*FIBROUS PROTEINS—NEURONAL ORGANELLES**

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At the turn of the century, thin fibrous structures, having thickness close to the resolution of the light microscope, were seen in silver-stained preparations of many kinds of nerve cells. Called "neurofibrils," these fibrous proteins were thought to be vital organelles of neurons and at first were believed to conduct the nerve impulse. After the surface membrane was found to be the site of bioelectric properties, the role of the fibrous protein had to be reassessed. Physiologists tended to regard neurofibrils as artifacts of fixation. Polarization optical evidence supported the existence in fresh axons of a fibrous protein having thickness small with respect to the wavelength of light.[1] At midcentury the fibrous protein was observed by electron microscopy in unfixed preparations[2, 3] and subsequently, by several investigators, in thin sections of fixed preparations. The protein became known as the *neurofilament;* the term *microfilament* is a generic term for similar structures found in many types of cells.

For many years the fibrous proteins of axoplasm have been studied in our laboratories and attempts made to characterize them physicochemically and to determine their function. Progress was slow because only small amounts could be obtained from the giant fibers of the squid *Loligo pealii* from Cape Cod waters during the summer months. Brain as a source of protein was not practical at that time because of the lack of an identifying marker constituent such as a particular amino acid (as collagen is characterized by hydroxyproline, for example). The problem of year-round supply of adequate amounts of fresh axoplasm was solved by procuring it from the large squid *Dosidicus gigas*, available during most of the year at the Marine Biological Station of the University of Chile near Valparaiso.

Microtubules, recently shown to be present generally in plant and animal cells, represent a portion of the fibrous proteins of neurons and probably play a role qualitatively similar to that played in other cells, i.e., to subserve processes of motility and transport of solutes and particulates directionally within cells. Microtubules are also thought to provide mechanical strength and rigidity which could be of importance in neurons, with their enormously extended axonal and dendritic processes. A more detailed presentation of the evidence concerning the probable function of microtubules and neurofilaments will be given in a report of a Neurosciences Research Program work session by Schmitt and Samson.[4]

Electron-Microscopic Characterization of Neurofilaments and Microtubules.—With the electron microscope two fibrous forms are observed in neurons: neurofilaments and microtubules. The two types resemble each other in fundamental ultrastructure and in chemical composition. Both types are shown in the electron micrograph of Figure 1. Neurofilaments are about 100 Å in diameter; microtubules are 240 Å in diameter, with a wall thickness of 50 Å. In longitudinal sections both structures are straight, apparently rigid, and unbranched, extending

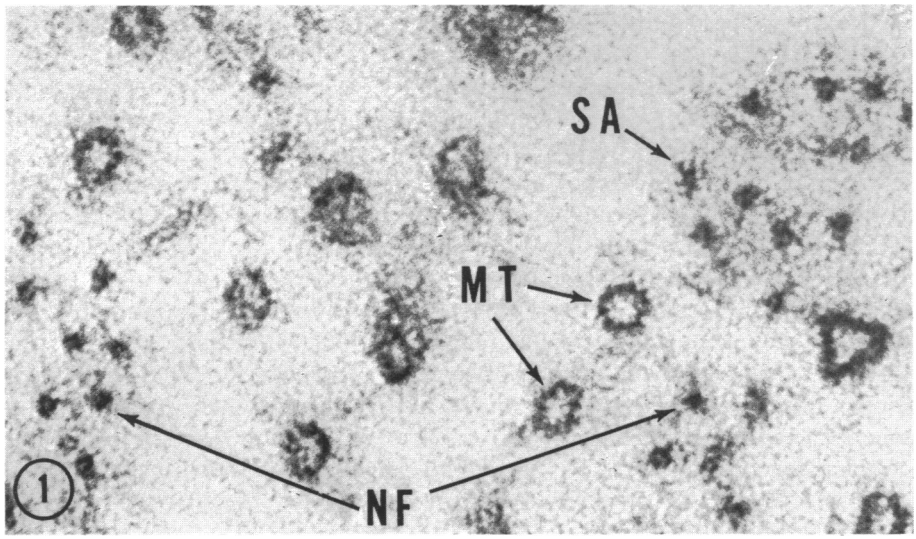


FIG. 1.—Electron micrograph of anterior horn cell of the rat, showing microtubules (*MT*) and fascicles of neurofilaments (*NF*) in a dendrite. Neurofilaments are similar to those in cell bodies and axons. Notice subunits in neurofilament walls, also radiating side arms (*SA*). Magnification, 280,000. Original electron micrograph courtesy S. L. Palay and comparable with those shown by Wuerker and Palay.[5]

continuously in the axon or dendrite. Cross-bridges between microtubules have been observed by Kohno,[6] who found them particularly evident in the region of the axon hillock. Radially directed outgrowths like spokes, which may represent cross-bridges, are frequently seen on the neurofilaments (Fig. 1). As in the case of muscle, cross-bridges may be important functionally.

Overproduction of fibrous protein is characteristic of brain cells in the presenile dementia called Alzheimer's syndrome and in certain abnormalities such as neurolathyrism, produced experimentally in animals by feeding compounds like iminodipropionitrils or by treating the brain with aluminum salts.[7] Characteristically, twisted microtubules or bifilar neurofilaments pack the cells.[8, 9] This overproduction of fibrous protein may be so great as to lead to the exclusion of other vital organelles, such as mitochondria, and to subsequent cell death.

Because some ten million people in this country alone suffer from senile manifestations of various kinds, the problem is one of much clinical concern. It is also of importance in aging processes. Investigation of factors controlling the production of fibrous proteins is therefore a critical one of great human concern as well as scientific interest.

Biophysical and Biochemical Characteristics of Neurofilaments and Microtubules.—During the last decade improved methods have been developed for the purification of the fibrous proteins from axoplasm extruded from the giant nerve fibers of *Dosidicus gigas*. In the presence of 0.1 *M* mercaptoethanol the unrefrigerated material is stable for shipment from Chile to our laboratories in Cambridge. Neurofilaments such as those shown in the electron micrograph in

Figure 2 may be obtained by zone sedimentation in a sucrose gradient or by freezing, which causes the filaments to separate upon thawing.

Dried cylindrical extrudates of axoplasm have high positive birefringence.[12] However, wide-angle X-ray diffraction patterns obtained from such preparations show no evidence of orientation of the fibrous proteins; only two rings characteristic of unoriented backbone and side-chain spacings are recorded. These findings indicate that neuronal fibrous protein is not of the α or β type.

P. F. Davison and E. W. Taylor,[13] of our laboratory, suggested in 1960 that neurofilaments are built of globular subunit protein molecules, somewhat along the lines of tobacco mosaic virus (TMV). Their model is illustrated in Figure 3. Globular subunits of 30–40 Å in diameter are depicted as forming strands which are wrapped about each other to form a filament 80–100 Å in diameter. Unlike TMV protein, the neurofilament has neither RNA nor nucleotide component. A central aqueous channel *ca.* 30 Å in diameter, though unnecessary for the theory, was pictured.[14] Several investigators have confirmed the general features of the model by high-resolution electron micrographs obtained from transverse sections of axons.

Because the protein subunits, as in TMV and other viruses, have a strong affinity for each other, isolation of monomer subunits in the native state is extremely difficult. This pronounced affinity may be due primarily to bonding of hydrophobic residues within the subunit molecules. Increase of ionic strength or of pH leads to partial depolymerization of neurofilament strands. Reversible aggregation of these subunits into the native helical structure has not yet been achieved in such preparations.

In our laboratory, Davison and F. C. Huneeus[15] have studied the protein subunits constituting the neurofilaments. Dissociation of the filamentous structure may be accomplished by treatment with detergents, by dissolution in concentrated 6 *M* guanidine hydrochloride, or by succinylating the protein and blocking the thiol groups. Chromatography of such dissociated preparations on agarose columns[16] has resolved two components, the smaller of which has an amino acid composition similar to that of microtubules and probably makes up the subunit skeleton of the neurofilament. The second component, also an acidic protein, has a high content of serine (*ca.* 12%) and may be an asymmetric protein (giving hypersharp peaks in the ultracentrifuge pattern). This protein may conceivably represent the fuzz, spikes, or cross-bridges intimately associated with the neurofilament protein as seen in the electron micrographs of thin sections of neurons of various kinds.[5, 6, 17] The function of this material remains unknown.

Microtubule protein has not yet been isolated from squid neuroplasm, though its colchicine binding is high.[18] Colchicine binds strongly to microtubule protein (as originally demonstrated for the microtubules of the mitotic apparatus) and also to neuroplasmic protein, presumably microtubule protein, according to Borisy and Taylor.[18] Weisenberg and Taylor[19] used this property to isolate microtubule protein from mammalian brain homogenates. According to Taylor,[4] the protein thus isolated, having a molecular weight of about 60,000, has amino acid composition similar to that of microtubules generally, to squid neurofilaments, and to actin. Microtubules in certain tissues are unstable at

low temperatures and high pressures, but are stabilized by D_2O, suggesting that subunit interbonding is primarily by hydrophobic forces.

Recently, Kirkpatrick[20] has fractionated microtubules, having characteristic appearance in electron micrographs, from homogenates of mammalian brain.

For comparison with the model of neurofilament structure, the model for microtubules is shown in Figure 4 (see also Tilney and Porter[21]).

Functioning of Microtubules in Cells Generally.—Microtubules are thought to give rigidity to long slender cell processes such as the axopods of Heliozoa;[21] to provide motility in the case of cilia and sperm tails, perhaps by some kind of sliding-filament mechanism;[22] and to mediate transport of cell particulates, e.g., in the movement of melanin granules into and out of the elongate arms of melanocytes[23] and of secretory vacuoles from the Golgi apparatus to the periphery of the rhabdite in *Planaria*.[24]

One of the most graphic examples of microtubule-implemented intracellular transport has been demonstrated by Rudzinska[25, 26] in the feeding process of the protozoan *Tokophrya*. The feeding apparatus in this sessile suctorian consists of tentacles—fine, thin, stiff tubes which broaden considerably at the tip. At the periphery of the knoblike tip are complex missilelike particulates containing enzymes. When a swimming ciliate such as *Tetrahymena* touches the knob of the tentacle, the ciliate becomes attached and immobilized. Apparently the protruding ends of the particulates are sticky and release a toxic substance into the prey upon contact. At the same time a stream of additional particulates starts to move rapidly outward through the tentacle from the cytoplasm of *Tokophrya*. Shortly thereafter, connection is made between the tentacle and the prey cell; constituents of the cytoplasm of the prey pass through the tentacle into the body of the predator. The two streams move in opposite directions, though not simultaneously: the outward stream of the particulates precedes the inward flow of the prey's cytoplasm. Forty-nine microtubules, arranged in seven clusters each consisting of seven microtubules, divide the thin cylindrical tentacle roughly into an outer tube, in which the missile-shaped particulates travel centrifugally, and an inner tube, in which cytoplasmic constituents pass as food from the prey centripetally into the predator cell.

It seems improbable that such directional movement is due to flow resulting from gradients of hydrostatic pressure; rather the microtubules might provide a structural basis for the active transport of food from the prey to *Tokophrya*.

Neuroplasmic Transport.—During the last 20 years, P. Weiss and his collaborators[27–29] have shown that neuroplasm constantly flows unidirectionally from the cell body down the axon. It is estimated that neuroplasm equivalent to one to three times the volume of the nerve cell is synthesized per day. Weiss has interpreted the phenomenon as one of maintaining metabolism of parts of the axon remote from the cell center. This discovery, which has given a new dynamic dimension to neurobiology, calls for explanations at the molecular level. The phenomenon has now been confirmed by radiolabeling experiments in many laboratories.[30] In addition to the slow, 1–6 mm/day velocity,[29–32] much faster rates (hundreds of millimeters/day[33–35] and even up to 2800 mm/day[36]) have been observed. This fast movement, which appears to be specific with respect

to the substance transported, may be saltatory, i.e., discontinuous translatory movements, quite different from Brownian movement.[37] Neuroplasmic transport, which is bidirectional in thin channels containing a colloidal system of high structural viscosity, is not fully understood at the present time. However, it is improbable that particulates are transported by gradients of hydrostatic pressure; rather, a local topochemical reaction on the surface of the microtubule seems responsible. One of the most striking cases in support of this conclusion is the rapid (100–200 mm/day) movement of catecholamine storage granules down the axons of sympathetic C-fibers which are only 0.5–1.0 μ in diameter.[33] Hydrostatic pressure gradients can probably be ruled out; they would have to overcome great shear forces to move particles so rapidly in such thin tubes. Microtubules appear to be the chief or only fibrous structures seen with the electron microscope in such thin nerve fibers. It is reasonable, therefore, to suggest that a local interaction on the surface of the microtubules is responsible for such rapid, probably saltatory movements. The hypothalamic neurons, in which secretion granules move at high velocities (e.g., 2800 mm/day or 32 μ/sec[36]), have recently been shown to contain many microtubules;[38] this finding supports the view that the transport is caused by the interaction between microtubules and secretion granules.

Mechanochemical Coupling in Transport.—How can rapid, saltatory transport of particulates such as vesicles, secretion granules, melanine granules, and even whole nuclei be explained at the molecular level? Clearly a transduction of chemical to mechanical energy is involved. The detailed mechanism by which mechanochemical coupling occurs, though unknown, is of great biological interest.

Perhaps by reference to muscle—the tissue differentiated for mechanochemical coupling—we may gain some insight into the coupling process. According to the sliding-filament theory,[39–42] the fibrous proteins myosin and actin do not themselves contract when muscle shortens. Rather the filaments slide by each other due to the thrust exerted on the actin by the heavy meromyosin cross-bridges. At the end of the cross-bridge is the enzyme adenosine triphosphatase (ATPase). Bound to the actin is ATP, the source of energy for the contraction. At the binding site, actin combines with myosin, forming actomyosin. The ATPase, activated by Ca^{++}, presumably at another site on the actin, splits off

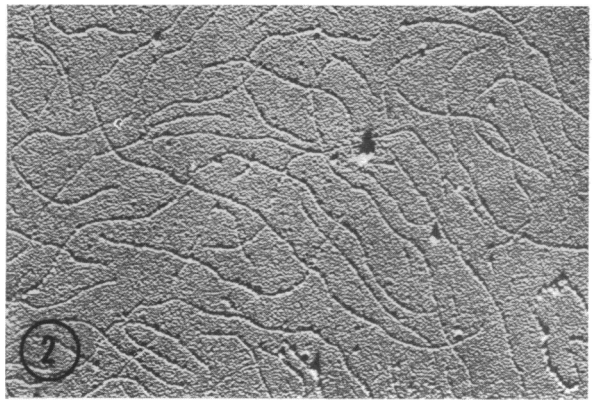
FIG. 2.—Electron micrograph of neurofilaments isolated from axoplasm of squid giant axon, as described by Maxfield.[10, 11]

FIG. 3.—Model of subunit organization of neurofilament, after Davison and Taylor[13] and Schmitt and Davison.[14] (*A*) Monomer subunit; (*B*) subunit strand; (*C*) neurofilament, longitudinal view; (*D*) transverse section.

FIG. 4.—Model of subunit organization of microtubules.

FIG. 5.—Postulated mechanism of active transport of vesicles by interaction with microtubules; model based on the sliding-filament hypothesis of mechanochemical coupling in muscle. On the left is shown a subunit strand of a microtubule; on the right are subunits in the protein wall of a vesicle. Significance of sites for binding the two proteins and for activating the ATPase (or GTPase) is described in the text.

FIG. 6.—Diagrammatic illustration of the roles of the axonal surface membrane in propagating the action potential and of the axoplasm in transporting certain vesicle-enclosed transmitters to synaptic endings by means of fibrous proteins.



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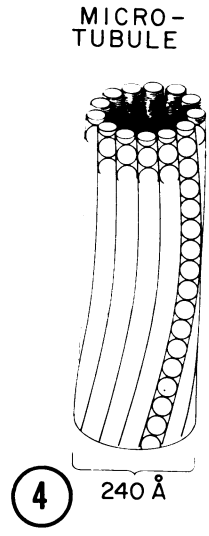
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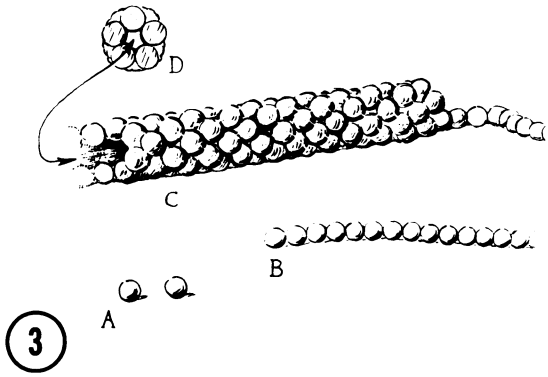
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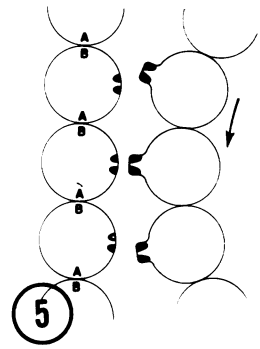

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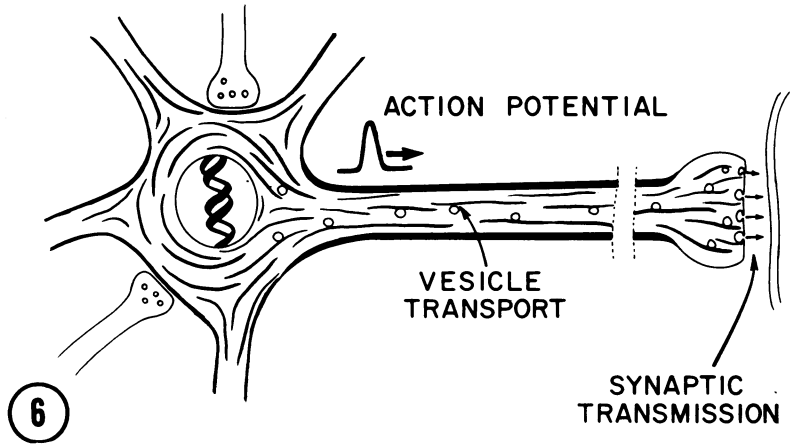
ACTION POTENTIAL
VESICLE
TRANSPORT
SYNAPTIC
TRANSMISSION

6

ATP; and energy is transferred, causing conformational change of the myosin cross-bridge of a kind that delivers a thrust to the actin, which then moves with respect to the myosin. In this mechanism, precise dimensional and steric conformance is required between actin and myosin subunits.[42]

It is not obvious how a sliding-filament system could cause transport of substances in neurons; nor is there evidence that the microtubules and neurofilaments interact through a sliding movement of one with respect to the other (though cross-bridges have been observed between microtubules[6]). However, on the basis of the circumstantial evidence of the presence of microtubules and their conjectured ability to transport particulates in other tissue, a speculative model (Fig. 5) may be suggested as a tentative working hypothesis.

Substances to be transported, such as certain transmitter molecules and enzymes or secretions, are contained in protein vesicles. It is suggested that the walls of the vesicle contain protein monomers, the diameters of which are congruent with those of the monomer subunits of the microtubule which presumably contain the source of energy, ATP or guanosine triphosphate (GTP). On the vesicle monomers, it is postulated, is a binding site for the microtubule subunit and another site where the ATPase (or GTPase) is activated. When the ATP is hydrolyzed, energy is coupled, and conformational changes occur which provide a thrust, moving the vesicle, so that a different vesicle subunit is in apposition with a different subunit of the microtubule, and the thrusting process is repeated. The vesicle thus moves or jerks along the microtubule or neurofilament in a direction dependent on the sense of the helix of the microtubule or neurofilament.

A similar mechanism may be responsible for the saltatory and directional movement of the particulates within cells generally. Holmes and Choppin[43] find that infection of hamster kidney cells with parainfluenza virus SV5 produces multinucleate syncytia in which nuclei, arranged in long parallel rows, migrate straight to the center of the syncytium. The cytoplasmic channels between the nuclear rows are birefringent, probably due to bundles of microtubules, shown by electron microscopy to be present in the channels and intimately associated with the nuclei. Colchicine treatment blocks nuclear movement. Holmes and Choppin suggest that the microtubules may determine the direction of nuclear migration and may provide the motive force for their movement. Such a transport of vesicle-enclosed, biochemically potent compounds directionally to particular intracellular locations may be evolutionarily older than the sliding-filament mechanism of muscle, which was presumably differentiated to develop large contractile force rapidly and on a macroscale.

Role of Fibrous Proteins in Molecular Neurobiology.—The slow, 1–6 mm/day, unidirectional, nonspecific flow may be effected by a mechanism different from the fast, specific, potentially bidirectional transport. Weiss[29] attributes the former to hydrostatic forces exerted upon the axoplasmic column as a whole, presumably by peristaltic contraction of a sheath structure; alternatively the slow movement may be actuated by neurofilaments. The fast transport, however, is most reasonably attributable to the microtubule system, which is associated with saltatory transport in cells generally.[37] Whether there is any coupling

between the propagated action potential wave and the transport of particulates, a process which may seem reasonable teleologically, is unknown; according to Kerkut *et al.*,[34] fast transport of glutamate occurs only in stimulated nerves.

As is illustrated diagrammatically in Figure 6, transport of transmitters down the axon to synaptic terminals is as vital to interneuronal transmission as is the action potential, at least in nerve-fiber types where the transmitter is not synthesized in terminals and must make the journey down the axon. Vesicles are in this category, and it is interesting that Weiss[44] has observed accumulation of vesicles in axons, the peripheral flow of which had been dammed by compression. The situation is clear in the sympathetic neurons in which catecholamine storage granules carry the transmitter norepinephrine, which is thought to be synthesized in the cell body and perhaps also within the storage vesicle that is transported down the axon to the ending. Lysosomes containing enzymes, possibly involved in protein metabolism at terminals, are known to be transported down the axon.[45]

Dendrites are rich in microtubules. Experiments have not yet been devised to test whether transport occurs in dendrites, but it seems probable that such may be the case.

If the synthesis of specific proteins is involved in memory consolidation, their transport to synaptic endings may play a vital physiological role in the plastic regulation of cell-to-cell interaction, as in learning, for example. In connection with this transport, Barondes[46] recently found that protein synthesized from labeled amino acid precursor passed down the axon to endings where, he suggests, the protein combines with mucoid constituents at the synapse; these proteins, he thinks, may be concerned with plastic alterations on which learning depends. Bogoch[47] has also speculated along these lines.

By means of the fibrous proteins of microtubules or neurofilaments, materials biosynthesized in the cell center may be directionally transported to various sites on or within the neuronal membrane; thus the neuron's ongoing bioelectric activity may be modulated not only by synaptic inputs, but also by the neuron's genetic and biosynthetic mechanisms. Substances impinging extracellularly on the neuronal membrane or generated near it may be rapidly transported to the cell center, there influencing gene expression. Such processes may be especially important in the development of the brain, possibly through the action of specific nerve growth factors.

Further study of the fibrous proteins may lead to a better understanding of neuronal processes at the molecular level and provide a basis for a more effective investigation of the biological, neurophysiological, and psychological phenomena.

Summary.—Neurofilaments, *ca.* 100 Å in diameter, and microtubules, *ca.* 240 Å in diameter, form the basis of the fibrous structures in neurons described in classical neurohistology. Not unique to neurons, these two fibrous types are found in cells generally and function as important organelles.

Neurofilaments from squid axoplasm have been isolated, their probable ultrastructure has been deduced, and the composition and properties of the protein subunits have been determined.

It is suggested that neuronal microtubules are functionally concerned with the movement of particulates and organelles, specifically with the fast (10^2 to 10^3 mm/day) transport of specific neuroplasmic constituents down the axon and possibly also within the cell body and dendrites. A tentative hypothesis is proposed for the molecular mechanism of such transport. Neurofilaments may also be involved in neuroplasmic movement, possibly of the slower (1–6 mm/day) type of neuroplasmic flow.

Transport of certain transmitters, neurosecretions, and trophically active substances from the site of synthesis in the cell body to axon endings appears to be mediated by the fibrous proteins. Regulation of the synthesis of these proteins is therefore vital to normal neuronal function.

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