# COLCHICINE INHIBITION OF NERVE FIBER FORMATION IN VITRO

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ABSTRACT

Inhibition of nerve fiber (neurite) formation by colchicine and Colcemid was studied in monolayer cultures of dissociated spinal ganglia of the chick. Replica cultures were fixed after appropriate incubation and alkaloid treatment. Quantitative estimates of the mean total neurite length per neuron (MNL) were made by use of camera lucida tracing. MNL values plotted against time of incubation gave control curves with an initial lag period, a phase of rapid increase, and a final phase in which MNL increase was retarded. Colchicine at 0.01–0.05 $\mu$g/ml ($2.4 \times 10^{-8}$–$1.2 \times 10^{-7}$ M) caused reversible, concentration dependent, inhibition of the increase in MNL when applied during the lag period or phase of rapid increase. At the highest concentration there was a net decrease in MNL. The effect of Colcemid at 0.05 $\mu$g/ml was similar to that of colchicine, but more rapidly reversible. In most experiments there was no loss of neurons during the period of inhibition of MNL increase by colchicine or Colcemid. Therefore selective destruction of neurons was not involved in the inhibition of neurite growth. Prolonged incubation after treatment with the highest concentration used resulted in a 50% loss of neurons, in part through detachment of viable cells. Quantitative radioautography of the alkaloid-treated neurons with leucine-$^{14}$C indicated little or no inhibition of incorporation into protein during inhibition of MNL increase. The results strongly suggest that inhibition of neurite growth involves a specific effect of colchicine, presumably the disruption of microtubules. They are thus consistent with the hypothesis that the polymerization of microtubules is essential to the formation of nerve fibers.

## INTRODUCTION

The predominant linear components of developing vertebrate nerve fibers are the neurotubules (Peters and Vaughn, 1967; Lyser, 1968; Tennyson, 1970) which appear to be homologous to the widely found cytoplasmic microtubules of other cell types (Gonatas and Robbins, 1964). There is a large body of evidence indicating that microtubules act as supporting structural elements or as mediators of transport in a variety of cell types. For example, in the heliozoan *Echinosphaerium* the long axopodia are rapidly withdrawn upon treatment with agents which specifically disrupt the central axonemal bundle of microtubules (Tilney, 1968). This is good evidence for the proposed structural role. A role in transport is suggested by the finding by Holmes and Choppin (1968) that the migration of nuclei in virus-induced syncytia of cultured hamster cells is prevented by colchicine treatment. Untreated syncytia possess microtubules which are aligned parallel to the migrating rows of nuclei; these are

absent in the colchicine-treated syncytia. Similarly, microtubules have been implicated in the migration of pigment granules of melanophores (Bikle et al., 1966; Wikswo and Novales, 1969).

It would seem likely, by analogy with these other microtubular systems, that microtubules play a role either in the development and maintenance of the elongate shape of nerve processes (Porter, 1966), or in axonal flow (Schmitt and Samson, 1968), or possibly in both. In a few studies microtubule-disruptive agents have been shown to produce effects on nerve fibers. Goldring and Landau (1961) induced reversible beading of growing nerve fibers in culture by application of high hydrostatic pressure. Angevine (1957) demonstrated the destruction of hamster nerves by perfusion with high concentrations of colchicine. Kreutzberg (1969) used colchicine to interrupt axonal flow in vivo. Apropos to the present study was Hoffman's (1952) demonstration of colchicine inhibition of axon sprouting in the severed sciatic nerve of the rat.

A growing literature indicates that colchicine is bound specifically by microtubule protein in a wide variety of tissues. Investigations on the relationship between mitotic arrest and the binding of colchicine to cellular protein (Taylor, 1965; Wilson and Friedkin, 1967) and on the identity and properties of the colchicine-binding protein (Borisy and Taylor, 1967 *a*, *b*; Shelanski and Taylor, 1967, 1968) have led to the conclusion that the primary action of colchicine is to bind noncovalently to the 6S protein subunits of the microtubules, thereby preventing assembly of the microtubules and creating equilibrium conditions favorable to subunit dissociation.

The 6S colchicine-binding protein is very abundant in vertebrate nervous tissue (Weisenberg et al., 1968; Olmsted et al., 1970). Neurotubules of motor neurons (Wisniewski et al., 1968) and spinal ganglion neurons (Bunge and Bunge, 1968) disappear during colchicine treatment. In all probability, then, the neurotubules are made of 6S colchicine-binding protein and can be expected to behave like other cytoplasmic microtubules.

If the polymerization of neurotubules is indeed essential for the formation of nerve fibers, treatment with colchicine should inhibit this formation. Depending on the stability of the neurotubules, colchicine might also be expected to cause the retraction of nerve fibers which have already developed. Hoffman (1952) demonstrated inhibition of nerve fiber sprouting in vivo. The present report deals with the ability of colchicine and Colcemid to inhibit nerve fiber sprouting and elongation and to cause retraction of nerve fibers in cultures of dissociated spinal ganglia, with the dynamics of these effects, and with their reversibility.

## MATERIALS AND METHODS

For convenience both neurons and neuroblasts, often indistinguishable after dissociation in trypsin, are called neurons.

### *Cell Culture*

Dorsal root ganglia from the sacrolumbar region of 11-day old White Leghorn chick embryos (Hamburger and Hamilton, 1951, stage 37) were dissected out under Tyrode's solution. One to four sets of dorsal root ganglia No. 26–30 (Watterson, 1949), easily identified by their location and their connection to the sciatic nerve, were used exclusively. In accordance with the method of Moscona (1961), the ganglia were washed once with Tyrode's solution and 3 times with calcium-magnesium-free Tyrode's solution (CMF), then preincubated for 10 min in CMF at 38°C under water-saturated 5% $CO_2$ and 95% air ($CO_2$-air). The CMF was replaced with 1.5 ml of a freshly prepared solution of trypsin, 25,000 NF units/ml (Tryptar, supplied by Armour Pharmaceutical Co., Chicago, Ill.). Ganglia were incubated in this solution at 38°C under $CO_2$-air for 30 min, the minimum time for almost complete dissociation into single cells. The trypsinized ganglia were washed twice with CMF and once with culture medium. The ganglia were transferred to 1.2 ml of fresh culture medium and disrupted by 20 vigorous passages through the reduced bore (about 0.7 mm) of a siliconized Pasteur pipette, excluding air from the pipette to avoid frothing.

After a hemocytometer cell count of the suspension, 0.3–1.0 ml was added to a 125 ml Erlenmeyer flask containing 12–40 ml of culture medium preequilibrated with $CO_2$-air to give about 12,000 cells/ml. This figure included all cell types in the ganglia. The culture medium contained 78% Eagle's basal medium, 50 units per ml each of penicillin and streptomycin, 2 mM L-glutamine (all from Microbiological Associates, Inc., Bethesda, Md.), 10% unfiltered horse serum (Colorado Serum Co., Denver, Col.), and 10% 10.5-day old chick embryo extract. The extract was prepared by homogenizing the embryos in 3 vol of Tyrode's solution and removing the larger particulates by sedimentation in a clinical centrifuge. The cell suspension was added in 1.8 ml portions to 35-mm plastic tissue culture dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) containing glass cover slips previously cleaned in

nitric acid–ethanol, shadowed with carbon, and overcoated with collagen. The collagen solution was prepared essentially as according to Hauschka and Konigsberg (1966). A crude preparation of acid-extracted calf-skin collagen (given to us by Dr. D. Kirk) was reextracted with 0.05% acetic acid, lyophilized, and frozen. A 0.05% solution of the stock collagen was dialysed exhaustively against 0.45 M NaCl at 5°C and then centrifuged in sterile tubes at 105,000 *g* for 45 min to sediment possible contaminating particulates. The sterile supernatant was stored in small portions at −15°C for up to 6 months. 12 hr before culturing, the carbon-shadowed cover slips were placed in the dishes and coated with collagen solution by pipetting and removing the excess. The solution gelled upon incubation overnight at 38°C in a moist chamber. Before use, the collagen-coated cover slips were washed 3 times with glass-distilled water and once with culture medium. 6 drops of medium were left in the dishes which then were equilibrated with $CO_2$-air and kept at 38°C, ready to receive the cell suspension. The completed cultures were incubated in a sealed moist chamber with $CO_2$-air at 38°C.

### *Fixation and Staining*

For morphological studies the cultures were fixed in 10% buffered formalin, pH 7.3. A few cultures were stained by a modification of Richardson's reduced silver method for nerve fibers (Sevier and Munger, 1965).

### *Neurite Growth Assay*

Cultures were fixed as described above, rinsed with water, dehydrated in an ethanol series, cleared in xylene, and mounted in Permount (Fisher Scientific Co., Pittsburgh, Pa.) for examination by phase microscopy. At least two of the three 15-mm round cover slips from each dish were scanned at × 400 magnification. All intact neurons were counted whose cell bodies fell within an evenly spaced series of horizontal strips, the vertical component of which (approximately 150 μ) was defined by the diameter of the ocular field. Enough strips were taken to cover from one-half to one-eighth of the total area of the cover slips. Sample sizes were between 100 and 300 neurons per single point. When one of the neurons counted had nerve fibers (neurites), these were traced by use of a Wild camera lucida (Wild Heerbrugg Instruments, Inc., Farmingdale, N.Y.), an attachment which permits unhampered binocular viewing and accurate drawing of the specimen. The neurites were completely traced, even when they extended outside of the strip being scanned. To complete the assay we measured the neurite drawings with a map measurer, summed the measured lengths, and divided this sum by the neuron count (including all neurons, with or without neurites), thus obtaining a value equal to the mean combined neurite length for a neuron in the culture. This value is hereafter referred to as the MNL. It was used in preference to one based only on the population of neurons with neurites because the frequency of neurons with neurites changed continuously during the initial outgrowth. The MNLs were then plotted against time elapsed from explanation to give the curves presented. The neurites of more than half the neurons scored could be traced unambiguously. In the ambiguous cases, which were especially frequent in the later stages of neurite growth when intercellular contact was common, certain decisions were made on the basis of objective criteria. These criteria were derived from many observations of individual neurons and have been described in detail elsewhere (Daniels, 1970). In general, the decisions tended to underestimate neurite length.

### *Radioautography*

Radioautography was used to study the relative rates of protein synthesis, as incorporation of amino acid-$^{14}C$ into insoluble protein, in control and inhibitor-treated neurons. Leucine-$^{14}C$, 160–310 mCi/mmole, (Schwarz Bio Research Inc., Orangeburg, N.Y.) was added to each culture to give 1 μCi/ml. $^{14}C$ was used instead of $^{3}H$, since it was thought that the stronger beta particle would provide a label representative of the deeper portions of the cell. After appropriate labeling periods the cultures were chilled on ice, washed with chilled Eagle's medium, and processed according to one of the two following schedules:

(*a*) Fixation for 1 hr at 4°C in 10% phosphate-buffered formalin, pH 7.3, followed by washing with water and 5% trichloracetic acid (TCA) at 4°C, then rinsing in three changes of 70% ethanol for 24 hr to remove traces of TCA.

(*b*) Fixation for 5 min in 70% ethanol followed by 95% ethanol for 15 min and two changes of 3:1 ethanol–acetic acid for 5 min. The fixed cultures were washed 15 min in hot (90°C) 5% TCA to remove nucleic acids and 15 min in hot 2:1 chloroform-methanol, then rinsed in 70% ethanol as in (*a*) (Prescott, 1963).

All cultures were dehydrated in an ethanol series, air dried, mounted cell side up on gelatin-coated glass slides (Caro, 1964), and coated with Kodak AR-10 radioautographic stripping film. Because its relatively even emulsion thickness was more suitable for quantitative radioautography, stripping film was used instead of liquid emulsion. Variation in the thickness of the liquid emulsion was found to cause

large variations in grain counts with $^{14}C$. After 2–7 days of exposure the radioautographs were developed 2 or 3 min at 18°C in Kodak D19 developer. The radioautographs were mounted in immersion oil for examination and grain counting. Preparations were scanned at × 400 with a phase microscope. Grains were counted within a 30 μ diameter circle centered on the perikaryon of each neuron. The number of grains associated with neurites was negligible with the 30 min labeling period used in our experiments. Background grains were counted within a 30 μ diameter circle about 60 μ from the neuron.

### *Treatment with Inhibitors*

Colchicine (ICN Nutritional Biochemicals Div., International Chemical and Nuclear Corp., Cleveland, Ohio) and Colcemid (Grand Island Biological Co., Grand Island, N.Y.) were made up at 0.01 mg/ml aqueous and stored in small lots frozen in the dark at −15°C. There was no apparent loss of activity in the 22 months of successive experiments with colchicine and the 11 months with Colcemid. Before use, the stocks were diluted appropriately with culture medium and 0.10 ml was added to each culture to make 0.05, 0.02, or 0.01 μg/ml. The maximum dilution of the culture medium by this method was 0.5%. For investigations of recovery from treatment the cover slips were rinsed once in place with alkaloid-free medium and transferred to new culture dishes with 2 ml of fresh medium for continued incubation. In experiments concerning protein synthesis, cycloheximide (ICN Nutritional Biochemicals Div.) dissolved in Tyrode's solution at 160 μg/ml was added to the cultures to make 2.0 μg/ml.

## RESULTS

### *Light Microscope Morphology of the Cultured Neurons*

The general morphology of the dissociated neurons was similar to that reported by Nakai (1956) for those of ganglia dissociated in proteinase A. The neurons had spheroidal cell bodies, 15–35 μ in diameter (Figs. 1–3). Neurites, when present, numbered from one to five per neuron, most commonly two (Figs. 2–4). They were generally uniform in thickness (0.5–2.0 μ) and were smooth surfaced except for occasional varicosities. The neurites usually followed straight paths interrupted by branches or bends at obtuse angles. They terminated, after a variable degree of branching, in expansions showing a wide range of form (Figs. 2–3). The neurites were clearly stained by Richardson's reduced silver method (Fig. 5).

### *Dynamics of Normal Outgrowth*

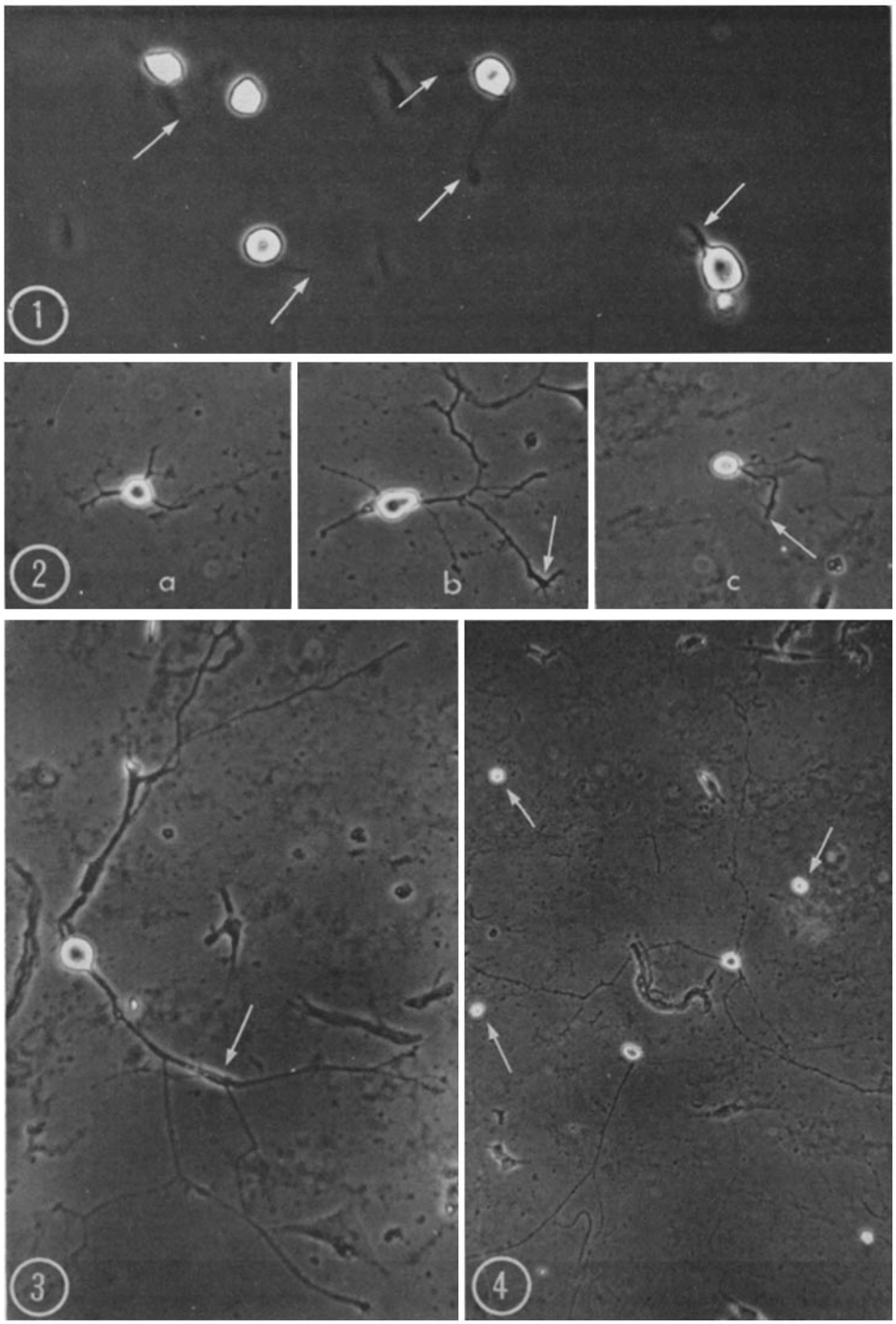
In the initial suspension a majority of the neurons had one or two stumps from neurites which were removed during dissociation of the ganglia (Fig. 1), most of which disappeared in the first few hours of incubation. At 6 hr only a few neurons had sprouted neurites. In the more rapidly growing cultures about 10% of the neurons had neurites after 12 hr. This frequency increased to about 20% at 17 hr, 30% at 20 hr, 50% at 26 hr, and 60% at 33 hr where it generally stabilized. In more slowly growing cultures the corresponding frequencies were about 7% at 12 hr, 10% at 17 hr, 20% at 20 hr, 30% at 26 hr, and 40% at 33 hr. Neurites elongated at an average rate of about 0.2 mm/day. This elongation, plus the formation of new processes, yielded increases in the MNL which showed a lag during the first 12–24 hr of incubation, followed by a phase of relatively rapid increase in MNL which persisted about 12 hr before the values began to level off (Fig. 6).

### *Variability of MNL Values*

All the cultures used in a given experiment were plated from the same cell suspension, and MNL values from duplicate cultures were in good agreement, variation being from negligible to 50% (Fig. 7). However, there was wide variability in the rate of MNL increase between the untreated cultures from different experiments (Fig. 6). This was reduced by careful control of the embryo stage, specific ganglia, and freshness of the trypsin solution used in preparing the cultures. Since we did not succeed in determining all the sources of this variability, however, we used a control series of cultures in each experiment and repeated most experiments one or more times.

### *The Effects of Colchicine and Colcemid on Neurite Elongation and Sprouting*

Inhibition of neurite outgrowth by colchicine or or Colcemid was best observed at 20–24 hr after explantation, at the beginning of the phase of rapid increase of MNL. The addition of 0.05 μg/ml of colchicine at 20 hr after explantation caused partial inhibition of the increase in MNL after 3 hr, followed by a sharp decrease in MNL (Fig. 7 *a*). Inhibition was reversed, after a 16 hr lag, when the cultures were transferred to colchicine-free medium after 6 hr treatment (Fig. 7 *a*). A similar




FIGURES 1–4 Phase photomicrographs of living cultures at various times of incubation. Fig. 1. Initial cell suspension. Some neurons have stumps of neurites (arrows). × 340. Fig. 2 *a–c*. 12–13 hr. The early outgrowth of neurites is often complex. Notice the variation in form and complexity of the growth cones (arrows). × 170. Fig. 3. 24 hr. This neuron has two branched neurites and an associated Schwann cell (arrow). × 190. Fig. 4. 48 hr. These two neurons show extensive neurite elongation. Apparent irregularities in the neurites are due to adhering debris. Notice the neurons without neurites (arrows). × 110.

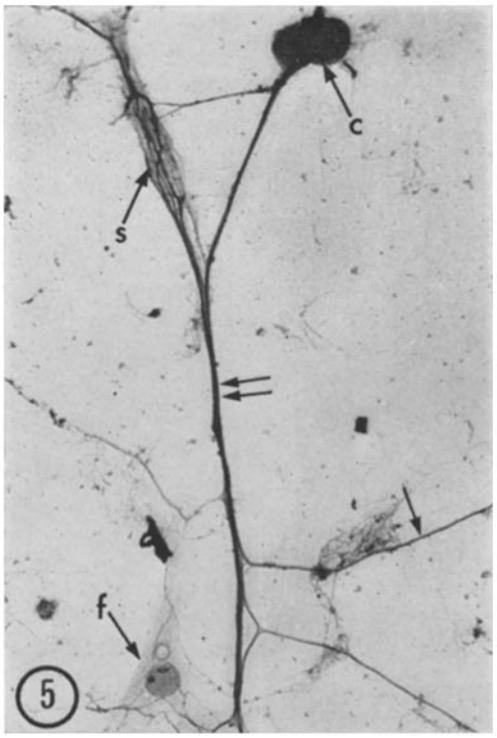



FIGURE 5 Photomicrograph of a silver-stained, 48 hr culture. Single (arrow) and multiple (double arrow) neurites and the cell bodies (*c*) are heavily stained; "fibroblasts" (*f*) and the Schwann or spindle cell (*s*) are lightly stained. × 1380.

effect was obtained with 0.05 μg/ml of Colcemid (Fig. 7 *b*). There was a decrease in MNL between 20 and 26 hr, and therefore marked net inhibition of neurite elongation. The MNL values increased slightly in the next 6 hr but there was no recovery in the presence of Colcemid. Recovery after transfer to Colcemid-free medium was rapid, with no significant lag (Fig. 7 *b*), in contrast to the 16 hr lag in recovery from colchicine (Fig. 7 *a*). Recovery from treatment with either alkaloid persisted for at least 5 days.

Reversible inhibition of the increase in MNL was also observed in cultures treated with 0.01–0.02 μg/ml of colchicine. However, in order to obtain marked effects at these lower concentrations it was necessary to use earlier or more prolonged treatment, for example, addition of colchicine at 12 hr after explantation, or addition of colchicine at 24 hr after explantation, followed by 12 hr treatment. The comparison between these results and those with 0.05 μg/ml, in which case treatment beginning at 20 hr after explantation caused marked inhibition within 6 hr, indicates concentration dependence of the inhibition of neurite elongation between 0.01 and 0.05 μg/ml.

MNL, as graphed in Fig. 7 a, is a function of both the total neurite length of each neuron and the number of neurons with neurites. Both of these factors were involved in the colchicine effects. Not only were neurites prevented from elongating and induced to become shorter, but in some cases their

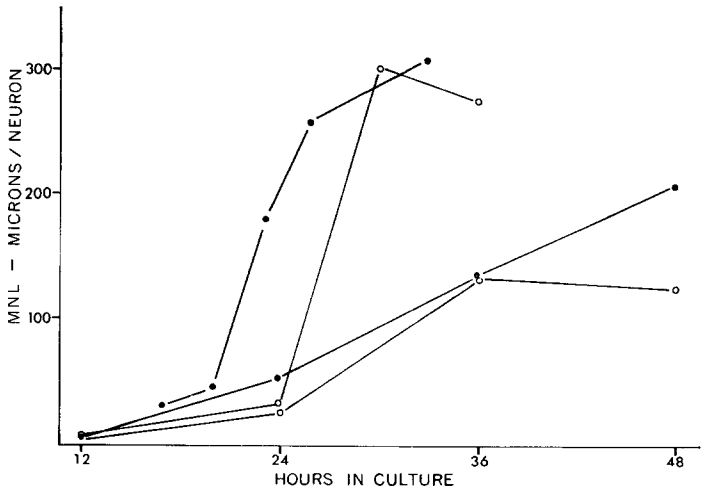



FIGURE 6 Changes in MNL in four different experiments, all untreated controls, illustrating the variation in neurite growth rates encountered in different preparations.

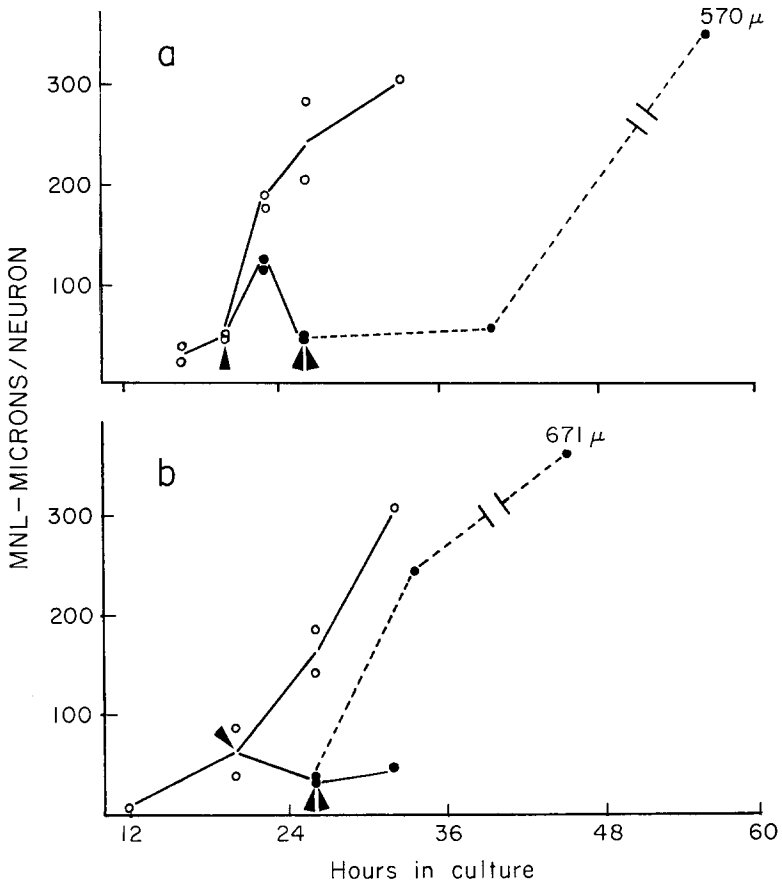



FIGURE 7 Changes in MNL in control (——○——) and colchicine- or Colcemid-treated (——●——) cultures. Single arrows indicate the time of alkaloid addition; double arrows indicate the time of alkaloid removal. Dashed lines (--●--) indicate recovery. (*a*) 0.05 μg/ml of colchicine was added at 20 hr and removed from some cultures at 26 hr. (*b*) 0.05 μg/ml of Colcemid was added at 20 hr and removed from some cultures at 26 hr.

initial sprouting was effectively inhibited and those neurites present were completely lost. To illustrate this point, the distributions of total neurite length per cell for the data points from Fig. 7 *a* are shown in Fig. 8.

In interpreting the foregoing results, it was necessary to take into consideration any losses of neurons due to drug treatment, with reference to the possible toxicity of colchicine and Colcemid and to the possibility that the observed reduction of average neurite length was partly the result of selective destruction of those neurons most active in neurite elongation. Neuron counts of treated and control cultures were made to estimate the loss of neurons under various conditions. The results of these counts are summarized in Table I. The lower concentrations of colchicine used, 0.01–0.02 μg/ml, caused no detectable loss of neurons when added to cultures at 20 or 24 hr after explantation. However, addition of the same concentrations at 12 hr after explantation caused significant losses after 12 hr or more of treatment. Application of 0.05 μg/ml colchicine or Colcemid at 20 hr after explantation led to the loss of about 50% of the neurons. At least one-fourth of these losses could be attributed to detachment of viable neurons from the substrate when the cultures were being transferred to alkaloid-free medium after 6 hr treatment. This was determined by counting neurons in cultures made by replating the medium used to rinse the cover slips before transfer to new culture dishes. In four out of five experiments, the losses of neurons caused by treatment with 0.05 μg/ml of colchicine or Colcemid occurred after neu-

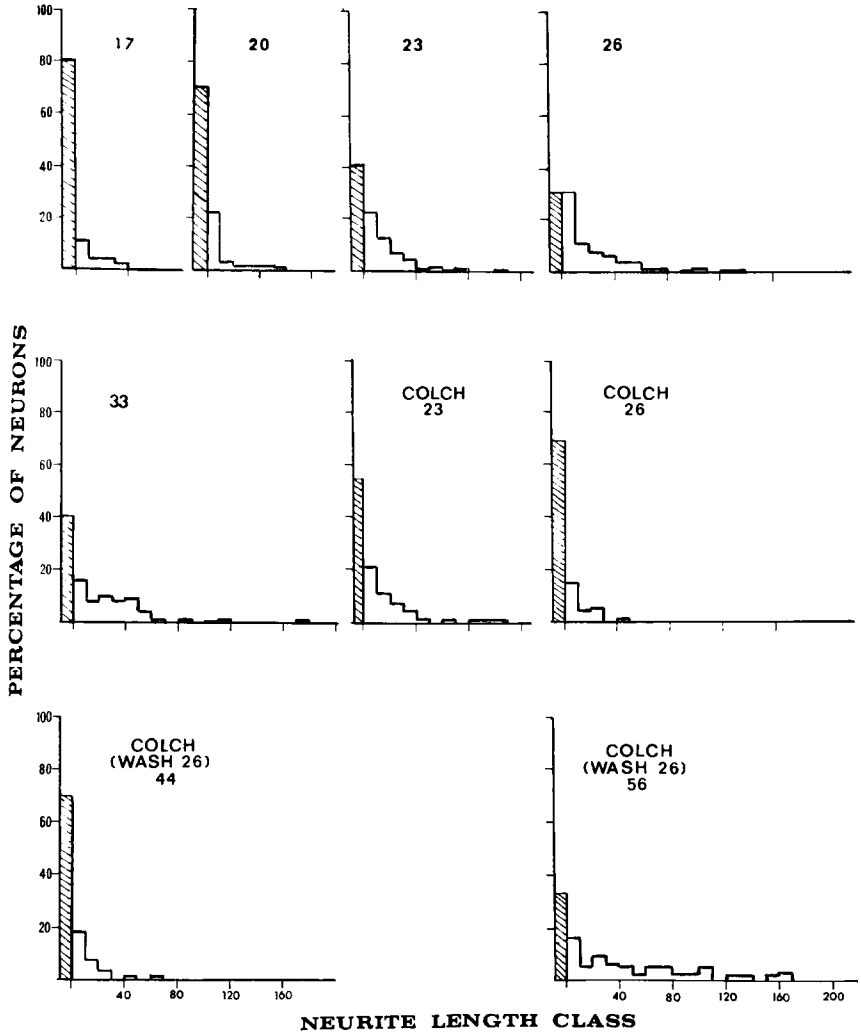



FIGURE 8 Frequency distributions of combined neurite lengths of individual neurons from the data points of the experiment shown in Fig. 7 *a*. The combined neurite lengths of all neurons in the sample were grouped into classes which were plotted against their frequency of occurrence. The blocked-in, far left-hand bar represents the zero-length class. All distributions are plotted to the same scale (10 horizontal scale units = 164 $\mu$ in real length). Numbers near the upper left corner of each graph represent hours of incubation at fixation time corresponding to Fig. 7 *a*. (*WASH* . . .) indicates the time of colchicine removal from the medium.

rite outgrowth inhibition was complete. Therefore it was concluded that neither selective detachment nor differential cell death could account for the colchicine or Colcemid effects on MNL such as graphed in Fig. 7.

## *Protein Synthesis*

The experimental results in other cellular systems have supported the hypothesis that colchicine and Colcemid act by binding to the protein subunits of the microtubules, thus preventing their assembly. However, it is possible that the alkaloids inhibited neurite elongation by interfering with the synthesis of the protein subunits of the microtubules or of other structures rather than, or in addition to, interfering with their assembly. It is also possible that these alkaloids might have other effects on cell metabolism reflected in a decrease in the rate of protein synthesis. To test these possibilities we estimated rates of protein

TABLE I

*Reduction in Intact Neuron Count due to Colchicine or Colcemid Treatment*

| Alkaloid used and concentration | Time alkaloid added (hr after explanation) | Time cultures fixed (hr after explanation) | Number of experiments | Reduction in neuron count over controls* |
|---|---|---|---|---|
| *μg/ml* | | | | % |
| Colchicine | | | | |
| 0.01–0.02 | 12 | 36–60 | 4 | 29–63 |
| 0.01–0.02 | 20–24 | 23–36 | 3 | Insignificant |
| Colchicine | | | | |
| 0.05 | 20 | 23–26 | 2 | Insignificant |
| 0.05 | 20 | 26 | 1 | 32 |
| 0.05 | 20 | 32–60 | 3 | 19–50 |
| Colcemid | | | | |
| 0.05 | 20 | 26–32 | 2 | Insignificant |
| 0.05 | 20 | 45–60 | 2 | 18–57 |

* Neurons were counted in the same preparations used to obtain MNL values as described in the text. Differences in numbers of neurons between alkaloid-treated and control cultures were considered insignificant if they were less than twice the standard error of the mean for three to nine control cultures fixed between 12 and 48 hr after explantation.

synthesis, as incorporation of leucine-$^{14}$C into insoluble protein measured radioautographically, in treated and control neurons. Two tests were used to demonstrate that we were measuring incorporation into protein as opposed to nonspecific binding of amino acid. First, grain counts from both acid-alcohol– and formalin-fixed cultures taken at 5, 15, 30, and 60 min after addition of leucine-$^{14}$C showed a steady increase in incorporation from near background (Fig. 9). Grain counts at 30 min were about 10% higher for acid-alcohol than for formalin fixation in cultures labeled and fixed simultaneously. Second, we fixed cultures with formalin after 30 min preincubation with 2 μg/ml cycloheximide followed by 30 min incubation with cycloheximide and leucine-$^{14}$C. Grain counts on cycloheximide-treated neurons equalled 6% of the control counts. We therefore concluded that the incorporation of leucine adequately reflected protein synthesis.

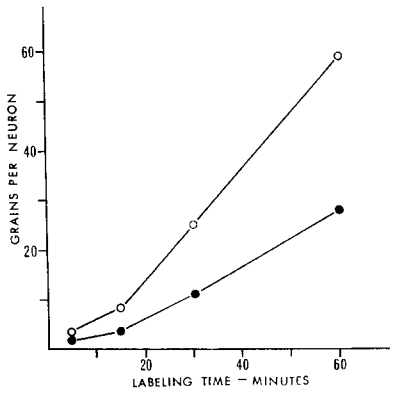



FIGURE 9 Grain counts of leucine-$^{14}$C radioautographs. Isotope was added at zero time and cultures were fixed either with formalin (○) or with acetic acid–ethanol (●) at the indicated time points. The 50% difference in absolute grain counts between the acetic acid–ethanol- and formalin-fixed cultures is mainly due to a corresponding difference in specific activity of the leucine-$^{14}$C used in the two experiments.

In each of three experiments (Table II), 0.05 μg/ml of colchicine or Colcemid was added to 20 hr cultures. In experiment No. 1, colchicine-treated cultures were labeled between 23.5 and 24.0 hr. Differences in leucine-$^{14}$C incorporation between treated and control cultures were negligible. In experiment No. 2, colchicine-treated cultures were labeled between 25.5 and 26.0 hr. There was no significant difference in incorporation rates between treated and control cultures, although neurite elongation had been severely inhibited. In experiment No. 3, colchicine-treated cultures were labeled between 23.5 and 24.0 hr or 25.5 and 26.0 hr, and Colcemid-treated cultures were labeled between 25.5 and 26.0 hr.

TABLE II

*Amino Acid-$^{14}C$ Incorporation into Protein in Colchicine- or Colcemid-Treated Neurons**

| Time leucine-$^{14}C$ added (hr after explantation) | Experiment No. | Treatment* | Mean grain counts‡ | | |
|---|---|---|---|---|---|
| | | | Neurons with no neurites | Neurons with $\Sigma$ neurites $< 150\ \mu$ | Neurons with $\Sigma$ neurites $> 150\ \mu$ |
| 23.5 | 1 | Colchicine | 58§ | 59§ | 83§ |
| 23.5 | 1 | Control | 54§ | 69§ | 69§ |
| 23.5 | 3 | Colchicine | 50 ± 1 | 45 ± 3 | 75 ± 4 |
| 23.5 | 3 | Control | 51§ | 48§ | 90§ |
| 25.5 | 2 | Colchicine | 43 ± 11 | 49 ± 3 | 82 ± 6 |
| 25.5 | 2 | Control | 49 ± 3 | 56 ± 1 | 69 ± 8 |
| 25.5 | 3 | Colchicine | 28 ± 1 | 35 ± 6 | 48 ± 2 |
| 25.5 | 3 | Control | 42 ± 1 | 45 ± 3 | 61 ± 3 |
| 25.5 | 3 | Colcemid | 29 ± 1 | 24 ± 2 | 59 ± 6 |

* Colchicine or Colcemid, 0.05 $\mu$g/ml, was added to cultures at 20 hr after explantation; leucine-$^{14}C$ was added to the colchicine- or Colcemid-treated cultures and to control cultures at 23.5 or 25.5 hr after explanation. All cultures were fixed 30 min after the addition of leucine-$^{14}C$, and prepared for radioautography (see Materials and Methods).

‡ Corrected for background, ± standard error of the mean from two to four replicate cultures. Before grain counts were made, the individual neurons were classed according to the total length of their neurites. This prevented statistical error due to the differences in the proportions of the three classes in different cultures, and the higher grain counts in the class of neurons with total neurite lengths over 150 $\mu$.

§ Single cultures.

No effect of colchicine on incorporation was detected at the earlier time period. At the later time period, however, there were decreases in incorporation rates in both colchicine- and Colcemid-treated cultures, from 21 to 47%. In summary, there was no detectable effect on the rate of amino acid incorporation after the first 3.5 hr of treatment with 0.05 $\mu$g/ml of colchicine. At this time, neurite outgrowth was already inhibited (Fig. 7 *a*). After 5.5 hr treatment with colchicine or Colcemid at the same concentration, there was partial inhibition of amino acid incorporation in one of two experiments. The inconsistency of this effect and the absence of detectable inhibition of amino acid incorporation at the earlier time point suggest that effects on protein synthesis are not important in the neurite outgrowth inhibition caused by colchicine.

## DISCUSSION

### *Inhibition of Neurite Elongation*

The present results confirm and extend Hoffman's (1952) report on the inhibition of axon sprouting in the severed sciatic nerve of the rat. First, our results show that the effect occurs directly on the neurons and not indirectly through some action on the surrounding cellular elements. Although Schwann cells and "fibroblasts" are present in our cultures, their degree of association with the neurons during initial outgrowth does not seem sufficient to have a strong effect on neurite sprouting and elongation. Furthermore, we have shown that colchicine or Colcemid treatment can cause a decrease in the length of neurites already present. It is not known to what extent this decrease represented retraction of neurites into the cell body, as opposed to breakdown of the neurites without retraction. Time lapse studies would be required to distinguish between these two phenomena. In any case, the present results are consistent with the hypothesis that the continued polymerization and integrity of the neurotubules are essential to the formation and maintenance of nerve fibers. Yamada et al. (1970) have demonstrated rapid retraction of nerve fibers in a culture system similar to the present one, using 0.1–1.0 $\mu$g/ml of colchicine or Colcemid. They report that the growth cones were not affected during the initial phases of retraction. This would be expected, since microtubules are not major constitutents of the growth cones and are not at all present in the

filopodia, the motile extensions of the growth cone (Tennyson, 1970; Yamada et al., 1970). Fine structural data (Daniels, 1970) indicate that the present results are indeed correlated with effects on the polymerization and integrity of the neurotubules. It is noteworthy that, in terms of their tendency to retract or break down during colchicine treatment, the developing nerve fibers are more stable than such structures as the axopodia of *Echinosphaerium* (Tilney, 1968), but less stable than the cilia of *Tetrahymena* (Rosenbaum and Carlson, 1969).

### *Effective Concentration Range*

Concentration dependence of the inhibitory effect was demonstrated in the 0.05–0.01 μg/ml ($1.2 \times 10^{-7}$–$2.4 \times 10^{-8}$ M) range. This is the same minimum range effective in other cell culture systems. Taylor (1965) reported $5 \times 10^{-8}$ M colchicine as close to the minimum concentration for complete mitotic arrest. In his system the lag time before the beginning of accumulation of blocked metaphases was just over 1 hr with $10^{-7}$ M colchicine. Considering the limited resolution of our system of MNL measurements, the appearance of inhibition as early as 3 hr after addition of $1.2 \times 10^{-7}$ M colchicine is in good agreement with Taylor's results. This suggests that a similar mechanism operates in the two systems. The effective concentrations reported by Bischoff and Holtzer (1968) for myoblasts overlap with the effective range reported here. The higher concentrations of alkaloids needed to inhibit other systems may reflect differences in cellular permeability or metabolism. For example, 4 mg/ml colchicine or 0.5 mg/ml Colcemid was required to block regeneration of cilia in *Tetrahymena* (Rosenbaum and Carlson, 1969).

### *Reversibility*

The reported effect of the alkaloids is reversible for about 50% or more of the neurons. The occurrence of about a 12 hr lag before recovery from colchicine treatment, as opposed to an insignificant lag with Colcemid, is in agreement with the results of Kleinfeld and Sisken (1966) on recovery from Colcemid-induced mitotic arrest in cultured mammalian cells and of Bischoff and Holtzer (1968) on colchicine and Colcemid arrested myoblasts in culture. Taylor (1965) reported the persistence of mitotic arrest for at least 24 hr after 6–8 hr treatment with $10^{-7}$ M colchicine. This was apparently related to persistent binding of the colchicine to the cellular protein. In the above mentioned report of Bischoff and Holtzer, it was also shown that when abnormal "myosacs" were formed by the fusion of myoblasts for several days in the presence of $10^{-8}$ M colchicine, formation of normal myotubes by elongation of these sacs proceeded within 3 hr after the drug was removed. However, when myosacs were formed by disruption of previously formed myotubes, this disruption requiring the presence of $10^{-6}$ M colchicine for 4–24 hr, the lag before recovery was about 2 days and many myosacs failed to recover and subsequently degenerated. Insofar as such comparisons can be made between different systems, partial recovery after treatment for up to 12 hr with $1.2 \times 10^{-7}$ M colchicine (0.05 μg/ml), as reported here, indicates conditions of reversibility similar to those reported by Bischoff and Holtzer.

In the present system, up to about 50% of the neurons were lost due to degeneration and detachment as a result of colchicine or Colcemid treatment and subsequent transfer of the cultures to alkaloid-free medium. It should be kept in mind that about 90% of the neurons in the present culture system degenerate within 2 wk, even without drug treatment. The degeneration occurring with colchicine or Colcemid treatment may represent an acceleration of this process and not a specific effect of these compounds. Colchicine or Colcemid inhibition of neurite elongation always occurred before neuronal degeneration or detachment. Therefore, these losses of neurons cannot explain the results obtained. Nevertheless, the relationship between colchicine inhibition and subsequent degeneration suggests the possibility that sublethal toxicity took part in the inhibition of neurite elongation. There is evidence against this suggestion. The rate of protein synthesis should be a good measure of the state of cellular metabolism and therefore an indicator of possible toxicity. There was little inhibition of protein synthesis due to the presence of colchicine or Colcemid. This occurred inconsistently, and then only when neurite elongation was already severely retarded. At the earlier treatment time examined, when inhibition of elongation had begun, there was no measurable inhibition of protein synthesis. This suggests that metabolic interference was not the primary effect of the alkaloids. Taylor (1965) reported that a low degree of inhibition of RNA and protein synthesis began after

about 20% of the cells had been arrested in mitosis, at which stage in the cell cycle macromolecular synthesis is normally lower, suggesting a secondary effect. Rosenbaum and Carlson (1969) observed no inhibition of protein or RNA synthesis with concentrations of colchicine sufficient to prevent regeneration of cilia in *Tetrahymena*. As mentioned above, Yamada et al. (1970) detected no early changes in the activity of the growth cone in neurons treated with colchicine at 2–20 times the concentration used in the present study, yet the highly active growth cones should be a sensitive indicator of toxicity. Although sublethal toxicity cannot be entirely ruled out as a contributing factor, it is clearly not the cause for the inhibition of neurite elongation. We conclude that the colchicine effects in the present system, as in others, depend on the alkaloid's tendency to disrupt microtubules or prevent their assembly.

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