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An Electron Microscopic Study of the Etiology of Hybrid Sterility in *Drosophila paulistorum*

I. Mycoplasma-like Inclusions in the Testes of Sterile Males*

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Abstract. Crosses between the semispecies of the *Drosophila paulistorum* complex produce fertile female but sterile male hybrids. An hypothesis is put forward that the hybrid sterility is in this case a result of discordance between a cytoplasmic symbiont and the genotype of the host. An ultrastructural analysis has been made of the testes of sterile male hybrids between the Andean and the Amazonian, and also other semispecies. Spermatid bundles undergo degenerative changes resulting in the loss of the axial filament complex and of associated mitochondrial derivatives. Numerous single or multiply clustered elements closely resembling Mycoplasma are observed in association with the degenerating spermatid bundles. Similar inclusions are observed also intruding into the wall of the distal parts of the testes and/or vasa deferentia. Some Mycoplasma-like bodies are observed also within the developing spermatids. These organs may be crowded with degenerating bundles and clusters of the Mycoplasma-like bodies. Each body is enclosed in a membrane, and may show a central reticular network and peripheral ribosome-like granules. The testes of the fertile males of the parental stocks reveal the presence of similar Mycoplasma-like inclusions, but not in such profusion.

Introduction

In the course of adaptation to a specific environment, a group of individuals usually develops an array of isolating mechanisms which protects the population's gene pool from disruption owing to mating with individuals not so adapted. Often reproductive restrictions are imposed by the sterility of the progeny from such "mixed" matings, where incompetent gametes are produced by the hybrid progeny. Matings between races or incipient species of *Drosophila paulistorum* (Dobzhansky and Pavlovsky, 1966, 1967) produce only sterile male progeny. Crosses between females of the Santa Marta strain and males of the Mesitas strain produce in the F_1 generation fertile females but sterile

* Dedicated to Professor Theodosius Dobzhansky on the occasion of his seventieth birthday, and in gratitude for our introduction to *Drosophila paulistorum*.

males. The reciprocal cross Mesitas ♀♀ × Santa Marta ♂♂ produce at least some fertile offspring of both sexes. Apparently the factors fostering this hybrid sterility are carried by females, transmitted maternally through the egg cytoplasm, causing sterility only in the male progeny. One possible explanation is that the Santa Marta strain carries a symbiont or parasite, with the host and infecting agent mutually adapted to one another. When maternally transmitted through the egg cytoplasm and exposed to a hybrid genotype, male sterility results.

Hybrid females produced from crosses Santa Marta ♀♀ × Mesitas ♂♂ may be backcrossed to either parental strain. The backcross to Mesitas males produces sterile sons but fertile daughters; sterile sons are produced for at least six serial backcross generations. If a cytoplasmic symbiont is involved in this unique type of male hybrid sterility, the maintenance of this factor is not prohibited in the hybrid females and at least six backcross generations are necessary for the dilution of the agent to a tolerable level or for suppression of the sterile male phenotype by the Mesitas genome. Ehrman and Williamson (1967, 1969) and Williamson and Ehrman (1967) have shown that the sterility may be transmitted by injection. Extracts prepared from either the Santa Marta strain, or from the progeny of the Santa Marta ♀♀ × Mesitas ♂♂ cross, were injected into Mesitas females, which were then mated to Mesitas males. In all instances some sterile sons were produced. Considered genetically, the progeny of the injected females mated to males of their own strain could only produce non-hybrid progeny with pure Mesitas genes. The sons were nontheless sterile, presumably because the causative agent and the Mesitas genome were not mutually adapted. This artifically induced sterility, however, is not transmitted to further generations as is the naturally occurring sterility.

Further support for the symbiont hypothesis derives from genetic tests conducted with another related strain, Llanos. For several years Llanos behaved as a member of the Orinocan semispecies, producing fertile progeny in crosses to other members of the same semispecies. In the laboratory this strain now produces sterile hybrid sons in crosses with all of the same strains with which it had originally produced fertile progeny. The Llanos strain appears to have acquired the capacity to produce sterile progeny (Dobzhansky and Pavlovsky, 1966, 1967). Further studies by Williamson and Ehrman (1968) have shown that extracts prepared from such sterile hybrid males are competent to cause sterility in experiments similar to those previously described.

More circumstantial evidence is derived from experiments conducted by Ehrman (1968) utilizing sperm motility as an index of fertility, where Santa Marta females were raised on a medium containing the antibiotics gliotoxin or toyocamycin and used in crosses to Mesitas males.

Treated females produced a higher proportion of fertile males than did untreated controls; apparently the antibiotic suppressed the maintenance or multiplication of the causative agent in the parental female.

Because of the circumstantial evidence suggesting involvement of an infectious agent, an ultrastructural study at the electron microscope level has been initiated in an attempt to identify this agent in the male reproductive tract. Initial observations were made upon the structure and contents of the paired accessory glands, the paragonia, since these glands had been implicated as a source of a cytoplasmic agent as previously described by Williamson and Ehrman (1967). These results have been reported elsewhere (Kernaghan and Ehrman, 1968; Tandler *et al.*, 1968). The results presented below describe symbionts observed in gonial tissue and possibly implicated in the infectious male sterility in *Drosophila paulistorum*.

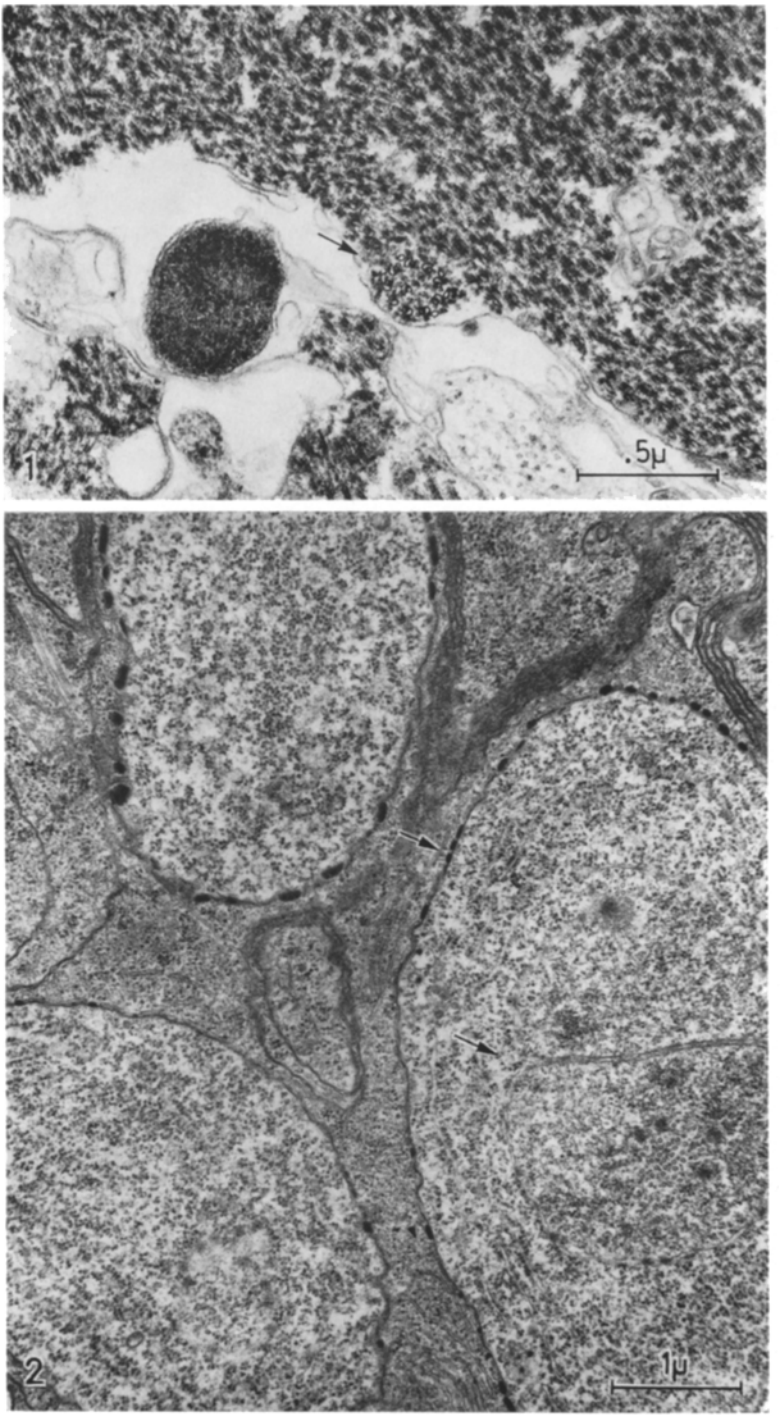
Materials and Methods

The *Drosophila paulistorum* tissues were fixed with 3% glutaraldehyde in 0.15 M phosphate buffer at pH 7.4 for 30 minutes at room temperature (Sabatini *et al.*, 1963), washed in buffer, postfixed with 1% Millonig's (1961) osmium tetroxide for 1 hour, dehydrated, and embedded in Epon 812 (Luft, 1961). Silver gray sections were cut with an LKB microtome, mounted on Formvar-coated grids, stained with 50% alcoholic uranyl acetate (Gibbons, *et al.*, 1960), and lead citrate (Reynolds, 1963) and observed with a JEM 6C electron microscope.

Strains of the superspecies *Drosophila paulistorum* used in this study were (1) Santa Marta, Colombia, Transitional semispecies; (2) Mesitas, Colombia, Andean-Brazilian semispecies; (3) Llanos, Colombia, Interior semispecies; (4) Georgetown, British Guiana, Orinocan semispecies; (5) Sarare, Venezuela, Transitional semispecies; (6) Angra, Brazil, Andean-Brazilian semispecies; and (7) Belem, Brazil, Amazonian semispecies. (See Dobzhansky and Spassky, 1959; Dobzhansky *et al.*, 1964; Dobzhansky *et al.*, 1969, for details of the origin of these strains.)

Observations

In *Drosophila paulistorum* the spermatids develop in membrane enclosed cysts, each cyst containing 64 cells. Usually two cysts are clustered together and all spermatids are observed in a synchronized state of development. Each cyst is derived from a single primary spermatogonium. Regularly, a syncytial arrangement is observed in which two or more spermatids each exhibiting the usual axial filament complex and mitochondrial derivatives, develop within the same limiting membrane. On a fine structure basis, at this stage in spermatogenesis it is not possible to distinguish the sterile hybrids from normal fertile males. Beyond this point in the maturation of the spermatids, degenerative changes may be observed to begin in the spermatid bundles of sterile hybrid males. Subtle changes may be observed such as the disruption of the peripheral fibers of the axial filament complex with a resultant destruction of the spherical



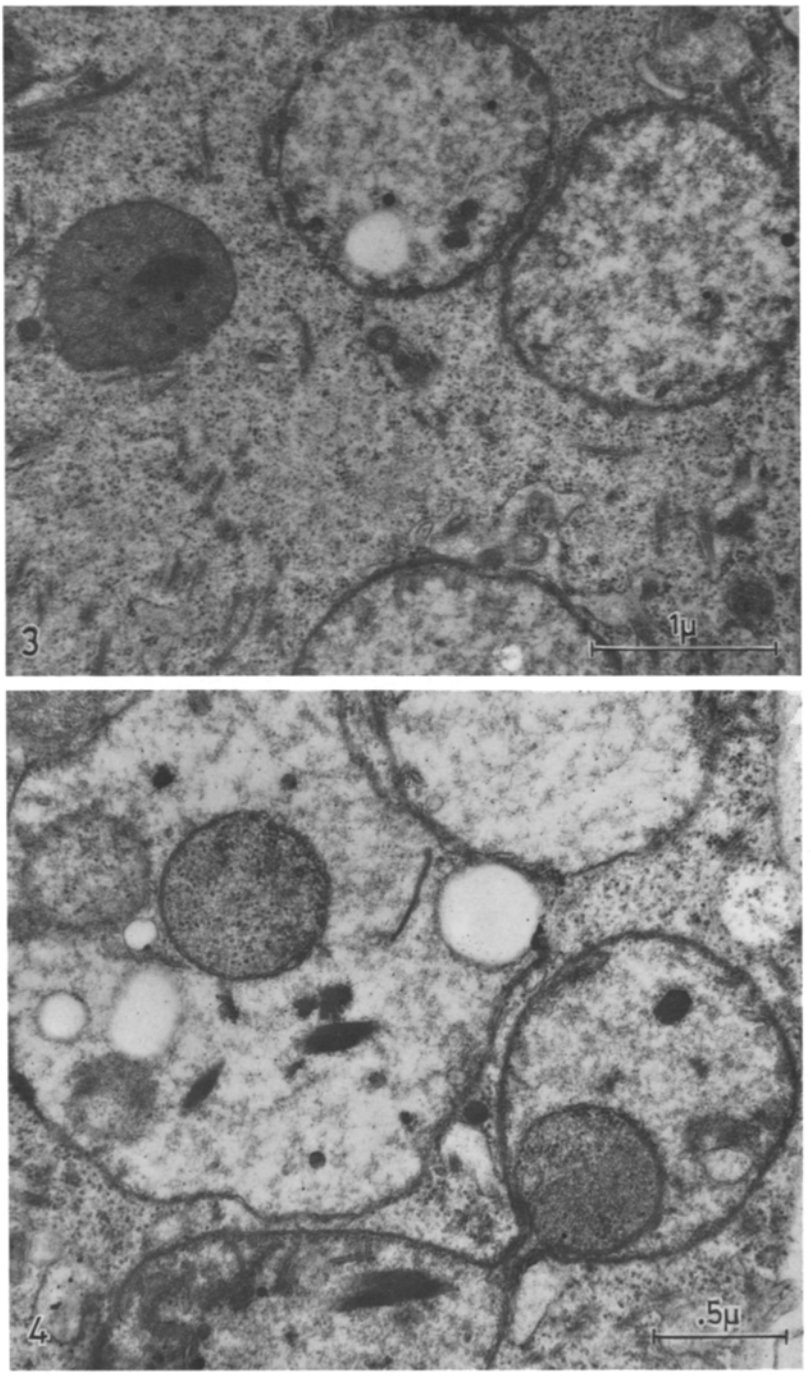
Figs. 1 and 2

symmetry. However, in the more severe cases, spermatid cysts appear ruptured, the axial filament complex and satellite fibers are disoriented and the mitochondrial derivatives are disorganized. Myelin figures, cell contents including mitochondria and ribosomes are observed interspersed among spermatids. In cases of severe degeneration, the lumen of the testes may be congested with aggregations of the contents from ruptured spermatids. Often dozens of axial filaments are observed condensed in syncytium minus normal spermatid structures (Fig. 1). Also, regularly dispersed in the luminal debris are membrane-bound vacuolated structures half a micron or more in diameter which contain one or more crystalline lamellar arrays. Infoldings of the limiting membrane are observed which resemble mitochondrial cristae and the crystalline inclusions are similar to paracrystalline bodies usually present as accessory structures in the neck of spermatids (Fig. 3). Widespread degeneration as described above has been observed in testes of F_1 sterile males obtained from crosses of Angra ♀♀ × Belem ♂♂; Sarare ♀♀ × Marco ♂♂, and Llanos ♀♀ × Georgetown ♂♂.

Regularly one or more kinds of inclusion may be observed in electron micrographs of testes of sterile hybrid males. Included within the luminal contents of the testes or protruding between developing spermatid cysts or even attached externally to the testicular membrane are groups of large cells often exhibiting a doubled nucleus-like structure accompanied by a granular reticulum (Fig. 2). Several apparent stages of maturity may be exhibited by various cells of a single cluster. Some members are bounded by a simple plasma membrane with no external coating evident while other cells exhibit an external tufting of dense material. Continued aggregation of this material envelopes the cell in a cyst-like electron dense capsule. Containment by this envelope is accompanied by internal morphological developments with the appearance of a helically wound filament embedded in a dense matrix. Comparison with the description provided by Burnett and King (1962) of a parasite infecting ovaries of *Drosophila willistoni*, shows that structures observed are microsporidia. These microsporidia have been observed more or less frequently in individuals of all fertile and hybrid stocks examined.

Fig. 1. Random aggregation of axial filaments in degenerating spermatid bundles in a sterile F_1 testis from the cross Llanos ♀♀ × Georgetown ♂♂. Arrow indicates a symmetrical complex. ×42,000

Fig. 2. Large binucleate cells (lower arrow) undergoing encapsulation by aggregation of electron dense material at the periphery (upper arrow). These are microsporidians of the genus *Nosema*. Section of the testis of F_1 males from cross Santa Marta ♀♀ × Mesitas ♂♂. ×17,500



Figs. 3 and 4

In the sterile testes numerous pleomorphic inclusions, either singly or in clusters may be observed interposed between spermatid cysts within the lumen of the testes, distributed between spermatids of the same cyst or intruding into the wall of the testes. These membrane bound bodies display a irregular peripheral ribosomal granulation (Fig. 8) bounding a central fibrillar network. No organized nucleus is evident. With an average diameter of 0.5 μ these inclusions closely resemble a Mycoplasma, and are morphologically similar to those described as C-type *Mycoplasma hominis* (Anderson and Barile, 1965). In regions of severe degeneration, Mycoplasma-like bodies are observed dispersed among the cellular debris. Other apparent variants in Mycoplasma morphology, e.g. elementary bodies, (minimal reproductive units) and transitional forms have also been seen. It is, however, necessary to be cautious in specific identification, because of possible abnormal morphology produced by spermatid degeneration (Fig. 4). No filamentous forms have been observed.

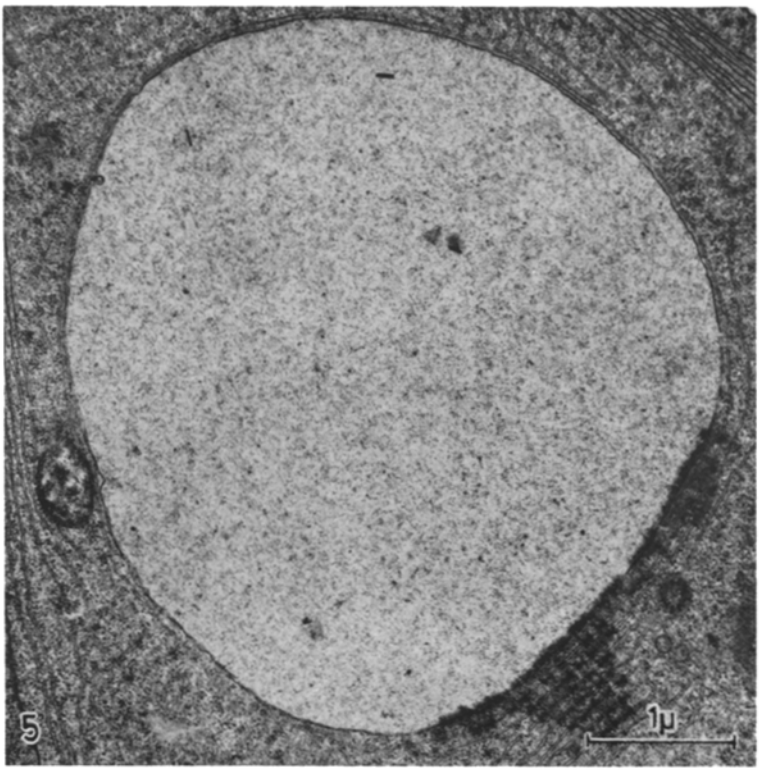
That the Mycoplasma-like inclusions may indeed be intracellular contaminants in developing spermatids can be seen in Figs. 5, 6 and 7. At the ultrastructural level, testes of all sterile hybrids exhibiting advanced stages of degeneration have been observed to contain Mycoplasma-like inclusions. Similarly, such inclusions have been observed in the testes of males of the fertile parental stocks of Angra, Belem and Llanos. In contrast to the sterile hybrid testes, none of the advanced types of degeneration, nor intense clustering of these organisms is evident.

Discussion

Although atypical cytoplasmic inclusions have been observed in the male reproductive tract of *Drosophila paulistorum*, the evidence associating the maternally transmitted hybrid male sterility with these inclusions is still not definitive. In paragonial cells filamentous structures contained in large cytoplasmic vacuoles have been described by Kernaghan and Ehrman (1968); these structures and the much larger luminal filaments reported by Tandler *et al.* (1968) probably play no direct role in male hybrid sterility. Structures similar to the vacuolar type have been

Fig. 3. Developing paracrystalline body in mitochondria presumably released from ruptured spermatids, accompanied by swollen mitochondrial derivatives. F_1 testis Santa Marta ♀♀×Mesitas ♂♂. ×24,500

Fig. 4. Distended mitochondria containing paracrystalline derivatives and membrane bound bodies resembling granular Mycoplasma. F_1 testis from Santa Marta ♀♀×Mesitas ♂♂. ×35,000



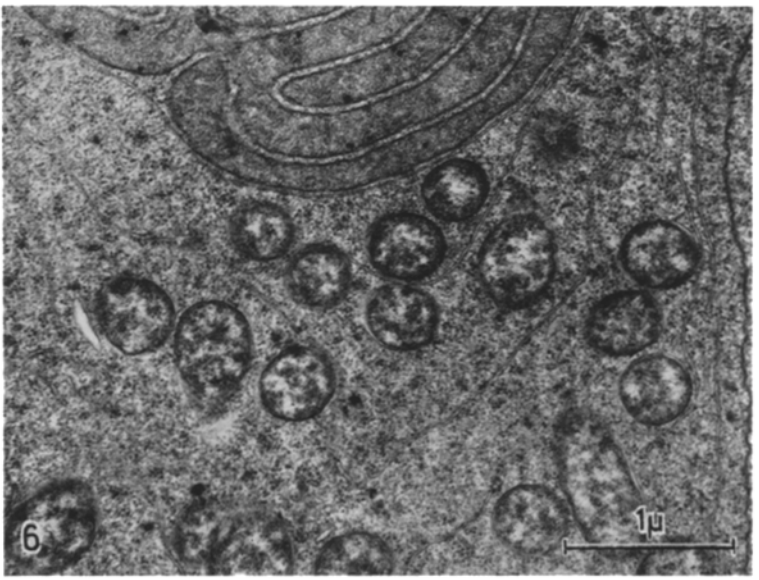


Fig. 5. A Mycoplasma-like inclusion near the nucleus in the head of a spermatid in a F_1 hybrid (Sarare ♀♀×Marco ♂♂). ×19,500

Fig. 6. Mycoplasma-like bodies clustered about large nebenkern derivative in an F_1 Angra ♀♀×Belem ♂♂ sterile male hybrid. ×22,500

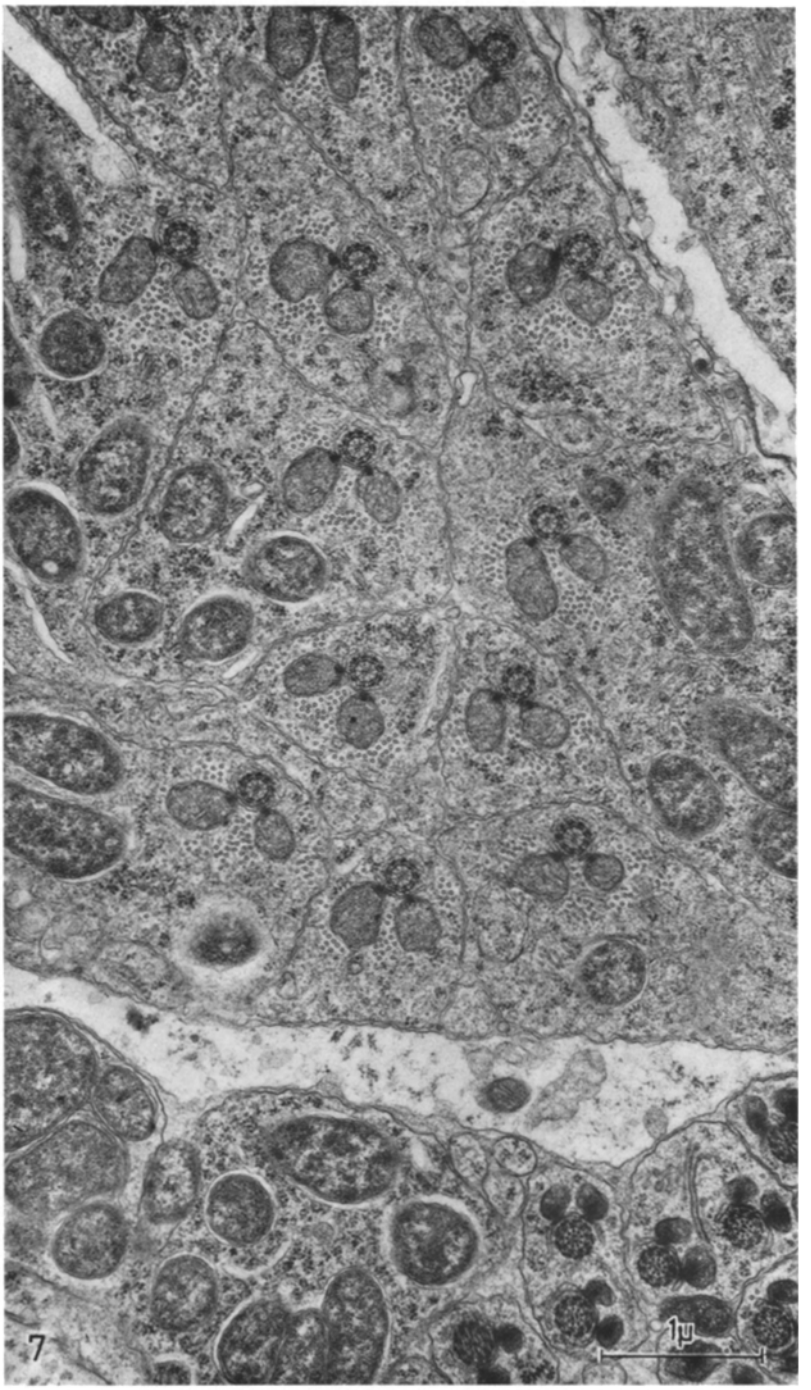


Fig. 7. Section through sperm bundles in a testis of an F_1 Angra ♀♀ × Belem ♂♂ sterile male hybrid. Note normal arrangement of the axial filament complex and flanking mitochondrial derivatives. Clusters of Mycoplasma bodies are observed between and also within spermatid bundles, displacing spermatids of the same bundle. ×21,500

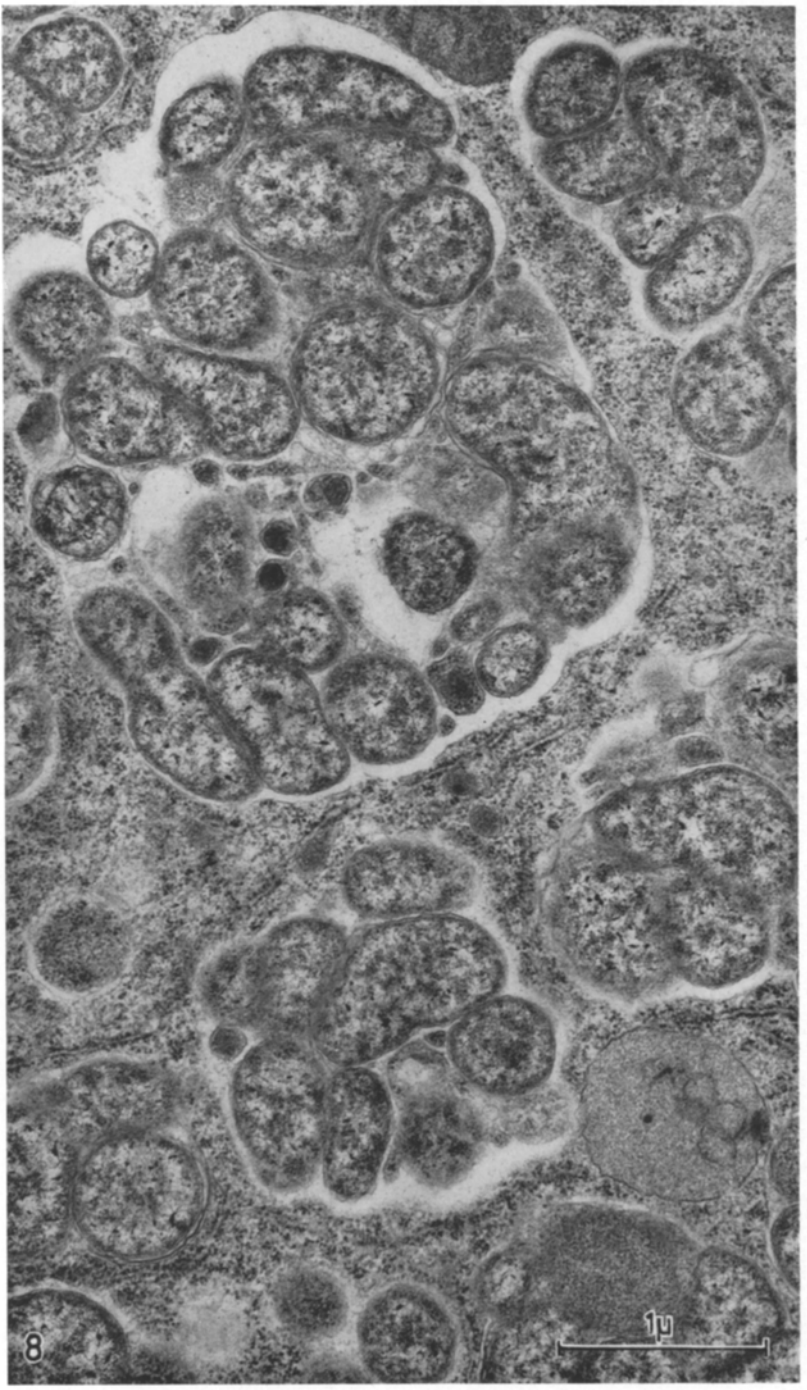


Fig. 8. Clusters of Mycoplasma-like bodies in the lumen of a sterile F_1 Angra ♀♀ × Belem ♂♂ testis. Clusters of these bodies are dispersed in mixture of ruptured cell contents. ×27,500

observed also by Bairati (1966), mixed with the sperm in the genital ducts of *Drosophila melanogaster*. Whether these structures are virus particles or some contractile elements concerned with sperm mixing is unknown. In the present study paragonia taken from sterile hybrids and parental stocks all show the filamentous structures and no differences in the cytology of the accessory glands of either sterile or fertile males has been observed.

The occurrence of a microsporidian parasite in the testes of *Drosophila paulistorum* likewise should not be considered related to hybrid sterility, since Burnett and King (1962) have observed a heavy infection by this organism in every major organ of *Drosophila willistoni* with little reduction in the viability. Kramer (1964) described this monosporoblastic parasite as *Nosema kingi*. In a personal communication, Kramer has identified this microsporidian of *Drosophila paulistorum* as similar or identical to that infecting *Drosophila willistoni*.

The Mycoplasma-like inclusions observed in the testes of *Drosophila paulistorum* are more likely to be the causative agent. These inclusions occur in large numbers, and are contained intracellularly in early spermatids close to essential structural elements of the developing spermatid. The degeneration of the spermatid bundles may plausibly be supposed to be a direct result of the Mycoplasma multiplying at the expense of the infected cells, or secondarily as a consequence of the production of a toxin. Aleu and Thomas (1966) have ascribed a similar cytopathic effect to a neurotoxin produced in mice infected with *Mycoplasma neurolyticum*.

The designation of the observed inclusion as a Mycoplasma is obviously tentative in the absence of complementing isolation and culture of the organism; excellent ultrastructural identity can, however, be recorded for cultured *Mycoplasma hominis* (Anderson and Barile, 1965). Granados *et al.* (1968) have observed Mycoplasma-like inclusions in the leafhopper *Dalbulus elimatus* and also in maize affected with Stunt disease. These Mycoplasma-like bodies have been implicated as the etiologic agent transmitted by the leafhopper vector in this plant disease. Mycoplasma have been implicated in other plant diseases on the basis of susceptibility of Aster Yellows disease to treatment with antibiotics (Whitcomb and Davis, 1969) and by ultrastructural studies by Doi *et al.* (1967), Bowyer *et al.* (1969), Hampton *et al.* (1969), and Shikata *et al.* (1968). In the case of the Mycoplasma isolated from infected peas, Hampton *et al.* (1969) have reported a serological relationship between this agent and an avian and a human Mycoplasma. Moreover, Koch and King (1966) and Koch *et al.* (1967) have described the widespread occurrences of pleomorphic inclusions, termed "A" bodies, in various tissues, particularly in the ovaries of *Drosophila melanogaster*.

Although no sterility or other effects have been attributed to these bodies, their resemblance to Mycoplasma structure is striking.

Dr. Patricia Smith, then of Professor R. C. King's laboratory at Northwestern University, first discovered the Mycoplasma-like symbiont in the eggs of the Mesitas *Drosophila paulistorum* strain in 1968 (personal communication). It was her preliminary work which prompted a more thorough re-examination of the sterile males.

Recently in micrographs of developing follicles taken from the ovaries of F_1 females from the cross Angra ♀♀ × Belem ♂♂, similar Mycoplasma bodies have been observed in the oocytes. The presence of such bodies in the young unfertilized egg and the correlation with the maternal effect known from the genetic studies of Ehrman (1962a, b), favors the view that this organism may be involved in the male hybrid sterility phenomena in *Drosophila paulistorum*.

The positive identification of this Mycoplasma-like inclusion as the agent responsible for the male hybrid sterility in *Drosophila paulistorum* awaits isolation, culture and typing of this organism.

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