UNUSUAL FILAMENTOUS STRUCTURES IN THE PARAGONIA OF MALE DROSOPHILA

BERNARD TANDLER, DAVID L. WILLIAMSON, and LEE EHRMAN

From the Sloan-Kettering Institute for Cancer Research, New York, the Woman's Medical College of Pennsylvania, Philadelphia, Pennsylvania, and The Rockefeller University, New York. Dr. Tandler's present address is the School of Dentistry, Case Western Reserve University, Cleveland, Ohio 44106

ABSTRACT

Drosophila paulistorum is a complex of five incipient species which when crossed produce sterile hybrid males and fertile females. Sterility of the male progeny can sometimes be induced by injecting females of one strain (Mesitas) with a homogenate of males of another strain (Santa Marta) or of hybrids between these strains, and then crossing the recipient females to Mesitas males. Filamentous structures have been found in cytoplasmic vacuoles in paragonial cells in males of these and other similar strains and their hybrids. These structures, which contain RNA, possess a helical substructure and resemble certain viruses. Large filamentous structures found in the lumen of the paragonia are also described.

INTRODUCTION

Four different but related kinds of hybrid sterility of males occur within the superspecies *Drosophila paulistorum* Dobzhansky and Pavan. The superspecies consists of at least five races or incipient species (11). The F_1 hybrids between these races are fertile if females and sterile if males. This sterility is the genic type, since the degenerative changes begin before meiosis, and the meiotic metaphases show at least some paired chromosomes.

The second kind of sterility occurs in males in backcross progenies. This sterility depends upon the genetic constitution not of the males themselves but rather of their mothers. As a rule, all the sons of a female carrying any mixture of the chromosomes of the parental races are sterile, even though some of these sons themselves carry only the chromosomes of a single race. This sterility operates via a maternal effect; the genes responsible are distributed in all three pairs of chromos somes which *D. paulistorum* possesses. The sterilitie- of the F_1 hybrid and of the backcross males are due obviously to different causes, since F_1 hybrids are sons of pure rather than of hybrid mothers. The different causation is shown also by the fact that the two kinds of sterility are sometimes dissociated; although in most crosses both the F_1 and the backcross males are sterile. In some exceptional cases the F_1 may be sterile and the backcrosses fertile, or vice versa (11).

The third kind of sterility is known thus far only in hybrids between strains of the transitional race, the ancestors of which were collected at Santa Marta (see legend for Fig. 3) and at Mesitas (see legends for Figs. 1 and 2), Colombia, respectively. The male progeny from the cross Santa Marta female × Mesitas male is sterile, while the reciprocal cross gives fertile progeny of both sexes. When the hybrid females from the Santa Marta female × Mesitas male are backcrossed to Mesitas males, some sterile male progenies are obtained in at least six successive generations (13, 14, 23). The sterility appears to be due to interactions between the Santa Marta cytoplasm

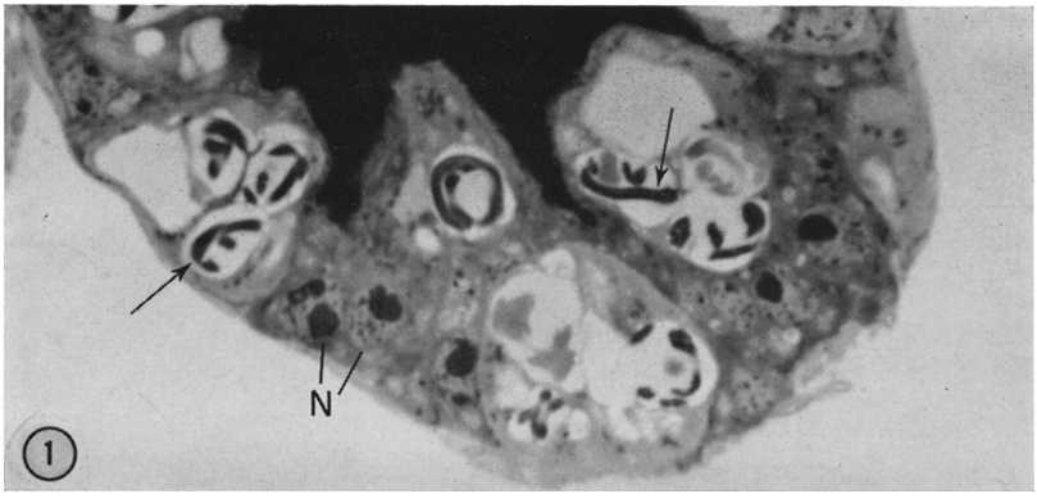

FIGURE 1 Photomicrograph of a portion of a transversely-sectioned paragonium from a male belonging to the Mesitas, Colombia *Drosophila paulistorum* strain. The cells are binucleate with prominent nucleoli. Many of the cells display large, clear cytoplasmic vacuoles which contain dark, rodlike structures (arrows). The luminal contents are deeply stained. *N*, nuclei. Toluidine blue. × 1600.

and the Mesitas Y chromosome (see legends for Figs. 6–8). Whatever the factor transmitted by the Santa Marta cytoplasm, it is eventually overcome by the Mesitas genome, since fertile sons are obtained by the seventh backcross.

The following working hypothesis was considered worthy of testing. Suppose that the Santa Marta strain carries a substance or an associated microorganism, a symbiont or a parasite of some kind, which is regularly transmitted in this strain from generation to generation via the egg cytoplasm. Suppose further that the genome of the Santa Marta strain and the cytoplasmic associate are coadapted, so that the viability and fertility of the flies are not adversely affected by the association. There is, however, no such mutual adaptation between the associate of the Santa Marta strain and genome of the Mesitas strain, particularly the Y chromosome of the latter. What results is a sterility of the male progeny from the cross Santa Marta female × Mesitas male. This sterility continues for as many generations as required to have the cytoplasmic associate of the Santa Marta strain suppressed by the Mesitas genome.

This hypothesis was first tested by an attempt to induce the sterility by injection. Can the agent which causes the sterility be transmitted by infection, as well as through the egg cytoplasm? Females of the Mesitas strain were injected with homogenates of Santa Marta flies or of Santa Marta female × Mesitas male hybrid flies. The injected females were allowed to incubate the "infection" and then were crossed to Mesitas males. Their offspring had only Mesitas genes, but the genetically nonhybrid male offspring were nonetheless sterile. Injection of Santa Marta females with similar homogenates leads to no sterility; nor do injections into males sterilize either the recipients or their sons (14, 23). Homogenates of whole male flies, of paragonia, and of the male internal reproductive organs induce sterility, while homogenates of whole female flies or injections of the hemolymph do not. This induced, or "infectious," sterility seems to be the fourth kind of sterility found in *D. paulistorum*. In contrast to the sterility in the hybrids, this fourth type is confined to the progeny of the injected mothers and is not transmitted to further generations.

And finally, the origin in the laboratory of a sterility of male hybrids between strains which were previously fertile has been recorded (11, 12). The strain involved is the "new Llanos" which descended from a progenitor collected in the Llanos of Colombia (see legends for Figs. 4 and 5). This strain gave fertile hybrids with the Orinocan incipient species of *Drosophila paulistorum*, but it is now producing sterile male hybrids. This unique phenomenon, plus the infectious nature of ho-

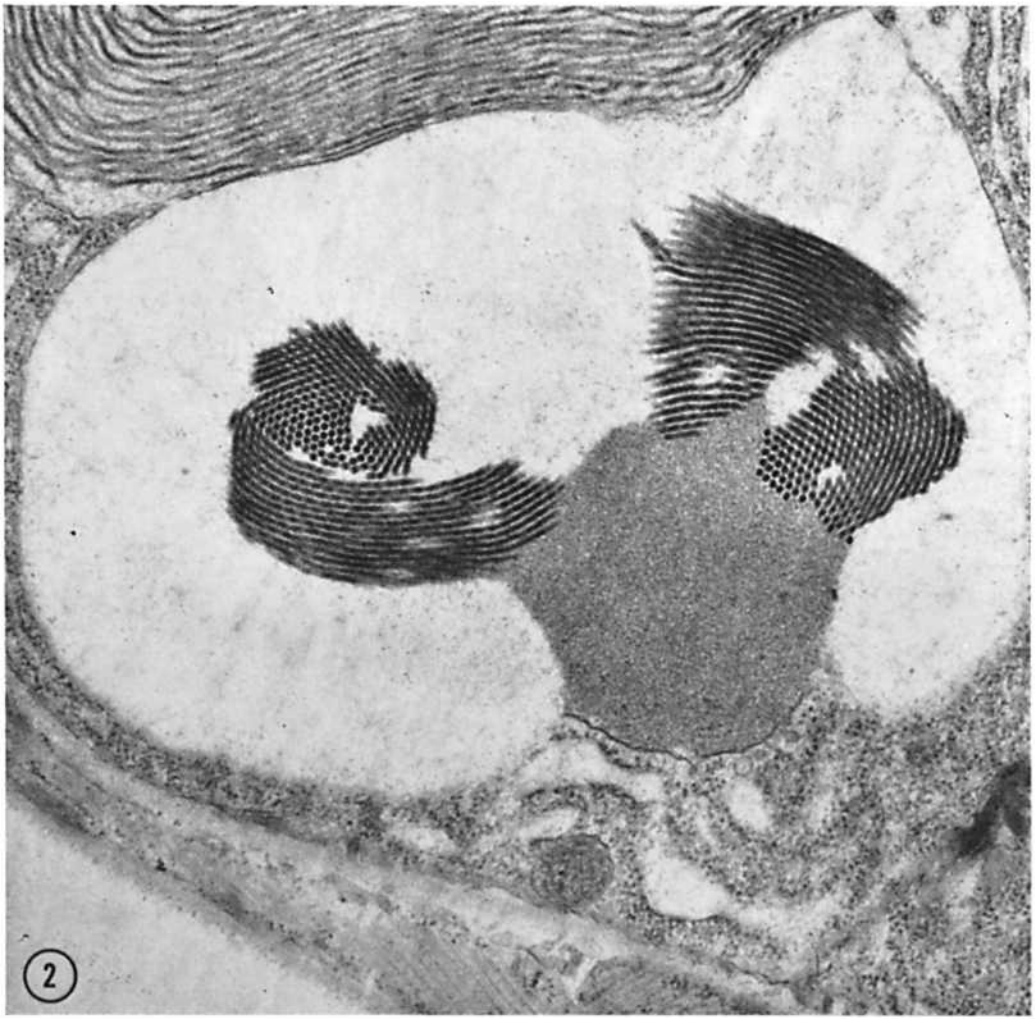

FIGURE 2 Electron micrograph of a cytoplasmic vacuole. The rodlike structures, in a cell from the paragonia of a Mesitas *D. paulistorum* male, are seen to consist of numerous, closely packed, parallel filaments. These structures are in close relation to a large, moderately dense, finely fibrillar mass. A portion of another vacuole may be seen at the top of the micrograph. It contains numerous, loosely arranged filaments which appear to be of lower density than those in the adjacent vacuole. A process of a visceral muscle cell is present at the bottom of the micrograph. × 40,000.

mogenates of Llanos material, suggests a hitherto scarcely suspected method of initiation of species formation, i.e., the establishment of a symbiotic relationship between the fly and some associated microorganism. The latter becomes integrated with and transmitted regularly through the female sex cells of the host species. The symbiont need not, however, be integrated with the genes of other populations of the host; the hybrids thus become sterile. Natural selection then promotes the formation of an ethological isolation between the populations carrying different symbionts. Whether this situation is very special and is confined only to the superspecies *Drosophila paulistorum*, or is distributed more widely, is for future investigations to discover.

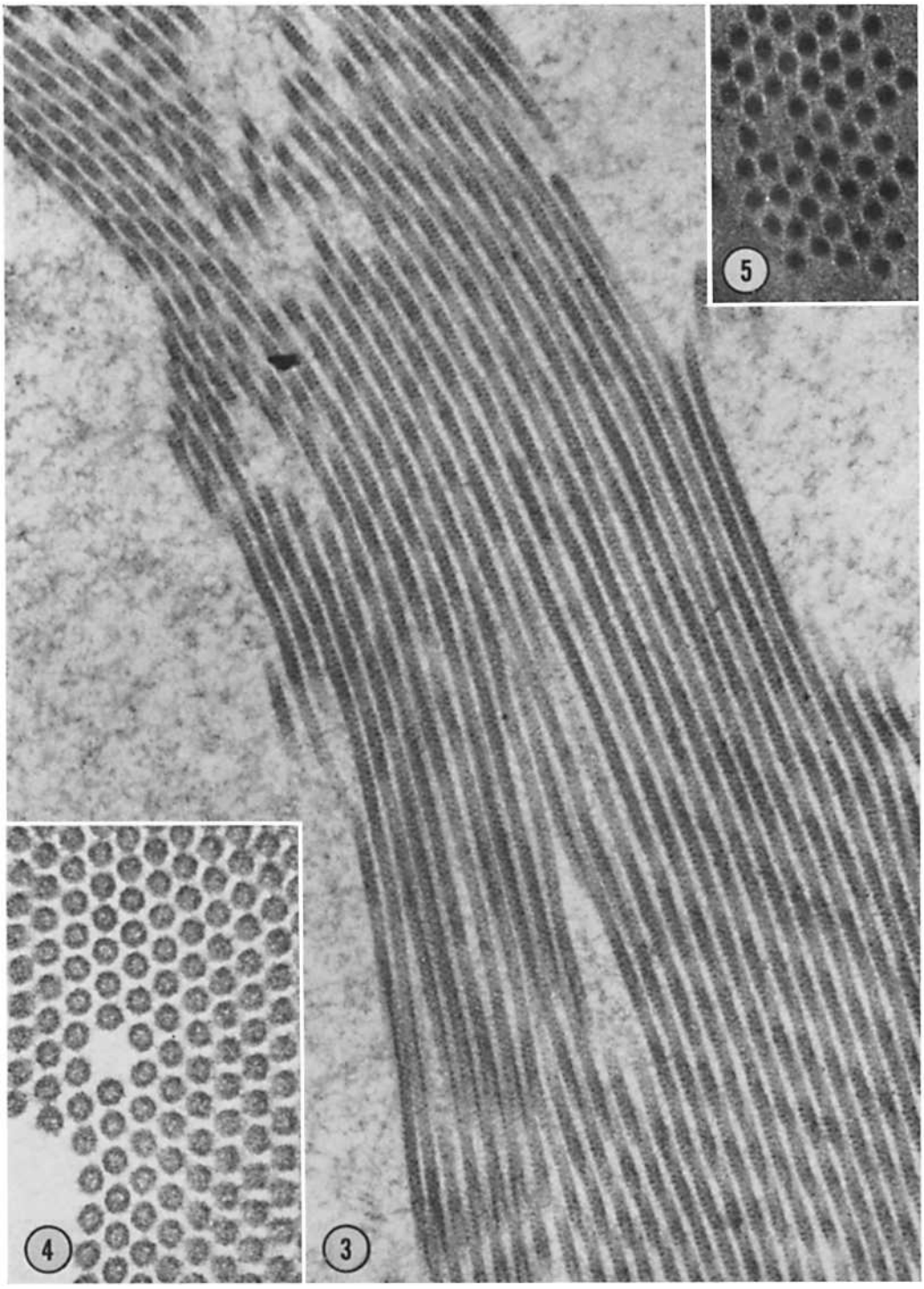

FIGURE 3 A longitudinal section of a packet of intravacuolar filaments. A helical substructure is apparent in many of the filaments. Tissue from a male of the Santa Marta, Colombia strain of *D. paulistorum*. × 74,000.

FIGURE 4 A transverse section of intravacuolar filaments which shows their regular hexagonal arrangement. Each filament has an electron-lucent core. Tissue form a sterile F_1 hybrid *D. paulistorum* male from the interracial cross, Llanos, Colombia ♀ ♀ × Caripe, Venezuela ♂♂. × 110,000.

FIGURE 5 Transverse section of a packet of luminal filaments. These structures, which also show hexagonal packing, consist of an extremely dense core which is surrounded by a thin halo of low density. F_1 sterile hybrid ♂ (Llanos ♀ ♀ × Caripe ♂♂). × 43,000.

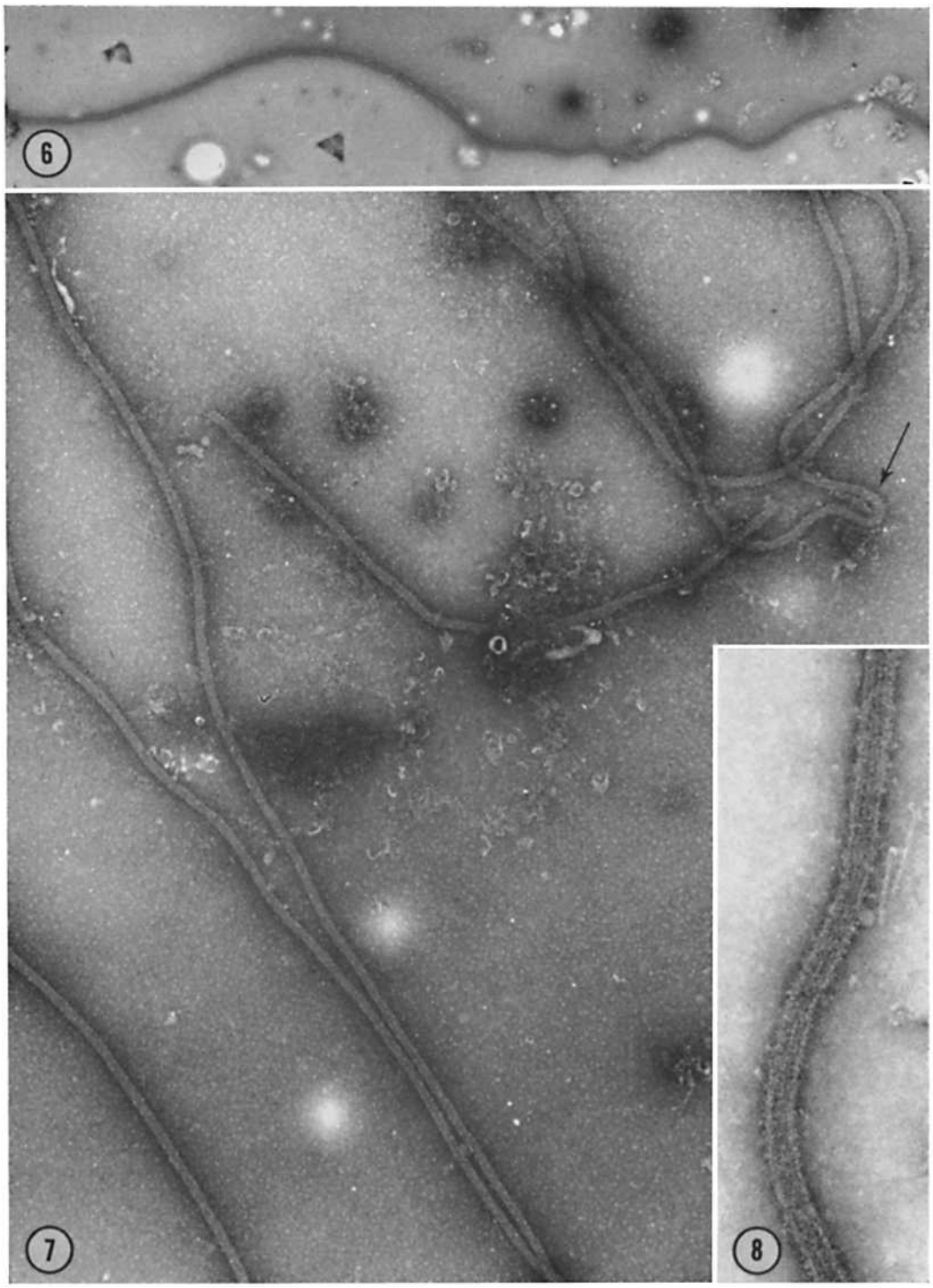

FIGURE 6 A negatively stained luminal filament demonstrating its great length. Isolated from sterile F_1 hybrid male produced by the cross Santa Marta ♀ × Mesitas ♂. × 4,750.

FIGURE 7 Negatively stained luminal filaments. These structures are extremely flexible and may be sharply bent (arrow). F_1 sterile hybrid ♂ (Santa Marta ♀ × Mesitas ♂). × 21,000.

FIGURE 8 A negatively stained luminal filament at higher magnification. Fine longitudinal fibrils are faintly apparent within the core. F_1 sterile hybrid ♂ (Santa Marta ♀ × Mesitas ♂). × 72,000.

MATERIALS AND METHODS

Flies were immersed in fixative, and the paragonia were dissected out. The initial fixative was a mixture of 2% acrolein and 6% glutaraldehyde (20) buffered with Veronal-acetate. The paragonia were postfixed in 2% osmium tetroxide in Veronal-acetate buffer (6), dehydrated in ethanol, and embedded in a mixture of Maraglas and Dow Epoxy Resin 732 (15). Thin sections were doubly stained with uranyl acetate (22) and lead tartrate (18) or were stained with lead tartrate alone. Stained sections were examined in a Siemens Elmiskop 1 or 1A electron microscope.

For purposes of orientation, 1 μ thick sections were stained with toluidine blue (4) and examined and photographed with a Zeiss Standard WL microscope equipped with planapochromatic objectives. Other sections were stained for RNA by the method of Flax and Himes (16), or for DNA by means of a fluorescent Feulgen technique (17).

Paragonial extracts were examined by the negative staining technique (5). For these studies, 30 F_1 sterile males from the cross Santa Marta female $\times$ Mesitas male served as the source of paragonia. Testes and paragonia were dissected in cold 0.24 M sucrose, Tris buffered at pH 7.4. The paragonia were carefully separated from the testes and transferred to a small glass homogenizer containing 0.1 ml of the sucrose solution. The paragonia were lightly homogenized at 4°C, and droplets of the crude homogenate were placed on electron microscope grids. These droplets were diluted with an equal amount of 2% phosphotungstic acid (pH 6.8); the excess fluid was withdrawn with filter paper, and the grids were allowed to air dry.

OBSERVATIONS

The paragonial glands are tubular organs consisting of a single layer of epithelial cells which enclose an expansive lumen, whose contents display an extreme affinity for toluidine blue (Fig. 1). In the plane parallel to the long axis of the gland, the epithelium varies from cuboidal to columnar. In transverse sections, the lower lateral boundaries of the epithelial cells are parallel, while the upper portions of the cells bulge into the lumen or taper to a blunt point. The luminal surfaces are covered by rather long, irregular microvilli, and many of the cells rest on processes of stellate muscle cells which invest the paragonia. The paragonial cells are binucleate, the two nuclei being identical in appearance. The nuclei contain prominent nucleoli and chromatin masses and are located near the cell base.

Huge cytoplasmic vacuoles are present in some of the paragonial cells (Fig. 1). The vacuolar contents are clear, but some vacuoles contain densely staining, rod-shaped structures, both straight and curved. These structures, which may be as long as 15 μ, are Feulgen-negative. They stain intensely with azure B at pH 4.2; this staining reaction is noticeably diminished after hydrolysis with hot trichloracetic acid, indicating that the rods contain RNA.

In the electron microscope, the vacuoles are seen to be delimited by a single membrane (Fig. 2). The rodlike structures consist of numerous filaments measuring about 280 A in diameter. The length of individual filaments is undetermined, but it is in excess of 2 μ. At higher magnifications of longitudinal sections (Fig. 3), each filament appears to consist of several plectonemic helices with a pitch angle of approximately 20° and a distance of about 120 A between successive gyres. In transverse sections (Fig. 4), the filaments are disposed in hexagonal array with a center-to-center distance of 450 A. Individual filaments have a central core of low density with walls that measure about 65 A in thickness. These walls appear to be made up of an undetermined number of subunits. The core frequently contains one or more tiny granules, which may represent cross-sectional profiles of filar structures.

The packets of filaments are surrounded by a flocculent matrix and are often associated with irregular masses of moderately dense, fibrillar material (Fig. 2). The packets may be partially buried in the fibrillar masses or may extend out from the masses at right angles. The masses themselves show no preferential location within the vacuoles.

In some vacuoles the filaments are not organized in regular arrays but are disposed in loose skeins (Fig. 2). Such filaments are usually indistinct in outline, and their substructure can be distinguished only with difficulty.

Both aggregated and single filaments are present in vast numbers in the paragonial lumen (Fig. 5). However, such filaments, which measure approximately 600 A in diameter, are considerably thicker than the intravacuolar filaments. The luminal filaments consist of a core of extremely dense material surrounded by a thin layer of electron-lucent material. This outer layer is quite obvious in thin sections (Fig. 5), because the high density of the surrounding luminal content permits visualization of this layer in negative contrast. Aggregates of the luminal filaments show the

same hexagonal packing in cross-section as do those in vacuoles.

Homogenates of paragonia examined by negative staining were found to contain only luminal filaments. These filaments are about 600–630 A in diameter and are extremely long, some measuring more than 30 μ (Fig. 6). They are not rigid rods but instead are extremely flexible. Many of the filaments describe hairpin bends, and some may be entangled or intertwined (Fig. 7). The negatively stained filaments consist of an undetermined number of parallel fibrils (Fig. 8) and give only a faint indication of a longitudinal repeating pattern. At broken ends of filaments, the fibrils do not fray out as they do in disrupted sperm tail microtubules, but they appear to terminate at the level of the breach.

DISCUSSION

In a fine structural study of testes and accessory sex glands of *Drosophila melanogaster*, Bairati (3) found numerous filaments which appear to be morphologically identical with the intravacuolar filaments of *D. paulistorum*. These structures were present within vacuoles in the paragonial cells and also were intermingled with spermatozoa in the genital duct. No filaments corresponding to the luminal filaments of *D. paulistorum* were noted. Bairati suggested that the filamentous structures may be contractile elements concerned with the transfer of sperm along the female reproductive tract. However, no evidence that the filaments possess motility was adduced.

A similar function was ascribed by Acton (1) to an unusual cilium-like process found in the lumina of seminal receptacles of inseminated female *D. melanogaster*. These elongated structures, which measured about 500 A in diameter, were described as having an outer ring of 10 elements and as lacking an encircling membrane. On this basis, they bear a superficial resemblance to the luminal filaments in *D. paulistorum*. However, structures similar to those reported by Acton have been observed in the seminal vesicles of sexually mature male *Gerris remigis* (Tandler, B. Unpublished observations.), and in well-preserved preparations they were seen to be bounded by a unit membrane. These long processes thus are clearly different from the filaments of *D. paulistorum* which lack a limiting membrane.

The nature of the intravacuolar filaments of *D. paulistorum* is not clear. The cytochemical tests reported in this study demonstrated the presence of RNA in these structures. This finding, coupled with their characteristic morphology, suggests that the filaments may be viruses. Although the filaments are extremely long, they are not unique since other viruses or viral components which attain unusual lengths have been described. In insects, a rodlike virus associated with granulosis may be as long as 4.5 μ (calculated from Fig. 4 of reference 21). In mammals, the tubular nucleocapsids of SV5 virus have been estimated to reach 8 μ in length (10). Segments of nucleocapsid measuring 2–3 μ have been isolated from disrupted SV5 virions (7–9). Similarly, the nucleocapsids of WB virus, a myxovirus, may be as long as 1.5 μ (calculated from Fig. 12 of reference 19). The granulosis virus is about 440 A in diameter, the nucleocapsids of both SV5 and WB viruses are about 170 A in diameter, and the *D. paulistorum* intravacuolar filaments are about 280 A in diameter. Each of these structures has a helical substructure. In addition, a recent report by Arnott and Smith (2) describes an RNA plant virus which closely resembles the intravacuolar filaments of *D. paulistorum*. These plant viruses are about 1.4 μ in length, 140–150 A in diameter, and they have a helical substructure. Furthermore, they display hexagonal packing. Unlike the *D. paulistorum* filaments, they are found in both the cytoplasm and nucleus.

The possibility that the *D. paulistorum* filaments are viruses is also supported by the presence, within the vacuoles, of dense, amorphous areas. These areas closely resemble the so-called "factory areas" seen in many virus-infected cells. The filaments appear to be assembled at the periphery of these dense areas.

The relationship of the luminal filaments to the intravacuolar filaments is also unclear. The former are present in the paragonial lumina in vast numbers, often exhibiting a packing arrangement similar to that of the intravacuolar filaments. It seems reasonable to suppose that the luminal filaments are intravacuolar filaments that have been liberated into the lumen. However, no vacuoles were observed discharging their contents, nor were vacuolated cells observed which showed signs of autolysis. In addition, the luminal filaments are twice as large in diameter as the intravacuolar filaments and have a different substructure. For these reasons, it is suggested that the two forms of filaments are unrelated. Since the

paragonial lumina contain high concentrations of mucopolysaccharide, the luminal filaments may represent spontaneous condensations of this material.

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REFERENCES

1. ACTON, A. B. 1966. An unusual ciliumlike process. *J. Cell Biol.* **29**:366.
2. ARNOTT, H. J., and K. M. SMITH. 1968. Electron microscopic observations on the apparent replication *in vivo* of a plant virus. *Virology*. **34**:25.
3. BAIRATI, A. 1966. Filamentous structures in spermatic fluid of *Drosophila melanogaster* Meig. *J. Microscopie.* **5**:265.
4. BJORKMAN, N. 1962. Low magnification electron microscopy in histological work. *Acta Morphol. Neerl. Scand.* **4**:344.
5. BRENNER, S., and R. W. HORNE. 1959. A negative staining method for high resolution microscopy of viruses. *Biochem. Biophys. Acta.* **34**:103.
6. CAULFIELD, J. B. 1957. Effects of varying the vehicle for OsO_4 in tissue fixation. *J. Biophys. Biochem. Cytol.* **3**:827.
7. CHOPPIN, P. W., and W. STOECKENIUS. 1964. The morphology of SV viruses. *Virology*. **23**:195.
8. COMPANS, R. W., and P. W. CHOPPIN. 1967. Isolation and properties of the helical nucleocapsid of the parainfluenza virus SV5. *Proc. Natl. Acad. Sci. U. S.* **57**:949.
9. COMPANS, R. W., and P. W. CHOPPIN. 1967. The length of the helical nucleocapsid of Newcastle disease virus. *Virology*. **33**:344.
10. COMPANS, P. W., K. V. HOLMES, S. DALES, and P. W. CHOPPIN. 1966. An electron microscopic study of moderate and virulent virus-cell interactions of the parainfluenza virus SV5. *Virology*. **30**:411.
11. DOBZHANSKY, T., and O. PAVLOVSKY. 1966. Spontaneous origin of an incipient species in the *Drosophila paulistorum* complex. *Proc. Natl. Acad. Sci. U. S.* **55**:727.
12. DOBZHANSKY, T., and O. PAVLOVSKY. 1967. Experiments on the incipient species of the *Drosophila paulistorum* complex. *Genetics*. **55**:141.
13. EHRMAN, L. 1967. A study of infectious hybrid sterility in *Drosphila paulistorum. Proc. Natl. Acad. Sci. U. S.* **58**:195.
14. EHRMAN, L., and D. L. WILLIAMSON. 1967. Transmission by injection of hybrid sterility to nonhybrid males in *Drosophila paulistorum. Proc. Natl. Acad. Sci. U. S.* **54**:481.
15. ERLANDSON, R. A. 1964. A new Maraglas, D.E.R. 732, embedment for electron microscopy. *J. Cell Biol.* **22**:704.
16. FLAX, H. M., and M. H. HIMES. 1952. Microspectrophotometric analyses of metachromatic staining of nucleic acids. *Physiol. Zool.* **25**:297.
17. LILLY, R. D. 1965. Histopathologic Technic and Practical Histochemistry. McGraw-Hill Book Company, New York. 3rd edition. 272.
18. MILLONIG, G. 1961. A modified procedure for lead staining of thin sections. *J. Biophys. Biochem. Cytol.* **11**:736.
19. PROSE, P. H., S. D. BALK, H. LIEBHABER, and S. KRUGMAN. 1965. Studies of myxovirus recovered from patients with infectious hepatitis. II. Fine structure and electron microscopic demonstration of intracytoplasmic internal component and viral filament formation. *J. Exptl. Med.* **122**:1151.
20. SANDBORN, E., P. H. KOEN, J. D. MCNABB, and G. MOORE. 1964. Cytoplasmic microtubules in mammalian cells. *J. Ultrastruct. Res.* **11**:123.
21. SMITH, K. M., and R. M. BROWN. 1965. A study of the long virus rods associated with insect granuloses. *Virology*. **27**:512.
22. STEMPAK, J. G., and R. T. WARD. 1964. An improved staining method for electron microscopy. *J. Cell Biol.* **22**:697
23. WILLIAMSON, D. L., and L. EHRMAN. 1967. Induction of hybrid sterility in nonhybrid males of *Drosophila paulistorum. Genetics.* **55**:131.