# ANESTHETIC-INDUCED TRANSFORMATION OF AXONAL MICROTUBULES

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## INTRODUCTION

Tubular structures measuring 310–520 A in diameter have been observed in heliozoan axopods and dividing root tip cells of *Allium sativum* subjected to low temperatures (10), digitonin (3), and halothane (1), a widely used volatile anesthetic. Since microtubules disappear as enlarged tubules form, the enlarged tubular structures are thought to represent a neopolymerizational state of microtubular elements. However, no structural intermediate between microtubules and the enlarged tubular forms have been observed, nor has the microtubule protein (tubulin)-like nature of the enlarged tubular forms been demonstrated.

Previously, we showed that halothane (Fluothane; Ayerst Laboratories, New York) in appropriate concentrations increases the number of formed microtubules in isolated rabbit vagus nerves (4). This observation and those of Seeds et al. (9) suggest that a pool of dissociated microtubular subunits may coexist with formed axonal microtubules within the framework of an alterable equilibrium similar to that described by Inoué and Sato (5) for the mitotic apparatus. Moreover, recent antigenic evidence suggests that microtubular protein may be an integral component of neuronal and synaptic vesicle membranes (11). In view of our previous observations we employed halothane in attempts to accelerate microtubule reformation in crayfish ventral cord axons after heat treatment. However, instead of accelerating microtubular repolymerization, halothane treatment produced enlarged tubular structures in the axoplasm similar to those observed by Allison et al. (1) in halothane-treated *Actinophrys*.

In this communication we report the anesthetic-induced formation of enlarged tubular forms in nervous tissue, the tubulin-like nature of these entities, and suggest a mechanism for their formation from microtubules.

## MATERIALS AND METHODS

Isolated interganglionic segments of crayfish ventral nerve cord were incubated at 40°C for 1 hr in crayfish Ringer's solution (12) pH 7.0–7.4 and then reincubated for 1 hr at room temperature (23°–25°C) in Ringer's containing 10 mM halothane (2-bromo, 2-chloro, 1:1:1 trifluoroethane; Ayerst Laboratories). Other segments were not prewarmed but incubated in 10 mM halothane for 1–3 hr. Segments were then immediately prepared for electron microscopy or treated with 0.2 mM vinblastine (VLB) or 5 mM colchicine for 1 hr at room temperature.

All preparations for electron microscopy were carried out at room temperature. Nerve segments

were fixed in 3% glutaraldehyde buffered with 0.1 M HCl-cacodylate pH 7.4, postfixed in 1% osmium tetroxide, dehydrated, and embedded in Epon 812. Sections were observed in an RCA-3F or Hitachi 11-ES electron microscope.

Since halothane is a volatile anesthetic, solutions containing it were prepared just before use, and incubations were performed in glass stoppered flasks.

## RESULTS

Axonal microtubules (235 A in diameter) rapidly disappeared at 40°C and were replaced by short filamentous strands measuring 50–60 A in diameter (Figs. 1 and 2). Upon reincubation at room temperature (1–3 hr), microtubules transiently reappeared in the axoplasm with a simultaneous decrease in filamentous material. Axons warmed at 40°C and then reincubated in 10 mM halothane contained numerous large tubular forms but no typical microtubules (Fig. 4). These enlarged tubules, hereafter called "macrotubules", averaged 420 A in over-all diameter and had a wall thickness similar to that of microtubules, measuring about 60 A. In contrast to control microtubules, macrotubules had few filamentous projections radiating from their walls (Fig. 6) and displayed no preferred orientation within the axons.

Halothane caused microtubules to disappear from unwarmed axons and produced unusual C-shaped filaments measuring about 60–70 A in thickness and 500–900 A in length (Fig. 3). Prolonged halothane treatment (3 hr) resulted in the appearance of macrotubules in unwarmed axons and the concomitant disappearance of C-shaped filaments.

To determine whether macrotubules are composed of microtubular protein, and are thus of microtubular origin, both macrotubules and microtubules were subjected to VLB and colchicine treatment and their responses to these tubulin-binding alkaloids were compared. Within 1 hr VLB caused microtubules to disappear from control nerves and induced large paracrystalline aggregates in the axons (Fig. 7). VLB also caused macrotubules to disappear and induced the formation of small crystalloidal elements in the axoplasm (Fig. 8). These crystalloidal forms have a distinctive ladder-like profile composed of 60 A particles with a center-to-center spacing between rungs of about 210 A (Fig. 9). In cross-section the small crystalloids resembled the VLB-induced arrays in control segments, having a center-to-center spacing of about 300 A. However, the crystalloidal forms were limited to three to five circular or hexagonal profiles.

Colchicine (5 mM: 1 hr) had little effect on the structure of microtubules from unwarmed nerve segments, but prevented microtubular reassembly after heat treatment. Macrotubule formation was also blocked by colchicine (Fig. 10) but, in contrast to microtubules, macrotubules were sensitive to colchicine, undergoing complete disintegration within 1 hr (Fig. 11).

## DISCUSSION

Structurally, macrotubules are thought to be a neoconformational state of microtubular elements since microtubules disappear as macrotubules appear and the wall thickness of both tubule types is similar (10). Our studies support this hypothesis by demonstrating the microtubule protein-like nature of macrotubules.

VLB concentrations which produced typical paracrystalline arrays in control nerve segments caused the disappearance of macrotubules and the formation of numerous small crystalloids which occupy the approximate position and orientation of macrotubules in the axoplasm. Thus, on the basis of VLB experiments, macrotubules appear to be chemically similar to microtubules. Wilson et al. (14) showed that membrane proteins, actin, and hyalin layer proteins, are precipitable by high concentrations of VLB. However, the concentration of VLB required to precipitate these proteins was more than fifteen times greater than that used in our experiments. Thus, we feel that the concentration of VLB used here significantly reduced the possibility of a precipitate contamination from nonmicrotubular sources. The small crystalloids observed after VLB treatment of macrotubules do not coalesce with time. This observation may reflect subtle structural modifications of the microtubular protein induced by halothane.

The observation that colchicine prevents the formation of macrotubules by halothane is consistent with the hypothesis that colchicine binds to the unassociated phase of microtubular protein (7). However, unlike microtubules which are structurally unaltered by 5 mM colchicine, macrotubules disintegrate into filaments after colchicine treatment. These filaments are similar to those observed when macrotubule formation is prevented by previous treatment with colchicine. Halothane-induced conformational changes within the subunits comprising macrotubular structures

may make the colchicine-binding site more accessible and, therefore, render the macrotubules more labile to colchicine treatment.

Hydrocarbon molecules (13) and anesthetics, including halothane (2), can induce conformational changes in globular proteins. Although the mechanism by which anesthetics alter globular proteins is unknown, the reversibility of the process suggests that anesthetic molecules may bind by hydrophobic forces to the protein moieties (8). Balasubramanian and Wetlaufer (2) found a correlation between the potency of an anesthetic and its effect on protein structure. Thus, methoxyflurane, chloroform, and halothane, which are efficient anesthetics, produce large structural changes in beta-lactoglobulin and bovine plasma albumin, whereas weak anesthetics such as nitrous oxide induced small changes. Although there is considerable evidence that halothane reversibly affects proteins, it is uncertain whether halothane causes microtubular disruption by a direct effect on the protein subunits or acts indirectly by modifying the microtubule-associated microfilaments or the intra-axonal environment. In view of the reversible, direct effects of halothane on globular macromolecules and the results of our studies, it is tempting to speculate about the origin of the halothane-induced C-shaped elements and their incorporation into macrotubular structures.

Tilney and Porter (10) suggested that macrotubules observed in cold-treated *Actinosphaerium* are derived from microtubules by a relaxation of the microtubular structure. This "registering subunit" hypothesis proposes that an increase in tubule diameter (accompanied by shortening) is accomplished by subunits sliding past one another while maintaining a tubular structure. As an alternative, Tilney and Porter (10) acknowledged the possibility that the microtubules might be degraded into subunits and then repolymerized into macrotubules. Since macrotubules can be induced after the heat-dissociation of microtubules and since halothane treatment produces C-shaped filaments in unwarmed segments, we favor the latter explanation of macrotubule formation. Thus, it appears that the C-shaped filaments are probably halothane-modified microtubular subunit strands which are capable of reassociating into macrotubular structures. Halothane may cause conformational changes in the globular subunits comprising microtubules and may alter the bonding angle between adjacent subunits. Modification of either the longitudinal (with respect to the long axis of the microtubule) or circumferential bonding angles holding the subunits in the microtubule wall could lead to microtubular disruption and the formation of free C-shaped filaments. Warming may accelerate the transformation of microtubules into macrotubules by converting the microtubules into subunit polymers which can then be readily altered by halothane to form C-shaped strands. Since the C-shaped elements appear to have a diameter similar to that of macrotubules and since these elements disappear as macrotubules form, we suggest that macrotubules are formed through an end-to-end helical association of C-shaped filaments, in a fashion analogous to the stacking of lockwashers. This mechanism of macrotubule formation by halothane fits the available ultrastructural data,

FIGURE 1 Cross-section of a control axon. Microtubules measure 235 A in over-all diameter, and delicate microfilaments (arrow) were frequently observed radiating from their surface. Bar, 0.5 $\mu$. × 39,530.

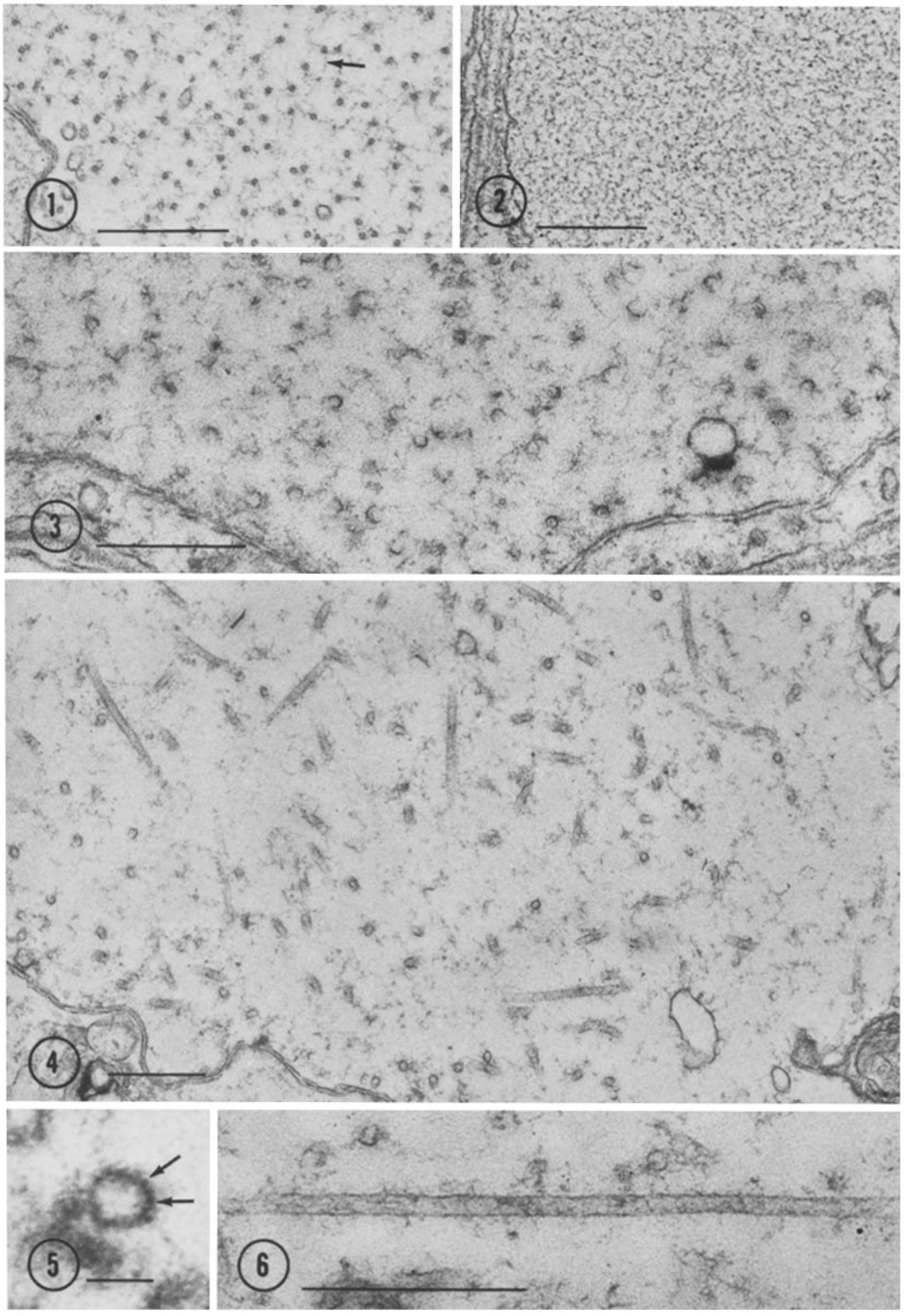
FIGURE 2 Cross-section of an axon incubated at 40°C for 1 hr. Microtubules were replaced by short filaments and particles measuring 60 A in diameter. Bar, 0.5 $\mu$. × 34,140.

FIGURE 3 Portion of an axon incubated in halothane for 1 hr containing numerous C-shaped filaments. Bar, 0.5 $\mu$. × 47,420.

FIGURE 4 Macrotubules induced by halothane after incubation at 40°C for 1 hr. Tubules varied between 380 and 480 A in over-all diameter, lacked specific orientation within the axon, and occasional macrotubules contained a dense "dot" in their lumen. Bar, 0.5 $\mu$. × 30, 790.

FIGURE 5 High magnification cross-sectional view of a macrotubule. In favorable sections, the walls of both microtubules and macrotubules were observed to be constructed of 60 A particles (arrows). Bar, 0.05 $\mu$. × 198,400.

FIGURE 6 Longitudinal section of a macrotubule induced by halothane. Few filaments were observed radiating from the surface of macrotubules. Bar, 0.5 $\mu$. × 74,940.



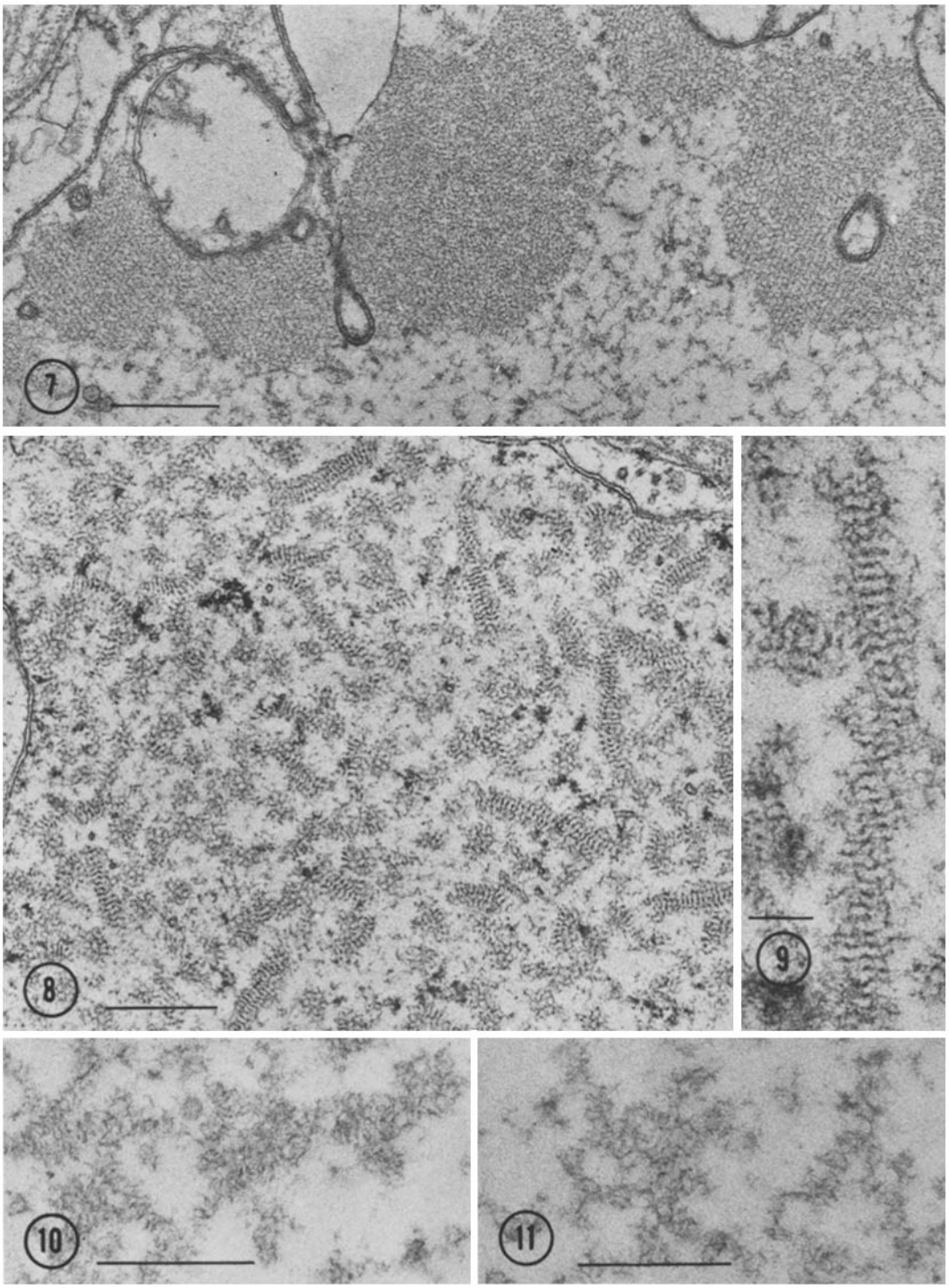



FIGURE 7 Portion of an axon incubated in 0.2 mM vinblastine for 1 hr. Large paracrystalline arrays appeared in the axoplasm. Bar, 0.5 $\mu$. × 32,310.

FIGURE 8 Portion of a vinblastine-treated axon after the induction of macrotubules by halothane. Discrete crystalloidal forms developed in the axoplasm in place of the large arrays. Compare with Fig. 7. Bar, 0.5 $\mu$. × 33,070.

FIGURE 9 High magnification micrograph illustrating the substructure of a crystalloid induced by vinblastine from macrotubules formed by halothane. Note the distinctive ladder-like appearance. Bar, 0.1 $\mu$. × 95,740.

FIGURE 10 Macrotubule formation was inhibited by pretreatment with colchicine. Bar, 0.5 $\mu$. × 46,530.

FIGURE 11 In contrast to microtubules, macrotubules were colchicine labile. Bar, 0.5 $\mu$. × 46,530.

maintains the spatial binding specificities of the subunits, and does not require extensive depolymerization of subunit strands. If halothane directly affects microtubular structure by inducing conformational changes in the subunits, optical rotation experiments similar to those of Laasberg and Hedley-Whyte (6) on halothane-induced conformational changes in hemoglobin and polypeptides should support this mechanism of macrotubule formation.

However, the possibility that halothane may modify microtubular structure indirectly by affecting the microfilaments radiating from the exterior wall of microtubules or by altering the intra-axonal environment must be considered. The microtubule-associated microfilaments are thought to be of a glycoprotein nature. Thus, it is interesting that few microfilaments are associated with macrotubules, and that hyaluronidase treatment can induce macrotubule transformations similar to those observed here (H. Fernandez and P. Burton, personal communication). Since macrotubules have been induced by low temperatures, digitonin, and hyaluronidase, in addition to halothane, it appears that other mechanisms for macrotubule formation are possible. Whether these microtubule-macrotubule transformations reflect compositional differences in the microtubular protein, and perhaps adjunct microfilaments, or are common phenomena due to fundamental changes in the microtubular environment has yet to be determined.

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