CONFIGURATION OF A FILAMENTOUS NETWORK IN THE AXOPLASM OF THE SQUID (*LOLIGO PEALII* L.) GIANT NERVE FIBER

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ABSTRACT

High-resolution electron microscopy is integrated with physicochemical methods in order to investigate the following preparations of the giant nerve fibers of the squid (*Loligo pealii L.*): (*1*) Thin sections of fibers fixed in four different fixatives; (*2*) fresh axoplasm stained negatively in solutions of different pH and composition; (*3*) chemically isolated threadlike elements of the axoplasm. A continuous, three-dimensional network can be identified in all these preparations of the axoplasm. The network is composed of coiled or looped unit-filaments ~30 A wide. The unit-filaments are intercoiled in strands ~ 70–250 A wide. The strands are oriented longitudinally in the axoplasm, often having a sinuous course and cross-associations. Microtubules are surrounded by intercoiled unit-filaments and filamentous strands. Calcium ions cause loosening and disintegration of the network configuration. UO_2^{++} ions of a 1% uranyl acetate solution at pH 4.4 display a specific affinity for filamentous protein structures of the squid giant nerve fiber axoplasm, segregating the filamentous elements of the axoplasm in a coiled, threadlike preparation. The uranyl ions combine probably with the carboxyl groups of the main amino acids of the protein—glutamic and aspartic acids. It is proposed that by coiling/decoiling and folding/unfolding of the unit-filaments, shifts in physicochemical properties of the axoplasm are maintained.

INTRODUCTION

The three-dimensional configuration, and the changes of the configuration of protein macromolecules and their supramolecular aggregates, represent central elements in cell structure and function (Lehninger, 1967). From this viewpoint, the following report on a highly ordered filamentous protein space-network and its configurational pattern in the axoplasm of the squid giant nerve fiber is of interest. Such a space-network configuration has not previously been observed as a structural pattern of supramolecular aggregates in cells.

The nervous system should possess a high degree of structural order, especially because of its immense capacity for information storage (Lehninger, 1967). Such an order has been observed in the nervous system only at the tissue and cellular levels of organization. At the subcellular level, structural arrays specific to the neuroplasm remain, up to date, an unresolved problem. However, Metuzals and Izzard (1969) have observed a highly ordered space-network configuration of intercoiled threadlike elements in the axoplasm of the squid giant nerve fiber. It was proposed by

these authors that changes of the physical properties of the axoplasm can be achieved by coiling and decoiling and folding/unfolding of the hierarchically ordered threadlike elements of such a structural organization. This must result from conformational changes of the constituent protein macromolecules with consequent changes in their electrical and electromagnetic properties. In such a way, coupling between the activities located in the excitable membrane with those in the axoplasm could be maintained (see Ungar et al., 1957; Ling, 1960; Ungar, 1963; Katschalsky et al., 1966; Cohen et al., 1968). The results of the present paper represent an integral part of those published in the preceding paper in this journal by Metuzals and Izzard (1969).

The detailed configuration of the axoplasmic network and its filamentous components was disclosed by high-resolution electron microscopy of thin sections and of negatively stained preparations of the axoplasm of squid giant nerve fiber. The pertinent observations will be reported in the present paper. The configurational changes of the elements of the axoplasmic network, caused by calcium ions, have been investigated in negatively stained preparations. Several observations suggest that calcium ions are essential for both the structural integrity of the axoplasm and the normal functioning of the axon (see Hodgkin and Keynes, 1956; Metuzals and Izzard, 1969). Furthermore, a method to obtain preparations from the axoplasm, which consist of the filamentous network components, will be described. Some results of physicochemical analyses of such preparations will be reported.

Usually, neurofilaments are considered to be characteristic structures of the neuron. In the vast literature on this subject, the neurofilaments are known as protein structures having a diameter of the order of 100 A and extending as individual units throughout most of the neuron (Schmitt and Davison, 1961; Davison, 1967). It has been reported, however, that in vertebrate axons the individual neurofilaments are associated with each other by cross-bridges (Metuzals, 1966 *a* and *b*). In the leech (Gray and Guillery, 1963; Coggeshall and Fawcett, 1964; Metuzals, 1967), the filamentous material in the axon is arranged in 0.5-μ-thick bundles which represent a morphological unit of coherent and hierarchic substructure. The bundles consist of 40-A-wide, heavily beaded filaments associated in aggregates of varying thickness; these filamentous aggregates are intertwined in a super-helix to form the whole bundle. The results reported in the following indicate that, in the axoplasm of the squid giant fibers, individual threadlike elements corresponding to the neurofilaments do not exist. Thus, the neurofilament has to be considered as an artifactitious product of the preparation techniques.

Preliminary accounts of the results of this investigation have appeared elsewhere (Metuzals, 1968 *a* and *b*).

MATERIALS AND METHODS

Living squid (*Loligo pealii L.*) obtained at the Marine Biological Laboratory, Woods Hole, Mass. were used throughout. All the experiments were carried out during the summers of 1966, 1967, and 1968 at the M.B.L., Woods Hole, with animals that had spent no more than several hours in the laboratory tank containing running seawater. The sectioning, the main part of the electron microscopy, and the physicochemical analyses were carried out at the University of Ottawa, and at the Department of Energy, Mines, and Resources, Ottawa, Canada.

High-resolution electron microscopy was combined with a number of physicochemical methods in the investigation of the following preparations of the giant nerve fiber of the squid: (*a*) thin sections of fibers fixed in four different ways; (*b*) freshly extruded axoplasm stained negatively in solutions of different composition and pH; (*c*) chemically isolated threadlike elements of the axoplasm.

The animals were decapitated, the mantle was opened, and one of the dorsal giant nerve fibers was dissected free under running seawater. This was done on a dissecting table with a glass window permitting illumination from below, in the way usually practiced at the Marine Biological Laboratory in Woods Hole.

Fixation and Electron Microscopy

The giant nerve fibers of the squid were fixed and treated according to one of the procedures described below, dehydrated in increasing concentrations of acetone, and embedded in Vestopal W. The blocks were sectioned with an LKB Ultratome. Most of the sections were double stained: first, in 1% uranyl acetate solution at 45°C for 1 hr, followed by staining in lead citrate according to Reynolds (1963) for 5–9 min (cf Pease, 1964). Some of the sections have been stained either with the uranyl acetate or with the lead citrate–staining method.

Preparations were examined in a Siemens Elmiskop I with an accelerating voltage of 80 kv, double condenser illumination, a 200-μ aperture in condenser II, and a 25-μ objective aperture.

The fixation solutions were prepared in the following way: to solutions of 1.5% osmium tetroxide or 2.5% glutaraldehyde, both in 0.1 M sodium cacodylate, triple strength seawater was added so as to obtain a final solution for fixation having the same osmolality as seawater—1010 millios-mols/1. The osmium tetroxide concentration of this final solution was 0.9%; the concentration of the glutaraldehyde was 1.7%. The osmolality of fixative solutions was measured with a freezing-point depression device, the Advanced Osmometer, Model 31 LAS (Advanced Instruments, Newton Highlands, Mass.).

The pH of the final fixation solutions of glutaraldehyde and osmium tetroxide was adjusted with 1.0 N HCl and varied from 7.2–7.6 in different experiments.

In several series of experiments, the fixing solutions were cooled to +2°C before the fixation. In these experiments, the final fixation and the first washing in seawater were carried out in a cold room at +2°C. The specimens were brought to room temperature before the dehydration. The fixing solutions in other series of experiments were kept at room temperature, and all the stages of fixation and dehydration were carried out at this temperature.

In all experiments, first the fixative was dripped slowly for 15 min, with a syringe, on the surface of the exposed fiber while the squid remained on the dissecting desk containing seawater. Afterwards, both ends of a 2-cm-long portion of the giant fiber were ligated, and the corresponding portion of the giant fiber was removed from the animal and placed in the solution for further fixation. The specimens were washed in artificial seawater for 15 min between fixation in glutaraldehyde and that in osmium tetroxide.

The following procedures of fixation have been used:

Procedure I: 1¼ hr in glutaraldehyde (after 5 min of dripping, the fiber becomes yellow). 1 hr in osmium tetroxide.

Procedure II: 1¼ hr in osmium tetroxide.

Procedure III: 1% uranyl acetate solution, pH 4.4 at +2°C, was dripped on the exposed fiber for 15 min. Afterwards, 1 hr in osmium tetroxide.

Procedure IVa and b: as in procedure I, except that before the fixation in glutaraldehyde, 1% sodium phosphotungstate (PTA) at +2°C was dripped on the exposed fiber for 15 min.

In IVa: the pH of the PTA solution was 5.4.

In IVb: the pH of the PTA was 8.0.

After the fixation, the preparations were changed four times in artificial seawater, washed in distilled water, and dehydrated.

Negative Staining

Axoplasm from a 2-cm-long portion of freshly dissected giant fibers was extruded into a number of different solutions. About 2–5 drops of a solution was placed in the concavity of a microslide, and pure axoplasm was squeezed into the solution with forceps, avoiding any contamination from the sheath material. The axoplasm can be recognized first as a viscous translucent rod which, depending on the solution, shortly becomes more or less opaque in appearance.

The axoplasm was extruded into the following solutions: (*1*) 0.5 and 1% solutions of phosphotungstic acid (PTA), pH adjusted with 0.1 N NaOH or KOH to 5.2, 7.2, 7.8, 8.0; (**2**) artificial seawater solution, pH 7.8, M.B.L. (Marine Biological Labs.) formula; (*3*) artificial seawater solution without Ca^{++}, pH 7.8, M.B.L. formula; (*4*) molluscan (marine) physiological saline solution, pH 6.6, M.B.L. formula.

The best negatively stained preparations have been obtained by squeezing the axoplasm into several drops of 0.5 or 1% phosphotungstic acid solutions. After stirring slightly with a glass rod, the axoplasmic rod disappears and the final mixture becomes homogeneous. This happens faster in PTA solutions of pH 8.0 than in solutions of pH 7.2 or lower. However, often fragments of a veil-like membrane remain floating in the final solution for a longer period of time (see Schmitt, 1950). This veil-like membrane corresponds probably, to the ectoplasm of the axon (see Metuzals and Izzard, 1969).

Grids coated with a formvar film were drawn through this homogeneous fluid. A drop of the fluid remained attached to the grid. This drop was removed by a filter paper from the grid immediately or after different intervals of time. A number of grids were allowed to dry on a microslide without prior removal of the drop. In this way, grids covered with different amounts of material were obtained.

In artificial seawater or in isotonic molluscan saline, which both contain calcium ions, the axoplasm rod disappears immediately. Grids coated with a formvar film were drawn through such dispersions of the axoplasm. Afterwards the grids, with a drop of the mixture attached, were allowed to dry for a brief period on a microslide. Then a drop of 0.5 or 1% PTA, pH 7.2 or 8.0, was placed on the grid by a micropipette. The drop of PTA was removed by filter paper immediately or after different periods of time.

Isolation Experiments

Portions of the giant nerve fiber, about 2 cm long, were dissected free from the connective tissue and removed from the mantle. One or several portions were placed in the concavity of a microslide containing 5–10 drops of one of the solutions listed below. Before being placed in these solutions, the portion of the fiber, as well as the forceps, were briefly washed in

distilled water so as to remove the traces of the seawater, and then filter paper was used for removing the excess of distilled water. The following solutions have been used: (*A*) 0.1, 0.2, 0.5, and 1% uranyl acetate each at pH 2.0, 3.0, and 4.4; (*B*) 10 cc of 1% uranyl acetate + 10 cc of 0.1% ammonium acetate + 10 cc of 0.5% ethylenediamine tetraacetate (EDTA), pH adjusted to 7.8 or 8.6 with ammonia; (*C*) 1% solutions of Cd, Fe, Zn, Co, Pb acetates, of $LaCl_3$ and $HgSO_4$; the pH of these solutions was adjusted to 4.4 with acetic acid.

The thread preparations, which have segregated in about 30 min from axoplasm placed in 1% uranyl acetate solution at pH 4.4., were washed in distilled water for 1 hr and stored in 50% glycerol at −15°C. A drop of toluene was added for preventing bacterial growth.

Grids coated with formvar film were dipped in the flocculent axoplasmic precipitate which develops around the thread segregating in the 1% uranyl acetate solution at pH 4.4. Negatively stained preparations were prepared from this flocculent axoplasmic material as described in the section on negative staining.

A number of thread preparations, after storage for several months in 50% glycerol at −15°C, were washed for several hours in six changes of double-distilled water and dehydrated in increasing concentrations of acetone. They were embedded in Vestopal W. Thin sectioning, staining of the sections, and electron microscopy were carried out as described above.

Physicochemical analysis of the thread preparations, segregated from the axoplasm during 30-min treatment with 1% uranyl acetate at pH 4.4, was carried out by the following methods. (The preparations had been stored in 50% glycerol at −15°C for several months and, before the analysis, were washed for 3 hr in numerous changes of double-distilled water.)

(*A*) The amino acid composition of the thread preparation was determined with an amino acid analyzer by the procedure of Spackman et al. (1958), in collaboration with Dr. L. Benoiton, Dept. of Biochemistry, University of Ottawa.

(*B*) The Dumas combustion method was used for the determination of nitrogen, fluorimetry for determination of uranium, and colorimetry for determination of phosphorus. The analyses of uranium and phosphorus were carried out in collaboration with Mr. J. B. Zimmerman et al., Department of Energy, Mines and Resources, Ottawa. A detailed description of these analyses has been published elsewhere and is available on request (Zimmerman et al., 1968).

(*C*) Calcium and magnesium concentrations were measured by atomic absorption spectrophotometry, Atomic Absorption Spectrophotometer, E.E.L. Model 140 (Arthur H. Thomas Co., Philadelphia, Pa. 19105), in 0.5% lanthanum chloride ($LaCl_3$), in collaboration with Dr. B. I. Hunt, Department of Physiology, University of Ottawa. Preliminary studies had shown that the 0.5% concentration of $LaCl_3$ was adequate to suppress the depressant action of uranium salts on magnesium and calcium absorption.

(*D*) Assay of the axoplasmic thread for nucleic acids and nucleotides was carried out according to the diphenylamine method of Burton (1956) and the orcinol procedure of Dische and Schwarz (1937), in collaboration with Dr. P. S. Fitt, Department of Biochemistry, University of Ottawa.

RESULTS

The results of this investigation will be presented in accordance with the main lines of the methodological approaches. The results obtained by all methods are in good agreement and can be well integrated in support of the main conclusions. Not only the configuration, but also the dimensions, of the network elements were basically the same in the electron micrographs of the different preparations.

In high-resolution electron micrographs of the material from all the different preparative methods outlined above (see also Materials and Methods), the smallest filamentous unit of the axoplasmic network was constantly identified as a 20–30-A-wide filament of helical, coiled, or looped outline. Therefore it is termed in the present investigation the *unit-filament*. No well defined filamentous unit 100 A in diameter, corresponding to the neurofilament of previous investigations, could be identified in high-resolution electron micrographs of properly prepared preparations. However, in all the different types of preparations, two or more unit-filaments were associated by intercoiling into elements measuring from 70 A up to 250 A in diameter. These elements will be termed *unit-filament strands*. Furthermore, 0.1–0.3-μ-wide strands could be delineated (see Metuzals and Izzard, 1969).

Investigation of Thin Sections of Fixed Fibers

The following structural components of the axon can be identified in electron micrographs made from thin sections of squid giant nerve fibers: (*1*) the filamentous network; (*2*) the microtubules; (*3*) the mitochondria; (*4*) the agranular endoplasmic reticulum; (*5*) the plasma membrane. The

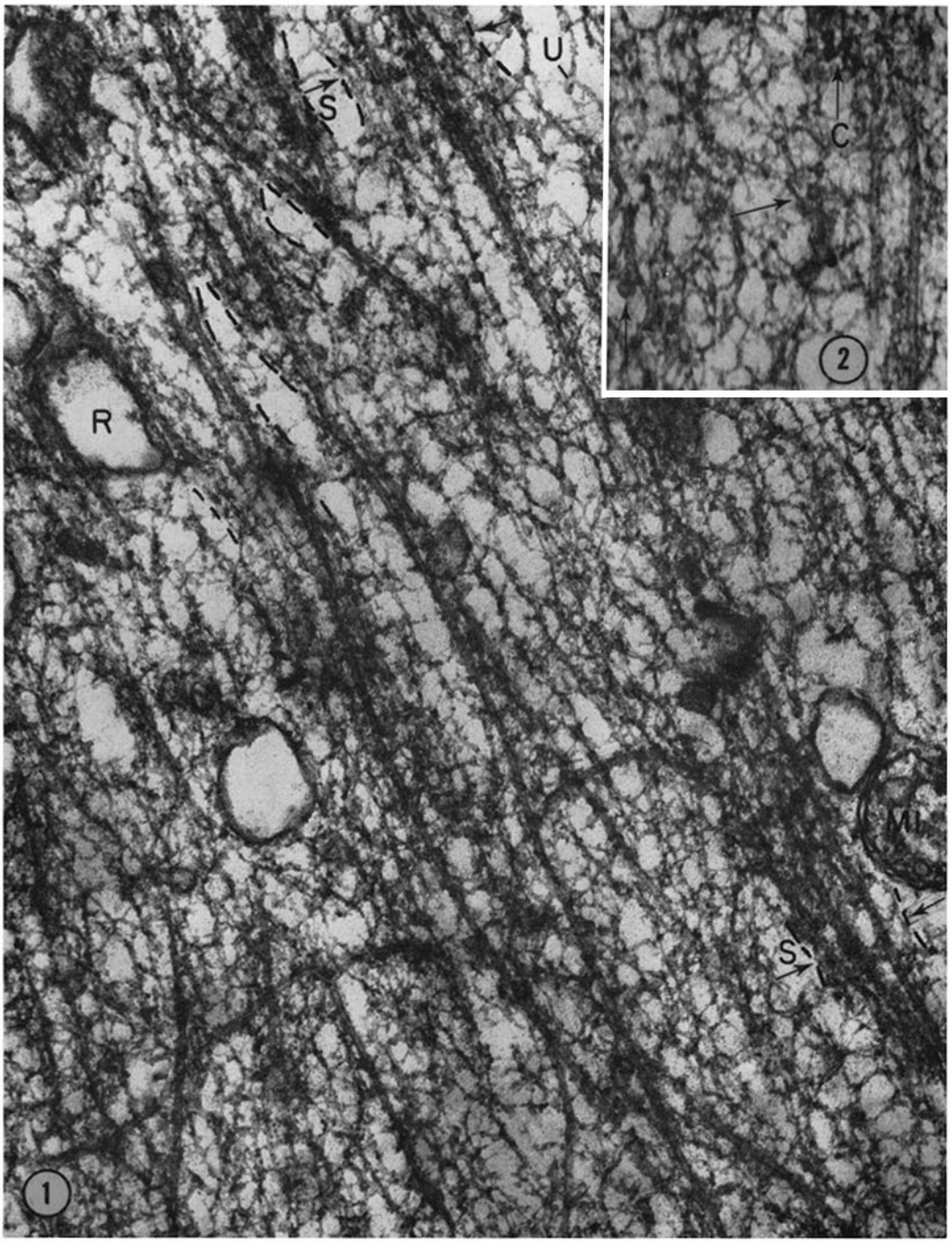


FIGURE 1 A survey electron micrograph illustrating the structural components of the ectoplasmic region of the axoplasm: profiles of the smooth-surfaced endoplasmic reticulum (*R*), mitochondrion (*MI*), microtubules, and the filamentous network. The 0.1–0.3-μ strands (*S*) are delineated by interrupted lines and indicated by opposed paired arrows; unit-filament strands (*U*). Fixed PTA/glutaraldehyde/osmium tetroxide (IVb); stained uranyl acetate and lead citrate. × 60,000.

FIGURE 2 Cross-sectioned microtubules (arrows) interwoven with filamentous elements of the axoplasmic network. At the right margin of the figure, two microtubules are surrounded by the filamentous network. *C*, a cross-bridge between two microtubules. Fixed PTA/glutaraldehyde/osmium tetroxide (IVa); stained uranyl acetate and lead citrate. × 80,000.

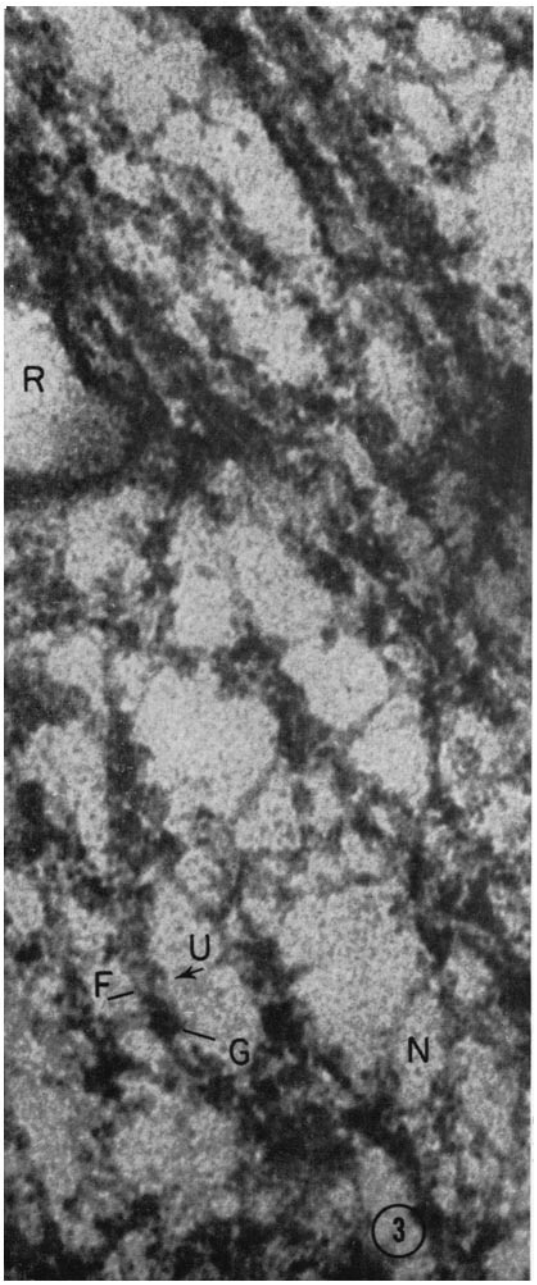


FIGURE 3 High-resolution electron micrograph of the ectoplasm. Several unit-filaments (*F*) intercoil in a unit-filament strand (*U*). A group of dense 50-A granules (*G*) are associated with a unit-filament strand; a profile of the smooth endoplasmic reticulum (*R*). *N* indicates an area in which the pattern of intercoiling and looping of the unit-filaments is loose and therefore clearly visible, displaying a continuous network configuration. In the upper right-hand portion of the figure, unit-filaments winding helically around two microtubules can be seen. Fixed PTA/glutaraldehyde/osmium tetroxide (IVb); stained uranyl acetate and lead citrate. × 200,000.

general arrangement of the axon and the surrounding sheath elements are illustrated in the survey electron micrographs (Fig. 1 of the present paper and in Fig. 20 of the preceding paper by Metuzals and Izzard, 1969).

The network was preserved best by fixation procedures IVa and b: 15-min pretreatment with 1% PTA at pH 5.4 or 8.0 followed by fixation in glutaraldehyde and osmium tetroxide solutions at +2C°. The best preservation of membranous structures was obtained by fixation in osmium tetroxide solution. Microtubules were preserved in the axoplasm by fixation procedures I, III, and IVa and b at +2C°, but not in fibers fixed in osmium tetroxide at +2C°.

A continuous network, composed of intertwined threadlike elements of different diameter and appearance, can be observed extending throughout the axoplasm (Fig. 1). A careful analysis of medium and high magnification electron micrographs indicates that the conformations of the axoplasmic structures in Figs. 1–4 represent *in toto*, a continuous three-dimensional structure. Most of the crossing points of this structure are not superimposed images, but junctions formed by twisting together of its threadlike elements. The network can be discerned in axons fixed by all the different procedures described in Materials and Methods.

In spite of the continuous character of the network formation, two hierarchic orders of associations of threadlike elements can be identified readily in medium-power micrographs (see Fig. 1), namely (*1*) the 70–250-A-wide unit-filament strands (*U*), and (*2*) the 0.1–0.3-μ-wide strands (*S*) (see also Metuzals and Izzards, 1969).

The detailed pattern of associations of the unit-filaments into the unit-filament strands and their interconnections can be revealed only in high-resolution micrographs (Figs. 3, 4). The high-resolution micrographs prove unequivocally that the threadlike elements of the axoplasm are arranged in a true network. The characteristic feature of the network is expressed at the junction of its elements. For example, where two unit-filament strands interconnect, their constituent unit-filaments regularly interchange from one

strand to the other so that the two strands are intimately joined. This feature is clearly displayed in sites in which the intercoiling and looping of the unit-filaments are loose (*N*, Fig. 3).

The 70–250-A-wide unit-filament strands consist predominantly of two or three intercoiled unit-filaments, each about 30 A in diameter. In some instances, four intercoiled unit-filaments can be identified. The variations of the diameter of the unit-filament strands can be related to coiling/decoiling and folding/unfolding of the unit-filaments. For example, an abrupt coiling and

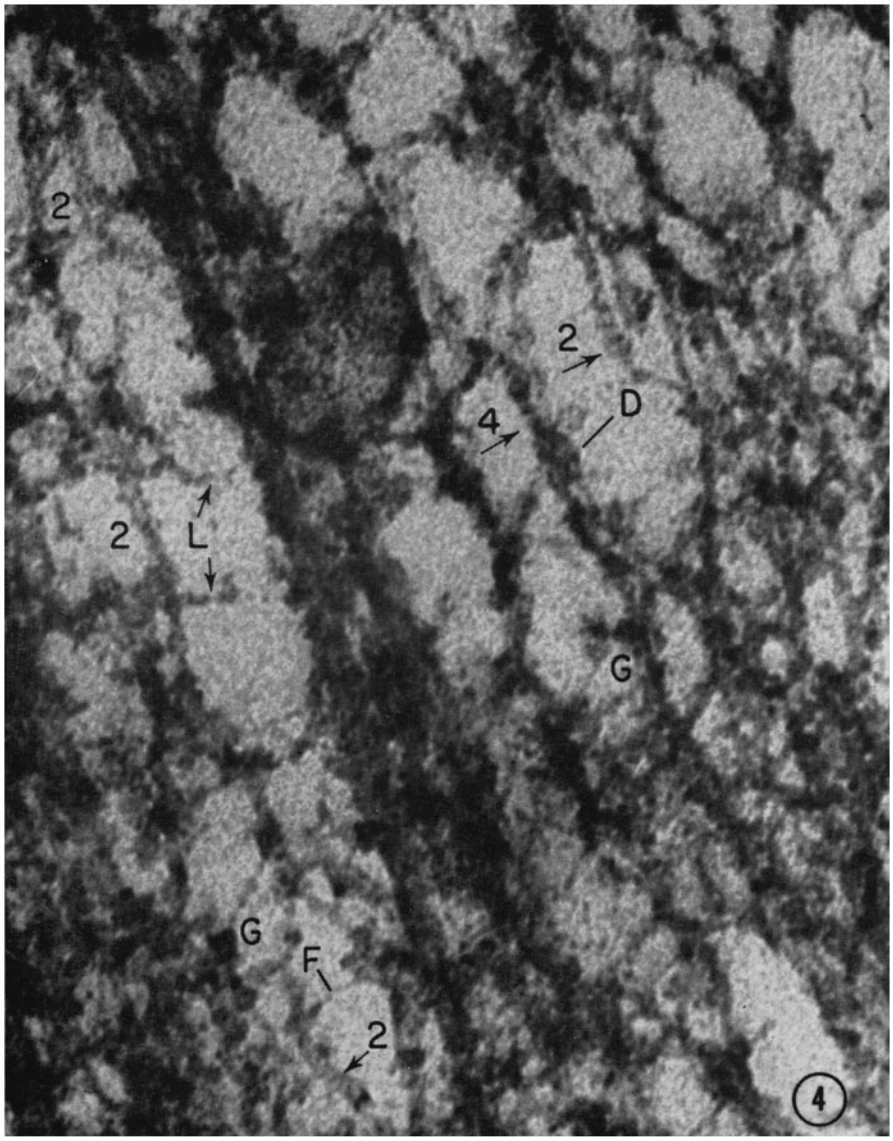


FIGURE 4 High-resolution electron micrograph illustrating the pattern of association of unit-filaments (*F*) into unit-filament strands; ladder-like associations (*L*) between unit-filament strands. *2* indicates unit-filament strands consisting of two intercoiled unit-filaments. *4*, a portion of a unit-filament strand consisting of four intercoiled unit-filaments. Single dense granules (*D*) are attached to the unit-filaments. Often such granules are aggregated in groups (*G*). The axoplasmic matrix is free of the granules. Fixed PTA/glutaraldehyde/osmium tetroxide (IVb); stained uranyl acetate and lead citrate. × 200,000.

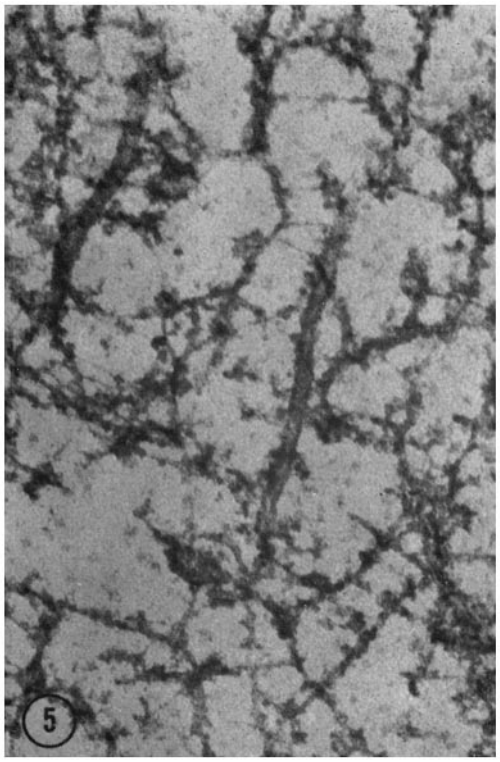


FIGURE 5 Electron micrograph of the endoplasm. Note the irregular, reticular pattern of the unit-filament strands without any predominant orientation. Both segments of the microtubules are associated closely with the unit-filament strands. Fixed PTA/glutaraldehyde/osmium tetroxide (IVa). × 80,000.

folding of the unit-filaments would cause a loosening of the strand and an increase of its diameter.

Dense granules ~50 A in diameter (*D*, Fig. 4) are attached to the unit-filaments. Often such granules are aggregated into groups (*G*, Fig. 4).

The microtubules frequently represent a structural component of the 0.1–0.3-μ strands. In high-resolution micrographs, one can regularly observe unit-filaments winding helically around microtubules and forming cross-associations between the microtubules (Figs. 2, 3).

The unit-filament strands have a predominantly longitudinal orientation in the axon and are interconnected by single unit-filaments oriented perpendicularly or obliquely to the main direction of the strands. In this way, ladder-like patterns originate (*L*, Fig. 4). However, the unit-filament strands may appear sinuous or curved and be interwoven into an irregular network without any predominant orientation.

In the endoplasm (see Metuzals and Izzard, 1969), the axoplasmic network is arranged much more loosely among the widely spaced microtubules. In this region of the axon, an open reticular array of the unit-filaments and the unit-filament strands predominates (Fig. 5), such that in certain areas of the endoplasm no predominant orientation of the threadlike elements can be discerned.

Numerous mitochondria can be observed regularly in thin sections. Their size and their structure vary considerably. The smallest profiles of mitochondria measure about 0.2 μ in diameter and have few tubular cristae (see Metuzals and Izzard, 1969, Fig. 20). Profiles of mitochondria measuring up to 2 μ can be observed. In these larger profiles, numerous tubular cristae are densely packed in the interior of the mitochondrion (see Figs. 6, 7).

Membrane-bounded profiles of irregular form and size, and corresponding to the components of the agranular endoplasmic reticulum, represent a constant structure of the axoplasm. The variability of size and form of the profiles may represent different states of activity of the agranular reticulum and may not entirely be due to the different procedures of fixation. The profiles are often arranged in rows extending in the longitudinal direction of the axoplasm and displaying the same appearance as the agranular reticulum observed by a number of authors in the axons of vertebrate nerve fibers.

Another component of the axoplasm is represented by large bodies measuring up to 4 μ in diameter and 12 μ in length. These bodies consist of membrane-bounded profiles of variable size and form, which are densely packed. The bodies are not separated from the surrounding axoplasmic matrix at all sites by a continuous membrane (see Fig. 7). Often mitochondria are closely associated with the bodies. From the available observations, the exact nature of the large bodies cannot be decided with certainty. Tentatively, however, it is proposed that these structures represent special differentiations of the agranular endoplasmic reticulum (see Fawcett, 1966).

Negative Staining

In preparations of fresh axoplasm, extruded in 1% PTA solutions, the structural patterns and

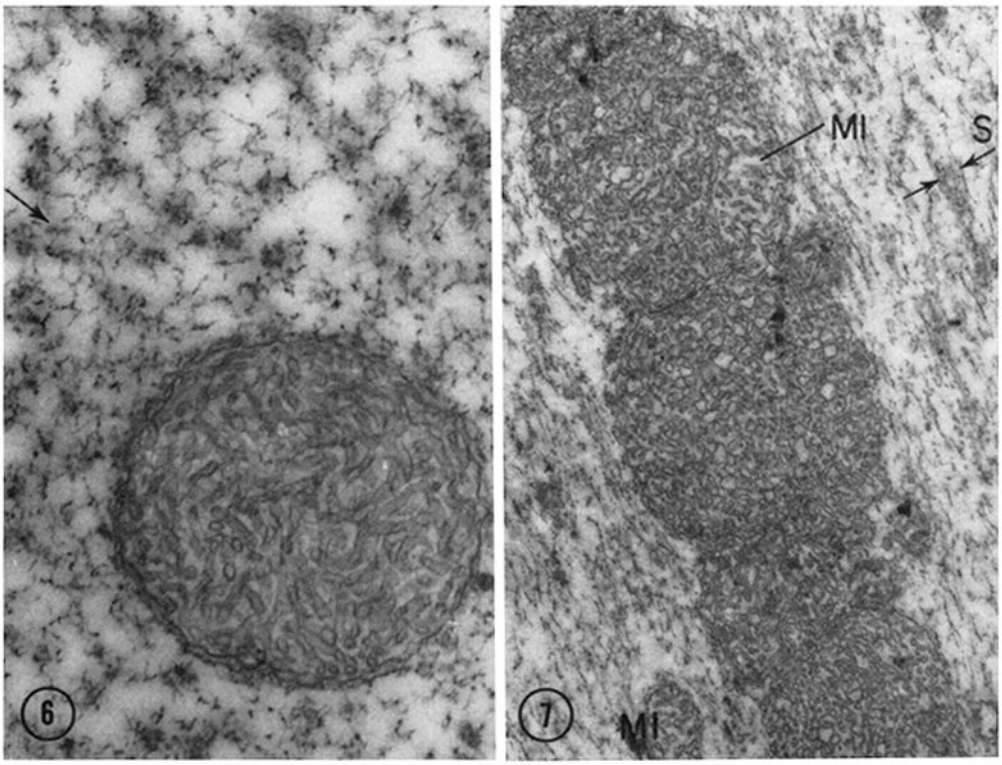


FIGURE 6 Electron micrograph of an oblique section of the axoplasm illustrating an area of the ectoplasm close to the endoplasm. A mitochondrion about 2 μ in diameter and unit-filament strands can be seen. The arrow indicates unit-filament strands interwoven in a network. Fixed osmium tetroxide (II); stained uranyl acetate and lead citrate. × 25,000.

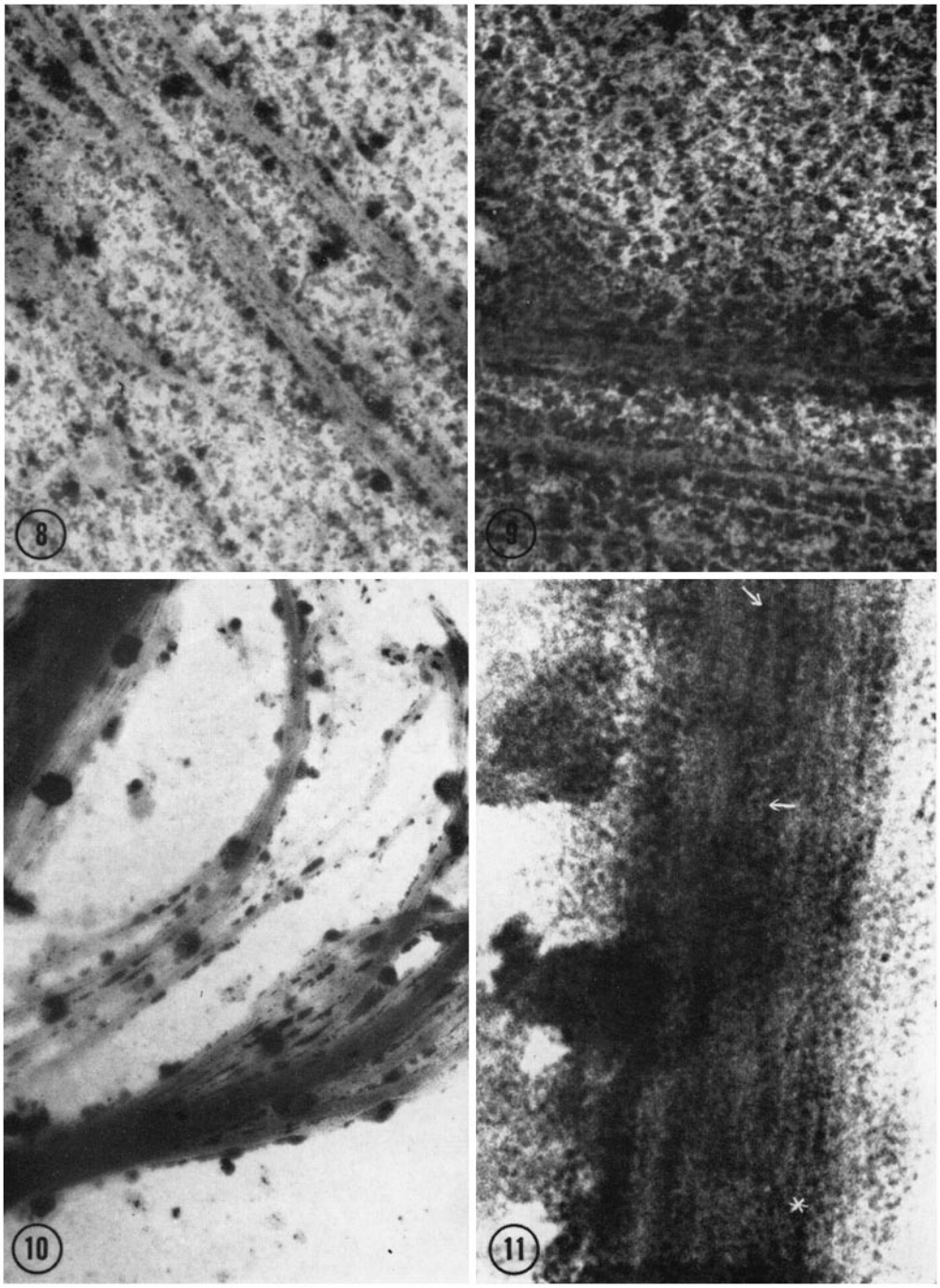
FIGURE 7 Electron micrograph of a longitudinal section of the ectoplasm. A body, consisting of aggregates of tubular elements, is closely associated with a typical mitochondrion (*MI*) and surrounded by threadlike elements. The body is not at all sites surrounded by a continuous membrane. Opposed arrows indicate a 0.3-μ-wide strand (*S*). Fixed osmium tetroxide (II); stained uranyl acetate and lead citrate. × 12,500.

FIGURE 8 Electron micrograph of a negatively stained preparation of the axoplasm extruded into 1% PTA, pH 8.0. Straight, 0.1–0.3-μ-wide strands are separated by areas of irregular, precipitate-like appearance. Because of light staining, no detailed structure of the strands or of the areas can be resolved. × 24,000.

FIGURE 9 The same preparation as Fig. 8, illustrating an intensely stained area. A continuous network formation consisting of irregularly interwoven threadlike elements can be discerned. At the lower margin of the figure straight strands can be observed. × 36,000.

FIGURE 10 Survey electron micrograph of a negatively stained preparation of the axoplasm extruded into 0.5% PTA, pH 7.2. Strands having smooth outline and diameter of 0.1–0.3 μ are joined into larger bundles. × 12,500.

FIGURE 11 High-resolution electron micrograph of a negatively stained preparation of the axoplasm extruded into 1% PTA, pH 7.2. Arrows indicate sites in which the intercoiling and looping of the unit-filaments into unit-filament strands is clearly displayed. Asterisk indicates an area in which the continuous network character of the unit-filaments predominates. × 250,000.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their dimensions are essentially the same as disclosed from thin sections. Such preparations can be obtained repeatedly, in spite of the unavoidable necessity of partially disrupting the structures during the preparation for obtaining an adequate cover and contrast over the grid. No essential differences in the filamentous structural patterns and in their dimensions can be discerned between

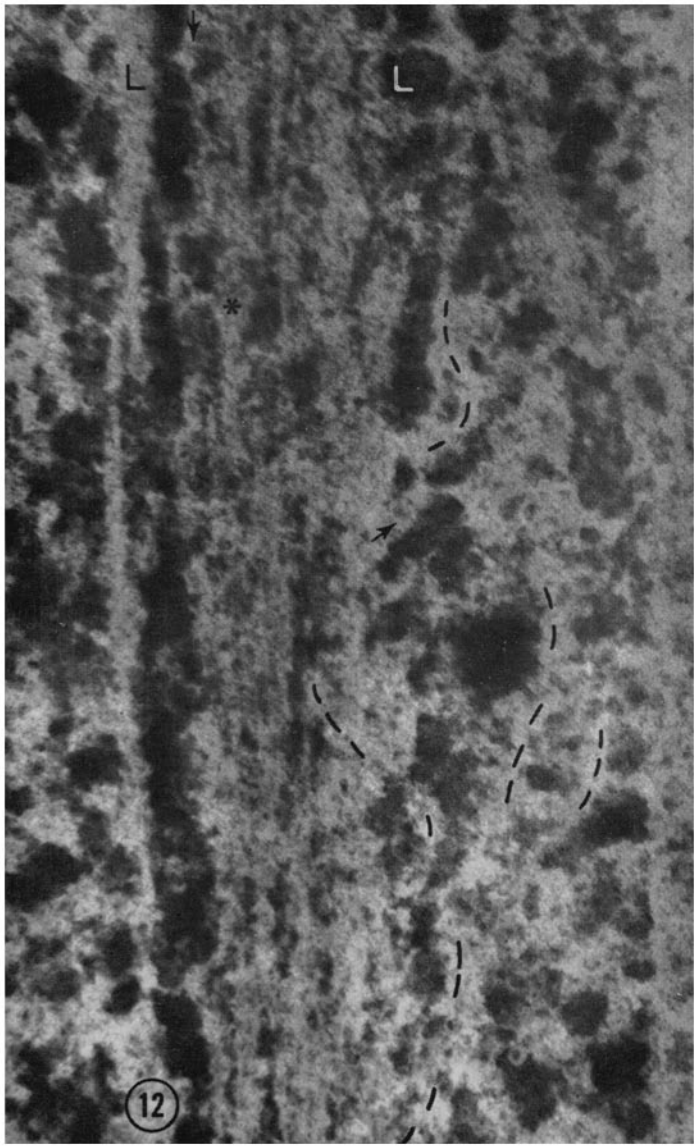


FIGURE 12 Electron micrograph of a negatively stained preparation of the axoplasm extruded into 1% PTA, pH 8.0. The continuous character of the threadlike elements, associated by intercoiling and interlooping into entities of larger size, is well illustrated. Vertical arrow at the upper margin of the figure indicates a well defined unit-filament strand displaying intercoiling and interlooping finer elements. Asterisk, a screw-like branch of the strand. Cross-bridge associations forming ladder-like patterns (*L*) among the threadlike elements can be seen. Arrow in the central portion of the figure indicates intercoiling and separation of two unit-filament strands. Sinuous paths are indicated by interrupted lines in areas of the network in which detailed structures are not revealed. × 80,000.

the preparations stained in PTA solutions of pH 7.2 or 8.0. In survey, and medium magnification, electron micrographs show aggregates of different size and consisting of threadlike elements, which represent a characteristic feature (Figs. 8–10). Often 0.1–0.3-μ-wide strands are separated by areas in which a reticular configuration of smaller threadlike elements predominates (Fig. 8). In intensely stained areas, a continuous network of distinct pattern can be discerned consisting of 100–200-A-wide, interwoven elements (Fig. 9).

As in thin sections, intercoiling and interlooping of threadlike elements of a smaller diameter into structures of a larger diameter, and cross-associations between these elements, are characteristic features of the negatively stained preparations (Fig. 12). In high-resolution micrographs (Fig. 11) unit-filaments 30 A in diameter displaying a sinuous, helical, or looped appearance can be resolved. The unit-filaments are intertwined in strands of 70–250 A diameter.

CALCIUM EFFECT: Calcium ions represent a causal factor determining the gel-state properties of the axoplasm and the properties of the excitable membrane. As described by Bear et al. (1937) and Hodgkin and Katz (1949), extruded axoplasm disperses rapidly in calcium-containing solutions but not in solutions free of calcium ions. Therefore, electron microscopy of negatively stained preparations of axoplasm which has been extruded into molluscan physiological saline containing calcium ions, provides important data for the understanding of the calcium effect on the nerve fiber. Besides that, it also gives important clues for the elucidation of the morphology of the axoplasm.

In survey electron micrographs of such preparations, granular filamentous material covers uniformly large areas of the grids (Fig. 13). The material is distributed in irregular reticular patterns or is aggregated in globules of different size. Profiles of microtubules are interwoven in this material. Straight or sharply bent microtubules may extend for relatively large distances on the grid. Several short microtubular segments are often aggregated in groups.

High-resolution electron micrographs (Fig. 14) of such preparations reveal the filamentous axoplasmic network in a dispersed form which, nevertheless, maintains intact the basic elements and patterns of the network. Threadlike elements of varying diameter and form, corresponding to unit-filament strands, can be identified. These

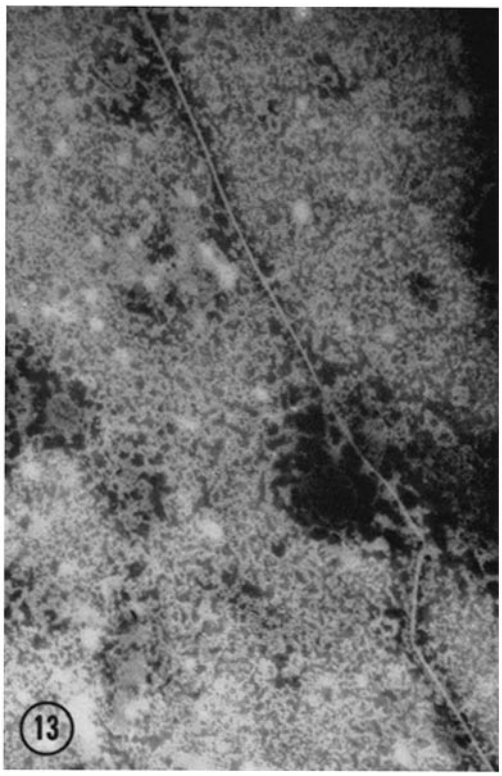


FIGURE 13 Survey electron micrograph of fresh axoplasm extruded into molluscan physiological saline containing calcium ions. The grid was stained negatively with 1% PTA, pH 7.2. For details, see the text. × 26,000.

elements consist of straight or sometimes also coiled, 30-A unit-filaments and of additional granular material. The strands and the unit-filaments are associated in a loose, yet continuous network and are also wound around the microtubules. The large globules are composed of densely interwoven unit-filaments.

Isolation Experiments

If a 2-cm portion of the giant nerve fiber is dissected free, removed from the mantle, and placed in 5–10 drops of 1% uranyl acetate solution at pH 4.4, a thread segregates in the axoplasm. In the light microscope, the axoplasmic thread can be readily recognized: it lies separated from the sheath and surrounded by an abundant flocculent precipitate (Fig. 15). During the segregation period, the thread displaces, coils, and forms bulbous enlargements outside the two cut ends of the

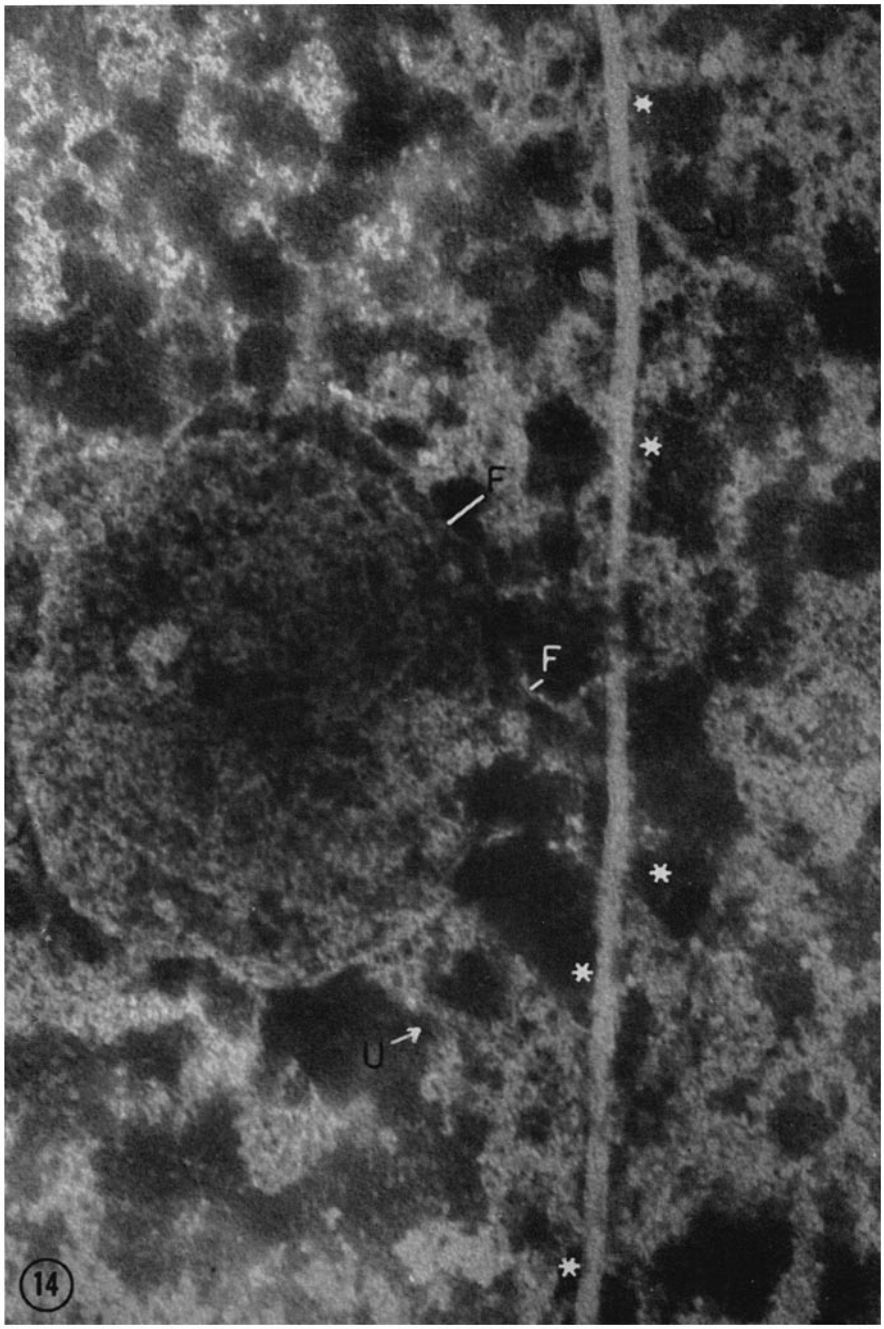


FIGURE 14 High-resolution electron micrograph of the same preparation as Fig. 13. Dispersed filamentous network elements and a microtubule are displayed. The basic structural components of the network—the 30-A unit-filaments (*F*), as well as their intercoiling pattern into unit-filament strands (*U*)—can be discerned. For details, see the text. The unit-filaments and the unit-filament strands are wound around the microtubule (See asterisks). × 150,000.

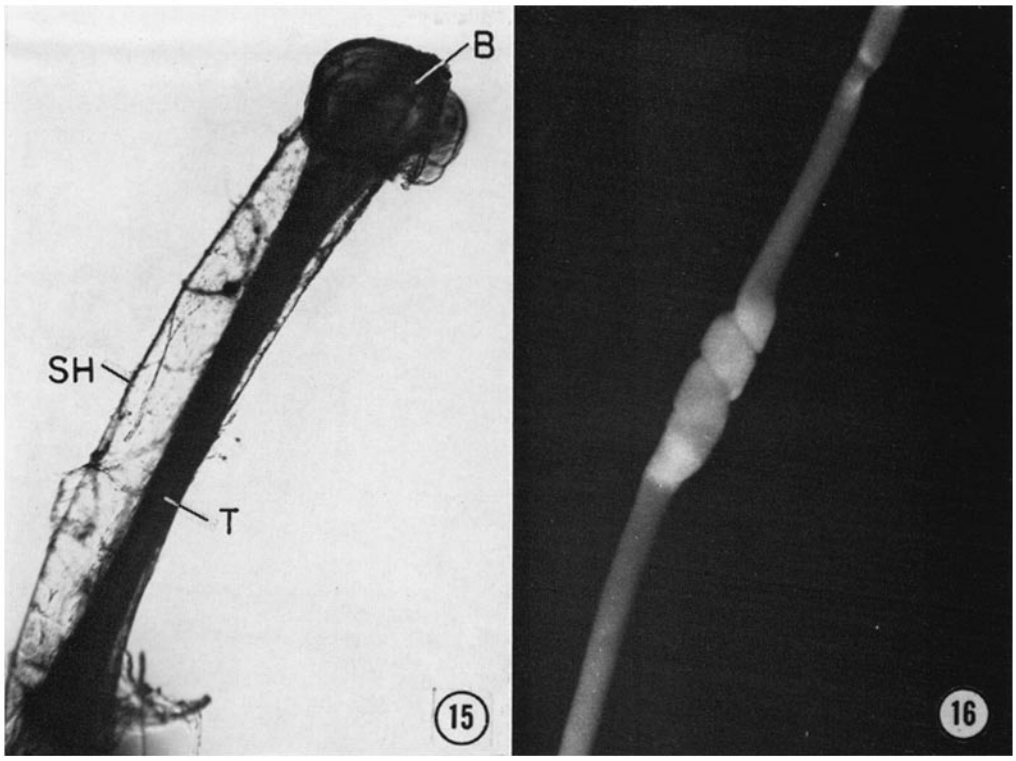


FIGURE 15 A portion of the giant nerve fiber dissected free, removed from the mantle, and placed for 30 min in 1% uranyl acetate solution, pH 4.4. An axoplasmic thread (*T*) has segregated and is separated from the sheath (*SH*). Bulbous enlargements (*B*) extend outside the cut ends of the sheath tube. For the photomicrography, the preparation was placed in the concavity of a microslide containing distilled water, after having been washed in several changes of distilled water. Coverslip was not used. × 25.

FIGURE 16 Thread preparation segregated from the axoplasm by the uranyl procedure. The thread was removed from the sheath after being exposed to 1% uranyl acetate solution for several hours. Note the distinctly coiled portion of the thread. The surface of the rest of the thread is smooth. For the photomicrography, the preparation was placed in the concavity of a microslide containing distilled water, after having been washed in several changes of distilled water. Coverslip was not used. The photomicrograph was taken through a Leitz binocular dissecting microscope, the microslide having been placed on black glass and illuminated obliquely from above. × 23.

sheath. After about 20 min, the thread can be pulled out from the sheath easily. The bulbous enlargements separate readily from the straight thread. Portions of such threads may coil extensively (Fig. 16). Thread preparations often display on their surface continuous coiled grooves and show in the light microscope a conspicuous fibrillation.

At pH 7.2 and 8.0, the segregation does not occur and no precipitate develops in 1% uranyl acetate solution. At pH 3.0 and 2.0, the thread segregation and the development of the precipitate in 1% uranyl acetate is considerably less pronounced than that in uranyl acetate solution at pH 4.4. The same is true when concentrations of the uranyl acetate less than 0.5% are used. Slight segregation of a thread, and the formation of bulbous enlargements and of some precipitate, occur in 1% $AgNO_3$ solution at pH 7.0.

No segregation and precipitate formation can be observed when the axoplasm is exposed to 1% solutions of Cd, Fe, Zn, Co, Pb acetates at pH 4.4, or to 1% solutions of $HgSO_4$ and $LaCl_3$ at pH 4.4. In all of these solutions, the axoplasm only becomes more or less opaque without retracting from the sheath and without forming any bulbous en-

largements at the ends of the preparation. The behavior of the axoplasm is the same in 1% PTA solutions of pH ranging from 5.2 to 8.0, and in 0.3% acetic acid.

ELECTRON MICROSCOPY OF THREAD PREPARATIONS: Thread preparations of the axoplasm, obtained by the uranyl procedure, have been dehydrated and embedded in Vestopal W. Electron micrographs of thin sections of such preparations have an exceptional sharpness and high contrast and, accordingly, the preparations are very suitable for high-resolution microscopy. In these micrographs, all the basic structural features of the axoplasmic network can be identified that have been revealed from thin sections of fixed fibers and from negatively stained preparations. Thus,

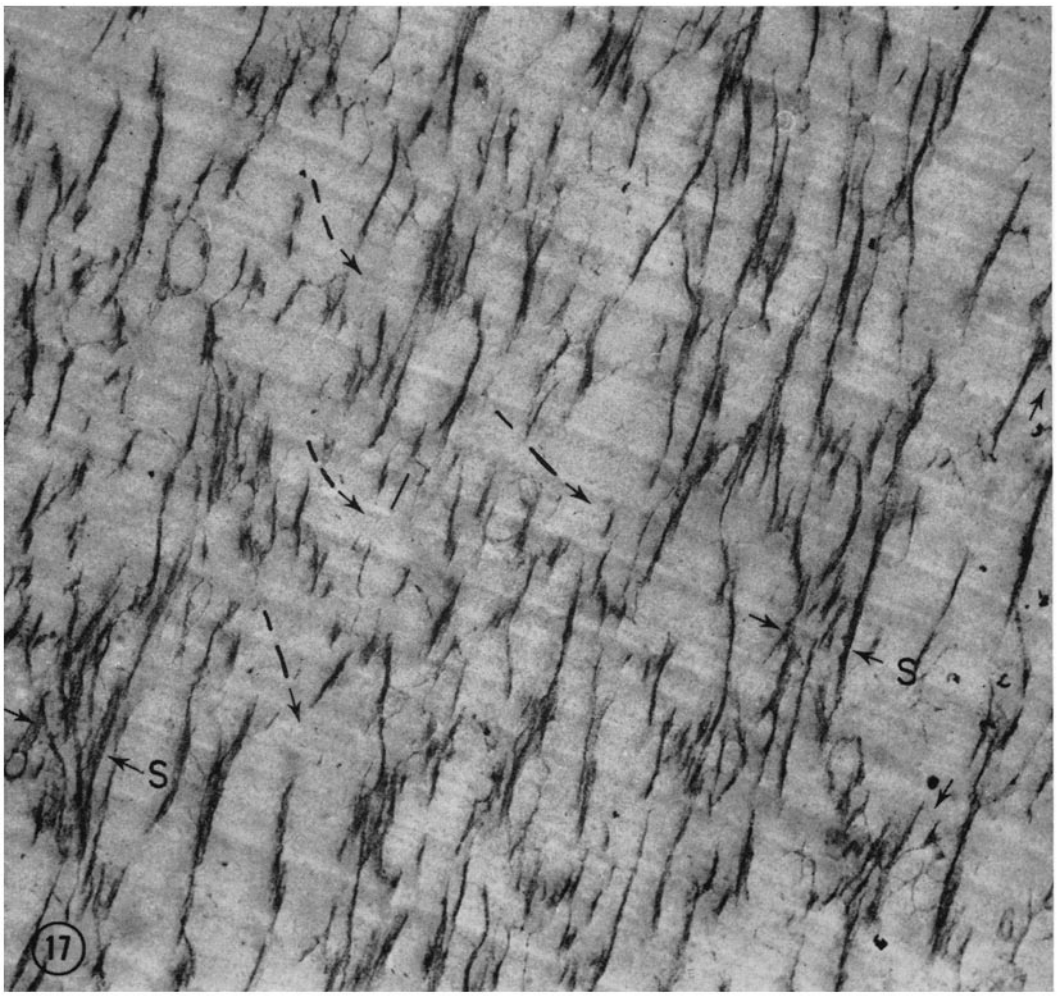


FIGURE 17 Survey electron micrograph of a thread preparation segregated from the axoplasm by the uranyl procedure. The preparation was embedded in Vestopal W without further fixation. The section has been stained with lead citrate after Reynolds. The figure demonstrates the continuous network composed of intercoiling threadlike elements of different diameter. Threadlike elements measuring from 200 up to 500 A in diameter and corresponding to single or groups of unit-filament strands can be identified. These elements often appear aggregated in 0.2–0.5-μ-wide strands (opposed arrows, *S*). Cross-associations (arrows) between the strands can be constantly observed. The threadlike elements are curved and do not extend continuously over the whole figure. Structureless zones (interrupted arrows) extend obliquely throughout the figure and divide the threads into narrow segments. × 22,500.

threadlike elements having the same morphology and dimensions as those described in previous sections can be identified also in Figs. 17–19. The helical configuration of the thread-like elements, as well as their patterns of associations into network formations, have been preserved. For example, in Fig. 18, the 0.1–0.3-μ strands (*S*) can be delineated for a short distance, and the strands are interconnected with each other. Narrow, structureless zones (interrupted arrows, Fig. 18) extend over the micrographs, producing a characteristic segmental pattern of the axoplasmic structure (see Fig. 21, Metuzals and Izzard, 1969). This pattern probably results from the sectioning of helical strands.

In high-resolution micrographs of the 0.1–0.3-μ-wide strands in the thread preparations (Figs. 18, 19), unit-filament strands and unit-filaments can be identified easily. In such micrographs, especially the pattern of intercoiling of unit-filaments into strands is displayed: two, three, or more ~30-A-wide unit-filaments are intercoiled in strands measuring from 70 up to 500 A in diameter. Dense granules about 50 A in diameter are attached to the unit-filaments. No other structural components, such as microtubules or fragments of membranes and mitochondria, can be found in the straight thread preparations (Figs. 19, 17).

ELECTRON MICROSCOPY OF BULBOUS ENLARGEMENTS: The bulbous enlargements (*B*, Fig. 15) formed outside the cut ends of the tubular sheath, during the segregation of the thread in 1% uranyl acetate at pH 4.4, have been dehydrated and embedded in Vestopal W. Survey electron micrographs of thin sections of such preparations show a dense outer layer and less dense core. The main structural components are microtubules surrounded by a dense filamentous network. Furthermore, large vacuoles surrounded by a membrane and fragments of mitochondria can be found (Figs. 20, 21). The surface of the bulbous enlargements is convoluted and, therefore, the main orientation of the elements in the outer layer of the bulbs differ considerably.

Macroscopically, the bulbous enlargements are intensely stained—yellow in uranyl acetate and brown in silver nitrate—in contrast to the thread preparations which have a whitish appearance.

ANALYTICAL DATA: The amino acid composition of pure thread preparations, obtained by the treatment with 1% uranyl acetate at pH 4.4, is given in Table I. The bulbous enlargements have not been included in the analysis.

The elementary analysis data of the preparations segregated from the axoplasm by treatment in 1% uranyl acetate at pH 4.4, are presented in Table II.

The thread preparation, including some bulbous enlargements, were dried on filter paper, weighed, and extracted with 7% (v/v) perchloric acid at 90°C for 20 min. The extract was assayed for deoxynucleotides by the diphenylamine method (Burton, 1956) and for ribonucleotides by the orcinol procedure (Dische and Schwarz, 1937). No detectable deoxynucleotides were present, but the orcinol reaction showed that the preparations contain 1–2% by weight of ribonucleic acid or ribonucleotides. Insufficient material was available for a complete separation to be carried out (e.g., by the Schneider procedure), so it is not yet certain what proportion of the orcinol-positive material was present as ribonucleic acid.

THE FLOCCULENT PRECIPITATE: As mentioned at the beginning of this section, an abundant flocculent precipitate develops during the segregation of the thread from the axoplasm in 1% uranyl acetate at pH 4.4. In the light microscope under oil immersion, fresh preparations of the precipitate appear to consist of filamentous granular material.

Negatively stained preparations have been prepared from the precipitate in the usual way. High-resolution electron micrographs of such preparations show that the precipitate consists of a dense, irregularly interwoven matrix of filaments. The filaments appear beaded or smooth, and their diameter measures about 20 A or less (Fig. 22). Their appearance is completely different from that of the unit-filaments in the thread preparations (see Fig. 18).

Comparison of the Observations Based on Different Methods of Preparation

Structural entities of the same patterns and dimensions exist in all the preparations obtained by the different methods used in this investigation. This conclusion can be justified by comparing the corresponding structures demonstrated in Figs. 18, 23–27. The dimensions of the unit-filaments and of the unit-filament strands are of the same order and their arrangement is the same in axons treated either: (*a*) *in toto* by procedure *IVa* (Fig. 23), or (*b*) fixed in osmium tetroxide (Figs. 25, 27),

or (*c*) in negatively stained fresh preparations (Figs. 24, 26), or finally (*d*) in thread preparations (Fig. 18).

DISCUSSION

Electron microscopy has always had the need to approach two frontiers of biological research. One of these frontiers is represented by investigations of structural configurations in the living, undissected organism. The other frontier is characterized by the analysis of the structure of organisms in terms of macromolecular components. Recent developments of new instruments and methods provide new opportunities to expand electron microscopic research closer to these two frontiers. It appears that the axoplasm of the squid giant nerve fiber provides a very suitable material for the successful extension of research towards the two frontiers mentioned above. Thus, Metuzals and Izzard (1969) have integrated the data obtained by electron microscopy of fixed squid giant nerve fibers with the data obtained by observations of living fibers. In the present paper, an attempt has been made to correlate high-resolution electron microscopy of the axoplasm of the squid giant fiber with physicochemical methods in order to approach the second frontier.

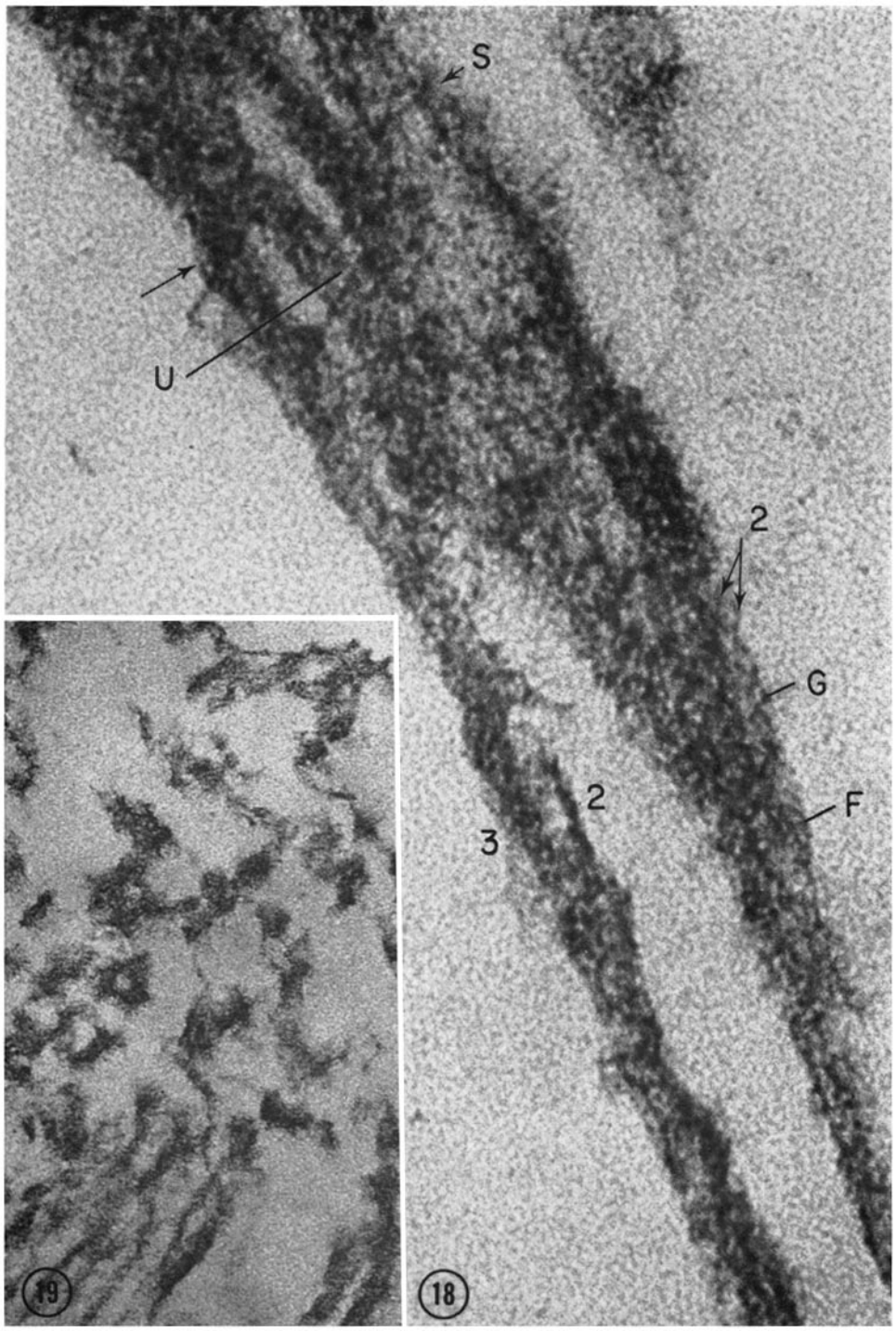
In survey and medium power electron micrographs of the axoplasm of the squid giant nerve fiber, the diameter of the well defined threadlike elements, the unit-filament strands, varies from 70 up to 250 A. The entity unit-filament strand corresponds to the neurofilament of previous investigations. In previous reports, the same order of variations of the diameter of neurofilaments has been reported. Thus, according to Schmitt and Geren (1950), the filamentous material of the axon, fixed in formalin or osmium tetroxide, occurs in electron micrographs of thin sections as beaded filaments ranging in diameter from 100 to about 200 A. Villegas and Villegas (1960 *a* and *b*) comment briefly that the axoplasm of the giant fibers of the tropic squid *Doryteuthis plei* consists of 50–70-A-wide filaments, running in all directions but predominantly longitudinally.

The first investigations of the squid giant nerve fibers with the electron microscope were carried out on freshly extruded axoplasmic filaments (see Richards, Steinbach, and Anderson, 1943). The pioneering observations of these authors can be reconciled in many respects with the results of the present investigation. Thus, Richards et al. (1943), using smear preparations of pure axoplasm, were able to demonstrate regular particles and fibrils. In smears, fibrils having diameters of 150–200 A and of 500 A could be recognized. The thin fibrils appear as zigzag lines, while the thick fibrils indicate an internal spiral. The fibrils can be associated in a network formation. Also, the investigations of Schmitt (1950), using fragmented axons fixed in formalin, can be correlated with the results of the present investigation. Thus, the filaments appear nodose, having widths ranging between 75 and 200 A. Frequently, the filaments have a contorted appearance resembling a linked chain, a loosely wound helix, or a zigzag arrangement. In preparations of axoplasm dispersed in distilled water, the filaments are interwoven to form a mat.

Another important aspect resulting from the

FIGURE 18 High-resolution electron micrograph of a longitudinal section of a thread preparation. Method of preparation the same as in Fig. 17. The following hierarchic levels of the threadlike elements can be discerned: (a) 0.14-μ-wide strand (*S*, opposed arrows) consisting of (b) 70–500-A-wide unit-filament strands (*U*), and (c) ~30-A unit-filaments (*F*) with dense granules attached (*G*). *2* indicates a unit-filament strand consisting of two intercoiled unit-filaments. *3* indicates a unit-filament strand consisting of three intercoiled unit-filaments. × 280,000.

FIGURE 19 High-resolution electron micrograph of a cross-section from a thread preparation. Method of preparation the same as in Fig. 17. No microtubules or nonfilamentous structures of the axoplasm can be identified (cf. Fig. 21). Note the irregular profiles of the cross-sectioned 0.1-μ-wide strands and their cross-connections into a formation of a continuous character. In the lower left corner, the strands are sectioned longitudinally. × 120,000.


S
U
2
G
2
F
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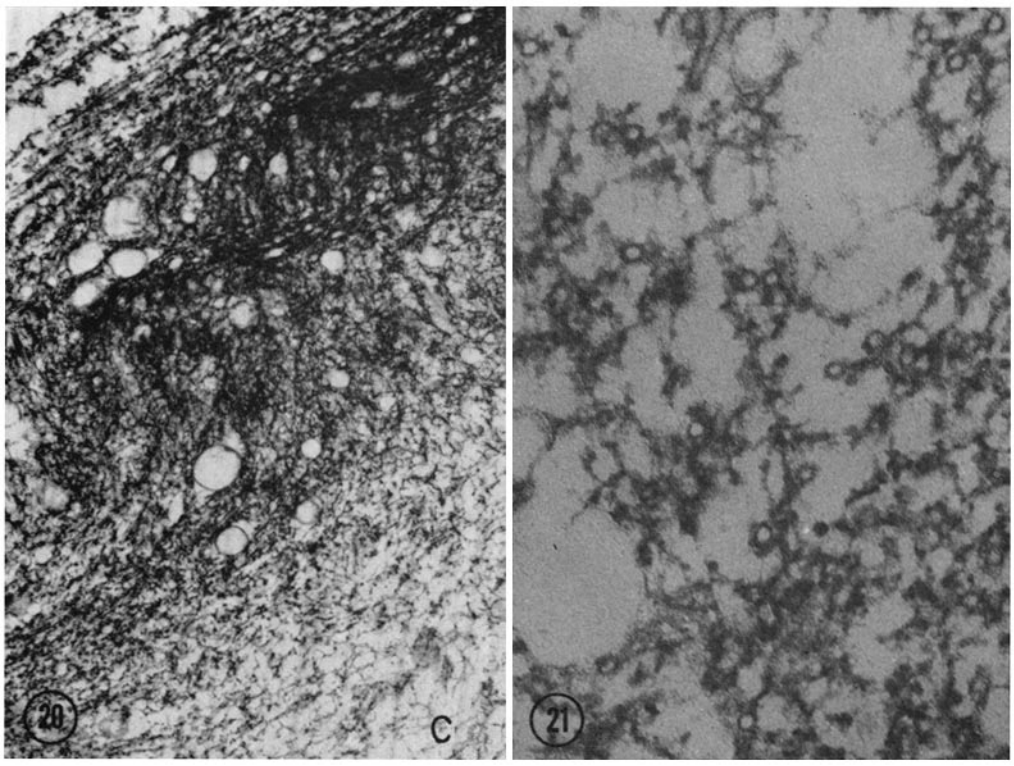
19
18



FIGURES 20 and 21 Electron micrographs of a bulbous enlargement (cf. *B* in Fig. 15). Method of preparation the same as in Fig. 17. Fig. 20, note the dense outer layer of the enlargement consisting of a convoluted filamentous network and large vacuoles surrounded by membranes; the less dense core (*C*) of the bulb. × 11,200. Fig. 21, microtubules surrounded by a filamentous network (cf. Fig. 19). × 120,000.

present investigation is the resolution of unit-filament strands in smaller threadlike entities about 30 A in diameter—the unit-filaments. Already Davison and Taylor (1960) have proposed that the neurofilament may consist of smaller subunits. These authors investigated the filaments isolated from the axoplasm of *Dosidicus gigas*. From physicochemical experiments, the authors propose that the intact filament may contain one or several continuous protein backbones or protofibrils. These protofibrils are constrained into a 50–80-A-wide helix. Dissociation would involve the breakdown of the helical organization, allowing the protofibrils to separate and assume a less extended form. According to Maxfield and Hartley (1957), the 70–200-A-wide neurofilaments, isolated from the axoplasm of the squid giant nerve fiber, split laterally into thinner filaments under the influence of changes of pH between 6.0 and 7.7. This process is reversible.

As described in detail in the Results of the present paper, the unit-filaments and the unit-filament strands are wound helically around the microtubules. The microtubules represent an integral part of the filamentous space-network configuration of the axoplasm. In this connection, the electron microscopic investigations of cilia and flagella should be mentioned (Gibbons and Grimstone, 1960; Gibbons, 1961): the so called central sheath of these structures is composed of one or two filaments about 45 A wide and coiled around the pair of central microtubules. If Fig. 3 of the present paper is compared with Fig. 28 of the paper of Gibbons and Grimstone (1960), the similarity of the morphological constructions in both figures is obvious. Several authors have described cross-bars among the microtubules. The question arises whether in such instances components of a continuous filamentous network have remained properly fixed only among the microtubules (cf. Fig. 5 in the paper of Palay et al.,

TABLE I

Amino Acid Composition of the Axoplasmic Thread Prepared by the Uranyl Acetate Procedure*

Alanine	9.0	Lysine	6.4
Arginine	3.5	Methionine	2.5
Aspartic acid	11.0	Phenylalanine	3.5
Cysteine	0.8	Proline	5.7
Glutamic acid	14.4	Serine‡	9.6
Glycine	10.8	Threonine§	5.7
Histidine	1.5	Tyrosine§	2.5
Isoleucine	2.5	Valine	3.9
Leucine	6.8		

* Expressed as moles per cent. An 0.81-mg sample was hydrolyzed in 6 N HCl in a sealed tube at 110° for 20 hr. Amino acids were determined with an amino acid analyzer by the procedure of Spackman, Stein, and Moore (1958); see also Moore and Stein, 1963. Tryptophan was not determined.
‡ Corrected for 10% destruction.
§ Corrected for 5% destruction.

TABLE II

Elementary Analysis Data of the Axoplasmic Preparation Prepared by the Uranyl Acetate Procedure*

Elements	%	Methods used
N	10.8	Dumas combustion‡
U	18.8	Fluorimetry
P	4.6	Colorimetry
Ca	0.6	Atomic absorption spectrophotometry
Mg	0.08	Atomic absorption spectrophotometry

* Samples dried to constant weight at 100°C in platinum crucible.
‡ 0.301 mg of ash remained from a 0.860-mg sample.

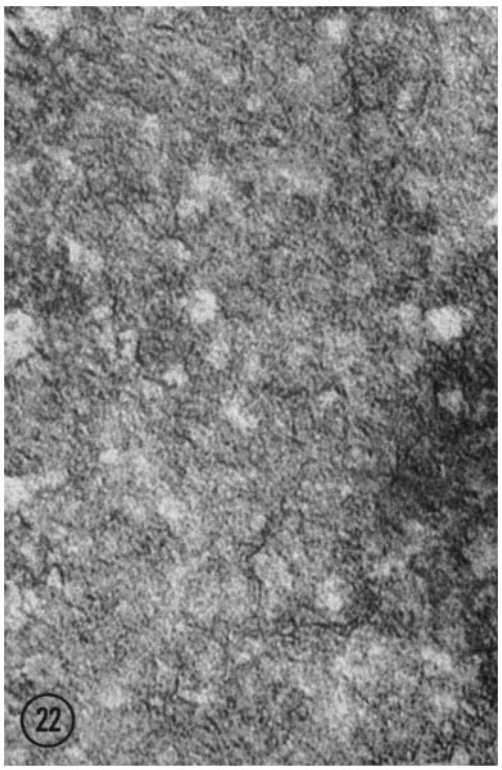
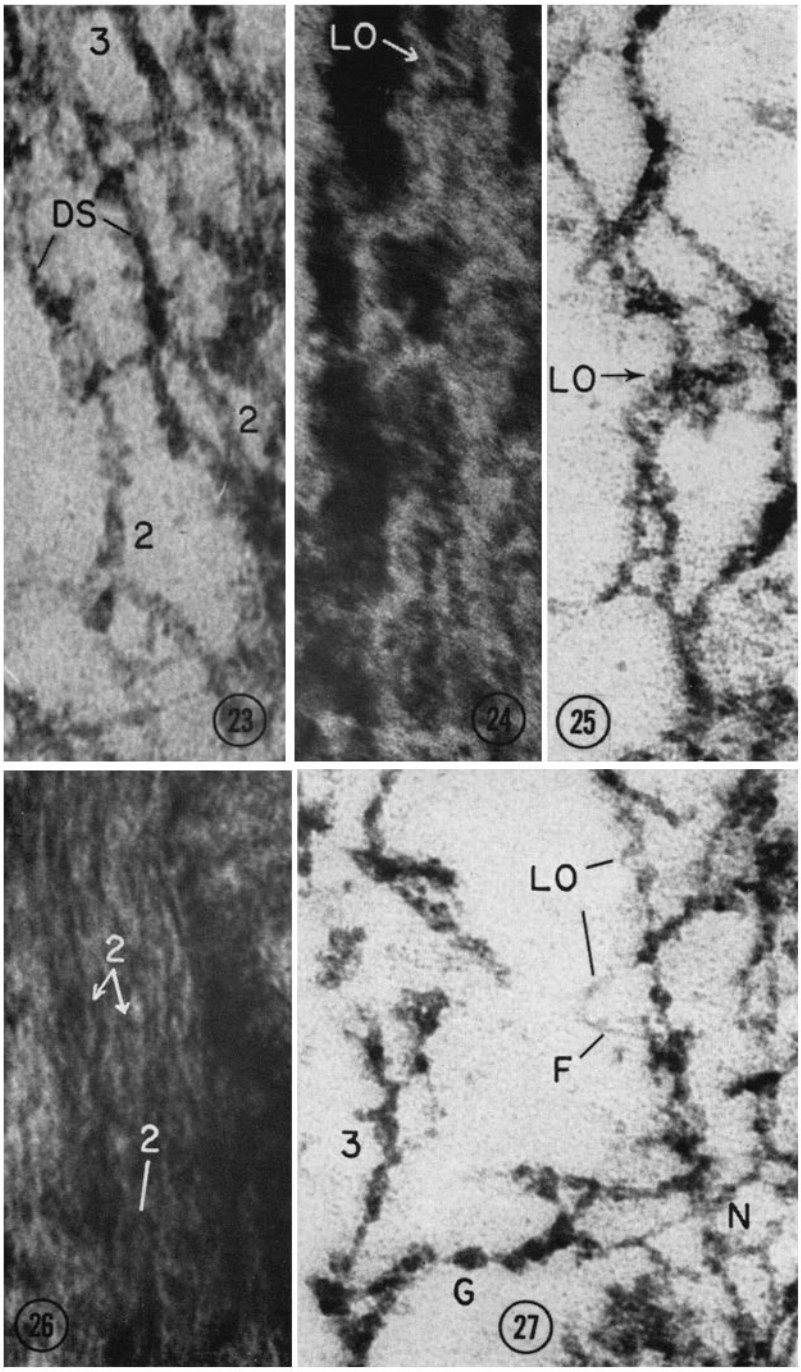


FIGURE 22 Electron micrograph of the fluocculent material precipitated from the axoplasm during thread formation in 1% uranyl acetate at pH 4.4. Note the dense irregular texture consisting of smooth and beaded filaments measuring ~20 A or less in diameter. Negatively stained preparation. × 203,000.

1968, with Figs. 2 and 3 of the present investigation).

The microtubules are not preserved in the axoplasm fixed in osmium tetroxide at +2°C, but the filamentous network is excellently preserved in these preparations. This observation indicates that the properties of the proteins composing the microtubules and the filamentous network are different in respect to their response to osmium tetroxide fixation. Ions of other heavy metals, such as those of uranyl acetate and phosphotungstic acid, exert a stabilizing effect on the filamentous network elements as well as on the microtubules. Independent from differences of pH and temperature, solutions containing these ions preserve the threadlike elements of the axoplasm very satisfactorily. The PTA anion measures about 10 A in diameter, and it could fit easily into the meshwork of the protein framework and achieve stabilization (Huckel, 1950; Kühn et al., 1958; van Bruggen, 1962). Therefore, it is understandable that no significant differences can be observed between the preparations stained negatively with PTA at pH 7.2 and 8.0. The cytoplasmic pH of the squid giant nerve fiber is 7.35 (±0.1) (see Spyropoulous, 1960).

Certain heavy metal ions show specificity for reactive groups of protein molecules and can be incorporated into the molecular framework of a protein without affecting its arrangement. By virtue of such a specificity, the proper choice of metals can be very useful in fractionation, crys-



FIGURES 23–27 Comparison of the 30-A unit-filaments and the unit-filament strands in preparations of the axoplasm prepared by different preparative methods. The corresponding strands composed of two or three unit-filaments are indicated in the figures by *2* and *3*. Decoiled strands (*DS*); unit-filaments (*F*); loops of unit-filaments and unit-filament strands (*LO*); network associations between the unit-filaments (*N*); dense granules (*G*). Fig. 23, fiber fixed *in toto* by the procedure IVb (PTA, glutaraldehyde, osmium tetroxide); stained uranyl acetate and lead citrate. × 200,000. Fig. 24, negatively stained preparation of the axoplasm extruded into 1% PTA, at pH 8.0. Higher magnification of Fig. 12. × 150,000. Figs. 25, 27, fiber fixed *in toto* in osmium tetroxide (II). Endoplasm. Stained uranyl acetate and lead citrate. × 140,000. Fig. 26, negatively stained preparation of the axoplasm extruded into 1% PTA, at pH 7.2. × 210,000.

tallization of desired proteins, determination of protein structure, and labeling of active sites of protein. (Keller and Block, 1960; Singer. 1967). The careful control of pH greatly reduces the chance of irreversible denaturation of proteins by heavy metals. For example, in this way even mercury may precipitate proteins without denaturation and facilitate crystallization. Warburg and Christian (1941) employed mercuric sulfate for the crystallization of enolase from brewer's yeast. Scott (1934) prepared zinc-insulin crystal with the aid of zinc. Polis and Meyerhof (1947) used lanthanum acetate for the purification of muscle adenosine triphosphate.

According to the results of the present investigation, UO_2^{++} ions of a 1% uranyl acetate solution at pH 4.4 display a specific affinity to the filamentous protein structures of the squid giant nerve fiber axoplasm. At pH 4.4, the uranyl ions segregate the filamentous elements of the axoplasm in a coiled, threadlike preparation surrounded by a flocculent precipitate. Considerable part of the uranyl ions combine probably with the carboxyl groups of the main amino acids of the protein—the glutamic and the aspartic acids (see Table I)—forming saltlike associations. According to Dounce and Lan (1949), such saltlike associations between the uranyl ions and carboxyl groups of a protein have no damaging influence on the protein if the pH is kept within the limits that are safe for the protein. As a general rule, the precipitability of proteins by uranyl acetate appears to be maximal in the pH range of 4.0–5.5 (see also Dirr et al., 1957).

The amino acid composition of the thread preparations segregated by the uranyl procedure resembles closely that of the neurofilament protein obtained by selective salting-out with ammonium sulfate (Schmitt and Davison, 1961). The isoelectric point of the neurofilament protein was given by Schmitt and Davison to be ca. 4–4.5. It has to be noted that the pH of the 1% uranyl acetate solution has to be about 4.4 to obtain optimal segregation of the thread preparation. It is well known that at their isoelectric point the proteins show the greatest stability. Accordingly, considerable evidence supports the conclusion that the protein structures of the thread preparation, as revealed in high-resolution micrographs (Fig. 18), represent closely their state in the living axoplasm and that the thread preparations consist of a protein material of considerable homogeneity. Other protein fractions of the axoplasm have been precipitated in the flocculent material surrounding the thread preparation and separated in the bulbous enlargements.

It appears that the experiments on the isolation of the filamentous components of the axoplasm by uranyl acetate provide a basis for a very promising method of protein separation from small and heterogenous samples. The method provides good possibilities for isolating filamentous proteins from the axoplasm without denaturation in a rather simple and inexpensive way. Dirr et al. (1957) employed uranyl acetate for the isolation of pure α- and γ-globulins from human serum. Because of the fixing effect of uranyl ions and the simultaneous increase of contrast, the preparations obtained by the method are well suited for an integrated study with both high-resolution electron microscopy and a number of physicochemical methods. Further work, especially at the limit of the resolving power of the electron microscope and on the quantitative aspects of the associations between the uranyl ions and the isolated protein, is in progress.

The data of the elementary analysis and of the ribonucleotide concentration of the thread preparation cannot be correlated at present with definite structural components of the axoplasm. Besides the thread preparations which constitute the main material for the elementary analysis, some bulbous material has been included in the analysis too. This was necessary because of lack of sufficient material. Therefore, the data are of a preliminary character and will be extended further when a larger amount of samples will be available. The calcium content of the samples is very high—0.6% dry weight corresponding to 150 mmole/kg dry weight. According to Keynes and Lewis (1956), the calcium concentration in the axoplasm of the squid giant nerve fiber is of the order of 0.5 mmole/kg wet weight. Hodgkin and Keynes (1957) conclude that in squid axons almost all the calcium in the axoplasm is in bound form, and that the intracellular concentration of ionized calcium may be less than 0.01 mM. With reference to the 1–2% content of ribonucleotides and ribonucleic acid in the samples, attention has to be drawn to the 50-A-wide dense granules associated constantly, either singly or in groups, with the unit-filaments (see Figs. 4, 18). Such granules could never be observed free in the axoplasm as, for example, glycogen granules are observed in other

tissues. In this context, the question has to be raised whether the 50-A granules in the axoplasm may not be ribosome-like particles in association with the protein of the unit-filaments (see also Davison, 1967).

It appears that in 1% uranyl acetate at pH 4.4, the filamentous network of the endoplasm is segregated from components of the ectoplasm (see Metuzals and Izzard, 1969). The thread preparation consists of the filamentous network. Contrary to this, the bulbous enlargements contain, besides a dense filamentous network, numerous microtubules and fragments of mitochondria and membranes. It can be reasoned that during the segregation period in the uranyl acetate, microtubules and other structures have been squeezed out from the filamentous network of the endoplasm. This can be understood because of a partial collapse of the large-scale configuration of the endoplasm which is considerably looser as compared with the ectoplasm. The coiling of the thread preparations as well as the helical grooves on its surface demonstrate convincingly the existence of a torque in the axoplasm (see also Metuzals and Izzard, 1969). The observations of Solomon and Tobias (1950) are of interest in this connection. When the axoplasm is gently compressed along the long axis of the axon by an inserted quartz needle, alternating light and dark bands form in the axoplasm ahead of the rod.

In high-resolution electron micrographs of sections, negatively stained preparations, and thread preparations, the unit-filaments and their associations by intercoiling and looping in unit-filament strands are clearly displayed. It can be proposed that by coiling/decoiling and folding/unfolding of the unit-filaments different degrees of orientation of the protein macromolecules can be achieved. Thus, shifts in the electrical properties of these macromolecules (e.g., with respect to interaction at exposed ionized sites) and/or their electromagnetic properties (e.g., refractive index or birefringence) could be brought about and maintained (see Scheraga, 1963; Weber and Teale, 1965; Doty et al., 1957; Ungar et al., 1957; Ungar, 1963; Cohen et al., 1968). One may speculate that such shifts may be of such a magnitude that they may interact with the processes associated with the excitable membrane and impulse conduction. Conversely, depolarization of the excitable membrane may influence the electrical and electromagnetic properties of the filamentous protein macromolecules which thereby achieve changes in their configuration. In such a way, a causal link between activities associated with the excitable membrane and those located in the axoplasm could be maintained and coupling among the different levels of functional activities of the nerve fiber achieved—i.e., impulse conduction, metabolism, and the bidirectional displacement of the axoplasm (see also Katchalsky et al., 1966). Several observations indicate that calcium ions may play an essential role in such a coupling mechanism. The effect of calcium on the filamentous network elements, described on page 491, support the conclusion that calcium ions, acting on the attachment sites between the units of the network, cause loosening and disintegration of the network configuration. Hodgkin and Keynes (1957) observed that stimulation increases the rate of entry of calcium into the axoplasm. The authors suppose that the calcium entry during the spike may not be accidental accompaniment of activity, but is somehow connected with the development of the state of increased sodium permeability. It might be imagined that when the fiber is suddenly depolarized, calcium ions are enabled to discharge from the membrane into the axoplasm and that this liberates carriers which are then able to transport sodium for a limited period. The present observations suggest that in such a process, configurational changes of protein macromolecules of the axoplasmic network are involved (see also Luxoro, Rojas, and Wittig, 1963).

In the proposed mechanism of the conformational changes of the filamentous protein structures in the axoplasm, sliding of the unit-filaments over each other and over the microtubules may be involved, whereby the microtubules would act as guides and surfaces (see also Porter, 1966). Such a mechanism could be involved in the production of the motive force of axonal streaming.

The main observations of the present paper, namely, the continuous character of the filamentous space-network in the axoplasm and the predominantly coiled arrangement of the elements of this network, lead to two final conclusions. First, shifts in the configuration of the unit-filaments with respect to each other and to the microtubules must be reflected at all levels of structural organization of axoplasm. This will result in ecto-/endoplasmic transformations as well as in changes in the degree of twist of the whole axoplasm (see Metuzals and Izzard, 1969). Secondly, configura-

tional shifts and displacements of the unit-filaments, compensated by changes in the degree of coiling of the 1–3-μ threads (Metuzals and Izzard, 1969) of the axoplasm, do not necessarily result in significant alterations of the diameter and length of the axon. In other words, a bidirectional axoplasmic streaming must not necessarily be associated with dimensional changes of the axon.

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