

CYTOLOGICAL EFFECTS OF J2581 (5-ETHOXY-6-ETHOXY-6-[4-METHOXYPHENYL] METHYL-1,3-BENZODIOXOLE) ON SPERMATOGENESIS IN THE ORIENTAL FRUIT FLY, *DACUS DORSALIS* HENDEL

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(Received 11 December 1989)

Abstract—1. Examination by phase-contrast microscopy of testes from J2581-treated flies showed (1) the presence of spermatocytes and the absence of normal meiotic divisions, and (2) abnormal development of spermatids and sperm bundles.

2. Examination by electron microscopy of flagella from spermatozoa of J2581-treated flies revealed abnormal development of axonemes.

3. Possible interference in microtubule assembly by J2581 is discussed.

INTRODUCTION

Topical or oral administration of several methylenedioxy derivatives of substituted benzylphenols (benzyl-1,3-benzodioxoles) (BBDs) to insects resulted in temporary or permanent interference with reproduction (Jurd *et al.*, 1979; Rawlins *et al.*, 1979; Flint *et al.*, 1980; Chang *et al.*, 1980; Langley *et al.*, 1982; Van Mellaert *et al.*, 1983; Chang *et al.*, 1984; Nelson *et al.*, 1985; Song, 1988; Hsu *et al.*, 1989, 1990). The mode of action of BBDs is not known. However, an anti-juvenile hormone function has been proposed (Van Mellaert *et al.*, 1983).

Hsu *et al.* (1990) reported that eggs oviposited by untreated females of the oriental fruit fly, *Dacus dorsalis* Hendel, mated to males exposed as prepupae to a BBD, J2581 (5-ethoxy-6-ethoxy-6-[4-methoxyphenyl]methyl-1,3-benzodioxole) in soil, did not hatch. Further examination revealed that eggs that failed to hatch were not fertilized. In an effort to discover the reason for lack of fertilization, an examination of testes from J2581-treated flies was conducted. This revealed that spermatozoa from J2581-treated flies were immotile while those from acetone controls were fully motile.

We have examined, by phase-contrast microscopy, the spermatogenic stages in testes from *D. dorsalis* treated with J2581. We have further examined flagella from spermatozoa of J2581-treated flies by transmission electron microscopy with special attention to possible interference in spermatid differentiation. The results are presented here.

MATERIALS AND METHODS

Insects

Late third instar *D. dorsalis* larvae were obtained from the mass rearing facilities of the USDA Tropical Fruit and Vegetable Research Laboratory in Honolulu. Prepupae were allowed to pupate in soil containing J2581 (concentration range of 0.83–13.20 mg/g soil) for 1–7 days as described by Hsu *et al.* (1990).

Chemicals

J2581 was synthesized by Dr Leonard Jurd (USDA Western Regional Research Laboratory, Berkeley, CA) and its structure confirmed by elemental (C, H), proton NMR and mass spectral analysis (Jurd *et al.*, 1979). A purity of >99% for J2581 was established by thin-layer chromatography on silica gel and later detection and quantitation by charring.

Preparation of testes for phase-contrast microscopical examination

Within 12 hr after adult eclosion, testes from J2581- or acetone-treated *D. dorsalis* were dissected out into a drop of Ringer's insect saline on a microscope slide and squashed with a coverslip. The preparation was then examined with an AO Reichstar phase-contrast microscope.

Preparation of testes for transmission electron microscopic examination

A slightly modified procedure from Matthews (1981) was followed to prepare the testes for transmission electron microscopic examination. Within 12 hr after adult eclosion, testes from J2581- or acetone-treated *D. dorsalis* were fixed in 2% glutaraldehyde in 0.05 M sodium phosphate buffer, pH 7.2, for 1 hr post-fixed in 1% osmium tetroxide in phosphate buffer for 1 hr, stained with 0.5% aqueous uranyl acetate for 16–20 hr, and rinsed in three changes of distilled water. The samples were then dehydrated in an ethanol series consisting of 20 min intervals in 25, 50, 75, 95 and 100% ethanol. Testes were then cleared with two changes of propylene oxide, 30 min each, and then brought through a series of plastic: propylene oxide mixture (1:2 and then 2:1, 8 hr each) until 100% plastic was reached. Testes were then embedded in Spurr's plastic (a mixture consisting of 26 g nonenyl succinic anhydride, 10 g vinyl cyclohexene dioxide, 4 g Dow epoxy resin 736) and hardened by catalysis with 0.4 g dimethylaminoethanol. The plastic was allowed to polymerize at 70°C for at least 8 hr.

Embedded testes were then sectioned on a Hitachi ultramicrotome equipped with a glass knife. Grid sections of interest 70–100 nm thick were poststained with 2% uranyl acetate at 37°C followed by Reynold's lead citrate. Specimens were then examined with a Hitachi 300 transmission electron microscope at 50 kV.

RESULTS AND DISCUSSION

Treatment of *D. dorsalis* with BBDs affected either males or females depending on the developmental stage of the flies (Hsu *et al.*, 1990). Of particular interest was the finding that males previously exposed as prepupae to J2581 in the soil apparently were unable to fertilize females (Hsu *et al.*, 1990). Combination matings between acetone-treated and untreated males and females resulted in failure of eggs to hatch only if one of the pairs was a BBD-treated male (Hsu *et al.*, 1990).

Phase contrast microscopical examination of testes from acetone-treated flies showed normal development, with cysts from all stages present. The morphology and arrangement of primary

spermatocytes, spermatids (Fig. 1a), and sperm bundles (Fig. 1b) appeared identical to those from untreated controls. However, examination of testes from at least ten J2581-treated flies showed differing degrees of abnormality in sperm development. Sections from the central area of the testis showed the presence of spermatocytes (Fig. 1c). However, spermatocytes undergoing normal meiotic divisions were not seen. Furthermore, spermatid cysts were not observed in the area between the primary spermatocytes and sperm bundles (Fig. 1d). The proximal region of the testis lacked the distinct sperm bundles with sperm heads in close lateral alignment seen in sections from acetone-treated or untreated controls (see Fig. 1b). Instead, randomly dispersed structures resembling

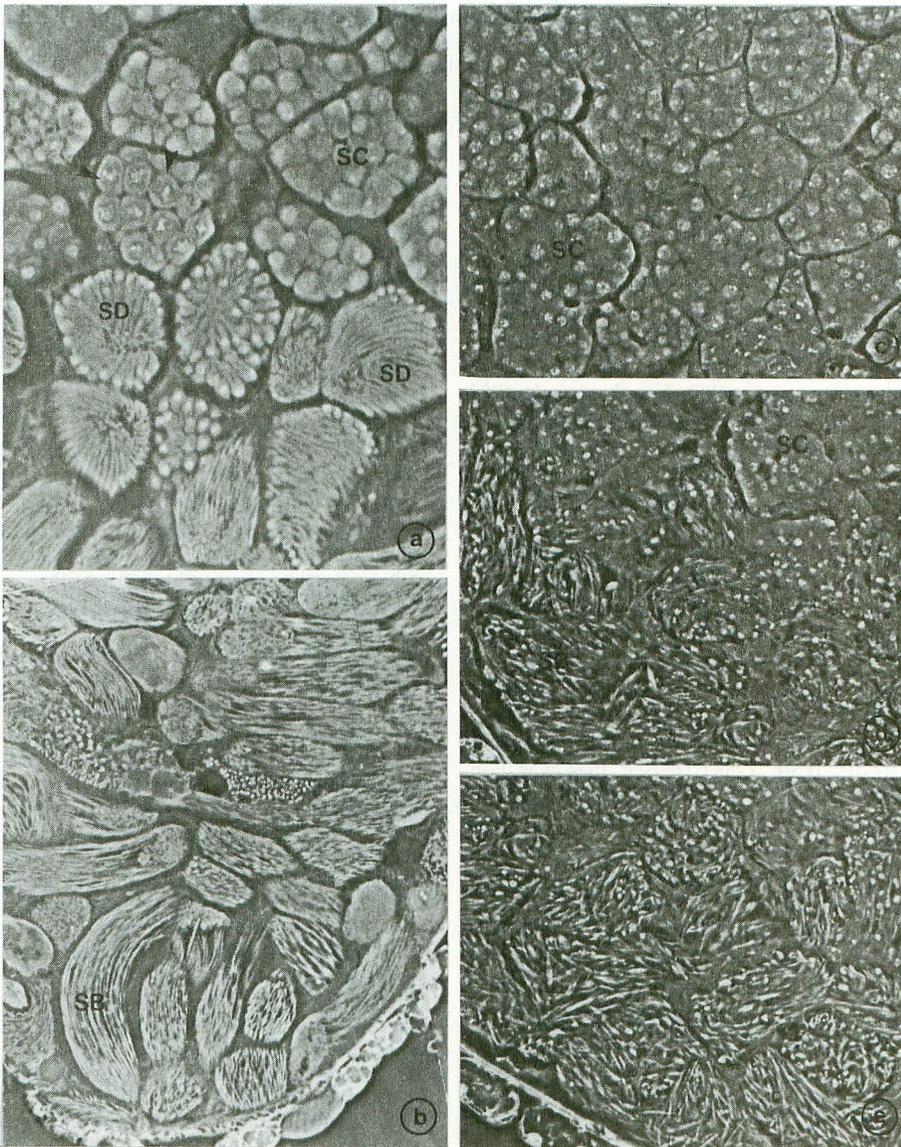


Fig. 1. Phase contrast photomicrographs of the sequential stages of spermatogenesis in testes of *D. dorsalis* treated with acetone only (a-b) or J2581 (c-e). Sections from the central area of the testis showing (a) a primary spermatocyte (SC), spermatids (SD), and a cyst undergoing meiosis (arrows), and (b) elongated sperm bundles (SB) in the proximal region. Other sections showing (c) primary spermatocytes, (d) normal (SC) and aberrant (SB) sperm bundles in the proximal area. (a) $\times 7143$ (b) $\times 7143$ (c) $\times 7143$ (d) $\times 8333$ (e) $\times 8333$.

spermatids, with shortened flagella, were observed (Fig. 1e).

Transverse sections of sperm tails from acetone-treated or untreated *D. dorsalis* revealed a central core consisting of an axoneme with a 9 + 2 array of microtubules (nine doublets surrounding a central pair of singlets) (Fig. 2a). This arrangement is consistent with those seen in cilia and flagella from other eukaryotic organisms (Gibbons, 1981). Associated with each axoneme were two mitochondrial derivatives, each containing an occlusion (Fig. 2a). This occlusion was first described by Andre (1962) and

later termed a paracrystalline body by Phillips (1970). These features, which have been found in several other insects as well (Bawa, 1964; Phillips, 1966; Payne, 1966; Hoage *et al.*, 1968; Pratt, 1970), have been described in detail for *D. dorsalis* by Lee (1982).

Transverse sections of sperm tails from J2581-treated *D. dorsalis* revealed differing degrees of abnormality of axonemes. These consisted of the appearance of more than one axoneme in each flagellum, with or without an abnormally elongated mitochondrial derivative (Fig. 2b), three paracrystalline bodies in an axoneme-associated mitochondrial

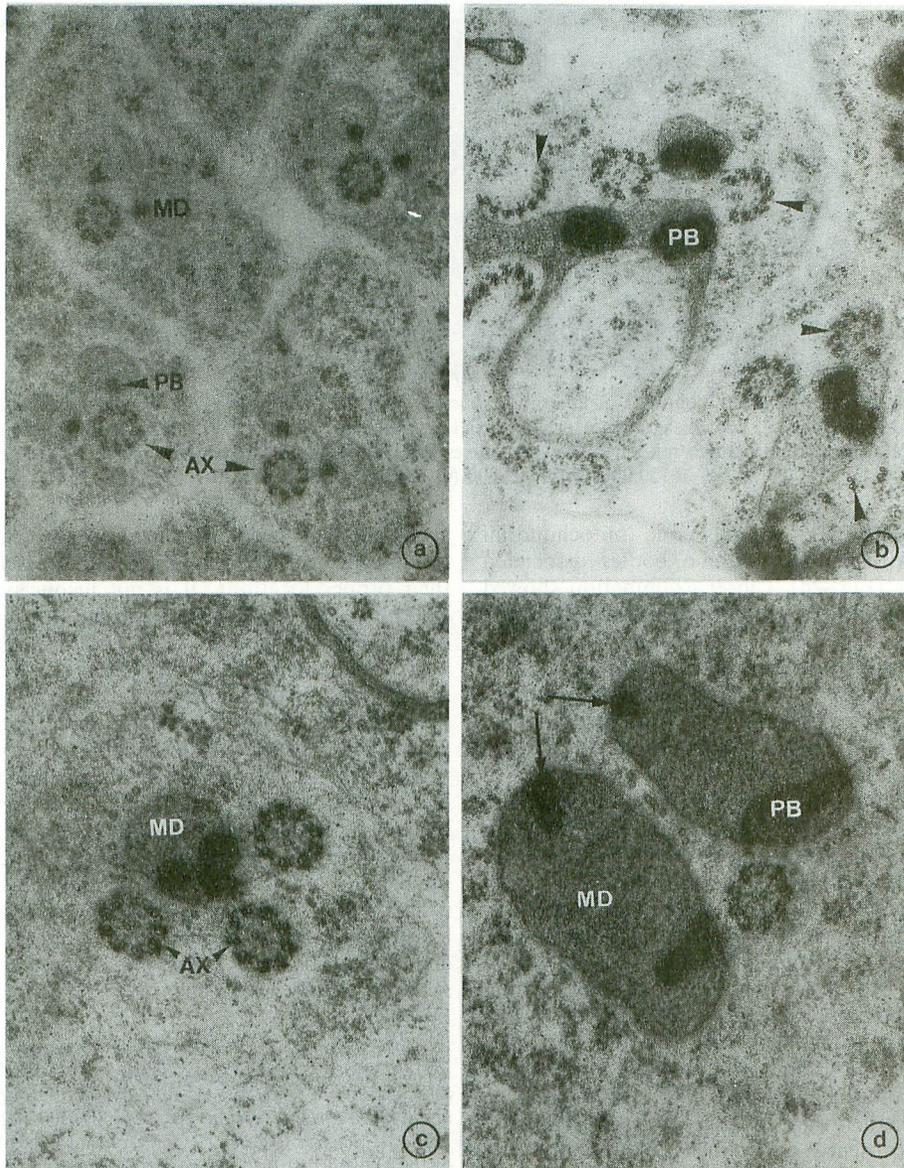


Fig. 2. Electron photomicrographs of transverse sections of sperm tails from *D. dorsalis* treated with acetone only (a) or J2581 (b-d). Section of several sperm tails showing (a) an axoneme (AX) in each tail (with a 9 + 2 array of microtubules) accompanied by a pair of mitochondrial derivatives (MD), each containing a paracrystalline body (PB). Sections showing (b) incomplete axonemes (arrows), a fused mitochondrial derivative containing two paracrystalline bodies (PB), (c) three axonemes (AX) enclosed within a sperm tail accompanied by a mitochondrial derivative (MD) containing three paracrystalline bodies and (d) an axoneme accompanied by two mitochondrial derivatives (MD), each with an extra paracrystalline body (PB). (a) $\times 36,000$ (b) $\times 32,550$ (c) $\times 53,550$ (d) $\times 53,550$.

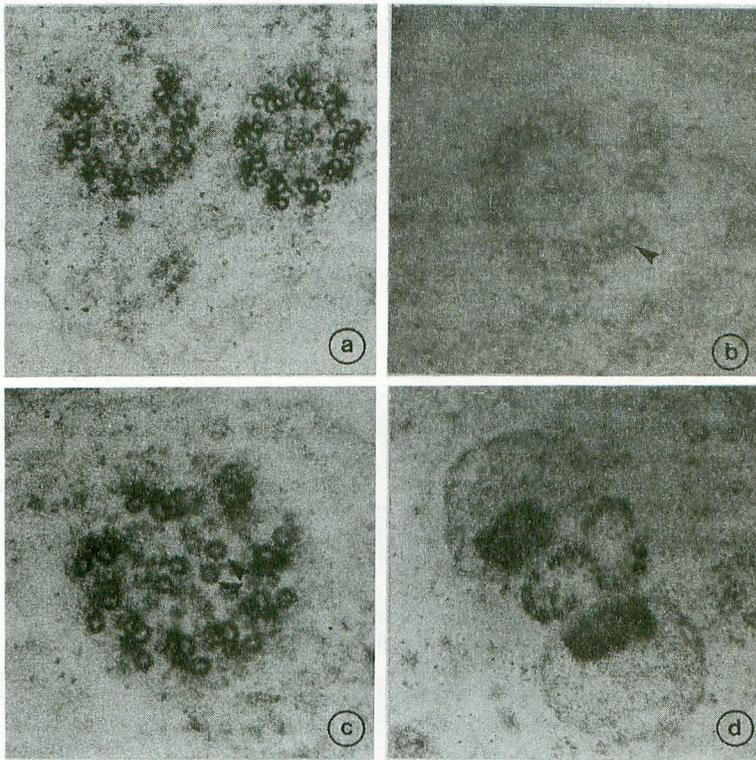


Fig. 3. Electron photomicrographs of aberrant axonemes in sperm tails from J2581-treated *D. dorsalis*. Sections showing (a) broken axonemes or (b) partial loss of outer doublets, (c) more than one pair of central singlets, and (d) two mitochondrial derivatives associated with an incomplete pair of axonemes. (a) $\times 120,000$ (b) $\times 109,000$ (c) $\times 129,600$ (d) $\times 38,570$.

derivative (Fig. 2c), or only one mitochondrial derivative with multi-crystalline bodies associated per axoneme (Fig. 2d). Closer examination showed broken axonemes (Fig. 3a) or missing doublets (Fig. 3b), more than one central pair of singlets associated with the outer doublets (Fig. 3c), or two mitochondrial derivatives associated with a pair of incomplete axonemes (Fig. 3d). The outer dynein arms and radial spokes were observed in some sections (Figs 3b,c).

The "dynein-walking" model, first proposed by Afzelius (1959) and later supported by Satir (1965) in cilia and Gibbons (1975) in sea urchin spermatozoa, has been used to explain the generation of movement in intact flagella and cilia of eukaryotic organisms. This model is based on the making and breaking of cross bridges between adjacent outer doublets, catalyzed by hydrolysis of ATP, and enabling sliding of one doublet past another, effecting movement (Witman and Minervini, 1982). Although outer dynein arms and radial spokes were observed in axonemes from J2581-treated flies, it is difficult to ascertain whether the structures were functionally intact. The absence of doublets or the presence of supernumerary pairs of singlets, as found in axonemes from sperm tails of J2581-treated flies, could prevent movement of the flagellum if the "dynein-walking" model is correct.

The immotile-cilia syndrome, an inborn human (or animal) disorder, characterized by inability of cilia or flagella to beat normally, results in respiratory disease and decreased fertility (Afzelius, 1985). This

syndrome has been attributed to the inability of the dynein arms to be synthesized or assembled on their proper locations. It is not inconceivable that J2581 may interfere with assembly of microtubules during spermiogenesis in *D. dorsalis* leading to aberrant changes in axoneme structure. This would prevent proper attachment of the dynein arms to their proper locations. Furthermore, Batra *et al.* (1984) showed that BBDs inhibited *in vitro* microtubule assembly from purified calf brain tubulin by partially inhibiting tubulin-dependent GTP hydrolysis. BBDs were also shown by these workers to be potent competitive inhibitors of colchicine binding to tubulin. We are presently investigating whether BBDs will also inhibit *in vitro* microtubule assembly from tubulin isolated from *D. dorsalis* embryos which may help to uncover the mode of action of BBDs in insects.

Acknowledgements—This research was supported by the USDA under CSRS Special Grant No. 85-CRSR-2-2652 managed by the Pacific Basin Advisory Group (PBAG) and NSC Research Grant No. 79-0409-B005-02 from the National Science Council (ROC). Journal Series No. 34207 2427 of the Hawaii Institute of Tropical Agriculture and Human Resources.

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