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NEURONAL FIBROUS PROTEINS

A Review Based on Two NRP Conferences

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NEURONAL FIBROUS PROTEINS

A report based on two NRP Conferences held
August 16, 1967, and January 15, 1968

by

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I. INTRODUCTION

The existence in living cells of fibrous structures, thin with respect to the wave length of light, was indicated from early polarization optical studies (Schmidt, 1937). A "cytoskeleton" composed of a framework of submicroscopic fibrous material was postulated in the early thirties by some investigators on the basis of certain rheological properties of living cells (see, for example, Needham, 1936). After-fixation methods were improved, the presence of well-defined microtubules was demonstrated by electron microscopy.

Microtubules, about 240 A in diameter, are now known to be composed of globular protein molecules or subunits which, through their particular mode of aggregation, determine the characteristic morphology of the microtubule. The even thinner fibrous structure, the microfilament, about 40 to 100 A in diameter, has also been observed in various types of cells by electron microscopy. Microfilaments, such as neurofilaments, are composed of subunits and may be essentially tubular in nature. Although the ubiquity in living cells of these fibrous structures is now established, little is known concerning their role; they may be regarded as organelles vital to cell function. The status of our knowledge about these organelles is comparable with that about mitochondria a generation ago.

Because these fibrous structures, which are prominent in neuronal and glial cells, may have specialized functions in brain cells, the Neurosciences Research Program scheduled a one-day conference on microtubules to assess the state of the art and to discuss their possible functional roles. Several months later, another one-day conference was held to discuss the relationship of microtubules and microfilaments to microscopically visible fibrous components, such as neurofibrils, which have been widely discussed in the classical neurohistological and neurophysiological literature.

This report of the two conferences, as well as the chairman's essay, has been expanded to provide a discussion of a subject that is clearly of burgeoning significance for the neurosciences. For example, evidence, still mostly circumstantial in nature, closely links the fibrous organelles with the movement of products synthesized in the perikaryon down the axon to synaptic terminals and probably also out into the dendritic tree. Among such transported products are those having certain transmitter, neurosecretory, and trophic functions, all vital to the operation of the neuron and innervated tissues.

Most of the literature relating to microtubules has thus far been of a descriptive and morphological kind. Recently the isolation of certain types of microtubules in fairly homogeneous preparations from mitotic spindles and sperm tails has provided the opportunity for detailed physicochemical studies. These have shown that the microtubule* and the neurofilament are crystallographically defined fibrous structures and are not tubules of widely varying diameters within individual cells, nor does the individual microtubule characteristically vary in diameter along its length.

At this time of increasing knowledge from widening areas of investigation, it is hoped that this report, which surveys the high points of the subject of neuronal fibrous proteins, may stimulate further research and conceptualization concerning the role of these organelles in brain-cell functions.

*Microtubules are sometimes confused with tubules of varying diameter (usually substantially larger than microtubules), which may derive from the endoplasmic reticulum. These, together with characteristic microtubules, are referred to collectively by some authors as "neurotubules."

II. THE MOLECULAR BIOLOGY OF NEURONAL FIBROUS PROTEINS

An Essay by Francis O. Schmitt

With the Editorial Assistance of H. E. Schwenk

The problem of the physicochemical properties and the physiological function of the fibrous proteins of the neuron has long been a major concern of many neurobiologists and of investigators in this laboratory in particular. The subject has been considered in a previous NRP Work Session on neuronal proteins (Schmitt and Davison, 1965). In the last few years, progress has been rapid in three areas that, when considered together, suggest new theoretical and experimental approaches to molecular neurobiology. These areas are: 1) slow neuroplasmic flow and fast specific transport of substances in the nerve axon; 2) the active role of microtubules in transport of particulates within cells generally; and 3) isolation and characterization of neuronal fibrous proteins and of their subunits.

The first of these topics was the subject of a Work Session already reported (Barondes, 1967); the second and third were considered in consecutive NRP conferences and are reported in this issue of the *NRP Bulletin*.

In this essay the writer attempts to articulate information communicated at these conferences with other data in order to present a point of view concerning this important and fast-moving field. A brief exposition of this view was presented in a symposium paper before the National Academy of Sciences (Schmitt, 1968). The Symposium on Frontiers of Molecular Neurobiology included papers by Bullock (1968), Lehninger (1968), Whittaker (1968), and Schmitt (1968).*

Historical: The Neurofibril Hypothesis

Eighteenth- and early nineteenth-century theories of brain function portrayed the brain as a giant reticulum of interconnected structures

*These proceedings also appear in a Supplement (to Volume 6) of the *NRP Bulletin*.

which somehow subserved the processing of psychological information. The discovery of fibrous structures, later called *neurofibrils* (Valentin, 1836; Remak, 1837; Müller, 1838; and Purkinje, 1838), and their characterization microscopically by Schultze (1871) led to the so-called neurofibril hypothesis, which became fairly prominent at the time. According to this hypothesis, neurofibrils were considered one of the fundamental structures of the nervous system in propagating nerve impulses throughout the reticular network of the brain.

Important in this development was a technique, introduced by Apáthy (1889), of silver and gold staining that, though poorly reproducible, did occasionally reveal the neurofibrils with great clarity as delicate long strands with widths near the resolving power of the light microscope (Apáthy estimated the thickness to be about 0.25 μ). It was believed that neurofibrils consist of a parallel alignment of thin elongate structures considered to be fundamental, vital, and self-replicating units. This idea was subscribed to by the great authorities of the day, each of whom seemed to have a different name for the fundamental elongate subunit, e.g., Apáthy's *neurotagmen*, Heidenhain's *protomeres*, and Cajal's *neurobiones*. As related by Peterfi (1929), these units were thought to possess autonomous powers of growth and division by which they achieved microscopically visible size as elementary fibrils.

Adherents of the neurofibril hypothesis accepted the continuity theory stating that neurofibrils are continuous across the synaptic gap and, indeed, may pass outside the neuronal cell body into intercellular space; the impulse was thought to be conducted in the neurofibrils whether their course was intra- or extra-neural.

Later Bielschowsky (1902) developed an ammoniacal silver method and Cajal (1903) introduced his reduced silver method. These techniques led to new investigations of the ubiquitous neurofibrillar organelle of nervous tissues. Cajal carefully studied the distribution and intracellular course of neurofibrils as preserved by his method, and many of his figures are reproduced in standard textbooks to this day. If modern molecular neurobiology could assign specific functions to the neurofibril as an organelle, the drawings, made at the level of the light microscope, by Cajal and his successors might provide important clues to the function of the fibrous protein in different parts of the neuron, i.e., dendrites, soma, axon, and endings.

A powerful proponent of the neurofibril hypothesis was the eminent German general physiologist Albrecht Bethe (1900). With his methylene blue and toluidine blue method for vital staining of neuro-

fibrils, he demonstrated an important redox relationship between neurofibrils and bioelectric processes which he attributed to a hypothetical "fibrillary acid," thought to be a component of neurofibrils.

Proponents of the neurofibril theory believed that the delicate strands not only cross synapses, but also form reticular structures in the extracellular space, particularly in neuropiles seen at that time as a jungle of poorly resolved fibrous structures, and now shown by electron microscopy to be very thin nerve fibers and glial processes. The nerve impulse was visualized as passing along the delicate neurofibrillar strands and passing into and out of neurons in the giant reticulum of the brain.

In opposition to the neurofibril-continuity theory was the neuron doctrine, as it is now known. First clearly enunciated by Kölliker (1891, 1896) and Waldeyer (1891), it stated that the nervous system is composed of discrete cellular neurons, contiguous but not continuous with each other at synapses, and representing the true units of the nervous system. In the intellectual contest between proponents of the neurofibrillar, continuity, reticular theories and those favoring the neuron theory of contiguity, Sherrington (1906) took a middle ground; he suggested that continuity may exist in invertebrates, but that in vertebrates, particularly the mammals whose CNS had been extensively studied, discontinuity prevailed, in agreement with the neuron theory.

Meanwhile physiologists and biochemists were beginning to doubt that the fibrils seen in fixed and stained histological preparations truly represented structures in the living cell; many investigators regarded the fibrils as gross fixation artifacts. Marinesco (1914) had pointed to the extreme lability of neurofibrils, although Boeke (1926) and Cowdry (1928) believed that there must be a fibrous reality in living neurons represented by the neurofibrils of fixed preparations. Critics like Bayliss (1927), a leader in general physiology of his day, pointed to the work of Hardy (1899) in demonstrating the coagulating power on colloidal proteinaceous systems of reagents such as were used in cytological techniques. From his detailed review, Peterfi (1929) concluded that elongate micelles exist in the living neuron and that the appearance of neurofibrils depends on the tendency of the elementary micelles or subunits to aggregate linearly, a tendency readily influenced by electrical polarization and other physical factors, as well as by the action of reagents. At this juncture in the transition from the nebulous concepts of "colloidal" chemistry to the modern understanding of macromolecular properties, the best guess was that nerve impulse propagation depended upon a subtle, transient interaction of elongate protein micelles, aggregating linearly in excitation.

In some forms and under certain conditions such as in tissue culture, fibrous structures were observed in the living, unfixed, though not necessarily normal, state (Bozler, 1927; Speidel, 1935; Weiss and Wang, 1936).

Even in dark-field illumination no fibrous structure was seen in fresh axons. However, immediately upon treatment by certain salts, by alcohol, or by similar reagents commonly used in fixatives, the neurofibrillar structure appeared (Ettisch and Jochims, 1926). Polarization optical methods for investigating molecular orientation in living cells was applied also to neurons (see Schmidt, 1937; Chinn, 1938). Early evidence (Apáthy, 1897) of dichroism in gold-stained axons suggested that the submicroscopic structures possess considerable anisotropy. Application of imbibition methods for the study of form birefingence led Bear, Schmitt, and Young (1937) to conclude that a fibrous protein exists in living axons (at least in the squid giant axon which was under investigation) and that the partial volume occupied by this oriented fibrous component of the axon must be small. These conclusions were in general confirmed later by Thornburg and De Robertis (1956).

In an excellent review of the function of neurofibrils, Parker (1929) pointed out that neurofibrils follow a course, with respect to the neuronal cell body and axon, which might be expected of the flux of metabolites, rather than the route of propagation of the nerve impulses. He doubted that neurofibrils demarcate "streams of materials emanating from the region of the nucleus and percolating throughout the neuron"; nevertheless he suggested that neurofibrils "are the parts of the neuron concerned with the metabolism of its more distant parts." It would appear that Parker may have come close to the truth in supposing that the fibrous protein transmits metabolic influences to the farthest reaches of the axon.

Ultrastructural Characteristics of Neuronal Fibrous Proteins

In addition to the neurofilaments, which since 1950 have been identified as the fibrous protein of the neuron (Schmitt, 1950; Schmitt and Geren, 1950; Fernández-Morán, 1954, 1957), a second fibrous type, the microtubule, must now be added. Microtubules had indeed been observed in neurons as long ago as 1956 by Palay, but only in the last half dozen years has the microtubule been identified as a cellular organelle present in most tissue cells. A detailed description of microtubules is

contained in this issue of the *NRP Bulletin.* Here are summarized only those general features essential to the development of the thesis of this essay.

Neurofilaments and microtubules resemble each other in molecular organization and in chemical composition. In electron micrographs of longitudinal thin sections (see Figure 1), neurofilaments are seen as 80-100-A-wide filaments. They are straight and unbranched, extending presumably continuously in the axon or dendrite. Radially directed

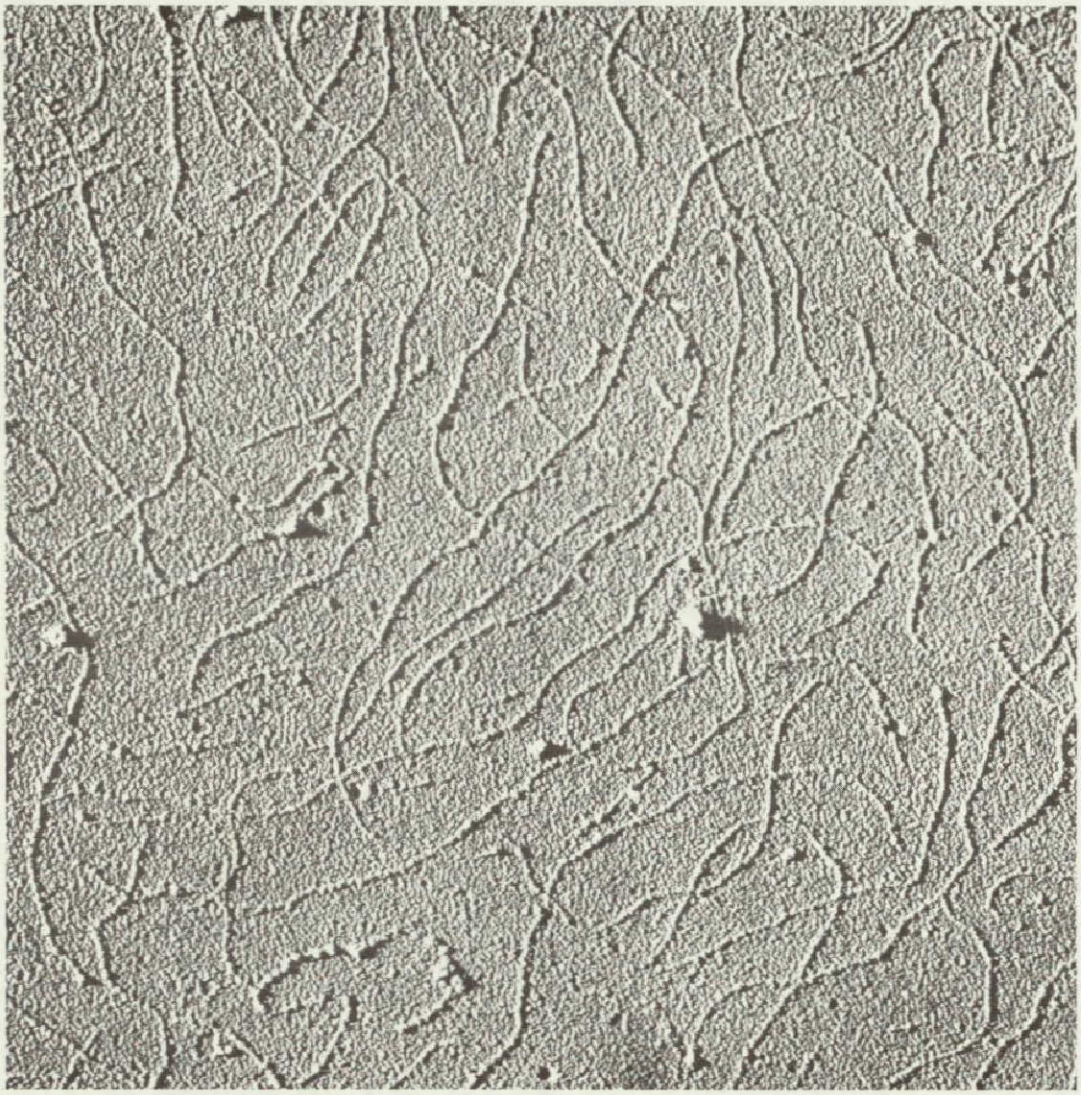

Figure 1. Neurofilaments isolated from axoplasm of squid giant axon. Magnification, X 26,000. [Maxfield, 1953]

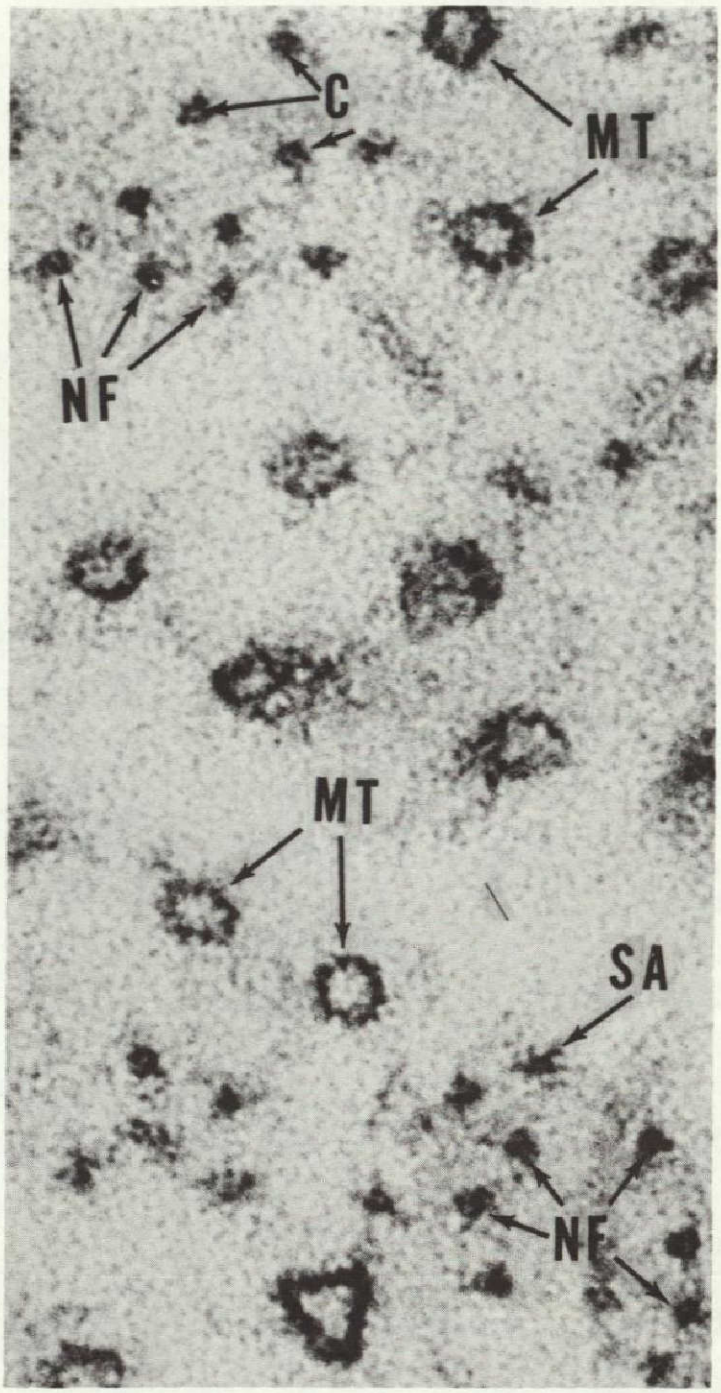

Figure 2. Electron micrograph of dendritic region of anterior horn cell of the rat, showing microtubules (MT) and fascicles of neurofilaments (NF). Notice subunits in neurofilament walls, also central regions of low density (C) and side arms (SA) extending radially from neurofilaments. Magnification, X 320,000. [Original electron micrograph, courtesy of S. L. Palay, comparable with those shown by Wuerker and Palay, 1968]

outgrowths or side arms, like spokes, shown in Figure 2 taken from Wuerker and Palay (1968), have been noted to extend from neurofilaments. In cross-section, globular subunits 30-40 A in diameter have been observed to form the wall of the neurofilament, and favorable electron micrographs show a central region of low density about 30 A in diameter. Whether the central region is literally a canal, as indicated in the model of Schmitt and Davison (1961), is not certain. Variations in width of negatively stained neurofilaments seen by Davison (unpublished) suggest a possible bifilar structure. Solution of this problem must await refinements of preparation and electron microscopic techniques.

Microtubules in neurons, as in other cells, have a diameter of about 240 A, a wall thickness of 50 A, and a cylindrical core of low density, 140 A diameter. They appear to be rigid and unbranched structures extending continuously in axons and dendrites. Cross-bridges, usually only a few hundred A long, between microtubules have been observed by several authors. Kohno (1964) describes them as particularly prominent in the axon hillock, and Palay et al. (1968) draw attention to the possible significance of this structure in a region thought to trigger the regenerative action potential; the speculation suggests that contraction exerted by the fascicles of microtubules may cause mechanical constraints on the membrane, leading to excitation. Whether such cross-bridges are analogous to those between actin and myosin in muscle or to the dynein-containing extensions of microtubules in cilia is not clear; in both cases the presence of adenosine triphosphatase (ATPase) or guanosine triphosphatase (GTPase) has been demonstrated or surmised. From the studies of microtubules already published on this very young subject, one gains the impression that these fibrous constituents are not placed "haphazardly" in cells, but are directed along specific (metabolic traffic) routes, and in transverse sections frequently indicate patterns of distribution in which individual microtubules are fairly equally spaced in clusters with dark-staining fuzzy material in the intertubular space. Perhaps future analyses will reveal whether or not spacers, as a second component, exist between tubules. In other cases the pattern of intertubular arrangement may vary greatly. For example, there are the 7 clusters each containing 7 microtubules with relatively invariant positioning of subclusters of 3 and 4 microtubules seen by Rudzinska (1965,1967) in the tentacles of *Tokophrya.* Thick, compact bundles of tubules form the structural basis for contraction in the stalk of a flagellate (Grimstone and Cleveland, 1965).

Overproduction of Fibrous Proteins in Neurons: Alzheimer's Syndrome, Neurolathyrism, and Aging

Overproduction of fibrous protein sufficient to displace vital organelles such as mitochondria is characteristic of brain cells in presenile dementias such as Alzheimer's syndrome and in certain abnormalities experimentally induced in animals by feeding compounds such as β-iminodipropionitrile causing artificial neurolathyrism (Ule, 1962), or by treating the brain with aluminum salts (Terry et al., 1964b; Terry and Peña, 1965). According to Kidd (1964), the fibrous structures are neurofilaments twisted about each other in regular bifilar array with constant helical pitch. Terry suggested that the fibrous structures in Alzheimer's disease may be twisted microtubules. Overproduction of fibrous protein may be a major cause—or result—of neuronal degeneration. For further description of the work of Terry's group, see pages

Because of the widespread occurrence of various senile manifestations, investigation of factors controlling the synthesis and the polymerization of fibrous proteins is of great clinical concern as well as of scientific interest. For example, seemingly trivial biochemical defects or alterations of the composition of the subunits (or of the environment) may drastically change the degree and mode of aggregation of subunits into strands, neurofilaments or microtubules. The variation could result from complete failure of subunits to aggregate or from disaggregation of already formed tubules, production of aberrant forms of tubules, overproduction of tubules from normal precursor subunit pools, or through a change of gene expression. Apparently only one amino acid difference in the composition of hemoglobin alters the highly soluble molecules into ones which spontaneously form a kind of microtubule responsible for the mechanical deformation of erythrocytes in sickle-cell anemia, according to Murayama (1966a,b). Conceivably such a variant in neuronal protein might also greatly alter the transport of substances (e.g., transmitters, neurosecretions, trophic substances) to the target tissue and thus lead to neuropathies and other pathological manifestations.

Biophysical and Biochemical Characterization of Neurofilaments and Microtubules

From the giant axons of the large squid *Dosidicus gigas,* axoplasm can be extruded in quantities sufficient for physicochemical charac-

terization. Over a period of years, studies have been made in our laboratory of the neurofilament protein isolated from squid axoplasm by Maxfield (1953), Maxfield and Hartley (1957), Schmitt (1957), Davison and Taylor (1960), Schmitt and Davison (1961).

Maxfield succeeded in purifying the protein and obtaining electron micrographs of the isolated filaments (Figure 1). A great improvement was achieved when the source of axoplasm was changed from the squid *Loligo pealii*, available only in the summer months in New England waters, to the large squid *Dosidicus gigas* caught in Chilean waters during most of the year and processed in our laboratories at the Marine Biological Station at Montemar near Valparaiso. With much larger quantities of axoplasm thus available, Davison and Taylor (1960) were able to make a more concerted attack on the problem of the nature of the fibrous protein and to propose a structure radically different from that previously visualized. Their model, suggested by the tobacco-mosaic-virus (TMV) structure, depicts the neurofilament as being composed of globular protein subunits strung together in strands that, in turn, are wound helically about each other to form a rigid rod; see Figure 3. Such a structure would explain why repeated efforts in previous years to obtain a fiber diagram in the wide-angle X-ray diffraction pattern failed despite the high positive birefringence of dehydrated, oriented axoplasm preparations. The protein is not of the α-type (like keratin, myosin, epidermin, or fibrin). New methods may succeed in crystallizing the protein or in orientating the neurofilaments parallel to a common fibrous axis; it should then be possible to obtain a diffraction-rich pattern presumably resembling that of tobacco mosaic virus.

Figure 3 shows a model, proposed by Schmitt and Davison (1961) and based on the work of Davison and Taylor (1960). Electron microscopic evidence for the presence of a central core or canal with a diameter ca. 30 A, as well as subunits of the filament wall, seen in transverse sections of neurons has been obtained (Palay, 1964; Wuerker and Palay, 1968; and Fernández-Morán*). The central core resembles that in TMV structure, though neurofilaments contain no RNA (Davison and Taylor, 1960). Because the neurofilament protein is acidic, it is likely that the microenvironment of the aqueous channel of the neurofilament is also acidic and that the counterions composing the diffuse double layer projecting from the inner wall of the canal is composed of cations, organic

*Personal communication.

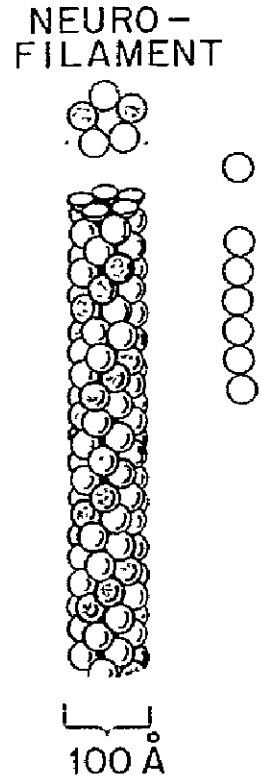

Figure 3. Neurofilament subunit organization, showing monomer, subunit strand, and neurofilament in longitudinal view and in transverse section (depicting subunits and central region of low electron density that may possibly represent a central canal). [After Schmitt and Davison, 1961]

as well as inorganic; whether these include the biogenically active quaternary ammonium ions is unknown (see Schmitt and Davison, 1961).

Further important advances have been made by Davison and Huneeus* in the purification and the analysis of neurofilament protein. Their discovery that, in the presence of 0.1 M mercaptoethanol, unrefrigerated axoplasm is stable at room temperature and can be shipped from Chile to our laboratories in Cambridge, Massachusetts, permits stockpiling the material in adequate supply for analytical purposes.

Purified neurofilaments, such as those shown in Figure 1, may be obtained by zone sedimentation of axoplasm in a sucrose gradient, by repeated freezing and thawing, or by fractional precipitation. Dissociation of the filamentous structure may be accomplished by treatment with detergents, by dissolution in concentrated 6 M guanidine hydrochloride, or by succinylation of the protein and blocking the thiol groups.

*Unpublished.

Chromatography of such dissociated preparations on agarose columns by the method of Davison (1968) 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. Davison and Huneeus have shown that the second component, also an acidic protein, has a high content of serine, a fraction of which occurs as serine phosphate, and may be an asymmetric protein (suggested by hypersharp peaks in the ultracentrifuge pattern). This second 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 various kinds of neurons (Kohno, 1964; Peters and Vaughn, 1967; Metuzals, 1966; and Palay et al., 1968).

Characteristic is the strong attractive interaction of the subunit protein monomers, making preparation of the monomers in native form extremely difficult. Neurofilament protein shares this property with certain other proteins such as tobacco mosaic virus and apoferritin (Hofmann and Harrison, 1963). As in the case of microtubules, this pronounced affinity may be due primarily to bonding by hydrophobic residues. Microtubules, e.g., those of the mitotic apparatus or of the axopods of Heliozoa, are unstable at low temperatures or high pressures, but are stabilized by D_2O. Others, e.g., those of cilia and sperm tails, are relatively more stable under such influences. Certain proteins, such as catalase, have been observed to crystallize in microtubular form (Kiselev et al., 1967).

Though microtubules have been observed in thin sections of squid axoplasm rapidly fixed in glutaraldehyde,† they have not yet been isolated from squid axoplasm as a purified component. Further studies of factors determining their metastability are required. Using methods effective in isolating microtubules from other tissues, Kirkpatrick (1968) has succeeded in fractionating microtubules from mammalian brain which have characteristic appearance in electron micrographs. These experiments do not prove that the isolated microtubules are derived from neurons rather than from glia or endothelial cells. Preliminary fractionation of neurons may overcome this difficulty.

Colchicine binds strongly to microtubule protein, and it is this property that, by disrupting microtubules of the mitotic apparatus already formed or by preventing their formation, causes colchicine to arrest mitosis in the metaphase stage. The mechanism of action of colchicine

†B.G. Uzman, personal communication.

when applied to cells is under active discussion. There is some question as to whether colchicine when added to already formed microtubules, through its strong binding power, causes their depolymerization. An alternative view is that colchicine primarily prevents the polymerization of subunits from the subunit pool, because subunits to which colchicine is bound lack the intersubunit affinities characteristic of the normal microtubule protein. Statements should be made only with respect to particular material under specified conditions, because the stability properties vary widely from one tissue situation to another. Borisy and Taylor (1967a,b) have shown that squid axoplasm strongly binds colchicine and that, by colchicine treatment, it is possible to isolate microtubule protein from mammalian brain homogenates. The protein thus isolated (Weisenberg and Taylor, 1968; Shelanski and Taylor, 1967, 1968) has an amino acid composition similar to that of microtubule proteins generally, particularly in the content of acidic amino acids and in relatively high values of glycine and proline. The molecular weight is about 60,000. For comparison with the hypothesized structure of neurofilaments, a model for microtubules is shown in Figure 4 (see also Tilney and Porter, 1967).

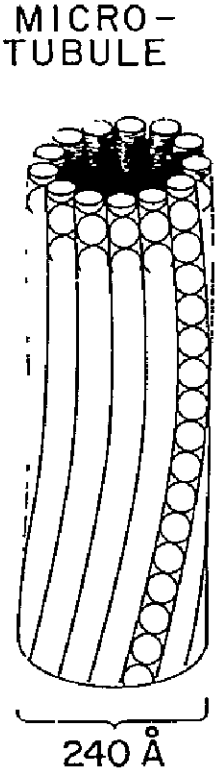

Figure 4. Model of microtubule subunit organization. In the case of sea-urchin flagella, according to Shelanski and Taylor (1968), the unit of structure is the subunit dimer with molecular weight of 120,000.

These experiments suggest, but do not prove, that colchicine depolymerizes preformed microtubules yielding colchicine-bonded monomer subunits which are extracted and recovered in that form. However, since quantitative evidence is lacking, it is possible that only subunit molecules present as monomers in the pool are bound by colchicine and recovered. Because of the metastability of the monomer ⇔ polymer ⇔ tubule transition and the lack of electron microscopic or chemical means of accurately determining the quantities of each of the three types without grossly changing the system by the reagents and fixatives used or conditions imposed, we have little real knowledge of the true metabolism of microtubule protein even in the most favorable systems, such as in the mitotic mechanism. In the case of neurons our ignorance is appalling, yet various workers have already used colchicine effectively to block axoplasmic transport.

It may indeed be this very metastability—this tantalizing ability of subunits to change from isolated monomers in a pool to a fibrous, microtubule form or possibly to a membranous form (structure protein)—that is basic to the structuration processes in cells, particularly in neurons. Obviously *in vitro* experiments with purified microtubule protein, of the kind pioneered by Oosawa and his colleagues (1966, 1967) at Nagoya, in the case of bacterial flagellar protein, are badly needed. Experiments along these lines offer attractive opportunities for young investigators to develop comprehensive programs in molecular neurobiology likely to be highly rewarding in the future.

Function of Microtubules in Cells Generally

The function of microtubules was ably reviewed by Porter (1966), who was one of the earliest to recognize the biological significance of these structures. His discussion at the conference is dealt with in detail in this NRP report. Porter emphasized the importance of microtubules in conferring mechanical strength and rigidity in cells generally, but particularly in slender processes such as the axopods of Heliozoa (Tilney and Porter, 1967). For the same reason, microtubules might be thought of as stabilizing the long, slender, dendritic, and axonal outgrowths of neurons. This view is supported by the studies of Lyser (1964, 1966, 1968), who followed the appearance of microtubules and neurofilaments in early stages of development of motor neuroblasts in the chick embryo.

Mechanical force capable of propelling protozoa or of producing

aqueous current flow over the surface of epithelia is exerted in the microtubular systems of cilia, flagella, and sperm tails. The mechanism of this contractility is unknown, though Satir (1967) proposes that a kind of sliding-filament process of two kinds of microtubules may be involved. Although his analysis is suggestive regarding the mechanical forces involved, there is still little knowledge of the molecular mechanisms by which neighboring filaments exert thrust upon each other; particularly needed is knowledge about the ATPase (GTPase) which, as the protein dynein, may serve as a cross-bridge in the mechanochemical coupling.

A major function of microtubules is thought to be the translocation or transport of cell particulates, as in the movement of chromosomes in mitosis or the movement of melanin granules into and out of the elongate processes of melanocytes in response to nervous, hormonal, or ionic stimulation. In the multinucleate syncytium induced by virus treatment of hamster kidney cells, Holmes and Choppin (1968) found that whole nuclei might be propelled in directions determined by microtubules which were subsequently observed by electron microscopy to be embedded between the nuclei; the movement of nuclei continued after cell rupture by osmotic shock.

Perhaps one of the most graphic and instructive examples of the transport or propulsion of cellular particulates is that by which the suctorian protozoan *Tokophrya* feeds on protozoan prey such as *Tetrahymena,* as described by Rudzinska (1965, 1967). The long hairlike tentacles (see Figure 5) constitute the feeding apparatus. Although only 0.5 μ in diameter and ca. 50 μ long, the tentacles are remarkably stiff and straight, properties which may be referable to the microtubules. Near the tips of the tentacles are little knoblike enlargements which include missile-shaped enzyme-containing particulates. The tentacular knobs form the attachment mechanism by which free-swimming ciliates, such as *Tetrahymena,* become stuck and immobilized, presumably by the release of a toxic substance. Additional particulates then move rapidly from the cytoplasm of *Tokophrya* through the tentacle toward the knob. There the particulates change their appearance, becoming clear, a process that Rudzinska interprets as due to release of substances, including digestive enzymes, into the prey. Shortly thereafter, connection is made between the tentacle and the prey because cytoplasmic inclusions are seen to pass from the prey through the tentacle into the body of the predator. Bidirectional movement of particulates thus occurs within the thin cylindrical tentacle: first a centrifugal flow of missile-shaped particulates, then the centripetal flow containing cytoplasmic material from the prey; the movement in opposite directions is not simultaneous.

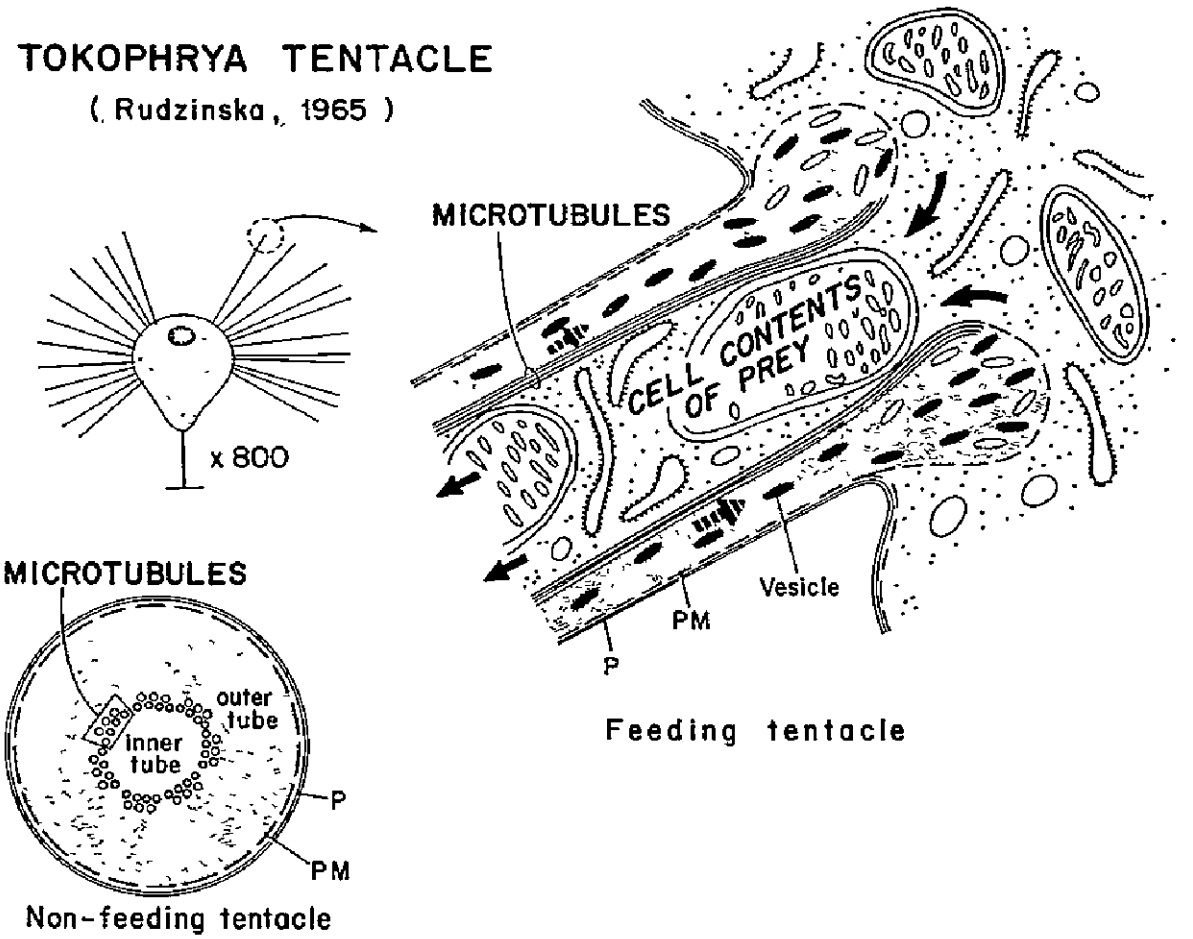

Figure 5. Microtubular structure of *Tokophrya* tentacles thought to be involved in the process of feeding upon prey such as *Tetrahymena*. [Based on descriptions of Rudzinska, 1965, 1967]

Conspicuous within the tentacle are 49 microtubules, arranged in 7 clusters each having 7 microtubules, dividing the thin cylindrical tentacle roughly into an outer tube, in which the centrifugal flow of particulates occurs, and an inner tube, in which the centripetal flow of prey cytoplasm occurs. It seems highly improbable that such rapid bidirectional movement of particulates could occur as a result of gradients of hydrostatic pressure from predator or prey cells, because this would require overcoming very high shear forces within a microcapillary system of high structural viscosity. More likely, an active topochemical role seems ascribable to the microtubules. The nature of this process is unknown. Rudzinska* is inclined to ascribe it to a process of microperistalsis.

*Personal communication.

However, the movement may well be saltatory and may belong in the same class with other phenomena of fast transport of intracellular particulates, of which a possible molecular mechanism is suggested later.

In the classical literature of cell physiology, intracellular movement has been considered a phenomenon of contractility related to that of muscle. This was supported by the facts discovered by ultrastructure research, e.g., by polarization optical analysis (Schmidt, 1937). Although the detailed molecular mechanism of cell motility could not be specified at that time, it was generally assumed that the mechanism was related to that of muscle contraction. Now that electron microscopy permits the structures to be visualized at or near the molecular level and the fibrous proteins of muscle have been isolated and characterized, it may be possible to determine more accurately the molecular mechanism of contraction. Circumstantial evidence suggests that microtubules are directly, probably causally, related to transport processes because microtubules are the most prominent structural features in preparations in which transport has been demonstrated with the use of appropriate histological techniques. Possibilities by which the mechanochemical coupling may occur are considered below.

Transport of Neuroplasmic Constituents in Nerve Axons

Following Gerard's (1932) suggestion that certain substances may move distally in axons, Weiss and Hiscoe (1948) produced the first evidence for a cellulifugal movement of axoplasmic constituents. The phenomenon of axoplasmic flow has since been confirmed by radiolabeling experiments in many laboratories, as reported in the NRP Work Session on *Axoplasmic Transport* (see Barondes, 1967).

Progress in the study of axoplasmic flow by Weiss and his collaborators over the last two decades is summarized in a recent essay (Weiss, 1967). He calculates that neuroplasm equivalent to one to three times the volume of the cell body is synthesized per day and passes peripherally down the axon at a velocity of 1 to 6 mm per day. This discovery revealed a biochemical dynamism not previously conceived by neurobiologists, especially neurophysiologists, who were concerned primarily with membrane-mediated bioelectric phenomena. Axoplasmic flow was consistent with the facts that the neuronal cell body has the biosynthetic equipment of an actively secreting cell and that the RNA content is also high. In the last few years, axoplasmic flow and the

transport of particular substances in neurons have assumed important roles in molecular neurobiology.

In addition to the slow, 1-6 mm per day, unidirectional flow, there have been observations of transport of specific substances at much faster rates, as is shown in Table I. Because the velocity of transport of different substances varies over a wide range even in the same nerve type, it seems improbable that this phenomenon can be explained on the basis of bulk flow of axoplasm implemented by hydrostatic pressure, generated perhaps by peristaltic contraction of sheath components.

There is some evidence that, at least under certain experimental and abnormal conditions, transport (but not bulk flow) may be bidirectional (Lubińska et al., 1963). Moreover the necessity of overcoming large frictional shear forces in moving substances by hydrostatic pressure through very small-bore capillary systems of such high structural viscosity poses a severe rheological problem. Dense-core catecholamine storage granules ca. 500 A in diameter were found by Dahlström (1965, 1967) to pass down sympathetic motor neurons, 0.5 to 1.0 μ in diameter, at velocities of 100-200 mm per day. The fast specific transport seems more likely to be mediated by local, topochemical, perhaps saltatory, reactions with or upon the only obvious structural constituents available for the purpose, the fibrous proteins, i.e., microtubules, neurofilaments, or both. Microtubules seem to be associated with the fast transport, but neurofilaments may also participate. Sympathetic motor neurons, in which transport occurs at the rate of 100-200 mm per day, contain predominantly microtubules. The neurons in which the highest rate of transport has thus far been noted, that of secretion granules in hypothalamic neurons (rates of 2,800 mm per day or 32 μ per sec), according to Jasinski et al. (1966), contain a large complement of microtubules (Bergland and Torack, 1968).

Mechanism of Transport by Microtubules

Although the evidence that microtubules participate in the movement of structures such as chromosomes, melanin granules, secretion granules, and vesicles is indirect, it is nevertheless compelling. Yet there is no consistent theory for the physical mechanism by which chemical energy, presumably that of "high energy" groupings in ATP or GTP, can be utilized by macromolecular structures such as microtubules to exert force sufficient to move cellular objects and transport them sometimes with high velocities (10-30 μ per sec).

TABLE I
VELOCITY OF AXOPLASMIC TRANSPORT

Neuron Type	Substance Transported	Transport Velocity		Measurement Technique	Investigator
		mm/day	μ/sec		
Axolotl lateral line; cat dorsal root; cat and rat ventral root; rat sciatic	Protein	1 to 6	0.01 to 0 07	Radioactivity of axon segments; ^{3}H-leucine, autoradiography	Weiss, 1948-1967; Lasek, 1967a; Droz and Leblond, 1962; others
Goldfish optic nerve	Protein	0 4 40 - 80	0.005 0.5-1.0	^{3}H-leucine; scintillation counting of axon segments	Grafstein, 1967; McEwen and Grafstein, 1968a,b
Rabbit hypoglossal Rabbit vagus	Phospho-lipid	40 72	0.5 0.9	$^{32}PO_4$, radioactivity of axon segments	Miani, 1962 Miani, 1964
Cat ventral root		30-100 930	0.4-1.2 11.2	^{3}H-leucine; radioactivity of segments; autoradiography	Ochs et al., 1967, 1968
Rabbit sympathetic	Norepi-nephrine	48	0.6	Histochem. fluorescence of norepinephrine	Dahlström and Häggendal, 1967
Rat sympathetic	storage	120	1.4		Dahlström, 1967
Cat sympathetic	granules	240	2.8		Dahlström, 1967
Rat ventral root	Protein	100	1.2	^{3}H-leucine; scintillation counting of axon segments; autoradiography	Lasek, 1967a
Cat dorsal root		500	6.0		Lasek, 1968
Snail motor neurons	Glutamate	720	9 0	Radioactive glutamate, appearance at muscle	Kerkut et al., 1967
Growing fibers of explant of chick spinal ganglion	(Particles)	700-1000 (saltatory movements)	8.4-12.0	Optical, particles observed	Burdwood, 1967
Hypothalamic preoptic neurons	Secretion granules	2800	33.0	Depletion of secretion granules in 1 min	Jasinski et al., 1966

The problem of intracellular transport and cytoplasmic flow has been dealt with in several recent reviews. The paper by Rosenberg (1966) focuses the problem well. He refers to molecular itineraries within erythroblasts by which hemoglobin is formed, as Bessis strikingly portrays (1963). An analogous problem of major interest to molecular neurobiologists would be the synthesis of storage granules, vesicles, and transmitters in the perikaryon, their transport down the axon, and their liberation at the synapse. In consideration of the mechanochemical transduction by which cytoplasmic flow and transport are effected by the agency of microtubules, Rosenberg (1966) suggests microtubular undulation, linear contraction, torsional changes in pitch, reversible crystallization, and pumping actions.

An alternative possibility is here proposed as a tentative working hypothesis by the writer. Formation of the microtubular mitotic apparatus is thought to occur by polymerization of soluble subunits from cytoplasmic pools. Force might conceivably be exerted upon intracellular objects such as vesicles by quaternary configurational changes of constituent subunit molecules. However, no such reversible polymerization changes are known to occur in structures such as the *Tokophrya* tentacle or in certain nerve axons where transport nevertheless is vigorous.

Perhaps the chemomechanical coupling by which objects and substances are moved within the cell is much the same in molecular idiom almost everywhere in living cells with modifications suitable to each special situation. It is conceivable that motive force might be delivered to substances in the microtubule surround by the beating of protein side-arms projecting cilialike laterally from the microtubules. It is not clear how, in such a system, conformational changes in the laterally projecting side-arms which produce the beating and energy from bound nucleotide could occur cyclicly and metachronously so as to produce unidirectional movement in the surround.

More logically we may turn to the contractile process in muscle, the most widely studied contractile system, for hints concerning microtubule mechanisms. The most cogent present theory of muscular contraction is the sliding filament theory proposed by Hanson and H.E. Huxley (1953-1955)*and by A.F. Huxley and Niedergerke (1954). This theory was later elaborated by H.E. Huxley (see reviews by Pringle, 1967, and by Perry, 1967; also a detailed ultrastructural analysis by Huxley and Brown, 1967). According to the sliding filament model the constituent fibrous proteins actin and myosin do not themselves contract when muscle shortens; intrasarcomeric filaments slide by each other due to thrust

*See Huxley (1953), Huxley and Hanson (1954), and Hanson and Huxley (1955).

exerted on the actin by the heavy meromyosin cross-bridges. It is assumed that the regularly spaced cross-bridges are not in contact with actin in resting fibers. However, in rigor or in contraction there is interaction at particular binding sites on the actin and myosin subunits. ATP, the source of energy for the contraction, is bound to actin subunits. When the ATPase, which is located at a site different from the binding site, is activated by calcium ions that enter the muscle substance with excitation, the ATP is split and energy is transferred to the myosin subunits; this causes substantial conformational change of the cross-bridge subunits which, in turn, deliver a thrust to the actin subunits; the result is movement of the bifilar actin filament with respect to the myosin filament. The detailed X-ray analysis of Huxley and Brown (1967) shows precise dimensional and steric conformance between actin and myosin subunits, as would be required for such a mechanism to work according to the process postulated by Huxley. This theory—still only an ingenious working hypothesis—has the advantage of suggesting a specific mechanism by which intramolecular energy may be transferred to a protein molecule as fast molecular conformational change; this does work on the system.

Microtubules could conceivably move intracellular objects through similar conformational changes of their protein subunits. In that case the microtubule would play a role analogous to that of actin in muscle contraction, i.e., to provide a source of energy (probably GTP) for conformational changes with which to move objects with respect to the microtubule. It is not obvious how a sliding filament mechanism could play a role in the rapid transport of substances in axons. Nor is there evidence for (or against) the view that microtubules and neurofilaments interact, in analogy to the myosin-actin model, through a sliding movement of microtubules with respect to neurofilaments. Cross-bridges have been observed, but these extend between microtubules (Kohno, 1964). Along the entire length of the axon, microtubules appear to course individually, showing no connections with neurofilaments or other structures. Spikelike side arms, which may be cross-bridges, have been observed to extend from neurofilaments (Palay et al., 1968), but whether these interact with microtubules or other neurofilaments is unknown. Neurofilaments with their side arms may be related to the slow (1-6 mm per day) movement of axoplasm visualized originally by Weiss and now widely confirmed by other workers. However, a different mechanism seems to be demanded to account for the fast, possibly saltatory, specific, sometimes bidirectional transport in axons.

A speculative model that may prove useful pending further

experimental evidence is shown in Figure 6. The essence of this model is that microtubules are relatively stationary objects, extending continuously down the axon and containing in bound form the chemical energy (ATP or GTP) source for the chemomechanical transduction. Most substances, such as transmitters, neurosecretions, and enzymes, which are to be transported from the perikaryon, the site of synthesis, to nerve endings at the synapse, are enclosed in thin-walled (perhaps monomolecular) vesicles. The membrane does not give the appearance of a "unit membrane." Lysosomes (Gordon et al., 1968) and catecholamine storage granules of sympathetic neurons are enclosed in such vesicles, and the same may be

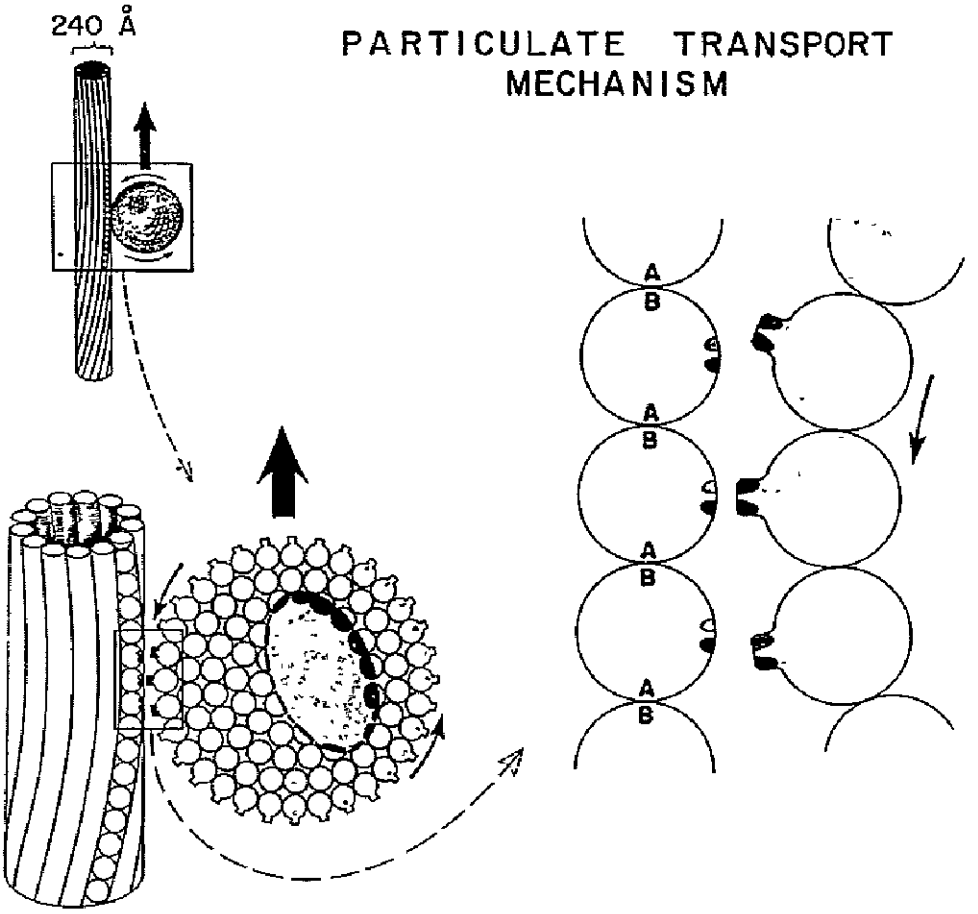

Figure 6. Hypothetical sliding-vesicle model of mechanism of fast specific transport of vesicles by (saltatory?) interaction with microtubules; principle of mechanochemical coupling based on sliding filament hypothesis of muscle contraction. Binding between subunits of microtubule and of vesicle wall at one of paired binding sites; other site relates to binding between nucleotide of microtubule with ATPase (GTPase) of vesicle subunit.

true of other particulates and transported solutes. It is suggested that transport may occur by a saltatory "sliding vesicle," rather than by a sliding filament mechanism as in muscle.

Suppose the subunits of microtubules (possibly also neurofilaments) contain bound ATP or GTP. Suppose also that the protein subunits forming the wall of the vesicle (or other object to be transported) contain a binding site for microtubule protein and an ATPase or GTPase that, after activation by divalent cation, can split the ATP or GTP bound to the microtubule subunits. As in muscle, the energy coupled with this hydrolysis could conceivably cause substantial conformational change in the subunits of the vesicle, thereby producing a thrust sufficient to propel the vesicle in a saltatory jump. Interaction with another binding site on the same or a different microtubule would produce a similar thrust, and the vesicle would be propelled down the system of more or less parallel microtubules. Directionality of movement may be provided by the sense of the helical packing of subunits in the microtubule. Such directionality seems to characterize striated muscle in which actin filaments are thought to slide from the Z-band toward the M-band in each segment, i.e., in opposite directions within individual sarcomeres.

The suggested transport mechanism would be applicable to any situation in which effector molecules, whose functional site is remote from their site of synthesis, are enclosed in or associated with a membrane-limited vesicle. The Golgi apparatus, frequently characterized as the "packaging department" of the cell, is believed to enclose synthesized particulates with a paucimolecular surface film. The protein subunits of the film thus provided may serve the role, analogous to the myosin in the actomyosin system, pictured in connection with rapid intracellular transport.

Various investigators have suggested that cells may be provided with a microcirculatory system—though possessing no heartlike force pump—through their complement of microtubules which also give directionality to the movement (Sandborn et al., 1965 and Du Praw, 1968). To the extent that the limiting membranes of particulates contain protein subunits having properties described in the present model, the particulates may be transported to regions in the cell determined by the orientations and lengths of the microtubules.

Such a microtransport system may be very old evolutionarily considered, antedating striated muscle from which the present model was adapted. Microtubules, and possibly microfilaments (neurofilaments) would be organelles, and so also would be the vesicles which

serve as the vehicle of transport, according to the hypothesis here proposed.

Role of Fibrous Proteins in Molecular Neurobiology

Because dendrites, frequently rich in microtubules, and axons may extend far from the biosynthetic center of the cell, the presence of microtubules (and neurofilaments) in these structures may be explicable in terms of transport of effector molecules from biosynthetic cell centers to the periphery and vice versa.

Kerkut and his co-workers (1967) observed fast transport of glutamate in snail axons to occur only when the nerve was stimulated, suggesting a coupling between bioelectric processes and transport mechanisms. Such a coupling of transport with the action potential (or current) would seem reasonable teleologically because the amount of transmitter substance available at axonal terminals for synaptic transmission would then be a function of the number of impulses passing down the nerve fibers. Confirmation of Kerkut's results or demonstration of coupling between excitation and transport is necessary before further speculations about mechanisms would be profitable.

Figure 7 illustrates the fact that, because synaptic transmission is

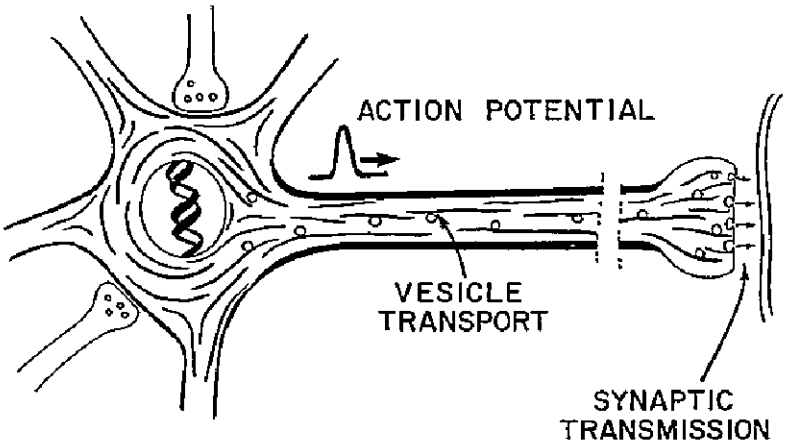

Figure 7. Dual nature of transmission of impulse to target tissue: 1) fast (meters per second) propagation of action potential wave; 2) slow (microns per second) transport of vesicle-enclosed transmitter from site of synthesis in cell body to synaptic ending where transmitter molecules excite or inhibit postsynaptic membrane. Where transmitter, e.g., acetylcholine, is synthesized at endings, vesicles (which are synthesized in the cell and journey down the axon) are nevertheless necessary for the transmission process.

humoral and because humoral or trophic agents, at least in certain cases such as the catecholamine transmitters, must be transported from the site of synthesis in the cell center via the axon to synaptic terminals, the transport mechanisms are as necessary for synaptic function as is the action potential which triggers the release of the transmitter. Weiss* observed accumulation of vesicles in axons, the peripheral flow of which had been dammed by compression. The situation is clearest in the sympathetic neurons in which catecholamine storage granules carrying the transmitter norepinephrine, thought to be synthesized in the cell body (as well as within the storage granule), are transported down the axon at relatively high velocities to the terminals (Dahlström, 1965, 1967). Acetylcholine is generally thought to be synthesized within nerve endings, but vesicles are essential, in a poorly understood way, for the synthesis, storage, release, and perhaps reabsorption of cholinergic transmitters.

Transport from the cell center to synaptic endings is important not only for transmitters, neurosecretions, and trophic substances, but possibly also for proteins or other macromolecular constituents playing a physiological role in the plastic regulation of cell-to-cell interaction, as in learning, for example. Barondes (1968) has found that protein synthesized from labeled amino acid precursor passes down the axon to endings where, he suggests, the protein combines with mucoid constituents at the synapse. These proteins, according to Barondes, may be concerned with plastic alterations on which learning depends.

In the biochemical economy of the neuron, transport of materials other than those destined for the synapse via the axon may also be important. Dendrites are reported by several authors (Palay, 1964; Porter, 1966; Peters and Vaughn, 1967) to be rich in microtubules; neurofilaments are less obvious there. Such microtubules, in addition, may play a mechanical, structural role. However, if they delineate routes and destinations of intracellular transport, this function would constitute an important role in cellular metabolism; vigorous search for experimental evidence of such a process would be warranted. Thus far, no experiments designed to examine this question critically have yet been devised. Substances impinging upon the neuronal membrane extracellularly, such as trophic and growth substances, neurosecretions, and some transmitters, may modulate the ongoing activity of the neuron by affecting metabolic processes through gene expression or by more peripheral reactions. Genetic factors probably play an important role in determining the

*Personal communication.

ongoing pattern of bioelectrical activity of neurons as well as special patterns of activity related to the performance of innate behavior. Since the neuron's manner of manifesting such responses is by bioelectrical processes at the membrane, biochemical determinants would have to pass from the synthetic centers, which translate genetic messages, to the membrane. If microtubules mediate transfer of this vital information, the possibility of directional transport—in contrast to diffusional processes—may guarantee speed of arrival at precise loci on the membrane with a minimum of mixing and interacting with other cellular constituents en route. In some highly convergent systems where there may be 10^4 to 10^5 inputs on a dendritic tree, the problem facing the cell in computing the appropriate response to these inputs, or modulating its own ongoing activity, may be greatly facilitated by an intracellular transport system to and from the cell center, as indicated graphically in Figures 7 and 8. Bullock's (1968) exposition of the language of the neuron makes it clear that plasticity is not determined alone at synaptic junctions, but by many other structures, some of which may be located in cytoplasmic regions, as

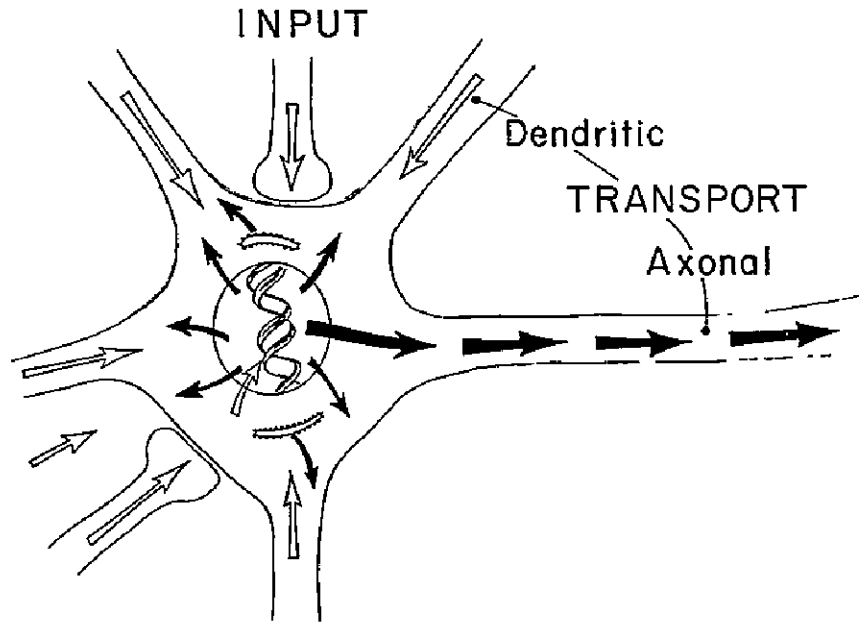

Figure 8. Molecular traffic routes (see arrows), in dendrites and perikaryon as well as axon, suggested as following directions specified by the presence and orientation of fibrous protein organelles.

in the synthetic center. M.J. Cohen* has adduced similar evidence in the case of arthropod neurons.

It is appealing, as a working hypothesis and as a basis for design of experiments, to explore the role of the fibrous proteins along lines here discussed, i.e., as a means for directing and conducting metabolic determinants between dendrites, cell body, axon, and synaptic endings.

*Personal communication.

III. MICROTUBULES AND FIBROUS CONSTITUENTS OF NEURONS

A. GENERAL MORPHOLOGY OF MICROTUBULES: DISCUSSION LED BY K. R. PORTER

Morphological Characteristics

Microtubules are widely distributed in the cytoplasm of both plant and animal cells and are especially numerous in some neurons, their intraneuronal quantity varying with the physiological or developmental state of the cells. In all cases their morphology is characteristic and constant. In cross sections they are about 240 A in diameter (extreme figures ranging from 180 to 300 A have been reported); in longitudinal sections they appear to be straight, long tubules having a less dense central canal 100 A in diameter. The limitations of thin sections used in electron microscopy restrict the determination of their actual length; but they have been traced for several microns in some preparations and it is not unlikely that some microtubules traverse the entire distance of a dendrite or even an axon for several millimeters or possibly centimeters.

Microtubules are generally straight and unbranched structures and show no evidence of blebbing. When properly prepared, they never have a bent or sharply curved shape. Rather, they appear as broken fragments, giving the impression of a fairly rigid structure broken by fracture forces. Tubules often cluster to form bundles in parallel arrangement. In some instances, however, they form a dense lattice of randomly oriented tubules (Porter, 1966). They usually are not seen attached to other organelles and, when such attachments have been noted, it is reasonable to suspect superimposition. Nonetheless, it should be noted that ribosomes have been observed attached along the sides of microtubules in the mitotic spindle (Rebhun et al., 1967) and nuclei attached to microtubules in infected syncytial cells studied by Holmes and Choppin (1968). Cross-bridges extending between neighboring tubules are commonly present, as, for example, in the initial segment of the neuron (Palay et al., 1968) or in the axons of the Purkinje cell (Kohno, 1964). Other finer structures, such as the radial "spokes" in the axial unit of the sperm tail or the highly organized "spokes" connecting the microtubules in the axostyles of *Saccinobaculus*, are often seen attached to tubules (Grimstone and Cleveland, 1965). In intranuclear microtubules, Behnke and Forer (1966a)

find bridging material between the microtubules and the chromosomes (in metaphase and anaphase) similar to that found in telophase nuclei. In some preparations the microtubules are studded with small protuberances, but it is not clear from the information currently available how common these protuberances are or what function they perform.

The walls of microtubules are composed of individual linear or spiralling filamentous structures about 50 A in diameter. Most investigators count 13 filaments in cross sections of the cylindrically shaped tubule wall (see Figure 1), but some workers report finding fewer filaments (e.g., see Burton, 1968). In cilia and flagella, the microtubules, which are arranged in 9 pairs or doublets around a central pair, were earlier reported to be constructed of 10 or 11 filamentous subunits, but counts obtained from negatively stained preparations are uncertain. The fine filamentous structure of the walls suggests to some investigators (Grimstone and Klug, 1966; Tilney and Porter, 1967) that the microtubule is composed of axially or helically oriented filaments which, in turn, are formed by a firm linear adhesion of subunits. If the lateral adhesion between filaments is reduced, as by treatment with colchicine and other mitotic inhibitors, the microtubules appear to break up into their constituent filaments (Wiśniewski et al., 1968). Similar effects of colchicine on microtubules of the mitotic apparatus have been shown by Robbins and Gonatas (1964a).

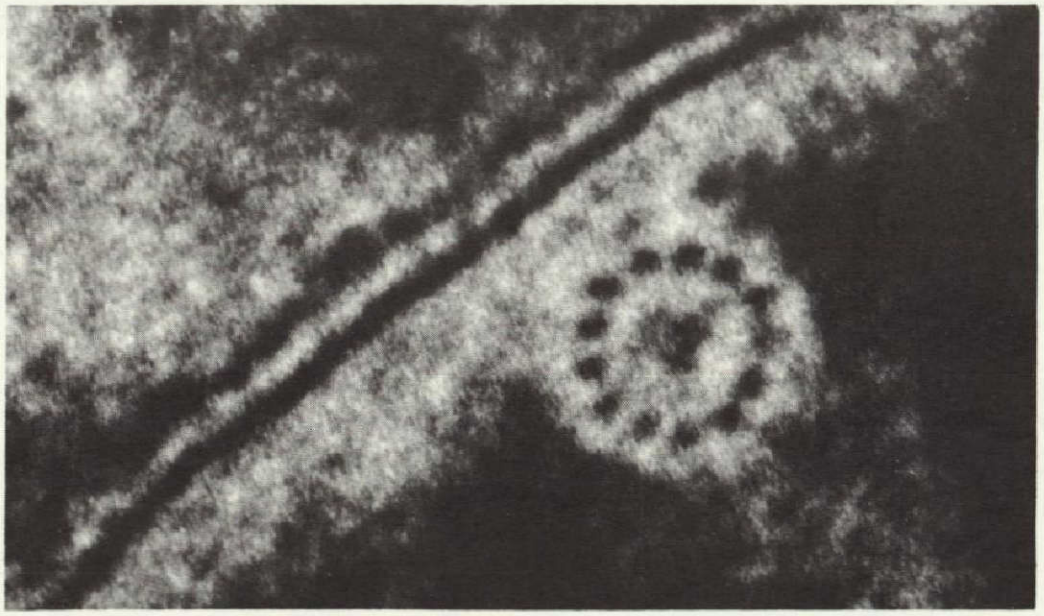

Figure 1. Transverse section of a single microtubule in a meristematic cell of *Juniperus chinensis*. Micrograph shows 13 subunits in the wall of the tubule. Magnification, X 800,000. [Porter]

Before proceeding further with this discussion, some remarks should be made about the usage of terms. Care in the use of terminology employed in regard to fibrous structures may prevent unnecessary conceptual confusion. The fibrous structures to which microtubules are converted are strands, protofilaments, or microfilaments; they are not neurofilaments for these are composed of some 5 or 6 subfilaments twined about each other in helical array. The thin fibrous constituents of microtubules are sometimes referred to as protofilaments. It is of course conceivable that microtubules and neurofilaments are but two different manifestations of the quaternary conformational states of the same subunit protein molecules; i.e., two different aggregation states of the same constituent protofilaments. The similarities of their amino acid composition is consistent with this view, but neurofilament protein contains no bound nucleotide and does not bind colchicine as the microtubule protein does (see page 179).

Several modifications in the fine structure of the tubule walls, such as a beaded appearance (Pease, 1963; Grimstone and Klug, 1966; Porter, 1966) have been reported, but it is possible that these may be artifacts of preparation or of geometric optics. What is now clear is that the microtubule walls do not have a "unit membrane" structure as proposed earlier by some investigators. In the 9 double pairs of microtubules seen in flagella, the strikingly characteristic appearance of the melded walls along a sizable fraction of their length emphasizes the high frequency of wall modification in tubules.

Since centers of tubules usually show no electron-dense material, it has been suggested that the tubules are "hollow" and are filled with "solutions" of small or even sizable molecules. The fact that these solutes are not observed may be due to their small contribution to electron density or their disappearance in tissue preparation. Dense "dots," which on longitudinal view sometimes appear as rods in the center of the tubules, have been seen by some investigators (e.g., see Echandía et al., 1968) and it is possible that they will be observed with greater frequency as the methods of preparation improve. Since the protein subunits are acidic (pK = 4), it is possible that the dense core material represents constituents of the diffuse double layer which are chiefly cationic in nature, e.g., K^+, Ca^{++}, or quaternary ammonium compounds which are of special importance in the nervous system (Schmitt and Davison, 1961).

Microtubules are usually distributed in cytoplasm in distinctive patterns. The axial unit with its 9 pairs of tubules and a central pair is the well-known pattern observed in motile appendages such as cilia, flagella,

and sperm tails (Fawcett and Porter, 1954; Fawcett, 1961). Cross sections of the tentacle of the suctorian, *Tokophrya infusionum,* show an array of 7 bundles, each bundle containing 7 microtubules arranged in a highly ordered circular pattern; i.e., in 2 rows, an external row of 3 and an internal row of 4 microtubules (Rudzinska, 1965) (see Figure 5 on page 133).

B. FUNCTIONAL PATTERNS OF MICROTUBULES: DISCUSSION LED BY K. R. PORTER

The greater part of the accumulated knowledge about microtubules is of a descriptive and morphological kind, and it is mainly from this literature about their structure, shape, and size, along with their presence or absence in different environmental conditions, that certain tubule functions have been proposed. Among these are mechanical and skeletal functions, motility, sometimes producing tension (as in the mitotic apparatus), intracellular transport, and sensory transduction.

Mechanical, Skeletal Functions

The rigidity suggested by the appearance of microtubules and their common occurrence in thin cellular extensions, such as axopods, indicate an intracellular skeletal function. The form and shape of some cell protuberances have been shown to be related to the orientation and distribution of microtubules with respect to cell walls, and, in some instances, it is known that microtubules anticipate the location of secondary wall thickenings in plant cells (Hepler and Newcomb, 1964; Cronshaw and Bouck, 1965). From their studies on microtubules in the primary mesenchyme cells of sea urchin embryos, Porter and his collaborators suggest that "microtubules are a morphological expression of a framework which operates to shape cells and redistribute their contents" (Byers and Porter, 1964; Gibbins et al., 1966, 1968).

The heliozoan, *Actinosphaerium*, has many slender axopods 400 to 500 μ long and only 10 to 15 μ in diameter which extend radially from the cell body. Cross sections of the axopods reveal a striking pattern in the arrangement of the microtubules (see Figure 2); the axoneme, an axial filament extending along the central axis of the axopod and which shows positive uniaxial birefringence, is found to consist of a double-coiled array of microtubules (Kitching, 1964). These remarkable extensions of the cell

surface are membrane-limited; only mitochondria and other particulates can move freely between the microtubules and the limiting membrane. According to Porter (1966), the inference is strong that microtubules play a central role in the development and maintenance of form of these axopods.

Further support for the theory that microtubules play a critical role in the shaping of cells, particularly the long, thin, cellular extensions, comes from the studies of Tilney and his collaborators. They exposed *Actinosphaerium* to various conditions, e.g., low temperatures and high hydrostatic pressures (see Tilney and Porter, 1967; Tilney et al., 1966). After exposure for 2.25 hours to 4°C, the microtubules disappeared (depolymerized?) and the axopods collapsed into strands of small blebs which finally withdrew into the cell (see Figure 3). The reaction is reversible for, if the cells are returned to room temperature or normal pressure within as short a time as 10 minutes, the microtubules rapidly reform, birefringence returns, and axopods reappear. Another similar example can be cited; for example, a marginal band comprised of a cluster of microtubules has been observed in white blood cells and the configu-

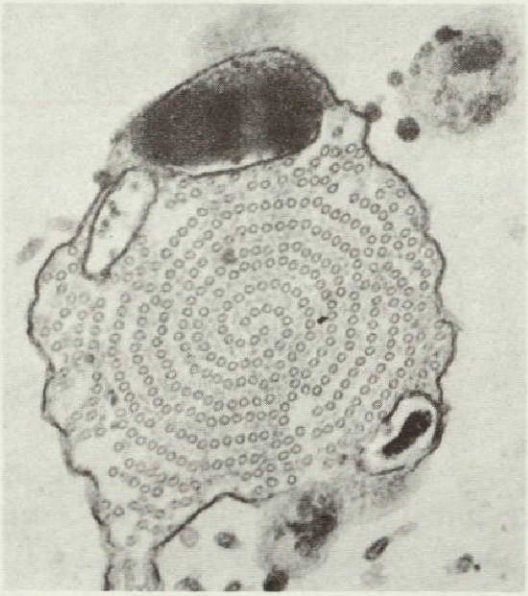

Figure 2. Transverse section through axopodium of *Actinosphaerium* showing ordered arrangement of microtubules normally present in fully extended axopode. This is a control for cold-treated cell shown in Figure 3. Magnification, X 36,000. [Tilney and Porter, 1967]

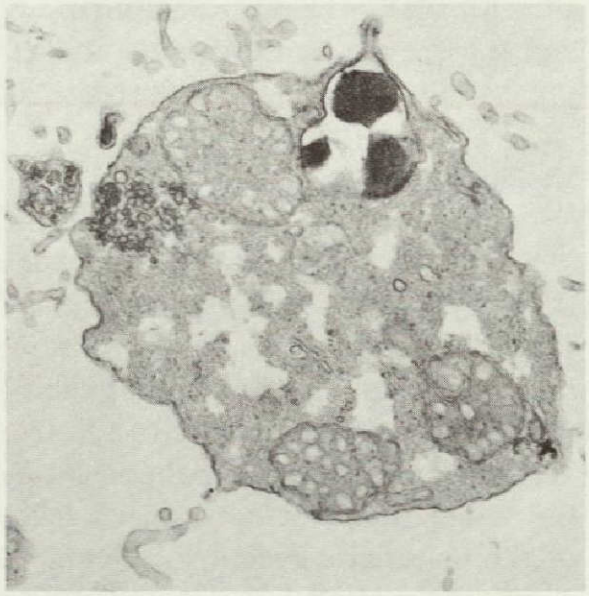

Figure 3. Transverse section through axopodium of *Actinosphaerium* fully retracted after 2.25 hours at 4°C. There remains no evidence of microtubules. Magnification, X 36,000. [Tilney and Porter, 1967]

rational relations suggest that microtubules may mechanically determine the shape of these cells.

The neuron is highly specialized with respect to the size, shape, and detailed form of its many delicate extensions. The elongated dendritic tree of many neurons or the great asymmetry of elongated axons might depend on microtubules' functioning as cytoskeletons. That the dendrites of the sensory neuron of the insect olfactory sensillum (K.-D. Ernst,* see also page 189) reveal a striking pattern of one centrally located microtubule in each dendrite is highly suggestive of such a function. The greater incidence of microtubules than of neurofilaments in small nonmyelinated axons or small dendritic branches on one hand, and the greater occurrence of neurofilaments than of microtubules in large myelinated neurons on the other, suggest a difference between the mechanical or structural function of microtubules and neurofilaments.

If microtubules play a role in determining the shape of cells or cellular extensions (including those of nerve cells), not only their distribution but their origin, position, and growth must follow a coordinated pattern. The basal body and centrioles are the centers best known for the initiation of microtubules. Cortical densities of the same structural texture as centrioles have been demonstrated in a variety of differentiating cells, and these same densities are known to initiate microtubule formation and serve as anchoring points (Porter, 1966).

Motility Functions

There is no direct evidence that the microtubule itself is contractile or has within itself enzymatic activity associated with motility. The prominent, widespread occurrence of the 9 doublet tubules in sperm tails, flagella, and cilia implicates them in the motility of these structures.† Cross sections of the sperm tail show it to have a fairly elastic "cage" of microtubules around its periphery. When the cell is in motion, the "cage" is distorted from its rigid straight form by local displacements of the material adjacent to the microtubules. Although the mechanism by which the microtubules are involved in motility is not clear, some form of mechanochemical energy coupling must clearly occur. This coupling probably involves the side arms which have an ATPase termed dynein (Gibbons, 1966; see also page 159).

*Manuscript in preparation.
†See Table on "Biological Movements" in Allen, 1967, page 330.

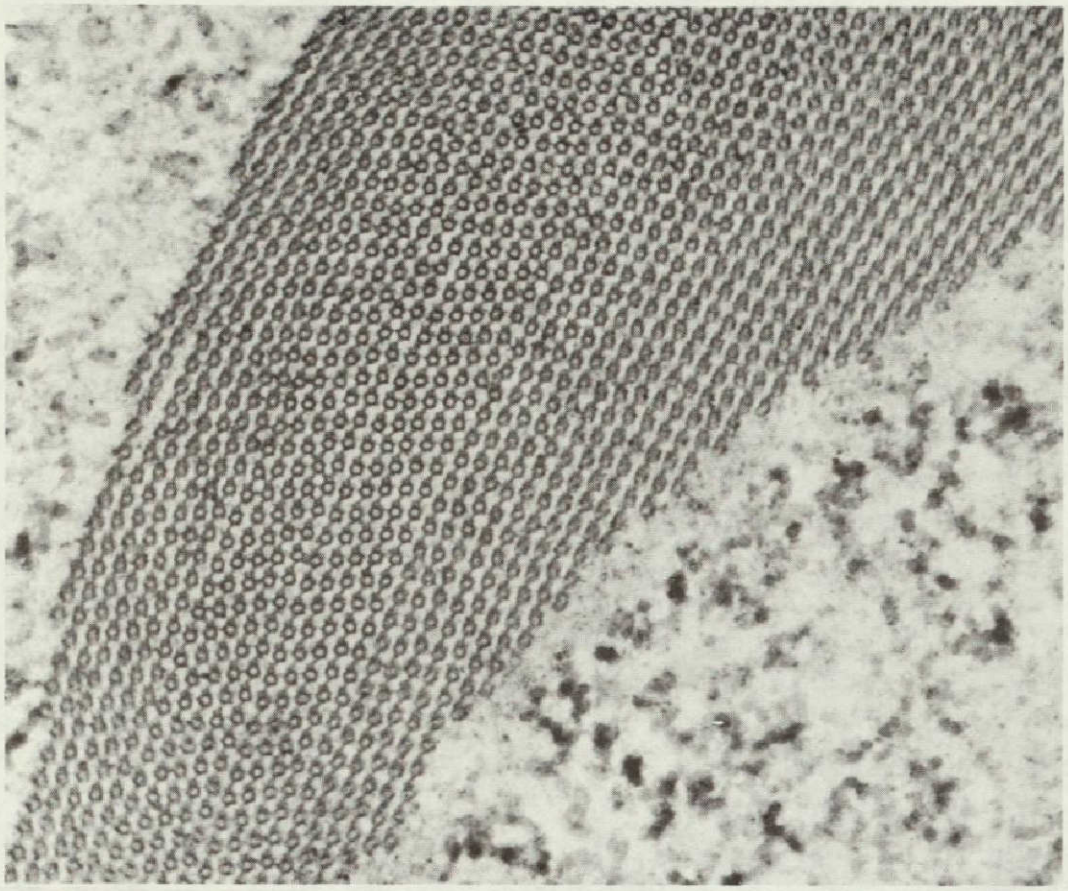

Figure 4. Cross section of axostyle of *Saccinobaculus*. The micrograph, besides depicting the ordered arrangement of microtubules, shows the existence of bridges of at least two types between the tubules. Magnification, X 80,000. [Porter]

In addition, a variety of highly motile cells possess the characteristic organization of microtubules which has come to be associated with motile processes. This is illustrated by the structure of a protozoan, *Saccinobaculus*, that lives in the intestinal tract of termites and displays a vigorous undulatory motion which appears to depend upon an unusual array of microtubules and peritubular material in its axostyles (see Figure 4). There are many cross-bridges, uniformly spaced and extending about 240 A between the microtubules. These cross-bridges may be an integral component of the motile apparatus; they may play a role analogous to that of the cross-bridges between myosin and actin filaments in striated muscle. On the basis of current theories of mechanical motion in cells, it is not obvious how a single component protein structure might function or transduce chemical to mechanical energy within itself.

The Mitotic Apparatus

The fibrous proteins of the mitotic apparatus provide supporting structures with directionally specific motility to bring about the sorting and aggregation of chromosomes in the division process (Robbins and Gonatas, 1964b; Inoué and Sato, 1967). There is convincing evidence that microtubules form the substance of the classical spindle fibers seen with the light microscope in stained preparations of the mitotic apparatus. Microtubules are said to account for 10 percent of the spindle protein (Bibring and Baxandall, 1968) and to be responsible for the birefringence of the spindle and astral rays which comprise the mitotic apparatus (Inoué and Sato, 1967; Rebhun et al., 1967).

In the later stages of cell division (cytokinesis), peristaltic waves have been observed to move along the stalk connecting the two daughter cells; undulating motions appear to move residual cytoplasm which contains a large amount of microtubules out of the stalk into the daughter cells (see Figure 5). Electron microscopic examination showed that the stalk is rich in microtubules which may be responsible for the isolated peristaltic movement.

Among the proteins which have been isolated from the mitotic apparatus is a 6S protein (discussed in greater detail on page 156) which characterizes microtubules in other cell types. Unlike microtubules in many other cells, those of the mitotic apparatus are unstable and dissociate, presumably to monomers or dimers, at low temperature (4°C) or high pressure.

Colchicine has long been known to block cell division in metaphase. This blocking action is thought to be due to the strong binding of colchicine to the microtubule protein, after which the subunits no longer aggregate in the characteristic helical array of protofilaments (see page 157). Details of the chemical basis of the inhibiting action are still unknown.

Intracellular Transport

The intracellular site of synthesis of proteins and other critical molecules is, in many cases, relatively remote from their point of utilization. Therefore efficient intracellular transport of metabolites is a critical activity of cells. Furthermore, effective coordination of metabolic and central processes would seem to require that transport be directional, specific, and rapid, distributing substances to their appropriate

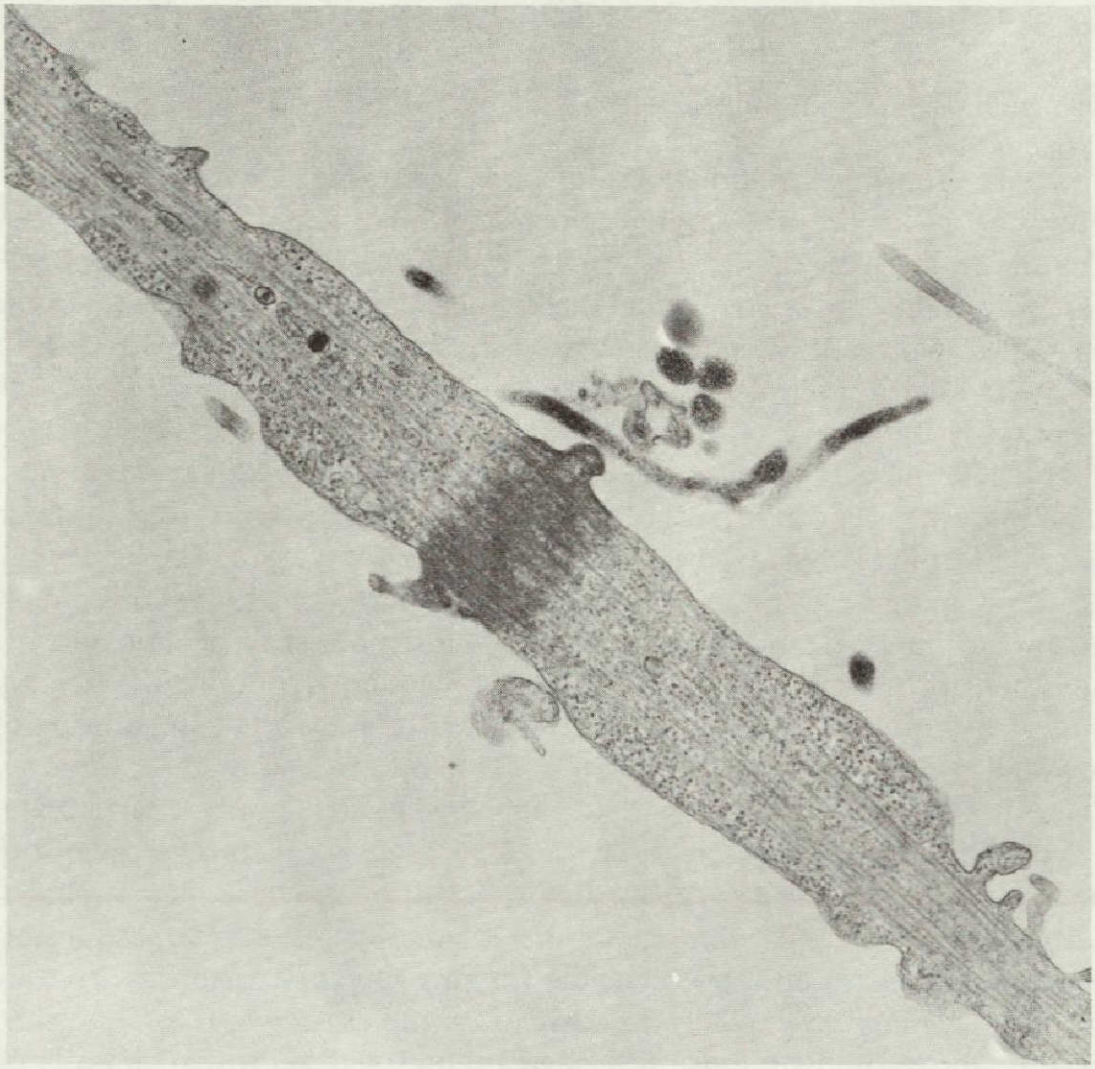

Figure 5. Longitudinal section of stalk and mid body connecting two cells at end of cytokinesis. The mid body is the dense band in the center. From it microtubules extend in opposite directions along the stalk toward the daughter cells. Two narrow regions of the stalk, equidistant from the mid body, represent zones of constriction which move along the stalk and away from the mid body during the final stages of cytokinesis. Magnification, X 32,000. [Micrograph courtesy of B. Byers]

destinations without undesirable mixing and interaction with other substances en route.

It is possible that several kinds of motile systems exist for intracellular transport. Prominent among these are the saltatory movements (discontinuous jumps) characteristic of the melanin granules in melanocyte processes, the streaming, both unidirectional and bidirectional, observed in protists and even in some cultured neurons (see Allen, 1967; Burdwood, 1965), and the transport of organelles (e.g., mitochon-

dria and lysosomes) and secretory granules in a wide variety of cells. Less is known about the active translocation of solute molecules in the absence of a membranous, vesicular enclosure by which tracking can be made with light optics.

In some cells transport or translocation is mediated by an actomyosin system rather than by microtubules. Slime mold is such a case according to Adelman and Taylor (1967) who have isolated typical actin- and myosin-like proteins from these cells. This kind of macroscopic streaming involves, however, nuclei as well as smaller inclusions and results from peristaltic contractions of filaments oriented normally to the direction of flow.

The importance of intracellular transport in neurons is self-evident in view of the separation of the metabolic center in the cell body from synaptic terminals by a sometimes enormously long axon. The volume of the axon may be a thousand times that of the cell body and simple diffusion would be much too slow and nondirectional to account for the movement of material in the axon. Data from many laboratories, recently published in an *NRP Bulletin* (Barondes, 1967) show that, in addition to the slow, 1 to 6 mm per day, cellulifugal axoplasmic flow discovered by Weiss, there is a fast, 10^2 to 10^3 mm per day transport of particulates which is directional and selectional with respect to substances present in the axoplasm. Hydrostatic forces such as might be generated by peristaltic contractions of sheath components, postulated by Weiss to be responsible for the slow flow, could not account for this type of transport. The only structures which extend continuously over the entire route of transport are the fibrous constituents, the microtubules and neurofilaments. Thus far no function has yet been conclusively demonstrated for these ubiquitous structures in neurons. Therefore it seems attractive to speculate on the possible role of microtubules and neurofilaments in both the slow flow of axoplasm and the fast, specific transport of certain axoplasmic constituents.

A source of enlightenment on this problem is the case of the transport of cytoplasmic constituents in both directions in the tentacles of *Tokophrya* (Rudzinska, 1965). This organism feeds by the use of tentacles which puncture the protective membranes of the prey (*Tetrahymena*) and act as a conduit for the transport of the prey cytoplasm as food to the predator cell. The tentacle possesses a group of 49 microtubules arrayed in a circular pattern of 7 sets of 7 tubules that separate the two streams of cytoplasm. The prey cytoplasm moves in the inner channel roughly surrounded by the microtubules while cytoplasmic constituents, e.g.,

granules, move peripherally in the outer channel between the microtubules and the tentacle membrane. Thus there is clearly two-way, bidirectional traffic and it is possible that it is the microtubules which are supplying the effective motive force or are a component of the system which does. Early observations on living *Tokophrya* included descriptions of peristaltic waves in the tentacles resembling (possibly in their action) those noted recently in the stalk connecting cells completing cytokinesis.

The melanocyte is another example of a cell in which microtubules are present in profusion and in which transport of particulates is a primary function (Bikle et al., 1966). The melanocyte contains many large, pigmented melanin particles, which move in or out of the arm-like extensions of the cell in response to neural, hormonal, or chemical stimulation. The arms of the melanocyte are populated with microtubules arranged so as to create "tracks" or channels along which the granules move centrifugally or centripetally depending on the nature of the stimulus. The melanin granules move rapidly and in a saltatory manner when "outward bound" but move smoothly and at a uniform rate when "inward bound." In some pigment cells, the granules have been observed to move rapidly and concentrate around the nucleus when small quantities of norepinephrine have been added. These movements can be reversed by the addition of acetylcholine. Because the granules seem to be moving between the microtubules, it has been suggested that microtubules may be guiding the direction of their movement (Green, 1965; Bikle et al., 1966; Allen, 1967).

In such bidirectional systems, if the force is not extended hydrostatically, it would seem that specific transport must result from movement occurring at the surface of the microtubule probably by a topochemical reaction. Generally, the microtubule alone (represented by the protein of its wall) is not sufficient since it presumably lacks the enzyme ATPase or GTPase necessary to liberate energy from ATP or GTP. In cilia such an ATPase, dynein, is present as side arms protruding from the microtubule and could liberate the necessary energy. As the reader can readily surmise, no comprehensive transport theory has yet been developed comparable to that for muscle contraction.

C. PROTEIN CHEMISTRY OF MICROTUBULES: DISCUSSION LED BY E. W. TAYLOR

Preparation and Physicochemical Characterization of Microtubule Protein

Although some knowledge exists about the chemical nature of microtubules, a clearer picture of their chemical and ultrastructural properties must emerge before their functions can be understood in detail and with certainty. Most of the chemical evidence has come from investigations of microtubules in sperm tails (Shelanski and Taylor, 1967, 1968) and in cilia (Renaud et al., 1968).

The protein from the central pair of tubules in the sea urchin sperm tails has been isolated (Shelanski and Taylor, 1967, 1968) and shown to be essentially one component with a sedimentation constant of 6S. Its molecular weight, determined by a variety of procedures including short column equilibrium, sedimentation and diffusion, and the meniscus-depletion method, is 120,000. The protein is relatively unstable and dissociates into 60,000 molecular weight subunits. Treatment with 6 M guanidine hydrochloride also yields a 60,000 molecular weight unit.

The outer doublet tubules of *Tetrahymena* cilia (Renaud et al., 1968) and sea urchin sperm tails (Shelanski and Taylor, 1968) can be dissolved at high ionic strength and the preparation yields a similar protein having a sedimentation constant of 6S and a molecular weight of 120,000, which can be converted to a 60,000 subunit in 6 M guanidine hydrochloride. The subunit proteins from central pairs and outer doublets possess a binding site for guanine nucleotide and there appears to be one binding site per 60,000 molecular weight dimer. The intact outer doublets of cilia and sperm tails contain one mole of guanine nucleotide per 60,000 gm of protein (Stephens et al., 1967).

Comparison of Microtubule Protein with Actin

The similarities between the microtubule subunit protein and actin have been noted by Renaud and his co-workers (1968). The amino acid compositions and electrophoretic mobilities of both proteins are very similar and each possesses a nucleotide-binding site. However, the differences appear to outweigh the similarities; the relative affinities for the nucleotides GTP and ATP are different; the molecular weights appear to differ by more than the experimental errors in the measurements; the microtubule protein binds colchicine whereas actin does not (Borisy and

Taylor, 1967a); actin contains one residue per mole of the rare amino acid 3-methylhistidine whereas the colchicine-binding protein from brain does not have measurable amounts of this compound (Weisenberg et al., 1968). The two proteins, though similar, do not appear to be identical. Nevertheless the similarities are interesting and may indicate some evolutionary relationship.

Colchicine and Microtubules

Colchicine is known to interfere with a number of cell functions dependent on microtubules, including mitosis (Eigsti and Dustin, 1955), movement of melanin granules (Porter, 1966), and saltatory movements (Freed, 1965). By the use of ^{3}H-colchicine it has been shown that colchicine forms a specific complex with a protein found in dividing cells, mitotic apparatus, cilia, and nervous tissue (Borisy and Taylor, 1967a). In all cases the protein has a sedimentation constant of 6S and in the case of the sperm tail it has been shown that the 6S colchicine-binding protein is the microtubule subunit described in the previous sections (Shelanski and Taylor, 1967).

Thus colchicine binding can be used as an assay for microtubule protein. By using this property, a protein has been isolated from mammalian brain which in its sedimentation constant, molecular weight, amino acid composition, and GTP-binding capacity closely resembles the cilia protein (Weisenberg et al., 1968). Presumably this protein is the subunit of neurotubules.

D. PROTEIN SUBUNITS OF MICROTUBULES: DISCUSSION LED BY R. E. STEPHENS

Subunit Proteins of the Mitotic Apparatus

A number of protein fractions from both the microtubules and the associated matrix in the mitotic apparatus have been described in the literature. The microtubule subunits are presumably present as a pool of dissolved monomers in the resting phase pending activation to cell division, at which time the monomers polymerize into microtubule spindle fibers. When isolated in the presence of dithiodiols, the principal protein component of the mitotic apparatus is a 3.5S subunit which can be reduced to two 2.5S subunits of about 34,000 molecular weight

(Kiefer et al., 1966). A 4S and a 22S component are obtained from hexylene glycol isolates (Kane, 1967). The 4S component from the microtubules is probably the monomer of a 6S component, but this value is an approximation and may represent a mixture of 3S and 6S proteins since the material is not homogeneous. The 4S component has a molecular weight of 66,000 (Stephens, 1968a). A different protein with a sedimentation constant of 22S and 880,000 molecular weight (Stephens, 1967; Kane, 1967) is not a subunit of the microtubule, nor a precursor, but appears to be associated with the surrounding matrix. Interestingly, no 22S component has been isolated from sperm tail microtubule preparations. However, a digitonin extract of sperm tails dissolved in 0.6 M KCl and ultracentrifuged will yield a 25S to 30S heterogeneous fraction. Fortuitously, the amino acid composition of this material corresponds almost exactly to that of the 22S peak, differing only in glycine and alanine content.* The 3S protein or its 6S dimer is the only one which is always present when the microtubule protein is isolated from different cell types.

Subunits of Flagellar and Ciliary Microtubules

When flagellar or ciliary outer fibers are isolated by selective solubilization (Gibbons, 1965b), a 3S subunit of 55,000-60,000 molecular weight can be obtained by dissolving the fibers in 8 M urea or 5 M guanidine hydrochloride (Renaud et al., 1968; Stephens, 1968a; Shelanski and Taylor, 1968). Solubilization in 50 mM Salyrgan or extraction of an acetone powder of whole cilia yields a 6S, 100,000-120,000 molecular weight dimer (Renaud et al., 1968; Stephens, 1968a). The central pair protein has properties similar to, if not identical with, this 6S outer fiber protein (Shelanski and Taylor, 1968). Regardless of source, ciliary or flagellar, central pair or outer fiber, the proteins show a strong resemblance to muscle actin in their amino acid compositions (Renaud et al., 1968; Stephens, 1968a; Shelanski and Taylor, 1968). The mitotic apparatus protein has a composition nearly identical to the ciliary and flagellar proteins (Stephens, 1968a).

The proteins are globular and contain 25-30 percent glutamic and aspartic acids, and a moderately high proline content, allowing little chance for α-helix formation (see Table I). Also noteworthy is that the electrophoretic mobilities of the proteins and that of actin are very similar (Renaud et al., 1968; Stephens, 1968a; Shelanski and Taylor, 1968).

*R.E. Stephens, unpublished.

TABLE I

AMINO ACID COMPOSITION*
(Moles per 100 Residues)

Amino Acid	Microtubules	Actins			Neurofilaments
	Cilia of *Tetrahymena*	Slime Mold	Rabbit Muscle	Squid Muscle	Squid Axon
Aspartic acid	9.4	8.4	8.2	11.8	10.5
Threonine	4.6	6.1	5.9	5.5	5.3
Serine	5.4	5.6	5.6	6.0	8.8
Glutamic acid	11.7	11.4	10.1	13.3	14.0
Proline	3.9	6.1	4.4	4.9	6.0
Glycine	8.0	7.4	6.7	9.8	6.1
Alanine	5.6	6.8	7.1	7.9	7.9
Valine	5.3	3.2	4.2	4.6	5.9
Cysteine	1.3	1.1	1.1	†	1.3
Methionine	2.6	2.8	3.0	2.8	2.7
Isoleucine	4.9	3.4	5.7	5.7	4.2
Leucine	6.6	6.1	6.3	8.8	7.8
Tyrosine	2.9	3.2	3.2	2.9	2.4
Phenylalanine	3.9	2.9	2.9	3.3	2.9
Lysine	5.1	4.1	5.2	5.7	6.0
Histidine	2.2	1.7	1.9	1.3	2.1
Arginine	4.1	4.0	3.8	4.9	4.7
Tryptophan	0.7	†	†	†	1.2

*See also the amino acid compositions in Renaud and co-workers (1968), Shelanski and Taylor (1968), and Stephens (1968a).
†Not determined.

Dynein

Dynein is a structural protein with a molecular weight of about 600,000 and high ATPase activity, an interesting property since 6S protein has no such enzyme activity. Dynein constitutes the so-called "arms" or "projections" which occur in pairs on the outer microtubules of cilia (see Gibbons, 1963, 1965b). The arms are all oriented in the same direction with respect to the radial axis of the cilium and with its direction of beat. Since dephosphorylation of ATP supplies the energy needed for motility, it is believed that dynein probably plays a functional

role in the motile process in cilia and flagella. Dynein has been reported to consist of two enzymatically active fractions which sediment at 14S and 30S, the latter being more physiologically active and probably representing polymers of globular 14S units (Gibbons and Rowe, 1965). Although both dyneins can be activated by either Ca^{++} or Mg^{++}, their Ca/Mg ratio differs (Gibbons, 1966). In neurons, however, no dynein-constituted side arms are found on the microtubules nor has any ATPase closely related to the microtubules been identified.

Nucleotide Interactions with Microtubule Proteins

The findings that the proteins of the outer fiber fractions of flagella from sea urchin sperms and of cilia from *Tetrahymena* bind guanine nucleotide in a 1:1 molar ratio of protein subunit (i.e., 1 mole per 60,000 g of protein) invite comparison to actin which similarly binds another nucleotide, adenosine triphosphate (1 mole of ATP per 60,000 g of G-actin)(Stephens et al., 1967). It has not yet been established whether the terminal phosphate of the nucleotide is split when the protein forms microtubules; the isolated outer fibers contain all three guanine nucleotides in the case of sperm tail flagella and also the free base and guanosine in the case of cilia, thus indicating that the nucleotide is susceptible to degradation (Stephens et al., 1967).

Repolymerization of Microtubules

So far, attempts to reconstitute microtubules from native subunits have met with limited success. Attempts of Renaud and his co-workers (1966, 1968) to form tubules from outer fiber protein resulted in the formation of fibrous material composed mainly of protofilaments. Various reasons for the failure have been suggested including possible degradation of the protein or its nucleotide moiety during isolation procedures. Stephens (1968a) found that treatment of outer fibers with agents such as guanidine hydrochloride, urea, and organic mercurials so alter the protein that polymerization from its subunits is impossible.

By treating the outer fibers of sea urchin sperm tails with low concentrations of Sarkosyl (sodium lauryl sarcosine), Stephens (1968b) has been able to reassociate microtubule protein subunits into ribbon-like strands 200 to 300 A wide and 2 μ long by dilution of the detergent solution. The addition of salt brings about aggregation of the ribbons into tubules up to 10 μ long. However, "nucleation" with some intact

microtubules was necessary for any polymerization to occur. This attempt at repolymerization recalls similar experiences of Oosawa and his co-workers (1966) and Asakura and his collaborators (1964, 1966) with the reconstitution of bacterial flagella from dissolved subunits. The efforts of the latter investigators at *in vitro* reconstitution resulted only in the growth, i.e., polymerization, of the monomeric flagellins onto the ends of added fragments or "seeds." Furthermore, they recently reported that this growth is in a unidirectional manner (see Asakura et al., 1968). Also, the earlier experiments of Davison and Taylor (1960) showed that the restoration of neutral conditions caused the lengthening of neurofilaments which had been partially disassociated by raising the pH of the solution. These experiments also could indicate the reconstitution of neurofilaments from subunits in a seeded preparation; for this protein, too, no neurofilaments have been reconstituted from subunits without seeding.

Although the reconstituted protein stains similarly to the microtubules from which it originated, the electron micrographs are not conclusive; in addition, the protein forms no doublets (the protein was prepared from the microtubule doublets) even after the addition of dynein. The nature of the subunit degradation responsible for this behavior is unknown. Stephens (1968b) noted that neither cold nor addition of colchicine prevented reassociation of the protein nor did D_2O encourage it.

X-Ray Diffraction of Microtubule Protein

X-ray diffraction studies of outer fiber microtubules reveal an equatorial spacing of about 50 A and a meridional spacing of 37 A.* If the subunits are wound in a helix, a pitch of at least 10 degrees is predicted from these data. There are difficulties with this approach since it has been suggested that a lipid component masks the subunit spacing of the outer tubule pairs, and, if the preparation is freed of lipid, the structure is destroyed. It is not known whether the lipid is closely associated with the globular subunit or if it is a residual of the matrix material. Tentatively, a model with 13 subunits per turn gives the right pitch and agrees with the available data. The lateral bonding is much weaker than the end-to-end bonding of the subunits.

*C. Cohen, S. Harrison, R.E. Stephens, and W. Longley, manuscript in preparation.

E. CHARACTERIZATION OF MICROTUBULES, NEUROFILAMENTS, AND CROSS-BRIDGES IN VARIOUS NEURONAL TYPES: DISCUSSION LED BY A. PETERS

Morphology and Distribution of Microtubules and Neurofilaments within Neurons

Three classes of fibrous structures have been described in neurons as pointed out in the first two sections of the essay accompanying this report (see pages 119-125). These structures are termed fibrillar, microtubular, and filamentous and are distinguished primarily by their morphological appearance and the methods by which they are visualized. Connecting strands referred to as cross-bridges are occasionally seen between neighboring microtubules and are typical accessories to the microtubules when they are organized into bundles of characteristic patterns.

The central dense dot seen inside some microtubules is common in neuronal microtubules and may be a universal structure that is easily lost in tissue preservation (see Figure 6). In longitudinal sections it appears to have a rod-like rather than a spherical shape. The presence inside microtubules of material which survives electron microscopic preparative techniques suggests that the material is macromolecular, and, conceivably, it could represent a strand of subunits unincorporated into the helical structure. No ideas have yet been expressed as to why this structure is observed in neurons but seldom in other cell types.

Tissue preparation, fixation, penetration of the fixatives, etc., in different locations of the neurons, length of time and temperature of the preservative, all might be important in determining the preservation and appearance of filaments and microtubules. Until their characteristics with respect to these variables are known, generalizations about their appearance and possible functions in neurons may be misleading. Glutaraldehyde fixation, which played such an important role in the revelation of microtubules in nervous tissue, was originally employed by histochemists (e.g. R.J. Barrnett) to preserve enzyme function, and it is possible that the hydrolytic enzymes thus preserved may affect fine structures depending upon the conditions prevailing. For instance, neurofilaments are known to be readily attacked by proteases.

Microtubules (neurotubules) and filaments (neurofilaments) are universal and prominent structures in neurons of all types. However, their distribution within the neuron or among cell types is not uniform.

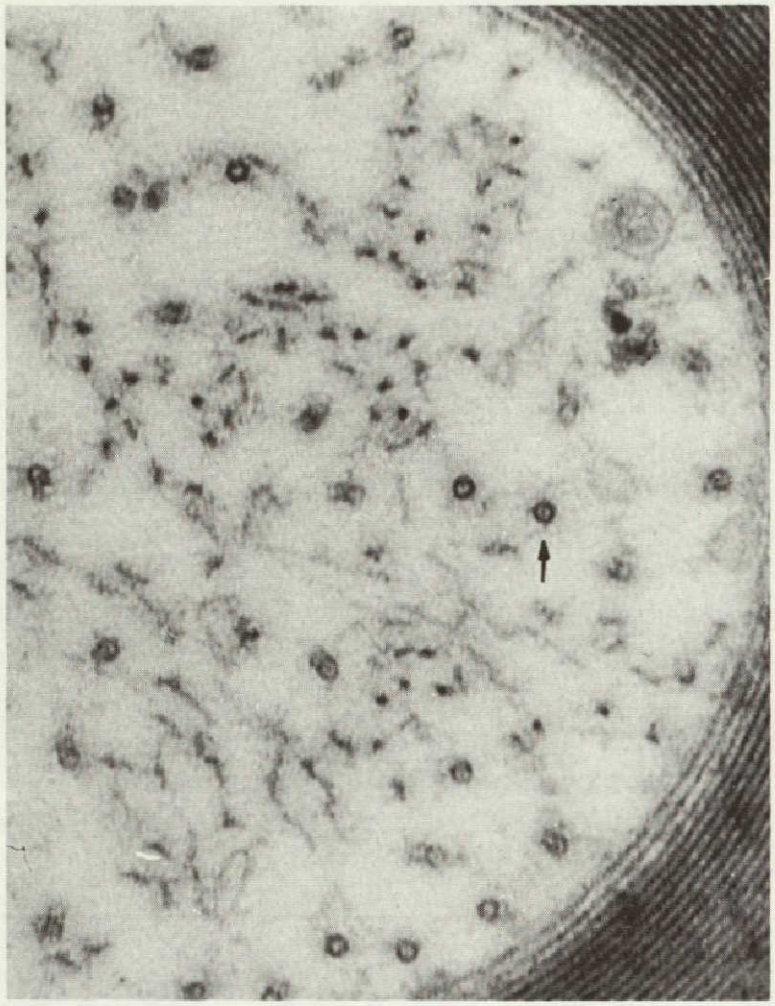

Figure 6. Myelinated axon (turtle vagus nerve) showing random distribution of microtubules and neurofilaments. Electron-dense dots can be seen in the cores (see arrow) of the microtubules. Magnification, X 213,000. [Courtesy of B. Lim]

Further, there is a roughly reciprocal relationship in the relative abundance of microtubules and filaments; when one is abundant, the other is usually scarce.

As a general rule, small nonmyelinated axons have microtubules but few filaments. Conversely, maturing or large myelinated axons have many filaments but few microtubules. For example, in axons of the rat optic nerve, neurofilaments first appear usually in clusters rather than being homogeneously distributed throughout the axoplasm when the axon starts to myelinate. Small myelinated axons frequently have both microtubules and filaments, and, in the myelinated axons of the cat, the neurofilaments do not always extend into the synaptic bulb in the characteristic ring or toroid configuration.

In those parts of the axon which are not myelinated, e.g., the initial segment (axon hillock), the node and paranodal regions, and the terminal parts of the axon, microtubules are clearly evident while in those parts of the axon covered by myelin they are more rarely seen. This is suggestive of a relationship between microtubules and the excitation processes which, in the ionic hypothesis, are limited to axonal membranes uncovered by myelin layers. Perhaps, it would be wise to await more detailed statistical data before speculating further on the possibility of this relationship.

Species differences account for variations in the general rule concerning the relative abundance of tubules or filaments in axons of various sizes and degree of myelination. For example, in the dorsal root of the cat and the frog sympathetic chain, the nonmyelinated axons as well as the large myelinated axons possess principally filaments. Axons in the same regions of the rat, on the other hand, more typically have a preponderance of microtubules rather than of filaments. These variations may reflect important, yet unknown, functional differences.

Microtubules are usually abundant in dendrites and may, in fact, occupy a large fraction of the dendritic cytoplasm, larger than that of any other organelle. Neurofilaments are uncommon in dendrites, but they have been observed in the dendrites of anterior horn cells. In the dendrites of other cell types, for example the pyramidal cell of the cortex, the cytoplasm is literally packed with microtubules that are very long and tend to be oriented parallel to the dendritic axis.

Although certain clusters of microtubules always contain cross-bridges with a well-defined size and shape, they are bound together by cross-bridges into fascicles only in the initial segment of the axon (Palay et al., 1968; Kohno, 1964). Beyond the initial segment, microtubules appear chiefly as individuals. In nonneuronal cells where microtubules seem to be involved in motility, they are characteristically bound together with cross-bridges (Grimstone and Cleveland, 1965; Rudzinska, 1965). The coincidence of the initial segment as the site of action potential initiation and the unique presence of microtubules held together in fascicles by cross-bridges has led to the speculation that the electrotonic current of the cell body may elicit contractility in the interconnected microtubules; this mechanical disturbance may, in the initial segment, cause molecular changes in the membrane leading to the firing of a self-regenerative action potential wave (Palay et al., 1968).

A halo or region of exclusion which is commonly seen around microtubules may represent an area that was occupied by some molecule

closely related to the microtubule and that was lost in tissue preparation. The spacing between fibrous structures is often characteristic and may be critical to normal function. In neurons at the hillock the microtubules are close together and definite cross-bridges extend between them, whereas in other regions such as axons they are spaced further apart. Spacing is also important in contrasting the filaments in glia with those in neurons. Glial filaments often appear in bundles whereas neurofilaments are spaced further apart. The factors that determine the spacing and the relative positioning of the fibrous structures in different physiological states of a cell are largely still unknown; thus this subject obviously needs further experimental illumination.

Distribution of Fibrous Proteins in Different Cell Types of the Central Nervous System

Some satellite cells are rich in fibrous structures, as, for example, the astrocytes which have many microtubules especially in the neonatal brain. At one stage in the maturing rat brain (11 days postnatally), astrocytes have many microtubules and no filaments, but, as the brain matures, filaments abruptly appear and microtubules tend to diminish in number (Vaughn and Peters, 1967). Although superficially similar to neuronal neurofilaments, the filaments of astrocytes have a slightly smaller diameter (see Figure 7).

Within the axons of the leech there are characteristic neurofibrils which in light microscope preparations have the same staining reactions as the neurofibrils of vertebrates but a quite different structure. In the electron microscope the leech neurofibrils are seen to be composed of bundles of filaments which appear to be joined by cross-bridges and the bundle as a whole has the appearance of a heavy rope. Whether each component filament within a bundle is equivalent to the neurofilament of the vertebrate neuron is not yet known.

Relation of Microtubules to Developmental Processes

During maturation the axon first contains microtubules homogeneously distributed; very few neurofilaments are evident at this time. As the stage of myelination approaches, microtubules give way to filaments except in those regions which are not myelinated (initial segment, node and paranodal regions, and terminal parts of the axon) and where the microtubules are to a large extent retained (Peters, 1967).

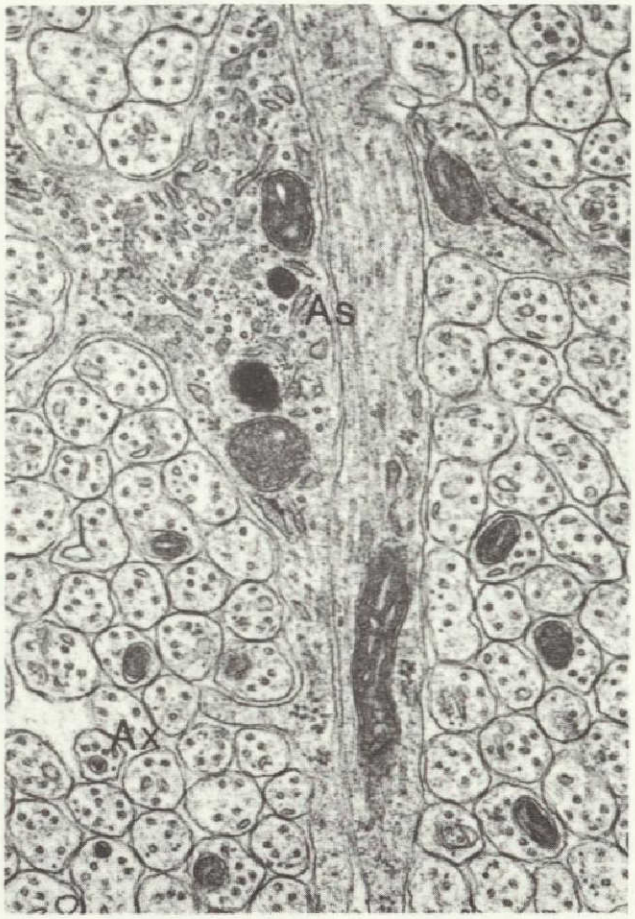

Figure 7. Electron micrograph of part of the optic nerve of a 3-day postnatal rat. Axons (Ax) and astrocyte processes (As) are present. Both contain microtubules and few filaments. Magnification, X 40,000. [Original micrograph by J. E. Vaughn and comparable to those shown by Vaughn and Peters, 1967]

According to Peters and Vaughn (1967) the cytoplasm in the optic nerve of the neonatal rat undergoes changes with maturation. Three days postnatally, the axons are very small (0.2-0.3 μ in diameter) and possess typical microtubules which appear in tissue fixed by aldehyde perfusion as circles about 230-250 A in diameter; sometimes there is a central "dot" inside the circles. Very few neurofilaments are seen at this stage. At 9 days postnatally, the axons are larger but they still contain chiefly microtubules, and a few filaments begin to appear at this time; at 11 days the relative number of microtubules and filaments changes considerably. Similar but more dramatic changes occur in astrocytes; in the mature

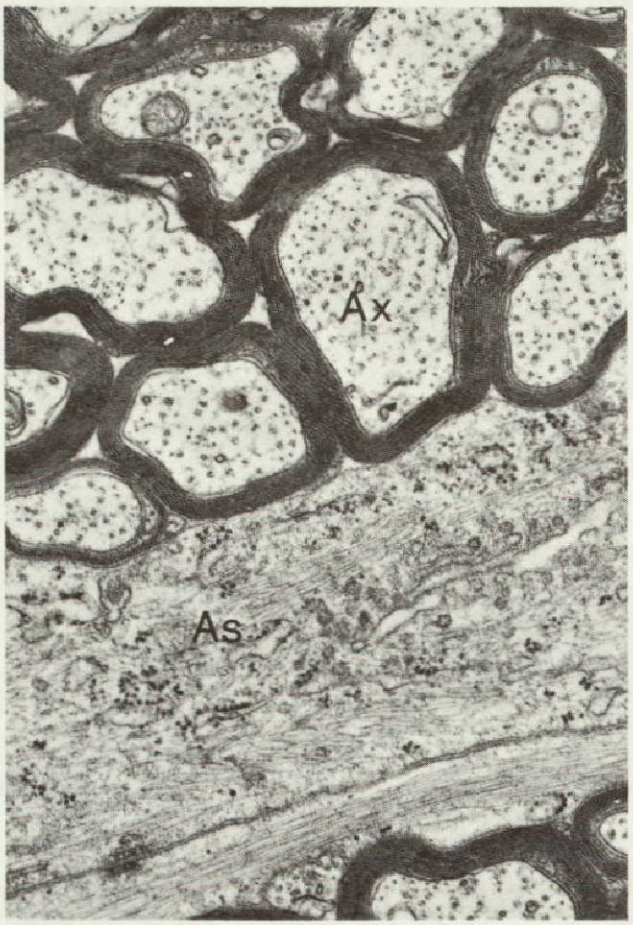

Figure 8. Electron micrograph of part of the optic nerve of an adult rat. The myelinated axons (Ax) contain neurofilaments and microtubules in their cytoplasm, and the astrocyte processes (As) have bundles of filaments. Compare with Figure 7. Magnification, X 35,000. [Original micrograph by Peters]

fibrous astrocytes, filaments pack the cytoplasm and microtubules are rare (Vaughn and Peters, 1967) (see Figure 8).

Pleomorphism of Microtubules, Neurofilaments, and Vesicles

The dynamics of the fibrous proteins is almost an unexamined subject. There is practically no information about their turnover rate and the degree and kinetics of their polymerization and depolymerization. It has been suggested from indirect evidence that microtubules and filaments are interconvertible, consisting of the same protein subunits aggregated in different ways. This view is supported by the reciprocal presence of

tubules and filaments and the events accompanying maturation discussed above.

It has been proposed (Peters and Vaughn, 1967) that microtubules, which are formed early in neuronal development, are converted into neurofilaments in later developmental stages; each cluster of neurofilaments presumably represents a microtubule precursor. However, it is possible that microtubules are not transformed into neurofilaments (which themselves may comprise some 5 helically wound protofilaments or strands) but rather into the elementary protofilaments or strands themselves, i.e., a linear aggregation of globular protein subunits. Obviously, it is important to be accurate about the terminology and conceptualization employed in connection with this hypothesized interconversion of fibrous types.

The amino acid composition of the microtubule protein is similar to that of the neurofilament protein, but this is inconclusive for identity or interconversion.

A crucial point for the transformation hypothesis mentioned above is that since the constituent subunit strands (protofilaments) of microtubules are about 45 A or 90 A in width, depending on whether the monomer or dimer is polymerized, they could hardly have the same structure as the neurofilaments which consist of about 5 subunit strands and show a 30 A hole (presumably a channel) in cross section. Conceivably, subunit strands of microtubules could first disaggregate, owing to changes in their chemical environment, and then reaggregate after the fashion of neurofilaments. The conversion of microtubules into vesicular structures by "a ballooning out" dilatation of the tubules has been suggested (Sandborn, 1966). The evidence for such a transition is entirely inferential from electron micrographs and is not widely accepted owing to the possibility of error from optical overlay and the angle of sectioning.

Being relatively insensitive to low temperature* or high pressure, microtubules of neurons appear to be among the stabler types. However, they are sensitive to compounds such as aluminum which effects fibrillar tangles and colchicine (and other mitotic spindle inhibitors) which causes them to disappear (Wiśniewski et al., 1968). Under certain conditions, their disappearance is accompanied by the appearance of filamentous material. These fibrous structures are highly pleomorphic and their relationship to normal structures is still not clear. (This subject is discussed in greater detail on pages 184 to 186.)

*Personal communication from B. Lim.

F. RELATIONS BETWEEN NEUROFIBRILS, MICROTUBULES, AND NEUROFILAMENTS IN SILVER-STAINED PREPARATIONS: DISCUSSION LED BY R. W. GUILLERY

Are Silver-Stained Neurofibrils Primarily Microtubules or Neurofilaments?

The question as to which structures seen in the electron microscope correspond to the silver-stained neurofibrils observed with light microscopy cannot be answered in an unqualified way. There was, in fact, some disagreement among participants at the conference on this subject. As seen with a light microscope in silver-stained preparations, neurofilaments in their distribution and occurrence correspond better with neurofibrils than do microtubules or any other subcellular substructure. As Bodian suggested, it is possible that the staining is quantitative rather than qualitative in character and depends on the details of the technique. Heimer proposed that the molecular alignment typical of filaments, as well as the tendency of filaments to occur in more or less compact bundles, may in itself increase stainability, in which case there would be no specific staining by silver methods of microtubules or neurofilaments but merely a quantitative difference in the amount of silver seen in different places. Cilia and the I and A bands of muscle stain with silver as do most polyionic compounds. Also, nuclei and nucleoli are easily encrusted with silver in most of the silver techniques. However, certain procedures would probably achieve a more selective staining than others.

The neurofibrillar stains reveal some nerve terminals with reticulated club-shaped endings and some ring-shaped endings. It can be shown that in these instances the histological stain reacts with neurofilaments, not with microtubules. Never have rings of microtubules been demonstrated in the terminal bulb. On the other hand, as Guillery has shown, rings or toroids of neurofilaments can readily be demonstrated at many sites where neurofibrillar rings can also be seen (Gray and Guillery, 1966; Guillery, 1967). Evidence of this kind convinces Guillery that silver stains primarily neurofilamentous structures.

Most of the axons in the developing nervous systems are unstained by the neurofibrillar technique as usually applied; electron microscopy shows that these axons contain microtubules but very few filaments. On the other hand, the nuclear cap on the developing neuron does stain with neurofibrillar techniques and possesses a high density of neurofilaments (see Guillery, 1965). Furthermore, an interesting observation is that

filaments are occasionally seen to be concentrated in postsynaptic regions, even forming rings in some dendrites of the lateral geniculate nucleus (Guillery, 1967). Although in light micrographs the neurofibrillar rings in this nucleus can be readily interpreted as axonal, electron microscopic evidence indicates that the neurofibrillar rings, being formed by bundles of filaments, lie in dendrites. Also worth noting here is that the neurofibrils of the leech axon stain histologically like classical neurofibrils. The relationship of these fibrils, which are characterized by a wide range of diameters, to neurofilaments is not yet clear.

It is obvious that the method by which the silver staining is done is critical since a wide variety of structures can be stained according to the modification used. Since there was consensus among the participants that bundles of neurofilaments have a neurofibrillar appearance, the controversy focused on how specific is the neurofibrillar staining procedure for neurofilaments. Would it stain individual filaments not organized into bundles? The resolving of this question would help to clarify the relationships between neurofilaments and neurofibrils.

The substantia gelatinosa (the relatively clear area capping the dorsal horn) is difficult to stain with neurofibrillar methods. This area is largely composed of very small neurons possessing many microtubules linearly arranged in their dendrites, yet the area is not argyrophilic. Admittedly, the small size of the dendrites presents a problem. Also, the substantia contains many nonmyelinated axons which possess linear arrays of microtubules but no neurofilaments. This appears to be an example of an area containing many microtubules in a linear array which does not stain with neurofibrillar methods. However, the region immediately adjacent to the substantia gelatinosa, where the constituent axons contain neurofilaments, stains with argyrophilic methods.

Some of the apical dendrites of the pyramidal cells stain with silver (Cajal, 1911) and many of these dendrites contain mainly microtubules. Were it not for the fact that neurofilaments are also present in some of these dendrites and that many dendrites do not stain with silver, this could be considered as a case in which microtubules had been demonstrated to take the silver stains.

The Bodian preparation of neurons from colchicine- or aluminum-treated animals, or of biopsies for diseases such as Alzheimer's (see page 184), shows a silver staining of the proliferated tubules which is essentially similar to that of tissue containing normal microtubules. Since the filamentous structures also show similar silver-staining properties, it is not possible to distinguish between the filamentous and tubular structures or their abnormalities by the Bodian method with the light microscope.

Silver probably does not stain microtubules generally in cells. Although there have not been any studies designed specifically to probe this question, the sizable number of cases where microtubules are known to be abundant but do not stain with silver supports this generalization.

Basis of Staining of Neurofibrils with Silver and Vital Dyes

The chemical basis of staining of proteinaceous structures with silver is not known in detail although the binding of heavy metals by acidic proteins well on the alkaline side of the isoelectric point may be expected to be extensive. The staining and the microscopic appearance of the stained structures are a function of both the method of tissue preparation and the nature of the subcellular structure. The ease of impregnation of neurofilaments and other fibrous structures with silver may be due to their compactness and anisodiametric organization rather than to specific chemical reactions.

Vital dyes, such as methylene blue and toluidine blue, have been used extensively to stain neurons *in situ*. Electron microscopy reveals that many of the fibrillar components described earlier from vital dye studies are actually individual axons with very small diameters. The increased staining after stimulation observed by Bethe (1920) and others may result from a local pH change or increase in some metabolite arising from axonal activity. There is no way of judging the state of a neuron at the moment of fixation because the fixation itself may impose such overwhelming chemical changes on the neuron as to mask any subtle changes related to excitability.

G. ROLES OF NEUROFIBRILS, MICROTUBULES, AND NEUROFILAMENTS IN DEGENERATION: DISCUSSION LED BY H. J. RALSTON, III

Typical degeneration of the axonal trunk does not appear to involve either neurofilaments or microtubules. An illustration of this is seen in cat spinal cord 3 days after dorsal root section (Ralston, 1968b). The degenerating myelinated axons, identified by an increased electron density of the axoplasm and a disruption of organelles, exhibit a floccular appearance which is not an increase in or a packing of neurofibrils or microtubules. Similarly, the nonmyelinated axons become electron-dense without direct involvement of the microtubules or filaments.

The terminals of axons react differently to axon sectioning. Some

undergo fibrillar hypertrophy whereas others show a "darkening" reaction, i.e., a marked increase in electron density (Gray and Guillery, 1966). This reaction to sectioning is not characteristic of neuronal types as seen in different species; in other words, the same neurons in different species respond differently. For example, in the monkey the neurons of the lateral geniculate show fibrillar hypertrophy, but in the cat there is the "darkening" reaction in some neurons and fibrillar hypertrophy in others (Colonnier and Guillery, 1964; Szentágothai et al., 1966). There are some indications that axon terminals which show the fibrillar hypertrophy survive longer (i.e., their mitochondria and vesicles do not clump as rapidly as those in electron-dense terminals) than those that exhibit the "darkening" reaction. The basis of the hypertrophy of fibrils is not known; it may be an accumulation of material that has been transported down the axon or the result of local fibrous proliferation.

Of the two types of degenerating synaptic knobs observed, the one showing a marked increase in electron density—and neither neurofilaments nor microtubules are involved—is by far the most common. These knobs may be of various sizes and are often distorted by an enveloping astrocyte. They are rapidly phagocytized by astrocytes and usually disappear 10 days after sectioning. Another type of degeneration, described first in chick optic tectum (Gray and Hamlyn, 1962), is an increase in the number of neurofilaments within the synaptic knob accompanied by a dispersion of synaptic vesicles to one side of the knob and a breakdown of mitochondria. The question can thus be posed: are these two distinct types of synaptic degeneration; i.e., neurofibrillar and dense, or are they two different stages of the same process; i.e., a synaptic knob first undergoes neurofilamentous degeneration followed by a marked increase in electron density?

In Ralston's view, neurofilaments and dense knob degeneration are distinct entities based on electron microscopic studies of the distribution of degenerated knobs in the six laminae of the dorsal horn (Ralston, 1968b). After dorsal root section in lamina III, there is an extremely heavy degeneration of synaptic knobs, all of an electron-dense nature, but neurofibrillar degeneration is never seen. On the other hand, deeper down in the dorsal horn (laminae IV to VI) there is not only dense knob degeneration after dorsal root section, but on the larger cells and dendrites in laminae IV and V, and on neuronal perikarya, dendrites, and other knobs in lamina VI, there are large knobs undergoing neurofibrillar degeneration.

The question arises as to why a synaptic knob undergoes one type

of degeneration and not the other. Some neurofibrillar degeneration occurs only in part of the spinal cord and along with dense degeneration. But in that area where the dense degeneration is the heaviest, neurofibrillar degeneration is never seen. One speculation based on anatomical relationships is that the fibers of the proprioceptive type undergo neurofibrillar degeneration while the cutaneous afferents undergo the "dense" degeneration. At this point it is not possible to say which normal knobs are predisposed to what type of degeneration.

H. FIBROUS CONSTITUENTS OF CELLS: DISCUSSION LED BY D.W. FAWCETT

There is no doubt about the close topographical relationship of filaments and microtubules in neurons and in many other cell types. Some investigators propose that filaments arise by disintegration of tubules into filamentous subunits whereas others would have them interchanging even more readily, with microtubules arising at the expense of filaments. Yet another view, derived from studies on insect spermatids, can be introduced. When the formation stages of microtubules are examined, as in the formation of the spermatid manchette of mammals or insects (e.g., the crane fly, see Behnke and Forer, 1966b), microtubules are not found intermingled with filamentous structures comparable to neurofilaments. There is, instead, a dense matrix material in the surrounding cytoplasm which seemingly can be resolved into very fine filaments. These may represent an aggregation of precursor materials which may be coming together to form the microtubules.*

In the spermatid manchettes, there are substantial, readily observable cross-bridges between the microtubules. Although the functions of these cross-bridges are not clear, they obviously determine the distance between neighboring parallel microtubules. No two microtubules are ever seen to be in contact in these structures. In one highly motile protozoan, *Pyrsonympha,* there is an orderly array of typical microtubules, arranged in multiple rows, extending perfectly straight for many microns (see Grimstone and Cleveland, 1965). As in the case of the spermatid manchettes, these microtubules are clearly connected with cross-bridges which are about 200 A in length. These cross-bridges are individual fibrillar connections rather than connecting sheets and recur at regular intervals along the length of the microtubules. Fawcett emphasized the

*D.W. Fawcett, unpublished.

distinction between these substantial cross-bridges and the other kind of ill-defined fuzzy material which may either be unincorporated precursors or simply protein precipitated out of the surrounding cytoplasm. An interesting feature of this protozoan is that during cell division the organized microtubule structures vanish and, simultaneously, the sinuous movements characteristic of the animal disappear only to reappear again when microtubules are again formed in the daughter cells.

It is possible to speculate that vigorous contraction or motility and not just skeletal shaping do accompany the presence of uniformly packed microtubules and well-developed cross-bridges as in the two cases just described. However, the molecular mechanism of the chemomechanical transduction in such systems remains completely unknown.

Relationships between Various Fibrous Constituents

According to Behnke and Forer (1967) there are at least four categories of microtubules that differ in their stability and structural relationships. They also differ in their susceptibilities to temperature (some microtubules disappear at low temperatures and others do not), to rate of digestion by pepsin, and to high pressure. Although these microtubules vary in their susceptibility to various agents, it does not necessarily mean that they are made up of different protein subunits but rather that their subunits are bound together in various ways.

Similarly, there may be more than one kind of neurofilament; e.g., those in the leech are clearly different from typical neurofilaments. Microfilaments resembling neurofilaments morphologically occur in many types of cells (see Figures 9, 10, and 11). The epithelial cells of the cow's muzzle, for example, have an abundance of filaments that seem to be very similar to neurofilaments (amino acid analyses and electron micrographs of these cells are available, e.g., see Matoltsy (1964, 1965)). Since skin cells and neurons both arise from ectoderm, could this common origin be in some way responsible for the similarity of these microfilaments? It should be pointed out that similarities in appearance of neurofilaments in the electron microscope do not necessarily mean identity of ultrastructure. No filaments have been as carefully examined in the electron microscope, biochemically, and biophysically as neurofilaments. Until such data are obtained no unequivocal answer will probably be possible concerning the identity of filaments in different cell types.

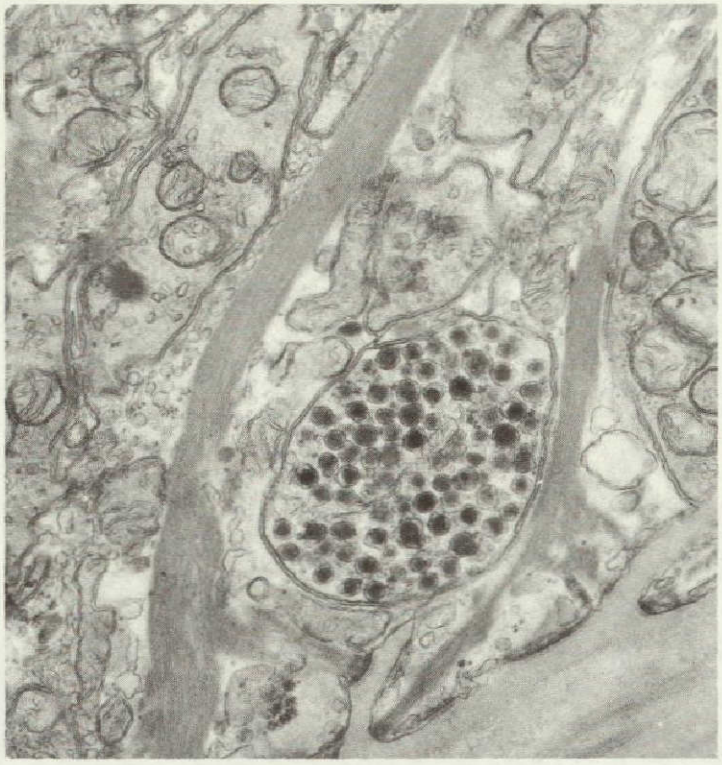

Figure 9. Glial filaments of the leech, *Hirudo medicinalis.* [Fawcett]

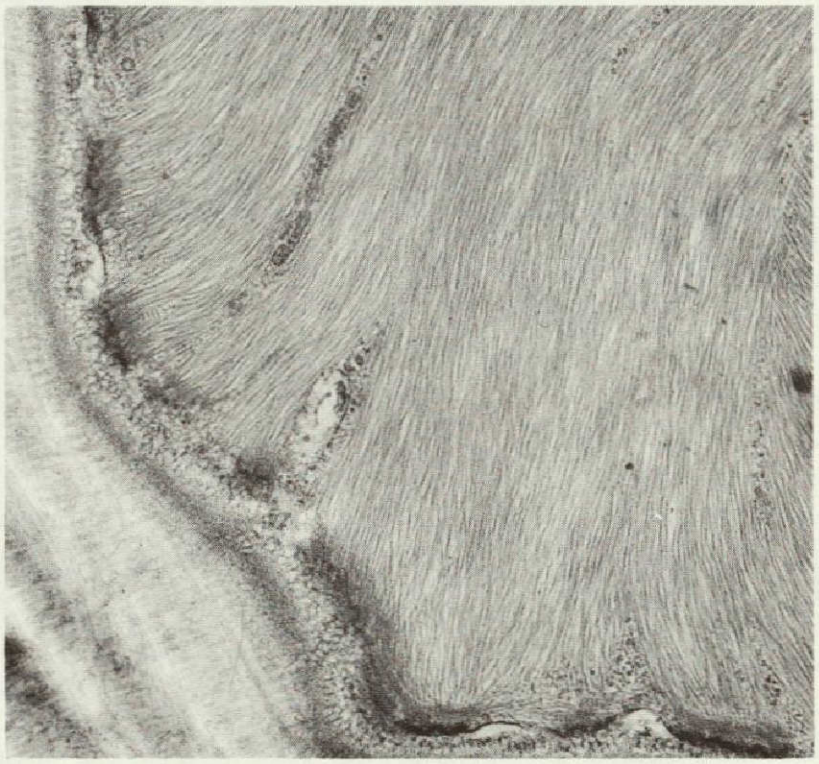

Figure 10. Filaments in epidermis of lamprey. [Fawcett]

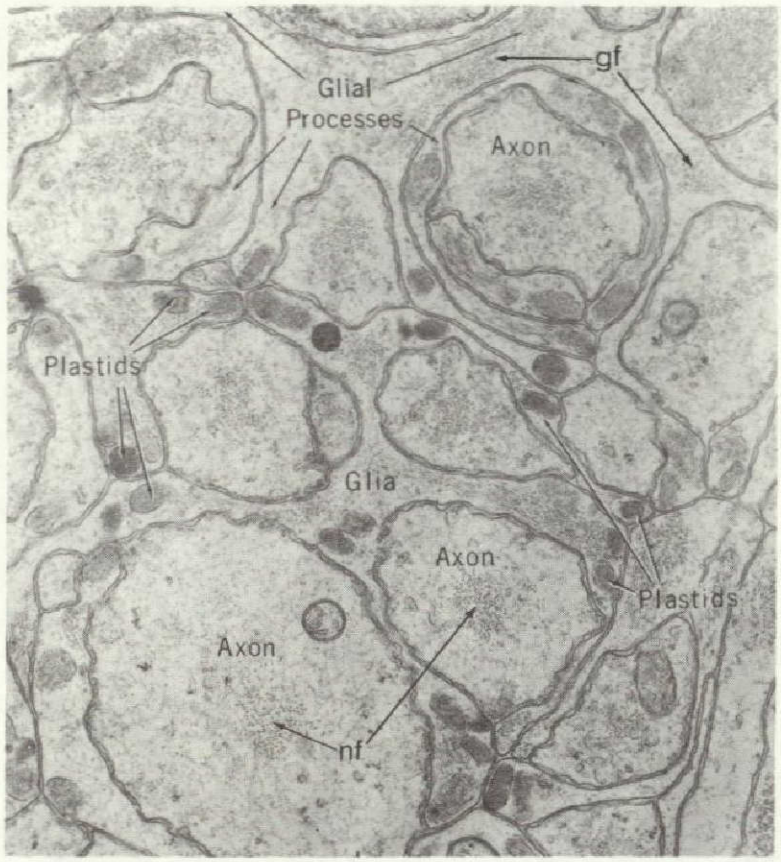

Figure 11. Neuronal (nf) and glial (gf) filaments of *Aphrodite*, a marine invertebrate. [Fawcett]

I. PHYSICOCHEMICAL PROPERTIES OF NEUROFILAMENTS: DISCUSSION LED BY P. F. DAVISON

The chemical investigation of neurofilaments has been confined almost entirely to the filaments of squid axoplasm since only squid axoplasm provides a sufficient amount of demonstrably intraneuronal material to make chemical characterization possible(Davison and Taylor, 1960; Schmitt and Davison, 1964). Thus the generality of the results obtained from these investigations is difficult to judge. An unknown but probably significant fraction of the fibrillar components of squid axoplasm is made up of microtubules but the predominant structure is the neurofilament. Therefore, several physical and chemical fractionation techniques have been devised to enrich the preparations with respect to neurofibrillar protein for further physical characterization.*

*P.F. Davison and F.C. Huneeus, unpublished.

The structure of the neurofilament as revealed by electron microscopy of nerve sections is a fairly stiff cylinder about 100-110 A in diameter, running apparently indefinitely the length of the axons. In transverse sections the filaments have the appearance of a hollow cylinder and Wuerker and Palay (1968) and Fernández-Morán* have photographs suggesting that four or five subunits are identifiable in the circumference of these cylinders. In negatively stained squid neurofilament preparations, a central dark line is observed frequently, an appearance which is compatible with the penetration of an electron stain into a hollow cylinder. On the other hand, the diameter of the filaments shows significant changes along the length of individual neurofilaments and at the meeting Davison discussed the possibility that the filament may be a bifilar structure composed of two protein chains, 60 A in diameter, of globular subunits. However, it appears at present that the diameter of the negatively stained neurofilament does not show a periodic variation consistent with the appearance of a regularly twisted bifilar thread and it is more likely that the structure is a hollow cylinder, but the association of the subunits is labile and local distortion can occur in the negative stain on the electron microscope grid (see Figure 12).

Attempts have been made to confirm the helical disposition of protein subunits in the neurofilaments from the squid axoplasm by X-ray diffraction, and, although oriented fibrils with strong optical birefringence can be obtained, there has been no evidence from any of the X-ray diagrams of protein orientation or of a regular structure. This is presumably due to the lack of crystalline perfection of the organization of the fibrous protein subunits.

In the shadowed preparations of axoplasm studied in the electron microscope originally by Schmitt (1950, 1957; Schmitt and Geren, 1950) and Maxfield (1953; Maxfield and Hartley, 1957), in the negatively stained preparations illustrated by Davison at the meeting, and in many sections of neurons, the neurofilaments appear to bear irregular filamentous or globular encrustations. Palay (see also Metuzals (1966)) pointed out that in certain circumstances there appear to be fine cross-bridges between adjacent neurofilaments. These observations suggest that one or more proteins that do not comprise the backbone structure of the neurofilament are frequently associated with it either as encrustations or as cross-bridges; their presence may offer a clue to the function of the filament.

*Personal communication.

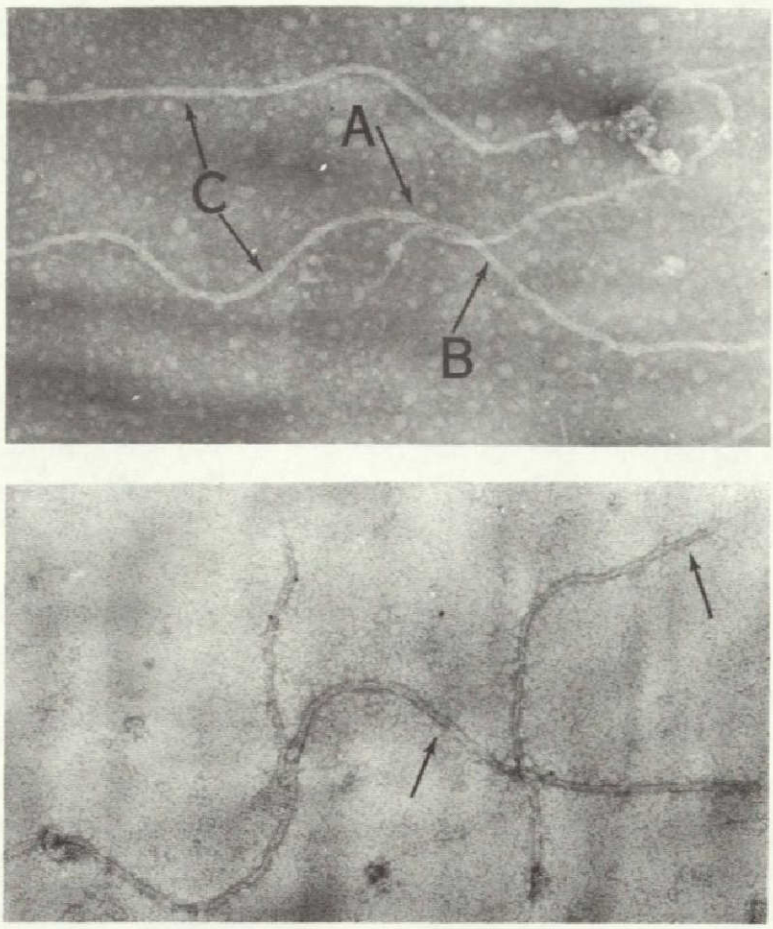

Figure 12. Squid neurofilaments negatively stained in uranyl acetate (X 140,000). Upper picture shows local suggestion of twisting bifilar structure at A, while at B and C the inconsistent diameter of the filaments is evident. The lower picture shows several lightly encrusted filaments and the arrows indicate regions where central dark staining is particularly apparent. [Davison]

Protein Subunits of Neurofilaments

The squid neurofilament protein molecules have a great tendency to aggregate; no monodisperse preparation of the native protein subunits has been prepared and even in the presence of strong denaturing reagents, such as guanidine hydrochloride or detergents, the dissociation of the protein to subunits is incomplete.

The major subunit protein from which the neurofilaments are built has a molecular weight of approximately 45,000 and 25 percent of the amino acid residues in the molecule are dicarboxycyclic acids, giving the molecule a predominantly acidic character. A second protein of higher

molecular weight has been consistently detected in the neurofilament fractions; this protein is similarly acidic but contains more than 10 moles percent serine and some of these serine residues are phosphorylated.*

Immunochemistry of Neurofilaments

Antibodies have been prepared against protein fractions from squid axoplasm enriched with respect to neurofilament subunits as well as against other proteins of the axoplasm. These antibodies do not cross-react with any proteins of squid blood, but they do cross-react with extracts from squid brain.† In these brain extracts, neurofilaments in the fibrillar form can seldom be identified and it is probable that, under the normal circumstances of homogenization of tissues which contain significant quantities of cathepsins, the native filamentous structure is destroyed. By complement fixation assay, these antibodies to squid neurofilaments have been shown to cross-react with neuronal extracts from several other invertebrate species but not with mammalian brain extracts.

Relationship between Neurofilaments and Microtubules

The reciprocal occurrence of neurofilaments and microtubules described elsewhere in several sections has stimulated the theory that neurofilaments and microtubules (neurotubules) are different forms of the same protein subunits (e.g., Wiśniewski et al., 1968). The evidence is largely morphological and inferential and the chemical characterization of these two protein species in the same animal has not been completed in order that the chemical relationship can be directly determined. Their amino acid compositions are similar but this is insufficient to establish identity. Perhaps the strongest evidence against their being identical is the observation by Borisy and Taylor (1967a) that frozen-dry preparations of squid axoplasm contain colchicine- and GTP-binding protein characteristic of microtubule subunits, whereas Davison and his co-workers find that there is no bound nucleotide to the subunits of the neurofilament. Since the nucleotide binding is labile, the identity of the microtubule and neurofilament subunits cannot be refuted on these grounds. Perhaps future work with antibodies against these proteins will settle this question.

*P.F. Davison and F.C. Huneeus, unpublished results.

†F.C. Huneeus, unpublished.

J. MECHANOCHEMICAL COUPLING IN MUSCLE: DISCUSSION LED BY M. REEDY

A variety of intracellular movements, such as that of chromosomes in mitosis, of pigment granules in melanocytes, of cilia, flagella, and amebocytes, cyclosis in plant cells, and the more recently discovered neuroplasmic flow and fast transport of particulates like vesicles or granules containing transmitters, secretory materials, or enzymes, require mechanical energy which must be directionally applied. It is generally believed that the chemical energy, which is coupled to the mechanical, comes from hydrolysis of a high energy-containing mononucleotide (ATP or GTP) bound to one fibrous protein of a pair of protein types by an enzyme built into the structure of the second member of the pair. In the case of muscle this pair is actin and myosin and the mechanism of the mechanochemical coupling whereby one filament type, actin, is made to move with respect to the other filament, myosin, via cross-bridges containing terminal ATPase has been well studied, particularly by Huxley (see Huxley and Brown, 1967) and by many others (for recent reviews see Pringle, 1967, and Perry, 1967). Muscle may therefore be a model on which to base concepts of the mechanism of movement generated in microtubule systems. This idea is supported by the chemical and structural similarity between actin and the proteins of microtubules and microfilaments. As a motile mechanism, microtubules have a wider distribution in animal and plant cells than does the actomyosin stereotype and indeed may be evolutionarily older.

One of the most highly specialized muscle types is the insect flight muscle (see Pringle, 1967). To characterize the mechanochemical coupling in this muscle (*Lethocerus*) and to place the discussion of contractility properly in the context of the more generalized microtubule problem, Reedy described his recent elegant electron microscopic studies (Reedy, 1967, 1968*; Reedy et al., 1965).

The contractile elements of muscle, as seen in the electron microscope, consist of 100 A "thick" filaments of myosin and 40 A "thin" filaments of actin hexagonally spaced about each myosin filament. These filaments do not themselves change length in contraction; rather the actin filaments slide by the myosin filaments, moving from the Z-band towards the M-band on either side of the myosin filament in each sarcomere. This is not due to some kind of long-range force but, according to the Hanson-Huxley model, is due to the thrust of the heavy meromyosin subunits delivered to the actin via a binding site. This action

*Papers II and III on ultrastructure of insect flight muscle are in preparation.

is made possible by a fast configurational change in the heavy meromyosin subunit when ATP bound to actin reacts with ATPase situated at a terminal region of the heavy meromyosin cross-bridges. Myosin can be visualized as a kind of shepherd's crook, the staff consisting of light meromyosin subunits and the head consisting of heavy meromyosin, the protruding part serving as the cross-bridge and bearing the ATPase. Actin filaments consist of a bifilar helix of linearly aggregated subunits of acidic protein. Each actin subunit is thought to have a binding site for the myosin cross-bridge and an ATPase-binding site. During rest the cross-bridges are mostly unattached to actin subunits. In rigor, a long-lasting form of contracture conveniently stimulated by treatment of striated muscle with glycerine, the cross-bridges remain maximally attached to actin subunits; this is shown beautifully in the accompanying electron micrograph of Reedy (Figure 13).

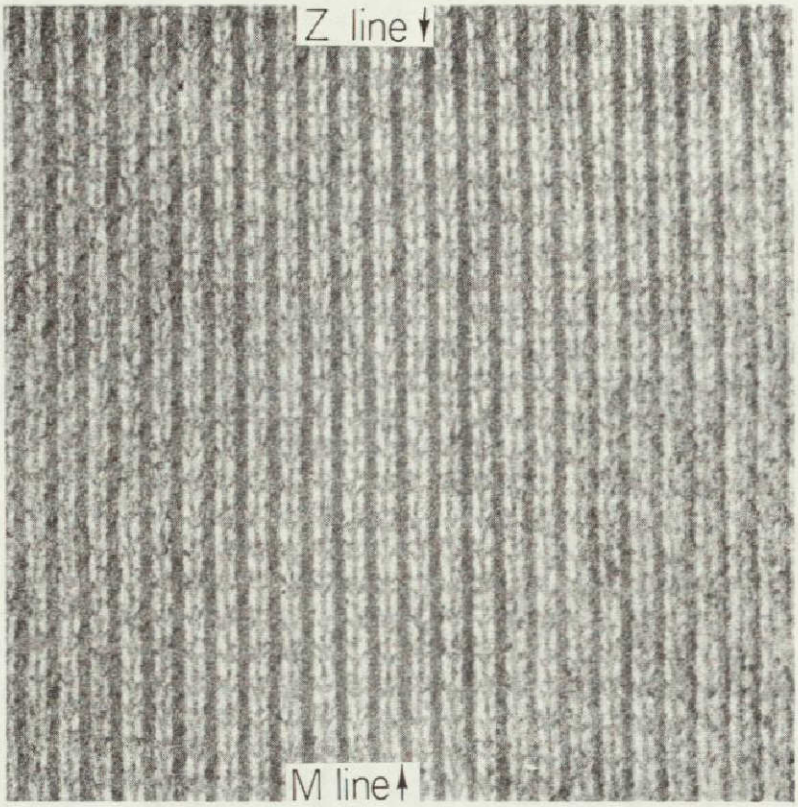

Figure 13. A 250-A thick section from glycerinated insect flight muscle in rigor state which shows a single layer of myosin (thick) and actin (thin) filaments, cross-linked by cross-bridges. In rigor, bridges attach to actin and slant to form chevrons, directed at center of sarcomere (M line). When ATP is added, bridges release actin filaments and lose their slant as they snap out perpendicularly to filament axis. These events help promote current notions of how ATP-dependent, cyclic cross-bridge movements and attachments may produce sliding of actin filaments past myosins and cause shortening. Magnification, X 192,000. [Reedy]

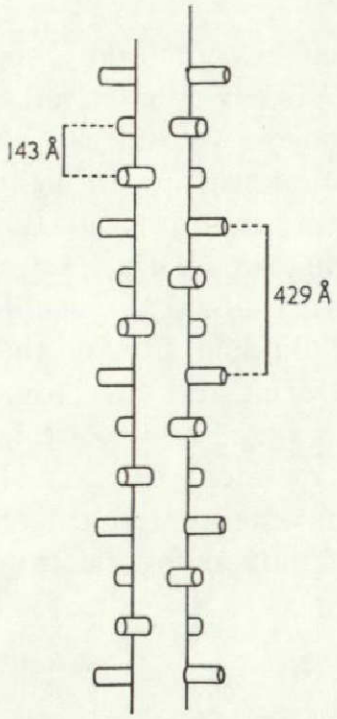

Figure 14. Schematic diagram showing arrangement of cross-bridges on 6/2 helix (successive pairs of subunits are related to each other by a 3-fold screw axis, i.e., each pair of subunits would be rotated relative to its neighbors by 120°; such an arrangement is known as a 6/2 helix). Helical repeat is 429 A, but true meridional repeat is 143 A. [Huxley and Brown, 1967]

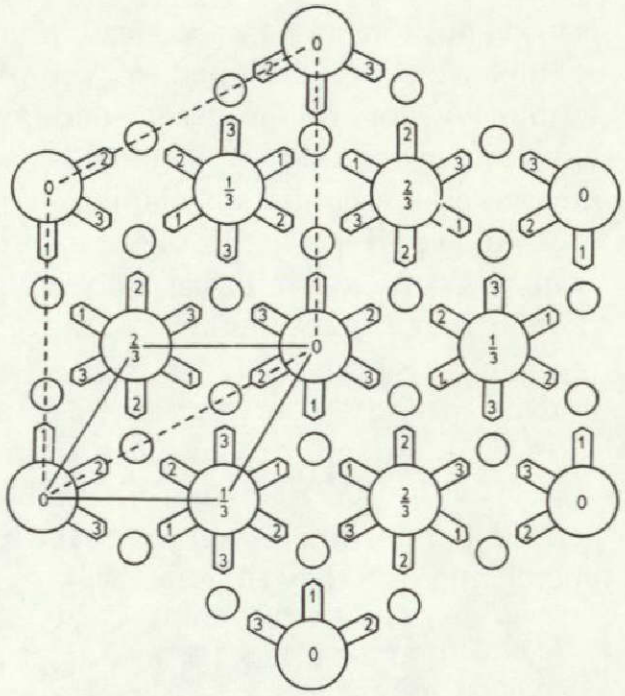

Figure 15. Arrangement of filaments and their cross-bridges in a superlattice, i.e., a hexagonal lattice of spacing $\sqrt{3}$ times that of the spacing of the standard lattice. Superlattice cell shown (– – – –); normal cell shown(———). Next-nearest neighbor myosin filaments have identical orientations, but nearest neighbors are rotated 1/3 or 2/3 of a revolution relative to each other, in the manner shown. The particular arrangement of cross-bridges shown is consistent with the symmetry requirements, but other arrangements are also possible. [Huxley and Brown,1967]

The sequence of events in muscle contraction may be briefly characterized as follows: when the wave of excitation spreads over the muscle membrane, calcium ions penetrate into the muscle substance because of permeability changes in the t-tube system at Z-bands. The calcium ions activate the myosin ATPases of the cross-bridges which, on contact with actin, undergo a conformational change, thereby exerting a thrust to actin filaments. According to Huxley and Brown (1967) (see Figures 14 and 15), cross-bridges extend across myosin filaments at 120° angles along a helical axis, the axial distance between them being closely similar to that between the binding sites on actin subunits, as was deduced from small-angle X-ray diffraction patterns of actin, myosin, and muscle. Actin filaments are pictured as relatively rigid invariant structures capable of sliding between trigonally located myosin neighbors. Myosin filaments

are also pictured as relatively rigid stationary structures from which protrude cross-bridges capable of fast changes in an azimuthal position and constituting the molecular effectors of the contractile machinery. Thus the whole highly ordered system can be instantly set into full activity by an electrical stimulus which influences enzyme sites responsible for activity.

Following isotonic or isometric contraction, tension exerted via Z-bands, muscle structures generally, and connective tissue, particularly at tendon junctions, restores the filament interrelations to that characteristic of the resting length. Calcium ions are pumped out of the cell, presumably by membrane-situated pump assemblies, ATP generated by the sarcosomal mitochondria rephosphorylates the actin-bound ADP, and the system is now ready for the next contractile cycle.

It is not clear whether the actomyosin system is merely a convenient model to depict one form of mechanochemical coupling which can cause movement and contraction or whether it represents a true analog. If the latter were the case, one would be inclined to regard the microtubule protein as analogous to actin not solely for the general similarity of its amino acid composition to that of actin, but also for its nucleotide-binding capacity. Even though in the case of cilia and flagella a sliding filament system may exist (Satir, 1967), it is difficult to visualize how this model can account for the fast transport of dense-core, catecholamine-containing vesicles in axons. Furthermore, although several particulate ATPases have been demonstrated in neuroplasm, their detailed relationship to the microtubules is not yet clear. Of possible, though presently unassessable, significance are the cross-bridges seen between microtubules in neurons (Kohno, 1964), the side arms visualized on neurofilaments by Palay (see also pages 125 and 138), and the recent finding by Davison and Huneeus* who have detected in neurofilament-enriched preparations from squid axonal protein a second protein which may have an anisodiametric shape.

A speculative formal model has recently been proposed (see Schmitt, page 138) in which the ATPase-bearing, myosin-like partner to the nucleotide-binding, actin-like microtubule protein is a protein component of vesicle coats. This represents a sliding or a saltatory vesicle-type model. This model does not exclude the possibility of mechanical energy transduction by the fibrous proteins of neurons, particularly the neurofilament component, that might be involved in slow, bulk movements of neuroplasm of the kind widely studied by Weiss and now confirmed by many investigators (see Barondes, 1967).

*Unpublished.

K. FIBROUS CONSTITUENTS IN SOME PATHOLOGICAL STATES: DISCUSSION LED BY R. D. TERRY AND H. WISNIEWSKI

Clinical Disorders

Several pathological conditions are characterized by increased amounts of filamentous and tubular structures in neurons. In some diseases, the abnormal aggregates are within the neuronal perikarya and to a lesser extent in the neuronal processes. In others, the axons are more severely affected with minimal or no change in the cell soma. The best example of the former type is Alzheimer's disease where the frontal and temporal regions of the human brain are especially involved. Vitamin E deficiency due to any one of several causes commonly induces the second type and involves the posterior tracts of the upper cervical spinal cord and medulla. Both types of lesion are argentophilic, but the electron microscope has revealed that in Alzheimer's disease (Terry, 1963; Terry et al., 1964a,b) and in certain other conditions the aggregate is composed almost exclusively of twisted microtubules; whereas in vitamin E deficiency (Lampert et al., 1964) or in vincristine intoxication (Shelanski and Wiśniewski, 1968) the aggregates are entirely filamentous. In the parkinsonism-dementia complex found in Guam (Hirano et al., 1968) many of the cells contain a mixture of twisted tubules and filaments.

As mentioned above, in contrast to normal microtubules, the tubules seen in Alzheimer's disease, Guam parkinsonism-dementia, and postencephalitic parkinsonism have a twisted appearance but otherwise are similar in morphology. They are within the dimensional range of ordinary microtubules, measuring about 220 A at their widest diameter, and manifest a hollow center which appears crescent-shaped in the twisted region. A small modification in the composition of the microtubule protein could alter the geometry of the protein subunit polymerization and this could give rise to the abnormally twisted tubules. The twist occurs regularly and is about 1600 A in length. A dense central core such as is frequently seen in ordinary microtubules is often observed. Since these tubules are well preserved in osmium-fixed tissue, they are probably more stable than ordinary microtubules (Figure 16). Side arms which may simply be a flocculum are often apparent.

In his description of the fibrillar structures characteristic of Alzheimer's disease, Kidd (1963) concluded that these structures are not tubular but are filamentous, are about 100 A in diameter and of indefinite length, occur in pairs coiled as double helixes, and make one full turn

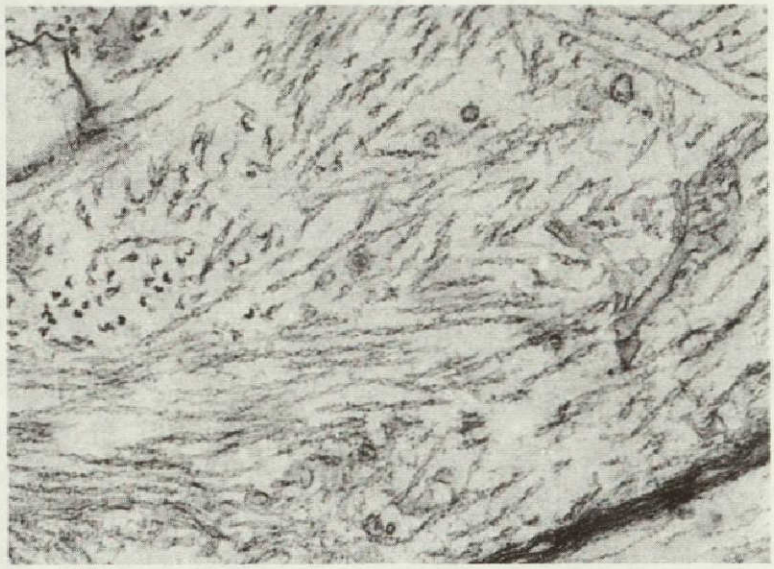

Figure 16. Twisted tubules within a dendrite in Alzheimer's disease. The narrowings are periodically arranged. Human brain biopsy. Magnification, X 86,000. [Terry and Wiśniewski]

every 1600 A. They are seen to sweep around the nucleus of the perikaryon and are also found in the neuronal processes. However, on the basis of their studies, Hirano and his co-workers (1968) believe that the structures are twisted tubules and have 12 or 13 protofilaments in their walls.

Disorders Experimentally Induced by Colchicine, Aluminum Phosphate, and Other Compounds

Following the injection of colchicine, vincristine, vinblastine, or podophyllotoxin into the subarachnoid space of animals, a large number of characteristic filaments possessing numerous side arms are observed; at this time the normal microtubules disappear almost entirely (Wiśniewski et al., 1968). This condition is later reversed by the appearance of large numbers of microtubules which look normal except for slight distortions.

The action of compounds such as vinblastine, vincristine or podophyllotoxin (all known to be inhibitors of the mitotic spindle) is approximately similar to that of colchicine as to the blocking of polymerization of 6S protein subunits. Under disturbed conditions, the 6S protein aggregates to form filaments but not tubules. These filaments, or more probably their subunits, assemble into tubules as the inhibitor disappears from the animal through metabolism or excretion.

The injection of aluminum salts directly into cerebrospinal fluid results in epileptic neuronal hyperactivity and the proliferation of intraneuronal fibrillar material which in the light microscope is very similar in appearance to the dense tangles seen in Alzheimer's disease (Klatzo et al., 1965; Terry and Peña, 1965; Wiśniewski et al., 1965, 1966). The anterior horn cells are particularly sensitive to the aluminum treatment. In the electron microscope the affected neurons show dense tangles made up of filaments with a diameter of about 100 A and no twisted tubules (see Figures 17, 18, and 19).

Although the fibrillar responses to colchicine and aluminum are similar, they differ in some aspects. In colchicine-treated animals, the nuclear membrane and the endoplasmic reticulum are often changed, whereas in the case of aluminum the tangles of filaments are apparently the only cellular abnormality. There is also a species difference in response to the above compounds, e.g., rats are not as susceptible to aluminum as cats, dogs, and rabbits.

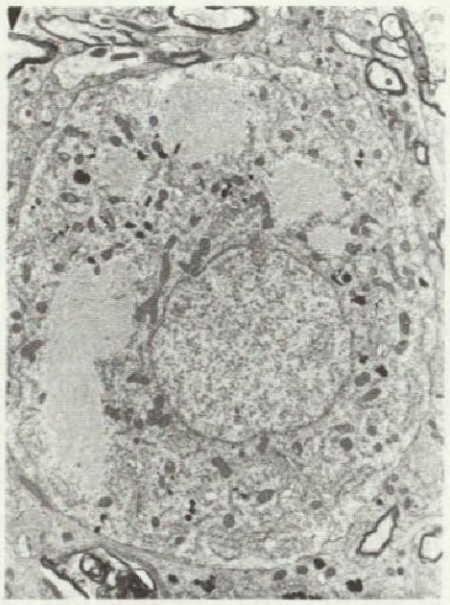

Figure 17. Cortical neuron from a rabbit treated with aluminum chloride. Several bundles of filaments are prominent in the neuronal cytoplasm. Magnification, X 3000. [Terry and Wiśniewski]

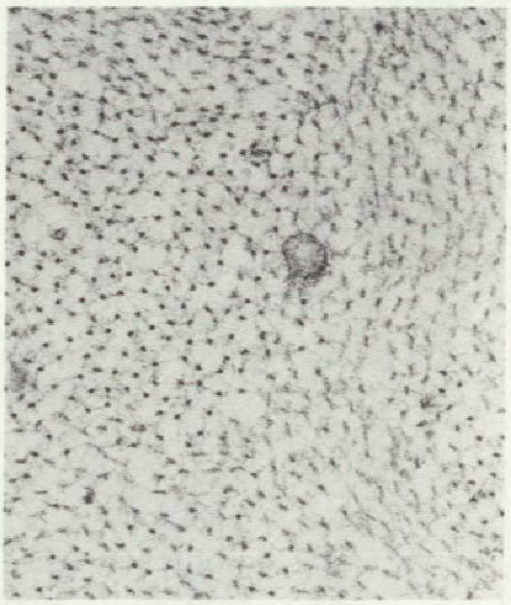

Figure 18. Cross section of filaments from a rabbit's neuron treated with aluminum. The filaments are about 100 A in diameter. Note the side arms linking the filaments. Magnification, X 82,000. [Terry and Wiśniewski]

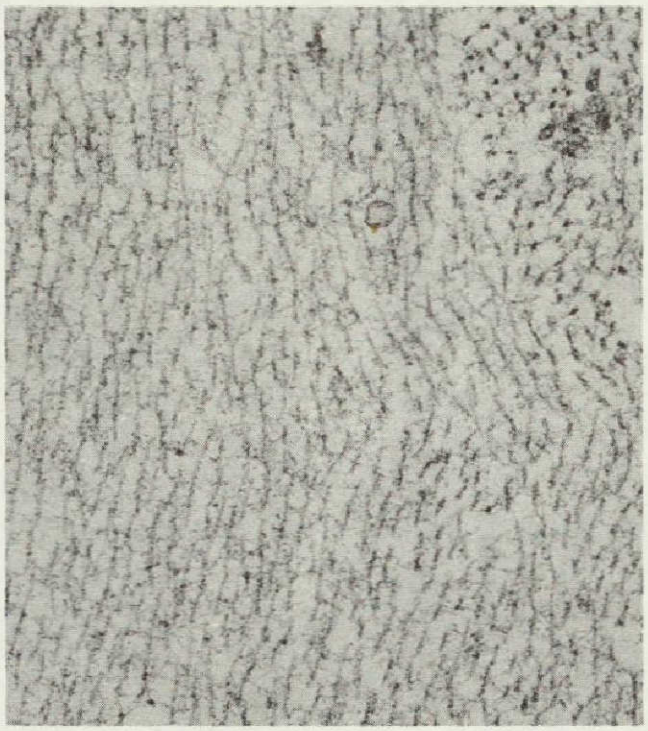

Figure 19. Longitudinal section of filaments from aluminum-treated rabbit neuron. Magnification, X 78,000. [Terry and Wiśniewski]

TUBULAR STRUCTURES IN SENSORY TRANSDUCTION

Microtubules and tubular systems are prominent features in many sensory cells where transduction from mechanical, chemical, thermal, and other forms of incident energy to impulses in neurons of the sensory system occur. In itself a complex subject, sensory transduction has been dealt with in several recent symposia.* Although it was not discussed in the two conferences here reported, several rather striking examples from recent work are presented as a short introduction to what will doubtless prove to be a very rewarding field of study.

In mechanoreceptors, e.g., the hair plate receptors of the honey bee, the general design is characterized by the separation of a specialized terminal segment of the neuronal process from the remaining distal fiber by means of a ciliary structure. Cross sections of the ciliary structure reveal the typical ring of nine pairs of microtubules (Thurm, 1965) seen in the axial unit of flagella. These microtubules continue and increase distally and may even connect with the tubular body at the distal end of the terminal segment. The tubular body, which is electron-dense, consists of about 50 to 100 microtubules lying parallel to one another and of the same diameter as those in the ciliary structure. Even though the separate tubules leave the tubular body at its proximal side and continue, independently of each other, toward the ciliary structure, it has not yet been possible to determine whether they are continuous with the tubules of the ciliary structure. Thurm has proposed the provocative idea that the normal stimulating event of the mechanoreceptor is a compression of this strategically located tubular body.

Investigations of tubular systems in the olfactory sensillum of the silk moth (*Bombyx mori*) and the carrion beetle (*Necrophorus*) are revealing interesting preliminary information. In the beetle, two kinds of tubular systems are found (Ernst†), one consisting of typical microtubules within the dendritic process of the olfactory sensory neuron and the other consisting of tubular structures, so-called "pore tubules," that are not microtubules but are similar enough to illustrate some general principles.

*See *NRP Bulletin* on *Sensory Transduction* (chaired by Werner Reichardt), in preparation. See also *Cold Spring Harbor Symposium on Quantitative Biology, Sensory Receptors* (Vol. 30, Cold Spring Harbor Laboratory of Quantitative Biology, 1966); *The Functional Organization of the Compound Eye* (Wenner-Gren International Symposium Series, Vol. 7, Oxford, Pergamon Press, 1966); *Olfaction and Taste II* (Wenner-Gren International Symposium Series, Vol. 8, Oxford, Pergamon Press, 1967).

†K.-D. Ernst, Max-Planck-Institut für Verhaltensphysiologie, Seewiesen, Germany, manuscript in preparation.

The microtubules within the terminal dendrite occur as one centrally located structure, having an overall diameter of 200 A, and appear to be continuous throughout the length of the outer segment of the dendrite (about 40 μ long) (Ernst†). Similarly, in the axons of the small nerve fibers of the silk moth antenna, from 1 to 5 microtubules have been observed whose cross sectional profile shows an over-all diameter of about 200 A (Steinbrecht‡). In longitudinal sections, they appear as straight structures with a minimal bending tendency. The function of such microtubules may be simply that of a supporting skeleton, but their location in these neurons indicates that they may play a more significant role in olfactory events.

The "pore tubules" constitute the extracellular tubular system in the cuticle of the sense hair of the beetle and have a diameter of about 150 A, a wall thickness of 25 A, a lumen of 100 A, and a length of about 1000 A (Ernst†). Extending from the cuticular pores, they penetrate the cuticle and come within 100 A of, but do not directly touch as once thought, the sensory neuronal dendrites. These "pore tubules" are open to the sensory pore and can serve as a model for the experimental testing of the passage of molecules down the lumen of microtubules. Electron-dense material such as ferritin or protargol placed on the antenna can be observed invading the tubules and proceeding along them to the terminal end. Schneider§ has suggested that this tubular system may act as a collector-and-receptor system for the olfactory stimulating chemicals; in other words, these microscopic tubular structures may act as "pipes" for the conveyance of small molecules, a role of possible relevance to that ascribed to typical microtubules.

A striking relationship between the number of "pore tubules" and their calculated receptor sites has recently been reported by Kaissling.‖ From the kinetics of the receptor potentials together with the threshold numbers of molecules, he has calculated the total number of acceptors for a single receptor cell. The calculated number of acceptors corresponds to the number of "pore tubules." Kaissling concludes that "the kinetics of the receptor potentials may be controlled by the 'pore tubules,' perhaps by the passage of odor molecules through these channels."#

†K.-D. Ernst, Max-Planck-Institut für Verhaltensphysiologie, Seewiesen, Germany, manuscript in preparation.

‡R.A. Steinbrecht, Max-Planck-Institut für Verhaltensphysiologie, Seewiesen, Germany, manuscript in preparation.

§Personal communication from D. Schneider.

‖ Paper presented by K.-E. Kaissling at the Third Symposium on Olfaction and Taste, New York City, August, 1968.

#Personal communication.

It is recognized that the equality of tubules and the number of receptor sites may be only a coincidence; on the other hand, these findings may open the door to more research and a fuller understanding of the mechanism of olfactory transduction.

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