SUBSTRUCTURE OF MICROTUBULES IN BRAIN NERVE CELLS AS REVEALED BY RUTHENIUM RED

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INTRODUCTION

The presence of neurotubules with a diameter of about 200–250 A was first described in nervous tissue by Palay (11). Similar components, the microtubules, have been observed as a universal component of cells and cell processes. Microtubular structures were also evident in spindle fibers as well as in fibers of flagella and cilia. Sectioning methods, however, reveal no details of structure down to the molecular level, and information about substructure has come mainly from observations on negatively stained preparations. The present study provides more information on the fine structure of the microtubules as revealed by ruthenium red, initially introduced into electron microscopy by Luft (9).

MATERIALS AND METHODS

Adult rats were anesthetized by intraperitoneal administration of nembutal. The brain of the animals was then perfused with fixative by a modified method of Palay et al. (12). After thoracotomy, the right atrium and then the cardiac apex were cut with a sharp knife, and a cannula was immediately inserted through the left ventricle into the ascending aorta. It is necessary to begin perfusion as soon as possible after the thoracotomy. After the perfusion with fixative was started, the descending aorta and the bilateral brachial arteries were clamped. After the perfusion was finished, the whole brain was removed, and the firm parietal lobe was cut into small pieces wihch were then immersed in fixative at 4°C for 1 hr. In another group of animals, the skull and then the underlying dura were carefully opened. Then the brain tissue was removed from the parietal lobe and diced into small pieces in fixative. Small fragments of the brain tissue were fixed for 2 hr at 4°C.

Staining of tissue with ruthenium red was carried out according to the method of Luft (9). The fixative available in the present study was 1.25% glutaraldehyde containing 5% sucrose and 500 ppm ruthenium red, and buffered with 0.1 M cacodylate at pH 7.4. All specimens, after fixation in the glutaraldehyde mixture, were briefly washed in the cacodylate buffer solution, and then postfixed with 1% OsO_4 containing 500 ppm ruthenium red buffered with 0.1 M cacodylate at pH 7.4 at room temperature for 2 hr. Samples for routine procedure were fixed in 1.25% glutaraldehyde solution buffered with 0.1 M cacodylate at pH 7.4 by the perfusion method. They were then washed with 0.1 M cacodylate buffer and immersed in 1% OsO_4 in 0.1 M cacodylate buffer (pH 7.4) at 4°C for 2 hr. After completion of postfixation, all specimens were dehydrated in a graded concentration of alcohol and embedded in Epon or Vestopal W. All of the blocks were cut with a Porter-Blum ultramicrotome (Ivan Sorvall Inc., Norwalk, Conn.) Thin sections were stained with uranyl acetate for 5 min and with lead citrate for 3 min on the grid, and examined with the HU-11 A electron microscope.

RESULTS

Ruthenium red–positive substances were found in the extraneous surface coat and the synaptic cleft (Fig. 1), as well as in the cytoplasmic organelles largely of neurons. Since ruthenium red does not cross the undamaged cell membrane (10), the intracellular ruthenium red–positive substances may become visible by way of penetration of the ruthenium red through damaged cell membranes, which probably occurs along the edge of the specimens during the immersion in the fixative. This possibility may be substantially supported by the observation that isolated intracellular precipitation of ruthenium red–positive substances occurred only in some dendrites (Fig. 1) or axons in deep portions of the specimens, where there was no

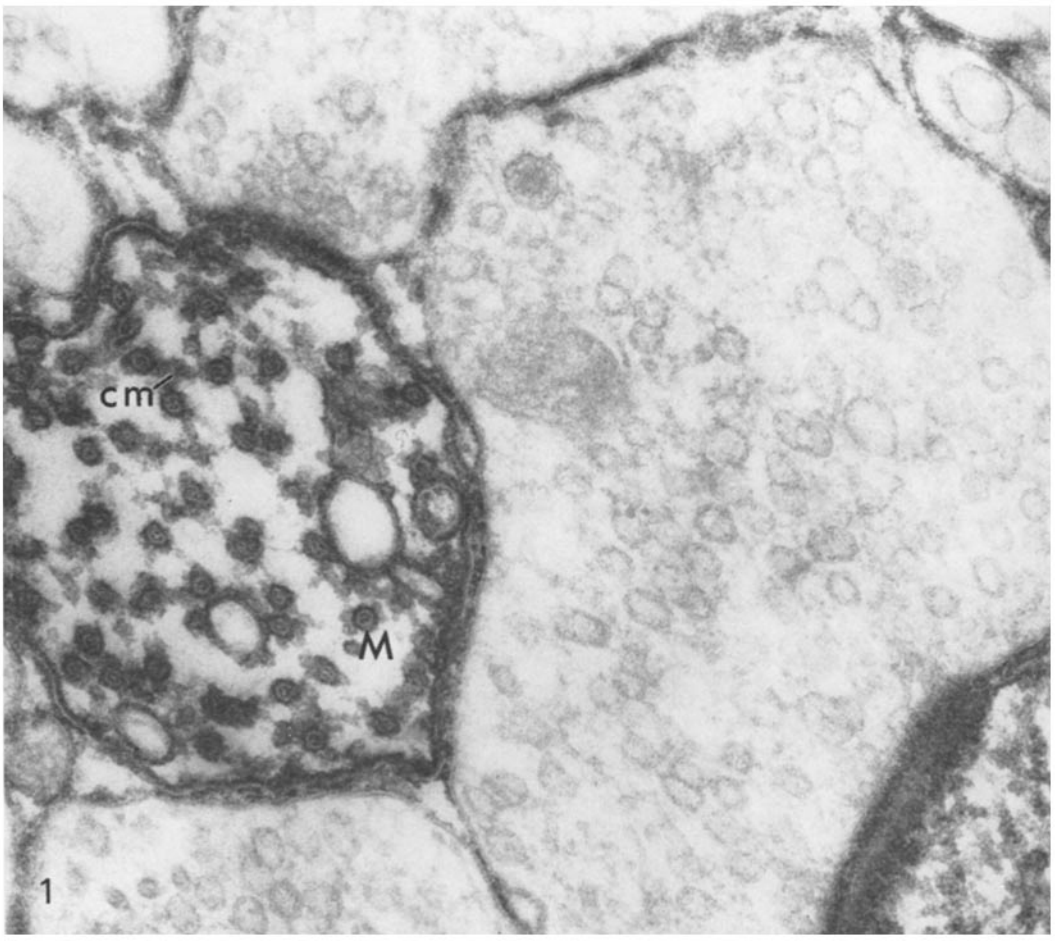

FIGURE 1 Ruthenium red–marked substances are seen in the extraneous cell coat and the synaptic cleft as well as in the cytoplasmic organelles of dendrites. Many microtubules (*M*) reveal two concentric layers, and are coated with cytoplasmic matrix (*cm*) with an intermediate density. × 79,000.

evidence of ruthenium red–marked materials in other cells or in the extraneous cell coat. Histological exploration has demonstrated the presence of mucopolysaccharides that are readily attacked by testicular hyaluronidase within the perikaryon as well as in the dendrites and the axons of the mammalian brain (17). In addition, intraventricular administration of hyaluronidase produced a removal of neural hyaluronic acid which in turn disrupted the electrical behavior of the cell to induce a decrease of the periventricular tissue impedance (18) and a seizure discharge (16). Since the ruthenium red precipitates a variety of acid polysaccharides (10), the association of the ruthenium red–positive substances with the cytoplasmic membranes of neuron is, therefore, of interest and will be reported in a subsequent paper.

Microtubules untreated with the ruthenium red (Fig. 2) generally revealed a circular profile, 120–150 A in inner diameter and 220–270 A in outer diameter in cross sections, and ran parallel to the length of the axons, the dendrites, or the axon hillocks, being relatively straight with no obvious dilatation for the most part in longitudinal sections.

When treated with ruthenium red, the ruthenium red–positive substances became visible on the outer wall of the microtubules. In addition, a concentric dense layer with an outer diameter of

about 140 A became visible within the microtubules in transverse sections. Consequently, two concentric dense layers were clearly distinguished in cross sections of microtubules in the axon hillock, axon, and dendrite (Figs. 1–5). The outer concentric layer was 220–260 A in inner diameter. A diffuse, intermediate density usually occurred within the microtubular matrix (Figs. 1–5), and some central matrix surrounded by the inner concentric layer was not stained with the ruthenium red and was rather clear in appearance (Figs. 3 and 4).

The inner concentric layer was rather irregular in width, averaging around 30–50 A thick and often composed of globules, about 30–50 A across (Figs. 3–5). However, some globules in the inner concentric layer became disorganized or obscure in orientation. The outer concentric layer, namely the outer wall of the microtubules treated with the ruthenium red, was also irregular in thickness, averaging about 40–50 A across in transverse sections, and composed similarly of ill defined globular subunits, about 40–50 A across (Figs. 3–5). Their outer border was embedded in a cytoplasmic matrix with an intermediate density and sometimes protruded into the surrounding cytoplasmic matrix. Consequently, the outer border of the outer concentric layer was rather irregularly outlined. The number of the globular subunits was not precisely determined. The inner and the outer concentric layers were not clearly demonstrated in transverse sections of some of the microtubules, irrespective of showing its constant inner diameter, and the globular subunits were rather irregularly oriented in the microtubular matrix or in the neighboring cytoplasmic matrix (Figs. 3–5).

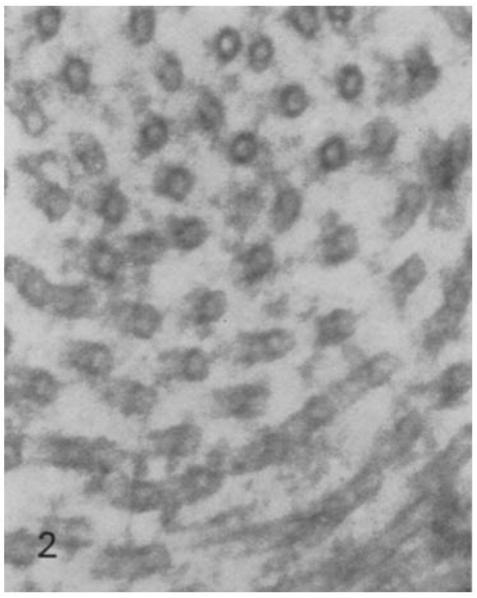

FIGURE 2 Numerous microtubules in a dendrite untreated with ruthenium red are sectioned transversely and obliquely. × 135,000.

The cytoplasmic matrix, which coated the microtubules in an irregular fashion and extended as a fluffy structure between the microtubules and was connected with the same material around the adjacent microtubules in transverse sections, was of rather intermediate density, amorphous in appearance, and composed at times of globular subunits (Figs. 3–5). The globules in the cytoplasmic matrix were similar in size and structure to the subunits in the outer and the inner concentric layers of the microtubules (Figs. 3–5).

The detailed structure of longitudinally oriented microtubules appeared somewhat less clearly defined than the structure of crossly sectioned units. These differences may be partly due to the fact that most usually the longitudinal appearance of the microtubules is the full image of these 220–260 A thick organelles, since most of them lie entirely within the range of the 300–600 A thick sections. In addition, the cytoplasmic matrix around the microtubules was seen frequently superimposed on the microtubules or projected as a spine from the wall of microtubules at intervals of 400–1000 A in longitudinal sections (Fig. 6). Nevertheless, the longitudinally arranged microtubules revealed four parallel linear structures in most cases (Fig. 6). The outermost linear structures were generally more clearly defined than other linear structures. The linear structures were often beaded in appearance. The width of the individual beads, which were mostly globular in form, could not be measured with great accuracy, but it was 40–50 A on the average. The beaded appearance in the linear structures became obscure if the cytoplasmic matrix around the microtubules was superimposed on them, for dense globular subunits with a diameter of about 50 A similar in size to the beads in the microtubules, were also present in the cytoplasmic matrix. In addition, the tndividual globular beads did not necessarily lie in a straight line and were rather randomly disiributed in places along the linear structures.

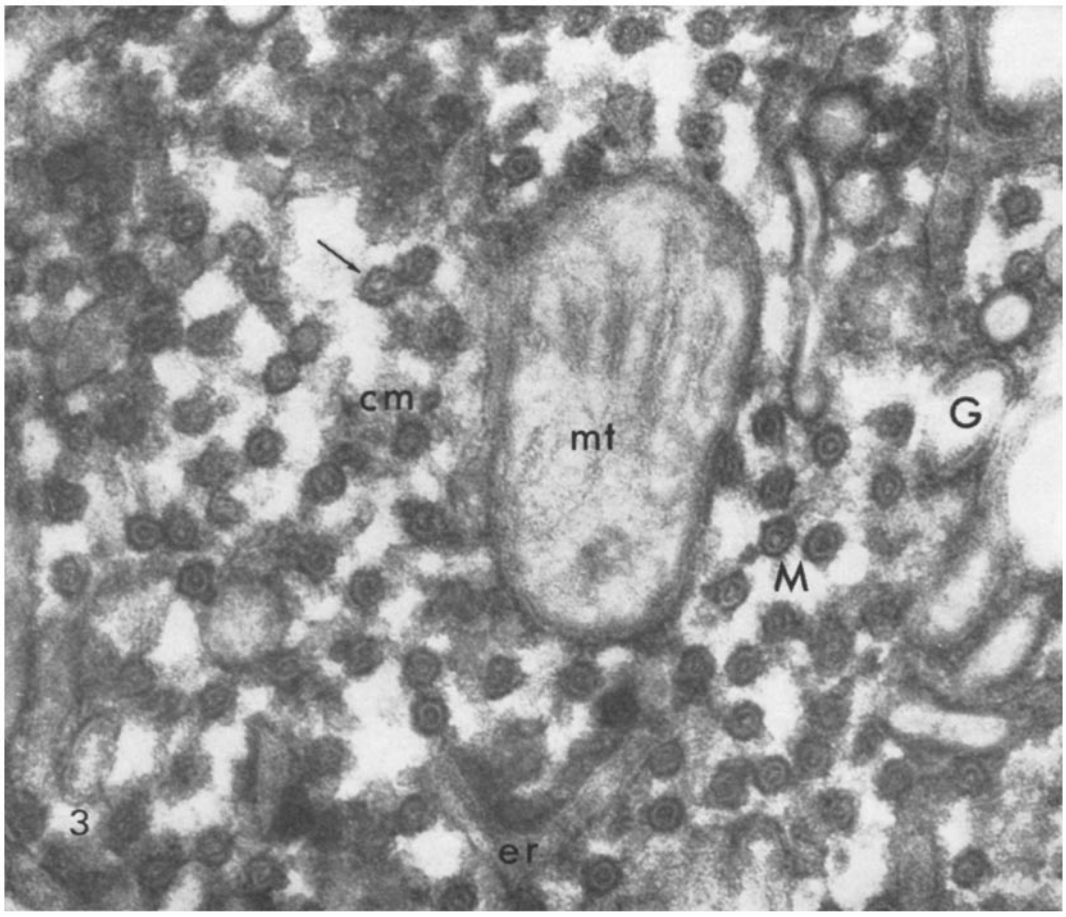

FIGURE 3 Cytoplasmic matrix (*cm*) coated around microtubules is visible as a fluffy structure in an axon hillock. The microtubules (*M*) consist of two concentric structures. The central matrix surrounded by the inner concentric layer in some of the microtubules is clear in appearance (arrow). Membranes of the endoplasmic reticulum (*er*), mitochondria (*mt*), and Golgi complex (*G*) also become strongly dense with ruthenium red. × 142,000.

However, the beads regularly oriented in the linear structures were likely to give rise to an axial repeat of about 50–70 A along the length (Fig. 6). A more accurate measurement was the center-to-center spacing of adjacent linear structures, for these were arrayed in more regular order. This spacing was about 90 A.

Nothing was seen of a trilaminar unit membrane in the wall of the microtubules, although the ruthenium red clearly revealed unit membrane structure of cytoplasmic membranes.

DISCUSSION

The two concentric dense layers, although reported in transversely sectioned flagellar fibers by Afzelius (1) and André (2), are not a universal structure of the microtubules when examined with conventional sectioning and staining. When the microtubules are treated with ruthenium red, the presence of two concentric layers is consistent in the transversely oriented microtubules in axons, dendrites, and axon hillocks. Consequently, the microtubules as a whole are composed of two cylindrical densities arranged in a concentric fashion. The inner diameter of the untreated microtubules, 120–150 A, is in conformity with the outer diameter of the inner concentric ruthenium red–positive layer. In addition, the outer diameter of the untreated microtubules, about 220–270 A, quite corresponds to the inner di-

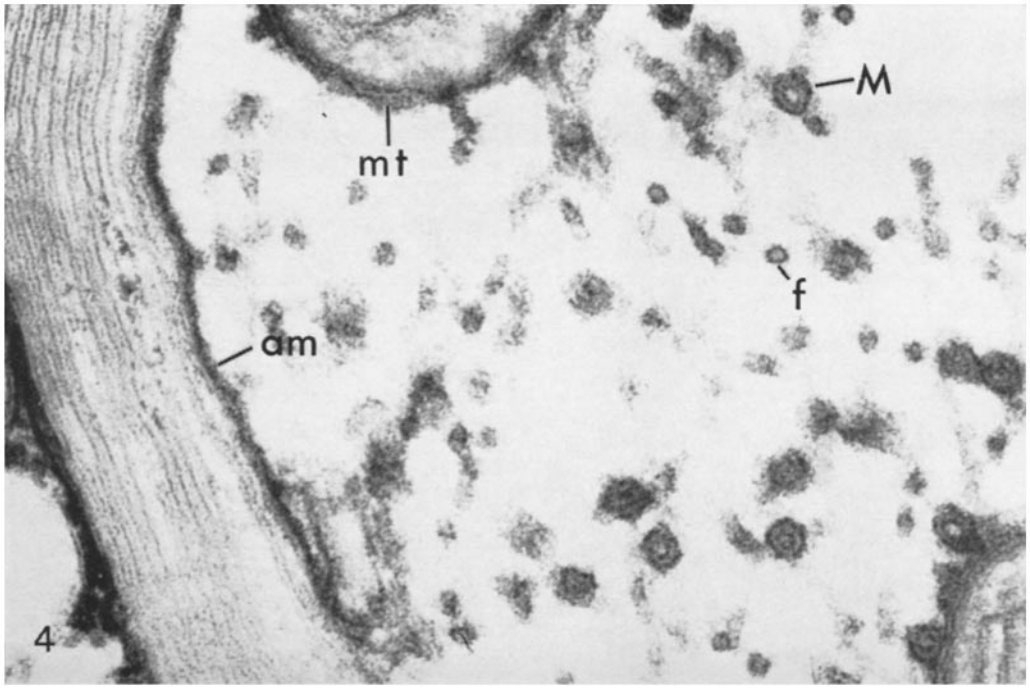

FIGURE 4 A transverse section of a myelinated nerve. Transversely oriented microtubules (*M*) demonstrate two concentric dense layers which are composed of globular subunits. Neurofialments (*f*) as well as axonal (*am*) and mitochondrial membranes (*mt*) also are deeply stained with ruthenium red. × 150,000.

ameter of the outer concentric ruthenium red–marked layer. It seems reasonable, consequently, to assume that the inner and the outer concentric ruthenium red–marked layers may be a precipitate of the ruthenium red–positive substances attached closely to the inner and the outer sides of the wall of cylindrical microtubules. No space is evident between the ruthenium red–marked layer and the wall of microtubules. It remains to be determined what the relationship is between the globular subunits in the outer and the inner concentric layers of the microtubules in the present study and the globules demonstrated in the rat optic (14) and the toad sciatic nerves (15). The ruthenium red–marked layer on the outer and the inner surfaces of the microtubules might represent a random precipitate of cytoplasmic matrix, probably induced by the rigorous ruthenium red procedure.

Periodicity specifically related to the longitudinally oriented microtubules has not been seen convincingly by the conventional technique, but several authors have reported a structural periodicity in flagella, cilia, and contractile axostyles (2, 6, and 7). On the other hand, with negative

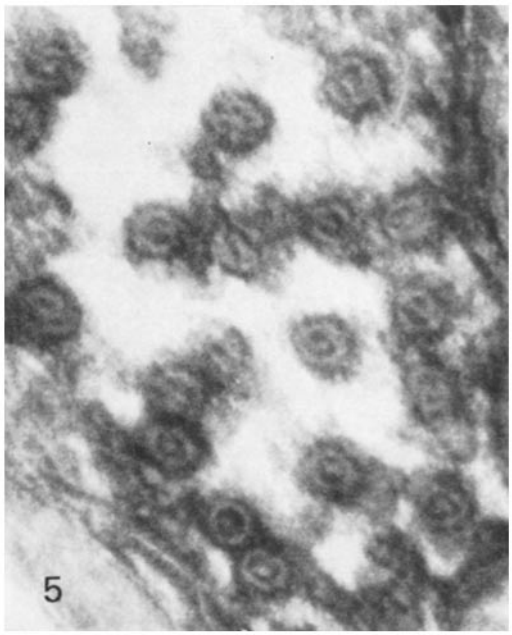

FIGURE 5 The outer and the inner concentric layers of microtubules are composed of globular subunits. The cytoplasmic matrix around the microtubules also demonstrates similar globular subunits. × 228,000.

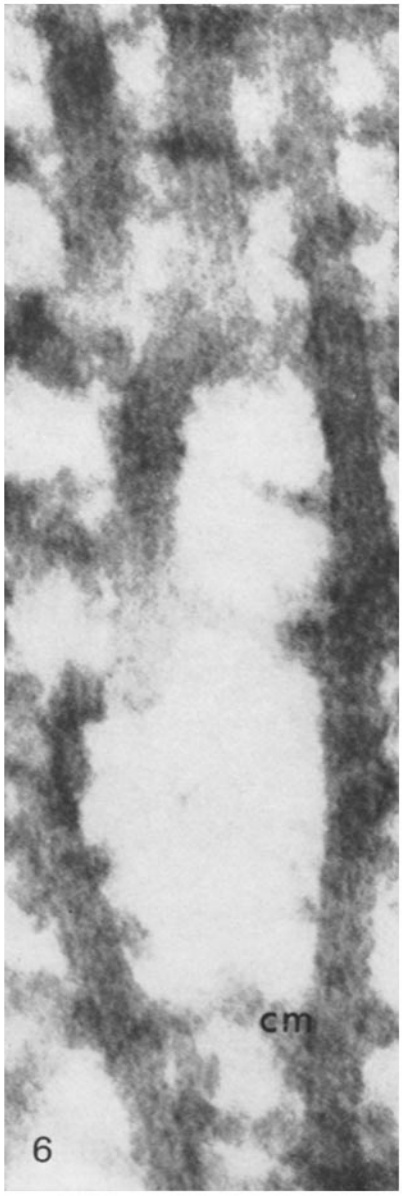

FIGURE 6 Longitudinally oriented linear structures in microtubules are beaded in appearance, the beads being represented as globular subunits. The cytoplasmic matrix (*cm*) is seen to be superimposed on the microtubules or to project as a spine from the microtubules. × 210,000.

staining, the longitudinally oriented filaments in the microtubules in sperm tails (3, 13), approximately 35–40 A thick, have a markedly beaded appearance, with a repeating period of 80–88 A, and the center-to-center spacing from one filament to the next is 55–60 A. The filaments of the peripheral and central fibers of the flagella of *Trichonympha* are about 40 A across, 40–50 A apart from center-to-center, beaded in appearance, and the subunits give rise to an axial repeat along the filaments of 40 A and a basic surface lattice of 40 × 50 A (8). The spindle fiber is bounded on either side by a layer about 50 A thick with an irregular, ragged outer surface. The central filaments have the appearance of rows of longitudinally connected granules about 35 A in diameter, and the distance between the centers of the granules in both longitudinal and transverse directions is 50 A (4). However, the use of an optical diffraction method has suggested a much more complex structure in negatively stained images of the outer wall of flagella than has hitherto been detected by direct observation (5). The individual beads of the microtubules in the present study seem to be similar in structure and size to the subunits reported hitherto, but the distance between the centers of adjacent longitudinal filaments is quite different. This difference in orientation of subunits might be in part due to the techniques used in the treatment of the microtubules: negative staining and ultrathin sectioning. However, an oblique sectioning of microtubules in the present study demonstrated four linear structures, the central two filaments of which obviously demonstrated an oblique view of the inner concentric cylindrical density. In addition, the spacing between the two central longitudinal filaments quite corresponds to the diameter of the inner concentric layer in transversely oriented microtubules. It might be suggested, therefore, that the longitudinal filaments in the present study might not represent the subunits on the outer wall of microtubules but the inner and outer concentric layers.

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