ELECTRON MICROSCOPE AND EXPERIMENTAL INVESTIGATIONS OF THE NEUROFILAMENTOUS NETWORK IN DEITERS' NEURONS

Relationship with the Cell Surface and Nuclear Pores

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ABSTRACT

The assembly of filamentous elements and their relations to the plasma membrane and to the nuclear pores have been studied in Deiters' neurons of rabbit brain. Electron microscopy of thin sections and of ectoplasm spread preparations have been integrated with physicochemical experiments and differential interference microscopy of freshly isolated cells. A neurofilamentous network extends as a continuous, three-dimensional, semilattice structure throughout the ectoplasm, the "plasma roads," and the perinuclear zone of the perikaryon. This space network consists of ~90-Å wide neurofilaments arranged in fascicles which are interconnected by an exchange of neurofilaments. The neurofilaments consist of intercoiled ~20-Å wide unit-filaments and are associated through cross-associating filaments with other neurofilaments of the fascicle and with microfilaments. The ~20–50-Å wide microfilaments display intimate associations with the plasma membrane and with the nuclear pores. Electron microscopy of thin sections from glycerinated and heavy meromyosin-treated Deiters' neurons shows that actin-like filaments are present in the pre- and postsynaptic regions of synapses terminating on these neurons.

It is proposed that the neurofilamentous space network serves a transducing function by linking plasma membrane activities with the genetic machinery of the neuron.

INTRODUCTION

At present one of the most important problems in neurobiology is to determine how the activities of the neuronal surface are linked to the genetic machinery in the nucleus of the neuron. A description of structures and mechanisms responsible for such a transduction is essential for an understanding of the functioning of the neuron. However, concrete data concerning the morphological and physicochemical organization of a plasma membrane-DNA axis in eukaryotic cells is scarce.

Experiments with the immune system have provided evidence for the existence of cellular mechanisms which transduce information from the cell surface to the genome. Several recent findings suggest that a specific state of assembly of colchicine-binding proteins in the cytoplasm of lymphocytes controls the distribution of surface receptors and the response of the genome to certain molecules with a surface restricted site of action (18, 21).

In the present paper we have investigated the problem of structures and mechanisms linking the surface of the neuron with the nuclear pores, utilizing the giant Deiters' neurons from the lateral vestibular nucleus of rabbit brain. Three lines of approach have been integrated: (*a*) electron microscopy of thin sections of Deiters' neurons fixed by intravascular perfusion; (*b*) correlated electron microscope and physicochemical experiments with isolated neuronal components; and (*c*) observations of freshly isolated cells by differential interference microscopy. Our studies indicate that a filamentous network establishes direct associations with the plasma membrane and the nuclear pores. It is proposed that such a network may be the substrate for mechanisms integrating the functions of the neuronal surfaces with those of the nucleus.

MATERIALS AND METHODS

Albino rabbits of both sexes weighing 2–3 kg were used. The animals were obtained from established commercial sources in Canada and Sweden.

Perfusion Fixation and Embedding for Thin Sectioning

16 rabbits were used in perfusion fixation experiments to determine optimal conditions. Each animal was anesthetized by injection of Nembutal (28 mg/kg) into a marginal ear vein. The trachea was connected to a small animal respirator (model 670-100, Harvard Apparatus Co., Inc., Millis, Mass.). A trochar with cannula (3-mm diameter) was inserted into the left ventricle, the trochar removed, and the cannula connected with perfusion solution. Immediately thereafter, the abdominal aorta was clamped and a large incision was made in the right ventricle.

First, 250 ml of prewash solution at 39°C were perfused at a pressure of 150 mm Hg (2–3 min). This was followed by the perfusion of 800 ml of fixative 1 at the same temperature and pressure (10–12 min). After perfusion, the brain with part of the skull still attached was placed in fixative 1 for 2 h at 4°C. After this, a transverse cut was made through the estimated center of the lateral vestibular nucleus and 2-mm thick sections adjacent to each side of the cut were removed (38). The regions which contained the lateral vestibular nucleus were cut into 1-mm blocks which were rinsed in buffer for 1 h and postfixed for 2 h in fixative 2. The blocks were then washed in buffer for 5 min, dehydrated, and embedded in Vestopal W (M. Jaeger, Geneva, Switzerland) as previously described (32). Ultrathin sections were cut on an LKB Ultratome using glass knives. The sections were mounted on copper grids coated with Formvar and carbon and double stained with saturated uranyl acetate in ethanol followed by Reynolds' lead citrate (45). The giant Deiters' neurons were located in the embedded tissue blocks by examining in the light microscope 1-μm thick sections stained with 1% toluidine blue (45).

A number of concentrations of glutaraldehyde and paraformaldehyde in various buffers were tested as fixatives. The best results were obtained using the following solutions (46).

PREWASH SOLUTION: 0.03% Tris(hydroxymethyl)amino methane (THAM) (Fisher Scientific Co., Pittsburgh, Pa.), 0.96% NaCl, 0.042% KCl, 0.02% $MgCl_2 \cdot 6\ H_2O$, 0.028% $CaCl_2$, and 0.075% dextrose, pH 7.35 (335 mosmol/liter).

BUFFER: 0.18 M sodium cacodylate, pH 7.4 (352 mosmol/liter).

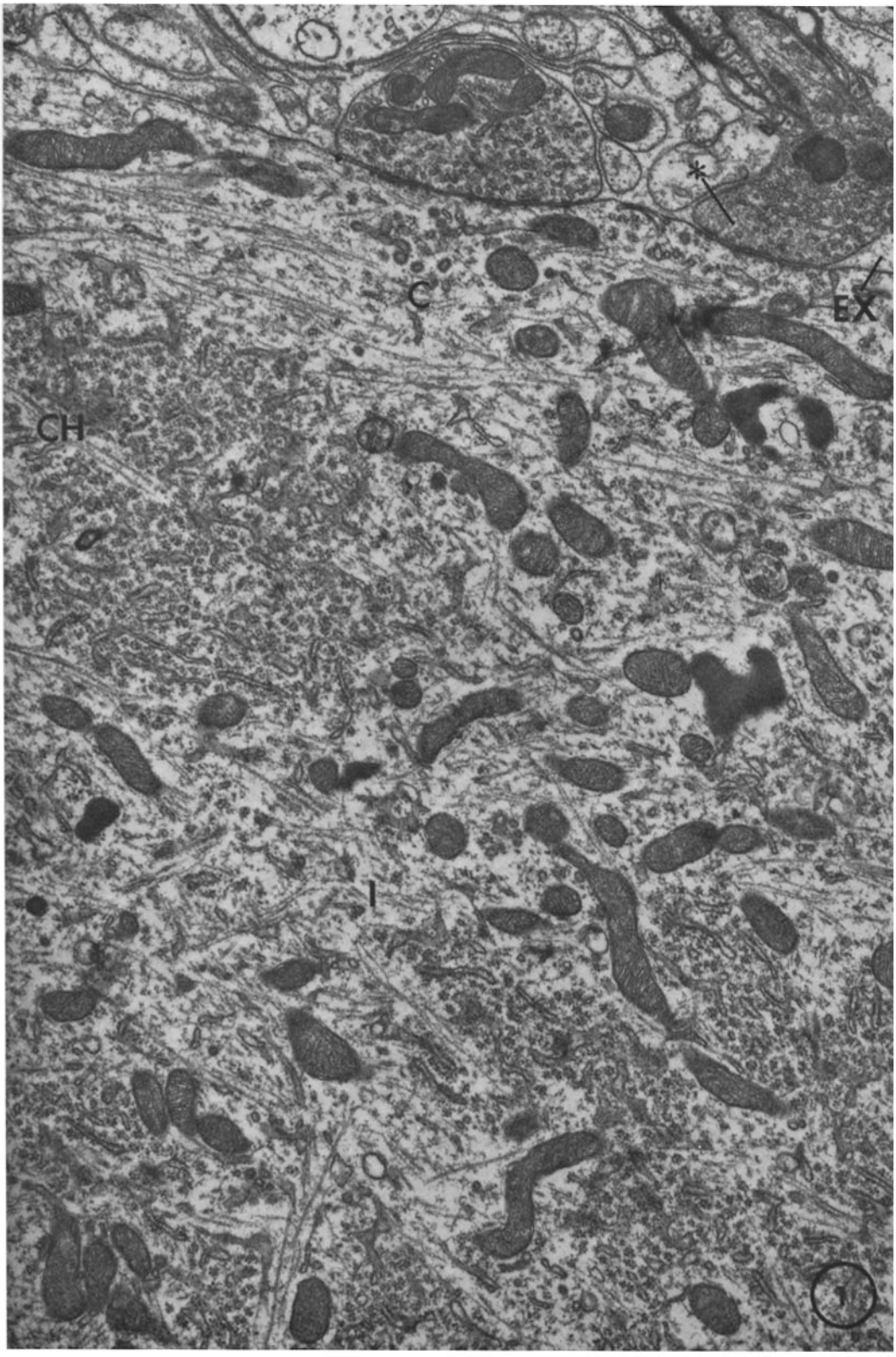
FIXATIVE 1: 1% glutaraldehyde, prepared from ampoules of highly purified 70% glutaraldehyde (Ladd Research Industries, Inc., Burlington, Vt.), 1% paraformaldehyde, in 0.18 M sodium cacodylate, pH 7.4 (820 mosmol/liter).

FIXATIVE 2: 2% osmium tetroxide in sodium cacodylate buffer. The osmolarity of fixative solutions was measured with a freezing-point depression device, the Advanced Osmometer, model 31LAS (Advanced Instruments, Inc., Needham Heights, Mass.).

Experiments with Freshly Isolated Deiters' Neurons

About 200 rabbits have been used in these experiments. This large number of animals was required because of the limited number of fresh Deiters' neurons that could be obtained from each rabbit, and the

FIGURE 1 Ectoplasm (*C*) displaying longitudinal profiles of threadlike elements. Ectoplasm-like neuroplasm can be traced from the cortex into deeper portions of the perikaryon between the Nissl bodies, where profiles of cross- and obliquely sectioned threadlike elements (*I*) can be identified. Threadlike elements in defined channels (*CH*) in the Nissl bodies; extracellular space (*EX*); microfilaments (*) in a presynaptic terminal. Thin section. × 24,000.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extensive number of repetitions and controls required for each experiment.

The rabbits were stunned by injecting 15 ml of air into a marginal ear vein and were immediately exsanguinated by cutting the major vessels in the neck. The cell bodies of giant Deiters' neurons were freshly isolated in 0.25 M sucrose as previously described (30). The differential interference microscopy of such cells was carried out as described earlier (42). An isolated cell was cut open with the sharpened end of a 10-μm wide stainless steel wire and flattened against the surface of a slide (16). The cortical layer of the Deiters' cell adheres firmly to the glass, thus allowing the contents of the cell to be removed. A layer of this cleaned cortical spread was then peeled off with a 10-μm steel wire and floated in 0.25 M sucrose onto a grid coated with Formvar and carbon. The spread adheres firmly to the film. An imprint of the spread, consisting of unknown elements, remains stuck to the slide (41).

The grids were treated with 1% uranyl acetate, pH 4.4, for 1–3 min and the excess solution was removed either by blotting with filter paper or by a brief water rinse. The grids were then dried in the air.

Treatment of Ectoplasm Spreads with Enzymes and Chemicals

Ectoplasm spreads adhered to grids were treated with the various substances mentioned in Tables I and II, by placing drops of the solution on the grids and incubating them in a humid chamber. After treatment, the grids were rinsed by brief immersion in water or 0.1 M KCl and the ectoplasm spreads were stained with uranyl acetate as described above.

ENZYME SOLUTIONS: Hyaluronidase (Sigma Chemical Co., St. Louis, Mo., type V), lecithinase C (Sigma Chemical Co., type I), and trypsin (Worthington Biochemical Corp., Freehold, N. J., lyophilized) were dissolved in 0.2 M sodium phosphate buffer, pH 7.3. Neuraminidase (Sigma Chemical Co., type V) was dissolved in 0.1 M sodium citrate, pH 5.1. Pronase B (Calbiochem, San Diego, Calif.) was dissolved in 0.01 M Tris-HCl, pH 7.5, at 1 mg/ml and predigested for 1 h at 37°C immediately before use. Ribonuclease A (Sigma Chemical Co., type IIIA) was dissolved in 0.15 M NaCl at 1 mg/ml and heated for 10 min at 80°C before use. Deoxyribonuclease I (Worthington Biochemical Corp., electrophoretically purified) was dissolved in 0.005 M $MgCl_2$, 0.005 M Tris-HCl, pH 7.5. DNase I treatment was carried out on both platinum and copper grids. The enzyme concentrations used to treat the ectoplasm spreads are given in Table I.

CHEMICALS: Cytochalasin B (Imperial Chemical Industries Ltd., Cheshire, England) was dissolved in 1% dimethylsulfoxide (DMSO) while colchicine (Sigma Chemical Co.) was in 0.01 M $MgCl_2$, 0.1 mM GTP, 0.01 M sodium phosphate, pH 6.5. [^{3}H]Actinomycin D (Schwartz BioResearch Inc., Orangeburg, N. Y.) dissolved in Millonig phosphate buffer at a concentration of 35 μg/ml (3.5 Ci/mmol) was placed on the grid for 15 min at room temperature. The grid was then rinsed twice with 400 μg/ml of unlabeled actinomycin D in the same buffer, incubated for 15 min with the unlabeled actinomycin D, rinsed, covered with photographic emulsion, and exposed for 3 mo. The grids were then processed for electron microscope autoradiography.

Heavy Meromyosin Treatment

In experiments involving treatment with heavy meromyosin (HMM), the isolation and dissection of Deiters' cells were carried out in 0.2 M sucrose, 0.05 M KCl. The ectoplasm spreads were treated with a drop of HMM (0.4 mg/ml) in standard salt solution (0.05 M KCl, 0.005 M $MgCl_2$, 0.006 M phosphate buffer, pH 7.0) for 3–5 min at room temperature. The grids were then rinsed in standard salt solution, fixed for 1 min with 1% formaldehyde in standard salt, rinsed in 0.1 M KCl, and negatively stained with uranyl acetate as described above.

Small blocks of lateral vestibular nucleus were glycerinated, treated with 1.4 mg/ml of HMM, and processed for electron microscopy as previously described (31).

Deiters' neurons isolated in 0.2 M sucrose, 0.05 M KCl were placed on depressed glass slides. The cells remain stuck to the slides throughout the following procedure. The neurons were treated with 50% glycerol, 50% standard salt solution (vol/vol), containing 400 μg/ml of HMM for 1 h; 25% glycerol, 75% standard salt solution containing 400 μg/ml of HMM for 30–60 min; and 5% glycerol, 95% standard salt solution containing 400 μg/ml HMM for 15 min. The cells were then treated with 1.4 mg/ml of HMM in standard salt solution for 2 h and washed with 0.1 M KCl for 15 min. All of these steps were carried out at room temperature. Control experiments consisted of the omission of HMM during incubation in the various glycerol/standard salt solutions or of the incubation of HMM-treated cells in the presence of 10 mM ATP in standard salt for 30 min.

The cells were fixed for 1 h at room temperature with 1% glutaraldehyde, 1% paraformaldehyde in 0.18 M sodium cacodylate, pH 7.4, and postfixed for 1 h at room temperature with 2% osmium tetroxide in the same buffer. This was followed by washing in 0.18 sodium cacodylate and then 0.05 M sodium-hydrogen maleate, pH 6.0. The cells were stained with 1% uranyl acetate in 0.05 M sodium-hydrogen maleate, pH 5.6, for 15 min, dehydrated with a graded series of ethanol solutions (50–100%), and embedded in Vestopal W.

Myosin from rabbit muscle was prepared according to Mommaerts and Parrish (43) and was subsequently treated with trypsin as described by Lowey and Cohen (34) to obtain HMM. The F-actin was prepared from rabbit muscle as previously described (53).

Electron Microscopy

All the grids were examined in Elmiskop 1A, Elmiskop 101, and JEM-100B electron microscopes equipped with a cold stage and operated at 80 kV with double

condenser illumination and a 30-μm, thin-foil objective aperture. All the high resolution electron micrographs were taken at magnifications ranging from 70,000 to 100,000.

RESULTS

Electron Microscopy of Thin Sections of Deiters' Neurons

Our light and electron microscope observations of sections of fixed Deiters' neurons are in agreement with previous investigations of large neurons (2, 6, 48, 56). For a general description of the morphology and fine structure of Deiters' neurons, see Sotelo and Palay (51).

In the perikaryon of the giant Deiters' neurons, a distinct peripheral zone ranging in thickness between 1 and 3 μm can be identified subjacent to the plasma membrane. This peripheral zone or cortex, often referred to as the ectoplasm, represents a well-defined morphological entity of the neuroplasm characterized by a distinct fine struc-

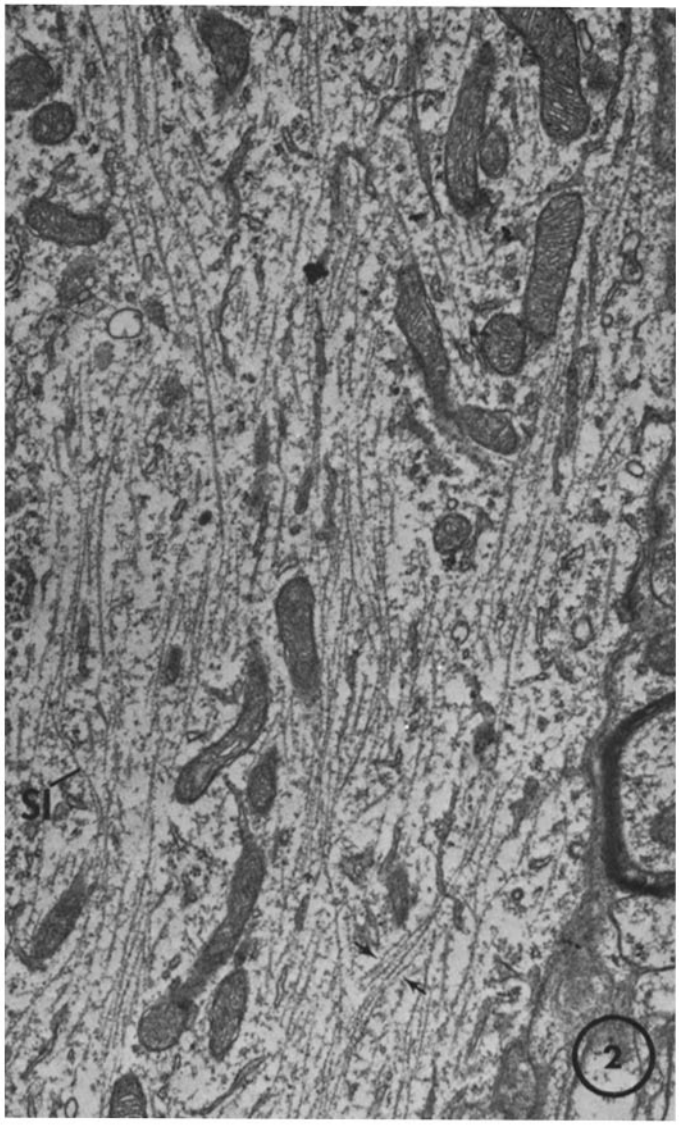


FIGURE 2 Curved and sinusoidal profiles of neurofilament fascicles (arrows) and of individual neurofilaments (*SI*) are arranged at various angles relative to one another. Thin section of ectoplasm. × 24,000.

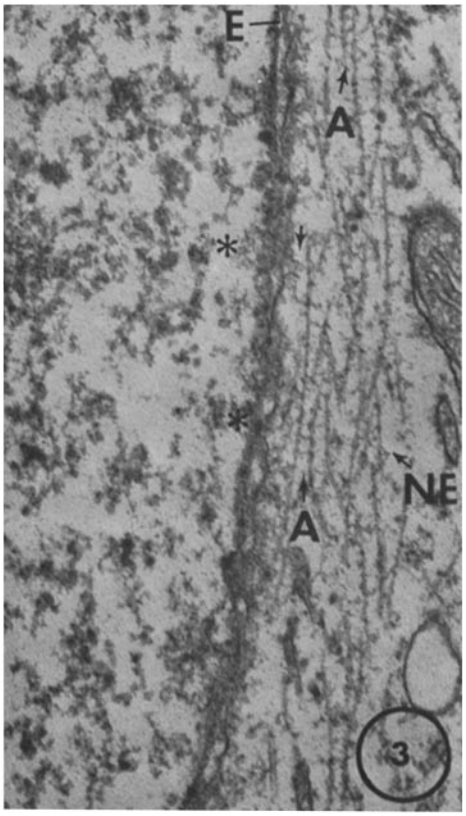


FIGURE 3 Neurofilaments (*NE*) arranged in a staggered cuneiform pattern in the perinuclear zone. (*A*) cross-associating filaments between the neurofilaments; microfilaments (arrow) between the neurofilaments and the nuclear pores (*); nuclear envelope (*E*). × 60,000.

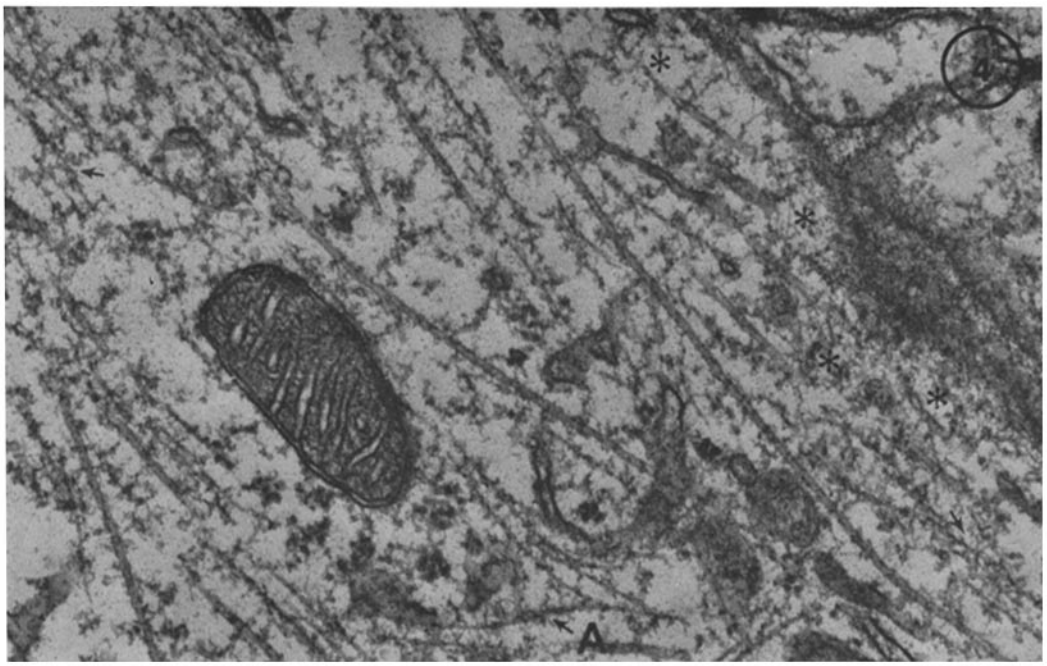


FIGURE 4 Microfilament tangles intimately associated with the plasma membrane (*) and with the neurofilaments (arrows) in the ectoplasm; cross-associating filaments (*A*) between the neurofilaments. × 60,000.

ture. It consists of assemblies of several populations of threadlike elements (such as neurofilaments, microfilaments, and microtubules), mitochondria, dense bodies, and different types of vesicles (Fig. 1). This ectoplasm is free of granular endoplasmic reticulum. Ribosome complexes are only rarely located in the ectoplasm.

The same ectoplasmic type of neuroplasm intrudes from the cortex into the deeper portions of the perikaryon (Fig. 1), surrounding the Nissl bodies as the so-called "Plasmastrassen" or "plasma roads" (2), and forms a distinct perinuclear zone (Fig. 8). The continuity between the ectoplasm, the plasma roads, and the perinuclear zone is clearly revealed in thick, stained sections investigated by light microscopy and in electron micrographs of thin sections.

In the perikaryon of medium- and small-sized neurons of the lateral vestibular nucleus all the cytoplasmic organelles are intermixed and the ectoplasm-like differentiation of the perikaryon cannot be observed.

The 90-Å wide neurofilaments in Deiters' neurons are not randomly distributed. They are arranged in a basic pattern characterized by fasciculation and interchange of neurofilaments among fascicles. Successive profiles of curved fascicles, measuring up to ~0.1 μm in diameter, can be seen to reverse alternately their curvature, indicating a helical configuration (Figs. 1, 2). The neurofilaments of individual fascicles fan out and join other fascicles, thus forming characteristic staggered cuneiform patterns (Fig. 2). This interchange of curved neurofilaments among fascicles indicates that the neurofilaments are assembled in a continuous three-dimensional network formation (42).

Most of the neurofilaments are curved and are arranged at acute angles relative to one another (Figs. 2, 3). Such neurofilamentous profiles are more or less evenly distributed in mutually close relationships throughout the ectoplasm, the plasma roads, and the perinuclear zone without any discernible boundaries between these regions. Thus, the neurofilamentous network extends throughout these regions as a continuous assembly of interwoven neurofilaments. In thicker sections the profiles of the neurofilaments are longer. Their curvature becomes more pronounced and the proportion of larger angles between the individual filaments increases correspondingly, revealing a predominant crisscross pattern.

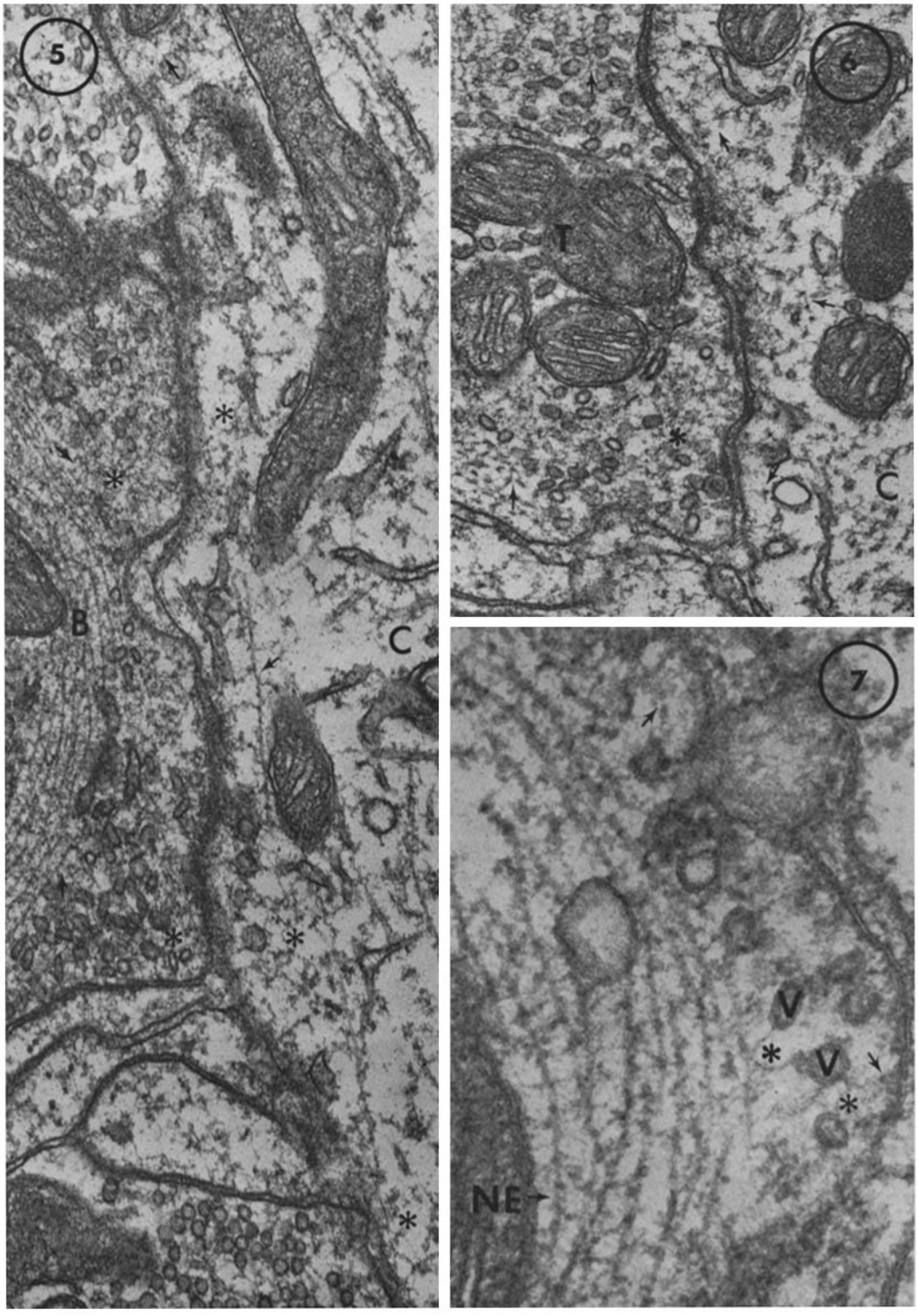
The neurofilaments have a barbed appearance in electron micrographs of intermediate resolution (Figs. 2, 3). Careful scrutiny reveals that the barbed ~90-Å filaments do not occur as independent entities. They appear to be associated with one another through cross-associating filaments which display great variability in their morphology. The cross-associating filaments, which are mostly thinner than neurofilaments, can be as little as 20 Å in width. The angles between the neurofilaments and the cross-associating filaments vary considerably (Figs. 3, 6).

A population of poorly defined filaments, which are convoluted and entangled with each other, is randomly distributed among the neurofilaments, the microtubules, and other organelles of the ectoplasm. These poorly defined filaments, which range in diameter from 20 to 50 Å, are the so-called microfilaments. The neurofilaments and their cross-associating filaments appear to be closely associated and perhaps even continuous with the microfilament tangles, as noted by other investigators (56, 55, 57). The microfilament tangles are in turn intimately associated with the plasma membrane (Figs. 4–6).

The close relationships between the neurofilaments and the microfilaments are especially well displayed in the preterminal and terminal regions of axons terminating on Deiters' neurons. In these regions, the microfilamentous tangle surrounds the synaptic vesicles and individual filaments display an intimate association with the axonal membrane (Figs. 5, 7).

The cytoplasmic zone adjoining the nuclear envelope displays all the structural elements characteristic of ectoplasm (Figs. 3, 8). In this perinuclear zone, the microfilaments appear to be closely associated both with the nuclear pore complexes as well as with the neurofilamentous network (Figs. 3, 9 A and B, 10). These 20–40-Å wide filaments often have a beaded appearance, the beads being smaller and less dense than ribosomes.

In grazed sections of the nuclear envelope the ~1,000-Å wide nuclear pore complexes reveal a considerable variability in their shape and detailed morphology (22). Among the circular profiles of the nuclear pores, profiles of oval, horseshoe, and rectangular shapes can be identified. This variable appearance of the nuclear pores is due to several factors. First of all, nuclear pore complexes are cut at many different angles in a tangential section because they are components of the sphere-shaped nuclear envelope. Differences in the fine structure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of nuclear pores may also reflect their differing functions.

The nuclear chromatin adjacent to the nuclear envelope appears different from that which is located further inside the nucleus. In the more central region of the nucleus the chromatin occurs as dense clumps and short fibers. In the zone adjoining the nuclear envelope the chromatin fibers are finer and are often arranged in rings and in rows of strands which are oriented perpendicular to the nuclear envelope (Fig. 9 A). Such rows of strands are composed of irregular, convoluted chromatin fibers, measuring 20–50 Å in diameter, which appear to be continuous with the nuclear pore profiles. This arrangement suggests that part of the nuclear pore complex is made up of a cylindrical assembly of interwoven chromatin fibers (51, 17, 33). From the foregoing it is evident that in the nuclear pore complexes both the chromatin fibers and the cytoplasmic filaments come in close proximity to each other (19).

With an increase in resolution of the micrographs, an identification and delineation of independent filamentous components of the neurofilamentous network become increasingly difficult. The complex distribution and irregular appearance of the filamentous structures resolved in high resolution micrographs is well illustrated in Figs. 11 A, 11 B, and 12. At favorable sites a filament with a diameter of about 20 Å, can be resolved as the smallest component of the neurofilamentous network, and may be considered as a unit-filament. The unit-filaments interweave to form strands with a diameter varying from 80 up to 150 Å. These strands extend as backbone structures throughout the neurofilamentous network and correspond to the neurofilaments referred to by numerous authors (40). Three morphological characteristics specify the neurofilaments. These are the ~90-Å wide smooth segments, also called "stalks," the ~150-Å wide "lumps" of irregular shape which are randomly distributed along the stalks, and the 20–50-Å wide side arms. When associated with other neurofilaments the latter represent cross associations (Fig. 11 A and B). In favorable locations it can be seen that the 90-Å wide smooth segments consist of two ~20-Å wide intercoiled unit-filaments (Figs. 11 B, 12). The lumps can be up to 300 Å in length. They consist of convoluted ~20-Å wide unit-filaments which appear to be continuous with those that make up the stalk portion of the neurofilaments. Thus, it appears that also at the supramolecular level of organization the neurofilaments are assembled into a network structure through interweaving, intercoiling, and intimate association of macromolecular components. The network character of the neurofilamentous assembly is especially pronounced in nerve terminals (*NE*, Fig. 12).

Ectoplasm Spreads

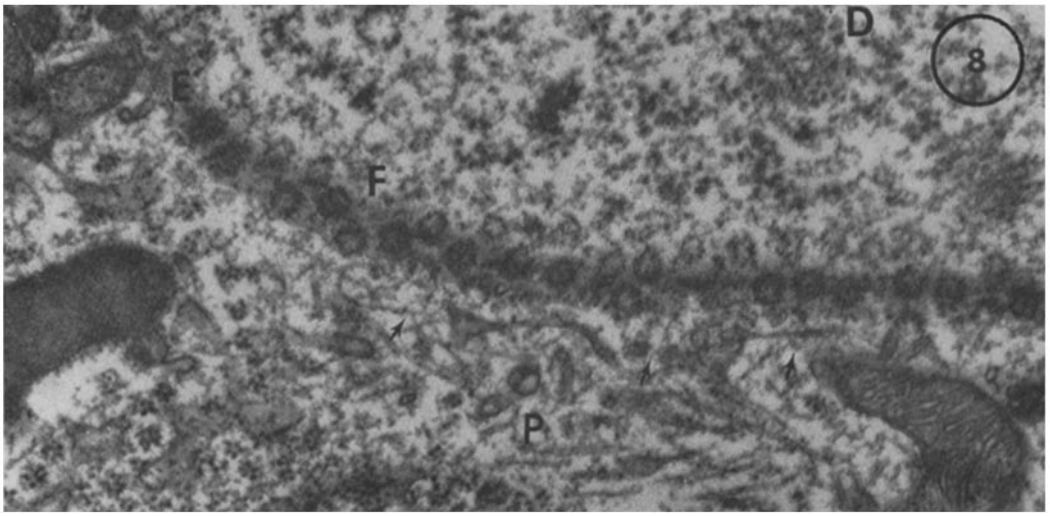
The threadlike elements in an ectoplasm spread preparation (see Materials and Methods) are organized rather regularly in a semilattice pattern (Fig. 13) (42, 5). Three groups of threads can be distinguished on the basis of their diameter. The main group consists of threads 70–150 Å in diameter and corresponds to the neurofilaments. The second group is made up of threads ~50 Å or less in diameter, representing microfilaments. The third group consists of threads which are 200 Å and wider and which represent fascicles of neurofilaments. Straight, curved, and folded neurofilaments appear to associate at numerous sites into fascicles forming in such a manner a network assembly of neurofilaments (Figs. 13, 14).

The association pattern of the neurofilaments is partially disrupted in the marginal zone of most of

FIGURE 5 Microfilament tangles intimately associated with neurofilaments (arrows) and with the plasma membrane (*) at pre- and postsynaptic sites; ectoplasm (*C*). A bundle of neurofilaments (*B*) with numerous cross-associating filaments in a presynaptic terminal. × 60,000.

FIGURE 6 Cross- and obliquely sectioned neurofilaments (arrows) displaying cross associations and contacts with microfilaments (*) which in turn are intimately associated with the synaptic vesicles and the plasma membrane at pre- and postsynaptic sites; ectoplasm (*C*), presynaptic terminal (*T*). × 60,000.

FIGURE 7 Neurofilamentous network (*NE*) in intimate association with microfilaments (*) which establish direct contacts with synaptic vesicles (*V*) and with the axonal membrane (arrows). Preterminal region of an axonal ending. × 60,000.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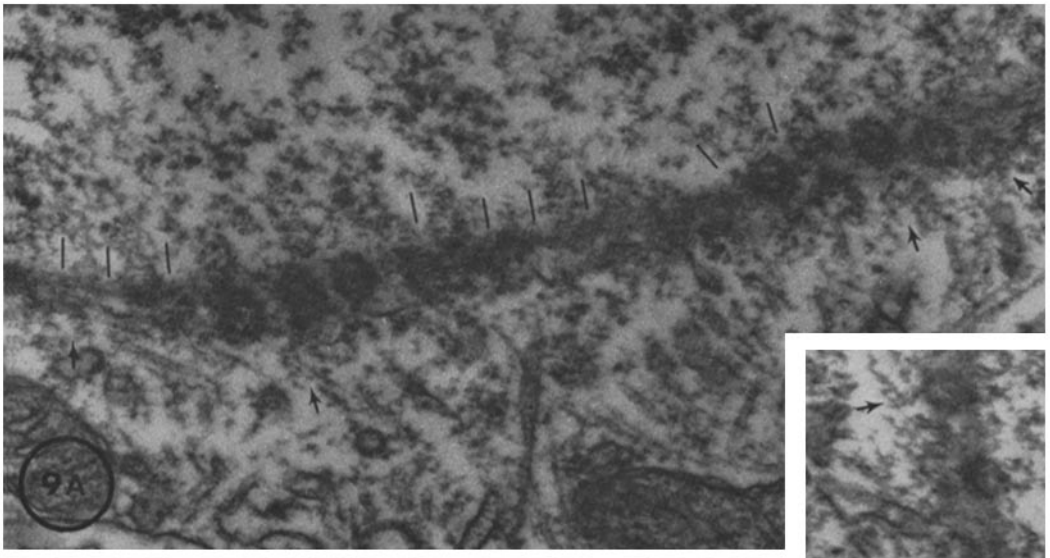
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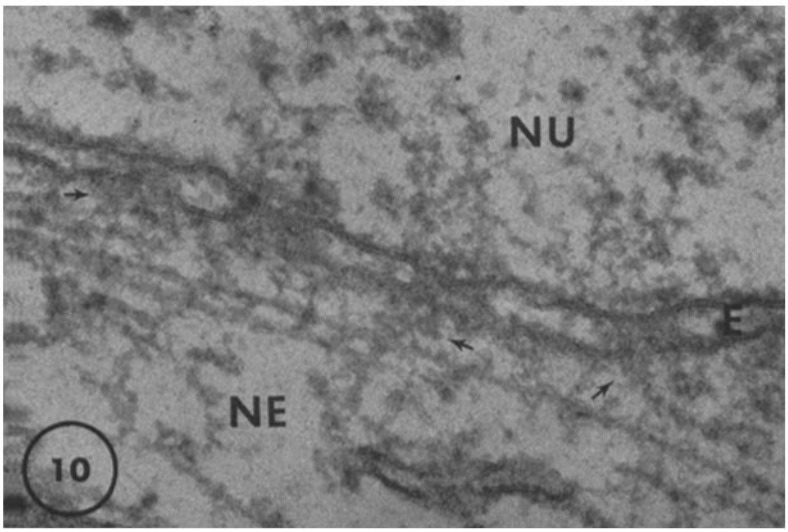
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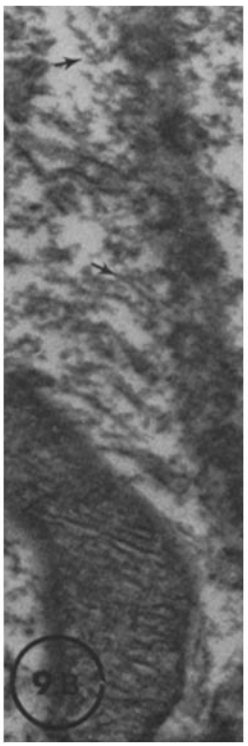
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the spread preparations as compared with their regular assembly in more central regions (Fig. 14). The neurofilaments in the marginal zone can be individually traced for long distances.

The helical intercoiling pattern of the ~20-Å wide unit-filaments making up a neurofilament, is often more prominent in spreads (*H*, Fig. 15 A) than in thin sections. The length of the pitch formed by the intercoiled unit-filaments varies considerably in different regions of the neurofilament and is often inversely related to the diameter of the neurofilament. In a number of spread preparations in which the neurofilaments are embedded in a homogeneous background of uranyl acetate, ~50-Å wide globules can be identified in the neurofilaments. Whether these globules are proteins located in the "gaps" between the intercoiled unit-filaments, cannot be decided at present.

The faintly stained 20–50-Å wide microfilaments can be seen in the meshes which are formed by the neurofilaments (Fig. 14; *, Fig. 15 A and B). At numerous sites the microfilaments appear to be intimately associated with the neurofilaments. Cross associations between the neurofilaments and their side arms can be identified at a number of sites in the spreads (*A*, Fig. 15 A–C). A lampbrush-like appearance of the neurofilaments can be observed at numberous sites (*LB*, Fig. 15 A and B). Whether the lampbush-like regions, as well as the intimate associations between the microfilament tangles and the neurofilaments, are the result of an in vivo disassembly of neurofilaments into their unit-filament components, cannot be decided at present. The possibilities of artifactitious disruption, adsorption, and precipitation must always be considered in preparations of this type.

Freshly isolated ectoplasm spreads were treated with various enzymes, stained with 1% uranyl acetate, and investigated by electron microscopy (cf. Table I). The disappearance of the neurofilaments in ectoplasm spreads after treatment with trypsin or Pronase, indicates that proteins are the major if not the only constituents of these filaments. Treatment with DNase, RNase, hyaluronidase, neuraminidase, or lecithinase C has no discernible effect on neurofilament structure.

The possibility that small amounts of DNA are associated with the neurofilamentous network was investigated further by means of autoradiographic experiments utilizing [^{3}H]actinomycin D of high specific radioactivity (cf. Table II). As judged by the lack of a preferential distribution of grains in autoradiographs of ectoplasm spreads, double-stranded DNA appears to be lacking in such preparations.

The treatment of ectoplasm spreads with 0.1 mM colchicine has no visible effect on neurofilament structure. In some preparations, treatment with cytochalasin B produces pronounced localized unravelling of the neurofilaments into tangles of unit-filaments, which appear to be continuous with the microfilaments (*U*, Fig. 16). Furthermore, round microfilamentous aggregates could be observed in these preparations. The individual 20–30-Å wide microfilaments in these aggregates appear to be intimately associated with neurofilaments, thus producing berry-like patterns (Fig. 16). Whether these changes represent a specific effect of the cytochalasin B on the ectoplasmic filaments still remains an open question because the experiments were difficult to reproduce. In control experiments treatment with 1% DMSO, the solvent for cytochalasin B, did not produce similar changes.

Ectoplasm spreads treated with 0.6 M KI or 2 M KCl tend to fold extensively but the structure of the individual filaments is not altered. Low concentrations of $CaCl_2$ have an immediate effect on ectoplasm spreads. The spreads are transparent when freshly isolated in 0.25 M sucrose and placed

FIGURE 8 A grazed section of the nuclear envelope and the adjoining structures: nuclear envelope with nuclear pore complexes (*E*), perinculear zone of the cytoplasm (*P*), fibrous chromatin (*F*), dense chromatin (*D*). Note the intimate associations (arrows) between the nuclear pore complexes and the filaments of the perinuclear cytoplasm. × 42,000.

FIGURE 9 A and B Filaments of the perinuclear cytoplasm are intimately associated with the nuclear pore complexes (arrows). Rows of strands of irregularly looped and convoluted chromatin fibers (‖) continuous with the nuclear pores. × 60,000.

FIGURE 10 Neurofilamentous network (*NE*) in the perinuclear cytoplasm associated through microfilaments (arrows) with the nuclear pore complexes. Cross section of nuclear envelope (*E*); nucleoplasm (*NU*). × 120,000.

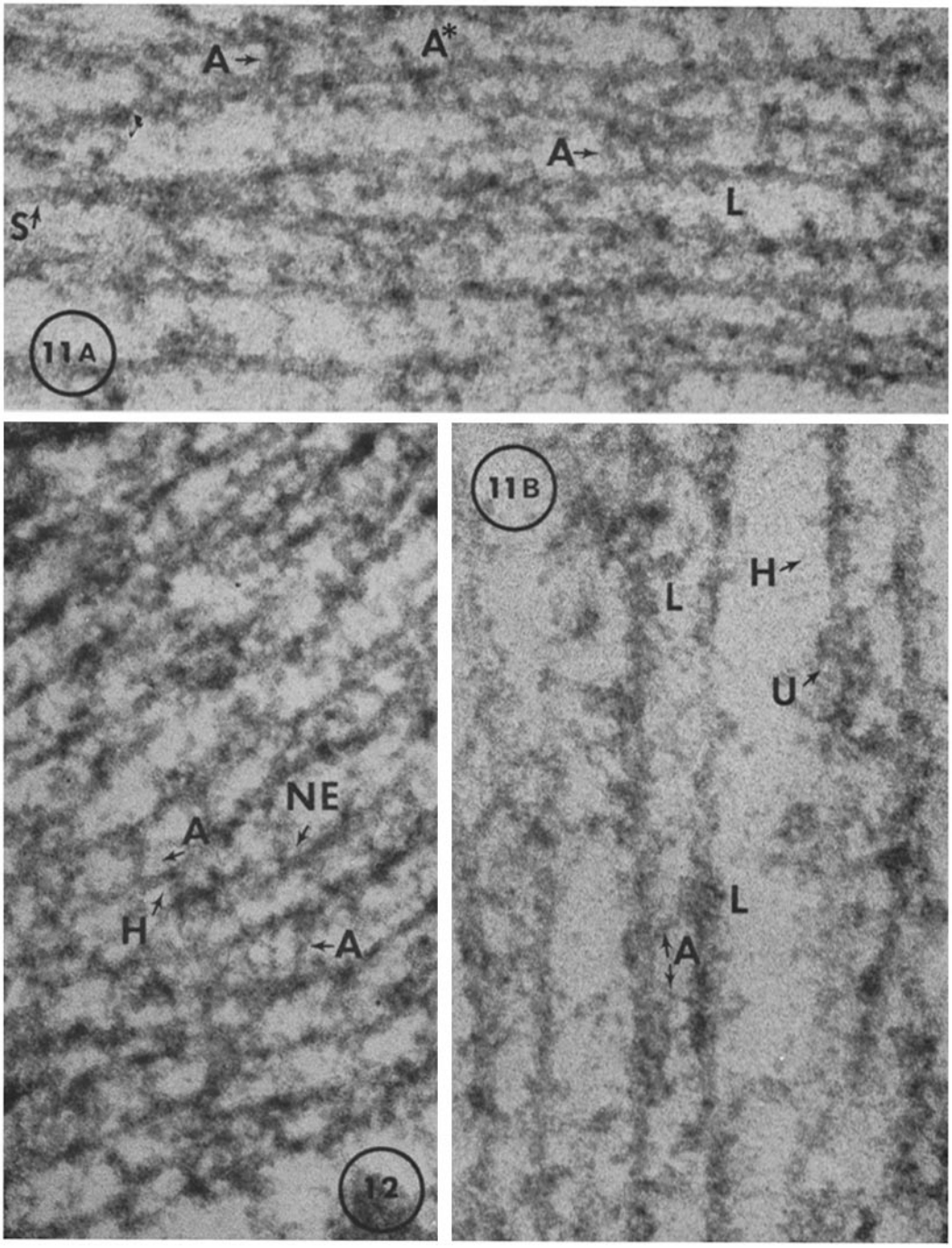


FIGURE 11 A and B Interwoven ~20-Å wide unit-filaments can be identified as components of the nurofilamentous network. The neurofilaments appear as strands characterized by stalk (*S*) and lump (*L*) segments and by side arms (*A**). The neurofilaments are associated through cross associations (*A*). Intercoiled unit-filaments (*H*); unravelling (*U*) of a neurofilament. Thin section. Fig. 11 A, × 250,000; Fig. 11 B, × 300,000.

FIGURE 12 The closely interwoven neurofilamentous network (*NE*) in the preterminal region of an ending on a Deiters' neuron. Intercoiled unit-filaments (*H*); cross associations (*A*) between the neurofilaments. This figure is a small area of a micrograph of the preterminal region of an axonal ending which includes synaptic vesicles, mitochondria, and the axonal membrane. × 210,000.

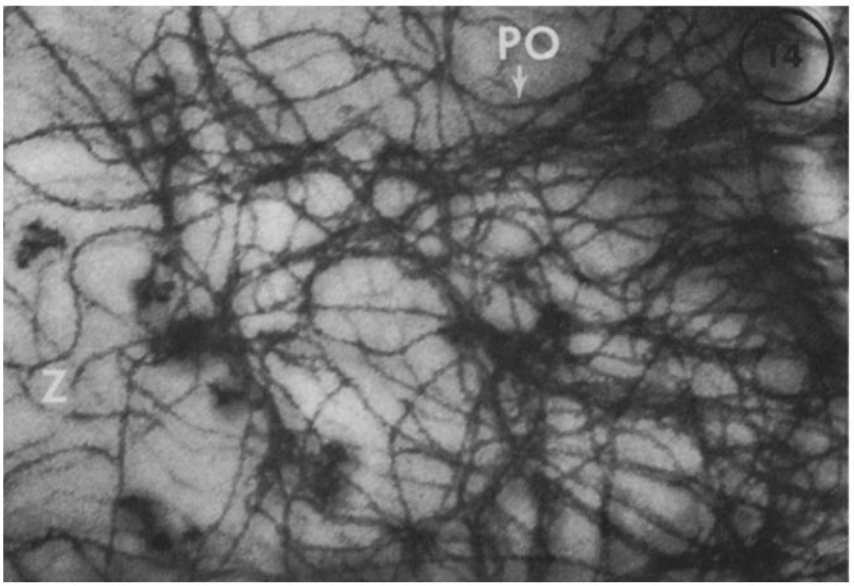


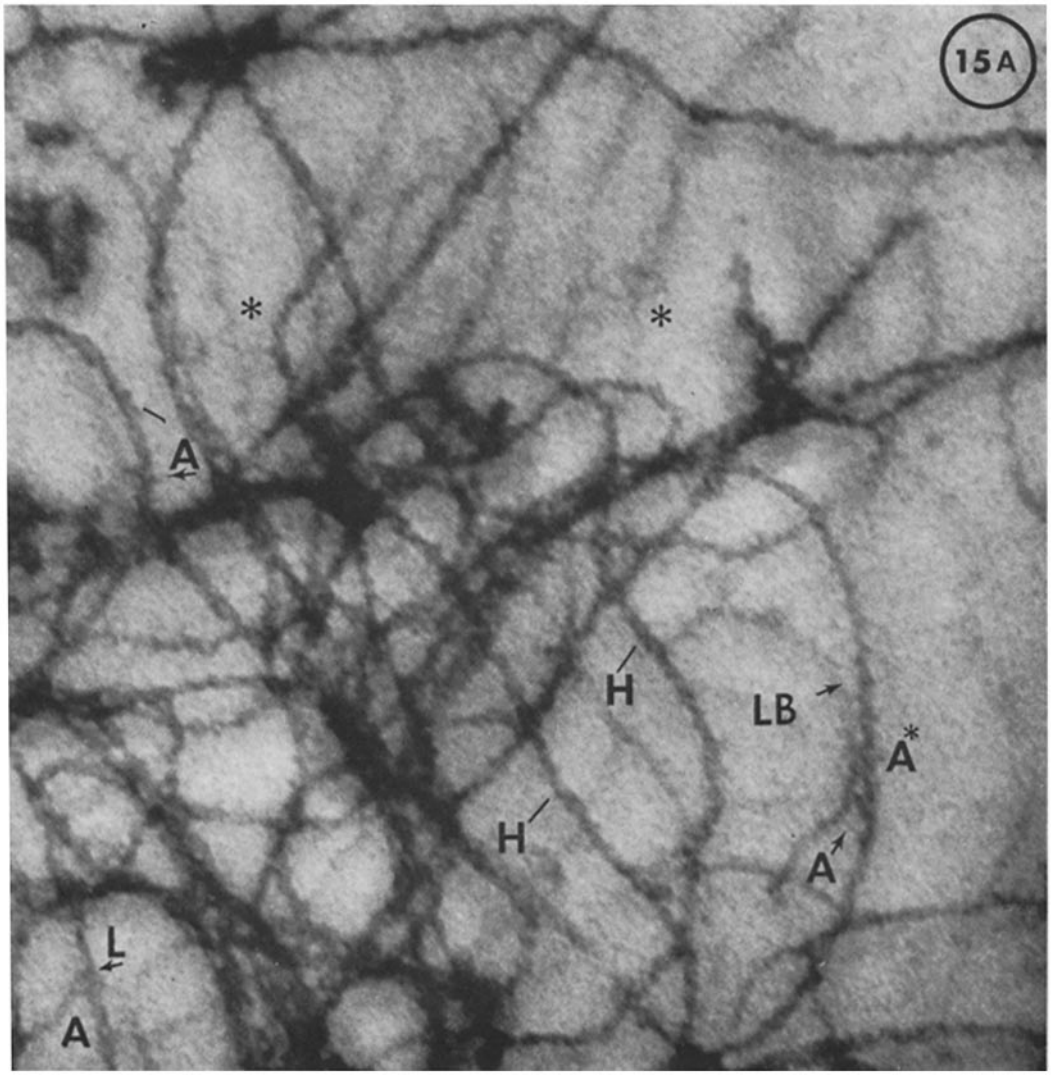
FIGURE 13 Ectoplasm spread prepared by dissection of an individual, freshly isolated Deiters' neuron, and stained with 1% uranyl acetate. The structure is composed of threads associated in a semilattice pattern. The dark, irregular ridges are folds in the preparation formed through flattening of the oval cortex of the perikaryon. × 45,000.

FIGURE 14 In the central portion (*PO*) of the spread preparation, the neurofilaments appear to be associated in a network assembly through fasciculation and filament interchange between the fascicles. In the marginal zone (*Z*) of the preparation, the neurofilamentous network is partially disrupted. × 90,000.

on a grid. The addition of 2 mM $CaCl_2$ in 0.25 M sucrose instantaneously causes the spread to appear opaque under the light microscope. This change in the light scattering properties of the ectoplasm spread may be due to binding of Ca^{++} to certain components of the spread or to changes in the hydration state of the preparation. Treatment of ectoplasm spreads with up to 25 mM $CaCl_2$ does not cause a disruption of the 90-Å neurofilaments.

Light Microscopy of Freshly Isolated Deiters' Neurons

A cross-lattice pattern formed by curved, threadlike components, 0.5–1 μm in diameter, extends as a predominant feature throughout the cytoplasm of freshly isolated Deiters' neurons viewed by differential interference microscopy (*X*, Fig. 17). Pear-shaped bodies several micrometers in diameter, which represent torn off presynaptic terminals, are located on the surface of the isolated cells. Preparations were made in which the nucleus and portions of the cell bodies were removed by microdissection and the remaining cytoplasm flattened on a glass slide in a relatively thick layer. In such preparations, a continuous, regular cross-lattice can be discerned over an area up to 60 × 60 μm due to the spreading out onto a plane of the curved and coiled space network elements of intact cells (Fig. 18). Ectoplasm spread preparations in



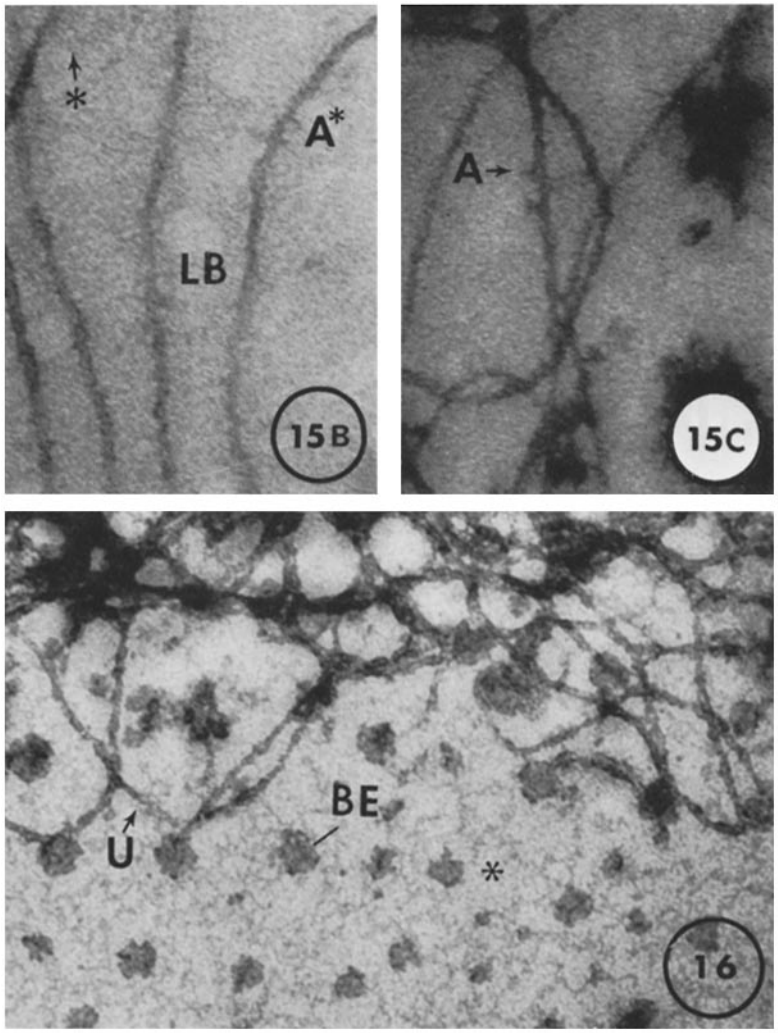


FIGURE 15 A–C Helically intercoiled stalk region (*H*) of the neurofilaments of an ectoplasm spread. Lumps (*L*), cross associations (*A*), and side arms (*A**) of the neurofilaments. Lampbrush-like (*LB*) appearance of the neurofilaments. Microfilament tangles (*) in areas between the neurofilaments. Fig. 15 A, × 210,000; Fig. B and C, × 150,000.

FIGURE 16 Ectoplasm spread treated with cytochalasin B and stained with 1% uranyl acetate. Note microfilament tangles (*) and berry-like aggregates (*BE*) closely associated with neurofilaments. Unravelling of neurofilaments (*U*). × 100,000. (This micrograph was made by Dr. H. A. Hansson in the laboratory of Dr. H. Hyden, Göteborg.)

sucrose viewed by differential interference microscopy display a similar cross-lattice pattern. Most probably the cross-lattice pattern revealed in Deiters' neurons by differential interference microscopy reflects primarily the arrangement of the neurofilament fascicles (42). This conclusion is in accordance with the results of electron microscopy of thin sections of Deiters' neurons and of ectoplasm spreads. A crisscross pattern of neurofilament arrangement can be observed in electron micrographs of thicker sections.

Localization of Actin-Like Filaments by Heavy Meromyosin-Binding Experiments

Actin-like filaments can be identified in the pre- and postsynaptic regions of some synapses on

Deiters' neuron cell bodies and basal dendrites after HMM treatment of glycerinated neurons (see Materials and Methods). In electron micrographs of thin sections, 150–250-Å wide filaments, which display regular arrowhead patterns for short distances, can be observed. Such a pattern is not evident over the entire length of these decorated filaments. However, their diameter and appearance is identical to that of HMM-decorated muscle F-actin which has been embedded and thin sectioned under the same experimental conditions.

Decorated filaments can be observed in a preterminal region (Fig. 19 A) and in postsynaptic areas of Deiters' cell bodies (Fig. 19 B and C). These decorated filaments, which are identical in appearance and diameter to HMM-treated muscle F-actin (cf. inset Fig. 19 A), could not be observed in other portions of Deiters' cell bodies. Control experiments consisted of the omission of HMM treatment or the incubation of HMM-treated cells with 10 mM ATP. In numerous thin sections made from these control preparations, filaments resembling decorated F-actin were never observed (Fig. 20). A large number of ectoplasm spreads freshly isolated in 0.2 M sucrose + 0.05 M KCl, were treated with HMM and negatively stained with 1% uranyl acetate. Filaments decorated in the characteristic arrowhead pattern were never observed in this series of experiments.

TABLE I

The Effect of Enzymes on the Filamentous Components of Ectoplasm Spreads

Enzyme	Concentration	Time of treatment at room temperature	Effect*
	mg/ml	*min*	
Trypsin	5.00	30	+
"	0.05	30	+
Pronase	0.10	30	+
Deoxyribonuclease I	0.01	15–30	–
Ribonuclease A	0.01	15–30	–
Hyaluronidase	0.10	20	–
Neuraminidase	1.00	30	–
Lecithinase C	0.01	20–30	–

* +, total disappearance of neurofilaments; –, no visible effect on neurofilament structure.

DISCUSSION AND CONCLUSIONS

Our observations show that in the perikaryon of Deiters' neurons a neurofilamentous network extends as a continuous formation from the ectoplasm throughout the plasma roads between the Nissl bodies into the perinuclear zone. The thread-like elements of the network are the ~20-Å unit-filaments, the ~90-Å neurofilaments, and the ~0.1-μm neurofilament fascicles. They are associated with each other at the corresponding levels of resolution through intercoiling, intimate contacts, and interchange and appear to be helically ordered. A network of this type may produce a torque in the cell body.

TABLE II

The Effect of Various Agents on the Filamentous Components of Ectoplasm Spreads

Agent	Concentration	Time of treatment at room temperature	Observations
		min	
[^{3}H]Actinomycin D	*	*	No binding
Colchicine	0.1 mM	10	No change
		60	"
Cytochalasin B	0.001%	5	Loosening of neurofilaments Aggregation of unit-filaments
KI	0.6 M	15	No change
KCl	2.0 M	15	No change
$CaCl_2$	2.0 mM	0.5	Spreads become opaque in light microscope
"	2–25 mM	5	No disruption of the neurofilaments

* See Materials and Methods.

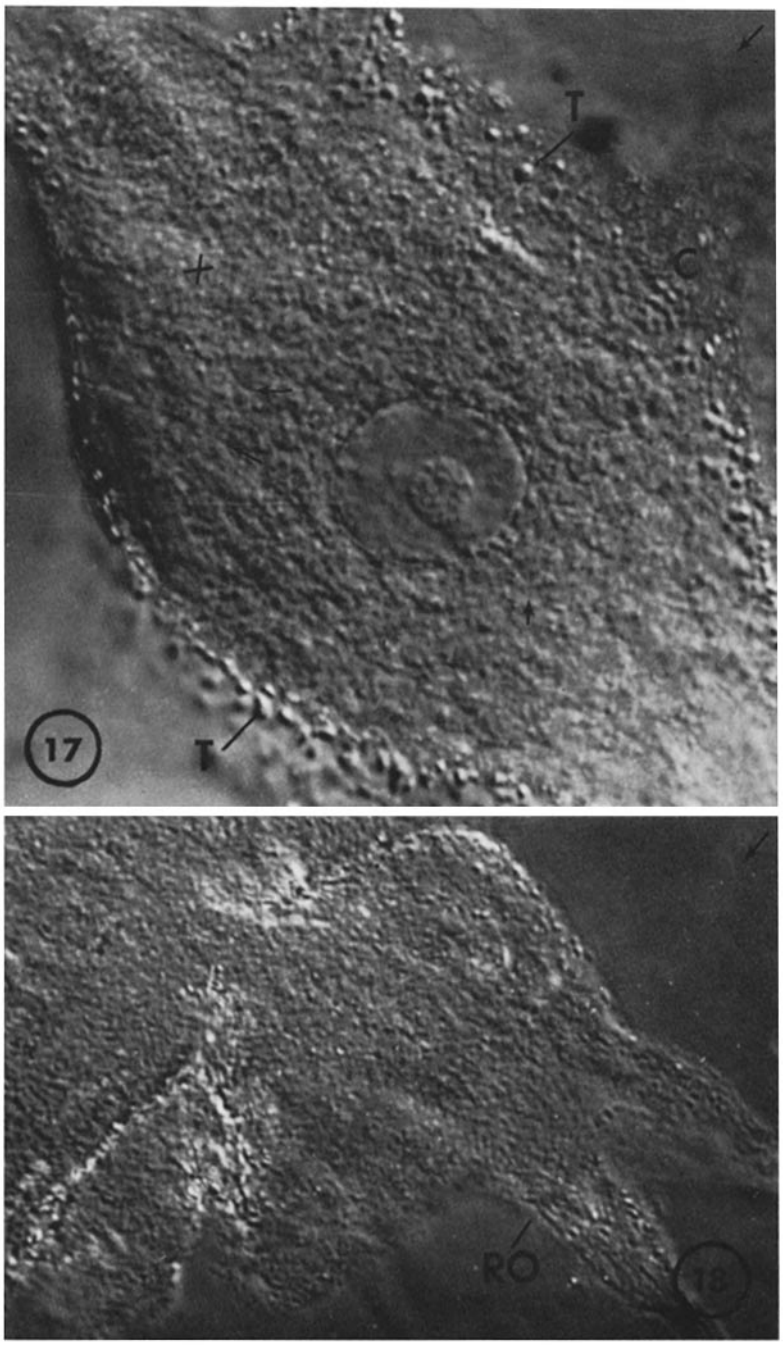


FIGURE 17 Optical section through the center of the cell body of a Deiters' neuron freshly isolated in isotonic sucrose. The arrows indicate threadlike elements. Cross-lattice pattern (*X*); presynaptic terminals (*T*); cortex (*C*). Differential interference 40/0.32. The arrow in the right upper corner indicates the direction of shear. × 1,200.

FIGURE 18 Optical section of a Deiters' neuron perikaryon flattened on a glass slide after partial transection of cell body and removal of the nucleus by microdissection. A regular cross-lattice pattern can be observed extending throughout the whole preparation. Ropelike intercoiling of threadlike elements at the origin of a process, probably an axon (*RO*). Differential interference 40/0.32. The arrow in the right upper corner indicates the direction of shear. × 1,200.

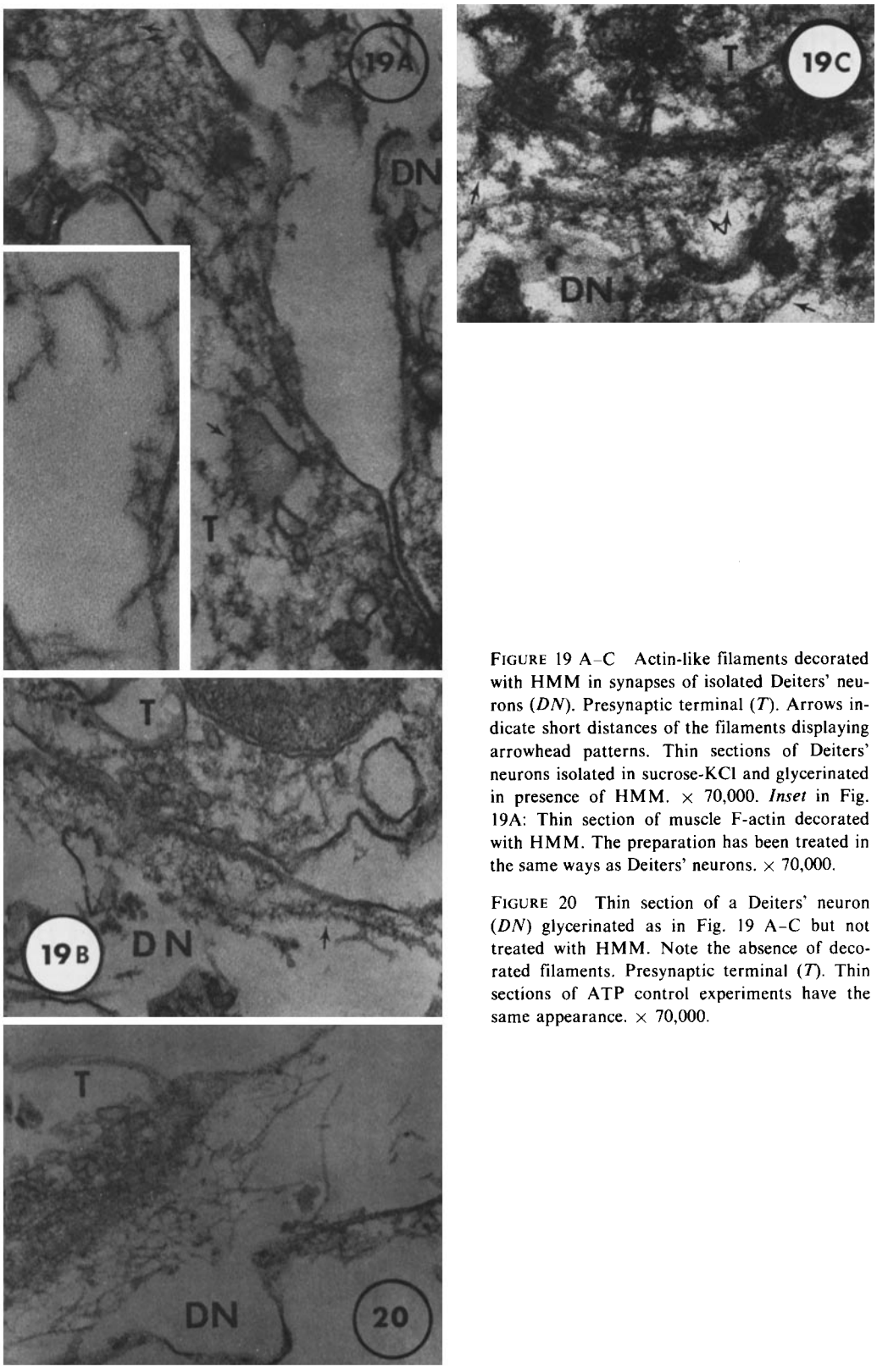


FIGURE 19 A–C Actin-like filaments decorated with HMM in synapses of isolated Deiters' neurons (*DN*). Presynaptic terminal (*T*). Arrows indicate short distances of the filaments displaying arrowhead patterns. Thin sections of Deiters' neurons isolated in sucrose-KCl and glycerinated in presence of HMM. × 70,000. *Inset* in Fig. 19A: Thin section of muscle F-actin decorated with HMM. The preparation has been treated in the same ways as Deiters' neurons. × 70,000.

FIGURE 20 Thin section of a Deiters' neuron (*DN*) glycerinated as in Fig. 19 A–C but not treated with HMM. Note the absence of decorated filaments. Presynaptic terminal (*T*). Thin sections of ATP control experiments have the same appearance. × 70,000.

Cajal (10) emphasized the regular network character of the fibrillar arrays in neurons ("reticulo neurofibrillar") and avoided the term "neurofibril." A cortical and a perinuclear layer, interconnected through an intermediate portion, has been identified in the neurofibrillar reticulum by light microscopy (7, 8). The axonal network is continuous with the perinuclear layer whereas that of the dendrites with the cortical layer (10). The dorsal root ganglion cells do not possess dendrites and do not receive any synaptic input on their perikaryon. There is no cortex in these cells (2) although the plasma roads and perinuclear zone are well developed (48).

High resolution electron microscopy of thin sections of Deiters' neurons and of ectoplasm spreads reveals ~20-Å wide unit-filaments as the supramolecular components of the neurofilamentous network. They intercoil to form ~90-Å wide strands, the neurofilaments (40, 29). The lampbrush-like appearance of portions of neurofilaments in spread preparations support this notion. These conclusions are based on observations of longitudinal profiles of neurofilaments. In interpreting the images of cross- and obliquely sectioned neurofilaments, many uncertainties are encountered due to the possibilities of differences in texture and staining of both surfaces of the sections (47). Stain aggregates, present only on one surface, may enhance the contrast of the image by refracting electrons (27). For these reasons, the supramolecular model of neurofilaments which is based solely on images of cross-sectioned neurofilaments (55) should be viewed with reservation.

A delineation of individual neurofilaments as independent entities is not possible in high resolution micrographs. In order to exclude image superimposition as one of the factors responsible for our observations, a comparison of images of all the structures in question, sectioned at different angles, was carried out. Numerous side arms and cross-associating filaments appear to be in intimate association with the neurofilaments and with the microfilaments in all orientations of the sections against the filaments (39). Intimate associations of the microfilaments with the plasma membrane and with the nuclear pore complexes can be verified at all angles of sectioning.

Electron microscopy of thin sections has limitations in studies of protein assemblies due to the changes caused by fixation, dehydration in organic solvents, and embedding. Information regarding spatial relations among threadlike elements is difficult to obtain from thin sections. We have attempted to avoid some of these drawbacks by isolating thin layers of ectoplasm by microdissection of unfixed Deiters' neurons and placing such preparations directly on grids. Obviously, a partial disruption and distortion of structures resulting from the microdissection of fresh cells, cannot be avoided and should always be considered a possibility. A correlation of observations of ectoplasm spreads with those of thin sections of Deiters' neurons fixed by intravascular perfusion, represent an essential aspect of the present investigation. Most of the basic morphological characteristics and dimensions of the neurofilamentous network in spread preparations, as revealed in electron micrographs at all levels of resolution, are the same as those seen in thin sections. They can be correlated, furthermore, with the results of differential interference microscopy of neurons freshly isolated in isotonic sucrose. The artifactitious origin of the intercoiled unit-filaments and the microfilaments can be excluded with reasonable certainty on the basis of their presence in both the thin sections and the spreads.

The results and conclusions of our structural studies of thin sections and ectoplasm spreads, as well as the results of the treatment of the spreads with various enzymes and solutions, are consonant with those of Shelanski et al. (50). These authors isolated two types of filament bundles from axons: a loose type and a dense type. Both types are chemically similar and appear to differ only in the extent of cross-linkage.

Localization of Actin-Like Filaments

Our observations on the presence of actin-like filaments in the preterminal region of some synapses terminating on Deiters' neurons are in accord with the biochemical findings of Berl et al. (3), who recently proposed that actomyosin may be involved in transmitter release. The exact structural relationship of the decorated filaments with the presynaptic membrane and synaptic vesicles remains uncertain in our preparations because of the relatively poor preservation of fine structure resulting from treatment with glycerol.

The significance of the localization of actin-like filaments in the postsynaptic region is at present obscure, although its participation in the establishment of cell surface contacts or the induction of membrane permeability changes (4), may be possi-

ble. The localization of actin-like filaments in close proximity to the surface membrane is also seen in neuroblastoma cells (12) and in other cell types (24). This may reflect a participation of actin-like proteins in cell surface activities unrelated to neurotransmission. The ultrastructural demonstration and localization of myosin is required for better understanding of the function of a contractile system in the neuron. In spite of the careful scrutiny of numerous preparations, HMM-decorated filaments could not be observed in regions of the Deiters' neuron other than the synaptic ones.

Linkage of the Neuronal Surface with the Nuclear Pores: a Hypothesis

According to our observations, the neurofilamentous network establishes through the microfilaments a direct morphological contact with both the plasma membrane and the nuclear pores. The association of biological membranes with protein networks is a widespread phenomenon (58, 37, 52) and may reflect the participation of such networks in the regulation of membrane functions (25, 18, 57). We have investigated the relationships among the neurofilaments, the microfilaments, and the plasma membrane in nonspecialized areas of the perikaryon as well as in the synapses on Deiters' neurons. In all these regions, the neurofilamentous network appears to be intimately associated and at certain sites even continuous with the microfilaments or with the subsynaptic web, which in turn are in direct contact with the plasma membrane (13). The presynaptic dense projections, which reportedly consist of filamentous aggregates (49), appear in our preparations to be continuous with the microfilament tangle of the preterminal axon.

The intimate association of the neurofilamentous network with the nuclear pore complexes is mediated by 20–40-Å wide microfilaments. The situation is analogous to that which occurs at the plasma membrane. The association of the cytoplasmic filaments with the nuclear envelope has been implied by electron microscope observations of various types of cellular preparations (23). The fact that we observe this contact to occur at the nuclear pores is especially relevant since it provides a basis for hypotheses concerning cell surface-nucleus interactions.

The nuclear pore complexes have a bipartite character consisting of a chromosome element with DNA (1), and a cytoplasmic element (19). The transcription of DNA and the control proteins associated with this process are thought to be located at the nuclear pores (19, 36). Thus, the cytoplasmic filaments which, according to our observations are in close proximity with chromatin fibers at the nuclear pore complexes, may represent regulatory proteins. Such proteins may control the transcription of DNA and also participate in other regulatory mechanisms of the nucleus.

In the early interphase nucleus a specific spatial order of the genome may be maintained by the attachment of the chromosomes to nuclear pores (14, 15). There is compelling evidence for a nonrandom distribution of the nuclear pores (35). The specific surface mosaic properties of the neuron, including its connections and location, could result from the projection of the genomic order to the level of the plasma membrane, thus allowing immediate transcriptional responses to cell surface-related activities. The mechanism underlying this form of transcriptional control could be based on the neurofilamentous network and its associations with microfilaments. Early hypothetical considerations on the significance of the "neurofibrillar lattice" in neuroblasts (28) and in adult neurons deserve mention in this context (44, 26, 9–11).

A dynamic equilibrium may exist between the random arrangement of filaments in tangles and the ordered state which occurs in neurofilaments and their regular semilattice associations. The neurofilaments could be transitory structures in a state of constant assembly from and disassembly into subunits arranged in a paracrystalline matrix (42, 40, 54). A major factor determining the equilibrium between assembled and disassembled states of neurofilaments could be the activity of the plasma membrane at the postsynaptic differentiations and at the axonal terminals. During plasma membrane depolarization, certain agents, such as Ca^{++} or cyclic AMP (20), may interact specifically with the filamentous proteins in the ectoplasm and cause changes in their conformation and a shift in the order of the neurofilamentous network. In this way, the membrane activities would immediately be fixed and transmitted to sites of DNA transcription at the nuclear pore complexes. The properties of the neurofilamentous network appear well suited for such a function.

This investigation was initiated in 1969 when J. Metuzals held a European Molecular Biology Organization

Professorship while on Sabbatical Leave in Göteborg. W. E. Mushynski is a Postdoctoral Fellow of the Medical Research Council of Canada. The isolation experiments were carried out in collaboration with Dr. H. Hydén. Dr. Hans-Arne Hansson participated in the electron microscope evaluation of drug-treated ectoplasm spreads. Perfusion experiments were carried out in cooperation with Dr. M. Colonnier, Dr. H. Rowsell, and Miss C. Beleydier. The technical assistance of Mr. D. Lee, Mr. P. Chow-Chong, and Mr. V. Redmond is gratefully acknowledged.

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