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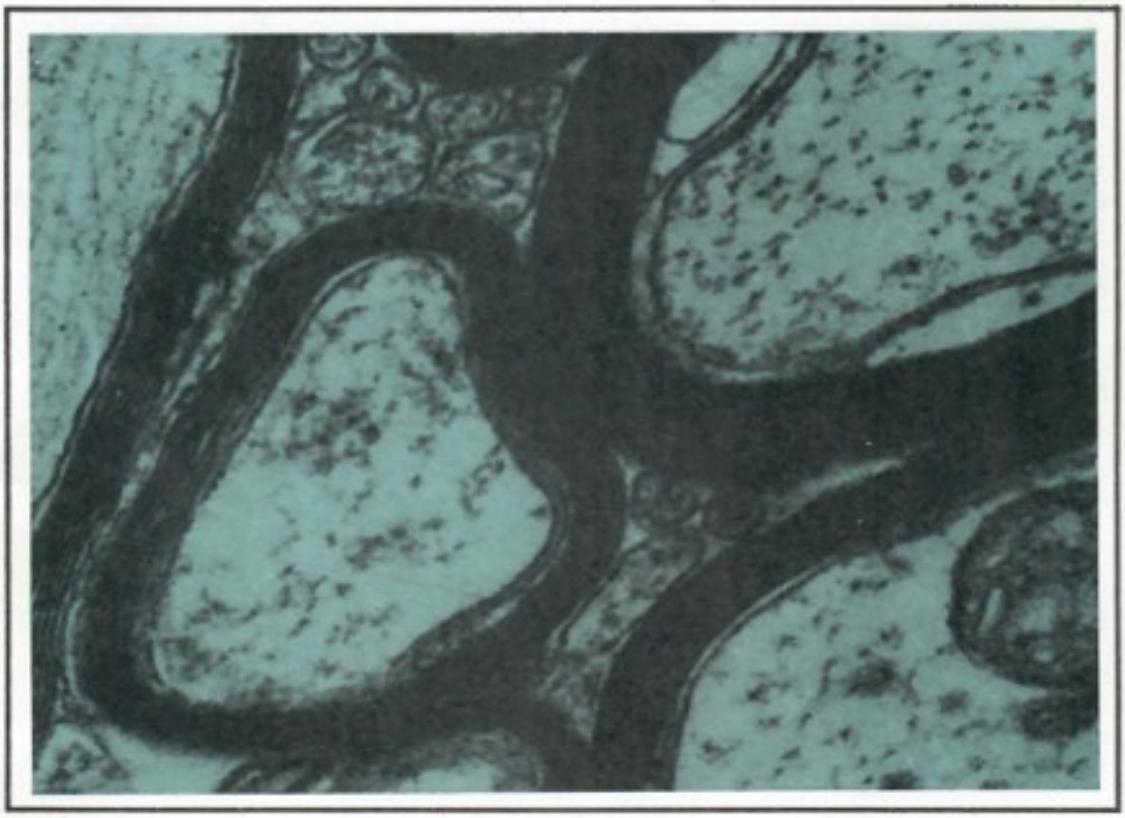

MYELINATION AND DEMYELINATION

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Myelination and Demyelination

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Acta cytobiologica et morphologica, 1

Sofia • 1989

Towards the frontiers of modem biology

(Preface)

A. A. Hadjiolov

Since the present issue, *Acta morphologica* of the Bulgarian Academy of Sciences is renamed *Acta cytobiologica et morphologica.* The decision was taken unanimously by the Scientific Council of the Institute of Cell Biology and Morphology at the Bulgarian Academy of Sciences. This decision reflects both the changes in the trends of development of modern biology and the efforts of our Institute to keep in pace with this development.

Biological science entered the 20th century solidly rooted in the cellular theory of structure, function and pathology of living organisms. Clearly, the organization and comportment of both unicellular and multicellular organisms could not be understood outside the frame of cellular structure. Therefore, the first half of the century witnessed our increasingly deeper understanding of the structural features of different tissues and organs in the highly diversified animal and plant organisms. The structural basis in the differentiation of more than 300 different cell types in mammalian organisms was unravelled and decisive steps were made allowing to outline the subsequent phases in cell differentiation taking place during embryogenesis. On the other hand, the development of new and powerful techniques, cell cultivation and electron microscopy in particular, permitted the elucidation of considerable details in subcellular structure. Moreover, it became possible to link specific functions with a given cell organelle: chromosomes as carriers of genes or mitochondria as the site of respiratory chains and oxidative phosphorylation. New subcellular organelles were discovered: ribosomes, lysosomes and cytoskeleton, etc. Each of these organelles was shown to constitute the structural basis for still further cell functions. In particular, studies on ribosomes and polyribosomes revealed these structures as the site of protein synthesis and their further analysis prepared the fruitful integration with biochemistry and genetics.

For a long time biochemistry has been scoring successes in elucidating the structure and metabolism of relatively simple organic molecules (carbohydrates and lipids) in the early stages and of nucleic acids and proteins during the fruitful 1950s and 1960s. When we understood that the gene is a segment of DNA coding for a specific polypeptide chain along the chain DNA ->RNA-»protein, and after

the genetic code was deciphered in 1961-1965, biological sciences were ready to enter the era of *molecular biology.*

It is now generally recognized that the advent of *molecular biology* in the second half of the 20th century is the landmark of the modern revolution in biology. For it represents the triumph of a new paradigm in biological sciences: life can be understood in molecular terms. On a philosophical basis, to understand means to know the structure, the function and the history of a given phenomenon. Yet, molecular biology provides just an integrated understanding at the molecular level of the structure, the function and the history (both genetics and evolution) of living cells and organisms. It must be added that for a long time the full cognitive power of molecular biology could be applied solely to bacteria and their viruses. Eukaryotes and multicellular eukaryotes in particular, were too complicated and their molecular genetics practically unexplored. Even the best studied organisms, like *Saccharomyces* and *Drosophila*, were for a long time too difficult to be understood as molecular machines. The breakthrough came in the mid-1970s, when the techniques of gene cloning and gene sequencing were introduced. Since then started the advent of *cell biology.* In my view, cell biology is the integrated understanding of cellular structure, function and genetics at molecular level. This is the basic characteristic which makes cell biology a major step in the ever-expanding field of modern biology.

The historical development of our Institute reflects the advancement of biological sciences and thinking outlined above. Founded in 1953 as an Institute of Morphology by Professor A. I. Hadjiolov, it has probed into different aspects of cellular and tissue organization and structure. Traditionally, the histochemistry of lipids has been at the center of interests of A. I. Hadjiolov and his coworkers. In line with the attempts at a deeper understanding of cell and tissue structure, Professor J. Jordanov introduced new techniques of tissue cultivation, while Dr A. Bojadjieva-Mikhailova pioneered the introduction of electron microscopy techniques in our country. The second director of our Institute, Professor I. Goranov developed further the study of blood tissue and started several studies in immunomorphology. Thus, we reached the presemt-day stage in the development of our Institute, where cell biology is becoming more and more its major area of research. This line of development justified the renaming of the Institute in 1986: Institute of Cell Biology and Morphology. The present issue reflects the attempts to develop some aspects of neurobiology in our Institute. It reflects also the attempts to link our activities with those of many leading laboratories in neurobiology and cell biology. Hopefully, this trend in the development of our journal will be expanded further in order to contribute towards the presently more and more clearly outlined frontiers of modern biology.

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Neuronal-glial cooperation in the production of myelin phospholipids

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The autor's experimental results and the data of other authors are summarized, showing convincingly the existence of cooperation between the neurons and the myelin-forming glial cells during the production of myelin phospholipids. The myelin alkenylacyl-glycerophosphoethanolamines, which correspond to ethanol-amine plasmalogens, are synthesized predominantly in the neurons. A compact myelin membrane with the characteristic lipoprotein composition of the mature myelin can be formed only when there are myelin-forming glial cells and neurons.

Key words', myelination, neuron, myelin-forming glial cell, phospholipids.

Myelin is one of the lipid-richest membranes containing 70-80% of lipids by weight. Its main lipids are cholesterol, phospholipids and galactolipids. On a dry-weight basis, the total phospholipids constitute a larger percentage of the myelin lipids and 18% of the hydrated myelin [22].

It is already generally accepted that the principal cells which participate in the formation of the myelin membrane during myelination and remyelination are the Schwann cells in the peripheral nervous system and the oligodendrocytes in the central nervous system. Parallel with this, however, convincing data have been accumulated, which show the existences of varied and close interactions between these glial cells and the neurons during the different phases of myelinogenesis: multiplication and proliferation of the glial cells, wrapping of the glial plasma membrane around the axon, production of myelin components and formation of compact myelin [1, 6, 10, 14, 16, 21, 26, 28, 29, 30, 32]. For the first time on the basis of histochemical studies on the myelination in chick brain, we expressed the view about the existence of such interactions in the production of the myelin components, more specifically of phospholipids [33, 34]. Using histochemical, histoenzyme, electronmicroscopic and autoradiographic methods, in our subsequent experiments we traced the cellular localization of phospholipid synthesis during myelination in the brain of guinea pigs, mice, rats and rabbits [7, 9. 35, 36, 37, 38, 39, 40]. Our experiments were carried out on a precisely defined

myelinating system (nerve or pathway, the oligodendrocytes located along the fibres, and the neurons whose axons form the respective nerve or pathway). It was shown that before and during the period of initial myelination active phospholipid synthesis is observed in the oligodendrocytes situated along the nerve fibres. In the neurons such a synthesis is detected during active myelination. Positive histochemical reaction for phospholipids in tbe oligodendrocytes and in the neurons is revealed only during these periods. No lipid granules are found during the routine electronmicroscopic investigations, whereas the ultastructural features are these of a cell in which active lipoprotein synthesis takes place.

In our most recent studies we applied more effective techniques for the preservation of lipids and for enhancing their electronmicroscopic density [8, 20]. Under these conditions, lipid granules are discovered at the site of their synthesis and transport. We have established highly osmiophilic granules in the perikarya of the oligodenrocytes and the neurons during the above mentioned periods of myelinogenesis (in the cisterns of the granular endoplasmic reticulum, in the Golgi apparatus and in some vesicles), in the axons and in the myelin sheaths [41].

Naturally, the question arises which of the myelin phospholipids are synthesized in the neurons, and which in the myelin-forming glial cells?

The pathways of phospholipid biosynthesis in the brain do not differ much from well-known pathways of lipid synthesis in other organs. The cytidine pathway discovered by K e n n e d y [17] is the key pathway of lipid synthesis in the nerve tissue. B i n a g 1 i a et al. [3] have studied *de novo* synthesis of phosphatidyl choline and phosphatidyl ethanolamine in isolated neurons and glial cells of rabbit cortex in vitro and have found much more active synthesis in the neurons. They concluded that the cytidine-dependent enzyme system is concentrated mainly in the neurons, as compared with the glial cells, and expressed the view that the lipid synthesis takes place mainly in the neurons and that the lipids synthesized in them are utilized by the glia.

F r e y s z et al. [13] have shown that neuronal phospholipids have a much faster turnover than glial phospholipids. G o r acc i et al. [15] have traced the incorporation of intraventricularly introduced radioactive ethanolamine into phosphatidyl ethanolamine and ethanolamine plasmalogen in isolated neurons and glial cells of rabbit cortex. In the neurons, maximum labelling of the two phospholipids was observed on the 7th hour after the precursor was introduced, in the glial cells — between the 20th and the 36th hour. Much faster turnover was also observed for the phospholipids in the neurons, as compared with the glial cells. These data give grounds to the authors to assume that the neurons are the site of the most active synthesis of ethanolamine phospholipids, with their subsequent transfer to the glial compartment.

Interesting are the attempts to follow up the further fate of the newly-sythesized neuronal phospholipids. Their selective transferring to the growing neurite has been observed by P f e n n i g e r and J o h n s o n [23] who have investigated the incorporation of [^{3}H]-glycerol on explant cultures of rat superior cervical ganglion. After the pulse with the phospholipid precursor they separated perikarya and neurites microsurgically and extracted their phospholipids. The phospholipid extract from the perikarya exhibited a steep rise followed by a rapid decline in specific radioactivity. In the neurites an increase in specific radioactivity was observed only after a lag period of 60 min. The rapid and massive accumulation of radioactivity in the neuronal perikarya identifies them as the major sites of phospholipid synthesis. The proximodistal transfer of phospholipids could be blocked with the microtubule drug colchicin and the metabolic uncouper, 2,4-dinitrophenol. Pfenniger and Johnson concluded that the data obtained by them indicate preferential export of newly-synthesized phospholipids

from the perikarya into the growing neurites, most likely by rapid axoplasmic transport of formed elements.

The possibility that axonally transported lipids might undergo transaxonal migration and become incorporated into surrounding myelin lamellae was studied by isolating myelin from optic tracts of 22 to 25-day-old rabbits (period of active myelination) at various times, following intraocular injection of [3-^{14}C] serine and [2-^{3}H]glycerol [16]. These studies have revealed that a portion of radioactive material passing down the axon moved into the adjoing myelin sheath, resulting in radiolabelling of specific myelin lipids. Two simultaneous processes were proposed to account for this phenomenon: a) axon-myelin transfer of intact lipids; b) biosynthesis of myelin lipids by myelin-localized enzymes utilizing radiolabelled substrate originating in the axon (reutilization mechanism).

The subsequent investigations have demonstrated the metabolic dependence of myelin on axon in the central nervous system not only in the period of active myelinogenesis [19]. Axon-to-myelin transfer of lipids and lipid precursors have been studied by intraocular injection of [^{14}C]glycerol, [^{3}H]glycerol and [^{32}P]orthophosphate into rabbits, approximately 43-46 and 57 days of age. Choline and ethanolamine phosphoglycerides and myelin showed increasing [^{32}P]-radioactivity between the 7th and the 21st day following injection, while [^{3}HJ- and [14C]-radioactivities remained relatively constant. The latter radioactivities decreased, however, in all the axon- and axolemma-enriched fractions during the same period. These results supported the concept of axon-myelin transfer of glycerol-labelled lipids during axonal transport. They showed that inorganic phosphate, possibly generated by catabolic activity within the axon, is able to enter myelin and participate in the reutilization mechanism previously described for serine, choline and acyl chains. The relative invariance of ^{3}H:^{14}C ratio suggested that the majority of glycerol is not reutilized in this manner but probably enters myelin through transfer of intact lipid.

The same two mechanisms were postulated to explain the axon-myelin transfer of phospholipids in the peripheral nervous system [10,11]. The kinetics of phospholipid constituents transferred from the axon to the myelin sheath were studied in the oculomotor nerve and the ciliary ganglion of chicken after injection with [^{3}H]glycerol and methyl-[^{3}H] choline into the cerebral aqueduct. Quantitative electron microscopic radioautography revealed that labelled lipids were transported in the axons mainly associated with the smooth endoplasmic reticulum. Simultaneously, the labelling of the myelin sheath was found in the Schmidt-Lanterman clefts and the inner myelin layers. The outer Schwann cell cytoplasm and the outer myelin layers contained some label with methyl-[^{3}H]choline, but virtually none with [^{3}H]glycerol. Since [^{3}H]glycerol incorporated into phospholipids is practically not reutilised, the occurrence of label in myelin results from a translocation of entire phospholipid molecules and from their preferential insertion into Schmidt-Lantermann clefts. In this way, the axon-myelin transfer of phospholipids contributes rapidly to the renewal of a limited pool of phospholipids in the inner myelin layers. When methyl- [^{3}H] choline was used as precursor of phospholipids, the rapid appearance of the label in the inner myelin layers was interpreted also as an axon-myelin transfer of labelled phospholipids. However, the additional labelling of the outer Schwann cell cytoplasm adjacent to Schmidt-Lantermann clefts and of outer myelin layers reflects a local reincorporation of the bases released from the axon.

The studies on the axonal transport of ethanolamine glycerophospholipids have demonstrated the preferential accumulation of transported ethnolamine plasmalogen in myelin [6, 12]. Two-week-old chickens were injected into the

cerebral aqueduct with [1'3H] ethanolamine, a suitable precursor to label the two classes of ethanolamine glycerophospholipids (diacyl-glycerophosphoethanolamine which corresponds to phosphatidyl ethanolamine, and alkenyl-glycerophosphoethanolamine which corresponds to ethanolamine plasmalogen). The axonally transported labelled lipids were identified by thin-layer chromatography and located in cell structures by light and electron microscopic autoradiography. The major part of axonally transported labelled lipids consisted of phosphatidyl ethanolamine and ethanolamine plasmalogen. The data obtained showed that axonal transport provides phosphatidyl ethanolamine to the axolemma and membranous elements of nerve endings. Part of them could be transferred from axon to myelin. In contrast, axonally transported ethanolamine plasmalogen could be preferentially and massively transferred to myelin.

Alkenylacyl-glycerophosphoethanolamine (ethanolamine plasmalogen) accounts for more than 30% of the phospholipid content in myelinated nerve fibre. Approximately 80% of the ethanolamine phospholipids of myelin are in plasmalogen form [22]. It was mentioned above that the neurons are the site of most active synthesis of ethanolamine phospholipids.

From all data presented so far it is evident that the existence of cooperation between the neurons and the myelin-forming glial cells for the production of myelin phospholipids has already been proved. This fact is very important and it should be borne in mind by the researchers of the processes of myelination and remyelination. Since the production of phospholipids of the myelin membrane requires the synthesis of part of them to take place in the neurons, then it is obvious that compact myelin cannot be produced by the oligodendrocytes or by the Schwann cells only. The results of all our comprehensive studies have shown that active phospholipid synthesis is observed in the neurons during the period of active myelination, i. e. when the compact myelin membrane is being formed. The transformation of the glial plasma membrane into a compact myelin membrane takes place through the incorporation of lipids and proteins during different periods of myelinogenesis. The term “myelin membrane” is understood to mean the compact myelin membrane which possesses the characteristic lipoprotein composition of the mature myelin, which differs both from the composition of the glial plasma membrane and from the loose myelin. The results of the studies on the formation of the myelin membrane in cell cultures are in support of this view.

P o d u s 1 o et al. [24] have observed the formation of membrane lamellae adjacent to the cell soma from oligodendrocytes obtained from bovine brain after their cultivation for 16 hours. When subcellular fractions were prepared from the cells in culture, three membrane fractions were obtained: a glial light fraction, an inermediate fraction that electron microscopically consists of whorls of membrane lamellae, and a plasma membrane fraction consisting primarily of small vesicles. In this way the authors were able to study the level of incorporation of the different precursors of the lipids and proteins into the three fractions. They have observed the highest level of incorporation for each lipid in the light glial fraction, and lower but parallel levels of incorporation in the membrane lamellae and plasma membrane fractions. Their incorporation study has shown that initially the cerebrosides, phosphatidyl choline and probably cholesterol were incorporated into these whorls of membrane lamellae. The authors concluded that these whorls of membrane may be the precursors of mature compact myelin.

S a r 1 î e v e et al. [27] have also obtained multilayer membranous material in dissociated cultures of mouse embryo brain, which they have called “myelin-like or premyelin structures”.

The production of extensive myelin-like membranes by highly purified oligodendrocytes in culture was demonstrated also in the study of Romeet al. [25]. The formation of membranous material around a carbon fibre placed in cultures of isolated oligodendrocytes has been observed [2]. After cultivation for about four weeks, the whole fibre was enwrapped by the processes of the oligodendrocytes. The sucrose gradient centrifugation of these cultures results in the separation of a fraction composed electron microscopically of lamellae and some unidentified debris. The authors also concluded that the membrane fragments might resemble premature myelin.

However, there are some researchers of the myelin formation in cell cultures [5]. who state that "cultured neonatal rat oligodendrocytes elaborate the myelin membrane in the absence of neurons". They isolated oligodendrocytes from the brains of 24-hour-old rats, maintained them in culture and performed transmission electron microscopic observations on 14- and 26-day-old cultures. In the 26-day-old cultures they have established the existence of intracellular membranes which were shown to be continuous with the oligodendrocyte processes. These membranes tended to form multilaminar whorls and apparent cylinders with "open" lumen. Some of these membranes were localized within indentations of the cell membrane. They often exhibited the appearance of loose myelin. The researchers have not studied the composition of the membranous fraction, nor the synthetic activity of the oligodendrocytes. They do not rule out the possibility of the existence of a small number of neurons in the cultures. Besides, they have demonstrated the presence of a large amount of membranous material in the cytoplasm of the oligodendrocytes, most of which is found to be in various stages of degeneration.

The tubular myelin in the pulmonary alveoles is also known to possess external features of the myelin membrane [31], as well as "myelin figures" resulting from poor fixation of the lipids.

Knapp et al. [18] suggested that "axons may play a vital role in orchestrating certain aspects of membrane production by oligodendrocytes." Evidence for this idea stems from both qualitative and quantitative differences between oligodendrocytes in vivo and in vitro. They have maintained primary cultures of neonatal mouse cerebra for up to 4 weeks in the absence of neurons. Oligodendrocytes in these cultures pass through a sequence of cytoarchitectural changes and antigen expression which "mimics" the differentiation of oligodendrocytes in vivo. The majority of them elaborate large sheets of membranous material from the tips and lengths of cell processes. These membranous sheets, which contain galactocerebroside and myelin basic protein, are reminiscent of unwrapped myelin profiles in vivo. They suggested that oligodendrocyte shape and membrane production are in part regulated from within the oligodendrocyte itself.

A role for neurons in regulating the production of myelin components is suggested by the investigations showing that the appearance of galactocerebroside and myelin basic protein are accelerated in neuron-rich embryonic cultures [4].

In conclusion, it can be pointed out that: 1) the hypothesis about neuronal-glial interactions during production of myelin phospholipids, put froward for the first time by us in 1966, has been successfully proved by studies on axonal lipid transport; 2) the existence of neurons and glial cells is necessary for the formation of a compact myelin membrane with the characteristic lipoprotein composition of mature myelin.

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Central nervous system myelination in vitro

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A revue concerning tissue culture methods and myelination in vitro is made on the basis of our own results and of the other authors' data. Different in vitro systems are used for revealing the complex mechanisms of myelination: organotypic, aggregated, dissociated cultures and cultures of bulk-isolated myelinating cells. Myelinogenesis in vitro corresponds to this process in vivo with respect to its morphological and biochemical parameters. The myelin marker lipids, the proteins and the enzymes responsible for their synthesis, have an ontogenetic profile and time-sequence which are similar to the described in vivo. In this connection the tissue cultures provide a convenient system to study factors regulating the cell interactions and expression of myejin components during the myelinogenesis.

Key words-. CNS, myelination, tissue culture, cell markers.

The myelin membrane is a unique structure characteristic of the nervous system which has definite morphological, biochemical, physiological and immunological properties. The isolated myelin contains 70-85% of lipids (cholesterol, phospholipids and galactolipids) and 15-30% of proteins [52]. About 50% of the total proteins are proteolipid proteins (PLP), including DM-20 proteins; 30-35% are myelin basic proteins (MBP); 5% are 2'-3'-CNP and 1% are myelin-associated glycoproteins (MAG) [25]. The myelin sheath is formed during ontogenesis as a result of a complex and multilateral process in the nervous system — myelinogenesis — whose mechanisms have not been totally clarified yet. The priority role in this process is attributed to the oligodendrocytes in the central nervous system and to the Schwann cells in the peripheral nervous system, but the interactions between the different elements in the nervous system are rather complicated. The introduction of new methods and techniques filled in many gaps concerning the mechanism of myelin-formation. Particularly important in this respect was the introduction of tissue and cell cultures in neurobiology. The importance of these systems is suggested by the fact that the participation of the satellite Schwann cell in the formation of spirally wrapped structures around the axons was demonstrated for the first time in cultures of spinal ganglia [31].

In vitro systems of myelination

Several methods can be applied, depending on the objectives of the study:

Organotypic cultures. Small organs (spinal ganglia) or tissue fragments, about 1 mm^3 in size, are explanted on a collagen or polylysine substrate under suitable conditions. The organotypic cultures are maintained in a medium with serum, embryonal extract, glucose, etc., most often in Maximov's assemblies at temperature of about 34,5°C [16, 20, 21, 55]. The explants preserve their optimal cell composition in the organotypic relations between the cells of which they consist. The shortcoming of this method is that the cells are not clearly visualized in a living state and selective access to them is not possible. The method is suitable for morphological studies and for inducing demyelination [2, 13, 34].

Aggregated cultures. Using the tendency of the freely dispersed animal cells in a liquid medium to migrate and form aggregates, M o s c o n a [48, 49, 50] introduced the method of aggregated cultures. S e e d s [68] modified the method by introducing eddy movement of the suspension with the aim of facilitating the aggregation processes. The aggregates are obtained in the first 24 hours and can be maintained under suitable conditions for up to several months. The orientation of the cells in aggregates is not accidental. They tend to take a natural position and orientation, characteristic of the histotypical organization of the original tissue [79], and to establish definite contacts which are closest to the contacts in vivo [69].

This system of cultivation is suitable for applying biochemical and morphological methods in the studies of myelination (TEM and SEM) [42]. It eliminates some of the shortcomings of the dissociated cultures: the cells occupy more suitable positions, but preserve some of the disadvantages of the organotypic cultures — difficult access for direct observation and manipulation.

Cultures of bulk-isolated myelinating cells. Methods have been proposed recently for obtaining enriched fractions of oligodendrocytes which could be preserved and investigated in vitro [28, 58, 59]. This system finds an ever wider application for studying myelination by adding other cell types (e.g. neurons) to the enriched fraction of oligodendrocytes and then using them for transplantation [51].

Dissociated cultures. The dissociation can be enzymatic or mechanical, using nylon sieves or tubes with definite dimensions. The suspension obtained is cultivated over substrate in flasks, at a temperature of 36-37°C and 95% humidity. The nutrient medium is chosen depending on the aims of the study [33, 42. 65].

The dissociated cultures have several advantages. This system is suitable for performing biochemical investigation for which large quantities of material are needed: there exists a possibility for direct observation of the cells and for studying the metabolic parameters, as well as for obtaining enriched or pure cell population.

When this culture system is used for investigating myelination in vitro, it is necessary to observe the following requirements: the most suitable object for dissociation should be the neonatal brain, because its cells are not yet differentiated, more specifically definite brain areas whose composition is known; obligatory identification of the isolated cell types should be made [46, 47].

Due to the big changes which the cells undergo in the processes of isolation and cultivation, one cannot rely on the morphological characteristics only of a concrete cell population. Specific immunological markers have been proposed, 'Ahich guarantee a reliable identification of the cells.

It is necessary to take into account the fact that when dissociated cultures are used, initially the neurons manifest a process of differentiation and then of regression to the primitive state of neuroblasts. Moreover, it is possible that no normal myelination would occur.

The main cell types in the composition of the nerve tissue — neurons, oligodendrocytes and astrocytes — repeat along general lines in vitro the morphological and metabolitic properties of the respective population, although certain peculiarities may also be observed depending on the culture system chosen.

Behaviour of CNS cells in culture

Neurons. An important condition for the normal development of the nerve cells in an in vitro system for studying myelination is the choice of a suitable stage in their development in the brain for isolation and explantation. The most suitable period for culturing neurons of rodents (mice and rats) has been proved to be the 14th-15t-16th embryonal day [40, 65]. By using cell markers for the neuroblasts and neurons, they can be identified even on the third day in vitro [37]. A number of specific markers have been proposed, the principal ones among them being the antisera against concrete substances or receptors contained in the nerve cells, only: 1) usage of antiserum against neurofibrils (NSF) [37] monoclonal antibodies RT 97 has already been proposed; 2) identification of neuron-specific enolase (NSE) [12, 40], considered to be an intracellular neuronal marker; 3) identification of receptors against tetanus toxin [45]; 4) labelling of the body of the neurons with antisynapsin I-antiserum, especially in the early terms [40]; 5) labelling of the GABA-receptors with ^{3}H-muscimol, which is a specific label for neurons, neuroblasts and neuronal processes [67]; $_{6}$) determination of the acetylcholinesterase activity during the later terms of cultivation [37].

Neurons develop an extensive network of neurites in vitro and form synaptic contacts. The closest morphological similarity with definite neuronal types in vivo is found in nerve cells cultivated in organotypic cultures. During the first week of organotypic culture the neurons are differentiated and acquire the morphological characteristics of those in vivo [20, 21]. They contain large light nuclei, well developed endoplasmic reticulum, free ribosomes scattered in the cytoplasm and multiple Golgi appartus. Numerous neurofibrils which pass into the processes are demonstrated in the cytoplasm.

In spite of their poor mobility, neurons in monolayer cultures tend to group, so that small cell aggregates can be seen in the first 24 hours. Processes begin to grow, becoming longer with the increasing duration of the culture, the dendritic network becomes denser and dendrite groups in bundles appear on the 15th day in vitro [33]. The neurons are differentiated during the first and second week in vitro [76], after which they tend to degenerate [$_{8}$, 33, 42, 40, $_{66}$].

The metabolic activities of the nerve cells in vitro are still insufficiently studied.

In organotypic culture from the central nervous system the glial cells are localized either in the dense zone close to the neurons, or they migrate to the growth zones where there are no nerve cells. Their numerous processes participate together with the axons in the formation of the growth zone [20, 21, 36].

Astrocytes. In dissociated cultures of all cell elements of the nervous tissue, the most frequent cell population obtained consists of astrocytes. Mechanical isolation through a nylon sieve with a definite mesh size results in a cell

suspension containing 95 per cent of the astrocytes. Their number increases during the culturing [5], consisting of large flattened cells with branching fibrous growths containing large light nuclei.

A characteristic feature of astrocytes is that they contain glial fibrillar acid protein (GFAP) which is detected as early as during the first week in vitro and is considered to be astroglial cell marker [46, 75]. Protein S-100 is also specific for the astrocytes, but it remains at a low level (compared with its content in vivo) throughout the entire culturing period [70]. The supporting and trophic function of the astroglia has been recognized for a long time. There is still no clarity about their participation in the process of myelinogenesis in the central nervous system. It has been demonstrated, however, that the astrocytes are localized among the neurons in cerbellum cultures on the 7th day in vitro, with numerous processes prior to the myelination [38] among which the neurons are localized. A description is also given of the process of fasciculation, which is associated with myelinogenesis [83].

Oligodendrocytes. In organotypic culture the oligodendrocytes are localized in the inner part of the explants. From the very first days of their cultivation they migrate and participate together with the astrocytes in the formation of the growth zone. The introduction of the methods of dissociated cultures resulted in the discovery of new possibilities for detailed study of the morphological and biochemical specificities of oligodendrocytes. Great difficulties are involved in the obtaining and maintaining of pure oligodendrocyte cultures, therefore, until recently the term “enriched” oligodendrocyte culture was used. On the basis of some properties of the oligodendrocytes, Araceli Espinosa de los Monteros et al. [27] recently proposed a method for obtaining and for maintaining a pure culture of metabolically active oligodendrocytes.

During the first days, in mixed cultures the oligodendrocytes lie over the layer of astrocytes [70]. In pure cultures they are scattered over the surface of the substrate, having long thin processes which exceed several times the diameter of the cell body. In an in vitro system the vital oligodendrocytes manifest the same specific features as in vivo. They are small cells, with dark nucleus and relatively dense cytoplasm, and numerous branching processes. Under phase contrast the cytoplasm is strongly granular [70]. Their ultrastructural pattern identifies them as cells with relatively electron-dense cytoplasm, with many mitichondria and free ribosomes having an electron-dense round nucleus and peripherally positioned heterochromation [59].

Cell criteria have been established for identifying oligodendrocytes in tissue and cell cultures, namely: 1) they have a positive response to the action of antigalactocerebroside serum, considered to be a surface cell marker [61, 62]; 2) the myelin basic protein (MBP) is considered to be an intracellular oligodendrocyte marker [74, 83]; 3) the enzyme 2'3'-CNP is found to be a marker for oligodendrocytes and for the myelin sheath [80, 87].

Both oligodendrocytes and neurons degenerate quickly in mixed cell culture and after the second week in vitro they are replaced by fibrous astrocytes [70].

The principal function of oligodendrocytes in the central nervous system is their participation in the formation and maintenance of the myelin sheaths.

CNS myelinogenesis in culture

Different in vitro systems have been used for revealing the complex mechanisms of myelination. In organotypic culture myelinogenesis corresponds to this process in vivo with respect to its morphological and biochemical parameters [15, 16, 20.56].

Growth cones are identified from the very first hours of the explanting [20, 21]. The proliferation and migration of glioblasts starts on the lst-2nd day in organotypic culture and around the 4th day in vitro in aggregated cerebellar culture [42], being induced according to Wood and Bunge [82] by the growing axons. The neurons end their differentiation before the beginning of myelination in organothypic cultures — around the 8th day in vitro, and slightly later in dissociated cultures. The amount of ceramideglu-cosyltransferase in them increases. This enzyme catalyses the formation of glucosylceramide a precursor of the ganglioside synthesis, considered to be a marker of the neuronal differentiation [42].

The onset of myelination depends, above all, on the brain area cultivated and on the culture system chosen. In organotypic cultures myelination starts around the 10th day [20, 73]. In dissociated and aggregated cultures the process of myelinogenesis is delayed, starting around the 2nd-3rd week [63, 65, 66]. Orientation of the glial cells along the nerve fibres is observed before the visualization of the myelin sheaths [20]. Cell nests are formed in monolayer culture [63], with filamenous surface and later with an uneven surface [66].

Most accurate data on the course of the synthesis processes during in vitro myelination have been obtained by applying radioactive precursors of the myelin components. Irrespective of certain variations resulting from the use of different systems and conditions of culture, the myelin-associated marker lipids, the proteins and the enzymes responsible for their synthesis, have an ontogenetic profile which is similar to the one described in vivo. They appear exactly at definite intervals and in a definite order, and perhaps the coordinating control is performed by a common central cell source, i. e. probably by the oligodendrocytes [66], because this order is preserved in a purely oligodendrocyte culture as well, although the quantities of the different components are smaller [63]. One cannot rule out the influence of some neuronal factors on the myelinating oligodendrocytes [6]. The cholesterol synthesis shows a manifold increase during myelination, with a peak of deposition in organotypic culture from newborn mouse cerebellum around the 15th day [57]. Galactolipids-cerebrosides and sulphatides are barely identifiable around the 4th day in vitro, but they increase considerably between the 20th and 30th day in vitro [17]. The peak in their deposition coincides with the period of active myelinogenesis [39, 63, 72]. As compared with cerebrosides, the sulphatides are synthesized and metabolized more actively [66]. The data on the synthesis of the myelin lipid components coincide with the data established in vivo for the respective animal species [22,43].

During the whole period of myelinogenesis, and especially during active myelination, there is a strong activation of the enzymes included in the synthesis of myelin lipids: galactosylceramide' sulfotransferase (E.C. 2.8.2.11) and ceramidegalactosyltransferase (E.C. 2.4.145) [42, 65, 66].

The main myelin proteins: myelin basic proteins (MBP), proteolipid proteins (PLP) and myelin-associated glycoprotein (MAG), are synthesized in vitro slightly later than the myelin lipids (about one week after the galactolipids) [25], but they follow the order established in vivo.

MAG is a minor transmembrane glycoprotein of the myelin in the central nervous system, which is limited to the periaxonal and noncompact areas of the myelin sheath [78]. Applying immunocytochemical techniques, it has been found that MAG appears in the oligodendrocytes later than MBP (on the 4th day in vitro in mixed cultures of dog cerebellum) and is assumed to play a certain role in the interactions between the action and the myelin-forming cells. Oligodendrocytes express MAG, establishing a contact with the axon as one of the first steps of

myelination, bofore the other components have been formed [90]. These assumptions were confirmed by the findings of D u b o i s - D a 1 c q et al. [25], who have found galactolipid-positive cells in oligodendrocytic culture from the brain of 16 to 17-day-old mouse embryos in the very first days, and MAG-positive cells around the 5th-7th day. MAG is localized perinuclearly and it is the first of the myelin proteins to be observed in the oligodendrocyte processes around the 10th day.

MBP is synthesized on the free ribosomes in the cytoplasm and in the processes of the oligodendrocytes [64], being identified immunocytochemically in myelinating oligodendrocytes, diffusely in the cytoplasm [74], and in the major dense line of the myelin sheath [53].

During the first days the MBP content in rat fetal brain aggregated culture is very low. After the 18th day their amount begins to increase — more than ten times until the 40th day [41]. In dissociated cultures in the cytoplasm of the oligodendrocytes MBP is seen during the first week [25], but only after the second week all cells have a positive reaction to anti-MBP antiserum [63] and this antigen is detected in the processses as well, i.e. one week after the first visualization in the cytoplasm [25].

PLP, the major myelin proteins, are involved in the process of increasing the density end stabilization of myelin. They are synthesized on membrane-bound ribosomes, mainly in the oligodendrocytic cell body, becoming acylated after passing through the Golgi apparatus and being incorporated in the myelin membranes [77]. They are demonstrable immunocytochemically in the myelinating cells 1-2 days after MBP [19], as well as in vivo. Maximum PLP synthesis is observed several days later, as compared with MBP.

The enzyme 2'-3'-cyclic nucleotide phosphohydrolase, (EC 3.1.2.37), which is specific for myelin, increases during the whole period of myelination, where its maximum activity coincides with the period of active myelinogenesis [42, 57].

The visualization of the myelin sheaths using different histochemical and electron microscopic methods depends on the cultivation system and coincides with the time of the deposition of myelin components in the sheath. It becomes denser with time and acquires a compact lamellar structure, with alternation of the major dense and intraperiod lines, as in the normal myelin [21].

The myelination process in organotypic cultures is completed towards the end of the third week [20]. In aggregated and dissociated monolayer cultures it is difficult to determine the exact terms, because a degeneration occurs in the neurons and oligodendrocytes after 3-4 weeks in vitro [42, 65, 66].

Many publications appeared recently on the formation of myelin membranes in pure oligodendrocyte culture, in the absence of neurons [1, 18, 25, 60]. The term “myelin-like” is most frequently used, because there is still no absolute clarity on the degree to which these membranes correspond to the mature myelin in vivo. They possess all components characteristic of the mature compact myelin, but the correlation between them is rather different. The evidence about CNP is contradictory [1, 60]. As compared to the proteins, the quantities of cerebrosides and sulphatides are twice higher in the myelin-like fraction than in the compact myelin [60]. The MBP synthesis is considerably delayed and reduced [1]. The morphological picture of the myelin-like membranes does not correspond always to that of the mature myelin. There often appear loosely wrapped lamellae, without a clearly identifiable major dense and intraperiod lines, referred to as “premature myelin” by Althaus et al. Many results indicate a similarity between the myelin-like lamellae and the mature compact myelin (density, enzyme activity

and lipids), but there are also distinct differences, mainly in the protein content, and these membranes may be precursors to mature compact myelin [60].

There are still no exact data on the participation of the cell elements in the synthesis of the myelin components and on their interaction during in vitro myelinogenesis. Similar to the in vivo system, the priority is given to the oligodendrocytes. There is no evidence about the direct participation of neurons in this in vitro process. Little is known about the nature of the processes which induce or regulate the changes in the oligodendrocytes, mainly with a view to their proliferation and the onset of the synthesis of the myelin 'components. Direct cell contacts are found to influence some biochemical parameters of the cell. Neuronal stimulation results in increased proliferation of the glial cells. The molecular mechanisms involved in the cell proliferation have not been studied yet, but it is assumed that the changes on the cell surface due to the “cell-cell” contact influence this process of increased synthesis of cell proteins [80], although it is not known to what extent these mechanisms are under genetic control. Transferrin, which is considered to be a neurotrophic factor [4], is synthesized by the oligodendrocytes and increases during myelination. It probably plays some special role in this process, with a stimulating effect on the neurons [9]. Evidence has been found recently about the stimulating influence of some growth factors on the individual cell elements of the nerve tissue, although they are not yet associated with the myelination process: nevertheless, they may influence its course indirectly. A stimulating effect on the astrocytes is observed for EGF [81], interleukin 1 [32], PDGF [35], AGF(A) and AGF(B) [56] and thrombin [54]. Oligodendrocytes are stimulated by the brain extract [23], FGF[8,26], IGFj [44]. The problem concerning the stimulating effect of some factors on neuroblasts and neurons is least studied. The action of two factors has been proved for the time being: of FGF(B) [29] and meningeal extract [30]. The unsuccessful attempts connected with the isolation, survival and maturing of the neurons can explain the fact that mature myelin sheaths are seldom formed in dissociated brain cultures [83]. The role of the neuron in this process is probably much more active. Zaprianova [88, 89] expressed a view about the existence of neuron-glia interactions in the product of CNS myelin phospolipids in vivo. The stimulating effect of the nerve cells in the proliferation of the glia, prior to the beginning of visible myelination, has been proved [10, 11]. Another important factor influencing the process of normal in vitro myelination is the obligatory presence of astrocytes in the cultivation system. The beginning of myelination is preceded by fasciculation of the glia, with the formation of bundles on which the mature oligodendrocytes are localized, which have a positive reaction to antiserum against galactocerebrosides [83]. The fasciculation results in the appearance of the necessary micromedium which leads to extensive myelination [83]. No fasciculation is observed in dissociated cultures in which the viable neurons are few or lacking, whereas astrocytes with their processes form a continuous network on which the oligodendrocytes are localized [70]. This fact clearly demonstrates the influence of the neurons on this process. The signal for the onset and for the discontinuation of myelination, and for the proliferation of oligodendrocytes probably comes from the neuron, either directly or through the axon, because myelination of all axons simultaneously is never observed and, moreover, the division of the oligodendrocytes is also asynchronous [5, 73].

There exist data on the influence of the thyroid hormones on myelination. The direct effect of 3'-triiodothyronin (T_3) on myelination, in its capacity of monitor of the synthesis of some lipids closely connected with myelin, is demonstrated by using an experimental model in which mixed cell cultures grow in a medium containing serum of calves after thyroidectomy. Low degree of

synthesis of sulphatides and CNP is observed in hypothyroid serum [6, 7, 71, 86] which is restored to its normal level after addition of normal serum or of T_3. The activity of the lipid sulphotransferases is disturbed by more than 30 per cent [6]. A dose-dependent effect is observed: the sulphatide synthesis increases with the increase of the doses of T_3 and T_4 (thyroxine). T_3 (the active form of the hormone) is 30 times more active than T_4. It has been proved that the maximum effect of the thyroid hormones on the expression of CNP and sulphatides is found in the earlier cultures.

In vitro myelinogenesis can be delayed or totally inhibited by changing the culture medium. Myelinogenesis is inhibited when antisera against components of the central nervous system are added to the nutrient medium, e. g. antiserum against the white matter of the brain [14], antiserum against galactolipids [84, 85], and antiserum against gangliosides [84, 85]. The mechanisms of action of the antisera on myelinogenesis are not yet known.

Although many studies have been devoted to the myelination in vivo and in vitro, many problems connected with this process still remain unsolved.

1. It has not been completely clarified where the myelin components and their precursors are synthesized, and what control of the synthesis is realized.
2. Which cell types participate in the synthesis of the myelin components and what interactions exist between them?
3. What is the exact role of the neuron in the in vitro myelinogenesis?
4. What biologically active substances participate in the regulation of the cell interactions during myelinogenesis?
5. What is the mechanism of action of the endogenous brain stimulators?
6. Which elements of the nerve tissue are influenced by the antiserum myelin-inhibiting factors?

In this connection, the tissue culture provides a convenient system to study factors regulating neuron-glia interaction and expression of myelin components during myelinogenesis.

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Transplantations of olygodendrocytes in the CNS

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The intracerebral transplantation in the newborn mouse central nervous system (CNS) is so far the only method allowing long-term investigations of identified populations of olygodendrocytes in the very complex spatial environment on the developing CNS *in situ.* This technique, coupled with immunohistochemical and ultrastructural studies provides a dynamic system which allowed the autors to study the myelination process *in situ.*

Key words: intracerebral transplantation, olygodendrocytes, myelination, homochronic grafts, heterochronic grafts.

From the first studies on myelination, most often performed in man, it has been clearly established that it does not proceed throughout the CNS at one and the same time, but seems to follow in some way the caudo-rostral gradient of the phylogenetic development of this system. The same gradient is observed in rodents which for practical reasons have been widely used as models to study the myelination process. In mouse, myelination starts at birth and is almost completely achieved at P35, allowing the study of the entire process in a very short period of time.

Three types of classical approaches have contributed to the knowledge of glial cell maturation and myelination:

— First, the biochemical analysis of myelin components and the sequential study of their deposition in the myelin sheath have been performed on normal and dysmyelinating mutant mice and provided much information to aid our understanding of myelin formation [3].

— More recently, the production of poly- or monoclonal antisera allowed to detect *in situ* the presence and localization of specific antigenic components. These immunohistochemical studies proved very fruitful in identifying the sequential synthesis of these components in the myelin-forming cells and in analysing their deposition during the myelin formation. Moreover, these techniques have allowed to identify the myelin-forming cells early in development [13] and to trace the different glial cell lines [1, 16, 17, 18].

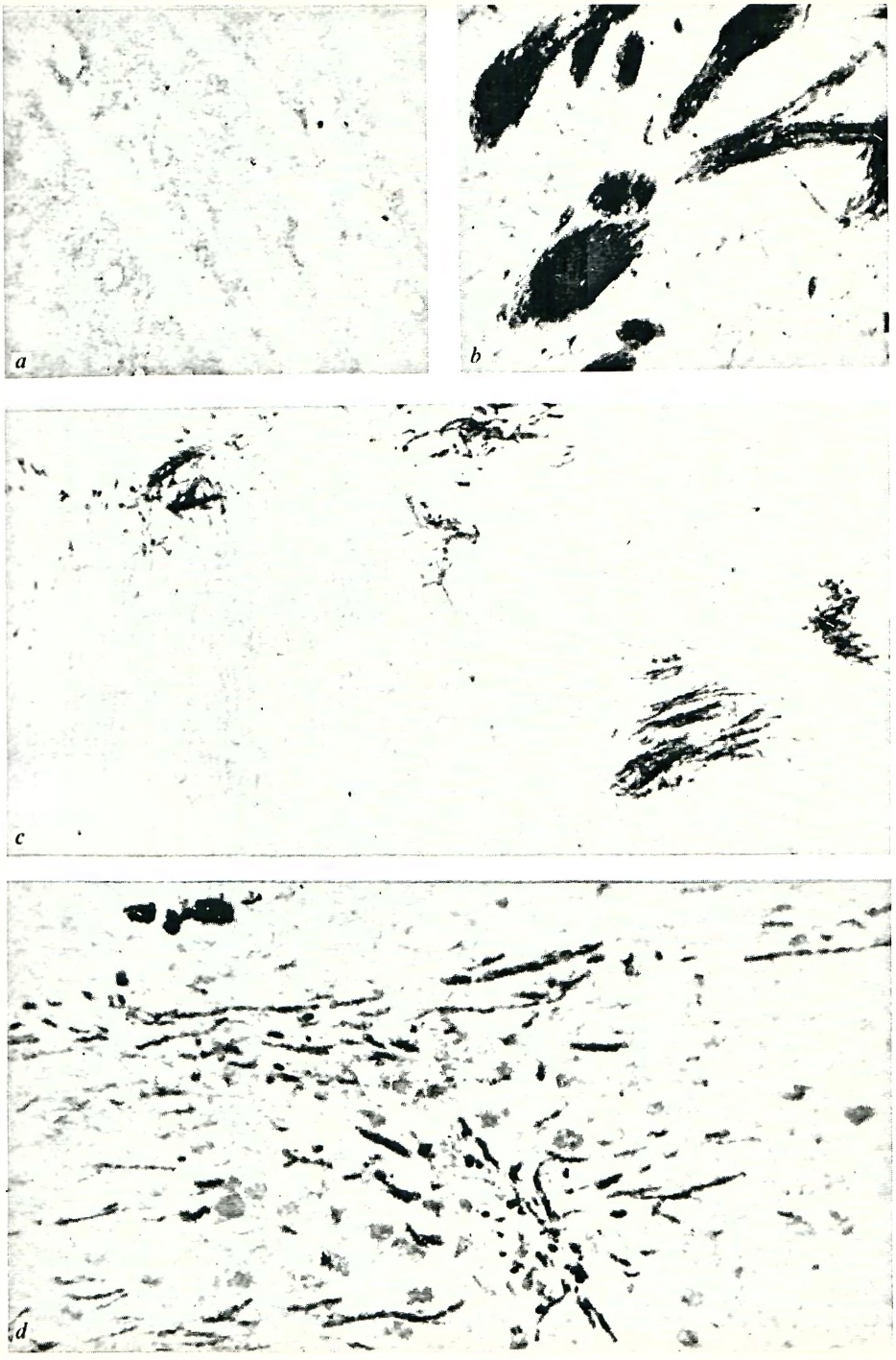

Fig. 1. Immunohistochemical study of the MBP-positive myelin synthesized by transplanted oligodendrocytes (PAP technique)

a absence of immunostaining with anti-MBP anti-serum in the striatum of an adult (2 months) shi/shi homozygote x 150); *b* strong immunostaining with the same anti-MBP anti-serum in the striatum of a control normal mouse of the same age. (PAP technique) x 150); *c* dispersion of the MBP-positive myelin patches in the shi/shi brain 70 days after graft. (Fragment of olfactory bulb from newborn control mouse into the newborn shi/shi brain) (x 300); *d* high magnification of a M BP-positive myelin patch: axons from different fascicles are myelinated by both normal myelin and shiverer myelin (x 700)

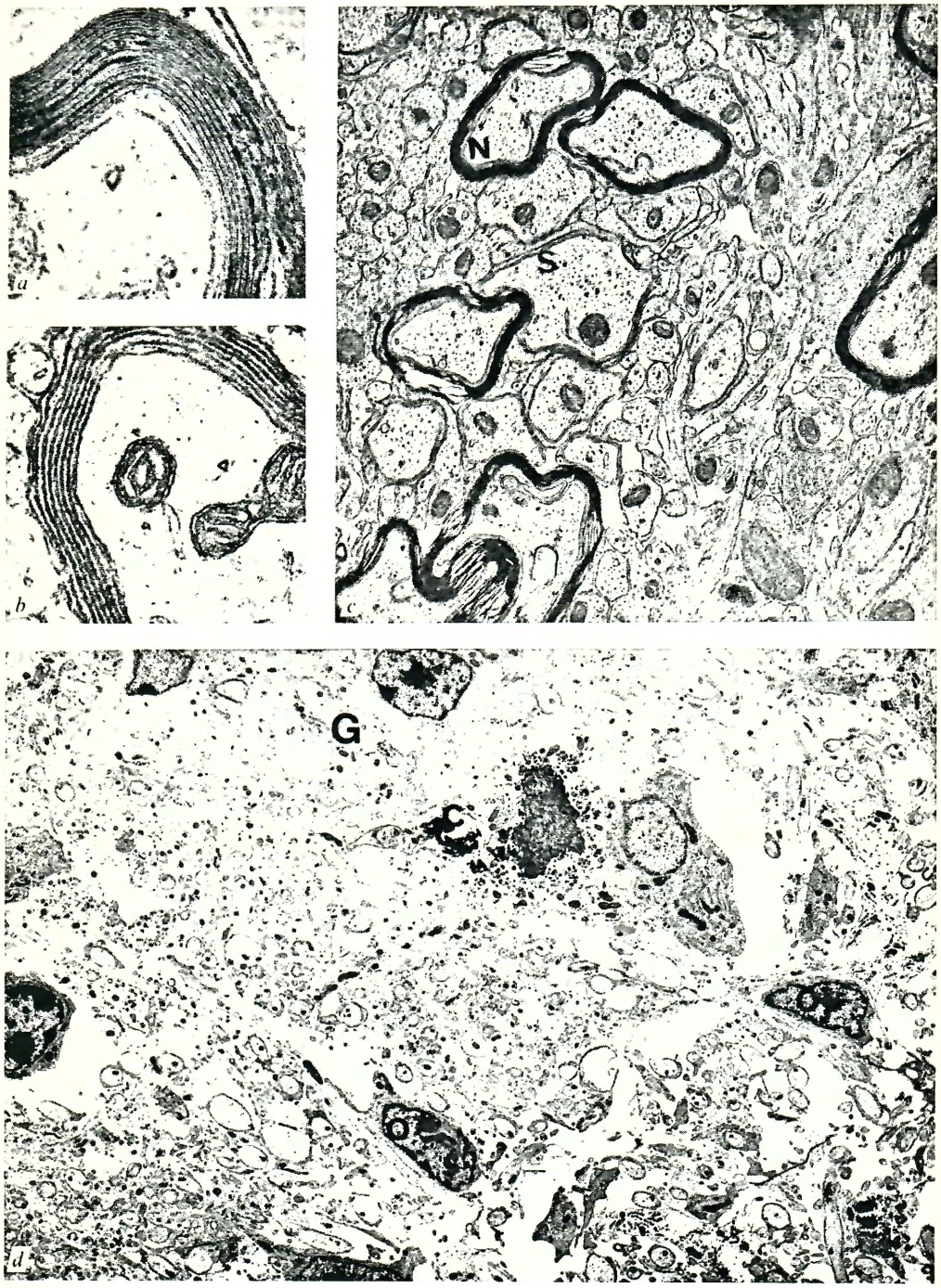

Fig. 2. Ultrastructural study

a the normal myelin, well compacted, presents a continuous major dense line (x 1000 000); *b* -» the shiverer myelin is poorly L-ompacted and devoid of the major dense line (x 100000); *c* – - normal myelinated axons (N) among shiverer-myelinated axons (S) in the same fascicles (x 20000); *d* — aspect of the ring of cell processes surrounding the graft (G); one can observe charcoal (C) and oligodendrocytes (O) leaving the graft 12 days after transplantation (x5000)

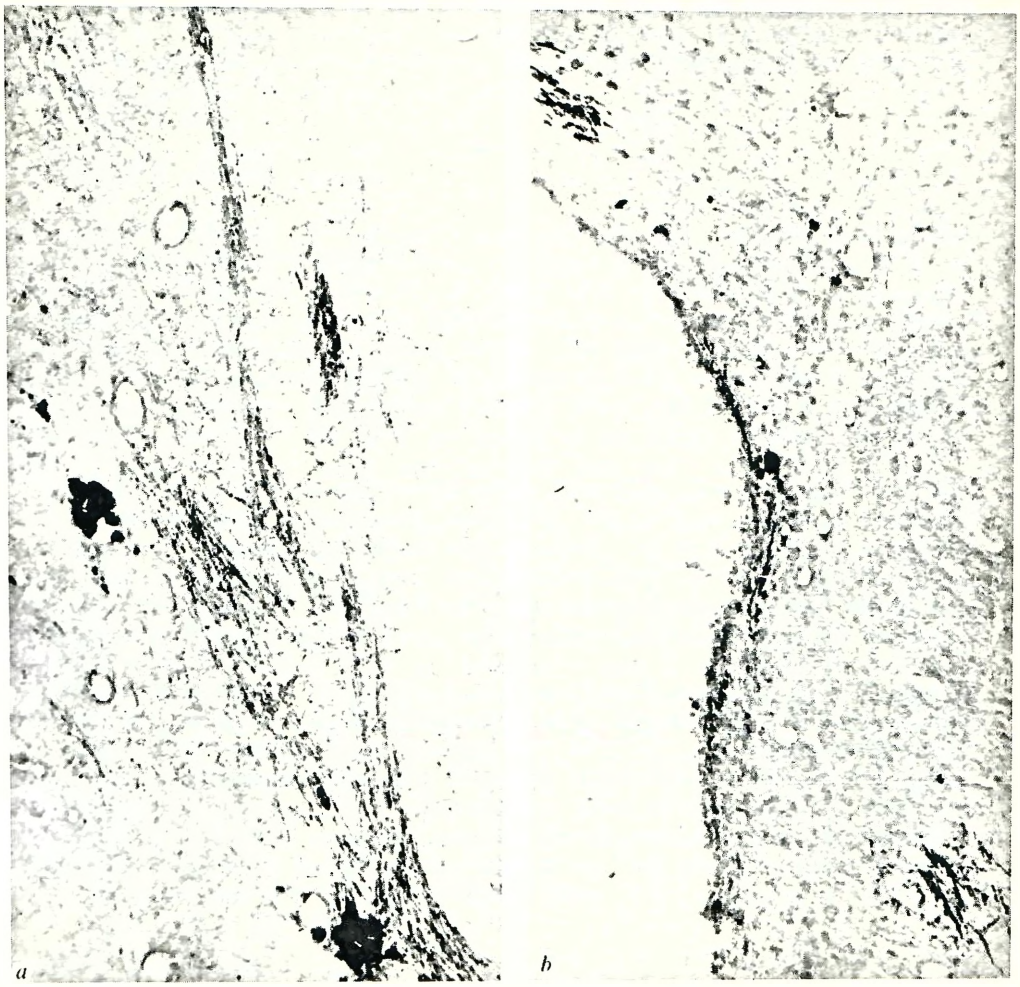

Fig. 3

a — oligodendrocytes from spinal cord of normal mouse synthesize M BP-positive myelin in the striatum of the shi / shi host brain (newborn in newborn 60 days after grafting) (x 190); *b* normal oligodendrocytes from cerebellum synthesize M BP-positive myelin in the lateral ventricle region of the shi/shi host brain (newborn 70 days after grafting) (x 190)

— At last, organotypic as well as dissociated brain cell cultures have been widely used to study the neuroglial relationships. They are very useful in defining the role of intrinsic factors regulating the differentiation and behaviour of myelinating cells [4, 21, 22].

The Shiverer model

This type of work can only be carried out with a clear identification of the implanted cells or their products in the host parenchyma. In order to identify the transplanted oligodendrocytes or at least the myelin they form post-transplantation, we have developed the "Shiverer model" using two phenotypical traits of the Shiverer mutation of the mouse. The autosomal recessive mutation Shiverer (shi) [14] has been identified to a deletion of five out of the seven axons of the structural gene encoding of myelin basic protein (MBP) [19] in chr. 18 [20]. Homozygotes are fertile but their lifespan is considerably reduced (90 to 150 days).

Biochemical as well as immunohistochemical studies have previously shown a total absence of MBP in the homozygote CNS [11, 5]. Thus, using an anti-MBP antiserum it is possible to detect the presence of the myelin formed by transplanted oligodendrocytes able to synthetize MBP [7, 12] (Fig. 1 *a, b, c*). Moreover, this defect is correlated with an absence of the major dense line (MDL), a reduced number of sheaths, and a defect of compaction of the myelin in the homozygotes CNS [15] (Fig. 2 *a, b).*

Electron microscopy (EMI allows the identification of the normal myelin
'hesized by the transplanted^ oligodendrocytes. This myelin is well compacted
presents a well defined major dense line [6] (Fig. 1 *b).* Different types of
dinating cells (oligodendrocytes, Schwann cells) have been grafted under
arious conditions (fragments of CNS tissue, pellets of reaggregated cells) into Shiverer mice CNS at different age. The examples discussed here will be limited to results obtained after implantation of fragments of neural tissue form different species into newborn shi/shi homozygote brains.

Grafting technique

The grafting technique used in all types of experiments has been extensively described in previous papers [7, 12]. Briefly, tissue from defined regions of the donor's CNS are dissected out and placed in saline on ice. The meninges are carefully removed and the tissues are cut up into 1 / 2 mm^3 fragments. Then, the fragments are rolled in charcoal used as a marker to localize the implant in the host parenchyma. After cold anaesthesia, the receiver's skull is incised with an iridectomy scissor and one or several fragments are introduced into the host's brain using a thin glass pipet connected to a peristaltic micropump.

Homochronic grafts of normal mouse CNS tissue into the newborn Shiverer CNS

Myelinating behaviour

In the first series of experiments, fragments of olfactory bulb from newborn normal mice were implanted into the rostral thalamus of newborn shi/shi homozygotes.

The myelinating behaviour of the grafted oligodendrocytes was evaluated by staining the M BP-positive myelin synthesized by these cells in the host mutant brain. The immunodetection was performed by classical immunohistological procedure on frozen sections of the CNS tissue of the host as described elsewhere [7, 12]. Sagittal sections were cut at the level where charcoal indicated the presence of the graft. The study was performed every 5 days from day 10th to day 30th after transplantation, and then at day 50th, 70th, 90th, 120th and 150th. In these homochronic conditions (newborn transplant into newborn host brain) normal oligodendrocytes were able to survive, migrate and synthesize MBP-positive myelin in the host parenchyma (Fig. 1 *c*). Simultaneously, "Sham experiments" were performed in which fragments of olfactory bulb from newborn shi/shi homozygotes were injected into the brain of newborn shi/shi homozygotes. In no case MBP-positive myelin was observed, whatever the age of the examined animals [2, 7, 8, 9, 12]. Chronological studies showed that the first M BP-positive patches were observed from day 15th after implantation. The amount of myelin formed and its dispersion in the host brain were maximum at day 20-25th and did not increase or decrease after this time during the host's lifespan.

Distribution of the MBP-positive myelin

In order to study the distribution of the M BP-positive myelin patches, semiserial horizontal sections of the entire grafted brain were performed 50 to 150 days after grafting [2]. This study showed that myelin patches were widely distributed throughout the brain in both hemispheres and in the commissures. They were generally isolated from each other and one could never reconstitute a continuum of M BP-positive myelin from the region of the graft up to the more distant patches. At last, whatever the extension and the dispersion of the patches, MBP-positive fibers were always visible at the periphery of the graft. When immunoreactive myelin segments were tightly aligned in the same fascicle, patches appeared rather dense, but in no case were all axons in one fascicle wrapped by normal myelin as proved by EM (Fig. 1 *d).*

Migration behaviour of the transplanted cells

The location of MBP-positive patches at long distances from the site of grafting suggests significant migration of transplanted cells. In several cases we found M BP-positivity in the rostral spinal cord even when the graft was placed in the thalamus [2]. In order to analyse the conditions of this migration, we designed two types of experiments. First, in order to evaluate the influence of the cellular environment, fragments of olfactory bulb were injected into different regions of the CNS [9]. Second, fragments from different regions of the CNS (spinal cord, cerebellum, roof of the lateral ventricle, olfactory bulb) were injected into the rostral thalamus. We thus tried to determine: a) if a regional heterogeneity of the oligodendrocyte population exists, and b) if privileged targets exist for oligodendrocytes from defined anatomical regions [2].

In both types of experiments the transplanted oligodendrocytes were able to survive, migrate and synthetise MBP-positive myelin after transplantation, and no behavioural trait could be correlated with the origin of the graft (Fig. 3 *a, b).* However, the migration appeared to be correlated in some way with the anatomical environment. More precisely, the dispersion of the oligodendrocytes was favoured by the proximity of large axonal pathways [2].

Host-graft interactions

This study has been performed on shi / shi homozygotes implanted at birth with fragments of olfactory bulb from normal mice.

The graft region was dissected and processed for EM, following standard procedures [6]. An ultrastructural examination of the graft showed an intensive membrane activity between graft and host tissue from 6 to 15 days. A ring of cell processes arising from all types of neural cells surrounded the graft. Cells from both graft and host tissue contributed to the formation of this ring. Moreover, isolated oligodendrocytes have been observed between the host and the graft, suggesting that these cells are leaving the graft (Fig. 2 *d).*

Healthy astrocytes, oligodendrocytes and neurons could be observed in the graft even 130 days after transplantation. At this stage the graft was surrounded by gliotic astrocytes and isolated from the host tissue by a basal lamina. No sign of acute rejection was observed.

Ultrastructural study of newly-formed myelin

The EM examination of the implanted region showed that in transverse sections axons myelinated by normal oligodendrocytes were dispersed among bundles of nonmyelinated axons or axons surrounded by Shiverer myelin. The normal myelin, morp compacted and more electron-dense could be easily distinguished from the Shiverer myelin (Fig. 2 *d).* Longitudinal sections showed that the same axon might be myelinated by both types of myelin. In this case the node of Ranvier seemed normal, although the number of oligodendroglial loops formed at the node was greater on the normally myelinated side than on the abnormal side.

At higher magnification, the Shiverer myelin was typically noncompacted, while the normal myelin was thicker and presented a well-defined continuous MDL.

Because of the very extensive dispersion of the normal myelin patches observed after transplantation, it was necessary to design a procedure allowing the immunohistochemical prelocalization of the M BP-positive regions in the host brain before their ultrastructural observation. We used in this case thick vibratome sections (60[*n*). One of two sections wastreatedfor immunohistolocalization of the M BP-positive patches and the corresponding region of the adjacent section was dissected and prepared for classical EM [6].

After coloration with toluidine blue, the normal myelin which was thicker and more contrasted, could be distinguished from the Shiverer myelin on semithin sections. Their EM examination showed that they could be recognized ultrastructurally as normal myelin and one similar to the myelin observed at short distances from the graft. No sign of rejection (inflammatory reaction, reactive gliosis) was observed in the vicinity of the normal myelin even 150 days after implantation.

Heterochronic grafts: transplantation of human embryonic CNS tissue into the newborn mouse brain

In order to investigate the chronological aspects of myelination, homochronic transplantations — newborn into newborn — were carried out.

These studies demonstrate that the timing of myelination by transplanted oligodendrocytes is similar whatever the origin of the graft and wherever the site

of implantation. However, the first appearance of myelin is delayed (15 days after transplantation), when compared with myelination *in situ* [10].

In order to demonstrate an inductive or modulating effect of the cell environment on the differentiation and the myelinating behaviour of the transplanted cells, we designed an experiment in which human embryonic CNS tissues were grafted into the newborn shi / shi brain. Indeed, in man, according to Yakovlev and Lecours [23], myelination starts at E150 in the motor roots of the spinal cord and at E180 in the brain itself. By contrast, it starts at E 20 and is almost completely achieved at P 35 in the mouse. The timing of myelin differentiation in these two species is so different that interspecies grafts may provide a useful model to demonstrate the effect of environment on oligodendrocyte differentiation.

The human MBP is cross-reactive with the antisera used in our homospecific experiments, and the human myelin can be recognized by EM because of its high degree of compaction and the presence of MDL. It was thus possible to study the myelinating behaviour of the transplanted oligodendrocytes [10].

In a preliminary series, fragments of human embryonic CNS tissue from different regions were grafted into newborn shi / shi rostral thalamus. Under these conditions human oligodendrocytes from all parts of the CNS were able to differentiate, migrate, and form M BP-positive myelin at distance from the graft. The ultrastructural examination confirmed the normal phenotype of MBP-positive myelinated fascicle. Transverse ultrathin sections through major fascicles showed axons with normal myelin adjacent to axons with Shiverer myelin [10].

The chronological study of the MBP-positive myelin was performed on a series of shi/shi brains implanted at birth with human embryonic CNS (fragments of the roof of the lateral ventricles). Tissue from 6,10,17 and 18-week-embryos was used for this study.

In these series, MBP-positive myelin was observed 20-30 days after transplantation. Under these conditions human cell lines differentiated and matured much more rapidly than *in situ.* These results showed a strong modulating influence of the cell environment on the timing of differentiation of the transplanted cells.

Conclusions

Intracerebral transplantations in the newborn mouse CNS is so far the only method allowing a long-term study of identified populations of oligodendrocytes in the very complex spatial environment of the developing CNS *in situ.*

The fact that normal (mouse or human) and Shiverer' oligodendrocytes compete to myelinate axons in the same fascicle suggests a recognition of similar myelination signals in different species. This lends support to the use of this model in the study of the behaviour of myelinating cells in vivo. This may be especially true in the case of oligodendrocytes, which seem to be sensitive to the biological environment for the expression of their sophisticated behaviour.

The long-distance dispersion of M BP-positive myelin patches attributed to migratory behaviour of the transplanted oligodendrocytes after birth reflects their usual behaviour. This can only be studied under in vivo conditions.

The study of the first appearance of MBP-positive myelin after transplantation suggests a strict timing of myelin expression under these conditions regardless of oligodendrocyte origin. Mouse and human implanted oligodendrocytes express MBP 15 to 20 days after transplantation. This is very

surprising in case of human oligodendrocytes which would not be expected to myelinate for 10 weeks after transplantation. Therefore, there may be a strong modulating effect of the host environment on the differentiation chronology of grafted cells.

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Reactive recombination of myelin nervous fibres in the process of normal myelination

(Hypothesis on the further development of myelination mechanism)

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N. n. tibiales were investigated in 2 to 11-day-old rats, using electron microscopy. All the main types of membrane junctions were found: serial desmosomal, continuous, septate, gap and tight junctions. The junctions were both of glio-glial and axonglial type. These types may be stages of a unified process of membrane interaction. They are located in multiple loci and form intermediate dense myelin line upon merging. Such junctions result from aggregation and retraction of outer paramembrane electron-dense material. The same mechanism of inner surface membrane coupling was observed in lamellopodies of lemmocytes. Thus, "inside-out" local junctions were fomed. Merging of these junctions forms the main dense line of myelin. Consequently, compact myelin, thus formed, should be considered as a gigantic complex membrane junction.

Key words: myelination, ontogenesis, membrane junctions, myelin fibres.

At present there exist two groups of hypotheses trying to elucidate the complex mechanism of myelin sheath formation. The first group supposes myelin to be the product of lemmocyte membrane of the Schwann cell [3, 9]. The second group of hypotheses was based on morpho-biochemical research. According to it, in the process of myelin formation equally important part play both the neuron and the gliocyte. Not only gliocyte but also neuron do participate in the phospholipid and protein provision of the biogenesis of myelin sheath [2, 22]. Both concepts presuppose the existence of certain intermembrane glio-glial or glio-neuron interactions. In this respect, frequently desorbed findings of glio-neuron and glio-glial intermembrane junctions are of special interest. The role of intercellular junctions in ontogenetic development of many organs has been known for a long time [16]. The part these junctions play in the process of myelination seems to be significant. We consider this problem to be of particular interest because our laboratory has shown the reactive nature of some glio-glial

and glio-neural intercellular junctions. They appear in the period of increased activity of the nervous system, during some changes in metabolism and upsetting of homeostasis of intercellular liquid. As one of the main conditions of the commencement of myelination is a certain signal coming from the axon to the glial cell [8, 10,], myelination may be considered a reactive process, a response to the axon signal followed by recombination of the glial membrane. It may well be that this axon signal is not a special factor, but an ordinary primary impulse activity of some developing nonmyelinized axons, connected with the release ol potassium ions by the axon and their assimilation by the glial cell [4, 10]. This study considers myelination as a reactive process accompanied by membrane alteration and membrane junction formation.

Materials and methods

Our concepts are based on the study of the myelination process in Wistar breed white rats aged from one to eleven days. The object of research was nervus tibialis. The rats were decapitated, a fragment of the nerve was segregated and fixed in 2,5% glutaraldehyde on phosphate buffer. Postfixation was then carried out in 1 % solution of osmium tetraoxide. The substance was enclosed in araldite and examined by means of transmission electron microscopy.

Results and discussion

Rats aged from 24 to 36 hours did not display any myelin nervous fibres (non-myelin period). Around non-myelin axons several flat and broad cytoplasmic processes of lemmocyte (windings) were formed. Forty-eight-hour-old rats showed a few fibres with myelin sheath having sometimes up to 6-8 lamellae (Fig. 1). In this period we succeeded in finding the first structures suggesting the formation of Ranvier nodes and Schmidt-Lantermann clefts. That was the earl} myelination period. It should be noted that transition from the first to the second stage takes not days but hours.

The period of active myelination was registered in 5 to 11-day-old rats, greal variations in the degree of myelination being observed. Some fibres already had a developed structure of nodes and clefts and a thick myelin sheath of up to 30-4C lamellae of myelin. Other fibres were in the incipient stages of myelination or did not have myelin at all. At all stages of nerve fibre myelination there is a greal number of membrane junctions. This suggests their sharing in the process ol myelin sheath formation.

Just as it has been shown for gliocytes or the central nervous system, the non-participating in myelinogenesis lemmocytes are, firstly, capable of forming all known types of membrane junctions and, secondly, they do it in two differenl ways. The first one represents a well-known junction between the outei membrane surfaces (Fig. 2) of two adjacent lemmocytes or two adjacenl cytoplasmic tongues of one and the same lemmocyte. We observed suet junctions at all stages of myelination throughout the fibre. All types of junction! are present: septate (sj), continuous (cj), gap (gj) and tight (tj) junction, as well as others. At later stages, however, the number and size of tj and gj increases. This happens probably as a result of gradual transformation of the various types ol junctions into tj and their gradual fusion. As a result of fusion of adjacenl junctions formed by their membrane outer surfaces, intermediate dense lines ol myelin are formed. Such elongated junctions (lines) may form independently ol

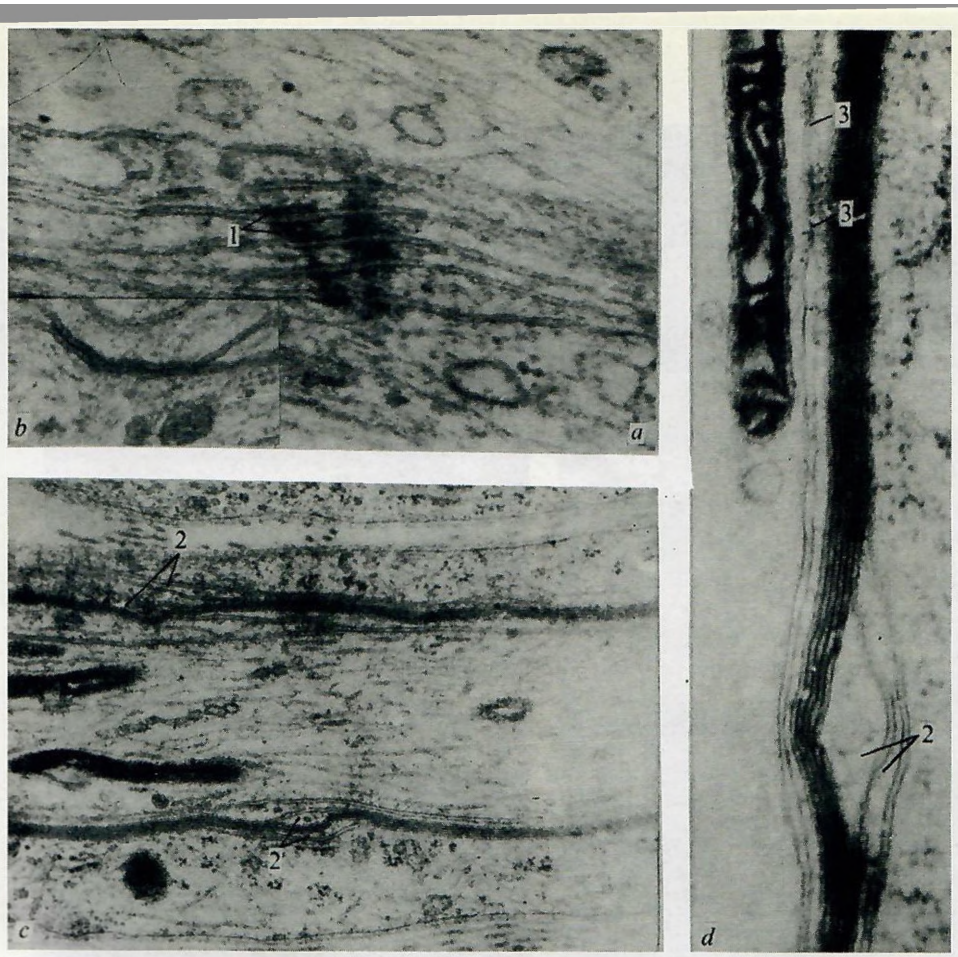
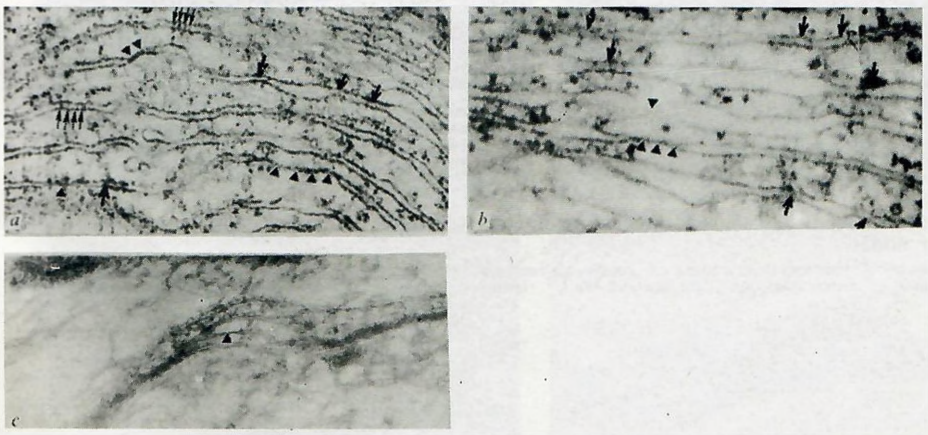


Fig. 1. Initial period of myelination (2 to 4-day-old animals)

a serial desmosome-like structures (1) in 6-layer glial non-myelin sheath; *b* — local "inside-out" junction glio-glial junction; c — beginning of myelination, cleft formation (2); *d* axonal-glial junctions (3) and a developing cleft; *a b d* x 40000; *c* — x 16000

Fig. 2. Formation of junctions between lemmocyte membrane outer surfaces

a adhesion aggregates and developing continuous junctions (thick arrows); *b* — septate junctions (fine arrows) and tight junctions (trangles); c — tight junction in the zone of a developing cleft; *a*. *b* — x 40000; *c* — x 18000



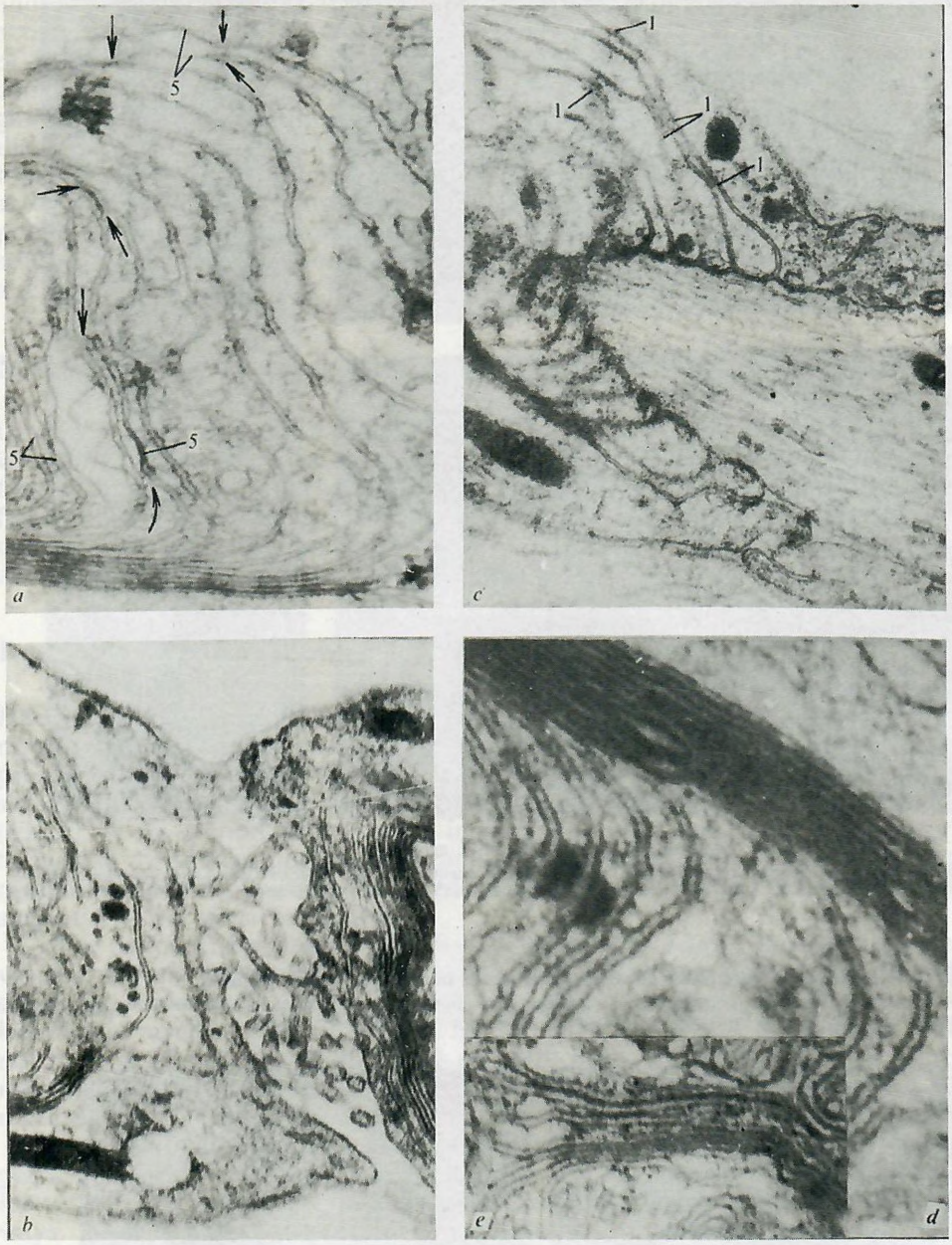


Fig. 3. Junctions of outer and inner glial membrane surfaces (“inside-out” junctions) in the zone of Ranvier nodes

a, *b* “inside-out” junctions (long arrows); *c* — continuous junctions (1); *d* septate junction passing into “insideout" desmosomal-like junctions; *e* serial “inside-out” tight junctions (5); 6 lemmocyte plasma: *a*, *e* x40000;/>, *c* x20000;rf x 60000

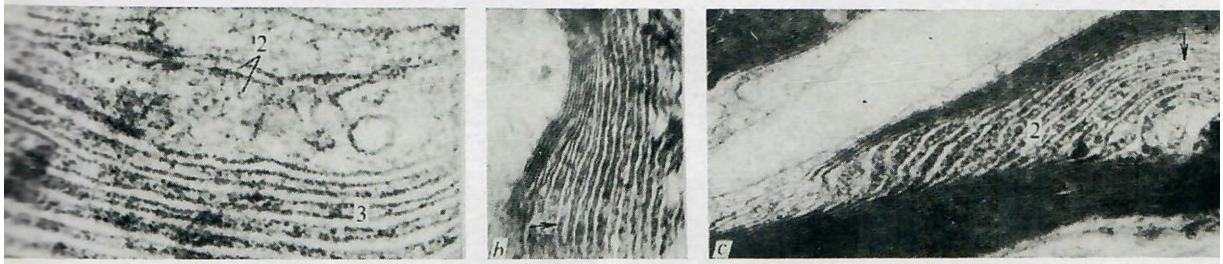
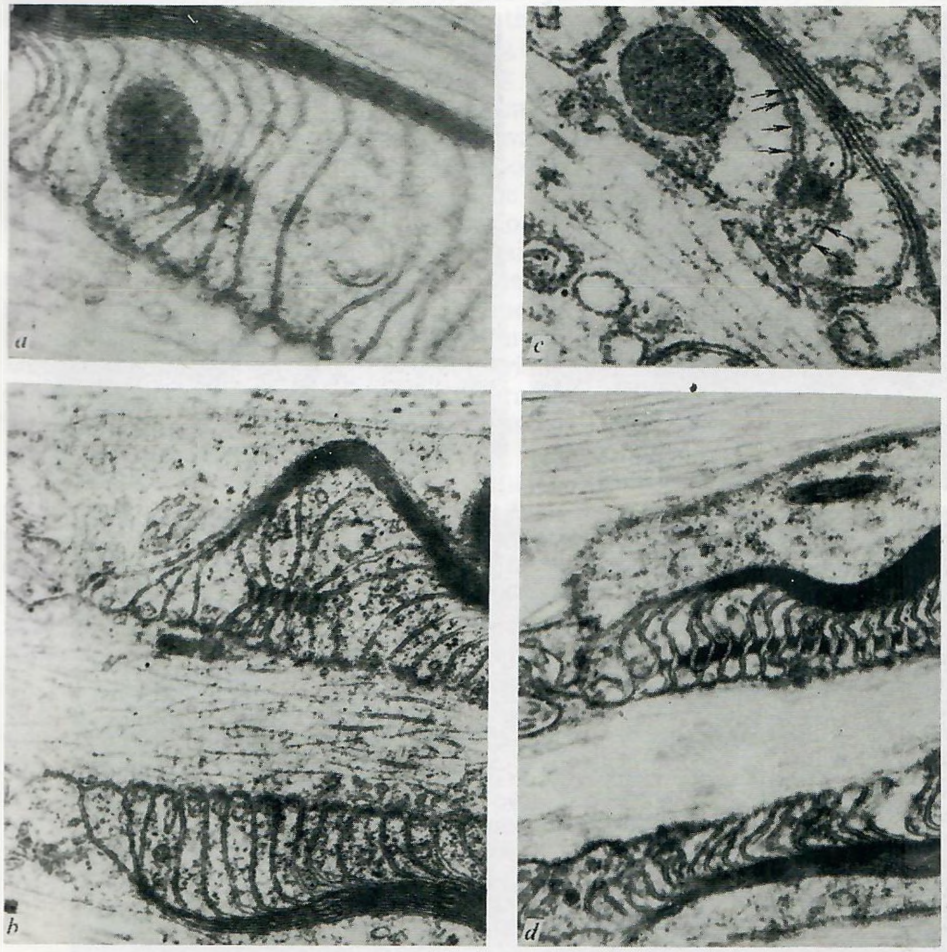


Fig. 5. Sites of membrane premyelin organization (arrows)

a second (2) and third (3) types of glial layer organization; *b* premyelin formation in paranodal zone; *c* formation of premyelin (serial "inside-out" continues junctions) in the zone of clefts; *a* x 48 000, *b*, *c* x 36000

Fig. 6. Serial desmosomal junctions

a. *h* retractive activity of serial junction submembrane aggregates; *c* stick-like junction structures participating in membrane "zip-fastening" (arrows); *d*—serial junction as indication of long-ran—local membrane interactions; *a*— 48000; *b*, *d*—x 18000; c— x 60000



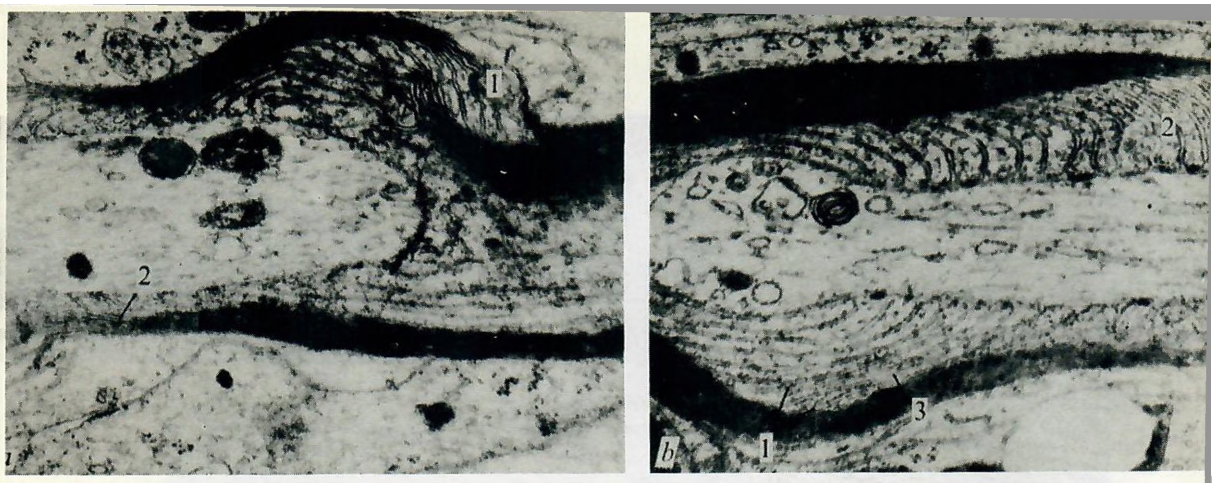
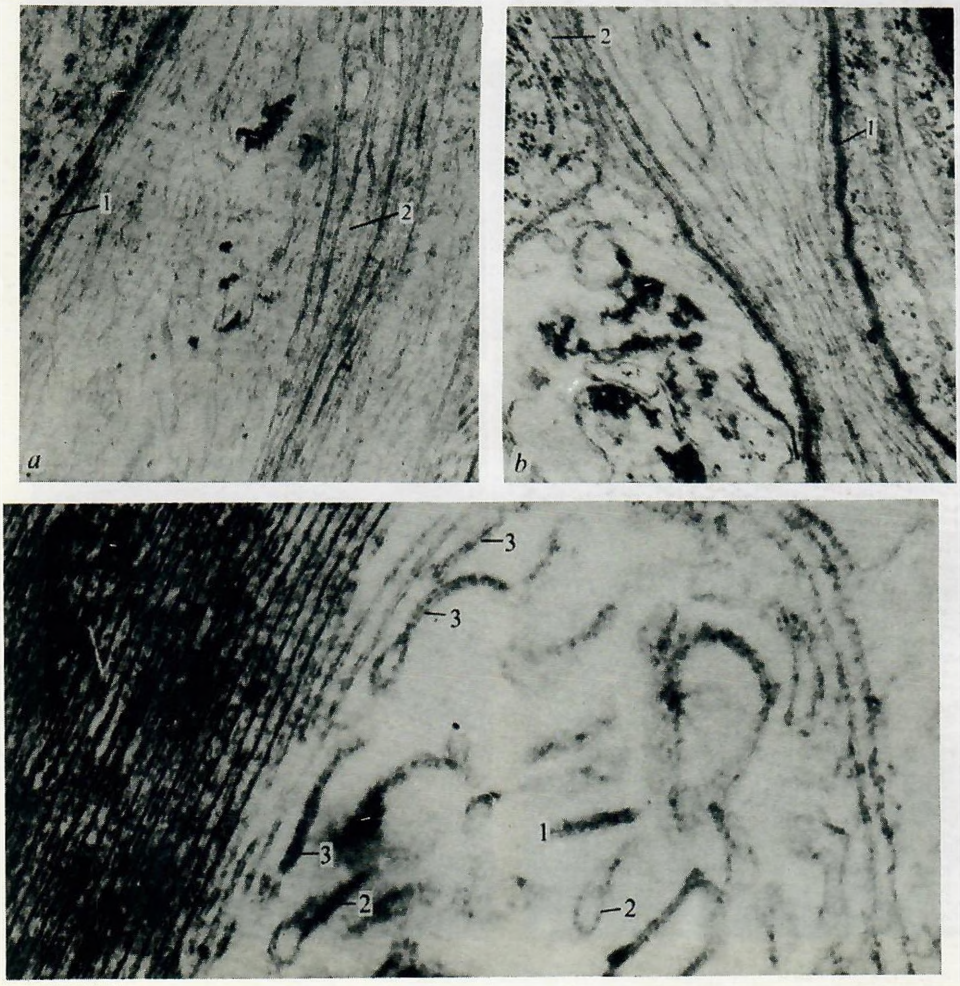


Fig. 7. Different types *(a, b)* of unusual structures (cleft-nodes) in developing myelin
1 developing cleft; 2 — developing node; 3 — developing membrane junctions, x 16000

Fig. 8. Asymmetry in main dense line formation *(a, b)*
1 main dense lines of developing myelin; 2 — non-myelin areas

Fig. 9. Building in of stick-like (1) and racket-like (2) structures into compact myelin; 3 — main dense lines



the main dense lines. Thus, the intermediate dense line may be considered to be the result of fusion of the local membrane junctions, with the formation of one gigantic intercellular junction.

The other way of junction formation is fusion of membrane inner surfaces of one and the same glial tongue. We call these "inside-out" junctions (Fig. 1 *b*, 3). The latter also include all types of junctions. "Inside-out" junctions of membranes appear early, when the axon is covered with only a few gliocyte cytoplasmic layers. Such layers are of varying thickness. At the sites of their narrowing, accumulations of aggregated electron dense material could be noticed. The higher the electron density of the material, the thinner is the layer and the closer the inner surfaces of two membranes of the same cytoplasmic layer. In this case, when the distance between gliolemma sheets drawn together reaches that of the intercellular gap, single submembrane aggregates unite, join the inner membrane surfaces together and form the sj pattern. In case the thickness of electron-dense cytoplasmic layer is less than the intercellular gap, we get a pattern not differing from gj.Finally, along the cytoplasmic processes of lemmocyte there are areas where complete membrane fusion takes place with the formation of tj-like structures. The only morphologic feature of these membrane junctions, as compared to ordinary intercellular ones, is that they are not formed by the outer but by the inner surfaces of the same cell. Many intermediate junction forms could be observed. The fusion of these junctions is actually equivalent to formation of the main dense line. Thus, the main dense line formation could be pictured as a mosaic process of appearing and fusing together of a great number of "insidd-out" junctions finally forming a single gigantic membrane junction.

On the basis of these data compact myelin could be supposed to be formed not only as a result of continuous Schwann wound membrane fusion as it is usually considered, but also by discontinuous mosaic-like formation and fusion of local membrane junctions. In this case the myelin sheath could be visualized as a model of a gigantic complex autojunction of the gliocyte membrane (Fig. 4).

Extended "inside-out" junctions (main dense lines) may form, topographically and in time, independently of the junctions formed by the membrane outer surfaces (intermediate dense lines). Frequently enough, however, one can notice bounded areas with distinct topical interrelations between several gliocyte membranes and the formation of local serial junctions of both kinds, the number of which increases not longitudinally but radially, transverse to the fibre axis. The formation of serial (multi-storey) junctions was found to be due to a certain type of gliocyte layer organization.

Four types of lemmocyte layer (tongue) organization, following one another in sequence, can be singled out. The first type is chartacterized by loose irregular winding of gliocyte layers around a neurite (Fig. 26). The layers vary in thickness and spacing. The outer layers display it more vividly. Thus, it can be supposed that in many cases true intercellular gaps have not yet formed. Single short glio-glial junctions appear. This primary type of gliocyte tongue organization was encountered by us at all stages of myelination investigated.

The second type of glial layers organization is characterized by the formation of a stereotyped intercellular gap (Fig. 1 *a),* i.e. stabilization of distances between the outer surfaces of gliocyte outer cellular membranes occurs. At the same time, some thinning out of cytoplasmic layers of the sheath was noticed, though their thickness may remain relatively irregular. This type of layer organization is accompanied by the formation of numerous outer and inner junctions.

The third type (Fig. 5) is characterized by unification of glial layer thickness. Crystal-like membranes are formed, which are not yet myelin. The important

feature of this type of organization is that the thickness of these cytoplasmic layers becomes equal to the width of intercellular gap. The intermembrane distance is equalled and stabilized not only between gliolemma outer surfaces, which was characteristic for the previous type, but the intermembrane distance between the inner surfaces of gliocyte membranes equalizes and reaches its critical level. This strictly geometrical type of gliocyte layers could by termed premyelin. Actually, premyelin sites are serial membrane junctions looking like single continuous or septate junctions (Fig. 2 *b*).

Two other general regularities have attracted our attention. First, the types of gliocyte layer organization mentioned are in no way developing simultaneously in different fibres. Second, they do not appear simultaneously along one fibre either. So the area occupied by each type of organization is usually limited, while different patterns of glial layer organitzation could be seen along one fibre (Fig. 5). Sites of true myelin formation in single fibres are also characterized by considerable time-straggling and restricted area.

The restriction of original premyelin site areas, as well as strict geometrical regularity in relative position of inner and outer surfaces of the membranes make it possible to suppose that among the myelination mechanisms an important place belongs not only to general cellular processes but also to local physico-chemical mechanisms of molecular interactions of membranes drawn together. It is well known that at the basis of intercellular junction formation there is an increase in adhesive property of conformly changed membrane protein macromolecules of increased hydrophoby [24]. Formation of serial membrane junctions shows the long-range and cooperative nature of the reaction of submembrane cytoplasmic, transmembrane and extracellular proteins of the intercellular gap.

It is in the loci of glial winding having a crystal-like premyelin type of organization that mass cooperative formation of gap and dense membrane junctions begins, accompanied by membrane convergence and fusion of the membrane inner and outer surfaces. However, it should be noted that serial junctions can be formed not only on the basis of premyelin organization but also when the 2nd and 1st types of glial layer organization are present. In the latter cese, more frequently junctions of serial desmosome type are formed (Fig. 1,3 *d).* They are usually seen at later stages in the zone of nodes and clefts (Fig. 6). Three features characterize the serial desmosome junctions. First, the inner layers of plasmolemma sites are usually connected by large aggregates of electron-dense material (membrane “inside-out” junctions). Second, the width of gliocyte tongue is narrowed here as a result of aggregated material retraction. Third, the intercellular gaps are locally broadened, adjacent membranes are stretched out in different directions by retracting aggregates of glioplasm. Local discrete formation of dense myelin lines is substantiated by the presence of specific structures, cleft-nodes, characteristic only of the developing myelin sheath (Fig. 7). These are complex non-differentiated structures consisting of broad lemmocyte tongues, some of which are narrowed and form membrane junctions (dense lines) on some area limited in length. A pack of serial membrane junctions is formed out of these single long junctions, which partially divides the primary complex structure into a node and a cleft. The other part of the lemmocyte tongues, however, remains broad and connects the zone of paranode and cleft (Fig. 4 *e).* The only way to completely divide the myelin sheath structures is formation of new local membrane junctions between them. Gradual narrowing of the lemmocyte tongue causes the inner gliocyte surfaces to draw together and form a local “inside-out” junction. Then, this junction elongates and “zips in” the

tongue space occupied by glioplasma like a zip-fastener, turning into the main dense line and separating cleft structure from node. The junctions thus formed supplement the pack of serial junctions in the zone of maximum thickness of the latter. Such processes contradict the existing notion of a gradual nondiscrete process of squeezing the glioplasma out of the spiral glial tongues during mesaxon rotation.

Heterogeneity and time straggling in the formation of local sites (main dense lines) are responsible for some unusual patterns of myelin fibres. Thus, at the beginning of myelination some asymmetry in the arrangement of myelin main dense lines on longitudinal sections is often observed, when there is a well-formed compact myelin of one side of the axon, and on the other side the axon is still covered with a few layers of gliocyte cytoplasm which have no formed dense lines (Fig. 8), or contain only single local "inside-out" junctions. Even the fibres with distinct myelination reveal this asymmetry by one-sided arrangement of clefts (there is compact myelin on one side of the axon, while on the other side there are islands of glioplasma deprived of any dense lines).

Common membrane and "inside-out" junctions are constantly found in relatively well-formed nervous fibres with developed myelin in the region of Ranvier nodes, i. e. in the zone of continuing myelination (Fig. 3, *4 e).* It is known that in the process of development nodes appear early and simultaneously with tjie commencement of myelin maturing [2, 17]. In our studies Ranvier nodes were registered in two-day-old animals. Paranodal loops of developing nodes are of considerable length and are like the cytoplasmic layers of a premyelin fibre glia cell. At this point, besides fusion of single membrane junctions, the main dense line formation takes place also by "sewing" the membranes together, just like "zip-fastening", at the edge of compact myelin (Fig. 6 *c*). This leads to gradual elongation of the compact myelin zone and simultaneous shortening of paranodal loops (Fig. 4 *c-e).* As a result, they acquire a rounder form and more uniform thickness, which is characteristic of mature nodes. This process is in synchronization with the formation of glio-neural junctions of paranodal loops and axial cylinder (Fig. 3 *c, 4 d),* which are likely to be those highly permeable sites through which low-molecular premyelin substances penetrate from the axon into the glial cell.

Along with the appearance of Ranvier nodes, Schmidt-Lantermann clefts are formed. First, several very long gliocyte cytoplasmic islands of irregular thickness appear at the site of the future cleft (Fig. 1 *c, d;* 4 /; 5c). Their matrix contains ribosomes, assembled microtubules, transparent and granular vesicles and vacuoles of different size. Later, glio-glial intercellular junctions are found in the zone of developing clefts. Between the membranes of adjacent cytoplasmic islands desmosome-like substances can be found. Glio-glial junctions of gj and tj type are of great importance in the formation of a mature cleft junction. These junctions are formed at the periphery of cytoplasmic islands of the cleft, where glial processes are thinning out or intercellular gaps are narrowed. Subsequently, these junctions fuse with one another, the extent of compact myelin being increased and the length of cytoplasmic islands diminishing respectively. The analysis of such patterns allowed us to suppose that the Schmidt-Lantermann clefts are formed as a result of intermembrane junction fusion and irregular "sewing together" ("zip-fastening") of the membranes along the main dense lines (Fig. 4 *f).*

Thus, different sections of the myelin sheath develop on the same principle—formation of an intermembrane junction system. In our opinion, the data obtained make it possible to render concrete the existing notion of myelination at the expense of gliolemma resources.

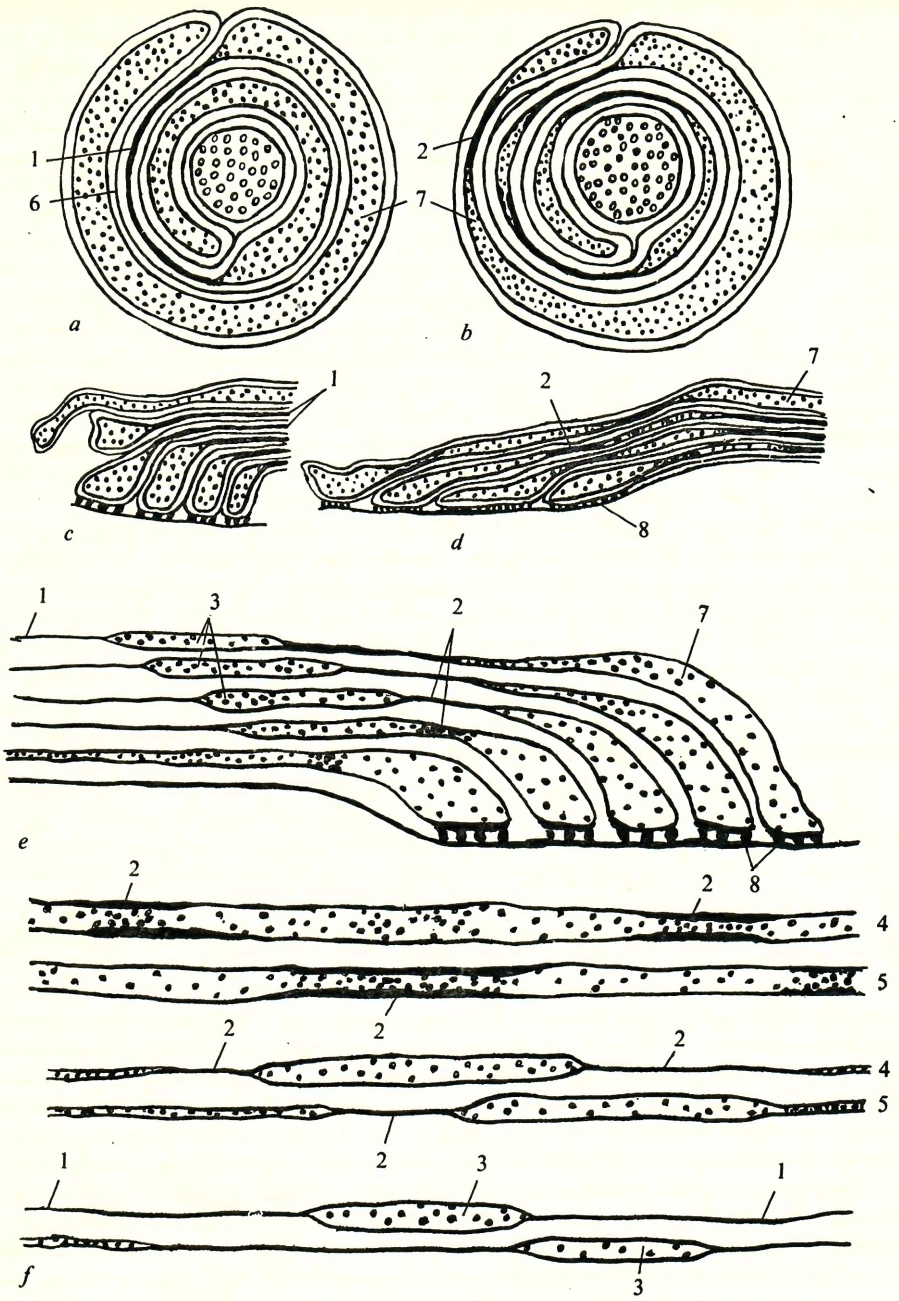


Fig. 4. Diagrammatic representation of dense line and myelin structure formation

a conventional concept of indiscrete formation of myelin continuous dense lines; *b* — diagram generalizing the data on discrete formation of main dense lines (1) by means of local "inside-out" junction fusion (2); *c* — diagram of compact myelin in a developed node; *d* diagram of junctions-forniing myelin in a developing node; *e* — cleft-node structure in a developing myelin sheath; / - diagram of formation of cytoplasmic islands (3) of clefts and main dense lines by two gliocyte flat layers (4, 5); 6 — intermediate dense lines; 7 lemmocyte plasma; 8 axonal-glial junctions

However, at the stage of active myelination (5th-7th day of development) patterns were observed that suggest a different process of myelin formation. The supposition of exact conformity of the number of myelin lamellae along the whole length of the myelin segment remains one of the unsubstantiated morphological aspects of myelination. The hypothesis of lemmocyte or mesaxon revolving around the axon demands such conformity. At the same time, while examining rat myelination, we observed patterns which testify the possibility of local self-assembly of myelin-like membrane structures in immediate proximity to myelin and their building into myelin in the internodal segment zone.

This process can be observed in the central zones of a myelin segment, at some distance from nodes and clefts. Glioplasma's inner layer invaginations into the axon that are often observed in this region, are abundant in vacuole-like structures which flatten, while the inner surfaces of their membrane are drawn together, forming something like a tight "inside-out" junction (Fig. 9). As a result, stick-like or racket-like membrane structures with developed basis are formed and approach with their main lines the compact myelin at a distance equal to the width of intercellular gap and gradually build into it from within. Such pictures were regularly observed in the 5 to 7-day-old animals, i. e. during the most active growth of the myelin sheath thickness. In all probability, at this stage of development the process of myelin sheath thickening may occur by way of membrane structures building into it on the principle of self-assembly. Such observations were not infrequent. Some researchers have also described the phenomenon of membrane structure formation in glial cell cytoplasma, which build into the myelin sheath [20, 23]. It should be pointed out that the process of self-assembly of lipid membrane structures (myelin blocks) was observed in our investigations only in the zone of glioplasma's inner layer located near the axolemma. This fact possibly suggests a high concentration of lipid components in this zone and their transportation from axoplasma through membranes.

The processes of interactions of both membrane surfaces observed in our research possibly suggest that during the studied period of nerve fibre development the degree of gliocyte membrane adhesion increases, which is manifested by the formation of membrane junctions and their fusion. According to the widely recognized concept of the mechanism of myelination, the essence of the process is in mesaxon elongation accompanied by the increase in the number of turns (in winding) and in lemmocyte concentric layers drawing together. Myelination starts near the site where mesaxon originates and spreads out gradually and unifirmly along the entire helix of a gliocyte tongue [18, 19].

It is possible for non-myelin nervous fibres to form local membrane junctions between the turns of glial winding. This ability had been demonstrated long before the notion of frequent occurrence of membrane junctions in the nervous system was developed [17]. The author observed the formation of a patchy (mosaic) pattern due to glial membrane adhesion, accompanied by filling up of the intercellular gap under the action of a stimulating hypotonic medium. At present glial membrane junctions are well-known [15]. Electrotonic interrelation between glial cells was also described [18]. The phenomenon of gliocyte membrane adhesion was described also in the zone of myelin fibres mesaxon [9]. A difference in local adhesive properties of glial membranes of myelin lamellae in some mutant and normal mice was discovered, when attempts were made to segregate the myelin intermediate and main dense lines [12]. The attention of researchers was drawn to the great heterogeneity and time straggling of the process of segregation. They also found out that after*this clearly marked dense-line segregation there are still non-segregated serial sites in myelin, propagating

radially towards the lamellae groups (radial myelin component). In the figures presented by the authors these remaining non-segregated dense lines loci do not differ at all from the serial membrane junctions described above, which demonstrate the cooperative and long-range nature of adhesive membrane processes.

The data obtained by us on heterogeneity and time straggling of myelin dense line formation on the basis of membrane junctions seem to be in conformity with the data presented in literature.

While considering the main and intermediate dense lines as gigantic intercellular junctions, it is extremely important to discuss the gliocyte adhesive membrane proteins participating in the formation of these junctions. There are the same antigens as in myelin on the outef and inner side of gliocyte membrane. They appear in gliocyte at different stages of its differentiation and then remain in myelin [11]. The intermediate dense line is shown to contain proteolipid protein (PLP). The latter is a highly hydrophobic macromolecule which has components released by membranes [6, 21]. These features make it possible to suggest that PLP may take part in providing the membrane outer surface protein adhesion. A second component in this process might be high-molecular Wolfgram — a protein which can also be located in the intermediate dense lines [18].

The princinpal protein of the main dense lines is myelin basic protein (MBP). The main dense lines do not form in mutant mice in the absence of MBP [5]. This suggests that MBP may participate in the membrane inner surface protein adhesion provision, i.e. in the formation of "inside-out" membrane junctions during myelination. The concept of myelination as a process of membrane junction formation allows us to consider this particular and highly specialized phenomenon from a wider biological stand. The general biological and evolutionary essence of this process is of considerable significance.

The importance of myelination for perfecting axon functions during phylogenetic and ontogenetic development is well known. Less attention is paid to the importance of this process for lemmocyte itself in the study of myelogenesis. Lemmocyte is known to be a unique cell. It is a matter of common knowledge that within one day the lemmocyte produces membranes to the weight which exceeds several times its own mass [13]. The consequences of this are very important. The formation of a multi-layer helix of exceedingly thin flat tongues leads to a considerable increase in gliocyte surface area (S) with relatively little change in its cytoplasmic volume (V). However, such increase in the relative surface area S / V is equivalent to a considerable decrease in the thermodynamic stability of the structure. From this follows, first, that the increase in the number of gliocyte cytoplasmic layers is apparently limited. Second, the structure of these glial processes must be highly unstable. This can actually be detected in experiments with homeostasis disturbance of isolated nerve fibre external medium [24]. How gliocyte "manages" to attain considerable stability of multi-layer thick fibre sheath is of great interest for many researchers [18]. It is known that in conditions of permanently varying external medium parameters single isolated cells find it difficult to keep up their integrity and the intracellular homeostasis of their inner contents. Structural stability of the system in multicellular aggregates in tissue culture or in multicellular organisms increases at the expense of decreased total cell surface area bordering on the external medium, i.e. at the expense of decreased S / V, and also in connection with the formation of intercellular junctions. It is only the formation of intercellular junctions that secures the organization and stability of multicellular associate and the formation of the system internal medium (intercellular gaps). Membrane junctions fill up

intercellular gaps and facilitate a partial segregation of the associate enternal medium from the external one. This also decreases the intensity of the external medium influence on live structures. The intercellular junctions integrating the cells mechanically and metabolically contribute to their increased total stability and increase the system's compensatory abilities. The response of cells acquires cooperative nature and long-range abilities [24].

It is this picture of membrane junction formation and gliocyte tongue aggregation that makes the basis of myelination. In connection with this, myelination might be supposed to be the necessary reactive compensatory process which helps to maintain the thermodynamic stability of gliocyte lamellae and preserve its integrity. A number of investigators also consider myelination to be a lemmocyte and oligodendrocyte response to a certain "signal" of the axon [1, 8, 10]. It is quite possible that the factor ("signal") causing this protective reaction — myelination — is a common initial bioelectric axon activity and the associated with it considerable changes in the medium homeostasis in axon-glial and glio-glial intercellular gaps. It has been previously shown that disturbances of intercellular space medium homeostasis connected with axon bioelectric activity lead to membrane junction formation [24]. Conformational restructuring of superficial proteins of the membranes proper, as well as of intercellular gap proteins is accompanied by their increased adhesive properties, gluing up and drawing together of adjacent membranes.

As regards myelination, it has also been shown that the axon signal is localized on the membrane surface and is associated with the external proteins as it can be removed by means of tripsin [1]. This feature has much in common with the factor causing axon electrophysiological action upon the glia [17] in non-myelin fibres. In this case the "signal" also comes from the axolemma surface and is also associated with protein as it can be removed by tripsin. The author's supposition is that such an effect can testify to the formation of axoglial membrane junctions. It has also been noted that for myelination to occur, components of extracellular matrix secreted by lemmocyte into the intercellular gap are necessary [1]. The important part of axoglial junctions in premyelin transfer has been repeatedly shown [7, 18].

Thus, the material presented supplements the traditional concepts of myelination with new data on the part played by the membrane junctions in this process. These data enable us to visualize the myelin sheath as a gigantic complex membrane junction. Its formation is probably of reactive nature and is directed towards increasing the thermodynamic stability of the differentiating neuro-lemmocyte structure.

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Myelin-associated glycoprotein in myelinogenesis and demyelinating processes

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The data concerning localization of myelin-associated glycoprotein (MAG) in myelin, functional significance of MAG and changes of MAG in demylinating processes are presented. In our studies we have not seen any appreciable changes in the content of MAG both in the early and later stages of central Wallerian degeneration in the optic nerve. We have also found that the decrease of MAG content in triethyl tin poisoning is proportional to the drop of total myelin proteins and is not indicative of a specific sensitivity of that glycoprotein. Changes in the content of MAG in brains of children who died from SSPE were also only negligible and myelin fraction was even relatively enriched in this glycoprotein.

Key words: myelin-associated glycoprotein, myelinogenesis, demyelinating processes.

Introduction

Glycoproteins, the less known constituents of myelin sheaths, are likely to be of importance for the formation, structure and perhaps for the pathological breakdown of myelin. The basic glycoprotein of the central myelin is a glycoprotein of high molecular weight, unique to myelin and referred to as the myelin-associated glycoprotein (MAG). Although MAG is quantitatively a minor constituent of the whole myelin fraction, it probably accounts for a considerably higher proportion of the protein molecules exposed on the surface of the myelin membrane, and plays the role of a receptor. Hence, it is possible to speculate about their significance in molecular events leading to myelinogenesis and demyelination.

The myelin-associated glycoprotein (MAG) is a 100 K-Dalton, integral membrane glycoprotein containing 30% carbohydrate that is in central and

peripheral myelin sheaths. MAG can be purified from isolated myelin by extraction with lithium diiodosalicylate, partitioning against phenol, and gel filtration on sepharose-C16B.

MAG in developing nervous system

In the early stages of development of the optic nerve the increase of MAG content runs parallel to the degreee of myelination of its axons, which indicates the function of this component in the formation of myelin sheaths. It can be, therefore, suggested that the external surface membrane components of myelin sheaths to which the glycoproteins belong may be involved in specific recognitation roles during the process of myelination.

The interpretation of the last findings is rather complicated for two reasons: firstly, each brain structure is myelinated at different times during ontogeny and, secondly, there are regional differences in myelin composition of the CNS.

Localization of MAG in the myelin

Based on immunocytochemical studies and radioimmunoassays using polyclonal antisera raised in rabbits against MAG, it appears that the myelin-associated glycoprotein is a nervous system specific protein. Furthermore, the immunocyto-chemical studies indicate that it is restricted to myelin-forming oligodendrocytes in the CNS and Schwann cells in the PNS. Recently, however, there have been a number of studies with monoclonal antibodies that bind to MAG, which have suggested that MAG or a MAG-like molecule is present in other cell types both in the nervous system and in the immune system. However, there is a conflict in the literature about whether or not MAG is a constituent of compact myelin, or whether it is restricted to non-compact membranes associated with myelin sheaths, including the periaxonal membrane of oligodendrocytes and Schwann cells. A variety of biochemical experiments on CNS tissue involving the subfractionation of myelin and myelin-related membranes had suggested that MAG was most concentrated in oligodendroglia membranes that were different from compact myelin, but biochemical experiments cannot precisely define the localization of this protein. Therefore, the immunocytochemical studies of S t e r n b e r g e r e t a l . [3], which first showed immune staining for MAG only in the periaxonal part of the central and peripheral myelin sheaths and not in compact myelin, were a very important step in defining the localization of this protein. Light microscopic immunochemical studies have shown that MAG is present in the periaxonal region of the developing myelin sheaths. On the contrary, some other experiments have proved on the basis of electron microscopic and immunochemical studies that MAG is localized within the compact CNS myelin. It was also shown that MAG increases in the whole brain during development. In the peripheral nervous system, where unlike in the CNS the major PO-glycoprotein constitutes a considerably greater part of myelin proteins, it is localized both in the intraperiod and major dense lines. In iminodipropionitrile neuropathy the presence of MAG in Schwann cell periaxonal membranes has also been demonstrated unequivocally by staining the axonal ingrowths of axolemma and Schwann cell periaxonal membrane. Quantitative biochemical studies on the mutants provide supporting data for the presence of MAG in membranes that are different from compact myelin in the PNS.

Functional significance of MAG

The presence of MAG in periaxonal Schwann cell and oligodendroglia membranes strongly suggests that this glycoprotein is involved in maintaining the junction between the myelin sheaths and the axon. Correlation of the presence or absence of MAG determined by immunocytochemistry with ultrastructural changes in the tissue continue to support a role for MAG in the formation and maintenance of glia-axon junctions. Since glycoproteins are known to be cell-surface antigens and receptors for some viruses, there are theoretical reasons to suspect that MAG could be involved in autoimmune or viral aspects of multiple sclerosis or other demyelinating diseases. The finding that a carbohydrate epitope on the surface of a subset of lymphocytes is shared with MAG and other glycoconjugates in the nervous system may be of significance with regard to autoimmune demyelination diseases. In any case, the existence of a shared epitope between lymphocytes and carbohydrates determinant on MAG and other glycoconjugates of the nervous system is a factor that needs to be considered with regard to autoimmune demyelinating diseases. The nature of the molecule in lymphocytes containing this epitope has not yet been established conclusively. An abstract has appeared indicating that the antigen consists of a protein or proteins with M similart to MAG. The involvement of glycoproteins in pathological processes connected with myelin breakdown is assumed from some experimental data, particularly from the great loss of MAG in the degenerating myelin isolated from hexachlorophene intoxicated rats [2].

MAG in demyelinating processes

In our own studies central Wallerian degeneration was evoked in rabbits' optic nerves by ophthalmectomy. The incorporation of radioactive fucose into MAG of the optic nerve undergoing Wallerian degeneration was studied [7]. In other groups of experimental animals, changes in the content of MAG were evaluated [6]. The results were compared with myelin lipid and protein studies.

Chemical evaluation of MAG. The soluble proteins of the delipidated myelin fraction were separated by means of polyacrylamide gel electrophoresis and stained with SchifFs reagent. The electrophoregrams were evaluated densitometrically. Results are expressed in of MAG per mg of total myelin protein.

Incorporation of radioactive fucose into MAG. Bilaterally ophthalmectomized rabbits were injected intracisternally with ^{14}C of ^{3}H-labelled fucose (40 and 100 (xCi respectively) Twenty-four hrs or 7 days after surgery both experimental groups were killed 20 hrs after injection of the radioisotope, and the isolated myelin fraction of the optic nerve was lyophilized. The soluble proteins of the delipidized fraction were electrophoresed on polyacrylamide gel and radioactivity of the electrophoregrams cut into 2 mm sections committed. The results obtained in central Wallerian degeneration showing an essentially unchanged metabolism of MAG, i. e. an only negligibly reduced incorporation of radioactive fucose (Table 1), as well as lack of changes in the content of this glycoprotein fraction (Table 2), thus do not support the assumption that MAG is seriously involved in biochemical events occurring during the early stages of all central demyelinating processes. In our studies we have not seen any appreciable changes in the content of MAG both in the early and later stages of central Wallerian degeneration of the optic nerve. Furthermore, tracing the rate of radioactive fucose incorporation into MAG, we were unable to demonstrate eminent changes in MAG metabolism in this experimental model.

Table 1. Incorporation of ^{3}H-fucose into myelin-associated glycoprotein in central Wallerian degeneration

	Control	24 hrs after enucleation	7 days after enucleation
MAG	39,0	40,8	34,3

Incorporation of ^{14}C-fucose into myelin-ass< aciated glycoprotein in cen tral Wallerian degeneration

	%	Control	24 hrs after enucleation
MAG		54,3	43,6

Note: Expressed in percentages of total protein radioactivity.

Table 2. Total protein and MAG content in the myelin of the optic nerve undergoing Wallerian degeneration

	Dry weight of myelin in *fig/mg* of optic nerve	Total myelin protein in mg per one optic nerve	MAG in arbitrary units per mg of total myelin proteins
Control	117,8± 7,9	1,54±0,06	4,95 ± 1,03
1 day after enucleation	113,5± 8,9	1,67±0,15	3,96±0,79
7 days after enucleation	139,3 ± 16,0	1,07±0,10	5,45 ± 1,06
14 days after enucleation	83,2± 3,6	1,06 ± 3,6-	5,15 ± 1,09
28 days after enucleation	94,5± 3,5	1,01±0,05	7,11±0,64

Number of animals in each group: 6; mean ± S. E.; significant differences underlined; one unit of MAG is equivalent to one *ng* of fetuin.

In central Wallerian degeneration of optic nerves there are other chemical events which are more pronounced. Following perikaryon-axon disconnection a marked reduction of the most sensitive protein fraction (the basic myelin protein) is observed. This reduction, however, is preceded by an early appearance of esterified cholesterol. From these results we are inclined to assume that undue esterification of cholesterol and not the alterations of MAG or basic protein could function at least as one of the primary factors injuring the molecular architecture of myelin membranes in the course of central Wallerian degeneration.

In the peripheral nervous system, where unlike in the CNS the major Po-glycoprotein constitutes a considerably greater part of the myelin proteins and is licalized both in the intraperiod and major dense lines, it was shown to disappear more rapidly than other proteins during Wallerian degeneration of the rat sciatic nerve [8]. The discrepant behaviour of the glycoprotein components in the central and peripheral nervous system are not surpising when the above mentioned differences in the structural localization of glycoproteins between the central and peripheral myelin are borne in mind. We have also found [5] that the decrease of MAG content in triethyl tin (TET) poisoning is proportional to the drop of total myelin proteins and thus is not indicative of a specific sensitivity of that glycoprotein to TET poisoning (Table 3). The slight decrease of the MAG component of total myelin proteins occurring on the top of the pathological process seems to result from the localization of MAG within the intraperiodic line, where the splitting of myelin lamellae takes place during TET poisoning.

Table 3. Total protein and MAG content in the myelin of the optic nerve in rats intoxicated with triethyl tin sulfate (TET)

	Total myelin proteins in *ng* per g of dry myelin	MAG in arbitrary units* per mg of total myelin proteins	MAG in arbitrary units* per mg of dry myelin
Control animals	204 ±8,90	20,7 ±1,14	4,22 ±0,41
5 hrs after intoxication	128 ±2,14	18,4 ±0,54	2,35 ±0,19
24 hrs after intoxication	137 ±5,50	17,1 ±1,04	2,34 ±0,24
7 days after intoxication	84 ±2,81	16,6 ±0,92	1,39 ±0,17
28 days after intoxication	135 ±3,93	24,8 ±0,88	3,35 ±0,23

Number of animals in each group: mean ± S. E.; significant differences underlined;
* one unit of MAG is equivalent to one *ng* of glucose oxidase (E.C.— 1.1.3.4.).

Table 4. MAG in SSPE cases (in arbitrary units per mg of total myelin proteins)

SSPE (4 cases)	Control (6 cases)
4,81 ±0,53	2,50 ±0,15

Myelin proteins in SSPE cases (in % of total myelin proteins)

	SSPE (4 cases)	Control (6 cases)
Wolfgram protein	27,0±1,7	22,6 ± 1,4
Proteolipid	50,2±0,7*	38,7±0,7
Basic protein	22,8 ± 2,1*	38,7 ±1,2

Note: Mean value for 5 brain pieces.

Table 5. CSF anti-MBP and anti-MAG antibody level (in cpm per 0,5*fig* of CSF IgG)

	SSPE 10 cases	MS 18 cases	Neurotics 6 cases
Anti-MBP	2721 ±764*	832 ±452*	384 ±221
Anti-MAG	1337 ±480*	42 ±225*	183± 90

Changes in the content of MAG in brains of children who died from SSPE were also only negligible (Table 4), and the myelin fraction was even relatively enriched in this glycoprotein. By contrast, a very high level of antibodies against MAG was found by solid-phase radioimmunoassay. in the CSF of SSPE cases (Table 5). That is why, we conclude that the alterations of MAG should not be considered as a trigger mechanism for demyelination in SSPE.

Itoyama et al. [1] reported a decreased MAG immunostaining in early acute multiple sclerosis lesions that extended far beyond the margin of acute demyelination. The loss of immunoreactivity could represent either a progressing loss of MAG molecules at the periphery of the developing plaque, or a great change in their antigenic properties, so that they no longer reacted

adequately with the appropriate antiserum. Contrary to that, immunocytochemical studies on leukodystrophies conducted by Ulrich and Heitz [4] on sections of brains from various forms of leukodystrophy showed that the distribution of MAG was remarkably little altered. In view of the results pertaining to the behaviour of MAG in various demyelinating processes, it should be stated that, although MAG shows considerable sensitivity towards definite pathological factors, it does not constitute a specifically weak chain in the molecular structure of the myelin sheaths that is affected independently by the agent that brings about demyelination.

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The role of proteins in demyelinating processes

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The old and the new findings concerning the role of proteins in demyelinating processes can be fitted together to produce a consistent notion. According to this notion, the primary change in both PNS and CNS demyelination, which results in the formation of spheroids and ovoids, is due to proteolysis. The stage of positive Marchi staining in centrifugal demyelination is due to two factors; a split between myelin-associated glycoprotein (MAG) and myelin basic protein freeing MAG to react; and somewhat later — esterification of cholesterol.

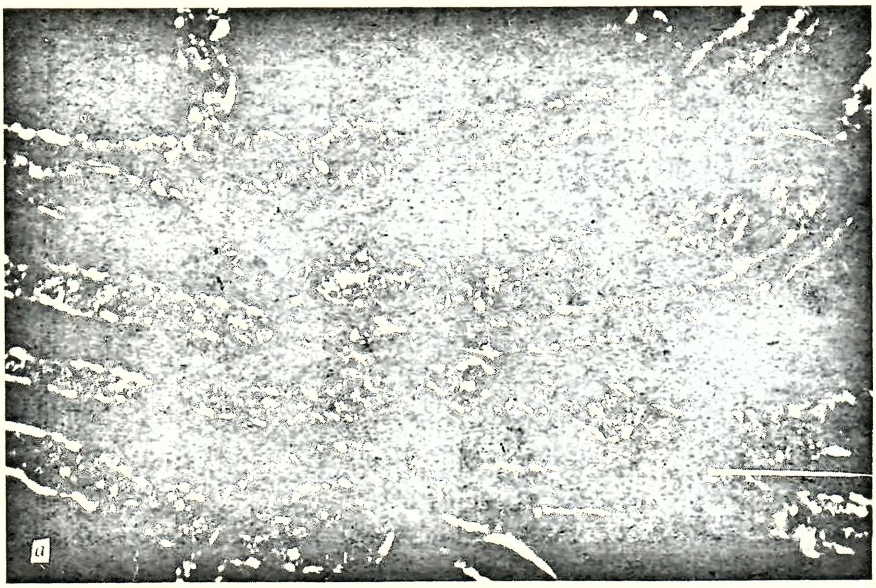
Key words: myelin proteins, PNS demyelination, CNS demyelination.

Common sense tells us that recent factual information and knowledge is mostly more accurate than old knowledge. In fact, modem scientists have at their disposal better technology than their predecessors and, furthermore, they have their own data *in addition* to those of their forerunners, who could not foresee future developments. This conclusion is not, however, always true, as modern scientists are often ignorant of their predecessors' studies and their technologies and technics are often different, but not always more effective than those of older scientists and we, therefore, have to test conclusions on their merits only.

Karl Popper [57],the famous philosopher of science, noted that the game of research is endless. A person who decides one day to stop testing scientific notions and to regard them rather as proven truths, is out of the game and stops being a scientist.

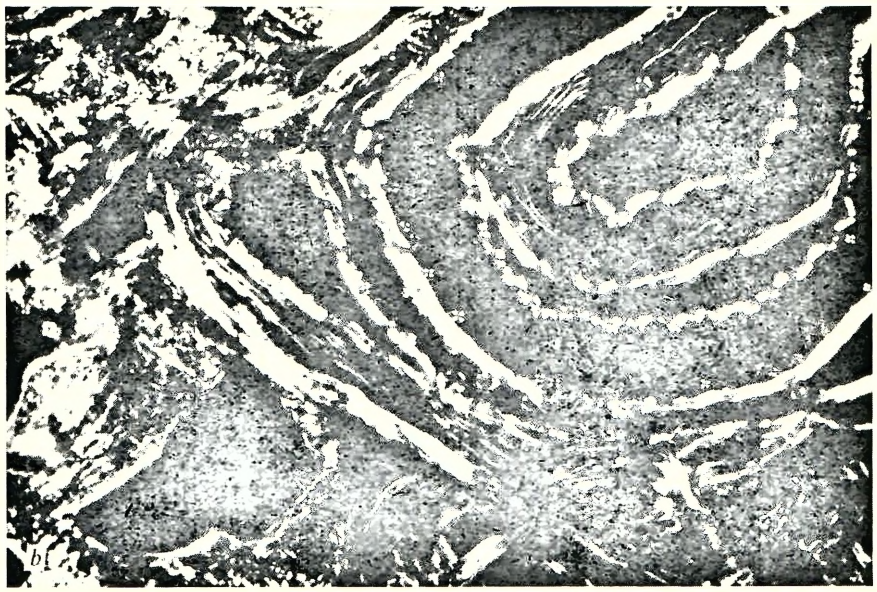
The fact that nerves are surrounded by a sheath was observed already in the 17th century by Leeuwenhoek [5], and the birefringence of the sheath by Ehrenberg in 1849 (cited by Göthlin, [30] ,Valentin [81] in a book published in 1861 and Klebs [45] in 1865 presented polarized-light evidence indicating that myelin is a quasi-crystalline ordered structure in which the constituents are aligned in a perpendicular direction to that observed in collagen. Göthlin [30] correlated these findings with the presence of polar lipids in myelin aligned radially around the axon. The presence of proteins in myelin was proven biochemically and morphologically by Ewald and Kühne in 1877 [24]. The change in the sign of birefringence of myelin after ether extraction (which indicates that in addition to the radially oriented lipids, myelin

Fig. 1. Types of in vitro breakdown of myelin (Polarized-light appearance of teased sciatic nerves of rats. Reprinted from an article of M. Wolman in *Biochim. biophys. acta*, ref. 93, by the kind permission of Elsevier Science Publishers B. V. Amsterdam)
a — granular degeneration of myelin; *b* - lamellar splitting of myelin; c - Wallerian-like myelin breakdown



contains longitudinally oriented solvent-insoluble components, presumably proteins, was first described by F r i e d l ä n d e r i n 1889 and by A m b r o n n in 1890.

It was only in the thirties of the present century that S c h m i d t by using polarization microscopy [66] and by combining polarized light microscopy and X-ray diffraction [67] could safely conclude from their findings that the myelin sheath consists of alternating layers of radially oriented lipid moieties and longitudinally aligned proteins. This constitution corresponds to the Danielli-Davson model of membrane structure and suggests that myelin is made of concentric layers of plasma membranes, a notion confirmed by innumerable electron-microscopic studies.



On physico-chemical grounds the preservation of the lamellar structure of myelin depends on a number of factors which are effective in both the hydrophobic and the hydrophilic layers [53]. In the hydrophobic layer binding forces between individual hydrocarbon chains of the lipids and between these chains and the hydrophobic domains of structural proteins are mainly of the short range (van der Waals) type. Kinking of chains due to unsaturation, lack of cholesterol and some other factors decrease packing and, therefore, stability. These forces are partly responsible for the adhesion between two layers of hydrocarbon chains, while the presence of hydrophobic peptide chains spanning across the membrane (from one main dense line to the next) is obviously a major

factor in the preservation of the multilamellar structure of myelin. Bonds between polar groups of lipids and proteins also contribute to stability, although excessive attraction between polar groups belonging to adjacent moieties as, for example, in the presence of excess of divalent ions can also cause membrane disintegration.

Demyelination occurring in different diseases and experimental models need not be a uniform process. The best studied one is Wallerian degeneration, in which the sequence of events could easily be followed in experimental animals. In many other naturally occurring and experimental demyelinating processes the changes follow a similar pattern. In Wallerian degeneration (and in other demyelinating processes of this pattern, processes which were often called "sudanophilic") the earliest step consists of loss of molecular stability of the sheath. The structure becomes disorganized at different sites with formation of globules and ellipsoids. These fragmented bodies preserve during the first few days the original birefringence, the electron microscopic lamellar structure and the ascertainable chemical composition of normal myelin [6, 43, 84]. The rupture of continuity occurs at irregular intervals so that the ellipsoids consist of fragments of different length of apparently unchanged myelin. This early phase of demyelination, in which the changes are almost only physical, interruption of continuity of the lamellae at random places, is followed by a second phase in which satellite cells play a major role: Schwann cells (or oligodendrocytes), as well as phagocytic cells originating in the blood stream engulf the globules and ovoids of myelin and digest them.

Myelin deisintegration can also be studied under in vitro conditions [93]. In one type of breakdown (Fig. 1 *a)* the sheath changes into an array of granules, some of which follow the original outline, while others lie inside or outside the contour of the sheath. This granular type of breakdown (Fig. 1 *a)* may be induced by changing the ratio between Na and Ca ions in the medium, by agents causing protein denaturation, by heat or cold, or by surface active agents. Such a change has been described by various authors, including S c h m i d t [66], who used isopropanol. In another type of disintegration produced by hypotonic media the myelin is split into separate lamellae along the intraperiod line [64]. This type of change (Fig. 1 *b)* has been extensively studied by N a g e o t t e already in 1922 [52], and occurs in vivo in tri-ortho-cresyl phosphate and alkyl tin poisoning [10, 11]. A third type of myelin breakdown in vitro is similar to changes occurring in vivo in Wallerian degeneration (Fig. 1 *c).* It can be produced by tryptic digestion, and also occurs spontaneously after variable periods of immersion in a balanced salt solution. This "autolytic" breakdown of myelin did not occur in nerves heated to 60°.

In 1886 M a r c h i reported that at a relatively early stage of demyelination, degenerating myelin could be differentiated from normal myelin by staining with a mixture of dichromate and osmium tetroxide [7]. Both osmium tetroxide and dichromate are oxidizing agents and the fact that the mixture of the two with or without dichromate pretreatment differentially stained degenerating myelin (which could not be differentiated from normal myelin by osmium tetraoxide alone) indicated that demyelination was associated with appearance or unmasking of new reactive groups or compounds. In 1956 I searched for compounds which would react with the Marchi procedure and might be responsible for the reaction [89]. Among the compounds tested only heparin fitted the requirements. Experiments done on rats [90] have shown that as from the day after transection of the scientic nerve of rats, the degenerating myelin stained more intensely than normal myelin by the oxidative-leukofuchsin procedures (such as PAS) and became more intensely metachromatic. In further studies,

positive Marchi staining was found to be associated with liberation or exposure of a weakly acidic hexosamine-containing glucosaminoglycan [91].

Biochemical estimations I performed on sheep nerves at various stages of Wallerian degeneration have shown that myelin contains hexosamine and hexuronic acid which can be extracted by water in the intact nerve after tryptic digestion. In demyelination, the amount of hexosamine extractable by water increased after the sixth day and also the total hexosamine was markedly increased for at least 3 weeks [92]. I called the polysaccharidic substance which became split off or available for reacting during demyelination "myelosaccharide" and the assumption was that peripheral demyelination (such as that occurring in Wallerian degeneration) starts in a split between the polysaccharide-containing component and other constituents.

Adams in 1958 and 1960 [2, 3] proposed a different mechanism for the Marchi reaction in degenerating myelin. It was based on an elegant new histochemical procedure he developed (OTAN procedure), which differentiates hydrophobic lipids, such as cholesterol esters, from those which are hydrophilic. The histochemical findings and biochemical estimations of cholesterol and cholesterol esters in demyelination led Adams to conclude that Marchi-positivity is due to the formation of cholesterol esters. Similar conclusions were reached by Wolfgram and Rose [87] and other authors [28, 29, 55]. According to Adams and Bayliss [4], myelin does not contain mucopolysaccharides, an opinion which cannot be held anymore after 1973 when the presence of glycoproteins as a major component in peripheral myelin and as a minor component in the central nercous system was proven [23, 61].

The observations made by Adams and collaborators in 1961-1962 that demyelination is associated with increased activity of proteolytic enzymes [9] opened the way to understand the pathogenesis of the process. This increase occurred in Wallerian degeneration as from the first day of nerve transection [32]. Adams suggested that the increased proteolytic activity in degenerating nerve (and CNS) might be due to liberation, or greater accessibility of the enzyme. The role of proteolysis in demyelination was confirmed by other authors [e. g. 58, 59] and was extended by Adams' group to plaques of multiple sclerosis [8]. The possibility that phospholipases rather than proteases may represent main factors in the early stages of some demyelinating processes has recently been proposed [80].

It is also possible that myelin breakdown may be initiated, at least in some instances, by peroxidative deterioration rather than by (or in addition to) proteolysis. Evidence supporting this possibility includes the observation that some myelin proteins are very sensitive to oxidative damage [17, 46], and the finding that the amount of products of lipid peroxidation in myelin increases with age [19].

The widely-accepted notion that proteolysis is the primary step in Wallerian degeneration and other demyelinating processes was, however, hard to reconcile with the following two observations: 1) normal myelin was found to be relatively poor in both hydrolytic and oxidative activities; 2) cellular infiltration (which brings additional enzyme activities) occurred later than the increased proteolysis. This difficulty was solved by Dr Bubis and Wolman [15] who asked the question: why should myelin which in peripheral nerves is part of a neighboring cell, disintegrate when the axon, which belongs to another cell, is severed from its often distant trophic center. We could show that lysosomes of the degenerating axons spilled their hydrolytic enzymes and these effected demyelination. Similar findings were reported by Weller and Meilick in both Wallerian degenertation and diphtheritic neuropathy [85].

The notion that axonal lysosomes are responsible for Wallerian degeneration was further strengthened by electron microscopic studies [e.g. 36,20], showing that within 24 hours of nerve transection the axons undergo severe changes with disintegration of the neurofilaments and appearance of numerous dense bodies at their periphery. These dense bodies are lysosomes presumed to be responsible for the axonal destruction and myelin breakdown. The partly fragmented myelin globules are then ingested by Schwann cells, where the main course of disruption occurs. The previously referred to our findings [92,93] are also consistent with the notion that proteolysis of trypsin-digestible proteins is responsible for the early phases of demyelination.

The studies of Hirsch and collaborators suggest, however, that lysosomal hydrolases might play a role in the disposal of myelin debris rather than in the pathogenesis of demyelination [34, 35].

Demyelination, as I have suggested [94], can be *centrifugal*, when the lytic factors destroying myelin spread outwards from the axon, or *centripetal*, when the lysis is due to cells or humoral factors spreading from the extracellular space, as can be seen in Fig. 2. It is obvious that in the first case the breakdown will spread at first along the main dense line, while in the second case — along the intraperiod line which is a continuation of the extracellular space. Of course, soon after the early changes occurred, activated Schwann cells and macrophages take over most of the lysis.

In fact, in experimental allergic encephalomyelitis (EAE) [67], in serum and complement-induced demyelination in a patient [71] and in tissue cultures [18], which represent centripetal processes, splitting along the intraperiod line of myelin was observed.

Myelin is a complex semi-crystalline structure which contains a number of different protein moieties. In the central nervous system myelin consists mainly of three major proteins [63]: proteolipids which account for 50-53%, basic (22-30%) and acidic (16.5-20%) proteins. Proteolipids, which were discovered in 1951 by F o l c h and L e e s [27] are characterized by their lipophilic nature expressed in good solubility in a chloroform-methanol mixture. Lipids constitute about one-third of the proteolipid and are presumed to be situated at the periphery of the molecule, but also the apoprotein is extremely lipophilic and has been appropriately termed lipophilin. Proteolipids are acidic and are rich in tryptophan [1, 88].

Recent studies [44, 48, 76] which included aminoacid sequencing of the lipophilin have shown that the apoprotein has clusters of hydrophobic aminoacids separate from hydrophilic domains. The trypsin resistance of this protein was found to be due to the fact that the enzyme-sensitive sites are not available for proteolysis in the tightly packed lamellar structure, and the protein itself is easily digestible by trypsin only ofter hypoosmotic shock. Lipophilin exhibits mainly negatively charged groups towards the intraperiod line (the extracellular space), while the main dense line (which represents the cytosolic side) is flanked mainly by cationic charges. A hydrophobic domain of lipophilin may cross the extracelluar space and become embedded in an opposite lamella. Such bridges might be responsible for the compaction of myelin and its known resistance to disruption into single bilayer structures [69]. Thus, proteolipids play a major role in stabilizing the lamellar structure of myelin and S t o f f e 1 et al. [76] suggested that demyelination, like tryptic digestion, may begin by proteolysis at the extracellular (intraperiod line) side.

The myelin basic protein (MBP) constitutes about one-quarter of CNS myelin proteins and is present in an approximately equimolar concentration to

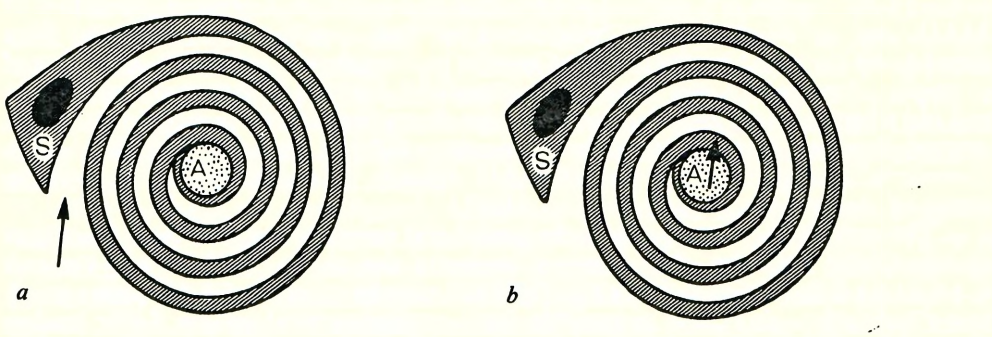


Fig. 2. Schematic representation of two types of demyelinating processes (Reprinted from an article of M. Wolman in the *J. Cytochem.* ref. 94, by the kind permission of the Histochemical Society Inc.)
a — centripetal spread of lysis; *b* — centrifugal spread

that of proteolipid. This protein is rich in polar groups, many of which are basic. The free cationic charges tend to form ionic complexes with acidic lipids, mainly phospholipids and sulfatides [63]. The binding to lipids is known to occur in vivo and allows folding and stabilization of the polypeptide chain [77]. MBP also contains domains of hydrophobic aminoacid sequences. The importance of the MBP-lipid interactions for the orderly arrangement of myelin layers and for the maintenance of multilamellar structure has been demonstrated repeatedly [68]. In fact, the loss of even a single cationic charge of the basic protein reduces markedly its capacity for binding lipids and destabilizes the orderly structure [51]. MBP has been further shown to exhibit a special and specific affinity to lipophilin [96]. The reactivity of myelin basic protein is not confined to lipids and other protein. Ikeda and Yamamoto [37] have shown that the basic protein has lectin-like properties, binds to a number of saccharides and exhibits hemagglutinating activity.

MBP is the most sensitive among myelin proteins to the hydrolytic action of trypsin and plasmin [39]. Studies of Roboz-Einstein and her group [63] have shown that the encephalitogenic protein responsible for EAE is formed by enzymic breakdown of MBP. This hydrolytic process has been found to be stimulated by acidic lipids [86]. It appears, therefore, that MBP, a protein which is very sensitive to acid proteinase (cathepsin D) digestion, serves as a main pillar in the stability of myelin, with bonds to lipids, proteins and carbohydrate moieties. In fact, digestion of MBP was observed outside active plaques of multiple sclerosis patients [8].

Years ago we [16] adduced evidence indicating that this protein is localized in the major dense line of CNS myelin. Furthermore, electrophoretic studies by Dr. London and myself on myelin treated by different concentrations of various salts to extract proteins selectively have also shown [50] that the intraperiod line contains mainly acidic moieties, while the main dense line is rich in basic groups. The localization of myelin basic protein in the major dense line was confirmed by other studies [33, 54].

In 1972 and 1973 the group of Quarles at the National Institutes of Health in Bethesda, apparently unaware of my studies, adduced evidence that a hexosamine-containing glycoprotein, called MAG is present in central and in peripheral myelin [23, 60, 61]. The group further showed that in active lesions of multiple sclerosis [40], as well as in progressive multifocal encephalopathy [41], MAG is lost before the basic protein is affected. Furthermore, incubation of

human CNS myelin at a neutral pH resulted in a rapid proteolytic degradation of MAG and a much slower breakdown of the myelin basic protein. This breakdown occurred more rapidly in myelin preparations obtained from brains of multiple sclerosis patients than from control brains [65]. These findings explain my old observations, showing spontaneous demyelination occurring in balanced salt solution and being inhibited by exposure to 60° [93]. In acute EAE [42] and in chronic relapsing EAE [83] loss of MAG did not, however, extend beyond the areas of demyelination and loss of MBP.

The constitution of myelin in the peripheral nervous system differs from that in the CNS, although their protein contents are similar [72]. F e i g i n and C r a v i o t o have shown years ago [26] that peripheral nerve myelin stains much more intensely with P.A.S. in paraffin sections than central myelin. Later studies [31, 95] have shown that the protein composition of peripheral myelin parallels that of CNS, as both contain basic proteins, but instead of the proteolipid of CNS, peripheral myelin contains a glycoprotein. This glycoprotein, termed Po, is a major constituent of peripheral myelin, accounting for 50-60% of the proteins of the sheath, but is not the only glycoprotein of peripheral nerve myelin. Other authors have identified 6-26 different glycoproteins in myelin of different animals [49, 70]. It is obvious that Po and the other glycoporteins are responsible for the intense staining with P.A.S. of peripheral myelin. P o d u s 1 o and Y a o [56] have recently shown that, although Po is relatively easy to extract, it is an integral membrane protein with hydrophobic domains crossing the hydrocarbon chain layer and hydrophilic domains responsible for its solubility characteristics.

B l a u r o c k a n d N e l a n d e r [12] studied the X-ray profile of frog peripheral nerve myelin and proposed on the basis of the findings that the Po protein is situated in the extracellular half of the membrane extending into the extracellular space. Peripheral nerve myelin contains two basic proteins, Pj and P_2, the first being most probably identical to MBP of central myelin [31].

Peripheral myelin also contains MAG as a minor but very important constituent. It has been shown [14, 74] that in IgM gammopathy peripheral demyelination is associated with the presence of antibodies to MAG. Thus, this minor constituent appears to be able to act as an antigen in rare cases of peripheral demyelination. The antigenic stimulation caused by MAG may be related to the presence of similar or identical epitopes on some blood cells [22, 75, 78] and nerve glycolipids [38].

In Wallerian degeneration of peripheral nerves (which represents a classical example of centrifugal demyelination spreading along the main dense line) it may be concluded that the first step of demyelination is proteolytic degradation of MBP. Degradation and solution of this molecule, posssibly together with the attached glycoproteins called X and Y or 23 K and 19 K [72], leave the glycoprotein Po free to react, confirming the notion that Wallerian demyelination begins with a split between a glycoprotein and another constituent [90].

In peripheral myelin centripetal demyelination, as in diphtheria neuropathy and in experimental demyelination caused by intraneural injection of lysophosphatidylcholine, it is likely that the damage spreads along the intraperiod line and affects at first Po and the other glycoproteins. The early occurrence of myelin vesiculation in lysophosphatidylcholine demyelination [73] and the sensitivity of Po to plasmin degradation [18] appear to fit this notion. In CNS centripetal demyelination, as for example in EAE, the process spreads along the intraperiod line and would be likely to split at first the bond between proteolipids and acidic lipids with the extrinsic part of the MBP molecule. In centrifugal demyelination in the CNS, for example in transection of the optic nerve, the process is likely to affect first the bonds between MBP and MAG.

The question whether demyelination in multiple sclerosis is centrifugal or centripetal cannot be answered with certainty at present. The previously-mentioned studies indicating that in multiple sclerosis and in progressive multifocal encephalopathy MAG is lost before MBP, while this is not the case in EAE, suggest that EAE differs in nature from the other two diseases. The data seem to indicate that in multiple sclerosis demyelination is centrifugal, possibly related to viral infection of axonal structures.

The problem is complicated, however, by the discussion concerning the licalization of MAG: whether it is an integral component of myelin [25, 82], or it is present in loose and absent in compact myelin [62, 79]. The following two points may serve as arguments for considering MAG an essential component of myelin. First, the lack of staining for MAG in compact myelin might be due to the strong electrostatic forces present in the narrow spaces, and a similar lack of staining was observed in fact in other immunohistochemical procedures [21]. Secondly, the observation that MAG is produced by oligodendrocytes before MBP [97] suggests that MAG is an integral part of myelin.

This discussion dealt mainly with the early phase of demyelination, in which myelin breaks down into globoids and ellipsoids. As noted above, this phase is followed by intracellular digestion which occurs in satellite cells and in macrophages. P e t r e s c u [55] noted that the lipid inclusions within the phagocytic cells were of two types: myelinic granules containing hydrophilic (in reality — amphiphilic) lipids,.on the one hand, and sudanophilic (hydrophobic) granules consisting mainly of cholesterol esters. These last-mentioned inclusions were stained by the Marchi procedure.

It is interesting to note how parallel paths of research begun 30 years ago and yielding divergent results complement each other and can be fitted together with more recent studies. According to my studies, a carbohydrate-containing Marchi-positive compound is present in myelin and plays a major role in the first extracellular step of demyelination. Adams, on the other hand, observed that demyelination begins with - proteolysis followed by esterification of free cholesterol. It appears today that the two notions are not mutually exclusive but rather complementary. Demyelination of the centrifugal type of peripheral nerves (such as Wallerian degeneration) is probably started by a split between MAG, Po, and other proteins, rendering the glycoproteins more extractable and reactive. The positive Marchi reaction at this stage is presumably due to the uncovering of reactive groups in the glycoproteins. After the continuity of myelin is interrupted, the spheres and ovoids are metabolized in macrophages and satellite cells with progressive esterification of cholesterol. The fact that some macrophages contain free, while others contain esterified cholesterol (and stain by the Marchi procedure) indicates that the first step of demyelination, which occurs *in situ* precedes the esterification. Thus, M^rchi-positivity of spheroids not ingested by satellite cells is due to reactivity of a glycoprotein. In the intra-phagocytic stage of demyelination cholesterol esters determine the Marchi-positivity.

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Proteolipid protein: Chemistry and role in demyelinating disorders

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Our studies provide fundamental information on the structural and dynamic properties of proteolipid protein (PLP) and its role in the development, maintenance and function of CNS myelin and in the pathological processes associated with demyelinating disorders, particulary those involving autoimmune phenomena. The immunological aspects of the PLP-induced model are comparable to those of whole CNS tissue- and myelin basic protein-induced experimental allergic encephalomyelitis in other species and is similar to that of multiple sclerosis. The characteristics of the PLP-induced disease make it a useful model for understanding autoimmune processes in the nervous system and the contribution of PLP to the pathophysiology of multiple sclerosis.

Key words: proteolipid protein, myelination, demyelination, experimental allergic encephalomyelitis.

The myelin proteolipid protein (PLP) is the major protein of CNS myelin, where it is present in greater amounts than the more familiar and well-studied myelin basic protein (see [16] for review). The myelin PLP is characterized by its solubility properties [10] and is extracted from brain white matter with organic solvents, specifically 2:1 chloroform-methanol. The extract contains both protein and lipid, but it is possible to remove all of the non-covalently bound lipid. The apoprotein thus obtained retains its solubility in chloroform-methanol mixtures but can be converted to a water-soluble form under specified conditions. Thus, an important characteristic of the protein is that it shows a conformational flexibility. An additional characteristic, which will be discussed below, is that the apoprotein contains 2-4% covalently bound fatty acid [30].

The proteolipid is generally considered a structural protein and, therefore, its identification depends on its mobility on sodium dodecyl sulfate-polyacrylamide gels. However, since many proteins can migrate to the same position on a gel, for specific identification the electrophoretic separation must be combined with an immunologic probe on immunoblots. Using an immunoblot procedure, we have been able to detect as little as 250 ng PLP and thereby have confirmed that PLP is

absent from the peripheral nervous system and from non-neural tissues [21]. We have also followed the developmental appearance of PLP in the rat and found that it can first be detected in the spinal cord at 2 days and in upper brain regions at 10 days, at about the same time as myelin basic protein is detected [23].

Structural features

The primary structure of PLP was initially determined by amino acid sequencing [18] and subsequently confirmed by nucleotide sequencing [24] (Fig. 1). The protein has a molecular weight of 29,869, but its apparent molecular weight on gels is only 24-25,000. The reason for this discrepancy is that PLP has a higher zero electrophoretic mobility than the water-soluble proteins used as standards [8]. The protein contains 276 amino acids arranged in a strong domain structure of alternating hydrophobic and hydrophilic regions (Fig. 1). The protein is highly conserved during evolution with 90% or greater homology among the species thus far sequenced (bovine, human, rat and mouse).

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                                                  10                                  20
Gly-Leu-Leu-Glu-Cya-Cya-Ala-Arg-Cya-Leu-Val-Gly-Ala-Pro-Phe-Ala-Sec-Leu-Val-Ala-
                                                  30                                  40
Thr-Gly-Leu-Cy3-Phe-Phe-Gly-Val-Ala-Leu-Phe-Cya-Gly-Cy3-Gly-Hia-Glu-Ala-Leu-Thc-
                                                  50                                  60
Gly-Thr-Glu-Lya-Leu-Ile-Glu-Thr-Tyr-Phe-Sec-Lya-Aan-Tyc-Gln-Aap-Tyc-Glu-Tyc-Leu-
                                                  70                                  80
Ile-Asn-Val-Ile-His-Ala-Phe-Gln-Tyr-Val-Ile-Tyr-Gly-Thc-Ala-Sec-Phe-Phe-Phe-Leu-
                                                  90                                 100
Tyr-Gly-Ala-Leu-Leu-Leu-Ala-Tyr-Gly-Phe-Tyr-Thr-Thr-Gly-Ala-Val-Arq-Gln-Ile-Phe-
                                                 110                                 120
Gly-Asp-Tyr-Lya-Thr-Thr-Ile-Cys-Gly-Lys-Gly-Leu-Ser-Ala-Thr-Val-Thr-Gly-Gly-Gln-
                                                 130                                 140
Lys-Gly-Arg-Gly-Ser-Arg-Gly-Gln-His-Cln-Ala-His-Ser-Leu-Glu-Arg-Val-Cya-Hie-Cye-
                                                 150                                 160
Leu-Gly-Lys-Trp-Leu-Gly-His-Pco-Asp-Lys-Phe-Val-Gly-Ile-Thr-Tyr-Ala-Leu-Thr-Val-
                                                 170                                 180
Val-Trp-Leu-Leu-Val-Phe-Ala-Cya-Sec-Ala-Val-Pro-Val-Tyr-Ile-Tyr-Phe-Asn-Thr-Trp-
                                                 190                                 200
Thr-Thr-Cys-Gln-Ser-Ile-Ala-Ala-Pro-Ser-Lya-Thc-Ser-Ala-Ser-Ile-Gly-Thr-Leu-Cye-
                                                 210                                 220
Ala-Asp-Ala-Arg-Met-Tyr-Gly-Val-Leu-Pro-Trp-Aan-Ala-Phe-Pro-Gly-Lya-Val-Cya-Gly-
                                                 230                                 240
Ser-Asn-Leu-Leu-Ser-Ile-Cya-Lys-Thr-Ala-Glu-Phe-Gln-Met-Thr-Phe-Hla-Leu-Phe-Ile-
                                                 250                                 260
Ala-Ala-Phe-Val-Gly-Ala-Ala-Ala-Thr-Leu-Val-Ser-Leu-Val-Thc-Phe-Met-Ile-Ala-Ala-
                                                 270
Thr-Tyr-Asn-Phe-Ala-Val-Leu-Lys-Leu-Met-Gly-Arg-Gly-Thc-Lya-Phe
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Fig. 1. Amino acid sequence of bovine white matter proteolipid protein. Hydrophobic regions are underlined

As a consequence of our knowledge of the amino acid sequence, it has been possible to develop a model of the orientation of PLP in the myelin membrane (Fig. 2) [15]. The model contains 3 helical trans-membrane segments which pass through the bilayer and two cis-membrane segments which enter and leave on the same side of the membrane. An alternate model has been proposed by Stoffel and collaborators [29]. The two models are similar in the orientation of the carboxyl terminal part of the molecule, residues 190 to 276. They are also similar in the localization of the amino terminus on the extracellular face and in the location of free sulfhydryl groups in hydrophilic loops. The major differences derive from our placement of a cis segment at the amino terminus, whereas Stoffel places it close to the middle of the molecule. In our model a p turn at proline residue 14 leads to the alignment of cysteine residues opposite one another to form 3 pairs of disulfide linkages. Since most of the cys residues of the protein are in the disulfide rather than the free thiol form, the proposed structure appears realistic. As a consequence, in our model the highly charged basic region (residues 90-151) is cytoplasmic, whereas it is extracellularly localized by Stoffel et al. At this point, both models remain hypothetical. Nevertheless, they are important since they bring to the fore several new features of the molecule: a) PLP shows internal homology, e. g. the transmembrane segments show a repeating structure [14]; b) a portion of the molecule shows structural similarity to myelin basic protein [19]; and c) the covalently bound fatty acid (see below) is located in a hydrophilic region. The fatty acid would increase the hydrophobicity of this region and could act to hold together the multilamellar myelin structure. Both models place this region at the extracellular face of the membrane, and our experimental evidence confirms this location [20].

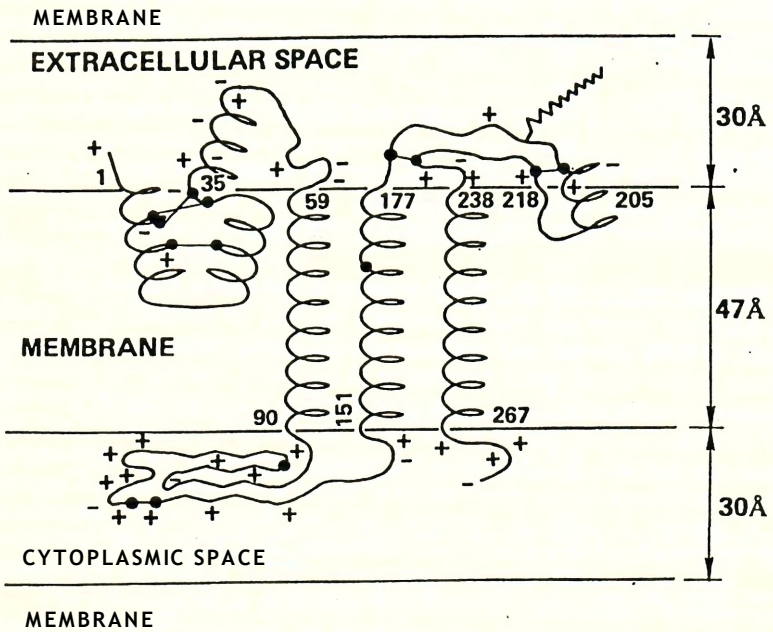

Fig. 2. Proposed orientation of the proteolipid in the myelin membrane, modified from Laursen et al. [15]. Numbers refer to residue numbers in the linear sequence of the protein

Acylation

PLP contains approximately 2 moles of covalently bound fatty acids per mole of protein, and these occur at specific sites in the protein. Thus far, only serine or threonine residue 198 has been identified as an acylated site. The fatty acids can be cleaved by hydroxylamine and are, therefore, bound in an O-ester linkage. The predominant fatty acid is palmitic (55%), with lesser amounts of oleic (26%) and stearic (19%) [30].

Acylation is the only known post-translational modification of PLP. Since acylation continues after protein synthesis is inhibited by cycloheximide, and since transport from the Golgi to the myelin membrane is not blocked with monensin or colchicine [6, 31], it appears that acylation takes place after synthesis, transport and incorporation of the protein into the myelin membrane. With Dr. Oscar Bizzozero, we have been studying the mechanism of the acylation reaction. First, we demonstrated that activated fatty acid is the immediate donor and that acylation occurs within myelin [1]. The in vitro acylation showed all the properties of an enzymatic reaction including Michaelis-Menten kinetics, fatty acid specificity and site specificity [3, 4]. Our more recent studies have shown that under suitable conditions, isolated, deacylated PLP can be acylated by palmitoyl-CoA with no requirement for the addition of any subcellular fraction or other source of enzyme [5]. Thus, fatty acid addition to PLP appears to be an autoacylation process, i. e., the protein acts as both the substrate and the acylating enzyme; a separate enzyme is not required. The mechanism by which the autoacylation occurs is not as yet known, but sulfhydryl groups and the conformational flexibility of the protein may be involved.

The covalently bound fatty acids may be of importance in certain demyelinating diseases. Adrenoleukodystrophy is an X-linked progressive neurological disorder characterized by demyelination, inflammation and an accumulation of very long chain fatty acid esters (C 24-30). PLP from adrenoleukodystrophy autopsy material shows a 2- to 3-fold increase in saturated C25 to C27 fatty acids and more than an 8-fold increase in C28 to C30 fatty acids, at the expense of oleic acid [2]. These fatty acid abnormalities do not represent the primary defect, but they may explain part of the pathophysiology of adrenoleukodystrophy. A study of this disease may well lead to important clues concerning the normal function of the covalently bound fatty acid. It is also of interest that among all of the genetic disorders of myelin adrenoleukodystrophy is the only one in which there is an inflammatory response. Thus, an understanding of this desease may be relevant to multiple sclerosis.

Role of PLP in experimental allergic encephalomyelitis (EAE)

It has long been recognized that myelin basic protein does not account for all the effects of whole tissue in the induction of EAE and that other components are involved. As the most abundant CNS myelin protein, PLP can be expected to play a role in autoimmune responses in the nervous system. Such speculation is reinforced by the fact that we have demonstrated that regions of the PLP molecule are on the extracellular face of the myelin membrane where they could be exposed to either normal or invading components of the immune system. A role for PLP in EAE was first suggested in the 1950s (see [12] for reviews of early studies), but at that time the presence of contaminating myelin basic protein could not be convincingly ruled out. However, modern methodological tools have

now demonstrated unequivocally that PLP preparations are free of myelin basic protein [17] and that PLP *per se* has an encephalitogenic effect [7, 26, 33, 36, 37,38].

For the last several years we have been studying the effects of immunization of rabbits with proteolipid apoprotein, along with complete Freund's adjuvant [7, 36]. Symptoms appeared one to six months after immunization and were characterized first by hind limb weakness and ataxia and then by flaccid paralysis, progressing to spastic paralysis and incontinence. In most animals the disease followed a chronic progressive course, but a relapsing course was observed in several rabbits. More recently, the disease has developed consistently 3 to 8 weeks after immunization. When the rabbits first showed clinical symptoms, a positive delayed type hypersensitivity was observed with the induration and erythema greatest in the sickest animals [34]. Histologically, the disease was characterized by meningitis with mononuclear lymphocytes accompanied by demyelination and reactive gliosis. The infiltrates were perivascular, and quantitation showed that sicker animals had a greater amount of infiltration. Immunocytochemical studies using antibodies against T cells and la showed both perivascular and diffuse infiltrates [28]. Our more recent studies have shown similar responses to PLP in mice [33]. S/JL mice show an acute form of the disease, whereas other strains show a more chronic form. Other investigators have also shown similar results in mice, Lewis rats and Hartley guinea pigs [37, 38]. Our studies with mice indicated that: a) the susceptibility of various strains of mice to PLP-induced EAE is not controlled solely by immune response genes; b) the strain susceptibility to PLP differs from that of myelin basic protein; and c) the varied clinical, histologic and genetic responses involved in PLP-induced EAE in mice may be comparable to the varied expression of multiple sclerosis in humans (T u o h y, unpublished).

Two characteristics of PLP may be relevant to its possible contribution to the complex series of events leading to multiple sclerosis. Since PLP is a transmembrane protein, exposed regions occur on both the cytoplasmic and the extracellular faces of the myelin membrane. The extracellular regions in particular provide potential for the involvement of mechanisms different from those found for myelin basic protein which is localized exclusively to the cytoplasmic face. Furthermore, we have found that the sequences of the exposed regions have extensive analogies with many viral sequences, even more than does myelin basic protein [27]. Thus, PLP may share similar epitopes with many different viruses. For example, an adenovirus protein contains a sequence identical to 7 of the first 9 amino acids at the amino Terminus of PLP and 11 of the first 19 amino acids:

PLP (1-20) GLLECCARCLVGAPFASLV
Adenovirus (561-579) GLLECHCRCNLCTPHRSLV.

The amino terminus and the region consisting of residues 135-153 show similarities to a particularly large number of viruses including Epstein-Barr virus and rodent polyoma viruses. Other regions show similarities to human adult T cell leukemia viruses, influenza virus and even the AIDS virus, although the latter is not as striking as some of the other similarities. These findings suggest that "molecular mimicry" may be occurring and that immunologic cross-reactivities between virus-induced antibodies or T cells and analogous epitopes in myelin proteolipid could be involved in the pathophysiology of multiple sclerosis of post-infections demyelinating syndromes.

Very little information is available on PLP in multuple sclerosis. Immunoreactive PLP activity has been demonstrated in CSF of patients with neurologic diseases [32], but since myelin fragments have been identified in spinal fluid sediments of multiple sclerosis patients, this is perhaps not surprising.

J o h n s o n et al. [11] found that peripheral blood lymphocytes from four of 19 multuple sclerosis patients showed a proliferative response to PLP but only after removal of suppressor cells. This suggests that at least in some patients the balance between suppressor and stimulator cells may be important in the appearance of a cell-mediated response to PLP.

Molecular biological studies

Molecular biological approaches to the study of PLP have recently progressed rapidly and provide new insights not only into PLP synthesis, but also into myelination in general. The PLP gene has been localized to the X-chromosome in the human and the mouse [35]. The gene consists of seven exons encompassing 17 kilobases of DNA [9]. In addition to conservation of the coding region, the regulatory regions also appear to be highly conserved [22]. It has now been shown that the primary abnormality in one of the mouse dysmydination mutants, namely the jimpy mouse, involves PLP [25]. The size of the PLP mRNA is shorter in the jimpy than in the wild type mouse, and this results in a deletion of amino acids 207-231 and the production of a highly abnormal protein as a result of a frame-shift. The importance of PLP to the regulation of myelin formation is indicated by the low levels of myelin basic protein mRNA and the essentially complete lack of myelin in the jimpy mice.

Pelizaeus-Merzbacher disease, a severe X-linked dysmyelinating disease, is the only human disorder in which proteolipid protein is implicated as a primary defect. Low levels of other myelin proteins can be detected, but PLP appears to be completely absent [13]. The nature of the gene defect remains to be elucidated.

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Models of chronic experimental allergic encephalomyelitis

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Based on recent neuropathological and immunological data, a hypothetical model of experimental allergic encephalomylitis (EAE) and experimental allergic neuritis (EAN) is presented. The discussed pathogenetic mechanisms suggest that the full picture of inflammatory demyelination is induced by a complex interaction of cellular and humoral immune rections. Furthermore, they show that in principle several different antigens of the CNS and PNS can mediate autoimmune inflammation or demyelination respectively.

Key words: experimental allergic encephalomyelitis, experimental ellergic neuritis, multiple sclerosis, inflammatory demyelination.

Introduction

Detailed clinical and pathological descriptions of neurologic complications following rabies and other vaccinations in the early decades of our century indicated that autoimmune reactions against neural antigens may induce an inflammatory or inflammatory demyelinating disease of the nervous system [32, 52, 85]. This concept was then formally proven by the induction of experimental allergic encephalomyelitis [62] and experimental allergic neuritis [88] by active sensitization of susceptible animals with tissue of the central and peripheral nervous system, respectively. The animal models of EAE and EAN have then been extensively studied and have lead to considerable new insights in basic problems of immunology and autoimmunity, as well as to more specific aspects of the pathogenesis of demyelinating diseases, including multiple sclerosis.

Studies in EAE models started from what at that time was believed to be a rather simple problem. Since active sensitization with neural tissue lead to disease in a broad variety of animal species and also in humans [73], the two key questions at that time were:

1. What is the antigen, against which the immune reaction is directed?
2. What immunological mechanisms lead to disease?

Interestingly, both problems are not yet solved today.

These two questions seemed to be answered in the middle of this century, when myelin basic protein (MBP) was found to be an encephalitogenic antigen [31] and when the disease was transferred to naive recipient animals by lymphocytes from EAE animals [59]. However, soon after these discoveries it became evident that EAE pathogenesis is much more complicated than expected before.

First, disease induction was in general more effective and the clinical and pathological manifestation more complete, when the whole CNS tissue was used for sensitization instead of MBP. This indicated that besides MBP other CNS antigens may be involved in the pathogenesis of the disease. Furthermore, not a single peptide epitope of MBP is responsible for disease induction, but different MBP epitopes are effective in different animal species and even in different strains of a given species [1].

Secondly, EAE susceptibility is under genetic control. The genetic control not only involves the T-cell immune reaction against MBP, but apparently also immune responses against other CNS antigens, as well as factors governing the susceptibility of the target tissue.

Thirdly, extensive studies using various different sensitization protocols showed that even subtle changes in the immunization procedure may profoundly change the disease. This suggests that complex imnmnoregulatory mechanisms are involved in disease induction and that additional immunological (e. g. antibodies) and non-immunological factors (e. g. neuropeptides or vasogenic amines) may modify the disease.

All these studies have lead to a large number of different models of autoimmune encephalitis and neuritis, with variable relevance for human inflammatory demyelinating diseases. In the following text it will be tried to clarify some principal aspects of EAE and EAN pathogenesis and to explore their relevance for research in human inflammatory demyelinating diseases.

Pathology of experimental allergic encephalomyelitis and neuritis

As mentioned before, EAE and EAN are inflammatory demyelinating diseases. This already characterizes the main pathological features:

Inflammation consists of perivenous infiltrates of inflammatory cells, which in active disease also disperse within the surrounding tissue (Fig. 1). They are mainly composed of lymphocytes and monocytes / macrophages, but in very severe disorders an additional considerable number of polymorphonuclear leucocytes can be present in the lesions. In early phases most of the lymphocytes are T-cells, phenotypically characterized as “helper / inducer” T-cells (Table 1) [26, 80, 90]. Later during the disease (in the recovery phase), “suppressor / cytotoxic” T-cells dominate (Table 1) [27, 39]. The majority of cells in the lesions, however, are stained by monoclonal antibodies against macrophage antigens (Fig. *4a)* and express Class II (la) histocompatibility antigens (Table 1; Fig. 1 *c*, *e*, *f*, 0; [26, 39, 75]. The composition of inflammatory infiltrates is in principle similar in acute EAE (Fig. 1), active lesions of chronic EAE [39] (Fig. 2), acute EAN [56] and PNS lesions of EAE [40].

The second main feature of EAE pathology is demyelination (Figs 2c, 3, 46). Demyelination in EAE and EAN in general is a selective process, leading to

Table 1. Quantitative evaluation of T-cell subsets and Ia-antigen in the spinal cords of SD-rats at different stages of acute and chronic EAE

	[illegible]	Early stage	Peak of disease	Acute recovery	[illegible]	[illegible]
W3/13	[illegible]	[illegible]	[illegible] ± 0,6	[illegible] ± 0,7	36 ± 0,5	[illegible]
OX8	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
OX6	[illegible]	37,[illegible] 6,8* 74,[illegible]	[illegible]	[illegible]	6,1 [illegible] 194,[illegible]	[illegible]
%OX8	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	59,1[ns]

W3/13: numbers of [illegible] cells/mm² (total T cells)
OX8: numbers of [illegible] cells/mm² ("suppressor/cytotoxic" T cells)
OX6: numbers of OX6⁺ cells/mm² (Ia⁺ cells)
%OX8: percentage of OX8⁺ cells compared to total (W3/13⁺) T-cells

[illegible]s: d[illegible] [illegible]nsitization
n: [illegible] of animals

Note: Statistical analysis was performed by using the U-test according to Mann and Whitney. The values of the "chronic progressive" group were compared with all other values; levels of significance are indicated as follows: ns = not significant, * = p 0,05; ** = p 0,01.

destruction of myelin sheats with sparing of axons (Fig. *3b)* and, when present, neurons. Myelin sheaths can be destroyed by several ways [33]. Most frequently macrophages with their processes invade between the myelin lamellae (myeliir stripping). In some models of EAE also attachment of myelin fragments to coated pits of macrophages is noted [17], a pattern similar to that described in chronic multiple sclerosis lesions [61]. In other lesions whole myelin segments are transformed into vesicular material (vesicular disruption of myelin [33]). Reactive gliosis and subsequent astroglia scar formation parallels with inflammation and demyelination. This pattern of tissue damage results in perivenous sleeves of demyelination or in the formation of large, confluent demyelinated plaques (Figs 3,4). In general, only minor loss of myelin sheaths is noted in the nervous system of animals with acute EAE and EAN, whereas demyelination is much more pronounced in models of chronic disease.

The structure of chronic EAE lesions and the extent of demyelination, however, vary from model to model: in guinea pigs selective primary demyelination is pronounced, leading to large confluent plaques (Fig. 3a), closely resembling those found in multiple sclerosis [36]. On the contrarty, SJL mice and rats with chronic EAE show relatively sparse demyelination [12, 34]. In mice, larger confluent lesions are associated with extensive inflammation and considerable unspecific tissue destruction. In rats, large demyelinated foci in the spinal cord may show additional pronounced destruction of astrocytes [34]. Subsequent remyelination is carried out by Schwann cells in these cases. Interestingly, a similar pathology with destruction of astrocytes has recently been described in Japanese multiple sclerosis patients [29]. Overall, remyelinating activity is much higher in EAE as compared to multiple sclerosis.

Summarizing the present knowledge on the pathology of EAE and EAN, the similarities to the alterations in human inflammatory demyelinating diseases, including multiple sclerosis, are striking [36]. Although this does not allow the conclusion that the etiology of these diseases is the same, it indicates that very similar pathogenetic mechanisms are responsible for the initiation and propagation of the lesions.

Basic pathogenetic principles of EAE and EAN lesions

As discussed above, the main alterations in the nervous system in EAE and EAN are the inflammatory process and demyelination. Since there are apparent differences in the mechanisms leading to inflammation and demyelination respectively, they will be discussed separately.

Inflammation

T-cell mediated immune reactions against MBP

The transfer of EAE by intravenous injection of lymphocytes from sensitized donors [59] suggested that T-lymphocytes are involved in the induction of the disease. More recently, monospecific MBP reactive T-lymphocyte lines and clones have been raised, which are able to transfer EAE to healthy rats and mice [2, 100]. Similarly, EAN can be induced by intravenous injection of T-cell lines reactive with P2-protein [44]. This new technology, now allows to address the following questions: how many cells are the minimum requirement to induce disease, how do these cells reach the norvous system, how are they locally activated and how do they induce tissue damage?

Titration experiments with MBP reactive T-cell lines showed that, regardless of the animal species, 10M0[6] encephalitogenic T-cells are required to induce clinical disease [3, 67, 100]. However, careful pathological analysis of the CNS of such transfer animals revealed that as few as 10[3] MBP reactive T-cells give rise to focal, clinically silent inflammatory infiltrates in the CNS (Lassmannet al., in preparation). Furthermore, in vivo stimulation of such T-cells by simultaneous intravenous injection of recombinant interleukin [2] potentiates clinical disease in a dose-dependent manner [68]. These studies thus show that extremely few specific MBP reactive T-cells are required to start autoimmune inflammation in the CNS. This may partly explain why it is so difficult to detect a correlation between autoreactive T-cells and disease activity in chronic relapsing EAE [18] and multiple sclerosis.

It is controversial at present how encephalitogenic T-lymphocytes reach the brain. One concept postulates a soluble pool of MBP in the brain extracellular space, which is transported through the blood-brain barrier and presented to MBP reactive T-cells on the luminal surface of cerebral endothelial cells. In principle, it has been shown that intrathecally injected proteins, including MBP can be transported through the blood-brain barrier to the vascular lumen [86]. In EAE, perivascular accumulation of MBP and "endothelial" staining has been reported [74, 81], although convincing ultrastructural evidence is lacking. In our own material we were not able to find MBP reaction product by light and electron microscopic immunocytochemistry in or around vessel walls in acute and chronic EAE lesions.

A second requirement for antigen recognition by T-cells in cerebral vessels would be expression of class II (la) histocompatibility antigens on endothelial cells. Indeed, induction of la-expression on cerebral endothelial cells by stimulation with gamma-interferon was suggested from in vitro studies [51]. A large number of light microscopic immunocytochemical studies have documented la-reaction product associated with the wall of cerebral vessels [15, 39, 50, 76, 80, 81, 87]. Whereas in some studies la-expression on endothelial cells is described [76, 80, 81], all studies on rat CNS tissue did not report endothelial la-staining [15, 39, 49, 50, 87, 90]. Similarly, by ultrastructural immunocytochemistry, la-antigen was found on the luminal surface of guinea pig cerebral endothelial cells [77] but not on those of rats [39, 87]. It has, however, to be considered that the amount of antigen, as well as of la-determinants which is needed for T-cell activation, is very small and may be far below the level of detectibility by immunocytochemical techniques.

There are, however, some observations which argue against the concept of antigen presentation and recognition on the surface of cerebral endothelial cells. These aspects have recently been summarized by Wekerle et al. [91]. If cerebral endothelial cells present autoantigens in normal animals, resting MBP reactive T-cells should recognize their antigen, become locally activated and start the disease. However, transfer studies with MBP-reactive T-line cells showed that only activated cells are able to initiate EAE, whereas resting cells, even when injected in extremely high numbers, do not even enter the CNS compartment. Furthermore, not only MBP-reactive activated T-cells may reach the brain tissue, but also activated T-blasts directed against irrelevant antigens like ovalbumin. These data suggest that the primary T-cell migration through the blood-brain barrier and immune surveillance of the CNS depends on peripheral T-cell activation and is antigen-independent [91]. Whether specific antigen recognition on the endothelial cells of cerebral vessels plays an additional role in augmenting the inflammatory response has yet to be determined.

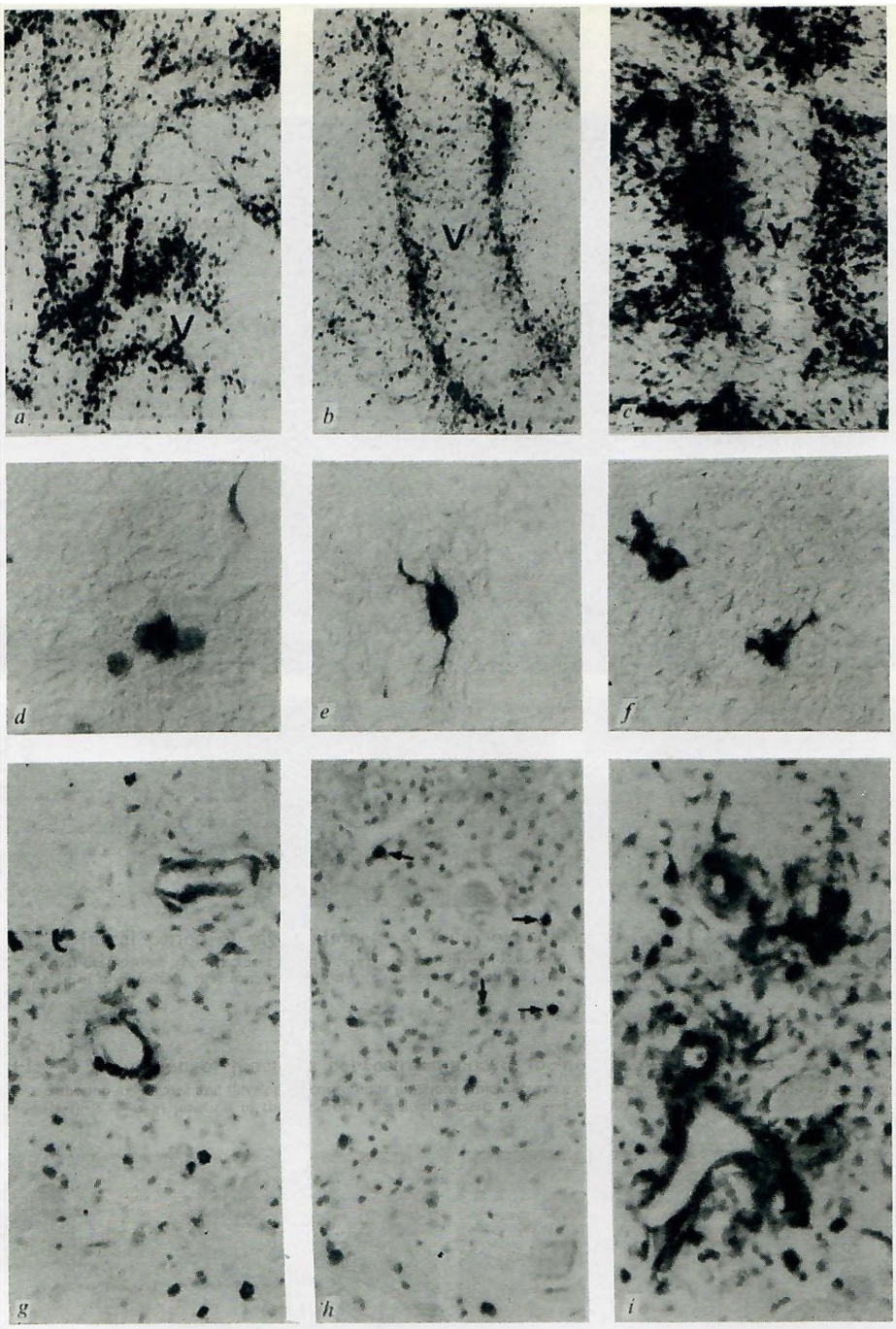

Fig. 1. T-lymphocytes and la-antigen in inflammatory lesions of acute rat EAE

a —15 days after sensitization (dps): many W3/13 $^{+}$cells (total T-cells) in the meninges, mainly in perivenous position; v — vene. Isolated leptomeninges, x 65; *b* — 15 dps: 0 x 8^{+} (suppressor/cytotoxic) T-cells in similar distribution as W3/ 13 $^{+}$ mononuclear cells. Isolated leptomeninges, x 65; *c* — 15 dps: intensive expression of la — (Ox6) antigen in the meninges. Isolated leptomeninges, x 65; *d* incubation period, 8dps: W3/13 $^{+}$ mononuclear cell in the spinal cord parenchyma. Paraffin section, x 650; *e, f* — incubation period, 8dps: 0 x 6^{+} cells in the spinal cord white matter with slender, ramifying proceses. Paraffin section, x650; g — acute, inflammatory EAE lesion, 15 dps: many W3 /13^{+} mononuclear cells in perivascular position and in the parenchyma. Paraffin seption, x 650; *h* same lesion as in Fig. lg: 0 x 8 $^{+}$ cells (arrows) are present in a similar distribution, although in lower numbers as compared; to W3 / 13 $^{+}$. Paraffin section, x 260; i — same lesion as in Fig. lg: massive expression of 0 x 6 antigen on cells in perivascular position and in the parenchyma. Paraffin section, x 260

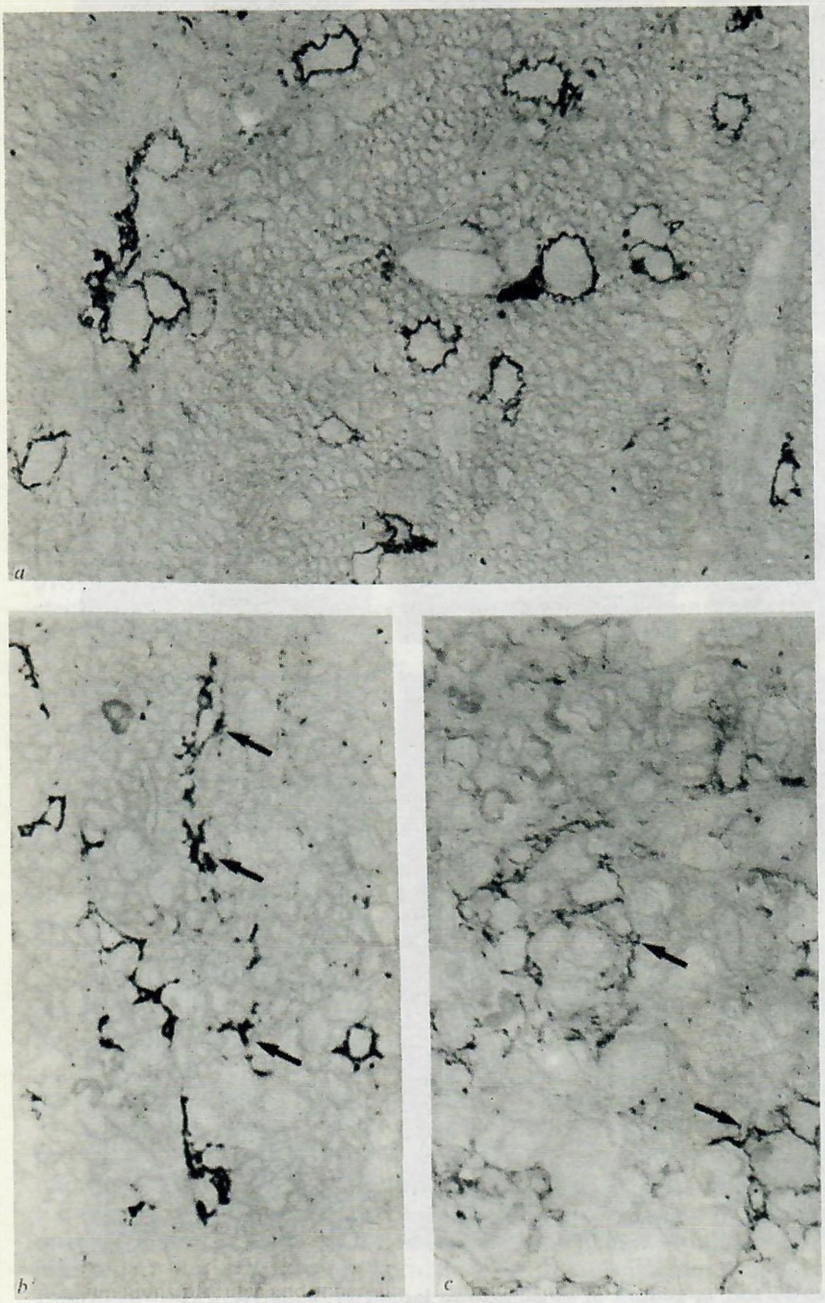

Fig. 2. Inflammatory infiltrates in mouse and rat EAE

a Lytl $^{+}$ T-cells dispersed within the relatively well preserved spinal cord white matter. SJL mouse, 310 dps, plastic section, preembedding staining method, x 1170; ft — strongly la $^{+}$ cells with stellate shape (arrows) at the edge of a demyelinating lesion; SD rat with chronic progressive disease course, 35 dps; plastic section, preembedding staining method, x 1170; c — la antigen in an actively demyelinating lesion from the same animal as in Fig. *2b;* 0 x 6^{+} cell processes (arrows) engulfing disintegrating myelin sheaths; plastic-section, preembedding staining method, x 1170

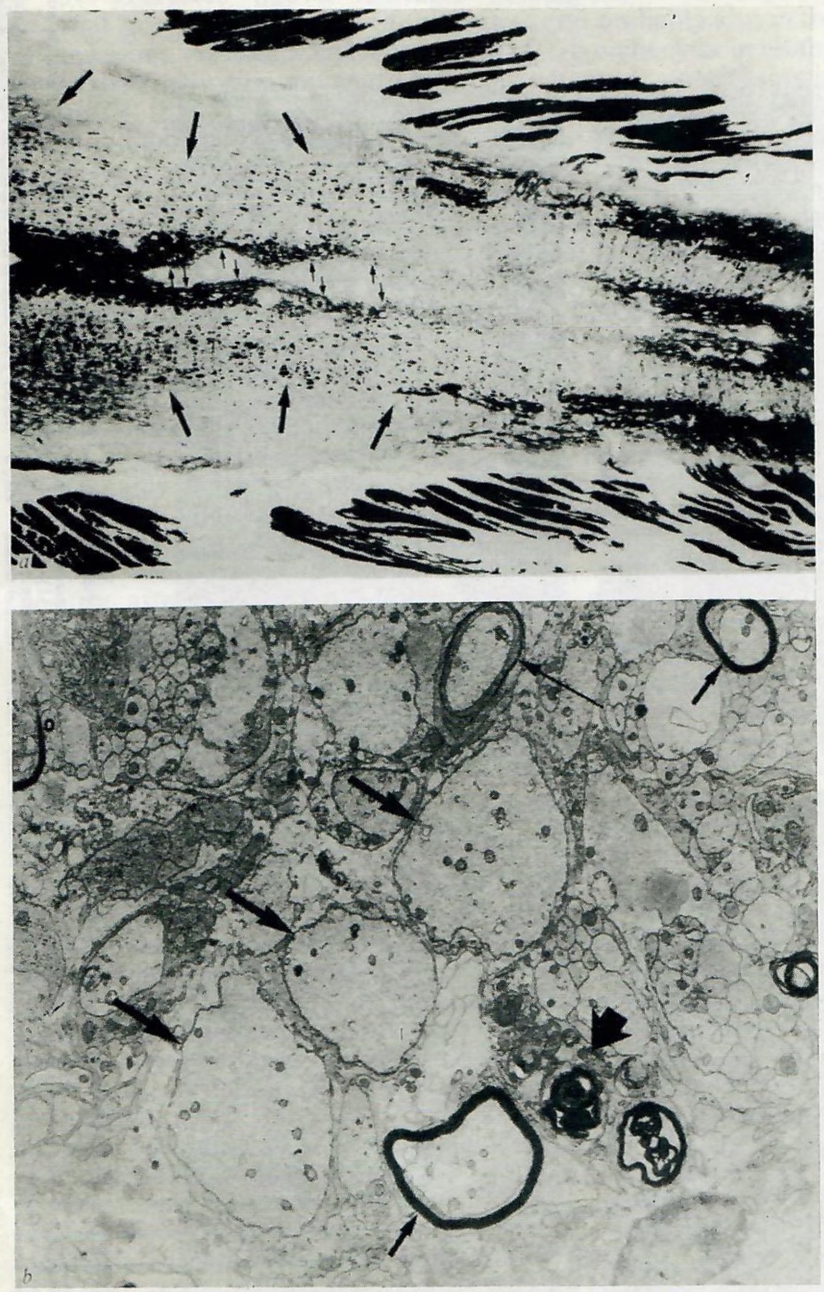

Fig. 3. Demyelination in EAE

a large, confluent perivenous (small arrows) and subpial (large arrows) demyelinated plaques in the medullary conus of a guinea pig with chronic EAE; 386 dps, paraffin section, Kliiver's myelin stain, x 35; *b* — demyelinated lesion in the spinal cord of a rat injected with encephalitogenic T line cells and a monoclonal antibody to a myelin / oligodendrocyte glycoprotein (MOG): a macrophage process with myelin debris (short thick arrow) among demyelinated axons (long thick arrow), two well-preserved nerve fibers (shorter arrow) and a remyelinated axon (long thin arrow), 4 days after antibody injection. EM, x 6000

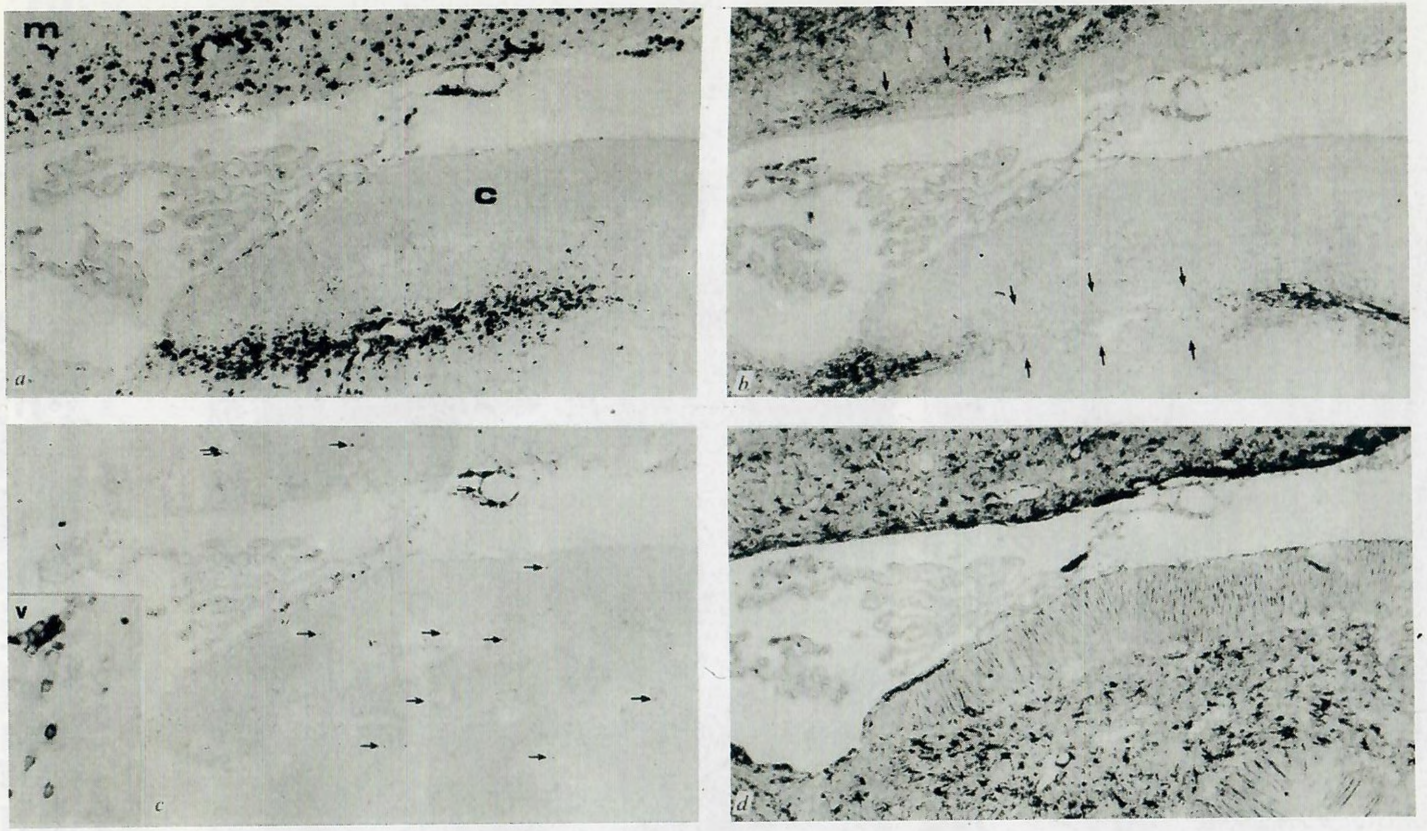

Fig. 4. MBP-reactive T line cells plus monoclonal antibody: a model to produce demyelination in acute EAE

a *d*: cerebellum (c) and medulla oblongata (m) of a Lewis rat injected with 4×10^6 encephalitogenic T line cells and 5 mg monoclonal antibody to MOG 4 days later. Perfusion 6 days after antibody injection. Serial paraffin sections, immunostained with a macrophage marker 2ED1, *a*), anti MBP (*b*), a T cell makrer (W3/13, *c*), and anti GFAP (*d*). × 100
a — numerous macrophages infiltrating the nervous tissue; *b* — large, party confluent zones of demyelination, corresponding to macrophage invasion (borders indicated by arrows); *c* — only few perivascular and infiltrating T cells 2some are indicated by arrows); insert: higher magnification of inflammatory T cells (v — vascular lumen; × 400); *d* — *reactive glosis parallels with inflammation and demyelination*

As discussed above, activated autoaggressive T-lymphocytes play an essential role in the induction of the disease and probably also in the propagation of the lesions. At least a high percentage of T-lymphocytes in established EAE lesions carry activation antigens (Lassmann, et al., unpublished). It is thus not surprising that therapy of EAE with monoclonal antibodies against T-cell activation antigens is effective and superior to that with antibodies against total T-cells or the "helper/inducer" T-cell subset [69, 70]. Considerable efforts during the last years have been devoted to the question, how encephalitogenic T-lymphocytes are activated and how their function is regulated within EAE lesions. Since CD4+ T-lymphocytes recognize their antigen only in conjunction with Class II (la) histocompatibility antigens, the presence and distribution of la-antigens within the CNS and especially in EAE lesion gives clues on possible sites of T-cell activation in the CNS. Indeed, EAE as well as EAN lesions show dramatic expression of la-antigens in the tissue [26, 56, 75] (Fig. *2b, c*). Abundant la-reactivity is present on macrophages and lymphocytes in the lesions and on stellate, microglia like cells in the surrounding tissue [50, 87] (Fig. 2 *b,c).* Interestingly, however, the number of la-positive cells was found to be considerably higher as compared to the number of cells stained with monocyte markers [15, 75; Lassmann et al., unpublished]. Since astrocytes have been shown to express la-antigens in vitro [19, 20], some of the stellate la-positive cells in EAE lesions could be astrocytes. Indeed, some authors have described la-positive astrocytes in EAE lesions by light microscopic immunocytochemical double staining, using glia fibrillary acidic protein as a marker for astrocytes [28] or by classifying la-positive cells by their morphology [80, 81]. Definite identification of la-positive astrocytes in light microscopic sections is, however, still problematic, even in double-staining experiments. By ultrastructural immunocytochemistry la-positive astrocytes have not been observed in acute EAE [87]; however, some weak la-reaction product has been found on astrocyte processes in chronic EAE lesions in rats [39]. No convincing evidence for la-expression on oligodendrocytes, myelin and neurons is available.

With recovery from acute EAE la expression in the lesions declines parallel with a decrease of "helper / inducer" T-cells and a relative increase of the "suppressor / cytotoxic" T-cell population (Table 1) [39]. It is not clear at present whether antigen-specific suppressor mechanisms downregulate the inflammatory reaction during recovery.

Another important question in EAE pathogenesis is how encephalitogenic T-cells interact with local tissue components and to what extent they by themselves exert tissue damage. In vitro, some demyelinating effects of EAE lymphocytes have been described in myelinated tissue cultures [5, 98]. More recently, demyelinating effects in vitro have also been reported to be induced by MBP reactive T-cell lines [47]. However, in these experiments the T-cell lines contained a considerable fraction of cells not reactive with T-cell markers. Furthermore, demyelination was also observed after exposure of cultures to lines directed against mycobacterial antigens [47]. A specific interaction of MBP reactive T-line cells with autoantigen-presenting astrocytes, leading to lysis of the presenter cells, has been described by Sun and Wekerle [78].

In vivo, T-cell line mediated acute EAE is a nearly exclusively inflammatory disease, with minimal or absent demyelination. In some models of chronic T-cell line mediated EAE demyelination may be present [79, 100]; it is, however, not yet excluded that during the chronic course of the disease additional immune responses are induced by endogenous liberation of antigens in the encephalitic

process. Direct injection of activated MBP reactive T-cell lines into the spinal cerebrospinal fluid did not induce structural lesions or demyelination in the adjacent white matter [91]. Thus, the present evidence suggests that MBP reactive T-cells induce vasculitis without significant direct damage to the CNS parenchyma. Clinical signs of T-cell mediated EAE are rather a consequence of vasculitis (edema, ischemia, etc.) than of immune mediated destruction of CNS tissue components like myelin.

The situation appears to be different in the peripheral nervous system. Contrary to the corresponding CNS disease, T-cell line mediated acute EAN is associated with significant .demyelination [30]. This suggests that peripheral myelin sheaths or Schwann cells could be destroyed by a direct cell-mediated mechanism. Interestingly, Schwann cells in contrast to oligodendrocytes are able to express la-antigens in vitro and to present MBP to MBP reactive T-cell lines [92]. Thus, it is possible that Schwann cells during T-cell mediated EAN are destroyed in the course of antigen presentation in a similar manner as described in vitro for astrocytes [78].

Other nervous system antigens and other immunological mechanisms involved in the pathogenesis of inflammation in EAE and EAN

Recently, evidence accumulated that MBP is not the only antigen which may induce EAE. Although already the early studies by W a k s m a n [89] suggested that proteolipid protein (PLP) may be encephalitogenic, only recently several models have been developed, which clearly document induction of acute or chronic EAE by highly purified PLP [13, 25, 48, 82, 95, 99]. Similar to MBP-induced EAE, the disease can be transferred to naive recipient animals by lymphocytes [96]. However, contrary to MBP-induced EAE, chronic disease courses are frequent [13, 82, 99], associated with widespread demyelination in the CNS. Another difference to M BP-induced EAE is that antibodies play a role in the pathogenesis of the disease [99], although no clear-cut correlations between clinical course and cellular or humoral immune responses were observed [82, 99].

In addition to PLP, other autoantigens have been described to induce disease by active sensitization. For the central nervous system they include gangliosides[14] and a yet not clearly defined membrane fraction of cerebral endothelial cells [84].

In the peripheral nervous system P2 protein is the main neuritogenic antigen [11], disease induction has also been reported by sensitization with galactocerebroside [65] and gangliosides [53].

In the central nervous system a cellular (T-lymphocyte mediated) immune response seems to be required to start an inflammatory or inflammatory demyelinating desease. At the present moment, there is no convincing evidence available that other immune reactions may start CNS disease in EAE. On the contrary, in the peripheral nervous system in addition to cellular immune reactions autoantibodies, either alone or in combination with immune complexes, may initiate lesions [65, 83].

Demyelination

Cellular immune reactions

Although, as discussed above, there is some demyelination in chronic models of MBP reactive T-cell line mediated EAE, there is overall little evidence for direct antigen-specific cellular immune responses leading to demyelination. This is further supported by ultrastructural immunocytochemical studies on the

interaction of inflammatory cells with tissue components during demyelination in central and peripheral nervous system lesions of EAE [39, 40, 77, 87]. These sudies did not find evidence for an interaction of T-lymphocytes with myelin sheaths during active demyelination. Demyelination was rather accomplished by la-positive cells with ultrastructural characteristics of monocytes and macrophages. It is thus not surprising that depletion of macrophages or inhibition of macrophage enzymes can inhibit demyelination in EAE [8, 9].

On the other hand, a T-cell mediated immune response could induce primary demyelination by an antigen unspecific way, in the sense of a bystander reaction [94]. In this situation monocytes and macrophages, activated in the course of the inflammatory reaction, secrete proteolytic enzymes, which could attack myelin sheaths (and other structures) in the surrounding. However, the importance of bystander demyelination, at least in the peripheral nervous system, has recently been questioned [23, 60]. In these experiments significant demyelination was only found, when in addition to an unspecific inflammatory reaction antibodies against epitopes located on the myelin surface were circulating. Similar studies in the central nervous system are lacking. The exact mechanism of myelin damage in EAE induced by transfer of MBP reactive T-cell lines is thus not determined. Furthermore, it is important to note that in these models significant demyelination is only observed after long-standing chronic disease course. It has thus to be considered that in the course of chronic CNS inflammation additional autoimmune responses are induced, which then are responsible for demyelination.

The lack of evidence for a direct, demyelinating T-cell cytotoxicity against MBP is not surprising, as MBP is located on the cytoplasmic side of the myelin membrane [58] and is thus not directly accessible for the immune system. Immune recognition of MBP can only take place when it is either processed and presented like in the peripheral nervous system [92], or when it is liberated into the extracellular space.

Antigen recognition in the CNS and subsequent demyelination is more likely for antigens located on the surface of myelin sheaths like PLP, galactocerebroside (GC) or gangliosides. Although T-cell mediated destruction of oligodendrocytes via galactocerebrosides has been described in vitro [54] and cellular immune reactions against gangliosides have been observed in EAE animals [55], their role in demyelination in active EAE lesions is not yet determined.

Demyelinating antibodies

Demyelinating activity of sera from EAE animals has first been described by Bornstein and Appel [4]. Complement dependent demyelinating activity was found in purified IgG fractions, as well as in IgG depleted fractions. This indicates that also IgM and (or) IgA may be involved in demyelination [24]. A similar demyelinating activity was also found in sera from animals with EAN [97]. More recently, demyelination was also reported to occur after injection of EAE sera into the cerebrospinal fluid [35], into the CNS tissue [93] or into the vitreous of the rabbit eye [7]. Similarly, EAN sera may induce demyelination when injected into peripheral nerves [64].

The immunological mechanisms by which demyelinating antibodies mediate demyelination in vivo are not fully elucidated. In the rabbit eye model, only IgG fractions are effective [24] and complement does not seem to play a role [7]. Demyelination is most likely mediated by an interaction of specific antibodies with macrophages, activated by lymphokines [7]. After injection into the cerebrospinal fluid or directly into peripheral nerves IgG, as well as IgM fractions are effective and the complement is at least partly involved in the demyelinating process [37, 66]. However, again in these models interactions with activated macrophages may play a role in demyelination [37].

An interesting feature of demyelinating antibodies is that they are not directed against the main encephalitogenic antigen, the MBP molecule [72]. This is not surprising, since MBP is located on the cytoplasmic side of the myelin membrane [58] and is thus not directly accessible for the immune system.

The main requirement for a myelin antigen as a target in antibody-mediated demyelination is its localization on the extracellular surface. Up to now, several such antigens have been identified. They include galactocerebroside [16] gangliosides GM1 and GM4 [63, 71], myelin / oligodendroglia glycoprotein (MOG) [41, 43] and possibly myelin-associated glycoprotein (MAG) [42]. Antibodies against epitopes, of PLP which are located on the surface of myelin have not yet been tested for demyelinating activity.

Role of demyelinating antibodies in the pathogenesis of EAE

EAE animals develop a humoral immune response against myelin antigens, which is most pronounced in the chronic stage of chronic relapsing EAE [21, 57]. In general, serum-demyelinating activity is highest in chronic disease, when large plaques of demyelination are formed in the CNS [35, 38], and in some models a correlation between myelination-inhibiting serum activity and demyelination in the CNS has been observed [6]. Furthermore, experimental induction of focal inflammatory infiltrates in the vitreous of the rabbit eye in galactocerebroside-sensitized animals induced primary demyelination in the retina [10, 22].

Most of the studies on antibody-mediated demyelination have been performed either with EAE sera or with antisera directed against galactocerebroside. However, in chronic relapsing EAE in guinea pigs, which is at present the model with the most widespread demyelination, the immune response against galactocerebroside is inconsistent and weak, and many demyelinating antisera from these animals do not contain measurable antibody titres against galactocerebroside [71]. More recently, however, we were able to show that demyelinating activity of guinea pig EAE sera correlate well with antibody titres against a novel myelin / oligodendroglia glycoprotein (MOG) [47], an antigen which is located in the surface of myelin sheaths and is a target for antibody-mediated demyelination in vivo [41].

We have used a monoclonal antibody against MOG to modulate the disease course and pathology of T-lymphocyte mediated EAE in rats [46] (Figs. 3*b*, 4): when anti-MOG antibodies are intravenously injected at the onset of EAE, induced by M BP-reactive T-cell lines, the severity of clinical disease is augmented and large confluent plaques of demyelination are formed, which closely resemble those found in models of chronic relapsing EAE. Furthermore, different disease patterns, reflecting the whole spectrum of inflammatory demyelinating diseases, can be reproduced by varying the balance between the number of encephalitogenic T-cells and monoclonal antibodies.

A similar augmentation of demyelination was also found, when anti-galactocerebroside monoclonal antibodies were injected in animals, in which EAE or EAN was induced by MBP or P2 protein reactive T-cell lines.

Summary

Based on recent neuropathological and immunological data, a hypothetical model of EAE and EAN pathogenesis is presented. The discussed pathogenetic mechanisms suggest that the full picture of inflammatory demyelination is induced by a complex interaction of cellular and humoral immune reactions. Furthermore, they show that in principle several different antigens of the CNS

and PNS can mediate autoimmune inflammation or demyelination, respectively. The basic principle, however, consists of two mechanisms:

1) An inflammatory response (vasculitis) which in most (if not all) instances is T-cell mediated and directed against an autoantigen, which is liberated from myelin sheaths, and presented to T-cells by perivascular presenter cells (monocyte / microglia-like cells) and possibly by local cells of the CNS. This process leads to perivascular inflammation with disturbance of the blood-brain barrier and activation of effector cells. Clinical disease in this purely inflammatory disorder results from secondary vascular problems (edema, ischemia) rather than from direct damage to the nerve parenchyma.

2) A demyelinating response, which is specifically directed against antigens or epitopes, which are exposed on the extracellular surface of myelin sheaths. A role of antibodies against myelin surface determinants in the demyelinating process is well documented. There is at present little evidence for direct T-cell cytotoxicity in the pathogenesis of demyelination. Destruction of myelin, opsonized by antibodies is mainly accomplished by monocytes / macrophages, which had been activated previously in the course of the T-cell mediated unflammatory process.

Although at present likewise detailed data are not available for multiple sclerosis lesions, the striking similarities between EAE and human inflammatory demyelinating diseases suggest that comparable mechanisms may be responsible for the human disorders.

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Book review

Glial-Neuronal Communication in Development and Regeneration. Edited by Hans H. Althaus and Wilfried Seifert. Springer-Verlag. Berlin, Heidelberg, New York, London, Paris, Tokyo, 866 pp. 1987

The development of the nervous system includes various processes resulting from a complex interaction between the genetic programme of the stem ectodermal cells and the surrounding humoral and cellular environment. Neuroglia differentiate at that in a number of different cell types. It has been accepted for many years that the functional communication between glial and neuronal cells, suggested already in 1928 by Roman y Cajal, confines only to the participation of glia in the regulation of intercellular contents of K + , in detoxification, nutrition and myelination of axons. Our understanding of glial cell physiology has increased greatly in the last decade. The widespread application of tissue culture techniques to examine glial and neuronal cell activity has revealed that glial cells, far from being inert or passive constituents of the brain microenvironment, are capable of participating in various physiological activities previously relegated iprimarily to neuronal populations. Thus, glia can participate in macromolecular, neurotransmitter and ion exchange regulation and, in turn, can influence neuronal survival, proliferation and differentiation. During ontogenesis, glial cells undertake a guiding function and provide an extracellular matrix necessary for axonal regeneration; they may also release neuronotrophic (NTF) and neurite promoting factors (NPF) which are of great importance for the reparative processes in the central and peripheral nervous system (CNS, PNS).

The book *Glial-Neuronal Communication in Development and Regeneration* succeds in bringing together authors with personal experience in these interesting aspects of present-day neurobiology, providing under the cover of a single volume of 866 pages a selection of subjects not otherwise available in one book. It represents the proceedings of a symposium under the same name held in Göttingen (FRG) in 1985. The book is divided into seven sections with three to thirteen contibutions in each section.

The first section deals with glial cell lineages. Chapter One is by S. Fedoroff and brings forward data about the phenotypic expression during development of cells from astrocytic family: radial glia, protoplasmic and fibrous astrocytes, ependimal cells, Bergmann glia, Müller's cells and pinealocytes. Good models to study reactive astrocytes and fibrous gliosis are astrocytes in primary culture, activation of Müller's cells under , light-induced retinal degeneration and fetal spinal cord transplantation into injured mature spinal cord (L. Eng.). In Chapter Three the group of G. Kreutzberg presents results of ultrastructural and immunochemical study on microglia in culture supporting the concept for a common mesodermal origin of macro- and microglia and that after injury microglia might become immunocompetent (E. Rieske-Shows et ala.). Further, R. Nirsky and K. Jessen give detailed information on the antigens (GFAP, Ran_2, N-CAM, L,) of peripheral glia (enteric glia, satellite cells, myelin-forming and non-myelin-forming Schwann cells).

The second section of the book is devoted to the receptors and antigens expressed by glial and neuronal cells during ontogenesis. It begins with a precise autoradiographic and electrophysiological study by L. Hösli and E. Hösli who demonstrate coexistence of a- and (3-adrenoreceptors, together with histamine receptors on glial membranes. In Chapter Three Zanetta et al. show that complementary molecules (glycoproteins-lectins) exist on the partner cells (Purkinje cells-radial glial fibers) in the molecular layer of cerebella of young rats at the period of synaptogenesis. These complementary molecules actually participate in a recognition phenomenon which serves to eliminate components of the

surface of one of the partner cells. The reported data by F. Omlin et al.. suggest that myelin-associated glyco-proteins (MAG) could be involved in mechanisms related to cell-cell' recognition or cell migration too.

The last two chapters of this section analyze the role of Müller's cells in the uptake and inactivation of GABA in the synapses of retina (N. Osborn) and the developmental^ regulated glial and neuronal antigens detected by monoclonal antibodies (M. Ghandour et al.)

The third section of the book examines the expression of recognition and adhesion molecules on the glial / neuronal cell surface. The first chapter by M. Albrechtsen et al. deals with biosynthesis of the neural adhesion molecules (N-CAM) in primary cultures of rat cerebellar granular neurons, cerebral glial cells and skeletal muscle cells. The properties of surface structures capable of enhancing neurite outgrowth of neuroblastoma cells and of neurons from embryonic rat brain in culture are considered by H. Rauvala et al. They present their recent attempts to solubilize and characterize membrane-bound neurite-promoting activities that might function in contact-dependent outgrowth of neurites in CNS. The last chapter by S. Carbonetto et al. is an account of the importance of the complementary interractions between the matrix adhesive molecules (located in the cellular membranes) and some components (collagens and non-collagens glycoproteins) of the extracellular matrix for a successful axonal regeneration in CNS.

The fourth section of the book is concerned with the glial / neuronal metabolic interractions. The elevation of K^+ concentration under neuronal activation or after application of monoamine transmitter substances increases the glycogen turnover in the glial cells of leech segmental ganglia (V. Pentreath et al.). In Chapter Two A. Schousboe et al. discuss the mechanisms through which neurons might modify the active uptake of GABA and glutamine in astrocytes. It is followed by the report of M. Tytell supporting the hypothesis that a protein transfer does occur from the surrounding glia into the axoplasm in squid giant axon through phagocytosis of the glial processes by axons. In the last chapter F. Mugnaini and M. Fiori consider the possibility that a glial-neuronal (satellite glia-afferent axonal terminals) competition may exist for apposition to neuronal surfaces during the establishment of intracellular contacts in avian cilliary ganglion.

The fifth and the longest section of the book contains a number of diverse chapters examining the influence on glial / neuronal cells by hormones, gangliosides, trophic factors and informational substances. The first three chapters deal with the problems of the effect of serum-containing media on the survival and maturation of neurons in culture (R. Balazs et al.), the role of glycolipid- and glycorpotein synthesis for neurite outgrowth (E. Yavin et al.), glial / neuronal communication (cell migration, interaction with extracellular matrix) during ontogenesis of cerebellum (G. Moonen et al.). Chapter Four by Ch. Richter-Landsberg andB. Jastorffisa review of the role of nerve growth factor (14GF) and cAMP analogues on neuronal differentiation in PC12 cells. Further, K. Unsicker and R. Lietzke provide a concise, well-written chapter updating the previous summaries on the neuronotrophic (NTF) and neurite-promoting factors (NPF) in developing chromaffin cells. The authors' concept is that chromaffin cells may be considered as modified neurons that are both targets and storage sites of NTF and NPF. The next five chapters take the reader into detailed reviews of the functional role of astrocytes during development of the nervous system and their interactions with neuronal cells in mature brain. In Chapter Seven the group of Seifert describes a model for biological testing and discrimination of different activities (for neuronal survival, neurite initiation and elongation) of a factor isolated from astroglial-conditioned medium which has a specific trophic effect only for hypocampal_ neurons (S. Beckh et al.). Conditioned medium from astrocyte cultures derived from the cerebellum of 7-day-old rats contains an autoregulative growth factor which stimulates the cell division of cerebellar and cerebral astrocytes to quantitative variable degree (A. Michler-Stuke). In Chapter Ten G. Fischer presents a method for serum-free cultivation of astrocytes and points the factors specifying the cellular heterogeneity, proliferation and differentiation in culture. This is followed by an article of the group of M. Sensenbrenner who describes their data on the biochemical characterization of an astroglial growth factor (AGF) isolated from bovine brain (B. Pettmann et al.). This factor is composed actually of two activities (AGF, and AGF2) which are identical to the acid- and basic fibroblast growth factors, respectively. AGF is localized in the nuclei of neurons and could play a role in the regulation of glial proliferation in ontogenesis, as well as in reactive gliosis after brain injuries. The remaining chapters are on the effect of epidermal growth factor on glial cell development in aggregating cell cultures as a function of developmental stage and culture conditions (P. Honegger and B. Guentert-Lauber), the trophic and metabolic coupling batween astroglia and neurons (S. Varon et al.), the effect of muscle-derived substances on survival and neurite outgrowth of spinal cord neurons and identified motoneurons in culture (Ch. Henderson).

The sixth section of the book summarizes the current undestandings of the mechanisms of axonal regeneration. It begins with there chapters outlining the structural and functional plasticity of CNS and PNS by neural tissue transplantation technique (B. Bregman and P. Reiner; O. Isacson et al., I. Zimmer et al.). Recently, it has been demonstrated by the group of Aquayo that the fate of transected neuron depends only on the type of the surrounding glia (other cells and matrix) irrespective of the cell

body's location in CNS or PNS. Thus, successful axonal regeneration occurs only in the environment of peripheral glia (Schwann cells and extracellular matrix) or of glia from immature CNS. In Chapter Four K. Crutcher examines the mechanism of axonal outgrowth of sympathetic neurons (with cell bodies located in PNS) into CNS-tissue (hypocampus). The results support an already generally accepted view that axonal growth in the mature nervous system requires a peripheral environment. The astrocytes, forming an important element of the neuronal environment, proliferate after trauma in all vertebrates. However, the generated astrocytic scar in fish and amphibia is permissive for growing axons, whereas in birds and mammals it is not. The deficiency of the astroglial / axonal interaction in avian and mammals might be due to the occurence of orthogonal arrays of particles only in the cell membranes of these species (H. Wolburg). Chapter Sixth by R. Lindsay et al. is a very good account of studies using tissue culture manipulations, cell marketing techniques and plasma clot method to follow the development of grafted neurons and glia in brain. The next chapter is concerned with models to study reactive gliosis after mechanical trauma or administration of neurotoxicants (kainic acid, trimethyltin). The treatment of lesioned rats with cytosine arabinoside or immunosupressant prior to lesioning greatly reduces the proliferative response of glia cells (M. Billingsley et al.). Further, H. Müller and E. Shooter report that the expression of the 37 kDa protein (apolipoprotein E) during development, as well as after injury of the PNS and CNS suggests that it plays a role in *de novo* nerve growth and in nerve repair, probably related to lipid metabolism and transport. The 37 kDa protein/apo E is expressed by macrophages in the PNS and astrocytes in the CNS of newborn rats, indicating a previously unexpected common function of these ontogenetically unrelated cell types in nerve development. In the last chapter a cell separation method is described which allows the simultaneous isolation and cultivation of neurons and oligodendrocytes from the same brain of young adult rats (A. Stoykova et al.). Mature CNS neuronal cells show low regenerative capabilities for neurite outgrowth in culture which increase considerably when cocultivated with oligodendroglial cells.

The final section of the book deals with the mechanisms of myelination and remyelination by oligodendrocytes in CNS and by Schwann cells in PNS. The removal of galactocerebrosides from the Schwann cell surface during the initial stages of myelinogenesis can prevent the myelination without significantly altering the other Schwann cell functions: formation of basal lamina and axonal ensheatment (B. Ranscht et al.). K. Jessen and R. Mirsky demonstrate that galactosocerebrosides appear to be a ubiquitous component of the mature Schwann cell membranes, irrespective wheather they form myelin or not. This suggests that the role of glycolipids in axon-glia interactions; is more general than currently envisaged and cannot be restricted to myelination only. This is followed by a very good account of the. molecular organisation of the cell membrane in normal and pathological axons (S. Waxman)., This section also has a chapter from N. Ratner et al., presenting evidence from culturing of neurons and Schwann cells that the burst of Schwann cell proliferation during development is regulated by a molecule (heparin sulfate proteoglicans?) on the neuronal surface. In Chapter Five R. Bansal et al. take the reader into detailed review of oligodendroglial differentiation in primary culture of dissociated fetal rat brain. Although oligodendrocytes undergo the main normal mylein-related differentiation, the synthesis of galactolipids, MBP, PLP, CNP proceeds at a lower level and the cells fail to complete the process with the elaboration of normal in vivo levels of myelin membranes. The chapter by S. Szuchet provides a good reading material on the mechanism of remyelination: a process of deposition of myelin on an axon that has lost its former myelin. The mature oligodendrocytes grown in pure culture synthesize myelin components and form multilamellar profiles with the ultrastructural and biochemical features of normal myelin. The process occurs in the absence of neurons and is designated by the term myelin palingenesis (reformation). In the last chapter H. Althaus.et al. describe an in-vitro system to study remyelination. Oligodendrocytes isolated from mature pig brain can be cultivated for several weeks in monolayer culture and enwrap with their new-formed processes the added carbon fibers. Several morphological and biochemical criteria provide evidence that this material represents in part mature myelin.

In conclusion, this is an important and useful book for beginners as well as advanced neurobiologists with interest in the neuro/glial interractions in development and regeneration of nervous system. The contributions are up-to-date, written by recognised authorities in their respective fields who point to problems of importance in the direction of future research.

A. Stoykova

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1. Tuohy, V. K., Z. Lu, R. A. Sobel.R. A. Laursen, M. B. Lees. A synthetic peptide from myelin proteolipid protein induces experimental allergic encephalomyelitis. — J. Immunol.. 141, 1988.1126-1130.
2. Norton, W. T., W. Cammer. Isolation and characterization of myelin. — In: Myelin (Ed. P. Morell), New York, Plenum Press, 1984, 147-180.

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