MICROTUBULES IN THE NERVE FIBERS OF THE TOAD *BUFO ARENARUM* HENSEL

Effect of Low Temperature on the Sciatic Nerve

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Recent studies on cytoplasmic microtubules have provided evidence for regarding them as gel components susceptible to solational weakenings. Microtubule structure in various cell types has been shown to be sensitive to temperature, pH, pressure, and other environmental conditions (Masser and Philpott, 1966; Tilney et al., 1966; Tilney and Porter, 1967). In the present work, the sciatic nerve of the toad was studied to determine whether low temperatures may also affect the integrity of neuronal microtubules. These microtubules were found to be sensitive to temperature and not to be dependent on the perikaryon for re-formation.

MATERIAL AND METHODS

Three abdominal roots of both sciatic nerves of 18 adult specimens of toad *Bufo arenarum* Hensel were used in the present series of experiments.

Control Preparations for the "In Vivo" and the "In Vitro" Experiments

Six adult toads are the material for controls. After demedullation, the abdominal wall was excised; two ligatures were applied on the sciatic nerves (abdominal course) at approximately 1.5 cm from each other. The ligated segments, with both ligatures, were removed and processed as follows. (*a*) Six nerves were immediately fixed at room temperature (control preparations for the in vivo experiments). (*b*) Six

nerves were incubated for 3 hr at room temperature in Ringer's solution, pH 7.1, with 0.2 g/100 ml of glucose added; then they were fixed for electron microscopy (control preparations for the in vitro experiments).

In Vivo Experiments

The specimens prepared for the in vivo experiments were six toads which had been cooled by covering them with cracked ice. On the basis of the work of Roth (1967), the duration of the cold treatment was 2 hr counted from the moment when the cloacal temperature reached 2°C. Then the animals were demedullated in the cold; their right sciatic nerves were removed and immediately were fixed in cold fixative. After suturing, the toads were warmed to 25°C for 1 hr; their left sciatic nerves were then removed and fixed at room temperature.

In Vitro Experiments

For the purpose of assessing the need for neuronal integrity, the sciatic nerves of six toads were removed, according to the technique described for the controls, were isolated in individual Ringer baths, and cooled at 2°C for 2 hrs. The right nerves were then fixed in cold fixative; for comparison the left nerves were warmed to 25°C for 1 hr and then fixed at room temperature.

The fixative used in all the experiments was glutaraldehyde-formaldehyde in cacodylate buffer with 50 mg percent of calcium chloride (Karnovsky, 1965) for 2 hr. After rinsing in cacodylate (to which 50 mg percent calcium chloride was added) the nerves were postfixed for 1 hr in 1% cacodylate-buffered osmium tetroxide, dehydrated in ethanol, and embedded in Epon 812 (Luft, 1961). In order to judge whether the fixation temperature may affect the integrity of the microtubular component, some control and experimental nerves were divided into two segments; one segment was fixed at room temperature, the other was fixed at 2°C. No fine structural differences were found.

In order to avoid the proximity of the cut ends of each nerve, which might have been damaged by the surgical procedure, only the middle third of each 1.5-cm segment was used for study. Sections were made with a Porter-Blum ultramicrotome, stained with uranyl acetate and lead citrate (Venable and Coggeshall, 1965), and observed in a Siemens Elmiskop I electron microscope.

RESULTS

Control Specimens

The control nerves, whether fixed immediately after removal or after 3 hr of incubation at room temperature, showed no significant fine structural differences in their microtubular component; they are, therefore, to be described in one group. Inasmuch as a majority of sciatic nerve fibers is myelinated, it seems paradoxical that most fibers selected for illustration are small, unmyelinated units. The reason for so doing is that most myelinated fibers are so large that they more than fill the field of a single electron micrograph at magnifications useful for identification of microtubules. Both myelinated and unmyelinated fibers appear richly supplied with similar packages of microtubules, oriented paralled to the fiber axis (Fig. 2). It seems clear that the number of tubules differs considerably from fiber to fiber; the averages per square micron are indicated in Fig. 1. The fine structure of the individual microtubules appears consistent with that described for other cell types. The microtubules are about 250 A in diameter, their dense wall being constructed of 6–11 subunits (Fig. 2, inset), as shown for other microtubules in the toad (Rodríguez Echandía et al., 1968). The tubules appear embedded in a cotton-like matrix of moderate density. Intermingled with the microtubules is a variable population of filaments, each 100 A across, and other organelles (Fig. 2).

Cold Treatment

After 2 hr of cooling, the in vivo and the in vitro preparations showed disintegration of the majority of the microtubules (Fig. 3). Ill-defined aggregations of cotton-like material, similar to that embedding the microtubules in control preparations, occur in cross-sections of all fibers (Fig. 3). Such aggregations are assumed to be made up of two components, the matrix which normally surrounds the microtubules and tubule subunits dispersed in it (Fig. 3, lower inset). Fig. 1 shows that just 10% of the microtubuels remains after the 2-hr treatment. Some of these microtubules have the normal fine structure; most, however, are wider than normal, and their subunits are not symmetrically associated (Fig. 3, upper inset). The filaments on the other hand, are apparently cold-resistant (Fig. 3).

Recovering

1 hr after the living animals and the in vitro preparations were warmed to 25°C, the microtubular component appeared completely reformed. It must be emphasized, moreover, that

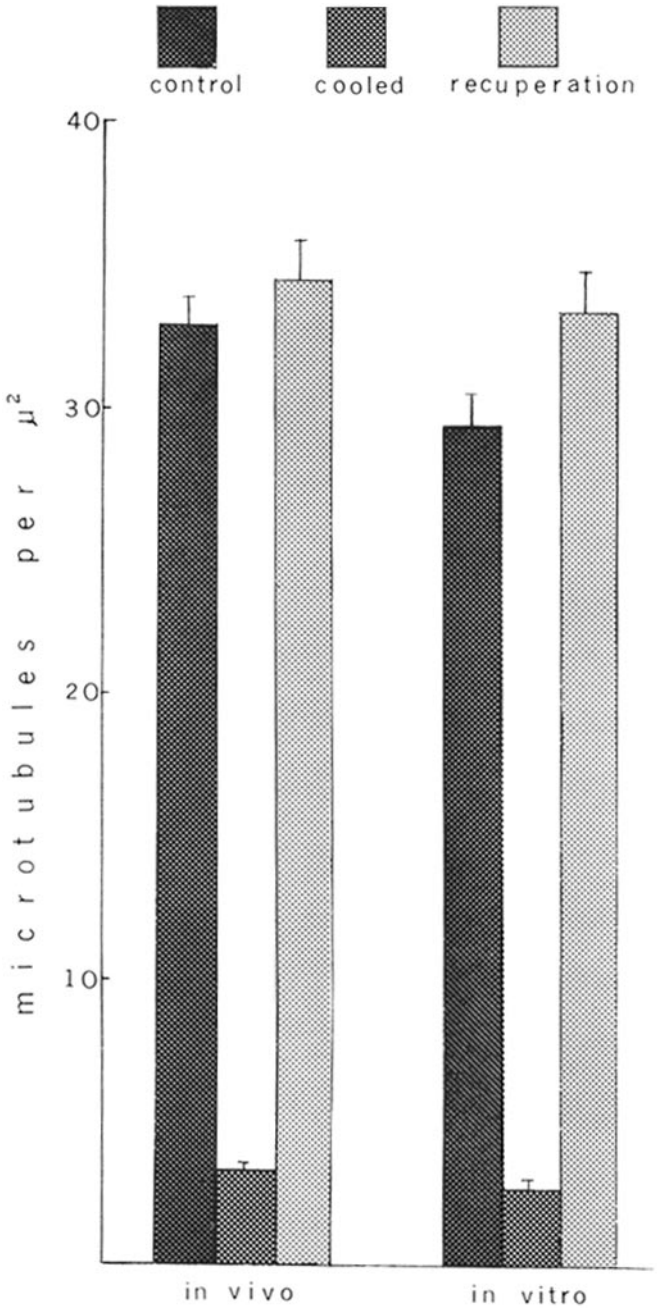

FIGURE 1 The histogram illustrates the number of microtubules per square micron of control, cooled, and recuperated unmyelinated nerve fibers, in vivo and in vitro. Averages were obtained by counting the number of microtubules in 100 μ^2 of cross-sectioned fibers. The electron micrographs selected for counting correspond to sections of six nerves from the six specimens of toad used in each experiment. In all cases the counts were made on topographically comparable sample areas (medial third of each nerve segment). The 90% decrease in the number of microtubules after cold treatment, and the reformation of these microtubules after warming are highly significant. Probability (P) between in vitro control and cooled fibers is <0.001; P between cooled and recuperated fibers is <0.001; P between control and recuperated fibers is <0.07. Probability (P) between in vitro control and cooled nerves is <0.001; P between cooled and recuperated fibers is <0.001; P between control and recuperated fibers is <0.05. Differences between corresponding bars of in vivo and in vitro experiments are not significant.

the shape, size, orientation (Fig. 4), and number of tubules in these fibers (Fig. 1) become close to normal. The recuperated nerve fibers resemble, therefore, the ones described for the control specimens.

DISCUSSION

From the experiments described above it appears that: (*a*) the microtubules in the toad sciatic nerve are affected by environmental temperature;

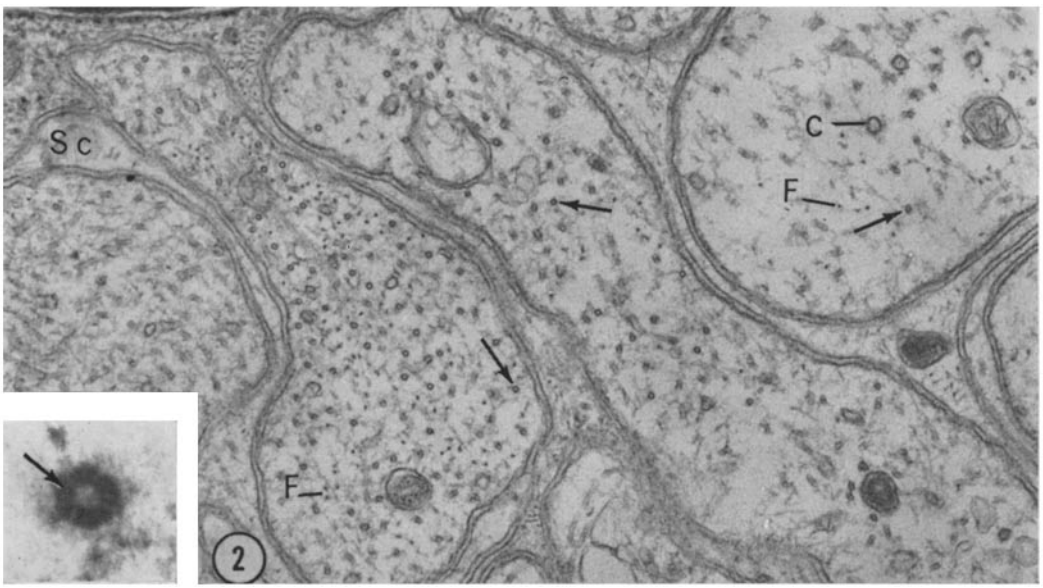

FIGURE 2 In vitro control nerve showing unmyelinated fibers richly supplied with microtubules (arrows) and filaments (*F*). *C*, canaliculi; *Sc*, Schwann cell processes. The inset illustrates a microtubule from a control preparation at high magnification (the picture was made by printing three superimposed negatives selected from a through-focus series). The microtubule subunits appear symmetrically arranged. × 35,400; inset, × 300,000.

(*b*) low temperatures (2°C) produce microtubule disintegration in both in vivo and in vitro preparations; (*c*) return of temperature to 25°C produces repolymerization of microtubules; and (*d*) cell integrity is not essential for a "temperature dependent" disorganization and re-formation of microtubules.

Depolymerization of microtubules by low temperature has been recently reported in a variety of cell types, as for instance mitotic apparatus in amebae (Roth, 1967), microtubular system of axopodia in Heliozoa (Tilney et al., 1966; Tilney and Porter, 1967), and microtubules of the marginal bundle of mammalian blood platelets (Behnke, 1967). In all these examples, warming of the cells yielded microtubule recuperation.

The experiments described here show that the microtubules in neurons are sensitive to environmental temperature in a manner similar to that reported for other microtubules. 10% of the microtubules was found to be resistant to the cold treatment. Some adaptation of microtubules to pressure (Tilney et al., 1966) and low temperatures (Tilney and Porter, 1967) has been reported also for other cell types.

A finding of interest is that disintegration of the microtubules may even occur if neuron integrity is not preserved. Moreover, cell integrity does not appear to be essential either for repolymerization of microtubule subunits or for a correct spatial orientation of the re-forming tubules within the nerve fibers. This is confirmed by the above reported experiments on isolated sciatic nerves. Such results may shed further light upon the problem of microtubule reconstruction. Since synthesis of proteins in neurons appers to be confined to the perikarya (Droz and Leblond, 1963; Petersen et al., 1967), the synthesis of new protein in re-forming tubules of isolated sciatic nerves is unlikely. The repolymerized microtubules must be constructed, therefore, of the same protein subunits that build the pretreatment tubules; or else, as suggested by Behnke (1967) for blood platelets, a pool of monomeric "microtubule protein" might exist in the cytoplasm.

The full process may be tentatively explained as follows. (*a*) Low temperatures would produce a

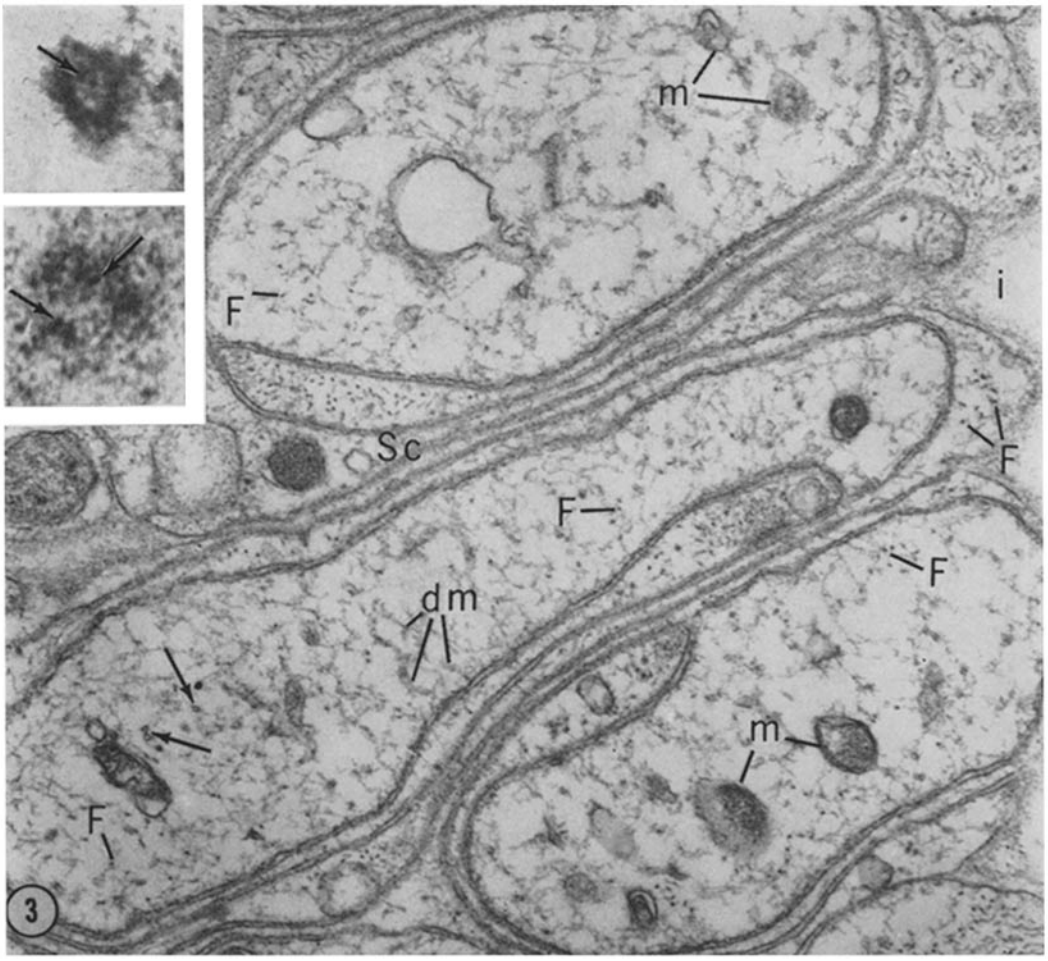

FIGURE 3 In vitro cooled nerve showing disorganization of the microtubular component. At arrows, cold-resistant microtubules; *m*, mitochondria; *Sc*, Schwann cell processes; *i*, interstices; *F*, filaments; *dm*, aggregations of a cotton-like material which probably contain the subunits of depolymerized microtubules. One of these aggregations is illustrated at high magnification in the lower inset; the condensations which are identified with microtubule subunits are indicated by the arrows. The upper inset illustrates a microtubule partially disorganized by the treatment; this microtubule is wider than normal, and its subunits (arrow) are not symmetrically arranged. × 35,400; upper inset, × 300,000; lower inset, × 350,000.

breakdown of the bonds which maintain the aggregation of microtubule subunits into well-defined patterns (Tilney and Porter, 1967). (*b*) Disorganization of microtubule structure would then occur, though their subunits would not migrate throughout the cytoplasm. The amorphous matrix which embeds the tubules may play a role in trapping the microtubule subunits within the region which had been occupied by the pretreatment tubules. (*c*) Warming would produce reaggregation of microtubule proteins according to the pattern required to build up tubular structures. The intermediate stages of disorganization of neuronal microtubules after cold treatment and their repolymerization after warming are to be described in detail in a forthcoming paper.

Though counts of filaments were not made, the

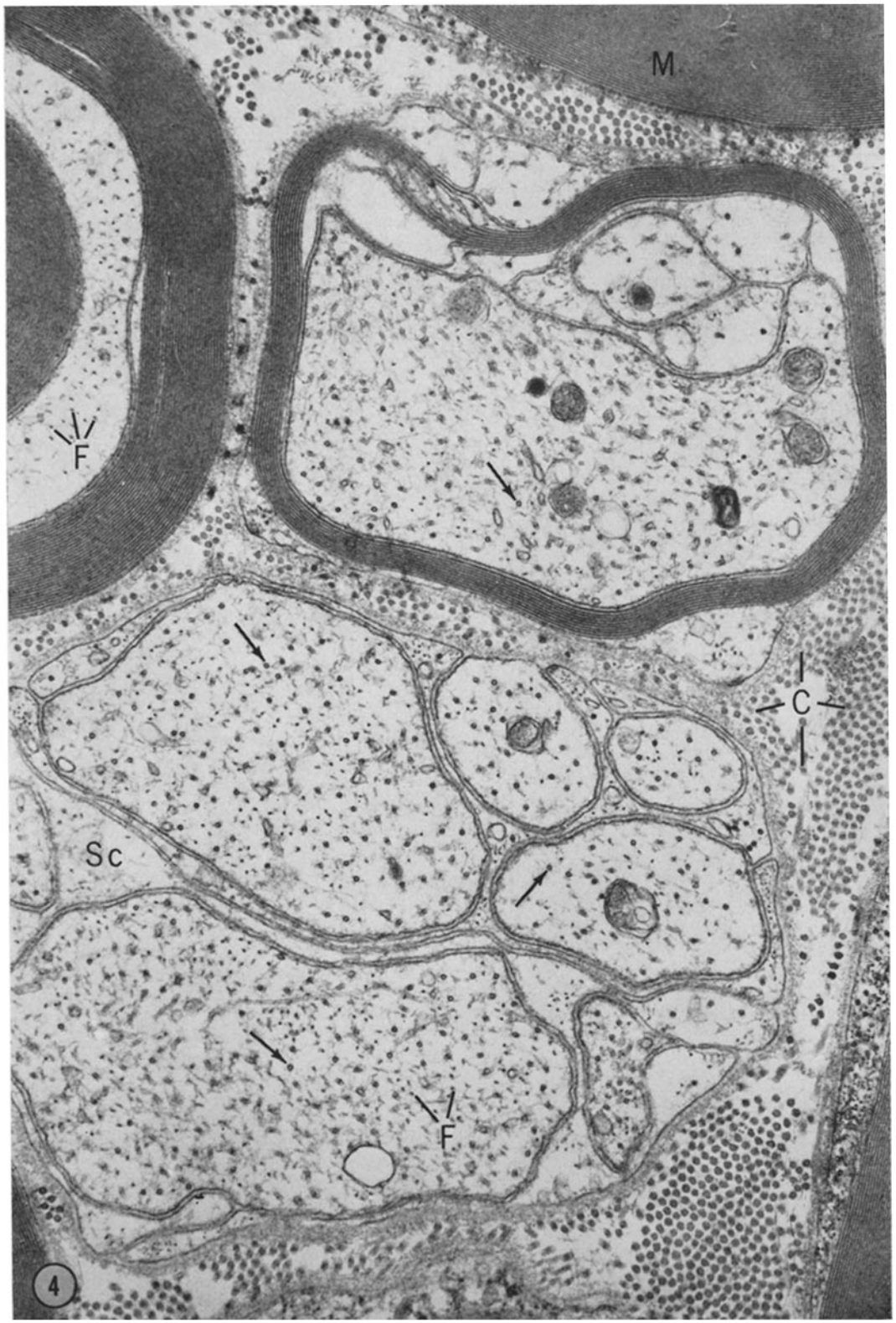

FIGURE 4 In vitro recovering. After warming to 25°C for 1 hr, re-formation of microtubules occurs in both myelinated (*M*) and unmyelinated sciatic nerve fibers (arrows). *F*, filaments; *Sc*, Schwann cell processes; *C*, bundles of collagen fibers. × 35,400.

study of about 1,000 electron micrographs does suggest that the filaments of nerve fibers are resistant to temperature changes under the experimental conditions described here. Peculiar physical properties should, therefore, be postulated to govern the aggregation of their subunits.

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