THE FINE STRUCTURE AND IMMUNOLOGICAL LABELING OF THE ACHROMATIC MITOTIC APPARATUS AFTER DISRUPTION OF CELL MEMBRANES

SAMUEL DALES, KONRAD C. HSU, and ARIAKI NAGAYAMA
With the assistance of TELLERVO HUIMA

From the Department of Cytobiology, The Public Health Research Institute of the City of New York, Inc., New York 10016 and the Department of Urology, Columbia University College of Physicians and Surgeons, New York 10032. Dr. Nagayama's present address is the Department of Microbiology, School of Dentistry, Kyushu University, Fukuoka, Japan.

ABSTRACT

After treatment of HeLa and L cells with vinblastine sulfate the material of microtubules (tubulin) was reorganized into (*a*) large paracrystals (PC) of tightly packed tubules; (*b*) smaller aggregates of tubules with greater diameter whose walls are constituted from well defined, helically arranged morphological subunits; and (*c*) microtubules associated with helices of polyribosomes of uniform size. All of these structures survived disruption of cellular membranes by means of a nonionic detergent. Following a thorough stripping of membranes there remained a subcellular fraction sedimenting at 1,500 *g* for 15 min, in which were contained nuclei, centrioles, and the above mentioned microtubular elements, maintained as a complex of organelles by an interconnecting network of 80 Å microfibrils.

As a result of membrane disruption it was possible to localize precisely in the electron microscope the binding of ferritin antibody conjugates. Specific labeling at the surface of PC and microtubule aggregates could be demonstrated. This result was substantiated by means of the immunoperoxidase method of labeling the PC.

A concentrated deposit of ferritin was also found in the vicinity of centrioles and related structures, the annuli of the nuclear pore complex and the annulate lamellae. However, the specificity of the label on these organelles remains questionable because ferritin, albeit in lower concentration, was also present on them in control preparations reacted with preimmune sera.

INTRODUCTION

Following the observation that alkaloids of *Vinca rosea*, including vinblastine and vincristine, cause a reorganization of microtubular elements resulting in formation of highly ordered paracrystals or PC (3), methods were devised for obtaining the PC in a concentrated, pure state for analytical purposes and to elicit specific antibodies. Work of several investigators established homology with respect to sedimentation value, molecular weight, electrophoretic mobility, amino acid composition,

and colchicine and guanosine triphosphate binding between tubulins of the microtubules and material derived from PC (4, 10, 16, 18, 20). Our immunological studies employing double-diffusion in agar and immune fluorescence revealed antigenic similarity or identity between the mitotic spindle and PC of the murine cell of origin (17). Furthermore, by using selected cell lines in culture it was possible to demonstrate the universality on the ascending evolutionary tree from fish to man of antigen(s) of the spindle and PC (5). We undertook the present experiments to substantiate at the fine structure level the specificity of immunological labeling, observed heretofore only at the level of the light microscope, and thereby to localize more precisely the antibody combining sites.

In connection with the current experiments it became necessary to devise procedures for disrupting cell membranes so as to allow free access to the antibody and marker substances. In the adopted method of choice, cell membranes were profoundly ruptured or stripped by a nonionic detergent and surprisingly the fine structure and interrelationships between components of the mitotic apparatus were revealed with greater clarity than seen within intact cells. Our coincidental observations on disrupted cells are, therefore, also described in this paper.

MATERIALS AND METHODS

Cells and Media

Continuous lines of strains HeLa of human derivation and subline L_2 of L_{929} murine fibroblasts were propagated in monolayer cultures using nutrient media as previously described (7). To elicit the formation of PC, cultures were incubated at 37°C for 18–24 h in Eagle's minimal essential medium (MEM) supplemented with 10% bovine fetal serum and 10^{-5} M vinblastine sulfate (Velban, Eli Lilly and Company, Indianapolis, Ind.), termed by us previously "NM A" (17). The medium used for suspending cells in preparation for rupturing with detergent and immunoferritin labeling, termed "NM B," consisted of MEM without sodium bicarbonate but with added 10^{-5} M vinblastine sulfate, adjusted to pH 6.5 with sodium hydroxide.

Antisera and Ferritin-antibody Conjugates

Antibody specific against a component of the microtubules was elicited by immunizing rabbits with purified PC material as described previously (17). It was demonstrated by both immunofluorescence and the Ouchterlony double diffusion method that antisera from immunized animals contained antibody with high binding affinity for the PC and mitotic apparatus of a variety of vertebrate cells (17, 5).

For direct immunological labeling the ferritin-antibody conjugates were prepared according to the procedures outlined previously (2). Indirect labeling with ferritin antibody was carried out by means of ferritin conjugated to sheep-antirabbit globulin as described by Andres et al. (2).

Disruption of Cells

After incubation in NM A, monolayers of 2–5 × 10^7 cells were chilled to 0°C by adding cold NM B scraped from Petri dishes, suspended in 20–50 ml NM B and centrifuged at 1,000 *g* for 2 min. After decanting the supernate the cell pellets were resuspended in 10 ml NM B and centrifugation repeated.

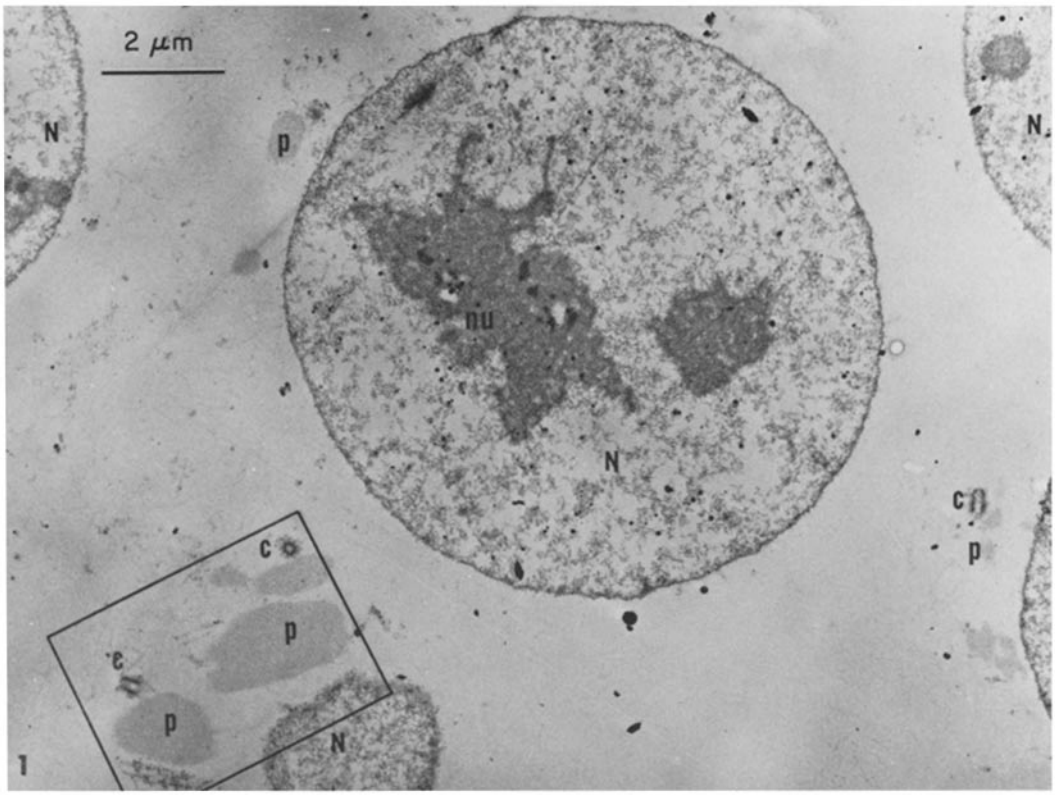
Figure symbols:

c, centriole
f, microfilaments
m, mitochondrion
N, nucleus
nu, nucleolus
p, microtubular paracrystals
t, microtubule
va, vacuole
v, virus-like particle

In the legends: H, HeLa cell; L, strain L cells.

FIGURE 1 Remnants of cells profoundly disrupted after addition 1% NP_{40}. Note the complete absence of membranes, including the nuclear envelopes. The centrioles lodged in the vicinity of PC and nuclei are most probably part of complexes of the three organelle components. L × 8,000.

FIGURE 2 Enlargement of the framed area in Fig. 1 reveals a connecting network of microfilaments between the nucleus, PC, microtubules and centrioles. The arrow points towards an aggregate of microtubules with polyribosomes. L × 30,000.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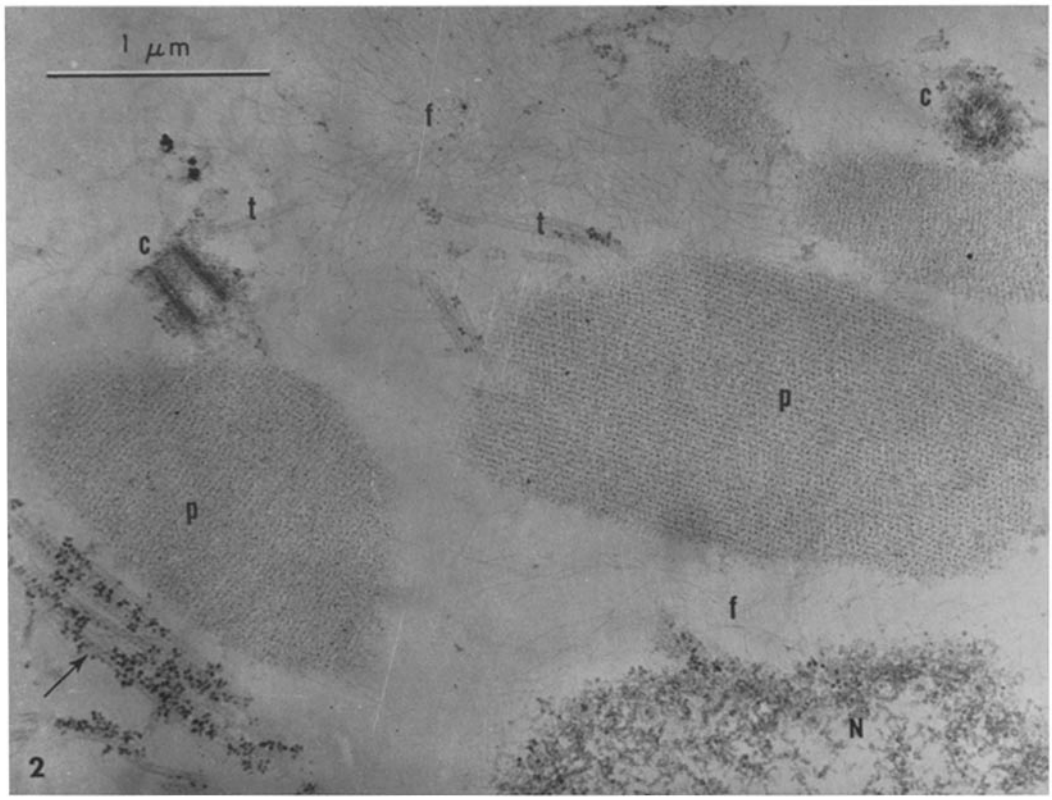
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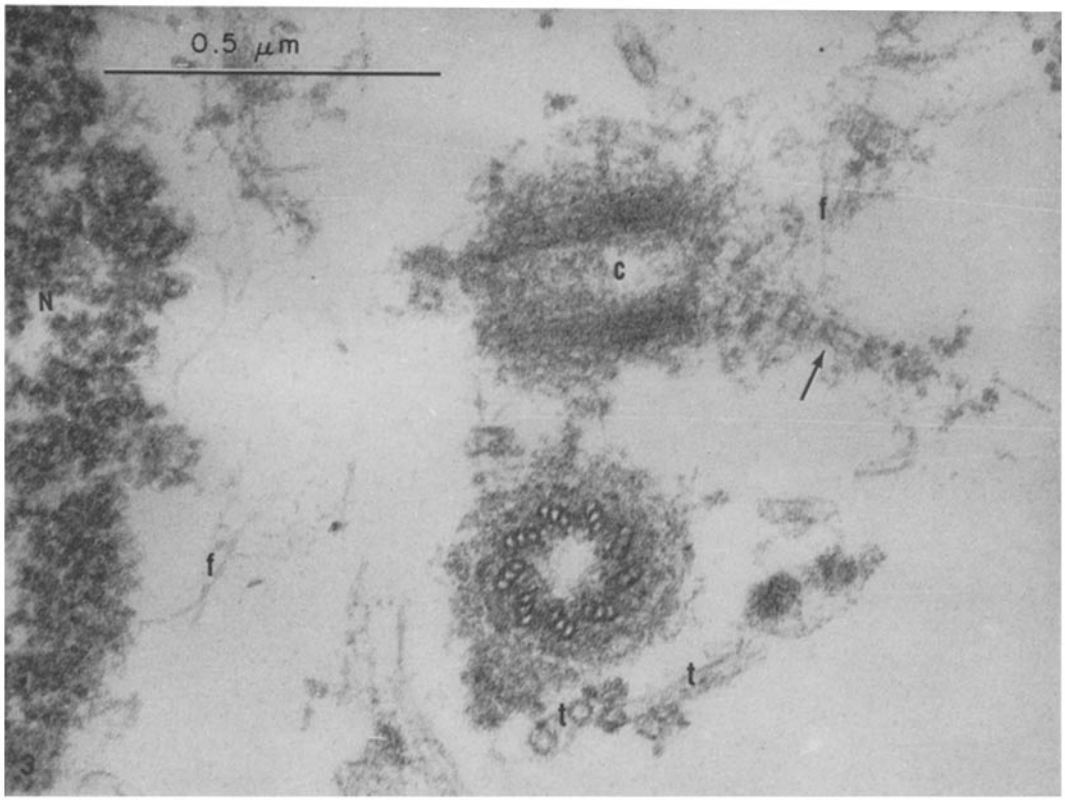


FIGURE 3 Another example of a centriole-microtubule-nucleus complex. The arrow indicates the rootlet structure of striated filamentous bundles. L × 90,000.

The cells were suspended by adding to the pellet 2 ml of NM B rapidly mixed for 10 s with 10–20 μl of the nonionic detergent NP_{40} (Shell Chemical Corp., New York) by means of Vortex mixer (Scientific Industries, Inc., Mineola, N. Y.) and immediately combined with 15–30 ml of NM B to dilute the detergent. More complete stripping of membranes was achieved with the higher concentration of NP_{40}. Suspensions containing cells with ruptured or stripped membranes were centrifuged at 1,500 *g* for 15 min and the pellets of cells were either fixed and processed directly for electron microscopy or employed in immunological labeling experiments.

Indirect Immunological Labeling with Ferritin

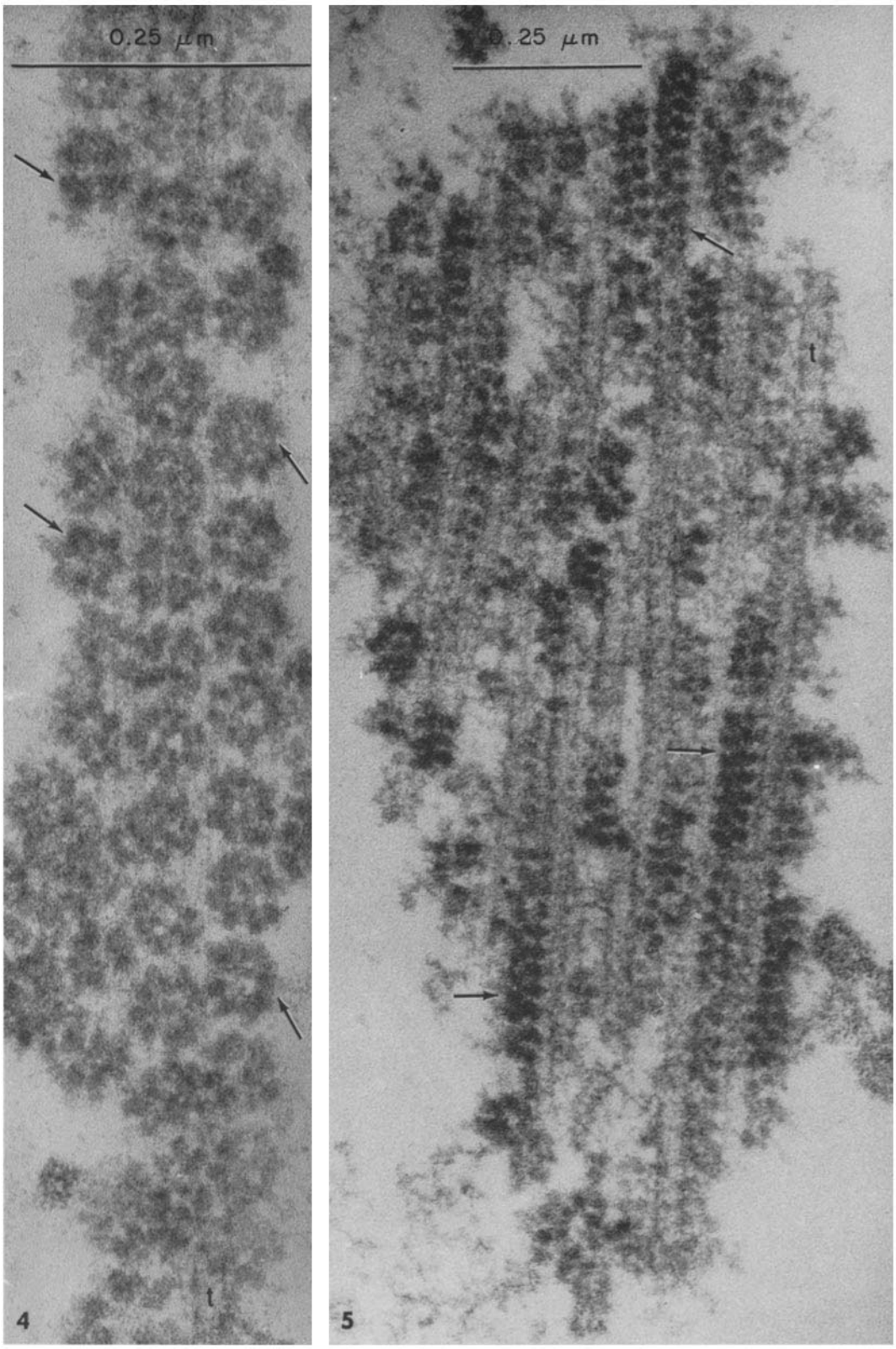
After cellular disruption and washing as described above, the pellets were resuspended in NM B and distributed in aliquots of 0.3 ml containing 3–5 × 10^6 cells. Each aliquot received 0.1 ml of either preimmune or specific antiserum and the mixture was incubated at 37°C for 45 min with frequent shaking. Unadsorbed antibody was removed by diluting each sample with 5 ml of NM B, centrifuging for 5 min at 1,500 *g*, decanting the supernate, suspending the pellet in 5 ml NM B, and repeating the sequence two times more. Pellets of washed cells were suspended in 0.3 ml of NM B, mixed with 30 μl of conjugates of sheep-antirabbit serum coupled with ferritin (2), incubated at 37°C for 45 min and subjected to three cycles of washing and centrifugation as described above. The final pellets were processed for electron microscopy.

Direct Immunological Labeling with Ferritin

The attachment of conjugates of specific rabbit-antibody coupled with ferritin (2) and removal of unadsorbed conjugates followed the protocol employed with the indirect method. The final dilution of the conjugate was 1:4.

Immunological Staining with Horseradish Peroxidase

Cultures of HeLa cells were grown for 18–24 h either directly on glass or on carbon-coated slides



FIGURES 4 and 5 Two examples of microtubule-polyribosome complexes. In Fig. 4, the plane of section was perpendicular and in Fig. 5, parallel to the long axis of the helically arranged polyribosomes indicated by arrows. L × 190,000 (4); L × 120,000 (5).

with NM A. The NM was washed away by three rinses in PBS (8) and the slides were plunged into 100% acetone chilled to −40°C with dry ice, fixed for 30–60 s, rapidly air dried, and stored at −70°C. Subsequently, from 1 wk to 6 mo later, the cultures were warmed to room temperature, washed twice for a total of 10 min in 0.01 M PBS, pH 7.2, and (*a*) covered with antiserum diluted 1:8 or 1:16, incubated in a humidified chamber at room temperature for 30 min, washed four or five times in PBS for 10 min at room temperature; (*b*) covered with a layer of the bridging antibody of sheep-antirabbit globulin diluted 1:50 in PBS, then incubated and washed as in (*a*); (*c*) covered and incubated with rabbit antiperoxidase serum diluted 1:50 in PBS, then washed as in (*a*); covered with 0.5 mg/ml solution of peroxidase type VI (Sigma Chemical Co., St. Louid, Mo.) in PBS, incubated, then washed as in (*a*); (*d*) placed in modified Karnovsky's DAB solution (23), consisting of 0.75 mg/ml of 3′,3′-diaminobenzidine (Sigma) in 0.01 M PBS, pH 7.2, with an addition of H_2O_2 to the freshly prepared and filtered DAB solution, at a final concentration of 0.003%, incubated for 10 min, and washed as in (*a*). For light microscopy the cells were dehydrated in alcohol and mounted in Permount (Fisher Scientific Co., Pittsburgh, Pa.). For electron microscopy the cultures were fixed either in 1% OsO_4 in PBS for 20 min or in 2.5% glutaraldehyde for 5 min, dehydrated, and embedded. After polymerization segments of embedded cells were sectioned as described previously (6, 11).

Electron Microscopy

Pellets of disrupted cells were fixed for 3–5 min with 2.5% glutaraldehyde buffered at pH 7.2 with M/20 PO_4 buffer, postfixed for 30 min with a solution of 1% OsO_4 in the same buffer, dehydrated, embedded in epoxyresin mixtures, sectioned, and stained conventionally with uranyl acetate and lead citrate. Both stained and unstained thin sections were examined in a Siemens Elmiscop I or Phillips EM300 electron microscopes equipped with anticontamination devices at magnifications of 4,000–60,000. Images were recorded on Kodak Electron Image fine grain plates (Eastman Kodak, Rochester, N. Y.).

RESULTS AND DISCUSSION

The Fine Structure of the Mitotic Apparatus and Interrelationship of Its Organelles Following Cell Disruption

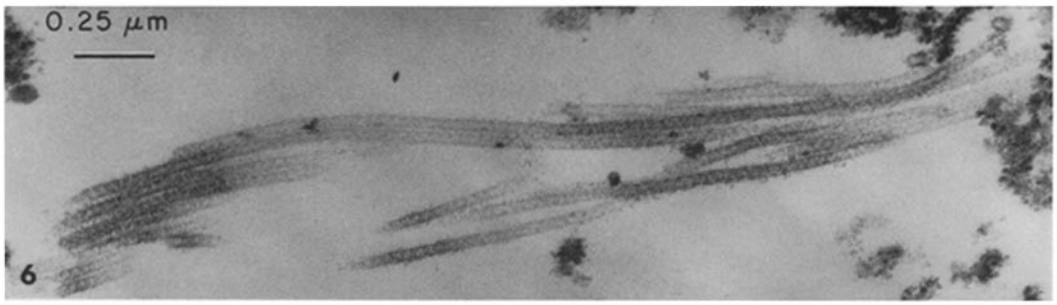
Disruption of vinblastine-treated cells by means of the nonionic detergent resulted in the removal of the plasma, nuclear, and cytoplasmic membranes, as well as membraneous organelles, leaving cell remnants that could be sedimented into pellets at 1,500 *g* for 15 min. Stripping of membranes by detergent, although somewhat variable from one experiment to the next, was greater using 1% than 0.5% NP_{40}. The pellets of subcellular material sometimes consisted almost entirely of nuclei, centrioles, PC, groups of microtubules, and bundles of 80 Å microfilaments. These organelles were closely associated with one another indicating that they had been sedimented as a unit (Figs. 1–3). Interconnections between the microfilaments and the other elements in each organelle complex were revealed at higher resolution. The microfibrils appeared to emanate from the peripheral chromatin, the PC, microtubules, and centrioles including the "rootlets" and to form links between these organelles (Figs. 2, 14, and 15). Rootlets or bundles of microfibrils such as those illustrated in Figs. 3, 14 and 15, are usually not recognizable within sections of intact HeLa or L_2 cells, presumably because the cytoplasmic ground substance obscures them. Identical rootlet struc-

FIGURE 6 A bundle of microtubules in which a regular periodicity indicates helical coiling of the walls. L × 42,000.

FIGURE 7 A ribosome-microtubule complex in which the periodicity of the wall is evident and uncoiling into a loosely helical configuration apparent (arrow). L × 80,000.

FIGURE 8 Predominantly cross section through a group of microtubules in which a subunit structure of the walls is clearly evident. The inset shows a single microtubule in which the "wall" consists of 12–14 morphological subunits. L × 120,000; inset, × 240,000.

FIGURE 9 A PC in cross section reveals the tightly packed tubules composing its mass. At the periphery are situated microtubules whose externally situated walls consist of the denser and larger subunits (arrows) evident also in loosely aggregated microtubules. (See Fig. 8.) A single microtubule of the latter type is also evident (double arrows) and may be part of the PC. L × 120,000.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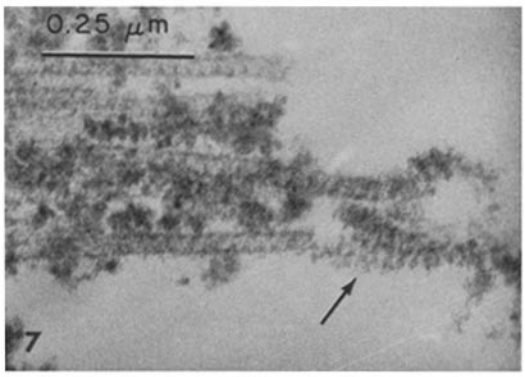
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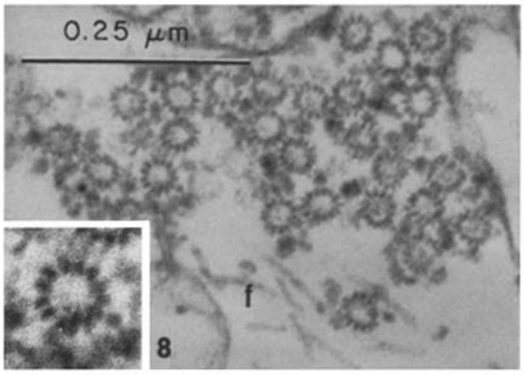
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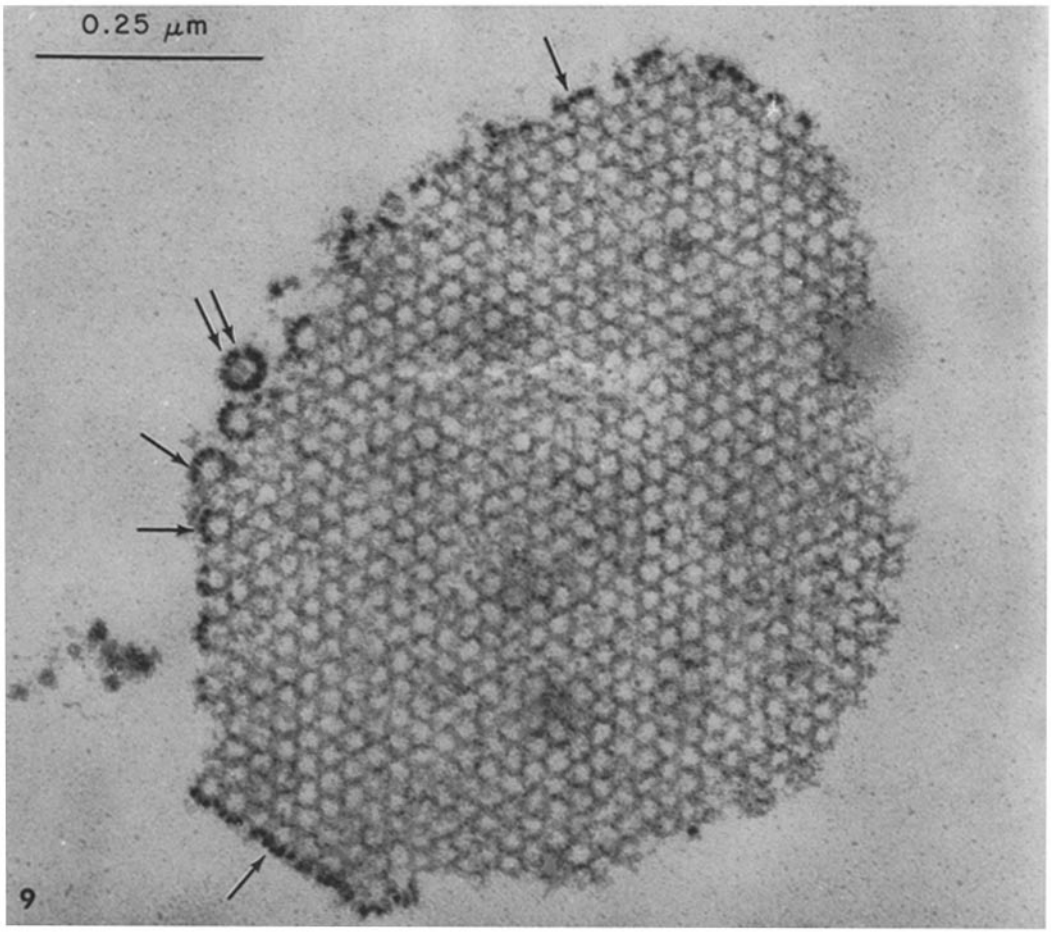
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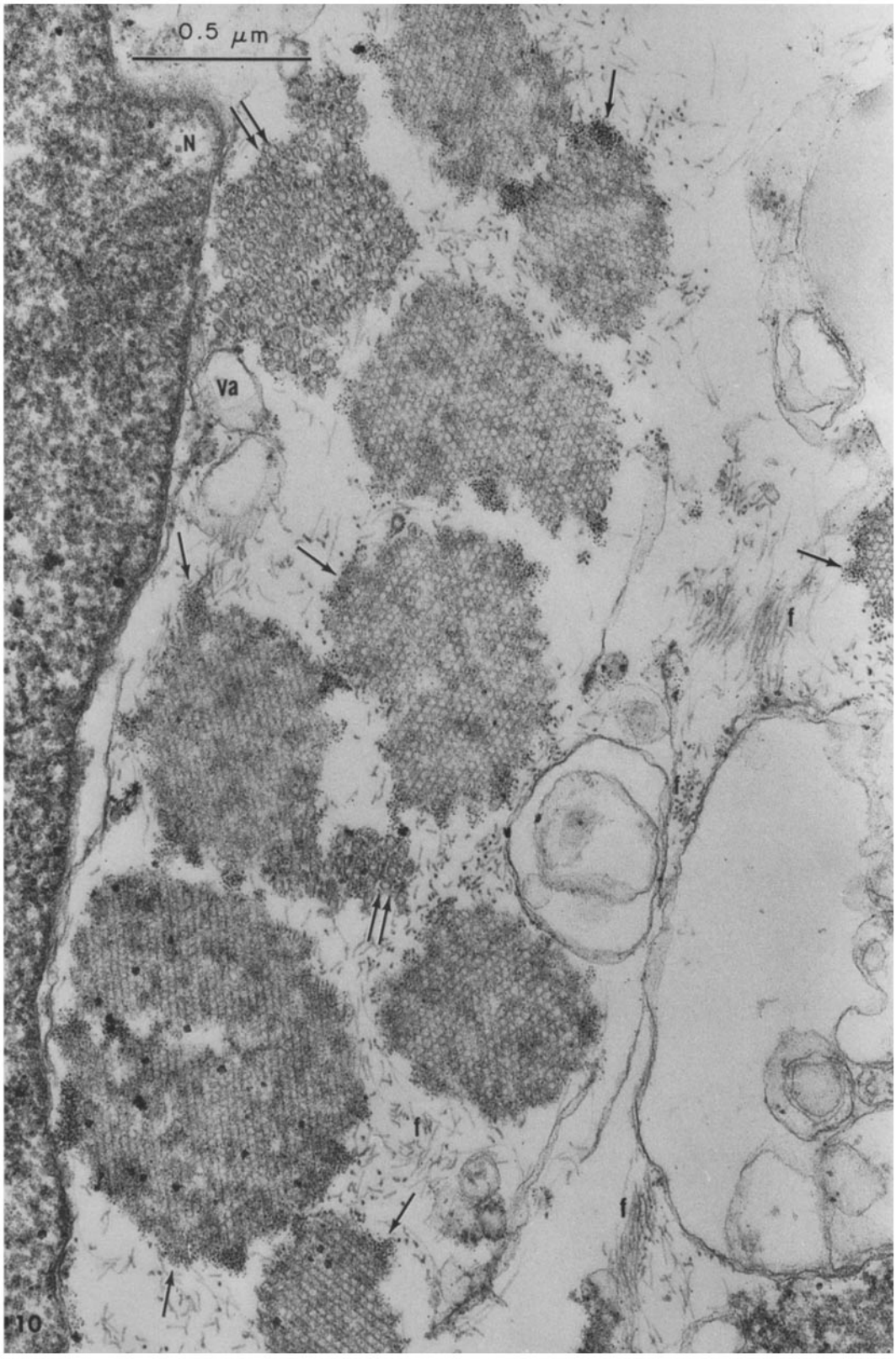


FIGURE 10 Low power image of a ruptured cell from a preparation subjected to labeling with ferritin antibody by the indirect procedure. Each PC has a coating of ferritin (arrows) frequently in large clumps. In the interior of each PC only a few, singly distributed ferritin molecules are discernable. Ferritin, albeit in lesser amounts, is also evident in the vicinity of loosely aggregated microtubules (double arrows). H × 56,000.

tures are commonly associated with the basal bodies of cilia and flagella (see 1 for additional references) and have recently been recognized within intact endothelial cells (15).

Following treatment with vinblastine, the tubulin(s), constituting the material of microtubules (22), were frequently organized as bundles of tightly aggregated microtubules associated with helices of polyribosomes (Figs. 4 and 5), or as loosely aggregated microtubules (Figs. 6 and 8), or as PC (Fig. 9).

An association between polyribosomes and microtubules identical to that illustrated in Figs. 1, 4, and 5 was previously observed within intact cells (12). Survival of these complexes after extensive disruption of cells, observed by us, implies that the polyribosomes have intimate structural links with the microtubules. The size of individual polyribosomes and their disposition among the microtubules was highly regular, as revealed by transverse and longitudinal sections (Figs. 4 and 5). Assuming that each turn of the helix contained about four ribosomes, it can be estimated that there were 30–40 ribosomes in each array. The biological significance of these complexes is at present unknown. It is, however, known that vinblastine causes a redistribution of the tubulins, giving rise to various types of microtubule assemblies. It seems plausible that nascent tubulin polypeptide chains, while still attached to the translational units that include the polyribosomes, are integrated by a process of self-assembly into polymeric structures of a higher order, namely the microtubules. Integration of the attached polypeptides would be expected to occur at widely separated intervals, so as to provide the space

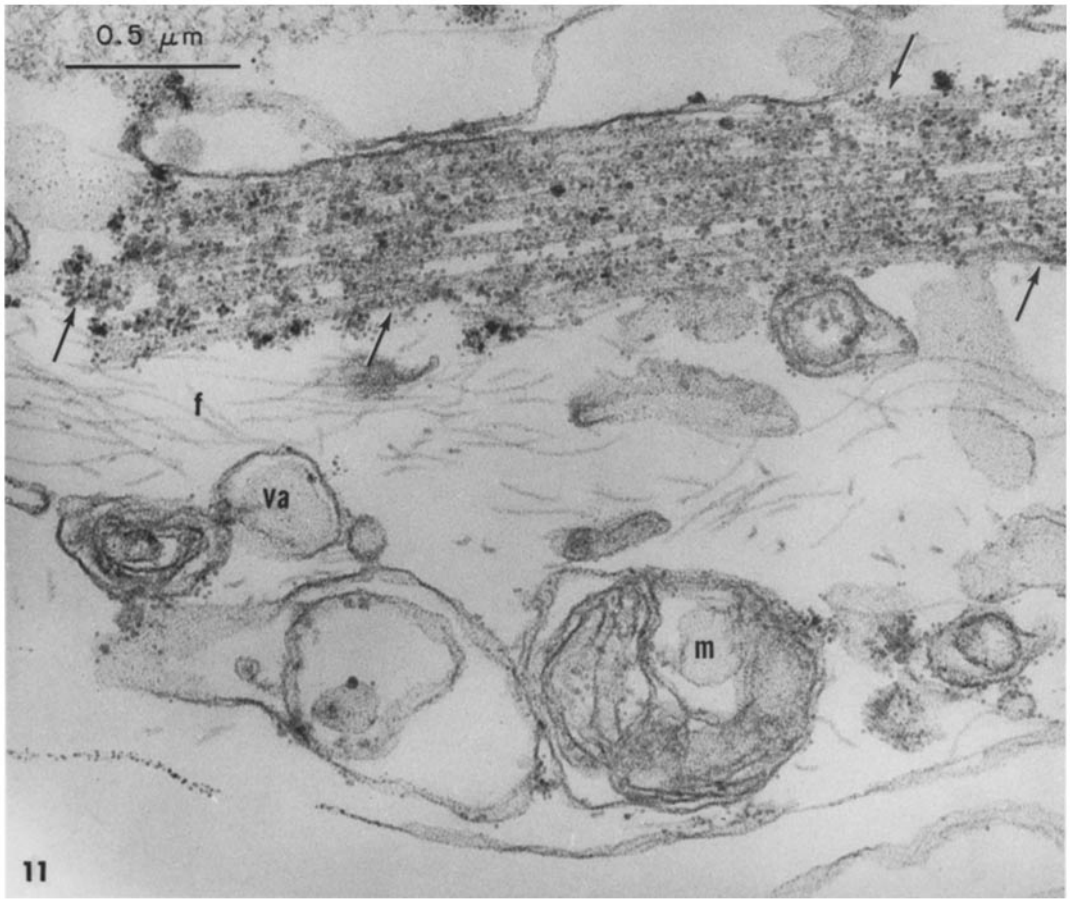
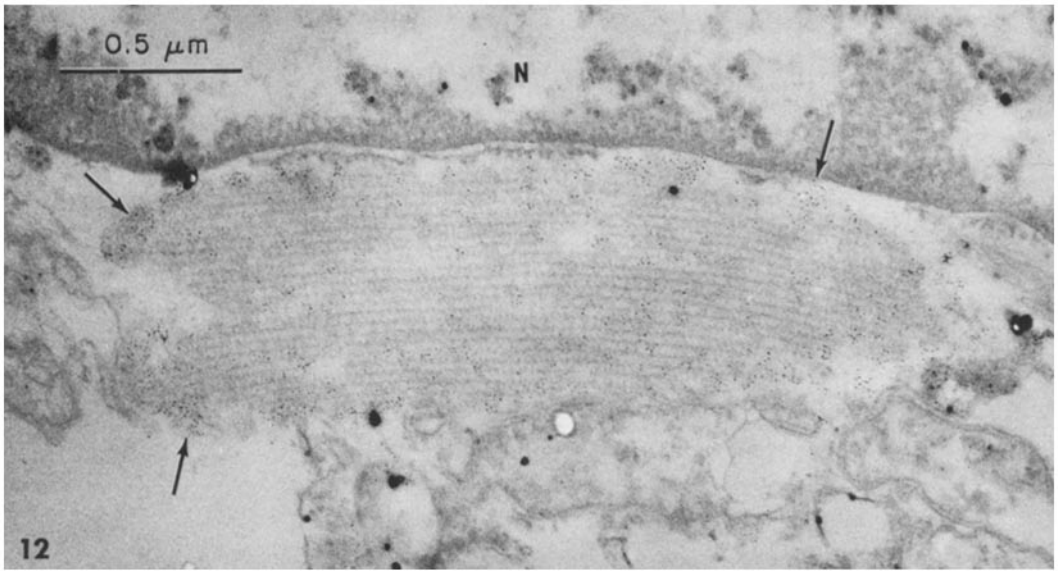


FIGURE 11 A bundle of loosely aggregated microtubules sectioned parallel to their long axis. The ferritin label, applied by the indirect method, is evident in association with the microtubules (arrows). L × 46,000.



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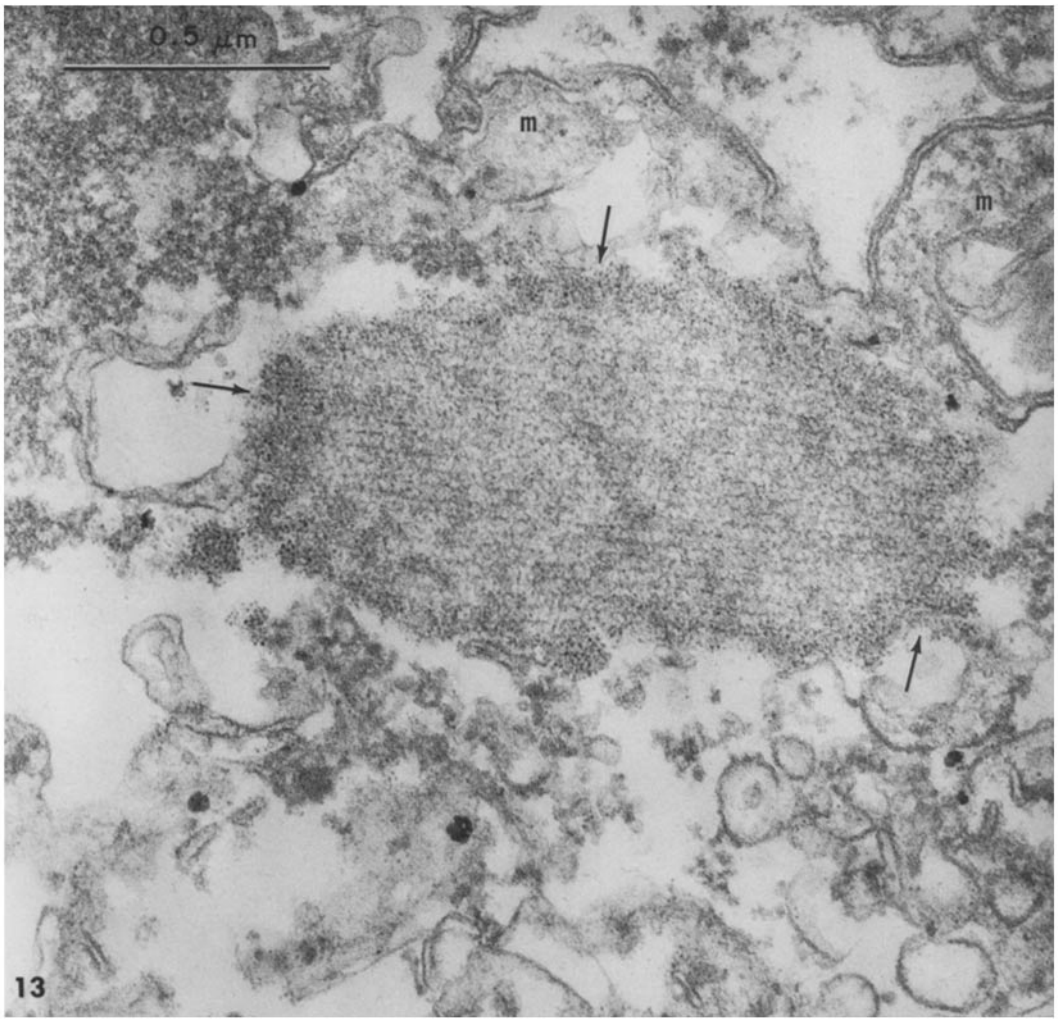

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necessary to accommodate the bulky polyribosomal helices. This idea implies that during self-assembly most of the subunits in the microtubules within such complexes are derived from free molecules and relatively few originate from molecules that are still bound to polyribosomes. From the foregoing it would be predicted that treatment of cells with inhibitors of translation should, upon the addition of vinblastine, abolish the development of ribosome-microtubule complexes. Such an effect can in fact be observed following application of the inhibitor streptovitacin A (our unpublished data). Partial inhibition of complex formation was observed by others employing cycloheximide and puromycin (13). Treatment with streptovitacin A did not, however, prevent the development of either the PC or the other type aggregate (unpublished observations).

The walls of loosely aggregated microtubules sometimes possessed regular periodicity when viewed in longitudinal sections (Fig. 6). Occasionally the ends of such microtubules appeared to be unfolded into a loose helix (Fig. 7). This observation suggests that in living cells the microtubules are wound into tight helices. Similar conclusions have been drawn from studies on whole mounts of isolated microtubules (20). A complex organization of microtubules was also suggested by their appearance in cross section (Fig. 8). Each tubule wall was composed of loosely aggregated dense subunits, about 12–14 in number, consistent with the 13 subunits that were observed in cross sections of other microtubules (19, 20), implygin that walls of microtubules in HeLa and L cells are organized from 13 protofilaments.

The PC observed in our preparation consisted of tightly packed microtubules (Fig. 9) arranged exactly like those described intracellularly in the original work by Bensch and Malawista (3). An additional feature of PC observed in the current study were peripheral tubules whose external walls contained distinctive subunits (Fig. 9), such as those that were evident in the transversely sectioned microtubules illustrated in Fig. 8. The diameter of microtubules within the PC was about 280 Å, while in the loose aggregates it ranged from 320 to 450 Å. By comparison, the width of microtubules of untreated HeLa or L cells examined at interphase or during mitosis, was approximately 230–250 Å. Our evidence, presented in Figs. 4–9, shows that the width of microtubules formed under the influence of vinblastine is highly variable, suggesting that the tightness of helical coiling of the protofilaments can vary depending upon the closeness of packing between the subunits. This type of pleomorphism has been recognized in other instances, as for example following hypothermia of the Heliozoan *Actinosphaerium*, in which short segments of wider than normal microtubules become evident (21).

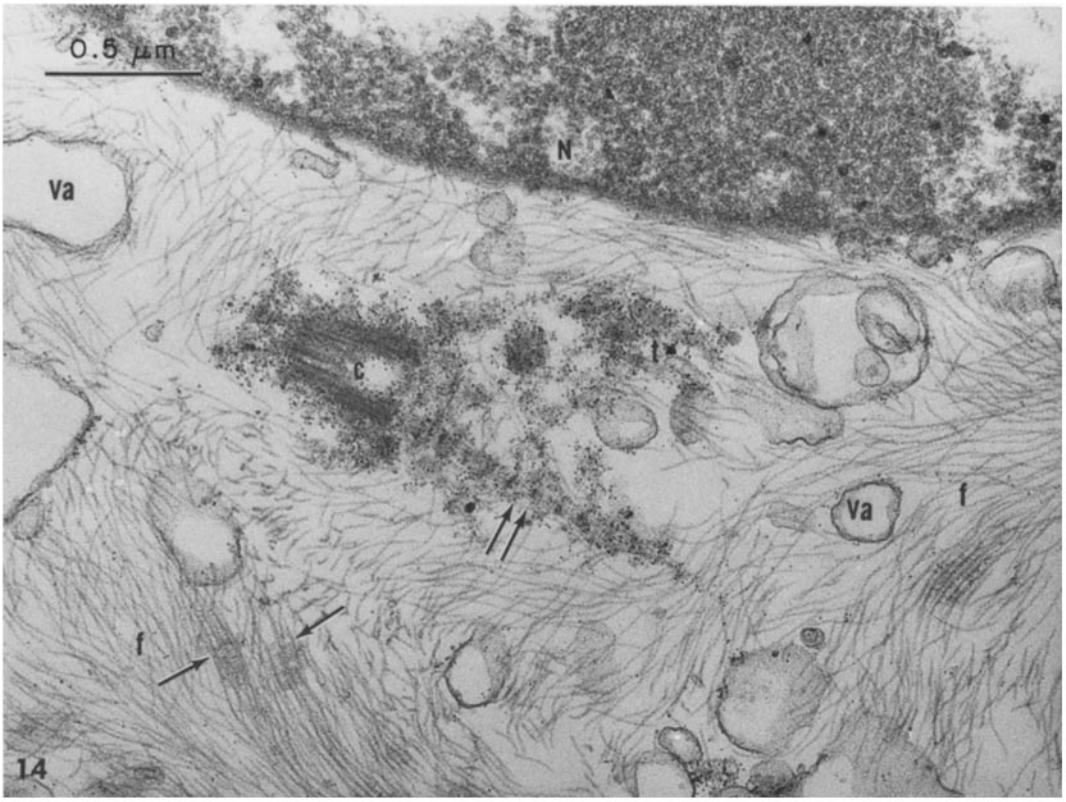
Immunological Labeling with Ferritin Antibody Conjugates

Dispersal of the cellular membranes by means of 0.5% NP_{40} removed the permeability barrier allowing for a free access of antibody and ferritin-antibody molecules to the microtubules and PC. Thus, it was possible to label specific antigen(s) employing rabbit antisera previously shown by means of fluorescence microscopy to be highly specific for the mitotic spindle and PC (17, 5).

Both direct and indirect techniques were used to demonstrate specific immune labeling in HeLa and L_2 cells. The ferritin marker became concentrated at the periphery of the PC or on the surface of microtubules (Figs. 10–13). When the indirect labeling procedure was used, ferritin molecules tended to be distributed in clumps (Figs. 10 and 11), but in the direct method the ferritin was dispersed more uniformly (Figs. 12 and 13). The central portions of PC were either devoid of ferritin or contained a few widely dispersed molecules, suggesting that microtubules at the interior of PC were inaccessible to binding by the antibody. When preimmune sera were employed as controls in either procedure, ferritin was either entirely absent (Fig. 18) or was present in rela-

FIGURE 12 A PC in the vicinity of a nucleus is sparsely coated by the ferritin label (arrows), applied by the direct method. A few ferritin molecules are also evident in the interior of the PC. The section was not stained. H × 48,000.

FIGURE 13 A similar example to the one illustrated in Fig. 12 with the exception that the section was stained. Arrows indicate a coating of ferritin on the surface of the PC. L × 70,000.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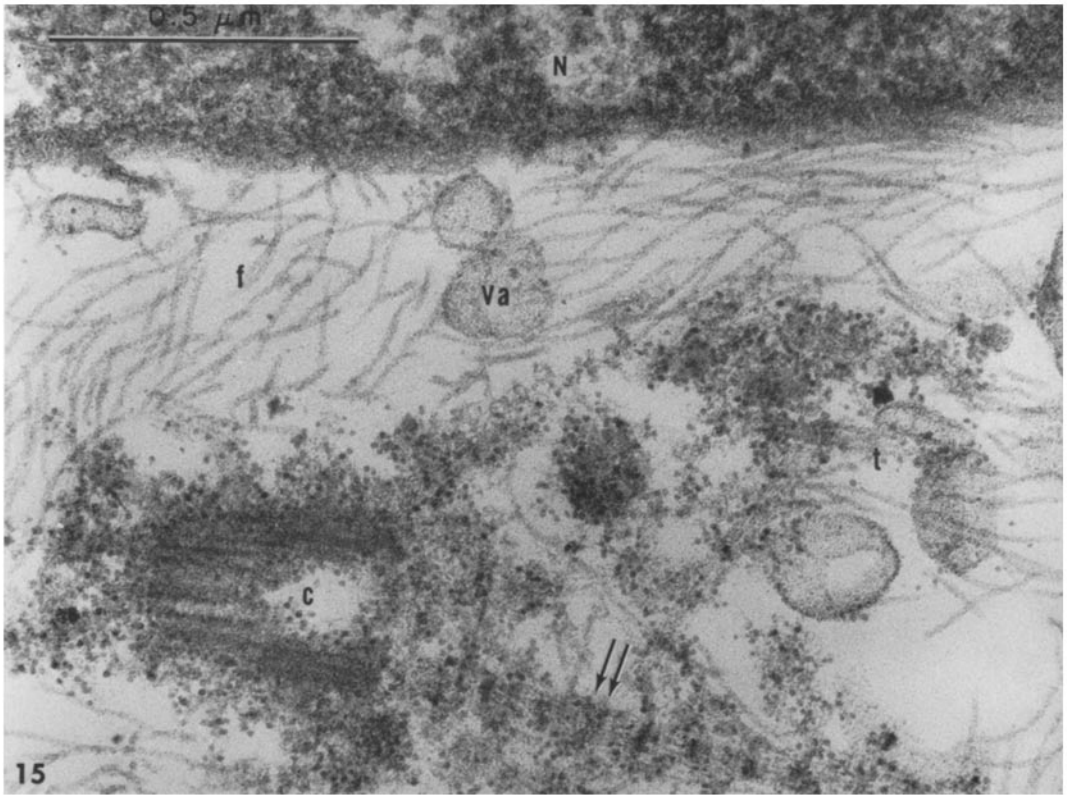
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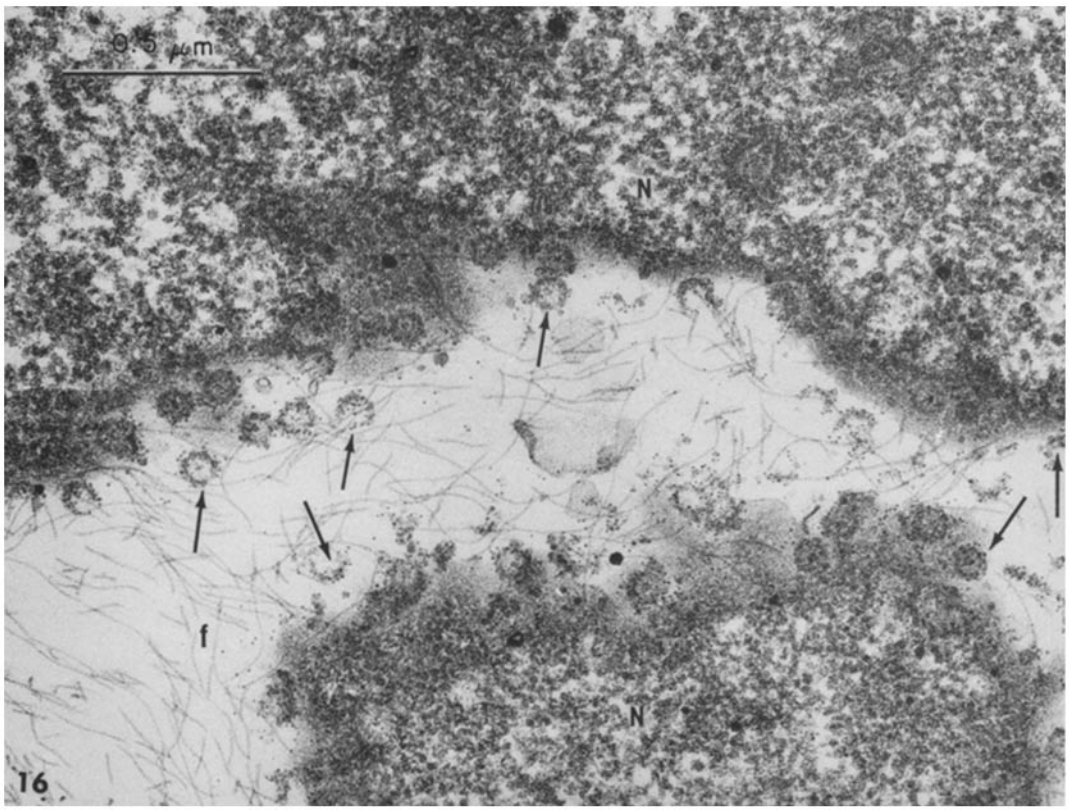


FIGURE 16 Portions of two nuclei showing numerous rings of the nuclear pore complex of which those situated peripherally (arrows) are heavily labeled with ferritin, after labeling by the indirect method. A similar disposition of ferritin was observed in preparations reacted with preimmune sera. L × 53,000.

tively small amounts as randomly scattered molecules. This result confirms previously demont strated specificity of the antibody in these rabbi-antisera (17, 5).

In addition to specific labeling of microtubules and PC the ferritin tag became concentrated on and near the centrioles (Figs. 14 and 15), on annuli of the nuclear pore complex (Fig. 16), and in the vicinity of the annulate lamellae (Fig. 17). Incidentally, the annulate lamellae increase in number after treatment with vinblastine (14). Ferritin was notably absent from the 80 Å microfilaments (Figs. 11, 14–17). The significance of the "labeling" of organelles other than the micro-

FIGURE 14 Ferritin labeling by the indirect technique. The marker is distributed in the vicinity of the centriole and associated structures, including the rootlet (double arrow) but is absent from the numerous microfilaments. In some regions the microfilaments are linked into bundles by finely striated cross-bridges (arrows). Some ferritin was observed in a similar location in preparations reacted with preimmune sera. L × 41,000.

FIGURE 15 An enlargement of the image in Fig. 14 reveals the ferritin labeling by the indirect procedure more clearly. Note the microfilaments emanating from the nucleus and apparently connecting the centriole-microtubule-nucleus complex of organelles, as in Figs. 1 and 2. The rootlet is identified by a double arrow. L × 82,000.

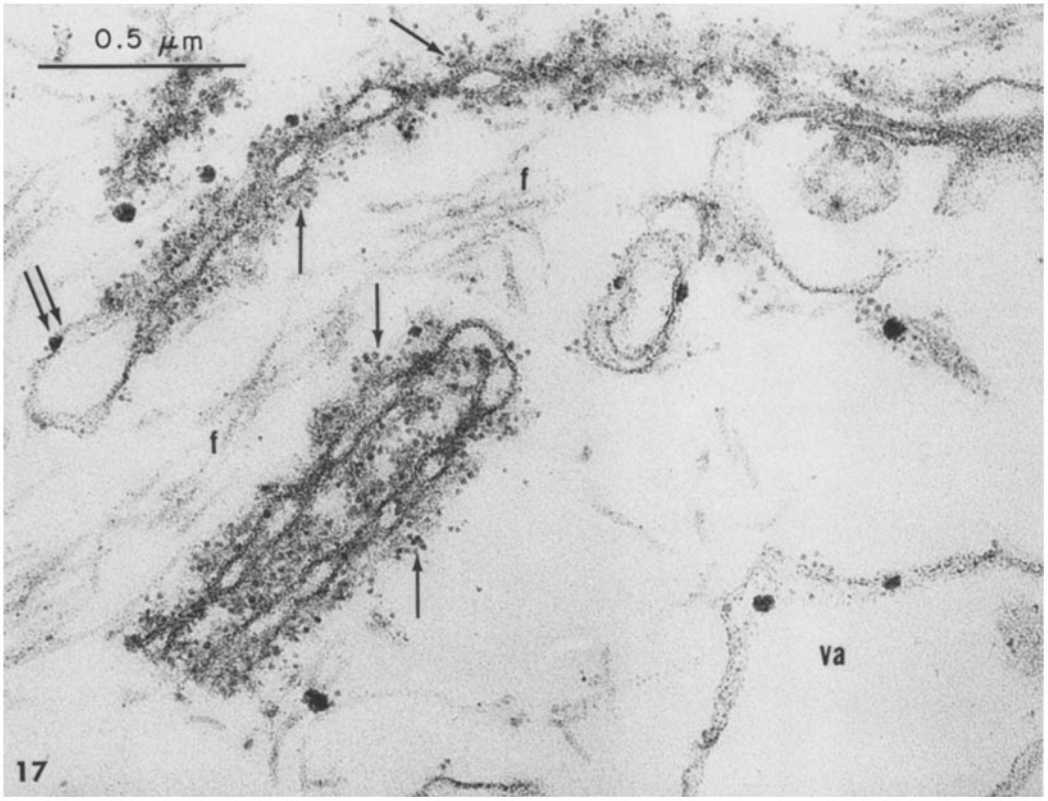


FIGURE 17 Annulate lamellae observed frequently in the cytoplasm of cells treated with vinblastine sulfate. In these profiles the ferritin, deposited when the cells were labeled by the indirect method, occurred in the vicinity of the pores (arrows) but was absent from contiguous segments of membrane (double arrow). Compare with the image in Fig. 19. H × 55,000.

tubules became problematical when the ferritin label was also detected in the controls that had been reacted with preimmune sera (Fig. 19). The possibility that specific labeling, especially of the centrioles and related components, was being visualized should not be completely discounted at this time because the number of ferritin particles observed in the controls was much less than in the experimental samples. In future work optimum conditions necessary to reduce the nonspecific binding of ferritin will have to be ascertained in order to demonstrate conclusively a specificity of binding or a lack of it due to the possible occurrence of a tubulin component in centrioles and/or the nuclear pores and the structurally related annulate lamellae. In considering the nonspecificity of ferritin binding it is worthwhile to mention that nuclear pores of the mature oocytes of *Rana pipiens* have been shown to possess a high avidity for colloidal gold particles (9), and perhaps also to bind indiscriminately ferritin molecules in the same manner.

Immunological Labeling by the Horseradish Peroxidase Procedure

As a further check on the specificity of the ferritin-antibody method we applied the more recently developed labeling with horseradish peroxidase (2). This technique is based upon the *in situ* formation of opaque deposits at the site of attachment of specific antibody to the antigen being localized. Because of the very numerous steps involved in the immunoperoxidase procedure (see Materials and Methods), use of suspensions of disrupted cells in the way they were employed for ferritin labeling would incur unacceptable losses of material and increase the chance

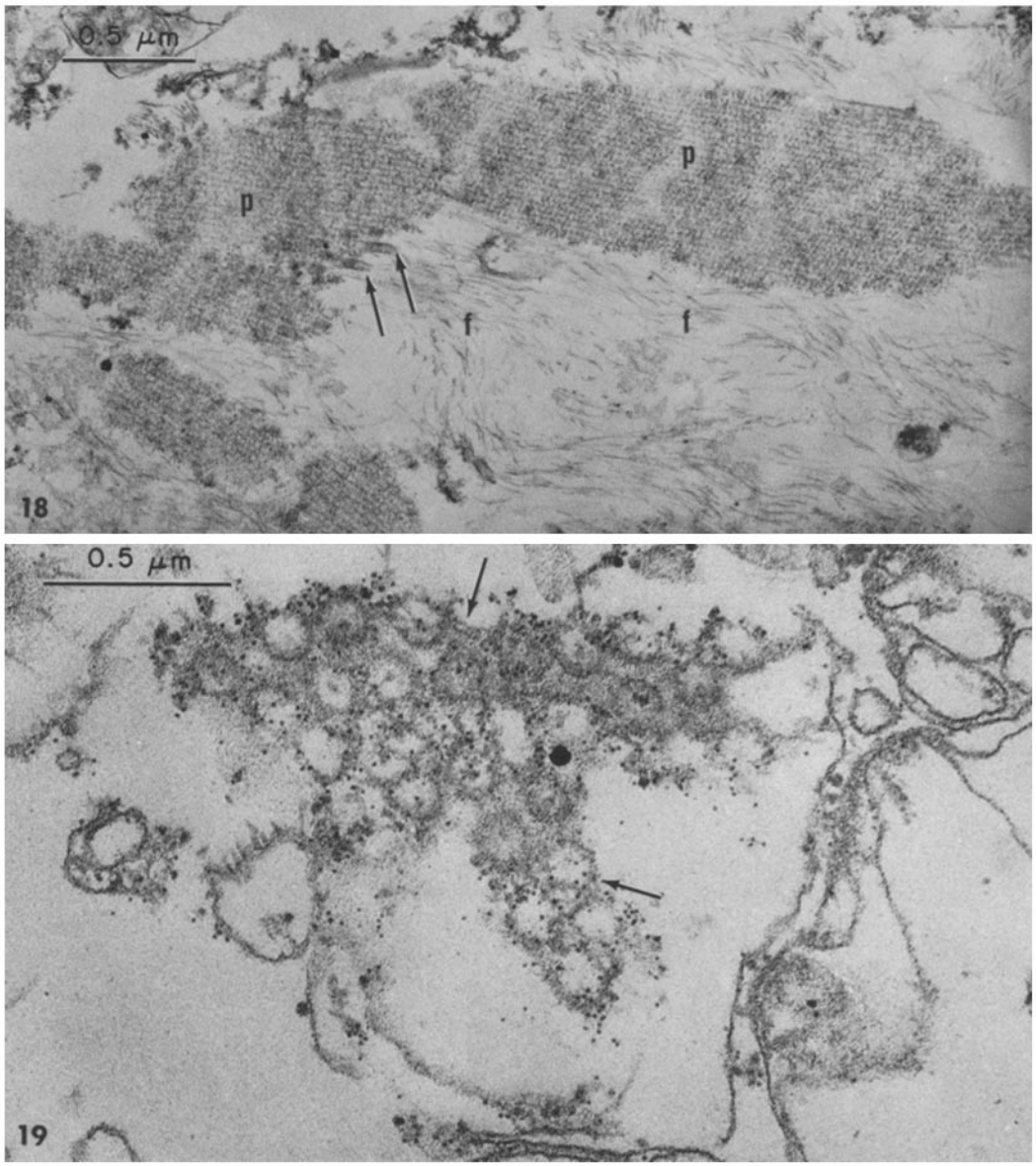
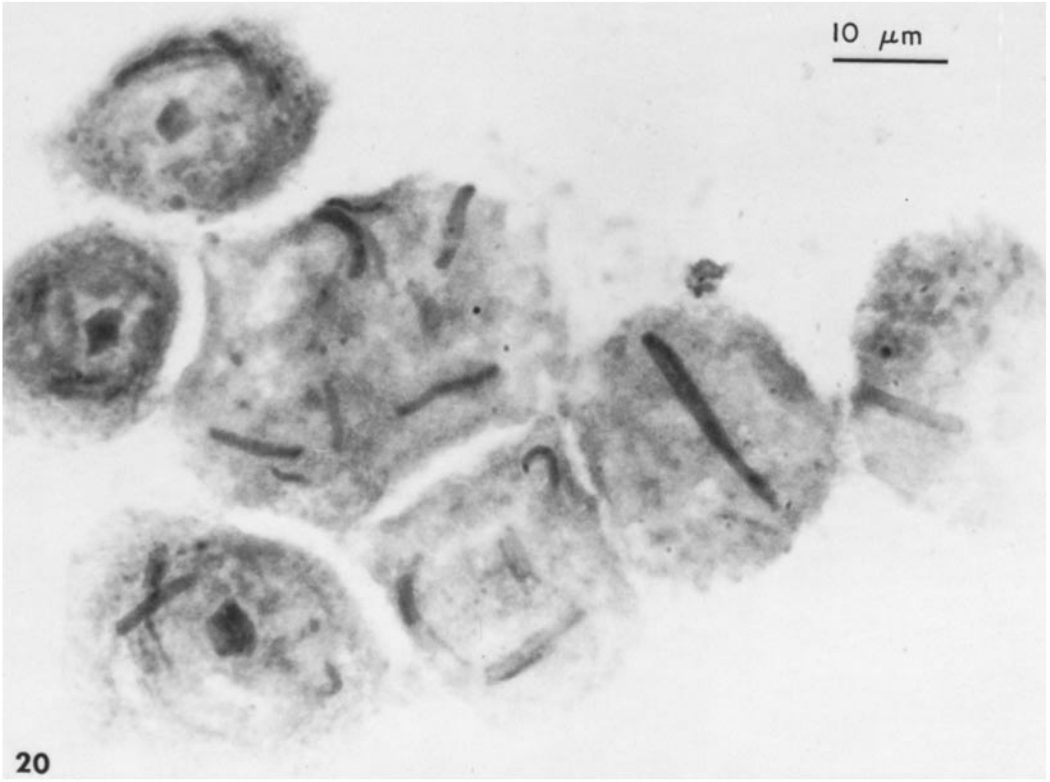


FIGURE 18 Cytoplasm of a cell labeled by the indirect immune procedure. In this case preimmune serum was applied before the addition of the sheep-antirabbit-ferritin conjugate. Note the absence of ferritin from the PC. H × 36,000.

FIGURE 19 A group of annulate lamellae exposed *en face* are encrusted with ferritin molecules, apparently indicating nonspecific labeling of these organelles. H × 50,000.

of damaging beyond recognition the structure of the organelles under investigation. For these reasons we instead fixed rapidly in cold acetone cells attached to glass slides, simultaneously rendering them permeable to the antibody. Following reaction with specific antisera the intracellular PC were densely stained and prominent when viewed by bright field light microscopy (Fig. 20). By comparison PC in control preparations reacted with preimmune sera possessed a



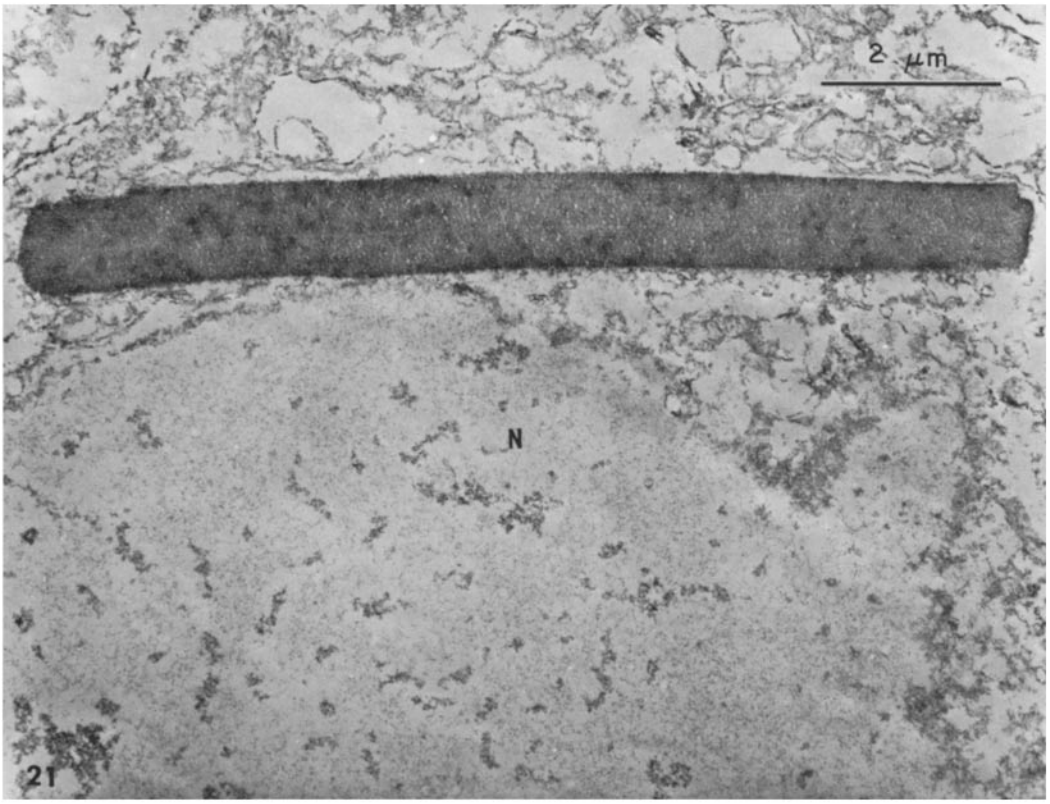


FIGURE 20 Staining by the horseradish peroxidase method. The dense PC are prominent in cells photographed in bright field. H × 1,500.

FIGURE 21 Thin section of a cell containing a dense, prominent PC, following immunological staining by the peroxidase technique. H × 12,000.

faint rim of stain and PC in controls maintained only in PBS as a substitute for either immune or preimmune serum were entirely devoid of precipitate. Examination by electron microscopy of thinly sectioned material from parallel cultures, whether postfixed with glutaraldehyde or OsO_4, revealed intracellular dense structures of the size and shape of PC (Fig. 21). It should be noted that fixation with acetone failed to preserve in any recognizable manner the fine structure of cell organelles, including the tubular nature of the PC. A uniform density throughout their mass indicates that after fixation with acetone the interior of PC became permeable to antibody. Therefore, our results using immunological labeling with peroxidase substantiated the specificity of labeling using ferritin-antibody conjugates reported here and in our previous work using immuno-fluorescence (17, 5).

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