Ultrastructural Studies of the Free Zoospore of the Rumen Phycomycete *Neocallimastix frontalis*

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The structure of the free zoospores of *Neocallimastix frontalis* has been examined by electron microscopy of thin-sectioned and negatively stained preparations. There are up to 15 flagella arranged in two rows. The free end of each flagellum is narrow and its tip does not contain microtubules. The flagella and the cell body are coated with distinct surface layers composed of regular arrays of particles and fibrils, respectively. The cell body contains a variety of inclusions. Near to the flagellar pole there are numerous membrane-bound electron-dense globules about 0.2 to 0.7 μm in diameter, between which are microtubules, particles and small vesicles. In the region of the centrally placed nucleus are arrays of helices of ribosome-like particles. These particles also occur in the form of globular aggregates, each partially enclosed within a membrane. The remainder of the cytoplasm is filled with material resembling glycogen. The zoospores stain positively for glycogen and contain ribonuclease-sensitive particulate material which is stained by toluidine blue.

Scanning electron microscopy shows that the zoospores attach to the substrate by the flagellar pole.

**INTRODUCTION**

The life cycle of the anaerobic aquatic rumen phycomycete *Neocallimastix frontalis* alternates between a motile flagellated zoospore and a non-motile, vegetative, reproductive stage, both of which occur within the rumen. The zoospores are released from sporangia in response to the increase in concentration of a substance derived from the diet (Orpin, 1975), soon after the host animal eats. The zoospores show a chemotactic response to a variety of carbohydrates, which apparently attract them to sites on plant material suitable for attachment and subsequent germination (although attachment is not an essential prerequisite for germination) (Orpin, 1977; Orpin & Bountiff, 1978). Movement through the liquid is normally brought about by the flagella, of which up to 14 have been detected by light microscopy. Movement over the plant surface is amoeboid.

We describe here the fine structure of the zoospore of *N. frontalis* up to the time of its attachment. Particular attention is given to the form of the surface layers of the zoospore, the nature of any energy reserves, and the presence of any features which could account for the ability of the zoospore to attach to a variety of surfaces and then grow rapidly.

**METHODS**

*Culture.* *Neocallimastix frontalis* was isolated from sheep rumen fluid as described by Orpin (1975). It was grown at 39 °C in the medium of Orpin & Bountiff (1978) supplemented with agar (1.0 g l⁻¹; Oxoid no. 3), dispensed under CO₂ into screw-capped tubes, each fitted with a rubber septum (Bellco Glass, Vineland, N.J., U.S.A.). Suspensions of zoospores were prepared from cultures in agar-free media using the methods of Orpin & Bountiff (1978). Occasionally, in older cultures, colony formation occurred, with the growth of ball-like clumps...
containing all stages in the life cycle. These clumps were used for the examination of freshly released zoospores. For the study of attached zoospores, culture tubes containing liquid culture medium with pieces of microscope slide cover-glass at the bottom were inoculated with a suspension of zoospores. The zoospores settled on the cover-glass, attached and germinated. The pieces of cover-glass were removed at intervals up to 18 h and processed for examination in the scanning electron microscope.

**Electron microscopy.** For the preparation of electron micrographs of thin sections, samples were fixed in suspension with a solution of 2% (w/v) glutaraldehyde in 80 mM-phosphate buffer pH 7.4, sometimes containing 1-4 mM-calcium chloride, for 1 to 2 h at room temperature and then overnight at 4 °C. The samples were washed with the phosphate-buffered medium, with centrifugation as necessary, and then post-fixed for 1 h with 1% (w/v) osmium tetroxide. The samples were dehydrated with ethanol, transferred to 1,2-epoxypropane and finally embedded in Araldite. Thin sections were stained with uranyl acetate and lead citrate. Zoospores for negative staining were separated from the culture medium by centrifuging and resuspended in glass-distilled water, in which some ruptured. The fragments thus obtained were negatively stained with 2% ammonium molybdate pH 6-9 or sodium tungstosilicate pH 7-2. For scanning electron microscopy, zoospores attached to pieces of cover-glass were fixed as above, dehydrated with ethanol and then transferred to amyl acetate. They were dried from carbon dioxide in a critical-point drying apparatus and sputter coated with gold.

**Histochemistry.** Zoospores were stained to show ribosomes essentially as described by Kusamrarn et al. (1975). Zoospores were fixed for 10 min at room temperature with glutaraldehyde, collected by centrifuging and washed twice by resuspension in 25% (v/v) aqueous ethanol. Drops of the suspension were dried on microscope slides at 50 °C. The slides were then incubated for 4 h at 37 °C in an aqueous solution of ribonuclease (1 mg ml⁻¹; Sigma) or in water. They were washed, redried and then stained for 60 s with 0-03% (w/v) toluidine blue in 25% ethanol at room temperature and de-stained with 95% ethanol.

Zoospores fixed with glutaraldehyde and dried on slides from ethanol were stained for glycogen with Best's carmine and counter-stained with Ehrlich's haematoxylin. The specificity of the staining was tested by pre-incubating some of the preparations with diastase.

**RESULTS**

As was expected from examination of live zoospores, their shape in thin sections was very variable, but they were approximately ovoid with an irregularly scalloped surface and with the flagella arising from one pole (Fig. 1). The cells contained a nucleus and a variety of inclusions: large globular aggregates and helical arrays of ribosome-like particles, amorphous electron-dense globules, glycogen, microtubules and some membranes were present. The sections of some zoospores also contained crystallites.

The amorphous globules were always at the end of the cell adjacent to the flagella between the basal bodies and the nucleus (Fig. 1). Most of the globular aggregates or ribosome-like particles were on the other side of the nucleus. The helical arrays of ribosome-like particles were predominantly in the zone of the globular aggregates, but they also occurred at the periphery of the nucleus.

**The flagella**

There were nine to fifteen flagella, alternating in two rows (Fig. 2a). They contained the usual (9 x 2) + 2 arrangement of microtubules (Fig. 2b), which extended for about three-quarters of the length of the flagella. The terminal part of each flagellum was narrow (Fig. 3a) and contained only a few microtubules. None of these microtubules extended very far into this region, so that the tips of the flagella were free of them (Fig. 3c). In sections, the outer electron-dense leaflet of the triple-layered flagellar plasma membrane was more prominent than the inner leaflet and was surrounded by, and apparently continuous with, an electron-dense layer about 17 nm thick. This layer normally appeared finely granular and in oblique sections there was sometimes a hint of a regular arrangement of the granules. In negatively stained preparations, the regular organization of the components of this layer was readily apparent. In all preparations there were prominent transverse striations 4-3 nm centre-to-centre at angles of 77° to 90° (Fig. 3b) to the long axis of the flagellum. This pattern was distorted in the region where the flagellum narrowed at the termination of the microtubules, but the layer was continuous. There were, in addition, less prominent striations.
at angles between 22° and 33° to the long axis (Fig. 3b). These families of striations are interpreted as arising from a regular arrangement of particles, each with a diameter of about 3.5 nm, unpenetrated by negative stain.

**Surface layers of the cell body**

Like the flagella the plasma membrane of the cell body was coated by a surface layer, but the two layers were not continuous where they met at the base of the flagella (Fig. 4a, 5c). The cell body surface layer was apparently composed of fibrils approximately 12 nm in diameter (seen in transverse section in Fig. 4c), arranged in rows to give a striated pattern when seen in oblique section (Fig. 4d). Unlike the layer coating the flagella, the cell body surface layer was not closely apposed to the plasma membrane but was about 25 nm from it. There was some regularly repeating material connecting the two so that they remained parallel. The fibrils making up the layer appeared to be about 12 nm centre-to-centre (Fig. 4c, d).

The plasma membrane appeared normal. In a limited region near to the insertion of flagella, but we do not know precisely where, the plasma membrane had an array of electron-dense filaments about 5.5 nm wide, 22 nm centre-to-centre, extending 40 nm into the cytoplasm (Fig. 4b). About 90 nm beneath this area of plasma membrane there was a discontinuous electron-dense layer (d', Fig. 4b) with another, less electron-dense layer (d'', Fig. 4b) a further 110 nm into the cytoplasm.
Fig. 2. (a) Section through the two rows of flagella near their bases; the adjacent cytoplasm contains amorphous electron-dense globules. (b) Transverse sections through four flagella; the surface layers of the plasma membrane, on both the flagella (fl) and the cell body (cl), are clearly visible.

Fig. 3. (a, b) Negatively stained preparations showing (a) the form of the flagella, and (b) the surface lattice over the narrow part of a flagellum. (c) Transverse section showing the narrow tips free of microtubules. p, plasma membrane; m, microtubule.
Fig. 4. (a and inset) Longitudinal section of a basal part of a flagellum; the layer (fl) coating the flagellum is not continuous with the layer (cl) coating the cell body (arrows). (b) The plasma membrane near the flagella has a series of fibrils (arrow) projecting into the cytoplasm and two diffuse layers of electron-dense material (d' and d'') approximately parallel to the surface membrane. (c) The surface layer (cl) on the cell body appears granular in cross-section. (d) In oblique section the surface layer over the cell body appears as an array of parallel lines.

The amorphous globules and adjacent cytoplasm

The amorphous globules were confined to the pole of the zoospore adjacent to the basal bodies (Fig. 5). There were some 30 to 50 per section. The globules were about 0.2 to 0.7 μm in diameter and were filled with amorphous material. The electron density of their contents was variable (Fig. 5a). The globules with less electron-dense contents were seen to be surrounded by a single membrane (Fig. 5b) and some also had a single membrane within them, apparently enclosing part of the contents. The cytoplasm between the amorphous globules contained small vesicle-like structures (v, Fig. 5c), some electron-dense particles about 40 nm in diameter (p, Fig. 5c), and microtubules which appeared to arise from electron-dense fibrous structures associated with the kinetosomes (Fig. 4a). The microtubules extended mostly in the plane at right angles to the long axes of the kinetosomes and are seen only in transverse or oblique section in Fig. 5(c).

The nucleus

The nucleus was about 2.5 to 3 μm in diameter and positioned near the centre of the zoospore (Fig. 1). Usually it contained, near the centre, a region about 1 μm in diameter of greater electron density than the rest of the contents. The nucleus was covered with a fibrous
Fig. 5. Amorphous globules (ag) at the flagellar end of the cell. (a) The contents of the globules are variable in electron density. (b) The globules with less electron-dense contents are membrane-bound and occasionally there are other membranes (arrows) within the globules. (c) Microtubules (m), electron-dense particles (p) and small vesicles (v) are present between the globules. cl, cell surface layer; fl, flagellar surface layer; k, kinetosome; rg, globular aggregates of ribosome-like particles.
layer with a minimum thickness of about 30 nm (Fig. 6). Examination of the many sections in which the plane of sectioning crossed the plane of the surface at various angles, and examination of sections tilted to a range of angles in the electron microscope, failed to reveal anything other than traces of a true limiting membrane lying beneath the fibrous layer. This appearance is in marked contrast to the clarity of the two nuclear membranes (and nuclear pores) in zoospores developing within the sporangia which, in some preparations, were present in the same sections as the free zoospores (Fig. 6).
Whole zoospores stained with toluidine blue and examined with the light microscope showed an overall faint blue colour with some densely staining particles stained a deeper blue to purple. Controls incubated in water before staining gave the same reaction with toluidine blue, but zoospores incubated with ribonuclease showed no staining with toluidine blue. From this it may be inferred that the zoospores contain ribosomes (or at least ribonucleoprotein particles) in aggregated form(s) as described for Entamoeba (Kusamrarn et al., 1975). We
have used the term 'ribosome-like' in the following description because the identification is not absolute.

The ribosome-like particles were prominent components of the zoospores and were present as globular aggregates and helical arrays (Fig. 1, 7). The globular aggregates (Fig. 7c) were mostly in the half of the cell away from the basal bodies but some were present around the centrally placed nucleus and extended as far as the region containing the amorphous globules (Fig. 1). Usually 20 to 30 globular aggregates were present per section. The individual aggregates were variable in shape but typically were ovoid. Their axial dimensions were maximally 0.5 by 1.0 μm. In most sections the particles (16 nm diameter) at the surface of the globules were aligned in a row 22 nm centre-to-centre around the periphery (Fig. 7c). In some sections it was clear that the particles in adjacent rows were also aligned although there was no unequivocal evidence that all the particles were ordered like this. Each aggregate was partially bounded by a single membrane and this was enclosed within an amorphous layer which was also membrane-bound; these membranes were presumably continuous. The apparent width of the amorphous layer depended on the plane of the section, with a minimum of about 25 nm. Occasionally it was seen that the non-membrane-bound part of an aggregate was continuous with some of the helices.

The bulk of the helices were present in a group among the globular aggregates near the nucleus (Fig. 7a). Some of the helices were perinuclear (Fig. 7a) and there were a few individual helices at other places in the zoospore. Each helix was composed of four particles with dimensions of about 16 by 20 nm in square array, when viewed down the axis of the helix, and was up to 10 gyres long. The pitch of the helix was 20 nm. Frequently four helices were themselves in the form of a square array (Fig. 7b). Each helix was more or less curved; it was not clear whether the variation in the curvature was real or was due to differences in the angles of the helices relative to the plane of the section.
The other cell components

Glycogen was shown to be present histochemically and it was identified by its characteristic appearance in sectioned (Fig. 1, 4d) and in negatively stained (Fig. 8a) preparations. It was present in every zoospore examined and occupied all areas of the cytoplasm which were not occupied by the other structures.

Some 5% of the sections of zoospores contained crystallites of the type shown in Fig. 8(b) in the pole away from the flagella. The crystallites were between 500 and 900 nm in length with a length-to-width ratio of 3-5. The exact appearance of the striations depended on the position of the crystallite relative to the plane of sectioning; one appearance could be changed to another by tilting in the microscope. The longitudinal striations were about 8 nm wide and 15 nm centre-to-centre, and the oblique striations were about 7 nm wide and 11 nm centre-to-centre. Small membranous elements were usually present along one of the longitudinal edges of the crystallites (arrow, Fig. 8b). Stacks of three or four similar membranous elements were occasionally found elsewhere in the cell.

Attachment of zoospores

Examination of numerous attached zoospores by scanning electron microscopy established that they attached by the flagellated pole with the release of fibrillar material which radiated out from the point of attachment (Fig. 9).Shortly after attachment many of the flagella separated from the zoospore body but remained on the cover-glass adjacent to the cell from which they were derived.

DISCUSSION

We here compare the structure of the Neocallimastix zoospore with that of the zoospores of aerobic phycomycetes and in particular with that of Callimastix cyclopsis, the only other fungal zoospore with numerous flagella (Vavra & Joyon, 1966) so far described. The genus Neocallimastix (Vavra & Joyon, 1966) was originated to accommodate the multilagellated zooflagellates found in the alimentary tracts of animals, with the rumen organism Neocallimastix frontalis as type species. The decision to classify the rumen species as a zooflagellate was based on the incorrect belief that reproduction was by binary fission, and
on studies made by light microscopy which suggested an internal structure not normally associated with fungal zoospores. We now know that the rumen species is a true fungus, on the basis of gross morphology, life cycle and presence of chitin in the cell walls of the vegetative stage (Orpin, 1977), but have retained the generic name *Neocallimastix*. It is noteworthy that *C. cyclopus* was itself originally classified among the zooflagellates but the ultrastructural studies of Vavra & Joyon (1966) clearly established its affinities with the Blastocladiales (Fuller, 1977). Ultrastructural studies of a great variety of aerobic uniflagellate zoospores have been reviewed by Lange & Olson (1979).

The appearance of the *Neocallimastix* zoospores as described here was constant from the time of their release from the sporangia until their attachment. During this period they are actively motile. Presumably the glycogen which occupies a large portion of the cytoplasm (Fig. 1, 4 d, 8 a) is the prime energy reserve. The metabolism of the *Neocallimastix* zoospores is, of course, strictly anaerobic and, as expected, in contrast to *Callimastix cyclopus* and other aerobic zoospores, they lack mitochondria. Even the zoospore of *Blastocladias ramosa*, which is wholly fermentative, apparently retains a vestigial mitochondrion (Held et al., 1969; Fuller, 1977). The most striking differences between the zoospores from the two genera are in the presence of the surface layer in the rumen fungus, in the form and position of the nucleus, and the distribution of the ribosome-like particles.

It is remarkable that two structurally distinct surface layers, each with regular substructure, coating the flagella and cell body respectively (Fig. 3, 4), should occur, and that they are apparently so different that they do not join where they meet at the bases of the flagella (Fig. 4 a, 5 c). The layer over the flagella must be highly flexible, and that over the cell body must also have a considerable degree of flexibility since the cell readily changes shape (Orpin, 1975). The layers are reminiscent of structures coating various algae (Roberts, 1974), but as far as we know the occurrence of extracellular layers of this kind has not been described before for fungal zoospores.

The small crystallites seen in some sections of *Neocallimastix* zoospores may be the equivalent of the 'baguette anterieure' in *C. cyclopus* described by Vavra & Joyon (1966).

In *C. cyclopus* the nucleus is triangular in section with one apex associated with the kinetosomes. An unusual feature of the nucleus in *Neocallimastix* is the apparent absence of two limiting membranes, although these are quite clear in the developing zoospores (Fig. 6). It is possible that there are two nuclear membranes present in the free zoospore but that they are obscured, the outer one completely and the inner one almost completely, by the fibrous layer which is characteristically present around the nucleus at this time. In the maturation of *C. cyclopus* the large, paranuclear body which contains the ribosomes is enclosed within a lattice of fibres derived from the kinetosomes (Vavra & Joyon, 1966).

The groups of helices or ribosome-like particles and the aggregates described here for *Neocallimastix* are presumably the equivalent of the paranuclear conglomerate of *C. cyclopus*. In zoospores from other genera the ribosomes are distributed in a variety of ways including (in *Physoderma*) packaging into several entities by double membranes within the nuclear cap (Lange & Olson, 1979). Among the fungi, only the ribosomes in *Neocallimastix* show any evidence of regular packing, but regular arrays of ribosomes have been reported from a variety of other sources: hypothermic chick embryos (Crain et al., 1964; Byers, 1967, 1971), ovarian cells in a lizard (Porte & Zahnd, 1961; Ghiara & Taddei, 1966; Taddei, 1972) and encysted forms of *Entamoeba* (Morgan & Uzman, 1966; Rosenbaum & Wittner, 1970; Barker & Swales, 1972 a, b; Lake & Slayter, 1970, 1972). In none of these is the packing at all like that seen in the *Neocallimastix* ribosome-like globules (Fig. 7c). Arrangements of ribosomes in tetramers occur in chicks and lizards, commonly as planar sheets, but also as three-dimensional arrays (Byers, 1967; Lake et al., 1974). The arrangement of the tetramers from these sources gives a lattice of the p4 plane group extending over comparatively long distances. In *Neocallimastix* the tetramers are the end-on views (transverse sections) of the helices (Fig. 7 b) of which only rarely do more than four show any regular packing, although...
the curved helices are arranged in stacks. In *Entamoeba* the ribosome helices are regularly packed in the chromatoid bodies and the arrangement of the particles in individual helices is quite different to that seen in *Neocallimastix*. Transverse sections of the helices in the chromatoid bodies of *Entamoeba* show discs about 60 nm in diameter in which individual particles cannot be distinguished, because with 12 particles in 1 helix repeat, 5 turns long (Lake & Slayter, 1970, 1972) they overlap in end-on view. Ribosome helices occurred scattered throughout the cytoplasm of *E. histolytica* as shown by Rosenbaum & Wittner (1970). Tetrads, interpreted as cross-sections of the helices were seen, as were pentads and hexads.

Our inability to see the ribosome aggregates in broken zoospores by negative staining, although we could readily see glycogen particles (Fig. 8a), may be explained if the aggregates behave in the same way as the ribosome polymers from *Entamoeba*, simple extracts of which, when examined in the same way, contain abundant glycogen and few ribosomes (Morgan et al., 1968). This implies that the *Neocallimastix* zoospores contain ribonuclease to which the particles are susceptible. This proposal has been borne out in preliminary experiments using a ribonuclease inhibitor in the extracting medium; ribosome-like particles are then seen in the negatively stained preparations. We suppose that these aggregates represent a store ready for rapid protein synthesis during germination, normally triggered by attachment of the zoospore to a suitable substrate. The presence of two distinct storage forms is particularly remarkable; they may be utilized at different stages in the germination process.

We propose that attachment of the zoospores involves the surface layer and/or release of the material in the amorphous globules which are at the pole which attaches the zoospore to the substratum; either of these might be the source of the finely fibrillar material seen at the point of attachment (Fig. 9).

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**REFERENCES**


ORPIN, C. G. (1977). Invasion of plant tissue in the
Structure of Neocallimastix zoospore


