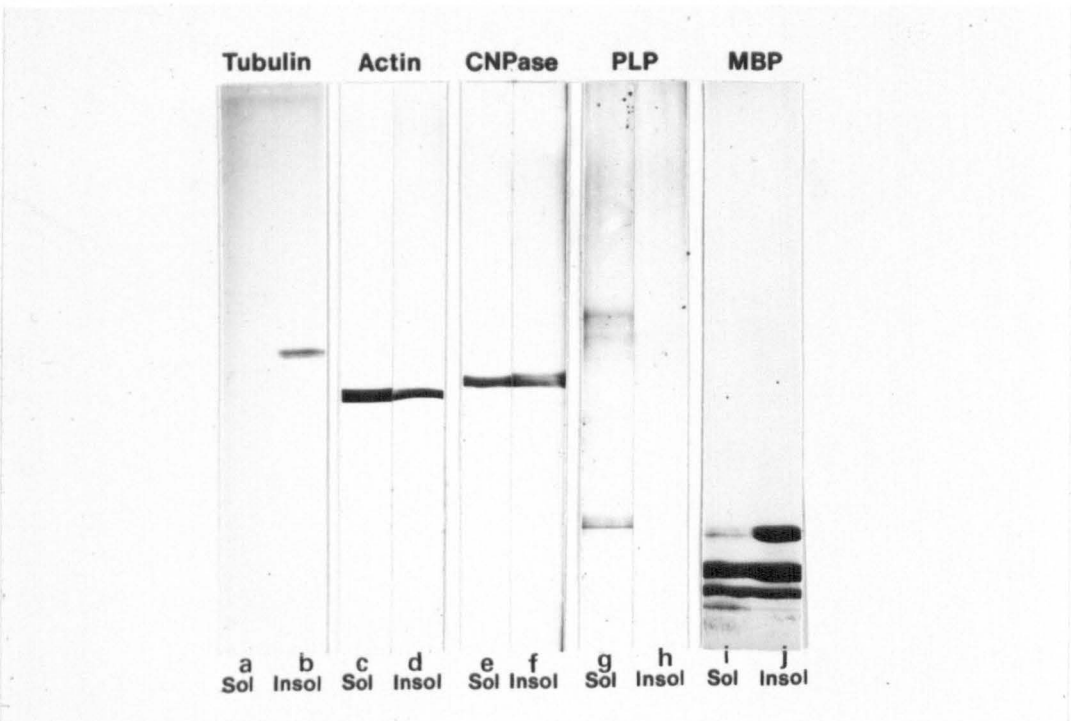
x10,000 magnification.

Scale bar: 1 μm x 10,000

Fig. 41. Protein distribution in the detergent-soluble and -insoluble fractions derived from rat brain myelin.

Rat brain myelin was extracted with Triton X-100 as described in Materials and Methods and equal volumes of the detergent-soluble and -insoluble fractions electrophoresed in 5-15% gradient gels and the proteins electro-transferred to nitrocellulose paper. Immunoblots were obtained (lanes a-j) using anti-tubulin, anti-actin, anti-CNPase, anti-PLP and anti-MBP antibodies at the dilutions described in Figure 38.




Tubulin
Actin
CNPase
PLP
MBP
a Sol
b Insol
c Sol
d Insol
e Sol
f Insol
g Sol
h Insol
i Sol
j Insol

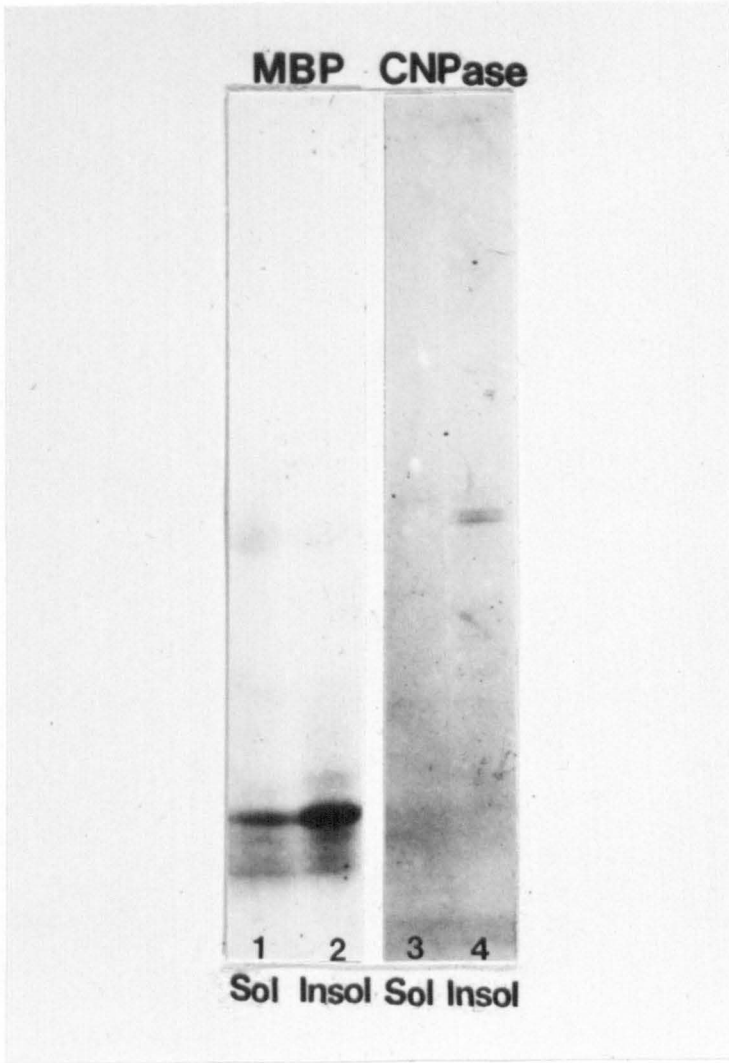
contrast, was completely extracted as would be expected of an intrinsic membrane protein. The distribution of PLP agrees entirely with that found earlier from oligodendrocytes (Figure 38). The distribution of MBPs within the soluble and cytoskeletal fractions was found to be approximately equal. However, of considerable interest was the finding that the pre-large (21.5kDa) MBP was found almost exclusively with the cytoskeleton. The other MBP species did not show this preferential association with the cytoskeleton.

3.6 CYTOSKELETAL INVOLVEMENT IN MBP AND CNPase SYNTHESIS

Soluble and cytoskeletal fractions were isolated from single-cell suspensions of rat brain as described in Materials and Methods (Section 2.6 i). RNA was extracted from these fractions and translated *in vitro* in a wheatgerm translation system. CNPases and MBPs were immune-precipitated from translations containing identical numbers of TCA-precipitable cpm and the relative amounts of the proteins in translations programmed with detergent-soluble fraction RNA and -insoluble (cytoskeletal) fraction RNA were compared. Figure 42 clearly shows an increased synthesis of CNPases and MBPs from RNA recovered with the cytoskeleton. The enrichment for CNPases and MBPs was not quantitated but was estimated to be at least 5-fold over that for the same proteins translated using RNA from the detergent-soluble fraction.

The data suggests that the majority of mRNAs coding for these proteins are associated with the cytoskeleton during the very active period of myelination (ie myelin protein synthesis) from which the single-cell suspensions were derived. Unfortunately, due to lack of material/

Fig. 42. The association of MBP and CNPase mRNA with the detergent-insoluble (cytoskeletal) pellet. Single-cell suspensions from rat brain were extracted with Triton X-100 as described in Materials and Methods and the RNA extracted from the detergent-soluble (1.7 OD U/ml) and -insoluble (2.7 OD U/ml) fractions used to programme a wheatgerm translation system. After translation, the samples were immune-precipitated (4.5×10^5 cpm per sample) using anti-MBP (lanes 1 and 2) or affinity purified anti-CNPase (lanes 3 and 4) antibodies.


MBP
CNPase
1
2
3
4
Sol
Insol
Sol
Insol

material attempts to try and determine the distribution of total poly(A)$^+$RNA between the detergent-soluble and cytoskeletal fractions proved unsuccessful.

3.7 LIPID ANALYSIS OF THE MYELIN-ASSOCIATED CYTOSKELETON

Preliminary experiments were carried out to determine if there were any residual lipid in the myelin-associated cytoskeleton and if so, were any particular lipids enriched in this fraction. The composition of the individual lipids contained in rat brain myelin and the Triton X-100-insoluble pellet were quantitated and the results are shown in Table 4. As in the case of the proteins, the associated lipids represent a subset of the total myelin membrane lipid. Most of the phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol are solubilized by the detergent while essentially all of the sphingomyelin remains associated with the detergent-insoluble pellet. The phosphatidylcholine and cholesterol content of the detergent-insoluble pellet was found to resemble that of the myelin membrane. This lipid content and composition between the membrane and the matrix is similar to that found in other systems (Davies et al., 1984; Apgar et al., 1985). A striking feature of the lipid analysis is the considerable enrichment of cerebrosides (other than sulphatide which essentially remained the same) found associated with the detergent-insoluble pellet. Within this cerebroside enrichment an increase in hydroxy cerebroside content over that in myelin was observed and conversely, the non-hydroxy cerebroside content of the matrix was less than that of myelin./

Table 4 Lipid composition of the Triton X-100 insoluble cytoskeleton residue compared with that of myelin

Component	Myelin	Triton X-100 pellet
Total lipid (μmol/mg protein)	2.46	0.73
Cholesterol (mol % total lipid)	39.8	38.4
Cerebroside (mol % total lipid)	11.4	35.6
Non-hydroxy cerebroside (mol % cerebroside)	32	23
Hydroxy cerebroside (mol % cerebroside)	68	77
Sulphatide (mol % total lipid)	0.13	0.08
Phospholipid (mol % total lipid)	1.07	0.11
Phosphatidylethanolamine (mol % phospholipid)	45	13
Phosphatidylcholine (mol % phospholipid)	32	39
Phosphatidylserine (mol % phospholipid)	13	ND
Phosphatidylinositol (mol % phospholipid)	4	ND
Sphingomyelin (mol % phospholipid)	6	48

All values are the means of measurements made on at least three different preparations. ND: not detected.

myelin. In order to ensure that the lipid extracted from the cytoskeletal fraction was genuinely associated with the cytoskeletal proteins, purified rat brain myelin and the detergent insoluble pellet were subjected to continuous sucrose density gradient centrifugation. Measurements of the OD_{280} of the myelin and cytoskeletal fractions revealed two distinct protein peaks (Figure 43) at 0.64M sucrose and approximately 0.98M sucrose. The protein fraction peaking at 0.64M sucrose was taken to be that of myelin and is in agreement with previously published results (Waehneldt, 1978). The denser protein peak (ranging from approximately 0.85-1.1M sucrose) represented the purified myelin-associated cytoskeleton. The results from this experiment also indicated that both the myelin and myelin-associated cytoskeleton fractions were basically homogeneous since the vast bulk of each sample was obtained throughout a relatively narrow sucrose density range.

The identity of the fractions obtained from these experiments was characterized by subjecting equal portions of the fractions to SDS-PAGE and Coomassie blue staining. Figure 44 clearly shows that the peak corresponding to fraction 10 was authentic rat brain myelin since the protein profile observed is very similar to that seen in SDS-PAGE profiles of purified rat brain myelin (see Figure 21). That the second denser peak corresponds to the myelin-associated cytoskeleton is shown in the lower part of Figure 44. A considerable enrichment in the cytoskeletal protein tubulin was observed, less so with actin. By Coomassie blue staining, fraction 24 was seen to contain most protein agreeing well with the OD_{280} profile (Figure 43). Also of interest was the presence of residual, tightly/

Fig. 43. Sucrose density gradient profile of rat brain myelin and the detergent-insoluble fraction derived from it.

Rat brain myelin (2mg protein) and Triton X-100-insoluble pellet (from 10mg myelin protein) were layered on top of 0.3-1.2M sucrose gradients and centrifuged for 16h at 50,000g. Fractions (0.5ml) were collected and 50µl aliquots of each analysed for protein content (OD_{280}). Rat brain myelin (O—O); detergent-insoluble pellet (●—●); sucrose concentration (□—□).

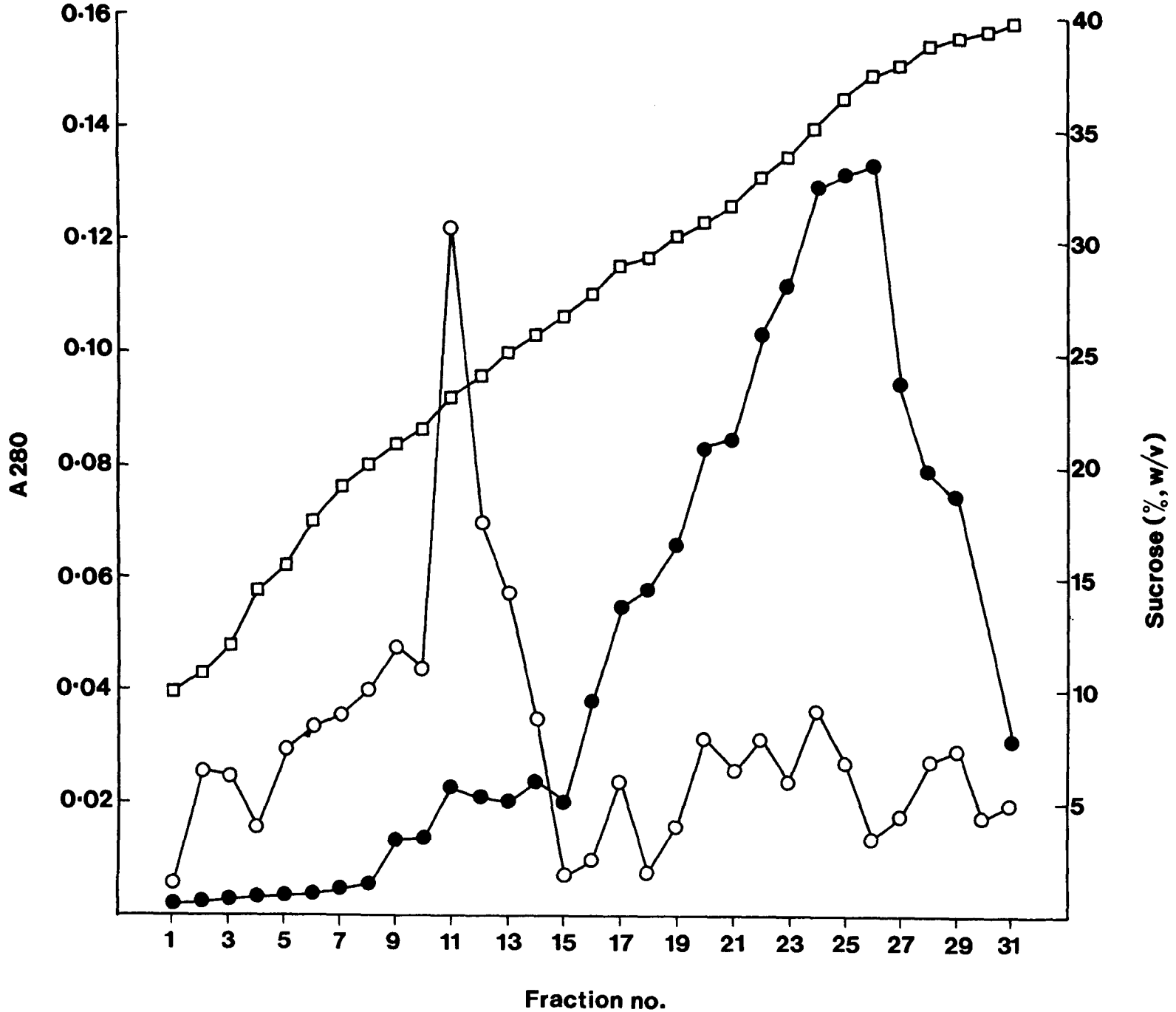
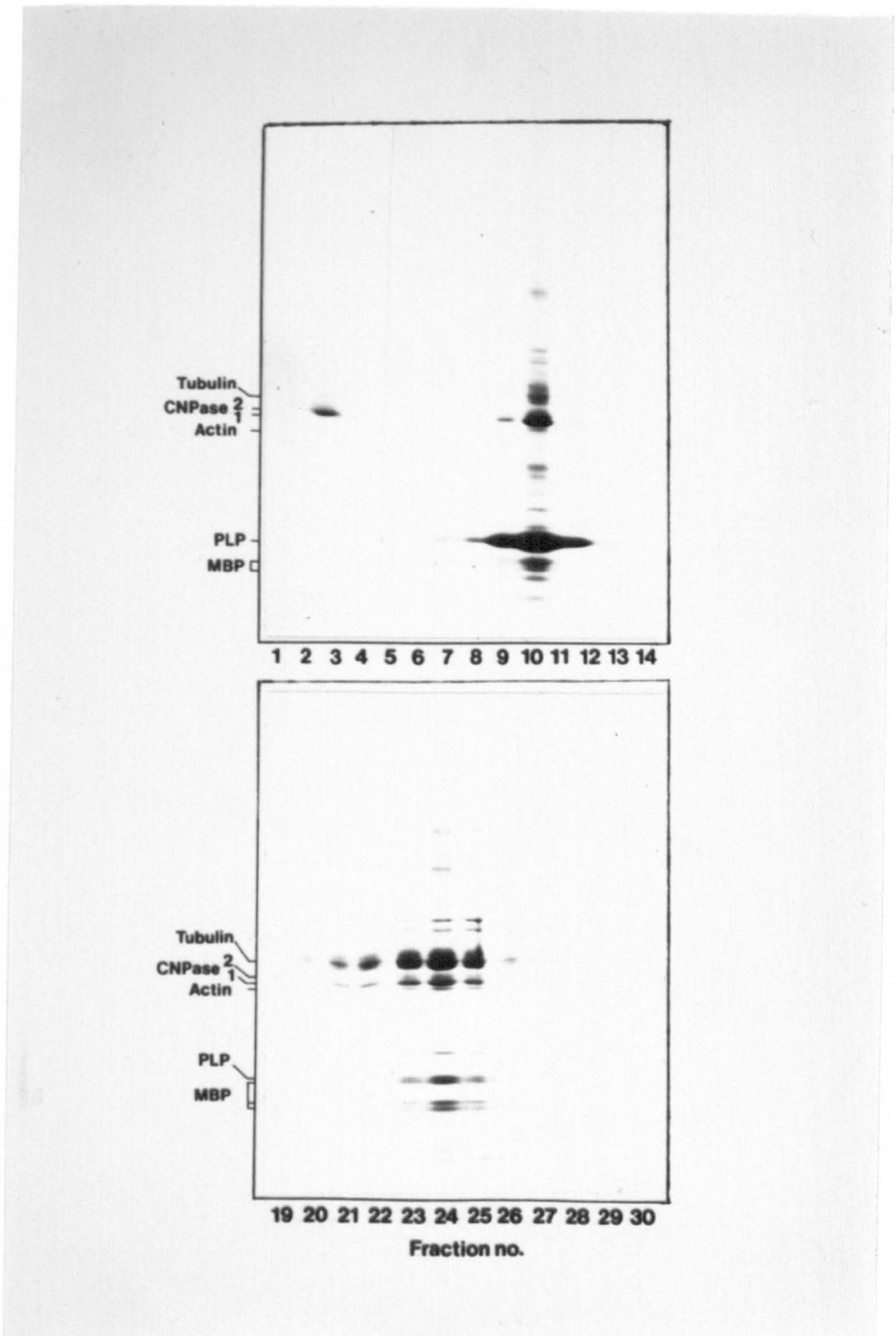

0·16
0·14
0·12
0·10
0·08
0·06
0·04
0·02
A 280
40
35
30
25
20
15
10
5
Sucrose (%, w/v)
1
3
5
7
9
11
13
15
17
19
21
23
25
27
29
31
Fraction no.

Fig. 44. Protein profile of fractions obtained from sub-fractionation of rat brain myelin and the detergent-insoluble pellet.
Aliquots (50µl) of the fractions obtained from linear sucrose density centrifugation (Figure 43) were analysed by SDS-PAGE in 5-15% gradient gels and the proteins stained with Coomassie blue.


Tubulin
CNPase 2
1
Actin
PLP
MBP
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Tubulin
CNPase 2
1
Actin
PLP
MBP
19 20 21 22 23 24 25 26 27 28 29 30
Fraction no.

tightly bound CNPases I and II and MBP. A small amount of PLP was also observed within this fraction although the amount was insignificant when compared to that contained in the myelin fraction. The amounts of CNPases I and II and MBPs found associated with the cytoskeleton relative to that in pure myelin were in agreement with that found when the detergent-soluble and -insoluble fractions from rat brain myelin were analysed by immunoblotting (see Figure 41).

These results were encouraging and further confirmed, since continuous sucrose density gradient centrifugation did not alter the protein profile of the fraction (compare Figure 39 (lane 3) and Figure 44 (fraction 24), for example), the assumption that the detergent-insoluble pellet obtained after optimal extraction of rat brain myelin was a true representation of the cytoskeleton and that these myelin proteins are tightly bound to it.

Also shown in Figure 44 is the presence of the CNPases (Fraction 2). This fraction (termed the floating 'pad') represented the top of the sucrose gradient (approx. 0.3M sucrose) and is of interest since it has been observed when PNS myelin is subfractionated on continuous sucrose gradients (Matthieu et al., 1979). In the case of the PNS the 'pad' has been shown to consist of single-shelled membrane vesicles which contained CNPase activity four times higher than that of purified PNS myelin. If this is the case for CNS myelin then the 'pad' contains membranes which are morphologically and biochemically different from that of myelin suggesting that a considerable amount of the CNPases are localized in/

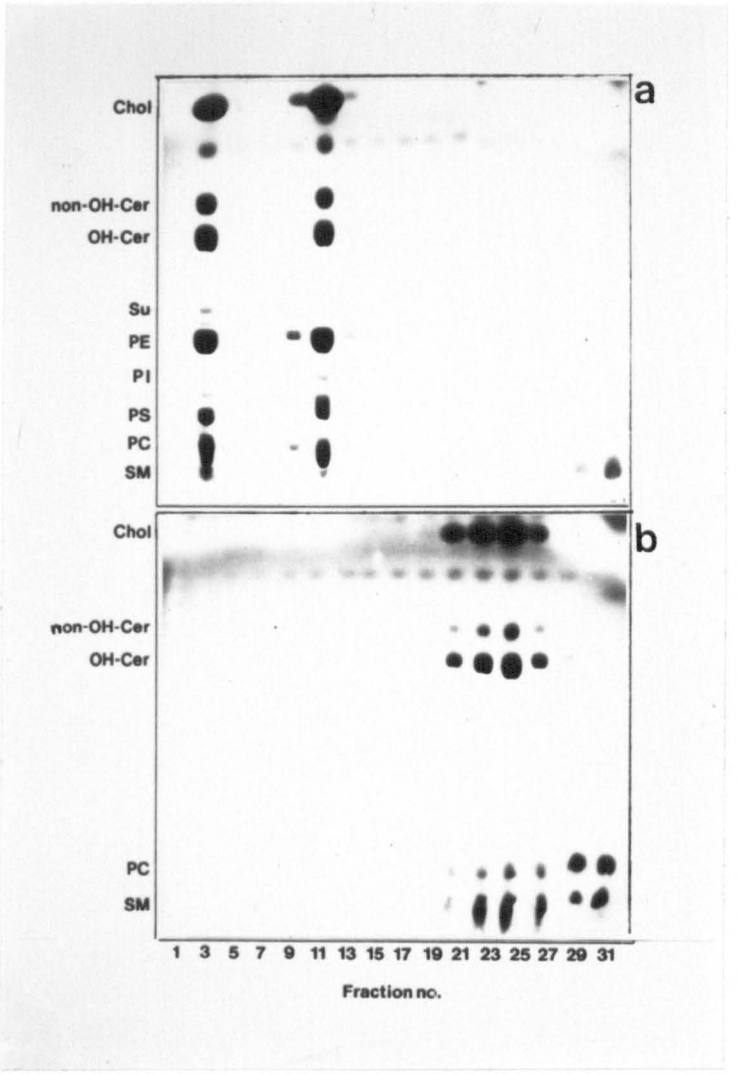
in the oligodendrocyte plasma membrane since it has been suggested that the floating 'pad' membranes of the PNS are derived from the Schwann cell plasma membrane (Matthieu et al., 1979).

TLC analysis of the sucrose density fractions clearly showed that some of the myelin membrane lipids remained associated with the detergent-insoluble fraction (Figure 45b) discounting the possibility that, since the cytoskeleton is isolated by pelleting the Triton X-100-insoluble material, lipid was simply cosedimenting with the protein and not actually associated with it. Comparison of the myelin lipids (Figure 45a, fraction 10) with the lipids associated with the cytoskeleton (Figure 45b, fraction 24) shows that the compositions were different and that a definite enrichment of specific lipids with the cytoskeleton existed. The comigration of a discrete set of proteins and lipids associated with them in the detergent-insoluble fraction suggests that this material may represent a submembranous protein matrix that persists following removal of most of the myelin membrane protein and lipid rather than being simply insoluble protein and/or lipid aggregates.

Also of interest in Figure 45a is the presence of myelin lipids in the floating 'pad' (fraction 2). As stated earlier this fraction may represent the oligodendrocyte plasma membrane however, since the lipid composition of the oligodendrocyte plasma membrane is very different from that of myelin, the lipid analysis results of this fraction tend to rule out this possibility. The presence of myelin lipids has also been observed in the 'pad' when PNS myelin was subjected to continuous sucrose density gradient centrifugation (Linington et al., 1980).

Fig. 45. Lipids associated with rat brain myelin and the detergent-insoluble pellet derived from it.

Lipids were extracted from alternate sucrose density fractions (400µl), dried and subjected to analysis by TLC as described in Materials and Methods. Lipids were visualized by briefly exposing the TLC plates to iodine vapour. a) rat brain myelin fractions; b) Triton X-100-insoluble fractions.


Chol
non-OH-Cer
OH-Cer
Su
PE
PI
PS
PC
SM
a
Chol
non-OH-Cer
OH-Cer
PC
SM
b
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31
Fraction no.

Discussion

4.1 BIOSYNTHESIS OF THE MAJOR CNS MYELIN PROTEINS AND MECHANISMS FOR THEIR ASSOCIATION WITH THE MEMBRANE

The experiments on the sites of synthesis (*in vitro*) and kinetics of incorporation (*in vivo*) demonstrate that there are vastly different mechanisms whereby extrinsic and intrinsic membrane proteins are synthesized and become associated with the growing myelin membrane.

MBPs, CNPases and P2 were all shown to share the same site of synthesis on free polysomes which is consistent with their being extrinsic membrane proteins, ie. proteins which do not penetrate the lipid bilayer to any great extent. The results obtained from the labelling studies *in vivo* reinforce this view. The rapid incorporation of MBPs and CNPases into developing myelin suggests that these proteins are discharged from free polysomes into the cell sap and are then incorporated post-translationally into myelin.

In contrast to MBPs and CNPase the major intrinsic membrane protein of myelin, PLP, was observed to incorporate into developing myelin only after a significant lag period. The presence of covalently attached fatty acid to PLP (Stoffyn and Folch-Pi, 1971) strongly suggests that the protein traverses the Golgi complex since this is the site at which viral envelope glycoproteins appear to be modified (Schmidt and Schlesinger, 1979). If this is the case with PLP then the protein probably reaches the oligodendrocyte plasma membrane via Golgi-derived vesicles. Once the vesicles fuse with the plasma membrane, transport of PLP to the myelinating process would require lateral diffusion. Alternatively, the Golgi-derived vesicles could transfer PLP to the myelinating process where it could/

could participate in myelin formation directly.

The membrane-binding experiments in which membranes were added either during synthesis or post-translationally to in vitro translations demonstrate striking differences in the behaviour of each of the in vitro synthesized polypeptides. When DPM and SRP were added during translation, PLP underwent normal co-translational insertion into the membranes. This result was expected since Colman et al. (1982) have shown that PLP is synthesized on bound polysomes. Of interest is the fact that the primary translation product was of the same electrophoretic mobility as the mature polypeptide indicating that the signal sequence is not cleaved during membrane insertion. It would thus appear that the uncleaved amino terminal signal sequence may be one of a series of insertion and halt signals, situated along the polypeptide chain, which enable the complex membrane disposition of PLP to be attained.

On the other hand, the binding of MBPs to the microsomes demonstrates that these highly charged proteins will readily and non-specifically (since the microsomes were derived from non-neural sources) associate with acidic lipids or phospholipid vesicles. That this non-specific association almost certainly does not occur in vivo has been shown by the observation that rough microsomes labelled in vivo were never found to contain labelled MBPs (Colman, D. R., personal communication). The finding that the CNPases did not associate with the microsomes suggests that the oligodendrocyte possesses very different mechanisms for targetting the CNPases and MBPs to the myelin membrane since it would have to prevent MBPs from associating with other intracellular membranes. The results involving/

involving the post-translational addition of DPM and myelin to newly synthesized MBP and P2 tend to re-inforce this view. MBP was observed to have an equal affinity for both types of membranes, therefore even in the presence of its target membrane (myelin) the degree of specific association that MBP exhibits with myelin is no more than that of non-specific sticking to, most probably, any type of membrane that exists in the cell. P2, on the other hand, displayed a similar pattern to that of the CNPases with added microsomes although the fact that this protein did not associate with myelin may mean that the association of P2 with the myelin membrane is very weak. Since the function of P2 may be that of a lipid carrier (see Section 1.3 ii e) it may require the presence of lipid in order for it to maintain a strong interaction with myelin.

Possibly the most interesting result obtained from the in vitro translation experiments was the finding that an RNA fraction isolated from purified myelin was enriched in messages encoding MBPs but was not enriched in messages that coded for the CNPases and P2. This is surprising since the three proteins are similar in that they are all of the extrinsic type and synthesized on free polysomes and all are highly charged with pIs of 9 or greater. Therefore these results suggest that there exist mechanisms for the transport of newly synthesized MBPs to the myelin membrane that differ considerably from that of the CNPases and P2. This discovery has important implications for the cell biology of myelination since it suggests that free polysomes synthesize the MBPs close to the myelin membrane which may serve to ensure that nascent MBP polypeptides associate/

associate rapidly (in vivo labelling results) and specifically (membrane-binding results) with the correct membrane.

A number of mechanisms could be involved in the establishment and maintenance of a segregation of those free polysomes synthesizing MBPs to the oligodendrocyte periphery (ie. the myelinating process). The cellular cytoskeleton would appear to be an attractive candidate for a specific association with MBP mRNAs in the myelinating process. Once this segregation is established the nascent MBPs could interact directly with other protein or lipid constituents of the growing sheath.

4.2 CYTOSKELETAL INVOLVEMENT IN MYELINOGENESIS

Many nucleated cells have been shown to have present within them extensive cytoskeletal systems that include microtubules, microfilaments and intermediate filaments (Timasheff and Grisham, 1980; Lazarides, 1980). Non-ionic detergent extraction of cells and isolated membranes from several mammalian sources has demonstrated the occurrence of detergent-insoluble components associated with the plasma membrane. Partial characterization of the components of these detergent-insoluble fractions has led to suggestions that they represent submembranous cytoskeletal complexes associated with the membrane. Studies of the detergent-insoluble plasma membrane fraction of murine tumour and lymphoid cells have led to the proposal that this material represents a membrane skeletal structure distinct from the filamentous cytoskeletal systems previously characterized (Apgar et al., 1985). The results presented here on the characterization of both the oligodendrocyte (whole cell) and myelin-associated (isolated membrane) cytoskeletons show that there is/

is a discrete association of some of the major CNS myelin proteins with this detergent-insoluble matrix. The fact that the unique polypeptide distribution of the detergent-soluble and -insoluble fractions was found to remain unaltered in both the oligodendrocyte and myelin-associated cytoskeletons even after prolonged extraction with Triton X-100 means that the complex represents a limit-solubility product. On comparing the PLP distribution from both types of cytoskeleton it was observed that none of this protein was found associated with the detergent-insoluble matrix. This suggests that PLP either has no direct association with cytoskeletal elements or that if an interaction exists it is through extrinsic-type 'linker' proteins as is the case with the erythrocyte cytoskeleton (Gratzer, 1981).

In contrast to PLP, significant differences in the distribution of MBPs and CNPases were observed within both the oligodendrocyte and myelin-associated detergent-soluble and -insoluble fractions. In the case of the MBPs the fact that the vast majority of all the four polypeptide species are seen to be extracted by the detergent in oligodendrocytes suggests that these may be newly synthesized MBPs which are en route from their site of synthesis to the myelin membrane proper. Such cytoskeletal association that exists might be transient reflecting cytoskeletal transport of these proteins rather than a stable cytoskeletal association that may be involved in some dynamic event such as the maintenance of myelin membrane stability. Evidence that post-translational cytoskeletal transport of MBPs may exist was shown by the fact that the majority of mRNAs coding for MBPs are attached/

attached to the cytoskeleton during synthesis and it is not unreasonable to assume that once synthesis has been completed the nascent polypeptides (still attached to the cytoskeleton) are then targetted to the myelin membrane using this transport system.

The MBP distribution within the myelin-associated cytoskeleton is more puzzling. Why should there be a significant enrichment of the pre-large MBP within the cytoskeletal fraction? This question is difficult to answer but may reflect the role that this MBP species has to play in the stabilization of the shape and/or structure of individual myelin bilayer leaflets rather than in interactions between the many membrane layers themselves. The distribution of the three smaller MBP polypeptides between the deteregent-soluble and -insoluble fractions was found to be about equal and may reflect the different role that these MBPs have as opposed to the pre-large MBP. It could be envisaged that maintenance of the multilamellar structure is due to the association of these three MBP species with one another (via dimerization for example) and forming the well known major dense line.

The CNPase distribution within the detergent-soluble and -insoluble fractions of both oligodendrocytes and myelin, when examined closely, appears to resemble that of the MBPs in that most of the proteins (both CNPase I and II) were solubilized by the detergent in oligodendrocytes but a selective enrichment of one of the CNPases (CNPase II) was seen to be associated with the myelin-associated cytoskeleton. Again, as with the MBPs, the 'soluble' oligodendrocyte-derived CNPases may be newly synthesized protein which is in the process of being cytoskeletally transported from/

from the cell body to the periphery of the cell since most of the CNPase mRNAs are attached to the cytoskeleton during synthesis. The fact that it is CNPase II which is enriched in the cytoskeleton may be due to phosphorylation since this appears to be the only post-translational difference between CNPase I and II which has been detected so far.

It has been shown recently that the phosphorylation of tubulin enhances its interaction with membranes and that this effect is reversible (Hargreaves et al., 1986). A strong and stable association between the cytoskeleton and the myelin membrane, mediated by CNPase II (and perhaps pre-large MBP), could therefore be envisaged with a flexibility of movement between the structures allowed for by the reversible phosphorylation and dephosphorylation of these proteins.

The lipid analysis results have shown that there is a considerable enrichment of sphingomyelin and cerebrosides (other than sulphatides) associated with the detergent-insoluble matrix of purified myelin. If this reflects an association that occurs in the native membrane then the matrix may have significant influence on the properties of the lipid bilayer. There is some evidence for a role of the spectrin matrix of erythrocytes in maintaining the lipid asymmetry in these membranes (Williamson et al., 1982) and the myelin cytoskeletal matrix may have a similar role. The binding of lipids directly to cytoskeletal elements would therefore be seen to shift the equilibrium distribution of sphingomyelin, cerebrosides and phosphatidylethanolamine. Cerebrosides, although not specific to the myelin membrane, are much enriched in this structure and have been/

been thought of as the most 'myelin-like lipid'. The observation that so much of this particular class of lipid is found associated with the detergent-insoluble fraction isolated from purified myelin suggests that this lipid may have a dynamic rather than a purely structural role to play in myelin membrane formation and/or stabilization.

It must be stressed that work on establishing a role for the cytoskeleton in myelinogenesis is at an early stage and although these results are consistent with the idea that the myelin membrane contains a detergent-insoluble matrix (or cytoskeleton) that may play a role in myelin formation and/or stabilization via a direct interaction between cytoskeletal and myelin-specific proteins and the membrane itself much more research will be needed to determine how these proteins interact with each other and to elucidate the true function of the myelin-associated cytoskeleton.

The experiments outlined here reveal that there must be a remarkable diversity of mechanisms that the oligodendrocyte must employ to organize the developing myelin membrane and, once formed, to stabilize what is, after all, an extremely complex structure. The fact that the oligodendrocyte is so extended in space, and that it must in the course of myelination rapidly generate large amounts of membrane, probably has resulted in a higher degree of development of the general subcellular mechanisms for targetting of protein and lipid and their interaction with subcellular components than exist in other cells.

Future Work

5 SUGGESTIONS FOR FUTURE WORK

It is crucial for our understanding of myelination and remyelination that we understand the factors that promote the activation of the genes responsible for the synthesis and assembly of the myelin membrane. Modern molecular biological techniques provide powerful tools for the study of such events. This methodology allows one to detect the subtle modifications in cellular behaviour and organization that control the expression of genes and therefore it is envisaged that the continued study of myelinogenesis will, in the future, utilize these techniques to the full.

The technique of *in situ* hybridization, using either cloned cDNAs of the major CNS myelin proteins or synthetic oligonucleotides could be used to probe the appearance and localization of specific mRNAs encoding these proteins. Allied to this would be the use of Northern blot analysis of RNA isolated from both the developing brain and cultured oligodendrocytes to study the relationship between the timely expression of genes encoding the major CNS myelin proteins and the process of myelination itself. If the initial cause of multiple sclerosis was found to be due to a virus (and therein lies a major goal for future work itself) then techniques like *in situ* hybridization could be used to test the effect of the virus on oligodendrocyte gene expression. A novel way in which molecular biological techniques could be put to use is in determining the (presumed) internal signal sequences(s) of PLP. This could be done by a coupled cell-free transcription/translation assay using fragments of the cloned PLP DNA and looking for the segment(s)/

segment(s) of the polypeptide chain which cotranslationally insert into microsomes.

From a biochemical point of view it would be interesting to purify and sequence rat brain CNPase II and also to map the phosphorylation site(s) contained within it. Since it appears that the only proteins contained in the floating 'pad' from sucrose density gradient centrifugation of myelin are CNPase I and II then this fraction could be used in the purification process. Until sequencing of CNPase II is carried out then the true nature of the relationship between CNPase I and II will remain unknown. Are CNPase I and II the products of separate mRNAs or is the difference between them due to some major post-translational modification other than phosphorylation?

There are many areas to explore with regard to the involvement of the cytoskeleton in myelinogenesis. It would be interesting to establish whether any of the major CNS myelin proteins bind to purified elements of the cytoskeleton, for example pre-large MBP and CNPase II with purified tubulin and actin. If binding was detected then the addition of antibodies to those proteins and their effect on myelinating oligodendrocytes in culture could be examined. Once the myelin process has been extended from the oligodendrocyte it must be able to recognize a nerve axon in order for spiralling of the process to commence. A major aim of research in the future would be to identify these recognition proteins since it may turn out that these cell-surface proteins might be responsible for the tight envelopment of the nerve axon.

The involvement of the cellular cytoskeleton in myelin membrane/

membrane protein biosynthesis could be studied using the microfilament-disrupting drug cytochalasin. Cell-free translation of RNA isolated from the cytoskeletal fractions of cytochalasin treated cultured oligodendrocytes or single-cell suspensions from brain would determine if the mRNA was truly bound to microfilaments during synthesis since the mRNA would then be found predominantly in the detergent-soluble fraction.

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