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Occurrence of lipofuscin pigment granules and «dense microspheres» in the spinal cord of young cats treated with ß,ß'-iminodipropionitrile (IDPN)

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Summary. Spinal cords of cats treated with the neurotoxic compound ß,ß'-iminodipropionitrile (IDPN) were observed to contain rounded homogeneous bodies, 1-12 μm in diameter, termed «dense microspheres» (DMS). These bodies, absent in control animals, were consistently found only in the ventral horns. No relationship with blood vessels was evident. When stained with PAS and a modified von Kossa's silver nitrate technique, DMS remained negative, showing only very slight metachromasia in some toluidine blue-stained sections. They were consistently acidophilic as evidenced by destaining and differentiation investigations. DMS were observed more frequently in the proximity of nerve cell bodies or closely adjacent to dendrites and their location was mainly extracytoplasmic: with the electron microscope, however, some DMS were also found in glial processes. Rounded osmiophilic bodies, 0.1-0.8 μm in diameter, were noticed in mitochondria of both neurons and glial cells; however, whether they were special forms of DMS or different inclusions was not assessed. Both intra- and extracytoplasmic DMS were similar in ultrastructure, appearing as single membrane-bound spherical or pear-shaped bodies containing a cottony or finely granular matrix. Additionally, both perikaryon and processes of large motoneurons were found to contain pigment granules identified as lipofuscin, which seemed to increase in number and to spread centrifugally in the processes in correlation with duration of the intoxication and size of axonal swellings induced by IDPN. While function and significance of DMS remain unknown, their occurrence in other pathological conditions as diverse as Alzheimer's disease, Parkinsonism, and schizophrenia, suggests that the appearance of DMS may be related to some derangement of protein metabolism, further evidenced by the increased number of lysosomes and lipofuscin granules appearing in the neuronal cytoplasm.

Key words: Dense microspheres, Lipofuscin, Neuropigments, Cat spinal cord, ß,ß'-iminodipropionitrile (IDPN)

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Introduction

The synthetic compound ß,ß'-iminodipropionitrile (IDPN) has been extensively used during the last decade to induce swellings in the proximal segment of motor and sensory axons in several areas of the central nervous system (CNS). The interest in the neurotoxic effects of IDPN was mainly due to the striking resemblance of IDPN-induced axonal swellings to those found in some human neurologic disorders of unknown origin, such as amyotrophic lateral sclerosis (ALS) and Hallervorden-Spatz disease (Hartmann et al., 1983).

It is now accepted that the swellings induced by IDPN result from the accumulation of neurofilaments at the first internodes, just beyond the unmyelinated initial segment of the axon, following impairment of slow axoplasmic transport (Griffin et al., 1978; Papasozomenos et al., 1981). Further studies, however, have shown that the supposedly specific action of IDPN on the proximal portions of the axon has notable exceptions, since neurofilamentous accumulations may be found at different stages of the intoxication in the distal axon (Griffin et al., 1982) as well as in the perikaryon and dendrites (Chou, 1984; Fiori and Lowndes, 1986 a,b). These findings, along with some peculiarities of IDPN neuropathy (e.g., lack of degenerative axonal changes in spite of steadily enlarging axonal swellings; unique segregation of axonal organelles), have questioned the use of this neurotoxic substance as a model for some human neurodegenerative diseases; both the similarities and the marked differences, however, have made the IDPN neuropathy

useful for investigating more basic neurobiological problems.

In the course of a study on the evolution of axonal swellings in the spinal cord of cats treated with IDPN (Delio et al., 1985), the occurrence of several pigment-like inclusions and of dense microspheres (DMS) was observed in the ventral horns. Since several types of dense bodies have been reported in association with axonal swellings in human pathological conditions affecting the CNS, an analysis of the DMS occurring in IDPN intoxication was undertaken in the attempt to correlate their presence with some of the morphological and functional features reported in the spinal cord of IDPN-treated cats (Fiori and Lowndes, 1984, 1986, 1988).

Materials and methods

The present study was a complementary part of a more extensive investigation, the results of which have been published elsewhere (Delio et al., 1985). Adult mongrel cats of either sex weighing between 2 and 4 kg were housed separately and fed commercial pet food and water *ad libitum*. Experimental cats were injected intraperitoneally (i.p.) with a single weekly dose of IDPN (50 mg/kg in a 3% (v/v) solution of 5mM phosphate buffer; Shimono et al., 1978) for one to five weeks. Other cats, caged for corresponding amounts of time, were used as controls and injected i.p. with weekly doses of phosphate buffer. Further details on the clinical manifestations of IDPN intoxication in cats have been reported (Delio et al., 1985). Although the age of the cats used in the present investigation could not be assessed with certainty, all of them were young adults presumably aged 2 years or less, determined by the thickness of the skull and bony tentorium.

For morphological study, animals were anesthetized with sodium pentobarbital and perfused transcardially with 2 liters of a phosphate-buffered aldehyde mixture (1% paraformaldehyde and 1.25% glutaraldehyde, pH 7.4, at 37°C) followed by 4 liters of 2.5-4% glutaraldehyde, similarly buffered (Friedrich and Mugnaini, 1981). The entire CNS was removed and cut in coronal, parasagittal or trasverse planes; sections representative of different regions were trimmed to areas about 1 mm in diameter under a dissecting microscope. Blocks were postfixed with buffered 2% osmium tetroxide, stained *en block* with uranyl acetate, dehydrated through a graded series of ethyl alcohols, washed in propylene oxide and embedded in an Epon-Araldite resin mixture.

«Semithin» (1-2 μm) sections were stained with 1% toluidine blue, counterstained with eosin, and examined by light microscope for further selection of the blocked areas. Serial sections were then obtained from identified segments of the spinal cord and, after resin removal, stained alternatively with PAS (periodic acid-Schiff) for polysaccharides, von Kossa's (1901) silver nitrate technique for calcified deposits, modified according to the technique of Gallyas and Wolff (1985), and Bodian's method for neurofilament polypeptides (Gambetti et al., 1981). Some sections were progressively destained by successive passage through 70%-100% ethyl alcohols; in order to ascertain the acidophilicity or the basophilicity of the structures present in a given section, additional differentiation was achieved by immersion in alcohols containing concentrated ammonium hydroxide and hydrochloric acid, respectively (Sheehan and Hrapchak, 1980). Sections for ultrastructural examination were placed on uncoated copper grids, double stained with uranyl acetate and lead citrate, and scanned in a Philips 300 electron microscope operated at 60 kV.

Results

A survey of semithin sections cut at different levels through the spinal cord of cats injected with IDPN for one and two weeks revealed the presence of small, globoid, eosinophilic structures, 1-12 μm in diameter, in the neuropil of the ventral horns, without apparent consistent relationship to blood vessels or particular cell types. A specific search in blocks obtained from other CNS regions (frontal and parietal cerebral cortices, hippocampus, medial thalamus, substantia nigra, periaqueductal gray matter, cerebellar vermis and flocculus) failed to identify similar bodies in either gray or white matter. Similarly, no DMS were found in any part of the CNS, including spinal cord, in both controls and cats treated with IDPN for five weeks.

When particularly small (Fig. 1), DMS may escape recognition or be regarded as a staining artifact. They are intensely eosinophilic but do not stain with PAS, von Kossa's or Bodian's method, suggesting that they do not contain polysaccharides, calcium phosphate or argyrophilic materials. Although frequently found in the proximity of nerve cell bodies or closely adjacent to dendrites (Fig. 3), DMS were not related to the formation and evolution of axonal swellings. They occurred with different frequencies and dimensions regardless of the number and size of axonal swellings in any individual section. In particular, DMS were never observed near axon segments with reduced or absent myelin sheaths, unlike the myelin whorls or debris occasionally found near some motor axons at early stages in IDPN intoxication (Delio et al., 1985). Because of the large variability in DMS diameters, serial sections (0.5-1 μm) were cut from blocks where these bodies were encountered with greater frequency; it was ascertained that small DMS did not appear to be part of larger DMS which had been sectioned tangentially, nor did they appear to be derived by splitting from larger DMS.

Electron microscopic examination of serial sections revealed the presence of small dense bodies in astroglial processes (Fig. 2). The fine structural appearance of both extra- and intracellular DMS was similar, suggesting that they may share a common origin. A dense microsphere had the appearance of a round, electron-dense corpuscle, surrounded by an organelle-like single membrane, containing a cottony or finely granular matrix. The content was consistently homogeneous, without any paracrystalline arrangement or structured

components such as filaments or tubules; the limiting membrane was single, suggesting that mitochondria were not involved in the formation of intracellular DMS. In some sections, however, rounded electron-dense bodies with homogeneous osmiophilic content were observed in mitochondria of both neurons and glial cells (Fig. 4). Intramitochondrial bodies too were surrounded by a single membrane; it is remarkable that these bodies were never observed immediately outside the organelle.

Staining characteristics and ultrastructural features of DMS in both intra- and extracellular location were consistent with a proteinaceous content; however, no specific immunohistochemical techniques were used to characterize the chemical composition of the electron-dense matrix.

The frequency of DMS in the ventral horns of IDPN-treated cats could not be accurately assessed in the material used, because of the excessive number of sections needed to obtain enough data for statistical analysis. Large DMS were apparently more numerous than small ones; DMS in extracellular locations outnumbered those within astroglial processes by a factor of 3:1 in selected sections from different levels in the spinal cord.

Whereas DMS appeared morphologically unrelated to location and size of axonal swellings, a striking increase in intracytoplasmic dense bodies, different from both DMS and intramitochondrial osmiophilic inclusions, was observed in spinal motoneurons with neurofibrillary accumulations in their proximal axons. These dense bodies were identified, at ultrastructural level, as lipofuscin granules and lysosomes (Fig. 5). Their

Fig. 1. An extracytoplasmic DMS, about 4 μm in diameter (arrow). Semithin section from plastic-embedded specimen stained with toluidine blue and eosin. × 400. Inset: serial section showing the same DMS after differentiation. An eosinophilic core surrounded by a destained halo is clearly visible (arrow). × 1,000

Fig. 2. Electron micrograph of a DMS in the cytoplasm of an astrocytic process close to a large blood vessel. The content is finely granular and homogeneous and slightly less dense than basal lamina materials (asterisks). The dense body (DMS) is enclosed by a single membrane (small arrows). N: nucleus of a neighboring pericyte. × 38,000

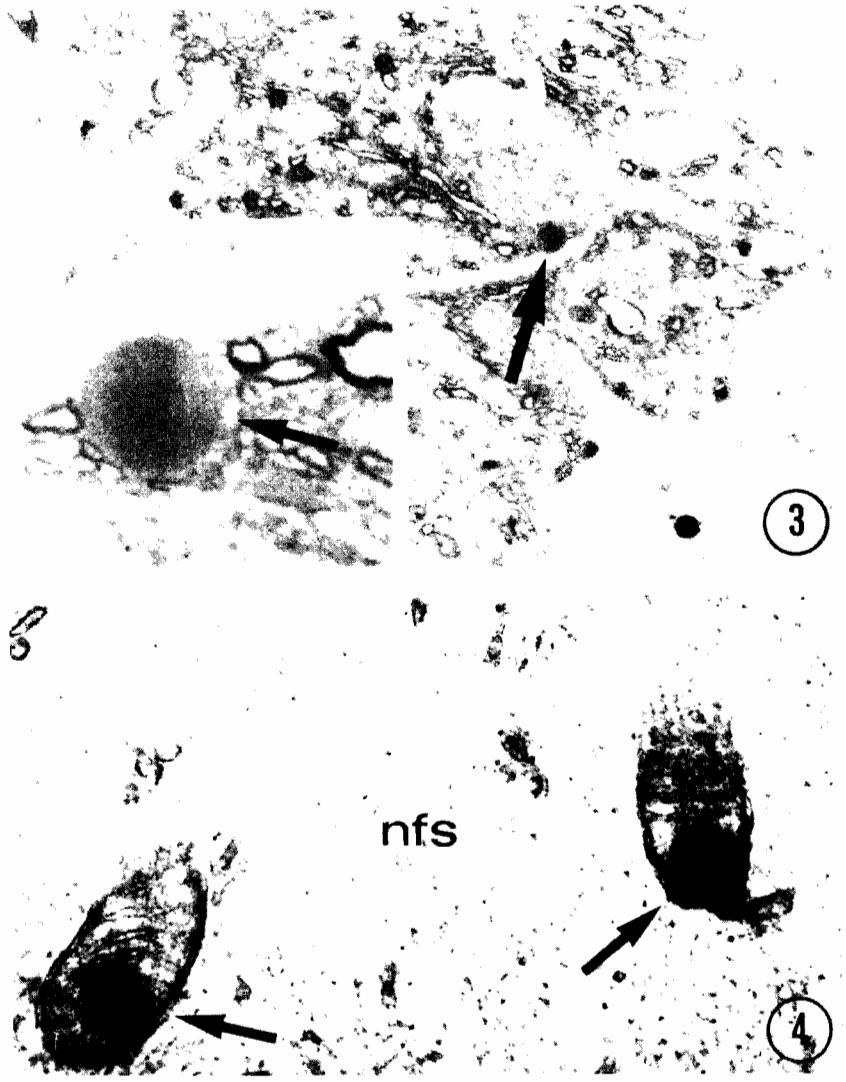


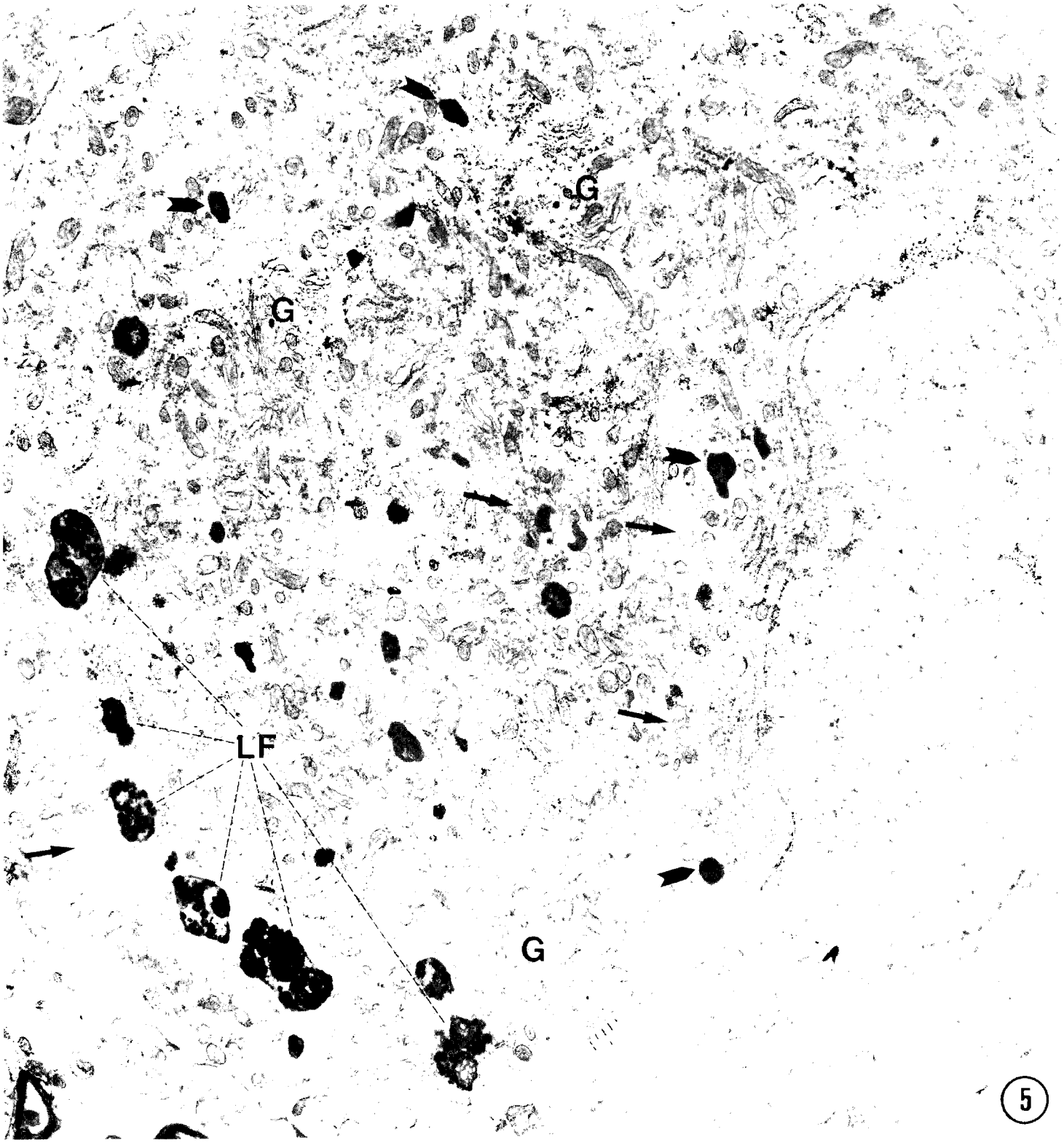
Fig. 3. A very large (about 11 μm in diameter) extracytoplasmic DMS (arrow) sandwiched between the perikaryon of a motoneuron and a dendritic process. × 100. Inset: at higher magnification, the same DMS (arrow) is shown to compress the dendritic profile. × 800

Fig. 4. Electron micrograph of an axonal swelling in the left ventral horn at L_5 level. Skeins of maloriented neurofilaments (nfs) occupy most of the field; two mitochondria (arrows) are shown to contain rounded, osmiophilic, homogeneous bodies enwrapped by a single membrane. × 35,300

Fig. 5. Low-magnification electron micrograph of a medium-sized motoneuron in the left ventral horn of a cat treated with IDPN for three weeks. Lysosomes (thick arrows), multivesicular bodies (long arrows) and lipofuscin bodies (F) are arranged concentrically to the nucleus, with the largest lipofuscin bodies concentrated in the outer regions of the cytoplasm. The distribution of other organelles is fairly normal, although a redundancy in Golgi apparatus can be noted (G) × 12,000

Fig. 6. Early stage in the transformation of a lysosome into a lipofuscin body. Parallel membrane arrays are clearly visible in the upper right pole of the organelle; a clump of electron-dense granules is gathering in the center, between two vacuoles originally containing materials of probable lipidic nature. × 46,000

Fig. 7. Portion of the perikaryon of a motoneuron with neurofibrillary accumulations in the proximal axon. A bundle of neurofilaments (nfs) is visible in the bottom part of the micrograph, while other neurofilaments appear irregularly scattered within the cytoplasm. Lipofuscin bodies of various size and maturation stage tend to cluster without significantly displacing the other organelles. × 33,000



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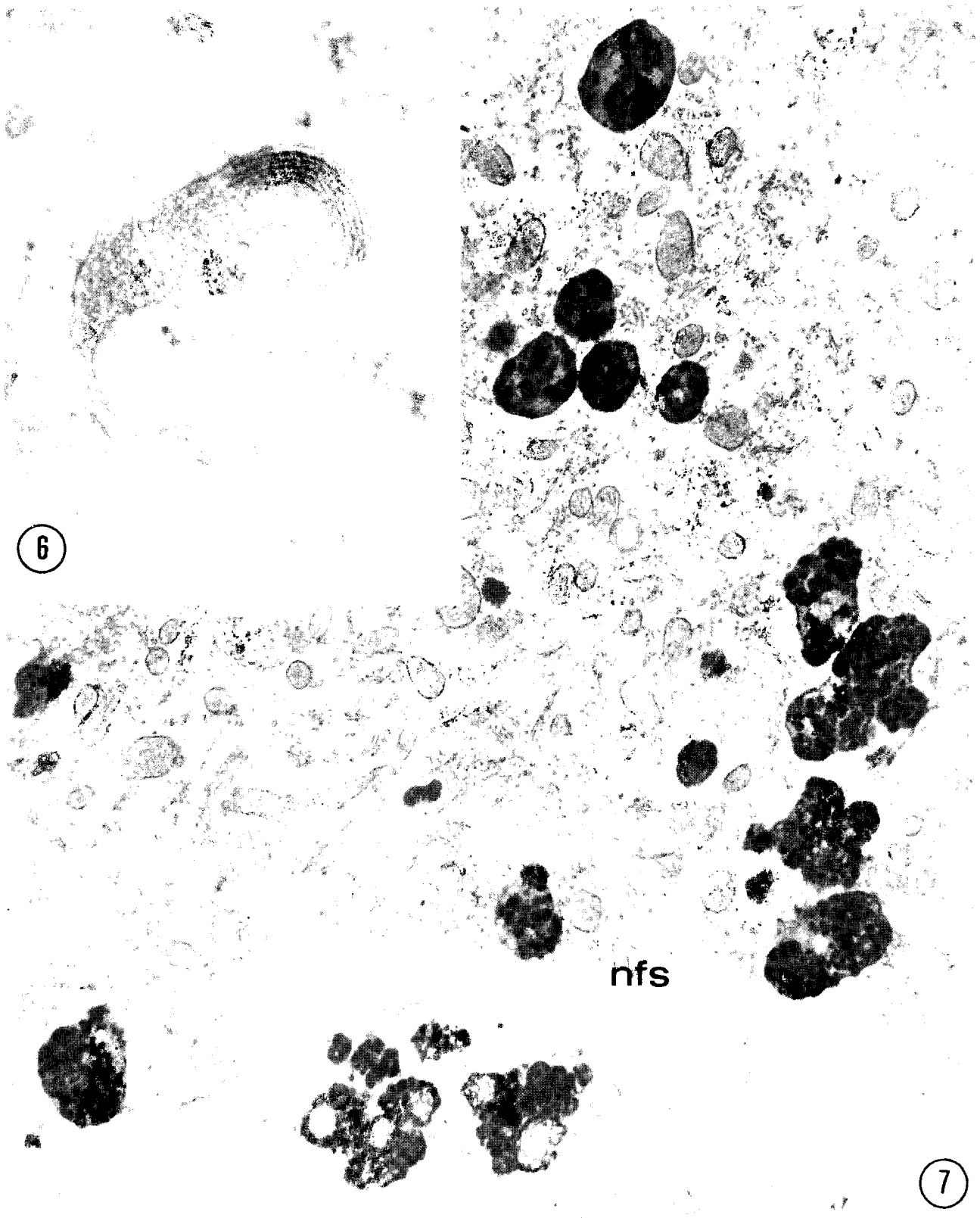
6
nfs
7

number and size appeared to correlate with the duration of the intoxication, nerve cells of cats treated with IDPN for five weeks containing more and larger electron-dense organelles than those from cats injected once or twice (Figs. 5 and 7). Lipofuscin bodies tended to be usually displaced to the cortical perikaryal cytoplasm and to extend also into the processes, while other dense bodies of lysosomal appearance were mainly concentrated in the perinuclear cytoplasm. Such a differential distribution (Fig. 5) allowed, in several instances, observations of all the stages in the transformation of lysosomes and microperisome-like multivesicular bodies into irregularly shaped, granule-containing lipofuscin bodies (Hasan and Glees, 1972; Figs. 5 and 6).

Notwithstanding the marked acumulation of lipofuscin, the other organelles did not show peculiar rearrangement, compared to their appearance in motoneurons from non-affected CNS areas. In particular, the highly ordered parallel cisterns of the rough endoplasmic reticulum, which are characteristic of normal young neurons and become dispersed during aging and chromatolysis (Hinds and McNelly, 1978; Johnson et al., 1985), retained their normal arrangement (Fig. 5). Occasionally, however, neurofibrillary bundles in the perikaryon (Fig. 7) and an apparent increase in the Golgi apparatus (Fig. 5) were observed in some neurons. That the lipofuscin pigment accumulation and coincident perikaryal remodeling were not age-dependent phenomena, as usually described (Sekhon and Maxwell, 1974), was further inferred from the structure of mitochondrial inclusions in IDPN-treated neural cells: when present (Fig. 4), they were always homogeneous and occupied large portions of the mitochondrion, unlike the paracrystalline inclusions reported as a consequence of prolonged mitochondrial aging *in situ* (Cheah and Cheah, 1977), which on the contrary are structured and mainly localized in the intracristal spaces.

Discussion

A considerable number of intracytoplasmic and intranuclear bodies occur in several neuropathological states. Some of these have been regarded as pathognomonic of a specific disease and have been characterized both morphologically and biochemically. In most cases their significance remains to be elucidated, and it is possible that they represent epiphenomena of non-specific cell reactions to various harmful factors. In this context, the dense microspheres observed in the ventral horns of the spinal cord of cats intoxicated with IDPN could represent another example of the wide spectrum of dense bodies encountered in various pathological conditions.

When located intracytoplasmically, DMS may be mistaken, on light microscopical examination, for such inclusions as Bunina bodies of the ALS-dementia complex (Hart et al., 1977), Hirano bodies, Rosenthal fibers, Lewy bodies, and so-called nucleolus-like bodies (Katoh and Shimizu, 1982). Some of these bodies are associated with neurodegenerative disorders in which neurofilamentous accumulations similar to those induced by IDPN may be found. DMS and other dense bodies are readily differentiated at the ultrastructural level, since DMS do not contain structured components such as filaments (characteristic of Hirano bodies and Rosenthal fibers: Okamoto et al., 1982; Towfighi et al., 1983), are limited by an organelle-like membrane (absent in the nucleolus-like bodies), and are usually confined to the periphery of astrocytic processes.

Extracellular DMS are less likely to be mistaken for other structures on the basis of morphological characteristics and staining properties. They do not, for instance, stain with von Kossa's silver nitrate technique and therefore contain no phosphates or carbonates (Pearse, 1980). Von Kossa's-positive calcifications usually appear as laminated, irregular, basophilic corpuscles surrounding or obscuring a blood vessel (Tonge et al., 1977; Saal et al., 1978), while DMS are round, eosinophilic bodies with no relationship to vessels. DMS remain unstained with PAS and can thus be easily distinguished from Lafora bodies and other polyglucosan inclusions, such as corpora amylacea (Palmucci et al., 1982; Szirmai et al., 1983). Although larger DMS, in the 6-8 μm range, could resemble extravasated erythrocytes, they do not have a bilenticular shape and are usually found at a considerable distance from the closest blood vessel (cf. Figs. 1 and 3).

Although DMS in both intra- and extracytoplasmic locations have a similar ultrastructural appearance, it is not known whether those located within glial processes are the same as those encountered in the extracellular space. Even if DMS found in astrocytic cytoplasm resemble distended vesicles of the smooth endoplasmic reticulum, it is unlikely that segments of this organelle would reach the dimension of the larger intracellular DMS (3 μm), particularly in peripheral expansions of an astrocytic process. Since the organelle from which the DMS membrane is derived has not been determined, it is possible that all DMS have an intracellular origin and are then extruded into the extracellular space by an exocytotic process. In this regard, the occurrence of dense osmiophilic bodies bound by a single membrane within mitochondria of both neurons and glial cells is suggestive. They could just be a coincidental structure or represent an early stage in the DMS maturative cycle, through which these bodies might be transferred from the organelle to the extracellular spaces. Likewise, the occurence of a larger number of lipofuscin granules and other dense intracytoplasmic bodies in the perikarya of spinal motoneurons in the course of IDPN intoxication could be explained as an activation of intracellular mechanisms that ultimately result in the transformation of cell organelles such as mitochondria and lysosomes into lipofuscin bodies (Brunk and Ericsson, 1972).

Although there is no structural evidence that DMS move across the plasmamembrane, it is generally conceded that exocytosis is either too rare or too rapid an event to be documented with any frequency (Willingham et al., 1981; Holstein and Tardent, 1984). On the other hand, since lipofuscin granules tend to accumulate in the

neuronal cell body while DMS can be found in both intra- and extracytoplasmic locations, it may be hypothesized that either a) the occurrence of lipofuscin bodies is a consequence of the inability of neurons for exocytosis of residues accumulating in lysosomes, whereas astrocytes may more easily eliminate particulate materials; or b) the appearance of DMS and lipofuscin pigments is a temporally coincident manifestation of two different metabolic phenomena taking place in glial cells and neurons, respectively.

This latter hypothesis seems to be supported by circumstantial evidence that a change in the rate of formation or degradation of neurofilaments may occur in IDPN neuropathy. On these grounds, in IDPN intoxication either a) the neurofilament-specific proteolysis is enhanced in the attempt to regulate amount and organization of the cytoskeleton in the neuronal segments, or b) an inhibitory message is delivered to the synthetic machinery of the cell body to reduce specifically the neurofilament production. It is not known whether the synthetic capability of the perikaryon is significantly affected by IDPN treatment: the neurofilaments, however, although inhibited in moving down the axon, do not usually tend to backfill the cell body. While the presence of neurofilament-specific Ca^{2+}-activated neutral proteases in lysosomes and lipofuscin granules has not been assessed (Goldfischer et al., 1966), it is suggestive that leupeptin, a potent inhibitor of these enzymes, induces a dose- and time-dependent formation of lysosome-associated granular bodies similar to the classical lipofuscin pigments (Ivy et al., 1984). In this framework, the occurrence of numerous lipofuscin bodies in IDPN-treated spinal motoneurons could be the morphological expression of an enhanced storage of inactivated proteolytic enzymes in lysosomes which, being incapable of autophagocytosis, eventually transform into dense bodies. It is interesting to note that the appearance of osmiophilic granules identified as lysosomes has been recently reported in the spinal cord of cats treated with acrylamide (Goldstein et al., 1984), a drug known to induce a central-peripheral distal axonopathy accompanied by neurofilament derangement.

DMS, whether or not formed as an intraorganellar material and then released outside the cell, display histochemical and ultrastructural features which are consistent with a proteinaceous content, although it seems highly speculative that they may possess an enzymatic activity. It is noteworthy that no obvious relationship exists between the occurrence of DMS and the formation and evolution of axonal swellings. DMS, however, are not unique to IDPN intoxication, since Averback (1983) has reported the presence of dense microspheres in human brains of normal individuals as well as in autopsy material obtained from patients suffering from Alzheimer's disease, Parkinsonism, and schizophrenia. More recently, Hara (1986) has confirmed Averback's findings, with the notable difference that DMS are described to be fewer in number in neurological disorders characterized by mental retardation or dementia. These dense microspheres were not found in newborn brains and showed a significant numerical reduction in brains from individual aged 70 years and over. Histochemical and ultrastructural aspects of the DMS reported by both Averback and Hara are similar to those of the DMS observed in IDPN-intoxicated cats. The presence of DMS in normal human brains is puzzling: it is possible that these dense bodies are dynamic structures which increase or decrease their content and density according to particular metabolic situations in the CNS. The large variability in DMS dimensions and their simultaneous occurrence in both intra- and extracellular locations in IDPN intoxication suggest the hypothesis that DMS become detectable when changes in protein metabolism occur. In this connection the absence of DMS in cats intoxicated for five weeks may reflect particular variations in the ventral horns of the spinal cord at early stages of IDPN treatment.

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