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MICROTUBULES IN RELATION TO FLAGELLOGENESIS IN *PTERIDIUM* SPERMATOZOIDS

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SUMMARY

Differentiating spermatocytes have been examined in a diploid culture of *Pteridium* gametophytes. Spermatogenesis is similar to that in haploid antheridia. Although the nucleus of the diploid gamete is larger, no differences have been detected in the dimensions of components such as microtubules and flagella.

Microtubules scattered in the cytoplasm of the young spermatocytes follow an irregular course and show no particular relationship to the blepharoplast, even during the differentiation of its periphery into basal bodies with characteristic triplets. Subsequently short lengths of notably straighter microtubules appear in quantity adjacent to the osmiophilic material lying along the microtubular ribbon of the developing gamete. These microtubules are particularly frequent around the distal ends of flagella beginning to develop from basal bodies, and often appear to converge upon them.

The view is put forward that the osmiophilic material adjacent to the microtubular ribbon, ontogenetically continuous with that originally at the centre of the differentiating blepharoplast, is responsible for the organization of the flagella. This material remains close to the ribbon throughout the differentiation of the gamete. If contact between the osmiophilic material and the flagella remains essential throughout their development, it would follow that they complete most or all of their growth parallel to the microtubular ribbon, possibly becoming emergent structures only as differentiation of the gamete is completed.

INTRODUCTION

The mature spermatozoid of *Pteridium*, after the shedding of the cytoplasmic vesicle (Bell, Duckett & Myles, 1971), is helical in form, the helix consisting of 4–5 gyres (Manton, 1959). The first gyre is occupied by the multilayered structure, which is closely appressed to a conspicuously large mitochondrion (Bell *et al.* 1971). The upper layer of the multilayered structure consists of microtubules which, in the differentiated gamete, extend beyond the plates of the multilayered structure as a ribbon, and run along the outer edge of the nucleus (Bell *et al.* 1971). The flagella, arranged in oblique lines (Manton, 1959), emerge from bases embedded in a layer of osmiophilic material on the outer surface of the microtubules. A peg of this material ascends into each basal body (Manton, 1959).

Studies of spermatogenesis in *Pteridium* and other ferns (for example, of *Ceratopteris* (C. F. Cave, unpublished observations)) have shown that in general the differentiation of the helical gamete follows the same course as in *Equisetum*, investigated earlier in this laboratory (Duckett & Bell, 1969; Duckett, 1973). One of the striking features of this differentiation is the abrupt appearance of fully formed flagella. In numerous sections of *Pteridium* spermatocytes, for example, no instances of half-grown flagella have been certainly identified, and flagellogenesis is evidently different from that seen in *Chara* (an alga) (Pickett-Heaps, 1968), where the flagella grow out from the differentiating spermatozoid in a manner recalling the regeneration of flagella in *Chlamydomonas* (Rosenbaum, Moulder & Ringo, 1969). In *Pteridium* many examples have, however, been found of flagella just beginning to develop from basal bodies. This early stage of flagellogenesis has been encountered in spermatocytes in which, the blepharoplast having broken up (Bell, 1974), the basal bodies have become scattered along the osmiophilic material adjacent to the outer surface of the microtubular ribbon (Fig. 2). A detailed examination of the cytoplasm adjacent to the osmiophilic layer, and of the distal ends of the basal bodies, has given new indications of the manner of growth of the flagella, and a possible explanation of the rapidity of their development.

MATERIALS AND METHODS

A diploid strain of gametophytes was raised as described previously (Bell & Richards, 1958). Fixation of antheridial fragments was in 2·5 % glutaraldehyde (TAAB Laboratories, U.K.) in 0·05 M phosphate buffer (pH 6·9) for 3 h at room temperature. Following overnight washing in ice-cold buffer, the material was osmicated (2 % aqueous) for 2 h at 4 °C, and subsequently dehydrated in acetone. Embedding was in Durcupan ACM (Fluka AG, Switzerland).

The searching of the material by means of 4-μm sections, remounting of the sections for fine-sectioning, and staining of the sections for electron microscopy were all by methods already described (Bell, 1974).

The use of diploid gametophytes

Diploid gametophytes have been used in this and a previous investigation (Bell, 1974) because the gametophytic tissue which proliferates freely from the margins of juvenile leaves of normal sporophytes laid upon a nutrient agar surface bears a mass of antheridia in various stages of development. Haploid gametophytes developing from spores tend to produce antheridia in flushes, all developing more or less simultaneously. The diploid antheridia are larger than the haploid, the diameter reaching or exceeding 60 μm (as opposed to about 40 μm). The spermatocytes are correspondingly larger, and this has assisted in the accurate identification of the stage of development of the spermatocytes before remounting for fine-sectioning.

OBSERVATIONS

Microtubules in young spermatocytes

In young spermatocytes, the protoplasts of which are beginning to become rounded as a consequence of the secretion of mucilaginous wall material (Cave & Bell, 1973), the periphery of the blepharoplast has already differentiated into basal bodies. At this stage isolated microtubules, unconnected with those of the basal body triplets, are seen in the surrounding cytoplasm, sometimes running up to, and occasionally penetrating, the mass of basal bodies (Fig. 1). These microtubules, about 15 nm in diameter, follow an irregular course, and are quite similar to those seen elsewhere in the cytoplasm (Fig. 1, upper left). There is no evidence that they are associated particularly with the blepharoplast.

Microtubules in association with the growing multilayered structure

The multilayered structure, very soon after it is released from the differentiated blepharoplast (Bell, 1974), comes to lie along a crest of the nucleus, and is closely associated with a single mitochondrion (Fig. 2). The multilayered structure is meanwhile growing, both the number of plates in the lower part, and the number of microtubules which form the upper layer, increasing in step with elongation of the associated mitochondrion. Frequently at this stage small vesicles can be seen between the edge of the nuclear crest and the plates of the multilayered structure (Figs. 3, 4: *v*), and there is often clear evidence that these are formed by blebbing of the outer membrane of the nuclear envelope.

Shortly after the emergence of the multilayered structure from the blepharoplast the upper layer of microtubules extends as a ribbon beyond the plates and on to the surface of the nucleus (Fig. 2). Osmiophilic material, which is ontogenetically continuous with that lying within the blepharoplast at an earlier stage (Bell, 1974), is now spread along the outer surface of the microtubular ribbon, and the basal bodies are partially embedded in it. Short lengths of microtubules, either isolated or in tufts, are often seen associated with this osmiophilic material. They sometimes lie parallel to it (Fig. 3), but more often appear to run into it at various angles (Figs. 4, 5). There is no evidence that these microtubules are associated with those of the ribbon, or with the basal bodies. Although of the same order of diameter as the microtubules seen earlier (Fig. 1), these microtubules are straighter, and give the impression of greater rigidity.

Microtubules in association with basal bodies

Although when the basal bodies first become distributed along the microtubular ribbon they are irregularly arranged, they soon come to lie oriented in one direction with their axes parallel to each other, and to the plane of the microtubular ribbon. A section transverse to the axes of the basal bodies is thus also perpendicular to the plane of the ribbon (Fig. 2, arrows). As this orientation is acquired, so does the development of the flagella become conspicuous. A cloud of fragments of microtubules is now present in the cytoplasm around the distal ends of the flagella (Fig. 6, between arrows). There is every indication that they are in the process of being incorporated into flagella (Fig. 6, inset). No similar concentration of microtubular fragments has been detected elsewhere in the cytoplasm.

The orientation of the section shown in Fig. 6 cannot be identified with certainty, but the distal ends of the flagella are evidently close to the osmiophilic material above the microtubular ribbon. The ribbon is cut obliquely at the right of the section and the osmiophilic material, with scattered microtubules, lies to its left (Fig. 6: *os*), separated from the ribbon by a less-opaque region.

The impression that growth of the flagellum takes place in close contact with the osmiophilic layer is strengthened by the peculiar inclination of the flagella seen in sections such as that shown in Fig. 7. This section is parallel to the anterior-posterior axis of the differentiating spermatozoid, and shows the nucleus beginning to be constrained into the helix characteristic of the mature gamete. The distal end of a growing flagellum (Fig. 7, arrow) is closely apposed to the osmiophilic material, whereas the proximal end of the basal body lies away from it. This distance may amount to as much as 0·3 μm. The distal ends of the growing flagella are often bent (Fig. 7, inset) as if weak, not yet having acquired the rigidity of the fully formed axis. A conspicuous feature of such flagella is that the radial links connecting the doublet tubules to the central tubules are clearly distinguishable, even though the doublets may be incomplete (Fig. 7, inset, arrow).

DISCUSSION

The effect of chromosome number on spermatogenesis

The course of differentiation of the diploid spermatozoids seems to agree in all essentials with that of the haploid. The diploid spermatozoids are larger, but this appears to be accounted for principally by the greater volume of the nucleus. No significant differences have yet been detected in the dimensions of components such as microtubules and flagella. Further, the diploid spermatozoids are fully motile, and experiments here and elsewhere (for example, see Takahashi, 1962) have shown that tetraploid and triploid sporophytes can be readily produced by allowing them to fertilize the appropriate female gametes.

There are no indications, therefore, that the observations presented here are likely to be in any way unrepresentative of flagellogenesis in fern spermatozoids as a whole.

The site of flagellar growth

In many flagellate unicellular organisms there is cytological and ultrastructural evidence (reviewed by Rosenbaum *et al.* 1969) that flagella grow at their tips. In *Ochromonas* it is particularly striking that if, after amputation, flagella are allowed to complete their regeneration in the presence of tritiated leucine the incorporated radioactivity is confined to the distal regions (Rosenbaum & Child, 1967). Similar results have been obtained with *Chlamydomonas* (Rosenbaum *et al.* 1969).

Consideration of the microanatomy of *Pteridium* flagella suggests that here, too, growth is more likely to be at the tip than elsewhere. Certainly there have been no indications in the present study of the intercalary incorporation of microtubular fragments. The incorporation of microtubule monomers, not visible in the electron microscope, along the lengths of the doublet and central tubules of the flagella cannot be excluded, but the finished appearance of the flagella in the proximal regions, and the well defined and regularly spaced radial connexions (Fig. 7, inset) speak against such generalized growth. Further, the tufts of microtubules apparently converging on the distal ends of the flagella (Fig. 6, inset) can be most simply interpreted in terms of incorporation and growth. The closeness of the approach of these microtubules to the ends of the flagella suggests that they are undergoing a direct end-to-end linkage to the peripheral and central tubules in the portion of the flagellum already organized.

The rate of flagellar growth

A possible explanation of the absence, despite the many spermatocytes examined, of instances in which clearly half-grown flagella were present in the cytoplasm is that flagellogenesis, once initiated, proceeds very rapidly. The regeneration of the principal flagellum of *Ochromonas*, comparable in length to the flagella of *Pteridium* (15–20 μm), is almost complete in about 6 h (Rosenbaum & Child, 1967), and that of the flagella of *Chlamydomonas* in about 2 h (Rosenbaum *et al.* 1969). In both these instances flagellar growth is dependent upon protein synthesis. In the fern *Ceratopteris*, however, and most probably in *Pteridium* (where spermatogenesis is in all respects similar), the final differentiation of the spermatozoid, including flagellogenesis, occurs without the incorporation of amino acid (Schedlbauer, Cave & Bell, 1973). This suggests that in the spermatocyte flagellogenesis might consist of little more than the assembly of preformed monomers, and consequently proceed faster than where synthesis is also involved. If the time required is a matter of minutes rather than hours, intermediate stages of flagellar growth might have been missed.

The role of the osmiophilic material

Despite a likely similarity in tip growth, flagellogenesis in differentiating fern spermatozoids may differ considerably in detail from that in regularly flagellate unicellular organisms, and in algal spermatogenesis. In *Chlamydomonas*, for example, little is visible in electron micrographs, and certainly nothing resembling that shown in Fig. 6 (inset), which could be interpreted as indicative of active incorporation into the growing flagellum (Rosenbaum *et al.* 1969). The same appears to hold for *Chara* (Pickett-Heaps, 1968). Presumably in these instances the enzymes and macromolecules concerned with growth cannot be distinguished from the general cytoplasmic background. In *Pteridium* spermatocytes, however, in addition to the fragments of microtubules, there is constant, and possibly significant, association between the distal ends of the growing flagella and the osmiophilic material adjacent to the microtubular ribbon. Developmentally this material shows continuity with that which appears at the centre of the differentiating blepharoplast (Bell, 1974), where it may play some part in the organization of the basal bodies and multilayered structure. That it should also be concerned with the assembly of microtubules and the organization of flagella would be in line with the experience of animal cytologists that structural features associated with the generation of specialized microtubules, such as dense aggregates and plaques, also seem to accompany procentriole formation (Wolfe, 1972). In *Pteridium* spermatocytes the microtubules of the basal bodies, and those which occur in association with the osmiophilic material and distal ends of flagella, are visually distinct from the microtubules scattered throughout the cytoplasm (Fig. 1), but it remains to be tested whether, as in lung fluke spermatozoa (Burton, 1968), they respond differently to colchicine and other treatments.

If the osmiophilic material retains an organizing function throughout the growth of the flagella, then their apices may necessarily remain in contact with it. Since this material does not become displaced from the microtubular ribbon and move out into the cytoplasm, it is conceivable that the flagella are organized, wholly or largely, parallel to the surface of the ribbon, following the helical course of the nucleus as they elongate. In the mature gamete the basal bodies are inclined away from the surface of the microtubular ribbon at an angle of about 40°, a distinct change in orientation from that seen during differentiation (cf. Fig. 7). Possibly this movement of the basal bodies frees the flagella from the surface of the microtubular ribbon and causes them to become the emergent structures seen as differentiation is completed (Manton, 1959). This could provide an alternative or contributory explanation of their seemingly abrupt appearance.

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Fig. 1. Section (close to median) of a blepharoplast in a young spermatocyte of *Pteridium* shortly after the differentiation of its periphery into basal bodies (for example, *bb*). Osmiophilic material (*os*) lies at the centre; *mt*, microtubules; *n*, nucleus. × 100000.

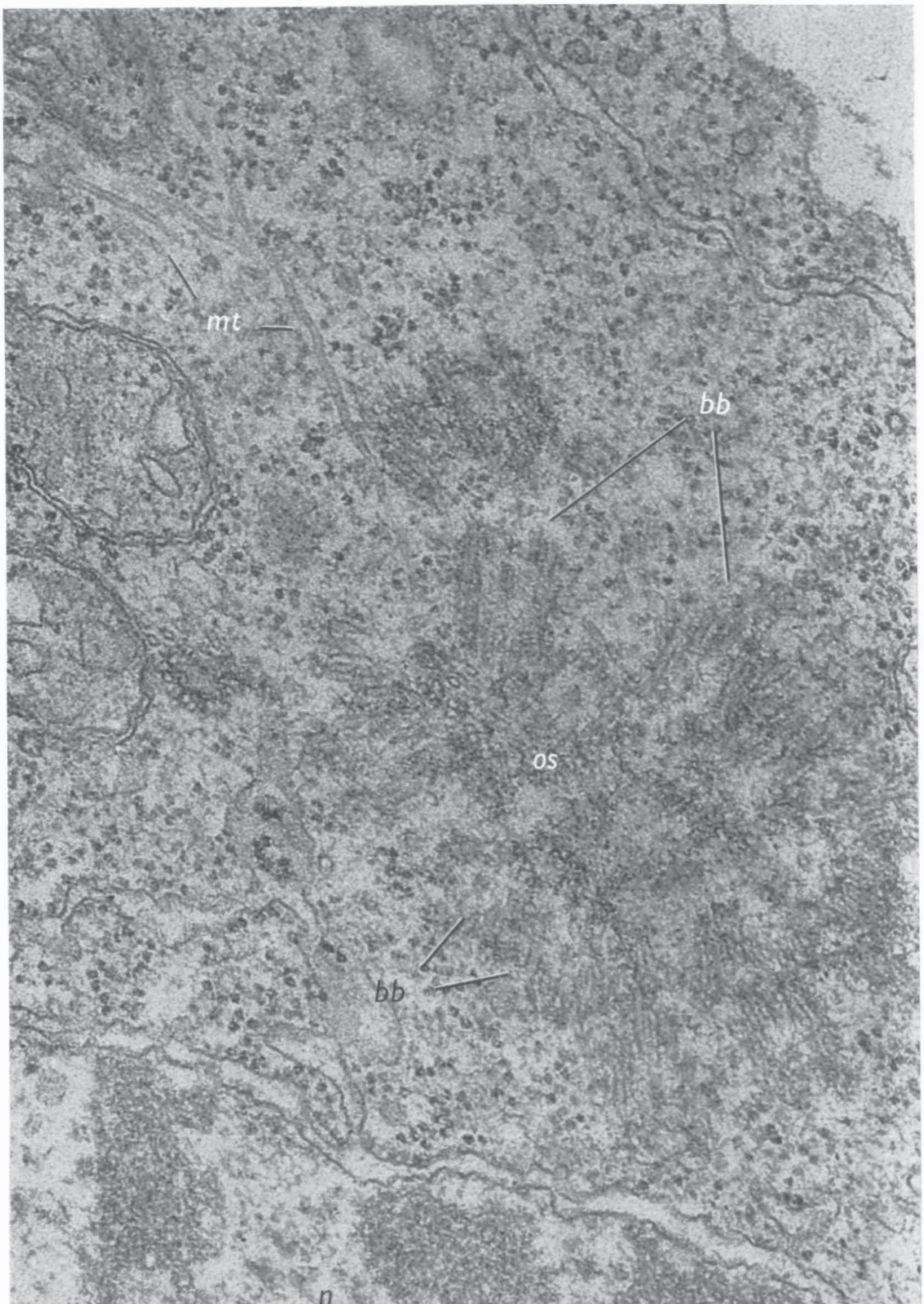
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Fig. 2. Portion of a differentiating spermatocyte after the formation of the nuclear crest (*nc*). The multilayered structure (*mls*) is completing its growth, and the microtubular ribbon (*mtr*) runs down on to the nucleus. Basal bodies (arrows) are scattered along the microtubular ribbon, partially embedded in osmiophilic material (*os*) separated from the ribbon by a narrow clear space. × 25000.

Fig. 3. Section (almost transverse to the microtubules of the upper layer and ribbon) of the multilayered structure, associated mitochondrion, and nuclear crest (*nc*) in a differentiating spermatocyte. A vesicle (*v*) is present between the edge of the crest and the plates of the multilayered structure, and a short length of microtubule (arrow) is lying parallel to the osmiophilic material. × 40000.

Fig. 4. As Fig. 3, taken from Fig. 2. A vesicle (*v*) is again present near the edge of the nuclear crest, and between it and the plates a less distinct region where plates are possibly forming (arrow). Short lengths of microtubules run into the osmiophilic material in the upper part of the micrograph. × 40000.

Fig. 5. As Fig. 4, the section almost parallel to the microtubules forming the upper layer of the multilayered structure. Short lengths of microtubules are visible near the edge of the multilayered structure (arrow). Lower in the micrograph short microtubules run through osmiophilic material (here not very distinct) up to the microtubular layer of the multilayered structure (*mls*). × 75000.

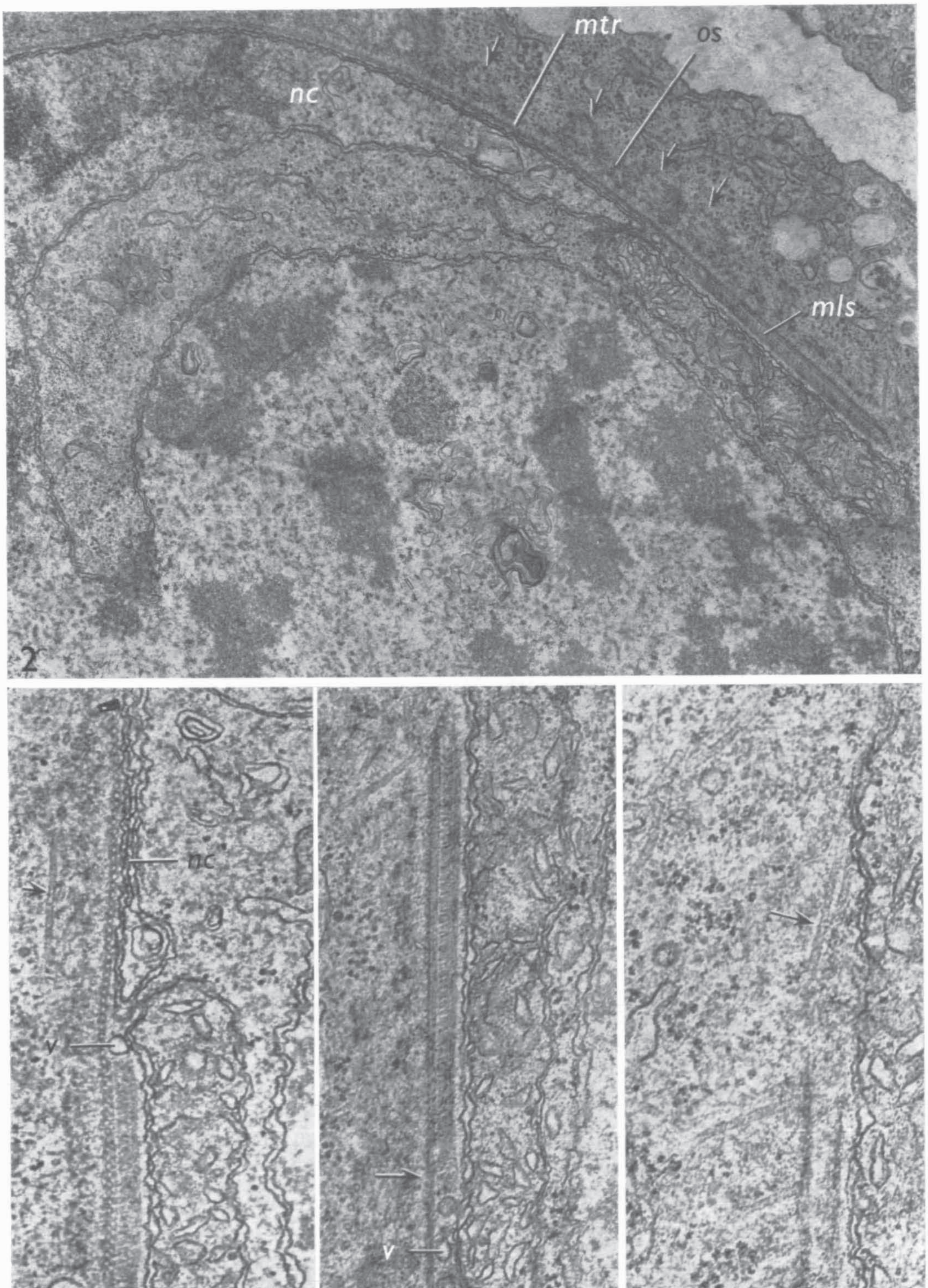
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Fig. 6. Section of a differentiating spermatocyte cutting the nucleus (*n*) near its surface. To the left of the nucleus is a glancing section of the microtubular ribbon, and a little further left osmiophilic material (*os*) separated from the ribbon by a clear space. On the left of the micrograph is a series of longitudinal sections of flagella developing from basal bodies (*bb*). Around their distal ends, and continuous with the osmiophilic material, is a cloud of short lengths of microtubules (between arrows). × 50000. Inset: detail of the microtubules at the distal ends of 2 flagella; × 100000.

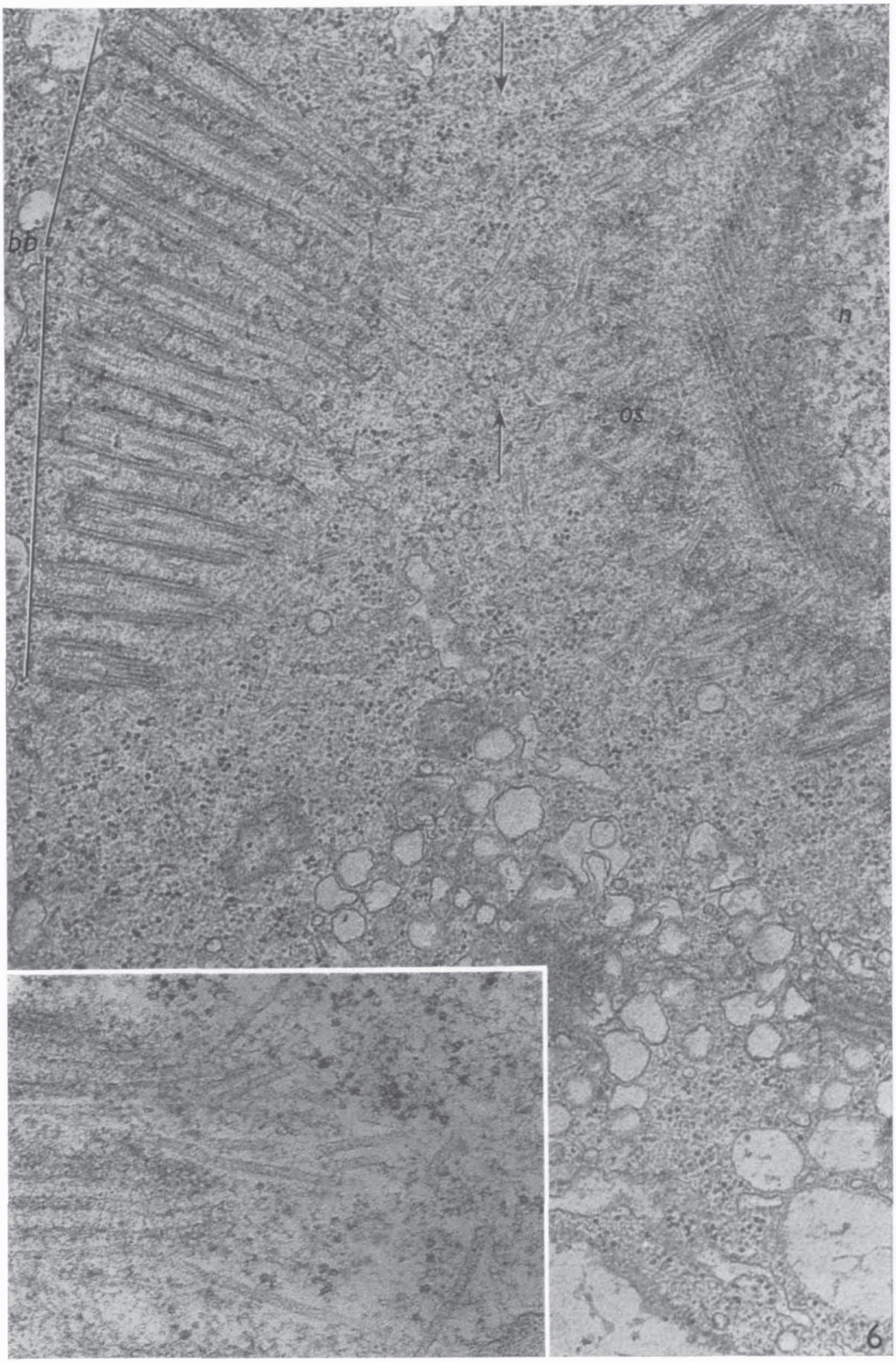
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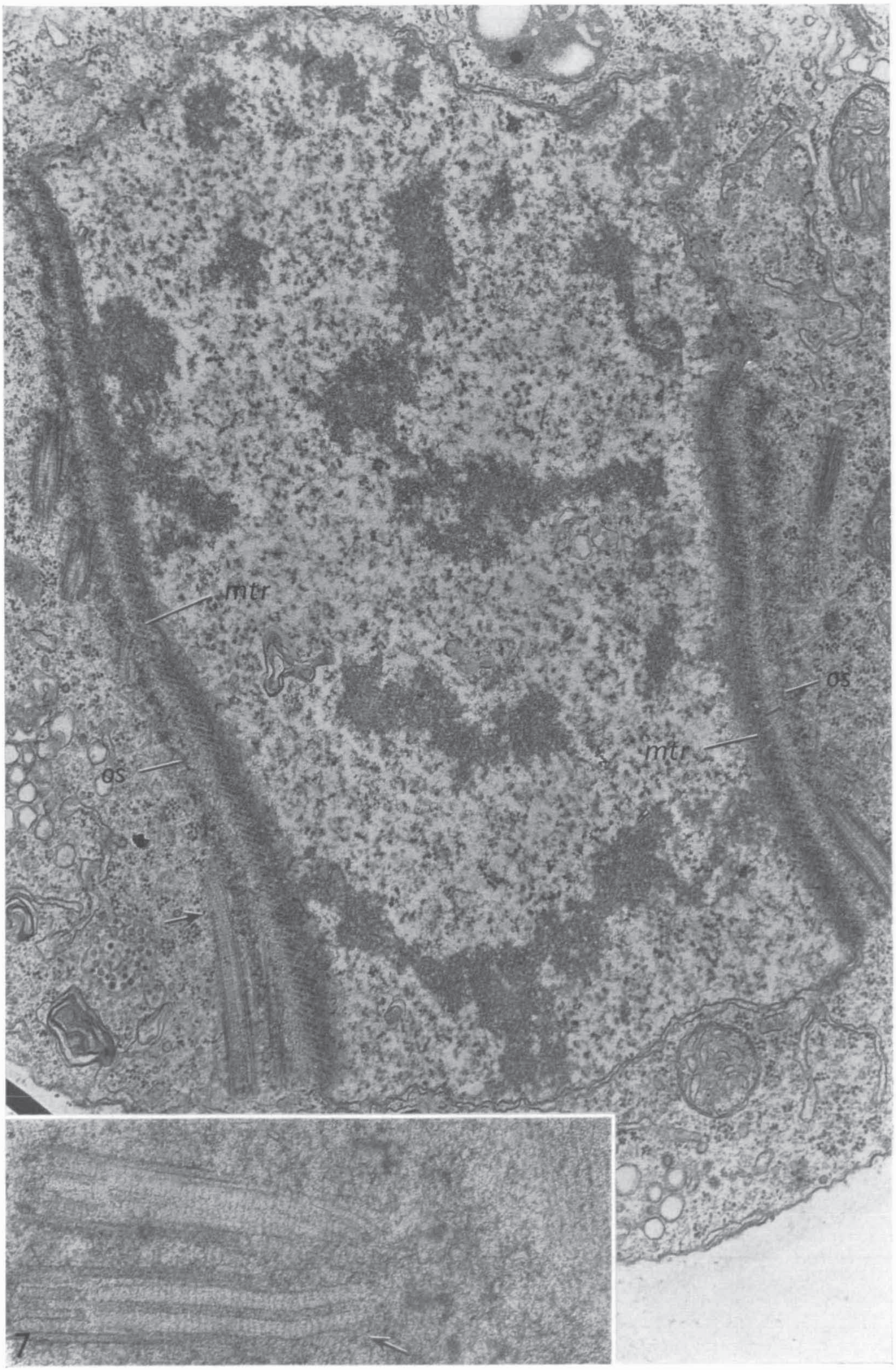
Fig. 7. Section parallel to the anterior-posterior axis of a developing spermatozoid. The microtubular ribbon (*mtr*) is cut obliquely on both sides of the nucleus. Flagella are beginning to develop from the basal bodies. The distal end of one flagellum (arrow) lies close to the osmiophilic material (*os*). × 30000. Inset: detail (from an adjacent section) of 2 developing flagella showing distal ends in contact with the osmiophilic material. Although radial connexions are clearly present close to the tips (arrow), the peripheral doublets are incomplete; × 75000.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