

EFFECTS OF CALCIUM ION CONCENTRATION ON THE DEGENERATION OF AMPUTATED AXONS IN TISSUE CULTURE

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ABSTRACT

Light and electron microscope studies were conducted on the nature of the degenerative changes in amputated nerve fibers of cultured rat sensory ganglia and on the effects of media with differing calcium concentrations upon these changes. With glucose-enriched Eagle's media (MEM) containing 1.6 mM calcium, the amputated myelinated and unmyelinated axons undergo a progressive granular disintegration of their axoplasm with collapse and fragmentation of myelin sheaths between 6 and 24 h after transection. With MEM containing only 25–50 μ M calcium, the granular axoplasmic degeneration does not occur in transected fibers and they retain their longitudinal continuity and segmental myelin ensheathment for at least 48 h. Addition of 6 mM EGTA to MEM (reducing the estimated Ca^{++} below 0.3 μ M) results in the structural preservation of both microtubules and neurofilaments within transected axons. A transient focal swelling of amputated axons occurs, however, in cultures with normal and reduced calcium. These observations suggest that an alteration in the permeability of the axolemma is a crucial initiating event leading to axonal degenerative changes distal to nerve transection. The loss of microtubules and neurofilaments and the associated granular alterations of the axoplasm in transected fibers appears to result from the influx of calcium into the axoplasm.

INTRODUCTION

Observations on amputated nerve fibers may be viewed as a study of nonviable isolated segments of specialized cytoplasm. Survival under conditions of separation from the neuronal soma depends upon the ability of the amputated tissue components to maintain homeostasis. This process undoubtedly involves the maintenance of ionic gradients across the plasma membrane with the exclusion of certain ions and the conservation of others. Of particular interest is the calcium ion,

known to be present at very low concentrations in normal axoplasm (35).

The intact axonal membrane serves as an effective barrier to calcium ions, maintaining an intraxoplasmic concentration of approximately 0.3 μ M (2). It is believed that over 98% of axoplasmic calcium is present in bound form (24), much of which is probably sequestered in a separate intraxonal compartment (4). The small quantities of calcium ions which enter the axoplasm during

depolarization (24) are quickly and effectively bound and/or removed from the axoplasm and may be limited to the axoplasm immediately beneath the surface membrane (2).

Amputated axons, in time, undoubtedly fail in their maintenance of ionic homeostasis, causing an increase of intra-axonal calcium concentration. Exposure of the axoplasm within excised nerve segments to elevated calcium levels produces a granular precipitation of their constituent neurofilaments and microtubules which closely resembles the axoplasmic changes occurring in transected fibers (48). Furthermore, damaged regions of squid axonal membranes are believed to be sites of rapid intracellular penetration of calcium ions, causing a local opalescence of the underlying axoplasm (17).

Highly organized, long-term cultures of peripheral sensory neurons have been useful in the study of Wallerian degeneration (44). This culture system permits the manipulation of extracellular ionic constituents with minimal disturbance of other physiological parameters. Alterations of extracellular ionic composition surrounding amputated axons might alter their burden in maintaining ionic gradients and thus influence the nature of their degenerative alterations. The present study demonstrates that lowering or chelation of extracellular calcium ions in the tissue culture medium markedly reduces the degenerative axoplasmic changes and retards the subsequent myelin sheath changes in amputated axons. A preliminary report of this work has appeared in abstract (50).

MATERIALS AND METHODS

Fetal rat dorsal root ganglia were grown on a collagen substrate within Aclar (Allied Chemical Corp., Morristown, N. J.) plastic dishes in a special chamber (8) with maintenance media consisting of 65.7 ml of Eagle's medium (MEM) (15), 25.0 ml of human placental serum, 10.0 ml of 9-day chick embryo extract (50% in balanced salt solution), 3.0 ml of 20% glucose, and 0.4 ml of Achromycin (400 $\mu\text{g}/\text{ml}$). Cultures were incubated in 95% air and 5% CO_2 at 35°C. Well-organized cultures develop within 2-3 months in this culture system (7) and those containing compact myelinated fascicles in the outgrowth zone (as diagrammed in Fig. 1, reference 7) were selected for experimentation.

Aseptic transections of selected myelinated fascicles were performed under a dissecting microscope using two no. 11 blades in a scissors-like fashion. Results of

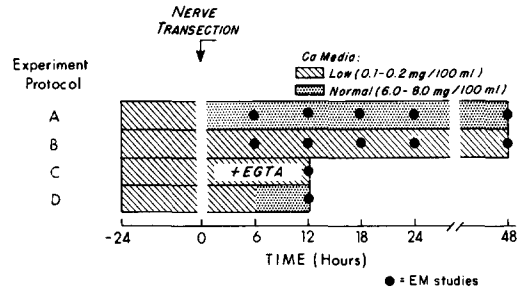


FIGURE 1 Schematic outline of experimental plan. For details see Materials and Methods.

these operations were monitored by phase and bright-field microscopy.

Changes in culture media in relationship to the time of nerve transection are indicated in the accompanying experiment schema (Fig. 1). All cultures were placed in commercial calcium-free MEM (Microbiological Associates, Inc., Bethesda, Md.) supplemented with 600 mg glucose/100 ml for 24 h before transectioning. This serum-free medium is not adequate for maturation of these cultures or for long-term maintenance, but it is adequate to sustain fully developed cultures for at least 1 week without detectable change. Some cultures were transferred to glucose-enriched complete MEM at the time of transection and examined at 6, 12, 18, 24, and 48 h after transection (protocol A). Other cultures were examined at the same time intervals but were continued in glucose-enriched calcium-free MEM (protocol B). In other cultures ionized calcium was lowered after transection to an estimated level of 0.3 μM (2, 45) by the addition of 6 mM EGTA to glucose-enriched complete MEM (protocol C). Finally, a reversal experiment was undertaken by transferring the cultures from calcium-free MEM to complete MEM 6 h after transection (protocol D).

All experimental media were collected and analyzed for total calcium by atomic absorption on a Perkin-Elmer 303 apparatus (Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.). Complete MEM and calcium-free MEM contained 6.0-8.0 and 0.1-0.2 mg/100 ml of total calcium, respectively.

Microscope observations of all cultures were undertaken periodically throughout the experiment. Selected cultures were fixed in 2% OsO_4 in balanced salt solution and stained with Sudan black for detailed light microscope examination.

For ultrastructural studies several methods of fixation were compared. The best preservation of axoplasm was obtained with an initial 30-min fixation at room temperature in 1% glutaraldehyde and 1% paraformaldehyde in a 0.1 M sodium cacodylate buffer with 2.5 mM CaCl_2 , pH 7.2, followed by a 4-h fixation in 2% glutaraldehyde and 2% paraformaldehyde solution in the same buffer with 5

mM CaCl_2 . After a wash in buffer solution the cultures were osmicated with 2% OsO_4 in cacodylate buffer and dehydrated in graded ethanol and propylene oxide. En bloc uranyl acetate staining in

maleate buffer preceded dehydration and embedding in a 1:1 mixture of Araldite and Epon. The procedures were performed within the Aclar plastic cups of the tissue culture dish. After the polymerization

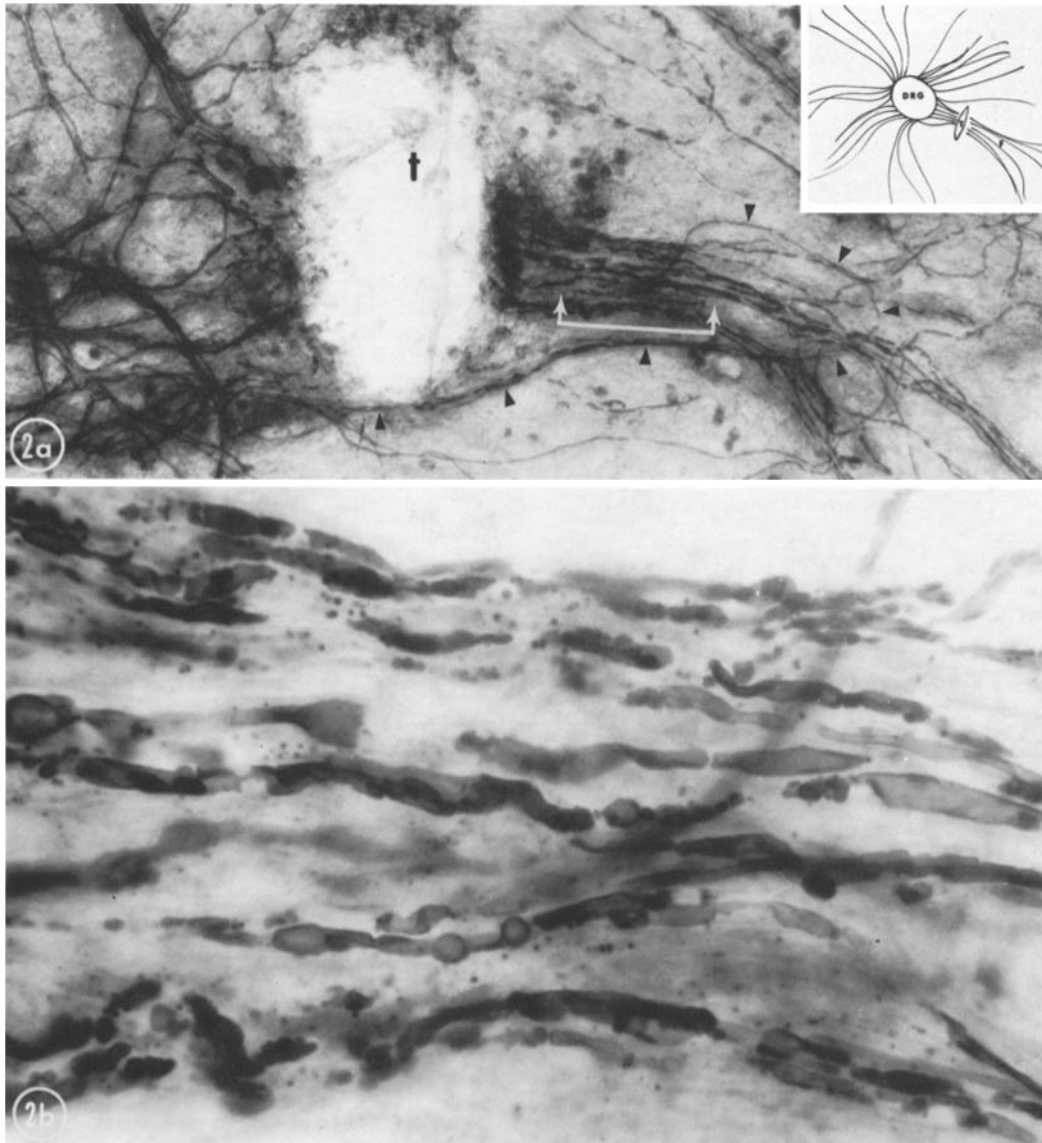


FIGURE 2 Nerve fiber degeneration in medium with normal calcium levels. The *inset* is a sketch of the overall configuration of a mature dorsal root ganglion in culture with the grouped neuronal somas (*DRG*) sending fascicles into the outgrowth zone; one of these fascicles (*F*) is shown transected at *T*. Photomicrograph *a* shows a site of a transection (*t*) 24 h after cutting; the fascicles proximal to the cut are at the left, fascicles distal are at the right. Note that the fiber marked by a series of arrowheads escaped the cut and curves back to enter the distal fascicle; this may explain the presence of an occasional normal fiber distal to the cut. The region within the bracketed arrows is enlarged in *b* to illustrate the breakdown of myelin segments. Sudan black staining after osmium fixation. *a*, $\times 125$; *b*, $\times 830$.

tissue blocks were easily separated from their plastic cups and undercoated with a thin layer of resin to provide a polymer layer on both sides of the tissue.

Transected fascicles were readily visualized within the polymerized blocks and the overlying surfaces were scored with transverse scratches 200 and 300 μm distal to the transection. Each amputated fascicle was then excised from the block and embedded in epoxy resin so that the ultrathin transverse sections of the fascicles were consistently obtained 200–300 μm distal to the transection. Unsupported thin sections were stained with lead citrate before examination in a Siemens Elmskop I.

RESULTS

Cell Cultures under Experimental Conditions (Light Microscopy)

Alterations of neuronal perikarya in living cultures were monitored by bright-field and phase microscopy during maintenance on glucose-enriched MEM with and without calcium deprivation. Peripheral nuclear displacement, central chromotolysis, and cytoplasmic granular alterations occurred more frequently with low calcium media. The onset of these changes varied among different cultures; they were often noted at 48 h and became prominent at 72 h. Longer periods in low calcium medium resulted in some neuron loss. The myelinated neurites of uncut fascicles and those proximal to nerve transections retained their continuity and segmental myelin ensheathment under normal and low calcium conditions (Figs. 2 and 3).

A laminar splitting of compact myelin was seen throughout the cultures maintained on low calcium levels (Fig. 4). This change appeared within 24 h after exposure to low calcium media and became progressively more severe. Segmental ensheathment remained intact, even in the presence of marked splitting of the internodal myelin structure. Detailed descriptions of these changes of myelin with calcium deprivation will be presented in a separate report.

Amputated Neurites in Complete MEM (Light Microscopy)

A progressive fragmentation of myelinated axons in amputated fascicles was observed in living cultures and in Sudan black-stained preparations of fixed cultures. These changes could be detected at 6 h but became more prominent 12

and 18 h after transection, involving all but an occasional fiber of the cut fascicle (Fig. 2). Intermittent swelling and collapse distorted the myelin internodes throughout the entire extent of the amputated neurites without apparent proximo-distal gradient.

Rows of translucent blebs or varicosities appeared along cut fascicles, representing the transient focal swelling of unmyelinated fibers previously described (38). These changes occurred throughout the extent of severed fascicles in the 6–24-h interval after transection.

Amputated Neurites in Cultures with Low Calcium Levels (Light Microscopy)

The fiber continuity and segmental arrangement of myelin sheaths were maintained with remarkable fidelity in amputated neurites distal to nerve transections under conditions of calcium deprivation (Fig. 3). Progressive fragmentation of amputated myelinated fibers was not observed in cultures in calcium-free MEM (total calcium, 25–50 μM) or with the addition of 6 mM EGTA to complete MEM (total calcium, 1,500 μM ; estimated ionized calcium, below 0.3 μM). Infrequent myelinated fibers in the process of degeneration before the time of transection remained largely unchanged in low calcium medium (Fig. 3).

Focal swellings of unmyelinated fibers occurred along the cut fascicles in low calcium media during the 6–24-h interval after transection (Fig. 4). These arrays of translucent blebs were similar to those which appeared along cut fascicles of cultures in complete MEM. Swelling of myelinated fibers distal to nerve transections was obscured by the lamellar splitting of compact myelin within the myelin internodes. Bulbous configurations of some amputated myelinated fibers were seen in areas of minimal myelin splitting and were suggestive of focal axonal enlargements within these fibers.

Amputated Neurites Maintained for 6 h in Calcium-Free MEM and 6 in Complete MEM (Light Microscopy)

Replacement of normal extracellular calcium levels in calcium-deprived cultures brought about a progressive fragmentation of transected myelinated fibers. At the time of calcium replacement,

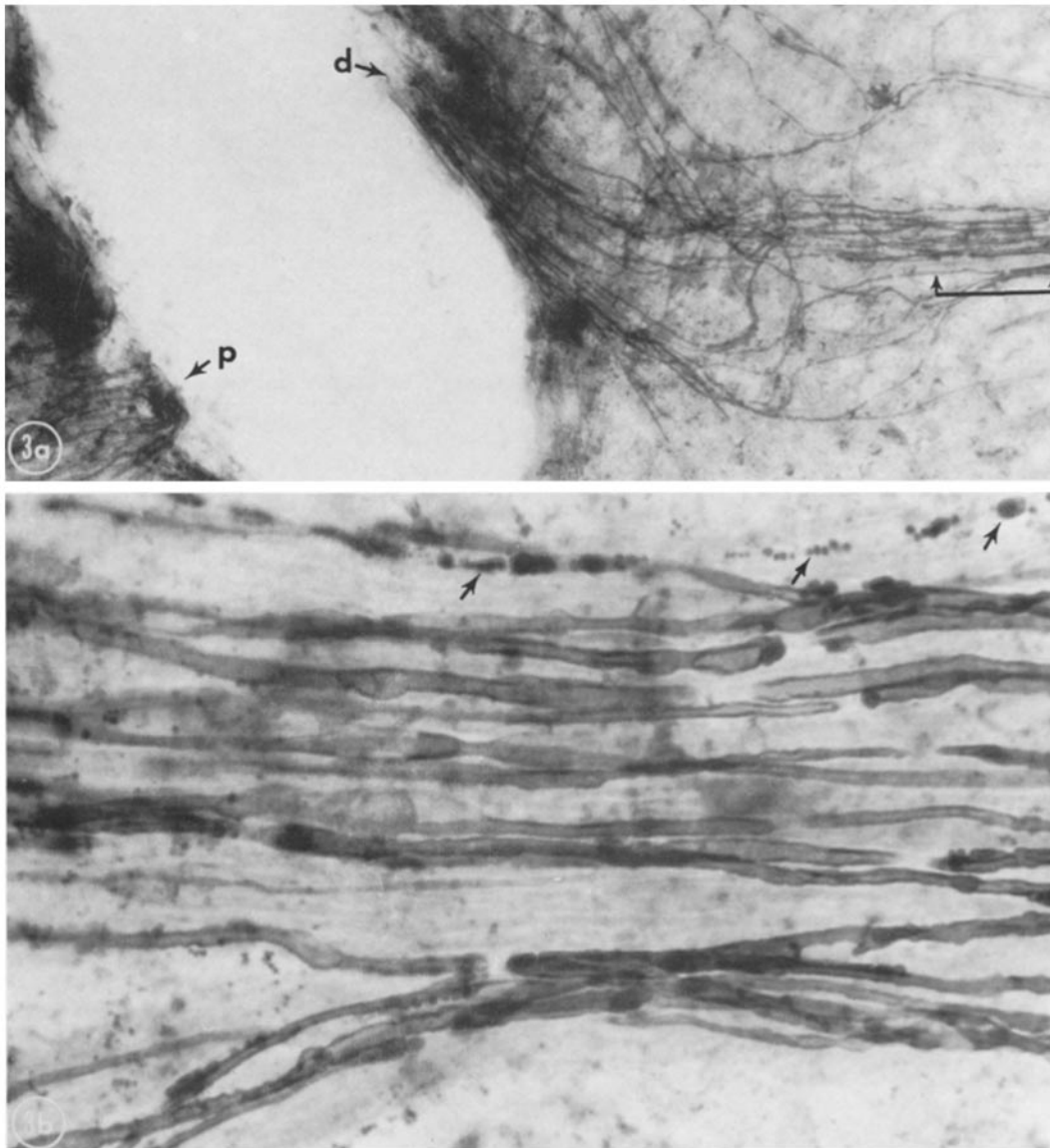


FIGURE 3 Nerve fiber degeneration in low calcium medium. As in Fig. 2, the site of fascicle transection is shown with the proximal fibers on the left and amputated fibers on the right. The course of the main fascicle has been displaced from *p* to *d* by the cut. The region in bracketed arrows is enlarged in *b*, showing swelling of axons and splitting within myelin sheaths (see also Fig. 4). The general form of the myelin internodes is retained. The remnants of an earlier fiber degeneration (about 4 days previously) are marked by a series of arrows. Sudan black staining after osmium fixation. *a*, $\times 125$; *b*, $\times 830$.

the transected myelinated fibers appeared intact when visualized by light microscopy. After an additional 6-h interval with normal calcium levels, the collapse and fragmentation of myelinated fibers distal to the transection were comparable to those observed in cultures maintained in normal calcium media from the time of transection.

Nonamputated Neurites in Complete MEM (Electron Microscopy)

Transverse sections of uncut nerve fascicles from cultures maintained for 48 and 72 h on glucose-enriched MEM revealed numerous myelinated and unmyelinated nerve fibers organized

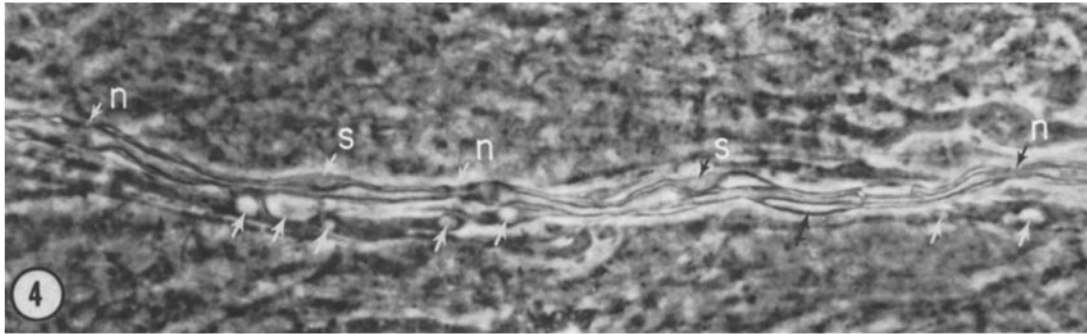


FIGURE 4 This small fascicle has been amputated 24 h earlier in a culture maintained in low calcium medium. It contains one myelinated fiber with two complete myelin internodes (*n*, node of Ranvier; *s*, Schwann cell nucleus). The splitting within the myelin sheath is particularly well shown at the crossed arrow. Below this myelinated fiber are seen the characteristic focal swellings (arrows) of degenerating unmyelinated fibers. Phase-contrast of living culture. $\times 480$.

in the histiotypic fascicles which characterize this highly organized culture system (7). Their axoplasm contained a normal admixture of microtubules and neurofilaments along with mitochondria and smooth membraneous vesicular structures. No degenerative axonal changes were seen.

Amputated Neurites in Complete MEM (Electron Microscopy)

A disintegration of microtubules and neurofilaments with granular transformation of axoplasm was seen 6 h after transection in many myelinated fibers of the amputated fascicle (Fig. 5). These axoplasmic changes were generally accompanied by a collapse or distortion of the myelin sheath and the axon, often with protrusion of membranous or myelin loops into the adjacent Schwann cell cytoplasm. Occasionally, these degenerating axons appeared focally enlarged and contained areas of increased axonal translucency which were either admixed with or displaced the granular axoplasmic content. No consistent early alterations were noted among the axonal mitochondria.

Granular disintegrative axoplasmic changes were also seen in transected myelinated fibers at 12, 18, 24, and 48 h after transection. Progressive distortion occurred in the myelinated fiber configuration, sometimes obscuring the location of the axoplasm. With more advanced changes, few identifiable myelinated fibers or remnants thereof could be found in a single cross-sectional area of the cut fascicle. Very rarely, intact myelinated fibers were encountered in the cut fascicle after

24 h, these presumably represented fibers of circuitous course which had escaped sectioning (Fig. 2).

Degenerative changes in unmyelinated fibers were similar to findings in myelinated axons. Granular disintegrative alterations of axoplasm was also seen at 6 h in these fibers. Some degenerating fibers were markedly swollen during the initial 24-h interval after transection (Fig. 5). Occasional unmyelinated fibers with intact neurofilaments and neurotubules were found at all periods of observation and probably represented fibers which escaped transection.

Amputated Neurites in Low Calcium MEM (Electron Microscopy)

A disintegrative granular transformation of axoplasm of myelinated fibers in transected fascicles was not observed when cultures were sustained in Ca-free MEM containing 25–50 μM of total calcium. The axons of these cut neurites retained their neurofilaments throughout the 48-h period of observation (Fig. 6). In some fibers the neurofilaments were aggregated within the axoplasm with reduced interfilamentous spacing but with their longitudinal orientation retained (Fig. 7).

In spite of the preservation of neurofilaments in transected neurites with low calcium media the axonal microtubules were disrupted or markedly distorted. A marked loss of axonal microtubules was noted 6 h after transection as well as at later periods of observation (Fig. 7). Microtubules were always readily seen in adjacent Schwann cell cytoplasm.

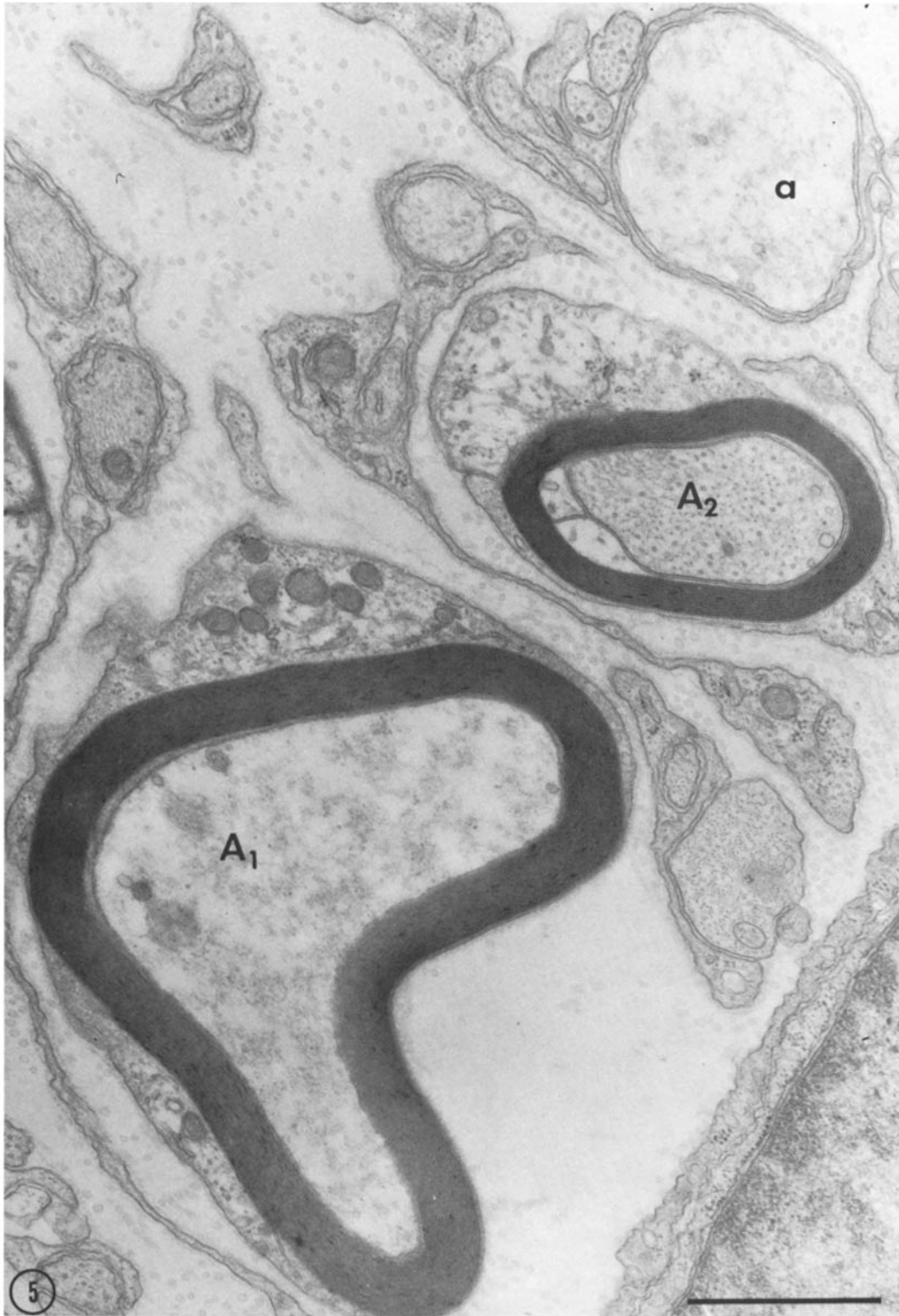


FIGURE 5 Granular disintegration of axoplasm with loss of microtubules and neurofilaments in myelinated (A_1) and unmyelinated (a) axons of amputated fascicle, maintained for 6 h in complete MEM after transection. The degenerating unmyelinated fiber is dilated. Intact microtubules and neurofilaments can be seen in another myelinated axon (A_2). $\times 30,000$.

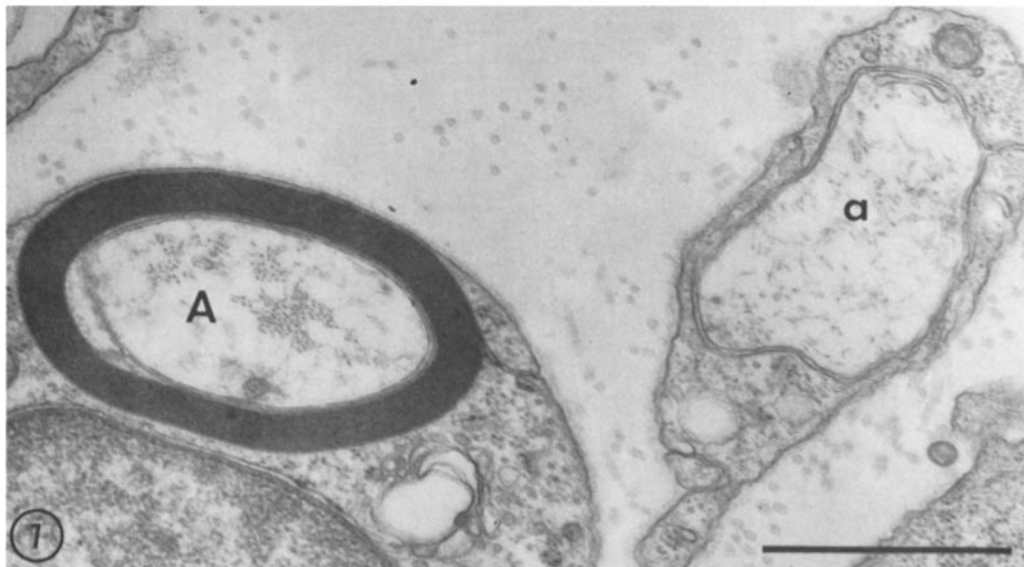
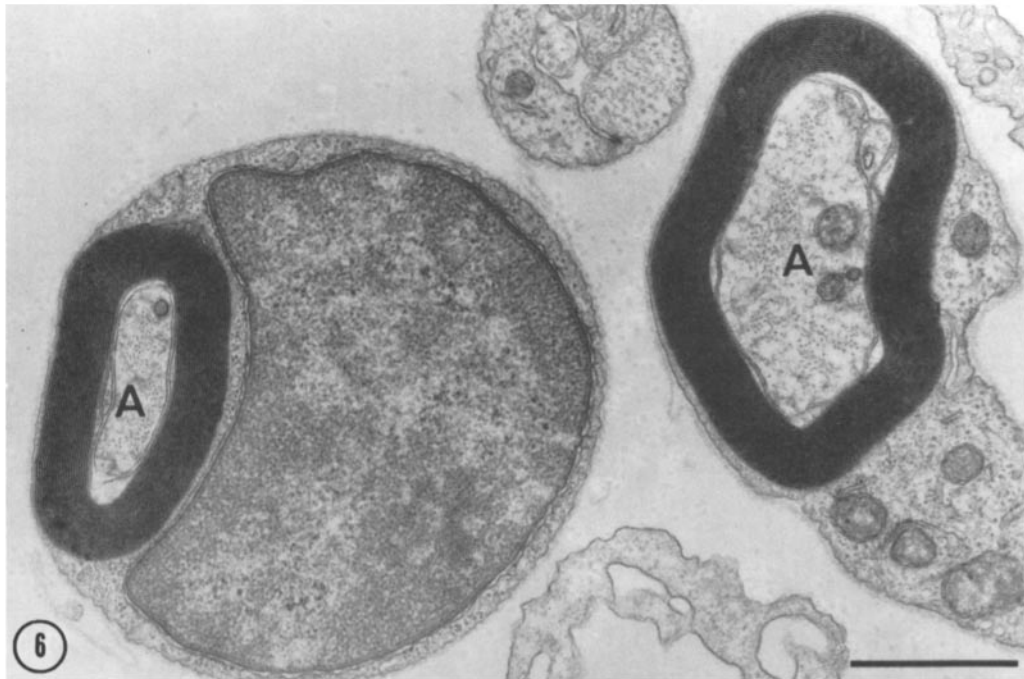


FIGURE 6 Axons (*A*) of amputated myelinated fibers with intact neurofilaments, maintained for 48 h in Ca-free MEM after transection. Microtubules are not present within these axons but may be seen in surrounding Schwann cell cytoplasm. $\times 22,500$.

FIGURE 7 Myelinated (*A*) and unmyelinated (*a*) axons of amputated fibers with intact neurofilaments but without microtubules, maintained for 12 h in Ca-free MEM after transection. Neurofilaments are aggregated within the myelinated axon. The unmyelinated axon is dilated. Microtubules can be seen in surrounding Schwann cell cytoplasm. $\times 32,600$.

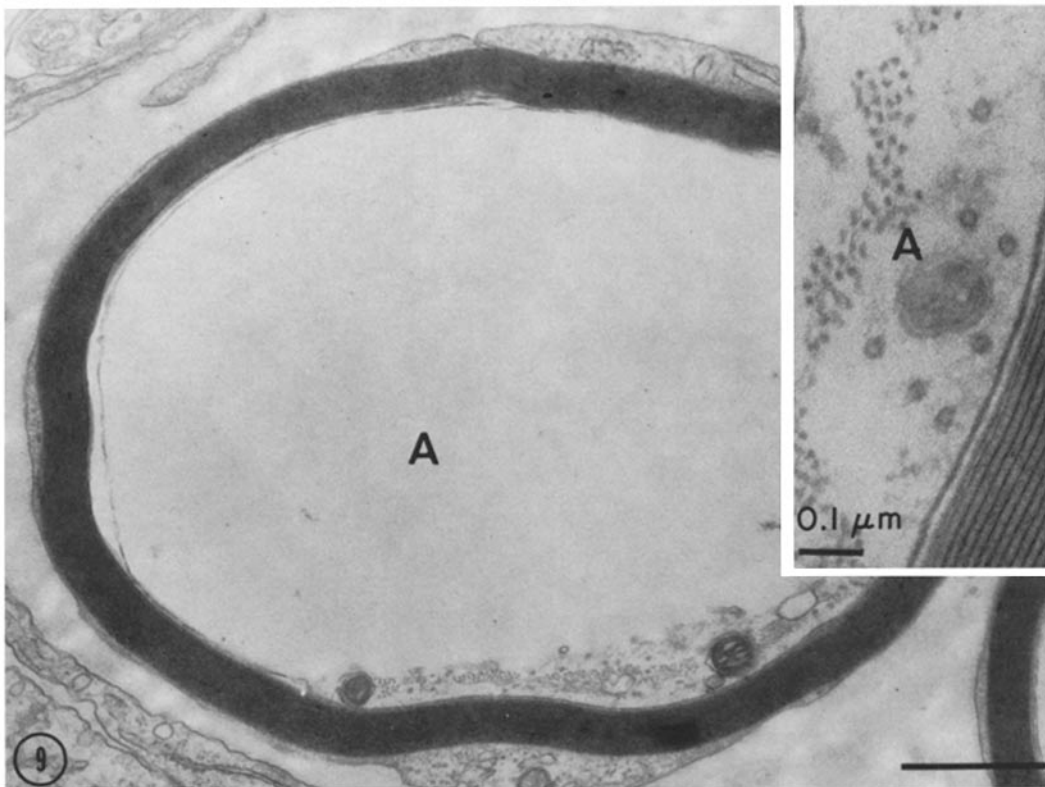
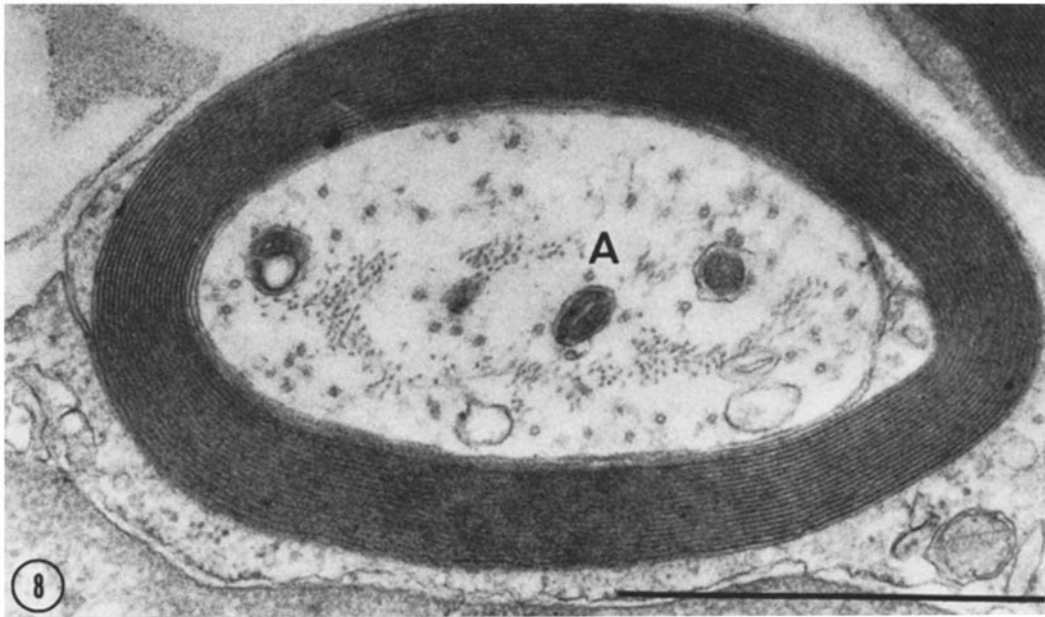


FIGURE 8 Myelinated axon (*A*) with intact microtubules and neurofilaments in an amputated nerve fascicle, maintained for 12 h in complete MEM with 6 mM EGTA after transection. Neurofilaments are aggregated in clusters apart from the microtubules. $\times 56,000$.

FIGURE 9 Marked dilatation of amputated myelinated axon (*A*), maintained for 12 h in complete MEM with 6 mM EGTA after transection. $\times 19,000$. Intact microtubules and neurofilaments of this axon are displaced to the periphery and can be seen within the *inset* taken from an adjacent section of this fiber. $\times 82,000$.

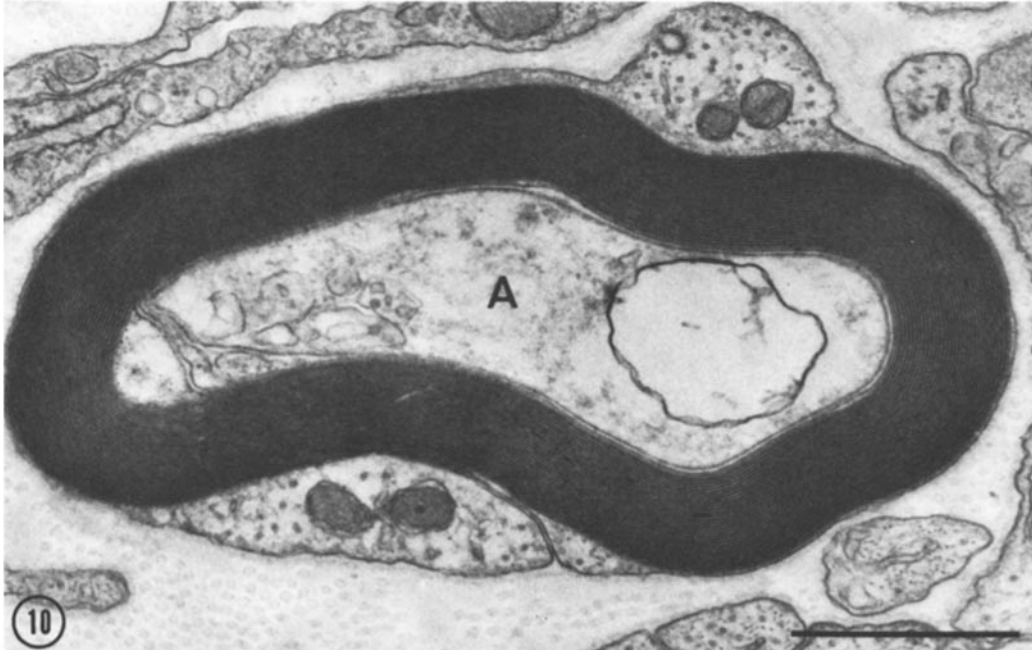


FIGURE 10 Granular disruption of axoplasm with loss of microtubules and neurofilaments in amputated myelinated axon (A), maintained for 6 h in Ca-free MEM and another 6 h in complete MEM after transection. Microtubules are present in surrounding Schwann cell cytoplasm. $\times 30,000$.

Axonal swellings were seen during the initial 24-h interval after transection of fascicles in low calcium media. These changes were most readily detected among unmyelinated fibers (Fig. 7). Neurofilaments and other organelles of these dilated axons were often displaced to a peripheral location as has been depicted in degenerating unmyelinated axons in reference 38.

Amputated Neurites in Complete MEM with EGTA (Electron Microscopy)

Axonal microtubules in transected neurites were preserved if ionized calcium of the culture media was markedly reduced by the addition of EGTA to the medium (Fig. 8). Neurofilaments were also preserved under these conditions and tended to aggregate into clusters apart from the axonal microtubules. Both axonal microtubules and neurofilaments maintained their longitudinal orientation.

Swollen myelinated (Fig. 9) and unmyelinated amputated fibers were also seen after the addition of EGTA to MEM culture medium. Within the dilated axons neurofilaments and microtubules were usually displaced peripherally. Occasionally,

these swollen axons contained enlarged vesicles or irregular membranous profiles.

Amputated Neurites Maintained for 6 h in Low Calcium MEM and 6 h in Complete MEM (Electron Microscopy)

Ultrastructural changes of the amputated neurites in reversal experiments were identical to those seen after a 6-h posttransectional interval in MEM. Granular disintegrative changes were present in both myelinated and unmyelinated fibers (Fig. 10). In addition, focally swollen unmyelinated fibers could be seen.

DISCUSSION

Calcium and Degeneration of Amputated Axons

The importance of calcium in the progression of degenerative changes in the distal segments of transected neurites has been demonstrated by comparing these phenomena under conditions of normal and low calcium. At physiological con-

centrations of extracellular calcium (1.6 mM), the amputated portions of neurites in tissue culture undergo a granular disintegration of axoplasm with subsequent collapse and fragmentation of myelin sheaths, the same changes which have been observed in vivo (27, 36, 40, 41, 55). This sequence of change does not occur at reduced levels of calcium, but may be induced by the addition of calcium to the medium of calcium-deprived cultures. These findings corroborate the interesting classical observations of Nageotte reported in 1910 (39), confirmed by Cajal (9), that excised nerve segments undergo in vitro myelin fragmentation only in the presence of bivalent metals such as calcium chloride.

While direct evidence of a calcium influx during axonal degeneration is lacking, the calcium-dependent degradative changes of neurofilaments and microtubules in amputated neurites closely resemble the alterations produced in excised nerves upon exposure of their axoplasm to 1 mM of calcium ions (48). The rapidity of calcium induced disruption of neurofilaments and microtubules in short excised nerve segments (48) is in contrast to that of amputated axons and is probably because of the more direct accessibility of extracellular calcium to the axoplasm through the cut and open ends of the nerve preparations. Similar changes are restricted to the ends of longer segments of excised and immersed nerves (unpublished data). Rapid disintegrative changes of the axonal segments abutting the sites of nerve transection have also been noted in electron microscope (60) as well as in light microscope studies of Wallerian degeneration (9, 52).

The preservation of axoplasmic structures in amputated axons under conditions of calcium deprivation is less dependent upon time than on the absolute concentration of calcium. Axoplasmic microtubules, in particular, are only well preserved in the presence of an EGTA-calcium ratio reported to reduce ionized calcium to 0.3 μM (2, 45), equivalent to the resting levels of intra-axonal ionized calcium (2). Neurofilaments were resistant to higher calcium levels (25–50 μM) supporting the view that their constituents differ from those of microtubules (28).

The selective disruption of axonal microtubules in amputated axons at low calcium concentrations seems compatible with a direct interaction of calcium ions with these structures. The converse process, namely the polymerization of purified microtubular subunits into the tubular configura-

tions, is largely dependent upon the removal of calcium ions below μM levels (5, 57), an in vitro process which may be reversed by the addition of calcium (5). Furthermore, at higher calcium levels, purified microtubular subunits undergo reversible precipitation (58, 59).

A spontaneous calcium influx into amputated axons presumes some degradative alterations in axonal surface membranes or their calcium-excluding mechanisms. A similar increased calcium entry through isolated axonal membranes occurs in degenerating synaptic endings, characterized by a calcium-dependent spontaneous release of nor-adrenaline (18). Accordingly, calcium (and not magnesium) has been found to promote and hasten in vitro degenerative changes in synaptic endings at frog myoneural junctions which closely resemble degenerating synapses after axotomy (23). It is of interest that the influx of calcium associated with transmitter release from synaptic endings has been likened to the late phase of calcium entry during depolarization of squid axonal membrane, suggesting a similarity of calcium entry sites at these locations (2, 32–34).

Axonal Fluid Accumulation in Transected Fibers

Degenerative alterations of amputated nerve fibers are also manifested by focal axonal swelling, a change indicative of axonal fluid accumulation which correlates with the increased H_2O content of transected nerve (6, 30, 37). In transected unmyelinated fibers, these changes are characterized by focal varicose dilatations along the fiber (Fig. 4) which are seen in electron micrographs to contain low density material (Figs. 5 and 7). Similar swellings have been observed along transected unmyelinated fibers in vivo (14). An early enlargement of transected myelinated fibers has also been described in vivo (51); however, these changes are often obscured by the concomitant collapse and fragmentation of myelin sheaths. In our experiment the swelling of amputated axons has been shown to occur under low calcium conditions and in the absence of progressive axoplasmic degeneration.

A manifestation of axonal fluid accumulations secondary to energy depletion is exemplified by the focal swellings along intact unmyelinated fibers in tissue cultures deprived of glucose and oxygen, changes which are readily reversed with correction of the deficient state (38). Identical swellings

along transected unmyelinated axons observed in the present study are indicative of similar fluid accumulations, but probably arise from a different mechanism. The following observations indicate that a significant energy depletion does not occur in transected nerves: (a) Conductivity, an index of energy-dependent pump activity, remains in transected mammalian nerves for 3–4 days (16, 21, 25), and for an even longer time in transected amphibian nerves (25, 43), long after marked degenerative changes have occurred in the composite nerve fibers (21, 25). (b) The ultimate failure of conductivity in transected nerve is not accompanied by a drop in ATP content of the degenerating nerve and is commensurate with the decrease of O_2 consumption due to stimulation (19). In contrast, failure of conductivity in excised nerve incubated in nonnutrient medium is preceded by both a loss of ATP and activity respiration (19). (c) Absolute values of ATP and creatine P (correcting for increases of H_2O) in the distal segments of transected rabbit tibial nerves were 90–107%, 77–75%, and 74–71% of normal control nerve on the 2nd, 3rd, and 4th days after transection (54), declines which could represent breakdown and loss of some constituent fibers. Over 80% of the residual creatine P (and ATP, to a lesser extent) of these nerves was lost during short periods of *in vitro* anoxia, a response which is similar to that of freshly excised nerve (10, 53, 54). (d) Anoxic failure of conductivity of excised nerve is associated with a rapid and marked depletion of creatine P and ATP (11, 42), a condition which is readily reversible (42) and not associated with ultrastructural alterations (29).

The lack of an energy depletion state indicates that other mechanisms lead to the axonal swellings in transected nerves. Baker et al. (1) have suggested that the liberation of lysosomal enzymes may account for the loss of ouabain-sensitive Na efflux in perfused squid axons. Increases of acid phosphatase have, indeed, been demonstrated in transected axons (26). Fluid accumulation in amputated axons, however, could also involve alterations in the Na influx component of net membrane permeability. Such an explanation would be compatible with the persistence of conductivity and associated pump activity in transected nerves. In fact, the increase in resting respirative activity which precedes loss of conductivity of transected nerves (19) in part might be because of increased pump activity which results from elevated levels of intra-axonal Na. The

relatively greater surface area of unmyelinated fibers renders them subject to proportionally larger Na influx during stimulation (20) and might account for their proclivity for swelling if their entire surface membrane became abnormally permeable to Na.

An increased membrane leakiness of amputated axons may be rather nonspecific. A concurrent influx of both Na and Ca ions is suggested by the approximate concomitant occurrence of axonal swelling and Ca-induced axoplasmic alterations. Yet, the presence of axonal swellings in low calcium experiments would indicate that degradative membrane changes are not calcium dependent and that Na influx may occur unaccompanied by Ca entry. Furthermore, the granular disruption of transected axoplasm upon the addition of calcium to calcium-deprived cultures suggests that the degradative membrane changes leading to increased Ca permeability occur concomitant to those producing the increased Na influx.

Speculation on the Mechanisms of Axonal Membrane Changes in Amputated Axons

It seems reasonable to believe that the degradative membrane changes which lead to ionic and fluid influx into amputated axons are because of a depletion of “membrane-sustaining factors” derived from the perikarya via axonal flow. Disruption of axonal flow by local injections of vincristine into peripheral nerve leads to the same pattern and time sequence of degradative changes in the distal nerve segments as seen after nerve transection (49). The more rapid morphological (9, 52, 56) and physiological (21) degeneration of small myelinated fibers in transected nerves is consistent with a depletion basis for membrane breakdown because the greater membrane surface/axoplasmic volume ratio of these fibers would provide relatively less inherent reserve. Spontaneous release of noradrenaline from degenerating nerve terminals is also delayed by leaving a longer segment of nerve (with larger axonal reserve) attached to the neuro-muscular preparations (18).

The identification of axonal membrane-sustaining factors which are necessary for the maintenance of membrane integrity remains entirely speculative. One might presume from the relative concomitant occurrence of disintegrative changes which take place along the entire length of ampu-

tated axons that there is a quickly mediated equilibrium of the depletion state or adverse conditions and that the membrane-sustaining factors are components of fast axonal flow or smaller molecular constituents which equilibrate even more rapidly through the axoplasm. They may consist of membrane constituents, such as the polyphosphoinositides which have been strongly implicated in membrane permeability (3, 12, 13, 22, 31, 46, 47), or they may serve to maintain membrane integrity by furnishing components necessary for local membrane synthesis or, perhaps, for the stabilization of lysosomes. In the latter instance, the rapid progression of degradative changes throughout the amputated axon could be explained by a liberation of various disruptive enzymes.

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