MULTIPLE SYMBIOSIS IN A LEAFHOPPER,
HELOCHARA COMMUNIS FITCH
(CICADELLIDAE: HOMOPTERA): ENVELOPES,
NUCLEOIDS AND INCLUSIONS OF THE
SYMBIOTES

K. P. CHANG AND A. J. MUSGRAVE
Department of Zoology, University of Guelph, Ontario, Canada

SUMMARY

Mycetomes in Helochara communis are paired syncytia each enclosed by an epithelium. Adjacent are loose aggregates of mycetocytes. Mycetomes harboured 3 kinds of inclusions: companion, a- and t-symbiotes according to Buchner's classification of homopteran symbiotes. In the mycetocytes, a fourth kind of inclusion was found and named g-symbiotes. All inclusions were Feulgen and Gram negative. Nucleoids were revealed in companion symbiotes by osmium-HCl-Giemsa; and, in some g- and t-, but not in a-, symbiotes by RNase-papain-Giemsa. DNase digestion caused loss of basophilia in g- and t-, but not in a-, symbiotes. By electron microscopy, using Kellenberger's fixative, companion, g- and t-symbiotes had lucid areas with reticular strands 4 nm thick; a-symbiotes were in general, homogeneously densely granular. Companion symbiotes had Gram-negative profiles with additional surface material; a- and t-symbiotes had 3, g-symbiotes 2, peripheral, trilaminar membranes. Parallel striations and regularly arranged granules occurred in companion symbiotes; crystalline inclusions in g- and t-symbiotes; and fibrillar elements and dense bodies in a-symbiotes.

Companion symbiotes may be Gram-negative bacteria or rickettsiae; g- and t-symbiotes, L-phase bacteria; but a-symbiotes are anomalous, apparently lacking DNA, and probably being derivatives of t-symbiotes, thus requiring that Müller's hypothesis of homopteran evolutionary pathways be rephrased.

INTRODUCTION

Many insects possess mycetomes and/or mycetocytes that harbour symbiotic microorganisms, presumably beneficial to their hosts (Musgrave, 1964).

Homopteran insects are often multisymbiotic, and various forms of intracellular symbiotes have been categorized by letters of the alphabet by Buchner (1965), who describes in leafhoppers, a-, H-, t-, and companion symbiotes present in certain combinations. The a-symbiotes apparently occur in all species (Buchner, 1965, p. 756). The insects become di- or tri-symbiotic when they further acquire H- or t- and/or companion symbiotes. The a-symbiotes have been described as pleomorphic, often elongated entities, existing in paired mycetomes or a-organs; the t-symbiotes as irregular, often spherical, structures; and H-symbiotes are yeasts. The companion symbiotes, however, are more like typical bacteria.

In most species of leafhoppers symbiotes are transmitted by ovarian infection and are present throughout the insect's life. They have been regarded as degenerate bacteria by Buchner (1965). In insects, generally, such micro-organisms have been
K. P. Chang and A. J. Musgrave

classed as ‘plasmids’ (Lederberg, 1952), ‘Blochmann bodies’ (Lanham, 1968) and, in an even wider sense, as ‘symbiotes’ (Steinhaus, 1967). Interpreting the word symbiosis in its broadest sense, we have adopted the word symbiote here.

References in the literature to in vitro cultivation of leafhopper symbiotes are rare and controversial. Resihr (1938) was unable to culture the symbiotes of Cicadella viridis, from which, however, Mahdihassan (1939, 1947) claimed to have grown two kinds of bacteria. Mitsuhashi & Maramorosch (1964) observed, in their culture of leafhopper tissues, structures that multiplied very slowly and were considered to be symbiotes; and, in an ultrastructural study of virus-infected leafhopper tissue cultures, Mitsuhashi (1967) regarded intracytoplasmic and intranuclear entities as ‘bacterial symbiotes’. Thus, our knowledge of leafhopper, and many other insect, symbiotes (Buchner, 1965) is derived mainly from in situ studies of their morphology. Indeed, Murray (1962), in referring to cockroach symbiotes, wrote ‘difficulties of cultivation place the burden of their identification on morphology . . .’.

Körner (1969) has studied the ultrastructure of the symbiotes in the disymbiotic leafhopper Euscelis plebejus, in which the α- and β-symbiotes show triple peripheral membranes and certain intrasymbiotic components, but no DNA structures. The microbial nature of such leafhopper ‘symbiotes’ may therefore be questioned.

The present work describes a light- and electron-microscope study of various symbiotic forms in a leafhopper, Helochara communis, especially their peripheral envelopes and nuclear materials, and attempts to reconcile these findings with Buchner’s (1965) and Müller’s (1962) hypotheses.

MATERIALS AND METHODS

Organisms

Leafhoppers, Helochara communis, were collected in Guelph and were briefly maintained, when necessary, on potted lawn grasses in a greenhouse (approx. 25 °C, 40 % relative humidity) or in a growth chamber (approx. 26 °C, 50 % relative humidity, 16-h photoperiod/day). Over 100 individuals, mainly mature female adults, were used. Ovarian eggs, nymphs of all stadia and male adults were also examined.

Smears

Mycetomes and mycetocytes were removed from insects and squashed on slides in tap water or Drosophila Ringer’s solution (Buck, 1953). For general observations, such preparations were either directly examined by phase-contrast microscopy or air-dried and treated with Gram stain, or fixed for 4 min in Carnoy’s mixture (Lillie, 1965) and stained for 15 min in 1:10 Giemsa solution in phosphate buffer at pH 6.8.

Air-dried smears were used in 3 methods to demonstrate DNA or nucleoids in the symbiotes: Feulgen (Lillie, 1965); osmium-HCl-Giemsa (Robinson, 1966); or enzyme digestions. For digestions (Thompson, 1966; Lillie, 1965), smears were fixed in Carnoy, rinsed several times in appropriate buffers and incubated at 37 °C by one of the following methods: (i) 0.1 % trypsin in phosphate buffer at pH 7.6 for 15, 30, 60 or 120 min; (ii) 0.01–0.001 % RNase at pH 6, or 22 units/ml DNase (Whorthington) at pH 7.5 in phosphate or Tris buffer for 2, 4, 8 or 16 h; (iii) trypsin for 25 min and RNase for 2 h; (iv) papain (prepared like trypsin) for 25 min and RNase for 1, 2, 3 or 4 h.

After digestion, slides were stained with Giemsa. For consistent technique, treated and control slides were processed identically and simultaneously, except that control slides were treated with corresponding buffers without enzymes.
Sections for electron and light microscopy

Method (a). Mycetomes and mycetocytes, dissected in either Drosophila Ringer's solution or
in fixative, were transferred into fresh 5% glutaraldehyde in cacodylate or phosphate buffer at
pH 7.3 or 7.4 for 2–16 h at 4 °C. With or without rinsing in sucrose washing buffer, tissues were
then postfixed for 2 h in 1% osmium tetroxide in corresponding buffer at pH 7.4 and treated
with saturated uranyl acetate aqueous solution for 1 h.

Method (b). Some mycetomes and mycetocytes dissected in the Ringer's solution were also
fixed by Kellenberger's method (Kellenberger, Ryter & Séchaud, 1958), except that pre-fixation
and construction of agar blocks were omitted.

Methods (a) and (b). After dehydration in graded acetone or ethanol, specimens were infil-
trated and embedded in Maraglas (Freeman & Spurlock, 1962) or Epon 812 (Luft, 1961) epoxy
resin. Ultrathin sections were cut with glass knives mounted on an ultramicrotome and
collected on 400-mesh copper grids or, for serial sections, on Formvar-coated R 150 copper
grids. They were then stained for 2 min with lead citrate (Reynolds, 1963) and examined with
Philips electron microscopes (Series EM 100, 200, 300) operating at 60 kV.

Thick (about 1 μm) sections of mycetomes and mycetocytes were also cut from Epon-
embedded blocks and stained with 1% methylene blue in 1% sodium borate solution for 15 s
at 90 °C.

Observations

Descriptions below apply to adults and nymphs.

General

Dissection showed that H. communis had paired mycetomes located antero-laterally
in the abdomen. Adjacent to each was an aggregation of mycetocytes which were giant
cells in racemose formation.

Light microscopy of sectioned material – methylene blue stain

From these observations, the general nature and location of the mycetomes, myceto-
cyes and symbiotes were established.

Each mycetome consisted of a central syncytium surrounded by a peripheral epi-
thelium. Each had 3 kinds of inclusions resembling the symbiotes categorized by
Buchner (1965) in other leafhoppers as:

(i) α-Symbiotes (Fig. 4) – large, irregular, often strap-like structures, that stained
heterogeneously blue and co-existed with β-symbiotes in the syncytium. Some occasional-
ally possessed spherical inclusions.

(ii) β-Symbiotes – spherical or irregular, sometimes vacuolated bodies, staining pale
purple and located in the syncytia (Fig. 1).

(iii) Companion symbiotes – deep-purple-stained coccobacillary entities located
mainly in the cytoplasm of the epithelial cells. The entities were indistinguishable
from mitochondria, except ultrastructurally (see below, cf. Fig. 22).

Mycetocytes contained a fourth kind of inclusion, morphologically similar to the
β-symbiotes but differing from them slightly in ultrastructure (see below), and
tentatively categorized as γ-symbiotes in the present study. They were either irregular
or globular and stained deep blue or pale purple. Most mycetocytes were replete with
symbiotes, which had irregular contours (Fig. 2). Symbiotes in the few less-crowded
mycetocytes were globular in shape (Fig. 3). Deeply stained crystal rods were sometimes observed in the symbiotes and in the cytoplasm of the mycetocytes.

Fresh smears for phase-contrast microscopy

In Drosophila Ringer's solution, α-symbiotes appeared mostly as fragile irregular 'straps'. Often they formed local swellings, or at times, larger globular bodies. The straps occasionally contained spherical inclusions. The g- and t-symbiotes never formed straps but appeared as globules, approximately 2.5-7 μm in diameter (Fig. 7). Sometimes they formed pleomorphic irregular bodies. The various symbiotic forms existed singly, in pairs, or arranged in clusters. In tap water, α-, g- and t-symbiotes burst. Companion symbiotes were not identifiable in fresh smears.

Stained smears for general observations

Gram stain. All symbiotes were negative.

Giemsa stain. The α-symbiotes were convoluted straps approximately 1.5-4 μm wide and 7-63 μm or more long (Fig. 5). Though most of them stained homogeneously purple or deep blue, some lighter-stained straps had dark purple cores (Fig. 6).

The g- and t-symbiotes appeared as spherical bodies approximately 1.5-4 μm in diameter and stained dark blue. Pleomorphic forms were frequent (Fig. 8). It seems unlikely that this pleomorphism resulted from distortion caused by Carnoy's fixative as such forms were also frequent in Epon sections stained with methylene blue. Many symbiotes possessed a pink-staining vacuole (Fig. 10).

Companion symbiotes were lightly stained rodlets (approximately 0.3 by 1.0-1.5 μm), occurring mostly in the cytoplasm of the epithelial cells.

Stained smears for the demonstration of DNA or nucleoids

Feulgen test. The nuclei of mycetomes and mycetocytes stained pink to red; all symbiotes stained evenly green, except a few g-symbiotes, which were tinted purple—probably an unspecific colour reaction resulting from diffusion of DNA from host nuclei. The symbiotes were therefore considered to be Feulgen negative.

Osmium-HCl-Giemsa. Many companion symbiotes had centrally located nucleoids surrounded by pale blue cytoplasm. The g- and t-symbiotes were mottled, the α-symbiotes evenly blue.

Protease and/or nuclease digestions. Companion symbiotes were unidentifiable after these digestions. Reactions of α-, t- and g-symbiotes are given below.

Trypsin digestion caused deforming and progressive loss of stain intensity. Cores and spherical inclusions of the α-symbiotes seemed resistant to trypsin treatment. Digestion longer than 30 min caused disruption and produced no further effect on the symbiotes.

RNase digestion did not cause deformation but was associated with loss of basophilia, which was evaluated as degree of change in stain reaction from bluish to reddish. It varied among symbiotes. With increasing digestion period, α-symbiotes became progressively paler pink, indicative of considerable loss of basophilia, whereas other
Ultrastructure of leafhopper symbiotes

Symbiotes remained light purple even after 16 h digestion. RNase treatment revealed a granular appearance in some of the symbiotes, but no additional subcellular structures. Incubation for 8 h or longer caused excessive loss of smears and growth of contaminant bacteria.

After RNase-trypsin digestion a-symbiotes stained pink or light blue but their inclusions remained. Most g- and t-symbiotes were light purple, but some remained blue. A few of those staining purple had centrally located dark grains which were poorly defined, but were not seen in the control.

RNase-papain-digestion at all incubation periods, revealed nothing further about the a-symbiotes. Although most g- and t-symbiotes stained homogeneously light purple after 25 min in papain and 3 h in RNase, some appeared to have well differentiated dark central grains (Fig. 9), not seen in symbiotes in control slides (Fig. 10).

After DNase digestion for all periods, a-symbiotes (including their spherical inclusions and cores) remained unchanged, while most g- and t-symbiotes progressively lost basophilia. After 8 h digestion, some of the latter developed a paler area which had approximately the same dimensions as the dark grain revealed by RNase-papain-Giems.

The following conclusions were drawn. The a-, g- and i-symbiotes contained RNA and protein. As loss of basophilia after RNase digestion was greatest in a-symbiotes, they contained more RNA than the others. The dark grains of g- and t-symbiotes were nucleoids (not protein crystals) because they persisted after prolonged RNase digestion and were of similar dimensions to the pale area digested by DNase. Dark grains were not seen in the a-symbiotes, which were thus devoid of nucleoids.

From ovarian egg to adult insect there seemed to be an increase in the numbers of all symbiotes.

Ultrastructure

Descriptions are based chiefly on material from adult and nymphal stadia; some observations were also made on ovarian eggs.

Following fixation by method (a) or (b) (p. 277), nucleoids could be clearly seen in companion, g- and t-symbiotes, but not in a-symbiotes similarly and simultaneously processed. The nucleoids were manifested as electron-lucid areas with networks of fine fibrillae (approx. 3-5 nm) (Kellenberger et al. 1958). However, as better fixation of mycetomes, mycetocytes and symbiotes was obtained with method (a) and as nucleoid fixation was similar by method (a) or (b), most of the ultrastructural observations were made on material fixed by method (a).

a-Symbiotes. These appeared as irregular dark granular bodies (Fig. 11), each enclosed by 3 peripheral membranes (Fig. 12, inset). The symbiotes contained ribosome-like particles (Fig. 13), amorphous dense materials (Fig. 12) and, occasionally, in the less-dense areas, short fibres. The dense amorphous materials were sparse in some symbiotes, which thus appeared paler (cf. Fig. 4 - a light micrograph). Occasionally dense bodies, spherical inclusions or fibrillar elements were present. The dense bodies and spherical inclusions were not limited by membranes and appeared respectively as aggregations of dark blobs (Fig. 12), and fenestrated bodies stippled
K. P. Chang and A. J. Musgrave

with fine granular materials (Fig. 13). These spherical inclusions were probably equivalent to the spherical bodies observed by light microscopy (p. 278). The fibrillar elements consisted of parallel solid filaments approximately 15-20 nm in diameter (Fig. 14). Occasionally, they gradually disappeared and reappeared at a further short distance—suggesting undulation. There was never any evidence of DNA in any form in any a-symbiote in adult, nymph or ovarian egg stages of the insect.

The g- and t-symbiotes. These were confined respectively to the mycetocytes and the mycetomal syncytia. Both symbiotes appeared as large irregular or spherical bodies; and each consisted of a granular matrix, nuclear area and enclosing membranes (Fig. 17). The g-symbiotes were surrounded by only 2 (Figs. 18, 19), t-symbiotes often by 3, membranes (Fig. 16). The latter tended to be more pleomorphic. The following descriptions, unless otherwise specified, apply to both symbiotes.

The enclosing membranes of the symbiotes were trilaminates, each being 2 dense layers separated by a light space about 6.5-7.5 nm thick (Figs. 16, 19). The outer membranes sometimes appeared to have been torn apart from the inner membrane (probably fixation artifact) or to be folded against the smooth inner membranes, which closely followed the contour of the symbiotes (Fig. 17). Some symbiotes possessed large, centrally located, nucleoids filled with condensed fine fibrillae (Fig. 15). Such nucleoids were probably the dark grains revealed by papain-RNase-Giemsa treatment. The granular matrix of the symbiotes consisted of aggregations of ribosome-like particles: very sparse in some paler individuals. The t-symbiotes frequently contained (Fig. 17) lamellated membranous bodies, and (not shown) vacuoles, with or without bounding membranes, and, sometimes, polyribosome-like structures. Some symbiotes contained rod-shaped crystalline inclusions (Fig. 18). In cross-section these were hexagonal structures consisting of dense subunits approximately 21 nm in diameter (Fig. 19). Some g-symbiotes also had tubular extensions of the outer membrane, which were frequently beaded and contained dense granules.

Companion symbiotes. These oval or elongated bodies occurred in the cytoplasm without host-provided membranes or peripheral vacuoles (Figs. 20-22). The majority were located in the mycetomal epithelium, but a few were found in adjacent tracheal cells and mycetomal syncytia. The elongate symbiotes sometimes seemed to undergo binary fission (Fig. 21). Each symbiote possessed nuclear areas, ribosome-like particles and a multilayered envelope (Fig. 21). The cell envelope was composed of 3 parts: an inner membrane (p), an intermediate zone (in) and an outer membrane (o, Fig. 21, inset). The inner membrane was trilaminate (about 6.5 nm thick) and was thus regarded as the plasma membrane. The intermediate zone possessed little intervening materials and varied considerably in thickness (8.5-17.5 nm). The outer membrane itself also consisted of 3 layers: an outermost dark layer about 9-12 nm thick, a lucid layer about 4 nm wide and an inner thin dense layer about 2.5 nm thick (Fig. 21, inset). However, in some sections the inner surface of the outermost dark layer appeared to be delimited by an inner denser lamina (Fig. 21, inset), which, together with the lucid layer and the thin dense layer, formed a dark-light-dark tripartite structure, reminiscent of Gram-negative profiles (o, Fig. 21, inset). Thus, the outer membranes of these symbiotes resembled the cell walls of Gram-negative bacteria or
Ultrastructure of leafhopper symbiotes

rickettsiae, enclosed by some additional unknown surface material manifested as the outermost dark layer.

Parallel striations, arrays of dense particles and membrane-bounded vesicles (mesosomes?) were often observed (Figs. 20, 21). At higher magnification the parallel striations appeared to be composed of blunt-ended dense bands. One end of the dense bands often contacted the plasma membrane, with most of the structure extending into the cytoplasm. In serial sections, the dense bands persisted through two to three sections (about 120-270 nm), suggesting that the parallel striations were cross-sections of a lamellated structure. Continuation of the plasma and vesicular membranes was evident in places, suggesting that the vesicles were formed by the invaginations of the cytoplasmic membrane. In the electron-lucid area were arrays of dense particles: each measured approximately 13.5 nm in diameter (Fig. 20) and consisted of substructures about 3.0 nm in diameter (not shown).

DISCUSSION

Light microscopy

Symbiotes of *H. communis* were Feulgen-negative, and thus similar to some other insect symbiotes (Buchner, 1965). But even DNA of free-living bacteria may give a feeble Feulgen reaction (Brieger, 1963). Better results may be obtained by Robinow’s osmium-HCl-Giemsa (1960), which indeed, in many companion symbiotes of *H. communis*, revealed nucleoids, though it failed to reveal them in the *g* - and *t*-symbiotes. But nucleoids could be detected in some of these (in logarithmic growth phase?) after RNase-proteinase digestion followed by Giemsa (p. 276). This indicated the concomitant presence of RNA and protein and suggested that they may have masked the nucleoids, as occurs in the blue-green alga *Oscillatoria amoena* (Fuhs, 1958).

Electron microscopy

The ultrastructural studies confirmed that some companion, *g* - and *t*-symbiotes, in all stadia examined, possessed nucleoids. They lacked nuclear membranes and had DNA in the form of fine fibrillae; i.e. they were prokaryotic. Apparently the only insect symbiotes hitherto shown to possess such nucleoids are those of weevils, *Sitophilus* (Grinyer & Musgrave, 1966; Musgrave & Grinyer, 1968). Certainly it seems that, in most other homopterans studied, no nuclear materials have been detected by electron microscopy *in situ*, e.g. symbiotes in *Brevicoryne brassicae* (Lamb & Hinde, 1967), *Icerya purchasi* (Louis, 1969) or *Psylla pyricola* (Chang & Musgrave, 1969). Moreover, Körner (1969) could find no DNA in *t*-symbiotes of the leafhopper *Euscelis plebejus*. But Hinde (1971) has shown DNA fibres in symbiotes in a tissue culture of *B. brassicae*. For various reasons none of these authors rejected the symbiotes as microorganisms. Clearly there is sufficient evidence that the companion, *g* - and *t*-symbiotes of *H. communis* are micro-organisms.

The companion symbiotes were Gram-negative by light microscopy, but by electron microscopy were seen to have an outermost dark layer measuring 9.0-12.0 nm. It differed from the surface additional layer of certain free-living bacteria, from capsular
The companion symbiotes showed certain similarities with rickettsiae (Anderson, Hopps, Barile & Bernheim, 1965; Ignatovich & Gulevskaya, 1969; Maillet & Folliot, 1967; Ormsbee, 1969; Philip, 1957). However, rickettsiae rarely possess cellular organelles (Ormsbee, 1969), such as the lamellated structures. These, indeed, resembled the lamellar bodies of the Gram-negative bacteria, *Thio- bacillus* spp. (Shively, Decker & Greenawalt, 1970). The companion symbiotes thus resemble both Gram-negative bacteria and rickettsiae.

The *g* - and *t*-symbiotes might be rickettsiae, mycoplasma or L-phase bacteria. However, these symbiotes were too large to be rickettsiae, except perhaps the reticulate bodies of the unusual rickettsia *Chlamydia psittaci* (Tamura, Matsumoto, Manire & Higashi, 1971) – but this has a complicated life-cycle. Mycoplasma (Anderson, 1969) and the mycoplasma-like bodies found in leafhoppers by Maramorosch, Granados & Hirumi (1970), unlike the *g* - and *t*-symbiotes, are usually very small, have single peripheral membranes and lack conspicuous nucleoids. However, the *g* - and *t*-symbiotes of *H. communis* do resemble protoplasts or spheroplasts, which are fragile, pleomorphic and abnormally large (Dienes, 1968) and remain viable and multiple as L-phase variants (Hijmans, van Boven & Clasener, 1969). In these, the nucleoids are expanded (Cole, 1967; Dienes & Bullivant, 1967). The *g* - and *t*-symbiotes have strikingly similar nucleoids. Moreover, symbiotic bacteroids of some cockroaches (Milburn, 1966) and the mycetomal symbiotes of *P. maritimus* (Louis, 1967) at times strongly resemble protoplasts or spheroplasts in ultrastructure. Furthermore, ultrastructural studies of the symbiotes of *Macrosiphum rosae* and *I. purcJiasi* led Vago & Laport (1965) and Louis (1969), respectively, to equate them with type A and type B L-forms of *Proteus*, though these symbiotes appeared to lack nucleoids. Thus, it is suggested that the *g* - and *t*-symbiotes may well be L-phase bacteria. Possibly, the *g*-symbiotes are either protoplasts of Gram-positive or Gram-negative bacteria, bounded by 2 membranes representing host-provided membranes and plasma membranes; or, because of their pleomorphism, spheroplasts of Gram-negative bacteria without host-provided membranes. Similarly, the *t*-symbiotes may be spheroplasts of Gram-negative bacteria, lacking the rigidity supplied by a mucopeptide layer, their 3 peripheral membranes being a host-provided membrane, the lipoprotein layer of the cell wall and the plasma membrane.

Because Körner (1969) could not detect DNA in a-symbiotes of *E. plebejus* by electron microscopy and it could not be detected in the many host stadia examined in the a-symbiotes of *H. communis* by either light or electron microscopy, it is difficult to accept these ‘symbiotes’ as micro-organisms.

The number of peripheral membranes suggests that they are not an insect product (Chang & Musgrave, 1969; Locke & Collins, 1965; Threadgold, 1967, p. 111); yet the number of a-symbiotes increases as the host develops.

Rejecting Mahdihassan’s (1947) claim that a-symbiotes were not micro-organisms, Buchner (1965, quoting Müller, 1962) seems to consider that a-symbiotes are present in all leafhoppers and are the sole symbiotes in the insects (Coleorrhyncha) he regards...
Ultrastructure of leafhopper symbiotes

as ancestral to leafhoppers. This statement is, however, based on Müller's (1951) examination of material, 'insufficient for studying minute details', of the rare, moss-feeding Australasian peloridiid *Hemiodoecus fidelis*. Buchner presents the phylogenetic plan of homopteran symbiote relationships proposed by Müller (1962), in which a-symbiotes play an essential role, and t-symbiotes are a secondary acquisition.

We must then either assume that contemporary views on evidence of DNA are not completely satisfactory; or postulate that a-symbiotes are living organisms whose DNA has become degenerate as a result of long, intimate association with the insect host; or, reluctantly, accept the 'a-symbiotes' as non-living inclusions, and (whether or not the 'a-symbiotes' in *H. communis* and *E. plebejus* are typical of homopteran insects) rephrase the Buchner–Müller hypothesis. This last seems most likely, for observations made in this study (and anticipated additional evidence to be presented later) and descriptions given by Buchner (1965, p. 363), suggest that the 'a-symbiotes' may be derived from t- or other symbiotes. This notion would explain the apparent increase in numbers of the 'a-symbiotes' in *H. communis*. Perhaps the material of *Hemiodoecus fidelis* examined by Müller precluded recognition of t- or other symbiotes. We may thus rephrase the Buchner–Müller hypothesis so that the 'a-symbiotes' are understood to be symbiote-derived inclusions. Alternatively, we must adopt the less-likely notion that the symbiotes of *H. communis* and *E. plebejus* are atypical.

Various inclusions are present in the symbiotes of *H. communis*, e.g. fibrillar elements of a-symbiotes and the crystalline inclusions of g- and t-symbiotes. Similar inclusions have also been observed in corresponding symbiotes of *E. plebejus* (Körner & Feldhege, 1970). The fibrillar elements resemble striated inclusions found in a wide range of organisms including streptococcus L-forms (Corfield & Smith, 1970), fungi, insect and human cells (Beck, Decker & Greenawalt, 1970). The crystalline inclusions superficially resemble protein crystals in *Bacillus thuringensis* (Norris & Proctor, 1969). Their precise nature awaits further study.

To summarize, the evidence offered in this study suggests that the companion symbiotes are Gram-negative bacteria or rickettsiae, with an additional covering; the a-symbiotes may be non-living inclusions, perhaps derivatives of the t-symbiotes; the g- and t-symbiotes are L-phase bacteria.

The origin of these intimate relationships between insects and micro-organisms remains obscure. Presumably, the symbiotes were once free-living (Lanham, 1968), with rigid cell walls; or they may have been always host-dependent in accordance with ideas expressed by Hanks (1966).

We wish to thank Professor K. Ronald for facilities, Professors P. R. Sweeney and G. King for use of electron microscopes and Mr D. Macpherson for some technical help; the National Research Council of Canada for a grant-in-aid to A. J. Musgrave; and the Taxonomists at Entomology Research Institute, Canada Department of Agriculture, Ottawa, who confirmed the identification of *Helochara communis*.

* Peloridiidae – a Family in the Coleorrhyncha.
REFERENCES


Ultrastructure of leafhopper symbiotes


* Indicates that the reference was not verified in the original.
Figs. 1–4. Thick sections of Epon-embedded mycetomes and mycetocytes stained with methylene blue. \( \times 950 \). Scale = 10 \( \mu \)m.

Fig. 1. Portion of a mycetome, showing globular and irregular \( t \)-symbiotes (t). \( n \), nucleus.

Fig. 2. A mycetocyte crowded with irregular \( g \)-symbiotes (g). \( n \), nucleus.

Fig. 3. Portion of a mycetocyte containing globular \( g \)-symbiotes (g). \( n \), nucleus.

Fig. 4. Part of a mycetome showing irregular strap-like \( a \)-symbiotes (a). \( n \), nucleus.

Figs. 5–10. Smear preparations of various symbiotes stained with Giemsa (except Fig. 7). Scale = 10 \( \mu \)m.

Fig. 5. A convoluted strap-like \( a \)-symbiote (a). \( \times 950 \).

Fig. 6. Deeply stained core (c) in an \( a \)-symbiote. \( \times 950 \).

Fig. 7. Globular forms of the \( g \)-symbiotes (g) from a fresh unstained smear of a mycetocyte; phase contrast. \( \times 2500 \).

Fig. 8. Pleomorphic \( t \)-symbiotes (t) of a mycetome. \( \times 1300 \).

Fig. 9. Some \( g \)-symbiotes having dark grains probably nucleoids after papain-RNase-Giemsa. \( g \), \( g \)-symbiotes with nucleoids; \( g' \), \( g \)-symbiotes without nucleoids.

Fig. 10. The \( g \)-symbiotes treated with enzyme diluent serving as control. Note the homogenously stained symbiotes (g) and vacuoles (v) in some individuals. \( \times 1300 \).
Figs. 11–22. Ultrathin sections of mycetomes and mycetocytes. Glutaraldehyde and osmium tetroxide fixation; uranyl acetate and lead citrate stain.

Fig. 11. The a-symbiotes (a) in the syncytium of a mycetome. Note the absence of electron-lucid nuclear area in the symbiotes. db, dense body; m, mitochondrion; n, nucleus. × 13000.

Figs. 12, 13. The a-symbiotes surrounded by 3 peripheral membranes (tm), each being trilaminate, as shown in the inset of Fig. 12 (× 140000) and containing ribosome-like particles (r), dense amorphous material (dm) and 2 kinds of inclusions: (1) dense body (db) consisting of dark blobs; (2) finely granulated spherical body (sb). × 37000.

Fig. 14. Fibrillar elements consisting of solid filaments approximately 20 nm thick in an a-symbiote. cf, cross-section of a filament; lf, longitudinal section of a filament. × 92400.

Fig. 15. A lobulated g-symbiote having a large nucleoid (mu) filled with DNA fibrillae. × 21000.

Fig. 16. Portion of a t-symbiote (t) showing its 3 peripheral trilaminar membranes (between arrows). × 160000.
Fig. 17. A $t$-symbiote in the syncytium of the mycetome containing DNA filaments ($f$) as reticulated network in electron-lucid nuclear area (nu). $lb$, lamellar body. $\times 74\,000$.

Fig. 18. A crystalline inclusion ($cr$), cut longitudinally, traversing the long axis of a $g$-symbiote. $i$, inner membrane; $o$, outer membrane of the symbiote. $\times 48\,000$. 
Fig. 19. Portion of a g-symbiote with a crystalline inclusion (cr) cut transversely, appearing as a hexagonal structure with dense subunits (ds). Note the trilaminated appearance of the inner (i) and outer (o) membranes of the symbiote. × 140 000.

Fig. 20. Regularly arranged granules (gr) in the companion symbiote, each granule being 13.5 nm in diameter. × 45 000.

Fig. 21. A dividing companion symbiote lying freely in the cytoplasm of an epithelial cell. mv, membrane-bounded vesicle; nu, nuclear area; ps, parallel striations. × 92 500. Inset, an enlarged portion of the symbiote’s envelope: in, intermediate zone; o, outer membrane; p, plasma (inner) membrane. × 140 000.

Fig. 22. An epithelial cell of the mycetome containing companion symbiotes (arrows) in the cytoplasm. fr, free surface of the mycetome; m, mitochondrion; n, nucleus; pi, pigment granule; v, vacuole. × 13 000.
Ultrastructure of leafhopper symbiotes

19

0.1 μm

20

0.2 μm

21

0.1 μm

22

1 μm