EESTI NSV TEADUSTE AKADEEMIA TOIMETISED. BIOLOOGIA
ИЗВЕСТИЯ АКАДЕМИИ НАУК ЭСТОНСКОЙ ССР. БИОЛОГИЯ
PROCEEDINGS OF THE ACADEMY OF SCIENCES OF THE ESTONIAN SSR. BIOLOGY
1984, 33, 4

https://doi.org/10.3176/biol.1984.4.03

УДК 576.8.095.38:591.557

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ULTRASTRUCTURAL ASPECTS OF ENDOCYTOBIOSIS IN LEAFHOPPER (*INSECTA: CICADINEA*) CELLS

Specific endocytobiosis observed in insects is biologically a rather interesting phenomenon which presents intriguing problems concerning the morpho-functional integrity of organisms, their autonomous existence as well as several questions concerning the formation and development of interrelations of different organisms in their evolution.

The fact that there is a specific coexistence of a great variety of insect species with various intracellular microorganisms has been known for about one hundred years (Buchner, 1965). On the basis of light microscopic investigations, P. Buchner and his followers laid the foundation for a conception according to which this coexistence has been regarded as endosymbiosis and the corresponding microorganisms participating in it have been named endosymbionts. According to that conception, endosymbiosis is also regarded as a well-regulated coexistence of two organisms belonging to different levels of organization. The differentiated cells of the multicellular animal usually serve as a biotope for the unicellular endosymbiont, and their mutual adaptation is such that they both benefit from it (Buchner, 1965). V. A. Dogel (Догель, 1962) regards symbiosis as a kind of coexistence of organisms where both members of the association jointly participate in the regulation of their relations with the outer world (as being different from parasitism, where this function is controlled by the host). The important features of the symbiotic systems are their permanence as well as the specificity of partners involved, which both increase with the growth of the intricacy of their interconnections (Dubos, Kessler, 1963).

In insects, particularly in *Homoptera* with their most complicated endosymbiotic interconnections, we have to do with a peculiar system insect—microorganism, where interdependence of the partners borders on an utter loss of their functional autonomy. This, in its turn, practically results in the formation of compound organisms. As to microorganisms, this association generally results in the impossibility of cultivating them outside the cells of the host organism and they can be studied mainly by morphological descriptive methods which do not always satisfy the requirements set for traditional microbiological investigations. The endosymbionts of insects have been included into the order *Rickettsiales* in three genera (*Wolbachia, Symbiotes, Blattabacterium*), but they have not been divided into separate species (Buchanan, Gibbons, 1975). In papers on insect endosymbionts the microorganisms are denoted by Greek or Latin letters, and they are, as a rule, ascribed to a certain definite insect host (Müller, 1962).

Because of a close morpho-functional link between endosymbionts and the host cells, and because of the fact that they exist exclusively in

the host cells, W. Schwemmler (1980) suggested naming these microorganisms endocytobionts and the phenomenon as such—endocytobiosis, which is very befitting. The terminology is of great significance here, for it serves as a clue to the general interpretation of the interrelations of organisms involved in such associations. Both these terms—endocytobiosis and endocytobiont—are quite suitable, for they encompass a wider range of the corresponding phenomena than the terms "endosymbiosis" and "endosymbiont" that characterize mainly the mutual benefit of partners, whereas "endocytobiosis" denotes any forms of intracellular existence of symbiotic, parasitic and other organisms (Тийвель, 1982).

It is most difficult to carry out *in vitro* this cyclic process (changing vegetative and infectious stages) of the development of intra-ovarially transmitted microorganisms. This is also what probably considerably impedes experimental investigation of endocytobionts. Therefore, it was morphological investigations (now electron microscopical) that have served as a chief source of information about this unique biological phenomenon. Of considerable interest are, on one hand, the ultrastructure of the cells that harbour endocytobionts, and, on the other, the ultrastructure of various types of endocytobionts at different stages of their life cycle.

Material and methods

For this study we used the leafhoppers *Aphrophora alni* Fall. and *Philaenus spumarius* L. (*Cicadinae, Aphrophoridae*). The material was gathered in the park of Harku in the vicinity of the Institute of Experimental Biology of the ESSR Academy of Sciences, and in the meadows of Rutja village, Rakvere District, Estonian SSR. The gathered insects were killed with CO_2 and prepared in buffer solution containing some drops of glutaraldehyde. Fragments of the insect bodies as well as extracted mycetomes were fixed in a 6% solution of glutaraldehyde in insect Ringer (Shikata, Maramorosch, 1965), or in cacodylate buffer for 90 min at ph 7.0 and in a 2% solution of OsO_4 in insect Ringer or in a cacodylate buffer. To get a more effective impregnation of the eggs by the fixing solution, they were pricked with a needle in their fore end that contains no cytobionts.

This was followed by the dehydration of the material in a gradient of alcohol and acetone, staining it with uranyl acetate and phosphostungic acid, and embedding it in EPON 812 or in a mixture of EPON 812 and araldite. The sections were obtained on the microtome LKB III. For the light microscopic investigation the staining of the sections of 1 μ thickness was carried out in two stages. In the beginning, a double stain was used — a mixture of 1% water solution of methylene blue in 1% borax and 1% water solution of azure II, mixed in equal parts. During staining, the sections were slightly heated (t=50°C) for 3 to 5 min. At the second stage the staining was done with 1% basic fuchsin in 70% alcohol (Тийвель, 1981).

For electron microscopy, sections on grids and coated grids were stained with uranyl acetate for 15 to 30 min (t=50°C) (some sections were stained in a saturated solution of uranyl acetate in alcohol for 30 min at room temperature) and with lead citrate for 30 to 40 min. The samples were examined through the electron microscope Tesla BS-613.

Some electron microscopical preparations prepared in the usual way but without OsO_4 postfixation were treated with phospholipase, pronase and trypsin. With phospholipase A_2 from *Naja naja oxiana* venom

(produced at the Institute of Chemical and Biological Physics of the ESSR Academy of Sciences, Tallinn) (specific activity 300 units/mg) in concentrations of 0.2 and 1.0 mg/ml in the solution of 0.05 M Tris-HCl at pH 7.5, the time of treatment was from 1 to 24 hrs. With 0.4% pronase E (Merck 70,000 PUK/g) and 0.4% trypsin (SPOFA, specific activity 58 mM/min, mg) in 1×SSC buffer (0.15 M NaCl, 20 mM sodium citrate, 0.5 mM EDTA) at pH 7.4, the time of treatment at 37 °C was 8 to 24 hrs.

The structure of mycetomes and mycetocytes

Mycetomes of *Aphrophora alni* and *Philaenus spumarius* are paired structures on both sides of the insect's abdomen, closely fitted to the tergite and sternite epidermis in the segments from the 2nd to the 6th. Their sizes are about 1.0×0.2×0.2 mm. In the process of preparation they could be differentiated because of their various colour—the tissues of the abdomen are usually whitish, while mycetomes are discernibly yellowish. Mycetomes in female insects are much bigger than those in males, they are metameric, and their sizes and form change periodically. In mycetomes of *P. spumarius* there are various regions inhabited by morphologically different cytobionts. Such regions containing different types of microorganisms are often interlaced, presenting a formation of irregular shape (Fig. 1). The disposition of the mycetomes prompts their close morpho-functional connection with many tissues and organs of the insect, such as fat body, gonads, muscle tissue and integument. In male specimens the closest contact seemed to be between mycetomes and testicles (Fig. 2). No direct contact with the intestines was to be noticed. In several places mycetomes were transversed by dorsoventral muscles and tracheoles. As a rule, mycetomes are surrounded by single-layered epithelium consisting of flattened cells with several oval or round nuclei (Fig. 1). The epithelium, being somewhat thicker on the side of the fat body than on the side of the testicles, on the eggs and on integumentary tissues of the insects, continued between separate branches of mycetomes, but there it was thinner and less granulous. Some branches of the mycetomes penetrated rather deeply between the organs and tissues of the insects.

Mycetomes consist of mycetocytes which, being larger in size than the ordinary insect cells (up to 100 μ in diameter), have several nuclei as well as bodies of both round and irregular shapes—endocytobionts which considerably differ from the usual structural components of the animal cell. Beside these structures mycetocytes contain mitochondriae, ribosomes, Golgi complexes, lysosomes and endoplasmatic reticulum. One of the characteristic features of mycetomes was their big nuclei—up to 10 μ; being of irregular configuration they often looked as if compressed between endocytobionts (Fig. 3). Granulated karyoplasm contains thin fibrilles, but no nucleolus could be observed electron microscopically.

Mycetocytes contain numerous oblong mitochondriae 0.5—0.8 μ thick and up to 2 μ long, that are often closely fitted to the endocytobionts, being joined to the membrane of the bacteriophoric vacuole. Golgi complex was seldom noted in mycetocytes. When present, it consisted of flat little sacs of about 150—200 Å in diameter, with vesicules of 0.2 μ in diameter at their ends (Fig. 4). Cytoplasm of mycetocytes just as that of endocytobionts, is rich in ribosomes that are often united into polyribosomes. Mycetocytes have a clearly discernible granulous endoplasmatic reticulum. The cisterns of the rough reticulum formed concentric configurations as well as widened reticulum at various stages

of its swelling, which could be observed in several places. Besides the organelles typical of animal cells, various structures were noted in the mycetocytes that resembled secondary lysosomes. Those were multivesicular formations of the size 0.5—2.0 μ, containing vesicules, thin threads, rows of friable lamellae as well as phagosomes (Fig. 5). There is a considerable variety of such multivesicular structures that are surrounded by membranes in the mycetocytes of both *A. alni* and *P. spumarius*. In larger formations, up to 2 μ, the main components were twisted membranes and friable lamellae often ending with vesicules. Such structures occurred in cells containing cytobionts mainly of the first and third types. In *P. spumarius* cells containing cytobionts of the second type, such multivesicular bodies never exceeded 1.5 μ; besides vesicules, there were also threads and granulous substrate. The cytoplasm of mycetocytes of both species contained also vesicules surrounded by elementary membranes of 0.1—0.3 μ in size, with finely granulated matrix (Fig. 5).

In mycetocytes of *A. alni,* besides the structures described above, electron-optically very dense bodies of irregular configuration and of up to 10 μ in size, consisting of concentric myelin-like tightly packed lamellae with a regular period of about 50 Å, were observed (Тийвель, 1981). In regions of less regularly packed membranes, usually in the centre of the bodies, there were often lumps of a very electron-dense substance. In mycetocytes of *P. spumarius* similar myelin-like structures were more numerous, smaller in size (up to 2 μ), and of a more regular oval shape (Fig. 7). Concentric membranes were not so tightly packed here as they were in similar structures of *A. alni*.

In the ultrastructure of the mycetocytes of the larvae from the 3rd to the 5th stage of development, no differences were observed as compared to the corresponding cells of the adults. The only difference was that the mycetomes in the larvae were always surrounded with connective tissue cells, the epithelium of the larval mycetome was thicker, and the cytobionts in the cells of the larvae of these species did not adhere to each other too closely (there were more of the cytoplasm of the host cell) as compared to the corresponding structures in adults.

The types of cytobionts in cells of leafhoppers

As a result of electron microscopic investigations, three types of endocytobionts have been found in *Philaenus spumarius*, two of which were exclusively connected with mycetomes, and the third microorganism was localized both in mycetomes and in the connective tissue cells, whereas in *Aphrophora alni* only two types have been stated, corresponding to the 1st and 3rd types found in *P. spumarius*. The first of the types of endocytobionts was very large—10 to 20 μ, of irregular shape, and was surrounded by a cytoplasmic membrane, a membranous cell wall typical of gram-negative bacteria, and, on the outside, by an additional membranous casing, most probably a derivative of the mycetocyte, which forms a kind of space around each cytobiont—a bacteriophoric vacuole (Figs 4, 10, 12).

The vacuoles considerably differ in size, beginning with a slit-like space between two cytobionts up to a rather big vacuole, the size of which may exceed the size of microorganisms by some micrometres. The ultrastructure of this type of endocytobionts in both species is quite uniform—the cells are homogeneously filled with ribosomes of 100—150 Å in size. In the disposition of the ribosomes there is a certain condensing which cannot be connected with any particular regions of

the cells. It was not often that we came across dividing cells as well as cells with noticeable fibrillous structures—components of nucleoid. In these endocytobionts crystal-like organized structures (in light-microscopic preparations they were just dark patches) of the size of 0.1—0.4 μ, consisting of particles of up to 300 Å, were observed. Usually they occupied a central position in the microorganism cell. They could not be observed in all preparations, but if present, they seemed to occupy several microorganisms (Fig. 12). In some cells lamellar structures were observed of about 1×4 μ in size (Fig. 11). In some endocytobionts of the 1st type, vacuoles of up to 1 μ, surrounded by a single-layer membrane, and large inclusions of about 0.5 μ with a less electron-dense content were observed. In some cases near the cytoplasmic membrane of the microorganism one could observe round and pear-shaped structures, with membranes around them, 0.1 μ in size, with the surrounding cytoplasm of the microorganism being less electron-dense. The content of such structures was, as a rule, homogeneous (Fig. 13).

The cytobionts of the second type of *P. spumarius,* 10 μ in size, in general resembled the first type, but in adult insects they were always localized in different cells. They were usually round or oval in shape; the irregular form, characteristic of the first-type endocytobionts, was not observed. Their cytoplasm was less electron-dense and less homogeneous than that of the first type; the ribosomes in the microorganism cytoplasm formed islets with electron-light regions with thin fibrilles between them (Figs 6, 7). These endocytobionts were also surrounded by a three-component border structure. The bacteriophoric vacuole to be formed usually exceeded the size of the cytobionts in it somewhat more than was the case in the vacuole of the first type. It was often possible to see even several second-type cytobionts as well as dividing bacteria in one and the same vacuole (Figs 6, 14). The latter narrowed down in some places, producing a dividing line so that finally typical membranous layers were formed between the two microorganisms. There were often vesicules as well as pieces of membranes to be found in places of such narrowings.

In the cytoplasm of the second type of cytobionts there were inclusions as well as membranous structures. No crystal-like structures characteristic of the first-type cytobionts have been observed. However, rows of some kind of particles could be seen in some cells, which resembled ribosomes in their size and contrast, but differed from the ribosomes in the cytoplasm of cytobionts by their orderly rows (Fig. 16).

Among other intracytoplasmic structures there were a lot of those with homogeneous contents, surrounded by a membrane. Their size was 0.1—0.8 μ. Most of them occupied peripheric sites of the microorganism cytoplasm; however, no direct contact with the cytoplasmic membrane of the cytobiont has been noted (Figs 14, 15).

In many cells there were filamentous structures, which probably constitute a part of the nucleoid of the microorganism. The diameter of the nucleoid fibrilles was about 0.01—0.02 μ. The nucleoid regions often had a central, more electron-dense zone that could be up to ten times bigger in diameter than the fibrilles branching off that zone. The nucleoid region was particularly clear-cut in the cells about to divide (Figs 6, 14). In several places a certain contact could be observed between the described structures that were surrounded by membranes and a region of nucleoid with fibrilles (Figs 14, 15).

The third type of cytobionts, 1—2×10 μ in size, was located in cells which already contained cytobionts of either the first or the second type and, in some cases, in the connective tissue cells as well (Figs 8, 10).

They looked like typical bacteria—they had both central electron-dense nucleoid regions and dense cytoplasm adhering to the walls, which, as a whole, were somewhat less electron-dense than those in the first and second type endocytobionts. The ribosomes were, as a rule, evenly dispersed. The walls of the third-type endocytobionts were also three-layered, thus corresponding to those of gram-negative bacteria; the size of the bacteriophoric vacuoles here usually considerably exceeded those of the cytobionts.

The third type endocytobionts differed in their structure—beside the typical bacteria described above, the mycetomes of both species of the leafhoppers contained bodies of the same size but with a more electron-dense content and with wrinkled border membranes lacking nucleoid regions with fibrilles. There were indications of numerous intermediate cases as well (Fig. 9). The third-type cytobionts did not seem to have any particular inclusions.

Beside the forms described, the mycetocytes contained structures of about 2 μ of two different kinds—some were electron-dense, thickly filled with granulated matrix, while the others were surrounded by electron-dense border-structures of lighter contents, full of particles resembling the ribosomes of cytobionts. Such bodies were observed in the cells filled with ribosomes and a rough endoplasmatic reticulum, near the nucleus of the mycetocyte and the endocytobionts of the second type only (Figs 17, 18).

After a 2.5 h phospholipase treatment, the membranes of the cytobionts became blurred, and the clear-cut contours of the vesicular formations in the mycetocyte cytoplasm disappeared (Fig. 19). After a 24-h treatment, the cytoplasm of the first type of cytobionts became lighter as compared with that of the insect cell. The ribosomes of the cytobionts remained more or less normal, while only the contours of their membranes could be seen. Following the 18 h pronase and trypsin treatment, the fine granular material disappeared, only particles of about 200 Å in size remained, the membranous coats of bacterial cells became disrupted and the distances between them became greater. The cytoplasm of the cells as well as the lamellar inclusions in them became electron-optically much lighter.

The fine structure of the embryonic mycetome

In order to elucidate the morphological peculiarities of the embryonic mycetocytes, the unlaid eggs of *Philaenus spumarius* were studied electron microscopically.

The cell containing cytobionts was surrounded by yolk, and was situated at the posterior end of the egg (Fig. 20). Two types of cytobionts were found. They were similar to the corresponding forms in the adult insects, being only somewhat smaller. The cytoplasm of the first type, abounding in ribosomes, was electron-optically rather dense. Some of those cytobionts contained various inclusions, such as, for instance, electron-optically very dense regions without any surrounding membranes as well as vesicular formations of 0.02—0.5 μ in size, which most likely represent certain stages in their development (Tiivel, 1983a). The dense regions resembled corresponding structures of the endocytobionts of the first type of adult insects. Vesicular formations were of two different forms—some consisted of several vesicules of 0.05 μ in size, the others were large (up to 1 μ) structures of light contents (Fig. 21).

In the embryonic mycetome the cytobionts of the first type appeared to be packed together more closely than those in the adult specimens

which fact accounted for their form not being particularly regular, with numerous invaginations. Every single microorganism was surrounded by two membranous coats of bacterial origin as well as by an additional coat enveloping several cytobionts, obviously a derivative of the mycetocyte. There was no typical bacteriophoric vacuole around each cytobiont, and in several places between them there were membranes folded many times, probably belonging to the microorganisms, although in some cases the existence of the host cell cytoplasm might be suspected between these folds (Fig. 21).

The other type of cytobionts were bacteria-like microorganisms of 1—2 μ in size with three membranous coats around each of them, and with ribosomes scattered unevenly in their cytoplasm. As a rule they were situated near the border of the embryonal mycetome, and the mycetocyte cell walls often formed cisternae beside the cytobionts of this type (Fig. 22).

After a phospholipase treatment, the picture was very much the same as in the adult cells, where the most drastic changes were observed in the first-type cytobionts and in their membranous coats (Fig. 23).

Discussion

Endocytobiosis has been, up to the present, studied electron microscopically in about 20 species of *Homoptera* (see Houk, Griffiths, 1980; Тийвель, 1982; Schwemmler, Kemner, 1983). However, it must be pointed out that those were chiefly various aspects of the ultrastructure of mycetocytes and the microorganisms inhabiting them that the investigators were concerned with. The life cycle of *Homoptera* endocytobionts is rather complicated — in many cases we have to do with different types and probably with different species of microorganisms and forms of their existence in insect cells (both vegetative and infectious). Because of this, a comparison of all the data obtained is far from simple. So, we have to be content with the fact that endocytobiontic interrelations with microorganisms have been studied in greater detail in only one species, namely *Euscelis plebejus*, and even here a lot of problems remain yet to be solved (Houk, Griffiths, 1980).

While studying the ultrastructural aspects of the specific coexistence—endocytobiosis of microorganisms with cells of two species of leafhoppers, the cells of microorganisms as well as the eukaryotic insect cells that served as specific biotopes for them, were subjected to a closer examination.

In the mycetocytes of those species, the cytobionts were in a vegetative stage of their development, which could be concluded when the data obtained were compared with those in literature (Körner, 1972; Schwemmler, 1974).

We came to a conclusion that the first type of cytobionts of the cicadae *A. alni* and *P. spumarius* evidently correspond to the so-called "chief" or "a"-cytobiont described in several species of *Homoptera* (Louis, Nicolas, 1976; Müller, 1972).

Further, there does not seem to be any justified caution for ascribing the second and third type of cytobionts to any type of cytobionts already described in literature, for even in leafhoppers the number of various types of cytobionts exceeds several dozens, so that to identify them is still almost impossible. As to the "t"-cytobionts in *E. plebejus*, they actually resemble the second type of the cytobionts of *P. spumarius* as described by us (Тийвель, 1979). There are, however, certain differences

in the general morphology of those microorganisms, namely in their inclusions; besides, the hosts themselves are not very close to each other systematically, which is not to be disregarded while different types of cytobionts are being described (Müller, 1962). To recognize the affinity of these microorganisms, it would be necessary not only to study their life cycle in greater detail but also to isolate them, investigate their DNA, and infect other leafhopper species with them. Until this has been done and until some valid proof of their identity has been obtained, we have no right to denote them with one and the same letter.

The third type of cytobionts is bacillus-like bacteria, rather similar in both species under investigation, which most probably belongs to the so-called "co"-cytobionts described in several species of *Homoptera* (Schwemmler, 1974).

The present data show that the first type of cytobionts of *A. alni* and *P. spumarius* are on more or less the same level as mycetocyte organelles, i. e. they are in close contact with insect cells, which is morphologically expressed in a close contact of the membranes of the bacteriophoric vacuole with the cell wall of the endocytobiont as well as in its functions being more co-ordinated with the vital activities of the mycetocytes. The second and obviously the third type of endocytobionts are less dependent on concrete insect cells (the third type has been found, besides mycetomes, in connective tissue cells), which can be inferred from a larger bacteriophoric vacuole, and they are evidently more autonomous.

The situation where several second- and third-type cytobionts were found in one and the same bacteriophoric vacuole can perhaps be accounted for by the presence of cytobionts failing to separate after their division. It is quite possible that in their later development these cytobionts will separate; there will be formed a layer of mycetocyte cytoplasm between them, and every separate microorganism will be surrounded by a bacteriophoric vacuole. No such situation where two "a"-cytobionts existed in one and the same bacteriophoric vacuole has been observed in adult mycetocytes. This may also testify to the above-mentioned closer connection of the vital activities of "a"-endocytobionts with the life cycle of the mycetocyte than is the case with the second- and third-type cytobionts, i. e. the division of "a"-endocytobiont cells is accompanied by the formation of peribacterial membrane around the cytobiont immediately after their division.

Intercellular contacts observed between the third-type cytobionts can be characterized by some kind of sticking together of the outer membranes of bacterial cells and the appearance of an electron-dense layer at the point of contact. Such a situation differs from intercellular contacts in eukaryotic cells, where specific contact structures have been noted but where to so-called desmosomes have certain similar features with the intercellular contact of cytobionts in those cells (Тийвель, 1981); it is not plasmatic membranes that interact there, and the "desmosome" results from the merging of the cellular walls of the microorganisms.

In the investigated cells we could observe electron-dense bodies (Figs 17, 18) that looked very much like the infectious forms of some cytobionts described in literature (Chang, 1983). Still, it seems reasonable to regard them with a certain amount of caution, as the infectious stage of cytobionts is of a comparatively short duration, and data obtained in other species may somewhat differ as compared to those of the species under investigation.

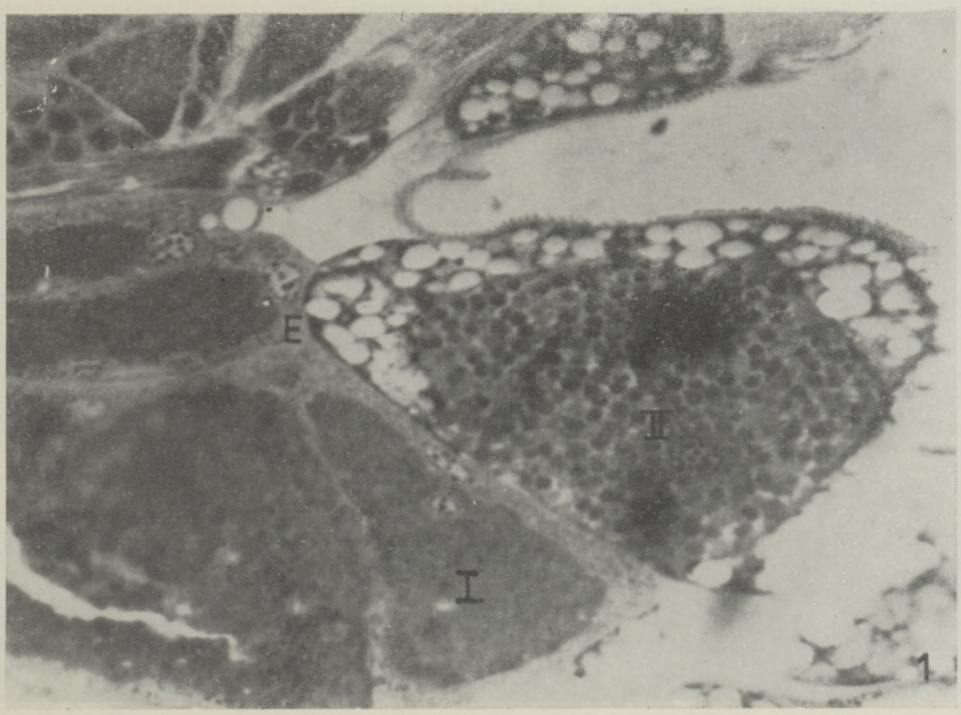
The presence of vacuoles containing finely granulated substrates and glycogenic inclusions (Körner, 1972), some membranous bodies as well as other inclusions of unknown origin (Chang, Musgrave, 1972;

Chang, Musgrave, 1975; Griffiths, Beck, 1975; Körner, Feldhege, 1970; Louis et al., 1976) have also been mentioned in connection with cytobionts. In the endocytobionts of *E. plebejus* there were electron-dense bodies with a diameter up to 1 μ without membranes, while similar structures in the mycetocyte cytoplasm are usually surrounded by membranes (Körner, Feldhege, 1970; Louis et al., 1976). These authors are of the opinion that such structures are derivatives of endocytobionts, which are able to penetrate through the membranous system of the cytobiont into the mycetocyte cytoplasm. The variety of inclusions and membranous structures observed in *Homoptera* mycetocytes as well as in cytobionts, is remarkable indeed. For example, in cytobionts of *Acyrthosiphon pisum* mesosome-like structures were found (Griffiths, Beck, 1975), similar to those of gram-negative bacteria (Remsen, 1982). In "co"-cytobionts of *Helochara communis*, a lot of parallel microtubules, that are directed towards the cytobiont plasma membrane, have been described (Chang, Musgrave, 1972). The structures described above in mycetocytes and endocytobionts of *A. alni* and *P. spumarius* might contribute to some extent to the general morphology of those cells. The slight differences in the fine structure in adult and embryonic mycetocytes can obviously be explained by the difference in the functions performed by endocytobionts in these stages of the insect life cycle.

As to the crystal-like regular structures in the cytoplasm of the first-type cytobionts of both *A. alni* and *P. spumarius* (Fig. 12), those are most probably conglomerations of viruses already described (Авакян, Быковский, 1970). The presence of viruses in leafhopper mycetocytes has also been described by S. Nasu (1965) who found out that in *Nemphotettix cincticeps* the transmission of rice dwarf virus to the next generation occurs with the endocytobionts. The lamellar structures (Fig. 11) are most probably of protein origin as have been frequently described (Threadgold, 1976) which was also testified with pronase treatment of our material.

During a long period of time several researchers have been trying, but without success, to expose DNA as proof of autonomous genetic information in endocytobionts of various species of insects, doubting the real nature of these microorganisms (see Houk, Griffiths, 1980), and questioning their being regarded as microorganisms (Lanham, 1968). Such proof has now been obtained (Chang, Musgrave, 1975; Houk et al., 1980; Schwemmler et al., 1975). Whether the described types of cytobionts belong to different species and are not just different forms of the existence of one and the same species, is difficult to say as yet. The cytobionts clearly differ in their morphology not only in the mycetomes of adult leafhoppers but even in the embryonic mycetomes. According to literary data on other species, the authors have preferred up to now to speak of the types of cytobionts and not of different species. K.-P. Chang and A. J. Musgrave (1975) did not succeed in finding DNA in "a"-endocytobionts of *H. communis*, and therefore they regard "a"-endocytobionts as derivatives of "t"-endocytobionts that have lost DNA in their evolution. Thus, they conclude that "a"-cytobionts cannot be regarded as live microorganisms. Other authors (Houk, Griffiths, 1980) think that this information is insufficient, because the methods used for exposing DNA in both free-living and intracellular microorganisms do not always yield satisfactory results (Murray, 1978); according to other data (ultrastructure, early development), "a"-cytobionts may undoubtedly be regarded as microorganisms.

The nucleoids of endocytobionts as typical prokaryotes devoid of nuclear membranes can be observed in the cell electron microscopically



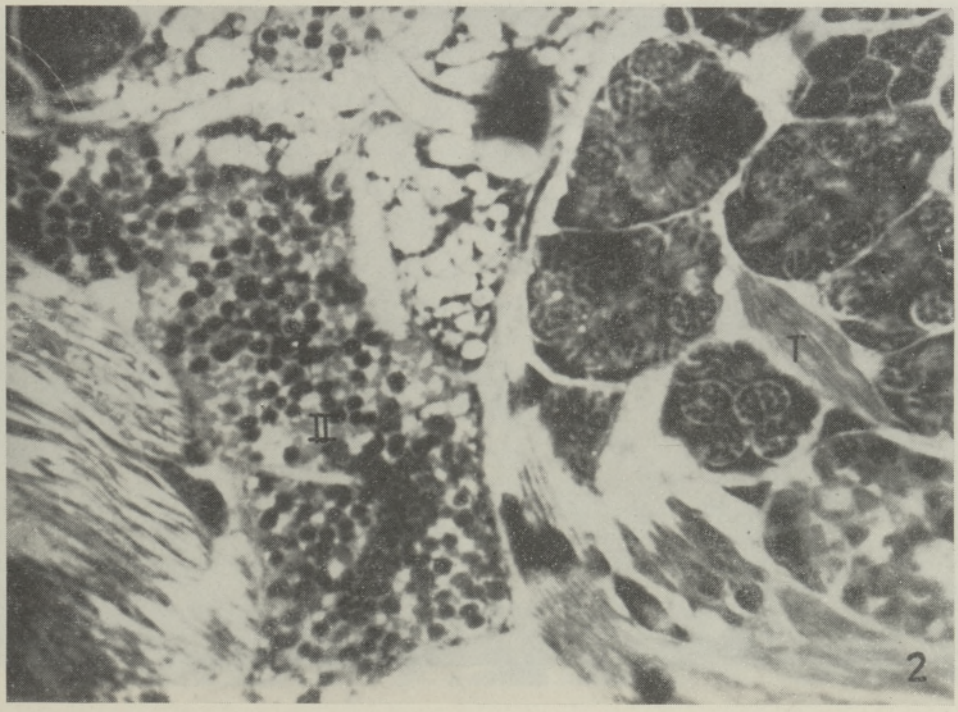
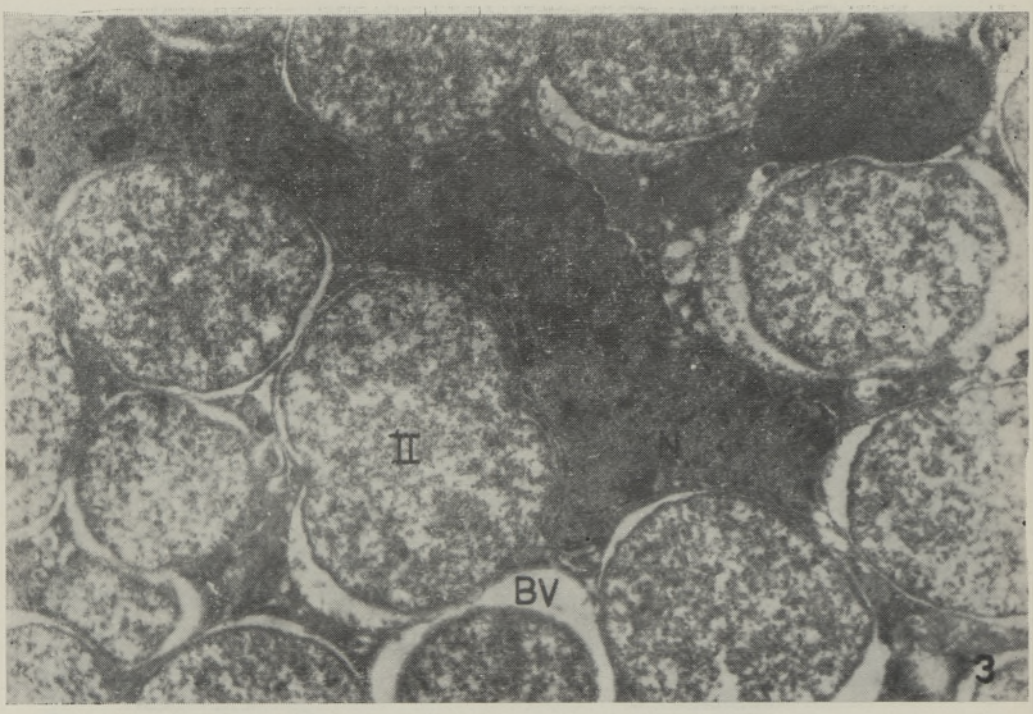


Fig. 1. Mycetome containing different types (I&II) of endocytobionts surrounded with epithelium (E) in *P. spumarius,* ×1,100.

Fig. 2. A lobe of a mycetome between various *P. spumarius* tissues (T — testicles), ×1,100.



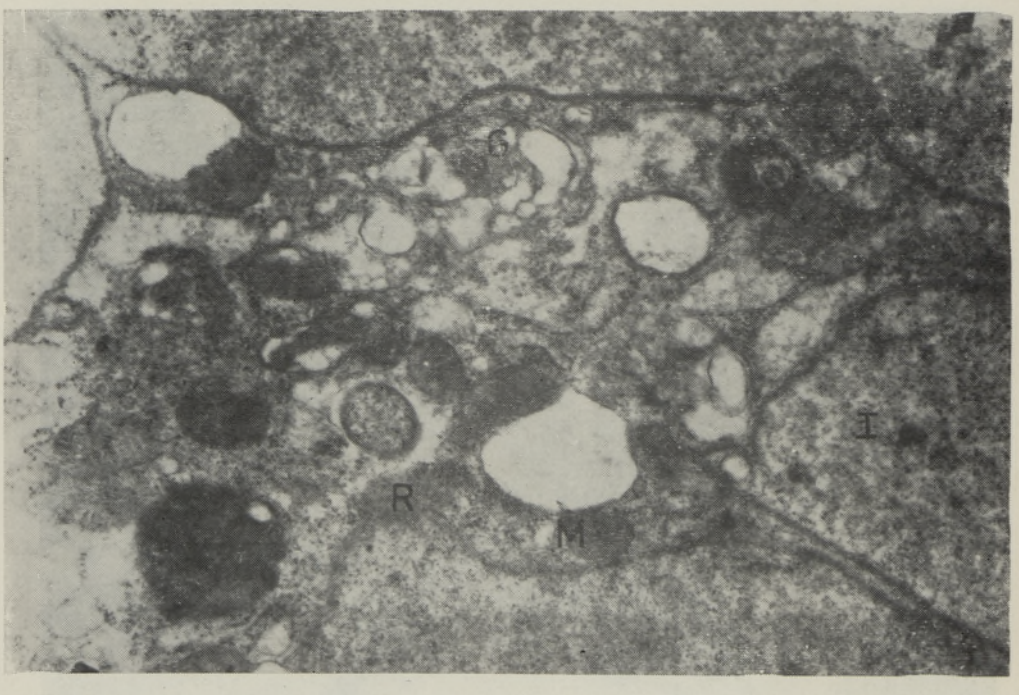
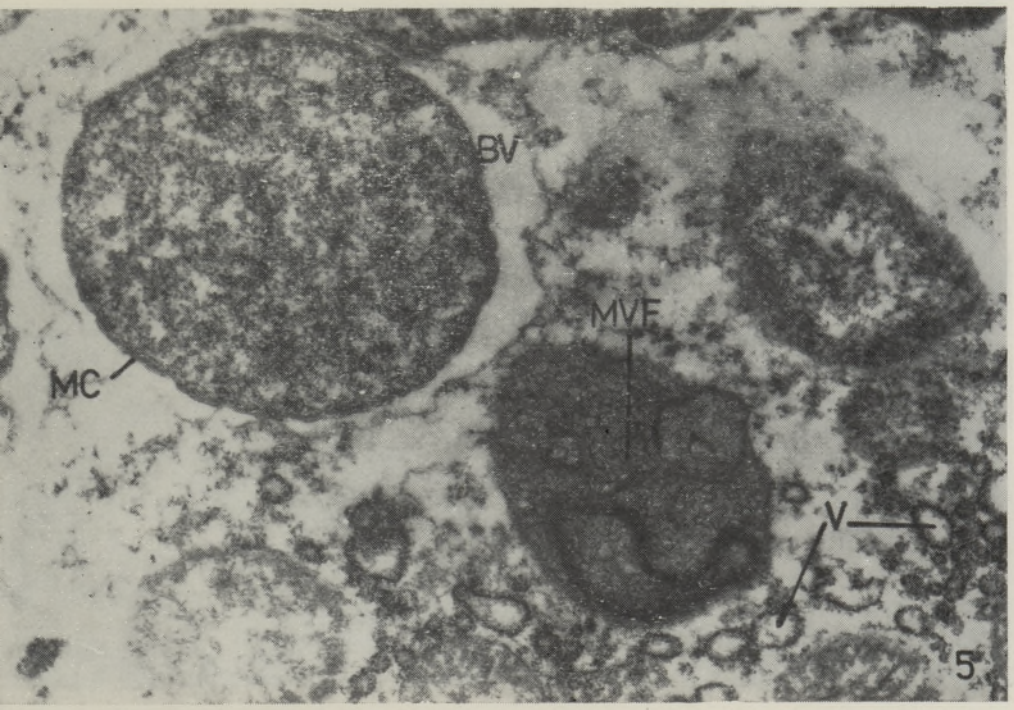


Fig. 3. Endocytobionts of the second type (II) in the mycetocyte of *P. spumarius* (N — mycetocyte nucleus, BV — bacteriophoric vacuole), ×9,400.

Fig. 4. *A. alni* mycetocyte cytoplasm containing endocytobionts of the first type (I), mitochondria (M), Golgi complex (G), and ribosomes (R), ×19,000.



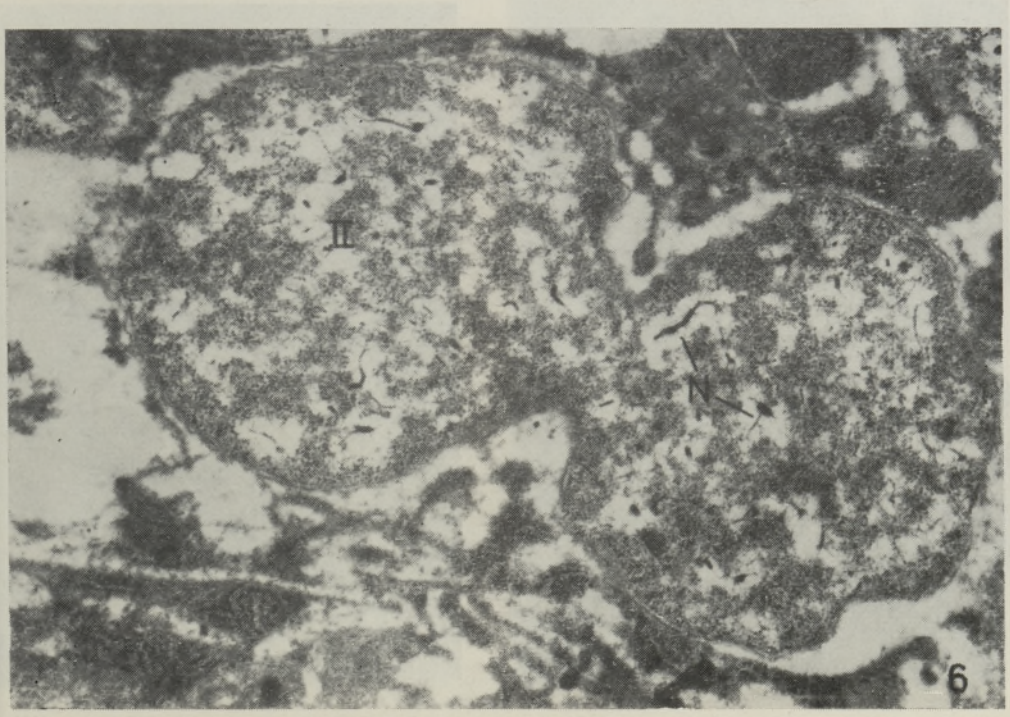
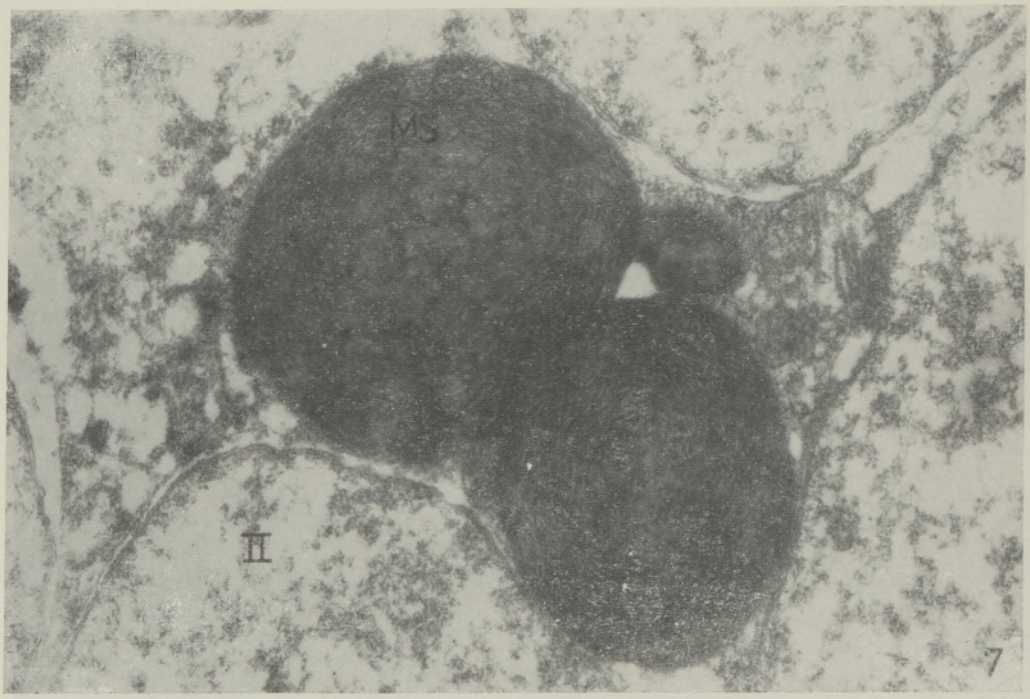
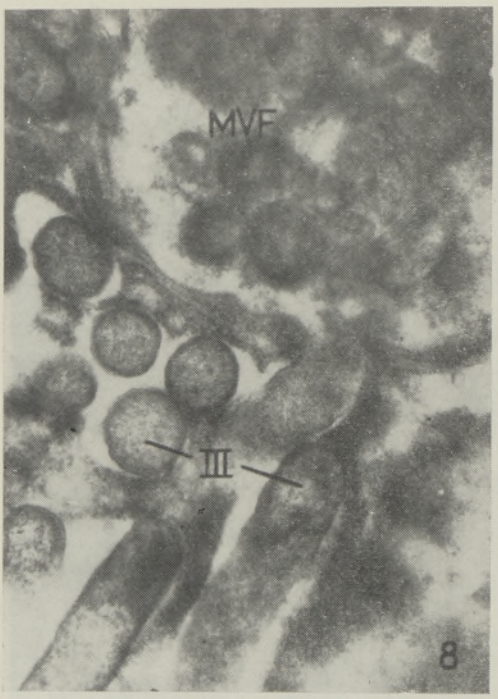


Fig. 5. Multivesicular formation (MVF) and vesicules (V) in *P. spumarius* mycetocyte with endocytobionts of the second type (II) (BV — bacteriophoric vacuole, MC — membranous coats), ×20,000.

Fig. 6. An endocytobiont of the second type (II) about to divide. Note the nucleoid regions (N) with thin fibrilles, ×28,000.





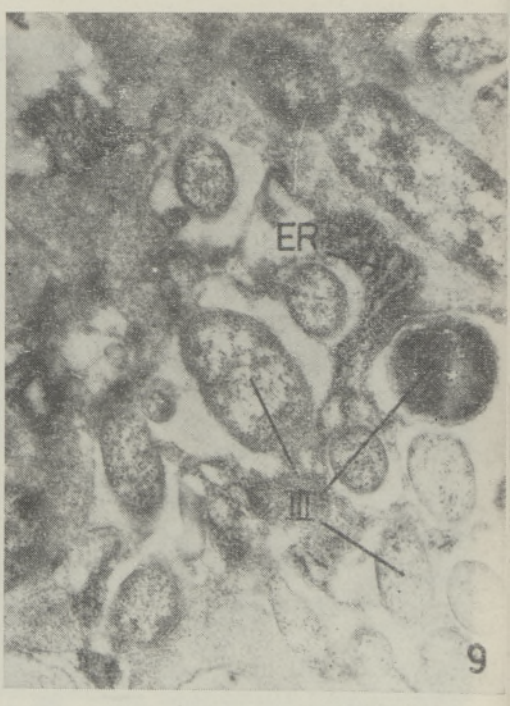
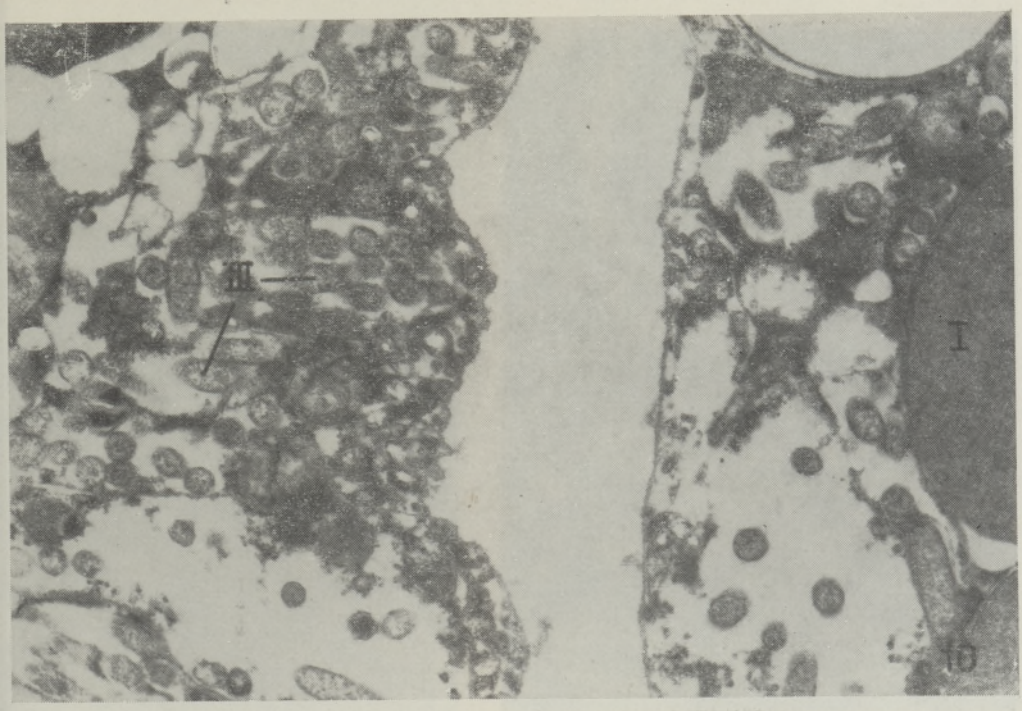
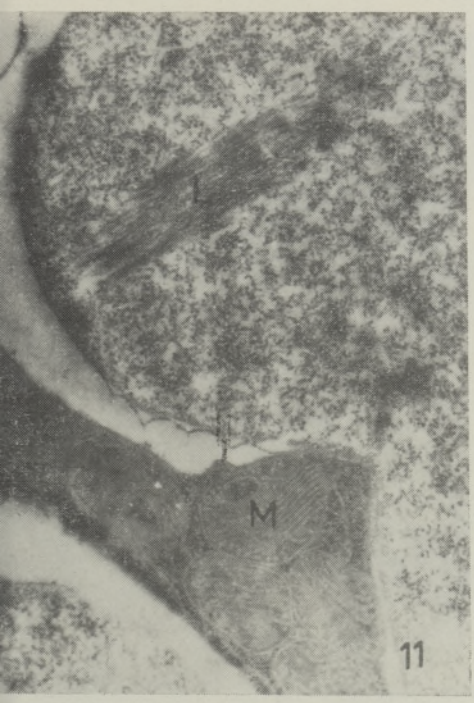


Fig. 7. Myelin-like structures (MS) between endocytobionts of the second type (II) in *P. spumarius* mycetocyte, ×25,000.

Fig. 8. Endocytobionts of the third type (III) and a multivesicular formation (MVF) in *A. alni* mycetocyte, ×30,000.

Fig. 9. Various kinds of endocytobionts of the third type (III) in *P. spumarius* mycetocyte (ER — rough endoplasmatic reticulum), ×31,000.





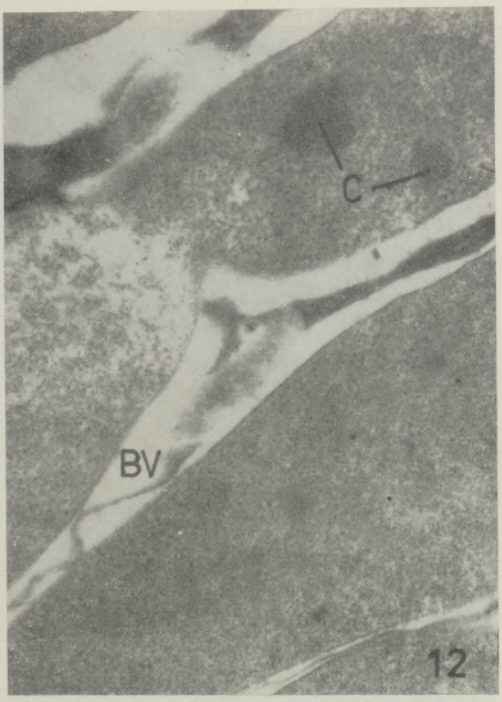
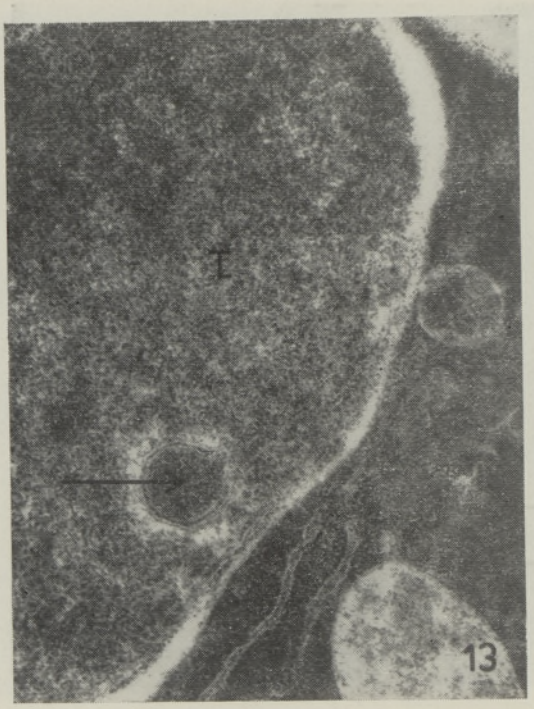
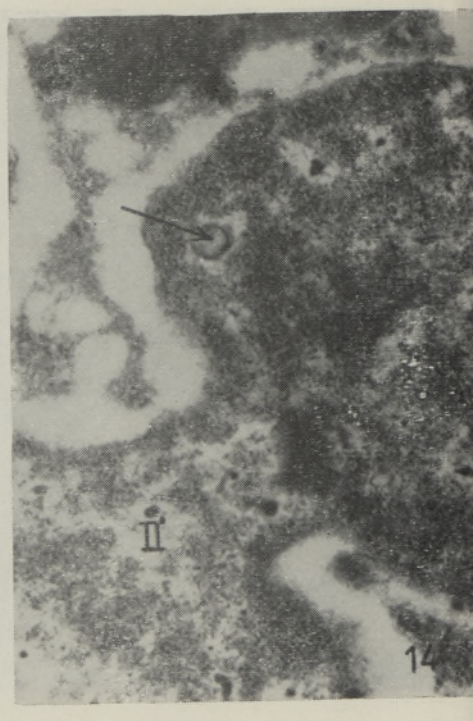
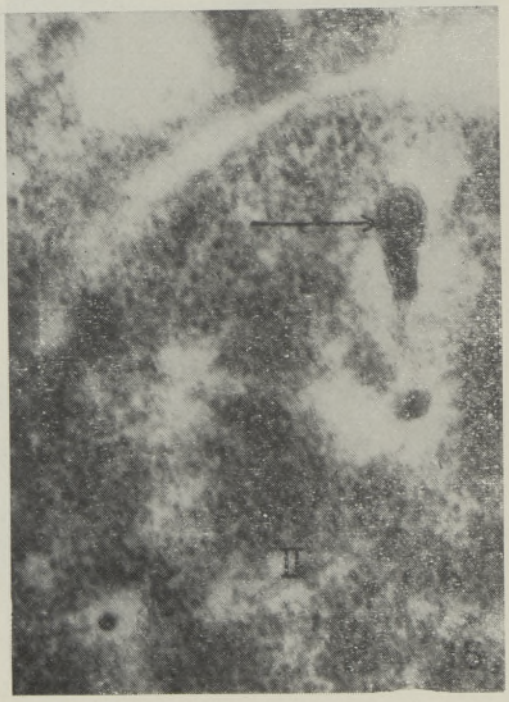


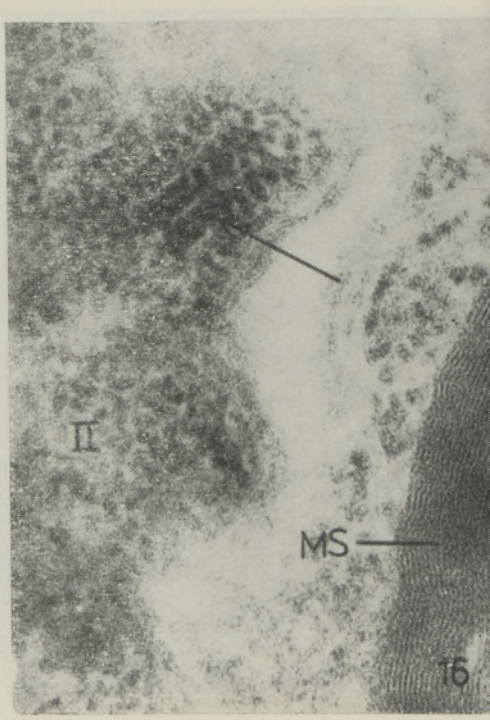
Fig. 10. Endocytobionts of the first (I) and third (III) type in *A. alni* mycetocytes, ×12,000.

Figs 11—12. Lamellar structures (L) and crystal-like structures (C) in endocytobionts of the first type (BV — bacteriophoric vacuole, M — mitochondria), ×26,000, ×20,000.





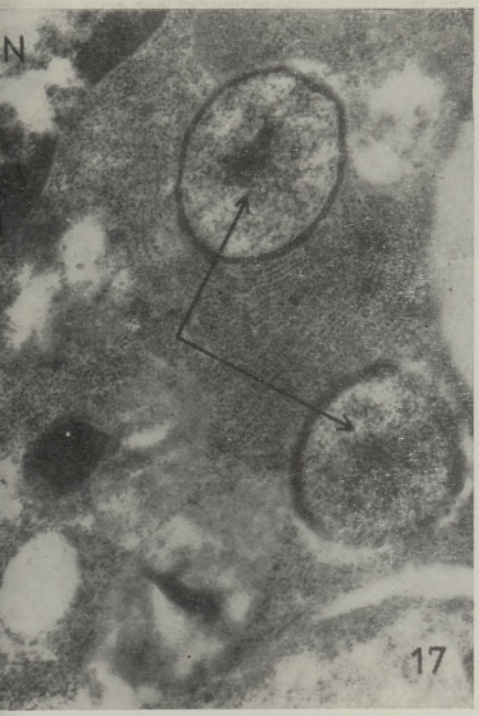


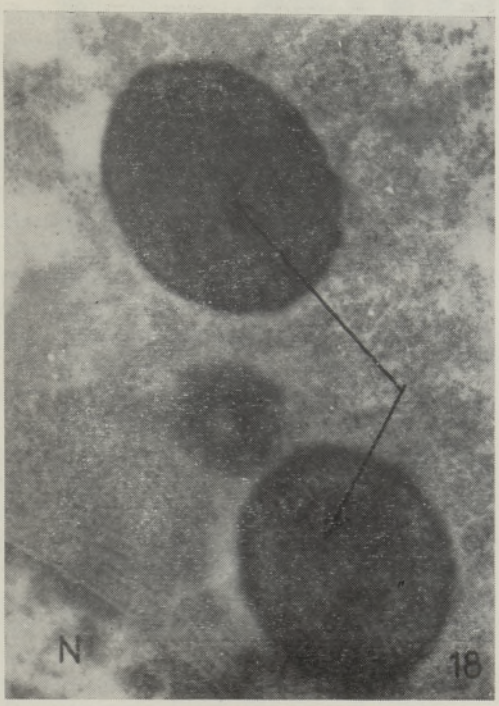


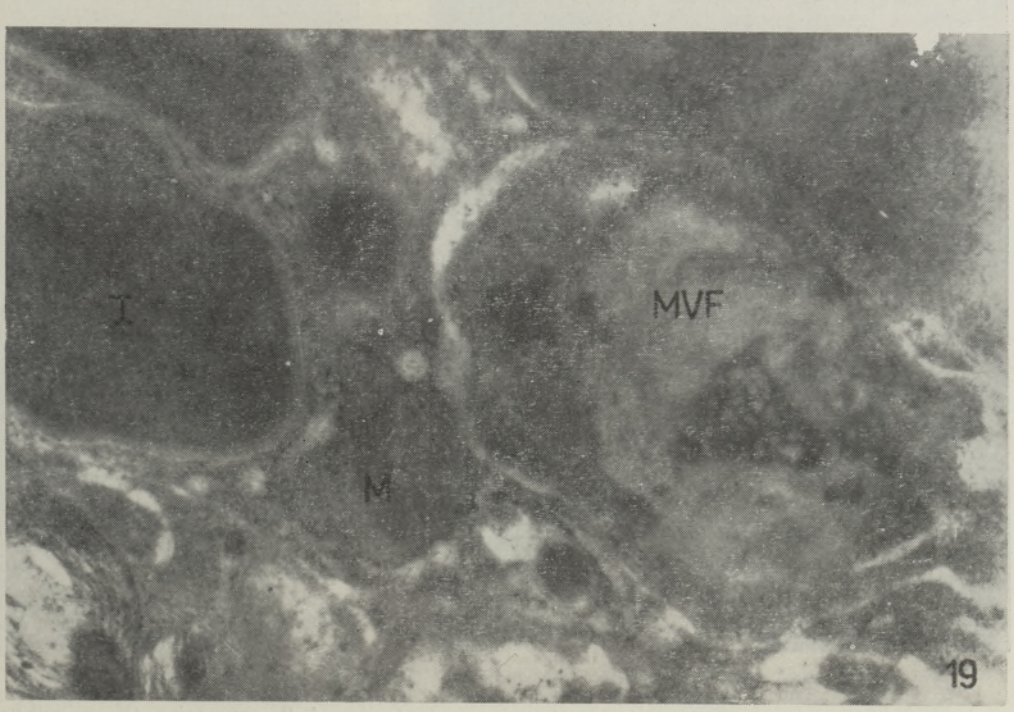
Figs 13—14. Structures surrounded by membranes (↑) in *P. spumarius* endocytobionts of the first (I) and second (II) type, ×50,000, ×40,000.

Fig. 15. A structure surrounded by membranes (↑) in *P. spumarius* endocytobiont of the second type (II), ×70,000.

Fig. 16. Particles in rows (↑) in endocytobionts of the second type (II) and lamellae of myelin-like structures (MS) in *P. spumarius* mycetocyte, ×110,000.

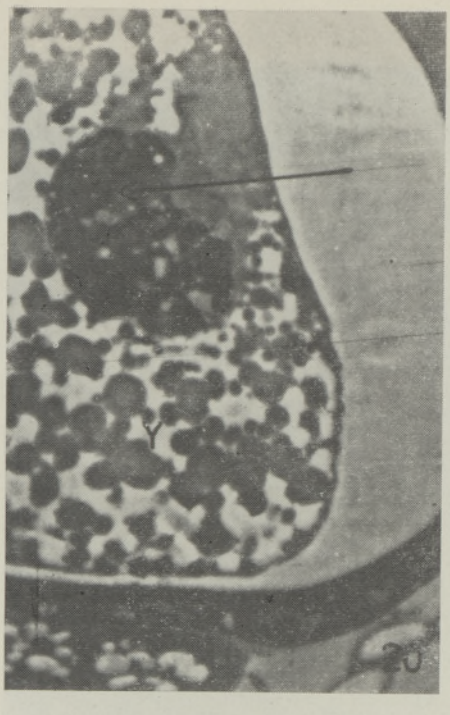


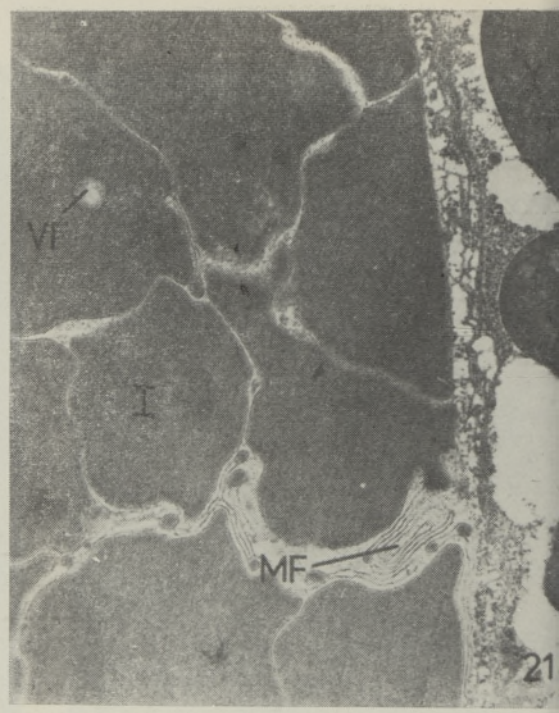


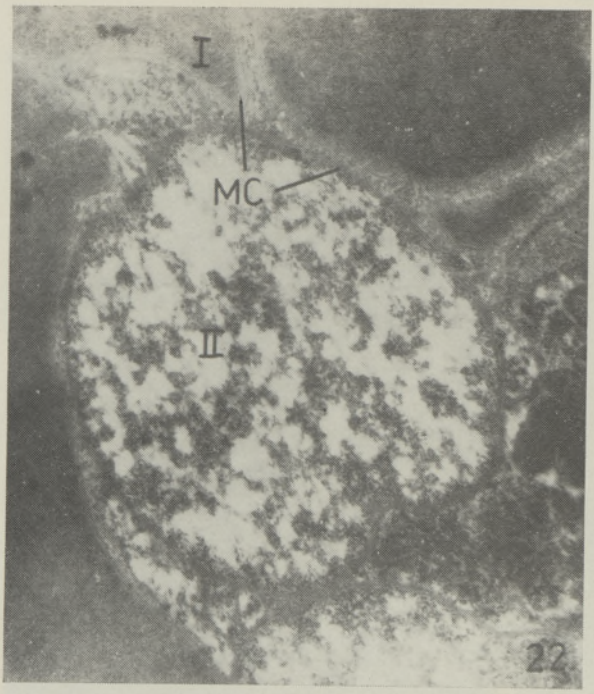


Figs 17—18. Structures (↑) with electron-dense border structures in *P. spumarius* mycetocyte with second type endocytobionts (N — mycetocyte nucleus), ×28,000, ×28,000.

Fig. 19. Endocytobionts of the first type (I) and the mycetocyte cytoplasm with mitochondria (M) and a multivesicular formation (MVF) after a 2.5 hour phospholipase treatment, ×25,000.







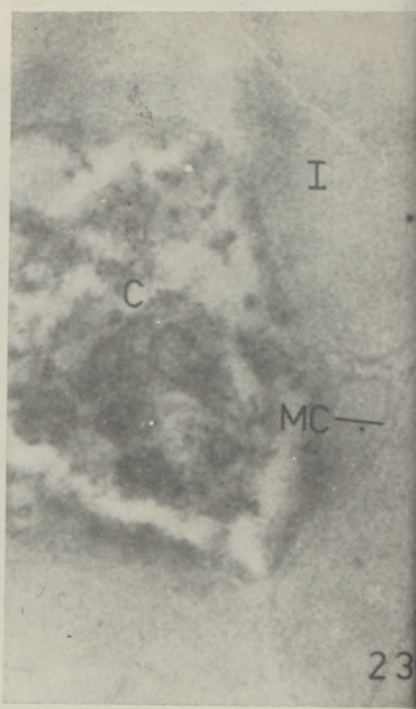


Fig. 20. An embryonic mycetome (↑) in an unlaid egg of *P. spumarius* (Y — yolk), ×1,600.

Fig. 21. Endocytobionts of the first type (I) with membranous folds (MF) between them in the *P. spumarius* embryonic mycetocyte (Y — yolk, VF — vesicular formation), ×10,000.

Fig. 22. First- (I) and second-type (II) endocytobionts surrounded by their membranous coats (MC) in the embryonic mycetocyte of *P. spumarius*, ×22,000.

Fig. 23. Endocytobionts of the first type (I) containing ribosomes, surrounded by their membranous coats (MC) and the mycetocyte cytoplasm (C) after a 24-hour phospholipase treatment, ×24,000.

as fibrillous structures which may be seen only at certain stages of their life cycle (Chang, Musgrave, 1972; Schwemmler, 1974) which was also the case in the species under investigation. A similar situation has been described by D. V. Ossipov (Осипов, 1981) in *Holospora undulata* (Ω-particles)—symbiotic bacteria of the micronucleus of *Paramecium caudatum*, where in vegetative cells between ribosomes there were regions transparent to electrons with fibrillous material. These regions probably correspond to the patches of DNA condensation (Осипов, 1981).

The fact that there were still ribosomes to be found in the cells after the phospholipase, pronase and trypsin treatment, confirms the opinion that they are microorganisms, i. e. they are autonomous structures and not conglomerations of lipoproteids. This is also supported by the three-component border structure around all the three types of cytobionts and by a clear-cut region of bacterial nucleoid with DNA fibrilles in the endocytobionts of the second and third types.

In general, the material presented above should demonstrate that the coexistence of microorganisms and insects has in the process of evolution led to endocytobiosis which is of decisive significance for the existence of a great number of insects (leafhoppers among others), where endocytobionts have become components of their organisms. The process of isolating endocytobionts by mycetocytes into bacteriophoric vacuoles serves probably as the ultrastructural basis for forming three-component border structures. This is expressed in the so-called membranous reaction of the insect cells upon microorganisms. On the other hand, despite the functional impossibility to separate endocytobionts from the insect organism, the appearance of the bacteriophoric vacuole undoubtedly testifies to both a lesser homologousness and to a greater autonomy of endocytobionts as compared to mycetocyte organelles proper.

On the basis of the hypothetical endosymbiotic evolution of the eukaryotic cell (Margulis, 1970; Margulis, 1981; Taylor, 1974), W. Schwemmler supposes (1975, 1979) that endosymbiosis of insects and microorganisms may serve as a suitable model for studying the evolution of organelles, for it seems to represent several stages of their formation. This model demonstrates the two basic stages in the hypothetical endosymbiotic evolution of organelles—the reduction of the cell wall and the changes in DNA of the prokaryote cell due to its long coexistence with the eukaryotic cell (Schwemmler, 1979; Schwemmler, 1980). This opinion is based on the fact that endocytobionts of different types can be arranged according to the complexity of their links with the eukaryotic cell, that is with the host (Tiivel, 1983b).

To ascertain the potential possibilities of that model as well as to study more profoundly several moot points in endocytobiotic associations, more detailed investigations (among those morphological ones) of various insect groups are needed.

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Received
May 3, 1984

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ENDOTSÜTOBIOOSI ULTRASTRUKTUURSEID ASPEKTE TIRTIDE (*INSECTA: CICADINEA*) RAKKUDES

On uuritud endotsütobiontidega seotud rakkude ja endotsütobiontsete mikroorganismide peenehitust tirdiliikidel *Aphrophora alni* ja *Philaenus spumarius* putukate mitmes arengustaadiumis. Tirdi *P. spumarius* mütsetotsüütides leiti kolme tüüpi endotsütobionte, *A. alni*'l kahte tüüpi. Esimene ja teine tüüp olid seotud ainult spetsiifiliste rakkudega putukate mütsetoomides, kolmandat leiti aga ka sidekoerakkudes. On kirjeldatud mütsetotsüütide ja endotsütobiontide membraanstruktuure ja sisaldisi ning mikroorganismide piirstruktuure ja nukleoidi. On käsitletud ka nende struktuuride seost mikroorganismide elutegevusega ning putukate rakkudes sedastatud endotsütobioosi uurimisel saadavate andmete kasutamist eukarüootsete rakkude evolutsiooni paremaks mõistmiseks.

Тоомас ТИЙВЕЛЬ

К УЛЬТРАСТРУКТУРЕ ЭНДОЦИТОБИОЗА В КЛЕТКАХ ЦИКАДОК *INSECTA: CICADINEA*

Электронно-микроскопически исследована ультраструктура клеток, содержащих цитобионты и эндоцитобионты двух видов цикадок *Aphrophora alni* и *Philaenus spumarius* в разных стадиях развития насекомых. В клетках *P. spumarius* было найдено три типа эндоцитобионтов, два из которых связаны исключительно с мицетомами, третий же локализован как в мицетомах, так и в клетках соединительной ткани; в клетках *A. alni* обнаружено два типа цитобионтов (соответственно первый и третий). Описаны также включения и мембранные структуры в этих клетках, мембранные оболочки и нуклеоид цитобионтов. Высказано предположение о связи этих структур с жизнедеятельностью внутриклеточных микроорганизмов, а также о возможностях использования данных о эндоцитобиозе микроорганизмов в клетках насекомых для более глубокого понимания эволюции эвкариотной клетки.