Fine-Structure of Haplosporidian Kernstab, a persistent, intranuclear mitotic apparatus

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FINE STRUCTURE OF THE HAPLOSPORIDAN 
KERNSTAB, A PERSISTENT, INTRANUCLEAR 
MITOTIC APPARATUS

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SUMMARY
The fine structure of the haplosporidan mitotic apparatus is described from observations of plasmodial nuclei of Minchinia nelsoni, M. costalis, Minchinia sp., and Urosporidium crescent. The apparatus, which is the Kernstab of light-microscope studies, consists of a bundle of microtubules terminating in a spindle pole body (SPB) at each end of the bundle. A few microtubules extend from SPB to SPB, but most either extend from an SPB and terminate in the nucleoplasm or lie in the nucleoplasm, free of either SPB. The bundle lengthens during mitosis, increasing the SPB-to-SPB distance by a factor of 2 to 3 as compared to interphase nuclei. SPBs are not in contact with the nuclear envelope, being found always in the nucleoplasm which is delimited by the nuclear envelope throughout mitosis. The mitotic apparatus is persistent through interphase, at least in a form which is not significantly different from that found in mitotic nuclei.

INTRODUCTION
Light-microscope studies of Minchinia spp. and Haplosporidium spp. (Sporozoa: Haplosporida) have shown that in the nuclei of many species there is a bar-like, achromatic structure or intradesmose which bisects the nucleus (Granata, 1914; Debaisieux, 1920; Jirovec, 1936; Sprague, 1963; Couch, Farley & Rosenfield, 1966; Haskin, Stauber & Mackin, 1966; Myhre, 1969). Jirovec (1936) called the structure a Kernstab and noted that it expanded to form the mitotic spindle during nuclear division. The structure was not seen in all plasmodial nuclei nor in all stages of sporogony, but was considered by the above workers (except Couch et al. 1966) to be a regularly occurring structure in plasmodial nuclei, even in those nuclei which appeared to be in interphase judging from their shape. Couch et al. (1966) suggested that the bar appeared only during or just before mitosis.

In studies of the oyster pathogen, Minchinia nelsoni, Perkins (1968) first noted that the bar-like structure of interphase plasmodial nuclei consists of a bundle of microtubules with an electron-dense, granular plaque at each end. During nuclear division it was observed to serve as a mitotic spindle. Identical mitotic apparatuses were later observed in sporogony of other haplosporidans, Urosporidium crescent (F. O. Perkins, unpublished data), M. costalis (Perkins, 1969), and Minchinia sp. (Perkins, 1975).

Myhre (1969) applied numerous cytochemical tests to plasmodia of M. nelsoni and found that the Kernstab is Feulgen-negative, PAS-negative, and is not revealed in
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cells stained for protamines and histones using the alkaline fast green procedure of Alfert & Geschwind (1953) and the picric acid-cosin Y procedure of Block & Hew (1960).

The present study was conducted to determine whether the Kersttob is a persistent mitotic apparatus and to determine changes in its structure during mitosis. The haplosporidians M. nelsoni, M. costalis, Minchinia sp., and U. crescens were studied. They have not been established in culture; therefore, all information was derived from observations of uncultured, infected oyster, crab, and trematode tissues. M. nelsoni and M. costalis parasitize oysters (Crassostrea virginica), Minchinia sp. is a parasite of the mud crab, Pasippeus herbstii, and U. crescens parasitizes the metacercariae of Carneophallus sp. which is, in turn, found in blue crab (Callinectes sapidus) tissues.

MATERIALS AND METHODS

Minchinia nelsoni-infected oysters were derived by placing uninfected James River, Virginia oysters in the York River at Gloucester Point, Virginia. After a suitable holding period, dependent on time of entry into the river (Andrews, 1966), infections occurred and were detected by finding plasmodia in squashes of living gill explants. M. costalis-infected oysters were obtained by similar techniques except that susceptible oysters were held at Wachapreague, Virginia. Mud crabs infected with Minchinia sp. were obtained from natural populations in the York River, and blue crabs, parasitized with infected metacercariae, were obtained from natural populations at Wachapreague. Pieces of gill and hepatopancreas, size 1 mm³, were fixed for 30 min in 2.5 % glutaraldehyde containing 0.2 M Millonig's phosphate buffer. Three buffer rinses, over a period of 45 min, were accomplished in 0.2 M Millonig's phosphate buffer in 0.15 M NaCl followed by postfixation for 3 h in 1 % OsO₄ containing 0.2 M Millonig's phosphate buffer and 0.2 M NaCl. All solutions were used at pH 7.0-7.2 and 22-24 °C.

The embedding medium was Epon 812. For serial section studies sections were mounted on Formvar-coated, single-hole grids. The mounting techniques were those of Galey & Nilsson (1966) modified by Moens (1970). Sections were stained 15-20 min in 2% aqueous uranyl acetate followed by 5-10 min in Reynolds' lead citrate. Micrographs were obtained from a Hitachi HU-11B electron microscope calibrated with a carbon replica of a 54864-line block shadowed with germanium (Ladd Research Industries, Inc.).

For light microscopy oysters were either fixed in Zenker's fixative (Humason, 1962) for 24 h, Davidson's fixative (Shaw & Battle, 1957) for 2-3 days, or formol-ethanol (Humason, 1962) for 3 days. Sections of material embedded in Paraplast (Sherwood Medical Industries, Inc.) were deparaffinized and stained in Harris' haematoxylin (Humason, 1962) and counter-stained in eosin Y.

The Feulgen technique (Humason, 1962) was applied to sections using a 1 N HCl hydrolysis time of 12 min at 55 °C. Both 0.1 % acid fuchsine in 2 % acetic acid and 0.005 % acid fuchsin in 1 % acetic acid (Robinow, 1963; Robinow & Marak, 1966) were used on smears fixed in Helley's fixative and on sectioned material fixed in either Zenker's, Davidson's, or formol-ethanol fixative. Smears were stained for 2-5-3.5 min using the techniques of Robinow & Marak (1966). Sections were stained for 5 min and rinsed, then mounted in 1 % acetic acid for observation. Live cells were mounted in 0.1 % methylene blue in estuarine water and pressed beneath a coverslip for observation.

RESULTS

Light microscopy—Minchinia nelsoni

Interphase nuclei (herein considered to be those nuclei with round or nearly round profiles) were 1.5-8.0 μm in diameter in plasmodia after fixation in formol-ethanol and staining in Harris' haematoxylin and eosin Y. The smaller nuclei (1.5-
2·0 μm) were generally densely stained with haematoxylin and contained a single peripheral nucleolus resembling a skullcap. Larger nuclei showed the nucleolus, 2–5 small, Feulgen-positive regions against the nuclear envelope, and a single dark band, the Kernstab, which extended across the middle of the nucleus (Figs. 1, 2).

With bright-field optics the Kernstab of plasmodia can be visualized after several different staining procedures are used: (1) Harris’ (Fig. 1) or iron haematoxylin staining of Zenker’s-, Davidson’s-, or formol-ethanol-fixed and sectioned cells; (2) acid fuchsin staining of Helly’s-fixed and smeared cells; and (3) methylene blue staining of live cells. The structure was not seen in live cells using bright-field, phase-contrast, or Nomarski interference optics. As already noted by Myhre (1969), preparations stained with the Feulgen technique do not show the Kernstab, only peripherally located clumps of chromatin (Fig. 2).

The frequency with which Kernstäbe were observed in interphase nuclei varied greatly depending on nuclear size, fixative used, and size of specimen fixed. Fewer than 10% of the nuclei, 2·0–3·0 μm in diameter, were seen to contain the bars. They were rarely observed in nuclei of the size range 1·5–2·0 μm. In about 40% of interphase nuclei of > 3·0 μm diameter the Kernstab was easily seen as a result of its larger size, probably because nuclear enlargement leads to a lower-density nucleoplasm and consequently higher contrast between the bar and background in the fixed and sectioned material observed in this study (Fig. 3).

The marked changes in plasmodial nuclear size of haplosporidians have been the subject of much speculation (Granata, 1914; Debaisieux, 1920; Perkins, 1975); however, the significance in the life cycle is still unknown. Since smaller nuclei (< 3·0 μm) divide without marked enlargement, the increase in nuclear size is apparently not a prerequisite for mitosis (Perkins, 1975).

Mitosis in nuclei with diameters of 2·0–3·0 μm consists of lengthening of the Kernstab and change in shape of the nucleus from round or oval to fusiform with the ends of the nuclear bar embedded in the apexes of the nucleus (Figs. 4, 5). After lengthening to about 7–9 μm (2·0–3·0 μm diameter interphase nuclei) the nuclear ends become rounded, the medial region collapses, and pinching in half occurs to yield 2 daughter nuclei. The nucleolus is located medially beside the Kernstab and appears to pull in half without dispersing during nuclear division. I was not able to reconstruct movements of the Feulgen-positive regions; however, they may migrate from their peripheral location against the nuclear envelope during interphase to the Kernstab, forming a plate-like configuration during metaphase (Fig. 6). Prophase and anaphase figures were not detected.

Electron microscopy—Minchinia nelsoni

Interphase nuclei of M. nelsoni plasmodia contain a fibrogranular, electron-dense nucleolus with ribosome-sized granules scattered throughout (Fig. 7). The Kernstab consists of a bundle of microtubules which average 25·5 nm diameter (range: 22·4–28·5 nm; N = 50; Sx = 0·3). In the mid-region of the nucleus most of the microtubules are parallel to one another and number 33–53 (N = 8). At each end of the bundle is a spindle pole body (SPB) located in the nucleoplasm free of the nuclear
envelope (Fig. 8). In addition to those forming the bundle, microtubules splay from the side of the SPB facing the nuclear interior (Figs. 8, 9). In interphase nuclei the splaying is more pronounced than in obviously dividing nuclei (Figs. 8–10 vs. 11–13, 18–20).

SPBs are electron-dense, granular units with the microtubules terminating and embedded in the matrix. Although a suggestion of layering was present in a few SPBs, it is not a normal characteristic of M. nelsoni plasmodial nuclei.

In an attempt to determine whether the Kernstab is persistent through interphase, 113 plasmodial nuclei of M. nelsoni with round or nearly round profiles were selected at random from 2 oysters and examined for Kernstäbe. When one section per nucleus was considered, 67% had 10 or more microtubules and/or at least one SPB. When 1–9 sections per nucleus were examined, 76% had the same characteristics as in the 67% group.

During nuclear division changes in degrees of aggregation and in movement of chromatin could not be related to classical stages of mitosis; however, since the nuclear envelope is persistent throughout division, its shape was useful as an approximate indicator of stages. The first indications of mitosis are bulging of the nuclear envelope overlying the SPBs and subsequent elongation of the nuclear profile (Fig. 11). Force is apparently exerted against the nuclear envelope, as evidenced by the change in shape which progresses until the nucleus is spindle-shaped (Figs. 11, 12). At no time does the SPB fuse or make obvious contact with the nuclear envelope. The distance from outside surface of SPB to nuclear envelope narrows from about 240 nm (N = 10) during interphase to about 72 nm (N = 15) in presumptive anaphase when the nucleus is spindle-shaped. The distance increases again to about 550 nm (N = 5) in telophase when the ends of the division figure become rounded prior to daughter cell delimitation (Figs. 14, 15). The SPB-to-SPB distance lengthens from a mean of 2.8 μm (range = 2.6–3.1 μm; N = 12) in interphase nuclei to a mean of 6.3 μm (range = 5.7–8.3 μm; N = 9) in telophase nuclei.

Nucleoli do not disperse during mitosis, but rather elongate while situated in the mid-region of the nucleus on one side of the Kernstab. Continued elongation leads to pinching in half and relocation of each half in a daughter nucleus.

Although chromosomes were not positively identified, fibrogranular aggregates of finer texture than the nucleolus and presumed to be chromatin were observed near the nuclear envelope in interphase cells. During mitosis the largest aggregates of chromatin appeared to remain in a peripheral location and appeared to move apart as the envelope elongated. Smaller but similar aggregates were scattered throughout the nucleoplasm. Some microtubules terminated in the small aggregates (Figs. 16–20), but not in the larger peripherally located ones. At the termination points there was no kinetochore-like differentiation.

In serial, longitudinal and cross-sections of the Kernstab, microtubules were not followed from SPB to SPB due to technical problems. However, two were followed 97% of the distance from attachment at one SPB toward the other SPB in 3 longitudinal sections; therefore it is believed that pole-to-pole microtubules are formed. Seventeen serial sections of an apparent interphase nucleus, normal to the bundle of
microtubules, showed that some of the microtubules extend only a few hundred nanometres, originating and terminating in the nucleoplasm free of any obvious chromatin-like aggregates.

Movement of nucleoplasm to the poles results in rounded instead of pointed ends for the nuclear division figure. This is accompanied by constriction of the mid-region (Fig. 15). Upon completion of mitosis there is presumably only one SPB which divides, the daughter SPBs migrating to opposite sides of the nucleus (Fig. 22).

Other haplosporidans

Mitosis in the plasmodial nuclei of other haplosporidans (M. costalis, Minchinia sp., and U. crescens) was identical to that of M. nelsoni with a few exceptions. Occasionally kinetochores were seen in dividing Minchinia sp. nuclei where 2 electron-dense layers were present separated by an electron-light zone (Fig. 21). Microtubules attached along the outside face of one layer and fibrogranular, chromatin-like material along the outside face of the other layer.

In M. costalis the SPBs were usually homogeneously granular as in the other species; however, they sometimes consisted of 2 or 3 electron-dense layers separated by electron-light bands and oriented at right angles to most of the Kernstab microtubules (Perkins, 1969).

DISCUSSION

Mitosis in Minchinia spp. plasmodia appears to differ in at least two characteristics from that of any other organism thus far described: (1) spindle pole bodies are formed which are free of the nuclear envelope and function throughout mitosis within an intact envelope; and (2) the mitotic apparatus persists through interphase. The descriptions of mitosis in plasmodia of Physarum polycephalum by Sakai & Shigenaga (1972) and Tanaka (1973) appear to be the only other reports in which nucleoplasmic SPBs are found in the nucleoplasm free of the nuclear envelope; however, those SPBs are found only in prophase. It was suggested that they may serve as microtubular organizing centres for the mitotic spindle, which lacks any discrete structures at the poles when fully formed.

If one accepts the existence of 10 microtubules and/or one or two SPBs as indicators of the mitotic apparatus, then persistence of the apparatus in Minchinia spp. can be accepted. Whether its form is always as organized during interphase as during mitosis cannot be determined at present, because only 4 nuclei were serially sectioned to reveal the whole apparatus. Each of the 4 nuclei appeared to be in interphase or early mitosis on the basis of their spherical shape and the apparatuses appeared to be complete (i.e. 2 SPBs were present and there were more than 30 microtubules arranged in a bundle). Determining when a nucleus is in interphase and not early in mitosis is not presently possible, since shape of the nuclear envelope is the only available criterion. Nevertheless, the high incidence of microtubule sightings in nuclei indicates persistence. It is difficult to imagine that non-persistent apparatuses would be seen as frequently as they were in a randomly selected population
of nuclei. Assuming the Kernstab is persistent, if one considers 60-nm-thick sections of a 3-μm-diameter, spherical nucleus in which the apparatus is centrally located, the apparatus would be seen in 94% of serial sections at right angles to the microtubular bundle, in 14% of serial sections parallel to the bundle, and 64% of those at 45° to the bundle. The assumption was made that all of the microtubules are organized into a bundle which is 0.4 μm in diameter and 2.8 μm long, based on measurements of a 3.0-μm-diameter nucleus. Each SPB was assumed to be 0.4 μm in diameter and 0.13 μm thick. Therefore, it is not surprising that 67% of randomly oriented sections (one section per nucleus) contained evidence of the Kernstab in the form of > 10 microtubules and/or one or two SPBs. Persistence appears even more likely when one considers the results of observing 1–9 sections of each nucleus in the same group of nuclei. The number of sightings then rose to 76%.

Nuclear division in other Sporozoa has been studied revealing a great diversity of structure in the taxon. Plasmodium spp. (Aikawa & Beaudoin, 1968; Aikawa, 1971) form 2 SPBs in nuclear pores at the spindle poles early in mitosis and disassemble them after nuclear division. The coccidians, Eimeria spp., also form 2 spindle pole bodies in nuclear pores, but each SPB is located in a cone-shaped chamber formed by the nuclear envelope and 2 centrioles are associated with each SPB (Dubremetz, 1973; Hammond, Roberts, Youssef & Danforth, 1973). The centrioles are unusual, having a ring of 9 singlet microtubules and a hub in the centre. Presumably cartwheel spokes are also present; however, they were not clearly demonstrated. The SPB material is persistent through interphase as a single mass in a large, flattened cytoplasmic chamber partially delimited by the nuclear envelope (Dubremetz, 1973).

Basically all other reports of SPBs have involved formation of 2 electron-dense, fibrogranular or granular aggregates which are located either in the cytoplasm or embedded in the nuclear envelope during nuclear division (for examples other than Sporozoa see Lerbs & Thielke, 1969; Pickett-Heaps, 1969; McLaughlin, 1971; Moens & Rapport, 1971; Peterson, Gray & Ris, 1972). In most species the nuclear envelope is intact throughout nuclear division. In all of these species the nucleoplasmic portion of the spindle apparatus disappears during interphase. Whether the SPBs disappear during interphase has not been determined for most of the species; however, some fungi are known to retain them (Aist & Williams, 1972; McCully & Robinow, 1972a).

M. nelsoni mitosis resembles mitosis in some fungi in that anaphase figures consist of a spindle-shaped nuclear envelope with pointed ends followed by telophase figures with rounded ends (Aist & Williams, 1972; McCully & Robinow, 1972a, b). It has been suggested by Aist & Williams (1972) and others that the pointed ends indicate that force is being generated by the spindle microtubules against the nuclear envelope. The rounded telophase ends, they believe, represent completion of elongation followed by migration of nucleoplasm into the ends.

The suggestion of force generation by microtubules to explain the spindle-shaped nuclei appears reasonable for organisms where the SPB is attached to the nuclear envelope; however, in organisms such as M. nelsoni and the heterobasidiomycetous yeasts where the SPB is free in the nucleoplasm, McCully & Robinow's (1972a)
suggestion of localized membrane growth appears more reasonable. They propose that
the SPBs may direct formation or breakdown of nuclear membrane which would
explain the configurational changes that occur in the envelope. If force were being
generated by the spindle microtubules and were transferred to the SPBs to yield
pointed anaphase figures, then there would have to be some rigid structure in the
nucleoplasm to transfer force to the envelope. If this intermediate structure is present,
it is not obvious in glutaraldehyde- and osmium tetroxide-fixed material.

Although distinct chromosomes were not resolved in M. nelsoni, diffuse fibro-
granular regions were observed in mitotic nuclei which could have been chromatin.
They were distinguished from the nucleolus by the lack of ribosome-size particles;
however, the nucleolus also had chromatin-like regions mixed with it. It is striking
that the chromatin-like regions were nearly all located against the nuclear envelope
throughout division or at least outside the centrally located bundle of microtubules.
Aist & Williams (1972) also noted that chromosomes of Fusarium oxysporum were
located outside the bundle. There did not appear to be more or denser aggregation of
chromatin-like regions in dividing nuclei as opposed to nuclei with circular profiles.
Obviously future mitotic studies of organisms such as M. nelsoni will require better
fixation or staining techniques to resolve the chromosomes.

Since the chromatin-like regions were most often found near the nuclear envelope
of M. nelsoni, movement of chromosomes possibly occurs by expansion of the nuclear
envelope if they are attached to the membrane. Such movement has been suggested
for dinoflagellates (Kubai & Ris, 1969) and bacteria (Cuzin & Jacob, 1967). In
M. nelsoni, however, microtubules from SPBs were found to terminate in small, electron-
dense, fibrogranular regions, some of which were stratified like kinetochores of animal
cells (Pickett-Heaps, 1969). The regions were both within the bundle and around the
periphery. Only rarely were they in continuity with the presumptive chromatin
aggregates.

In haplosporidan plasmodial nuclei, therefore, there is apparently a mitotic
apparatus with SPB-to-SPB microtubules and SPB-to-chromosome microtubules.
However, an explanation is needed to establish why the only aggregates which appear
to be chromosomes are located against the nuclear envelope and rarely connected
to microtubules. These aggregates are most probably the peripherally located,
Feulgen-positive regions seen in light microscopy. Answers to such questions must
await the time when the organisms can be cultured and some control over mitotic
activity can be imposed to obtain synchrony of division.

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REFERENCES


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Figs. 1–6. Light micrographs of *Minchinia nelsoni* plasmodia. Figs. 1 and 3–6 represent haematoxylin- and eosin-stained sections.

Fig. 1. Interphase nucleus showing Kernstab \( (k) \) and nucleolus \( (n) \). \( \times 1800 \).

Fig. 2. Interphase nuclei showing peripheral aggregates (arrows) of Feulgen-positive material against nuclear envelope. \( \times 1800 \).

Fig. 3. Pair of enlarged vesicular nuclei showing Kernstäbe \( (k) \) and nucleolus \( (n) \). \( \times 1800 \).

Figs. 4, 5. Probable anaphase nuclei showing nucleoli \( (n) \) and Kernstäbe \( (k) \). Note pointed ends of nuclear envelope. \( \times 1800 \).

Fig. 6. Spindle-shaped nucleus with basophilic band \( (b) \) across medial region. Band may represent a metaphase plate. \( \times 1800 \).

Figs. 7–22. Electron micrographs of *M. nelsoni* plasmodial nuclei, except Fig. 21 which is from *Minchinia* sp.

Fig. 7. Cross-section of bundle of microtubules in interphase nucleus. \( n \), nucleolus. \( \times 59000 \).
Figs. 8, 9. Spindle pole bodies (s) of interphase nuclei. Fig. 8, oblique section of spindle pole body showing microtubules extending towards centre of nucleus, ne, nuclear envelope. Fig. 9, frontal section showing radiating, stellate pattern of microtubules. Fig. 8, x 67000; Fig. 9, x 68000.

Fig. 10. Interphase nucleus showing Kernstab consisting of bundle of microtubules and 2 SPBs (s). x 32000.
Fine structure of Kernstab
Fig. 11. Nucleus early in mitosis. SPBs (s) have moved closer to nuclear envelope as microtubular bundle lengthens. Nuclear envelope has extended or been pushed outward in region of SPBs. \( \times 37000 \).

Fig. 12. Probable anaphase nucleus comparable to those in Fig. 5. Nuclear envelope has extended further in the region of the SPB (s) resulting in a spindle-like profile. \( \times 44000 \).

Fig. 13. Higher magnification of SPB in a nucleus similar to the one in Fig. 12. Note close proximity of SPB to nuclear envelope. \( \times 46000 \).
Fine structure of Kernstah
Fig. 14. Telophase nucleus in which medial region of nuclear envelope has begun to collapse inward. Presumptive nucleolus (n) has divided and presumptive chromatin (c) is peripherally located outside the bundle of microtubules (m). s, SPB; nu, adjacent nucleus. × 25,000.

Fig. 15. Telophase nucleus later in mitosis than the one in Fig. 14. nu, adjacent nucleus; s, SPB. × 25,000.

Figs. 16, 17. Adjacent sections at right angles to bundle of microtubules in interphase nucleus. Note within circles the termination of a microtubule in electron-dense aggregate of chromatin-like material. × 62,000.
Figs. 18–20. Consecutive serial sections of nucleus in metaphase or anaphase. One microtubule can be seen to extend from SPB (s) to chromatin-like aggregate (c). Another, less certain, termination is indicated by unmarked arrow. Hollow arrows indicate identical regions in adjacent sections. No kinetochore-like differentiations are visible in the aggregates. $\times 40000$. 
Fig. 21. Kinetochore (kt) in mitotic nucleus of Minchinia sp. with attachment of at least 4 microtubules. The kinetochore was in the mid-region of the nucleus. × 83,000.

Fig. 22. Possible formation of secondary SPB (j) from side of larger SPB following mitosis. From nucleus believed to be in interphase. × 48,000.