

## Hagfishes of Taiwan (II): Taxonomy as Inferred from Mitochondrial DNA Diversity

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**Kao-Feng Huang, Hin-Kiu Mok and Pien-Chien Huang (1994)** Hagfishes of Taiwan (II): Taxonomy as inferred from mitochondrial DNA diversity. *Zoological Studies* 33(3): 186-191. Parallel studies based on morphological characteristics suggest the classification of four new hagfish species (Kuo et al. 1994). We compared restriction patterns of hagfish mitochondrial DNA and found that two species (*Paramyxine sheni* and *P. wisneri*) have homologous but distinct mitochondrial genomes in contrast to other species in this genus. However, *P. sheni* individuals display a high frequency of heteroplasmy. Thus there is agreement between morphological and molecular taxonomic classifications of hagfish.

**Key words:** Hagfish, *Paramyxine*, Mitochondrial DNA, Phylogeny, Taiwan.

Hagfishes of the order Myxiniiformes dwell mostly in deep subtropical waters. They have become increasingly important economically as both edible and ornamental fish. That they thrive at great depths is interesting from a marine biology viewpoint since little is known about genetic stability under conditions of great pressure. Mitochondrial DNA heteroplasmy has recently been noted in several deep-sea organisms (Bentzen et al. 1988, Bermingham et al. 1986); although no single species mitochondrial DNA sequence has been completely determined. Sequence size has been measured at about 17 Kb similar to that found in mammals, suggesting an efficient genetic economy (Attardi 1986). Because of its precise organization, mitochondrial DNA has been shown to be useful to taxonomists as an index for classifying and detecting changes in the episomal genome under evolutionary constraints.

Until now, inquiries into the systematics of hagfish have relied on morphological characteristics. Three major hagfish genera (*Myxine*, *Paramyxine* and *Eptatretus*) were classified according to gill aperture, efferent duct, slime pores and dental

formulae (Dean 1904, Matsubara 1937, Okada et al. 1948, Bigelow and Schroeder 1952, Strahen and Honma 1961, Shen and Tao 1975, Fernholm 1982, McMillan and Wisner 1984, Hensley 1985). Species from the first two genera have been sighted in Taiwan waters (Shen and Tao 1975). We recently collected a large sample of hagfish within which at least four new species were classified employing the traditional taxonomical criteria listed above (Kuo et al. 1994). The validity of these classifications was uncertain since morphological differences could be explained by intraspecific phenotype variation. This study was undertaken to provide another independent criterion: mitochondrial DNA restriction patterns for justification of these new species. Moreover, mtDNA information has enabled us to reconstruct the phylogenetic relationship of the six species examined.

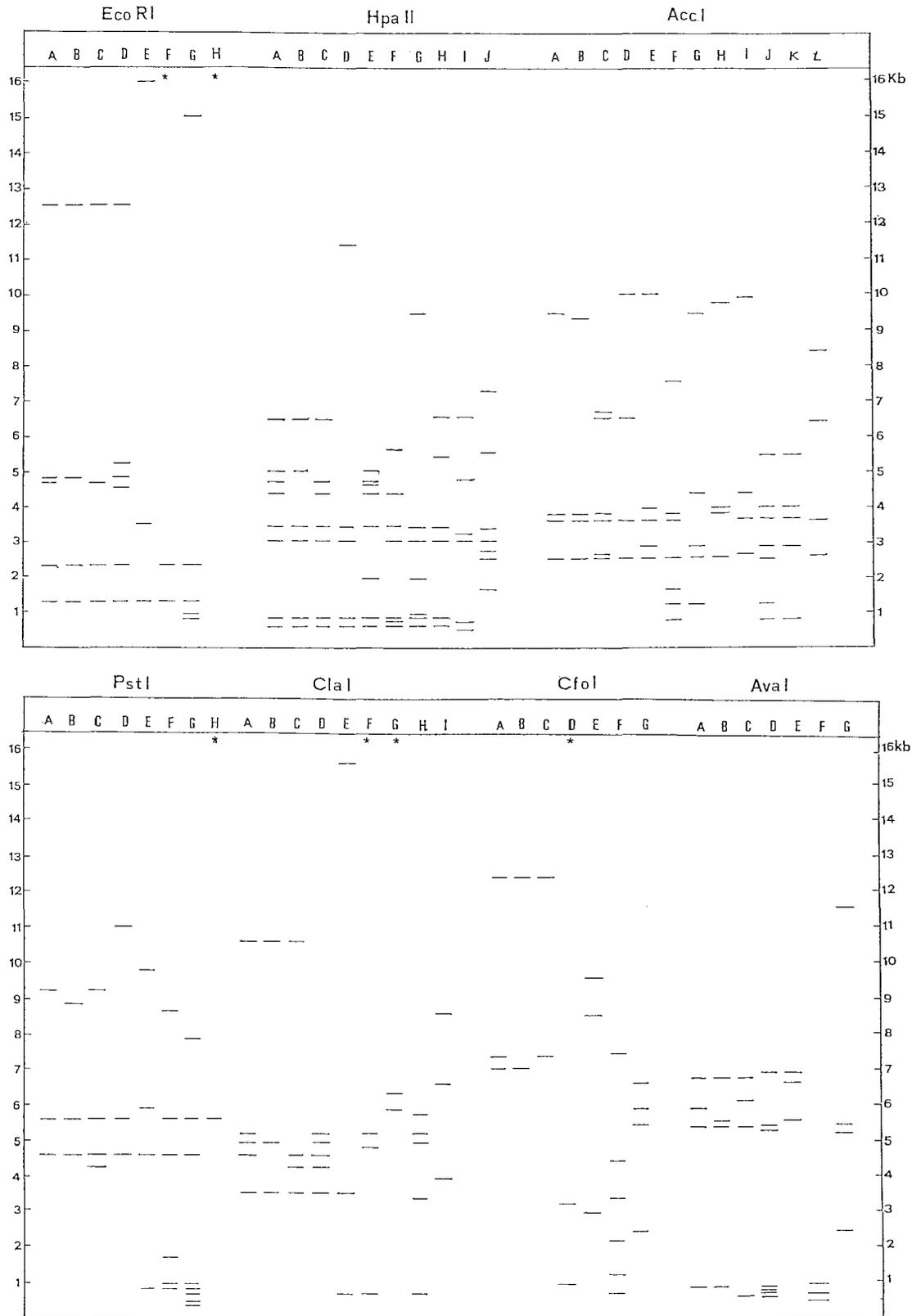
### MATERIALS AND METHODS

Hagfish were collected by shrimp trap (Hensley 1985) from the northeastern, southwestern and

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**Fig. 2.** Digestion patterns of hagfish mtDNA for individual restriction endonucleases. Various restriction endonuclease digestion of individual hagfish mtDNA resulted in a range of patterns. The results of seven enzymes are depicted with tracing lines. Each letter represents a pattern for a given enzyme. The combination of individual patterns resulted in 19 unique combinations which are summarized in Table 1. Asterisk = mtDNA fragment barely visible.

*E. burgeri* and *E. okinoseanus* ( $F = 0.174\text{--}0.219$  and  $0.075\text{--}0.080$ , respectively), since two different genera are involved. In light of this, the contrast between *E. burgeri* and *E. okinoseanus* ( $F = 0.0606\text{--}0.0625$ ) is notable.

**Table 1.** Composite mtDNA genotype observed in 6 hagfish species

Species	Restriction patterns	No.	%
<i>Paramyxine sheni</i>	1 A A A A A A A	20	74.07
	2 A D A A D A A	1	3.70
	3 A E C A A A A	1	3.70
	4 D A A C D A A	1	3.70
	5 B B A A B B A	2	7.40
	6 C C B B C C B	2	7.40
<i>P. wisneri</i>	7 E F D D E D C	1	11.11
	8 E F E D E D C	3	33.33
	9 E F F D E D C	1	11.11
	10 E F G D E D C	4	44.44
<i>P. taiwanae</i>	11 F G H E F E D	4	100.00
<i>P. yangi</i>	12 F H I F G C E	13	100.00
<i>Eptatretus burgeri</i>	13 G I J G H F F	8	50.00
	14 G I K G H F F	1	6.25
	15 -- J -- H F --	4	25.00
	16 G -- J G H F --	1	6.25
	17 -- J G H F --	1	6.25
	18 -- I J -- H F --	1	6.25
<i>E. okinoseanus</i>	19 H J L H I G G	1	100.00

Letter designations are for the following restriction endonucleases (from left to right *EcoRI*, *Hpa II*, *Acc I*, *Pst I*, *Cla I*, *Cfo I*, *Ava I*). See Fig. 2 for gel pattern, -- denotes data not available.

**DISCUSSION**

Compared with nuclear genes, mitochondrial DNA undergoes a relatively rapid base-pair change (Upholt and Dawid 1977, Nei and Li 1979, Vawter and Brown 1986, Moritz et al. 1987). Thus, within a given species individuals can be expected to show a high degree of homology, while fixed differences are usually much higher between species (Brown et al. 1979), and limited samples can verify such an expectation. Our examination of 27 individuals may be considered sufficient for intra-specific determination. Studies of this nature include: Graves et al. (1984), skipjack tuna ( $N = 16$ ) and chimpanzees ( $N = 10$ ), Avise and Vrijenhoek (1987), *Bagre marinus* ( $N = 2$ ), *Opsanus pardus*, and *Opsanus beta* ( $N = 16$ ), and Brown et al. (1979), sheep ( $N = 1$ ) and goats ( $N = 2$ ) are examples of effective small sample use. These samples allowed us to observe a predominant form of mtDNA in *P. sheni* with which to deduce a basic physical map for the variation comparison.

Since heteroplasmy has been found in deep sea invertebrates (Bermingham et al. 1986), scallops (Synder et al. 1987), and shad (Bentzen et al. 1988), its presence in *P. sheni* was expected, but its absence in other species was not. A dyad symmetric sequence of GGGGGCATCCCC is present within a 206 base pair fragment constituting the basic repeating unit of heteroplasmic mtDNA from crickets (Rand and Harrison 1989). Whether or not such a sequence has evolved in hagfish

**Table 2.** Restriction fragment comparison between mtDNA from six hagfish species

	<i>Paramyxine sheni</i>						<i>P. wisneri</i>				<i>P. taiwanae</i>	<i>P. yangi</i>	<i>Eptatretus burgeri</i>	<i>E. okinoseanus</i>	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	19
1	26	24	24	26	26	23	12	12	12	10	9	9	6	6	2
2	0.941	25	23	24	24	21	12	12	12	10	10	9	7	7	2
3	0.889	0.868	28	24	24	22	12	12	12	10	9	9	6	6	2
4	1.0	0.941	0.889	26	26	22	12	12	12	10	10	9	7	7	2
5	0.962	0.941	0.889	1.0	26	20	12	12	12	10	9	8	6	6	2
6	0.885	0.824	0.815	0.846	0.769	26	12	12	12	10	9	10	6	6	2
7	0.444	0.453	0.429	0.444	0.444	0.444	28	27	25	24	8	6	6	5	2
8	0.436	0.444	0.421	0.436	0.436	0.436	0.947	29	26	24	9	6	8	7	2
9	0.421	0.429	0.407	0.421	0.421	0.421	0.847	0.867	31	26	10	6	7	6	3
10	0.364	0.370	0.351	0.364	0.364	0.364	0.842	0.828	0.867	29	9	7	7	6	3
11	0.316	0.357	0.305	0.351	0.316	0.316	0.271	0.300	0.303	0.300	31	10	8	8	3
12	0.340	0.346	0.327	0.340	0.302	0.377	0.218	0.214	0.207	0.250	0.345	27	8	8	2
13	0.180	0.212	0.174	0.209	0.179	0.179	0.174	0.229	0.194	0.200	0.222	0.235	41	39	2
14	0.185	0.219	0.179	0.215	0.185	0.185	0.149	0.206	0.171	0.176	0.229	0.242	0.975	39	2
19	0.078	0.080	0.075	0.078	0.078	0.075	0.074	0.107	0.111	0.107	0.107	0.077	0.0606	0.0625	25

Number 1-19 on the X and Y axes denote patterns as defined in Table 1 and Fig. 3. Integers are number of restriction fragments shared by patterns. Fractions are computed per equation of Kessler and Avise 1985.

is still undetermined.

With seven restriction endonucleases, this study was able to establish a set of digestion patterns for the estimation of relatedness between individuals within and between hagfish species. The results support a classification of *P. sheni* and *P. wisneri* as new species based on morphological features. The authenticity of a third species, *P. nelsoni*, also classified as new (Kuo et al. 1994) remains tentative as there were insufficient samples available for the mtDNA analysis.

Despite the close similarity between *P. sheni*, *P. wisneri*, and *P. taiwanae*, they can be distinguished by differences in their fused unicuspid and slime pores (Kuo et al. 1994). The high coefficient of mtDNA relatedness ( $F = 0.364-0.444$ ), further supports their being congeneric. Possibly diverging early in evolution, but retaining certain morphological features, *E. burgeri* and *E. okinoseanus* differed greatly in their mtDNA restriction patterns.

The following conclusions (Huang 1989) are based on an attempt to trace the phylogenetic relationship between hagfish in Taiwan employing a traditional morphological analysis:

1. *Paramyxine* is monophyletic and *Eptatretus* is probably paraphyletic; the phylogenetic status of *Myxine* is uncertain.

2. Within the *Paramyxine* genus, *P. yangi* and *P. nelsoni* constitute a sister group, while *P. taiwanae*, is the primitive sister species of the *P. yangi* — *P. nelsoni* group.

3. *E. okinoseanus* is the most primitive hagfish species examined.

Some of these conclusions have been supported by mitochondrial DNA analysis in this study. The monophyly of *Paramyxine* is evident by the relatively high  $F$  values between different *Paramyxine* species, as compared to those between *Paramyxine* and *Eptatretus* species. A higher  $F$  value for *E. burgeri* with all *Paramyxine* species examined than with its congeneric species, *E. okinoseanus*, support the paraphyletic status of genus *Eptatretus*. The low  $F$  value of *E. okinoseanus* with *E. burgeri* and all *Paramyxine* species (Table 2) clearly shows its primitiveness.

The high  $F$  value between *P. sheni* and *P. wisneri* does not contradict the proposed close relationship between *P. taiwanae*, *P. yangi* and *P. nelsoni*. The restriction patterns of *P. taiwanae* and *P. yangi*, in particular, are distinctively different from that of *P. wisneri* (Table 2). The degree of similarity in the mtDNA restriction patterns of *P. sheni*, *P. taiwanae*, and *P. yangi* are approximate

(Table 2) and leaves their interrelationship unresolved.

Since, near the Taiwan coast *P. sheni* and *P. wisneri* are sympatric, morphological distinctions could have been considered intraspecies variations, were it not for an mtDNA analysis. Similarly, other hagfish species from a given geographical area may also be mixed; *Eptatretus stouti* displays a large variation in dental formulae (from 8/9 to 12/11), and in gill aperture (from 10 to 15), *Eptatretus polytrema* varies both in dental formulae (from 11/7 to 13/12) and in gill aperture (from 10 to 14), and *Eptatretus cirrhata* too varies in dental formulae (from 8/7 to 12/11). If dental formula and gill aperture are reliable markers for speciation, the variation in these species suggests the presence of species or subspecies. Such propositions can be justified with other supplementary identification methods such as mtDNA analysis.

The extremely economical organization of mitochondrial genomes render it a desirable resolution for taxonomical dilemma (Moritz et al. 1987, Hillis 1987). Geographically isolated individuals of the same species have been identified, using mitochondrial DNA restriction patterns. Thus the previously classified subspecies of *Mus musculus*, *M.m. vesonis* in Hokkaido, and *M.m. molossimus* in various Japan localities were shown to be genetically identical (Yonekawa et al. 1981). As were two fresh water species of *Crossostoma* (Tzeng et al. 1990). Awaiting further study is the possible reclassification of twenty-seven hagfish species.

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## 台灣的盲鰻(II)：從粒線體去氧核糖核酸推論其分類

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本文描述並比較台灣海域六種盲鰻的粒線體去氧核糖核酸限制酶切位形態，發現沈氏副盲鰻 (*Paramyxine sheni*) 及懷氏副盲鰻 (*P. wisneri*) 兩者種內限制酶切位形態相同性高，但均與同屬內其他種類有顯著的差別。沈氏副盲鰻的個體出現異質性 (heteroplasmy) 之機率相當高。從分子生物學與形態學的研究得到台灣副盲鰻種類的分類結果相當吻合。

關鍵詞：盲鰻，副盲鰻屬，粒線體去氧核糖核酸，種族發生史，臺灣。

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## Histopathological Effects of *Bacillus thuringiensis* $\delta$ -endotoxin on the Malpighian Tubules of *Pieris canidia* Larva

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**Jian-Bin Wang and Wilkin Wai-Kuen Cheung (1994)** Histopathological effects of *Bacillus thuringiensis*  $\delta$ -endotoxin on the Malpighian tubules of *Pieris canidia* larva. *Zoological Studies* 33(3): 192-199. The histopathological effects of *Bacillus thuringiensis* subsp. *kurstaki*  $\delta$ -endotoxin on the Malpighian tubule cells of *Pieris canidia* larva were studied after mucosal and serosal treatment. Results showed that tissues examined after one minute of exposure to the toxin had already undergone fine structural alterations. The number of vacuoles in the cytoplasm increased, and the apical microvilli and the basal intracellular channels became slightly disorganized. The mitochondria, however, appeared normal. After ten minutes of toxin treatment, the ground cytoplasm had large vacuoles or cytoplasmic spaces, the microvilli were damaged extensively, there was extrusion of apical cytoplasm, and the basal infoldings were damaged. After twenty minutes of toxin treatment general cell disintegration was observed. The microvilli were severely damaged, the apical membrane lysed, all the mitochondria became very swollen, the rough endoplasmic reticula and cytoplasm were extremely vacuolated, the basal infoldings were greatly disorganized, and the basement membrane was eroded. These results indicate that *Bacillus thuringiensis*  $\delta$ -endotoxin could effect cell leakage by forming large pores in cell membranes.

**Key words:** Bacteria, Insect excretory system, Ultrastructure.

*Bacillus thuringiensis* subsp. *kurstaki* (Btk) is a widely distributed, spore-forming, gram-positive bacterium (Faust and Bulla 1982, Whiteley and Schnef 1986). It produces a parasporal crystal during the sporulation cycle. When the spore or crystal is ingested by a susceptible insect (primarily lepidopterous larvae) the crystal is activated. This crystal injures the midgut epithelium (Sutter and Raun 1967, Fast and Morrison 1972, Ebersold et al. 1978, Endo and Nishiitsutsuji-Uwo 1980, Ellar et al. 1985, Chiang et al. 1986, Cheung et al. 1990, Cheung and Lam 1993).

The histopathological effects of Btk on Lepidoptera Malpighian tubules have not been reported except in the works of Reisner et al. (1989) on *Calpodex* and in Ryerse et al. (1990) on *Heliothis*. Insect Malpighian tubules possess a one cell thick epithelium generally similar to those of the midgut (Smith 1968, Wigglesworth 1972, Marjota and Ballan-Dufrancais 1984).

Investigating the histopathology of the Malpighian tubule as compared to that of the midgut

may help us further understand the mode of action of Btk on insect epithelial membranes in general.

### MATERIALS AND METHODS

#### Preparation of parasporal crystals of *Bacillus thuringiensis* subsp. *kurstaki*

Monoclonal strains of Btk, supplied by the Sandoz Company (U.S.A.), were grown in a modified CHES medium (Chestukina et al. 1980): Casamino acid, 0.5%; yeast extract, 0.4%; glucose, 0.2%; NaCl, 0.5%; MgSO<sub>4</sub>·2H<sub>2</sub>O, 0.01%; CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.05%; pH 7.4. Initially the Btk strain was grown for 7 hours at 28°C and then another 12 hours in a more concentrated form (1% v/v of the pre-culture). The cultured material was centrifuged at 8,000 rpm and 20°C for 15 min. The pellets collected were resuspended in sterile distilled water and incubated in a shaker at 28°C for 4 days to allow sporulation and autolysis. The mixture of

spores and crystals was then layered onto a 67% urografine solution (Millon and Delcour 1984).

The final solution was centrifuged at 6,000 rpm and 4°C for 2 hours. Btk spores were forced to the bottom of the centrifuge tube. A debris band and a parasporal crystal band were visible (Fig. 1). The crystals were isolated carefully with a Pasteur pipette and resuspended in distilled water (4x); later they were lyophilized and stored at -20°C. The sample contained over 99% pure  $\delta$ -endotoxin, as reported by Millon and Delcour (1984). Samples of these were viewed with a JEOL JEM-35 scanning electron microscope (Fig. 2).

Solubilization of crystal  $\delta$ -endotoxin was accomplished by incubating the crystals in 1% (w/v) sodium dodecyl sulfate (SDS), 2% (v/v)  $\delta$ -mercaptoethanol, 6 M urea, and equimolar ratio of  $\text{NaH}_2\text{PO}_4$  and  $\text{Na}_2\text{HPO}_4$  solution (pH 7.2) for 1 hr at 28°C.

Protein composition of the crystals was determined by the SDS-PAGE procedure (Laemmli 1970). A 3% stacking gel and a 7.5% resolving gel were run on a Mini-protein II electrophoresis cell (Bio-Rad) for 10 min at 50 volts and 1 hr at 100 volts, respectively. Different molecular weights of bovine serum albumin and ovalbumin were used as standards. Traces of 63 kDa toxin and 134 kDa protoxin were easily identifiable (Fig. 3).

### Activation of toxins

Five mg of purified crystals (containing the MW 134 and 63 kDa proteins) were suspended in 1 ml of a 1.0% protease (enzyme extract with trypsin, Sigma Company) and 0.2% dithiothreitol in 0.2% CAPS (cyclohexylaminopropane sulfuric acid) buffer, pH 10.5 (Yamamoto and McLaughlin 1981). The solution was incubated at 28°C on a rotary shaker for 24 hr and then assayed against

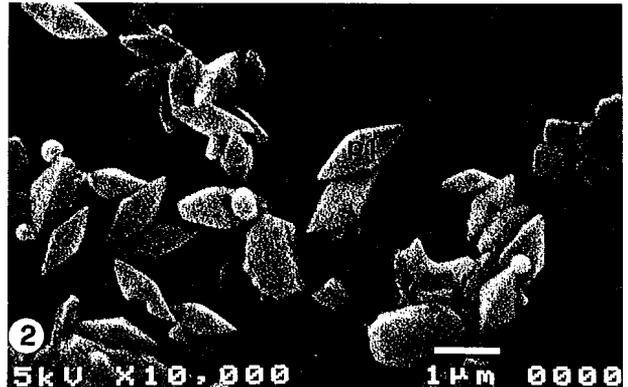


Fig. 2. Scanning electron micrograph of 134 kDa (P1) and traces of 63 kDa (P2) crystals before activation. x12,000.

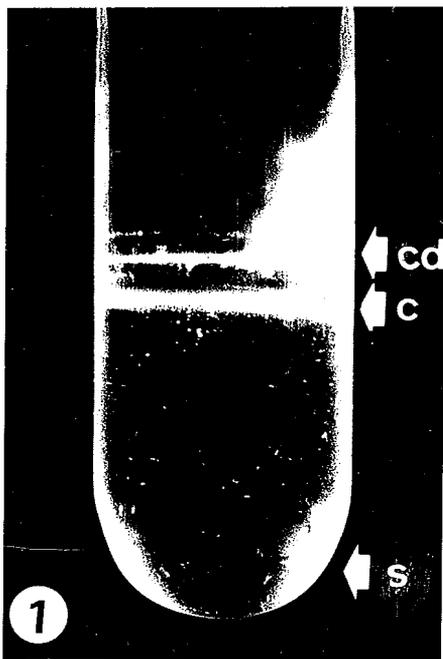


Fig. 1. Separation of spores, crystals, and cell debris of Btk in urografine solution. Shows spores (s), crystal band (c), and cell debris (cd).

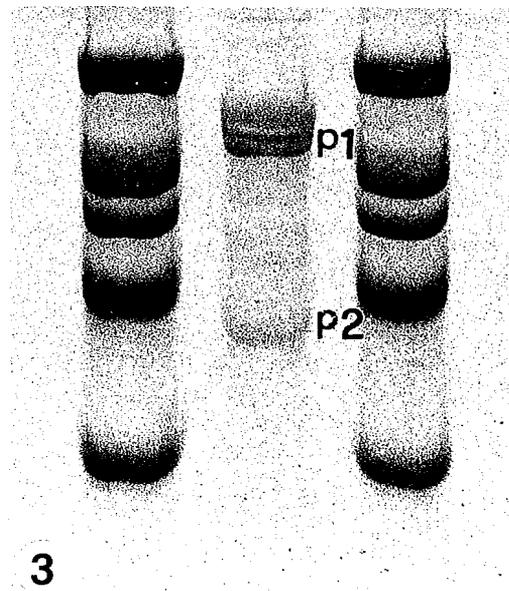


Fig. 3. Solubilized products of Btk crystals (before activation) analyzed by SDS-polyacrylamide gel electrophoresis. Shows 134 kDa protoxin (P1) and traces of 63 kDa toxin (P2). Molecular standards are at both sides.

the earlier mentioned Bio-Rad protein standards. Samples of known concentrations of the 63 kDa toxin were eventually used for treatment of *Pieris canidia* (Talbot 1939) Malpighian tubules. However, the 134 kDa had no noticeable effects on the Malpighian tubules.

### Experimental animals and tissue treatment

Larvae of *Pieris canidia* were reared in an insectary according to Cheung et al. (1990). Fourth instar larvae were used for the experiments. For mucosal exposure, 0.00001  $\mu\text{g/ml}$  toxin (in phosphate buffered saline, PBS) was injected into the Malpighian tubule lumen with a micropipette. For serosal exposure Malpighian tubules studied in vivo were immersed in 0.001  $\mu\text{g/ml}$  toxin (in PBS); and those studied in vitro were bent in a U-shape in order just to limit toxin exposure to the arc. Increased toxin concentrations offer results similar to experiments of luminal exposure. The toxin treatments were limited to one, ten, and twenty minute(s), respectively. Controls were performed with buffer only.

Control and toxin treated Malpighian tubules were then fixed in 1% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2, with sucrose). These were post-fixed in 1% osmium tetroxide in 0.2 M phosphate buffer (pH 7.2). Tissues were subsequently blocked in Spurr resin. Thin sections were cut with a Reichart Ultratome (OMU2 model) and were stained in uranyl acetate and post-stained in lead citrate. Sections were viewed under a Zeiss or JEOL JEM-100C electron microscope.

## RESULTS

### Malpighian tubule cell structure

Although each *Pieris* Malpighian tubule had regional differentiation in structure and functions (Cheung and Wang 1993) the cellular arrangements appeared principally the same in all four regions i.e. there were approximately one dozen principal cells making up the tubule circumference with only sporadic occurrence of stellate cells. Each principal cell had: 1) a large centrally placed nucleus, 2) numerous basal membranous infoldings forming intracellular channels extending deeply towards the cell apex, and 3) many apical microvilli with mitochondria extending inside them. Most histopathological studies have been carried out on the principal cells of the yellow region since it

performs both secretion and mineral storage functions and is the longest of the four regions.

### Histopathological effects

#### One minute post-treatment

Since the Btk endotoxin can bind to membrane receptors (Knowles and Ellar 1987) an exposure of 0.00001  $\mu\text{g/ml}$  of the toxin to the mucosal surface of principal cells for one minute effected damage. The apical microvilli showed slight swelling and the number of vacuoles in the cytoplasm increased (Fig. 4). The mitochondria suffered no notable damage. The nucleus had chromatin materials dispersed and the rough endoplasmic reticula swelled. Basally, the intracellular channels were rather disorganised, but the mitochondria appeared normal (Fig. 5). The basement membrane (0.4  $\mu$  thick) was apparently unaffected.

Serosal exposure of principal cells (Figs. 6, 7) with the same dosage of endotoxin did not show any damaging effect. The dosage had to be increased hundred-fold i.e. 0.001  $\mu\text{g/ml}$  before any damage could be noticed. With such dosage the basal channels were disorganised and there were numerous vacuoles in the ground cytoplasm. The apical microvilli also began to show swellings (Fig. 6). The basement membrane remained intact.

#### Ten minutes post-treatment

Mucosal exposure of the yellow region principal cells showed extensive microvilli damage and there was apical cytoplasm extrusion (Figs. 8, 9). The mitochondria cristae had been modified when compared to the control. The ground cytoplasm had large vacuoles or disorganised cytoplasmic spaces. Apparently the rough endoplasmic reticula had been seriously damaged (Figs. 8, 9). The basal intracellular channels enlarged drastically.

Serosal treatment of the cells resulted in the basal membranous channels becoming disorganised resulting in large vacuoles in the ground cytoplasm (Figs. 10, 11). The mitochondria cristae were seriously damaged. The apical mitochondria appeared to show less damage in their cristae. The whole mitochondrion became round in shape and apparently contracted in size. The microvilli also showed damage (Fig. 10).

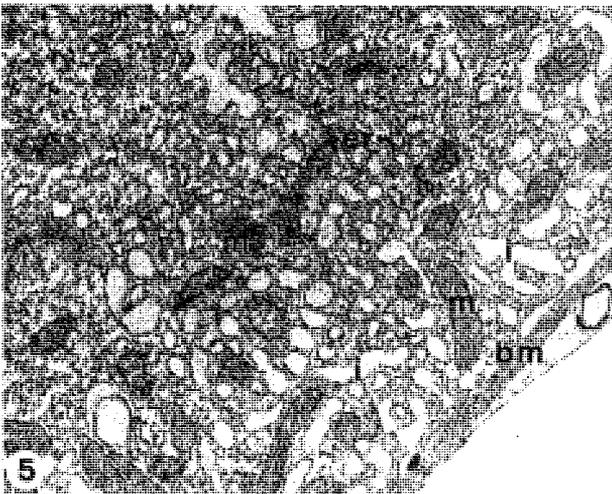
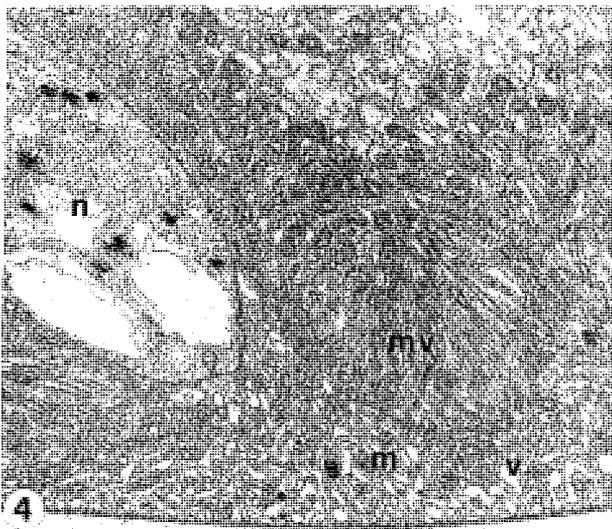
#### Twenty minutes post-treatment

Mucosal exposure of toxin showed complete damage of apical microvilli (Figs. 12, 13). The mitochondria cristae disintegrated while the

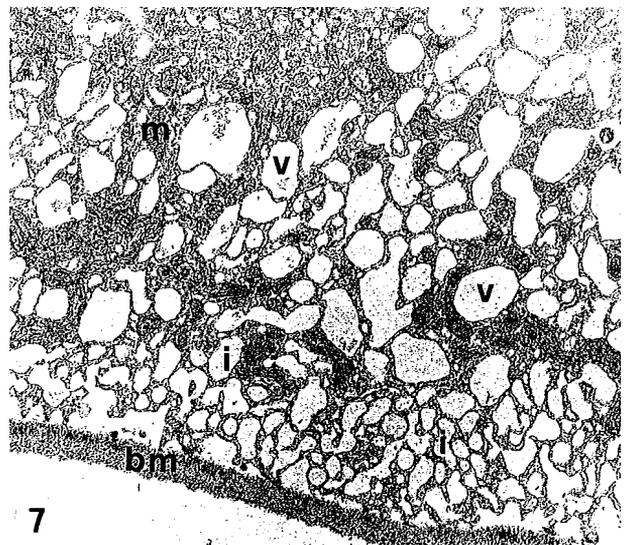
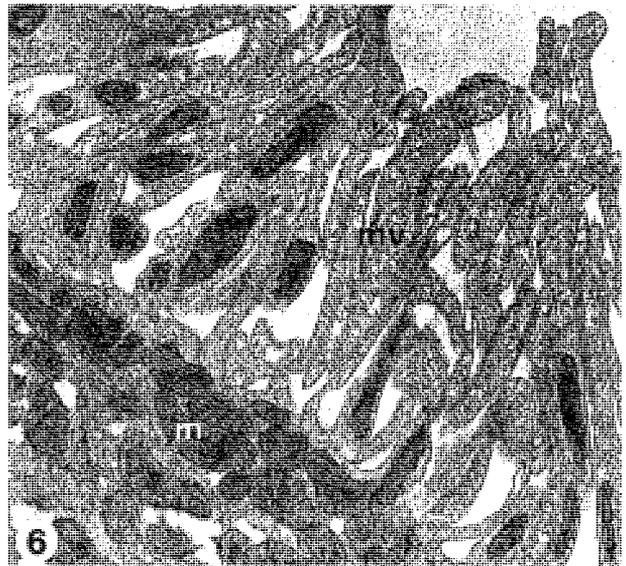
mitochondria themselves became very swollen. The cytoplasm had vacuolated to such an extent that no visible rough endoplasmic reticula could be recognised. Basally the mitochondria became bulbous in shape (Fig. 12) and the basal intracellular channels had suffered much damage. The chromatin materials in the nucleus had all been lost or dispersed. The nucleus had only the nuclear membrane left, resembling almost like an empty bag. The basement membrane, however,

could still be recognised.

Serosal treatment of the Malpighian tubule cells showed similar damage to that of the mucosal exposure (Figs. 14, 15). The basement membrane had been eroded to some extent. There was serious destruction of the basal intracellular channels. All the mitochondria had their cristae impaired and they became very much swollen. The cytoplasm was filled with large vacuoles. The apical microvilli had been completely destroyed.



**Figs. 4-5.** 4. Mucosal exposure of principal cell treated with  $0.00001 \mu\text{g/ml}$  toxin for 1 min. Shows numerous vacuoles (v), microvilli (mv), mitochondria (m), and the nucleus (n) with dispersed chromatin materials.  $\times 4,300$ . 5. As above. Basal region. Shows mitochondria (m), basement membrane (bm), rough endoplasmic reticulum (er), and disorganised intracellular channels (i).  $\times 6,600$ .

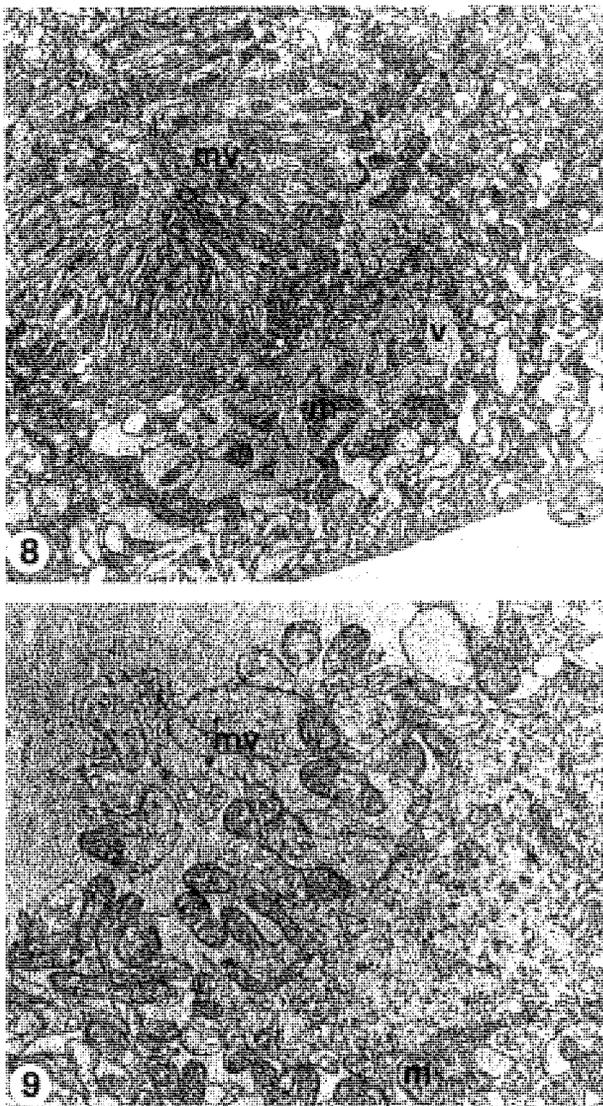


**Figs. 6-7.** 6. Serosal exposure of principal cell treated with  $0.001 \mu\text{g/ml}$  toxin for 1 min. Shows relatively undamaged microvilli (mv) and normal mitochondria (m).  $\times 7,600$ . 7. As above. Basal region. Shows disorganised intracellular channels (i), numerous vacuoles (v), and mitochondria (m).  $\times 7,600$ .

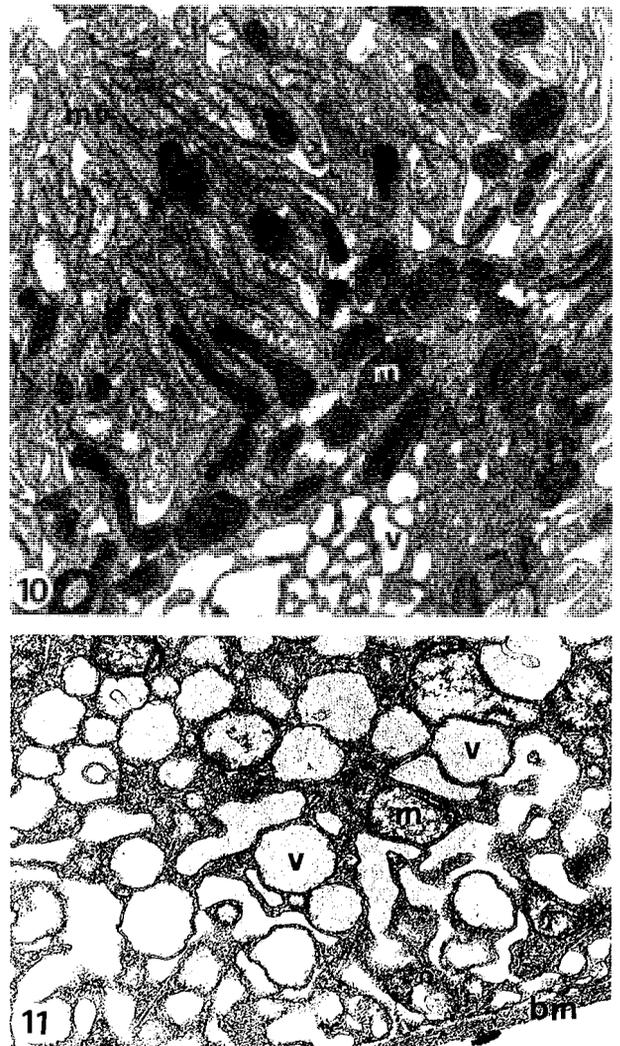
## DISCUSSION

Heimpel and Angus (1959) reported that the action of Bt on different lepidopterous larvae (primarily acting on the midgut) differed in their response. There were caterpillars which showed immediate response to Bt treatment (within one to seven hours). There were caterpillars which died within two to seven days and finally there were caterpillars which were killed by septicemia rather than the toxin.

Reisner et al. (1989) studied the histopathological effects of Btk  $\delta$ -endotoxin on *Calpodex* Malpighian tubules. At one hour post-treatment, the principal cells exhibited various cytopathological changes with a progression of severity from low to high toxin concentration. Mucosal exposure of cells to 16  $\mu\text{g/ml}$  63 kDa toxin completely inhibited urine secretion and induced massive cytolysis. Serosal exposure required a 20-fold higher concentration (314  $\mu\text{g/ml}$  toxin) to bring about a similar effect. Mucosal exposure resulted first in microvilli



**Figs. 8-9.** 8. Mucosal exposure of principal cell treated with 0.00001  $\mu\text{g/ml}$  toxin for 10 min. Shows damaged microvilli (mv), numerous vacuoles (v), and mitochondria (m). x4,600. 9. As above. Apical region. Shows damaged microvilli (mv) and mitochondria (m). x7,600.



**Figs. 10-11.** 10. Serosal exposure of principal cell treated with 0.001  $\mu\text{g/ml}$  toxin for 10 min. Shows microvilli (mv), vacuoles (v), and mitochondria (m). x7,400. 11. As above. Basal region. Shows numerous vacuoles (v), mitochondria (m) with cristae damaged, and basement membrane (bm). x8,700.

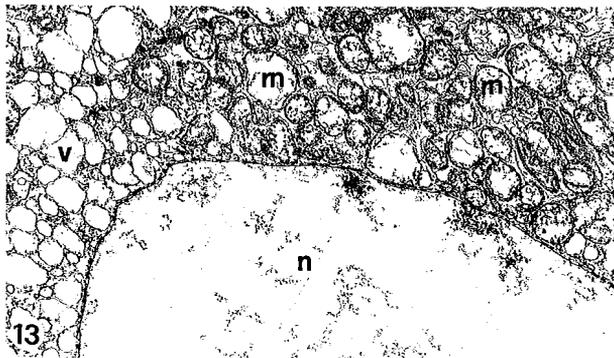
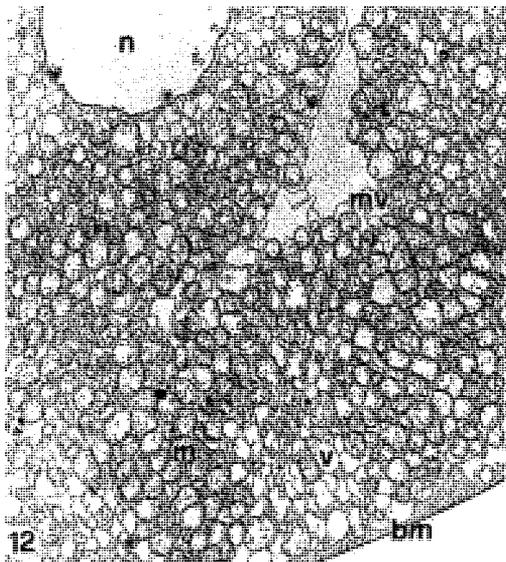
disruption whilst serosal exposure resulted in damage to the basal infolds first. The progress from low to severe damage could be broadly categorized into 3 stages: stage 1 had only slight enlargement of cytoplasmic spaces, stage 2 had further enlargement of these spaces and stage 3 had complete destruction of microvilli, basal infolds, endoplasmic reticula, and mitochondria.

Our results on *Pieris* larvae showed similar effects though the required toxin concentration was much lower and the time required to have comparable cytopathological damages was much shorter. This might indicate that *Pieris* larvae could be more susceptible to Btk  $\delta$ -endotoxin impairment than *Calpodex* larvae.

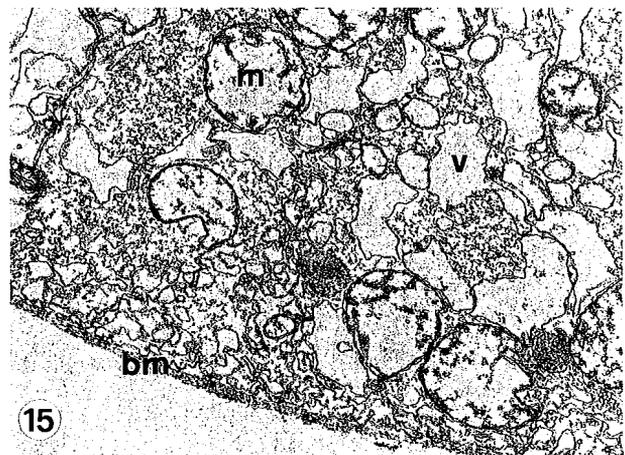
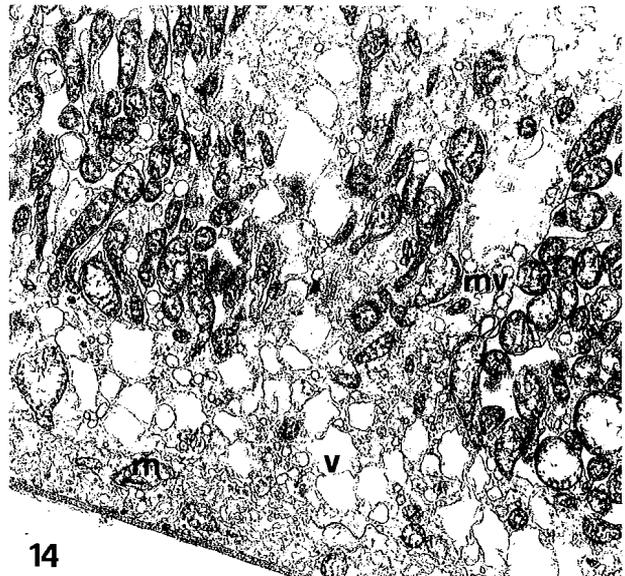
*Pieris* Malpighian tubules apparently show a

more sensitive response than its midgut cells to Btk treatment. Cheung et al. (1990) found that *Pieris* midgut cells required at least one hour of exposure in order to have similar organelle damage. The result discrepancies might be due to the fact that midgut cells were larger in size and the Btk toxin used in the midgut experiment was a crude spore-toxin mixture instead of purified  $\delta$ -endotoxin.

When purified  $\delta$ -endotoxin was used in experiments on other lepidopterous larvae, other toxicologists were able to demonstrate that the Btk  $\delta$ -endotoxin also acts quickly on midgut cells. For example, Percy and Fast (1983) noticed that



**Figs. 12-13.** 12. Mucosal exposure of principal cell treated with 0.00001  $\mu\text{g/ml}$  toxin for 20 min. Shows damaged microvilli (mv), nucleus (n), mitochondria (m), vacuoles (v), and basement membrane (bm).  $\times 3,100$ . 13. As above. Part of nuclear region. Shows numerous vacuoles (v), damaged mitochondria (m), and nucleus (n) with chromatin materials lost.  $\times 4,600$



**Figs. 14-15.** 14. Serosal exposure of principal cell treated with 0.001  $\mu\text{g/ml}$  toxin for 20 min. Shows damaged microvilli (mv), vacuoles (v), and mitochondria (m).  $\times 4,200$ . 15. As above. Basal region. Shows damaged basement membrane (bm), numerous vacuoles (v), and mitochondria (m) with cristae impaired.  $\times 8,500$ .

the *Bombyx* midgut cells exhibited cytopathological effects after a one minute treatment. However, slightly longer times were required to have similar results for *Manduca* (Gupta et al. 1985, Lane et al. 1989).

As pointed out by Reisner et al. (1989), response differences could be due to a variety of variables associated with the tissue type, time, and concentration of toxin exposure. Other factors such as the developmental stage and fixation conditions might add to the complications.

In general, it might be said that the primary effect of Btk  $\delta$ -endotoxin is cellular membranes damage. Several hypotheses have been proposed to explain the mechanism of the  $\delta$ -endotoxin: 1) it binds to a specific plasma membrane and forms lytic pores (Knowles and Ellar 1987), 2) it uncouples oxidative phosphorylation (Travers et al. 1976), 3) it specifically affects  $\text{Na}^+$  and/or  $\text{K}^+$  transport (Himeno et al. 1985), 4) it inhibits an epithelial  $\text{K}^+$  pump (Gupta et al. 1985), 5) it causes a general breakdown of cell permeability barriers (Nishiitsutsuji-Uwo and Endo 1980).

Our results showed that Bt  $\delta$ -endotoxin had damaging effects on epithelial membranes other than the midgut. It might create lytic pores by binding to specific membrane areas or receptors resulting in osmotic lysis of cells as proposed by Knowles and Ellar (1987).

Ryerse et al. (1990) demonstrated the presence of Bt binding areas on the midgut and Malpighian tubule membranes of *Heliothis* with an immunofluorescent method at the light microscopic level. These "receptors" were randomly distributed all over the exposed epithelial membranes. Thus future specific antibody probes at the ultrastructural level need to be carried out in order to better understand the nature and distribution of these receptors.

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## 蘇力菌 $\delta$ -內毒素對東方粉蝶幼蟲馬氏管之組織病理影響

王建斌<sup>1</sup> 張偉權<sup>1</sup>

東方粉蝶幼蟲馬氏管細胞經蘇力菌 $\delta$ -內毒素在粘膜面和漿膜面處理後，對其組織病理影響進行研究。結果顯示經過毒素一分鐘處理後的組織，其超微結構已經改變。這些變化包括細胞質中液泡增加，頂部微絨毛和基部細胞膜內管道輕微紊亂，但線粒體仍然顯示正常。經毒素10分鐘處理後，細胞質有些大液泡或大細胞間隙，微絨毛受到廣泛的破壞，並且頂部細胞質從細胞中擠出，同時基部細胞膜內折疊也受到損壞。經毒素20分鐘處理後，可觀察到整個細胞已經分解，微絨毛完全損壞，頂膜溶解，所有線粒體都變成了膨脹形，粗糙內質網和細胞質極大地液泡化，基部細胞膜內折疊十分紊亂，並基膜損壞。這些結果表明蘇力菌 $\delta$ -內毒素能通過和細胞膜結合，引至產生大孔，導致細胞漏泄。

關鍵詞：細菌，昆蟲排泄系統，超微構造。

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