HELICAL ARRANGEMENT OF THE SUBUNITS OF THE NEUROFIBRILLAR BUNDLES ISOLATED FROM LEECH NERVOUS SYSTEM

J. METUZALS. From the Department of Histology and Embryology, the Faculty of Medicine, University of Ottawa, Ottawa, Canada

INTRODUCTION

Since the early days of classical neurohistology, the nervous system of the leech has been and still is a favorite object of investigation. In particular, the neurofibrillar bundles or the neurofibrils have attracted much attention because they represent a characteristic and ubiquitous component of the neuron (1). The term neurofibrillar bundle has been introduced recently to designate the structures in the electron micrographs corresponding to the neurofibrils of light microscopy (2–4). In spite of the continuous efforts of numerous investigators the structure, chemical composition, and the functional significance of the neurofibrils remain obscure (5).

Apàthy (1) showed that the neurofibrils of the leech neuron are about 0.5–0.75 μ thick and have a helical course in the axon. Furthermore, he demonstrated with the light microscope that the neurofibrils consist of elementary filaments; when the neurofibril is highly coiled, the filaments could separate. It is noteworthy that already in 1897 Apàthy (6) was able to isolate the neurofibrils which appeared as rigid rods and showed, in polarized light, positive uniaxial birefringence. Recent electron microscopic studies of thin sections of leech nervous system (2–4) are in agreement with these observations. In transverse sections, the neurofibrillar bundles present a coherent structure of reticular or cribriform subunits. These subunits correspond to transversely sectioned filaments about 60 A in diameter bound together laterally. However, the subunits do not show as segregated dots, in contrast to the glial filaments, tonofilaments, and also the neurofilaments in vertebrates. According to Gray and Guillery (2), the angular structure of the subunits of the neurofibrillar bundles is probably produced by three or more closely packed finer filaments, and these filaments may have the same structure as the vertebrate neurofilaments. Hagadorn et al. (4) suggest a tubular structure of the subunits of the neurofibrillar bundles; the walls of these tubular subunits consist of "protofibrillae" about 60 A in diameter.

It is evident that methods of specimen preparation other than thin sectioning need to be applied in order to clarify the intricate, fine structure of the neurofibrillar bundles of the leech and to relate properly the neurofibrillar bundles to the vertebrate neurofilaments. Electron microscopy of isolated and negatively stained neurofibrillar

bundles is at present the best method for the study of this kind of problem. Furthermore, the nervous system of the leech presents a very suitable object for attempts to isolate and identify fibrous structures. The dimensions and the fine structure of the neurofibrillar bundles differ considerably from the morphological characteristics of other thread-like components of the leech nervous system, such as the glial fibrils, the tonofibrils, the microtubules, and the collagen fibrils. These differences are well documented by electron microscopy of thin sections.

In leech nervous system the glial fibrils are structurally identical with the tonofibrils. They consist of faintly beaded filaments about 50 A in diameter, easily recognized as individual units (2, 3). Furthermore, in leech the unit fibrils of the collagen are thinner than the unit fibrils of vertebrate collagen and show a simpler pattern of periodic cross-striation. Cross-sectioned leech collagen fibers appear always as separate dots and measure about 100 A in diameter (compare Gray and Guillery, reference 2, Fig. 18; Coggeshall and Fawcett, reference 3, Fig. 6). From this the conclusion is warranted that, using leech nervous system, the problem of identifying isolated neurofibrillar bundles in electron micrographs of negatively stained preparations can be met effectively. The difficulties encountered by previous investigators with different material (7) can be thus avoided in the present investigation.

MATERIALS AND METHODS

Neurofibrillar bundles were isolated from the nervous system of the turtle leech (*Macrobdella* sp.). The connectives and rami of the ventral ganglion chain were dissected free from their connective tissue sheaths and fragmented in an isotonic solution of the following composition (mM/liter): NaCl, 113.5; KCl, 4.3; $CaCl_2$, 1.8; Tris(hydroxymethyl)-aminomethane, 10.0; maleic acid, 10.0; NaOH, about 10.0; maleic acid, 10.0; NaOH, about 10.0 to bring the pH to 7.4 (reference 20). A drop of suspension was placed on a coated grid and was stained negatively with 1% Na-phosphotungstate solution at pH 7.5 (6). The preparations were examined in the Siemens Elmiskop I.

RESULTS

In electron micrographs of the preparations profiles of filamentous bundles, microtubules, endoplasmic reticulum, mitochondria, and membrane fragments can be observed. In electron micrographs of properly stained preparations the glial fibrils and the neurofibrillar bundles can be identified rather easily. This can be achieved by comparing the dimensions, the fine structure, and the general morphology of the profile in question with the corresponding descriptions provided by electron microscopy of thin sections and summarized in the introduction of this paper.

The principal differences of fine structure among the filamentous bundles can be clearly demonstrated in preparations in which the bundles have been partly disrupted during preparation and their components have been spread on the film (see Figs. 1 and 7). One type of bundle consists of faintly beaded filaments, about 50 A in diameter, which can be easily recognized as individual units distributed homogeneously (see Figs. 1 and 2). This fine structure corresponds to that of glia fibrils as described in electron micrographs of thin sections. As judged by the dimensions, the fine structure, and the general morphology, another type of fibrous bundle corresponds well to the neurofibrillar bundles as illustrated by Gray and Guillery, reference 2, Figs. 5–7 *a*, by Coggeshall and Fawcett, reference 3, Fig. 28, and by Hagadorn et al., reference 4, Figs. 5 and 9.

In the preparations the profiles of the neurofibrillar bundles measure about 0.2–0.6 μ in diameter and up to 20 μ in length. In electron micrographs of isolated and negatively stained neurofibrillar bundles, a general pattern of hierarchic levels of fine structural organization can be discerned. A basic structural unit of heavily beaded filaments with a diameter of about 40 A can be observed in micrographs of intensely stained neurofibrillar bundles. These beaded filaments are closely associated in structural units which I will call filamentous aggregates (Figs. 3 and 7). The filamentous aggregates correspond to the coherent reticular, cribriform, or tubular subunits of the neurofibrillar bundles described by previous authors (2–4). The diameter of the filamentous aggregate of the neurofibrillar bundle can vary considerably; however, 300 A is the most commonly observed width. The beads on the filaments are frequently seen to be in register, and thus cause cross-striation. For certain distances a globular subunit pattern predominates in the aggregates, and individual filaments cannot be discerned at these sites (Fig. 4). Fig. 4 represents a slightly underfocused micrograph of a

through-focus series. The same pattern remains at all focal levels.

In Figs. 6 and 7, the filamentous aggregates, about 300 A in diameter, can be clearly discerned as structural units of an isolated portion of a neurofibrillar bundle about 0.2 μ thick. In the middle part of the bundle the aggregates are tightly associated with each other. Because of mechanical disruption, the aggregates splay out at both ends of the profile; this indicates their character as a structural unit of the neurofibrillar bundle. The existence of the 40-A beaded filaments is demonstrated by the longitudinal striation of the aggregates in Fig. 7.

Single threadlike profiles of 200–300 A in diameter can be observed regularly in the preparations. These profiles are probably microtubules.

In the neurofibrillar bundle the filamentous aggregates are intertwined in a superhelix. The pitch of the helices of the filamentous aggregates varies in different portions of the same neurofibrillar bundle and from one preparation to another. Portions of the same neurofibrillar bundle are illustrated in Figs. 3 and 5. The filamentous aggregates in the center of this bundle are intertwined tightly into a superhelix of a ropelike appearance. The peripheral aggregates are intertwined and associated more loosely. That there is considerable variation in the degree of coiling of the filamentous aggregates may be appreciated by comparing Fig. 6 with Fig. 5. In Fig. 6 the degree of coiling of the filamentous aggregates is markedly lower than in Fig. 5.

The three-dimensional appearance of the neurofibrillar bundles can be observed best by viewing the electron micrographs at an oblique angle along the long axis of the bundle.

Among the filamentous aggregates, dense material in the form of spinous projections or cross-bridges can be observed in a number of electron micrographs of intensely stained specimens. This dense material is regularly spaced with a certain periodicity (Figs. 3 and 8).

DISCUSSION

The rodlike profiles which have been identified in the present investigation as neurofibrillar bundles represent a morphological unit of coherent and hierarchic substructure. The dimensions and the morphology of the 0.2–0.6 μ thick profiles as well as their subunits (the filamentous aggregates, about 300 A in diameter, and the heavily beaded filaments, about 40 A in diameter) correspond well to the dimensions and structure of the neurofibrillar bundles described in electron micrographs of thin sections. The organized helical arrangement of the filamentous aggregates in the neurofibrillar bundles, as illustrated in Figs. 3, 5, and 6, excludes the possibility that this kind of structure could be produced by artifactitious aggregations of certain threadlike elements, for example the microtubules, during preparation of the specimens.

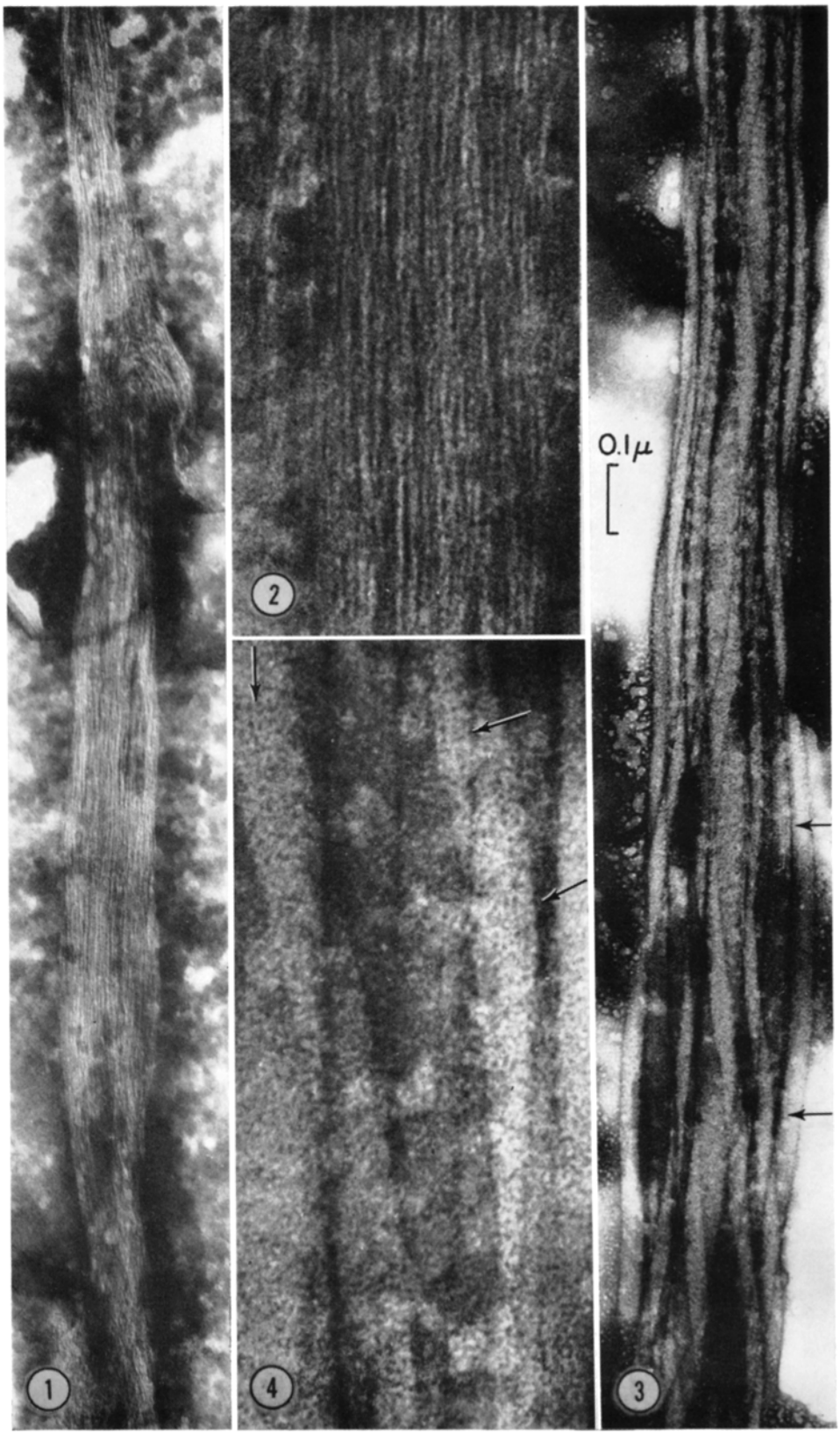
There is some resemblance in fine structure between the filamentous aggregates of the neurofibrillar bundles and the microtubules as observed in isolated and negatively stained preparations (compare Fig. 4 with the illustrations in references

FIGURE 1 Electron micrograph of a bundle of glial filaments. The individual filaments can be clearly discerned. They are distributed homogeneously throughout the bundle. Note the characteristic, uneven shape of the bundle as compared with the rodlike appearance of a neurofibrillar bundle. 1% Na-Phosphotungstate. × 91,000.

FIGURE 2 High resolution electron micrograph of glial filaments. Note the faintly beaded appearance of the filaments as separate individual units. 1% Na-Phosphotungstate. × 300,000.

FIGURE 3 An intensely stained neurofibrillar bundle displaying a detailed structure. A central rope of tightly intertwined, filamentous aggregates can be distinguished. The arrows indicate longitudinal and transverse substructure in the filamentous aggregates. 1% Na-Phosphotungstate. × 104,000.

FIGURE 4 Beaded filaments composing the basic structure of the filamentous aggregate of the neurofibrillar bundle. In certain portions the beads are in register, causing a cross-striated appearance of the aggregate (arrows). 1% Na-Phosphotungstate. × 343,000.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8–10). Indications of a tubular substructure of the neurofibrillar bundles have been reported by Hagadorn et al. (4) (see also 11). The reticular appearance of the subunits of the cross-sectioned neurofibrillar bundles could be explained, perhaps, by the possibility of the existence of spinous projections or cross-bridges among the subunits (see Figs. 8 and 7). Furthermore, the helical arrangement of the subunits, as illustrated in Figs 3, 5, and 6, could also be responsible for the peculiar reticular substructure of neurofibrillar bundles as seen in electron micrographs of sectioned material. Subunits oriented obliquely against the plane of sectioning may cause overlapping of their profiles in electron micrographs.

By comparing Figs. 1 and 2 with Figs. 3–8, the morphological differences between the glial fibrils and the neurofibrillar bundles are evident. Accordingly, the differentiation and the identification of these two types of fibrous bundles do not present any problem in the present investigation.

The present results are in good agreement with the classical observations made by Apàthy (1, 6) at the end of the 19th century. The helical arrangement of the filamentous aggregates may explain the peculiar appearance of the neurofibrillar bundles in electron micrographs of thin sections of fixed material. The observed, different degrees of tightness of coiling and the associations of the filamentous aggregates in neurofibrillar bundles may be due to mechanical disruption during specimen preparation (see Figs. 6, 7). On the other hand, there is an interesting possibility that this variation in coiling may be a structural correlate of the functional activity of the neurofibrillar bundles themselves. For example, one might speculate that the neurofibrillar bundles could generate the motive force for axonal streaming by the coiling and decoiling of their subunits (12).

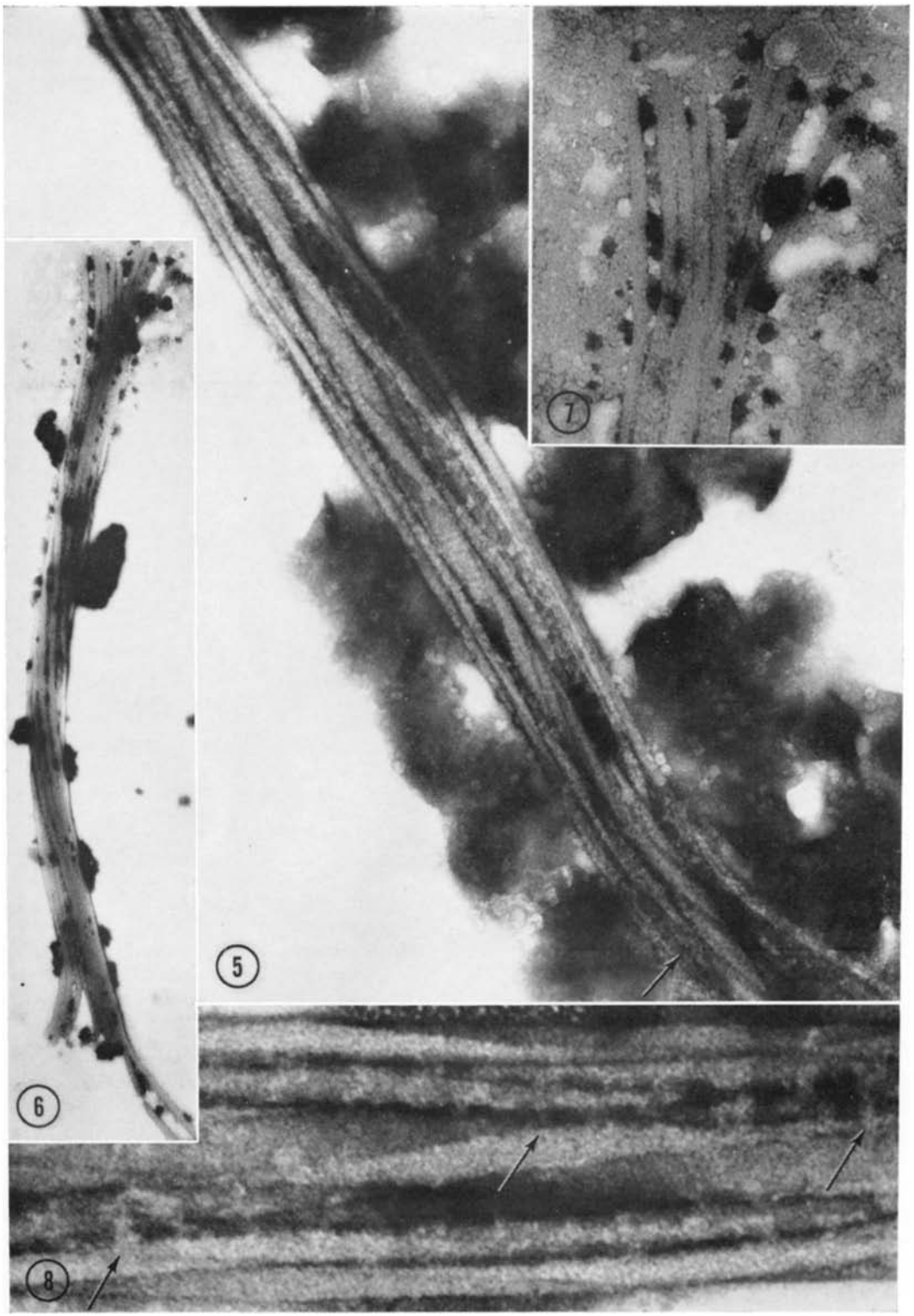
Although certain differences exist, the neurofibrillar bundles of the leech can be regarded as equivalent to the neurofibrils in the vertebrate nervous system (13). Under certain physiological and pathological conditions, large bundles consisting of numerous neurofilaments may also develop in vertebrate neurons (14). Several investigations suggest that the neurofibrils (or neurofilaments) may be causally involved in the movement, development, and regeneration of axons (15–17). Ramòn y Cajal (18) proposed that the neurofibrils consist of ultramicroscopic functional units, the neurobiones, to which he attributed the capacities for independent growth, multiplication, and movement. Thus, the present observations of the structure of the neurofibrillar bundles of the leech may prove to be of functional importance. Investigations of this favorable material are being continued by the present author. Especial emphasis is thereby placed to clarify the relationships among the neurofibrillar bundles, the microtubules of the leech, and the vertebrate neurofilaments, in order to evaluate the significance of the filamentous protein arrays of the neuron (see also 19).

FIGURE 5 Another portion of the same neurofibrillar bundle illustrated in Fig. 3. In the lower part of the bundle the filamentous aggregates are tightly coiled in a central rope. Arrow indicates transverse structures between the filamentous aggregates. 1% Na-Phosphotungstate. × 82,500.

FIGURE 6 Neurofibrillar bundle displaying a less tight coiling of its filamentous aggregates which splay out at both ends of the profile because of mechanical disruption of the bundle. In the lower part of the figure single filamentous aggregates are visible. 1% Na-Phosphotungstate. × 32,000.

FIGURE 7 Filamentous aggregates splayed out, probably because of artificial disruption of the neurofibrillar bundle during preparation. Higher magnification of the upper part of Fig. 6. 1% Na-Phosphotungstate. × 75,000.

FIGURE 8 Regions of high density (arrows) which might be interpreted as spinous projections or cross-bridges between the filamentous aggregates of the neurofibrillar bundle. 1% Na-Phosphotungstate. × 214,000.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SUMMARY

Neurofibrillar bundles were isolated from the nervous system of the turtle leech (*Macrobdella* sp.) and stained negatively with 1% Na-phosphotungstate solution at pH 7.3. Electron microscopy of the preparations reveals a hierarchically ordered substructure. Heavily beaded filaments of about 40 A in diameter are associated in filamentous aggregates measuring mostly about 300 A in diameter. The filamentous aggregates are intertwined into larger superhelices and associated in different degrees into neurofibrillar bundles.

The electron micrographs of this investigation have been exhibited at the Sixth International Congress for Electron Microscopy, Kyoto, 1966.

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ADDENDUM

Since the manuscript was submitted for publication, the present author has examined thin sections of leech ventral ganglion chain, fixed in glutaraldehyde and osmium tetroxide. Cross, oblique, and longitudinal sections of the neurofibrillar bundles have been investigated in high resolution electron micrographs. In these micrographs, the dimensions and the substructural configuration of the neurofibrillar bundles correspond well to the data of the present paper. In cross-sections, the filamentous aggregates have often a circular appearance. A matrix material can be discerned among the filamentous structures in the neurofibrillar bundles.

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