

THE FINE STRUCTURE OF THE AXON AND GROWTH CONE OF THE DORSAL ROOT NEUROBLAST OF THE RABBIT EMBRYO

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ABSTRACT

The centrally directed neurite of the dorsal root neuroblast has been described from the period of its initial entrance into the neural tube until a well-defined dorsal root is formed. Large numbers of microtubules, channels of agranular reticulum, and clusters of ribosomes are found throughout the length of the early axons. The filopodia of the growth cone appear as long thin processes or as broad flanges of cytoplasm having a finely filamentous matrix material and occasionally small ovoid or elongate vesicles. At first the varicosity is a small expansion of cytoplasm, usually containing channels of agranular reticulum and a few other organelles. The widely dilated cisternae of agranular reticulum frequently found within the growth cone probably correspond to the pinocytotic vacuoles seen in neurites in tissue culture. The varicosities enlarge to form bulbous masses of cytoplasm, which may measure up to $5\ \mu$ in width and $13\ \mu$ in length. They contain channels of agranular reticulum, microtubules, neurofilaments, mitochondria, heterogeneous dense bodies, and a few clusters of ribosomes. Large ovoid mitochondria having ribonucleoprotein particles in their matrix are common. Dense membrane specializations are found at the basal surface of the neuroepithelial cell close to the area where the early neurites first enter the neural tube.

INTRODUCTION

The growth cone of a dorsal root neuroblast as described by Cajal (1909) is a conical thickening of protoplasm that spreads out into prickly flanges or lamellar appendages. Neurofibrillae extend from the axon proper into the axis of the growth cone. This description, based on Golgi silver impregnations, is remarkably similar to the appearance of the growing nerve tip in tissue culture. Harrison (1910) states that the ending of each axon is a rhizopod-like structure with very fine processes or pseudopodia, which possess active amoeboid activity. The morphology and movement of the growth cones of nerve fibers growing in tissue culture have been recorded in phase-con-

trast cinematography by Pomerat et al. (1967). These investigators showed that growth cones derived from the dorsal root ganglion exhibit certain differences in morphology and in movement as compared with those of the spinal cord. The filopodia of dorsal root neuroblasts are thin membranous extensions that exhibit flaglike undulations; whereas those from spinal cord neuroblasts appear as multiple filaments, which are in a state of constant elongation and contraction. The pinocytotic vacuoles within the growth cone of dorsal root neurites, moreover, are larger and more numerous than those from the spinal cord. Neurites from both areas exhibit centrifugal and

centripetal axoplasmic flow of vacuoles, mitochondria, and granules (Pomerat et al., 1967).

Several electron microscopic studies have been done on the perikarya of the dorsal root neurons in the rabbit (Tennyson, 1965; 1969), the chick (Wechsler and Schmekel, 1967; Pannese, 1968 *a*, *b*), and on neuroblasts grown in tissue culture (Bunge et al., 1967*b*), but very little attention has been given to the growing tip of the neurite. Previous reports of structures presumed to be growth cones in the immature nervous system have made no mention of the filopodia. Bodian (1966) has described the growing tip of the axon in the spinal cord of the fetal monkey as a swollen bulb about 0.5 μ in diameter containing a cluster of large empty vesicles, but lacking mitochondria. A similar description was given of growth cones in the developing cerebellum of the rat (del Cerro and Snider, 1968 *a*). The absence of mitochondria in these embryonic growth cones (Bodian, 1966; del Cerro and Snider, 1968 *a*) is an unexpected finding, since growth cones of regenerating nerve fibers contain a number of mitochondria (Estable et al., 1957; Wechsler and Hager, 1962; Wettstein and Sotelo, 1963; Lampert, 1967; and Lentz, 1967). This discrepancy and the differences observed in tissue culture of growth cones from different areas of the nervous system (Pomerat et al., 1967) led us to examine the neurites of the embryonic dorsal root ganglion at the time when they first enter the neural tube, that is, from day 11 to day 12. This period was chosen because a high proportion of growth cones could be expected to be present, thereby making their identification more certain.

The present study describes the neurite of the dorsal root neuroblast, including the varicosity and filopodia of the growth cone, from the time of its initial entrance into the neural tube until a well-defined dorsal root has formed. The finding that certain changes take place within the growth cone during development may help to explain some of the discrepancies in the literature concerning these structures. In addition, this study will serve as a frame of reference for a cytochemical and microgasometric study of the development of acetylcholinesterase in these fibers (Tennyson and Brzin, manuscript in preparation). A brief summary of the characteristics of the growth cone has been given in a recent review article on the ultrastructure of the embryonic nervous system (Tennyson, 1969).

MATERIALS AND METHODS

Animals and Method of Fixation

110 Dutch rabbit embryos ranging in age from day 11 through day 12½ of gestation were used for this study. The age of the embryos was determined from the time of mating. Since the litters are not always uniform in development, reference to an atlas on the development of the rabbit (Minot and Taylor, 1905) proved useful in establishing the proper developmental stage of each embryo.

The dam was anesthetized with about 60 mg/kg of Nembutal (Abbott Laboratories, North Chicago, Ill.) given intravenously, and the uterus was exposed by midline incision. In the earlier studies, immersion fixation of small transverse blocks of the neural tube taken immediately after removal from the uterus was considered adequate, since blood vessels have not invaded the nervous system extensively during days 11 and 12. More recently, however, somewhat better preservation was obtained by a combination of vascular and cerebral ventricular perfusion for a short period of time, followed by immersion fixation. A 30 gauge needle was inserted through the transparent chest wall into the heart and about 2 cc of fixative were gently forced through a syringe over a period of about 2 min. The thin umbilical vessels, which are intact at the beginning of the perfusion, rupture at the surface of the placenta during this procedure, providing an outlet for the fixative. The fixative was then perfused through the ventricular system of the brain after cutting through the neural tube in the sacral region to provide an opening. The same procedure of vascular and ventricular perfusion was repeated, and then the brachial region of the neural tube was cut into transverse blocks, about 0.3–0.4 mm thick, and put into fresh fixative.

Fixatives and Other Preparative Procedures

This study took place over a period of 3 yr during which numerous fixing solutions were tried. They contained various concentrations of glutaraldehyde (Sabatini et al., 1963) or combination of aldehydes (Karnovsky, 1965; Sandborn et al., 1964), and particularly different buffer concentrations. As pointed out by Dossel (1966), it was necessary that the osmolality of the fixative and phosphate buffer combination for young embryos be lower than the osmolality of that used for the adult (Schultz and Karlsson, 1965). The most satisfactory fixative was 1% glutaraldehyde (purified by the method of Fahimi and Drochmans, 1965, and kindly supplied by Dr. Myron Tannenbaum and Mr. Joseph Ortiz) in a 170–180 milliosmolar phosphate buffer at pH 7.2–7.4, giving a total osmolality of about 300–310 milliosmols to the fixative. Satisfactory results are

also obtained when 0.1% paraformaldehyde is added to the above fixative. Total fixation time was 1–1.5 hr at room temperature. The specimens were then washed for about 1 hr in a 290 milliosmolar phosphate buffer or placed immediately into 2% OsO₄ (Palade, 1952) in a 290 milliosmolar phosphate buffer (Millonig, 1962) at pH 7.2–7.4 at room temperature for 3 hr. The tissue was then dehydrated through ascending concentrations of methyl alcohol (70%, 95%, absolute) and given a brief rinse in absolute acetone or propylene oxide prior to embedding in Durcupan (International Chemical and Nuclear Corp., Calif.). Some specimens were stained in the block by the addition of uranyl acetate to the methyl alcohol dehydration (Stempak and Ward, 1964).

Semithick sections were examined by phase microscopy, and the ventral portion of both the neural tube and the ganglion was removed to simplify the identification of the dorsal root fibers during electron microscopic study. Thin sections for electron microscopy were cut with glass or diamond knives on a Porter-Blum microtome (Ivan Sorvall Inc., Norwalk, Conn.). They were mounted on Formvar- or collodion-coated copper mesh grids, and were double stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963). In order to identify early dorsal root neurites before they can be recognized by phase microscopy, 10 blocks of segments of neural tube and ganglia from embryos of 11¼ to 11½ days gestation were sequentially thin sectioned, and near-serial sections throughout the blocks were examined. Electron micrographs were taken with a Siemens Elmiskop I.

OBSERVATIONS

The morphology of the centrally directed axoplasmic prolongation of the early bipolar neuroblast varies as the axon proceeds from the point of its emergence from the perikaryon to the tip; therefore, the following terminology will be used in this paper to avoid confusion. The word “axon” will refer to the elongated, roughly cylindrical portion between the axon hillock and the expanded tip. The expanded tip of the axon, which will be called the “growth cone,” consists of two portions: a central thickened core of cytoplasm, the “varicosity,” and a thin irregular membranous cytoplasmic expansion, the “filopodium.” The word “neurite” will be used in a more general manner and will include both portions, the axon and the growth cone.

Early Ingrowth of Neurites

AXONS: The centrally directed neurites of the bipolar neuroblasts of the dorsal root ganglion

generally grow into the neural tube during the early part of day 11 of gestation. The exact time of entrance varies somewhat from one litter to another and with the level of the neural tube examined. A phase micrograph (Fig. 1) shows that ganglion cell processes are directed toward the dorsolateral portion (arrow) of the neural tube at day 11¼. The presence of ingrowing axons could not be demonstrated by electron microscopy in a sequential section at this level, but were seen deeper in the block at one point (Fig. 10). Most of the neuroblasts in the ganglia have spindle-shaped or globular perikarya and elongated or ovoid nuclei with prominent nucleoli (Fig. 1, crossed arrow). A complete account of the perikarya of these cells, which are the precursors of the “small dark neuron” and the “large light neuron” of the adult ganglion, will not be described here since recent papers (Tennyson, 1965; 1969) have considered them in detail. Illustrations of the initial segments of their centrally directed neurites are given, however, since the proportion of microtubules to neurofilaments differs between the two cell types. The precursor of the small dark neuron, the first type to differentiate, is readily recognizable by day 11¼. Its cytoplasm contains an unusually large number of polyribosomes, but relatively few neurofilaments and other membranous organelles. The initial segment of its axon (Fig. 2) characteristically contains numerous microtubules (*T*), but few neurofilaments. The precursor of the large light neuron, which begins to differentiate by day 11½, has polyribosomes, granular endoplasmic reticulum, and neurofilaments in its perikaryon. Large numbers of neurofilaments extend into the initial segment of this cell (Fig. 3, *F*). In keeping with the findings on adult dorsal root neurons (Palay et al., 1968), the initial segment of these early neuroblasts does not exhibit the fasciculation of microtubules or the dense undercoating of the surface membrane characteristic of some adult neurons (Palay et al., 1968, Peters et al., 1968).

A study of the dorsal region of the early ganglion during day 11¼ reveals the cell bodies and processes of numerous undifferentiated cells, presumably the precursors of satellite and Schwann cells and possibly undifferentiated neuroblasts, but relatively few profiles that have typical axonal characteristics. The axons are not collected into a single large bundle forming a dorsal root as in the following stage, but are randomly dispersed. They

appear singly or in small groups of three or four axons (Fig. 5). They are often surrounded by the cytoplasm of several immature Schwann cells (*S*), but well-defined sheaths have not formed. Frequently only one side of an axon is apposed to another axon or undifferentiated cell, whereas the other surface is exposed directly to the mesenchymal fluid (Figs. 4, 7). One axon, a portion of which is illustrated in Fig. 4, was unusually well oriented so that it could be followed from its axon hillock for a distance of almost 60 μ through the mesenchyme. Over most of this distance, at least one surface of the axon was directly exposed to the mesenchymal area, with only intermittent attachment of Schwann cell processes. Some axons appear to be completely devoid of accompanying cellular processes, but usually for only a short distance. Naked axons may course for somewhat longer distances in the narrow space between the capillaries and the lateral border of the neural tube. The axons are usually irregularly ovoid or somewhat flattened, rather than circular in cross-section. They are of varying size, but are roughly in the range of 0.4–1.5 μ in diameter (Fig. 5). Close to their endings, axons occasionally exhibit offshoots (Fig. 7, crossed arrow), which contain a dense web of filamentous material. In a serial section one of the projections appeared to be elongated into a thin process, resembling the "microspikes" reported in other cells (Taylor, 1966).

Clusters of particles, 150–250 A in diameter, morphologically identical to ribosomes in the perikaryon, are present in small groups along the length of the early axons (Figs. 2–5, *R*). The segment of the axon illustrated in Fig. 4 was located more than 30 μ from its axon hillock, but its morphology was typical of the entire 60 μ length of the axon available for study. The probable cell body of origin, as determined by phase microscopy, was located 125 μ from the anlage of the oval fasciculus.

Microtubules (*T*) having the usual dimensions of 230–250 A in diameter with walls 50–60 A in thickness are the most common organelle in the axoplasm. Occasionally a central density is apparent in the core of the microtubule (Fig. 6), as was found by Gonatas and Robbins (1964). Filamentous projections (arrows) radiate from the walls of the microtubules toward other microtubules and their sidearms. Although connections may exist between them, these microtubules are randomly arranged in the axoplasm and are separated by a

distance of at least 400 A or more, and thus do not form distinct fascicles. Neurofilaments about 90–110 A in diameter are found at this stage, but they are much fewer in number than the microtubules. The presence of large numbers of microtubules in these early axons that have little external support is in accord with the suggestion that microtubules might form a cytoskeleton to add rigidity to elongated cytoplasmic processes (Tilney and Porter, 1965).

The mitochondria may be ovoid or greatly elongated and longitudinally oriented in the axon. The cristae run transversely or obliquely and fine 150 A particles and larger 300–600 A spherules are found in the matrix. One or more profiles of flattened or slightly dilated agranular reticulum (Figs. 4, 5, *Ar*) are seen in most cross-sections of axons in the earliest stages. The smooth reticulum is frequently located close to the surface membrane of the axon, suggesting the relationship of subsurface cisternae to the cell membrane of the perikaryon. A variable number of coated pinocytotic vesicles (Fig. 4, arrow), and smooth surfaced vesicles (*v*) are found in the axoplasm.

GROWTH CONES: No dorsal root axons are entering the neural tube in Fig. 1, but a study of near-serial thin sections of this segment and of other specimens at similar or slightly later stages of development shows that a few axons have gained access to the neural tube. Such axons are found infrequently, however, and only by an examination of numerous sequential sections. Their sparsity suggests that the ingrowth of these neurites has just begun. The following examples of processes entering the neural tube illustrate features that suggest these processes are neurites that are near their termination. The portion of the single neurite coursing through the mesenchymal area (Fig. 7) exhibits typical embryonic axonal (*Ax*) morphology. At its juncture with the neural tube, the axon loses its primitive sheath cell attachment and forms an expansion of cytoplasm or varicosity (*V*), which contains mitochondria and saccules of agranular reticulum (*Ar*). The varicosity is continuous distally with thin processes (*P*), which are filled primarily with a finely fibrillar matrix material. The offshoot of its axonal process (crossed arrow) contains the same fibrillar matrix material as the distal processes (*P*). In the four near-serial sections of this neurite available for study, the processes (*P*) did not extend farther than shown. A small longitudinally oriented bundle of axons

was located a few cells distant from the neurite, however, indicating that the anlage of the posterior fasciculus has already been formed.

The axon of the single neurite in Fig. 8 (*Ax*) also loses its sheath and swells to form a small varicosity (*V*). The varicosity is continuous with a broad flange of cytoplasm (*P*) containing a few organelles and a finely filamentous matrix material, which is more concentrated here than in the former growth cone in Fig. 7. The flange of cytoplasm did not extend deeper into the neural tube in the four near-serial sections of this neurite available for study, nor was a longitudinal tract of fibers present close to this area.

The small group of axons entering the neural

tube in Fig. 9 were seen in only two sections, but examination of other near-serial sections that showed no anlage of the posterior fasciculus suggested that it is unlikely that these neurites have penetrated deeper into the neural tube than is illustrated. Typical axonal (*Ax*) morphology is exhibited by some of the fibers in the primitive dorsal roots as they emerge from their sheath cells (*S*). The varicosity (*V*) has a bulbous tip containing ovoid and elongate vesicles, 450–700 Å in diameter, in a finely filamentous matrix. The channels of interconnected agranular reticulum (*Ar*) are extensive, and at two points (arrows) they fuse with the surface membrane. A few mitochondria are present.

FIGURE 1 A phase micrograph of the dorsal root ganglion and neural tube at day 11¼ of gestation. The dorsal root ganglion consists of a loose grouping of bipolar neuroblasts (crossed arrow) and undifferentiated cells. Although a narrow rim of cytoplasm can be seen in the dorsolateral region of the neural tube (arrow), a well-defined posterior fasciculus and dorsal root are not present. A few motor neuroblasts, which are also beginning to differentiate, form a slight bulge in the ventrolateral region of the neural tube. × 200.

FIGURE 2 The initial axonal segment of a spindle-shaped bipolar neuroblast, which contains primarily polyribosomes in its perikaryon. Although some neurofilaments are present, microtubules (*T*) predominate. Polyribosomes (*R*) and smooth-surfaced endoplasmic reticulum can be seen. An elongated mitochondrion extends into the initial segment from the axon hillock. × 32,000.

FIGURE 3 The initial segment of a filamented globular bipolar neuroblast contains numerous neurofilaments (*F*), some microtubules (*T*), polyribosomes (*R*), and smooth- and rough-surfaced endoplasmic reticulum. × 32,000.

FIGURE 4 Clusters of ribosomes (*R*), microtubules (*T*), flattened channels of agranular reticulum (*Ar*) smooth vesicles (*v*), and an occasional coated pinocytotic vesicle (arrow) are present along the entire length of the axon. One surface of the axon is apposed to another axon, the other to the mesenchymal area. There is only intermittent contact with Schwann cell processes. × 28,000.

FIGURE 5 A small bundle of axons are apposed to the cell bodies and processes of several satellite cells (*S*). The axons contain microtubules (*T*), clusters of ribosomes (*R*), and moderately dilated channels of agranular reticulum (*Ar*). × 27,000.

FIGURE 6 The microtubules frequently show a central density and side arms (arrows). × 123,000.

FIGURE 7 A single axon (*Ax*) containing numerous microtubules (*T*) expands within the neural tube (*Ne*) to form a varicosity (*V*), which bifurcates into thin processes (*P*) surrounding a longitudinally oriented axonal profile (*Ax'*). The processes contain primarily a finely filamentous material. Mitochondria and cisternae of agranular reticulum (*Ar*) are present in the varicosity. Within the mesenchymal area, a second process (crossed arrow) sprouting from the axon is directed toward a dense membrane specialization (arrow). Schwann cell (*S*) cytoplasm containing polyribosomes, straight cisternae of granular endoplasmic reticulum, mitochondria, and droplets (*D*) having low electron opacity partially ensheathes the axon. × 12,000.

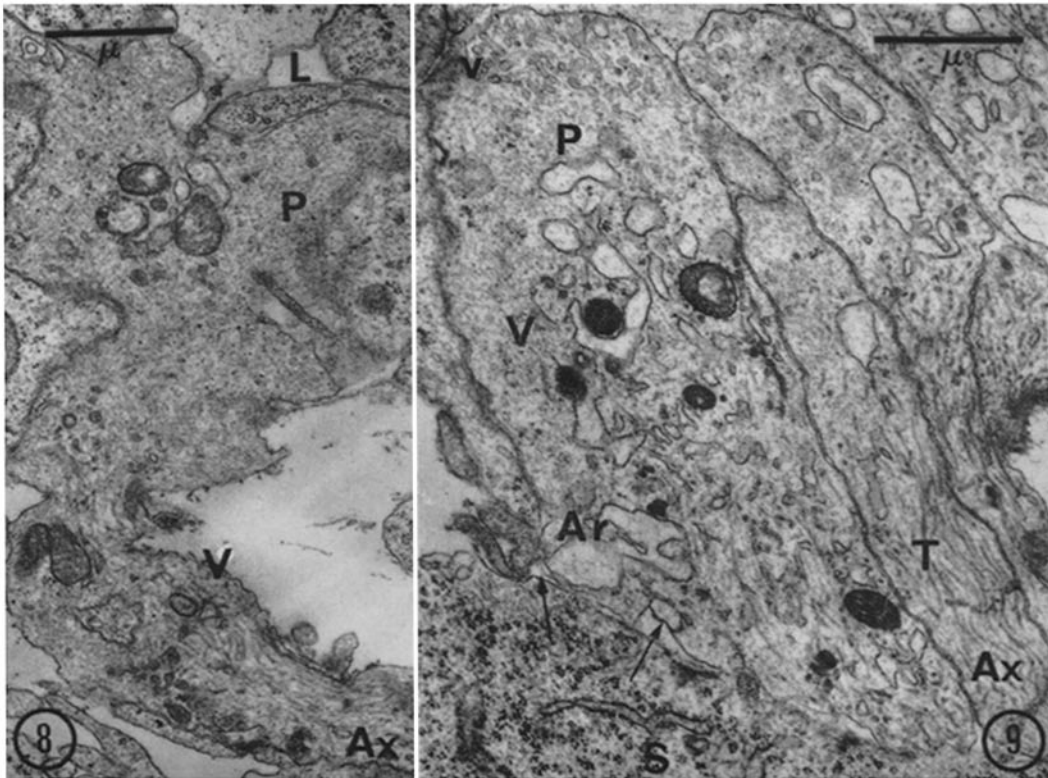


FIGURE 8 A single neurite entering the neural tube. An axon (*Ax*) loses its Schwann cell covering where it expands to form a varicosity (*V*), which contains vesicles having a moderately dense content. The process (*P*) within the neural tube is an irregular flange of cytoplasm containing a condensed finely filamentous matrix material and a few organelles. Small lacunae (*L*) of extracellular space are present. $\times 16,800$.

FIGURE 9 A small group of neurites lose their Schwann cell (*S*) before entering the neural tube. Microtubules (*T*) are present in the axons (*Ax*). The varicosity (*V*) has an extensive system of dilated agranular reticulum (*Ar*), which appears to fuse with the surface membrane (at arrows). The broad distal portion (*P*) of the varicosity contains a finely fibrillar matrix material and numerous small ovoid and elongated smooth vesicles (*v*). $\times 19,200$.

Fig. 10 is one of five near-serial sections that illustrates several axons entering the neural tube prior to the formation of the anlage of the posterior fasciculus. Only a small portion of a neurite having typical axonal (*Ax*) morphology is seen here, but further evidence of axonal structure in the portion coursing through the mesenchyme was evident in another section. The agranular reticulum (*Ar*) of the varicosity (*V*) is widely dilated and appears to fuse with the surface membrane (arrow). Small elongate or spheroidal vesicles, ranging from 300 to 500 Å in diameter, are present close to the surface membrane (crossed arrow) and in a process (*). In another section the process (*P*), which is

continuous with the varicosity (*V'*), extends deeper into the neural tube for only a short distance. The thin processes and much of the cytoplasm of the varicosities contain a finely filamented matrix material.

Fig. 11 illustrates a larger bundle of neurites that have entered the neural tube at a point where the anlage of the oval fasciculus (*Ax*) is well established. This tract can usually be recognized by phase microscopy in such specimens, but study of longitudinal sections shows that it does not extend the length of an entire segment. Continuity of these neurites with axons in the primitive dorsal root is present in some of the 32 near-serial sections of

these fibers available for study. The neurite having a varicosity (*V*), which branches into long thin processes (*P*) and an irregular bulbous expansion (*P'*), probably is very close to its ending, since these structures did not extend deeper into the neural tube in other sections. A finely fibrillar matrix material fills these processes and 450–1000 A vesicles (*v*) are present. The varicosity contains mitochondria and interconnected channels of agranular reticulum (*Ar*), which closely appose the surface membrane. Microtubules (*inset, T*) and clusters of ribosomes (*inset, R*) are evident in a near-serial section of the varicosity.

The ingrowing neurites can readily be distinguished from capillary sprouts that are also entering the neural tube at this time. Immature endothelium has a moderately dense cytoplasmic matrix and large numbers of polyribosomes. Usually the lumen can be identified. The capillary sprouts, moreover, tend to enter the lateral surface of the neural tube at a distance ventral to the ingrowing neurites.

MEMBRANE SPECIALIZATIONS: A feature noted in this material only during these early stages is the presence of focal dense membrane specializations (Figs. 7, 11, arrow) in the basal portion of neuroepithelial cells close to the region where the neurites or capillaries enter. The external limiting membrane is denser in these areas, and there is an accumulation of finely fibrillar material in the adjacent cytoplasm. The surface is usually undulated and is frequently thrown into folds or even into longer thin processes, resembling microspikes (Taylor, 1966). The basement membrane may follow the folds closely, or be somewhat out of register (Fig. 11, arrow). Processes of cells traversing the mesenchymal area are frequently directed toward, or are closely apposed to, the basal surface specializations. It is noteworthy that the offshoot of the neurite in Fig. 7 (crossed arrow) is directed toward an undulated membrane containing dense material. Mitochondria seem to be particularly numerous in the basal portion of most of the neuroepithelial cells during this period of early neural and capillary ingrowth.

EXTRACELLULAR SPACE: A small amount of extracellular space may be found between some of the bulkier processes in the dorsolateral portion of the neural tube, but in most cases this space is limited to small lacunae (Figs. 8, 11, *L*), as was found by Rosenbluth (1966). The large volume of extracellular space in the nervous system at the

perinatal period reported by others (Bondareff and Pysh, 1968; del Cerro and Snider, 1968 *b*; and Pysh, 1969) is not found in the oval fasciculus in this study. Most cell processes within the neural tube at the early and the later stages are closely applied to one another, permitting a narrow but variable extracellular space, as was found in spinal cord cultures (Bunge et al., 1967*a*). Occasionally adjacent membranes appear to be in direct apposition to one another.

SUPPORTING CELLS: The cell bodies of immature satellite and Schwann cells (Figs. 5, 7, 9, 10, 11, *S*) are often closely applied to a portion of the surface of early neuroblasts and their neurites, but they have not formed sheaths that completely surround the neural structures. The cytoplasm of the supporting cells contains numerous polyribosomes, but the cytoplasmic matrix material is somewhat less dense than that of the neuroblasts. Large droplets (Fig. 7, *D*) having a low electron opacity are common in the Schwann cells.

Later Ingrowth of Neurites

DORSAL ROOT AND OVAL FASCICULUS: A well-defined dorsal root ganglion, a dorsal root, and an oval fasciculus have formed by day 12 (Fig. 12). Studies of longitudinal sections during this period indicate that the oval fasciculus is continuous throughout the brachial region. The Schwann cells have become flattened along the surface of the dorsal root and form an almost unbroken sheath, but their processes do not grow extensively among the axons within the root. The nuclei of glia cells are not usually seen in the oval fasciculus during this period. Cross-sections of axons within the posterior portion of the ganglion (Fig. 13), as well as in the dorsal root, show that the axons generally contain many more neurofilaments (*F*) than in the earlier period (Fig. 5). The change in the population of microtubules and neurofilaments is in keeping with the findings in immature rat optic nerve (Peters and Vaughn, 1967). Because of the concomitant increase in the filamented type of neuroblast during this period, however, no statement can be made regarding the possibility of the interconvertibility of these organelles, which was suggested by Peters and Vaughn (1967). Ribosomes (Fig. 13, *R*) are still found in the axoplasm at this stage, and profiles of agranular reticulum (*Ar*) and longitudinally oriented mitochondria are conspicuous.

Varicosities and thin prolongations of cytoplasm

may be found in the proximal portion of the dorsal root, but most of them have already grown into the distal portion of the root or into the oval fasciculus (Fig. 14). The varicosities usually measure from 2 to 5 μ in width and from 6 to 13 μ in length. In opportune sections continuity can be traced between the varicosities (*V*) and the thin processes (arrows), as well as between the varicosity (Fig. 15, *V*), typical axonal cytoplasm (*Ax*), and thin processes (*P*). The narrow cytoplasmic processes vary, but are usually 500–4000 Å in diameter and have been traced for more than 12 μ in length from the varicosity. As in earlier stages, they are filled with a finely filamentous matrix material, and sometimes contain a few vesicles.

The varicosities exhibit a greater quantity of organelles than in earlier stages. Interconnected cisternae of agranular reticulum in either flattened (Fig. 14, *Ar*) or dilated form (Figs. 16, 18, *Ar*) are conspicuous, particularly near the surface membrane. Clusters of ribosomes (Figs. 16, 17, arrows) and scattered neurofilaments and microtubules are common. In some varicosities, the neurofilaments are concentrated (Fig. 18, *F*). Although microtubules usually appear as relatively straight longitudinally oriented structures in axons, they are often curved and randomly oriented in varicosities. Mitochondria and dense heterogeneous bodies are usually very numerous. The mitochondria may be narrow and elongated (Fig. 16) or more characteristically they may exhibit a large ovoid shape (Fig. 19). Spherules, 300–600 Å in diameter, and

small 110–150 Å particles are present in the finely granular and filamentous matrix of the mitochondria. The small mitochondrial particles have been seen in other areas of the embryonic nervous system (Mugnaini and Forstrønen, 1967) and in X-irradiated dorsal root neurons in culture (Masurovsky et al, 1967). They have been identified as ribonucleoprotein by indium staining (Watson and Aldridge, 1964), as well as by their removability after ribonuclease digestion (André and Marinozzi, 1965). Occasionally ovoid mitochondria have been observed that are partially or completely encircled by a cisterna of agranular reticulum, which may indicate a stage of autophagosome formation (Novikoff and Shin, 1964). Multiple lamellae often surround the heterogeneous bodies (Fig. 14, *inset*), and fine particles, membranes, and larger spherules may be present in their matrix. The appearance of these bodies suggests that they may have been derived from altered mitochondria.

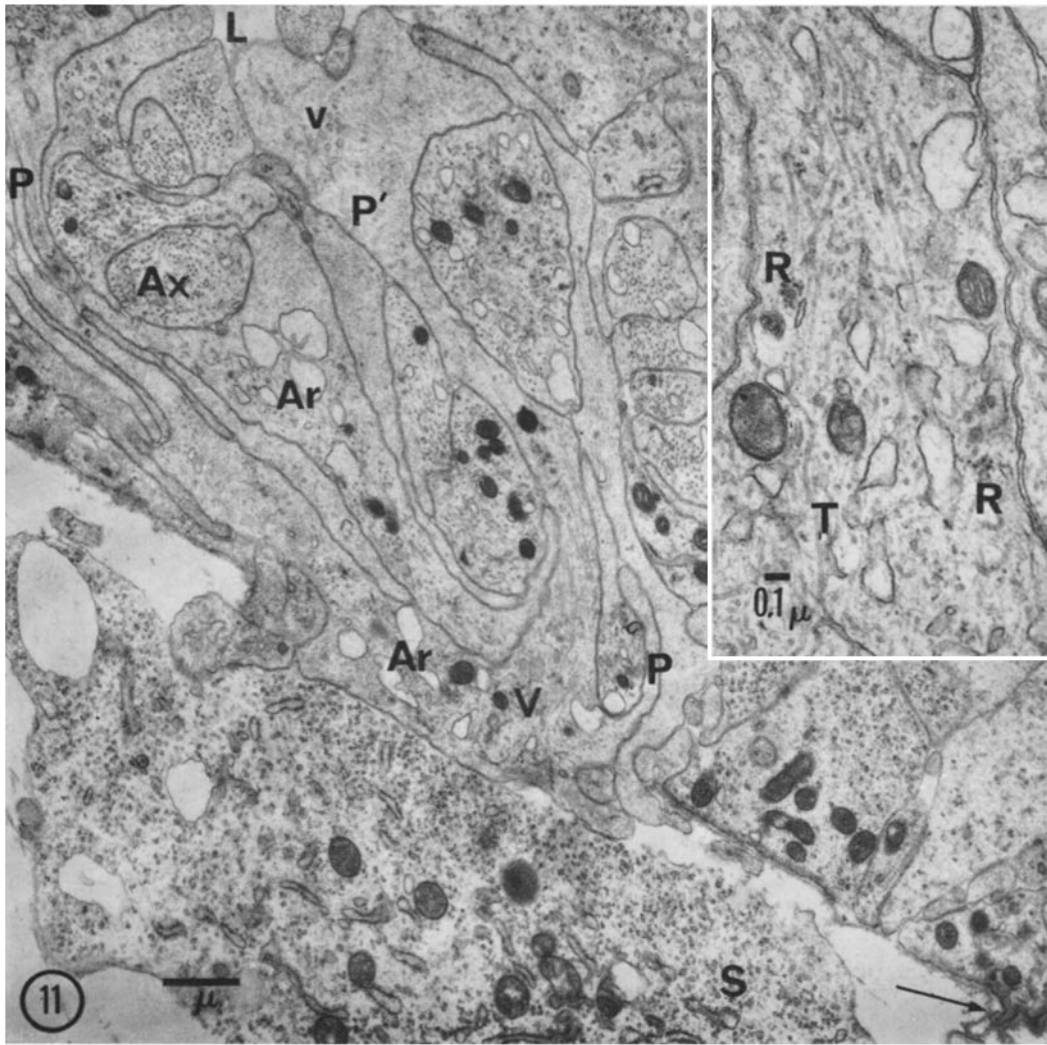
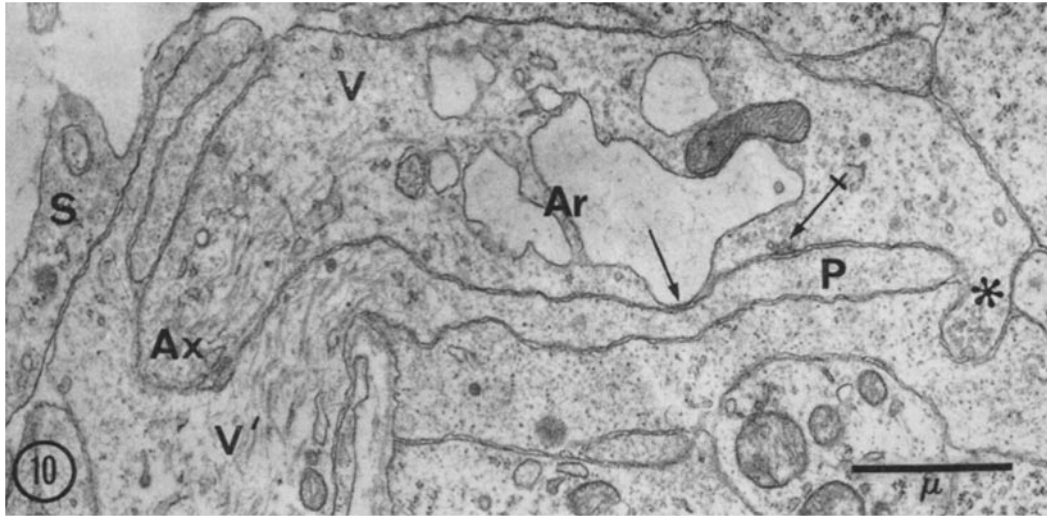
DISCUSSION

Identification of the Growth Cone

The expanded cytoplasmic mass and the thin flange of cytoplasm that are continuous with an axonal profile are interpreted here to be the varicosity and filopodium, respectively, of the early growth cone of the dorsal root neuroblast. This image is in keeping with the cinematographic records of the growth cone of dorsal root neurites

FIGURE 10 A small bundle of axons (*Ax*) entering the neural tube. The varicosity (*V'*) extends into long thin processes (*P*). A few vesicles (crossed arrow) are located close to the membrane of the varicosity (*V*) and are present in a short cytoplasmic projection (*). The agranular reticulum (*Ar*) is widely dilated and is closely apposed to the surface membrane (arrow). Schwann cell cytoplasm (*S*) surrounds the axons. $\times 21,000$.

FIGURE 11 A bundle of axons lose their Schwann cell (*S*) before entering the neural tube. The expansion of cytoplasm or varicosity (*V*) of a neurite extends outward to form two thin processes (*P*) and a broad flange of cytoplasm (*P'*), which has finely filamentous material and small vesicles (*v*). The varicosity contains interconnected channels of agranular reticulum (*Ar*), mitochondria, and microtubules. Cross-sectional profiles of axons (*Ax*) are fibers of the oval fasciculus. Most of the processes of this region are closely packed together, exhibiting only occasional lacunae (*L*) of extracellular space. An irregular evagination (arrow) of the basal surface of a neuroepithelial cell can be seen. An accumulation of moderately dense filamentous material is present in the evaginations and in other patches along the basal membrane. $\times 10,100$. *Inset*. Clusters of ribosomes (*R*), microtubules (*T*), agranular reticulum, and mitochondria from a near-serial section of the varicosity. $\times 32,000$.



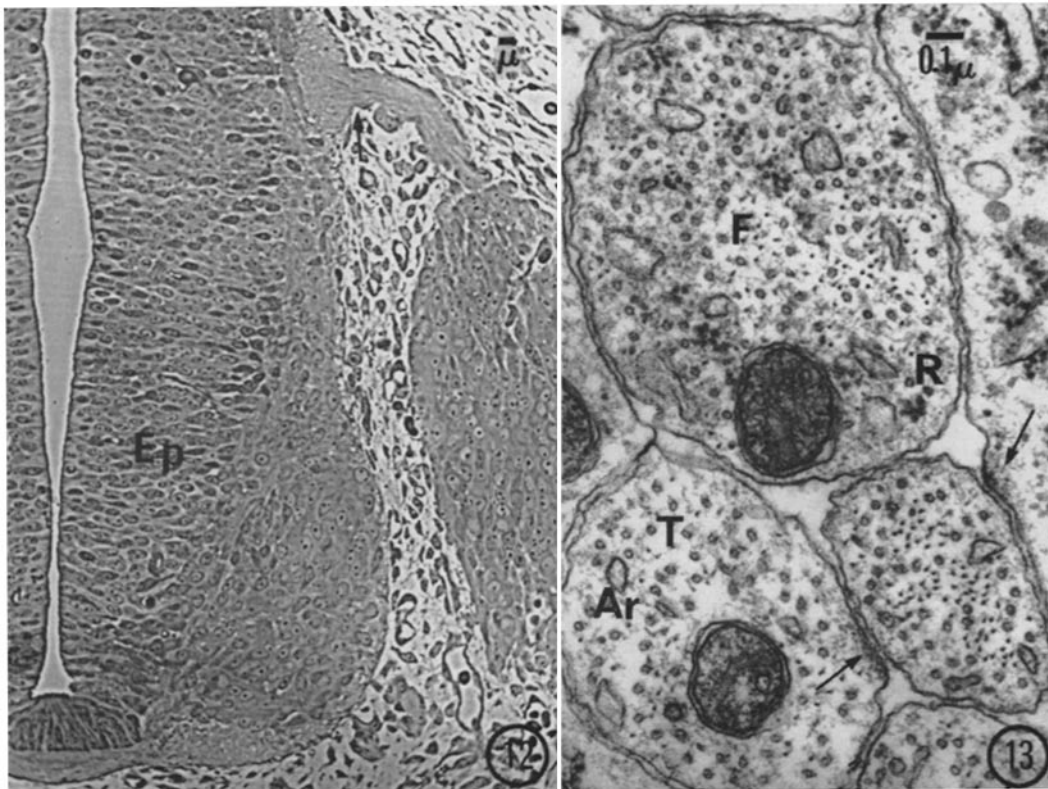


FIGURE 12 A phase micrograph of the neural tube and dorsal root ganglion at day 12 of gestation. A well-defined marginal and mantle layer can be distinguished from the ependymal zone (*Ep*) in the ventral region. The dorsal root ganglion consists of a closely packed grouping of well-differentiated bipolar neuroblasts with vesicular nuclei and prominent nucleoli, as well as immature sheath cells and capillaries. A well-defined dorsal root, surrounded by immature satellite cells, enters the neural tube (arrow) to form the oval bundle of His. $\times 200$.

FIGURE 13 Axons from the proximal portion of the dorsal root show numerous microtubules (*T*), neurofilaments (*F*), clusters of ribosomes (*R*), channels of agranular reticulum (*Ar*), and mitochondria. Patches of dense material are present beneath the surface membrane at some areas of close contact (arrows). $\times 52,000$.

in tissue culture (Pomerat et al., 1967), as well as with the illustrations by Cajal (1909, Figs. 239, 240) after Golgi silver impregnations. Cajal states that the fiber of the dorsal root ganglion has a short, thick terminal expansion at first, but that after entering the spinal cord the expansion gradually enlarges and becomes bicornuate, having two pointed appendages lying horizontally (Cajal, 1909, Fig. 244). The bifurcating processes of the early neurites entering the neural tube in this study correlate well with this description. Large spindle-shaped varicosities containing smooth reticulum, mitochondria, heterogeneous dense

bodies, neurofilaments, and microtubules are numerous during the subsequent period of rapid neuroblast differentiation, which results in the formation of the dorsal root and oval fasciculus. It is during this stage that the embryonic growth cone resembles the cone of regenerating nerve fibers (Estable et al., 1957; Wechsler and Hager, 1962; Wettstein and Sotelo, 1963; Lampert, 1967; Lentz, 1967). Although both cones are similar with respect to the bulbous shape of the varicosity and the large accumulation of organelles, the embryonic growth cone usually has a somewhat smaller varicosity.

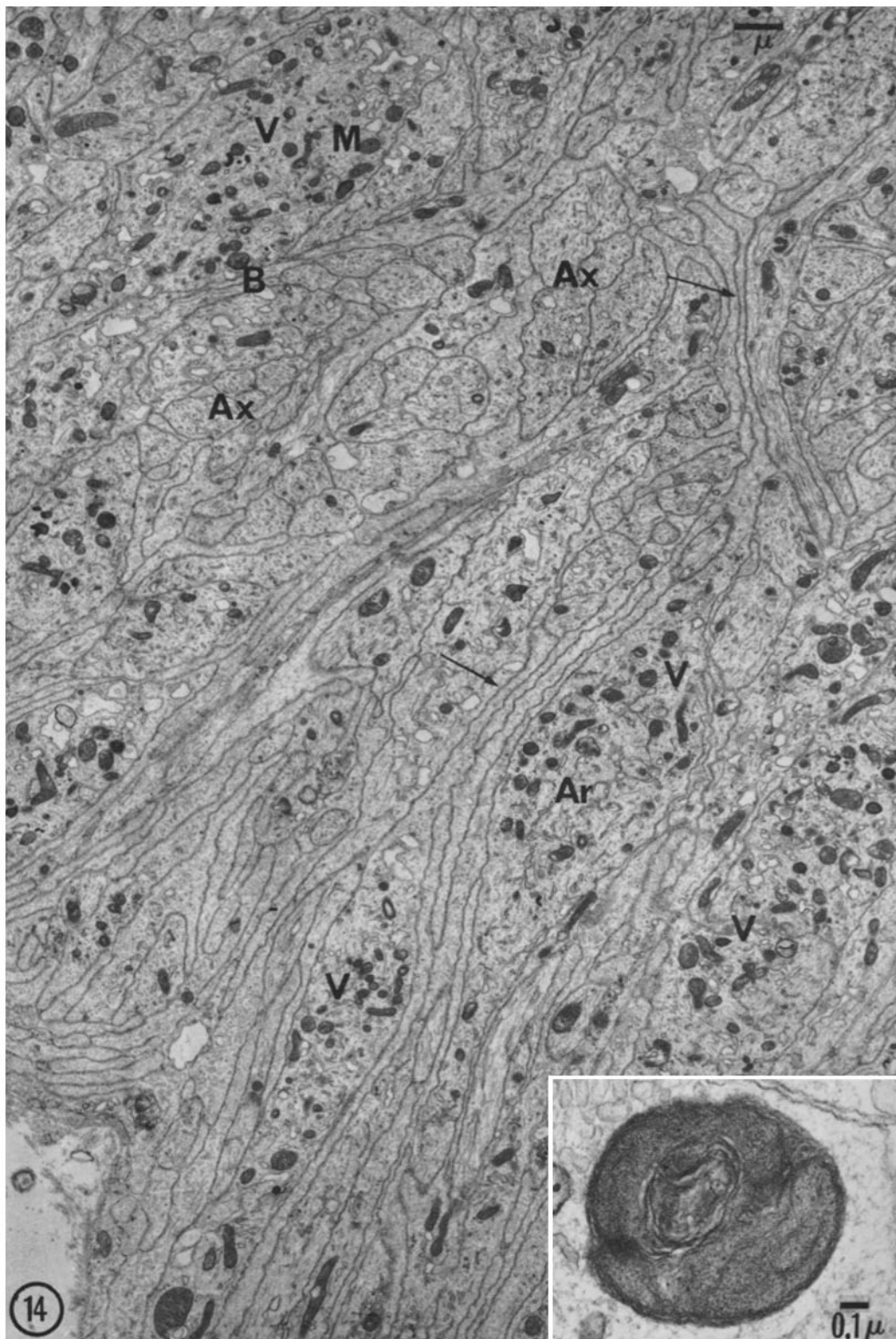


FIGURE 14 Junction of the dorsal root (bottom) with the oval fasciculus. For orientation see arrow in Fig. 12. Numerous varicosities (*V*) ranging from about 2 to 3.5 μ in diameter are present. They contain mitochondria (*M*), dense heterogeneous bodies (*B*), and dilated or flattened interconnected cisternae of agranular reticulum (*Ar*). Longitudinally oriented varicosities, entering from the root often show continuity with thin processes (arrows), which in some areas are less than 500 \AA in diameter. The processes contain primarily a finely filamentous matrix material. Oblique- and cross-sectional profiles having more characteristic axonal structure (*Ax*) are fibers that have entered at another level and are running longitudinally through the neural tube. $\times 7,500$. (*Inset*) A dense heterogeneous body is surrounded by several lamellae and contains membranes in whorled formation, as well as pairs of more parallel membranes that resemble flattened cristae. Isolated particles are present in the dense matrix. $\times 48,000$.

At first glance, there appears to be little correspondence between the growth cones described in this study and structures considered to be growth cones in the developing spinal cord (Bodian, 1966; Bodian et al., 1968) and cerebellum (del Cerro and Snider, 1968 *a*). Mitochondria were absent from the growth cones described in the latter studies, and the presence of large agranular vesicles in a mass of electron-lucent cytoplasm appears to be the primary identifying characteristic. Collections of ovoid or elongate smooth surfaced vesicles have been seen in the finely filamented cytoplasm of the filopodia of the growth cones in this study, but they generally tend to be of small to medium diameter. Moreover, similar vesicles have been seen in Schwann cells. Profiles resembling the structures illustrated in the studies of Bodian and coworkers (1966, 1968) and del Cerro and Snider (1968*a*) were found in the posterior fasciculus during day 16, when immature synapses were first seen (unpublished data). These profiles have not been identified in this laboratory, since they often appeared to be poorly preserved, and they could be interpreted as dendritic processes equally as well as axonal processes. In some cases, similar large vesicles could be seen in swollen processes of glial or satellite cells. Nevertheless, the possibility remains that at least some of these profiles may represent the endings of neurites prior to synapse formation, since Bodian (1966) has shown large vesicles in the preterminal portion of an embryonic bouton having typical synaptic vesicles

and a synaptolemma. A detailed study is in progress to characterize the further development of the growth cones into early synapses. The apparent inconsistencies in the description of the fine structure of the growth cone, therefore, may be due to the possibility that the morphology of the cone changes under different conditions. The growth cone could be expected to exhibit a differing ultrastructure, not only during maturation in embryonic development, but also during regeneration and growth in an altered environment, such as in tissue culture.

Comparison with Growth Cones in Tissue Culture

The structures identified as growth cones in this study can be correlated well with phase micrographs of their living counterpart in tissue culture (Pomerat et al., 1967). Precise estimates of the limits of the size of the living growth cone are not possible because of its dynamic character, but reference to Fig. 13 in the paper of Pomerat et al. (1967) would indicate that the following dimensions are at least in the proper order of magnitude: (a) The bulbous portion of the spindle-shaped varicosity varies from 10 to 12 μ in length and from 4 to 5 μ in its widest diameter. The varicosities of the embryonic growth cone during the stage of rapid ingrowth of nerve fibers are comparable i.e., they usually range from 6 to 13 μ in length and 2 to 5 μ in width. (b) The ruffled con-

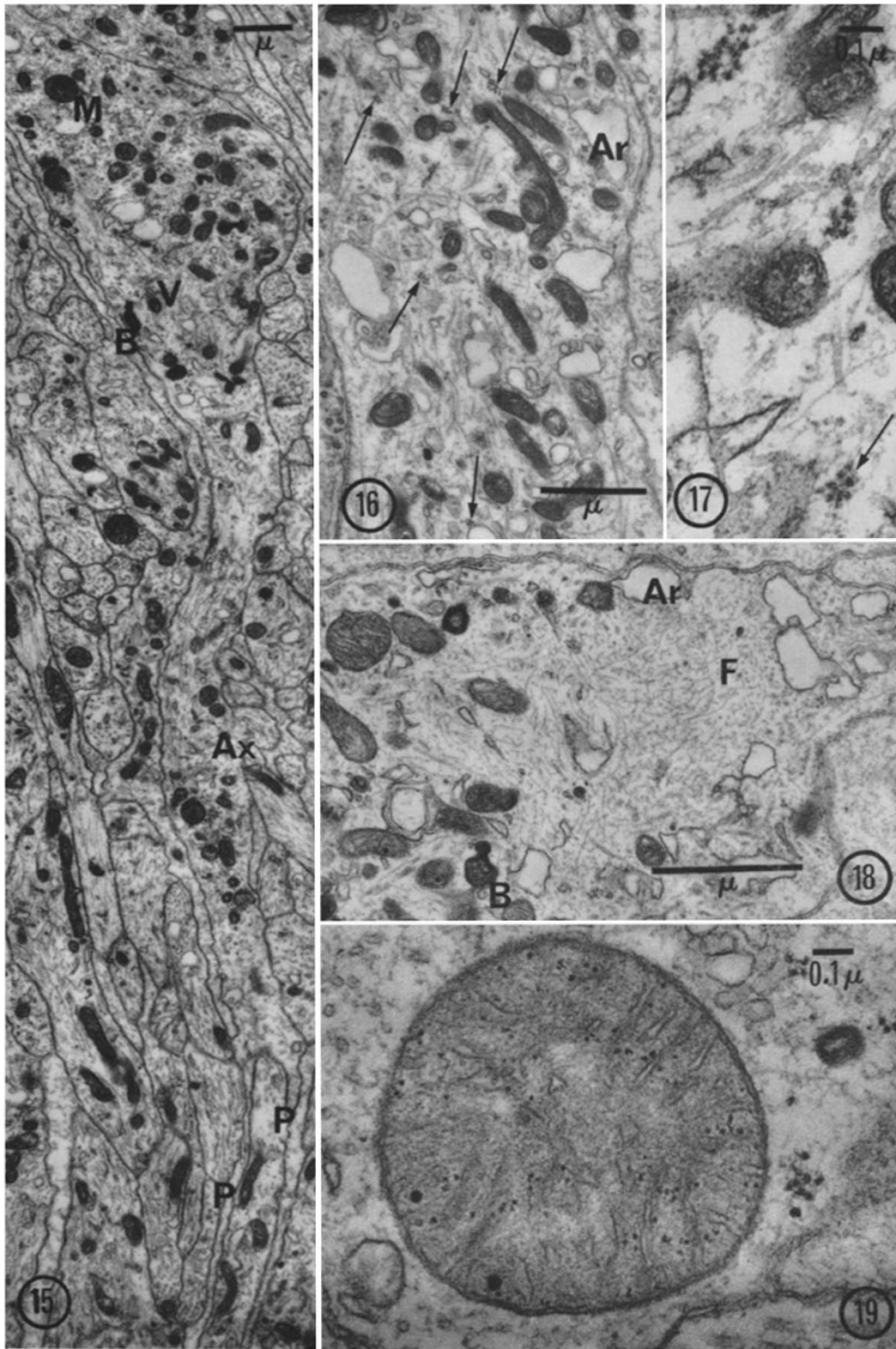
FIGURE 15 A varicosity (*V*) containing numerous mitochondria (*M*) and dense bodies (*B*) is continuous with a typical axonal segment (*Ax*) that bifurcates into processes (*P*), one of which narrows to about 550 Å in diameter. Varicosity, 6.4 μ long and 4 μ wide. \times 9,000.

FIGURE 16 A varicosity contains numerous mitochondria, dense bodies, dilated agranular reticulum (*Ar*), clusters of ribosomes (arrows), and randomly oriented microtubules and neurofilaments. Varicosity, 8.8 μ long and 3.3 μ wide. \times 16,200.

FIGURE 17 Higher magnification of three ribosomal clusters shown in the varicosity in Fig. 16. The ribosomes (arrow) are about 150–200 Å in diameter and are rather irregularly shaped. \times 64,000.

FIGURE 18 Numerous randomly oriented neurofilaments (*F*) fill a large area of a varicosity. A dilated cisterna of agranular reticulum (*Ar*) approaches the surface. Dense bodies (*B*) are present. Varicosity 6.3 μ long and 3.6 μ wide. \times 23,500.

FIGURE 19 A large ovoid axonal mitochondrion, about 0.95 μ in diameter, has 110–150 Å particles and dense 310–400 Å spherules in a moderately dense matrix. Cytoplasmic particles, 185–230 Å in diameter, resemble ribosomes. \times 64,000.



tour of the fanlike filopodium in tissue culture permits its easy visualization, although its thickness is probably just at or below the level of resolution of the phase microscope. Since this membrane is constantly changing in size and shape no measurement is meaningful, but it can be stated that lengths greater than $10\ \mu$ are visible in Fig. 13 of Pomerat et al. (1967). The irregular flange of cytoplasm and the thin processes $500\text{--}4000\ \text{A}$ in diameter and at least $12\ \mu$ in length in this study are in keeping with the image of the filopodium seen in culture. The finely dispersed filamentous matrix material containing few formed elements seen in filopodia and axonal sprouts in this study have also been found by Bunge and Bray (personal communication) in the filopodia at the tip of the outgrowing nerve fibers from cultured rat sympathetic ganglion cells. An even greater concentration of filamentous material was present in the cortical regions of other cultured cells (Buckley and Porter, 1967). These investigators pointed out that biophysical studies of gels (Frey-Wyssling, 1953) show that gel properties depend on a framework of interweaving fibrillar macromolecules that bind water. Increased viscosity results from an increased fibril density and on the junctions between fibrils. On the basis of this information, most of the filopodia illustrated in the present paper would appear to have a relatively low viscosity. It is of interest that Lewis (1950) proposed that local changes in viscosity of the ectoplasmic gel might be responsible for the undulating movements of the filopodial membrane of the neurite. (c) Small dense granules and spherical and irregular vacuoles, varying from less than $1\text{--}2.4\ \mu$ in diameter, are present in the endoplasm of the growth cone of Fig. 13 of Pomerat et al. (1967). The numerous mitochondria and heterogeneous bodies seen in the varicosity of the embryonic growth cones would appear as granules in the living growth cone. The interconnected system of agranular reticulum is the only organelle present in the embryonic varicosity that could dilate to the size of the endoplasmic vacuoles seen in the growth cone of the living neurites. This organelle, moreover, could readily account for the interconnected vacuoles that appear as a string of beads in Fig. 13 b of Pomerat et al. (1967). Bellairs (1959) previously noted that vacuoles connected by a narrow stalk were present in the axon and associated them with the pinocytotic vacuoles seen in tissue culture. Agranular reticulum was also found to be prominent in the

growing tips of cultured neurites (Bunge and Bray, personal communication).

Endoplasmic Vacuoles— Agranular Reticulum

The possibility that the agranular reticulum may be the organelle that corresponds to the endoplasmic vacuoles, which are often called pinocytotic vacuoles in the growth cone of the living neurite, raises the question of the origin of the membranes of the agranular reticulum and the mechanism of macropinocytosis. Agranular reticulum is generally assumed to arise in the perikaryon and move into the axon by axoplasmic flow. Pinocytotic vacuoles, on the other hand, have been reported to be taken up in cultured macrophages (Lewis, 1931) and neurons (Lewis, 1950; Hughes, 1953) at the periphery of the membranous pseudopodia. The vacuole is thought to be trapped in the folds of a ruffle, which then fuse and enclose the vacuole. The theory of macropinocytosis was supported in a study of the uptake of thorium dioxide particles by ameba (Brandt and Pappas, 1962). The particles in the surrounding medium adhered to the surface of the ameba and then were incorporated into the cell on the inner surface of the membrane of a large pinocytotic vacuole. If such a mechanism occurs *in vivo* in the embryo, at least some of the membranes of the agranular reticulum in the varicosity of the neurite would be derived from the surface membrane of the filopodium.

Although static pictures cannot provide evidence concerning the direction of movement, the vacuoles fused with the surface of the growth cones in Figs. 9 and 10 are at least consistent with the concept of internalization of fluid vacuoles *in vivo*. The membranes surrounding vacuoles, therefore, may have been derived from the surface of the growth cone. An alternative possibility, that the vacuolar membranes originated from agranular reticulum of perikaryal origin, should be considered, however. It is conceivable that, as the filopodium surrounds a volume of fluid, the agranular reticulum within the growth cone approximates and fuses with the surface membrane, forming a modified subsurface cistern. The fluid may then pass across the fused membranes and enter the channel of the agranular reticulum, thereby causing it to swell to the size of the pinocytotic vacuole. Once incorporated into the growth cone, the fluid may move centripetally through the axon

toward the cell body within continuous channels of agranular reticulum as implied by Bellairs (1959), or as discontinuous cisternae that move independently.

Certain lines of evidence suggest that the agranular reticulum within the axon may play a role in concentrating the enclosed fluid. Hughes (1953) noted that pinocytotic vacuoles get smaller as they move centripedally through the neurite in culture. This is supported in the present study in which it is shown that the agranular reticulum in the proximal portion of the axons is usually less dilated than that in the varicosity. In both sites, however, this reticulum is often located close to the surface membrane, like subsurface cisternae in the perikaryon. Rosenbluth (1962) has proposed that subsurface cisternae might be involved in the exchange of water and electrolytes between the perikaryon and the exterior. Further evidence that the agranular reticulum may be involved in fluid transport comes from studies of thallium poisoning of cultured neurons (Hendelman, 1969). This metal, which interferes with the pumping of potassium ions, causes severe dilation of the agranular reticulum in unmyelinated axons and in the peripheral cisternae of the endoplasmic reticulum of the perikaryon.

Axonal Ribosomes

Ribosomes are absent from most axons of the mature nervous system, but have been described recently in the initial segment of various adult neurons (Palay et al., 1968; Peters et al., 1968), as well as in the initial portion of embryonic or fetal axons (Lyser, 1964, 1968; Tennyson, 1965, 1969; Caley and Maxwell, 1968). In the present study, clusters of ribosomes were found along the entire length of a 60 μ segment of axon that was available for study, and also in the varicosities of numerous growth cones. These findings are consistent with earlier studies of embryonic axons in which ribonucleic acid was detected by ultraviolet absorption microscopy (Hydén, 1943; Hughes, 1955). The presence of ribosomes in the early embryonic axons

suggests that protein synthesis may continue in these segments at a considerable distance from the perikaryon.

Basal Membrane Specializations

The presence of dense elaborations of the basal membrane of the neuroepithelial cell close to the site of axonal and capillary ingrowth raises the question of their significance. These specializations are found only during the initial ingrowth period. Since axonal sprouts have been seen directed toward these membrane densities, the possibility arises that they may play some role in neurobiotaxis. Little is known about the factors responsible for the outgrowth of neurites or for the direction of their growth, but chemical agents seem to be implicated. For instance, a protein, nerve growth factor, causes a rapid outgrowth of dorsal root neurites that converge toward the source of the agent (Levi-Montalcini, 1966). Further studies are needed to investigate the possible role, if any, that the membrane specializations might play in attracting neurites to the proper entrance point in the neural tube, and also to attempt to elucidate the nature of the dense material within the basal cytoplasm.

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