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Miscellanea

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The Micro-Organisms of Tsetse Flies

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Abstract

Micro-organisms from tsetse fly mycetomes were maintained in culture, where they were more pleomorphic than in the mycetomes, but were in some cases very similar to those observed in ovaries by other authors. Agglutination tests on the cultured forms indicated an affinity to Rickettsia. They were sensitive to antibiotics introduced by feeding flies on hosts treated with Ampicillin; this reduced the longevity and fecundity of the tsetse flies and appeared to disturb normal digestion of bloodmeals.

Introduction

Until recently there have been comparatively few studies of the micro-organisms in tsetse flies (*Glossina* spp.). In 1907 Stuhlmann reported yeast-like organisms present in the crop, and bacteria in the intestine of the tsetse fly. This was followed in 1919 by Rouboud who described a mycetome-like organ which lay in the midgut and contained organisms showing a "budding" characteristic of yeasts. Wigglesworth (1929) declared these to be G^- bacteria, although Buxton (1955) called them bacteroids. In a review Brooks (1964) referred to them as a rickettsia-like organism. An attempt to grow these organisms in culture (Wallace, 1931) was unsuccessful. Most recently, while the present research and the conjoint studies of Drs. Pinnock and Hess were under way, a number of new investigations have been reported. Reinhardt, Steiger and Hecker (1972) reported ultrastructural studies on larger (up to 8 μm) bacteroids in the mycetome of *Glossina* spp.; they concluded these organisms were too large to be rickettsiae, but referred briefly to other sparse small rickettsia-like organisms within the mycetome. Hill, Saunders and Campbell (1973) reported preliminary observations on the treatment of tsetse flies with antibiotics in which the numbers of bacteroids in fly mycetomes were reduced and the fertility of the females fell markedly. Huebner and Davey (1974) found bacteroids in the ovaries of *G. austeni* indicating that transmission from one generation to the next was transovarian. Pinnock and Hess (1974) have recorded smaller (1.9 μm) rickettsia-like organisms in the midgut body and the developing oocytes of *Glossina* spp. These organisms appeared to be surrounded by a lytic region and Pinnock and Hess postulated they were parasitic and quite distinct from the larger (up to 9 μm) bacteroids found in the midgut epithelium (but not in developing oocytes). Ma and Delinger (1974) found symbiotic bacteria in the milk glands of *G. morsitans*.

The aims of the present study, commenced in 1971, were a) to determine the nature of the micro-organisms in the tsetse fly, b) to grow them *in vitro*, c) to determine their sensitivity to antibiotics administered through the bloodmeal of the fly, d) to find out if the antibiotic treatment could form the basis

for a mechanism of aposymbiotic control of the tsetse fly. The probability that these organisms are symbiotic in the fly and, as with other blood-feeding insects, play a vital role in its nutrition (BROOKS, 1964), suggests aposymbiotic control might be possible. This might be particularly appropriate with peri-domestic populations and would need to be based on a capsular or similar method allowing the successive release of the antibiotic in the favoured host.

Materials and Methods

Puparia of *Glossina morsitans* were obtained from the ODM Tsetse Research Laboratory at Longford, Bristol. These were usually hatched in our laboratories at Silwood Park, but experiments using antibiotic treatment were carried out in the laboratories at South Kensington. The flies used for all the experiments were fed on rabbits.

Examination of micro-organisms in situ

Flies both before and after drug treatment were dissected into Locke's medium and the gut transferred to 2.4 % glutaraldehyde in 0.1 M phosphate buffer pH 7.4 and fixed overnight at 4 °C. Following three washes in 0.15 M phosphate buffer pH 7.4 the specimens were postfixed in 1 % osmium tetroxide in Caulfield's buffer for 1 hour at 4 °C. After dehydration in ethanol, specimens were embedded in araldite. All sections were stained in uranyl acetate and lead citrate.

In vitro culture of micro-organisms

The flies (all non-teneral) were anaesthetised with carbon dioxide, surface sterilised with 0.0125 % vanadin, washed in sterile distilled water and dissected in sterile insect saline. The dissected gut was removed then chopped into pieces in insect saline then transferred to the culture media. Sterile procedure was observed throughout. Cultures were incubated at 37 °C for 10 days. This work was undertaken in two phases, in 1971 and again a year later. On both occasions a number of cultures were successfully established. The most successful isolations were made from flies in which the bloodmeal had blackened inside the gut, this suggesting the redox potential of the cells had risen sufficiently to enable the microorganisms to multiply. The only media in which the organisms were successfully maintained were:

a) Serum-saline medium which consisted of sterile insect saline and horse serum (Flow Laboratories 4-012D: pre-tested for ability to grow *Mycoplasma* spp.) mixed at a ratio of 18 : 2 respectively.

b) Broth-serum-yeast extract medium comprising 2.2 % Bacto PPLO broth dehydrated (Difco Laboratories) in deionised water, horse serum (as above) and 25 % yeast extract in deionised water prepared after the method of FALLON and WITTLESTONE (1969) mixed at a ratio of 15 : 4 : 2 respectively.

Some of these cultures were examined by electron microscopy. Preparations were examined following negative staining with uranyl acetate (Figs. 4, 6) or alternatively cultures were fixed in osmium tetroxide according to KELLENBERGER et al. (1958) and embedded in araldite and examined in thin sections (Figs. 5, 7).

Effects of feeding flies on hosts treated with antibiotics

Upon hatching female flies were divided into two groups. One ("the treated group") was fed on a rabbit (3.1 kg), given daily intramuscular doses of 500 mg

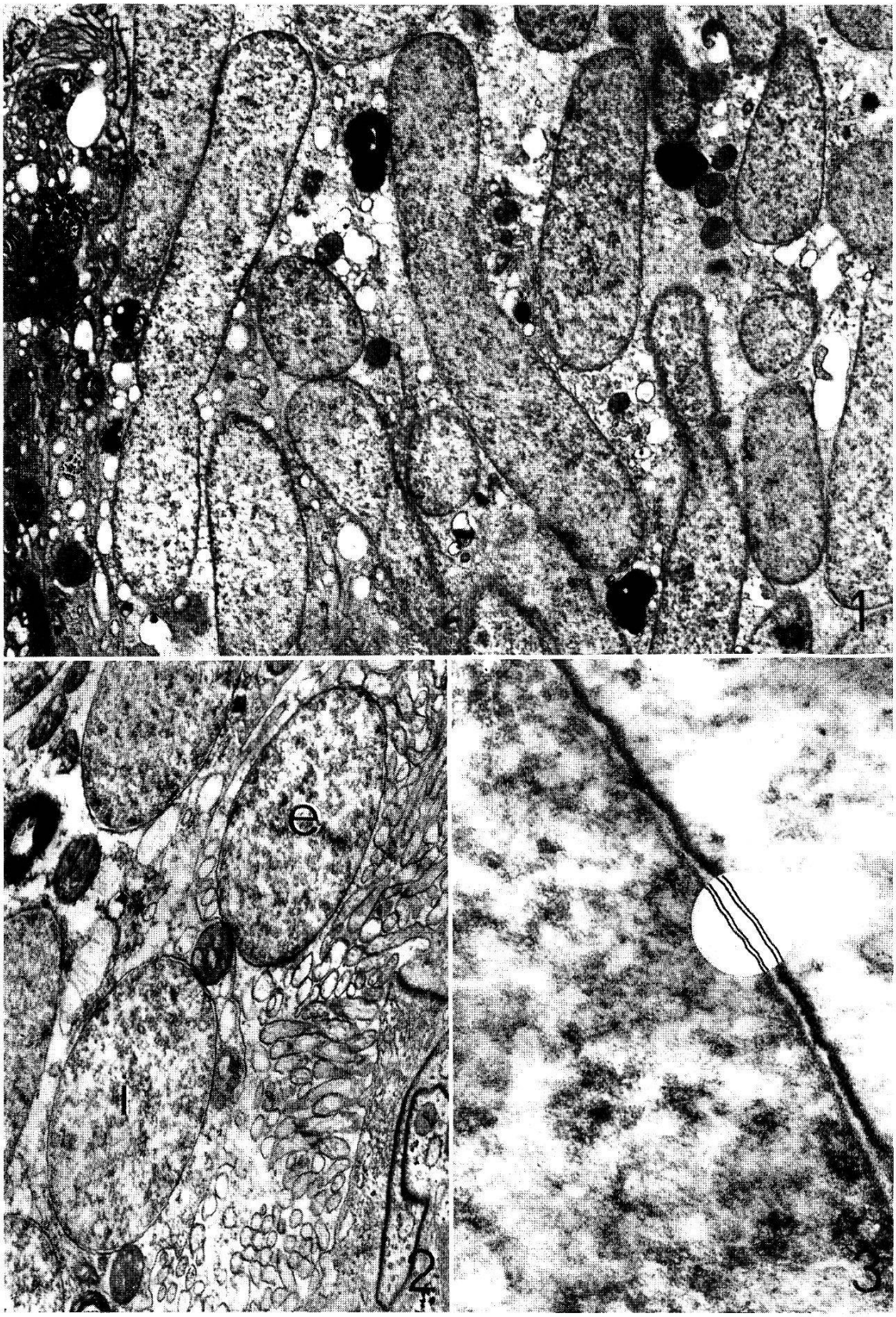

Fig. 1. Cross section of midgut mycetome of *G. morsitans* ♀ showing intracellular symbiotic micro-organisms, one of which is in division (8,627×).

Fig. 2. Section of brush border of midgut epithelium of *G. morsitans* illustrating the intracellular (i) and extracellular (e) locations of the micro-organisms (18,559×).

Fig. 3. Micrograph of cell wall of midgut form of micro-organisms showing the two tripartite membranes (75,240×).

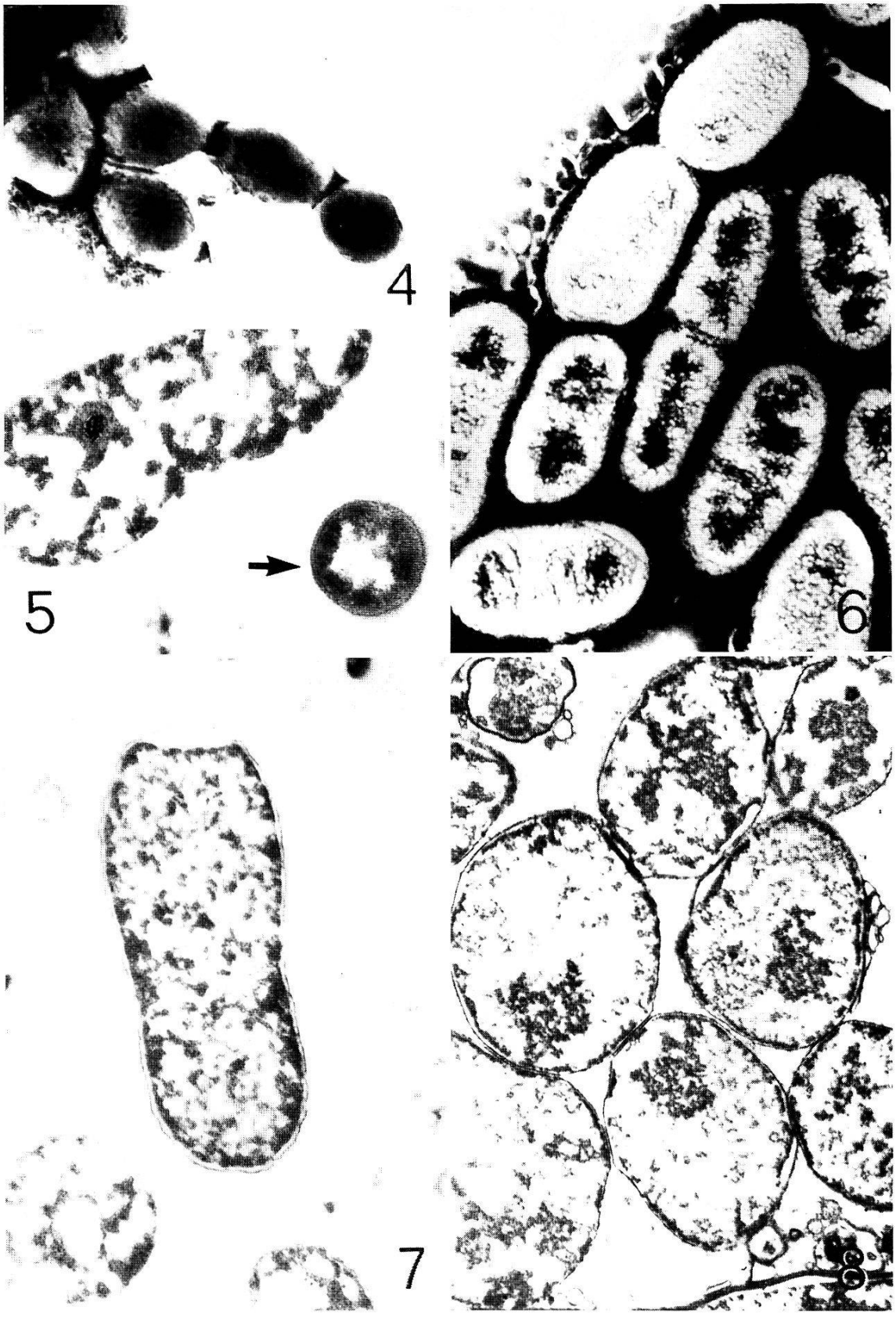

Fig. 4. Negatively stained preparation of small micro-organisms in a culture of symbionts isolated from a control fly (20,520×).

Fig. 5. Small micro-organism (arrowed) reminiscent of "les formes denses" (DEVAUCHELLE et al., 1972) and large bacteroid forms, in preparation of cultured micro-organisms (28,578×). →

of "Ampicillin" (Beecham Laboratories). Control flies were fed on a rabbit that had not been treated with antibiotics. Experimental and control groups were maintained in separate, but identical, cages in an incubator at 27 °C. Flies were removed, the mycetome isolated and transferred to culture media as described above; these cultures were maintained either at 27 °C or 37 °C. The remaining flies were fed at intervals on the appropriate rabbit. Throughout the experiment which continued for 33 days, note was made of any flies dying and whether their guts were full or whether they were apparently starved.

Immunological identification of cultured micro-organisms

Standard slide agglutination tests were made using portions of the cultures, which were tested against agglutinating sera *Proteus* OX2, OX19 and OXK[7] (AM series-*Proteus,* Wellcome Laboratories).

Results

1. The mycetome (Figs. 1, 2 and 3)

Sections of the midgut epithelium in the region of the mycetome showed the tissues packed with oblong micro-organisms similar to the bacteroids described by REINHARDT et al. (1972), HUEBNER and DAVEY (1974) and the large symbiotes of PINNOCK and HESS (1974). The wide range of section size suggested these organisms were of variable size up to a maximum length of 9 μm (Fig. 1). They were found in both intra- and extra-cellular positions (Fig. 2). The intracellular forms, some of which were dividing (Fig. 2), lay directly in the cytoplasm. They were limited by a typical trilaminar plasmalemma 8.6 μm wide which was surrounded by an electron lucent zone 12 μm deep, outside which lay a crenulate dense trilaminar membrane 12 μm thick, the outer surface of which appeared fuzzy (Fig. 3). These measurements it is noted correspond closely to those of REINHARDT et al. (1972), and are regarded as typical of rickettsial wall structure (HIGASHI, 1968).

2. The cultures (Figs. 4–8)

The cultured micro-organisms were not clearly distinguishable by light microscopy, further the slight opacity observed around the cultures on the agar plates might have resulted from the action of residual secretions from the tsetse gut tissues. Consequently cultured material was examined by electron microscopy to determine their appearance. Both negatively stained and sectioned material revealed numerous forms of micro-organism, from small dense oval organisms 0.7–1.04 μm in length (Figs. 4, 5) to large (1.43–6.97 μm) bacteroid forms (Figs. 6, 7, 8), there was some evidence of replication (Figs. 4, 5, 7). In some cultures (Fig. 8) the organisms appeared degenerate, the wide variety of form of the

Fig. 6. Negatively stained preparation of larger micro-organisms taken from a culture, prepared from the mycetome of a control fly, showing a crenated cell wall and dividing cells (16,720×).

Fig. 7. Thin section of larger micro-organism found in control cultures (23,488×).

Fig. 8. Distorted forms of the larger micro-organism present in control cultures (13,376×).

cultured micro-organisms contrasts with the mycetome forms and suggests they are pleomorphic.

3. Effects of antibiotics

36, of a total of 63, flies were treated with antibiotics: of these 14 had died by the 33rd day with their guts still full of blood (a further 7 had been removed for dissection and culturing). While flies from both control and treated groups died, apparently of starvation (for although offered rabbits every day their guts were empty), none of the control group died with blood in the alimentary canal. One "control" fly produced a puparium on the 28th day; none of the treated flies reproduced. There were no significant differences in mortality between flies given one control feed before exposure to the drug treated rabbit, those treated from the start and those treated from the 7th day.

So far as could be established by light microscopy no cultures of micro-organisms were established from mycetomes of the treated flies; control cultures were however successful. This was confirmed by electron microscopy.

4. Slide Agglutination Tests

Three "control" cultures were tested against the antisera as shown:

	OX 2	OX 19	OX K^7
1	?	–	+
2	–	–	+
3	–	–	+

The positive agglutination with OX K^7 suggests the cultured micro-organisms were rickettsia (see WILSON and MILES, 1966).

Discussion

Both REINHARDT et al. (1972) and PINNOCK and HESS (1974) recognised two types of micro-organisms in the tsetse flies they studied: "bacteroids" up to 8 μm (REINHARDT et al.) or 9 μm (PINNOCK & HESS) and small rickettsia-like organisms under 1.8 μm, the latter surrounded by a "lytic zone". The "bacteroids" found by HUEBNER and DAVEY (1974) in the ovaries of *G. austeni* were virtually of the same order of size as the small rickettsia-like organisms of REINHARDT et al. and PINNOCK and HESS, but apparently differed from them in that they lay in membrane limited vacuoles in the cytoplasm. While some aspects (e.g. the structure of the wall) of the micro-organisms studied here were remarkably constant *in vivo* and *in vitro*, other features particularly the size and general form were found to vary greatly. SIKORA (1942) and most recently DEVAUCHELLE, MEYNADIER and VAGO (1972) have described a number of different forms or stages of rickettsia, some of which compared closely to those seen *in vitro* in the present study. Indeed pleomorphism is a character of this group, one normally associated with arthropods. We believe our findings on the cultured forms and those (*in vivo*) of HUEBNER and DAVEY (1974), showing the considerable variation in size of the micro-organisms suggest that one pleomorphic organism may be involved. The agglutination tests indicated a rickettsia-type of organism in our cultures. However, STEINHAUS (1946) states that rickettsiae are normally around 1 μm in size, thus the large size of these micro-organisms, especially in the myce-

tome, and our apparent ability to maintain cultures in a relatively simple medium are unusual for rickettsia.

This organism is clearly sensitive to antibiotics and the tsetse flies are dependent upon it. Should flies feed on hosts with an antibiotic burden then their fertility (HILL et al., 1973) and longevity may be affected. The present studies suggested the actual process of digestion of blood may be affected. The aposymbiotic control of the tsetse fly is thus a theoretical possibility. It would be most appropriate with peri-domestic fly populations: the antibiotic might be "administered" via pigs, a favoured host.

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