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ULTRASTRUCTURE AND PERIPHERAL MEMBRANES OF THE MYCETOMAL MICRO-ORGANISMS OF *SITOPHILUS GRANARIUS* (L.) (COLEOPTERA)

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SUMMARY

The peripheral membranes of the micro-organisms of the mycetocytes of adult midgut caecae and of larval mycetomes of *Sitophilus granarius* (L.), GG strain, have been examined with an electron microscope. The majority of the mycetocytes were depleted of intracellular organelles but contained large numbers of mycetomal micro-organisms, most of which exhibited only one peripheral membrane. Some mycetocytes, however, had well-developed ultrastructure and harboured mycetomal micro-organisms which showed two peripheral membranes, namely a cell wall and plasma membrane. Intermediate conditions also occurred.

It is suggested that the absence of host-provided membranes around the micro-organisms categorizes them as obligate symbiotes.

INTRODUCTION

Musgrave (1964) in a review of insect mycetomes discussed much of the known information about these interesting organs and the pleomorphic micro-organisms which they harbour. The characteristic mycetomal micro-organisms of the GG strain of *Sitophilus granarius* (L.) have proved resistant to *in vitro* culture but may be studied in hanging-drop preparations, in which they maintain a lifelike condition for months (Musgrave & McDermott, 1961).

Buchner (1953), Steinhaus (1946) and authors reporting on observations with the light-microscope have depicted the mycetomal micro-organisms as lying freely in the cytoplasm of the cell they inhabit. This was later confirmed with the electron microscope by Musgrave, Grinyer & Homan (1962), who also remarked upon the thinness of the 'cell walls' of the micro-organisms.

The work presented here is an extension of the earlier paper by Musgrave *et al.* (1962), complements the study of the mycetomal micro-organisms carried out recently by Singh & Musgrave (1966) with the light-microscope, and represents a further contribution to our knowledge of mycetomal symbiotes.

MATERIALS AND METHODS

Initial failure to demonstrate two peripheral 'unit membranes' (cell wall and plasma membrane) around the mycetomal micro-organisms led to the use of several methods of fixation at two pH levels (7·3 and 6·0 to reveal respectively insect and micro-organism structure); and also to the use of dilute fixatives in the hope that a low tonicity would encourage rapid penetration and better preservation.

Larval mycetomes and adult midguts were dissected from weevils as described previously (Musgrave *et al.*, 1962) except that the dissections were done in Wyatt's (1956) solution and the material subjected to one of the following procedures:

(*a*) Treated at 4 °C for 2 h in 5% glutaraldehyde buffered at pH 6·0 with 0·1 M phosphate. After washing in three changes of 0·2 M sucrose in the same buffer the tissues were 'post-fixed' in buffered 1% osmium tetroxide at pH 6·0 for 1 h.

(*b*) Treated as in (*a*) except that all solutions were buffered to pH 7·3.

(*c*) Fixed in Kellenberger's standard fixative (Ryter & Kellenberger, 1958) for 16 h at 4 °C.

(*d*) As in (*a*) but fixed for 1 h in glutaraldehyde diluted 1:10 with distilled water.

(*e*) As in (*d*) but all solutions buffered to pH 7·3.

(*f*) Treated with Kellenberger's fixative diluted 1:10 with distilled water for 1 h, then as in (*c*)

After the above procedures all tissues were treated with 1% aqueous uranyl acetate for 1 h. Dehydration was accomplished in graded ethanol and the tissues embedded in 'Maraglass' epoxy resin (Freeman & Spurlock, 1962).

Sections were cut with glass knives held in a Porter–Blum ultramicrotome and mounted on 200-mesh, carbon-coated, copper grids. They were then stained with lead citrate (Reynolds, 1963) and examined in a Philips EM 200 electron microscope at an accelerating voltage of 60 kV.

RESULTS

The fine structure of the mycetomal micro-organisms in both mycetomal and midgut mycetocytes was, in general, best preserved in Kellenberger's standard fixative. Fixation for the first hour in diluted fixatives did not improve the preservation of the micro-organisms, so the following description of the ultrastructure of mycetocytes and mycetomal micro-organisms is based on specimens fixed in Kellenberger's standard fixative.

Mycetocytes

With the light-microscope studies (Singh & Musgrave, 1966) as a guide, three kinds of mycetocytes could be distinguished on the basis of differences in ultrastructure. The more common (type *a*) were those in which mitochondria were few and an organized endoplasmic reticulum scanty or absent (Fig. 1). In contrast, much higher concentrations of cytoplasmic organelles and free ribosomes were encountered in certain other mycetocytes (type *b*) (Figs. 3, 4). The division of the mycetocytes into types

was a matter of convenience to facilitate description. Intermediates between the two extreme types *a* and *b* could often be recognized and are referred to as type *c* (see below).

Micro-organisms

The mycetomal micro-organisms lay freely in the cytoplasm of mycetocytes and occupied most of it (Fig. 1), thus confirming the observations mentioned in the Introduction. In addition some mycetomes also contained some smaller bacteria-like structures perhaps similar to those mentioned by Musgrave & Miller (1956) and not necessarily a form of the organisms under investigation.

Most of the mycetomal micro-organisms in type *a* mycetocytes showed only one trilaminate 'unit membrane' (Robertson, 1959) and had cytoplasmic and 'nuclear' areas of low electron density (Fig. 2) but a small number had two 'unit membranes' while still showing low electron density in their cytoplasmic and nuclear areas. In contrast the smaller 'bacteria-like' structures mentioned above all showed two 'unit membranes'.

All the micro-organisms in the type *b* mycetocytes had double 'unit membranes' and cytoplasmic and nuclear areas of relatively greater electron density (Fig. 4).

Some of the mycetomal micro-organisms showed a layer (possibly mucopeptide) between the cell wall and plasma membrane, presumably similar to an intermediate layer described independently by Claus & Roth (1964) and Murray, Steed & Elson (1965). Cytoplasmic structures could be seen in some of the micro-organisms, presumably continuations of the plasma membranes extending into the cells. The nuclear elements were typically 'bacterial' in appearance showing fine strands of nuclear material in an area of low electron density. The greater part of the cytoplasm (Fig. 4) was filled with closely packed ribosome-like material (Schlessinger, Marchesi & Kwan, 1965).

The mycetomal micro-organisms found in the type *c* mycetocytes varied; some had the robust appearance of those in type *b*, whereas others looked like those in type *a*. Both kinds of organisms sometimes occurred in the same mycetocyte.

None of the micro-organisms described was surrounded by a host-provided membrane.

DISCUSSION

The light-microscope findings and the occurrence of apparently three kinds of mycetocytes with differing micro-organisms naturally led to some concern about the methods of fixation and other preparatory work. Three observations seem to substantiate the findings presented. First, the small bacteria-like structures always showed two peripheral 'unit membranes' even when in the same mycetocyte as, and close to, mycetomal micro-organisms showing only one membrane. Secondly, mycetomal micro-organisms with one and those with two peripheral membranes were at times seen in the same mycetocyte. Thirdly, as noted, certain mycetocytes (type *b*) were not only more like normal healthy cells in their ultrastructure but also harboured large numbers of mycetomal micro-organisms with two peripheral membranes.

The mycetomal micro-organisms with double membranes were comparable in appearance with those shown in recently published micrographs of well-preserved Gram-negative bacteria (Glauert, Kerridge & Horne, 1963; Poindexter & Cohen-Bazire, 1964; Claus & Roth, 1964; Murray *et al.*, 1965). In addition to the double membrane, Claus & Roth and Murray *et al.* have described an intermediate layer and suggested that it was responsible for the rigidity of the cell wall. Murray *et al.* also discussed the possibility that this layer might, in some micro-organisms, be more strongly associated with the plasma membrane; an idea which might explain the stability of the L-forms of some bacteria (which have only one peripheral unit membrane). As already mentioned, such a layer was visible in some of the mycetomal micro-organisms. However, since Claus & Roth suggested that the conditions for the fixation of this intermediate layer were very critical, speculations as to its presence and consequent responsibility for rigidity in single-membraned mycetomal micro-organisms seem inappropriate at this time.

Singh & Musgrave (1966), using the tannic acid/crystal violet technique for light-microscope studies, have found evidence of a distinct cell-wall-like membrane around some of the mycetomal micro-organisms of *S. granarius*, and indications of a plasma membrane. But the micro-organisms are pleomorphic and may have some kind of life-cycle (Musgrave, 1964). It is also possible that some of them have become almost a part of the host cell, or may be dying or dead, or being lysed by the host cell with consequent loss of the outer membrane. The tannic acid/crystal violet technique, strictly speaking, detects membranes (R. G. E. Murray, personal communication) and the cytologist is tempted to assume that if a number of cells in a field respond to this technique then the cells have cell walls. This, although not all the cells in the field may respond and although only a 'membrane' has been detected.

It seems that, in the present state of knowledge of 'bacterial' envelopes, conclusions as to their nature should not be drawn from any one method of research. Undoubtedly, full understanding of the true nature of the membranes of mycetomal symbiotes awaits further research and advances in technique.

On the basis of the present findings it is suggested that mycetomal micro-organisms in *S. granarius* have two unit membranes, but that one membrane may at times be lost.

The finding that mycetomal micro-organisms are not surrounded by host-provided membranes and their resistance to *in vitro* culture (Musgrave & McDermott, 1961) suggests an obligate mutualistic relationship and lends some support to an idea (advanced by Pearson, Freeman & Hines (1963) in discussing bacterial and viral parasites) that while facultative intracellular parasites are surrounded by host-provided membranes obligate intracellular parasites are not. Some symbiotic bacteria are known to be enclosed in host-provided membranes (Jordan, Grinyer & Coulter, 1963; Jordan & Grinyer, 1965). But the obligate intracellular parasites cited by Pearson *et al.* (1963) are all viruses which, by definition, contain only one type of nucleic acid. The mycetomal micro-organisms contain DNA (Musgrave & Singh, 1965; Singh & Musgrave, 1966) and their ultrastructure strongly suggests that they also contain RNA. If they do, then they are most unusual, for intracellular entities without host-provided

membranes rarely contain both nucleic acids. However, as Anderson, Hopps, Barile & Bernheim (1965) state that certain rickettsiae (which contain both nucleic acids) lie freely in the cytoplasm of infected cells, such a situation with mycetomal micro-organisms is not impossible.

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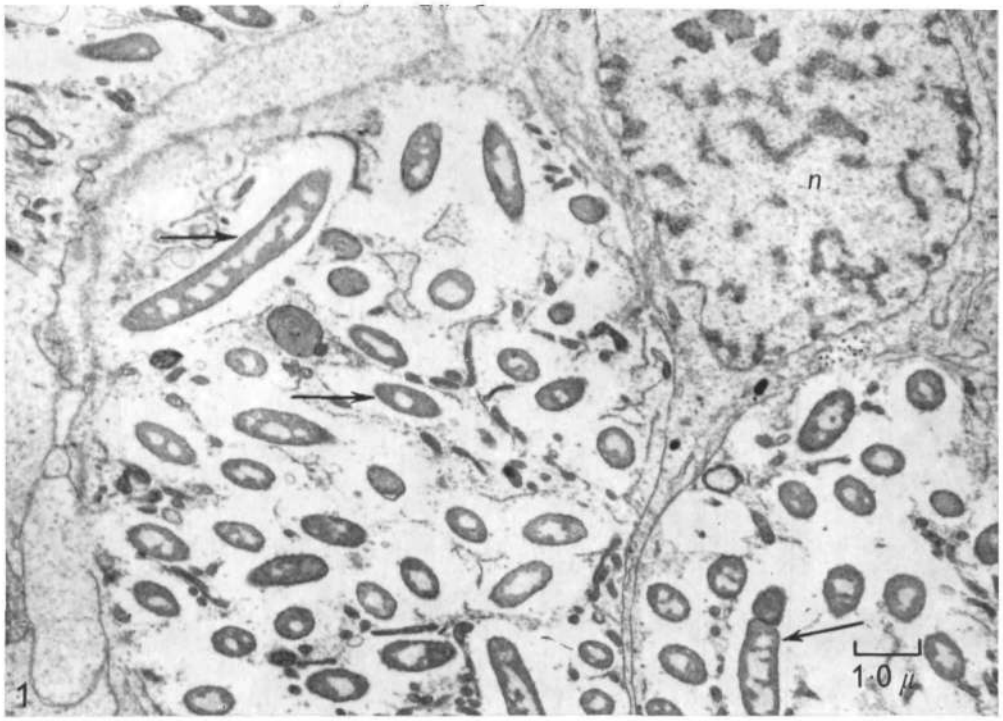

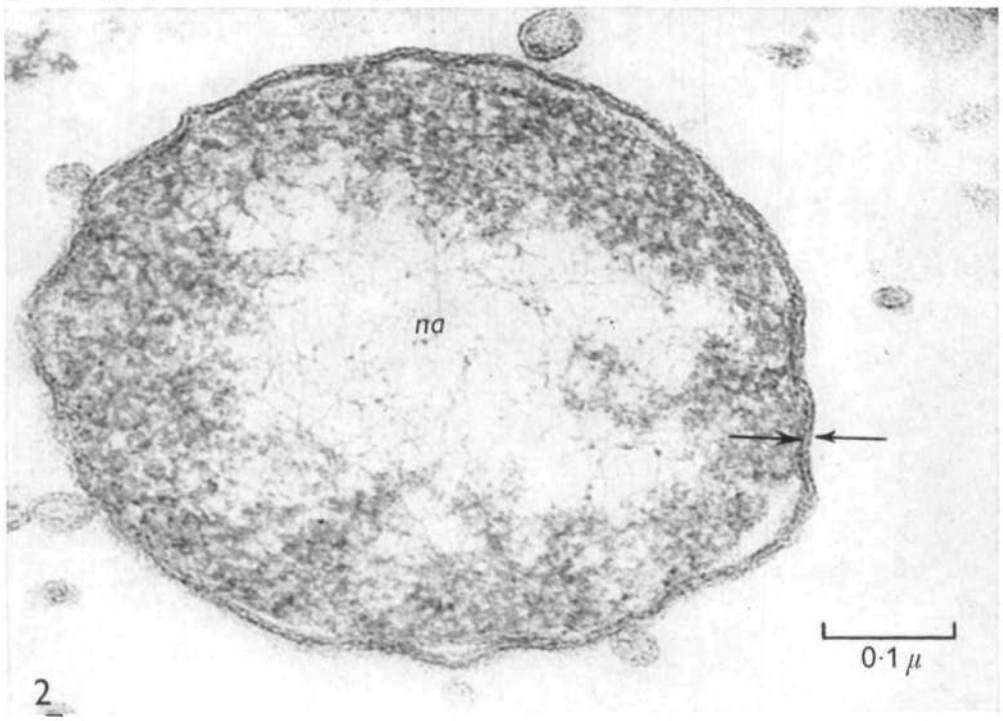

Fig. 1. Mycetomal mycetocyte (type *a*) at low magnification. Arrows indicate mycetomal micro-organisms (*n*, mycetocyte nucleus). × 9000.

Fig. 2. Mycetomal micro-organism at high magnification, typical of those found in type *a* mycetocytes (*na*, nuclear area). Note single 'unit membrane' between arrows. × 165000.

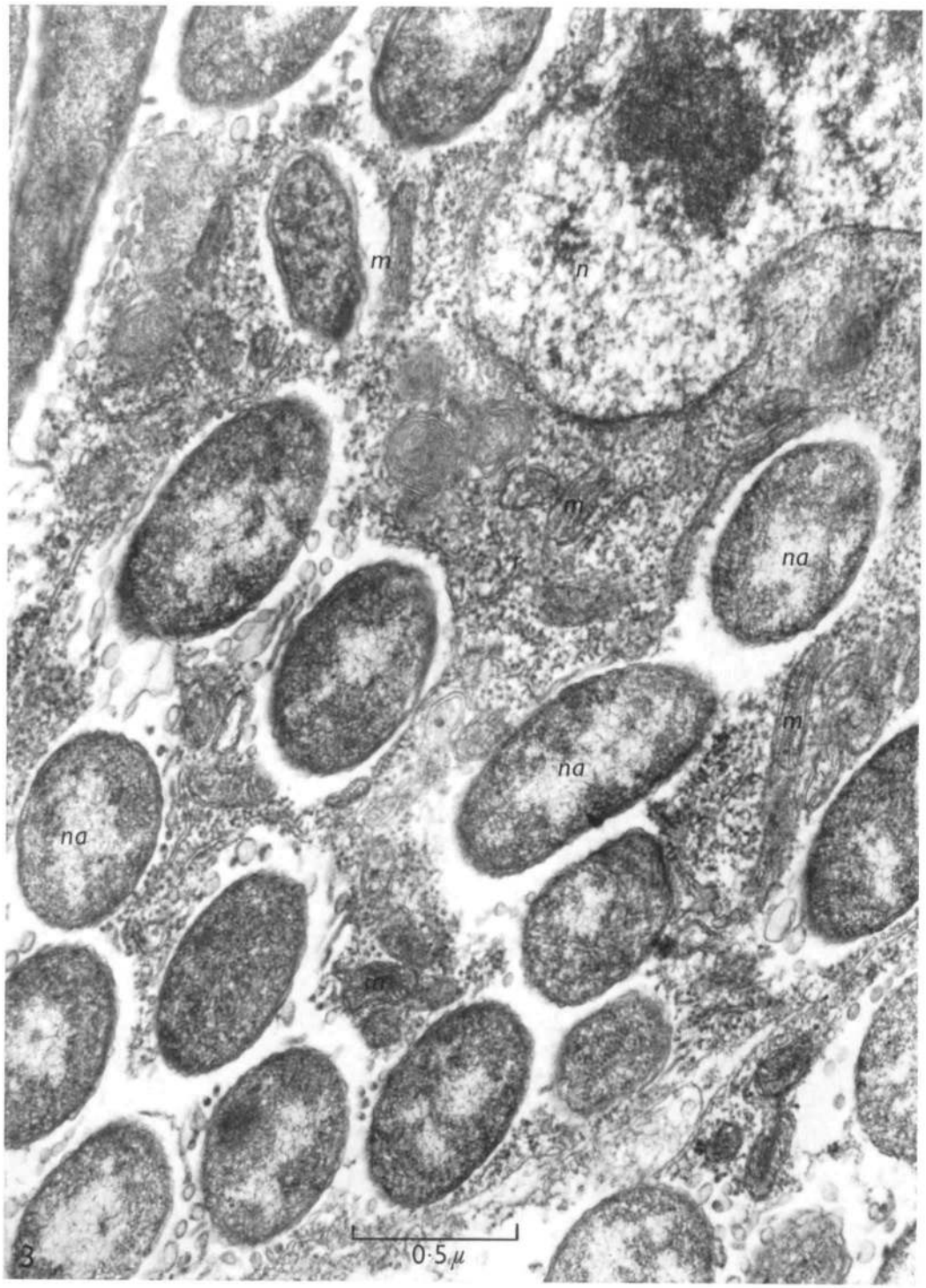

Fig. 3. Mycetomal micro-organisms in type *b* mycetocyte (*m*, mitochondria; *n*, mycetocyte nucleus; *na*, nuclear area of mycetomal micro-organisms; *r*, ribosomes). × 50000.

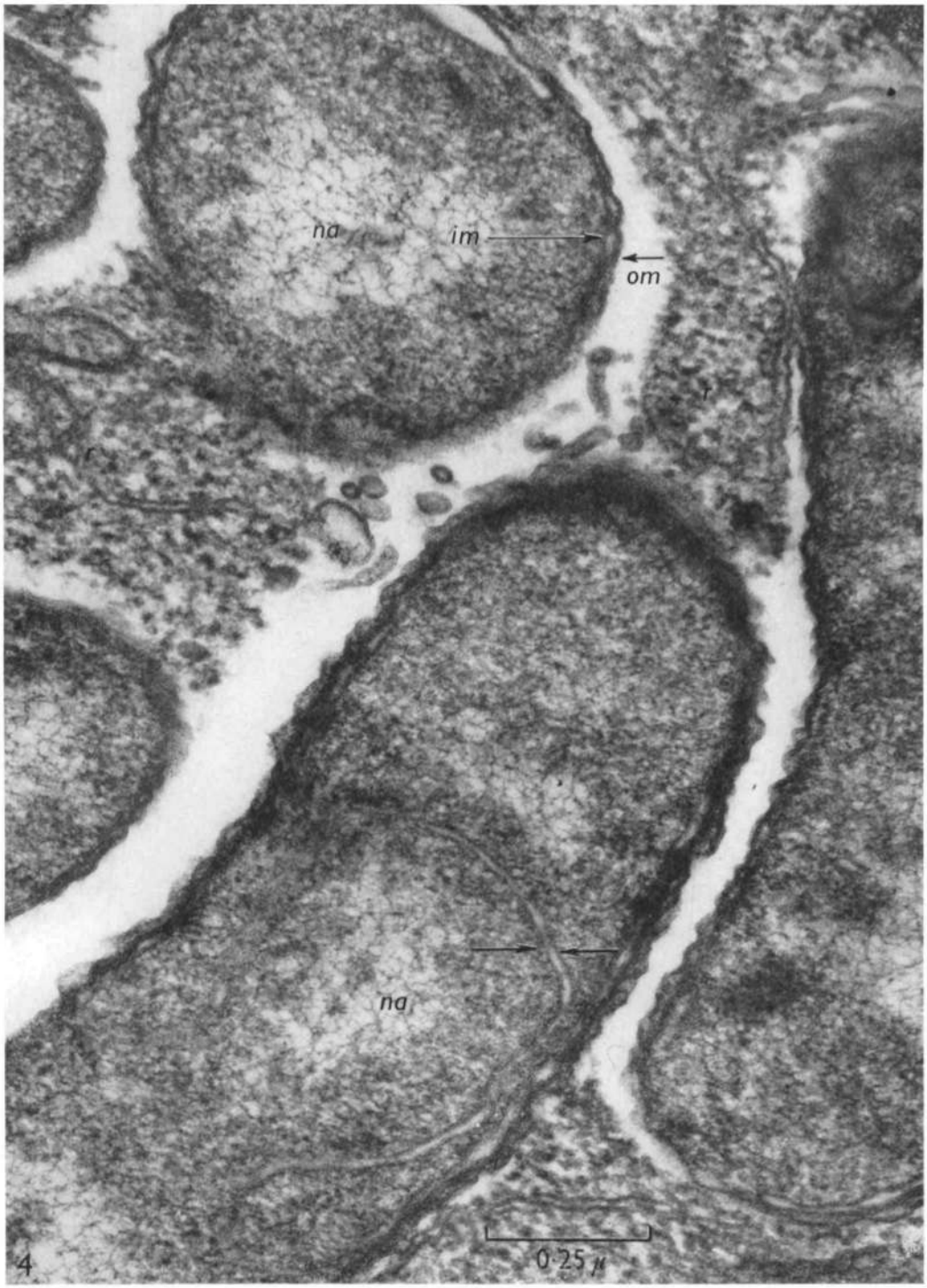

Fig. 4. Midgut mycetomal micro-organisms in type *b* mycetocyte (*im*, inner peripheral membrane; *na*, nuclear area; *om*, outer peripheral membrane; *r*, ribosomes). Note intracytoplasmic membranes between arrows. × 100000.