MICROTUBULES IN PROTOZOA N CELLS. I I I.
ULTRASTRUCTURAL CHANGES DURING
DISINTEGRATION AND REFORMATION OF
HELIOZOAN MICRO TUBULES

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SUMMARY

In the heliozoan, Echinosphe rium nucleofilum strain MA, cold temperature (2 °C) induced
axopodial retraction to about 36% of the initial length after 3 h. By electron microscopy, it
was found that such axopodial shortening is accompanied by degradation of axonemal micro-
tubules (25 nm in diameter), followed by the appearance of macro tubules (37 nm in diameter)
and filamentous structures (14 nm in diameter) of a tubular and twisted appearance. All of these
structures (microtubules, macrotubules and filamentous structures) were found to be depoly-
merized completely by applying 10 mM colchicine for 1–2 h, and to be replaced by regions with
low electron density. Axopodial re-extension was induced rapidly by returning the cold-treated
organisms to room temperature (20 °C). At a very early stage of axopodial re-extension, the
filamentous structures were often observed to be continuous with the macrotubules. At a late
stage of axopodial re-extension, the reforming axoneme was composed only of normal micro-
tubules, while the macrotubules and filamentous structures had disappeared. On the basis
of these results, the processes of disintegration and reformation of microtubules are discussed.

INTRODUCTION

Since Weisenberg (1972) succeeded in reassembling microtubules in vitro, poly-
merization systems from microtubule protein (tubulin) to integrated microtubules
have been much investigated. Kirschner, Honig & Williams (1975) showed that
fully formed microtubules consisted of protein which was initially both in the ring
form and in the tubulin-dimer (6-s) form, and also quantitatively demonstrated that
twisted ribbons were assembly intermediates, those with a full protofilament com-
plement presumably converting to microtubules by cylindrical folding. On the
other hand, short regions of open protofilament sheet or C-microtubule found in vivo
have also been observed during the in vitro polymerization of microtubules (Bryan,
1976). It therefore seems essential to compare in detail microtubule polymerization
in vitro with that seen in vivo.

In this respect, heliozoan cells appear to be an excellent model system that can be
easily experimented on to elucidate the mechanisms of disintegration and reformation

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of microtubules. Unlike those of cilia and flagella, the intra-axopodial axonemes are composed of a large number of microtubules, which are sensitive to various physical and chemical factors (Kitching, 1957; Roth & Shigenaka, 1970; Shigenaka, Roth & Pihlaja, 1971; Shigenaka, Kaneda & Tadokoro, 1973; Shigenaka, Watanabe & Kaneda, 1974; Shigenaka, Tadokoro & Kaneda, 1975; Shigenaka, 1976; Tilney, 1968). Furthermore, disintegration and reformation of microtubules occur repeatedly even in normal physiological and cultural conditions such as cell division, cell movement, and food capture and ingestion.

In the present study, low temperature or colchicine was applied to a large heliozoan, *Echinospheirium*, in order to clarify the actual process of microtubular disintegration and reformation in an *in vivo* system. The results show that macrotubules and filamentous and tubular structures are found in close association with microtubules, which suggests that these structures are closely involved with microtubular disintegration and reassembly.

**MATERIALS AND METHODS**

A heliozoan, *Echinospheirium nucleofilum* strain MA, originally collected from the campus of Fukuoka University of Education, was cultured continuously at 20 ± 1 °C. Before making new cultures each Petri dish was lined with a layer of 1% agar, 2–3 mm thick. As culture medium, 0.01% Knop solution containing small-sized ciliates and flagellates was employed in the present study. Subcultures were made at intervals of 7–10 days.

Just before the experiments 5- to 7-day-old heliozoans were washed twice with a large amount of culture medium. Cold treatment was carried out at 2 °C for 3 h, and then the organisms were returned to room temperature at 20 ± 1 °C. For observing the changes of axopodial length which occurred during cold and room-temperature treatments, each organism was transferred to a ring slide which was filled with fresh culture medium and covered by a coverslip. A series of light micrographs was taken in time sequence under a Nikon light microscope equipped with Normarski differential-interference optics. The light micrographs of each organism were taken just before the application of cold treatment, and then at 30-min intervals for the cold treatment and 10-min intervals for the subsequent room-temperature treatment. Measurements of axopodial length were made directly from the negatives with a calibrated magnifier, and the average axopodial length for 5 organisms at a given time was calculated.

Prior to colchicine treatment, the heliozoans were washed twice with fresh culture medium. They were then treated with various concentrations of colchicine (Sigma) between 6 and 20 mM for 120 min. Changes of axopodial length resulting from the colchicine treatment were measured in the same way as with cold treatment.

Specimens for electron microscopy were prepared after treatment with cold temperature for 1 and 2 h, and subsequent room temperature for 15, 30 and 90 min, and with 10 mM colchicine or 1 and 2 h. These organisms were fixed according to the method of Shigenaka et al. (1971), dehydrated through a graded ethanol series, and embedded in Spurr's low viscosity embedding medium (Spurr, 1969). Ultrathin sections were cut with a Porter-Blum MT-1 ultramicrotome, stained with 3% uranyl acetate aqueous solution for 10 min and lead citrate stain recommended by Reynolds (1963) for 5 min, and finally examined under a JEOL JEM-100S electron microscope.

**RESULTS**

When heliozoa were treated with cold the extended axopodia were not degraded completely in almost all of them, even after 3 h, but became shortened to about 36% of their initial length (Fig. 1). Furthermore, axopodial re-extension was found to be
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rapidly induced by returning the organisms to room temperature (Fig. 1). The axopodia recovered their initial length in about 60 min.

Electron-microscopical observations of organisms treated with cold for 1 h revealed that (i) a group of microtubules often became detached from the main axoneme;

(ii) the distance between neighbouring microtubules varied; (iii) microtubules themselves were degraded to some extent; and (iv) filamentous structures, thinner than the microtubules, appeared around the degrading axoneme (Fig. 2).

In the organisms treated with cold for 2 h, the axonemal pattern was nearly disintegrated and the microtubules were observed to be replaced by thicker tubule-like structures, here termed macrotubules. Although the normal microtubules varied from 23 to 28 nm in outer diameter, with an average of 25 nm, the macrotubules were about 1.5 times thicker than the microtubules, varying from 33 to 40 nm in outer diameter, average 37 nm. The macrotubules were always of a wavy and twisted appearance and their walls were less dense than those of microtubules (Fig. 3), but of the same thickness.

During the same period of cold treatment, filamentous structures were observed to appear near or at the place which the microtubules had occupied before (Figs. 2, 4).
They were often found to coexist with the above-mentioned macrotubules, and always to have a wavy, tubular and twisted appearance, like the macrotubules. They varied from 10 to 20 nm in outer diameter, with an average of 14 nm (Figs. 4, 5). The filamentous structures appeared to be more conspicuously wavy and have more electron-dense walls than the macrotubules.

Treatment with 6-20 mM colchicine also caused degradation of the axopodia. As illustrated in Fig. 6, the degradation was remarkably dependent on the concentration of this reagent. Complete degradation of axopodia was induced by about 30 min in 20 mM colchicine and about 60 min in 10 mM. In colchicine concentrations lower than 8 mM, axopodial degradation was incomplete; about 82% of the initial axopodial length was lost by 120 min in 8 mM colchicine and about 50% in 6 mM.

Electron-microscopical observations of organisms treated with 10 mM colchicine for 1 h revealed that the microtubules disappeared completely. However, filamentous structures (fs in Fig. 7), similar to those found in the cold-treated organisms, were observed and the same regions (ld in Fig. 7) with low electron-density. After more prolonged treatment of organisms with 10 mM colchicine, the filamentous structures could not be recognized any more inside the cytoplasm, and instead, regions with low electron-density appeared in the same areas that the filamentous structures had occupied.

These results indicate that the filamentous structures may be composed of colchicine-sensitive proteins and, therefore, the derivatives of microtubules, and that the region with low electron-density may correspond to the so-called tubulin dimer pool. Moreover, direct evidence for the close relation between macrotubules and filamentous structures was obtained here from serial sections of specimens in which the axopodia were just re-forming following release from cold treatment. In these the filamentous structures were evidently continuous with the macrotubules at many places, particularly at the very early stage of axopodial re-extension (Fig. 8). Furthermore, these filamentous structures also had a twisted and wavy appearance. At the late stage of axopodial re-extension, the macrotubules and the filamentous structures disappeared and were replaced by microtubules alone (Fig. 9). These showed the newly formed axonemal pattern resulting from rearrangement of microtubules and formation of inter-microtubular links.

Fig. 2. Electron micrograph of a cross-section through the proximal region of axopodium of the organism treated with cold for 1 h. A group of microtubular sheets (mt) detached from the main axoneme (ax) and some filamentous structures (arrows) are seen. x 60000.

Fig. 3. Electron micrograph of longitudinal section through the proximal region of an axopodium of an organism treated with cold for 2 h, showing wavy and less-dense macrotubules in the region previously occupied by microtubules. x 76000.
Figs. 4, 5. Electron micrographs of sections through the cell periphery of organisms treated with cold for 2 h.

Fig. 4. Wavy, twisted and filamentous structures in the place the microtubules had occupied before. \( \times 100,000 \).

Fig. 5. Filamentous structures also show a tubular appearance (arrows) with an outer diameter of about 14 nm. \( \times 200,000 \).
DISCUSSION

It is well known that the microtubule-containing axopodia of heliozoans are highly sensitive to various physical and chemical factors. For example, axopodial degradation is easily induced by application of dilute urea (Shigenaka et al. 1971), heavy metal ions (Roth, Pihlaja & Shigenaka, 1970; Roth & Shigenaka, 1970; Shigenaka, 1976), light metal ions (Shigenaka et al. 1974, 1975), and so on.

Fig. 6. Changes of axopodial length with time resulting from colchicine treatment. Axopodial length at each time is indicated as a percentage of the initial average length of axopodia. With 10–20 mM colchicine, all axopodia are degraded completely within 60 min, while 6–8 mM colchicine does not cause complete degradation of axopodia even after 2 h of treatment. △, 6 mM colchicine; ▲, 8 mM; ○, 10 mM; ●, 20 mM.

Tilney & Porter (1967) have shown that during cold treatment of heliozoans (Echinospaeraium nucleofilum, American strain EC) at 4 °C tubule-like structures, 34 nm in outer diameter (macrotubules) appear, but that when organisms are removed from the cold, these macrotubules disappear. They seem to be one of the disintegration products of microtubules. These authors have also reported that the walls of macrotubules are 5 nm thick, like those of normal microtubules. Moreover, Warfield & Bouck (1974) have suggested that the macrotubules consist of tightly coiled helices formed by longitudinal compacting of loosely coiled protofilament pair intermediates.

In the present study on E. nucleofilum, Japanese strain MA, it was found that in addition to 33- to 40-nm macrotubules, 10- to 20-nm filamentous structures appeared near or at the site of pre-existing microtubules during cold treatment at 2 °C. Further evidence of the direct relationship between macrotubules and filamentous structures was obtained in the present study, in the form of micrographs showing the connexion
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Fig. 9. Electron micrograph of a cross-sectioned axoneme at a late stage of axopodial re-extension, showing characteristic microtubule patterns in the main axoneme (upper) and a smaller axoneme (lower.) \( \times 105,000 \).

Figs. 7, 8. Electron micrographs of sections through the cell periphery.

Fig. 7. Organism treated with 10 mM colchicine for 1 h, showing the mass of filamentous structures \((fc)\) and the characteristic region with low electron-density \((ld)\). \( \times 47,000 \).

Fig. 8. Organism treated with cold for 3 h and then incubated at room temperature for 30 min, showing that the wavy filamentous structures \((fc)\) are evidently continuous with the macrotubules \((mt)\). \( \times 124,000 \).
or structural transition between macrotubules and filamentous structures. Typical sensitivity of the filamentous structures to certain concentrations of colchicine was also demonstrated, as well as in the microtubules, suggesting that the filamentous structures are derivatives of microtubules. As far as the fine-structural details are concerned, macrotubules and filamentous structures appear to be quite similar to the twisted ribbons found in \textit{in vitro} systems (Kirschner et al. 1975; Matsumura &

Fig. 10. Schematic drawing showing the suggested disintegration and reformation process of microtubules. The processes indicated by solid arrows can be considered as confirmed by the present data, while the other processes (arrows with dotted line) are believed to be possible. For further details see text.

Hayashi, 1976). However, it was found here that the filamentous structures are thinner than the twisted ribbons composed of 19 protofilaments (Matsumura & Hayashi, 1976). Therefore, the filamentous structures seem to be composed of very few protofilaments. On the other hand, the outer diameter of the filamentous structure (14 nm), was found to be approximately similar to that of the colchicine-induced polymer (17 nm) reported by Matsumura & Hayashi (1976). From these data, it is considered that the disintegration and reformation of microtubules, which occurs repeatedly \textit{in vivo}, may be very similar to the \textit{in vitro} process.
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In addition to these results, more detailed studies of the disintegration and reformation process led to the hypothesis illustrated in Fig. 10. At first, microtubule degradation may result in the appearance of macrotubules, filamentous structures or tubulin dimers. These seem to vary in amount depending on the degree of microtubule degradation. Then, the macrotubules and filamentous structures appear to be degraded finally into the tubulin dimers. It also seems possible that the microtubules can be directly degraded into tubulin dimers. However, in the present study, the macrotubules certainly appeared during the disintegration of the microtubules, and the filamentous structures are possibly one of the disintegration products of microtubules. The macrotubules and filamentous structures are both considered to be labile structures, because they always appeared wavy and twisted. All of these processes are also considered to be reversible. Thus, the in vivo reassembly process of microtubules may proceed directly from tubulin dimers or indirectly from them, i.e. through filamentous structures or macrotubules or both. At the early stage of axopodial re-extension, it was found that the macrotubules are continuously associated with the filamentous structures, suggesting that the latter may be structurally transformed into the former. Furthermore, these macrotubules may be transformed into microtubules, thus reaching the most stable state.

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