creasing sharpness of tuning with increasing CF.

In this lizard, there is a strong relationship between free-standing cilium length and the tonotopic organization of the nerve. As cilium length decreases in the basal and apical populations, fiber CF increases, even though this results in increasing fiber CF in both the apical and basal direction. To our knowledge, this pattern of tonotopic organization has not been found in any other vertebrate.

Hair cells in the central population have the shortest cilia, and the corresponding fibers have the lowest CF. Since this population has a tectorial membrane rather than free-standing cilia, different mechanisms may be responsible for fiber tuning in the two types of hair cell populations in this papilla. Cilium length varies within the central population, but not systematically along the papilla. Cilium length seems to increase in an abneural direction across the papilla. It is not possible from these data to determine the relation between cilium length and fiber CF for hair cell populations with a tectorial membrane.

In this lizard, the particular structures associated with the hair cells—tectorial membrane and free-standing cilia—seem more important for determining fiber CF than location on the basilar membrane. Within the free-standing cilia populations, cilium length is inversely related to fiber CF, even when cilium length decreases in the apical direction. These results support the hypothesis that the length of hair cell cilia influences auditory nerve fiber tuning.

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6 February 1981; revised 12 June 1981

Bodian's Silver Method Stains Neurofilament Polypeptides

Abstract. *Bodian's silver method was used to stain polypeptides of rat spinal cord or peripheral nerve separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The bands corresponding to the three polypeptide subunits of the neurofilaments were intensely impregnated. Two other polypeptides were stained inconsistently and less intensely. The tubulin band was stained weakly or not at all; other polypeptides, including glial fibrillary acidic protein, actin, and vimentin, remained unstained. This novel application of Bodian's method provides indirect proof that neurofilaments are the neuronal subcellular structure stained by the technique.*

Reduced silver staining techniques have been used to study nervous tissue since the beginning of the century. In 1936 Bodian (*1*) introduced a new staining procedure that is based on the combination of silver proteinate and metallic copper and is highly selective for axons. This procedure has been widely used by neuroanatomists to trace axonal pathways and by neuropathologists to study abnormal axons and to visualize pathological changes in the nerve cell characterized by an accumulation of abnormal fibrillary structures. These structures, called neurofibrillary tangles, are the hallmark of senile and presenile dementia (Alzheimer's disease) (*2*). When Bodian's staining method is applied to routine histology sections, axons, some dendrites, and fibrillary structures in large neurons are stained black (*1, 3*). Thin collagen fibers of vessels, probably type III collagen or reticulin fibers, are also often stained (*4*).

The subcellular structures stained by Bodian's method and the chemical reaction involved have not been determined. Potter (*5*) correlated the ultrastructure of axons, dendrites, and synaptic endings with the intensity of the staining by various reduced silver techniques and concluded that Bodian's method stains cell structures rich in neurofilaments. Electron microscopic studies (*6, 6a*) suggested that silver granules are selectively aligned along neurofilaments in axons and presynaptic endings. However, the poor preservation of the tissue and the unsatisfactory reproducibility make this approach difficult (*6a, 7*).

The relative specificity of the Bodian silver stain is probably due to the formation of insoluble silver nuclei on specific cell structures. During the formation of silver nuclei (a process similar to the formation of the latent image in photography), the metal is reduced at the staining sites (*8*). The reducing groups responsible for the silver impregnation in axons have not been identified, although carbonyl and sulfhydryl groups are probably involved (*9*).

During an immunocytochemical study of a toxic neuropathy in which antibodies to the 68,000-dalton subunit of the neurofilament were used (*10*), we observed a marked similarity in staining between immunoperoxidase and Bodian's silver method (Fig. 1). We used Bodian's method to stain polypeptides of whole spinal cord and peripheral nerve separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (*11*). Strips about 1 cm wide were cut from Coomassie-stained gels, immersed in distilled water for 1 hour to remove the fixative, and placed in a horizontal sliding freezing microtome (Lipshaw Manufacturing Corp.). Sections approximately 70 μm thick were cut, mounted under water on gelatin-coated slides (*12, 13*), and stained (*1, 14*).

The bands of the three polypeptide components of the neurofilament were intensely stained (Fig. 2). Two additional bands of approximately 92,000 and 74,000 daltons were stained inconsistently and less intensely. Tubulin was stained weakly or not at all. The weak and inconsistent staining of the tubulin

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band could be due not to tubulin but to a neurofilament polypeptide, probably a breakdown product (*12*). Other polypeptides of fibrous proteins, such as actin and glial fibrillary acid protein, remained unstained (Fig. 2). The staining was not related to the molecular weight of the polypeptides. At variance with the 68,000-dalton subunit, albumin, a protein with the same molecular weight, remained unstained. The staining of gels from whole spinal cord preparations by Bodian's method resulted in a pattern similar to that obtained by immunoperoxidase staining of the same gels with antiserums to the three subunits of the neurofilament (Fig. 2). Similar results were obtained for peripheral nerve.

The quest of anatomists and pathologists for staining procedures specific for certain cells or cell components is being fulfilled by modern immunocytochemical techniques. These techniques, however, are costly, time-consuming, and require the production of specific antiserums. The present study shows that Bodian's silver method may confidently be used to identify cell structures or proteins. Thus, since it intensely impregnates the neurofibrillary tangles in Alzheimer's disease, it may be applied to identify polypeptide components of these abnormal structures. That Bodian's method selectively impregnates the polypeptide components of neurofilaments is consistent with the suggestion that Alzheimer's neurofibrillary tangles are related to neurofilaments (*15*).

Other methods that stain certain cell populations or cell structures may also do so by reacting with specific proteins. Silver stains such as Fink-Heimer's, which impregnates degenerating axons (*16*), and Holtzer's, which stains fibrous astroglia with a pattern similar to that seen in immunostaining with antibodies to glial fibrillary acidic protein (*17*), should be investigated.

The finding that at least one reduced silver method stains specific polypeptides on polyacrylamide gels is a step toward clarifying the mechanism of silver impregnation. Peptide mapping should lead to the identification of the reducing groups responsible for the impregnation of neurofilaments and possibly of other argyrophilic structures, such as type III collagen (*4*).

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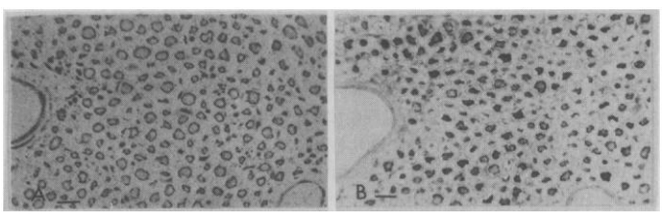

Fig. 1. Cross section of Epon-embedded sciatic nerve from rat intoxicated with β,β′-iminodipropionitrile (*10*). (A) Bodian's silver stain (*14*); (B) peroxidase-antiperoxidase immunostaining with antiserum to the 68,000-dalton polypeptide subunit of neurofilament (*10*). In peripheral axons intoxication with iminodipropionitrile causes displacement of neurofilaments toward the outside; the center of the axon is occupied largely by microtubules (*10*). Both methods stain the periphery while leaving the center weakly stained or unstained, creating a ringlike appearance. This model shows clearly that Bodian's method stains neurofilaments and not tubules. Other peripheral nerve structures such as myelin and endoneurium remain unstained. Vascular collagen is partially impregnated in the Bodian preparation (scale bar, 10 μm).

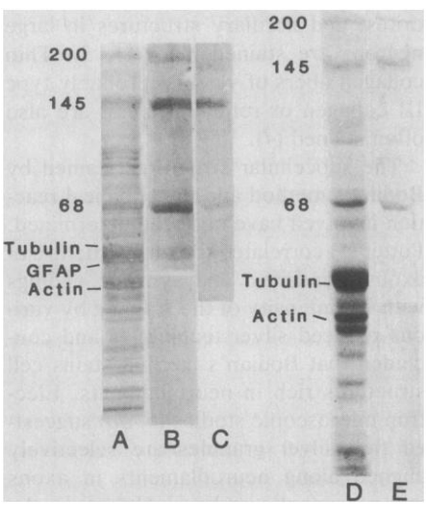

Fig. 2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of rat whole spinal cord homogenate. (A) Gel stained with Coomassie brilliant blue; (B) 70-μm-thick section of the gel in (A), stained with Bodian's silver method; and (C) 70-μm-thick section of the gel in (A), immunostained with the peroxidase-antiperoxidase method using a mixture of antiserums to the 68,000-, 145,000-, and 200,000-dalton subunits of neurofilament polypeptides (*12*). (D) Second gel of rat whole spinal cord stained with Coomassie dye; (E) 70-μm-thick section of the gel in (D), stained with Bodian's method: the neurofilament triplet is intensely stained. Other minor bands of 92,000 and 74,000 daltons and a band corresponding to tubulin are weakly stained [compare (B) and (E)]. The bands below tubulin, including glial fibrillary acidic protein (*GFAP*) and actin, remain unstained. The gels stained with Bodian's method and the immunostained gels are very similar [compare (B) and (C)]. The tubulin band is also inconsistently immunostained with antiserum to neurofilaments (*12*). The silver- and peroxidase-antiperoxidase–stained gels were cut below the tubulin band because of technical difficulty in slicing and impregnating gel sections longer than 8 cm. The lower portions of gels (B) and (E) were also stained with silver.

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14. Bodian's method was modified in our laboratory by introducing a second toning with gold chloride followed by a dip into the reducing solution. Only with this modification can gels be stained with sufficient intensity; in tissues the silver impregnation of axons is more intense and consistent than with the original method. The modified procedure includes the following steps: (i) impregnation in 1 percent Protargol-S (Winthrop) solution containing 5 g of metallic copper per 100 ml of solution for 30 hours at 37°C or for 60 to 72 hours at room temperature (the best results are obtained when the copper is placed at the bottom of the staining jar. Two polished U.S. pennies can be used. Water is then added and Protargol is spread evenly on the surface and left to dissolve before the slides are added); (ii) two rinses in distilled water; (iii) immersion in reducing solution (1 percent hydroquinone in 1.85 percent formaldehyde) for 10 minutes; (iv) rinsing in tap water for 5 minutes, followed by three changes in distilled water; (v) toning for 5 minutes in solution containing 1 g of gold chloride and 0.1 ml of acetic acid in 100 ml of distilled water; (vi) three washes in distilled water; (vii) one wash in 1 percent aqueous oxalic acid for 1 minute; (viii) three washes in distilled water; (ix) one dip in gold chloride followed by brief treatment with reducing solution until the gel becomes gray; (x) three washes in distilled water; (xi) rinsing in 5 percent aqueous thiosulfate solution for 5 minutes; and (xii) washing in tap water for 30 minutes. Steps (v) and (ix) may be carried out by adding the solutions to the horizontally placed slides so that the sections are covered.
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29 April 1981