Reorganization of Axoplasmic Organelles following β,β'-Iminodipropionitrile Administration

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ABSTRACT β,β'-Iminodipropionitrile (IDPN), a synthetic compound that selectively impairs slow axonal transport, produced a rearrangement of the axonal cytoskeleton, smooth endoplasmic reticulum, and mitochondria. Immunoperoxidase staining using an antiserum to the 68,000-dalton neurofilament subunit demonstrated a displacement of neurofilaments toward the periphery of the axons of IDPN-treated rats. This change occurred simultaneously along the entire length of the sciatic nerve. Ultrastructural morphometry of the axonal organelles confirmed the peripheral relocation of neurofilaments and also showed a displacement of microtubules, smooth endoplasmic reticulum, and mitochondria to the center of the axons. The overall density of axonal mitochondria was increased, whereas those of other organelles were not significantly changed. Axons were reduced in size by 10–24%, the large axons being more affected than the small ones. The observed rearrangement of axonal organelles may be due to an effect of IDPN on microtubule-neurofilament interactions, which could in turn explain the impairment of the slow transport. Axons in IDPN intoxication are a useful model to study the organization of the axoplasm and the mechanism of axonal transport.

The organization of the axoplasm and its role in axonal transport are highly controversial. Ochs and Worth (1) have proposed a model in which materials are transported intraaxonally as the result of their interaction with "transport filaments," which move along stationary microtubules (MT) by means of cross-bridges. Partial support to this hypothesis has come from recent ultrastructural studies showing a network of cross-bridges between MT, neurofilaments (NF), axonal organelles such as mitochondria and smooth endoplasmic reticulum (SER), and axolemma (2). On the basis of these findings, it has been suggested that transformations in the structure of cross-bridges between either NF or MT and other axonal organelles are responsible for the transport of the latter in both antero-grade and retrograde directions (2). Lasek and co-workers (3, 4) have postulated that each rate component of axonal transport is made up of groups of axonal organelles or structures which are transported coherently, as distinct complexes, rather than of proteins transported individually. Thus, according to this hypothesis, the slower component of the slow transport, the so-called slow component a (SCa), represents the movement of MT-NF network (5). The faster component of the slow transport, the so-called slow component b (SCb), is made up of a microfilament network to which are attached a variety of polypeptides, several of them enzymes. The fast component contains largely membranous structures such as SER and dense-core vesicles, whereas mitochondria move with an inter-mediate component (3, 6). According to this model, each structural complex migrates independently from the others.

Pharmacological agents have greatly contributed to the study of axonal transport and axonal cytoskeleton. The synthetic compound β,β'-iminodipropionitrile (IDPN) selectively impairs the axonal transport of NF proteins (7, 8), whereas the transport of tubulin and actin is either unaffected (8) or impaired to a lesser degree (7), depending on the mode of intoxication. The rate of retrograde and fast anterograde axonal transports remain unchanged (7). Morphological studies have shown that the intraspinal and proximal root segments of the axons are enlarged and contain an increased number of NF (9, 10). In the distal segment of peripheral axons no other specific alterations besides atrophy of the axon have been reported (11).

Using immunocytochemistry and light and ultrastructural morphometry, we have uncovered a novel change along the entire length of peripheral axons of IDPN-treated animals; NF are displaced toward the periphery of the axon whereas MT, SER, and mitochondria are largely relocated at the center. Part of this study has been reported (12).

MATERIALS AND METHODS

IDPN Administration

IDPN (Eastman Kodak, Rochester, N. Y.) was diluted 1:5 in saline and the

pH adjusted to 7.3 with hydrochloric acid. Male Sprague-Dawley rats weighing 180-220 g were given 2 mg/g body weight of IDPN according to two injection schedules: (a) animals killed no later than 1 wk after intoxication received a single intraperitoneal injection; (b) those killed at longer time intervals received the same dose divided into four equal injections, given every 3 d. Controls received an equal volume of saline.

Antiserum

An antiserum to the 68,000-dalton subunit of rat neurofilament was raised in rabbits and used for immunostaining at a dilution of 1:1,000. This antiserum reacted strongly with the 68,000- and weakly with the 145,000- and 200,000-dalton subunits of neurofilaments. Details on the preparation and specificity of this antiserum are described elsewhere (13).

Immunohistochemistry

Rats were sacrificed at 1, 2, 4, and 7 d, 2, 3, 4, 6, 8, 12, 16, and 20 wk, and 6, 8, 10, and 12 months after IDPN administration. Under ether anesthesia, animals were perfused for 10 min with a mixture of 1.5% paraformaldehyde and 0.25% glutaraldehyde in 0.12 M sodium phosphate buffer (pH 7.3).

Tissue samples were obtained from the spinal cord at the level of the fifth lumbar (L_5) segment, the L_5 ventral root at the exit zone, the L_5 ventral root just proximal to the dorsal root ganglion, the sciatic nerve at the intrapelvic portion and at the thigh before its trifurcation, the posterior tibial nerve at the popliteal fossa and just before its bifurcation, and the lateral and medial plantar nerves. The distance of each nerve sample from the spinal cord was recorded.

Additional fixation was carried out for a total fixation time of 90 min in a mixture of 4% paraformaldehyde and 0.25% glutaraldehyde in 0.12 M sodium phosphate buffer (pH 7.3). The tissue samples were dehydrated in graded alcohols and propylene oxide, and embedded in soft Epon 812. Sections, 2-3 μm thick, were treated with sodium methoxide to dissolve the Epon (14) and processed for the peroxidase-antiperoxidase technique (15, 16) and for Bodian's silver stain (17).

Morphometric Studies at the Light and Electron Microscope

2 wk after IDPN administration, three experimental and three control animals were perfused through the aorta with 4% paraformaldehyde for 30 s followed by 5% glutaraldehyde for 15 min. Both fixatives were buffered with 0.12 M sodium phosphate buffer (pH 7.3). Nerve segments from the L_5 ventral root, just proximal to the dorsal root ganglion, were further fixed overnight in 5% glutaraldehyde and postfixed in 4% OsO_4 for 2 h. They were dehydrated in graded alcohols and propylene oxide, and embedded in Epon 812.

For ultrastructural morphometric analysis, thin sections stained with uranyl acetate and lead citrate were photographed at random and enlarged $\times$ 27,000. Axons from both control and IDPN-treated animals were divided into three groups of comparable sizes. Each group included five control and five experimental axons. The total axonal densities of NF, MT, SER, and mitochondria as well as their densities in four concentric compartments were determined. Concentric compartments were drawn at 0.37-μm intervals, starting from the axolemma. The total axonal area and the fractional areas were calculated with an electronic digitizer (Numonics Model 1224) or by the technique of random points
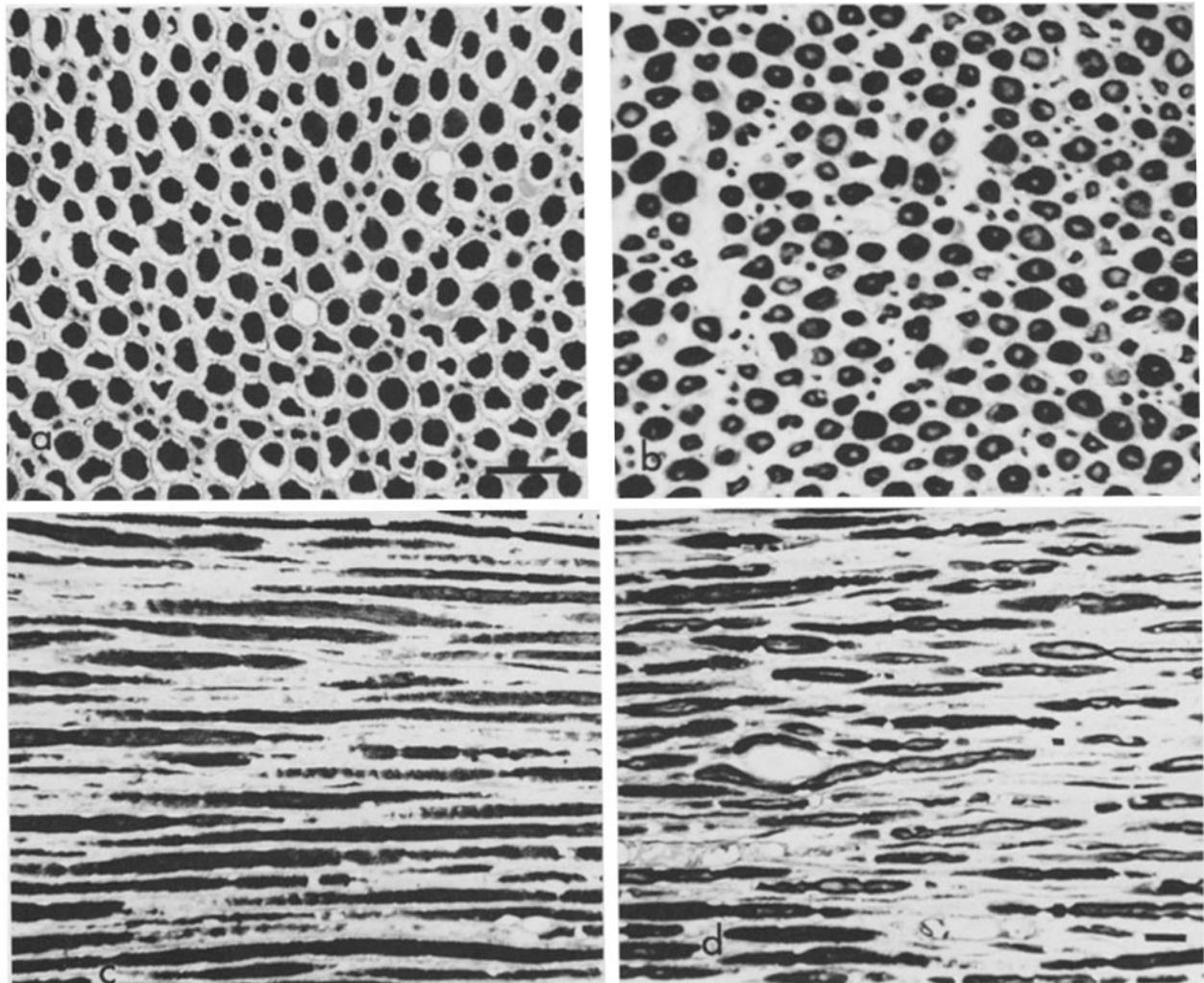
FIGURE 1   Cross and longitudinal Epon sections of rat distal ventral roots immunostained with antiserum against the 68,000-dalton neurofilament subunit using the peroxidase-antiperoxidase method. (a) Control. $\times$ 580; (b) 4 wk after IDPN administration. $\times$ 580; (c) Control. $\times$ 270; and (d) 2 wk after IDPN adminstration, $\times$ 270. While the control axons are uniformly stained, in the IDPN-treated rats the immune reaction is intense at the periphery and weak or absent in the center. Bars, 20 μm.

(18). Mitochondria were analyzed only in 32 axons of medium size.

Morphometric studies at the light microscope level were done in semithin cross sections stained with toluidine blue and enlarged to a final magnification of × 1,800. The area of each axon present was determined using the electronic digitizer.

The results for NF, MT, and SER were statistically analyzed by the two-tailed Student's t test. For the mitochondria, we used the formula $(M/A)/(\sqrt{1/M + 1/A})$, where M equals the number of mitochondria and A equals the sectional area in μm^2, to obtain the standard error. In comparing experimental with control densities, we used the formula $(D_1 - D_2)/\sqrt{(SE_1)^2 + (SE_2)^2}$ where D equals M/A and SE equals standard error, to determine the level of significance (18).

RESULTS

Immunohistochemistry

Using the antiserum to the 68,000-dalton NF subunit, axons of the sciatic nerves from control animals showed a uniform and intense immunostaining while the myelin sheath and connective tissue were not stained (Fig. 1 a and c).

In IDPN-treated animals, the immune reaction was unevenly distributed within the sciatic nerve axons, being very strong in a ring adjacent to the axolemma and weak or absent in the center (Fig. 1 b and d). These "doughnutlike" or "tubelike" axons appeared simultaneously along the entire length of the nerve 4 d after IDPN administration. They were not observed in the proximal portion of the root nor in the spinal cord. There was no difference between animals treated with either one of the two injection schedules. Similar axonal changes were observed in sections stained with Bodian's silver method (17).

This abnormal immunostaining disappeared between the sixth and sixteenth week after IDPN administration. A uniform axonal staining, similar to that of the control, was regained in a proximodistal direction, at a rate of ~1–2 mm/d (Fig. 2 a and b).

Electron Microscope Observation

Electron microscope examination confirmed the location of NF close to the axolemma and at the same time also showed displacement of MT, SER, and mitochondria toward the center of the axons, as compared to controls (Fig. 3).

Morphometric Studies at the Light and Electron Microscope

In axons of control animals the density of NF was uniform while that of MT showed a significant increase at the center ($P < 0.001$). The above statistical results were obtained when the densities of the innermost and subaxolemmal compartments were compared. The density of SER was significantly higher at the periphery ($P < 0.01$) with 31.5% of SER present at the subaxolemmal compartment. The density of mitochondria was highest in the intermediate area between the periphery and the center of the axon, but this increase was not statistically significant (Fig. 4).

In IDPN-treated animals, the density of NF was higher at the periphery and gradually decreased toward the center of the axons. In contrast, the density of MT was very low at the periphery and markedly increased at the center of the axons. There was also displacement of SER and mitochondria toward the center. This rearrangement of cytoskeletal elements and organelles in IDPN-treated animals was statistically significant when compared with controls (Fig. 4).

The total axonal densities of NF, MT, SER, and mitochondria in medium-size axons are shown in Table I. They were consistently higher in IDPN-treated animals but, with the exception of mitochondria ($P < 0.05$), not significantly different from the control. In smaller and larger axons the total axonal densities as well as the distribution of organelles were similar to those of medium size axons (data not shown).

Morphometric analysis at the light microscope level showed a reduction in the size of axons in IDPN-treated animals which was more severe in large axons (23.7%) than in small ones (10.2%) (Fig. 5).

DISCUSSION

Displacement of NF toward the periphery and of MT, mitochondria, and SER toward the center along almost the entire length of the axon has not been previously reported. However, careful examination of published micrographs suggests that a similar change may occur in other conditions such as chronic constriction of nerves (19), hexacarbon intoxication (20, 21),
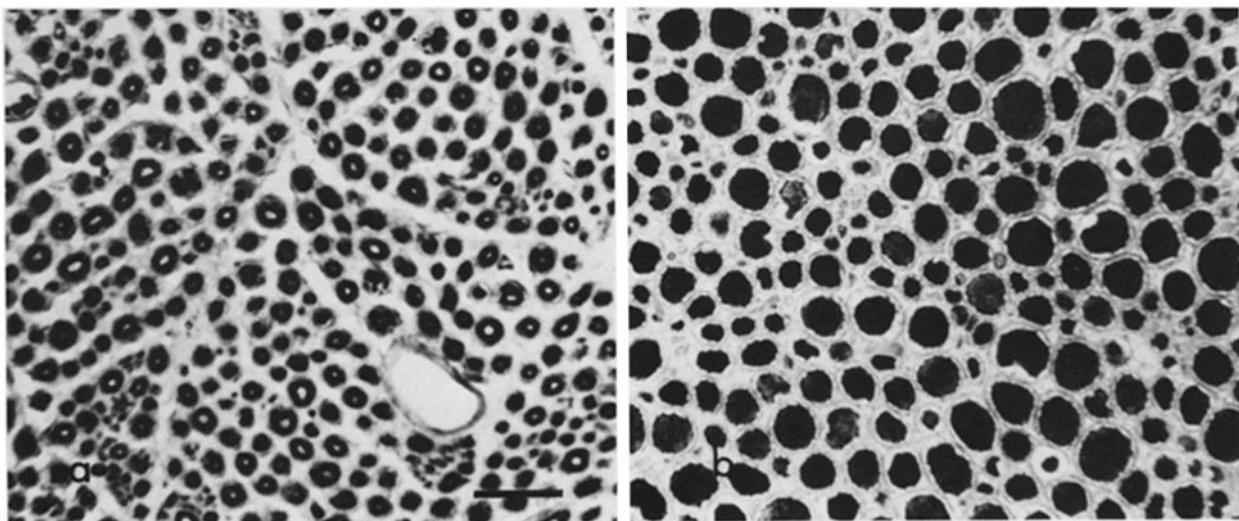
FIGURE 2 (a) Epon cross section of the posterior tibial nerve from a rat 12 wk after IDPN administration immunostained as in Fig. 1. The peripheral displacement of neurofilaments is still present. × 580. (b) More proximally, at the thigh, the axons are uniformly immunostained as the controls. × 580. Note also the difference in size of axons at the two levels; distally, the axons are still atrophic, while proximally they resumed normal dimensions. Bar, 20 μm.
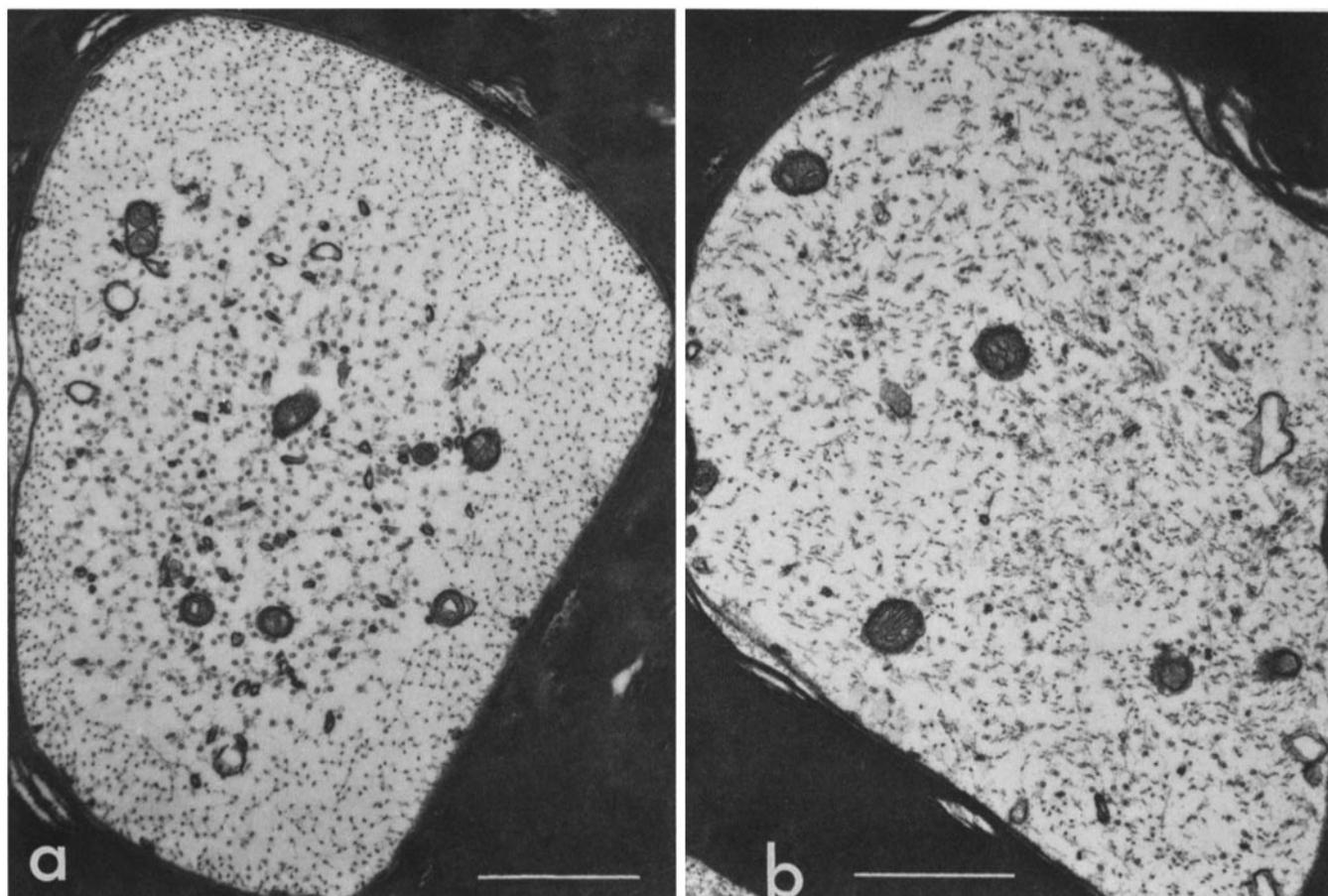
FIGURE 3 Electron micrograph of sciatic nerve at the level of the thigh. (a) 2 wk after IDPN administration. (b) Control. Neurofilaments are displaced toward the periphery of the axon while MT, SER, and mitochondria are largely found in the center. In the control axon, neurofilaments are uniformly distributed. Bar, 1 μm. × 22,000.
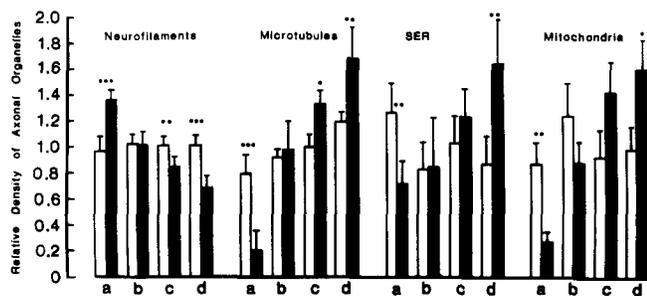
FIGURE 4 Distribution of organelles in four concentric axonal compartments designated a, b, c, and d from the axolemma to the center of the axon. Relative densities were obtained by dividing the densities in each compartment by the total axonal density. □, control, ■, IDPN. In IDPN-treated animals, the density of neurofilaments is higher at the periphery and decreases toward the center, while the densities of MT, SER, and mitochondria are lower at the periphery and increase toward the center of the axon. Note also the higher density of smooth endoplasmic reticulum in compartment a and of mitochondria in compartment b in the controls. Size of axons and experimental design are as in Table I. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ refers to IDPN data as compared with controls for each compartment.

and giant axonal neuropathy (22). Since in these studies this axonal lesion is neither described in detail nor commented on, it is not clear whether the lesion is focal or occurs along the entire length of the axon. As in the case of IDPN intoxication,

in chronic constriction there is also atrophy of the axons (19). These data suggest that redistribution of axonal cytoskeletal elements and organelles is not restricted to IDPN intoxication but is probably common to a variety of other conditions.

Except for an increase in the density of mitochondria, the densities of axonal organelles in IDPN axons of medium size were not significantly different from those of the control axons. The lack of diminution in number of NF is remarkable since, under our experimental conditions, the axonal transport of NF proteins is blocked (7, 8; M. G. Fiori, A. Bizzi, S. Ch. Papasozomenos, L. Autilio-Gambetti, and P. Gambetti, unpublished observations). It has been previously reported that NF proteins are very stable, and that unless a traumatic event such as severing of the axon takes place they are not degraded to any significant extent (23, 24). Our findings indicate that axonal NF are stable even after sustained block of their axonal transport. They suggest that in the absence of trauma there is very little, if any, turnover of NF proteins in the axon.

In IDPN-treated animals, SER and mitochondria are also displaced toward the center of the axon along with MT. Preliminary autoradiographic data show that proteins migrating with the fast component are also displaced toward the center (25). These findings are consistent with the model that MT mediate the distal migration of organelles and of fast component of axoplasmic transport (1, 2). It is remarkable that displacement of the migrating proteins toward the center of the axon does not impair the rate of their transport, which remains normal in IDPN intoxication (7).

	Neurofilaments*	Microtubules*	Smooth endoplasmic reticulum*	Mitochondria‡	Neurofilament/ microtubule ratio
Control	114.8 ± 8.7	17.8 ± 2.5	3.83 ± 0.51	0.187 ± 0.018	6.55 ± 1.37
IDPN	128.6 ± 20.3	20.6 ± 5.3	3.97 ± 0.60	0.250 ± 0.021	6.50 ± 1.43
P	NS	NS	NS	<0.05	NS

Data were obtained from the L$_5$ ventral roots of three control and three animals 2 wk after IDPN administration. Values are expressed as means ± standard deviations (*) or means ± standard errors (‡). NS, $P > 0.05$.
* The average size of the axons examined were: Control: 23.1 ± 0.6 μm^2; IDPN: 20.2 ± 2.5 μm^2.
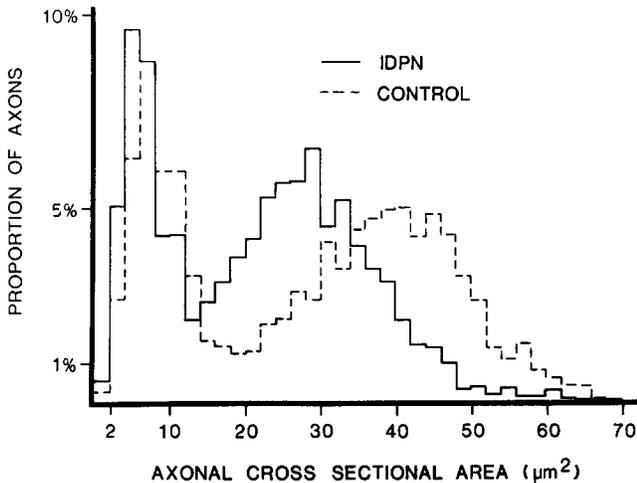‡ Control: 21.4 ± 0.4 μm^2, IDPN: 21.2 ± 0.4 μm^2.

FIGURE 5 Histogram of axonal cross-sectional areas of all axons present in distal ventral roots of a control rat (2,118 axons/whole root) and of a rat 2 wk after IDPN administration (2,225 axons/whole root). There was a 10.2% reduction in size of IDPN axons smaller or equal to 14 μm^2 and a 23.7% reduction in size of axons >14 μm^2 when compared with the respective groups of control axons. No statistically significant difference in the number of axons present in each group between control and IDPN-treated animals was found. Dashed line, control; solid line, IDPN.

Since the rearrangement of the axonal organelles takes place simultaneously along almost the entire length of the axon, it is likely that IDPN acts directly at the axon rather than at the cell body. This conclusion is supported by the recent finding that reorganization of axonal organelles, similar to that produced by us with systemic administration, can be produced locally by endoneurial injection (26). The site of action of IDPN within the axon can be only a matter of speculation. An attractive hypothesis is that IDPN affects specifically the side-arms connecting NF with MT (27). Recent studies suggest that in axons tau factor may be the cross-bridging molecules (27, 28) and that this cross-bridging may be regulated by a protein kinase (29). IDPN might impair this kinase activity. If IDPN does affect the side-arms, the following sequence of events can be postulated to explain the present results: MT and NF would dissociate and MT would move toward the center of the axon, carrying with them SER and mitochondria, still connected to the MT by unaffected side-arms. Pushing this hypothesis one step further, one can speculate that, through the side-arms, MT provide the driving force for the migration not only of SER and mitochondria but also of NF. This would explain the finding that with the dose of IDPN used in this study transport of NF proteins is blocked, whereas that of tubulin is less or not affected.

Regardless of the site of action, the local effect of IDPN on

the arrangement of axonal organelles appears to be irreversible since the NF resume a normal distribution only in a proximo-distal direction at a rate of 1–2 mm/d. This rate is consistent with that of SCa which carries NF proteins and tubulin (5). Thus, the normal distribution of NF is resumed only when NF are replaced by axonal transport. The IDPN axon lends itself to test most of these hypotheses; it is a useful model to provide a better understanding of the structural organization of the axonal cytoskeleton and of the mechanism of axonal transport.

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