Journal of Neurocytology 7, 251–263 (1978)

Immunocytochemical method to identify basic protein in myelin-forming oligodendrocytes of newborn rat C.N.S.

NANCY H. STERNBERGER, YASUTO ITOYAMA, MARIAN W. KIES* and HENRY deF. WEBSTER

*Laboratory of Neuropathology and Neuroanatomical Sciences, National Institute of Neurological and Communicative Disorders and Stroke and *Laboratory of Cerebral Metabolism, National Institute of Mental Health, National Institutes of Health, Bethesda, Maryland 20014, U.S.A.*

Received 12 October 1977; accepted 12 November 1977

Summary

An immunocytochemical method for detecting myelin basic protein in oligodendrocytes and myelin of newborn rat C.N.S. is described. C.N.S. tissue is perfused and fixed in $HgCl_2$–formaldehyde and 20 μm Vibratome sections are treated with antibodies to myelin basic protein using the peroxidase–antiperoxidase method. Oligodendrocytes in the newborn rat are intensely stained by antiserum to basic protein and multiple stained processes extend from the perikaryon to myelin sheaths. With this procedure it is possible to demonstrate the geometric relationships between a single oligodendrocyte and multiple myelin sheaths. Stained oligodendrocytes and myelin are present in newborn cervical spinal cord, medulla oblongata, pons and midbrain. By 25 days of age, staining in oligodendrocytes is less intense than in newborn rats and differences in amount of staining can be detected in areas that are myelinating at different rates. With anticerebroside serum, cerebroside, of newborn and developing rat C.N.S. tissue is localized only in myelin. In the developing P.N.S., myelin basic protein is localized in Schwann cell cytoplasm and myelin sheaths of the trigeminal ganglion. Cerebroside is found only in myelin.

Introduction

In the developing C.N.S., glial cells extend long, slender cytoplasmic processes that wrap around axons and form myelin sheaths (Bunge *et al.*, 1962; Peters, 1964; Bunge, 1968; Peters and Vaughn, 1970). Unlike the close Schwann cell–myelin relationship in the embryonic (Geren, 1954) and adult (Robertson, 1955) P.N.S., oligodendroglial processes extend for variable distances to their contiguous myelin sheaths. Direct connections between the oligodendroglial perikaryon and its myelin sheaths are not easily seen. Also unlike Schwann cells, oligodendroglia have been observed to form more than one internode of myelin (see Peters and Vaughn, 1970). Continuity between the outermost myelin lamellae and oligodendroglial surface

membrane has also been demonstrated in a compact sheath at the margin of a cystic lesion in adult rat C.N.S. (Hirano, 1968).

Myelin is a unique, multilayered membrane composed of 70% lipid and 30% protein. One protein component found in both central and peripheral sheaths is the myelin basic protein (MBP) (mol. wt. 18 500) which accounts for about 30% of the total protein in C.N.S. myelin (Kies *et al.*, 1965; Eng *et al.*, 1968). Since it was first localized in myelin by isolation (Laatsch *et al.*, 1962) it has been extensively studied both biochemically and immunologically. Biochemical studies of isolated myelin have shown that the appearance of MBP in the 5 day old rat spinal cord and brain stem correlates with the appearance of compact, multilamellar myelin (Banik and Smith, 1977). Using a radioimmunoassay of brain homogenates Cohen and Guarnieri (1976) showed that low levels of MBP can be detected in the developing rat spinal cord at 5 days but not in the medulla, pons or midbrain. Proteins with the same mobility as MBP on polyacrylamide gels were found in isolated, mature, human oligodendroglia (Iqbal *et al.*, 1977) and isolated bovine oligodendroglia (Fewster *et al.*, 1974) and the 'non-myelin' fraction of rat brain (Adams and Osborne, 1973). However, McDermott *et al.* (1977) have recently shown that oligodendroglia can absorb [^{125}I]MBP during isolation. They suggest that a significant proportion of MBP found in isolated oligodendroglia probably arises by absorption of solubilized MBP present in the tissue suspension.

Immunocytochemical efforts to study the electron microscopic localization of MBP in oligodendroglia and myelin of the developing C.N.S. have yielded little data because of fixation difficulties and poor penetration of immunoreagents into the myelin sheath. However, MBP has been found in oligodendroglia of 15 day old rat optic nerve (Eng and Bigbee, 1978) and *Xenopus* tadpole optic nerve (Sternberger *et al.*, 1977).

In this report we describe a light microscopic immunocytochemical method that can be used to identify MBP in both myelin and oligodendroglial cytoplasm in the C.N.S. of newborn and immature rats. Dense staining of newly formed myelin sheaths at the tips of heavily stained oligodendroglial processes indicates that the technique can be used to investigate the numbers and geometric relationships of myelin internodes formed by one developing oligodendrocyte. In the P.N.S. MBP can be detected in myelin sheaths and Schwann cell cytoplasm of developing rat trigeminal ganglion. In addition, observations using anticerebroside antibodies suggest that the method can be used to detect the presence of other myelin constituents and compare their distribution and time of appearance during the early stages of myelin formation.

Methods

Newborn to 25 day old Osborne–Mendel rats were anaesthetized with chloral hydrate and fixed by intracardiac perfusion for 10 min. The fixative used for immunostaining was prepared as follows: $HgCl_2$ was heated in distilled H_2O until dissolved and cooled to 0° C to prepare a

saturated stock solution which was kept at 4° C; just before use, 76 ml of the saturated $HgCl_2$ solution was mixed with 20 ml of 37% formaldehyde, filtered and allowed to come to room temperature. Cervical spinal cord, medulla oblongata, pons, midbrain and trigeminal ganglia were dissected and fixed for an additional 2 to 3 h at 4° C. Coronal sections, 20 μm thick, were cut on a vibrating microtome (Vibratome) and stored in 0.5 M tris–HCl buffer, pH 7.6, overnight at 4° C.

Sections were immunocytochemically stained by the peroxidase–antiperoxidase (PAP) method (Sternberger *et al.*, 1970) after a 30 min incubation in 0.25% Triton X-100 (Pickel *et al.* 1975). In summary, the sections were incubated sequentially in: (1) 3% normal sheep serum in 0.5 M tris pH 7.6 for 30 min; (2) rabbit antiserum to MBP or cerebroside, diluted 1:500 to 1:8000 in the same tris buffer containing 1% normal sheep serum, overnight at 4° C; (3) sheep antirabbit IgG, diluted 1:50 in tris buffer, for 3- min; (4) PAP, diluted 1:100 in tris buffer with 1% normal sheep serum, for 30 min; (5) 0.01% hydrogen peroxide and 0.05% 3,3′-diaminobenzidine tetrahydrochloride in 0.1 M tris buffer for 5 min; (6) Sections were then treated for 5 min with 2% OsO_4, infiltrated with glycerol and whole mounts made in glycerol (Webster *et al.*, 1974) for viewing in a Zeiss microscope using differential interference contrast (Nomarski) optics. As controls for specificity of staining, sections were incubated in step (2) with either serum collected from the rabbits prior to immunization or absorbed antiserum in which the specific antibodies were removed by precipitation with purified MBP in amounts sufficient to remove all anti-MBP reactivity detectable by radioimmunoassay.

To relate the distribution of immunocytochemical staining to the cytology of the developing nervous system, the light microscopic appearance of the same regions was studied in litter mates. The fixative used for perfusion contained 1.5% glutaraldehyde and 0.5% formaldehyde in .08 M phosphate buffer. Fixation was continued overnight at 4° C before the spinal cord, brains and trigeminal ganglia were dissected, processed, sectioned and stained by conventional techniques.

Antisera to two myelin components were used in this study. Antiserum to MBP (anti-MBP) was prepared by multiple injections of purified bovine C.N.S. MBP in complete Freunds adjuvant combined with injections of MBP in incomplete Freunds adjuvant. The injection schedule was similar to that described by Seil *et al.* (1975). The antigen binding capacity of the anti-MBP serum used was 150–200 μg/ml by radioimmunoassay. Anticerebroside serum was prepared by an initial injection of cerebroside emulsified in complete Freunds adjuvant followed by a booster injection of whole bovine spinal cord. The antiserum was obtained 39 days after the latter immunization when the rabbit was terminally ill with experimental allergic encephalomyelitis. No antibody to MBP was detected in this serum by radioimmunoassay.

Results

In the newborn rat, intense immunostaining was seen in myelin and oligodendroglia in C.N.S. tissue sections treated with antibodies to MBP. Staining was observed in the cervical spinal cord, medulla oblongata (Figs. 1–3), pons, and midbrain (Fig. 4). No reaction product was found in the diencephalon or cerebral hemispheres. That the MBP containing cells are oligodendrocytes was shown by intense staining of processes that could be traced from perikarya to terminal extensions located around or along axons and their myelin sheaths. Also, the shape and appearance of oligodendroglial nuclei and perikarya was similar in immunostained (Figs. 1–4) and Epon (Fig. 5) sections of the same regions. As expected, the undehydrated tissue in the immunostained sections contained slightly larger oligodendrocytes, axons, and myelin sheaths than the Epon sections.

The distribution of immunostaining along the neuraxis of the newborn rat (brain

weights 0.26–0.27 g) is illustrated in Fig. 6. Staining was most intense and widely distributed in the spinal cord and decreased progressively at more rostral levels. In the midbrain, staining was limited to a few oligodendrocytes and myelin sheaths in the medial longitudinal fasciculus (see also Fig. 4).

In appropriately oriented sections of newborn and neonatal C.N.S., we could determine the number and lengths of oligodendroglial processes that extended from the perikaryon to axons and developing myelin segments. In Fig. 3, for example, there is an oligodendrocyte with heavily stained cytoplasm and processes 12–32 μm long that attach to 7 densely stained myelin sheaths. In the pons of a 5 day old rat, an oligodendrocyte is shown at 4 levels of focus in Figs. 7–10. At each level several processes are seen extending to or surrounding myelin sheaths that differ in orientation. The reconstruction of this cell from all levels studied is shown in Fig. 11. Its processes, which are 4–38 μm long, attach to 10 myelin sheaths that surround axons belonging to both longitudinal and transversely oriented fibre tracts.

MBP localization was restricted to myelin sheaths and oligodendroglial cytoplasm during all stages of development studied. Enclosed axons and oligodendroglial nuclei were unstained. Further restriction of the MBP localization can be seen by the absence of reaction product in neurons, astrocytes, endothelial cells, perineuronal oligodendrocytes (Fig. 12) and ependymal cells (Fig. 13).

Specificity of the immunocytochemical stain was demonstrated by incubation of sections from 25 day old, heavily myelinated rat spinal cord (Fig. 14) with either pre-immune serum (Fig. 15) or MBP absorbed serum (Fig. 16). With the two control

---

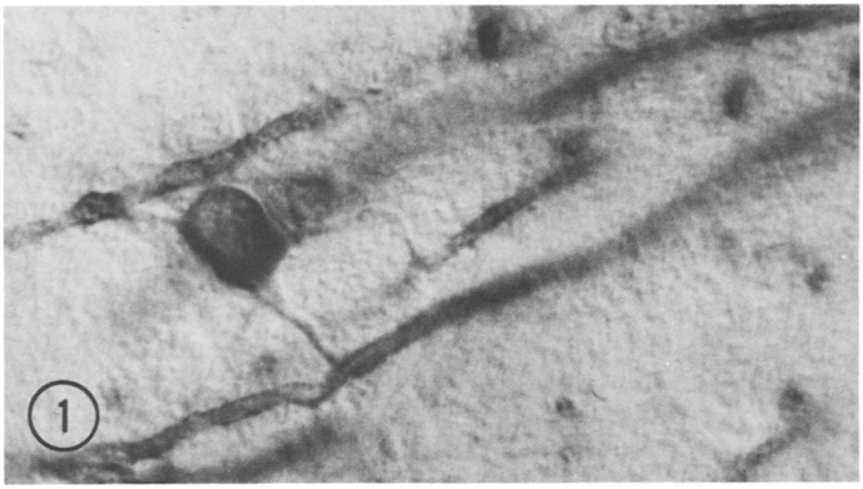
Fig. 1. Midline region of newborn rat medulla stained with 1:500 MBP antiserum. A densely stained oligodendrocyte has processes extending to 3 heavily stained longitudinally oriented myelin sheaths. Axons, the oligodendroglial nucleus and the remaining neuropil are unstained. Nomarski optics. x 1250.

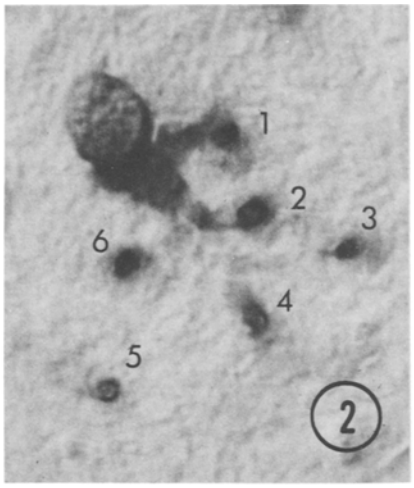
Fig. 2. Another oligodendrocyte in newborn rat medulla stained with 1:500 MBP antiserum. The very dense rings are 6 heavily stained myelin sheaths that surround unstained axons. At this level of focus stained processes terminate on sheaths 1 and 2. Processes joining sheaths 3–6 to the stained oligodendrocyte can be seen in this section at other levels of focus. Nomarski optics. x 1250.

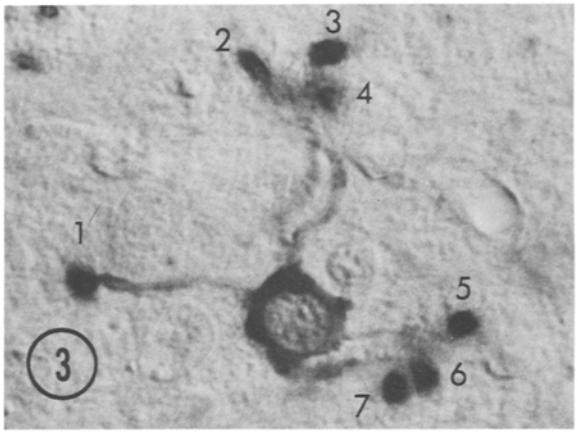
Fig. 3. Another oligodendrocyte in newborn rat medulla stained with 1:500 MBP antiserum. Processes belonging to this oligodendrocyte extend to 7 myelin sheaths in this section. Five (1, 2, 3, 5, 6) are shown at the level of focus illustrated. Nomarski optics. x 1000.

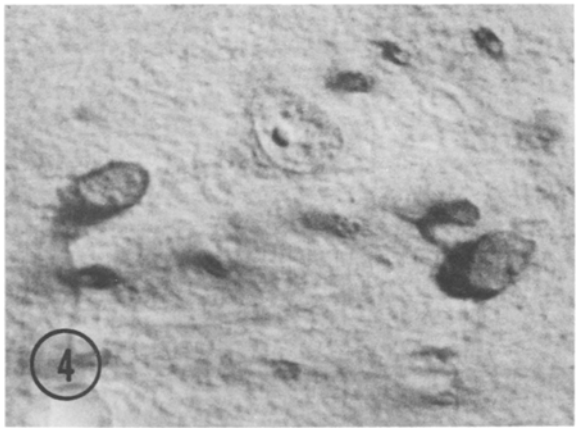
Fig. 4. Medial longitudinal fasciculus, newborn rat midbrain immunostained with 1:500 MBP antiserum. There is no reaction of newborn midbrain with MBP antiserum except in the area illustrated and a similar one located contralaterally. On the right, a heavily stained oligodendroglial process surrounds an axon; no dense ring of myelin is present. An obliquely sectioned myelin sheath is attached to the stained oligodendrocyte on the left and a narrow rim of staining is present around 4 other axons. Nomarski optics. x 1100.

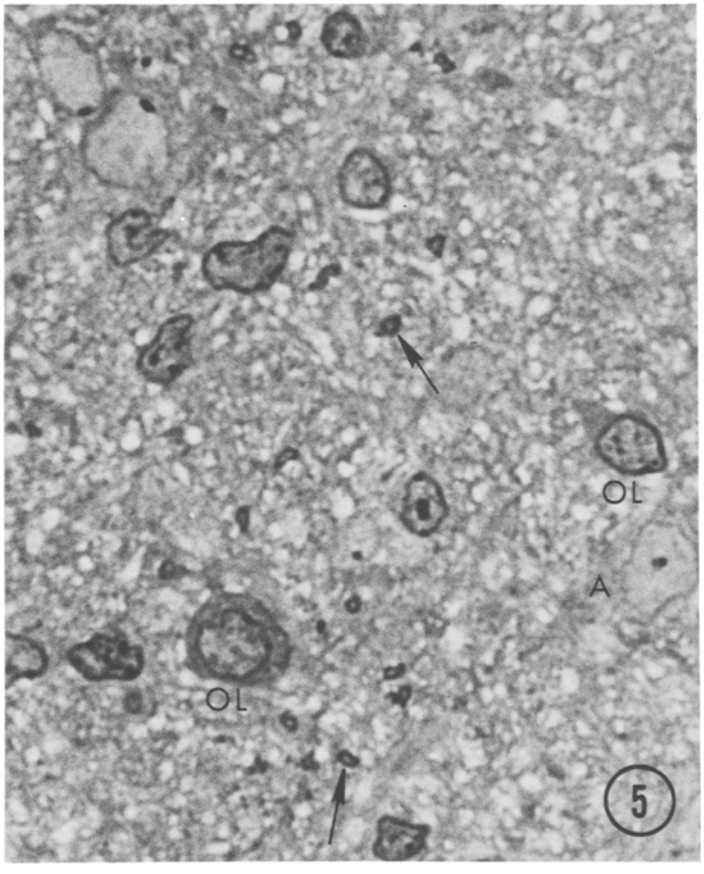
Fig. 5. The oligodendroglia (OL) in this Epon section of newborn rat medulla closely resemble those shown at the same magnification in Figs. 1 and 2. Astroglia (A) have more elongated, larger, more faintly stained nuclei. The smaller size of myelinated axons (arrows) in the Epon section may be a dehydration effect. Phase optics. x 1250.


1


1
2
3
6
4
5
2


2
3
4
1
5
6
7
3


4


OL
A
OL
5

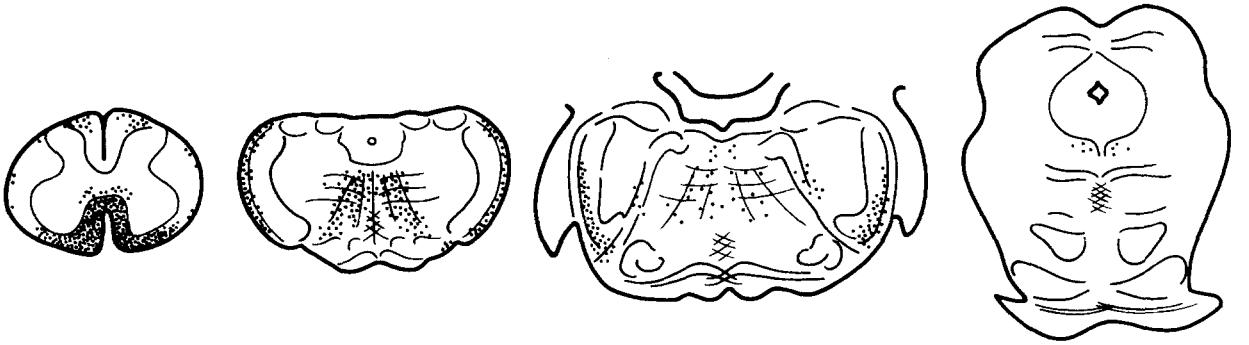


Fig. 6. Tracings of transversely sectioned newborn rat C.N.S. showing the localization and relative numbers of MBP-stained oligodendroglia and myelin sheaths. The most intense staining is seen in the ventral white funiculus of the cervical spinal cord. Staining of the medulla is less intense and is localized mainly in the medial longitudinal fasciculus (MLF), tectospinal tract, spinal tract of the V nerve, and the XII nerve. Even less staining is observed in the MLF, tectospinal tract and cranial nerves VII and VIII in the pons. In the midbrain, staining is limited to a few oligodendroglia and myelin sheaths in the MLF.

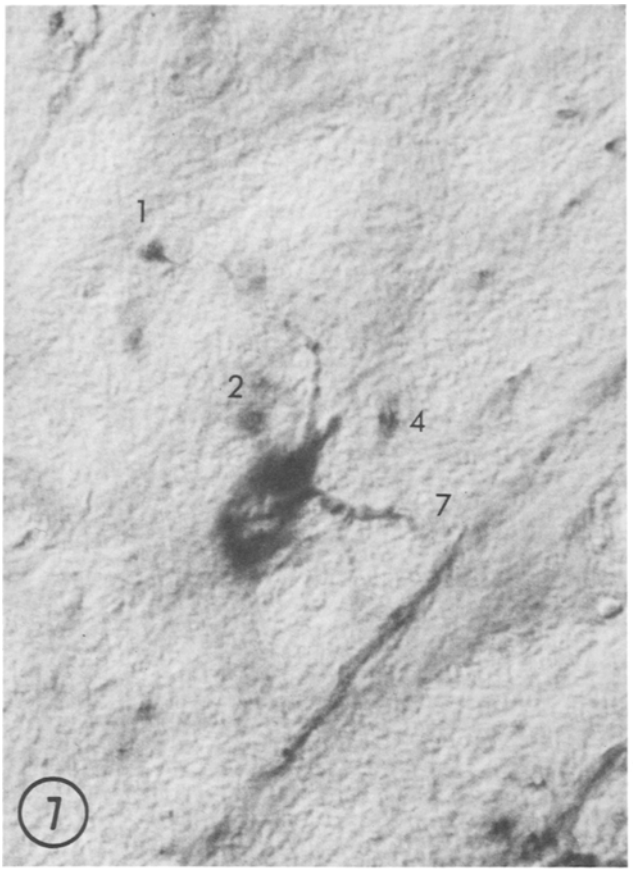
sera, there was a complete absence of reaction with the tissue. Neither sera contained any anti-MBP antibody detectable by radioimmunoassay.

Estimates of the relative amounts of MBP present in oligodendrocytes during each stage of development were derived from the concentration of antiserum required for staining. In the medulla, pons and midbrain of the 3 day old rat, oligodendrocytes and myelin are readily stained with antiserum diluted 1:8000, the highest dilution tested. By 25 days of age, more concentrated antiserum was required to demonstrate MBP in oligodendrocytes in rat medulla and pons. No detectable staining was obtained with antiserum diluted 1:2000 but there was a visible reaction with serum diluted 1:1000. In the midbrain, at 25 days, oligodendroglial MBP could be detected with antiserum dilutions of 1:2000 but not 1:4000. At both ages (day 3 and day 25), myelin was stained by all antiserum dilutions.

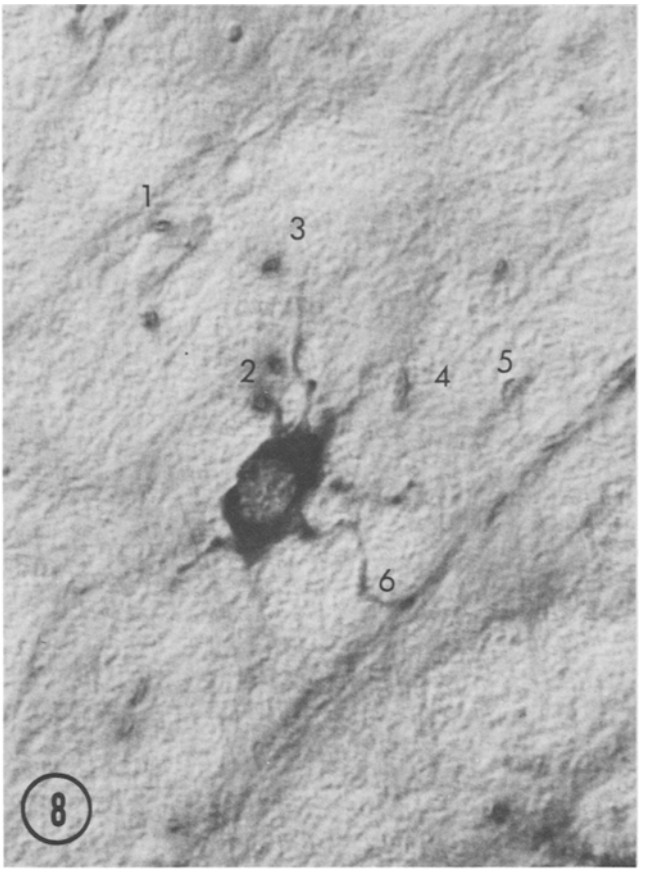
When sections were incubated with anticerebroside serum instead of anti-MBP, a consistent difference was observed in the staining pattern. At all ages studied, cerebroside was found only in the myelin sheath. No reaction with oligodendrocytes or any other cells was seen (Fig. 17).

To test the effectiveness of the method for studying the localization of MBP in the P.N.S., trigeminal ganglia from a 5 day old rat were stained with anti-MBP (Fig. 18). Stained Schwann cell cytoplasm surrounded even more heavily stained myelin sheaths. Neurons, their satellite cells and Schwann cell nuclei did not contain MBP as shown by their lack of reaction with antibody. In sections treated with anticerebroside, staining was confined solely to the myelin sheath.

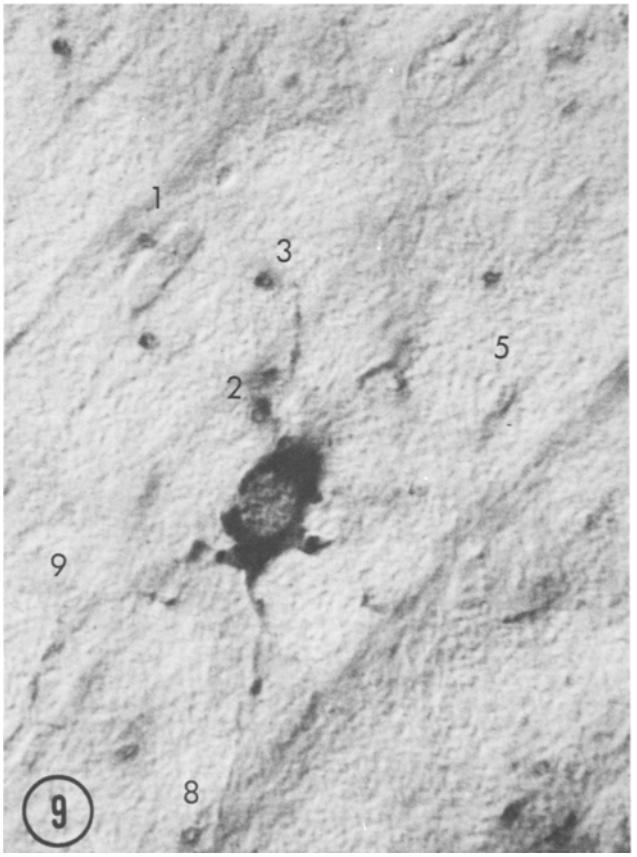
Figs. 7–10. Oligodendrocyte from 5 day old rat pons illustrated at 4 levels of focus to show extension of processes to transversely and longitudinally oriented myelin sheaths. In Fig. 7, processes extend to myelin sheath numbers 1 and 7. Attachment of processes to sheaths 2, 3, 4, 6 and 5, 8, 9 and 10, is shown respectively in Figs. 8, 9 and 10. Nomarski optics. x 1000.



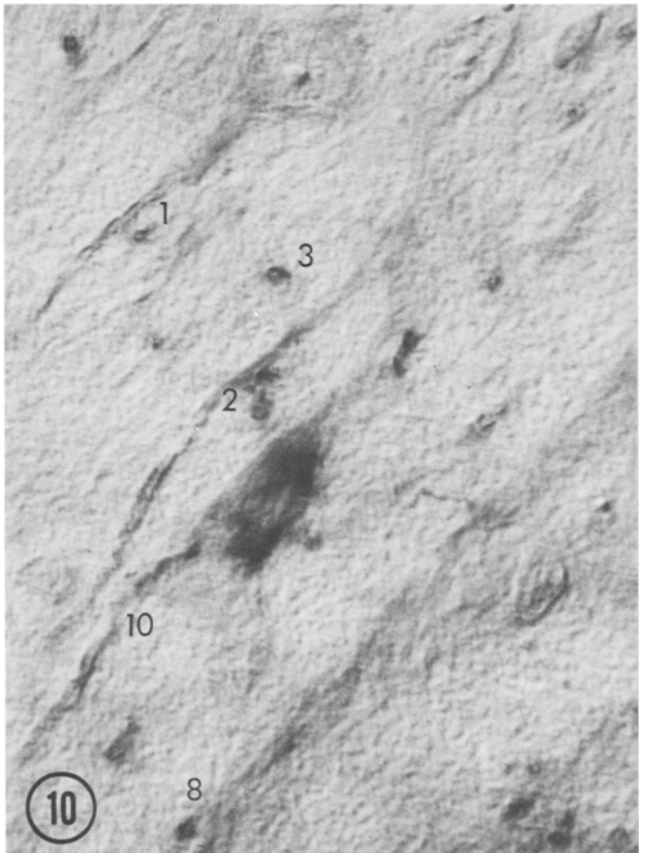
7



8



9



10

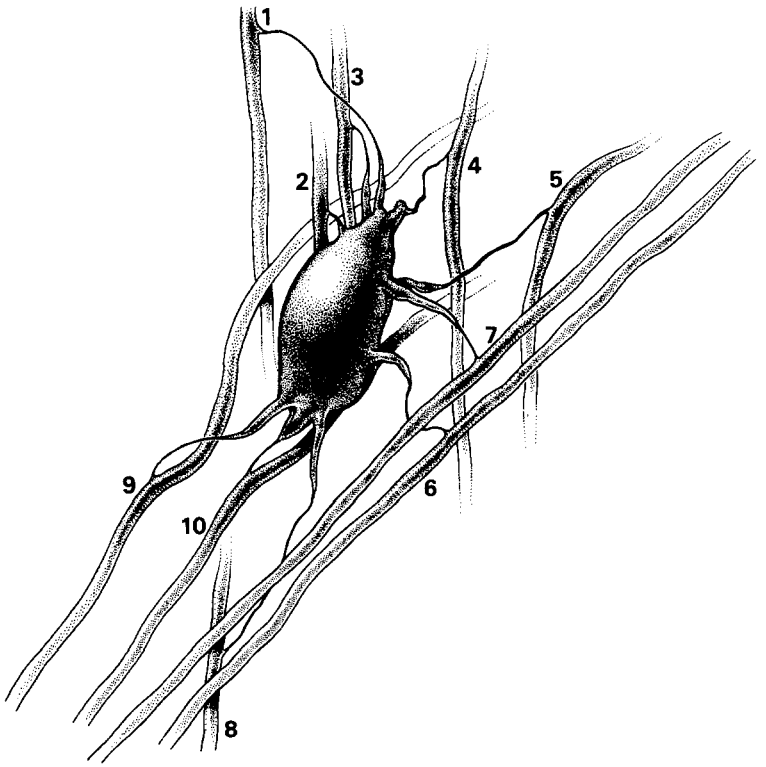


Fig. 11. Reconstruction of 10 myelin sheaths being formed by the oligodendrocyte illustrated in Figs. 7–10. Six that are transversely sectioned belong to the tectospinal tract and the other 4 that are longitudinally oriented probably surround pontocerebellar fibres.

Fig. 12. Grey matter, 7 day old rat spinal cord immunostained with 1:500 MBP antiserum. There is no staining of neurons (N), an astrocyte (A), endothelial cells (E) or an oligodendrocyte (arrow) next to a neuron. Two myelin-forming oligodendrocytes (OL) are intensely stained. Nomarski optics. x 760.

Fig. 13. Pontine ependyma, 5 day old rat, immunostained with 1:500 MBP. Staining is absent in ependymal cells (E) adjacent to heavily stained oligodendrocytes and myelin sheaths. Nomarski optics. x 450.

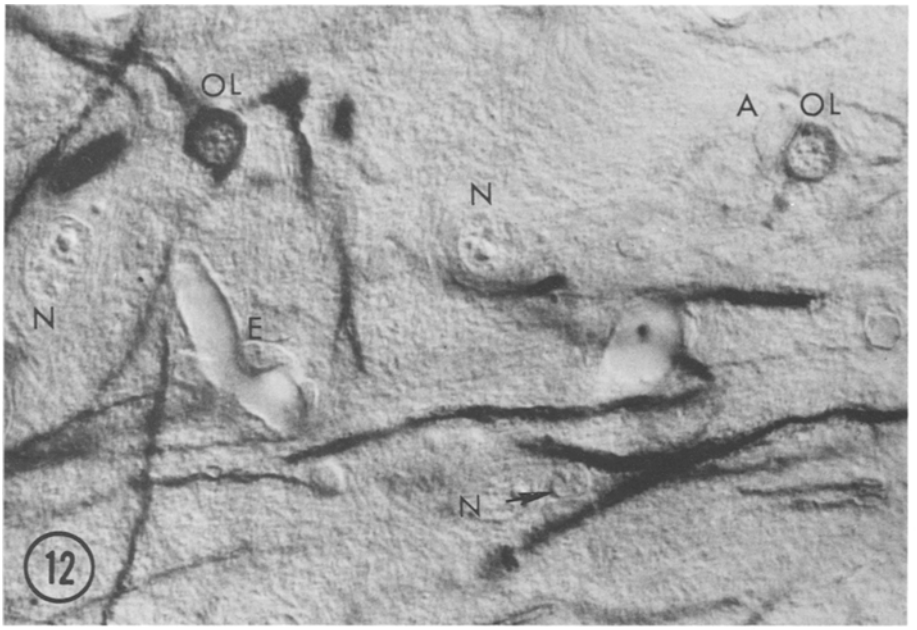
Fig. 14. Lateral white funiculus, 25 day old rat spinal cord, immunostained with 1:1000 MBP antiserum. Bundles of myelin sheaths are intensely stained. Nomarski optics. x 800.

Fig. 15. Same region as Fig. 14 in another section of 25 day old rat spinal cord treated with 1:1000 pre-immune serum. No staining of myelin or cells is observed. Nomarski optics. x 800.

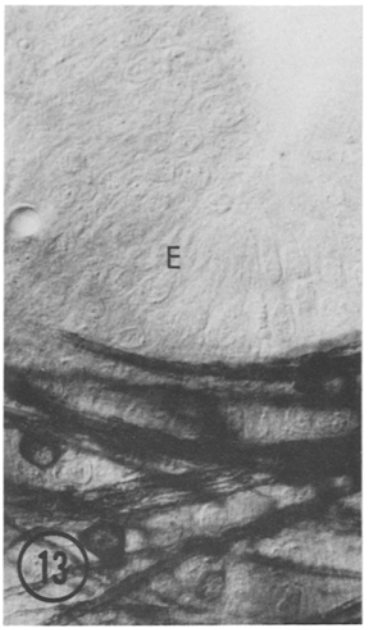
Fig. 16. Same region as Figs. 14 and 15 in another section of 25 day old rat spinal cord treated with MBP absorbed 1:1000 MBP antiserum. There is no staining of myelin sheaths or cells. Nomarski optics. x 800.

Fig. 17. Same region as Figs. 2 and 3 in another section of newborn rat medulla immunostained with 1:500 cerebroside antiserum. Between heavily stained myelin sheaths, there is an unstained oligodendrocyte (OL) with a process extending to two of the stained sheaths (arrows). Nomarski optics. x 1250.

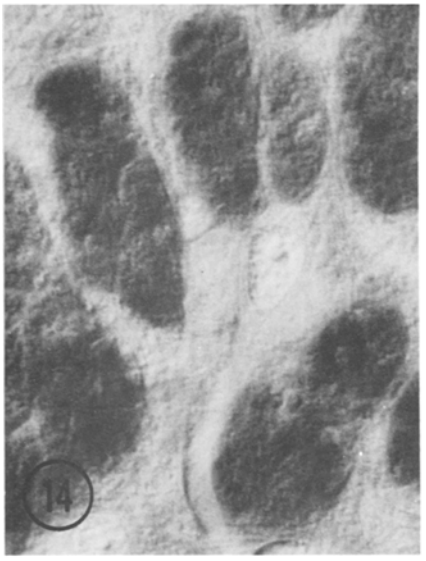
Fig. 18. Trigeminal ganglion, 5 day old rat, immunostained with 1:500 MBP antiserum. Myelin sheaths are heavily stained and staining of surrounding Schwann cell cytoplasm can be observed in myelin-forming Schwann cells sectioned through the nucleus. One of these cells (arrow) containing a dense ring of myelin and an unstained nucleus is shown at higher magnification in the insert. Neurons and their satellite cells are unstained. Nomarski optics. x 650; insert, x 1300.



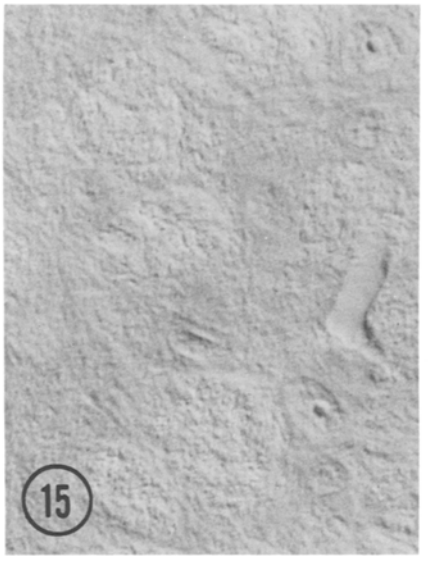
12



13

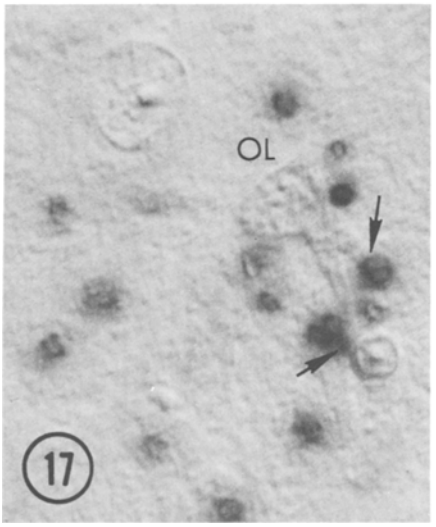


14

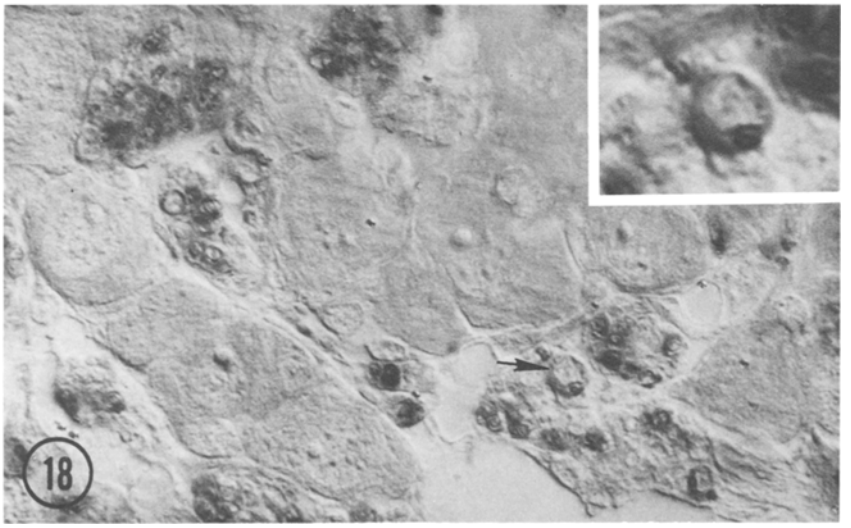


15

16



17



18

Discussion

We have described a new method that includes an $HgCl_2$-containing fixative and PAP immunocytochemistry and have identified MBP in oligodendroglia and myelin sheaths of newborn rat brainstem and spinal cord. Since myelin-forming oligodendrocytes are intensely and specifically stained with very low concentrations of MBP antisera, the method can be used to define their shape, dimensions and relationships with axons. Our results clearly demonstrate that the stained cells are oligodendroglia. The ovoid cell bodies contain round nuclei and have many slender processes that sometimes branch before surrounding axons or terminating along myelin sheaths. Using the criteria of Mori and Leblond (1970), these cells can also be identified as oligodendrocytes by the similarity in structure and location to cells seen in toluidine blue or paraphenylene diamine stained sections. In addition, their morphology is similar to the 'oligo-like-cell' found in young chick medulla oblongata with the Golgi method (Inoue *et al.*, 1973). By tracing the cell processes in one section stained with anti-MBP, we have observed up to 10 myelin sheaths attached to one oligodendrocyte. These processes extend for variable distances up to 38 μm from the nucleus. By using this method on serial sections, the numbers of myelin segments being formed by one oligodendrocyte could be studied along with approximate radial and longitudinal dimensions of oligodendrocyte territories.

We have shown that in some areas of the developing brain, one oligodendrocyte can myelinate axons belonging to different fibre tracts. If axons provide a 'signal' needed by glia to begin myelination, our data suggest that the signal may be present in functionally different axons. Another possibility is that oligodendrocytes in these areas may have enough plasticity to respond simultaneously to several kinds of axonal signals. Evidence relevant to axon–glial interactions that precede and accompany C.N.S. myelination can now be obtained by using our method to investigate the appearance of MBP in oligodendroglia, and to observe the branching pattern of processes as they extend to surround and myelinate axons.

With this technique we have also demonstrated unequivocally that oligodendroglia contain MBP before myelin sheaths are formed (Sternberger *et al.*, 1978). In transversely sectioned tracts, moderate periaxonal staining is apparent as axons become surrounded by oligodendroglial processes. Later, when myelin sheaths are first detected in Epon sections and electron micrographs, very dense rings are seen within immunostained periaxonal collars of oligodendroglial cytoplasm. The ability to identify these relationships by light microscopic examination of relatively thick sections of large regions should be helpful in studying the development of C.N.S. tracts.

By manipulating the amount of antiserum applied to the tissue, the relative amounts of MPB present in oligodendroglia and myelin can be estimated at different stages of development. MBP is easily detectable in oligodendrocytes of 3 day old rat C.N.S. tissue but subsequently, during the period of rapid myelin formation (9–25 days) the amount of MBP staining in oligodendroglia decreases progressively. Our observations are consistent with the times of rapid myelination in different areas of the C.N.S. reported by Cohen and Guarnieri (1976). They showed by radioimmu-

noassay that from 5 to 21 days postnatally, MBP appears in the developing rat medulla, pons and midbrain at the same rate. With further development, the rate of synthesis in the medulla and pons falls below that of the midbrain.

In the first light microscopic immunocytochemical study of MBP, Kornguth *et al.* (1966) used fluorescein-conjugated antibodies and found fluorescence in myelin of developing rat spinal cord but not in oligodendrocytes. Whitaker (1975) localized MBP in P.N.S. and C.N.S. myelin using horseradish peroxidase-conjugated anti-MBP. We have shown that the high concentrations of antisera used in these studies are not needed to detect MBP and when concentrations are much lower, non-specific staining is avoided.

In early experiments, we tried a variety of fixatives including many solutions containing formaldehyde, glutaraldehyde, or both. At best, myelin was only lightly stained and cell perikarya were so faintly stained that they could not be identified as oligodendrocytes. The addition of saturated $HgCl_2$ to formaldehyde appeared to be required to obtain the staining pattern shown here.

Finally, the method was also used successfully to detect cerebroside in newborn central and peripheral myelin. In contrast to the results obtained with MBP antiserum, no cerebroside was observed in oligodendroglia or Schwann cells. These data suggest that our method will be useful for investigating the sequence of insertion of other myelin components into the sheath. Currently, we are using the technique to investigate genetic disorders of myelin formation, remyelination, and myelination in human nervous tissue.

Acknowledgements

The authors acknowledge the excellent assistance of Mrs Kathryn Winchell and Ms Sue Larrick. We thank Dr Gloria Hoffman for providing slides of fixed brain.

References

ADAMS, D. H. and OSBORNE, J. (1973) A developmental study of the relationship between the protein components of CNS myelin. *Neurobiology* **3**, 91–112.

BANIK, N. L. and SMITH, M. E. (1977) Protein determinants of myelination in different regions of developing rat central nervous system. *Biochemical Journal* **162**, 247–55.

BUNGE, R. P. (1968) Glial cells and the central myelin sheath. *Physiological Reviews* **48**, 197–251.

BUNGE, M. B., BUNGE, R. P. and PAPPAS, G. D. (1962) Electron microscopic demonstration of connections between glia and myelin sheaths in the developing mammalian CNS. *Journal of Cell Biology* **12**, 448–53.

COHEN, S. R. and GUARNIERI, M. (1976) Immunochemical measurement of myelin basic protein in developing rat brain: An index of myelin synthesis. *Developmental Biology* **49**, 294–9.

ENG, L. F. and BIGBEE, J. (1978) Immunohistochemistry of nervous system – specific antigens. In: *Advances in Neurochemistry* (edited by AGRANOFF, B. W. and APRISON, M. H.) **3**. New York: Plenum Publishing Corp. (in press).

ENG, L. F., CHAO, F.-C., GERSTL, B., PRATT, D. and TAVASTSTJERNA, M. G. (1968) The

maturation of human white matter myelin. Fractionation of the myelin membrane proteins. *Biochemistry* **7**, 4455–65.

FEWSTER, M. E., EINSTEIN, E. R., CSEJTEY, J. and BLACKSTONE, S. C. (1974) Proteins in the bovine oligodendroglial cells at various stages of brain development. *Neurobiology* **4**, 388–401.

GEREN, B. B. (1954) The formation from the Schwann cell surface of myelin in peripheral nerves of chick embryos. *Experimental Cell Research* **7**, 558–62.

HIRANO, A. (1968) A confirmation of the oligodendroglial origin of myelin in the adult rat. *Journal of Cell Biology* **38**, 637–40.

INOUE, Y., SUGIHARA, Y., NAKAGAWA, S. and SHIMAI, K. (1973) The morphological changes of oligodendroglia during the formation of myelin sheaths – Golgi study and electron microscopy. *Okajimas Folia Anatomica Japonica* **50**, 327–44.

IQBAL, K., GRUNDKE-IQBAL, I. and WISNIEWSKI, H. (1977) Oligodendroglia from human autopsied brain: bulk isolation and some chemical characteristics. *Journal of Neurochemistry* **28**, 707–16.

KIES, M. W., THOMPSON, E. B. and ALVORD, E. C., Jr. (1965) The relationship of myelin protein to experimental allergic encephalomyelitis. *Annals of New York Academy of Science* **122**, 148–61.

KORNGUTH, S. E., ANDERSON, J. W. and SCOTT, G. (1966) Temporal relationship between myelinogenesis and the appearance of basic protein in the spinal cord of the white rat. *Journal of Comparative Neurology* **127**, 1–18.

LAATSCH, R. H., KIES, M. W., GORDON, S. and ALVORD, E. C., Jr. (1962) The encephalomyelitic activity of myelin isolated by ultracentrifugation. *Journal of Experimental Medicine* **115**, 777.

McDERMOTT, J. R., IQBAL, K. and WISNIEWSKI, H. M. (1977) The encephalitogenic activity and myelin basic protein content of isolated oligodendroglia. *Journal of Neurochemistry* **28**, 1081–8.

MORI, S. and LEBLOND, C. P. (1970) Electron microscopic identification of three classes of oligodendrocytes and a preliminary study of their proliferative activity in the corpus callosum of young rats. *Journal of Comparative Neurology* **139**, 1–29.

PETERS, A. (1964) Observations on the connections between myelin sheaths and glial cells in the optic nerve of young rats. *Journal of Anatomy* **98**, 125–34.

PETERS, A. and VAUGHN, J. E. (1970) Morphology and development of the myelin sheath. In: *Myelination* (edited by DAVIDSON, A. N. and PETERS, A.) pp. 3–79. Springfield, Illinois: Charles C. Thomas.

PICKEL, V. M., JOH, T. H. and REIS, D. J. (1975) Ultrastructural localization of tyrosine hydroxylase in noradrenergic neurons of brain. *Proceedings National Academy of Sciences of the United States of America* **72**, 659–63.

ROBERTSON, J. D. (1955) The ultrastructure of adult vertebrate peripheral myelinated fibers in relation to myelinogenesis. *Journal of Biophysical and Biochemical Cytology* **1**, 271–8.

SEIL, F. J., KIES, M. W. and BACON, M. (1975) Neural antigens and induction of myelination inhibition factor. *Journal of Immunology* **114**, 630–4.

STERNBERGER, L. A., HARDY, P. H., Jr., CUCULIS, J. J. and MEYER, H. G. (1970) The unlabeled antibody enzyme method of immunohistochemistry. Preparation and properties of soluble antigen–antibody complex (horseradish peroxidase–antihorseradish peroxidase) and its use in identification of spirochetes. *Journal of Histochemistry and Cytochemistry* **18**, 315–33.

STERNBERGER, N., TABIRA, T., KIES, M. W. and WEBSTER, H. deF. (1977) Immunocytochemical staining of basic protein in CNS myelin. *Transactions of the American Society of Neurochemistry* **8(2)**, 157. Abstract 180.

STERNBERGER, N. H., ITOYAMA, Y., KIES, M. W. and WEBSTER, H. deF. (1978) Myelin basic protein demonstrated immunocytochemically in oligodendroglia prior to myelin sheath

formation. (Submitted to *Proceedings of the National Academy of Sciences of the United States of America.*)

WEBSTER, H. deF., REIER, P. J., KIES, M. W. and O'CONNELL, M. (1974) A simple method for quantitative morphological studies of CNS demyelination: whole mounts of tadpole optic nerves examined by differential-interference microscopy. *Brain Research* **79**, 132–8.

WHITAKER, J. N. (1975) The antigenicity of myelin encephalitogenic protein: production of antibodies to encephalitogenic protein with deoxyribonucleic acid–encephalitogenic protein complexes. *Journal of Immunology* **114**, 823–8.