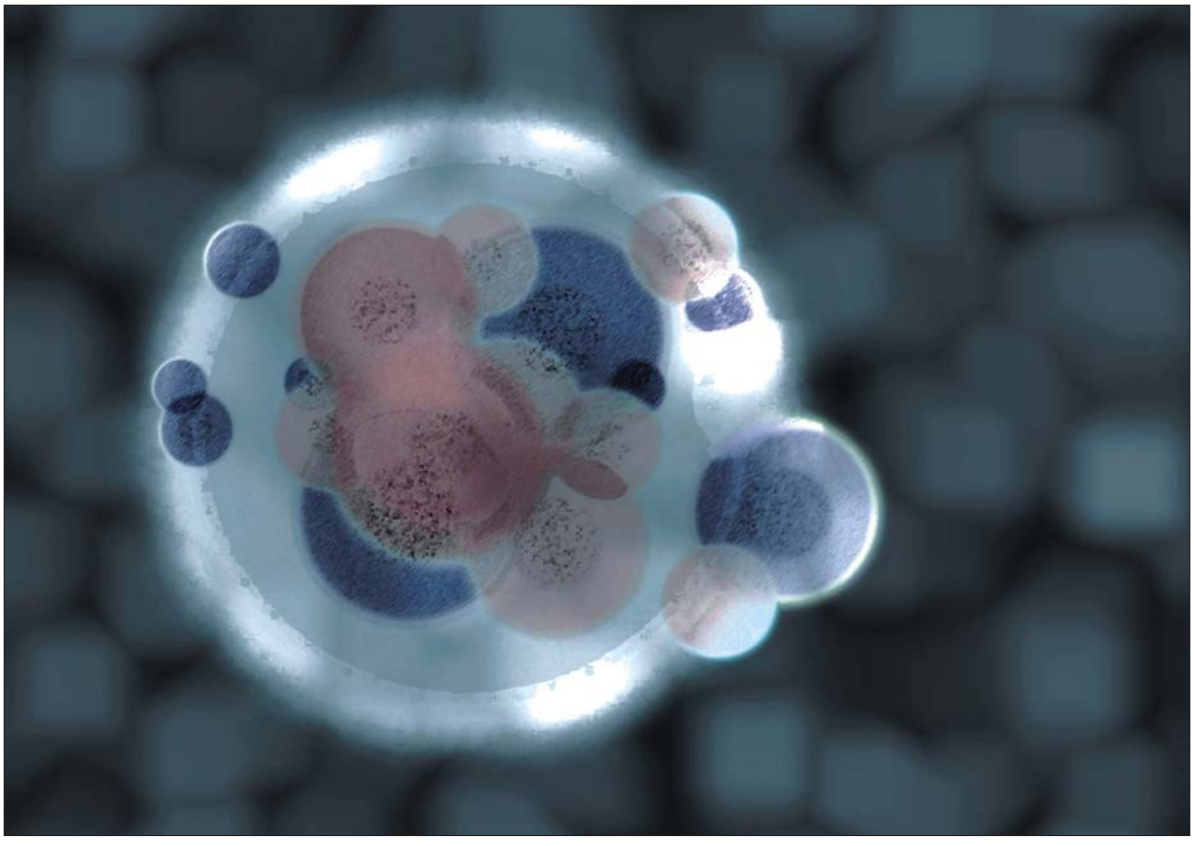

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Histological and Ultrastructural Changes Induced by Two Carbamate Molluscicides on the Digestive Gland of *Eobania vermiculata*

[1]Sherifa S. Hamed, [1]Nabila E. Abdelmeguied, [1]Amina E. Essawy,
[2]Mohamed A. Radwan and [1]Amira E. Hegazy
[1]Department of Zoology, Faculty of Science, Alexandria University, Moharram Bey, Alexandria 2151, Egypt
[2]Department of Pesticide Chemistry, Faculty of Agriculture (El-Shatby), Alexandria University, Alexandria 21545, Egypt

Abstract: Terrestrial snails are destructive agricultural pests as they cause a great damage to vegetables and crops. The present study was designed to evaluate the toxic action of two carbamate molluscicides, methomyl and methiocarb on the digestive gland of the land snail *E. vermiculata* which is the main site of accumulation and biotransformation of xenobiotics, using topical application and baiting techniques. Sublethal doses and concentrations of both pesticides were applied. After 1, 3, 5, 7 and 14 days of treatment, definite number of snails from each group were chosen and prepared for the aimed studies. Histopathological and ultrastructural alterations in the digestive gland were more obvious after topical application than after baiting technique and methomyl was found to be more toxic than methiocarb. These alterations included hemocyte infiltration, bizarre nuclei that ranged in their degenerative changes from karyolysis to severe karyorrhexis and complete pyknosis, after methomyl treatment and extensive destruction and disorganization of the intertubular connective tissue, after methiocarb treatment. In addition, severe cytoplasmic vacuolization, disruption and reduction of microvilli and formation of surface blabs, increased number of calcium spherules in calcium cells and an aberrant increase in the number of excretory cells containing large number of excretory granules or residual bodies were observed after treatment with both molluscicides. These results are important from the economical point of view since the use of low doses of molluscicides was shown effective, more feasible and less harmful to non-target species like vertebrate animals and human beings.

Key words: Digestive gland, *Eobania vermiculata*, methomyl, methiocarb

INTRODUCTION

Land molluscs including snails and slugs are considered of an economic importance among the animals and pests attacking different types of plants causing economic damage to a wide variety of plants including vegetables, forage crops, tree fruits, shrubs, flowers, ground green cover and newly sown lawn grasses. Moreover, they play an important role in transmitting and spreading diseases to cultivated plants (Godan, 1983). In Egypt, the land snail, *Eobania vermiculata* Müller (Mollusca: Gastropoda: Stylommatophora: Helicidae) is an important predominant agricultural pest and is becoming a real threat to crop and orchard production in different localities in northern coastal areas of Egypt (El-Okda, 1983).

Various attempts were made for detecting the molluscicidal effects using several pesticides including carbamates which are a potent class of molluscicides (Miller *et al*., 1988; Radwan *et al*., 1992; Schuytema *et al*., 1994). Methiocarb and methomyl are two carbamates that have been considered effective, little affected by environmental conditions and their toxicity increases in humid conditions which are favorable for terrestrial gastropods (El-Sebae *et al*., 1982).

Both topical application and toxic baits methods have been reported effective against terrestrial gastropods. However, the bait should be less repellent to them allowing more effective ingredients to be consumed before termination of feeding (Bourne *et al*., 1988). In topical application technique, the molluscicides can affect snails and slugs by absorption through the skin.

The digestive gland of molluscs is involved in extracellular and intracellular digestion of food material, absorption of nutrients; storage of lipids, glycogen and minerals and it plays also a major role in detoxification (Beeby and Richmond, 1988; Henry *et al*., 1991). The morphology and histology of the digestive gland of the

Corresponding Author: Sherifa S. Hamed, Department of Zoology, Faculty of Science, Alexandria University, Moharram Bey, Alexandria 2151, Egypt

land snail *E. vermiculata* have been described in a number of studies (Saad and Farag, 1988; Mersal, 1990; Aioub *et al*., 2000). The cytological features of the digestive gland were reported in several species of aquatic gastropods (El-Saadany *et al*., 1990; Muley and Mane, 1990; Taïeb and Vicente, 1999; Dimitriadis and Andrews, 2000; Lobo-da-Cunha, 2000; Taïeb, 2001). Although information is available on the morphology and physiology of the digestive gland cells, there are only few studies describe the number and types of the cells that constitute the digestive gland epithelium of terrestrial gastropods, probably because of its multifunctional nature and the significant ultrastructural changes during digestion.

The molluscicidal activity of plant extracts on the histology of the digestive gland of snails acting as intermediate hosts have been suggested in scattered cases in the literature (Bode *et al*., 1996; Brackenbury, 1999). In addition, several investigators have studied the histological and histochemical changes induced by molluscicides on the digestive gland of aquatic gastropods (Muley and Mane, 1990; Jonnalagadda and Rao, 1996; Rondelaud and Dreyfuss, 1996; El-Mehlawy and Rizk, 2000). Also, several investigations have been performed in order to enable a correlation of molluscicide-induced structural alterations in several cell types of slug tissue to functional aspects (Triebskorn, 1989; Triebskorn and Künast, 1990; Triebskorn and Köhler, 1992).

However, even today a detailed ultrastructural study dealing with the effect of molluscicides on the digestive gland of land snails is missing, in spite of the attention that this organ and these organisms have received from other points of view. Thus the consideration of studying the cellular and subcellular alterations in response to molluscicide action might be interesting.

MATERIALS AND METHODS

Adult herbivorous terrestrial snail, *Eobania vermiculata* (Müller) (Pulmonata: Stylommatophora: Helicidae) were collected from different gardens at Alexandria Governorate, Egypt, during spring and autumn seasons of (2002-2005) and transferred in plastic bags to the laboratory of Pesticide Chemistry Department, Faculty of Agriculture, Alexandria University. They were identified according to the key of Godan (1983). Adult snails having mean shell diameter of 20 mm±1.44 and mean body weight 2.55 g±0.23 were kept in open air cages (40×30×30 cm, with 100 individuals per cage), allowed to feed on fresh leaves of lettuce and kept to acclimatize under laboratory conditions (26-30°C and 63-64 R.H) over two weeks prior to the experiment.

Experimental design: Sublethal doses (¼ $LD_{50,}$ pesticides dissolved in dimethyl sulphoxide) and concentrations (¼ LC_{50}) of both methomyl and methiocarb were topically applied on the surface of the snail body inside the shell using a micropipette or provided as toxic baits to the snails, respectively. The animals were divided into six groups, with 250 animals per group (ten animals in each box.). Few milliliters of water were added daily into each box to provide humidity for snail activity.

N.B. LD_{50} and LC_{50} were calculated by the same authors in a manuscript under processing for publication.

Group I (-ve control): Animals of this group received no treatment (fed on bait-free from pesticides) and were considered as normal controls.

Group II (+ve control): Animals of this group received a single dose of 25 μL of DMSO was topically applied on the surface of the animal body and was considered as positive control.

Group III: Animals of this group received a single dose (25 μL) of ¼ LD_{50} of methomyl (i.e., 22.5 μg $snai^{-1}$) was topically applied on the surface of the snail body.

Group IV: Animals of this group were fed on toxic bait (wheat-bran bait, 0.05 metyelene blue dye and 2% molasses) containing ¼ LC_{50} of methomyl (0.08% w/w).

Group V: Animals of this group received a singe dose (25 μL) of ¼ LD_{50} of methiocarb (i.e., 103.3 μg $snail^{-1}$) was topically applied on the surface of the snail body.

Group VI: Animals of this group were fed on toxic bait (wheat-bran bait, 0.05 metyelene blue dye and 2% molasses) containing ¼ LC_{50} of methiocarb (0.23% w/w).

Dissection and tissue preparation: After 1, 3, 5, 7 and 14 days of treatment, definite number of snails from all groups were randomly chosen for histological and ultrastructural studies.

Shells of unanaethetized tested snails were removed by making a cut round the whorls in a continuous manner starting at the aperture opening using a bone scissors and the broken fragments of the shell were carefully removed. Dissection was carried out under Zeiss binocular microscope.

For histological studies, digestive glands from animals of different experimental groups were rapidly excised and immediately dropped in either 10% formalin or Bouin's solutions. Graded dehydration of the tissue was done by 70-100% alcohol in subsequent steps. Methyl

benzoate was used as a clearing agent. Tissues were embedded in paraffin (58.6°C) and sections were cut by a rotatory microtome (5 μm) and stained with hematoxylin and eosin or with Mallory's triple stain.

Cell size as well as nuclear diameter were measured with a calibrated ocular scale (10x), using a Zeiss microscope (40x) objective and a microcytometer (American Optical).

For electron microscopical studies, small pieces of fresh specimens of digestive gland were removed from animals after 14 days of treatment. The tissues were fixed by immersing them immediately in formalin-glutaraldehyde fixative ($_4F_1G$) in phosphate buffer solution (pH 7.2) at 4°C for 3 h. Specimens were then postfixed in 2% OsO_4 in the same buffer at 4°C for 2 h. Samples were washed in the buffer and dehydrated at 4°C through a graded series of ethanol. Specimens were embedded in Epon-araldite mixture in labeled beam capsules. Semithin sections (1 μm thick) were stained with toluidine blue. Ultrathin sections (60-70 nm thick) were picked upon 200 mesh naked copper grids and double stained with uranyl acetate for ½ h and lead citrate for 20-30 min (Reynolds, 1963). Specimens were examined under Jeol 100 CX TEM.

RESULTS

I-Anatomical and Histological observations of the digestive gland in control groups (GI and GII): The digestive gland of *E. vermiculata* is a bilobed tubulo-acinar gland located in the dorsal portion of the animal and it is surrounded by a thin membrane composed of a single layer of short columnar cells resting on a conspicuous basal membrane underlies with circular muscle fibers (Fig. 1a and b). The digestive gland tissue consists mainly of digestive tubules with spherical (108-165 μm in diameter) or oval (186- 306 μm length and 69- 174 μm width) shape separated by intertubular loose connective tissue containing hemolymphatic vessels and hemocytes (Fig. 1a and c). Each tubule is provided externally with circular muscle layer. Four different cellular populations were observed in the epithelium lining the lumen of the digestive gland tubules, three of which were easily identified than the fourth cell type. The cells are differentiated into (a) digestive cells, (b) calcium cells, (c) excretory cells and (d) thin cells (Fig. 1c).

Digestive cells: Digestive cells constitute the major cellular component of the digestive gland tubule epithelium and they show important morphofunctional alterations according to the digestive activity of the animal. Digestive cells at the absorptive and digestive phases are columnar measure 45±7.35 μm in height, with flattened or slightly rounded apical surfaces bearing well-developed brush border and their cytoplasm possesses abundant vacuoles (Fig. 1d). The basally-located nuclei of digestive cells are rounded or oval in outline (about 3.5-5 μm in diameter) with condensed chromatin and a single nucleolus. Two types of granules were demonstrated in Mallory's triple stain preparations. Green granules are found within vacuoles and are being limited towards the distal extremity of the cell and yellow granules appear in groups within cytoplasmic vacuoles in the basal region (Fig. 1c). Large round brown granules are considered as an advanced stage of yellow granules.

Calcium cells: Calcium cells are fewer than digestive cells and they occur either singly or in pairs in the corners of the tubules (Fig. 1c and e). They have pyramidal shape (40.95±8.13 μm in height) with narrow distal end and a marked broad base (35.70±5.74 μm). Calcium spherules are round, lightly refractive bodies with a diameter of 2.82±0.93 μm and give an orange-red colour in Alizarin red S preparations (Fig. 6a). Calcium cells possess apical secretory granules and large rounded nuclei (9.6±1.27 μm in diameter), rich in heterochromatin and possess a sharply outlined nucleolus usually located near the center or in the basal half of the cell (Fig. 1c and e).

Excretory cells: Excretory cells have globular shape (39.55±5.09 μm height and 25.2±7.77 μm width). They are characterized by the presence of a single large vacuole filling nearly the whole volume of the cell (Fig. 1c and e). The excretory products are accumulated in the vacuole often in the form of a large brown body. The free surface of the cell possesses a well developed brush border. The nucleus is small and pressed flat against the cell base.

Thin cells: Thin cells were rarely observed in our light microscopical preparations. They are distributed randomly between the other cell types.

Hemocytes: Different types of hemocytes were identified in the intertubular connective tissue (Fig. 1c). The blast-like cell is the smallest and displays round, oval or pear shape with a large nucleus occupying the entire cell volume. Avacuolar hemocytes are polymorphic with peripherally located rounded nucleus. The granular or vacuolar hemocytes dominate the hemocyte population. They are large, variably shaped cells, ranging from ovoid to kidney-like shape with many pseudopod-like protrusions. They possess a small central or eccentric nucleus and their cytoplasm is filled with granules.

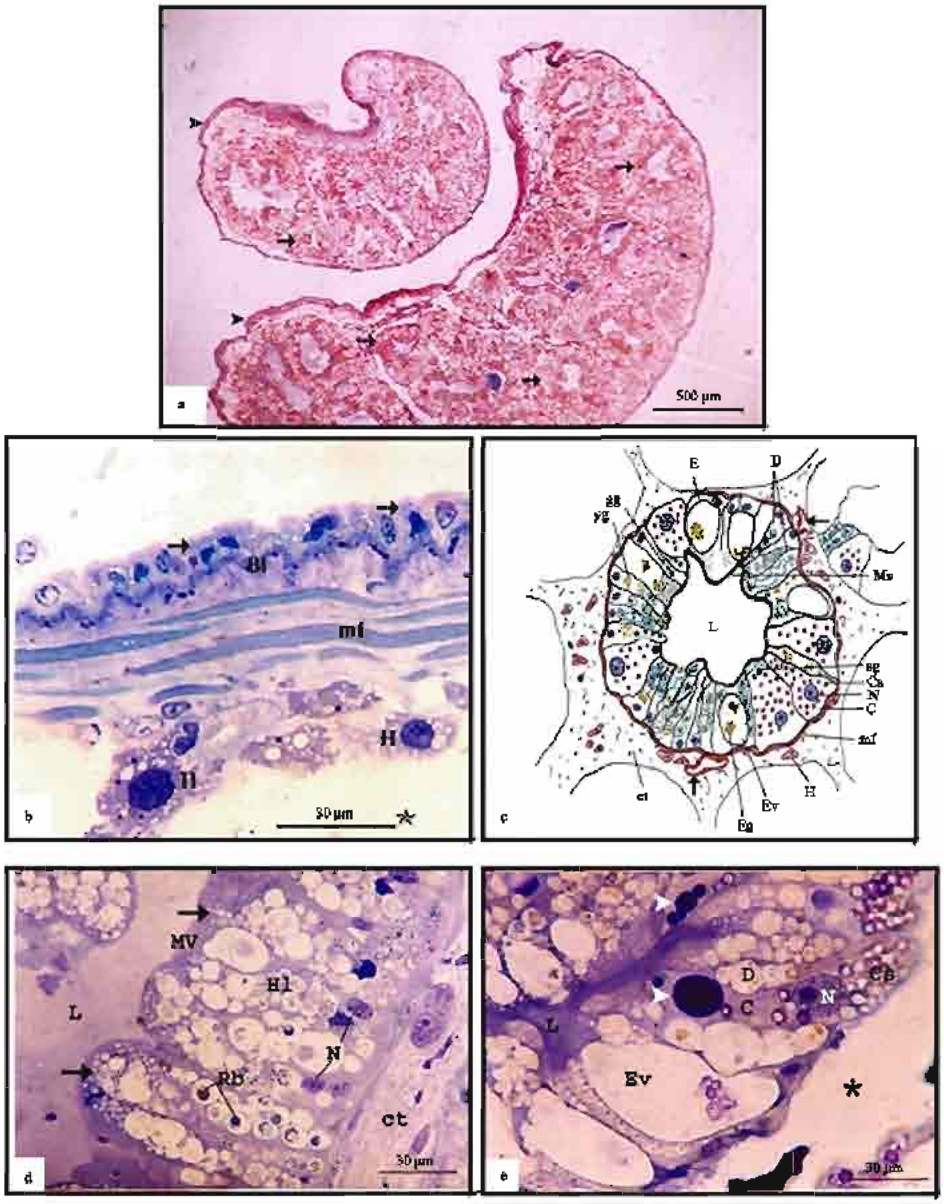

Fig. 1: T.S. of the digestive gland of control *E. vermiculata* (GI). (a) Light micrograph (L.M.) demonstrating the thin coat (arrowheads) surrounding the whole gland and the digestive tubules (arrows) with different size and shape (Mallory's triple stain). (b) Semithin section of the digestive gland coat with one-layered epithelium (arrows) resting on basal lamina (Bl). Muscle fibers (mf), hemocytes (H), hemolymph space (asterisk). ($_4F_1G$- toluidine blue). (c) Schematic diagram of a transverse section through a tubule of the digestive gland. Digestive cell (D); calcium cell (C); excretory cell (E); Calcium spherules (Cs); intertubular connective tissue (ct); excretory granules (Eg); excretory vacuole (Ev); green granules (gg); hemocytes (H); lumen (L); muscle fibers (mf); microvilli (MV); nucleus (N); secretory granules (sg); yellow granules (yg). Arrows point at hemolymphatic vessels. (d) Semithin section illustrating digestive cells at the absorptive and digestive phase. Note: apical small vesicles (arrows), heterolysosomes (Hl) and residual bodies (Rb). Intertubular connective tissue (ct); lumen (L); brush border microvilli (MV); nucleus (N). ($_4F_1G$- toluidine blue) and (e) Semithin section showing calcium cell (C) with nucleus (N), calcium spherules (Cs); apical protein granules (arrowheads); excretory vacuoles (Ev). Digestive cell (D); lumen (L); hemolymph space (asterisk). ($_4F_1G$- toluidine blue)

Table 1: Degree of histopathological alterations of the digestive gland of *E. vermiculata*, after treatment with methomyl and methiocarb

Treatment	Time of exposure	Dilatation of hemolymph space	Hemocyte infiltration	Narrowing of tubule lumen	Digestive-cell breakdown	Pyknosis of nuclei	Enlargement of yellow granules	Enlargement of excretory granules
Control		-	-	-/+	-/+	-	-	-
Methomyl	1	-	++/+++	++/+++	+/++	-/+	+/++	+
Topical application	3	+	+++	++/+++	++	-/+	++	+++
	5	-	+++	+++	++/+++	++/+++	+	-/+
	7	-/+	+/++	+++	++/+++	++/+++	-/+	+/++
	14	+	+++	-/+	+++	+++	-	+/++
Toxic bait	1	-/+	++/+++	++	++/+++	-	-	++
	3	+	++	++	+	-	+	+
	5	-/+	++/+++	+	++	-	++	+++
	7	-/+	++	++	+++	+++	++	+
	14	-		+++	+++	+++	-	+++
Methiocarb	1	+	-/+	+++	++	+++	+++	+
Topical application	3	+/++	-	+++	+++	+++	-	-
	5	+/++	+	+++	+++	+++	+	+++
	7	++	-	+++	+++	+++	+	-
	14	++	-	+++	+++	+++	+	+++
Toxic bait	1	+/++	-	+++	++/+++	+++	-	++
	3	++/+++	-	++/+++	++/+++	+++	++	-
	5	++	-	++	++/+++	+++	-	-
	7	+/++	-	++	+++	+++	-	-
	14	++/+++	+	+++	+++	+++	-	++

-: No change; -/+: Weak positive; +: Positive; ++: Middle; +++: Strong positive

At the light microscopic level, no histological changes were observed in the structure of digestive gland of snails treated with the solvent (GII). The gland tissue was more or less similar to that in (GI).

II-Histopathological changes of the digestive gland: The digestive gland tissue of *E. vermiculata* exhibited marked histopathological alterations in response to treatment by either topical application or toxic baits of both methomyl and methiocarb. The degree of histopathological changes is summarized in Table 1.

Methomyl-treated group (topical application) (GIII): One and three days after topical application of methomyl, digestive cells show a remarkable breakdown, vacuolization and increased number of large green granules (Fig. 2a). Some pyknotic nuclei were observed in calcium cells. There is also a remarkable increase in the number and size of excretory granules in the excretory vacuoles of excretory cells (Fig. 2a). After 5 and 7 days of treatment, digestive cells are severely affected, greatly vacuolated, have disrupted apical border and their cytoplasm show increased incidence of green granules and their nuclei appeared pyknotic. There is an increase in the number of calcium cells and in the size of most calcium spherules. Their cytoplasm is degenerated and their nuclei show shrinkage and karyolysis. Excretory cells show obvious alterations including disruption of their luminal border, disintegration of cellular boundaries and increased number of excretory granules. Fourteen days after treatment, severe infiltration of hemocytes was observed in the intertubular connective tissue and most of them appeared to contain deeply stained granules (Fig. 2b). There is complete destruction of digestive cells with nearly no visible cytoplasmic inclusions, breakdown of apical cell membrane, loss of cellular boundaries and karyolysis of nuclei (Fig. 2c). The cytoplasm of most calcium cells appear packed with large number of calcium spherules (Fig. 6b). In most tubules, excretory cells are hardly to be distinguished.

Methomyl-treated group (toxic baits) (GIV): One day after treatment, a few tubules with severe atrophy are mingled in some places. After 3, 5 and 7 days of treatment, hemocytic infiltration was frequently observed and the basement membrane of tubules appeared irregular and relatively thickened. The digestive cells show accumulation of large numbers of green and yellow granules and appear to undergo extensive breakdown into membrane-bound vesicles (Fig. 3a and b). Calcium cells are packed with enlarged calcium spherules (Fig. 6c) and they exhibit pyknotic nuclei. The cytoplasm of most calcium cells is replaced by large vacuoles containing yellow granules. Excretory cells show increased number of excretory granules. At 14 days of treatment, destruction of most tubules was complete and degeneration of tubular epithelium was evident (Fig. 3c). The thickened basement membrane and the intertubular connective tissue show severe structural disruption. Digestive cell cytoplasm appears to be nearly devoid of cytoplasmic granules and there is a marked reduction in the number of calcium cells and in the size of calcium spherules. In addition, calcium cells appeared with dissolute cytoplasm and karyolytic nuclei. Most excretory vacuoles of excretory cells appear with no obvious contents.

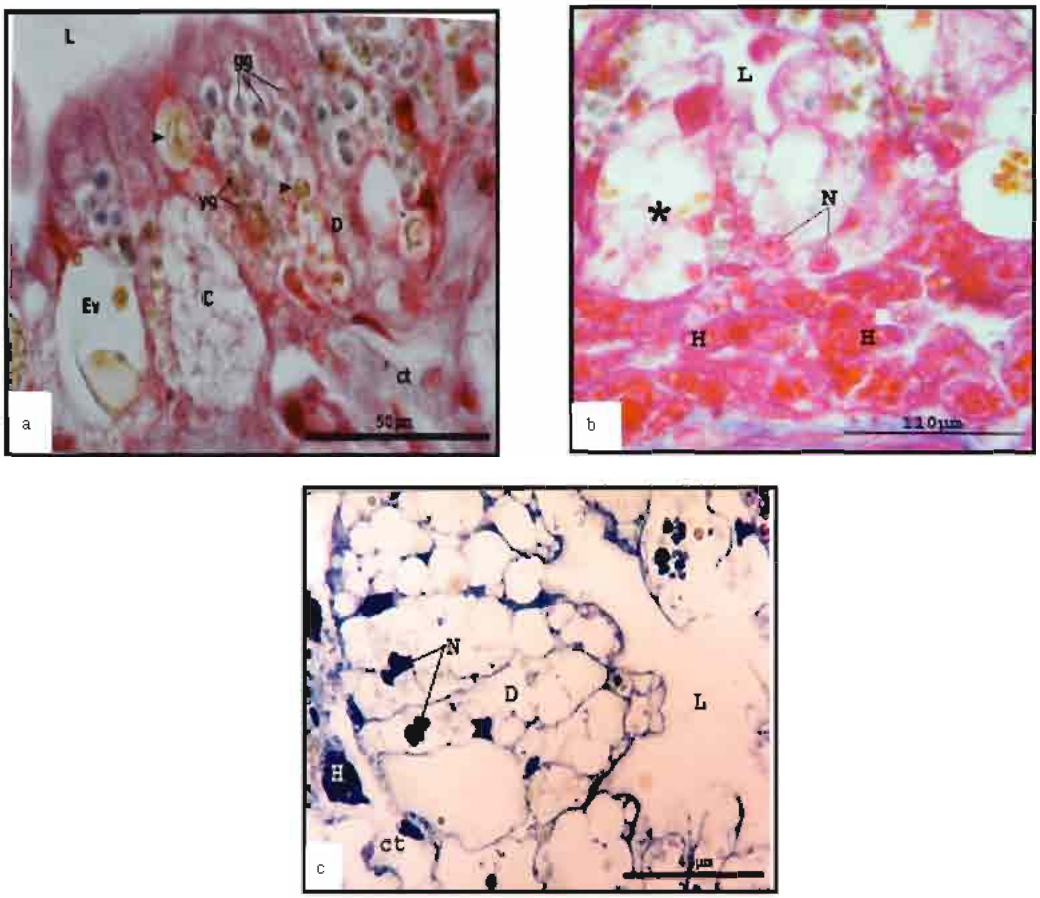

Fig. 2a-c: L.M. T.S. treated digestive gland of *E. vermiculata* (topical application of methomyl, GIII). (a) Three days after treatment, illustrating increased number of green granules (gg) and yellow granules (yg) in digestive cells (D). Note: Enlargement of some yellow granules (arrowheads). Calcium cell (C); excretory vacuole (Ev); lumen (L); intertubular connective tissue (ct). (Mallory's triple stain). (b) Fourteen days after treatment, showing large number of highly granulated hemocytes (H) and considerable vacuolation and necrosis of digestive tubule cells (asterisk); karyolytic nuclei (N); lumen (L). (Mallory's triple stain) and (c) Fourteen days after treatment, demonstrating severe vacuolization of digestive cells (D) with nearly no visible cytoplasmic inclusions and breakdown of apical cell membrane. Note: pyknotic nuclei (N); hemocyte (H); intertubular connective tissue (ct); lumen (L). ($_4F_1G$- toluidine blue)

Methiocarb-treated group (topical application) (GV): One day after treatment, some digestive cells appear packed with green and yellow granules or enlarged yellow granules. Severe reactions were mainly confined to calcium cells which possess swollen and dissolute cytoplasm and pyknotic irregularly-shaped nuclei with abnormal nucleolus (Fig. 4a). After 3 and 5 days of treatment, digestive cells possess karyolytic nuclei and their cytoplasm becomes pale with limited disruption and cytoplasmic vacuolation. Some calcium cells showed extensive pyknotic nuclei and dissolute cytoplasm; others possess karyolytic nucleus with marginated nucleolus. Excretory cells showed increase in the number and size of their excretory granules and disruption of their apical cytoplasm. Seven days after treatment, digestive cells show extensive fragmentation and their cytoplasm exhibit increased number of yellow granules. Calcium cells are greatly shrunken degenerated and their nuclei display severe pyknosis. In addition, most calcium cells show reduction in the number of calcium spherules (Fig. 6d). Excretory cells are frequently enlarged and possess increased aggregates of excretory granules. Fourteen days after treatment, a slight infiltration of hemocytes, especially granulocytes with deeply stained granules was

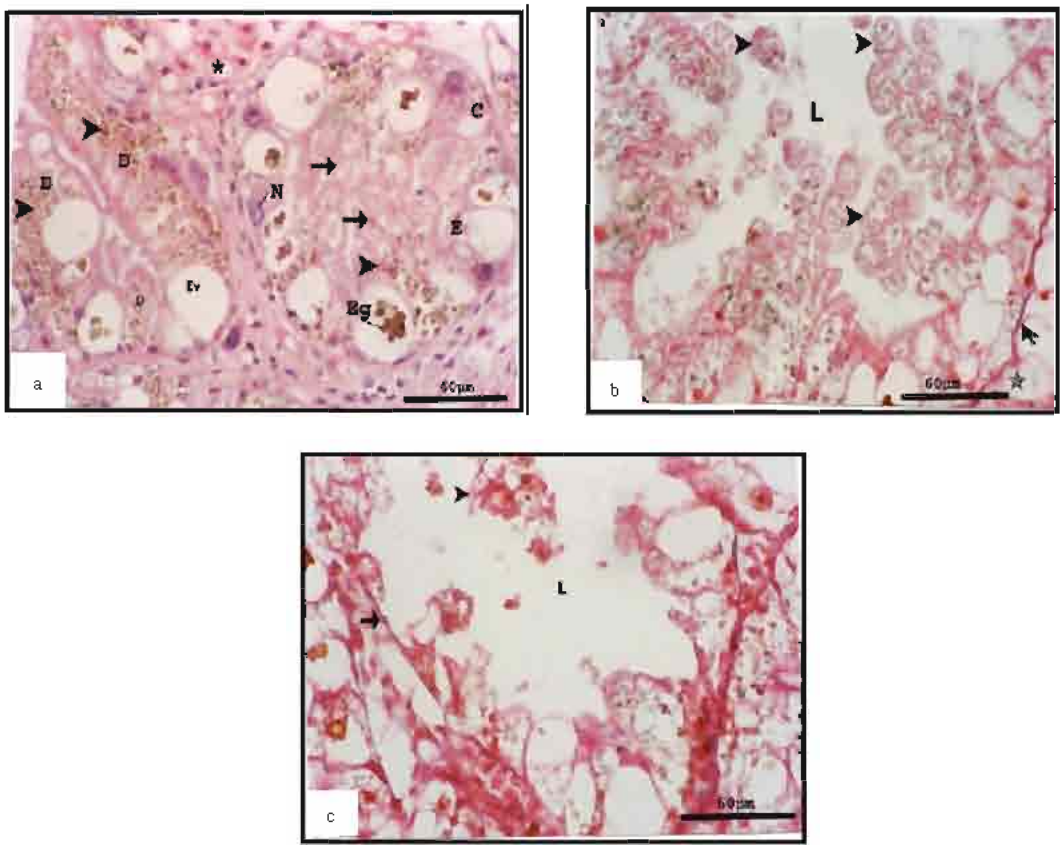

Fig. 3 a-c: L.M. T.S. treated digestive gland of *E. vermiculata* (methomyl bait, GIV). (a) Three days after treatment, illustrating large number of yellow granules (arrowheads) within digestive cells (D); increase in number and size of excretory vacuoles (Ev) and excretory granules (Eg); reduction in number of calcium cells (C); narrow lumen occluded by disintegrating cells (arrows); intertubular connective tissue filled with large number of small hemolymphatic vessels (asterisks); nucleus of calcium cell (N). (Haematoxylin and eosin). (b) Five days after treatment, showing extensive digestive-cell breakdown and the release of fragmented membrane-bound vesicles (arrowheads) into the Tubule lumen (L). Irregular, relatively thickened basement membrane (arrow); intertubular connective tissue (asterisk). (Mallory's triple stain and (c) Fourteen days after treatment, showing sloughing of disintegrating epithelial cells (arrowhead) into the lumen of digestive tubule (L). Note the clear view of the basal membrane (arrow) which is largely devoid of intact attached cells. (Mallory's triple stain)

observed (Fig. 4b). The basement membrane appears thickened and show extensive disruption. Digestive cells showed complete destruction of brush border, severe degeneration of cellular boundaries, extensive vacuolation of cytoplasm and karyolytic nuclei with poorly identified nuclear outlines (Fig. 4c). Calcium cells show reduction in their number, appear shrunken and exhibit dissolute cytoplasm and karyolytic nuclei. Moreover, excretory cells were not easily distinguished from the highly vacuolated digestive cells.

Methiocarb-treated group (toxic baits) (GVI): After 1 day of ingestion, moderate degeneration of digestive cells with lysed granules and pyknotic nuclei was observed. Calcium cells show swelling of cytoplasm and severe pyknosis of nuclei. No obvious pathological changes were observed in the structure of excretory cells, except for some pyknotic nuclei (Fig. 5a). Three and five days after treatment, digestive cells are greatly damaged and degenerated. They possess very low number of green and yellow granules and their nuclei are severely damaged

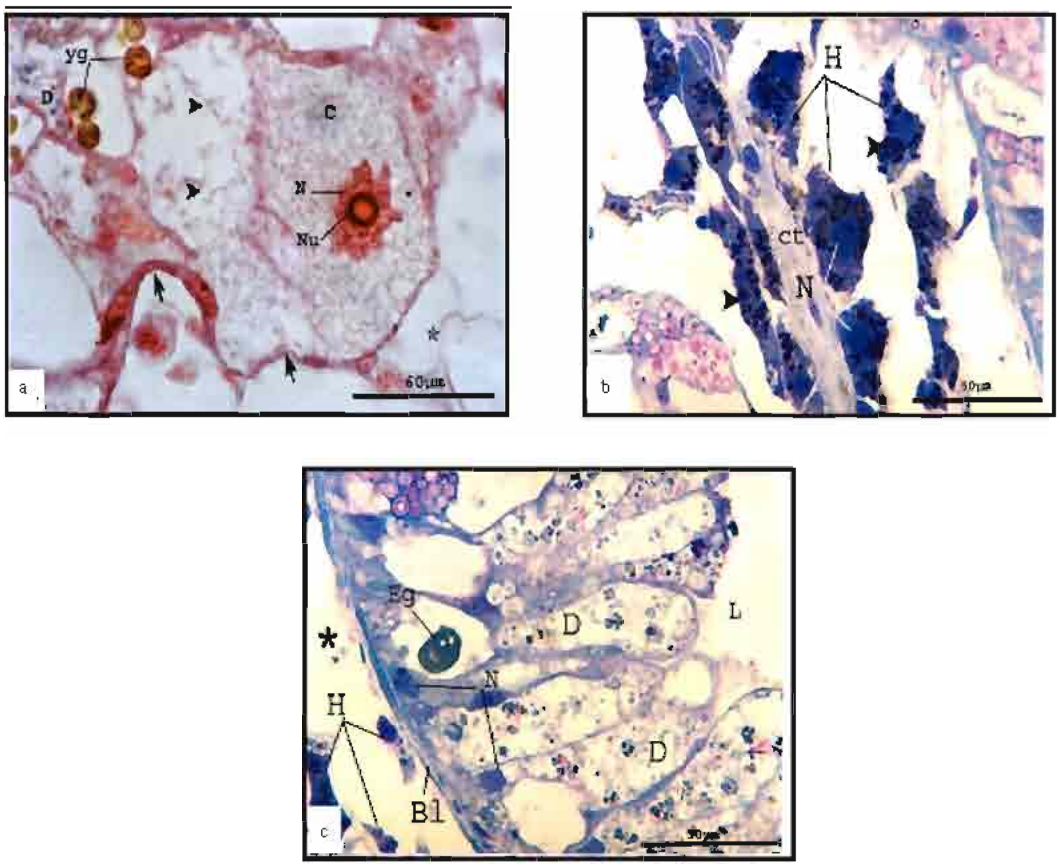

Fig. 4 a-c: L.M. T.S. treated digestive gland of *E. vermiculata* (topical application of methiocarb, GV). (a) One day after treatment, Showing calcium cell (C) with nucleus (N) having highly irregular nuclear membrane and abnormal nucleolus (Nu); parts of dissoluted cytoplasm (arrowheads). Note the folding of basal membrane (arrows); disorganized intertubular connective tissue (asterisk); enlarged yellow granules (yg) of digestive cells (D). (Mallory's triple stain). (b) Fourteen days after treatment, showing a number of hemocytes (H) in the intertubular connective tissue (ct). Note: pyknotic nuclei (N) and a vast number of large granules (arrowheads). ($_4F_1G$- toluidine blue) and (c) fourteen days after treatment, showing digestive cells (D) with degenerate cytoplasm and karyolytic nuclei (N); large excretory granule (Eg); lumen (L); basal lamina (Bl); hemolymph space (asterisk); hemocytes (H). ($_4F_1G$- toluidine blue)

(Fig. 5b). Calcium cells lost their distinct contours and show swelling of cytoplasm and pyknosis of their nuclei (Fig. 5b). In addition, they showed increase in the number and size of calcium spherules (Fig. 6e). Excretory cells became enlarged and their vacuoles assumed irregular outlines and appeared nearly with no visible contents. After 7 and 14 days of treatment, the cytoplasm of digestive cells appears completely degenerated with no obvious granules. In addition, cellular swelling, vacuolation and necrosis were observed for all calcium cells (Fig. 5c). Reduction in the number and size of calcium spherules was also observed. The nuclei appeared atrophied. Excretory cells were hardly to be identified in most tubule epithelia.

III-Ultrastructural observations of the digestive gland of *E. vermiculata*:

Control groups (GI and GII):

Digestive cells: The apical surface of these cells possess distinct microvillus border covered with a glycoprotein glycocalyx and the lateral surfaces of their apical regions are linked together by a zonula adherens followed by a long and pleated septate junction (Fig. 7a).

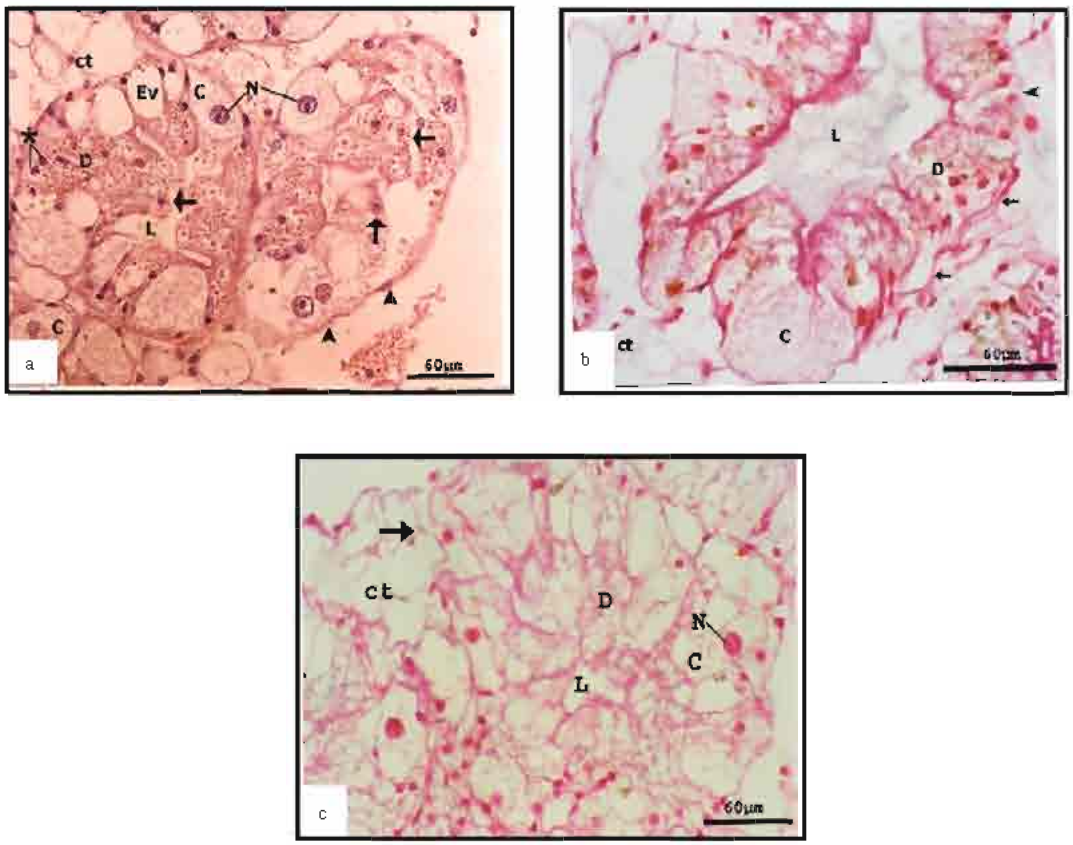

Fig. 5 a-c: L.M. T.S. treated digestive gland of *E. vermiculata* (methiocarb bait, GVI). (a) Three days after treatment, Showing extensive exfoliation of tubular epithelium leading to the presence of fragmented cells and nuclei (arrows) within the lumen of the tubule (L). Note: calcium cells (C) with dissoluted cytoplasm and karyolytic nuclei (N); digestive cells (D) with pyknotic nuclei (asterisk); excretory vacuole (Ev) of excretory cell; undistinguishable basal lamina (arrowheads); destructed intertubular connective tissue (ct). (Haematoxylin and eosin). (b) Five days after treatment, illustrating rupture and separation of the outer circular muscle layer from the epithelial lining (arrows); indistinguishable basal lamina (arrowhead); disorganized connective tissue (ct); degenerate digestive cells (D); swollen calcium cell (C); lumen (L). (Mallory's triple stain) and (c) Fourteen days after treatment, demonstrating intensive vacuolation and degeneration of digestive cells (D); necrotic calcium cells (C) with pyknotic nucleus (N); irregular basal lamina (arrow); destructed connective tissue (ct); occluded lumen (L). (Mallory's triple stain)

The most conspicuous organelles of the digestive cells are a well developed lysosomal vacuolar system consisting of numerous vesicles, cytoplasmic tubules and vacuoles. The apical region of these cells displayed vesicles (0.2- 0.5 µm in diameter) still associated with the plasma membrane and others located just beneath the membrane, both of these vesicles were typically coated. Some vesicular profiles were also found in deeper regions of the cytoplasm and it may correspond to green granules observed after light microscopic preparations. Beneath these organelles, are membrane-bound vacuoles or late endosomes (0.6-2.5 µm in diameter) containing homogeneous, moderate electron dense material. The subjacent cytoplasm is characterized by the presence of heterolysosomes containing heterogeneous material. Their shape could be almost spherical and usually had a size of about 2.5 µm, while some of them reached 6.4 µm. Residual bodies were frequent in the middle and basal regions of digestive cells. These bodies are believed to correspond to Yellow granules revealed with light microscopy (Fig. 7b).

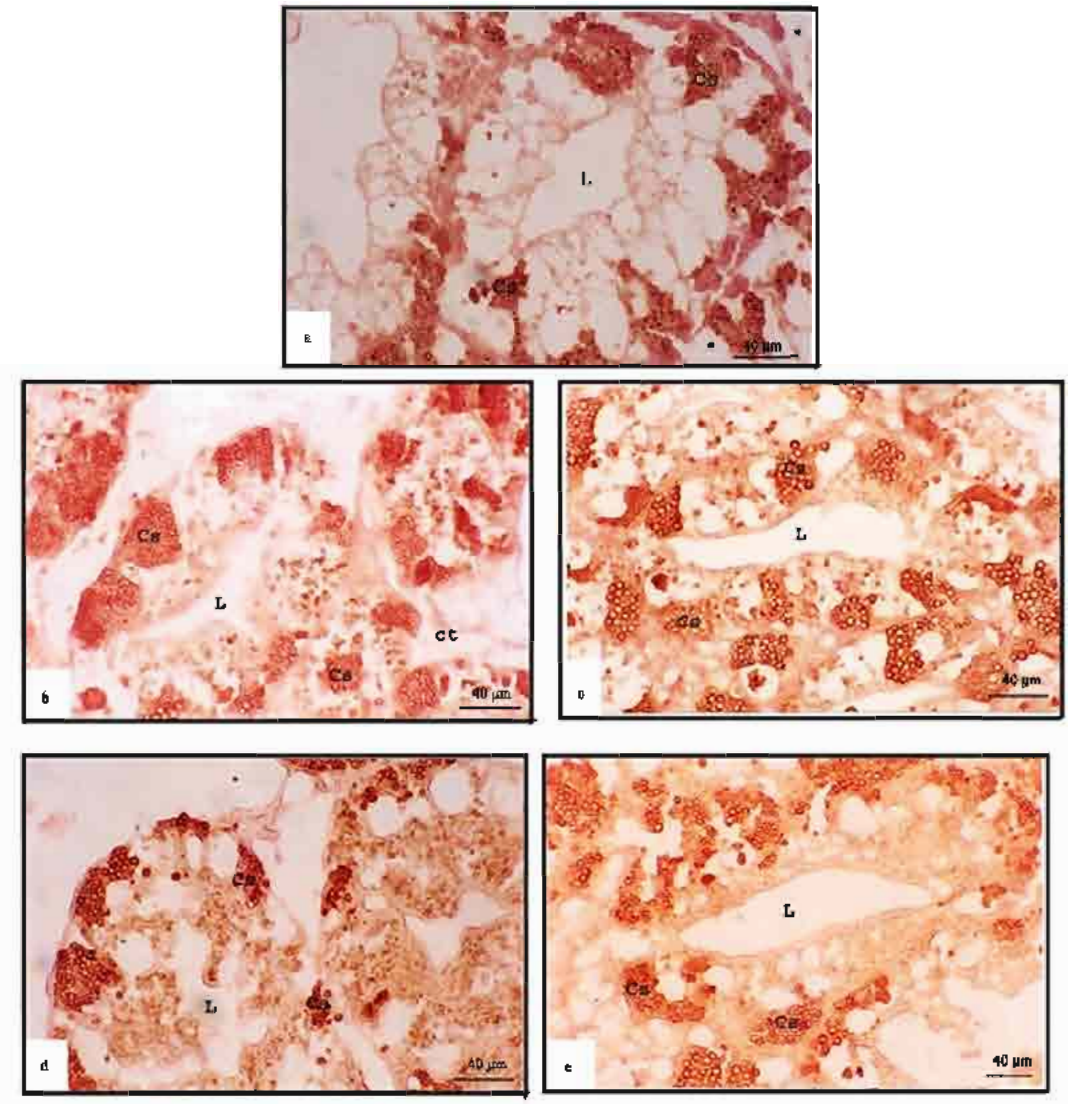

Fig. 6a-e: L.M. (a) Control digestive gland, demonstrating calcium spherules (Cs) with an orange-red colour after staining with Alizarin Red S. Hemolymph space (asterisks); lumen (L). (b) Treated digestive gland (GIII), revealing increased number of calcium spherules (Cs) in calcium cells; lumen (L); intertubular connective tissue (ct). (c) Treated digestive gland (GIV), showing enlargement of calcium spherules (Cs) of calcium cells; lumen (L). (d) Treated digestive gland (GV), showing general reduction in the number of calcium spherules (Cs); lumen (L) and (e) Treated digestive gland (GVI), revealing increase in the number and size of calcium spherules (Cs); lumen (L). (Alizarin Red S).

The nucleus of digestive cells contains scattered chromatin and a prominent nucleolus. In some cells the nucleus display an irregular shape adjusted to fit in the free space among the heterolysosomes and residual bodies. In addition, the position of the nucleus and the amount of heterochromatin in the nucleoplasm varies according to the different stages of the cell cycle (Fig. 7c). Golgi membranes and rough endoplasmic reticulum were rarely observed in the cytoplasm of digestive cells.

Calcium cells: The apical surface of calcium cell is covered with brush border microvilli. They are

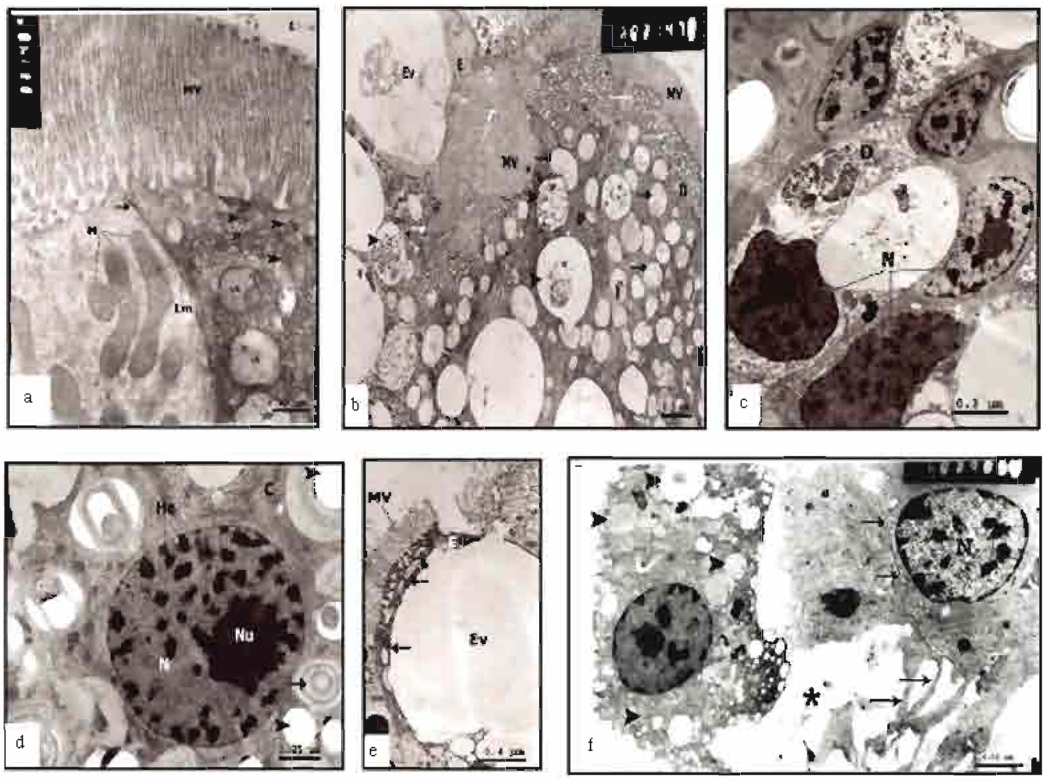

Fig. 7 a-f: Electron micrographs (E.M.) of the digestive gland of control *E. vermiculata* (GI), (a) Showing two adjacent digestive cells with different cytoplasmic density, one of which showing long, closely packed, intact brush border microvilli (MV). The other one shows many well organized rounded and elongated mitochondria with tubular cristae (M) and electron-lucent cytoplasm. Adhering junction (arrow); lateral plasma membrane (Lm); lumen (L). (b) Demonstrating apical parts of digestive cells (D). Note the well developed lysosomal vacuolar system consisting of tubulo-vesicular endosomes (white arrows), irregularly-shaped late endosomes with moderate electron dense contents (black arrows) and large heterolysosomes (arrowheads); microvilli (MV); excretory cell (E) with large excretory vacuole (Ev). (c) Demonstrating different shapes of nuclei (N) of digestive cells (D). (d) Demonstrating a calcium cell (C) with large rounded nucleus (N), prominent nucleolus (Nu) and heterochromatin (He); numerous holes formerly occupied by the mineralized granules (arrowheads); intact granule (arrow). (e) Showing excretory cell (E) with large excretory vacuoles (Ev) surrounded by a very thin dense cytoplasm (arrows); brush border microvilli (MV) and (f) Part of the intertubular connective tissue (asterisk), showing two different hemocytes; the avacuolar hemocyte characterized by large nucleus (N) with irregular chromatin pattern mainly arranged around the nuclear periphery; small arrows point at contact areas by which hemocytes adhere together; large arrows point at filopodia. The other hemocyte or vacuolar hemocyte showing an eccentric nucleus with large clumps of chromatin and extensive membrane-bound vacuoles of different size, texture and electron density (arrowheads)

characterized by a large nucleus with regular nuclear envelope, several clumps of condensed chromatin and a very prominent nucleolus and an extensive highly developed rough endoplasmic reticulum arranged in conspicuous stacks of parallel cisternae adjacent to the lateral cell membrane (Fig. 7d). Golgi complexes are located in the supranuclear cytoplasm and usually appear to consist of 3 to 6 cisternae with electron-dense contents. These cells are extremely rich in mitochondria, which extend throughout the cell, although being especially concentrated in the apical cytoplasm. The mitochondria assumed round or oval shape and their cristae are few and oriented transversely. Highly electron-dense apical granules (1.8-3.6 μm) were also observed.

Calcium spherules appeared to lie in membrane bound vacuoles and were observed to undergo a process of continuous growth. Immature or spherules in the early developmental stage showed a central electron-lucent core and only one or two electron-dense rings with clear spaces in between. Mature or spherules in late developmental stage were observed to consist of a great number of concentric layers of opaque and clear zones. Sometimes the interior of these granules had been torn out during the cutting procedure (Fig. 7d). The basement membrane exhibited many deep invaginations.

Excretory cells: At the Ultrastructural level, two forms of excretory cells were observed. The first form is characterized by the presence of one large vacuole surrounded by a thin layer of electron-lucent cytoplasm and a well developed microvillus border of 2.5 μm long. Many well organized rounded and elongated mitochondria were observed in the subapical cytoplasm. The other form of excretory cells appears with much denser cytoplasm contains a number of tubes which always appear bent. Furthermore, the apical cytoplasm contained a number of vacuoles which seemed to be endosomes and heterolysosomes, among which, mitochondria, dense bodies and free ribosomes were also present (Fig. 7e). In both forms of excretory cells, the nuclei were situated basally and appeared flattened with irregular shape because of the large excretory vacuoles.

Thin cells: In our electron micrographs, thin cells were rarely observed in the digestive gland tissue. They are characterized by elongated nucleus and very thin cytoplasm. Mitochondria with round or rod shape are the most prominent cytoplasmic organelles observed. They are located predominantly near the nucleus.

Outside the epithelial lining, a very thin circular muscle layer was observed. Small nerve fibers containing a number of vesicles were observed in and above the basal lamina.

Blast-like hemocyte displays centrally located, large nucleus with lightly stained perfuse chromatin and several large globules of heterochromatin associated with the nuclear membrane and within the nucleoplasm. Avacuolar hemocytes are larger and characterized by lack of vacuoles and the presence of extensive filopodia. Their nuclei have prominent nuclear pores and peripherally-located chromatin. Binding areas between these hemocytes were also observed (Fig. 7f). Vacuolar hemocytes are the largest hemocyte and are easily distinguished by the presence of a large number of vacuoles of different size, texture and electron density throughout the cytoplasm. At high magnification of the peripheral region, an interconnected network of plasma membrane and empty vacuoles was observed. The nucleus is somewhat eccentric and contains condensed chromatin around the periphery. Nucleolus is prominent and condensed (Fig. 7f).

It is worthy to mention that, in the present investigation, the ultrastructural characteristics of digestive gland cells of snails in GII (solvent-exposed snails), did not differ greatly from those of animals in GI (water-exposed snails). The most prominent difference was in the digestive cells which exhibited increased fusion of membrane-bound vacuoles. However, these cells appeared with organized microvillus border, lateral junctions and typical nuclear morphology.

Methomyl-treated group (topical application) (GIII): After 14 days of topical application of methomyl, the apical surface of digestive cells show reduction of microvilli with only some microvilli remnants and formation of surface blabs (Fig. 8a). Mitochondria show a hyperdensity of their matrix (mitochondrial pyknosis) and possess ballooned cristae (Fig. 8b). Most nuclei show compaction and margination of nuclear chromatin, dilation of the nuclear envelope and formation of multiple nucleoli, crystalline inclusions and nuclear clefts. Peripherally the nuclear membrane seems to break up into dense irregular fragments indicating severe karyorrhexis (Fig. 8c). In addition, an increase in the number of lipid droplets in the basal cytoplasm of digestive cells and a marked increase in the number of free ribosomes was also noticed.

Calcium cells possess abnormal nuclei with margination and condensation of heterochromatin and dilatation of the nuclear envelope. The nucleolus is more homogenous and show low electron density (Fig. 8d). Moreover, most mitochondria are enveloped with rER and were severely deformed and exhibited highly electron-dense matrix and disorganized few cristae. On the other hand, Golgi apparatus could not be distinguished in our electron microscopic preparations of this group.

The nuclei of excretory cells appeared pykvotic. Moreover, enlarged mitochondria closely aligned to lipid droplets were observed in the basal cytoplasm.

Thin cells showed abnormal nuclei with irregular nuclear envelope, marginated heterochromatin and segregated nucleolus in which the granular part is completely separated from the fibrillar component. A number of lipid droplets were observed in the cytoplasm of these cells (Fig. 8e).

The nucleus of blast-like hemocytes display condensed heterochromatin located both centrally and along the nuclear envelope. In addition, karyorrhexis was also observed. The cytoplasm of avacuolar hemocytes showed accumulation of a great number of lysosomes

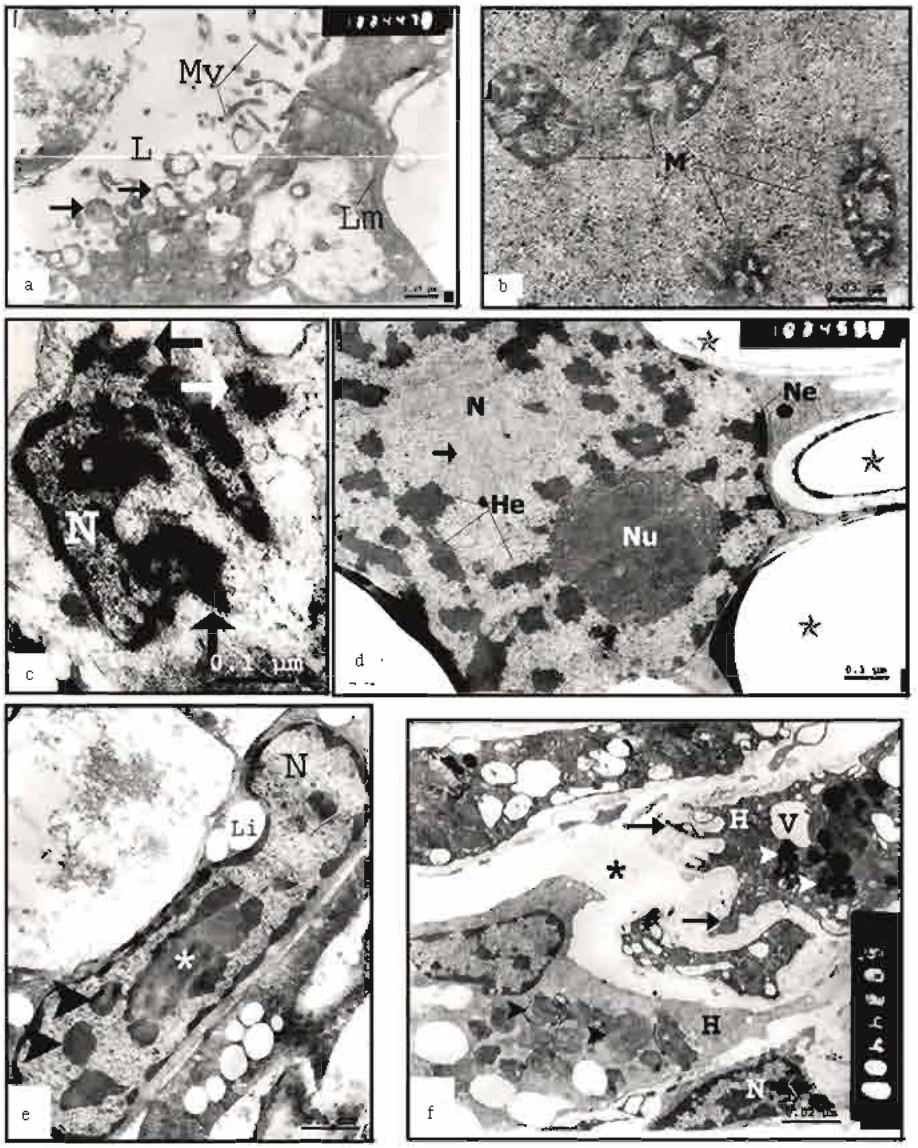

Fig. 8 a-f: E.M. Treated digestive gland (GIII). (a) Showing apical part of digestive cells with highly disrupted apical surface. Note: fragmented and dispersed microvilli (MV); lumen (L); surface bleb (arrows); lateral plasma membrane (Lm). (b) Showing a number of mitochondria (M) with intracristal swelling or ballooned cristae and hyperdensity matrix. (c) Showing nucleus (N) of digestive cell undergoes karyorrhexis (black arrows). The dark mass probably represents fragmented nuclear remnants (white arrow). (d) Showing nucleus (N) of calcium cell with more or less normal chromatin distribution (He) occurs in irregular clumps near to irregular nuclear envelope (Ne) and around nucleolus (Nu). Arrow points at lightened karyoplasm. Note: holes representing the regions formerly occupied by calcium granules (asterisks). (e) Showing abnormal nucleus (N) of thin cell with irregular nuclear envelope, segregated nucleolus in which the fibrillar component (arrowheads) is completely separated from the granular part (asterisk). Lipid droplets (Li). (f) Showing the intertubular connective tissue (asterisk) with a vast number of hemocytes (H). The cytoplasm of one hemocyte is thrown into pseudopod-like folds (arrows) and contains highly electron dense bodies (white arrowheads); the avacuolar hemocyte show accumulation of myelin bodies (black arrowhead), blast-like hemocyte showing nucleus (N) undergoing karyorrhexis

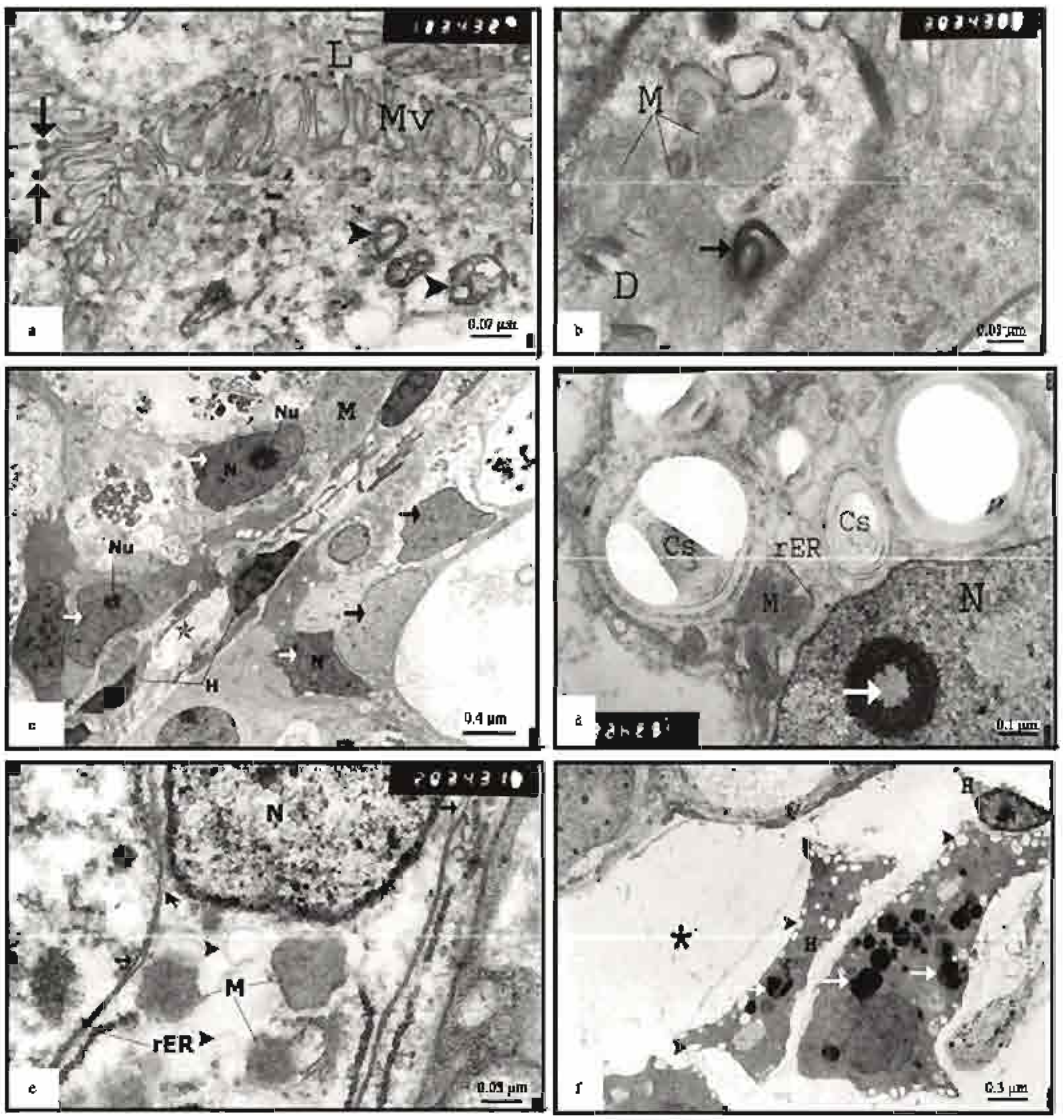

Fig. 9 a-f: E.M. Treated digestive gland (GV). (a) Showing apical part of digestive cell with irregular arrangement of dilated microvilli (MV); electron-dense small endo- or exo-cytotic vesicles being fused with the apical plasma membrane at the base of the microvilli (arrows); membrane-bound structures (arrowheads) which may be autophagic vacuoles; reduced or very narrow lumen (L). (b) Apical parts of adjacent digestive cells (D), showing extensive breakdown of cytoplasmic organelles. The mitochondria (M) show marked swelling with indistinct and completely disrupted cristea; myelin figure (arrow). (c) Showing irregularly-shaped nuclei (N) of digestive and excretory cells; some of which show lightening of the karyoplasm (black arrows), others show reduction of the heterochromatin (white arrows); nucleolus (Nu); large number of mitochondria (M) in the basal cytoplasm; blast-like hemocyte (H); hemolymph space (asterisk). (d) Showing nucleus (N) of calcium cell with irregular nuclear envelope and fading of the fibrillar component of the nucleolus (arrow); mitochondria (M) partially encircled by curved profiles of rough endoplasmic reticulum (rER); calcium spherules (Cs). (e) Showing part of thin cell with lytic cytoplasm containing disintegrated mitochondria (M); dispersed cisternae of rough endoplasmic reticulum (rER) heavily studded with ribosomes; membranous structures (arrowhead); nucleus (N) with unidentifiable nuclear envelope; disruption of lateral cell membrane (arrows). (f) Showing different hemocytes (H) in the hemolymph space (asterisk). The cytoplasm of the vacuolar hemocytes contains electron-dense cytoplasmic bodies which may be secondary lysosomes (arrows) and numerous pinocytotic vesicles (arrowheads)

with myelin-like membrane whorls and large lipid droplets. Vacuolar hemocytes appeared with large number of tubules, vesicles and vacuoles filling up most of their cytoplasm in addition to highly electron-dense granules. Mitochondria revealed signs of pathological alteration; their cristae increased in number and became closely packed and their matrix contained rounded electron-dense granule (Fig. 8f).

Methiocarb-treated group (topical application) (GV): The microvilli of digestive cells show shortening, reduction and vesiculation. Others appear dilated and fused together (Fig. 9a). Digestive cells exhibit disorganized, vacuolated cytoplasm with decreased cytoplasmic density and extensive breakdown of cytoplasmic organelles. In addition, an increase in number of cytoplasmic bodies mainly in the form of myelin figures or autophagic vacuoles containing remnants of cytoplasmic organelles was observed (Fig. 9a and b). The endoplasmic reticulum appears as dispersed cisternae or in the form of small vesicles which are often devoid of ribosomes. Mitochondria show marked swelling with non-distinct inner structure and disintegrated cristae (Fig. 9b). In addition there is a dramatic increase in the number of mitochondria especially in the basal cytoplasm (Fig. 9c). No lipid droplets were observed in the cells. The karyoplasm becomes less electron-dense and the nuclear membrane double layer structure turned into indistinct and disconnected envelope (Fig. 9c).

Concerning calcium cells, their nuclei show fading of the fibrillar component of the nucleolus, reduction of heterochromatin and irregularity of the nuclear membrane (Fig. 9d). The mitochondria showed a marked increase in number and they possess incomplete disorientation of cristae and appear partially encircled by curved profiles of rough endoplasmic reticulum (Fig. 9d). Some mitochondria appeared enlarged with dilated cristae and dense matrix. Cytoplasm appears vacuolated with a less dense cytoplasmic matrix. The endoplasmic reticulum showed degranulation and vesiculation. The Golgi complex could not be detected in the electron micrographs of this group. Thin cells appear with lytic cytoplasm containing disintegrated mitochondria and dispersed cisternae of rough endoplasmic reticulum heavily studded with ribosomes. The nuclei show unidentifiable nuclear envelope and lightening of karyoplasms with little heterochromatin (Fig. 9e).

A number of blast-like hemocytes were observed between the tubules; however, they possess no obvious ultrastructural changes. Furthermore, vacuolar hemocytes show euchromatic irregularly-shaped nuclei, reduction in the number of their vacuoles and a number of electron-dense bodies which may correspond to secondary lysosomes or phagolysosomes. Mitochondria displayed lysis and destruction of membranes (Fig. 9f).

DISCUSSION

The present study confirms the existence of four cell types in the digestive gland of *E. vermiculata* namely: digestive, calcium, excretory and thin cells. Controversy exists about the types and related nomenclature of the gastropod digestive gland cells and about the functions attributed to them Saad and Farag (1988) and Zaldibar *et al.* (2007) described two cell types; digestive cells and basophilic cells in the digestive gland of *E. vermiculata* and *Littorina littorea*, respectively. However, Al-Zahaby *et al.* (1993) described digestive and excretory cells in the digestive gland of the same snail. Three cell types: digestive, calcium and excretory cells have been observed in *H. lucorum* (Dimitriadis and Hondros, 1992), in *E. vermiculata* (Mersal, 1990; Aioub *et al.*, 2000), in *H. pomatia* (Chabicovsky *et al.*, 2004) and in *H. aspersa* (Snyman *et al.*, 2005). However, Beshr (2000) described digestive, calcium and thin cells and Heiba *et al.* (2002) reported the presence of digestive, excretory and secretory cells in the digestive gland of *E. vermiculata*. Four cell types: digestive, calcium, excretory and thin cells were described in *H. aspersa* (Sumner, 1965, 1966 and 1969) and in *A. reticulatus* (Walker, 1970).

In *E. vermiculata*, the digestive cells showed morphofunctional alterations which could be attributed to the difference in their different phases of activity. This is in accordance with Moore and Halton (1973) who identified successive phases of absorption, digestion and fragmentation in the cycle of the digestive cells in the tubules of *L. truncatula*. Recently, Dimitriadis and Konstantinidou (2002) reported that the cell changes observed in the digestive gland epithelium of *H. lucorum* were regarded as different phases of cell activity of the same cell type, the digestive cells. Our observations showed also that yellow granules are similar to those described for lipofucsin in other molluscs (Mathew and Damodaran, 1997). This is in accordance with the results of Walker (1970) who showed that yellow and green granules in the digestive gland of *A. reticulatus*, are quite distinct histochemically and structurally and that the transformation from green to yellow granules seems likely.

The present investigation confirms the absorptive role of digestive cells of *E. vermiculata* as they possess organelles involved in absorption and intracellular digestion. This is in accordance with earlier results by Roldan and Garcia-Corrales (1988) who suggested that the

presence of a dense microvillus border and the accumulation of lipid and glycogen reserves in epithelial cells of the digestive tract of gastropods indicate an absorptive function in such cells.

Ultrastructurally, neighboring digestive cells were observed to be firmly attached to each other through very elaborate intercellular junctions. The zonula adherens type of junctional complex is thought to facilitate ion transport between adjacent cells. Septate junctions or septate desmosome are unique to invertebrates. Much less is known about this type of junctions and they are probably best classified as anchoring junctions, for they act as connection sites for actin filaments; but it has been suggested that they can function as permeability barriers in some cases (Noirot-Timothee and Noirot, 1980).

In the present study, observations from semithin and ultrathin sections of the digestive gland showed that the single large vacuole of excretory cell seems to result from the fusion of all heterolysosomes and residual bodies of a digestive cell. Thus, it could be suggested that excretory cells originate from digestive cells during maturation. Similar results were reported in *H. lucorum* (Dimitriadis and Konstantinidou, 2002). In Lobo-da-Cunha (1999) found some basophilic (calcium) cells of *A. depilans* with a large number of vacuoles filling a great portion of the cell volume, but they maintain a characteristic pyramidal shape, some apical secretion granules and a large oval nucleus. Because of these morphological aspects, it was suggested that the vacuolated basophilic cells seem not to be precursors of the excretory type cells.

Thin cells were hardly identified in histological preparations; however, they were recognized in ultrathin sections with no indication of any cellular division. These cells were considered as undifferentiated cells and could be developed into other cell types. This interpretation is in agreement with Sumner (1965). Taïeb and Vicente (1999) identified three types of thin cells in the digestive gland of *A. punctata* and they suggested that these types correspond to various stages of development.

In the current investigation, two different types of hemocytes were observed in the intertubular connective tissue of the digestive gland of *E. vermiculata*, namely hyaline or agranular hemocytes and granulocytes. These cells are suspended in the coelomic fluid (Hamed *et al*., 2005) and are generally thought to play a role in shell repair in terrestrial gastropods by transporting calcium and organic shell matrix substances from the digestive gland to the sites of damage (Abolins-Krogis, 1976). The current trend considers the mollusk hemocytes as originated from the Amebocyte-Producing Organ (APO) which is a narrow band of stratified epithelial-like tissue, located within the renoepicardial region. However, Souza and Andrade (2006) postulated multicentric origin for *B. glabrata* hemocytes recapitulates earlier embryologic findings in vertebrates, when mesenchymal vascular spaces generate the circulating and phagocytic blood cells. It is also clarify the fact that the hemocytes can take origin from anywhere within the snail body, whenever an appropriate focal stimulus occurs (Pan, 1963), instead of having a central origin from a specific organ, the APO or amebocyte forming organ (Sullivan and Spence, 1994; Sullivan *et al*., 2004).

The digestive gland of molluscs is one of the target organs in toxicological prospects regarding its role in detoxification, biotransformation and excretion of xenobiotics. Thus, the present investigation reveals the impact of the two carbamate compounds methomyl and methiocarb on the histology and ultrastructure of the digestive gland cells of *E. vermiculata*. The results here indicated that the histopathological alterations in the digestive gland were more obvious after topical application than after baiting technique. This can be attributed to that molluscicides slow down the food transport (Wedgwood and Bailey, 1988; Bourne *et al*., 1991). Triebskorn and Künast (1990) showed that slugs ingest less cloethocarb-containing food when compared to controls. Furthermore, Triebskorn and Florschütz (1993) showed that the molluscicide cloethocarb interferes with the passage of food from the crop into the gut of the tested slugs. This effect had already been shown for other molluscicides (Wright and Williams, 1980; Bourne *et al*., 1988; Wedgwood and Bailey, 1988; Bailey *et al*., 1989). The assumption that molluscicides slow down or inhibit the food transport in the alimentary tract might be due to the possible influence of several molluscicides on the nervous system that could regulate muscle activity (Young and Wilkins, 1989).

In the current investigation, the digestive cells showed severe cytoplasmic vacuolization, especially after 14 days of topical application of both methomyl and methiocarb. These results confirm the investigations of Triebskorn and Künast (1990) and Triebskorn *et al*. (1996) who found that in the digestive cells of *D. reticulatum* treated with molluscicidal baits; intravacuolar digestive processes are intensified leading to large secondary lysosomes with fragile membranes. They added that the cells activate intracellular digestion, winning energy for reactive energy consuming and possibly detoxification processes that probably take place in the crypt (calcium) cells. It was also noted that treatment with methomyl and methiocarb resulted in extensive digestive-cell breakdown and release of cytoplasmic material into the digestive gland lumen of *E. vermiculata*. This is in accordance with

the results of Bowen and Davies (1971) and Oxford and Fish (1979) in *Arion hortensis* and *Cepaea nemoralis*, where release of hydrolytic enzymes from the cytoplasm of digestive cells was related to an increase in the rate of cell autolysis.

Both topical application and toxic baits of methomyl resulted in the enlargement of yellow granules or residual bodies of digestive cells of *E. vermiculata*. This apparent rise in the numbers of residual bodies suggests accumulation of the tested molluscicide in these granules and may reflect decreased excretory functions of these cells due to intoxication. Similar findings were observed in hepatopancreatic digestive cells of *Mytillus galloprovincialis* exposed to heavy metals (Domouhsidou and Dimitriadis, 2000) and in the digestive cells of *Littorina littorea* after exposure to organic pollutants (Cajaraville *et al*., 1995).

Calcium cells in the digestive gland of *E. vermiculata* treated with methomyl and methiocarb generally, express increased number of calcium spherules, suggesting that these cells are specialized for accumulation and elimination of toxic compounds. Chabicovsky *et al.* (2004) showed that heavy metal pollutants, such as Cd, Cu and Zn, induce increased number of calcium and excretory cells at the cost of digestive cell numbers. They also speculated that dying cells are phagocytosed by calcium cells. In addition, calcium show multiple roles in gastropods and other molluscs in which they are regarded as a component implicated in pH homeostasis (Jokumsen and Fyhn, 1982) in reproduction (Fournié and Chetail, 1982) in shell regeneration, in formation of the epiphragm during hibernation (Abolins-Krogis, 1965) and in detoxification of heavy metals (Mason and Simkiss, 1982).

The effect of methiocarb on the digestive gland of *E. vermiculata* resulted in extensive destruction and disorganization of the intertubular connective tissue, folding of the basement membrane and detachment of tubular epithelium from basal lamina. Triebskorn and Künast (1990) reported that carbamates, as nerve toxins, induce uncontrolled muscle contractions in slugs and as a consequence, the epithelial cells might be stretched, leading to basal cell extension, that leads to detachment of the cells from the basal membrane.

Hemocytic infiltrations especially granulocytes containing abnormal accumulation of granules was a remarkable feature in the intertubular connective tissue of the digestive gland of methomyl-treated snails. These granules may be undigested phagosomes and their accumulation suggests a problem in the disposal of either the toxic molluscicides or damaged cells. Histopathological alterations such as degeneration and the gathering of hemocytes in areas between the tubules have also been reported in the digestive gland of snails *B. dissimilis* exposed to endosulfan, methylparathion, quinalphos and nuvan (Jonnalagadda and Rao, 1996) *M. obstracta* exposed to lannate (Zedan *et al*., 1999) *E. vermiculata* exposed to oxamyl (Aioub *et al*., 2000) *Planorbarius corneus* exposed to endosulfan (Otludil *et al*., 2004) and *Galba truncatula* exposed to Thiodan (Cengiz *et al*., 2005).

At the ultrastructural level, the most important changes observed after treatment with methomyl were bizarre nuclei that ranged in their degenerative changes from karyolysis to severe karyorrhexis and complete pyknosis. However, the damage of the nuclei after topical application of methiocarb was less intense. Karyolysis is often described as a late reaction to intoxication in vertebrates and invertebrates (Bayne *et al*., 1985). Triebskorn (1989) stated that the reaction of nuclei does not seem to be an ultimate step revealing cell death, but a primary cell response that leads to cell death.

The most conspicuous cytopathological sign after treatment with methiocarb was the fading of the fibrillar component of the nucleolus which is generally considered as structural correlate of decreased rRNA synthesis (Goessens, 1984).

After treatment with methomyl and methiocarb, mitochondria of digestive gland cells displayed marked alteration in their shape and size. They showed a hyperdensity of their matrix (mitochondrial pyknosis) and possession of ballooned cristae. These alterations suggest a decrease in mitochondrial energy production (Goel and Dhawan, 2001) which is probably an important cause of the nuclear changes previously discussed. In addition, these alterations may be due to interactions of the toxin with membrane components and to changes in ion transport along the mitochondrial membrane.

Vesiculation and degranulation of rER have been described in the digestive gland cells after topical application of methomyl and methiocarb and this could be correlated with a disturbance in protein synthesis. Similar alterations were recorded by Triebskorn (1989), Triebskorn and Künast (1990) and Triebskorn and Köhler (1992) who related ultrastructural alterations in the endoplasmic reticulum in the crypt cells of *D. reticulatum* treated with molluscicides to activation of the NTR (NADPH-neotetrazolium reductase), which is known to be localized on the smooth endoplasmic reticulum (Netter, 1980; Lee, 1981) and might be active in oxidative detoxification.

The Golgi complex was affected by the two tested molluscicides and disappeared on day 14. The destruction

and disappearance of Golgi elements have been noted in tissues subjected to a variety of toxic influences or various physical and chemical agents (Im *et al*., 1980).

After topical application of methomyl and methiocarb, digestive gland cells showed disruption and reduction of the brush border microvilli, with the formation of surface blebs which suggest a possible decrease in absorption. Similar results were reported by Triebskorn and Künast (1990) in *D. reticulatum* fed with sublethal concentration of cloethocarb. Membrane blebs are spherical membrane extensions that are commonly seen at the periphery of cells as they spread on a substrate, during mitosis, or at the leading edge of migrating cells (Cunningharn, 1995). Alternatively, large, nonretracting blebs could be formed on cells injured by physical or chemical stress (Trump *et al*., 1971). Domnina *et al*. (2002) suggested that bleb formation might be considered as a protective reaction, attenuating general cell destruction, as they could be responsible for sequestration of some cell components involved into realization of apoptotic program.

REFERENCES

Abolins-Krogis, A., 1965. Electron microscope observations on calcium cells in the hepatopancreas of the snail, *Helix pomatia* (L.). Arch. Zool., 18: 85-92.

Abolins-Krogis, A., 1976. Ultrastructural study of the shell-repair membrane in the snail, *Helix pomatia* L. Cell Tiss. Res., 172: 455-476.

Aioub, A.A., S.A. Ismail and A.A. Mohamdein, 2000. Toxicological and histological studies on some pesticides-treated land snails. Proc. I. C. B. S., 1: 19-38.

Al-Zahaby, A.S., A.H. Abdel-Rehim and A.A. Al-Mahrouki, 1993. Histochemical features of the digestive gland of banded and unbanded phenotypes of the polymorphic land snail, *Eobania vermiculata*. J. Egypt. Ger. Soc. Zool., 12: 291-310.

Bailey, S.E.R., S. Cordon and S. Hutchinson, 1989. Why do not slugs eat more bait? A behavioral study of early meal termination produced by methiocarb and metaldehyde baits in *Deroceras caruanae*. Proc. Br. Crop Prot. Counc., 41: 385-390.

Bayne, B.L.O., K.R. Clarke, D.R. Dixon, A. Ivanovici, D.R. Livingstone, D.M. Lowe, M.N. Moore, A.R.D. Stebbing and J. Widdows, 1985. The Effects of Stress and Pollution on Marine Animals. Praeger Scientific, New York, pp: 384.

Beeby, A. and L. Richmond, 1988. Calcium metabolism in two populations of the snail, *Helix aspersa* on a high lead diet. Arch. Environ. Contam. Toxicol., 17: 507-511.

Beshr, S.M., 2000. Ecotoxicological studies on two species of snails and associated insects, infesting fruit trees, in three Egyptian governorates. M.Sc Thesis, Alexandria University.

Bode, A.U., C.O. Adewunmi, G. Dorfler and W. Becker, 1996. The effects of extracts from *Tetrapleura tetraptera* (Taub.) and Bayluscide on cells and tissue structures of *Biomphalaria glabrata* (Say). J. Ethnopharmacol., 50: 103-113.

Bourne, N.B., G.W. Jones and I.D. Bowen, 1988. Slug feeding behavior in relation to control with molluscicidal baits. J. Moll. Stud., 54: 327-338.

Bourne, N.B., G.W. Jones and I.D. Bowen, 1991. Endocytosis in the crop of the slug *Deroceras reticulatum* (Müller) and the effects of the ingested molluscicides, metaldehyde and methiocarb. J. Moll. Stud., 57: 71-80.

Bowen, I.D. and P. Davies, 1971. The fine structure and distribution of acid phosphatase in the digestive gland of *Arion hortensis* (F.). Protoplasma, 73: 78-81.

Brackenbury, T.D., 1999. Gross histopathological effects of an extract of *Agave attenuata* on the epithelium of the digestive tract of *Bulinus africanus*. Ann. Trop. Med. Parasitol., 93: 519-526.

Cajaraville, M.P., Y. Robledo, M. Etxeberria and I. Marigomez, 1995. Cellular Biomarkers as Useful Tools in the Biological Monitoring of Environmental Pollution: Molluscan Digestive Lysosomes. In: Cell Biology in Environmental Toxicology. Cajaraville, M.P. (Ed.), University of the Basque Country Press Service, Bilbo, pp: 29-55.

Cengiz, E.L., M.Z. Yildirim, B. Otludil and E. Unlu, 2005. Histopathological effects of Thiodan (R) on the freshwater snail, *Galba truncatula* (gastropoda, pulmonata). J. Applied Toxicol., 25: 464-469.

Chabicovsky, M., W. Klepal and R. Dallinger, 2004. Mechanisms of cadmium toxicity in terrestrial pulmonates: Programmed cell death and metallothionein overload. Environ. Toxicol. Chem., 23: 648-655.

Cunningharn, C.C., 1995. Actin polymerization and intracellular solvent flow in cell surface blebbing. J. Cell Biol., 129: 1589-1599.

Dimitriadis, V.K. and D. Hondros, 1992. Effect of starvation and hibernation on the fine structural morphology of digestive gland cells of the snail *Helix lucorum*. Malacologia, 34: 63-73.

Dimitriadis, V.K. and E.B. Andrews, 2000. Ultrastructural and cytochemical study of the digestive gland cells of the marine prosobranch mollusc *Nucella lapillus* (L.) in relation to function. Malacologia, 42: 103-112.

Dimitriadis, V.K. and V. Konstantinidou, 2002. Origin of the excretory cells in the digestive gland of the land snail, *Helix lucorum*. Malacologia, 44: 145-151.

Domouhsidou, G.P. and V.K. Dimitriadis, 2000. Ultrastructural localization of heavy metals (Hg, Ag, Pb and Cu) in gills and digestive gland of mussels, *Mytillus galloprovincialis* (L.). Arch. Envion. Contam. Toxicol., 38: 472-478.

Domnina, L.V., O.Y. Ivanova, B.V. Cherniak, V.P. Skulachev and J.M. Vasiliev, 2002. Effects of the inhibitors of dynamics of cytoskeletal structures on the development of apoptosis induced by the tumor necrosis factor. Biochemistry (Mosc), 67: 737-746.

El-Mehlawy, M.H. and E.T. Rizk, 2000. Toxicity of the organophosphorous pesticide diazinon on Schistosmiasis snail vector *Biomphalaria alexandrina*. Proc. ICBS, 1: 153-162.

El-Okda, M.M.K., 1983. Terrestrial snails and slugs (Pulmonata) destructive mollusca in ornamental plantages in Alexandria, Egypt. Proc. 5th Arab Pesticide Conf. Tanta Univ., 2: 369-378.

El-Saadany, M.M., S.A. Sakr and S.A. Gabr, 1990. Histological and ultrastructural studies on the effect of *Schistosoma mansoni* on the digestive gland of *Biomphalaria alexandrina*. J. Egypt. Ger. Soc. Zool., 1: 37-46.

El-Sebae, A.H., M.M.K. El-Okda and A.S. Marei, 1982. Local formulation of three carbanoylated oximes as terrestrial molluscicides. Agric. Res. Rev., 60: 85-91.

Fournié, G. and M. Chetail, 1982. Evidence for a mobilization of calcium reserves for reproduction requirement in *Deroceras reticulatum*. Malacologia, 22: 285-291.

Godan, D., 1983. Pest Slugs and Snails, Biology and Control. Springer-Verlag, Berlin, pp: 443.

Goel, A. and D.K. Dhawan, 2001. Zinc supplementation prevents liver injury in chlorpyrifos treated rats. Biol. Trace Element Res., 82: 185-200.

Goessens, G., 1984. Nucleolar structure. Int. Rev. Cytol. 87: 107-158.

Hamed, S.S., E. Kauschke and E.L. Cooper, 2005. Cytochemical properties of earthworm coelomocytes enriched by Percoll. Int. J. Zool. Res., 1 : 74-83.

Heiba, F.N., I.M. Al-Sharkawy and A.A. Al-Batal, 2002. Effects of the insecticide lannate, on the land snails, *Eobania vermiculata* and *Monacha contiana*, under laboratory conditions. Online J. Biol. Sci., 2: 8-13.

Henry, M., E. Boucaud-Camou and Y. Lefort, 1991. Functional microanatomy of the digestive gland of the scallop *Pecten maximus* (L.). Aquat. Living Resour., 4: 191-202.

Im, J., T. Sohn and P. Parky, 1980. Morphological changes of Golgi apparatus induced by actidione. Kyungpook. Univ. Med. J., 21: 550-556.

Jokumsen, A. and H.J. Fyhn, 1982. The influence of aerial exposure upon respiratory and osmotic properties of hemolymph from two intertidal mussels, *Mytillus edulis* and *Modiolus*. J. Exp. Mar., Biol. Ecol. (B), 61: 189-203.

Jonnalagadda, P.R. and B.P. Rao, 1996. Histopathological changes induced by specific pesticides on some tissues of the freshwater snail, *Bellamya dissimilis* Müll. Bull. Environ. Contam. Toxicol., 57: 648-654.

Lee, R.F., 1981. Mixed-Function Oxidases (MFO) in marine invertebrates. Mar. Biol. Lett., 2: 87-105.

Lobo-da-Cunha, A., 1999. Ultrastructural and cytochemical aspects of the basophilic cells in the hepatopancreas of *Aplysia depilans* (Mollusca, Opisthobranchia). Tiss. Cell, 31: 8-16.

Lobo-da-Cunha, A., 2000. The digestive cells of the hepatopancreas in *Aplysia depilans* (Mollusca, Opisthobranchia): Ultrastructural and cytochemical study. Tiss. Cell, 32: 49-57.

Mason, A.Z. and K. Simkiss, 1982. Sites of mineral deposition in metal accumulating cells. Exp. Cell Res., 139: 383-391.

Mathew, S. and R. Damodaran, 1997. Lipofuscin as physiological indicator of heavy metal stress in *Sunetta scripta* (yellow clam) and *Perna viridis* (green mussel). Indian J. Mar. Sci., 26: 64-67.

Mersal, H.T., 1990. Changes induced in one land snail of agriculture importance in Egypt. M.Sc. Thesis, Faculty of Girls, Ain Shams University.

Miller, E., D. Swails, F. Olson and R.T. Staten, 1988. White garden snail (*Theba pisana* Müller): Efficacy of selected bait and sprayable molluscicides. J. Agric. Entomol., 5: 189-197.

Moore, M.N. and D.W. Halton, 1973. Histochemical changes in the digestive gland of *Lymnaea truncatula* infected with *Fasciola hepatica*. Z. Parasitenk, 43: 1-16.

Muley, D.V. and U.H. Mane, 1990. Histopathological changes in body parts of the freshwater gastropod, *Viviparus bengalensis* Lam. due to pesticides. J. Environ. Biol., 11: 413-425.

Netter, K.J., 1980. Inhibition of oxidative drug metabolism in microsomes. Pharmacol. Therapeutics, 10: 515-535.

Noirot-Timothee, C. and C. Noirot, 1980. Septate and scalariform junctions in arthropods. Int. Rev. Cytol., 40: 63-97.

Otludil, B., E.I. Cengiz, M.Z. Yildirim, O. Ünver and E. Ünlü, 2004. The effects of endosulfan on the great ramshorn snail, *Planorbarius corneus* (Gastropoda, Pulmonata): A histopathological study. Chemosphere, 56: 707-716.

Oxford, G.S. and L.J. Fish, 1979. Ultrastructural localization of esterase and acid phosphatase in digestive gland cells of fed and starved *Cepaea nemoralis* (L.). Protoplasma, 101: 186-196.

Pan, C.T., 1963. Generalized and focal tissue responses in the snail, *Australorbis glabratus*, infected with *Schistosoma mansoni*. An NY Acad. Sci., 113: 475-485.

Radwan, M.A., H.B. El-Wakil and K.A. Osman, 1992. Toxicity and biochemical impact of certain oxime carbamate pesticides against terrestrial snail, *Theba pisana* (Müller). J. Environ. Sci. Health, B 27: 759-773.

Reynolds, E.S., 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol., 17: 208.

Roldan, C. and P. Garcia-Corrales, 1988. Anatomy and histology of the alimentary tract of the snail, *Theba pisana* (Gastropoda: Pulmonata). Malacologia, 28: 119-130.

Rondelaud, D. and G. Dreyfuss, 1996. The development of tissue lesions in the snail, *Lymnaea glabra* exposed to a sublethal dose of molluscicide. Vert. Res., 27: 79-86.

Saad, A.M. and E.A.S. Farag, 1988. Morphological studies on the digestive system of the land snail, *Eobania vermiculata* (Müller) (Gastropoda: Stylomatophora). Alex. J. Agric. Res., 33: 311-326.

Schuytema, G.S., A.V. Nebeker and W.L. Griffis, 1994. Effects of dietary exposure to forest pesticides on the brown garden snail, *Helix aspersa* (Müller). Arch. Environ. Contam. Toxicol., 26: 23-28.

Snyman, R.G., A.J. Reinecke and S.A. Reinecke, 2005. Quantitative changes in the digestive gland cells of the snail *Helix aspersa* after exposure to the fungicide copper oxychloride. Ecotoxicol. Environ. Safety, 60: 47-52.

Souza, D.S. and Z.A. Andrade, 2006. On the origin of the *Biomphalaria glabrata* hemocytes. Mem. Inst. Oswaldo. Cruz., 101: 213-218.

Sullivan, J.T. and J.V. Spence, 1994. Transfer of resistance to *Schistosoma mansoni* in *Biomphalaria glabrata* by allografts of amoebocyte-producing organ. J. Parasitol., 80: 449-453.

Sullivan, J.T., S.S. Piklos and A.Q. Alonzo, 2004. Mitotic responses to extracts of miracidia and cercaria of *Schistosoma mansoni* in the amebocyte-producing organ of the snail intermediate host *Biomphalaria glabrata*. J. Parasitol., 90: 92-96.

Sumner, A.T., 1965. The cytology and histochemistry of the digestive gland cells of *Helix*. Quart. J. Micr. Sci., 106: 173-192.

Sumner, A.T., 1966. The fine structure of digestive-gland cells of *Helix*, *Succinea* and *Tesacella*. J. Roy. Micr. Soc., 85: 181-192.

Sumner, A.T., 1969. The distribution of some hydrolytic enzymes in the cells of the digestive gland of certain lamellibranchs and gastropods. J. Zool., Lond., 158: 277-291.

Taïeb, N. and N. Vicente, 1999. Histochemistry and ultrastructure of the crypt cells in the digestive gland of *Aplysia punctata* (Cuvier, 1803). J. Moll. Stud., 65: 385-398.

Taïeb, N., 2001. Distribution of digestive tubules and fine structure of digestive cells of *Aplysia punctata* (Cuvier, 1803). J. Moll. Stud., 67: 169-182.

Triebskorn, R., 1989. Ultrastructural changes in the digestive tract of *Deroceras reticulatum* (Müller) induced by a carbamate molluscicide and by metaldehyde. Malacologia, 31: 141-156.

Triebskorn, R. and C. Künast, 1990. Ultrastructural changes in the digestive system of *Deroceras reticulatum* (Mollusca; Gastropoda) induced by lethal and sublethal concentrations of the carbamate molluscicide Cloethocarb. Malacologia, 32: 89-106.

Triebskorn, R. and H.R. Köhler, 1992. Plasticity of the endoplasmic reticulum in three cell types of slugs poisoned by molluscicides. Protoplasma, 169: 120-129.

Triebskorn, R. and A. Florschütz, 1993. Transport of uncontaminated and molluscicide-containing food in the digestive tract of the slug *Deroceras reticulatum* (Müller). J. Moll. Stud., 59: 35:42.

Triebskorn, R., I.F. Henderson, A. Martin and H.R. Köhler. 1996. Slugs as target or non-target organisms for environmental chemicals. BCPC Symposium Proceedings No. 66: Slug and Snail Pests in Agriculture.

Trump, B.F., B.P. Croslev and G.W. Mergner, 1971. Cell Membranes: Biological and Pathobiological Aspects. Richter, G.W. and D.G. Scarpelli (Eds.), Williams and Wilkins, Baltimore, pp: 84-126.

Walker, G., 1970. The cytology, histochemistry and ultrastructure of the cell types found in the digestive gland of the slug, *Agriolimax reticulatus* (Müller). Protoplasma, 71: 91-109.

Wedgwood, M.A. and S.E.R. Bailey, 1988. The inhibitory effects of the molluscicide metaldehyde on feeding, locomotion and faecal elimination of three pest species of terrestrial slugs. Ann. Applied Biol., 112: 439-457.

Wright, A.A. and R. Williams, 1980. The effects of molluscicides on the consumption of baits by slugs. J. Molluscan Studies, 46: 265-281.

Young, A.G. and R.M. Wilkins, 1989. The response of invertebrate acetylcholinesterase to molluscicides. Proceedings of the British Crop Protection Council, 41: 121-128.

Zaldibar, B., I. Cancio and I. Marigomez, 2007. Reversible alterations in epithelial cell turnover in digestive gland of winkles (*Littorina littorea*) exposed to cadmium and their implications for biomarker measurements. Aquat. Toxicol., 81: 183-196.

Zedan, H.A., A. Saleh and S.M. Ali, 1999. Bactericidal activity of *Bacillus thuringiensis* against snails, toxicological and histological studies. The 2nd International Conf. of Pest Control, Mansoura, Egypt, 6-8 Sept., pp: 489-497.