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Ultrastructural Effects of *Bacillus thuringiensis* var. *san diego* on Midgut Cells of the Cottonwood Leaf Beetle[1]

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Sequential observations of the ultrastructural effects of *Bacillus thuringiensis* var. *san diego* were made on midgut epithelial cells of the cottonwood leaf beetle, *Chrysomela scripta* F. Larvae imbibed a droplet of *B. thuringiensis* var. *san diego* containing δ-endotoxin and live spores. Sections of midgut were prepared for transmission electron microscopy at 15-min intervals during the first hour and then hourly for a duration of 3 hr. No signs of cellular disruption were observed until 2 hr after treatment. At this time midgut epithelial cells from treated larvae appeared more elongate and swollen than cells observed from control larvae, with the apical region of the cell bulging into the gut lumen. The cells contained large cytoplasmic spaces, expansion of the basal labyrinth, disruption of rough endoplasmic reticulum, and apical displacement of nuclei. No adverse effects were observed on microvilli, membranes, or organelles. After 3 hr cellular damage had increased, with cells ruptured at the apical surface and leakage of cytoplasmic materials into the gut lumen. *B. thuringiensis* var. *san diego* proliferated in the midgut lumen, producing additional δ-endotoxin crystals during sporulation. Although cellular swelling caused by *B. thuringiensis* var. *san diego* δ-endotoxin is similar to that reported for *B. thuringiensis* var. *kurstaki* δ-endotoxin in lepidopterans, some differences were observed. These differences included lack of membrane lesions and microvillar damage and comparatively slow response.

KEY WORDS: ***Bacillus thuringiensis* var. *san diego*; *Chrysomela scripta*; cottonwood leaf beetle; δ-endotoxin; ultrastructure; midgut epithelium.**

INTRODUCTION

Bacillus thuringiensis, aerobic soil-dwelling bacteria, produce insecticidal proteins during sporulation. The primary toxin is an alkaline-soluble crystalline protein known as δ-endotoxin. *B. thuringiensis* var. *san diego* is an isolate that is toxic to several species of Coleoptera, including the cottonwood leaf beetle, *Chrysomela scripta* F. (Coleoptera: Chrysomelidae) (Bauer, 1990). The nucleotide sequence of the gene encoding for *B. thuringiensis* var. *san diego* δ-endotoxin is identical to that of *B. thuringiensis* var. *tenebrionis* and *B. thuringiensis* var. EG2158, which are other coleopteran-toxic isolates (Herrnstadt *et al.*, 1987; Krieg *et al.*, 1987; Huger and Krieg, 1989; Donovan *et al.*, 1988). The insecticidal activity of these isolates in susceptible coleopterans results from the interaction of δ-endotoxin, an 64- to 68-kDa polypeptide (Herrnstadt *et al.*, 1986; Bernhard, 1986; Carroll *et al.*, 1989), with the midgut epithelial cells (Krieg *et al.*, 1983, 1984).

Much research has been done on the ultrastructural changes observed in the lepidopteran midgut epithelium after ingestion of δ-endotoxin (Ebersold *et al.*, 1977; Endo and Nishiitsutsuji-Uwo, 1980; Percy and Fast, 1983; Lane *et al.*, 1989). These studies show that δ-endotoxin causes swelling of the epithelial cells, disruption of microvilli, and cell lysis and sloughing; death results from gut lesions and hemolymph ion imbalance, followed by bacterial septicemia (Fast, 1981).

The overt symptomology of *B. thuringiensis* var. *san diego* intoxication in coleopterans is similar to that described for lepidopteran-toxic isolates (Krieg *et al.*, 1983); however, the effects at the ultrastructural level were not investigated. Differences in solubility (Koller *et al.*, 1992) and enzymatic activation of *B. thuringiensis* var. *san diego* δ-endotoxin (Bernhard, 1986; Herrn-

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stadt *et al.*, 1986; Carroll *et al.*, 1989), as well as an acidic midgut environment (Murdock *et al.*, 1987; Bauer *et al.*, 1990), suggest that mechanisms of action could differ.

We conducted a study to observe the dynamic ultrastructural changes in coleopteran midgut epithelial cells following ingestion of *B. thuringiensis* var. *san diego*. These findings will improve our understanding of *B. thuringiensis* var. *san diego* mode of action by describing the morphological events that occur during intoxication.

MATERIALS AND METHODS

B. thuringiensis var. san diego Spore/Crystal Complex and Purified δ-Endotoxin

A live preparation of *B. thuringiensis* var. *san diego* containing spores and native crystals was obtained from Dr. W. D. Gelernter (Mycogen Corp., San Diego, CA). δ-Endotoxin was quantified using laser densitometry scanning of SDS–polyacrilamide gels (Brussock and Currier, 1990). The stock suspension was diluted in sterile distilled water to the desired concentration for treatment.

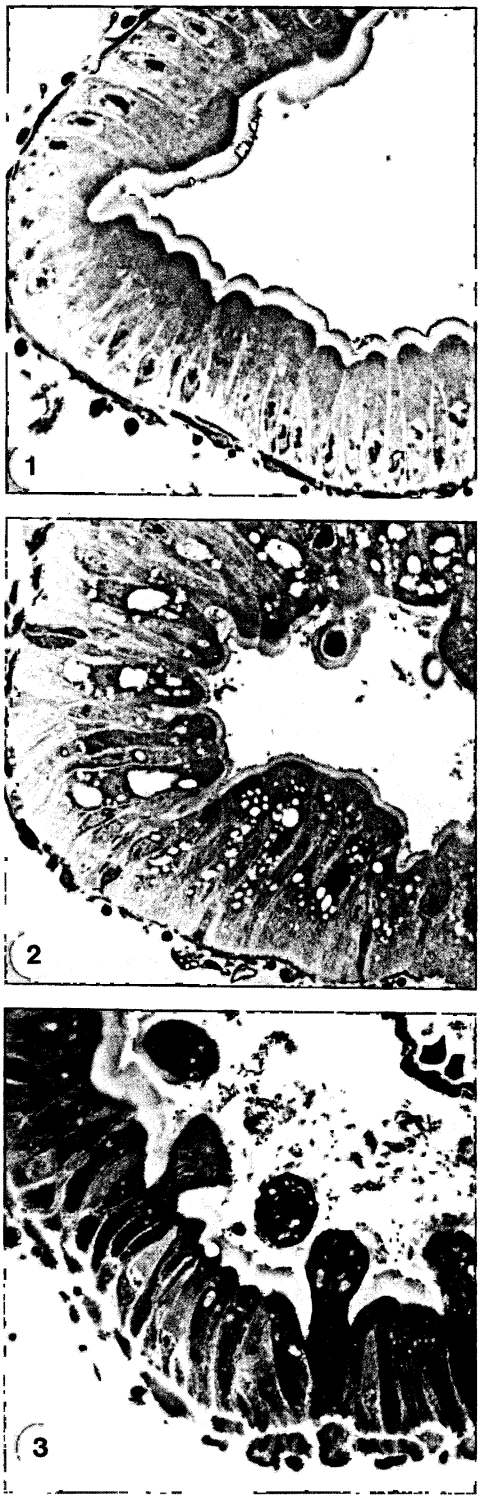
Insects and Assay

Field-collected cottonwood leaf beetles were reared on poplar foliage in the laboratory according to the methods described by Bauer (1990). Cohorts of newly molted third-instar larvae (last stage) were removed from foliage and starved for 24 hr before treatment.

Lethal doses of *B. thuringiensis* var. *san diego* were used which caused a short episode of diarrhea, paralysis for several minutes to hours after treatment, complete cessation of feeding, and death within a 48-hr period. Larvae imbibed either a 1-μl droplet of *B. thuringiensis* var. *san diego* containing live spores and 2 μg δ-endotoxin (spore/crystal complex) or sterile distilled water as a control. Larvae imbibing the entire droplet in less than 2 min were used for microscopy. Larvae treated with the live spore/crystal complex were held individually at 24 ± 1°C for time intervals of 15, 30, 45, 60, 120, and 180 min before dissection for microscopy. Each time interval was replicated twice.

Microscopy

After the allotted time, the alimentary canal was dissected directly into cold 2.5% glutaraldehyde in 0.1 M Na_2HPO_4–KH_2PO_4 buffer (pH 7.2) under a dissecting microscope. The midgut was cut into four segments (ca. 2 mm long), placed in individual vials of the cold glutaraldehyde solution, and fixed overnight. Following a buffer wash the segments were postfixed for 1 hr at room temperature in 1% OsO_4 in the same buffer as above. The segments were then dehydrated through an ethanol series, treated with propylene oxide, and embedded in Poly/Bed 812 (Polysciences, Inc., War-

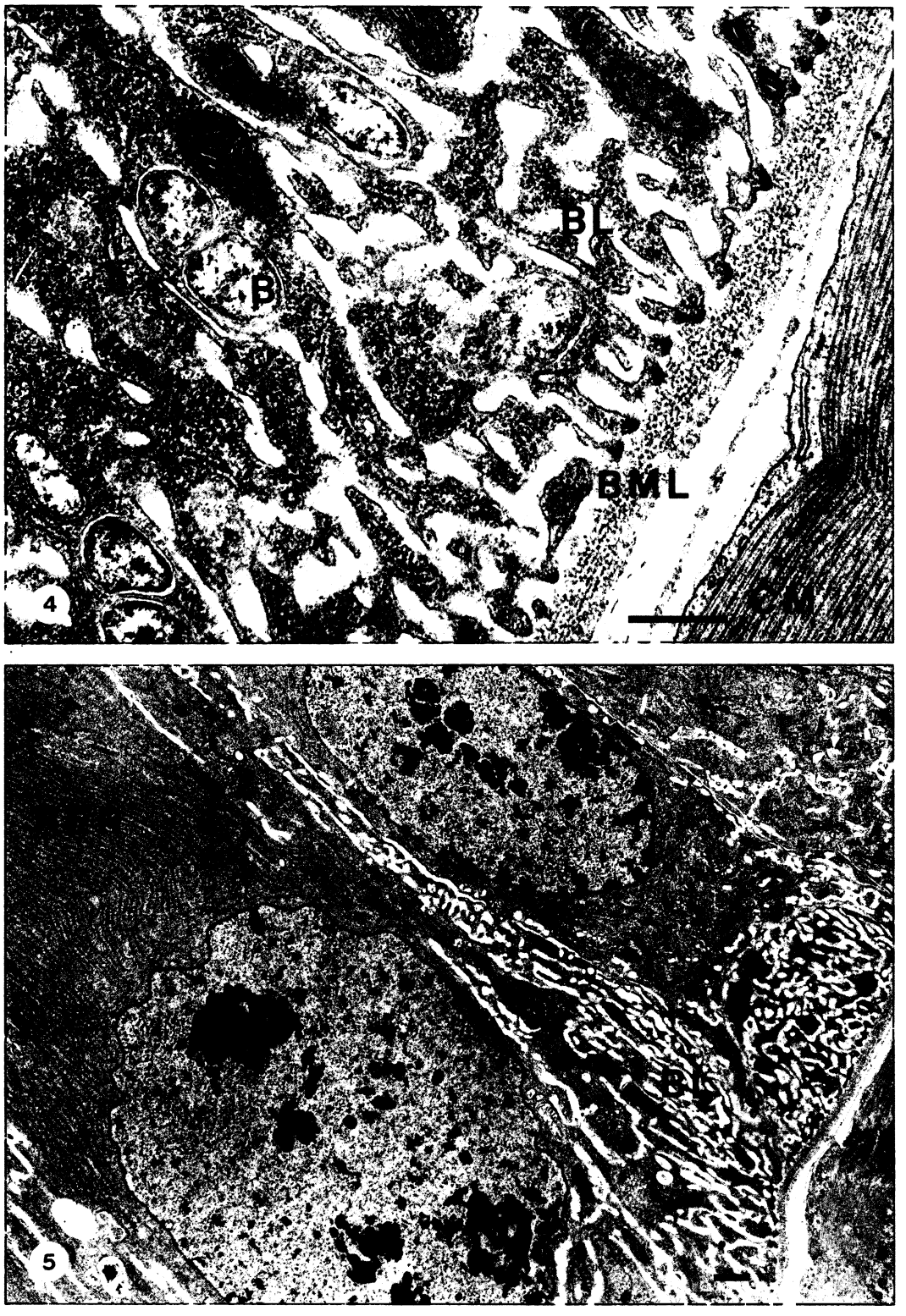


FIGS. 1–3. Light micrographs of midgut epithelial layer of third-instar *C. scripta* larvae.

FIG. 1. Control midgut epithelium 120 min after treatment with distilled water.

FIG. 2. Midgut epithelium 120 min after treatment with *B. thuringiensis* var. *san diego* containing 2 μg δ-endotoxin and spores.

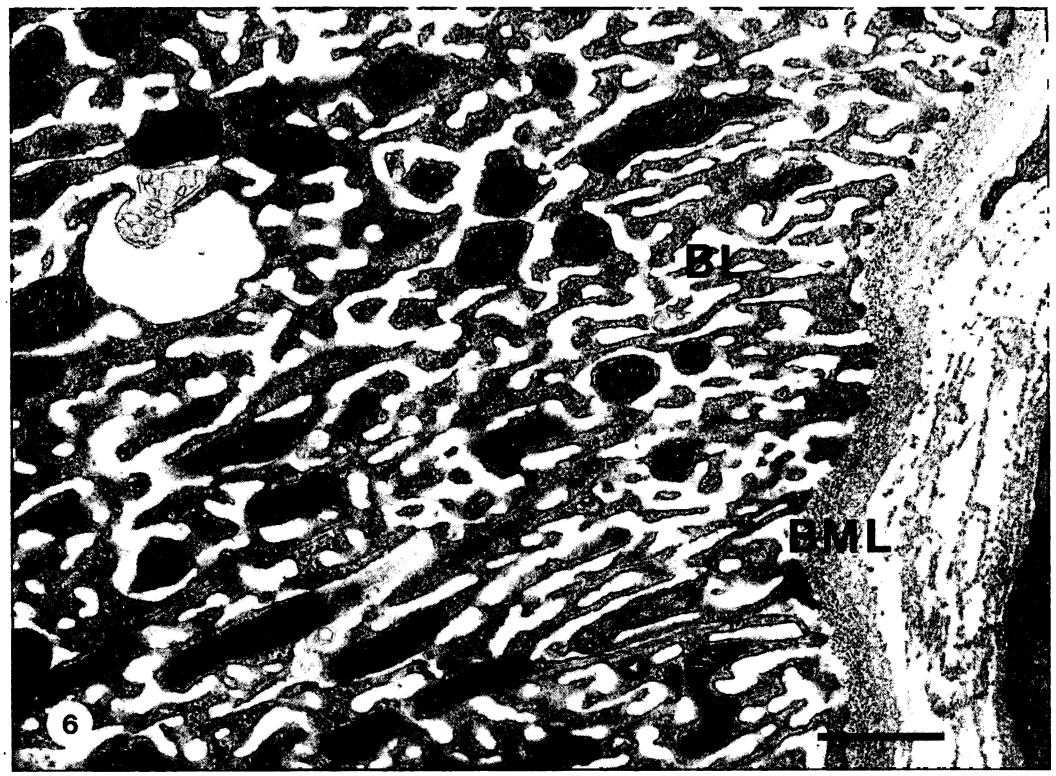
FIG. 3. Midgut epithelium 180 min after treatment with *B. thuringiensis* var. *san diego* containing 2 μg δ-endotoxin and spores.

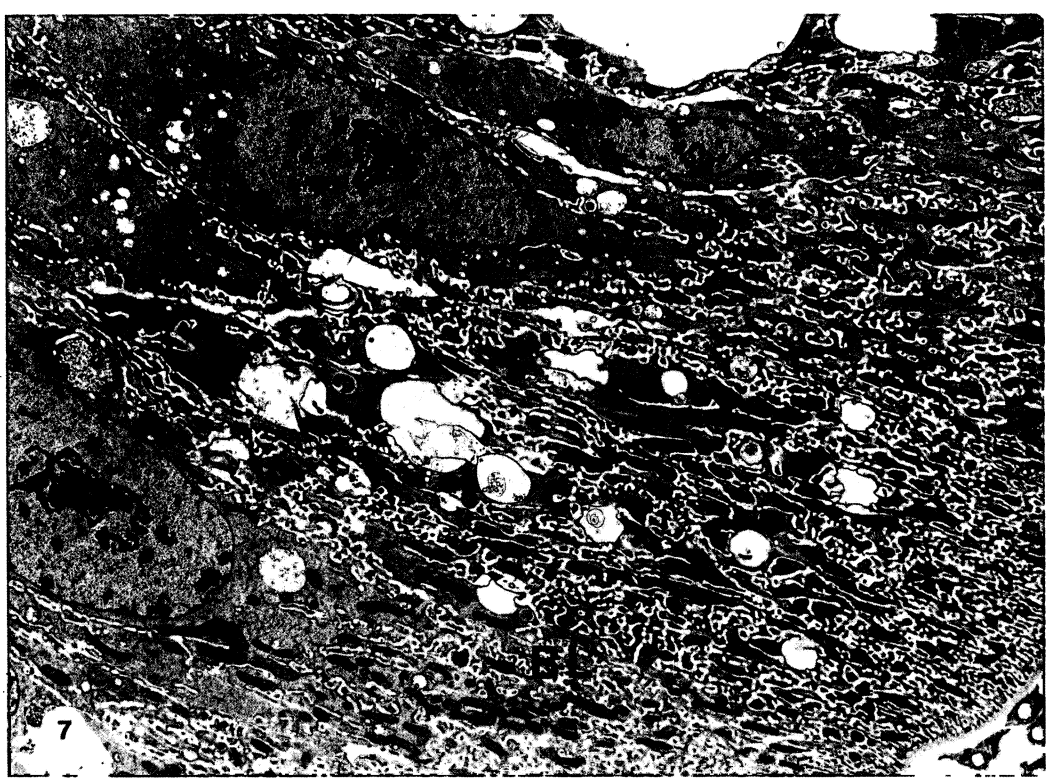


FIGS. 4 AND 5. Electron micrographs of control midgut epithelium of third-instar *C. scripta* larvae 120 min after treatment with distilled water.

FIG. 4. Basal portion of control midgut epithelial cell. BL, basal labyrinth; BML, basement lamina; CM, circular muscle; B, symbiotic bacteria. Bar = 0.5 μm.

FIG. 5. Basal region of control midgut epithelial layer. BL, basal labyrinth; RER, rough endoplasmic reticulum. Bar = 1.0 μm.





FIGS. 6–9. Electron micrographs of midgut epithelium of third-instar *C. scripta* 120 min after treatment with *B. thuringiensis* var. *san diego* containing 2 μg δ-endotoxin and spores.

FIG. 6. Basal portion of treated midgut epithelial cell after 120 min. BL, basal labyrinth; BML, basement lamina. Bar = 1.0 μm.

FIG. 7. Basal region of treated midgut epithelial layer after 120 min. BL, basal labyrinth. Bar = 1.0 μm.

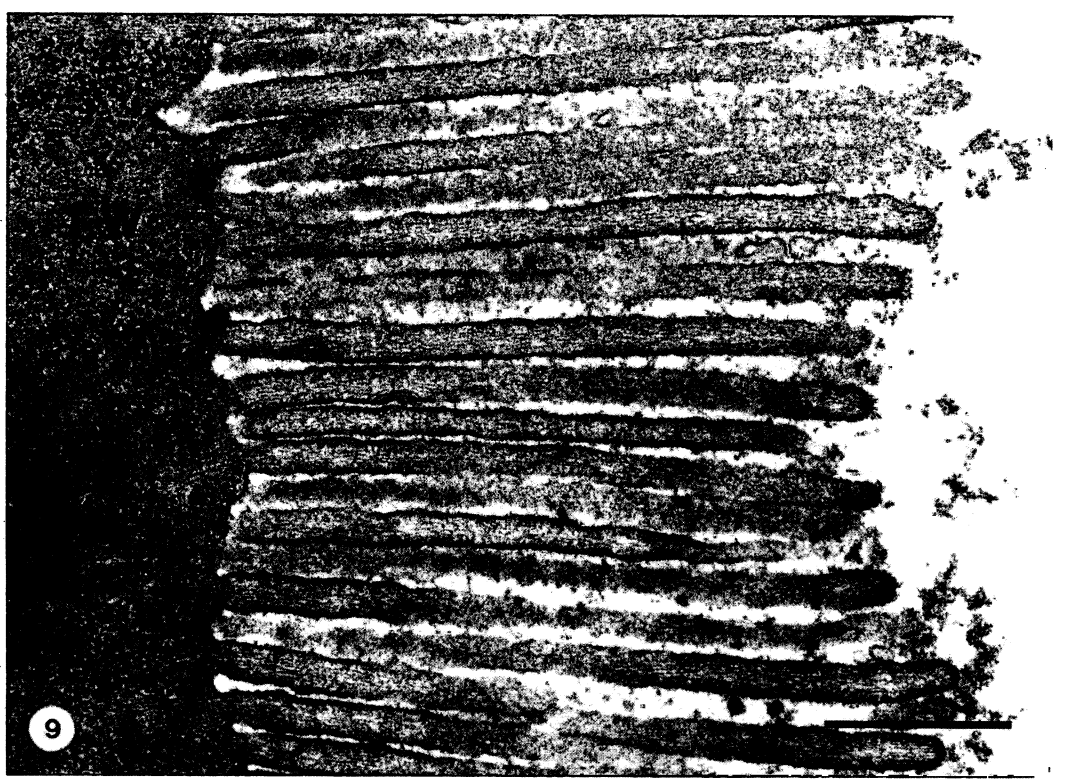


FIG. 8. Apical portion of treated midgut epithelial cell after 120 min. N, nucleus; RER, rough endoplasmic reticulum. Bar = 0.5 μm.
FIG. 9. Brush border of treated midgut epithelial cell after 120 min. TW, terminal web. Bar = 0.5 μm.

rington, PA). Thick sections were cut with a glass knife and stained with toluidine blue for light microscopy. Thin sections were cut with a diamond knife mounted on an Ultratome III (LKB Instruments, Inc., Rockville, MD), stained with uranyl acetate and lead citrate, and examined with an electron microscope (CM-10; Philips Electronic Instruments Co., Mahwah, NJ).

RESULTS

Preliminary observations of electron micrographs prepared from control midgut segments identified morphological differences along the midgut length. For comparative purposes, therefore, we chose to standardize our ultrastructural observations to a region that was immediately anterior to a characteristic bend in the midgut. This region is located in the anterior–middle region of the midgut and was easily identifiable at the time of dissection.

Light Microscopical Observations

Thick sections of this midgut region, observed from control larvae, showed typical columnar epithelial tissue with a monolayer of elongate cells lined with a brush border along the apical or lumenal margin. Nuclei are positioned basally along the basal or hemocoel margin (Fig. 1). Midgut tissue from larvae treated with the live *B. thuringiensis* var. *san diego* spore/crystal complex showed no gross structural changes until 120 min after treatment (Fig. 2). At this time, the epithelial cells appeared swollen, expanded into the gut lumen, and contained large cytoplasmic spaces. Nuclei were more centrally located and cells appeared to be swollen or extruded into the gut lumen. Disintegration of the midgut appeared more extensive after 180 min (Fig. 3).

Electron Microscopical Observations

Electron micrographs of untreated control midgut epithelial cells, immediately inside the circular and longitudinal muscles, revealed that the basement lamina, mitochondria, and symbiotic bacteria were closely associated with infoldings of the basal plasma membrane (Fig. 4). Nuclei were positioned basally, immediately interior to the region of basal infoldings (basal labyrinth) and a region of rough endoplasmic reticulum (RER) was located apically (toward the lumen) (Fig. 5). The apical region of the cell (not shown) included a region of free ribosomes interspersed with mitochondria and microvilli with terminal web region.

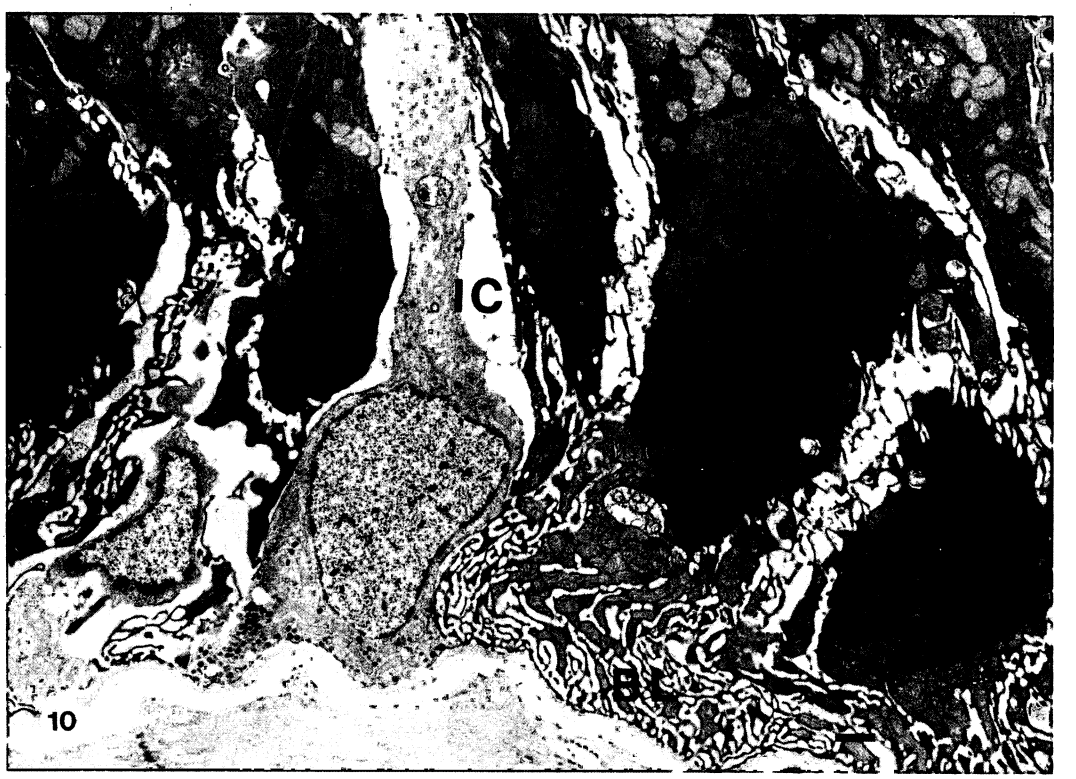
No ultrastructural changes were observed in midgut epithelial cells of larvae treated with the spore/crystal complex prior to 120 min post-treatment. At this time the most dramatic cytoplasmic disturbances observed were the extensive proliferation of the basal labyrinth and the development of large cytoplasmic and intercellular spaces (Figs. 6 and 7). In addition, organization of the basement lamina was disrupted (Fig. 6). Symbiotic bacteria did not appear to be directly affected (not shown). Nuclei appeared to be pushed apically by the increased vacuolization of the basal labyrinth (Fig. 7). The nuclear envelope and mitochondria showed no structural disturbances, although the region of RER was less organized and contained cytoplasmic spaces (Fig. 8). Despite swelling of the apical region of the cell into the midgut lumen, microvilli and terminal web showed no loss of structural integrity (Fig. 9). No increase in endocytotic activity was observed.

Ultrastructural observations 180 min after treatment showed more extensive cellular disintegration with notable increases in infoldings of the basal labyrinth and large intra- and intercellular spaces (Fig. 10). Response to the toxin was not homogeneous as damage to some cells was more advanced (less electron dense) than in adjacent cells. Although mitochondria appeared swollen and less electron dense, cristae and membranes remained intact (Fig. 11). The apical region of the cell contained large numbers of autophagic vesicles (Fig. 12). Swelling of the apical regions of the cells (Fig. 13) eventually resulted in lysis and leakage of cytoplasmic materials into the gut lumen (Fig. 14). At the site of lysis, only sporadic disturbances of microvillar membranes, microfibrillar core, and underlying terminal web structure were observed. The appearance of flat, rectangular crystals during bacterial sporulation confirmed the reproduction *B. thuringiensis* var. *san diego* in the lumen of treated insects (Fig. 15). δ-Endotoxin crystals were observed in various stages of development and dissolution within the gut lumen.

DISCUSSION

Cellular and subcellular morphological changes that occurred during *B. thuringiensis* var. *san diego* δ-endotoxin intoxication of the cottonwood leaf beetle were observed by light microscopy and transmission electron microscopy. The gross morphological changes of the midgut epithelial layer are similar to those reported for other chrysomelids treated with *B. thuringiensis* var. *tenebrionis* (Krieg *et al.,* 1983).

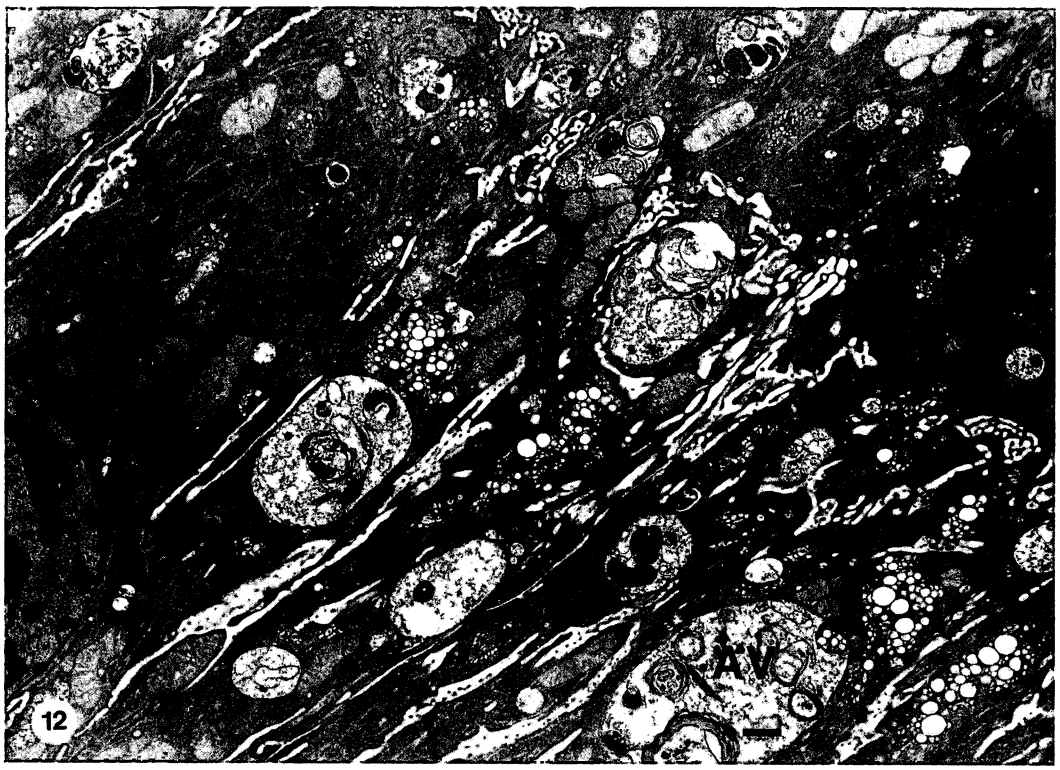
In the present study these changes, described as "*B. thuringiensis*-typical lesions" by Krieg *et al.* (1983), appeared as the swelling of the midgut epithelial cells into the gut lumen, the presence of large cytoplasmic and intercellular spaces, and cellular debris in the gut lumen. Transmission electron microscopy revealed the ultrastructural changes preceding cellular lysis and leakage of cytoplasmic materials lumenally. These included the dramatic expansion of the basal labyrinth, formation of intracellular and intercellular spaces, apical movement of nuclei, swelling of the apical cytoplasm and mitochondria, disruption of RER and basement lamina, and the presence of autophagic vesicles. These vesicles are typical of cells that are fated to die (Fain-Maurel *et al.,* 1973). Disruption of cytoplasmic



FIGS. 10–15. Electron micrographs of midgut epithelium of third-instar *C. scripta* 180 min after treatment with *B. thuringiensis* var. *san diego* containing 2 μg δ-endotoxin and spores.

FIG. 10. Basal region of treated midgut epithelial layer after 180 min. BL, basal labyrinth; ICS, intercellular spaces. Bar = 1.0 μm.

FIG. 11. Apical portion of treated midgut epithelial cell after 180 min. M, mitochondria; N, nucleus; RER, rough endoplasmic reticulum. Bar = 0.5 μm.



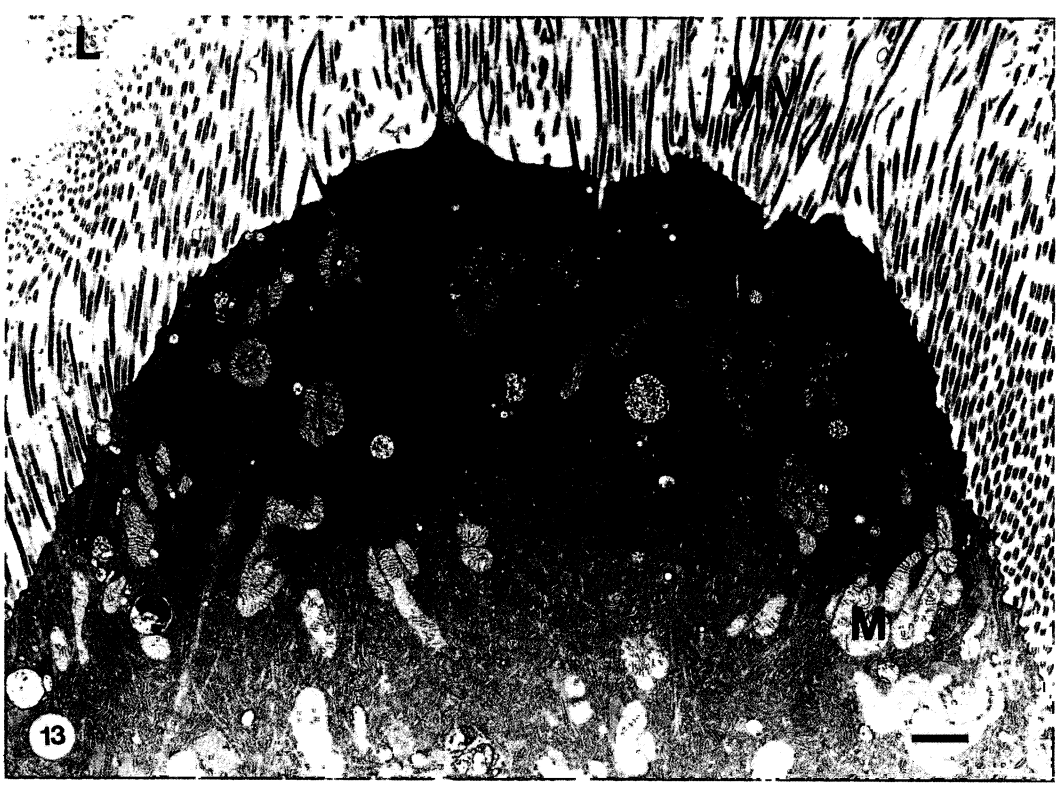


FIG. 12. Apical region of treated midgut epithelial layer after 180 min. AV, autophagic vesicles; M, mitochondria. Bar = 1.0 μm.
FIG. 13. Swollen brush border membrane and apical region of treated midgut epithelial cell after 180 min. L, lumen; M, mitochondria; MV, microvilli. Bar = 1.0 μm.

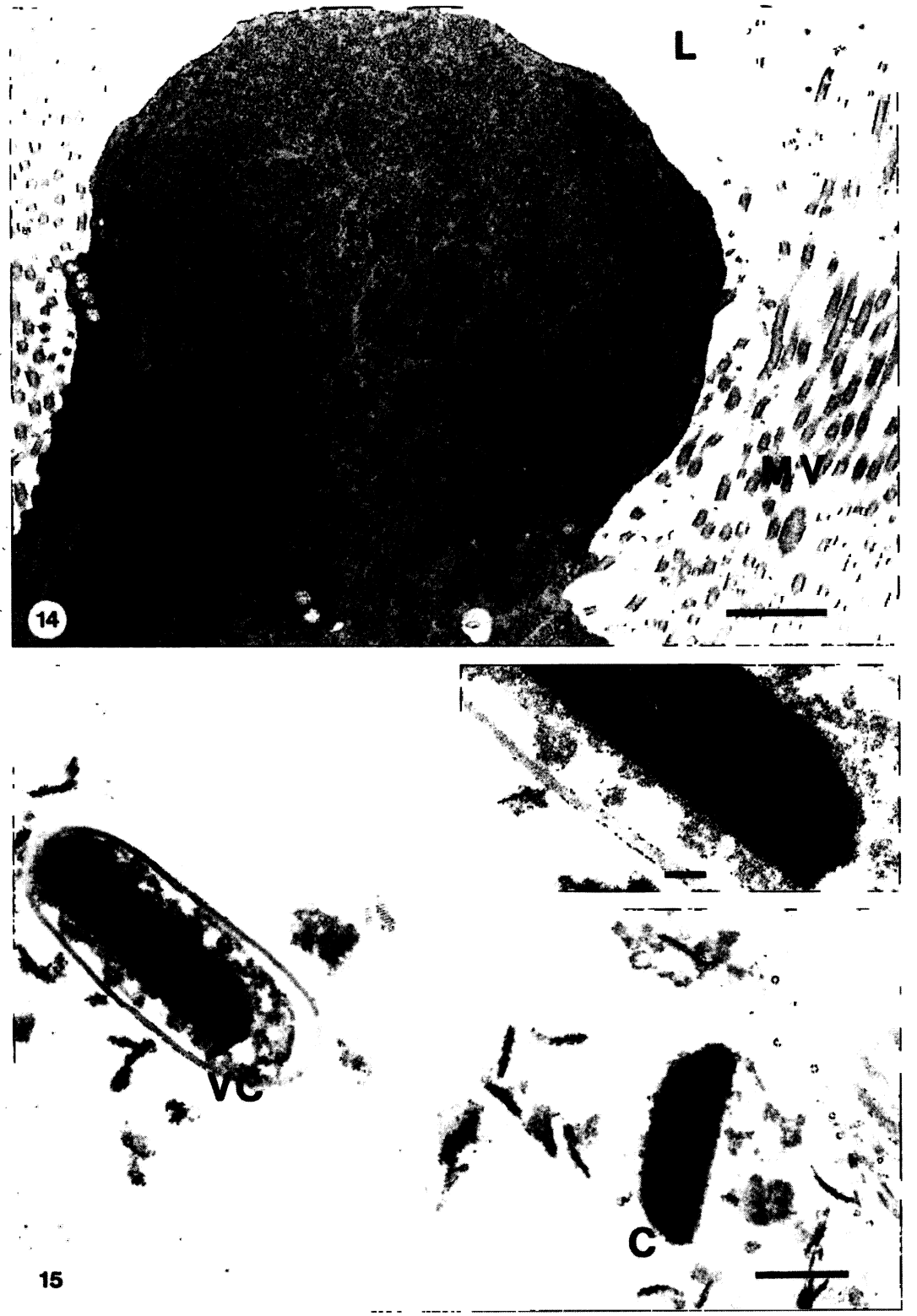


FIG. 14. Lysed brush border membrane of treated midgut after 180 min. L, lumen; LC, lysed cytoplasm; MV, microvilli. Bar = 1.0 μm.
FIG. 15. *B. thuringiensis* var. *san diego* in midgut lumen. Vegetative cell containing δ-endotoxin crystal. C, crystal; VC, vegetative cell containing crystal. Bar = 0.5 μm. (Inset) Enlargement of crystal showing periodicity. Bar = 0.1 μm.

structures appeared to result from the apical expansion of the basal labyrinth. Similar ultrastructural changes were observed in beetle epithelium 120 and 180 min after treatment with 10 μg purified δ-endotoxin crystals (Bauer and Pankratz, unpublished data).

The presence of bacterial vegetative stages, spores, and δ-endotoxin crystals within the midgut lumen indicated that the gut offered favorable conditions for growth and reproduction of *B. thuringiensis* var. *san diego*. The dissolution of these second-generation crystals within the insect midgut increased the toxicity of live preparations compared to purified δ-endotoxin crystals (Bauer, unpublished data).

The symptoms of cellular swelling and lysis are consistent with those described for *B. thuringiensis* var. *kurstaki* in several susceptible lepidopterans (Ebersold *et al.*, 1977; Endo and Nishiitsutsuji-Uwo, 1980; Nishiitsutsuji-Uwo and Endo, 1981; Percy and Fast, 1983). The ultrastructural observations presented in these lepidopteran studies, however, showed that *B. thuringiensis* var. *kurstaki* also causes disruption of plasma, nuclear, and mitochondrial membranes and loss of microvillar internal structure within 15 min of toxin ingestion (Percy and Fast, 1983; Lane *et al.*, 1989). Lepidopteran epithelial plasma membranes, exposed directly to the toxin, appear to suffer direct membrane disturbances prior to damage of cytoplasmic structures located basally (Reisner *et al.*, 1989; Lane *et al.*, 1989; Percy and Fast, 1983; Nishiitsutsuji-Uwo and Endo, 1981). Our observations differ from these findings in several ways: (1) the first cellular responses were slower (2 hr after treatment), (2) these responses occurred in the basal region of the cell with expansion of the basal labyrinth, and (3) microvillar structure was not compromised until cell lysis.

Some of the ultrastructural changes observed in the beetle are reminiscent of those reported during the osmoregulatory phase of the mosquito midgut after a blood meal (Billingsley, 1990; Houk and Hardy, 1982). These mosquito responses include a greatly expanded basal labyrinth and swollen, less electron dense mitochondria. These cellular changes are a generalized response of midgut epithelial cells following a luminal influx of fluids (Houk and Hardy, 1982). The increase in surface area of the basal labyrinth is indicative of enhanced transport of excess fluids into the hemocoel for excretion via the Malpighian tubules (Rudin and Hecker, 1979). This similarity suggests that the epithelial cells of the intoxicated beetle were responding normally to regulate water balance disrupted by the toxin.

Cellular swelling caused by *B. thuringiensis* var. *san diego* δ-endotoxin prior to cell lysis appears consistent with the colloid–osmotic lysis model for *B. thuringiensis* δ-endotoxin mechanism of action proposed by Ellar *et al.* (1986) and Knowles and Ellar (1987). They described a model in which activated δ-endotoxin binds to a receptor on the plasma membrane and inserts into the membrane, forming pores or leak channels. The presence of pores leads to an equilibration of ions across the membrane, resulting in a net influx of ions and accompanying water, causing cell swelling and lysis. However, the lack of membrane lesions, microvillar disruption, and comparatively slow response of beetle epithelium to treatment with *B. thuringiensis* var. *san diego* δ-endotoxin suggest that some differences in mechanisms of action may exist. These differences may result from variations in host gut environment, crystal solubility, toxin biochemistry, and toxin interactions with lipid bilayers or membrane receptors.

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