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ANTI-GONADOTROPIC EFFECTS OF BENZYL-1, 3-BENZODIOXOLE ON *DROSOPHILA MELANOGASTER*

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INTRODUCTION

Jurd *et al.* (1979) first reported a new class of insect chemosterilants, benzylphenols and benzyl-1,3-benzodioxoles, obtained by chemical modification of the biologically active constituents of panamanian hardwood, *Daibergia retusa*. The non-mutagenic and low toxicity nature of these chemicals have drawn interests from insect researchers because of their potential for insect control. The chemosterilant activity of benzodioxole series has been found in a number of dipterans and a few other insect species (Table 1).

Van Mellaert *et al.* (1983b) reported that benzodioxole disrupted ovarian development by interfering with the uptake of vitellogenin into ovaries. The anti-gonadotropic effect was attributed to the anti-juvenile hormone action of

Table 1
Chemosterilant activity of benzodioxoles on some insects

Insect species		References
Dipterans		
Face fly	*Musca autumnalis*	Broce *et al.* 1987
Flesh fly	*Sarcophaga bullata*	Van Mellaert *et al.* 1983b
House fly	*Musca domestica*	Jurd *et al.* 1979 Chang *et al.* 1980 Rawlins *et al.* 1982
Mediterranean fruit fly	*Ceratitis capitata*	Chang *et al.* 1984
Yellow fever mosquito	*Aedes aegypti*	Nelson *et al.* 1985
House mosquito	*Culex pipiens*	Readio *et al.* 1987
Screwworm fly	*Cochliomyia hominivorax*	Rawlins *et al.* 1979, 81
Tsetse fly	*Glossina morsitans*	Langley *et al.* 1982, 86
Other insects		
Colorado potato beetle	*Leptinotarsa decemlineata*	Van Mellaert *et al.* 1983a
Pink bollworm	*Pectinophora gossypiella*	Flint *et al.* 1980

benzodioxole. The experimental evidence of anti-JH activity came from the *Galleria* moth bioassy (Van Mellaert *et al.*, 1983c). Those benzodioxoles that had the most effective sterilant activity, also elicited the strongest anti-juvenile hormone reaction in the *Galleria* bioassay. Unfortunately, juvenile hormone replacement therapy failed to prevent sterility in both *Sarcophaga bullata* and *Glossina morsitants* by benzodioxole treatments (Van Mellaert *et al.*, 1983b; Langley *et al.*, 1986). The precise anti-JH action of benzodioxoles was not conclusive.

The role of juvenile hormone in regulating vitellogenesis of *Drosophila melanogaster* has been studied extensively by using genetic mutants (Postlethwait *et al.*, 1973; 1978), by the *in vitro* fat body and ovary assay (Postlethwait *et al.*, 1979), and by the direct measurement of juvenile hormone titers in different developmental stages (Bownes and Rembold, 1987). Therefore, it provides a good model for studying the action of a chemical which is proposed to have anti-juvenile hormone activity.

The objectives of this study were as follow: (1) to evaluate the sterility of benzyl-1,3-benzodioxole J2581 on both sexes of *Drosophila melanogaster*, (2) to check the effects of J2581 on vitellogenin production and uptake, (3) to demonstrate the anti-JH effects of J2581 by hormone replacement therapy, and (4) to evaluate the effects of J2581 on follicular epithelium patency formation.

CHEMOSTERILANT ACTIVITIES OF J2581 IN *DROSOPHILA MELANOGASTER*

LD_{50} and dose-response study

In order to obtain the LD_{50}, newly emerged female adults (0-10 min.) were immobilized in −20°C freezer for 3 min and temporarily held on crushed ice. Each fly was topically treated on the venter of the abdomen with different amounts of J2581 at 16, 8, 4, 2, 1 μg/fly in 0.5 μl of acetone respectively by a microsyringe, and control ones were treated with acetone. Thirty replicates for each treatment were carried out. The LD_{50} of J2581 was obtained from a plot of percent mortality versus log concentration. The LD_{50} of J2581 with topical application on *Drosophila melanogaster* females was 7.25 μg/fly (Fig. 1).

To obtain a dose-response curve on vitellogenesis and ovarian development, newly emerged female adults were treated with different amounts of J2581 within the sublethal range at 4, 2, 1, 0.5 μg/fly in 0.5 μl acetone respectively. Eight replicates for each treatment were carried out. The hemolymph and ovaries of treated females were collected in 50 μl of 0.15 M saline 24 hours after treatment. Individual YP titers in both hemolymph and ovaries were determined by the double antibody sandwich ELISA assay developed by Wu and Ma (1986) using a

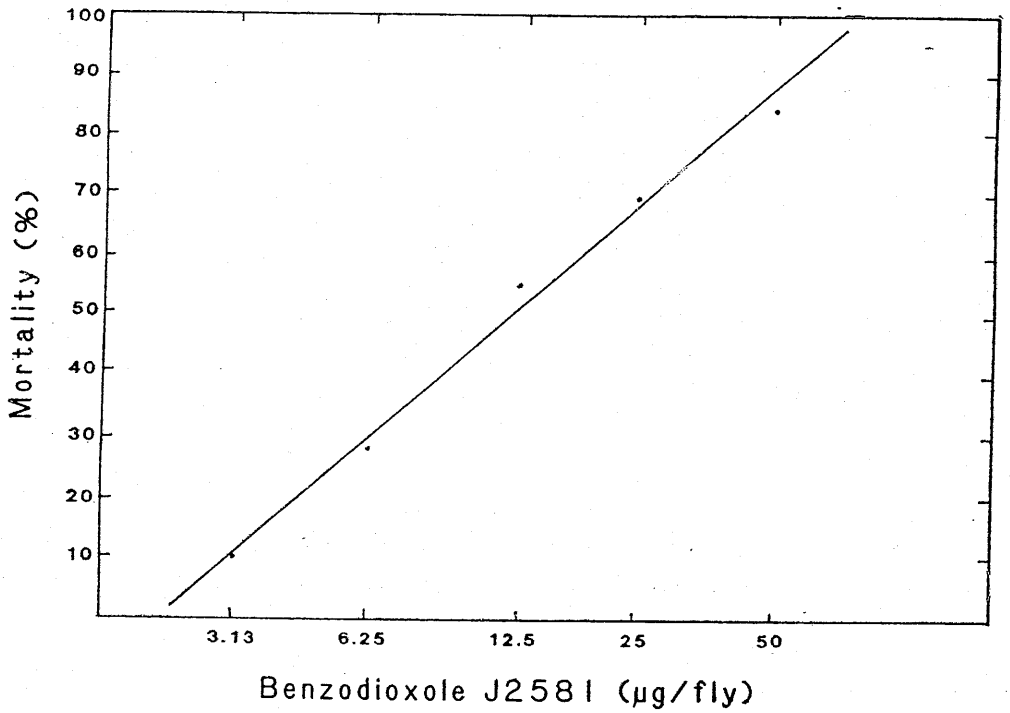

Fig. 1. Adult mortality of *D. melanogaster* following benzyl-1, 3-benzodioxole J2581 treatment. Each point represents thirty adults.

vitellogenin- or vitellin-specific antibody. The sensitivity of this ELISA permitted the design of experiments that required the quantification of vitellogenin in individual hemolymph or ovarian samples. The vitellogenin specific antibody used in the ELISA assay was developed by passing a rabbit antiserum against soluble yolk protein through (1) a protein A column, (2) a CNBr-activated Sepharose 4B column (Sigma, Louis, MO) coupled with whole male body proteins of *Drosophila melanogaster* to remove the crossreacting antibodies, and (3) another affinity column couple with whole body extracts of pharate females to remove all antibodies to adult female proteins except those bound to vitellogenin. The specificity of this antibody preparation was verified by the western blotting analysis (modified from the procedures developed by Towbin *et al.*, 1979 and Burnette, 1981) (Fig. 2).

The results of the dose response study showed that dosages exceeding 1 μg/fly disrupted ovarian development because vitellin was virtually undetected in the ovary samples (Table 2). However, vitellogenin synthesis was apparently not affected since substantial amount of vitellogenin in hemolymph was detected. Based on this dosage response study, 2 μg/fly was chosen to be the standard dosage in all subsequent physiological experiments.

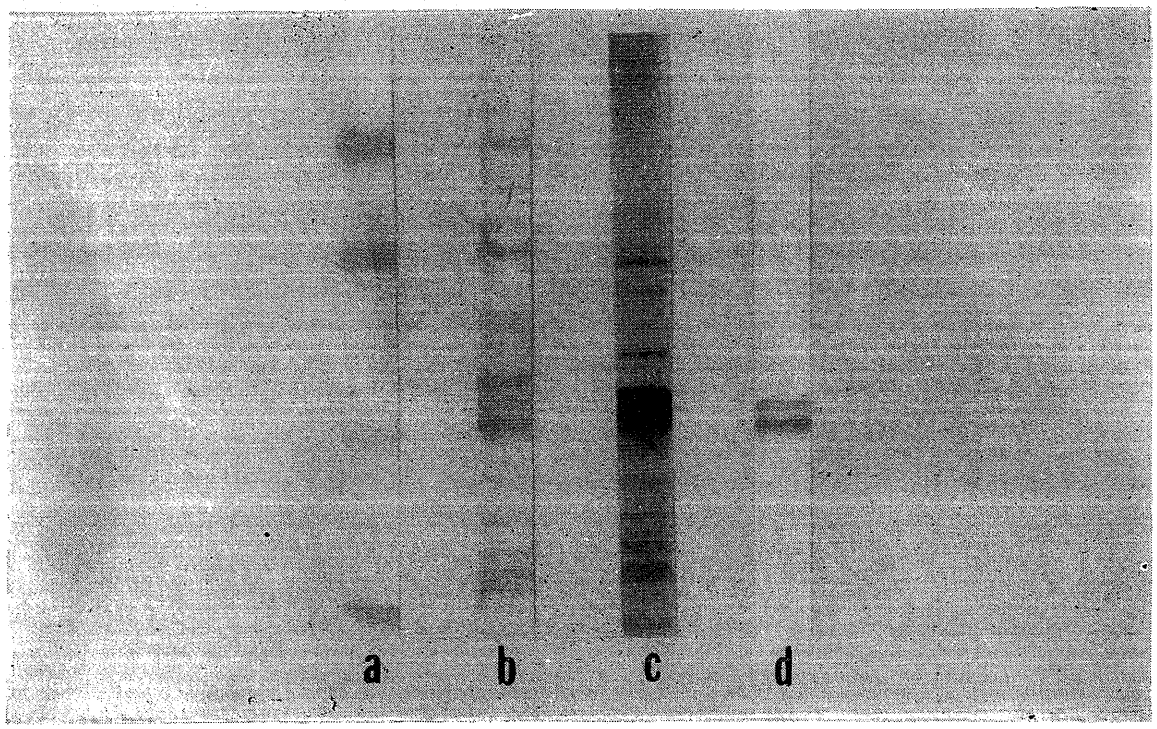

Fig. 2. Western blot analysis of the specificity of the purified antibodies to the three major yolk polypeptides. Amido black staining of (a) molecular weight standards and (b) soluble ovarian proteins of *D. melanogaster*. Binding patterns of (c) unpurified and (d) purified antibodies to soluble ovarian proteins.

Table 2
YP titers in hemolymph and ovaries of *D. melanogaster* 24 h after treatment with J2581

J2581 treatment	YP in hemolymph (ng/fly)	YP in ovary (μg/fly)
Acetone control	180.0± 3.0	9.3±2.6
8.0 μg/fly	3.7± 0.8	0.0±0.0
4.0 μg/fly	6.0± 5.4	0.0±0.0
2.0 μg/fly	340.7±49.9	0.0±0.0
1.0 μg/fly	324.0±30.3	0.0±0.0
0.5 μg/fly	261.2±17.4	8.4±1.1
0.1 μg/fly	195.0±30.0	8.8±1.2

Test of sterility in female and male flies

To investigate the sterilant action of benzodioxole J2581, newly emerged female or male flies (0-10 min old) were topically treated with 2 μg J2581/fly. The

treated females were placed in vials with three untreated males. Fecundity and percent egg hatch for these females were determined daily for fifteen days. Differences in fecundity and fertility between treated and untreated females were compared. Male fertility was determined by placing a treated male with three untreated females in a vial. A male was scored as fertile if larvae were present after 5 days of contact with the females.

The J2581-treated female flies, when crossed with untreated males, were 100% sterile (Table 3) while the acetone-treated females were not affected. No eggs were laid by J2581-treated females fifteen days after treatment compared to the 218±120 eggs laid by the acetone-treated females. Percent egg hatch was 90.8± 6.2%. In the acetone-treated females. As for the males, there was no significant difference in sterility between J2581-treated males (20.0%) and acetone-treated controls (16.1%) (Table 3).

Table 3
Chemosterilant activity of J2581 at 2 μg/fly on each sex of *D. melanogaster* treated topically

Treatment F×M	Number of eggs laid/female (mean+SD)	% egg hatch (mean+SD)	% sterility
J2581 UT	0	0	100.0
Acetone UT	218+120	90.8+6.2	3.3
UT J2581			20.0
UT Acetone			16.1

* UT=Untreated
SD =Standard deviation

Based on these results, it is clear that J2581 only significantly affect the female reproductive system of *Drosophila melanogaster* at the test concentration. These results were also consistent with those obtained by Chang *et al.* (1980) and Rawlins *et al.* (1979, 1982) using *Musca domestica* and *Cochliomyia homonivorax* as test animals respectively.

EFFECTS OF J2581 ON VITELLOGENESIS AND OVARIAN DEVELOPMENT

Effects on vitellogenin production

Vitellogenesis is an important process in insect reproductive physiology including the synthesis, transport and incorporation of yolk protein into the developing oocytes. The disruption of ovarian development after the benzodioxole treatment led to the speculation that benzodioxole may interfere with one of the

above processes. By monitoring the temporal events of vitellogenesis, we should obtain clues as to the specific point of attack by benzodioxole. Newly emerged female adults were treated topically with J2581 at 2 μg per fly and a control group was treated with acetone (0.5 μl/fly). The hemolymph samples were collected at 6 h interval from 0-72 hours after treatment. This method should allow the accurate monitoring of vitellogenin production in both J2581-treated and untreated individual flies.

The results showed no significant differences in hemolymph vitellogenin titers between the treated and control females during the first 24 h [210±92 ng/fly (T), 168±57 ng/fly (C)] (Fig. 3). From 36 h on, vitellogenin titers in the hemolymph of J2581-treated females were significantly higher than those in the controls [248±56 ng/fly (T), 180±45 ng/fly (C)], rising to about two and half times as much as the controls by 72 h [435±64 ng/fly (T), 180±47 ng/fly (C)]. To determine whether this compound could be reversed by the gonadotropic effects of JH, the JH analogue 7S-methoprene was co-applied with J2581 at 0.25 μg/fly to the newly emerged female flies. Vitellogenin titers in the hemolymph of J2581 and 7S-methoprene treated females were restored to normal (Fig. 3). These results demon-

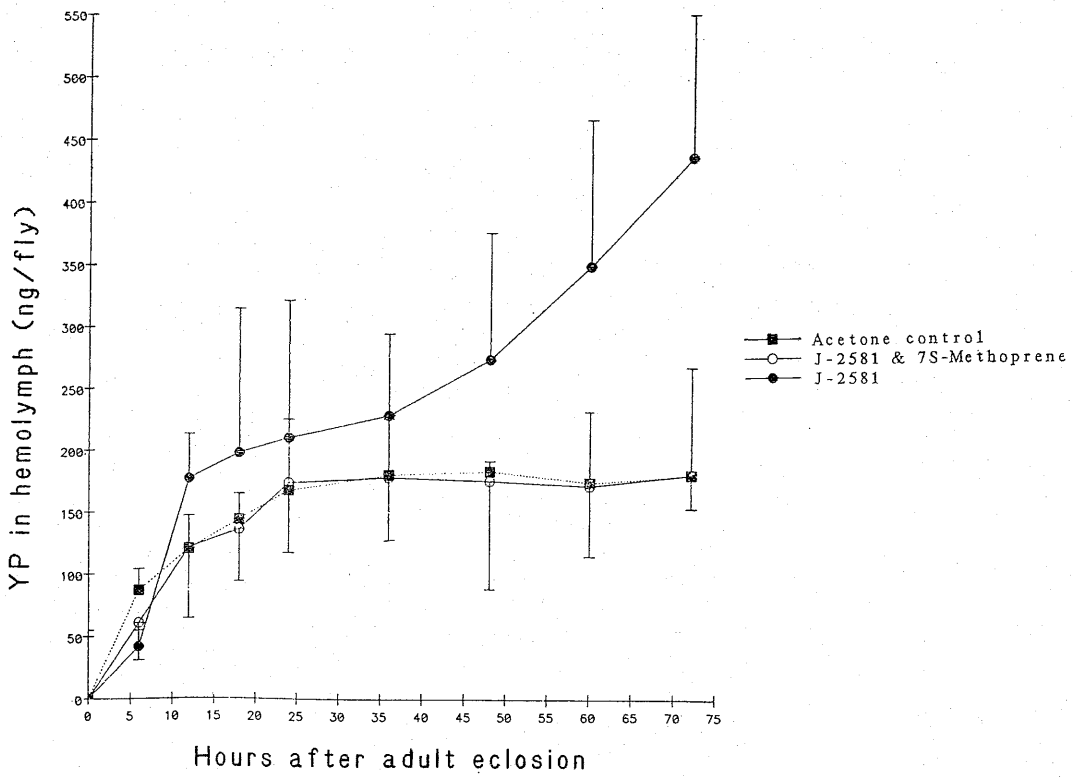

Fig. 3. YP titers in the hemolymph of *D. melanogaster* female adults collected at different times. The females were treated within 0-10 min. post eclosion. Each point represents the mean of eight insect and the represents standard deviations around the mean.

strated that benzodioxole J2581 did not appear to affect vitellogenin production in *Drosophila melanogaster* since vitellogenin continued to accumulate in the hemolymph.

Effect on ovarian development

To investigate the effect of J2581 on vitellogenin uptake and ovarian development, ovarian samples from the same group of flies were collected at 6 h intervals from 0-72 hours after treatment. The amount of vitellin in J2581-treated female flies was around $0.007 \pm 0.003\ \mu g$/fly at 72 h after treatment while $44.2 \pm 1.8\ \mu g$/fly in the control (Fig. 4). When 7S-methoprene was co-applied with J2581, the amount of vitellin in these flies was restored back to normal at around $47.9 \pm 2.9\ \mu g$/fly. The background level of vitellin in the undeveloped ovaries and the accumulation of vitellogenin in the hemolymph of the J2581-treated females strongly suggested that the pathway of vitellogenin from hemolymph into ovaries was disrupted. The restoration of ovarian development by 7S-methoprene was the first suggestion that the action of J2581 was anti-JH.

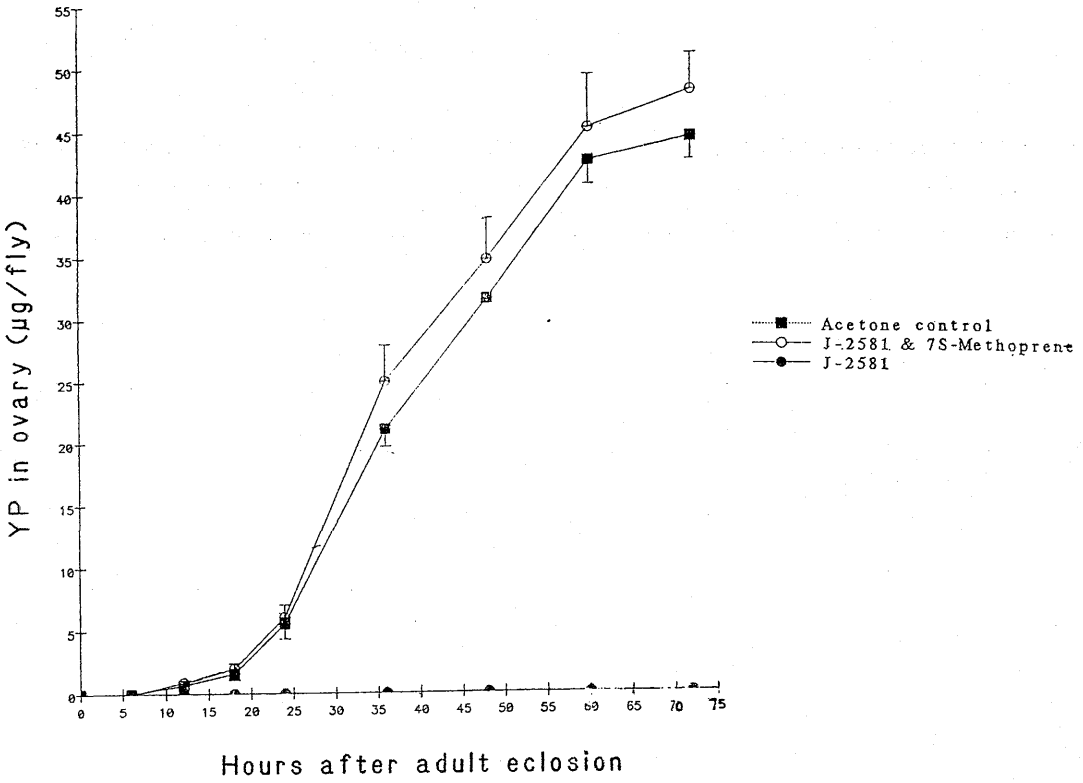

Fig. 4. YP titers in the ovaries of *D. melanogster* female adults collected at different times. The females were treated within 0-10 min. after eclosion. Each point represents the mean of eight insects and the bar represents standard deviation around mean.

The morphological changes of ovaries as a result of different treatments were found to be consistent with the above results (Fig. 5). The J2581-treated ovaries (c) were rudimentary compared to the fully developed (b) and acetone treated (a) ones. The co-application of J2581 with 7S-methoprene (d) restored the ovarian development to its normal size. The lack of vitellogenin internalization appeared to be the main cause of the premature state of the ovaries.

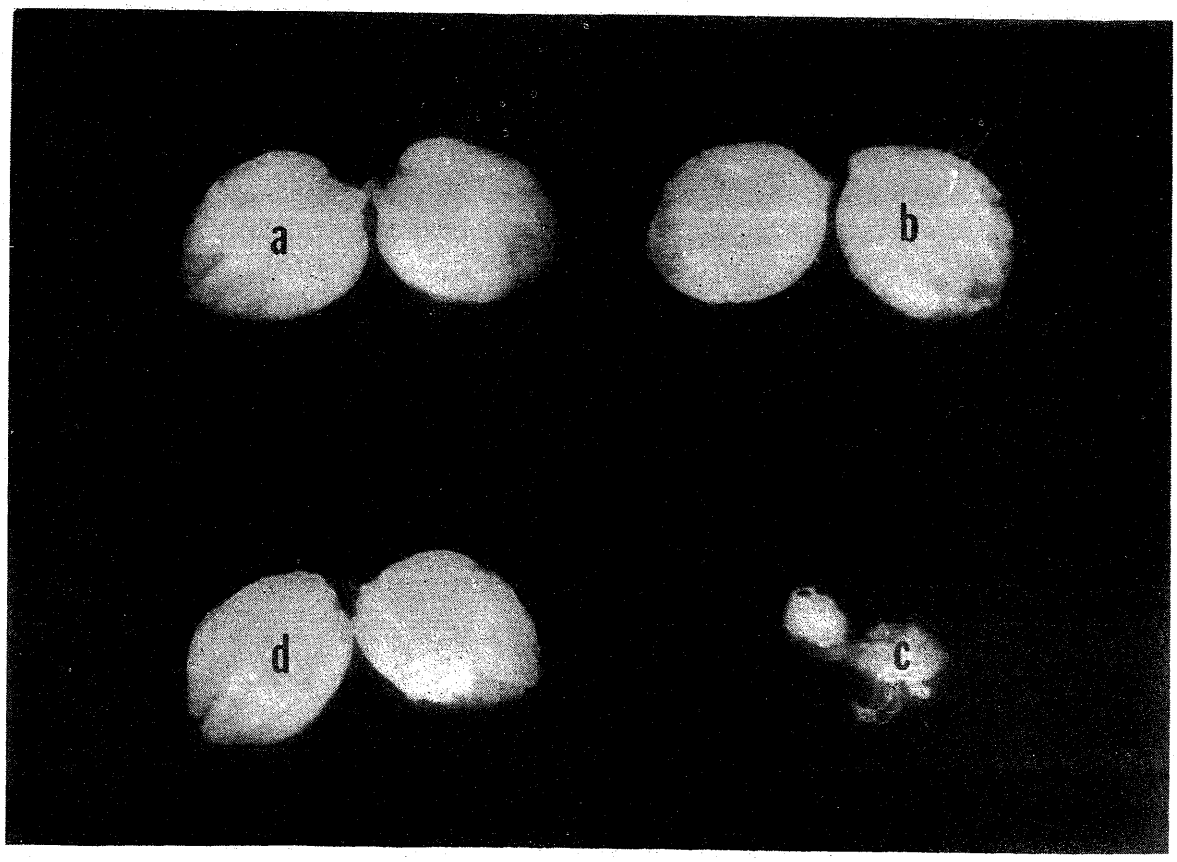

Fig. 5. Ovaries of *D. melanogaster* 72 h after treatment with (a) acetone (0.5 μl/fly), (b) no treatment, (c) J2581 (2 μg/fly) and (d) J2581 and 7S-methoprene (2 μg and 0.25 μg/fly).

Effects on patency

Patency, intercellular spaces between follicular cells, is a phenomenon which permits the entry of vitellogenin from the hemolymph into oocytes (Koeppe *et al.*, 1985). Any abnormality of patency would lead to problems in follicle growth. To investigate the effects of J2581 on the vitellogenin uptake process, ovaries from the treated and control females were collected and patencies of both groups were examined by the immunocytochemical staining of the ovary cryostat sections (Hsu *et al.*, 1981).

The differences in patency between J2581-treated and acetone-treated ovaries were compared by cryostat-sections stained with purified antibodies against vitellin (Fig. 6). No patency was observed in J2581-treated ovaries, and no vitellogenin deposition occured in the oocytes. Although a small amount of vitellogenin deposition was seen in the terminal oocytes, they never reached maturity (Fig. 6a). The space between folliclar epithelial cells (Fig. 6b) clearly showed that patency was formed in the untreated ovary and vitellogenin was internalized in the oocytes.

For ultrastructural examination of the effects of J2581 on patency formation and ovarian development, ovaries from treated and control females were dissected and fixed separately in 2.5% glutaraldehyde in 0.12 M sodium phosphate buffer (pH 7.4) for 3 h at 4°C. They were post-fixed in 1% OsO_4 in the same buffer containing 5.4% glucose, then dehydrated in a series of alcohol and finally embedded in Epon 812. Ultra thin-sections were obtained by cutting with LKB Ultratome I, followed by mounting on copper grids coated with Formvar membrane. The thin sections were stained successively with 2% uranyl acetate for 15 min, and lead citrate for 1 min. Finally, the thin sections were examined with Zesis EM 10 A Transmission Electron Microscope at 80 KV.

Electronmicrographs (Fig. 7) showed detailed difference between the treated and control ovaries. In the follicular epithelial layer, no obviously intercellular spaces between the follicular epithelial cells were observed in the J2581-treated ovaries (Fig. 7a), while well-formed patency occured in the controls (Fig. 7b). Vesicles were found within the follicle cells of the control ovaries which could be vitellogenin, but only a few were observed in the J2581-treated ones. The intercellular space between folliclar epithelial layer and the oocyte was approximately four times wider in the control ovaries than in the treated ones. Within this space, abundant microvilli were only observed in the control ovaries. The most prominent difference was the abundant yolk spheres and fat droplets that occurred in the oocytes of the control ovaries compared to the small number observed in the treated ones.

All the above results indicated that the ovaries from the J2581-treated female flies were nonpatent, resulting in the lack of vitellogenin sequestration from the hemolymph into oocytes. If 7S-methoprene had failed to restore ovarian development, it is possible that J2581 disrupted the internalization of vitellogenin by interfering with vitellogenin binding with its receptors which is vital to the process of endocytotic uptake. Otherwise, the failure of patency formation could be a result of juvenile hormone shortage in the hemolymph caused by J2581 treatment.

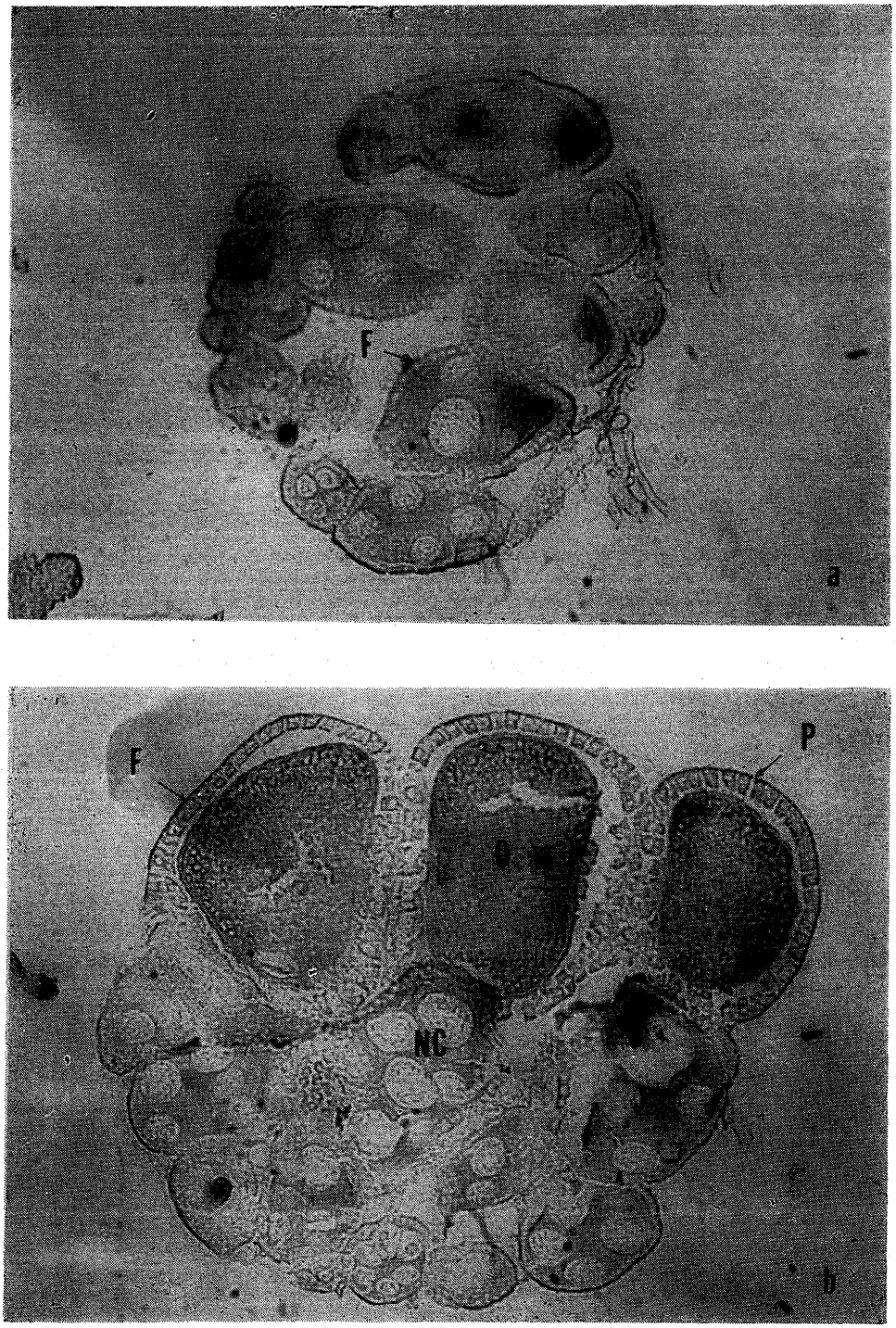

Fig. 6. Immunostained cryostat sections of the ovaries of *D. melanogaster*. YP granules with immunoreactivity are visible within oocytes: (a) ovary from the J2581-treated female and (b) ovary from the acetone-treated control. F: follicle cells. NC: nurse cells. O: oocyte. P: patency.

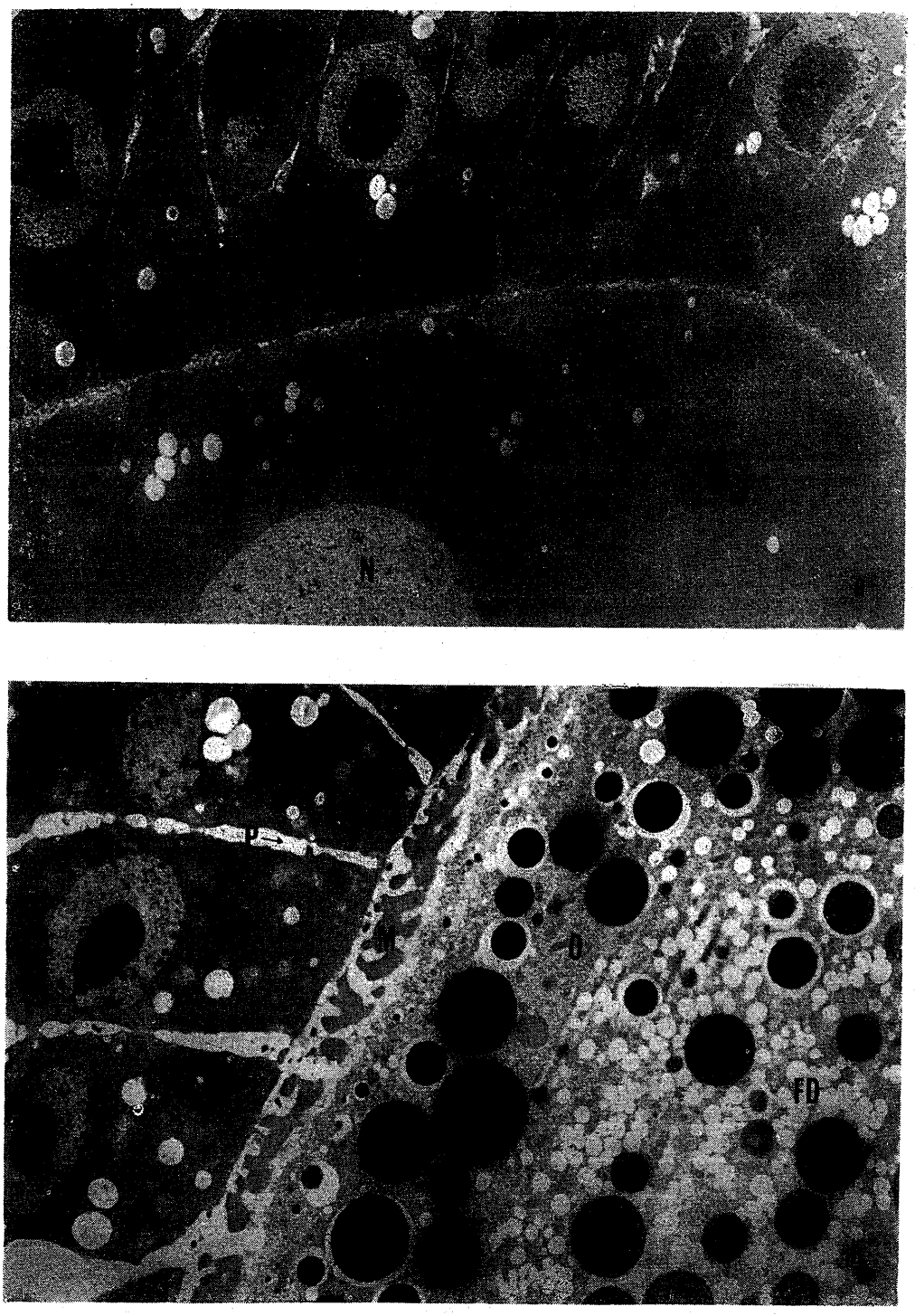

Fig. 7. Electron microscopy sections of the oocytes of *D. melanogaster* from (a) the J2581-treated female and (b) the control one. The difference of the patency and yolk particles between A and B is visible. F: follicle cells. FD: fat droplets. M: microville. N: nucleus. O: oocyte. P: patency. YS: yolk sphere.

Anti-JH action

In *Drosophila melanogaster*, vitellogenin sequestration has been shown to be under juvenile hormone control (Jowett *et al.*, 1980; Postlethwait *et al.*, 1973, 1979, 1981; Wilson *et al.*, 1983; Bownes *et al.*, 1984). Since JH reversed the action of J2581, it was important to understand whether the "anti-JH action" occurred at the site of vitellogenin synthesis, translocation or an anti-gonadotropic effect preventing uptake.

The accumulation of vitellogenin in the hemolymph of treated female flies in the previous studies suggested that J2581 did not interfere with vitellogenin synthesis by the fat bodies. To investigate the other possible target sites, a female decapitation test was carried out following the method developed by Wu and Ma (1987). Newly emerged females (0-10 min.) were decapitated immediately after emergence by severing the neck quickly with a pair of fine point forceps, thus eliminating the source of JH. They lived up to 72 h and frequent wing fluttering behaviors were observed. The ovaries in these decapitated females did not develop and vitellogenin titer in hemolymph declined to background levels at 24 h after decapitation (Wu and Ma, 1987). In our study, the decapitated females were topically treated with J2581 or a combination of J2581 and 7S-methoprene 24 h after the decapitation. Hemolymph and ovarian samples were collected 24 hr after the treatment followed by analysis with the double antibody sandwhich ELISA method.

The result showed that there was no significant difference in hemolymph vitellogenin titers between 7S-methoprene treated females and the ones co-applied with J2581 (Fig. 8). Both 7S-methoprene treated females and the ones co-applied with J2581 restored vitellogenin production. Also, applying J2581 and 7S-methoprene sequentially did not prevent vitellogenin production (Fig. 8). We concluded from these studies that the anti-JH action of J2581 was not targeted at the JH in hemolymph circulation.

No vitellin was detected in the ovaries of J2581-treated and control groups of decapitated females (Fig. 9). However, ovarian development was restored in all others treated with 7S-methoprene, J2581+7S-methoprene, and J2581 followed by 7S-methoprene. There were no significant differences in the amounts of vitellin between the 7S-methoprene-treated (1185±258 ng/fly), J2581±7S-methoprene-treated (1130±210 ng/fly) and J2581 followed by 7S-methoprene-treated (1060±510 ng/fly) groups. It was obvious that ovarian growth in decapitated females after 7S-methoprene treatment was not prevented by J2581, which again strongly suggested that J2581 did not act on the ovary as the target site.

Based on the results of the previous studies, J2581 has no direct action on the fat bodies and ovaries. The possible modes of action of J2581 in this test species

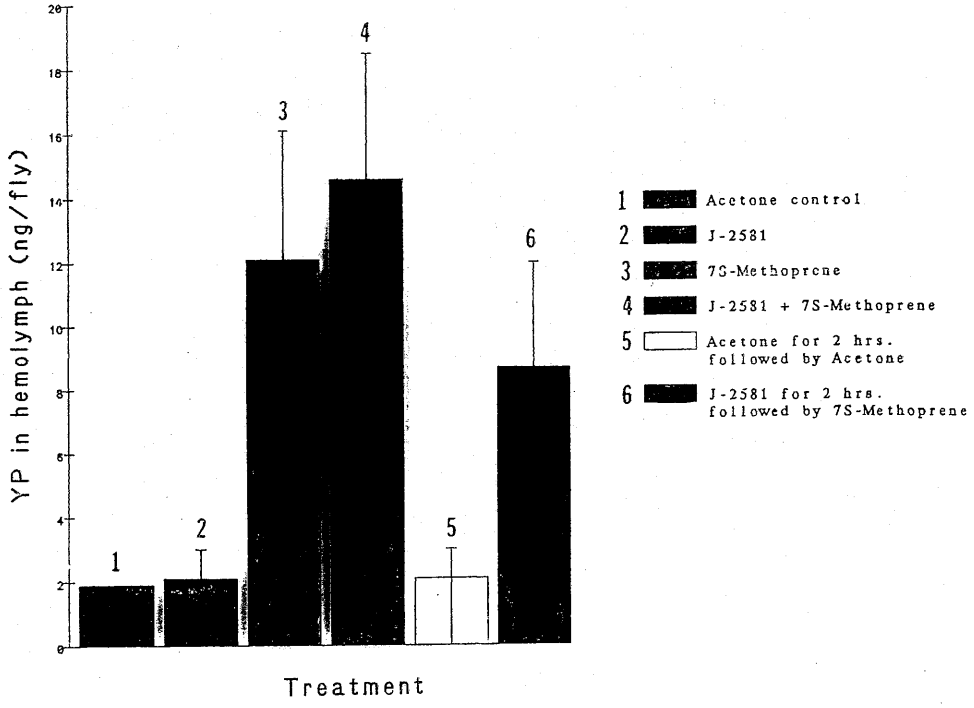

Fig. 8. YP titers in the hemolymph of decapitated female adults of *D. melanogaster* after different treatments.

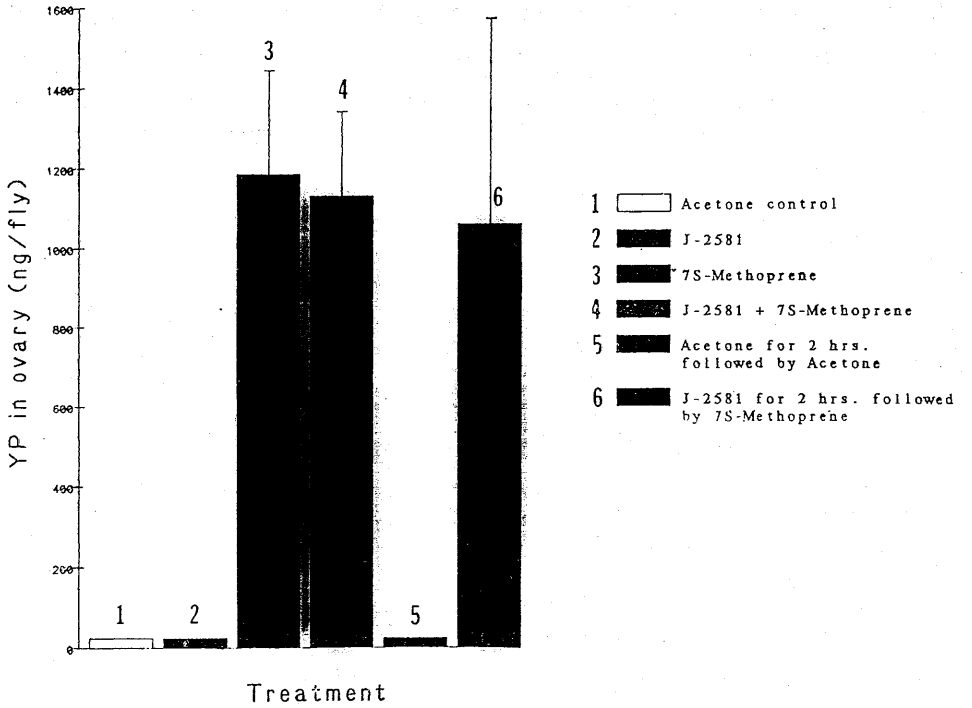

Fig. 9. YP titers in the ovaries of decapitated female adults of *D. melanogaster* after different treatments.

appears to be similar to that of precocenes which are well known as an anti-juvenile hormone agents in insect species, including *Drosophila melanogaster* (Landers *et al.*, 1980; Wilson *et al.*, 1983). Precocenes I and II were applied to the abdomens of intact newly-ecdysed adults of *Drosophila melanogaster* and inspected their ovaries 48 h thereafter. Both precocenes dramatically inhibited oocyte growth compared to the controls. The ovaries of the precocene-treated previtellogenic females also resembled those of the mutants ap^4 homozygotes which were deficient in juvenile hormone for vitellogenesis. Vitellogenin production and ovarian development in these short wing mutants could be restored to normal by treatment with JH-II. Although benzodioxoles and precocenes are different compounds, the similar symptom produced in *Drosophila* have suggested that their anti-JH mechanisms may also be similar. However, the final proof will depend on two future studies, (1) GC-MS measurement of JH to demonstrate the actual lowering of JH titer, (2) studies on the cytotoxic and allatatropic effect of J2581.

CONCLUSION

This paper evaluated the chemosterilant activity of benzodioxole J2581 on *Drosophila melanogaster* by following the normal course of ovarian development, quantification of vitellogenin by ELISA assay, immunocytochemical and electron microscopy techniques. The anti-juvenile hormone activity of J2581 was demonstrated by using the juvenile hormone rescue method. The results of the topical application with J2581 demonstrated that the females were completely sterilized by the compound. Substantial amount of vitellogenin accumulation was detected in the hemolymph of treated females while previtellogenic ovary remained rudimentary suggesting that the uptake of vitellogenin was disrupted. The observations of light and electron microscopy studies revealed that the lack of vitellogenin uptake was due to the failure of patency or the formation of intercellular spaces between follicle cells. The co-application of juvenile hormone analogue 7S-methoprene with J2581 restored ovarian development in this species which strongly suggested that J2581 resulted in the deficiency of circulating juvenile hormone in *Drosophila melanogaster*. Based on the evidence obtained, we suggest that the anti-juvenile hormone activity of J2581 is due to JH shortage, probably a result of the lowered synthesis by the corpora allata.

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