Histochemistry: The Widening Horizons
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Chapter
10

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Histochemical Contributions to the Study of Multiple Sclerosis

INTRODUCTION

Over the last 30 years, histochemistry has contributed substantially to the study of the demyelinating diseases. However, before considering the application to demyelinating disease, it is perhaps appropriate to consider some aspects of the developments of histochemistry over this period.

Around 1950, histochemistry was emerging from an occasional staining exercise towards an established scientific method and technology. Much of the impetus that established histochemistry can be attributed to the publication of the first edition of Pearse's textbook in 1953. Other groundwork has been accomplished, such as devising the early phosphatase reactions and the PAS method Z(see Gomori, 1952; Lillie, 1954). Although Gomori's book makes rewarding reading and Lillie's is an excellent reference book, the initial

impetus to set histochemistry on its way in the early stages was mainly achieved by Pearse's first edition. Its readability has doubtless stimulated many research workers into embarking into histochemistry—as it did with me—and the clarity of its presentation later resulted in a subsequent edition becoming one of the most cited texts in the scientific literature.

In its early days, histochemistry was largely concerned with methods based on chemical reactions that actually worked within tissue sections, but perhaps by the 1960s most of these possibilities had been explored. Much more emphasis was then placed on validation of existing methods, improved localization and quantification, both by autoradiography and later by microdensitometry. An exception has perhaps been in the development of fluorescence microscopy over the last 20 years. However, by 1970, the main direction of conventional histochemistry had become largely technical, namely the improvement of existing methods and their establishment as reliable technical methods.

Histochemical methods have spread over these years into biochemistry, with their use in electrophoresis and in thin-layer chromatography and vice versa. Subcellular fractionation led to the biochemists' interest in electron microscopy and electron histochemistry: in this, as well as other areas, the borders between histochemistry and biochemistry have become indistinct. It has always been claimed that the most valuable aspect of histochemistry is the capacity to localize at the histological level: this is a capacity quite beyond classical biochemical methods.

Over the last decade, the most striking development has been in histoimmunological methods, first with immunofluorescence and more recently with peroxidase-labelled antibody. In many ways this has revolutionized protein histochemistry, and now allows the precise recognition of a substance if it contains an antigenic locus. Even further advances may be expected when monoclonal antibodies become available for immunohistochemical use. Not only may particular tissue components be identified, but specific hormones and enzymes can now be detected and localized. Microbiology has a further practical application in utilizing fluorescence methods for the detection of specific parasites, organisms, and viruses: this application for detecting virus particles in tissues has an enormous future potential.

Standing back to see where histochemistry has travelled over the last 30 years leads one to consider whether it has lost its identity as a separate specialist discipline. Immunohistochemical methods are now the standard tools of cell-biologists, immunologists, and pathologists, while much of the subcellular and analytical aspects of histochemistry ('cytochemistry') have merged into biochemistry. Electron histochemistry—as far as it has managed to progress—now seems to be part of the electron microscopist's expertise.

The less complex histoenzymic methods and the standard chemical staining

reactions have now passed into the general armamentarium of histology, pathology, and cell biology. This means that, at a histochemistry meeting, much if not most of the work will be of an applied nature, with emphasis on the pathological or histological problem rather than on histochemical technology. Perhaps this is the way histochemistry is to go and indicates how successful its methodology has become, in that it has been absorbed into the standard grammar of histology, cell biology, pathology, immunology, and biochemistry. Histochemistry has now progressed beyond the stage of a novelty and has achieved its place as an integral part of many separate disciplines.

It is now time to consider the contributions that histochemistry has made to the study of a particular disease, namely multiple sclerosis. In passing, it may be mentioned that the histochemistry of the demyelinating diseases have been previously reviewed by Seitelberger (1960), Adams (1962a, 1972) and by Adams *et al.* (1965).

THE LIPIDS IN DEMYELINATION

Noback and his colleagues (Noback and Montagna, 1952; Noback and Reilly, 1956) had shown that myelin breakdown in Wallerian degeneration is preceded by a stage where the sheath undergoes fragmentation with the formation of ellipsoids and granular products. The staining characteristics remain normal at this stage, and it is somewhat later that sudanophilic lipid appears during myelin breakdown. In the last century, Marchi had devised a method using osmium tetroxide and an oxidizing agent to demonstrate degenerating myelin, but this reaction did not become positive until a later stage of demyelination (for references, see Adams, 1962a).

The Marchi reaction in degenerating myelin is due to the hydrophobic nature of the predominant cholesterol esters in the breakdown prodcuts (Fig. 1; Adams, 1958). Both osmium tetroxide and the polar oxidizing agent (potassium chlorate) penetrate the polar lipids of normal myelin, and potassium chlorate here prevents the reduction of osmium tetroxide by the lipid ethylene bonds. However, hydrophobic esterified cholesterol in degenerating myelin allows only osmium tetroxide and not polar potassium chlorate to enter and, thus, osmium tetroxide is reduced by lipid ethylene bonds to the black OsO_2 (Adams, 1958). Osmium that has entered normal myelin, but is held in unreduced state by potassium chlorate, can be demonstrated by its red–orange complex with α-naphthylamine, whereas esterified cholesterol reduces osmium tetroxide to black OsO_2. This appears to be the mechanism of the osmium tetroxide-α-naphthylamine (OTAN) method used for the simultaneous demonstration of normal and degenerating myelin (Adams, 1959).

Following the histochemical studies by Noback (cited above) and the chem-

ical investigations of Rossiter and colleagues (Johnson *et al.*, 1950), it was apparent that myelin breakdown in Wallerian degeneration went through a preliminary 8-day phase of physical disintegration before actual *chemical* breakdown of the component lipids. This chemical breakdown results in the formation of esterified cholesterol (Johnson *et al.*, 1950) and its presence was regarded by Cumings (1953, 1955) as the hallmark of demyelinating disease. Nevertheless, the formation of esterified cholesterol is a *secondary* event in myelin breakdown. As pointed out by Petrescu (1969), the OTAN method shows that myelin cholesterol is esterfied as a function of the mononuclear phagocytes (macrophages or microglia) in the multiple sclerosis lesion (Fig. 1). Such esterification is, of course, a well-known feature of reticuloendothelial cells involved in phagocytosis and storage (e.g. certain forms of experimental athersclerosis, lipoid pneumonia, hypercholesterolaemic xanthomatosis, etc). Hence, at this stage of the discussion, it can be stated that the eye-catching sudanophilic Marchi-positive esterified cholesterol in myelin breakdown has no pathogenic significance in multiple sclerosis: it merely represents a late stage of chemical degradation.

It should be stressed at this point that no distinction has so far been made in this chapter between Wallerian degeneration and multiple sclerosis. There is, however, a major difference in that multiple sclerosis is a primary demyelinating condition and the axon is spared, whereas Wallerian degeneration is a non-specific response of the whole neurone that results in degeneration of both the myelin sheath and axon.

Abnormal lipids have been detected in the 'normal' white matter outside plaques of multiple sclerosis. Some lipid is esterified cholesterol, perhaps representing mini-plaques. However, a metachromatic lipid has been iden-

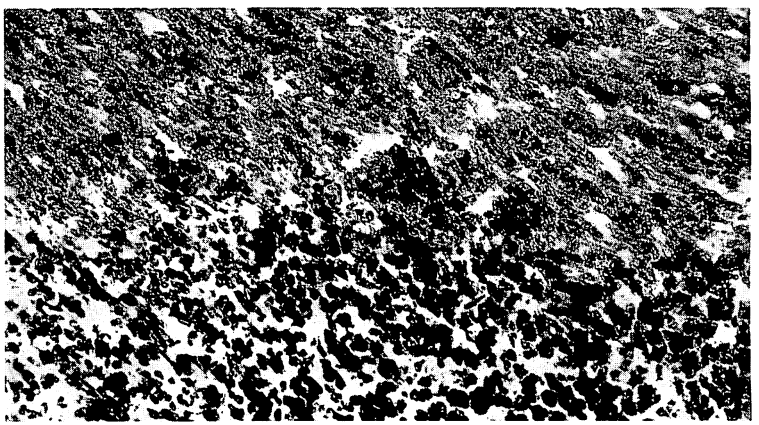

Fig. 1. Marchi reaction (OsO_2) for degenerating myelin within mononuclear phagocytes in plaque (bottom) of multiple sclerosis, OTAN × 90

tified histochemically in neuroglial cells outside plaques (Bayliss and Adams, 1971). It may possibly be a sulphatide, but its significance is obscure.

PROTEOLYTIC ENZYMES, PROTEIN LOSS, AND PHYSICAL FRAGMENTATION OF MYELIN

The early fragmentation of myelin in Wallerian degeneration seems to be of greater significance than the later lipid changes. The possibility was considered 20 years ago that the protein skeleton of the lamellar structure of myelin (the period or interperiod lines or both) may be damaged or partly digested in the early phase of myelin breakdown, hence accounting for the fragmentation and disruption of the sheath (diagrammatically shown in Adams, 1962b). It was shown that proteolytic enzymes are capable of removing lipid from myelin, and the local application of trypsin to brain sections produces an artificial plaque of demyelination (Fig. 2; Adams and Tuqan, 1961; Adams, 1962b). Histochemical methods have revealed that proteolytic activity increases in Wallerian degeneration (Fig. 3; Adams and Tuqan, 1961; Hallpike and Adams, 1969) and this is matched by corresponding increases in proteolytic and other lysosomal enzyme activities in the brain in multiple sclerosis (Fig. 4; Adams, 1968; Adams and Leibowitz, 1969; Hallpike and Adams 1969; Hallpike *et al.*, 1970a; Adams *et al.*, 1971b; Allen *et al.*, 1979). The histochemical studies confirmed and extended a previous biochemical study on Wallerian degeneration by Porcellati and Cutri (1960), who showed an increase in proteolytic activity at 2 weeks after nerve section. The histochemical findings in Wallerian degeneration and multiple sclerosis have apparently been the stimulus for a number of further biochemical studies on proteolytic and other lysosomal enzymes in this disease (e.g. Einstein *et al.*,

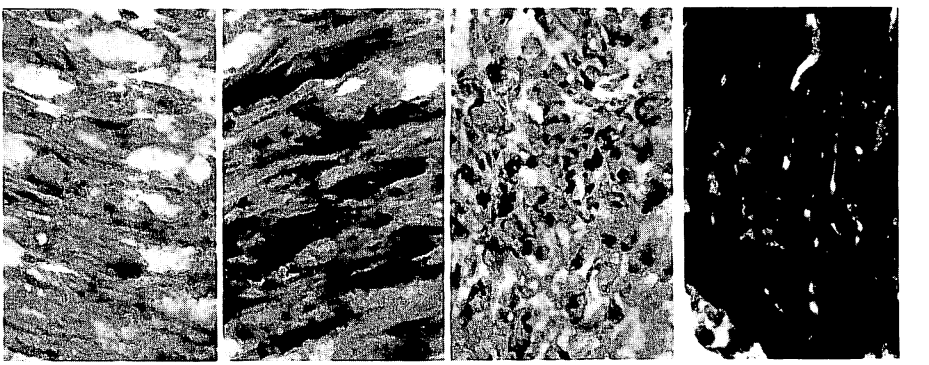

Fig. 2. The effect of tryptic digestion (0.1 per cent for 1 h at pH 7.0) on the staining of lipids in human white matter, each × 310. Left to right: Sudan Black B after trypsin; Sudan Black control; Baker's acid Haematein after trypsin; and Haematein control

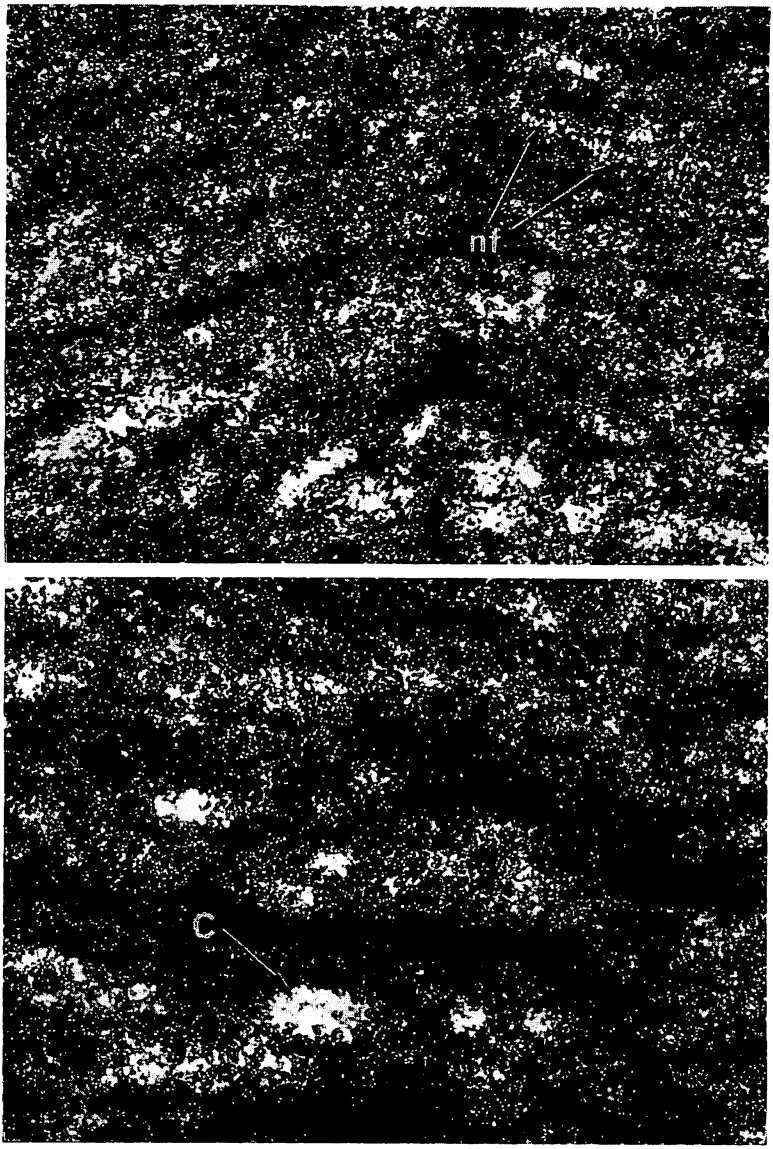

Fig. 3. Top: Acid proteinase activity in rat sciatic nerve, 4 days after section. Silver–gelatin autogram, pH 3.5, × 320. Bottom: As above but 8 days after section. nf = nerve fibre; C = Cajal 'digestion chamber'. (Reproduced with permission from *Histochem. J.*, 1970, **2**, 212)

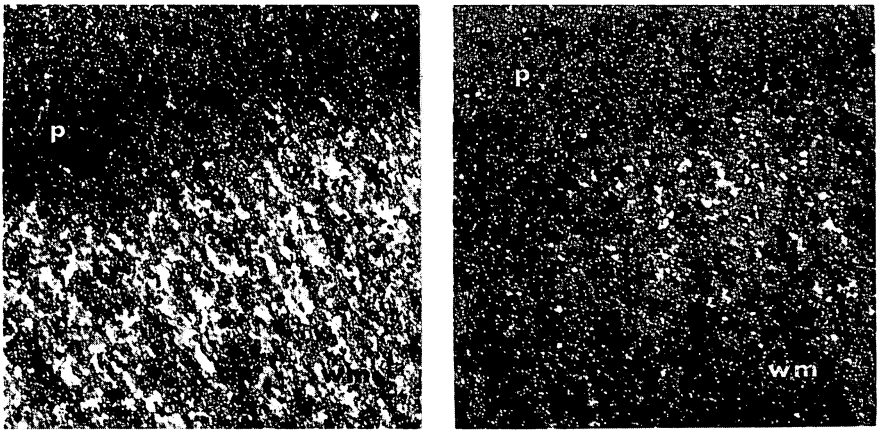

Fig. 4. Left: Acid proteinase at edge of active plaque of multiple sclerosis. Silver–gelatin autogram, pH 3.5, × 75. Right: As above, but at edge of inactive plaque. Wm = white matter; p = plaque. (Reproduced with permission from *Histochem. J.*, 1970, **2**, 202)

1970; 1972; Riekkinen *et al.*, 1970; Marks *et al.*, 1974. Cuzner *et al.*, 1978, Norton *et al.*, 1978, and others). Moreover, the histochemical approach discussed above has been extended to the study of neuroproteases in general, including their role in experimental allergic encephalomyelitis (Gabrielescu, 1978).

MYELIN PROTEINS IN DEMYELINATION

It is of interest that the component basic protein of myelin is a particularly good substrate for cerebral acid proteinase (Einstein *et al.*, 1968) and is lost from the plaque in multiple sclerosis (Einstein *et al.*, 1970, 1972; and others). Histochemical methods using Trypan Blue to stain basic proteins confirmed this loss of basic protein in multiple sclerosis and showed by quantitative means that a basic protein is lost in the early stages of Wallerian degeneration (Fig. 5; Hallpike *et al.*, 1970b; Adams *et al.*, 1971a, 1972). Interestingly, an extract of degenerating peripheral nerve was able to induce such loss of basic protein when used to incubate a section of normal nerve (Hallpike *et al.*, 1970c). Basic protein can also be localized by an immunohistochemical method using a Fluorescein-labelled specific antibody (Rauch and Raffel, 1964; Kornguth and Anderson, 1965).

'Neurokeratin' is a tryptophan-rich myelin protein (Adams, 1957) and can be clearly identified in cross-sections of the myelin sheath in the peripheral nerve. However, it does not seem to be primarily involved in Wallerian degeneration (Fig. 6).

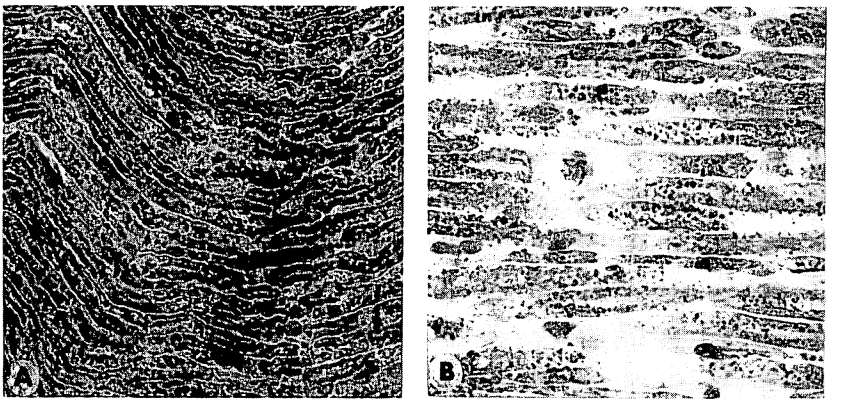

Fig. 5. Left: Basic protein in normal rat sciatic nerve, trypan blue, pH 5.0, × 240. Right: As above, but 1 day after nerve section (Reproduced with permission from *Histochem. J.*, 1970, **2**, 325)

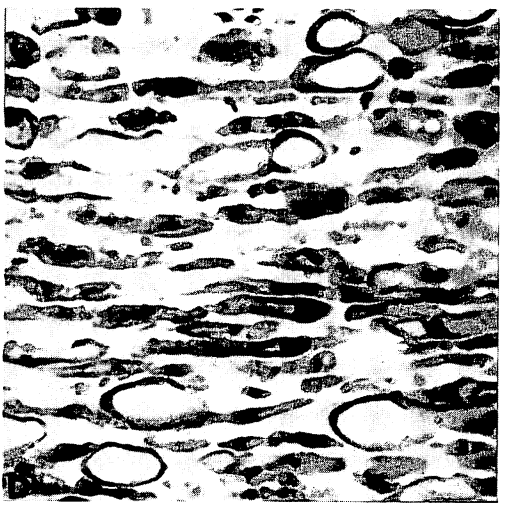

Fig. 6. Neurokeratin in rat sciatic nerve 4 days after nerve section. Note persistence of this protein. DMAB–nitrite method × 450

ORIGIN OF LYSOSOMAL ENZYMES IN MULTIPLE SCLEROSIS

Mononuclear phagocytes have been considered as a likely source of lysosomal enzymes in multiple sclerosis, but Allen *et al.* (1979) found that astrocytes contain considerable amounts of acid phosphatase and the inference has been made that they may be the source of other lysosomal enzymes (see Arstila *et al.*, 1973). However, mononuclear phagocytes within the plaque contain

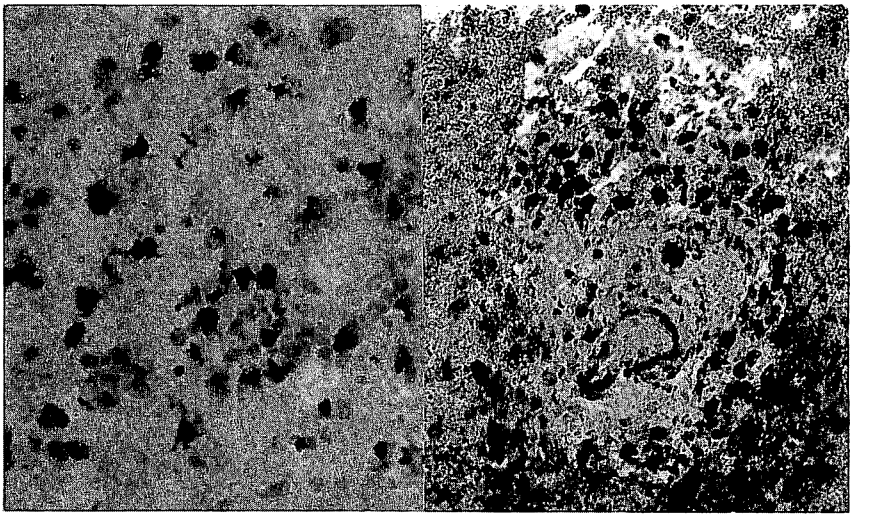

Fig. 7. Left: Acid phosphatase activity mainly in mononuclear phagocytic cells in plaque of multiple sclerosis. Naphthol-AS TR phosphate-hexazotized Pararosaniline pH 5.0, × 215. Right: Non-specific esterase activity in mononuclear phagocytes in perivenular infiltrate in multiple sclerosis, α-naphthyl acetate × 215

more acid phosphatase than do astrocytes (Fig. 7; Adams, 1982). The phagocytic cells in and around the plaque have been considered to be macrophages (Prineas and Raine, 1976), but both histo-enzymic and histo-immunological studies show that their enzyme pattern and surface antigens differ from the blood precursor of the macrophage, the monocyte (Adams *et al.*, 1976; Wood *et al.*, 1979; Oehmichen *et al.*, 1979). On the whole, these last studies suggest that the phagocytic cells within the plaque are microglia and are not immediately derived from monocytes. However, the mononuclear phagocytes in the perivenular infiltrates in multiple sclerosis do appear to be monocytes (Fig. 7). These conclusions are not firm and require further investigation with immunohistochemical and enzyme histochemical methods.

NEUROGLIA IN MULTIPLE SCLEROSIS

As indicated above, there is some difference of opinion about the exact nature of the mononuclear phagocytes in the multiple sclerosis plaque. On the other hand, the identification of astrocytes presents few problems. When activated in the early stages of multiple sclerosis (see Dawson, 1916a, b; Field 1967), astrocytes hypertrophy, become polyploid, and show intense oxidative activity with Nitro BT dehydrogenase and tetrazolium reductase methods. It must be admitted in all honesty, however, that special stains

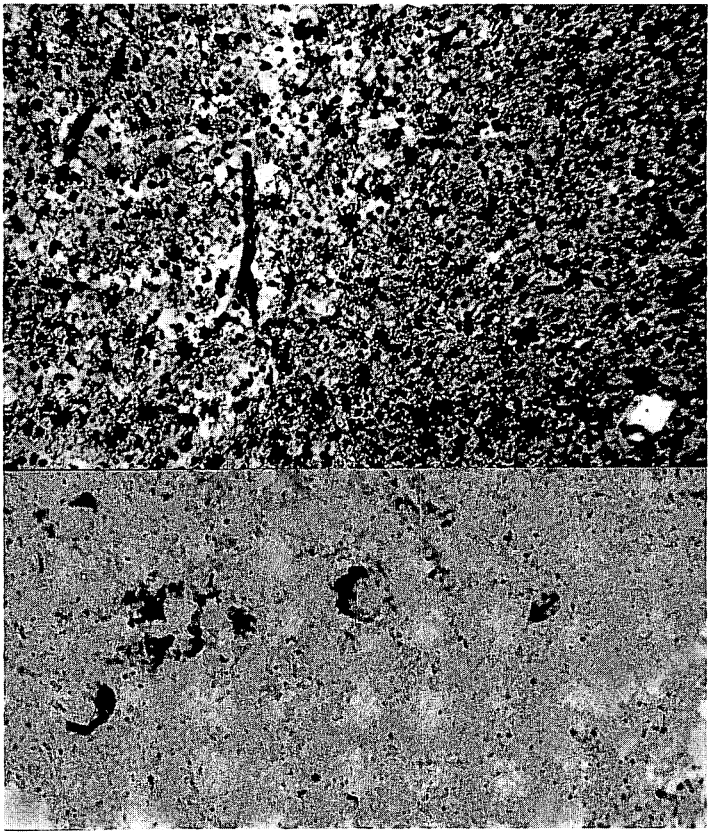

Fig. 8. Top: Astrocytic activation in and at edge of plaque of multiple sclerosis. Hortega's silver carbonate, × 40. Bottom: Hypertrophied oligodendrocytes at edge of plaque of multiple sclerosis. NADH-tetrazolium reductase × 500

and methods are often quite unnecessary to identify these characteristic cells (Fig. 8).

By contrast, oligodendroglia are most difficult cells to identify. Characteristically, they form chains to constitute the interfascicular oligodendroglia between bundles of nerve fibres. However, they do not necessarily always adopt this convenient configuration. Hortega's silver carbonate method sometimes stains them adequately, but is very dependent on the skill and whim of the operator. Probably the best histochemical marker method is pseudocholinesterase (Friede, 1966), but the most intense staining results are again obtained with Nitro BT dehydrogenase and tetrazolium reductase methods. These last show these oligodendroglia (oligo- = few; dendro- = branch) as intensely stained *round* cells with few branches (Fig. 8), in contrast to the characteristic triangular-shaped reactive astrocyte with its prominent branches and processes.

Neuroglia proliferate at the edge of active lesions, and may be demonstrated there by silver staining and enzyme histochemical methods (Friede 1961; Ibrahim and Adams 1963, 1965; Friede and Knoller, 1964). We identified at least part of these proliferating cells as oligodendroglia, even though some are clearly astrocytes and mononuclear phagocytes. Although Lumsden (1952) considered that loss of oligodendroglia is an initial event in multiple sclerosis, the earlier histochemical studies have now been confirmed by electron microscopy in that hypertrophic proliferating oligodendroglia are a feature of the acute lesion or the edge of the chronic active lesion (Raine, 1982). Nevertheless, all would agree with Lumsden that oligodendroglia largely disappear from the centre of the established chronic plaque of multiple sclerosis.

MECHANISM OF DEMYELINATION IN MULTIPLE SCLEROSIS

It has been suggested in a preceding section that lysosomal proteinases may be responsible for myelin breakdown in multiple sclerosis. The digestion of the protein framework of the myelin sheath would be the initial event in its slow destruction, as schematically represented in Adams (1962b). Lysophosphatides are also known to break up myelin by causing its membrane structure to disintegrate (Webster, 1957). Lysolecithin causes demyelination (Gregson and Hall, 1973), and can be formed by the action of certain lysosomal acid hydrolases on phospholipids.

Much evidence has accumulated that macrophages or other mononuclear phagocytes can digest myelin by direct phagocytosis, 'nibbling' or unzippering the myelin lamellae. This has been shown clearly in Wallerian degeneration (reviewed by Lampert, 1982), and instances have been detected in multiple sclerosis where mononuclear phagocytes are apparently directly phagocytosing myelin (reviewed by Raine, 1982). In multiple sclerosis, it is not clear whether the myelin is first damaged by release of lysosomal enzymes or whether phagocytosis is the primary event. However, in Wallerian degeneration, changes can be seen in the myelin before the entry of phagocytic cells (Hallpike *et al.*, 1970b) and, likewise, early changes outside plaques of multiple sclerosis are not necessarily accompanied by phagocytic activity (Adams, 1982). Myelin pallor and oedema, together with astrocytic activation is often seen as a diffuse condition outside plaques (Seitelberger, 1960; Jellinger 1969). The suggestion has been made by Wisniewski and Bloom (1975) that myelin is a vulnerable innocent bystander in multiple sclerosis, and that it is damaged as a result of a cell-mediated immune or inflammatory reaction in its neighbourhood. Certainly inflammatory perivenular cuffs of lymphocytes, plasma cells, and macrophages are frequently seen in multiple sclerosis (Figs. 9 and 10). Inflammatory oedema is a common event (Fig. 11; Adams 1980, 1982), and such fluid may well contain its complement of lysosomal enzymes. Macrophages or microglia (see above) may then go on to digest the

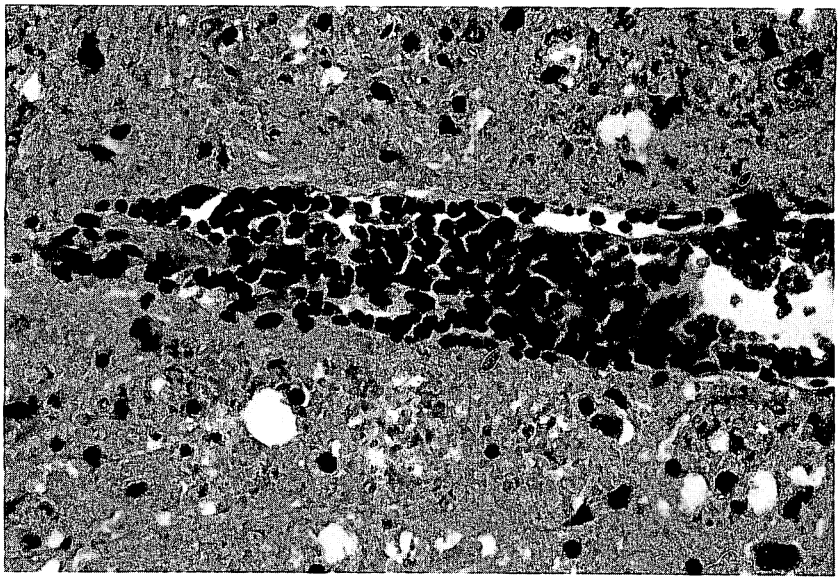

Fig. 9. Perivenular infiltrate, mainly of lymphocytes, in grossly normal white matter in multiple sclerosis. H. & E. × 250.

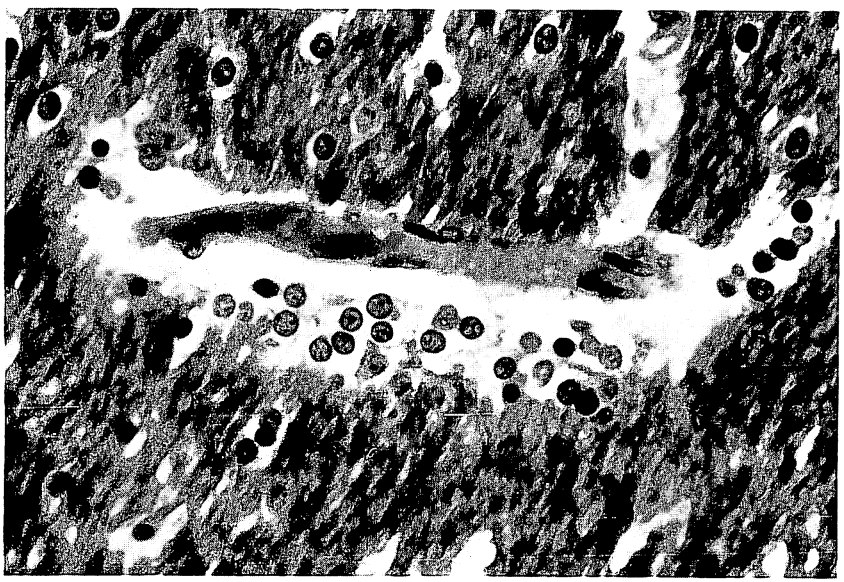

Fig. 10. Early myelin digestion and modest perivenular infiltrate in multiple sclerosis. Luxol Fast Blue–Neutral Red × 400

plaque is usually centred on a small vein (Fog, 1965), or on parallel rows of veins (Adams, 1975a,b). This inflammatory reaction is usually mild or moderate in intensity, but occasionally can be quite intense. It is frequently associated with a lymphocytic and plasmacytoid meningitis (Fig. 12; for references, see Adams, 1982). The lymphocytes concerned in these infiltrates seem to be mainly B-cells in that they do not contain the T-cell marker displayed by non-specific esterase. This is consistent with the observed synthesis of oligoclonal immunoglobulins within the central nervous system in multiple sclerosis (Cohen and Bannister, 1967; Thompson *et al.*, 1979; see Leibowitz, 1982).

Immunohistochemical methods have shown the presence of immunoglobulin within multiple sclerosis plaques. The early studies used immunofluorescence methods and showed IgG in perivenular lymphocytic cuffs, in myelin breakdown products and within neuroglial cells in the lesion and at its edge (Simpson *et al.*, 1969; Lumsden, 1971; Prineas and Raine, 1976). However, greater precision in localization can now be achieved with immunoperoxidase techniques and these clearly show that IgG is taken up by microglia and astrocyctes (Esiri, 1980), presumably as a result of pinocytosis or endocytosis by these cells. Woyciechowska and Brosoko (1977) showed that, in addition to IgG, a complement component was bound in the multiple sclerosis plaque and put this forward as evidence for the presence of an immune complex there.

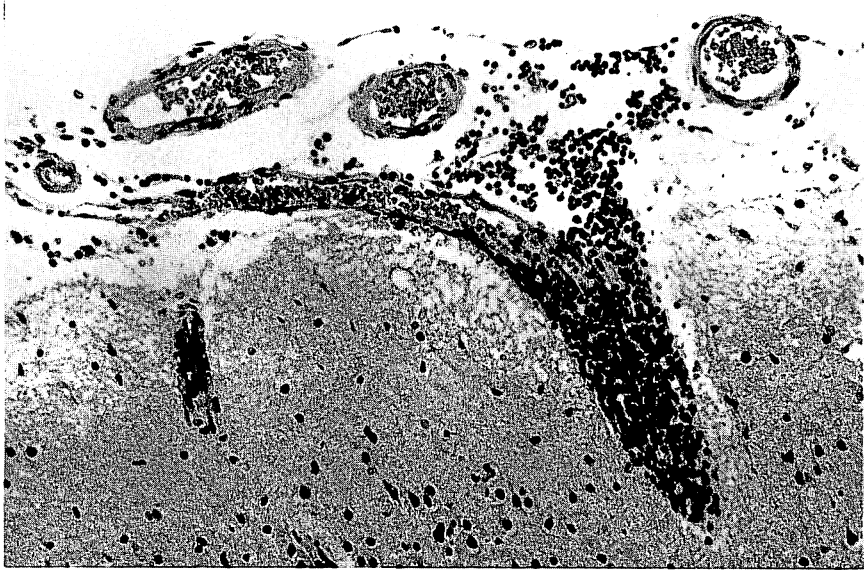

Fig. 12. Lymphocytic meningitis and meningeal perivenular infiltrate in multiple sclerosis. H. & E. × 45

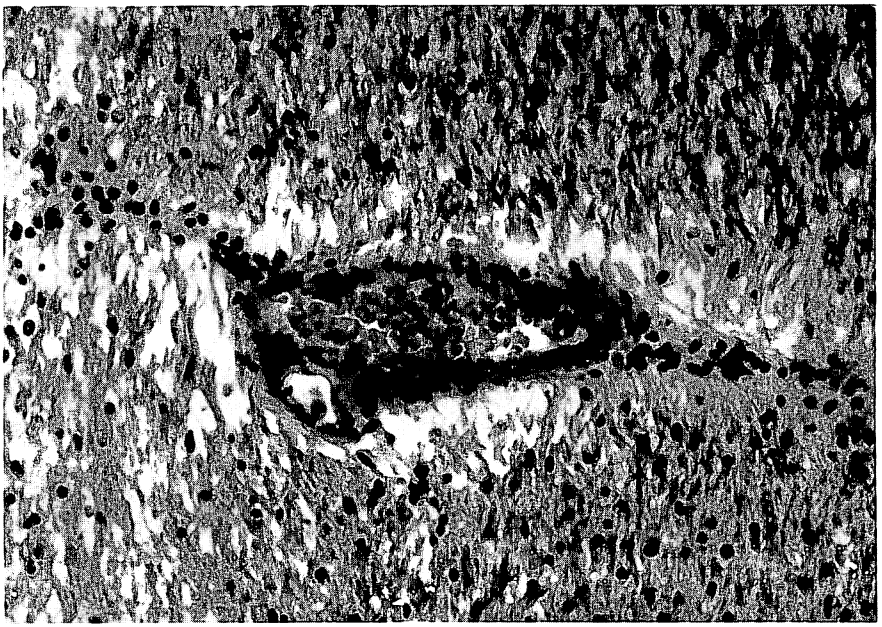

Fig. 11. Perivenular infiltrate and oedema in multiple sclerosis. H. & E. × 100

already damaged myelin. Histoenzymic methods might in future be able to distinguish between cytophagosomes involved in internal digestion and lysosomes releasing their contents to the exterior of the cell. In the meanwhile, as stated above, this remains a chicken-and-egg problem.

In spite of the uncertainty about the precise mechanism of the initial stage of myelin breakdown, it is worth while to consider the possibility that control of the inflammatory process in the vicinity of myelin may have considerable therapeutic implications. In this connection, Boehme *et al.* (1978) and Brosnan *et al.* (1980) have recently shown that a trypsin inhibitor prevents demyelination and the development of clinical signs in experimental allergic encephalomyelitis (an immune-mediated demyelinating condition). These observations have relevance to multiple sclerosis in regard to the control of myelin protein breakdown caused by the release of lysosomal enzymes. It is also a system that could be easily investigated by histochemical means, as localization would be an essential part in establishing the effect of an anti-trypsin agent.

TISSUE IMMUNE REACTIONS IN MULTIPLE SCLEROSIS

A lymphocytic and plasma cell infiltration is frequently seen around small cerebral venules in multiple sclerosis (Fig. 9). Indeed, the multiple sclerosis

2. A histochemical proteinase method was the original technique used to identify increased proteolytic activity in the very *early* stage of Wallerian degeneration and in the active multiple sclerosis plaque. This has led to considerable further biochemical investigation on proteolytic and other lysosomal enzymes in multiple sclerosis. Histo-enzymic methods have localized some of these lysosomal enzymes to mononuclear phagocytes and astrocytes.
3. Histochemical protein methods first identified protein losses in the early stage of Wallerian degeneration and confirmed the loss of myelin basic protein from the plaque in multiple sclerosis.
4. Histochemical lipid methods have localized some abnormal lipids in the brain outside plaques of multiple sclerosis, but the significance of these findings has not been established.
5. Histo-enzymic techniques have provided further methods for distinguishing different neuroglial cell types in and around the multiple sclerosis lesion.
6. Immunohistochemical methods have localized the site of synthesis and uptake of immunoglobulin in the brain in multiple sclerosis. Further studies may underline the importance of lymphocytes sequestered in the meningeal recesses.
7. Immunohistochemical methods may in future provide a method for identifying viral particles or aggregates within multiple sclerosis tissue.

Thus, it can be seen that histochemistry has provided some valuable information in the study of multiple sclerosis and has often led the way for subsequent deeper biochemical analysis. The value of histochemistry in localizing events at the histological level is in many ways paramount, and is an area in which modern biochemistry is largely defective. Considerable further advances can be anticipated with immunohistochemical methods for identification of proteins, antigens, antibodies, and micro-organisms.

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The significance of the immune reaction in the brain in multiple sclerosis is a more than a somewhat controversial question. The simplest explanation would be that it is a typical lymphocytoid response to a low-grade virus infection. However, because of the relationship between experimental allergic encephalomyelitis (EAE) and multiple sclerosis, many workers have tried to implicate an EAE-like immune reaction in multiple sclerosis. The antigen in EAE is myelin basic protein but, apart from one preliminary report, it has been impossible to date to detect any circulating antibody to myelin basic protein in multiple sclerosis or any other specific antibody not also found in other neurological diseases (Leibowitz, 1982). The simplest view is that the inflammatory reaction is a response to a virus of a slow type, taking probably many years to become clinically manifest. Certainly, similar perivascular inflammatory cuffs are seen in other viral encephalitides (see Adams and Richardson, 1961). Possibly, the viral reaction might cause a secondary auto-immune reaction so that individual perivenular lesions would then coalesce to form a larger lesion or plaque (Adams, 1975a,b, 1977).

VIRUSES AS A POSSIBLE CAUSE OF MULTIPLE SCLEROSIS

Since 1920, numerous inflammatory agents have been proposed as the cause of multiple sclerosis, such as a spirochaete, the scrapie provirus, the measles slow-virus, and many others. The topic has been recently reviewed by Ter Meulen and Stephenson (1982). The search for a specific virus as the cause of multiple sclerosis has for many years been an unenviable task, as the proof of pathogenicity is exceedingly difficult to establish. An isolated virus may only be a contaminant or harmless passenger in the tissue. Likewise, so-called viral profiles seen by electron microscopy may be artefactual and misleading (Raine, 1982).

An interesting new histochemical approach to the problem has been undertaken by Esiri and Porterfield (personal communication): they propose to look for virus particles in multiple sclerosis tissue by using a peroxidase-labelled antibody to a specific virus. This would have the great advantage of identifying the precise immunological characteristics of possible viral material situated within the multiple sclerosis plaque. Present results on multiple sclerosis plaques have, however, been negative with regard to SV5 virus (Esiri and Porterfield, personal communication).

CONCLUSIONS

Histochemical contributions to the study of multiple sclerosis and Wallerian degeneration may be summarized as follows.

1. Histochemical staining methods distinguished the early stage of physical fragmentation from the later stage of chemical degradation of myelin in Wallerian degeneration.

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