

FINE STRUCTURAL CHANGES IN NEURONS AND  
NERVE FIBERS ASSOCIATED WITH COLCHICINE INHIBITION  
OF NERVE FIBER FORMATION IN VITRO

MATHEW P. DANIELS. From the Laboratory of Biochemical Genetics, National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland 20014

INTRODUCTION

Neurotubules appear to be homologous to the cytoplasmic microtubules of other cell types in both their morphological (Gonatas and Robbins, 1964) and chemical (Weisenberg et al., 1968) characteristics. There is a large body of evidence indicating that microtubules have an important role in the development and maintenance of asymmetric form in cells and cell processes (reviewed by Tilney, 1968). For this reason it has been proposed that microtubules take part in the development and maintenance of the elongate form of neurites (Porter, 1966). There is some experimental evidence for this proposition, based on the effects of colchicine, which disrupts microtubules in vivo and in vitro (Weisenberg, 1972) through its specific binding to the protein subunit of that organelle (Taylor, 1965; Borisy and Taylor, 1967 *a, b*; Shelanski and Taylor, 1967, 1968). It has been demonstrated that colchicine disrupts the microtubules of spinal neurons (Wisniewski *et al.*, 1968), inhibits the sprouting of severed axons of the sciatic nerve (Hoffman, 1952), and both inhibits neurite outgrowth and causes retraction of neurites in monolayer cultures of chick dorsal-root ganglia (Daniels, 1968; Yamada et al., 1970). Daniels (1972) demonstrated that these effects of colchicine take place within 3–6 h of treatment with a low concentration of the alkaloid, 0.05  $\mu\text{g}/\text{ml}$ , and are reversible under these same conditions. The present report is concerned with changes in density of microtubules and neurofilaments in the neurons and neurites of these monolayer cultures during colchicine inhibition of neurite elongation and after reversal of this inhibition.

MATERIALS AND METHODS

*Cell Culture*

Monolayer cultures of trypsin-dissociated, 11-day-old chick dorsal-root ganglia were prepared and maintained exactly as described previously (Daniels, 1972), except that the glass cover slips were coated with a heavy carbon layer (Robbins and Gonatas, 1964) before the collagen substrate was added. 2,000 cells were plated in each 35-mm dish, yielding low-density monolayers.

*Electron Microscopy*

The cultures were fixed at room temperature in 2.5% glutaraldehyde in phosphate buffer, postfixed in 1% osmium tetroxide in buffer, and embedded in a 1–2 mm thick layer of an Epon-Araldite mixture (Luft, 1961). The polymerized epoxy, with the cell monolayer and substrate intact, was separated from the glass cover slip. Individual neurons were selected and marked by means of a device allowing the accurate marking of single cells without interruption of microscope observation. Selected neurons were classified for presence or absence of neurites. The selected areas were remounted and trimmed close to the desired cells and processes. Thin sections were cut parallel to the plane of the cell monolayer. The sections were stained with uranyl acetate (Stempak and Ward, 1964) and lead citrate (Venable and Coggeshall, 1965).

*Estimation of Microtubule and  
Filament Density*

Electron micrographs of random longitudinal sections of neurites, printed to a final magnification

of 35,000  $\times$ , were overlaid with a grid of 8–12 parallel lines perpendicular to the long axis of the neurites. The distance across the neurite and the number of intersections with either microtubules or filaments was recorded for each line of the grid. The mean number of intersections divided by the mean distance across the neurite gave an estimate of the density of microtubules or filaments in the neurite. This method of counting was presumed to give values equivalent to those obtained from cross sections of the neurites.

The micrographs used for this purpose were derived from 7 to 10 neurites (one to five different micrographs per neurite) from cultures of each treatment. Selection of cells with neurites for sectioning was random, except that neurons situated close to other cell types were excluded to avoid confusion of other kinds of elongate processes with neurites. The micrographs were also random as to depth of the sectioning plane and distance from the cell body (within the limitations of block-face size), excluding the tapered segment near the cell body.

Since many sections were taken obliquely to the plane of the cell monolayer and could not be used for quantitation, a larger sample was available for qualitative than for quantitative estimation of microtubule and filament density. Qualitative and quantitative estimates were found to be in agreement.

## RESULTS

### *Fine Structure of Untreated Neurons*

The fine structure of the cell bodies of the dorsal-root ganglion neurons in the cultures used for these experiments resembled that of neurons undergoing regeneration of damaged or removed neurites (MacKay et al., 1964; Price and Porter, 1972). The fine structure of nerve fibers (neurites) was similar to that described by Yamada et al. (1971). The primary constituents were elongate mitochondria, microtubules, and a smaller number of neurofilaments (Fig. 1).

### *Effects of Colchicine Treatment*

In a previous study (Daniels, 1972) it was shown that treatment of 20-hr-old cultures of dissociated 11-day chick dorsal-root ganglia with 0.05  $\mu\text{g}/\text{ml}$  colchicine caused measurable inhibition of neurite elongation after 3 h. After 6-h colchicine treatment there was both marked inhibition of elongation and retraction of neurites. Retraction continued during the subsequent 6 h of colchicine treatment. If cultures treated with 0.05  $\mu\text{g}/\text{ml}$  colchicine for 6 h were transferred to colchicine-free medium, at least 50% of the

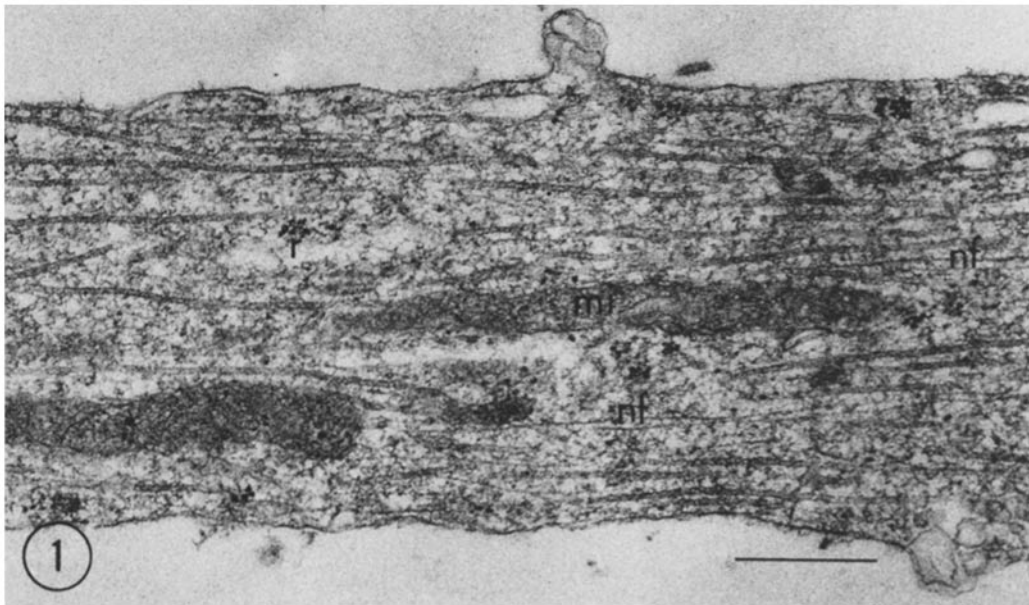


FIGURE 1 Neurite from a 26-h culture. Microtubules, elongate mitochondria (*mi*) and neurofilaments (*nf*) are the predominant organelles. Free ribosomes (*r*) are present. The distribution of organelles is homogeneous.  $\times 38,000$ . Reference bars in all figures equal 0.5  $\mu\text{m}$ .

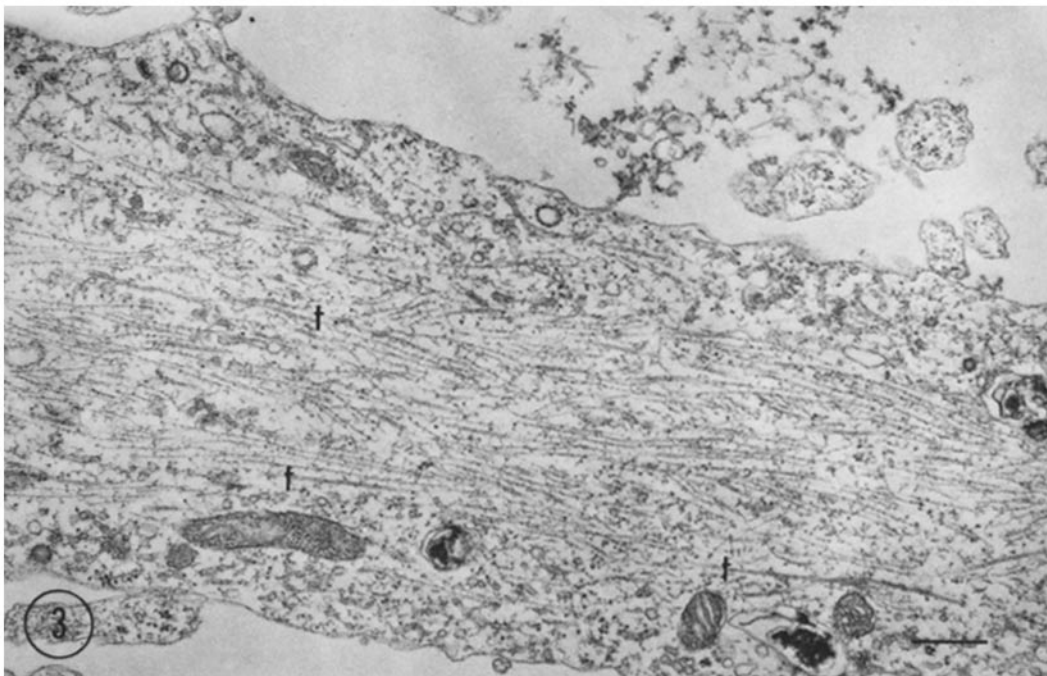
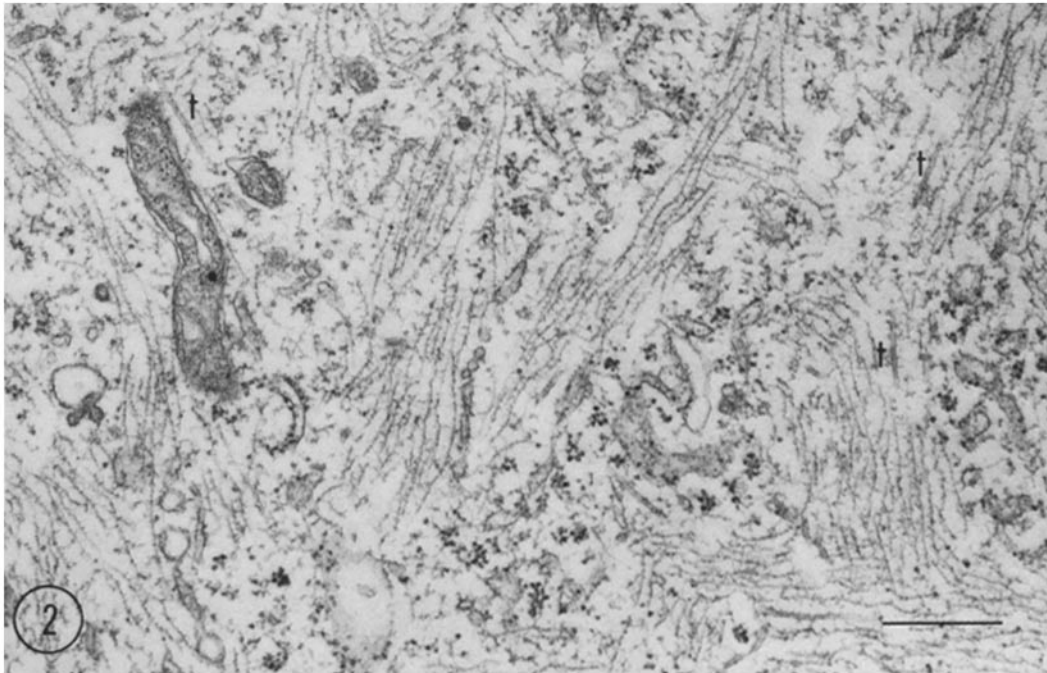


FIGURE 2 Cell body of a neuron from a 26-h culture after 6-h treatment with  $0.05 \mu\text{g/ml}$  colchicine. The cytoplasm contains bundles of  $70\text{--}100\text{-\AA}$  thick filaments. Some microtubules (*t*) remain.  $\times 32,000$ .

FIGURE 3 Neurite from a 26-h culture after 6-h treatment with  $0.05 \mu\text{g/ml}$  colchicine. The central portion contains large numbers of  $70\text{--}100\text{-\AA}$  thick filaments and some microtubules (*t*). Most mitochondria and vesicles are located peripherally.  $\times 21,000$ .

neurons survived, and, after a lag of about 18 h, neurite growth proceeded at a rate similar to that of untreated cultures.

In the present study the cultures were prepared and treated exactly as before (Daniels, 1972) and were fixed at 6 or 12 h after addition of colchicine. The pattern of inhibition of neurite outgrowth corresponded to that observed in the previous experiments (Daniels, 1972). Some cultures were transferred to colchicine-free medium after 12 h of treatment and fixed 30 h later. New outgrowth of neurites in these cultures was clearly less extensive than in those transferred to colchicine-free medium after 6 h of treatment. The advantage of this longer treatment, however, was that the continued retraction of neurites between 6 and 12 h after addition of colchicine yielded, in the recovered cultures, a population of neurites which were primarily the result of *de novo* growth. This simplified the interpretation of the fine structure of neurites fixed after recovery since the study did not involve observation of individual living neurons. Control cultures were fixed at 26 and 62 h of incubation.

Since the block faces selected for sectioning were approximately centered around cell bodies, most segments of neurites examined were located within 100  $\mu\text{m}$  of the cell body of origin. In some cases, however, neurites of neurons located several hundred  $\mu\text{m}$  outside of the selected areas were examined. These segments did not differ appreciably from the others in density of microtubules, filaments, or other constituents.

The fine structural changes in neurons both with and without neurites at 6 or 12 h after addition of colchicine were easily characterized (Figs. 2-4) although individual neurons varied widely in extent of change. The density of microtubules was lower in colchicine-treated neurons than in controls. This reduction was most clearly observed in the neurites. Disassembly was, however, incomplete, and all neurons observed contained some microtubules.

The most striking change was the appearance in the cell bodies (Fig. 2) and neurites (Fig. 3) of large numbers of 70-100- $\text{\AA}$  thick filaments indistinguishable from normal neurofilaments. These filaments often occupied discrete areas of the cytoplasm (Fig. 2) from which other organelles were virtually excluded. Some of the neurites remaining after 6-12-h colchicine treatment had a beaded form. A series of sections through

such a bead (Fig. 4) revealed the presence of a central bundle of filaments surrounded by vesicular elements which were not in the usual alignment with the long axis of the neurite. The separation of fibrous and vesicular organelles seen in Fig. 3 may represent an earlier stage of this disarrangement. Such misalignment and accumulation of organelles may have indicated a reduction in proximo-distal axonal flow resulting from colchicine treatment.

#### Recovery after Colchicine Treatment

The cell bodies and neurites (Fig. 5) of neurons fixed 30 h after transfer to colchicine-free medium had densities of microtubules and filaments intermediate between those of the 26-h untreated cultures and those treated with colchicine for 6-12 h. Apparently, microtubules were reassembled during recovery, but many filaments persisted. Neurofilaments also appeared qualitatively to be at higher density in untreated neurons after 62 h of incubation than after 26 h. In both recovered and untreated cultures, in the cell bodies of all intact neurons which had not formed neurites,

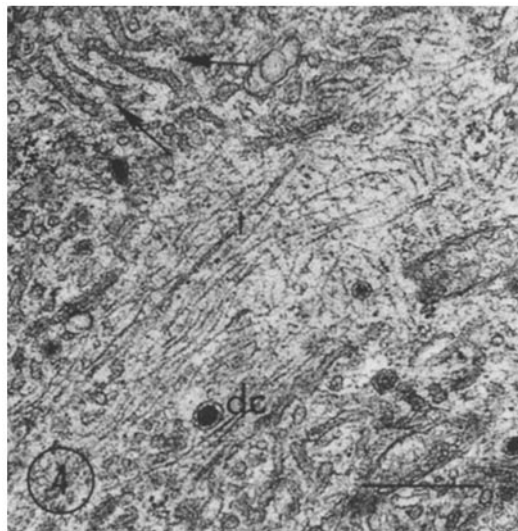


FIGURE 4 Section through the center of a large varicosity (bead) in a short neurite from a 32-h culture after 12-h treatment with 0.05  $\mu\text{g}/\text{ml}$  colchicine. The core of the bead consists of a bundle of 70-100- $\text{\AA}$  thick filaments with a few microtubules (*l*). There is an accumulation of dense-core vesicles (*dc*) and other vesicular organelles, some of which are oriented at right angles to the long axis of the neurite (arrows).  $\times 33,000$ .

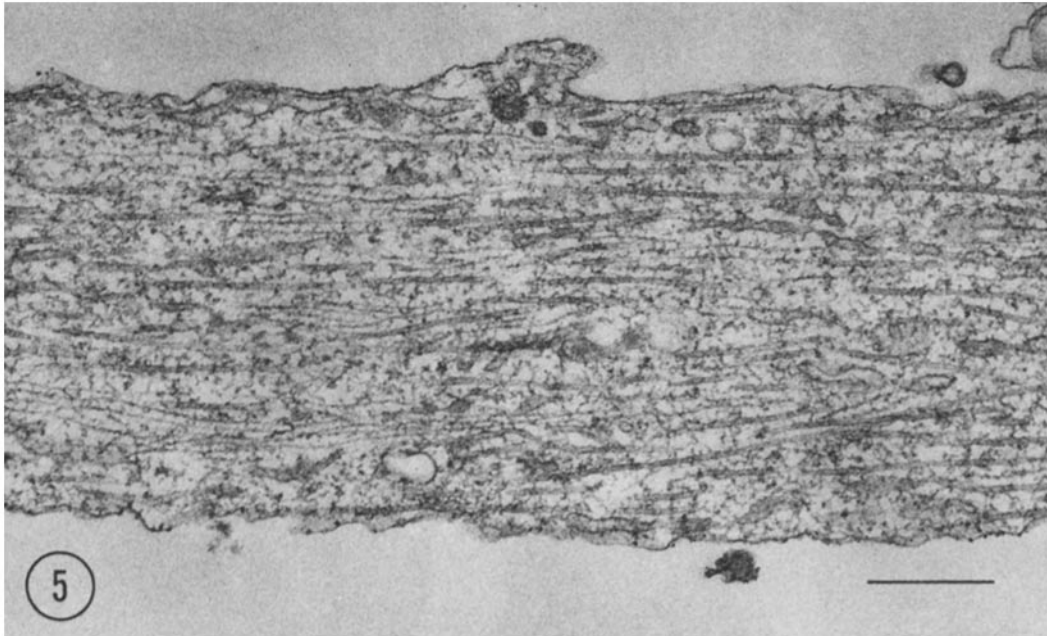


FIGURE 5 Neurite from a 62-h culture after 12-h treatment with  $0.05 \mu\text{g/ml}$  colchicine and 30-h recovery in colchicine-free medium. Microtubules are as abundant as in the controls. There are more 70-100-Å thick filaments than in the controls, but fewer than in the 6-h colchicine-treated neurites. The distribution of organelles is once again homogeneous.  $\times 34,000$ .

there was a striking accumulation of microtubules, but not of filaments, in the central cytoplasm (Fig. 6). This suggested that the overall rate of synthesis of microtubule subunits during and after colchicine treatment was similar to that of the controls.

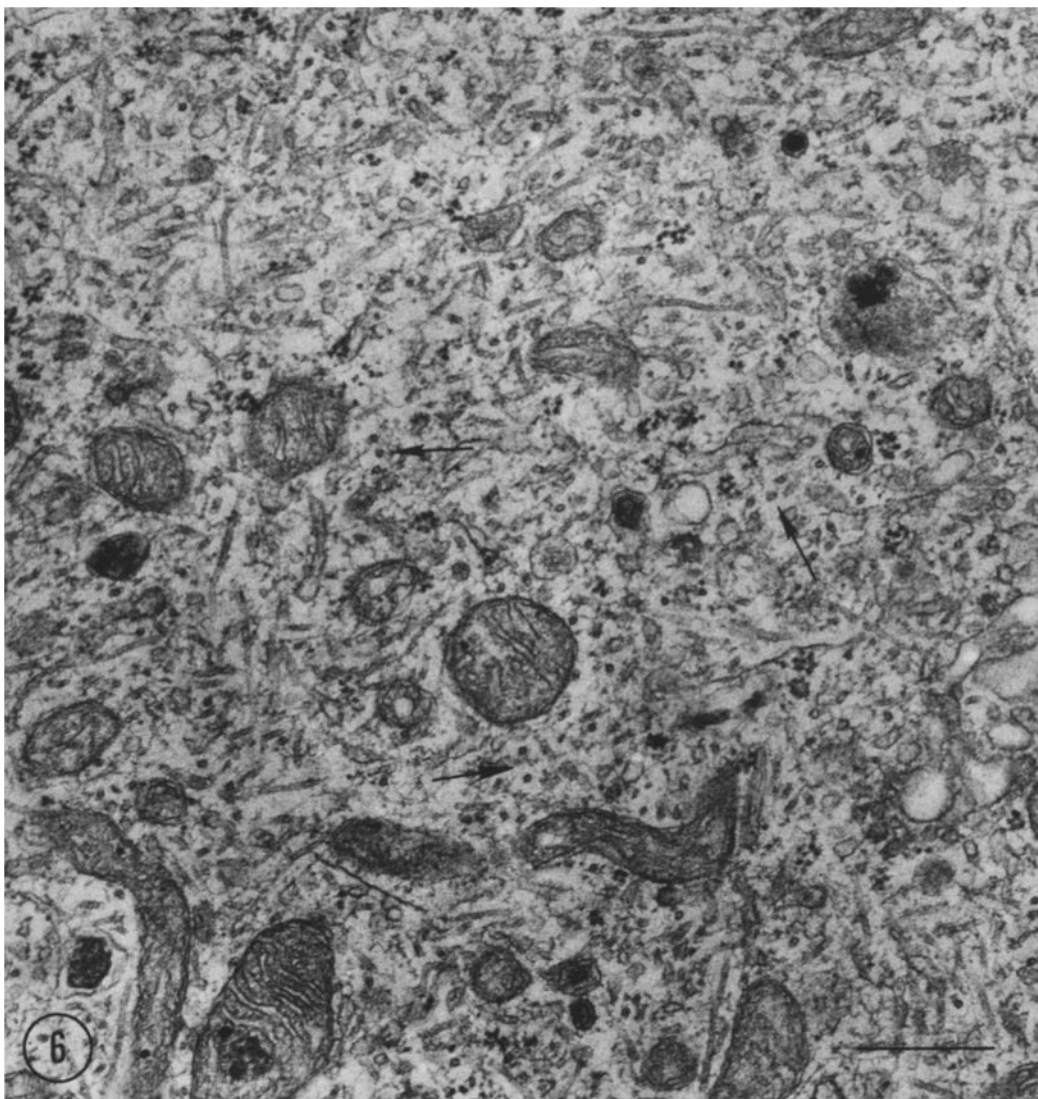
#### *Quantitation of Densities of Microtubules and Filaments in Neurites*

Quantitative estimates of the density of microtubules and 70-100-Å thick filaments in neurites of control, colchicine-treated, and colchicine-treated and recovered neurons were made, and were found to be in agreement with the qualitative observations described above. These results are shown in Table I. Treatment with  $0.05 \mu\text{g/ml}$  colchicine for 6 h caused a 42% reduction in density of microtubules in the remaining neurites and a 97% increase in density of filaments. After recovery from colchicine treatment, there was a 31% increase in density of microtubules and a 14% decrease in density of filaments in the newly formed and the few residual neurites as compared to the colchicine-treated neurites. These latter

changes were not statistically significant (U test) but were in the direction of a return to control densities. Clearly, as  $0.05 \mu\text{g/ml}$  colchicine inhibited neurite elongation and caused retraction, it interfered with the assembly of microtubules and was responsible for their partial disassembly. Since the neurite population examined after recovery represented primarily *de novo* growth and the density of microtubules remained the same or increased slightly, microtubule assembly must have proceeded after colchicine withdrawal, although the average density of microtubules required for this growth was apparently less than that found in control neurites.

#### DISCUSSION

Previous investigators have demonstrated colchicine effects on neurite growth and maintenance (Hoffman, 1952; Yamada et al., 1970) and on the microtubules and neurofilaments of neurons (Wisniewski et al., 1968). However, these studies have offered no temporal correlation between the two types of effect, and have involved concentrations of colchicine at least twofold that neces-



**FIGURE 6** Cell body of a neuron from a 62-h culture after 12-h treatment with 0.05  $\mu\text{g}/\text{ml}$  colchicine and 30-h recovery in colchicine-free medium. This neuron did not form a neurite during recovery. The cytoplasm contains many microtubules, seen in both longitudinal and cross(arrow) section.  $\times 39,000$ .

sary for reversible inhibition of neurite elongation. The present study has demonstrated that colchicine, at a concentration sufficient to cause both reversible inhibition of neurite elongation and reversible neurite retraction within 6 h, can, within the same time span, interrupt assembly of microtubules and promote their partial disassembly. It has also shown that renewed neurite outgrowth after colchicine withdrawal involves the assembly of microtubules. These results are consistent

with the hypothesis that the assembly of microtubules is essential for neurite growth and maintenance.

It is of interest that neurites grown after colchicine treatment contained fewer microtubules than the neurites of untreated neurons. Some continued retardation of microtubule assembly might have been expected, due to the presence of bound colchicine. This result may also indicate that the number of microtubules present in

TABLE I

*Density of Microtubules and Filaments in Neurites of Control and Colchicine-Treated Neurons\**

Treatment	Microtubules/cm‡	Filaments/cm‡
Control	1.30 ± 0.08 (13)	0.61 ± 0.11 (13)
Colchicine (0.05 µg/ml)	0.75 ± 0.12§ (26)	1.20 ± 0.15§ (26)
Colchicine (0.05 µg/ml for 12 h, recovery for 30 h)	0.98 ± 0.08§ (14)	1.03 ± 0.12§ (14)

\* Colchicine was added to the cultures at 20 h after explantation. Treated cultures were fixed after 6-h colchicine treatment or transferred to colchicine-free medium for recovery after 12-h colchicine treatment. Controls were fixed at 26 and 62 h after explantation.

‡ As measured in micrographs printed at 35,000 ×; ± standard error of the mean from 13 to 26 micrographs. The number of micrographs used is shown in parentheses.

§ Significantly different from the controls (0.05 significance level, U test). Microtubule and filament densities in the recovered neurites were not significantly different from those of the colchicine-treated neurites by the same test.

embryonic neurites is greater than that needed for outgrowth. It is possible as well that the 70–100-Å thick filaments which persist after colchicine treatment in some way take the place of microtubules.

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