Histopathological effects of an insect growth regulator, 4-phenoxyphenyl \((RS)-2-(2\text{pyridyloxy})\text{propyl ether}\) (pyriproxyfen), on the larvae of \textit{Aedes aegypti}

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Abstract: Histopathological effects of an insect growth regulator, pyriproxyfen, on the larvae of \textit{Aedes aegypti} were studied by treating with concentrations at 1 and 10 ppb for 24 and 48 hr. At the light microscopic level, vacuolation and inhibition of development of imaginal buds in various grades were seen. Electron microscopy revealed the lack of cuticular formation and vacuolation of epidermal cells. In the midgut and Malpighian tubules, disrupted mitochondria, abundant vacuoles and poorly-structured cytoplasmic organelles were observed. These findings suggest possible histolysis of the cells induced by pyriproxyfen.

INTRODUCTION

Pyriproxyfen, as a newly synthesized pyridyl ether compound, has been found to be effective against various insects such as mosquitoes, flies, tea scale and cockroaches (Cooper and Oetting, 1985; Estrada and Mulla, 1986; Mulla et al., 1986; Hatakoshi et al., 1987; Kawada et al., 1987, 1988, 1989; Langley et al., 1988). On the mosquitoes, it has been reported that pyriproxyfen inhibited adult emergence of \textit{Aedes aegypti}, \textit{Culex tarsalis}, \textit{Culex pipiens} pallens and \textit{Anopheles stephensi}. However, its effects on the tissue level have never been tested in any insect.

The present study intends to determine the histopathological effects of pyriproxyfen on the larvae of \textit{Aedes aegypti}, with special emphasis on their cuticle, fat bodies, midgut cells and Malpighian tubules. Imaginal buds in the thoracic region were also examined, since several morphological aberrations were reported on the pupae and adults of mosquitoes whose larvae were treated with juvenile hormone analogues (Arias and Mulla, 1975; Kawamoto et al., 1979; Awad and Mulla, 1984a, b).

MATERIALS AND METHODS

Third and fourth instar larvae of \textit{Ae. aegypti}, Surabaya strain, which were continuously reared in our laboratory were used. Fourth instar larvae were divided into four groups based on maturity of their compound eyes: early, middle, late and mature stages (Spielman and William, 1966). Each group containing 20 larvae was maintained in a polyethylene container \((30\times30\times15\text{cm})\) at \(24\pm1\text{C}\) and 60% RH, filled with 3 liters
of deionized water and pyriproxyfen at 1 or 10 ppb concentrations. All groups were fed on baby food powder containing liver extract and vegetables. Pyriproxyfen (purity, 97.2%) obtained from Sumitomo Chemical Co., was diluted with absolute ethanol to achieve proper concentrations. Control groups were given appropriate drops of ethanol.

**Light microscopy.** Both control and treated groups at 24 and 48 hr after treatment were fixed in Bouin’s solution for overnight, and rinsed in 70% ethanol three times before dehydration. In all groups only live larvae were fixed. They were embedded in paraffin. Cross sections (3-5 μm in thickness) were stained with haematoxylin-eosin.

Imaginal buds and fat bodies of all groups were taken from the thoracic region, which was removed carefully from the larvae after fixation, and serial sections were cut longitudinally from the dorsum in each group.

**Electron microscopy.** Control and treated groups were dissected in a drop of 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4). The sixth and seventh abdominal segments, midgut and Malpighian tubules were removed carefully and transferred to the fresh fixative. They were fixed overnight at 4°C, rinsed in the same buffer three times, and post-fixed in 1% osmium tetroxide for 2 hr. After dehydration, they were embedded in Spurr's resin. Thin sections were cut by a diamond knife on an LKB ultramicrotome.

Figs. 1–4 Light micrographs of imaginal buds of the larvae of *Aedes aegypti.*
1, 2: fourth instar larvae of control group. Both hypodermal cells are fusing with each other (arrow). 3: a mature fourth instar larva treated with pyriproxyfen 10 ppb for 48 hr. Bud of the wing appears in vacuolated form (arrow). In the bottom, a bud of leg does not appear to develop. 4: a mature fourth instar larva treated with pyriproxyfen 1 ppb at 48 hr. A bud of leg exhibits vacuolation. c, cuticle; d, diverticle; fb, fat bodies; h, imaginal bud of haltere; l, imaginal bud of leg; w, imaginal bud of wing. Bar: 10 μm.
mounted on copper grids and stained with uranyl acetate and lead citrate.

RESULTS

In this study, all of the third instar larvae in the control groups normally molted to fourth instar larvae. On the other hand, several fourth instar larvae in the treated groups died and failed to develop into pupae.

1. Imaginal bud

Sections of the thoracic region in the control groups revealed the buds of pupal siphon, wings, and halteres located in the dorsal part beneath the integument. In the ventral part, three pairs of buds of legs occupied the pro-, meso- and metathorax (Fig. 1). The buds of wings had already exhibited wing-like structure. The upper and lower hypodermal cells already fused with each other. Sections of the third and various stages of fourth instar larvae clearly demonstrated developmental sequences of the buds (Fig. 2). All of the buds seemed to be in the developing stage and full specialization appeared being undertaken by the bud of the wing.

In the treated groups, some morphological changes were noticed, particularly those treated for 48 hr. At 24 hr treatment, no significant morphological changes were found. Fourth instar larvae treated with pyriproxyfen 10 ppb for 48 hr revealed the lack of fusion of hypodermal cells, and...
vacuolation of imaginal buds (Fig. 3). On the other hand, in the larvae treated with 1 ppb for 48 hr, imaginal buds were seen to fail to undertake further specialization (Fig. 4).

2. Cuticle

Sections of 24 hr old third instar larvae of the control group varied in deposition of newly-formed cuticle. Some revealed only thin and electron-dense cuticulin layer, and others displayed already thickened epicuticle (Fig. 5). Old cuticle appeared to have been detached from the newly-deposited cuticle. Epidermal cells exhibited high activities as evidenced by cell membrane processes and a large amount of rough endoplasmic reticula in cytoplasm. Mitochondria were mostly located in the apical part, while Golgi bodies were found in the basal part of the cell adjacent to the nucleus. After 48 hr, old cuticles were found completely shed and newly-formed cuticle had been completed.

Fourth instar larvae of the control group which were examined after 24 hr exhibited an electron-dense and thin epicuticle (Fig. 6). A disoriented cement was detached from the epicuticle. Exocuticle apparently possessed a coarse, granular texture, and consisted of two or three lamellae. Endocuticle had a light and fine texture and consisted of four or five lamellae in which pore canals were seen. Cytoplasm was densely packed with organelles such as mitochondria, ribo-

Figs. 9–12. Electron micrographs of the midgut and Malpighian tubules of the larvae of *Aedes aegypti*.

9: midgut cell of the control group. 10: midgut cell after treatment at 10 ppb for 48 hr. Several mitochondria appear disrupted and distended, and vacuoles were scattered throughout cytoplasm. 11: Malpighian tubules of the control group. Protoplastic processes contain mitochondria with their cristae running mostly parallel to the process (arrow). 12: Malpighian tubules after treatment with pyriproxyfen at 10 ppb for 48 hr. Disrupted mitochondria are seen in protoplasmic processes and cytoplasm (arrow), cm, cell membrane; dm, disrupted mitochondria; er, rough endoplasmic reticulum; g, granules; gb, Golgi bodies; m, mitochondria; mv, microvilli; va, vacuole. Bar: 1 μm.
somes, smooth and distinct endoplasmic reticula, and a small and distinct membrane-bounded vacuoles. Other sections from 48 hr displayed similar morphological structure in their cuticle. In the treated groups, several morphological changes were noted. In the third instar larvae treated with 10 ppb pyriproxyfen for 24 hr, disrupted mitochondria and large vacuoles containing less electron-dense material were seen in cytoplasm (Fig. 7). The other organelles were well preserved. Old cuticle had already detached from the newly-formed cuticle. At 48 hr, third instar larvae were found to have failed to produce newly and well-structured fourth instar cuticle, and its cytoplasmic organelles were either vacuolated or poorly-structured. Third instar larvae treated with pyriproxyfen at 1 ppb for 48 hr exhibited similar morphological changes, however, those treated with 1 ppb for 24 hr exhibited no noticeable changes.

Fourth instar larvae continuously treated with pyriproxyfen 10 ppb for 48 hr displayed smooth epicuticle which partially detached from the underlying exocuticle (Fig. 8). In the endocuticle, some densely-stained material was deposited, and adjacent lamellae were disorganized and partially digested. In the ecdysial space, some densely-stained material occupied the borderline of the outer cell membrane. Cytoplasm exhibited such severe vacuolation that no cytoplasmic organelles could be identified. Those treated with 10 ppb for 24 hr and 1 ppb for 48 hr exhibited a disrupted mitochondria and other poorly-structured cytoplasmic organelles. No noticeable morphological changes were found in those treated with 1 ppb for 24 hr.

3. Fat bodies

Fat bodies in the thoracic region were found just beneath the epidermal layer, however, no significant morphological changes could be seen in either light or electron microscopic levels in the treated groups.

4. Midgut

Light microscopy of the sixth and seventh abdominal segment in both control and treated groups revealed midgut cells in the posterior one-third part. Each cell exhibited a single epithelial layer with typical microvilli. Nucleus was located in either the center or basal part of the cell. Endocrine cells resting on the basement membrane corresponded to the lumen. Both 1 ppb and 10 ppb groups appeared to have cell-damage in the form of disintegration of the cell membrane and cytoplasm. However, detailed elaboration of morphological changes could not be made.

Electron microscopy of the third and fourth instar larvae of the control group revealed similar histological structure. The apical part of midgut cell was densely packed with numerous mitochondria, frequently accompanying several small vacuoles (Fig. 9). Tubular-shaped microvilli containing less electron-dense microfilaments extended to the lumen. Smooth and rough endoplasmic reticula, and microtubules were seen throughout the cell. In some sections, degenerative midgut cells with fragmented microvilli were observed nearly detached into the lumen.

The third and fourth instar larvae treated with pyriproxyfen 1 and 10 ppb for 24 hr revealed no significant morphological changes of their midgut cells. In 48-hr treatment, however, disrupted mitochondria and large membrane bounded vacuoles were abundant in the apical part of cytoplasm (Fig. 10). Furthermore, other poorly-structured cytoplasmic organelles were also observed. Those changes were apparently severer in 10 ppb treatments for 48 hr. So far, microvilli have been found structurally similar to those in the control group, and in some sections, degenerated cells with fragmented form were also observed.

5. Malpighian tubules

Sections of sixth or seventh abdominal segment of the third and fourth instar larvae exhibited the posterior one-third part of the tubules and 2 or 3 relatively large epithelial cells resting on a thin basement membrane. Basal, intermediate and apical zones of each cell displayed a characteristic structural appearance.

In the apical zone which formed the brush border, a multitude of vertically arranged protoplasmic processes were seen. Each process contained a mitochondrion with its cristae running mostly parallel to the process (Fig. 11). In the intermediate zone, nucleus,
rough endoplasmic reticulum, Golgi bodies and several lime granules were seen. In the basal part, several mitochondria and double membrane vacuoles were located.

The third and fourth instar larvae which were treated with pyriproxyfen 1 and 10 ppb for 24 hr did not demonstrate noticeable morphological changes. However, after treatment for 48 hr, several large membrane bounded vesicles predominated the cell (Fig. 12). In protoplasmic processes, mitochondria appeared disrupted or distended and their cristae were evacuated. Several electron-dense granules were observed in the apical part adjacent to mitochondria. Basal part of the cells was so severely damaged that no cytoplasmic organelles could be identified.

**Discussion**

The histological effects of insect growth regulating compounds have been reported by many researchers on various kinds of insects (Radford and Misch, 1971; Kimura et al., 1974; Sedlak and Gilbert, 1975, 1976; Das and Gupta, 1977; Cocke et al., 1979; Kawamoto et al., 1979; Deb and Chakravorty, 1981). To date, the effects of IGRs on imaginal buds in either light or electron microscopic levels have never been revealed, except the study on Dimilin (Kawamoto et al., 1979). Awad and Mulla (1984b) reported no morphological changes on the hypodermis of the *Culex quinquefasciatus* after the treatment of cyromazine. In our study, the developmental sequences of the imaginal buds in the control group are in line with those described by Chapman (1975). In the treated groups, vacuolation of the imaginal buds as well as the lack of further specialization was revealed. These results suggest that pyriproxyfen may interfere with the developmental inhibition of the buds or directly cause cell vacuolation.

The cuticular formation in the third instar larvae of the control and treated groups was demonstrated in this study. In the control group, within 24 hr, the newly-formed cuticle was already deposited and epidermal cells exhibited high activities as evidenced by cell membrane processes, and large amounts of rough endoplasmic reticula and glycogen granules. In the treated groups, several disruped mitochondria and vacuoles were frequently seen. In addition, those treated with 10 ppb for 48 hr failed to produce a well-structured fourth instar larval cuticle and its epidermal cells were severely vacuolated. On the fourth instar larvae, the cuticle seemed so severely damaged that the epicuticle partially detached and the endocuticle was partially digested. Its epidermal cells were so severely vacuolated that no cytoplasmic organelles could be identified. These results suggest that pyriproxyfen may act to maintain the larval form of the cuticle and to prevent epidermal cells from producing newly well-structured cuticle which normally occurs during larval development. Cocke et al. (1979) tested two kinds of IGRs: methoprene and 5-((5-(dimethylamino)-1-naphtalenyl)sulfonyl)amino)-1,3-benzodioxole (DNSAB) in the larvae of *Ae. aegypti* and reported similar histopathological effects, though they treated with higher concentrations and for longer periods. Likewise Kawamoto et al. (1979) tested Dimilin (PH60-40) to the larvae of *Cx. pipiens* at the concentrations 50 and 100 ppb. In addition, they found the deposition of densely-stained granules and a decrease in lipid granules in the fat cells probably because of the disturbance of the lipid metabolism. In this study, the effects of pyriproxyfen on the fat bodies could not be seen. Nevertheless, at higher concentrations, it should not be discounted. Sedlak (1984) summarized that epidermis as a major target for both kinds of hormones, 20 hydroxyecdysone (20 HE) and juvenile hormone, responded with specific cellular changes. In *Hyalophora cecropia*, exogenous application of 20 HE accelerates normal development, including both cuticle and cell restructuring, whereas, an excess of juvenile hormone retards or prevents the differentiation by inhibiting the formations of autophagic vacuoles necessary for cell restructuring.

In the midgut and Malpighian tubules, disrupted mitochondria and vacuolation as well as poorly-structured cytoplasmic organelles were frequently seen. Fragmentation of the microvilli in our study was observed in both control and treated groups, and associated with degenerated midgut cells. These findings are not in accordance with
Cocke et al. (1979) which determined those fragmented microvilli as an effect of the insect growth regulator. Radford and Misch (1971) found that secondary lysosomes in the midgut cells of flesh-fly Sarcophaga bullata increased after injection of ecdysterone, and these lysosomes were normally associated with histolysis. The abundance of membrane-bounded vacuoles as well as poorly-structured cytoplasmic organelles, in the midgut and Malpighian tubules of the treated groups, therefore, may be suggested as a part of histolysis in mosquitoes in this study.

In conclusion, histopathological effects, including lethal effects, could be obtained by using low concentration of pyriproxyfen. It seems that concentrations and duration of treatment correlate with the severity of histological effects.

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References


