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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.

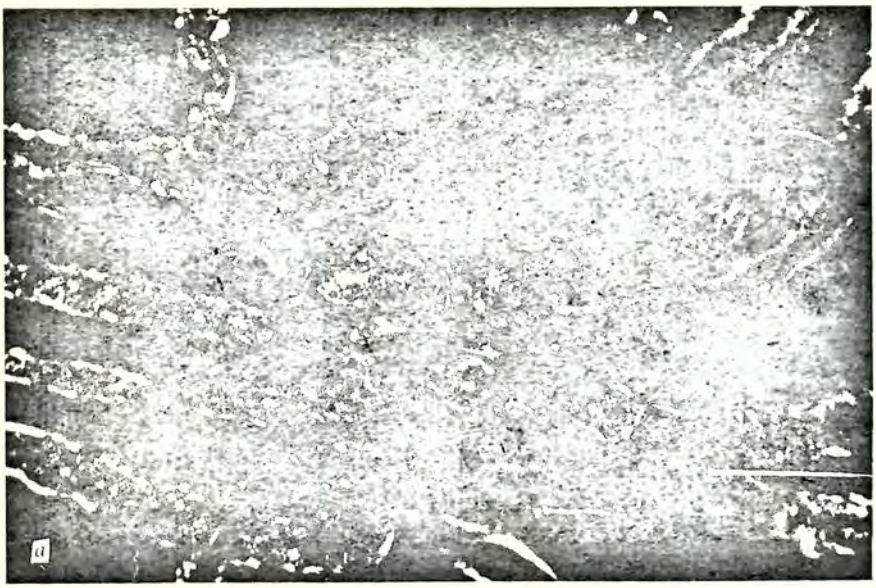
Key words: myelin proteins, PNS demyelination, CNS demyelination.

Common sense tells us that recent factual information and knowledge is mostly more accurate than old knowledge. In fact, modern 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 Friedländer in 1889 and by Ambronn in 1890.

It was only in the thirties of the present century that Schmidt 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 Mellick 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 l 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

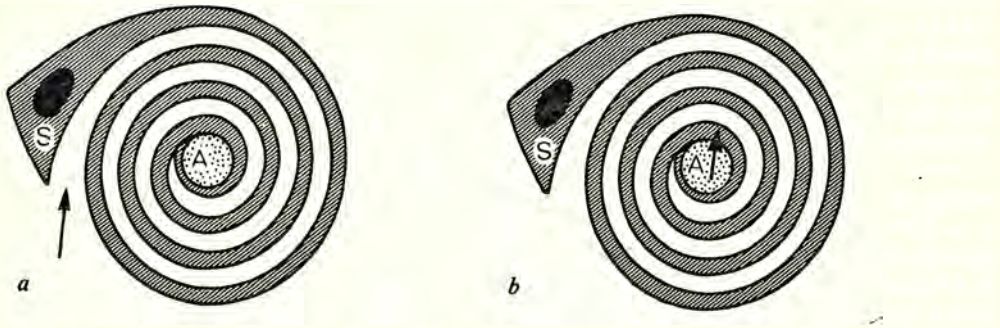


Fig. 2. Schematic representation of two types of demyelinating processes (Reprinted from an article of M. Wolman in the *J. Cytochem.* ref. 94, by the kind permission of the Histochemical Society Inc.) *a* — centripetal spread of lysis; *b* — centrifugal spread

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]. Feigin and Cravioto 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. Poduslo and Yao [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.

Blaurock and Nelander [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, P_1 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. Petrescu [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, Marchi-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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