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ASSESSMENT OF DEMYELINATION IN GLYCOL METHACRYLATE SECTIONS: A NEW PROTOCOL FOR CRESYL FAST VIOLET STAINING

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Abstract. **A simple staining technique for nervous tissue is described. Tissue perfused with glutaraldehyde and formaldehyde and postfixed with osmium tetroxide is embedded in glycol methacrylate. One-micrometer sections are stained with 0.05% cresyl fast violet aqueous solution at 60 C for 5 min, washed with tap water and air dried. With this method the details of all nervous tissue elements are clearly demonstrated. The technique is particularly useful for assessing demyelination because the staining of axoplasm allows demyelinated axons to be well visualized.**

We have recently described a simple method for high resolution light microscopy of osmicated nervous tissue using HistoResin (LKB Bromma), a new glycol methacrylate embedding medium (Pender 1985). We routinely use this method to assess the neuropathology of inflammatory demyelinating diseases such as experimental allergic encephalomyelitis (EAE), a widely studied animal model of multiple sclerosis. The method gives excellent preservation of tissue structure, yet does not require the time, expertise, and ultramicrotome needed to cut epoxy resin sections.

Primary demyelination, a cardinal feature of the inflammatory demyelinating diseases, is characterized by a loss of myelin and preservation of the axon, as opposed to secondary demyelination where the axon is damaged or destroyed. Therefore, to demonstrate primary demyelination, it is necessary to stain axons as well as myelin. We have found that conventional staining techniques using toluidine blue or cresyl fast violet stain osmicated myelin sheaths in HistoResin sections but stain the axoplasm poorly. Loss of myelin can be detected with such techniques if there is a group of demyelinated fibers; it is much more difficult to detect single demyelinated fibers because the poorly stained naked axons are difficult to distinguish from other structures such as small blood vessels, vacuoles, and dilated extracellular spaces. Furthermore, even when demyelination can be demonstrated, it is not possible to determine whether such demyelination is primary because the integrity of the naked axons cannot be assessed.

To overcome these problems, we have developed a new staining protocol for cresyl fast violet that stains not only the myelin sheath and the axon but also other nervous tissue elements, connective tissue structures, and invading inflammatory cells. It is simple, fast, and economical and allows a consistently accurate assessment of demyelination at the light microscopic level.

Materials and Methods

Rats with EAE were perfused through the left ventricle with modified Karnovsky's fixative consisting of 2.5% glutaraldehyde, 2% formaldehyde in

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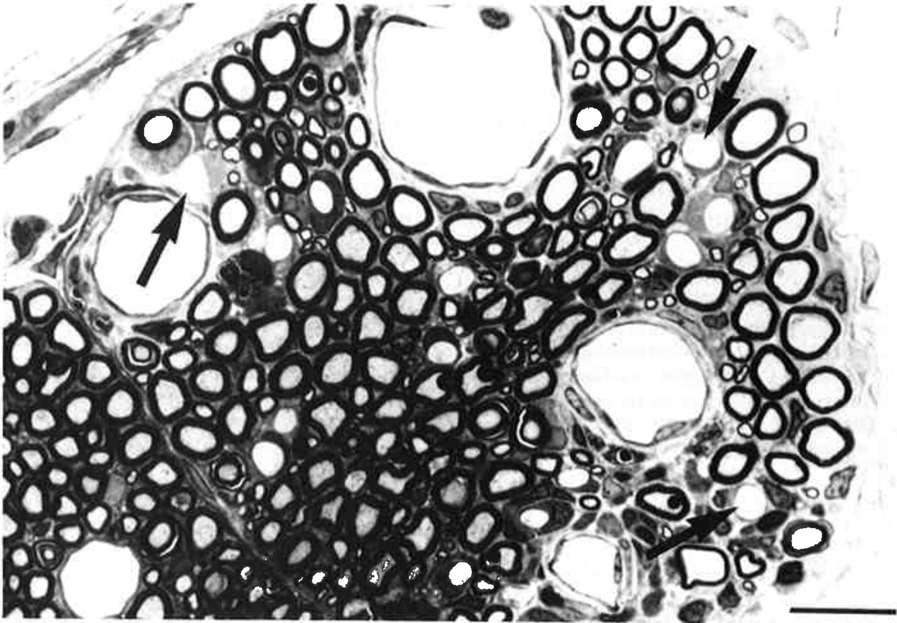

FIG. 1. Glycol methacrylate section through a sacral spinal root of a rat with acute EAE stained with toluidine blue. Demyelinated axons (arrows) stain poorly and inconsistently. The perineurium, Schwann cells, and fibroblasts are not well visualized. Bar = 25 μm.

0.1 M cacodylate buffer, pH 7.2–7.4 (Pender 1985). The brain, spinal cord, dorsal and ventral spinal roots, dorsal root ganglia, spinal nerves, and sciatic and tail nerves were removed and immersed in fixative for 3–4 hr. Specimens were postfixed in 1% osmium tetroxide in 1% dichromate buffer, pH 7.2–7.4 (Dalton's fixative; Glauert 1986), for 3–4 hr, dehydrated in ascending ethanols, infiltrated overnight with HistoResin and then embedded in HistoResin. One-micrometer sections were cut on a LKB retracting microtome with Ralph glass knives (Bennett *et al.* 1976) and dried in an oven at 60 C for 30 min.

For staining, a stock solution was prepared by dissolving 0.1 g of cresyl fast violet (Certistain, BDH England) in 100 ml of distilled water. A working solution was made by combining 20 ml of the filtered stock solution with 20 ml of distilled water. The final concentration of cresyl fast violet was 0.05%. The slide to be stained was immersed for 5 min in a screw-capped Coplin jar containing the working solution at 60 C. It was then washed in running water for 1 min, allowed to air dry and mounted with a resinous mounting medium (*e.g.*, Eukitt's). For comparison, sections were also stained with conventional toluidine blue (Culling 1974) and cresyl fast violet techniques (Heym and Forssmann 1981).

RESULTS

In glycol methacrylate sections of osmicated nervous tissue the conventional toluidine blue (Fig. 1) or cresyl fast violet techniques stain demyelinated axons

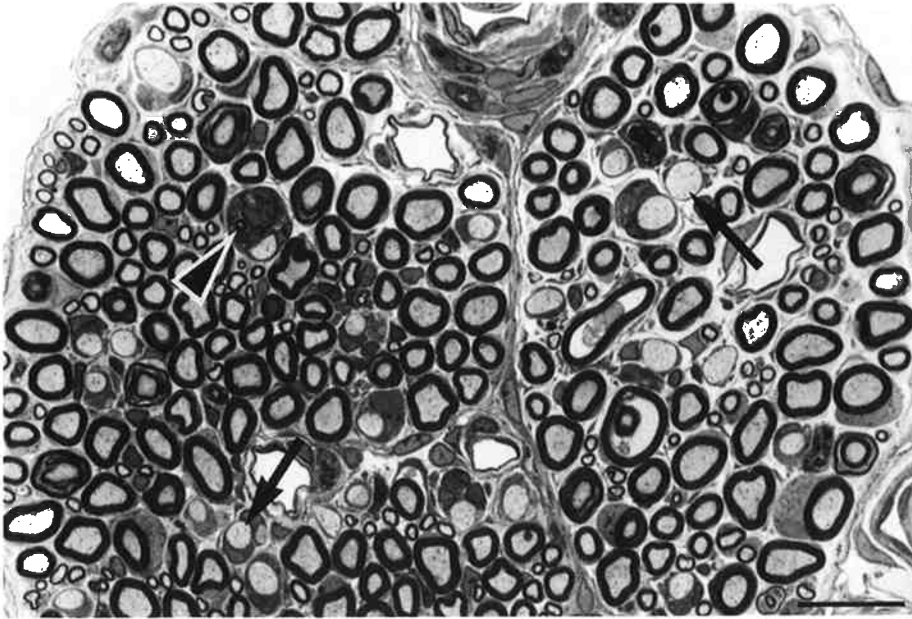

FIG. 2. Glycol methacrylate section through a sacral spinal root of a rat with acute EAE stained with the new cresyl fast violet protocol. Demyelinated axons are clearly defined (arrows). Other elements also stain better than in Fig. 1. Myelin debris (arrowhead) is present within macrophages. Bar = 25 μm.

poorly. In contrast, the new cresyl fast violet protocol stains these axons well and gives brilliant differential staining of all other central and peripheral nervous tissue elements and inflammatory cells (Figs. 2 and 3). Thus demyelinated axons can be readily distinguished from other structures such as capillaries, vacuoles, and dilated extracellular spaces. Furthermore, the occurrence of primary demyelination can be established by demonstrating the integrity of demyelinated axons (Figs. 2 and 3). Unmyelinated axons can also be visualized clearly.

Modification of the toluidine blue technique (*e.g.*, by avoiding the use of alcohol for differentiation and dehydration) results in consistently good staining of demyelinated axons in glycol methacrylate sections of osmicated tissue but does not produce the brilliant differential staining of nervous tissue elements achieved with the new cresyl fast violet protocol.

Discussion

We have demonstrated that, compared to the standard toluidine blue and cresyl fast violet techniques applied to glycol methacrylate sections of osmicated tissue, our new cresyl fast violet protocol stains demyelinated axons well and gives better differentiation of other nervous tissue elements. The standard cresyl fast violet technique stains mainly Nissl substance and the nuclei of nerve cells (Powers and Clark 1955, Lillie and Fullmer 1976, Heym and Forssmann 1981); it does not stain myelin sheaths or axons well, owing to the

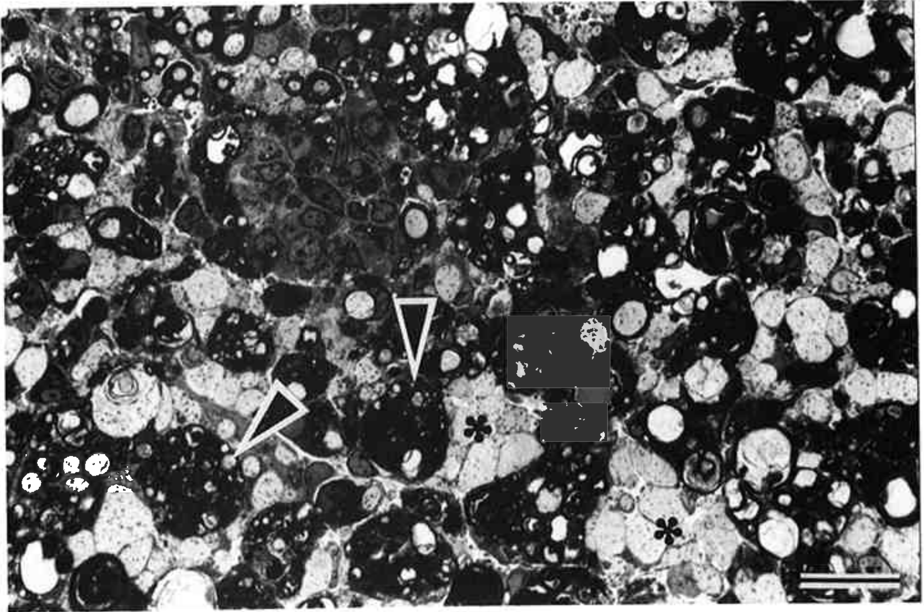

FIG. 3. Transverse section through the thoracic spinal cord of a rat with chronic relapsing EAE showing numerous demyelinated axons (asterisks) and macrophages containing myelin debris (arrowheads). Glycol methacrylate section stained with the new cresyl fast violet protocol. Bar = 25 μm.

acidic pH (usually 3–4) of the staining solutions used. At such a pH the affinity of cresyl fast violet for the axon and myelin is reduced. Furthermore, during the dehydrating step prior to coverslipping, cresyl fast violet dissociates from axonal cytoplasm and dissolves in alcohol. Therefore axons stain very faintly, making assessment of demyelination difficult.

We have modified the standard cresyl fast violet technique by 1) making the staining solution at a concentration of 0.05% instead of 1% and at a neutral pH instead of an acidic pH, 2) staining at 60 C instead of at room temperature, and for a shorter time (5 min instead of 20–30 min) and 3) omitting the dehydrating step with alcohol prior to coverslipping. These modifications give better staining of axons and myelin. All other nervous tissue elements are also clearly visualized. For best results, it is necessary to postfix the tissues with osmium tetroxide, as this gives better preservation of the myelin and intensifies its staining. Staining of sections of nonosmicated tissues still shows the details of all structures but the staining of myelin and axons is reduced. Satisfactory results also depend on complete polymerization of the glycol methacrylate resin, otherwise the tissue stains poorly, especially the myelin sheath. The shelf-life of the working solution is about two weeks. Forty milliliters of this solution are adequate for 50 slides, after which basophilic staining diminishes. The stock solution can be kept for six months at room temperature. So far, after two years we have not found any fading of the stained sections.

In conclusion, we have described a new cresyl fast violet staining protocol which is particularly useful for assessing demyelination in glycol methacrylate sections because it allows the demyelinated axons as well as other nervous tissue elements to be clearly visualized. It is simple, fast and economical and gives consistently good results.

Acknowledgment

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