# Redistribution of Proteins of Fast Axonal Transport Following Administration of β,β'-Iminodipropionitrile: A Quantitative Autoradiographic Study

SOZOS CH. PAPASOZOMENOS, MICHAEL YOON, REGINA CRANE, LUCILA AUTILIO-GAMBETTI, and
PIERLUIGI GAMBETTI
Division of Neuropathology, Institute of Pathology, Case Western Reserve University, Cleveland, Ohio 44106. Dr. Papasozomenos's
present address is the Division of Neuropathology, Department of Pathology, University of Virginia School of Medicine,
Charlottesville, Virginia 22908.

ABSTRACT   β,β'-iminodipropionitrile (IDPN) produces a rearrangement of axoplasmic organelles with displacement of microtubules, smooth endoplasmic reticulum, and mitochondria toward the center and of neurofilaments toward the periphery of the axon, whereas the rate of the fast component of axonal transport is unchanged. Separation of microtubules and neurofilaments makes the IDPN axons an excellent model for study of the role of these two organelles in axonal transport. The cross-sectional distribution of [$^3$H]-labeled proteins moving with the front of the fast transport was analyzed by quantitative electron microscopic autoradiography in sciatic nerves of IDPN-treated and control rats, 6 h after injection of a 1:1 mixture of [$^3$H]-proline and [$^3$H]-lysine into lumbar ventral horns. In IDPN axons most of the transported [$^3$H] proteins were located in the central region with microtubules, smooth endoplasmic reticulum and mitochondria, whereas few or none were in the periphery with neurofilaments. In control axons the [$^3$H]-labeled proteins were uniformly distributed within the axoplasm. It is concluded that in fast axonal transport: (a) neurofilaments play no primary role; (b) the normal architecture of the axonal cytoskeleton and the normal cross-sectional distribution of transported materials are not indispensable for the maintenance of a normal rate of transport. The present findings are consistent with the models of fast transport that envision microtubules as the key organelles in providing directionality and propulsive force to the fast component of axonal transport.

The role played by the axonal cytoskeleton in providing directionality and propulsive force to materials migrating with the fast axonal transport is poorly understood and controversial (1, 2, 3).

Ochs (4) has proposed that transport occurs along microtubules (MT) and is mediated by "transport filaments," made possibly of actin, bound to the transported materials and moved down the axon by MT side arms. According to Pollard (5), transport along the MT is mediated by an actomyosin system anchored to MT and to moving particles; propulsive forces would be generated by conformational changes similar to those of muscle contraction. In still another model, Gross (6) postulated that materials are transported by carrier streams generated by and around MT.

All these models emphasize the role of MT and are supported by numerous studies (7). However, the direct involvement of MT in fast transport has been questioned (8, 9), and in another model neurofilaments (NF) have been postulated as the possible mediator of fast transport: Willard and Simon (10) suggested that NF comprise a central core wrapped by a helix and might function as a rotary motor providing propulsive force to the fast transport.

We have recently shown (11) that, following administration of β,β'-iminodipropionitrile (IDPN) to rats, axonal MT and NF segregate: whereas MT with smooth endoplasmic reticulum (SER) and mitochondria are found in the central region of the axon, NF occupy the peripheral zone. Moreover, the slow component of the axonal transport is affected whereas the rates of the fast anterograde and retrograde transports remain normal (12). The IDPN axon, therefore, is an excellent model for investigation of the possible roles of MT and NF in the fast anterograde axonal transport. Using quantitative electron microscopic (EM) autoradiography, we determined the cross-sectional distribution of [$^3$H]-labeled proteins migrating with the front of the fast anterograde axonal transport in IDPN-treated and control rats. We found that in IDPN axons the fast-transported proteins occupy only the central region, where most of the MT and only few of the NF are located, whereas

in normal axons they are uniformly distributed within the axoplasm. Part of this study has been previously reported (13).

## MATERIALS AND METHODS

Male Sprague-Dawley rats (200 g) were used. IDPN (Eastman-Kodak, Rochester, NY) was diluted 1:5 in saline and the pH adjusted to 7.3. Each of three rats received 2 mg/kg of IDPN, divided in four equal doses, which were administered every third day by intraperitoneal injection. Three control rats received equal volumes of saline. 3 wk after the last injection, control and experimental rats were injected intraspinally with 6 $\mu$l of saline containing 1–1.5 mCi of a 1:1 mixture of L-(2,3,4,5-[$^3$H])-proline (139.1 Ci/mmol) and L-4,5[$^3$H]-lysine (74.3 Ci/mmol) (New England Nuclear, Boston, MA). Under halothane anesthesia, 3–4 microinjections were given into the left fourth, fifth, and sixth lumbar ventral horns according to a modification of the method described by Lasek (14).

Rats were sacrificed 6 h later by intracardiac perfusion of 50 ml of 1% paraformaldehyde followed by 1 l of 3.5% glutaraldehyde in 0.12 M phosphate buffer, pH 7.3, at 37°C. The left sciatic nerve was dissected out and cut into 2.5-mm segments. Radioactivity in every alternate segment was measured by liquid scintillation counting. (In preliminary experiments the unincorporated radioactivity extracted from unfixed nerves with 5% TCA accounted for ~10% of the total in both controls and IDPN-treated animals.) The region of the nerve containing the front of the transported proteins was processed for quantitative EM autoradiography. These segments were further fixed in 2.5% glutaraldehyde overnight, washed three times with 8% dextrose in phosphate buffer for 20 min each time, postfixed in 4% OsO₄ for 2 h, dehydrated in graded alcohols, propylene oxide, and embedded in Epon 812 (Ernest F. Fullam, Inc., Schenectady, NY).

Light and EM autoradiograms were prepared according to the technique of Salpeter et al. (15) as previously described (16, 17), except that autoradiograms were developed with Kodak D19. All myelinated axons with an approximate radius of 1.6 $\mu$m and a surrounding annulus 0.64 $\mu$m (4HD)[1] in width, containing three or more silver grains, were photographed and the negatives enlarged to a final magnification of × 27,000.

Histograms of grain density distribution were obtained according to the method of Salpeter (16, 17, 18). Because there were no statistically significant differences, data from controls (three rats) and from IDPN (three rats) were combined to obtain one histogram for each group. These experimental histograms were compared with theoretical curves for radioactive sources of size and shape similar to those of the axons examined, provided by Salpeter et al. (18), to analyze the distribution of radioactivity. Standard errors in the histograms were obtained using the formula $G/P \sqrt{1/G + 1/P}$, where $G$ = number of silver grains and $P$ = number of random points for each compartment (19). Statistical differences between IDPN and control densities were determined using the formula $(D1 - D2)/\sqrt{(SE_1)^2 + (SE_2)^2}$, where $D = G/P$ and $SE$ = standard error.

## RESULTS

The IDPN-treated animals showed reorganization of axoplasmic organelles with microtubules, SER, and mitochondria located at the center and neurofilaments at the periphery of the axons (Fig. 1). No significant difference was found in the rate of movement of the fast component of axonal transport along the sciatic nerve of IDPN-treated when compared to control rats (Fig. 2).

### Light and EM Autoradiography

Autoradiography at the light microscope showed focally heavy labeling of scattered axons and a background number of silver grains over the endoneurium (data not shown). In EM autoradiograms of IDPN axons, high concentrations of silver grains were present in the central zone of most axons whereas only a few grains occupied the neurofilament-containing peripheral zone (Fig. 3a). In control axons no preferential localization of silver grains in any axonal region was apparent (Fig. 3b).

Quantitative EM autoradiography demonstrated that the

---

[1] HD (half distance) is an experimental measure of resolution in autoradiography and is the distance from a radioactive line source within which 50% of the grains lie (18). In this study it is equal to 0.16 $\mu$m.
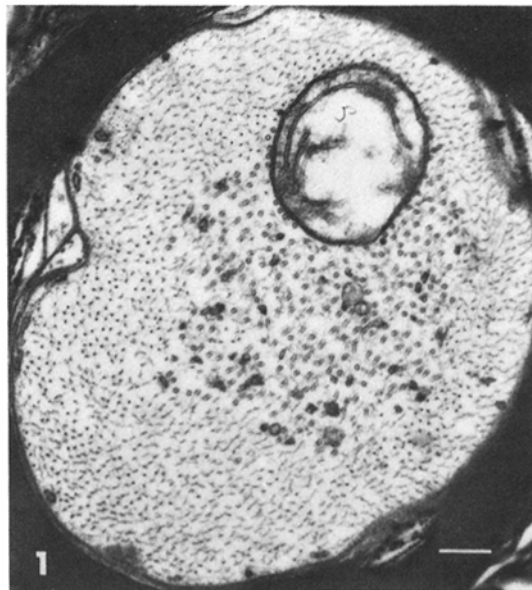


FIGURE 1  Cross-section of an axon from the sciatic nerve of an IDPN-treated rat. Microtubules, smooth endoplasmic reticulum, and mitochondria are clustered at the central region, whereas neurofilaments occupy the periphery. Note side-arms between neurofilaments and "wispy" material among microtubules. Bar, 0.5 $\mu$m. × 13,400.
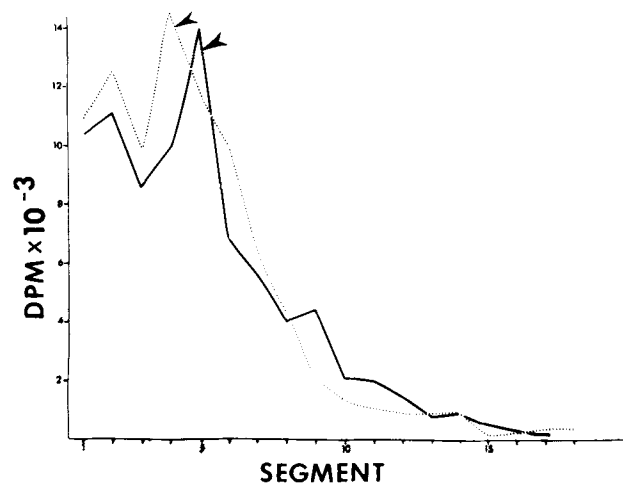


FIGURE 2  Distribution of radioactivity migrating with the fast axonal transport along the sciatic nerve, 6 h after intraspinal injection of 1:1 mixture of [$^3$H]-lysine and proline. (———): IDPN. (- - -): Control. The radioactivity is expressed in DPM/2.5-mm segment. Segment 1 corresponds to the initial segment of the sciatic nerve. Arrowheads indicate nerve segments used for quantitative EM autoradiography.

distribution of the intra-axonal radioactivity was strikingly different in IDPN and control axons (Fig. 4). As shown in the histograms of grain density distribution, each compartment of the IDPN axon was significantly different from the corresponding compartment of the control axon. In the IDPN axon, labeled proteins migrating with the front of the fast transport were present almost exclusively in the central region containing most of the MT, SER, and mitochondria. When compared to the theoretical curves, the distribution of radioactivity in this central region of the axon corresponded to that of a uniformly labeled disk whose edge was located 0.56 $\mu$m inside the axo-
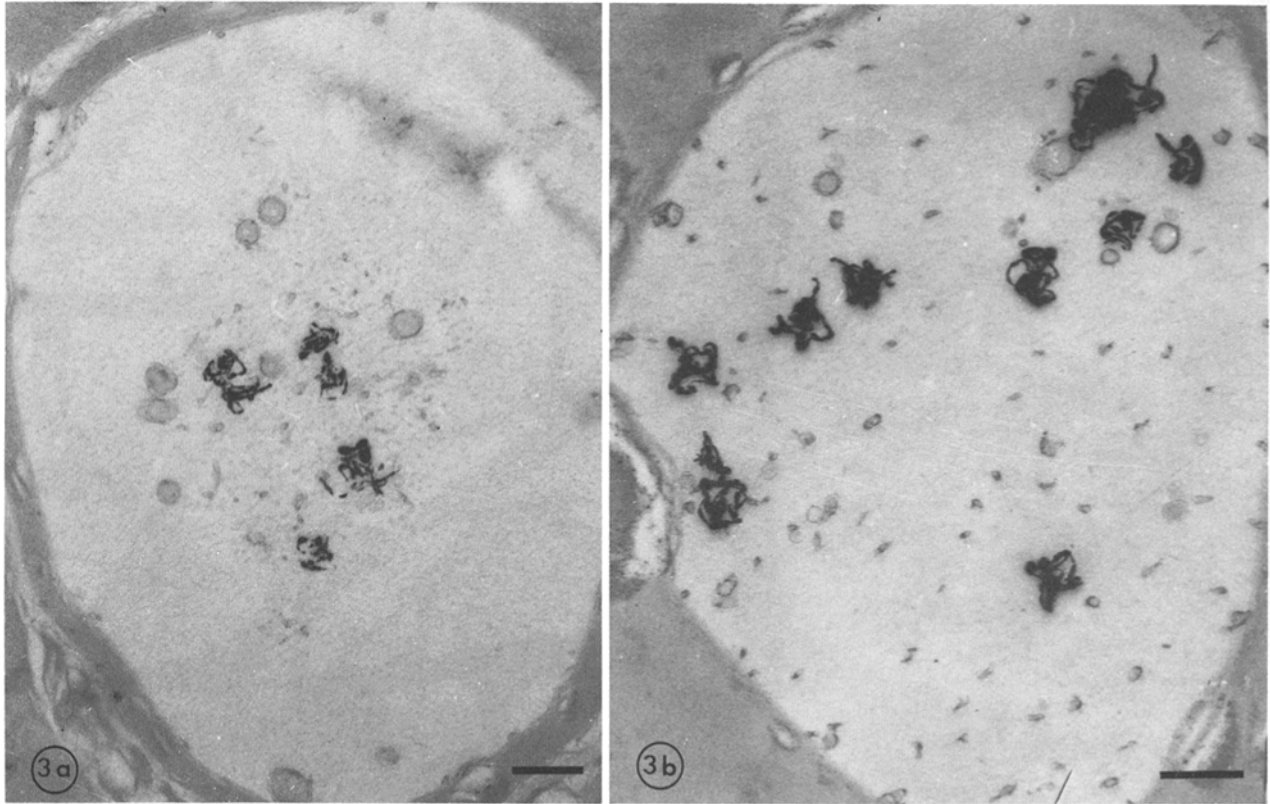
FIGURE 3 Representative EM autoradiograms of the front of the fast component of axonal transport. (a) IDPN. (b) Control. In IDPN axons silver grains are mostly concentrated at the central region of the axon, where microtubules, SER, and mitochondria are also found. There is virtually no radioactivity in the neurofilaments-containing peripheral zone. In the control axon silver grains show no preferential localization. Bars, 0.5 μm. a, × 19,300; b, × 22,300.
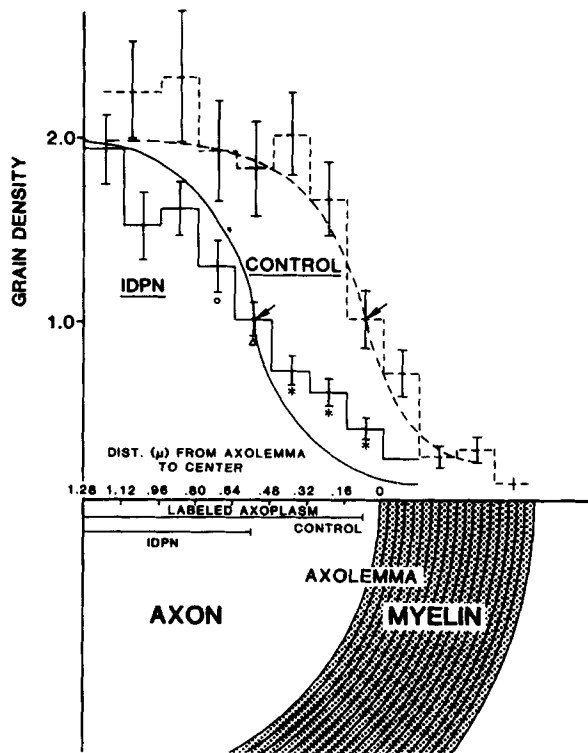


FIGURE 4 Histograms of normalized grain densities over and around IDPN (———) and control (- - -) axons, representing the cross-sectional distribution of the radioactivity migrating with the front of the fast transport. The theoretical curves that best fit each histogram are superimposed. In the controls this curve is that

lemma; the grain densities in the peripheral part of the axon were only slightly higher than those expected outside the edge of the radioactive source; this indicates that only little radioactivity was present in this region of the axon occupied mostly by NF. In the control, migrating labeled proteins were distributed uniformly within the entire cross-section of the axon, except for the axolemma, which contained little or no radioactivity.

## DISCUSSION

The present study demonstrates that, following IDPN administration, the displacement of MT, SER, and mitochondria toward the center and of NF toward the periphery of the axon is associated with a redistribution of the proteins migrating with the front of the fast component of the axonal transport; these proteins codistribute in the axon with the MT, SER, and

expected if the radioactivity is uniformly distributed over the axon. In the IDPN axon the theoretical curve fitting the histogram best is that expected if the radioactive source occupies only the central region of the axon and has its edge located 0.56 μm inside the axolemma. In this central region the radioactivity is uniformly distributed. Grain densities in the peripheral part of the axon are only slightly higher than those expected outside the edge of the radioactive source, indicating that very little radioactivity is present in this region occupied mostly by neurofilaments. Histograms were normalized at the edge of the radioactive sources (arrows). * P <0.001; Δ P <0.002; O P <0.05 refer to level of significance when grain densities in compartments of IDPN axons and controls are compared. (IDPN and control histograms are based on 940 and 463 grains, respectively.)

mitochondria whereas virtually none are found in the region occupied by NF. The present study also confirms that the rate of the fast transport is not altered in IDPN-treated animals (12).

These findings allow for several conclusions. The most important is that NF play no primary role in fast axonal transport. In addition it can be inferred that (a) a normal architecture of the axonal cytoskeleton and a normal distribution of the transported materials are not indispensable for the maintenance of a normal rate of fast transport and (b) the mechanism of transport of the fast component must be independent from that of the slow because after IDPN administration, as used in this study, the slow transport is impaired (reference 12 and unpublished data).

The present findings are consistent with the models of axonal transport that point to MT, in conjunction with actomyosin or other microfilament systems, as the structures providing directionality and propulsive force to the fast component of the axonal transport.

Available evidence indicates that SER and related structures serve as carriers for the proteins migrating with the fast transport (20–24). This study, however, did not show a very close correlation between the distribution of the SER and that of the fast transported proteins. Although there is a significant number of smooth membrane profiles in the peripheral region of the IDPN axon (11), this region contained a negligible amount of [$^3$H] proteins. This discrepancy might be explained with the finding of a component of the SER not migrating with the fast transport (25).

Ultrastructural studies over the last 10 years have emphasized the close relationships among mitochondria, SER, and MT in axons (1). Recently, this aspect of the axonal architecture has been reexamined by more sophisticated electron microscopic techniques.

Ellisman and Porter (26) have observed that axonal MT, NF, and bundles of microfilaments are connected to one another and to mitochondria, SER, multivesicular bodies, and plasma membrane by a fine filament meshwork. They suggested that this meshwork is the axonal counterpart of the so-called "microtrabecular lattice" described by Wolosewick and Porter (27, 28) in a variety of eukaryotic cells. This interpretation has been supported by a recent study of immature axons (29). The present findings suggest that, if in normal axons most SER and mitochondria are connected to both NF and MT, their connections with NF are more vulnerable to IDPN and are not necessary for the transport of the SER.

Evidence of a fairly stable association between MT and mitochondria has also been obtained by Ball and Singer (30) in cultured fibroblasts. These authors demonstrated that, following experimental segregation of MT from intermediate filaments, mitochondria codistribute with MT. On the other hand, Eckert et al. (31) observed that in cultured cells creatine phosphokinase (CPK) codistributes with IF. Whereas MT mediate the transport of axonal membranous organelles such as SER and mitochondria, NF might mediate the transport of CPK and other enzymes of the intermediary metabolism that are known to migrate with the component b of the slow transport (32). This hypothesis can be tested in the IDPN axon.

Another interesting finding of the present study concerns the distribution of the [$^3$H] proteins migrating with the front of the fast axonal transport in the normal axons; these proteins were found to be uniformly distributed within the axoplasm excluding the axolemma, which had little or no radioactivity. A detailed comparative study of the cross-sectional distribution of labeled proteins migrating with the front of the fast transport and of those left behind with the stationary phase in normal axons will be the subject of a separate report.

REFERENCES

1. Thoenen, H., and G. W. Kreutzberg. 1981. The role of fast transport in the nervous system. Neurosci. Res. Program Bull. 20:1–37.
2. Schwartz, J. H. 1979. Axonal transport: components, mechanisms, and specificity. Annu. Rev. Neurosci. 2:467–504.
3. Grafstein, B., and D. S. Forman. 1980. Intracellular transport in neurons. Physiol. Rev. 60:1167–1283.
4. Ochs, S. 1982. Calcium and the mechanism of axoplasmic transport. Fed. Proc. 41:2301–2306.
5. Pollard, T. D. 1981. Which organelles are necessary for fast neuronal transport? Neurosci. Res. Program Bull. 20:92–97.
6. Gross, G. W. 1975. The microstream concept of axoplasmic and dendritic transport. Adv. Neurol. 12:283–296.
7. Brimijoin, S. 1982. Microtubules and the capacity of the system for rapid axonal transport. Fed. Proc. 41:2312–2316.
8. Brady, S. T., S. D. Crothers, C. Nosal, and W. O. McClure. 1980. Fast axonal transport in the presence of high Ca$^{2+}$: evidence that microtubules are not required. Proc. Natl. Acad. Sci. U. S. A. 77: 5909–5913.
9. Byers, M. R. 1974. Structural correlates of rapid axonal transport: evidence that microtubules may not be directly involved. Brain Res. 75:97–113.
10. Willard, M., and C. Simon. 1981. Antibody decoration of neurofilaments. J. Cell Biol. 89:198–205.
11. Papasozomenos, S. Ch., L. Autilio-Gambetti, and P. Gambetti. 1981. Reorganization of axoplasmic organelles following $\beta,\beta'$-iminodipropionitrile administration. J. Cell Biol. 91:866–871.
12. Griffin, J. W., P. N. Hoffman, A. W. Clark, P. T. Carroll, and D. L. Price. 1978. Slow axonal transport of neurofilament proteins: impairment by $\beta,\beta'$-iminodipropionitrile administration. Science (Wash. D. C.). 202:633–635.
13. Papasozomenos, S. Ch., L. Autilio-Gambetti, and P. Gambetti. 1981. Redistribution of fast axonal transport of proteins in $\beta,\beta'$-iminodipropionitrile (IDPN) intoxication. An ultrastructural autoradiographic study. Society for Neuroscience. 7:486 (Abstr.).
14. Lasek, R. J. 1968. Axoplasmic transport of labeled proteins in rat ventral motoneurons. Exp. Neurol. 21:41–51.
15. Salpeter, M. M., and L. Bachmann. 1972. Autoradiography. In Principles and Techniques of Electron Microscopy. Biological Applications. M. A. Hyat, editor. Van Nostrand Reinhold Co., New York. 2:219–278.
16. Gambetti, P., L. Autilio-Gambetti, N. K. Gonatas, and B. Shafer. 1972. Protein synthesis in synaptosomal fractions. J. Cell Biol. 52:526–535.
17. Tessler, A., L. Autilio-Gambetti, and P. Gambetti. 1980. Axonal growth during regeneration: a quantitative autoradiographic study. J. Cell Biol. 87:197–203.
18. Salpeter, M. M., L. Bachmann, and E. E. Salpeter. 1969. Resolution in electron microscope radioautography. J. Cell Biol. 41:1–20.
19. Budd, G. C., and M. M. Salpeter. 1969. The distribution of labeled norepinephrine within sympathetic nerve terminal studies with electron microscope radioautography. J. Cell Biol. 41:21–32.
20. Rambourg, A., and B. Droz. 1980. Smooth endoplasmic reticulum and axonal transport. J. Neurochem. 35:16–25.
21. Souyri, F., M. Chretien, and B. Droz. 1981. "Acrylamide-induced" neuropathy and impairment of axonal transport of proteins. I. Multifocal retention of fast transported proteins at the periphery of axons as revealed by light microscope radioautography. Brain Res. 205:1–13.
22. Chretien, M., G. Patey, F. Souyri, and B. Droz. 1981. "Acrylamide induced" neuropathy and impairment of axonal transport of proteins. II. Abnormal accumulations of smooth endoplasmic reticulum as sites of focal retention of fast transported proteins. Electron microscope radioautographic study. Brain Res. 205:15–28.
23. Tytell, M., M. M. Black, J. A. Garner, and R. J. Lasek. 1981. Axonal transport: each major rate component reflects the movement of distinct macromolecular complexes. Science (Wash. D. C.). 214:179–181.
24. Hammerschlag, R., G. C. Stone. 1982. Membrane delivery by fast axonal transport. Trends Neurosci. 5:14–17.
25. Ellisman, M. H., and J. D. Lindsey. 1981. The axonal reticulum in myelinated axons is not rapidly transported and an asymmetry of cross-bridges exists in cisternae that are transported. J. Cell Biol. 91:91a (Abstr.).
26. Ellisman, H. M., and K. R. Porter. 1980. Microtrabecular structure of the axoplasmic matrix: visualization of cross-linking structures and their distribution. J. Cell Biol. 87:464–479.
27. Wolosewick, J. J., and K. R. Porter. 1976. Stereo high-voltage electron microscopy of whole cells of human diploid line, WI-38. Am. J. Anat. 147:303–324.
28. Wolosewick, J. J., and K. R. Porter. 1979. The microtubular lattice of the cytoplasmic ground substance: artefact or reality? J. Cell Biol. 82:114–139.
29. Stearns, M. E. 1982. High voltage electron microscopy studies of axoplasmic transport in neurons: a possible regulatory role for different cations. J. Cell Biol. 92:765–776.
30. Ball, E. H., and S. J. Singer. 1982. Mitochondria are associated with microtubules and not with intermediate filaments in cultured fibroblasts. Proc. Natl. Acad. Sci. U. S. A. 79:123–126.
31. Eckert, B. S., S. J. Koons, A. W. Schantz, and C. R. Zobel. 1980. Association of creatine phosphokinase with the cytoskeleton of cultured mammalian cells. J. Cell Biol. 86:1–5.
32. Brady, S. T., and Lasek, R. J. 1981. Nerve-specific enolase and creatine phosphokinase in axonal transport: soluble proteins and the axoplasmic matrix. Cell. 23:515–523.