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The nature of the clear zone around microtubules

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Summary. The clear zones seen around microtubules in transverse sections of nutritive tubes vary in size depending on whether a microtubule is bordered by ribosomes or by another microtubule. We consider that such a finding is not consistent with the current view, that the clear zone is maintained by microtubule-associated material. It can, however, be accounted for by an electrostatic repulsion between the surfaces of negatively charged microtubules and between microtubules and ribosomes which are also negatively charged. The experiments presented here, involving on the one hand the addition of cationic substances to microtubules and on the other the alteration in charge of the microtubules, support this hypothesis.

Key words: Microtubules – Clear zones – Cations

Almost since microtubules first began to be described in cells it was noticed that they are invariably surrounded by a clear zone which appears electron opaque, giving the impression of a "halo" around each microtubule when viewed in transverse section (Porter 1966). Naturally enough, early studies concentrated on the microtubules themselves and the clear zones around them were even dismissed as being artifacts of fixation (Maser and Philpott 1964). However, subsequent investigations using alternative fixation and staining techniques as well as freeze-etching (see Stebbings and Willison 1973) have confirmed that the region immediately surrounding a microtubule is seldom encroached upon by other organelles, and also that microtubules only rarely make contact with each other.

It became the presumption that the clear zone contained components which could not be visualised by the conventional preparative procedures and attempts to stain the clear zone using other techniques were quite numerous. It was found, for

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example, that ruthenium red and lanthanum hydroxide, which bind to polyanionic substances such as mucopolysaccharides, stained that part of the clear zone immediately surrounding the microtubule (Tani and Ametani 1970; Lane and Treherne 1970), but none succeeded in staining the entire clear zone and the emphasis on this approach waned when it was realised that such polycationic substances bind to tubulin itself.

More recently, attention has concentrated in a similar direction towards the possibility that the clear zone is occupied by a fine filamentous coating of protein which is of low average density compared to the microtubules, and the belief that this might be sufficient to prevent large particles approaching the microtubule walls (Amos 1979). In this connection it is pointed out that microtubule associated proteins (MAPs) appear as fine filamentous projections on microtubules assembled in vitro, and that the spacings of microtubules vary when tubulin is assembled with and without such MAPs (Kim et al. 1979).

The problem arises as to whether what has been demonstrated in vitro occurs in the living cell where projections are not seen with such regularity. In addition, although tubulin assembled in the absence of MAPs does indeed form microtubules which are more closely spaced than those assembled with MAPs, microtubules formed solely from tubulin do not actually touch (Herzog and Weber 1977) and for these reasons alternative explanations for the clear zones must be sought.

Clear zones are particularly conspicuous around microtubules which are bordered by other electron dense cytoplasmic components, as occurs, for example, in the nutritive tubes of insect ovaries. Here the microtubules are themselves quite closely packed in a parallel array and interspersed with ribosomes which are transported along their lengths (Macgregor and Stebbings 1970). Such a system lends itself well to experimental investigations of the clear zone since individual nutritive tubes containing their microtubule and ribosomal components can be teased from ovaries by micromanipulation (Hyams and Stebbings 1979a). The surrounding membrane can then be removed by detergent treatment, whereupon the ribosomes disperse leaving the microtubule bundle free in the isolation medium. In this way, microtubules which were known to be surrounded by clear zones can be dissected directly from the ovaries for experimentation.

In this study we have refocussed on the fact that polycationic substances associate with the surfaces of microtubules which are anionic at physiological pH and that they bind by non-specific electrostatic interactions (Erickson and Voter 1976). Our approach has been to determine the spatial arrangements of microtubules before and after their release from nutritive tubes, on addition of cationic substances, and after varying the negativity of the microtubules by adjustment of their pH. The significance of the spacings, and the experimental alteration in the spacings of the microtubules, to an understanding of the nature of the microtubule clear zones is discussed.

Materials and methods

Ovaries of *Notonecta glauca* were dissected in insect Ringer and individual ovarioles teased apart. These were desheathed and washed in 0.1 M Pipes (piperazine N,N'- bis 2-ethanesulfonic acid, 1 mM EGTA (ethylene glycol tetracetic acid), 0.1 mM $MgSO_4$ (PEM), pH 6.9. Nutritive tubes were isolated manually from the ovarioles using polarised light as already described (Hyams and Stebbings 1979a). Others were

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Electron microscopy

Comparison of isolated and extracted nutritive tubes. In all cases, isolated and extracted nutritive tubes were supported in 3% agarose (Type VII, low gelling temperature, Sigma) in the appropriate buffer prior to fixation in order to maintain them straight and so aid subsequent sectioning.

Isolated and extracted tubes were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 for 20 min, washed in buffer, and post-fixed in 1% osmium in the same buffer for 1 h.

Ruthenium red. Extracted nutritive tubes were transferred to 3% glutaraldehyde, 0.1% ruthenium red in 0.2 M sodium cacodylate buffer, pH 7.4 for 1 h and then to 1% osmium, 0.1% ruthenium red in 0.15 M cacodylate buffer for 3 h.

Cationized ferritin. Cationized ferritin (Polysciences, 25 mg/ml) was dialysed overnight against PEM. Extracted nutritive tubes were transferred to cationized ferritin for 20–40 min and then washed in PEM to remove excess ferritin. Controls were placed in normal ferritin and treated similarly. Preparations were then fixed in 3% glutaraldehyde in 0.1 M phosphate buffer for 20 min and then in 1% osmium in the same buffer for 1 h.

Effect of pH on spacings of microtubules. Extracted nutritive tubes were bathed in either 0.1 M Mes 2-N-morpholine ethanesulfonic acid), 1 mM EGTA, 0.1 mM $MgSO_4$ (MEM), pH 5.6 or MEM pH 7.2 for periods between 15 and 30 min. They were then fixed at these different pHs in 3% glutaraldehyde in 0.1 M cacodylate buffer for 20 min followed by 1% osmium in the same buffer.

All the fixed specimens were dehydrated in an acetone series and embedded in TAAB resin, formulation "C". Sections were cut on a Reichert Ultracut, stained in uranyl acetate and lead citrate and then examined using a JEOL 100S electron microscope.

Results

Transverse sections of in situ and isolated nutritive tubes show numerous microtubule profiles interspersed with ribosomes which are the only other components seen (Fig. 1). Where microtubules are surrounded by ribosomes these approach no closer than approximately 20 nm, giving the impression of an electron clear zone around each microtubule. On the other hand, where microtubules are seen adjacent to each other, they may be as close as approximately 10 nm.

On addition of the detergent mixture the membranes surrounding the nutritive tubes are removed and the ribosomes disperse leaving only the microtubules. In such extracted tubes the microtubules remain arranged in a parallel bundle and are sometimes scattered and other times clustered together (Fig. 2). In the latter case, although closely spaced, the microtubules do not make contact, but remain separated by distances of approximately 10 nm.

On addition of ruthenium red to extracted nutritive tubes the microtubules pack together closely into a crystal-like array (Fig. 3) with their walls separated only by the ruthenium red (Fig. 4). A similar result is seen with cationic ferritin (Fig. 5) although the microtubules do not pack so closely, being separated by the larger ferritin molecules (Fig. 6). Ordinary ferritin does not bind to the microtubules.

Where extracted nutritive tubes have been bathed and fixed in MEM at pH 5.6

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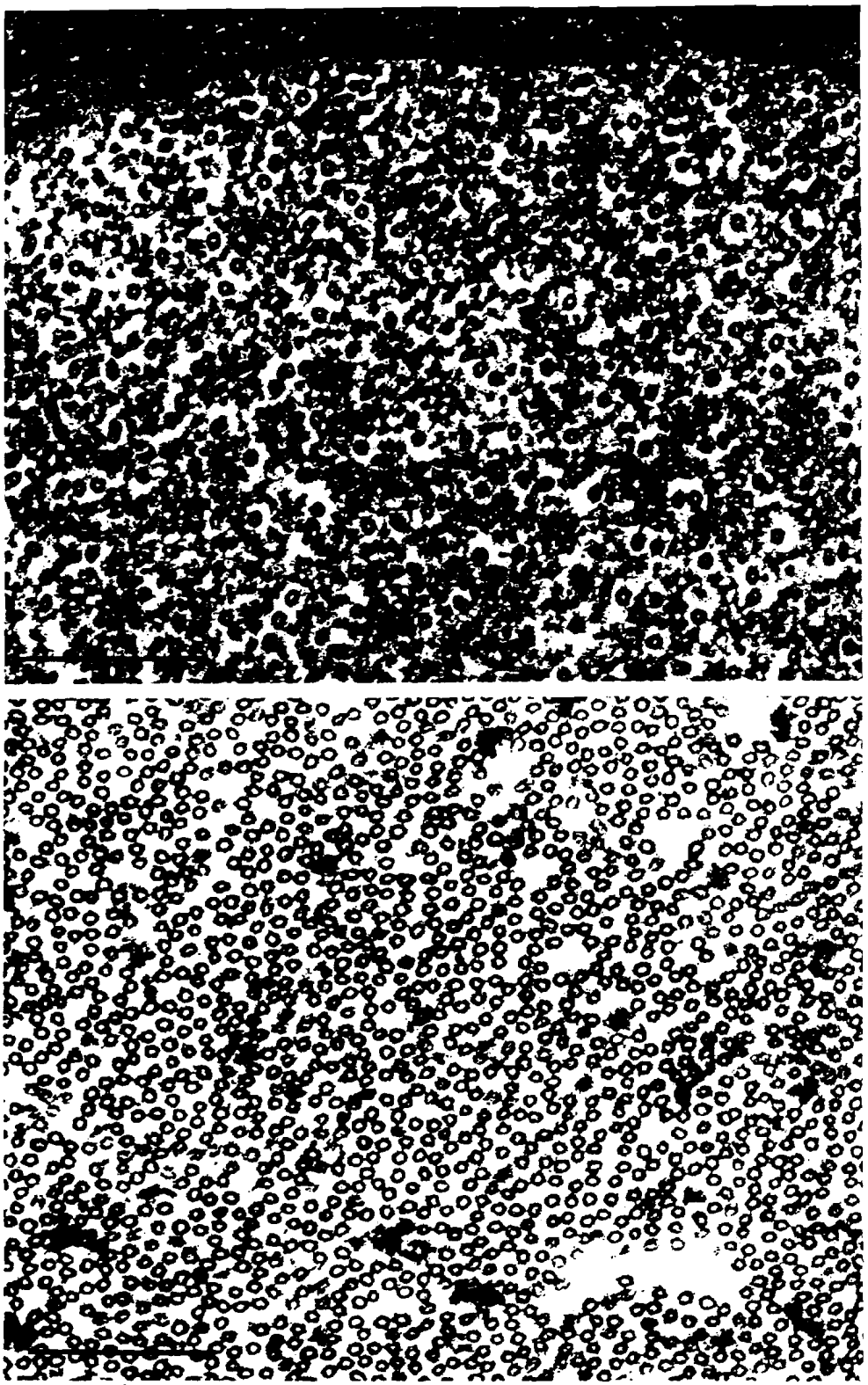
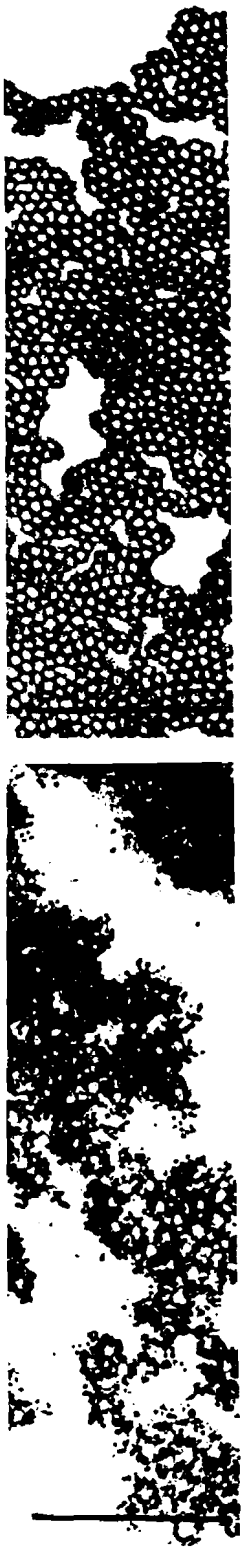


Fig. 1. Transverse section of a nutritive tube showing microtubule and ribosomal components. Clear zones of approximately 20 nm width can be seen around those microtubules bordered by ribosomes, while distances of as little as 10 nm separate adjacent microtubules. Bar = 0.25 μm

Fig. 2. Transverse section through an extracted nutritive tube. Here the microtubules approach to approximately 10 nm, but rarely touch. Bar = 0.25 μm



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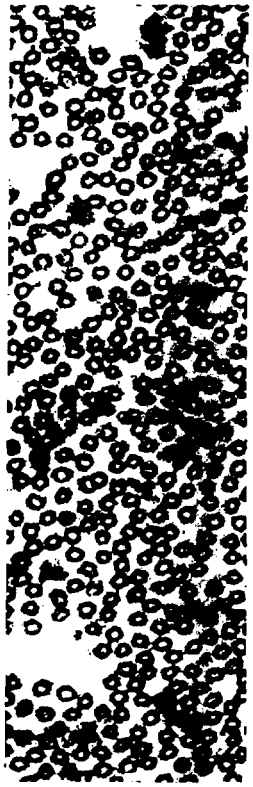

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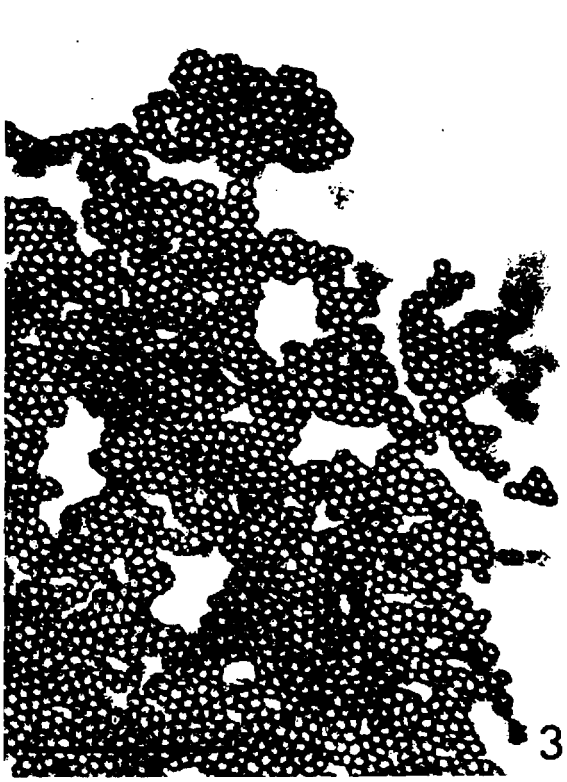

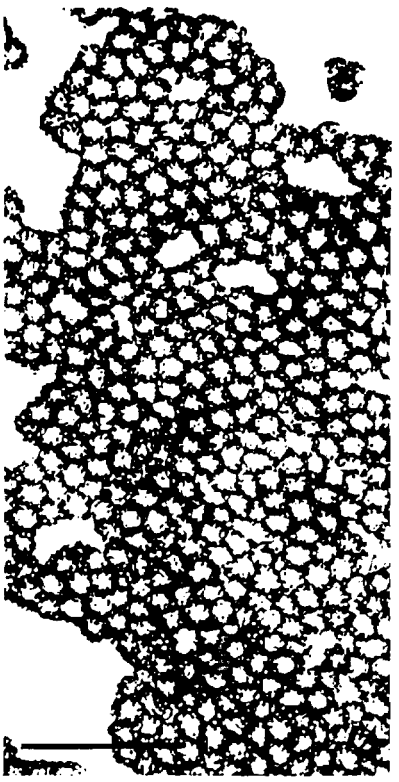

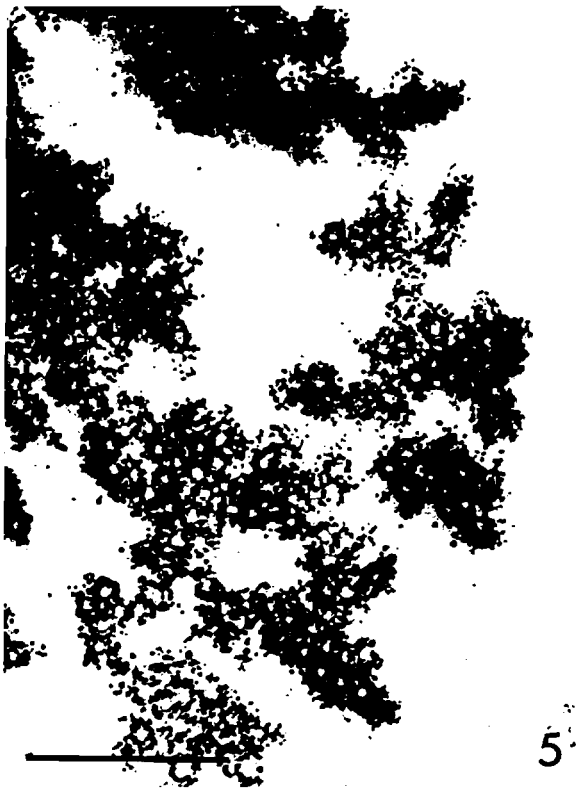

Figs. 3, 4. Transverse sections of extracted nutritive tubes treated with ruthenium red, in which the microtubules aggregated into closely packed crystal-like arrangements. Fig. 3 Bar = 0.25 μm. Fig. 4 Bar = 0.1 μm

Figs. 5, 6. Transverse sections of extracted nutritive tubes treated with cationic ferritin. As with ruthenium red the microtubules aggregate but are separated in this case by the much larger ferritin molecules. Fig. 5 Bar = 0.25 μm. Fig. 6 Bar = 0.1 μm

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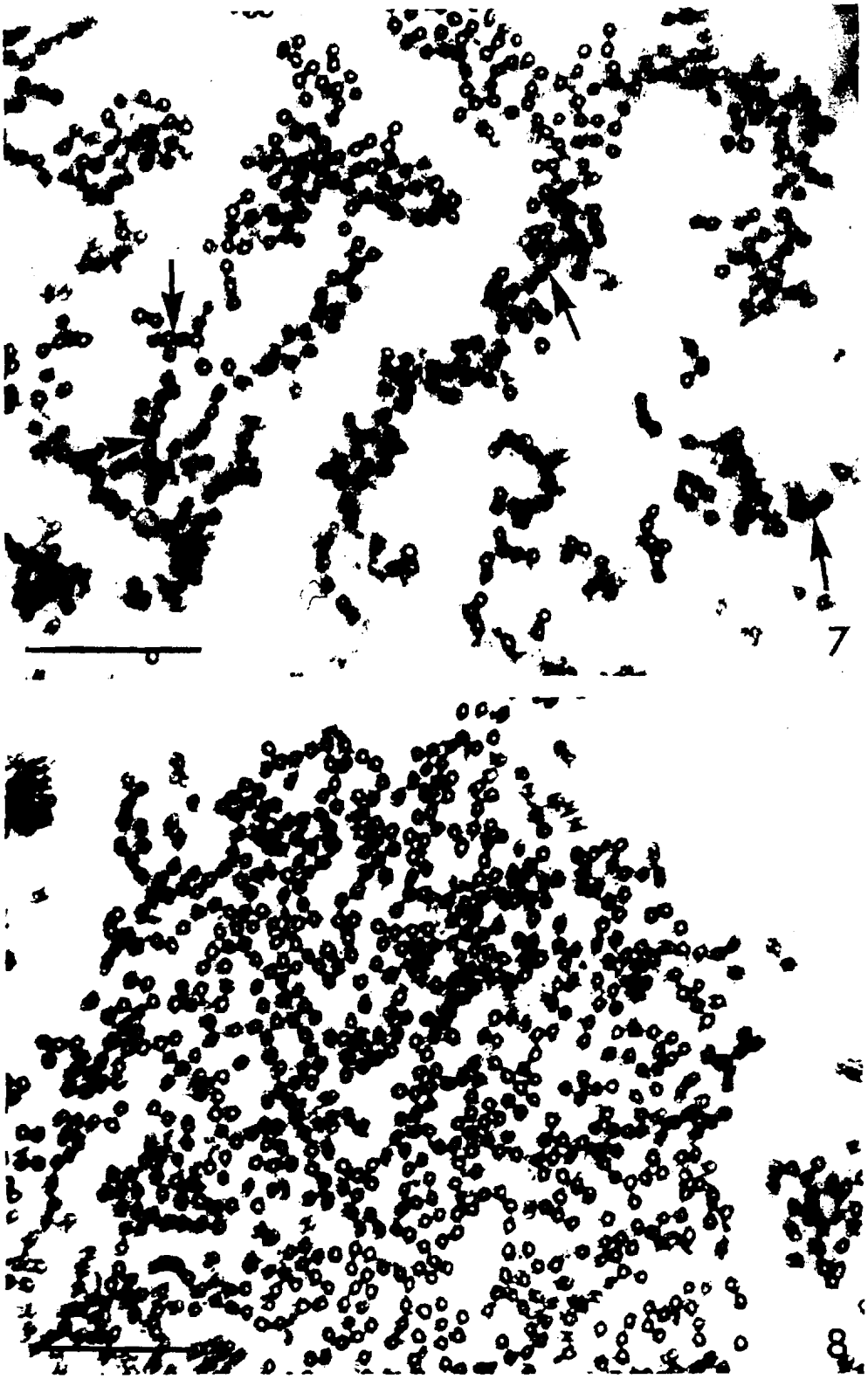

Fig. 7. Transverse section of an extracted nutritive tube bathed and fixed at pH 5.6. Microtubules can be seen to touch and indeed form rows and other groupings (*arrows*). Bar = 0.25 μm

Fig. 8. Transverse section of an extracted nutritive tube bathed and fixed at pH 7.2. In contrast with Fig. 7 the microtubules are not seen to touch. Bar = 0.25 μm

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Discussion

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there are many instances where microtubules actually touch and clusters of three or more microtubules in contact are not uncommon (Fig. 7). By contrast, at pH 7.2 the microtubules are never seen to touch (Fig. 8).

Discussion

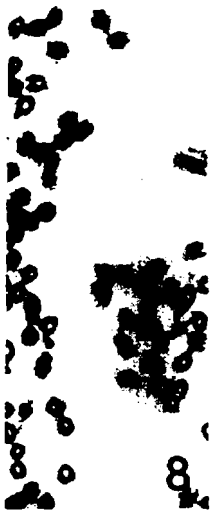
The microtubules in nutritive tubes when viewed in transverse section are seen to possess distinct peripheral clear zones. In this respect they are comparable to those of numerous other cells and tissues where microtubules with clear zones varying in width between 5–20 nm have been reported (see Amos 1979). Such observations are not confined to fixed and sectioned material since similar clear spaces have been seen around microtubules in nutritive tubes after freeze-fracturing (Stebbings and Willison 1973).

To date there has been no satisfactory explanation of the clear zone, and since it is not seen at all in negatively stained material and has been shown to have no structure independent of the microtubules themselves (Stebbings and Bennett 1976) the nature of the clear zone has remained a dilemma.

Most relevant, we feel, is that the periphery of microtubules is strongly anionic, since tubulin is an acidic protein by virtue of its high glutamate and aspartate content. Certainly the fact that their surfaces are negatively charged would explain the "staining" of microtubules by lanthanum hydroxide and ruthenium red (Lane and Treherne 1970; Tani and Ametani 1970), both of which are cationic and have a high affinity for tubulin. It would explain the thickening of the walls of blood platelet microtubules to form "an outer component" on addition of Alcian blue 8GX, protamine sulphate, polylysines and DEAE-dextran, all of which carry multiple positive charges at the pH of the stabilising media used (Behnke 1975). And it is in accord with the view that positively charged groups on MAPs bind to the microtubules by the same nonspecific electrostatic forces (Erickson and Voter 1976).

It seemed logical to ask to what extent might the surface charge on microtubules influence their positioning and arrangement in relation to each other and to other cellular components? In nutritive tubes, microtubules are mainly surrounded by ribosomes which are themselves strongly negative and bind cations and basic dyes. We believe that this could account for their spatial separation which manifests itself as a "clear zone" around each microtubule. Furthermore, in extracted nutritive tubes where the ribosomes are lost, and importantly the microtubules free to move, the latter often become very closely and even hexagonally packed without ever actually making contact – a configuration remarkably comparable to that of microtubules in redundant tubes (Hyams and Stebbings 1979b); and again this could be explained by their being held apart by an electrostatic repulsion between comparable charges of the same sign.

Our experimental addition of cationic substances to microtubules from extracted nutritive tubes supports this view. Here the negatively charged microtubules bind the added cationic components with the result that they associate into closely packed aggregates. This occurs most spectacularly on addition of ruthenium red, which is a small molecule with a molecular weight of 860 and an estimated molecular size of about 1 nm, after which the microtubules appear



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to fuse into a regular crystal-like pattern. Likewise, microtubules are bound and aggregated by cationic ferritin but are not seen to pack so closely in this case since, by contrast, the ferritin is a much larger molecule of approximately 500 times the molecular weight of ruthenium red and with a diameter of approximately 12 nm.

The theoretical isoelectric points of α and β tubulins calculated from the reported amino acid content of sea urchin tubulin (Luduena and Woodward 1973) are 5.7 and 5.6 while the values reported for calf brain tubulin are 5.4 and 5.3 respectively (Berkowitz et al. 1977). We hypothesised that if the negative charges on the microtubules are responsible for their separation, then a reduction in their negativity to zero, or almost zero, by lowering their pH to a value at, or close to, their isoelectric pH, should abolish the repulsion between adjacent microtubules, thus allowing contact. This is indeed the case. Extracted nutritive tubes bathed and fixed at pH 5.6 show a proportion of microtubules in close contact, forming in some instances clusters or rows, which are never seen at pH 7.2.

A particular feature which has not been adequately emphasised previously is the difference in dimensions recorded for clear zones in different tissues and even in the same tissue. In nutritive tubes, for example, in regions where microtubule profiles are completely surrounded by ribosomes they show a clear zone of approximately 20 nm in width. However, microtubules are also quite frequently seen adjacent to each other, and if the clear zone could be accounted for by material associated with the microtubules themselves one would expect them to approach no closer than 2×20 nm; but this is not the case, as microtubules separated by as little as 10 nm can regularly be seen. Such observations illustrate that the width of the clear zone is not constant, but that its size depends on what cellular component happens to border any particular microtubule. Such an observation could not easily be explained by the presence of microtubule-associated material, but is consistent with the findings reported here where the size of the clear zone might depend simply on the sizes of the opposing charges.

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