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EFFECT OF DIETHYLDITHIOCARBAMATE ON SOME BIOLOGICAL AND PHYSIOLOGICAL PARAMETERS OF *BIOMPHALARIA ALEXANDRINA* SNAILS

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Key words: Diethyldithiocarbamate (DDC), fecundity, vitellogenesis, meurosecration, oxidase, Pyruvate, enzymes, *Biomphalaria* snails.

.ABSTRACT

In this study, the chronic effects of diethyldithiocarbamate (DDC) on the fecunditiy and on some oxidative and anaerobic enzymes of *Biomphalaria alexandrina* snails, the intermediate host of *Schistosomiasis mansoni* in Egypt, were evaluated. The data obtained showed that the exposure of snails to sublethal concentrations of DDC reduced the egg laying capacity of the snails. The hatchability and survival rates of eggs, treated with very low concentrations of DDC were significantly reduced. All of the above effects were found to be concentration and time dependent. Also, these DDC concentrations resulted in a significant decrease in the activity of lactate oxidase (LO), succinate oxidase (SO), known to activate vitellogenesis, and pyruvate kinase (PK), the key enzyme of anaerobic metabolism. On the other hand, the levels of transaminases (AST, ALT) of the exposed snails to DDC concentrations tested were not affected. At the same time, the glycogen content of the treated snails was lowered significantly. Moreover, the tested DDC concentrations caused degenerative changes to

the histological architecture of hermaphrodite, albumin and neurosecretory glands of the exposed snails. All these findings may suggest that DDC has anti-fecundity and ovicidal effects on the examined snails, and affects the parasite – snail relationship by inhibiting some enzymes of both vitellogenesis and anaerobic respiration.

INTRODUCTION

Schistosomiasis is still considered as one of the important public health problems in tropical and subtropical countries particularly in Africa and South America (WHO, 1993). This chronic and debilitating disease is affecting more than 200 million people in 73 countries (McCullough and Mott, 1983) and from 500 – 600 million people are exposed to the risk of infection. *Schistosoma mansoni* is now more prevalent than *S. haematobium* among inhabitants of the Nile Delta, while the opposite case is found in Upper Egypt. The successful control of such disease should be based on an integrated approach which, of course, should include the control of the intermediate host snails (WHO, 1993).

For the above target, continued efforts are conducted to identify and produce more effective and selective molluscicides. Little is known about the mollusc – specific effects of commercial molluscicides owing to the fact that most research is restricted to LC_{50} tests, which provide information about lethal and non-lethal effects of such instances tested. For further information about the targets and biological mechanisms, which are essential for the development of new and selective molluscicide compounds, much basic research is required.

To date, the most efficient pesticides against slugs or snails are the carbamate compounds. From carbamate molluscicides: Methiocarb or Mesurol (Pessah and Sokolove, 1983); Cloethocarb (Triebskorn and

Koher, 1992) and recently Dithiocarbamates (Roa and Daughtie, 1984; Rizk and El-Bolkiny, 1997).

The carbamate compound tested herein is diethyldithiocarbamate (DDC), which has a variety of medical, industrial and agricultural applications (El-Bolkiny, 1995). It is also used as a chelating agent to separate copper from other metal solutions (Sunderman, 1964, 67) and it has been shown that DDC exhibits a potent antimalarial activity (Scheibel and Alder, 1980). The antimalarial activity of DDC was first noted in human by Scheibel *et al.* (1979) using disulfiram as a dimmer of DDC. Both compounds are potent inhibitors of the copper- and Zinc-containing superoxide dismutase, SOD (Heikkila *et al.*, 1979). It was found that DDC-treated SOD was highly toxic to malaria parasite *in vitro* (Meshnick *et al.*, 1990). Depending upon suppressed SOD by the application of DDC, it was used as antileishmanial (Meshnick and Eaton, 1981), antitrypanosomal (Meshnick *et al.*, 1986), antifilarial (Kohler *et al.*, 1990), antischistosomal (Rizk and El-Bolkiny, 1997), and in the chemotherapy for patients as well.

The present study was conducted to investigate the effect of DDC on the fecundity of *B. alexandrina* snails exposed to sublethal concentrations (much less than $_{1/10}$ LC_{50}) evaluated by Rizk and El-Bolkiny (1997). Biological monitored parameters were the egg laying capacity, ovicidal and hatchibility of snail's eggs. The physiological parameters focused on measurement of the activity of oxidizing and anaerobic enzymes involved in the process of egg production. The study was also extended to examine the histopathological changes in the hermaphrodite, albumin and neurosecretory glands of the snails following their exposure to DDC.

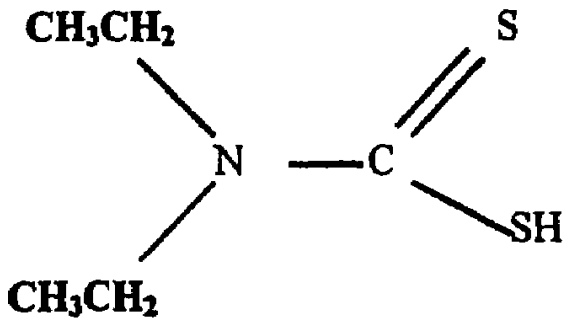
MATERIALS AND METHAODS

Source of snails:

Clean *Biomphalaria alexandrina* snails, the intermediate host of *Schistosoma mansoni*, were obtained from the snails control unit, Theodore Bilharz Institute Warrak Al-Arab, Cairo, Egypt. The snails were acclimatized to the laboratory conditions for at least one month before any experimentation. All snails were maintained in 30 liter-plastic aquaria at a water temperature of $26 \pm 2^{\circ}C$ under constant aeration. Snails were provided with a diet of fresh lettuce leaves and rabbit food pellets *ad libitum* and the water of the aquaria was renewed at fixed intervals to avoid deterioration.

Chemicals:

Diethyledithiocarbamic acid (DDC) is one derivative of dithiocarbamates. It was purchased from division of ICN biochemical (Sigma Chemical Co., St. Louis, MO). It exists in a purified form of white crystals. It is water soluble at the room temperature and also in other organic solvents as ethanol. It has an empirical formula of $C_5H_{11}NS_2$.



To prepare a stock solution, 1 gram of DDC was dissolved in 1000 ml of dechlorinated tap water to get a final concentration of 1000 ppm preserved in refrigerator. The tested concentrations were freshly prepared from the stock solution by further dilutions.

Experimental design and bioassay:

To achieve the objectives of this study, three experiments were carried out:

Experiment I: Effect of DDC on the egg laying capacity and on the histologic architecture of some glands of *B. alexandrina* snails:

This experiment was done to investigate the possible effects of the exposure with sublethal concentrations of DDC (much less than $_{1/10}$ LC_{50}) on the egg laying capacity of *B. alexandrina* snails. Clean adult snails were divided into three groups, 20 snails each, at a density of 10 snails / 2 liters of aquaria. The 1st group was left as a control while the 2nd and 3rd groups were exposed to 0.7 and 1.5 ppm of DDC respectively. The DDC concentrations were renewed every other day to avoid decomposition. All snails were provided every other day with lettuce and rabbit food pellets. The Clutches of eggs were collected twice weekly along the exposure period of 4 weeks. The egg laying capacity of each snail was estimated weekly and expressed as mean number of eggs per snail per week (E/S/W).

For the histological investigation, adult snails were exposed to 1.5 ppm of DDC solution against a control group. After one week exposure, snails were carefully dissected and the hermaphrodite, albumin and neurosecretory cells within nerve ganglia were taken out and fixed in Bouin's fluid. After fixation, the specimens were embedded in paraffin and sectioned at 5 µ. Sections were stained using hematoxylin and eosin, and the histologic changes were microscopically examined and photographed.

Experiment II: Investigation of the ovicidal potency of DDC against *B. alexandrina* ova and its efficacy on the hatchability rate of eggs:

To investigate the ovicidal activity of DDC, freshly deposited egg masses (35-40 eggs) were obtained from clean snails and subjected to

different concentrations (0.1 – 1.0 ppm) in small Petri dishes. The mortality rate was daily evaluated at a fixed time interval (24 h). Similarly, newly laid egg masses were exposed to the same concentrations of DDC till hatching to evaluate their hatchability rate.

Experiment III: Effect of DDC on the activity of some metabolic and oxidative enzymes involved in vitellogenesis of *B. alexandrina* snails:

In this experiment, three groups of clean snails were prepared in dechlorinated tap water. The 1st group was left as a control. The 2nd and 3rd groups were respectively exposed to 1.5 and 3.0 ppm of DDC along three weeks. The DDC concentrations were renewed every other day to avoid decomposition. At the end of the experiment, samples of hemolymph were collected according to Nduka and Harrison (1980) and tissue homogenates were prepared for enzyme activity and glycogen determinations respectively. In brief, the rinsed snails were wiped dry with a paper towel and placed on its left side (i.e. spiral clockwise) on the bottom of a dry Petri dish. Only the region over the heart was broken using a forceps to expose the heart chambers. A capillary pipette was used to collect the haemolymph. Using this method, about 35-70 µl could be collected from each snail (of 8-10 mm diameter).

Enzyme assays:

The activity of transaminases was measured according to Nabih *et al.* (1989). Determination of succinate oxidase (SO) activity was assayed according to Hammen (1973) in which an artificial hydrogen acceptor in the form of the blue dye dichlorophenolindophenol (DCPIP) was used. Lactate oxidase (LO) was measured according to Babson and Babson (1973) using 2- (B-iodophenyl)-3-(B-nitrophenyl)-5-phenyl tetrazolium chloride. Pyruvate kinase (PK) was measured using the method of Umezurike and Anya (1978). In this method, 2.9 ml reaction mixture containing 75 mM Kcl, 8 mM $Mgcl_2$, 2 mM ADP, 0.2 mM $NADH.Na_2$ and 0.5 mM PEP (phenol enol pyruvate) was incubated at 30 °C for 5

and 0.5 mM PEP (phenol enol pyruvate) was incubated at 30 °C for 5 minutes. The reaction was started by the addition of 0.1 ml of enzyme extract. The rate of reaction was measured at 340 nm and the initial absorbency was recorded. Measuring the absorbency at 340 nm after 5 minutes from the NADH oxidation. The activity of the enzyme was then calculated.

Statistics:

The data of the present work were analyzed and expressed as means ± standard deviation. Differences among treated and control groups were estimated by using Student's t-test.

RESULTS

Figures (1-3) show the total egg production of control and exposed snails to DDC along a period of 4 weeks. The total number of the egg masses, eggs and viable eggs was reduced after the exposure to 0.7 and 1.5 ppm of DDC respectively. This reduction in most tested parameters was significant, either at $P < 0.05$ or at $P < 0.01$ compared to those of control snails. Also, the values of the above parameters were significantly decreased with the exposure time versus those of control snails. These results suggest that the egg laying capacity of *B. alexandrina* snails was not only DDC concentration but also time dependent.

As shown in Table 1, DDC solutions exhibited a marked ovicidal activity against the newly laid eggs of *B. alexandrina* snails. The data obtained indicated that DDC concentrations displayed a time–concentration dependent ovicidal activity. A total death of eggs was observed at the application of 0.4 and 0.5 ppm of DDC after the 5^{th} and 4^{th} days of exposure respectively. No survivors were found at 0.9 ppm

The results obtained in Table 2 show that DDC caused a marked inhibition of the egg hatchability in a concentration dependent pattern. At a concentration of 0.3 ppm, DDC led to a retardation of the hatching about 5 days from the control by 88 %. At 0.4 ppm, no hatching was observed where DDC application impaired the hatching of eggs by 100 %. Generally, As the DDC concentration increased, the hatchability rate of eggs/snail was reduced.

Figure 4 shows slight fluctuations in the activity of transaminases, aspartate and alanine aminotransferases (AST, ALT), of the exposed snails to 1.5 and 3.0 ppm of DDC. These fluctuations were insignificant compared to the activity of such enzymes in the control snails.

Both DDC concentrations of 1.5 and 3.0 ppm caused significant ($P < 0.01, 0.001$) decrease in the activity of lactate oxidase (LO), succinate oxidase (SO) and pyruvate kinase (PK). In case of LO activity, the percent of decrease was –74.05 in both DDC tested concentrations compared to the control snails. Also, the percent of decrease in the level of SO was – 74.5 and - 71.0 due to the exposure of snails to 1.5 and 3.0 ppm of DDC respectively (Fig. 5). Likely, as shown in Figure (6) both DDC concentrations (1.5, 3.0 ppm) led to a fall in PK level by percentages of – 22.26 and – 28.05 respectively.

The glycogen content of the treated snails with 1.5 and 3.0 ppm of DDC was highly reduced than controls by percentages of – 58.78 and – 60.37 respectively (Fig. 7).

HISTOLOGICAL INVESTIGATION:

Neurosecretory cells:

The histological investigation of the neurosecretory cells of the control snails showed that they are included in the two cerebral ganglia that are connected by a cerebral commissure. Two dorsal bodies (DB)

that are connected by a cerebral commissure. Two dorsal bodies (DB) located medio- and latero-dorsally are attached to each cerebral ganglion. Each DB consists of a large number of small cells referred to as dorsal body cells (DBC). Besides, three types of neurosecretory cells are found in each DB. These cells are classified according to their size and locations into medio-dorsal light green cells (LGC), latero-dorsal cells (LDC) and ventral caudal dorsal cells (CDC). The LDC are formed of large number of small aggregated cells with very minute nuclei. The other tow types (LGC and CDC) are formed of large cells with large rounded nuclei. All types of cells are connected together with a fibrous connective tissue (Fig. 8a).

The exposure of snails to 1.5 ppm of DDC led to a great reduction in volume of the DB and in both the number and shape of DBC as well as disappearance of LDC. Moreover, necrotic changes and rupture of the cell membranes were observed in the other two cells (CDC and LGC) leading to a fibrosis (Fig. 8b).

Albumen gland:

The albumen gland tubules of the control snails are composed of cuboidal or pyramidal secretory epithelial cells. These cells have large rounded basal nuclei with apically located secretory granules. The tubules are connected together with a dense connective tissue and have very narrow lumina (Fig. 9a). Exposure of snails to 1.5 ppm of DDC caused marked degenerative changes in the histological architecture of the gland tubules. The apical membranes of the epithelial secretory cells were ruptured, leading to disappearance of secretory granules and to much wider lumina compared to the control (Fig. 9b).

Hermaphrodite gland:

The hermaphrodite gland of the control snails is composed of a number of acini connected together with a dense connective tissue. Each

gametes including spermatozoa and mature ova (Fig. 10a). After one week of 1.5 ppm of DDC exposure, a severe distruction of the germinal epithelia of the acini was observed and hence all stages of gametogenesis were affected. As shown in Figure 10b, gametocytes, spermatocytes, oocytes and spermatids were degenerated. Compared to the control, the number of both mature sperms and ova was markedly reduced. Moreover, the remnant mature ova of the treated snails appeared to be necrotized without ovarian membranes (Fig. 10 c).

DISCUSSION

Little attention has been paid to the fact that carbamate compounds possess molluscicidal activity (Pessah and Sokolove, 1983; Triebskorn and Koher, 1992 and; Rizk and El-Bolkiny, 1997). The data obtained in the present investigation showed that DDC (carbamate) affected the fecundity of snails exposed to sublethal concentrations. These concentrations reduced the egg production of snails and impaired the hatching of the viable eggs. Ovulation and egg laying are triggered by the release of several hormones from caudo-dorsal cells in the cerebral ganglia. It seems that a set of neuropeptides derived from a common polypeptide precursor was affected by DDC. Firstly, the egg-cell maturation in the ovotestis was stimulated by secretion of the gonadotrophic dorsal body hormone (Geraerts and Joosse, 1975; Sluiters, *et al.,* 1984). Secondly, ovulation was stimulated by two groups of cerebral, neurosecretory caudo-dorsal cells (CDC), which produce the ovulation hormone CDCH (Geraerts and Bohlken, 1976; Geraets *et al,.* 1988). Thirdly, the histological architecture of the ovotestis itself, where egg production takes place was considerably affected. The histological structure of the caudo-dorsal cells and dorsal body cells of the exposed snails showed complete damage and denaturation of the neurosecretory cell nuclei and surrounding cytoplasm. Previous studies showed that the

cell nuclei and surrounding cytoplasm. Previous studies showed that the egg production of *L. stagnalis* was also sensitive to treatment with carbamate insecticides, such as lindane and carbaryl (Seuge and Bluzat, 1983). Similar results were obtained about the reduction in the egg laying of *B. alexandrina* and *B. truncatus* due to chronic exposure to sublethal concentrations of various pesticides (El-Gindy and Rawi, 1991; Ibrahim *et al.*, 1992; Rawi *et al.*, 1994 and Rizk *et al.*, 1998).

Moreover, the results obtained also revealed that the longer the exposure period of snails, the higher the molluscicidal activity of DDC as monitored by inhibition of the egg laying capacity and blocking of oxidative enzymes. This result could be explained by either gradual release of DDC and/or its accumulation in the snail's body. This finding accords with the report of Triebskorn and Kunast (1990) that the effect of carbamate cloethocarb on the slug *Deroceras reticulatum* was a dose-time response relationship.

It was found that DDC blocked the oxidative enzymes responsible for the egg formation and production. It also inhibited the secretion of some enzymes of anaerobic respiration. Because phenoloxidase (PO) has been shown to be involved in egg formation in other invertebrate species, Bai *et al.* (1997) showed that the exposure of *B. glabrata* to DDC, PO inhibitor, affected normal *in vivo* egg production. This interesting result was confirmed by a significant decrease in the number of eggs laid in DDC-treated groups compared to non-treated groups. They added that DDC-exposure studies provide strong support for a crucial role of PO and other oxidases in normal egg production of the schistosomiasis snails.

The present study also revealed that the glycogen content of the treated snails was highly reduced and this may explain the decline in the egg laying capacity of the exposed snails. This also led to a decrease in the cell volume of the albumen and hermaphrodite glands, as well as an

increase in the intra-cellular connective tissue area. These results agree with those reported by Thomson (1988) and Bielefeld (1991) who demonstrated that a depletion of haemolymph, glycogen and lipids in starved *B. glabrata* snails caused inhibition of egg production and degenerative changes in its hermaphrodite gland.

On the other hand, Scheibel *et al.* (1979) showed that the chelating agents such as DDC combine with metal containing enzymes to form what is known DDC-metal complexes extracellularly. By virtue of the hydrophobicity of this complex, it penetrates the cell membrane. Once in the membrane, the dissociation of the complex was thought to occur, leading to the release of DDC to combine with enzymes-containing metals. Subsequently, the inhibition of a metal–dependent enzyme at its active site in the lysosomes may explain the molluscicidal properties of DDC solution (Meshnick *et al.*, 1986).

Accordingly, the mode of action of DDC may be due to one or more pathways. It may deprive the snail of essential metal(s) or it may cause an accumulation of free oxygen radicals released from suppressed oxidases (Heikkila *et al.*, 1979). Moreover, DDC form toxic DDC-metal complexes, which may impair the biological functions of the cell, particularly inhibition of CDCH, involved in the egg formation and production. In the view of the present results, it can be concluded that DDC due to its ideal water solubility and its unique mode of action can be recommended as a cheaper and effective new molluscicidal agent. Further investigations are continued in our laboratory to study the toxicity of DDC on the target and non-target species and to evaluate its antihelminthic activity.

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Table 1. The ovicidal activity of DDC concentrations against *B. alexandrina* fresh ova at standard laboratory conditions*

Hours / Conc. (ppm)	Mortality % of snail ova**						
	24	48	72	96	120	144	168
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.1	0.0	0.0	5.0	5.0	8.0	10.0	10.0
0.2	0.0	5.0	13.0	20.0	25.0	35.0	50.0
0.3	0.0	10.0	17.0	21.0	30.0	38.0	55.0
0.4	7.0	33.0	75.0	90.0	100	100	100
0.5	25.0	50.0	90.0	100	100	100	100
0.7	30.0	90.0	95.0	100	100	100	100
0.9	70.0	100	100	100	100	100	100

*At a water temperature of 25 ± 2 °C and pH 7.4

**Total number of eggs / each concentration is 40 eggs (in average).

Table 2. The effect of DDC concentrations on the egg hatchability rate of *B. alexandrina* snails at standard laboratory conditions*

Days / Conc. (ppm)	% of egg hatching**						
	8	**9**	**10**	**11**	**12**	**13**	**14**
0.0	95	100	100	100	100	100	100
0.1	0.0	0.0	38	80	83	90	90
0.2	0.0	0.0	0.0	0.0	30	35	40
0.3	0.0	0.0	0.0	0.0	0.0	12	23
0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0

*At a water temperature of 25 + 2 C and pH 7.4

**Total number of eggs / each concentration is 40 eggs (in average).

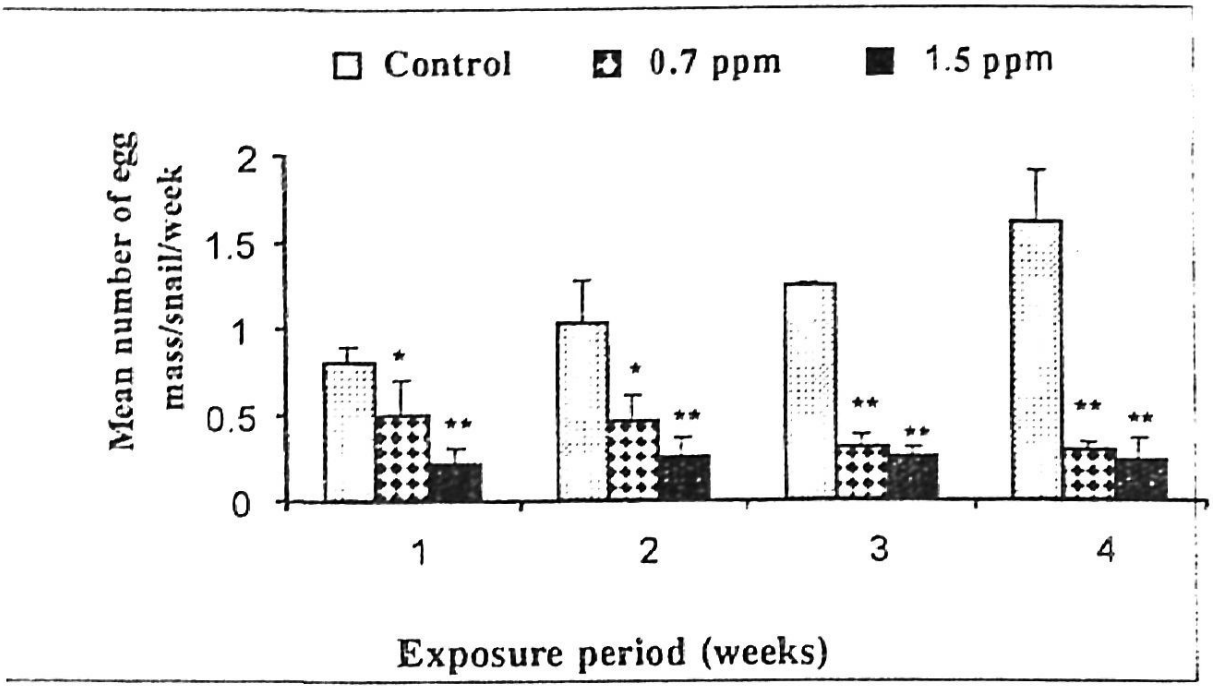


Fig. 1. Effect of sublethal DDC concentrations (0.7, 1.5 ppm) on the egg mass production of snails. *, ** Significant at $P < 0.05$ and at $P < 0.01$ (t-test).

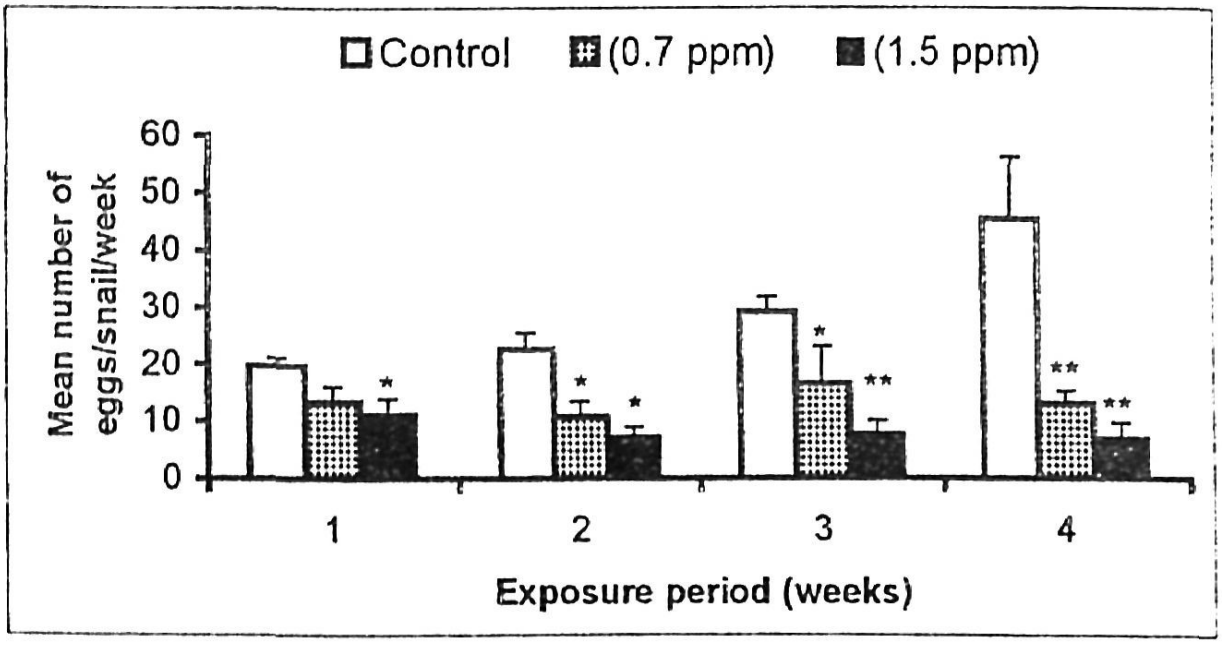


Fig. 2. Effect of sublethal DDC concentrations (0.7, 1.5 ppm) on the total number of eggs of snails. *, ** Significant at $P < 0.05$ and at $P < 0.01$ (t-test).

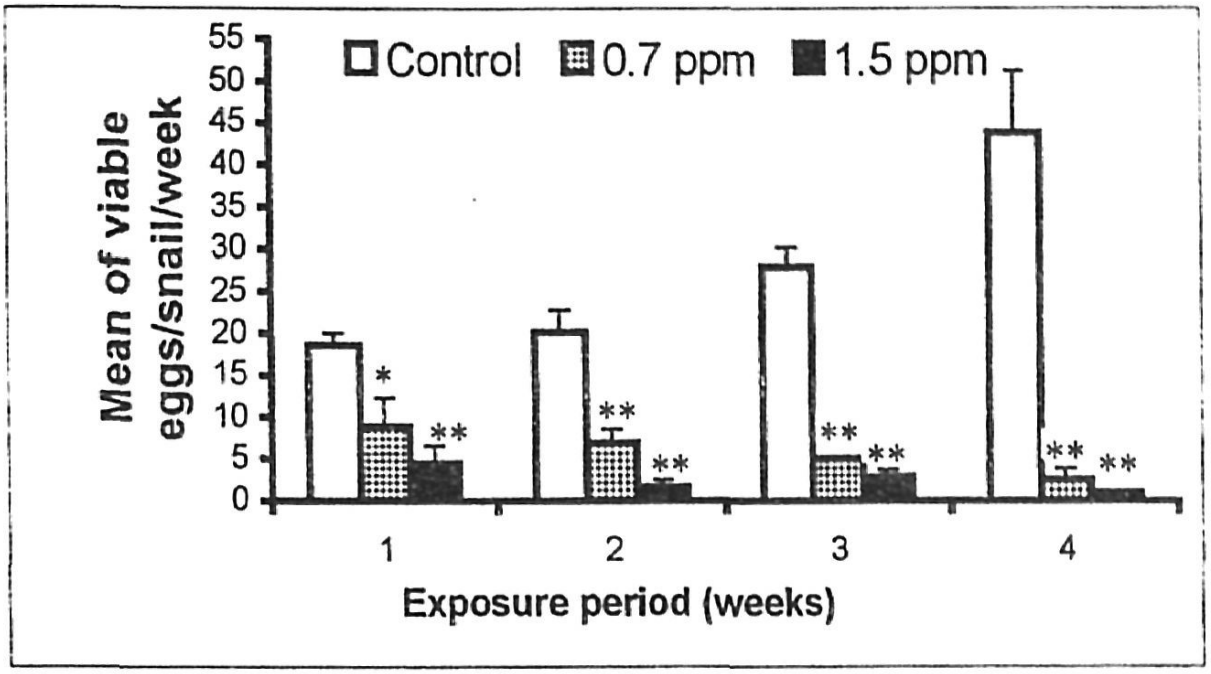


Fig. 3. Effect of sublethal DDC concentrations (0.7, 1.5 ppm) on the number of viable eggs of snails. ** Significant at $P < 0.01$ (t-test).

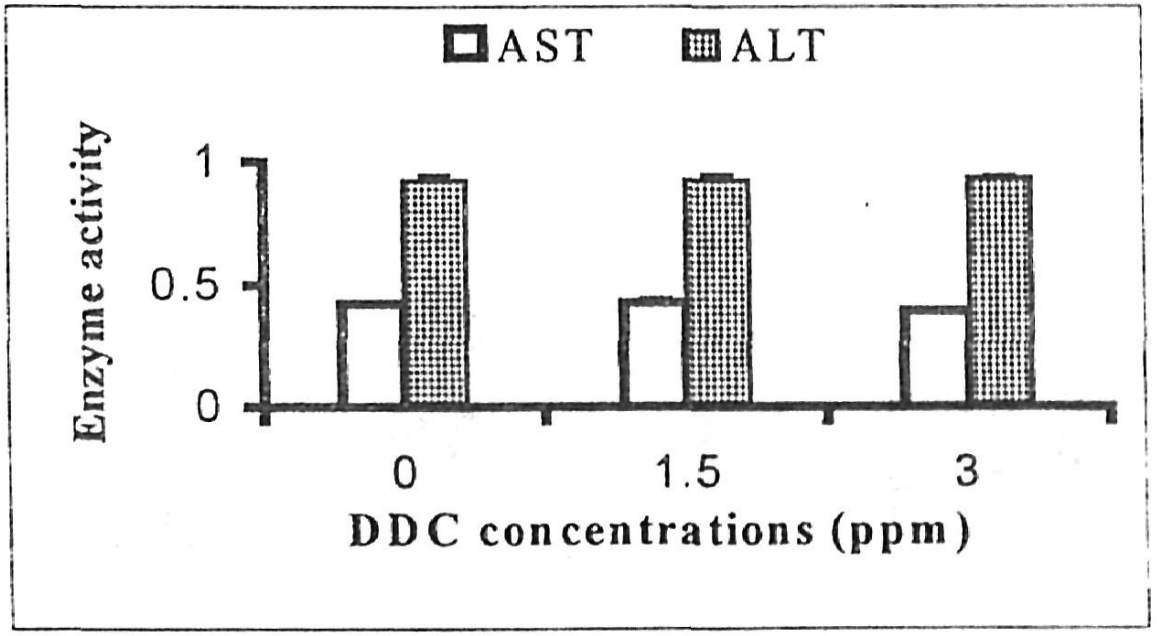


Fig. 4. Effect of sublethal DDC concentrations (1.5, 3.0 ppm) on the level of transaminases; aspartate aminotransferase (AST) and alanine aminotransferase (ALT) of snails.

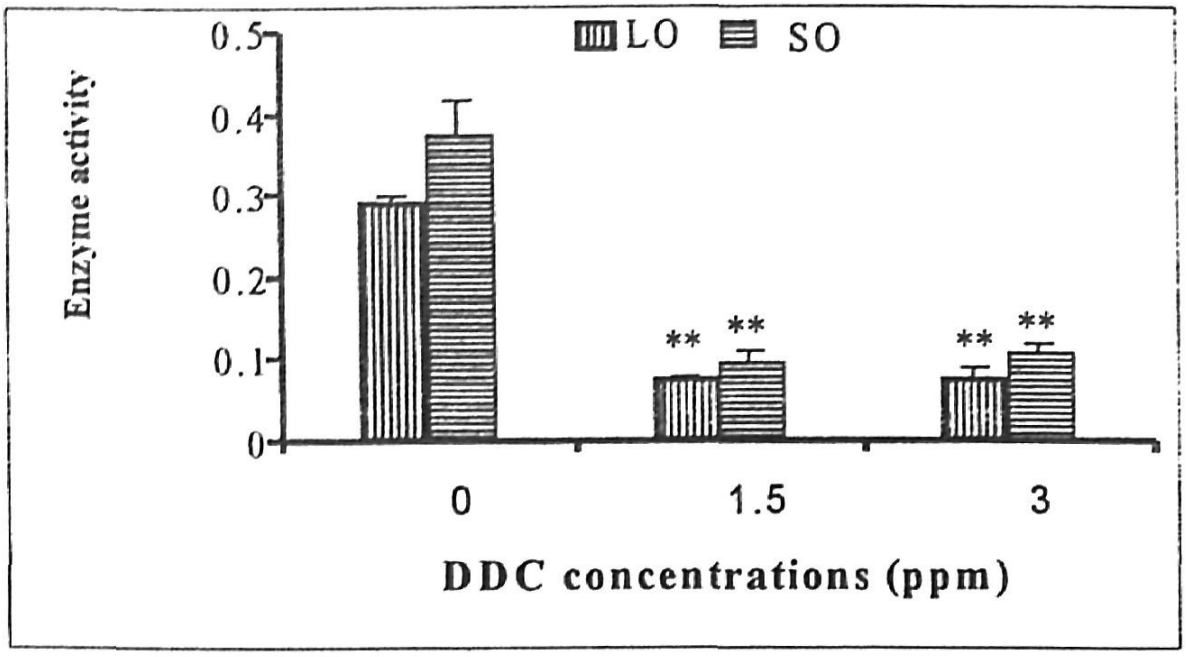


Fig. 5. Effect of sublethal DDC concentrations (1.5, 3.0 ppm) on the level of oxidases; lactate oxidase (LO) and succinate oxidase (SO) of snails. ** Significant at $P < 0.01$ (t-test).

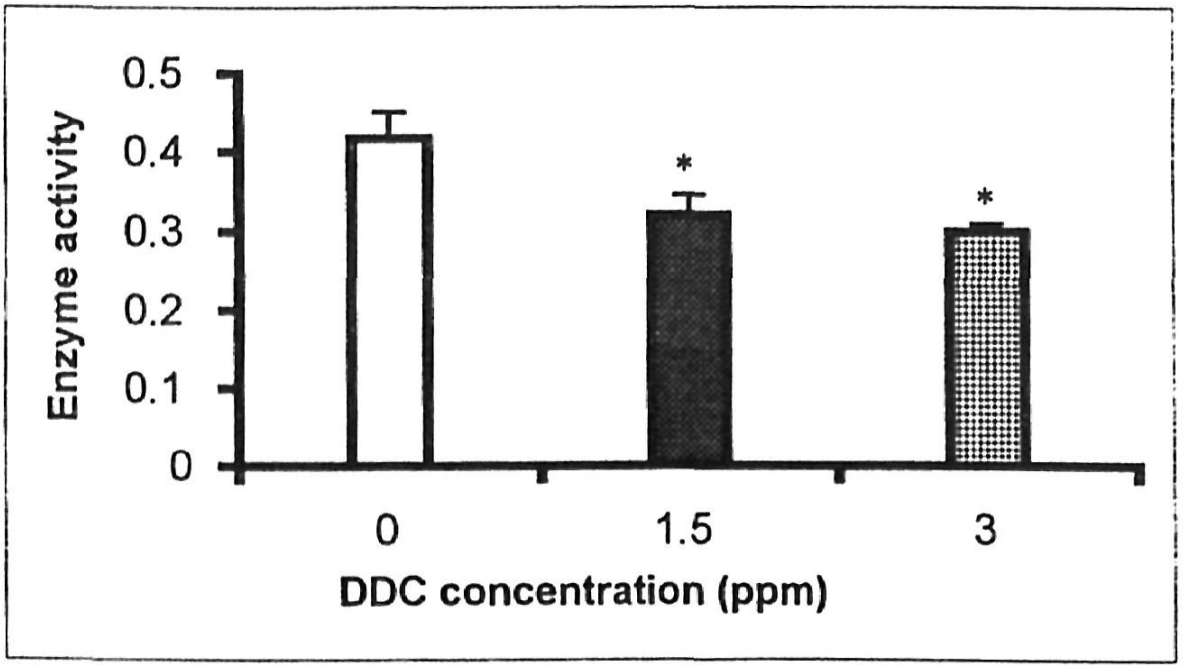


Fig. 6. Effect of sublethal DDC concentrations (1.5, 3.0 ppm) on the level of pyruvate kinase (PK) of snails. * Significant at $P < 0.05$ (t-test).

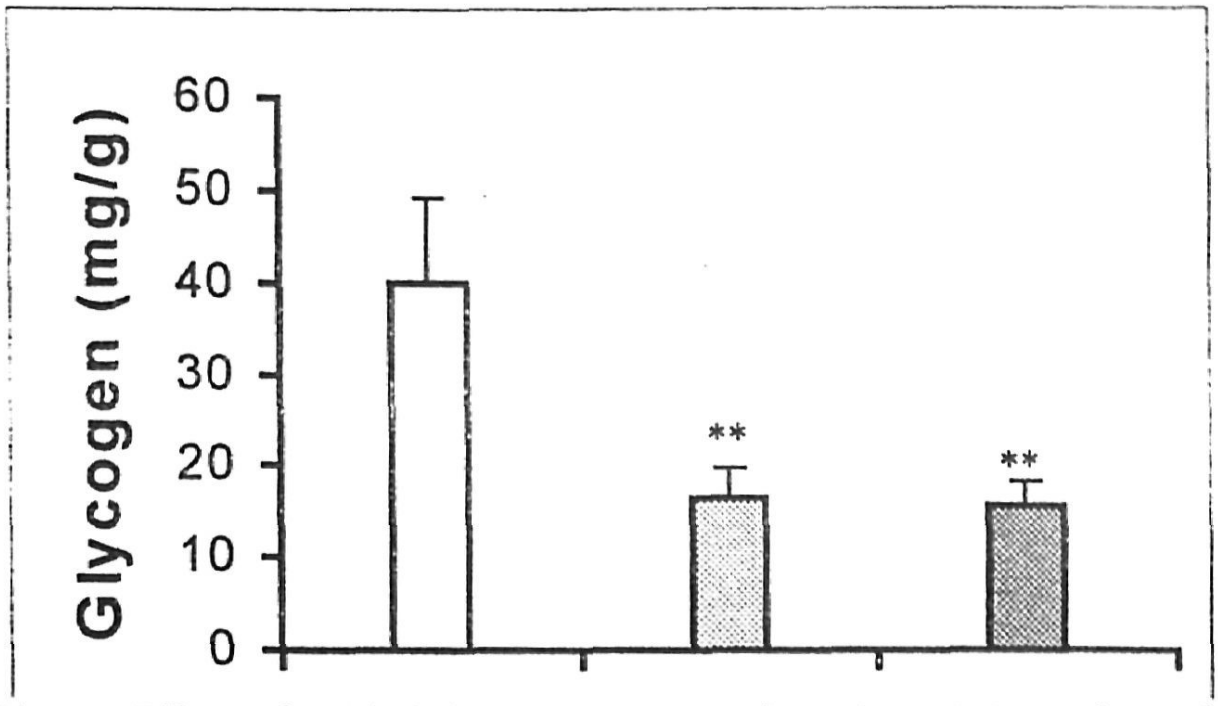


Fig. 7. Effect of sublethal DDC concentrations (1.5, 3.0 ppm) on the glycogen content of snails. ** Significant at $P < 0.01$ (t-test).

EXPLANATION OF HISTOLOGICAL MICROGRPHS

Figure 8 a: Photomicrograph in the neurosecretory gland of untreated *B. alexandrina* snails showing normal neurosecretory cell types; caudo-dorsal cells (cdc), lateral dorsal cells (ldc), light green cells (lgc), medeo-dorsal body cells (mdb) and intra-acini connective tissue (ct), X 400.

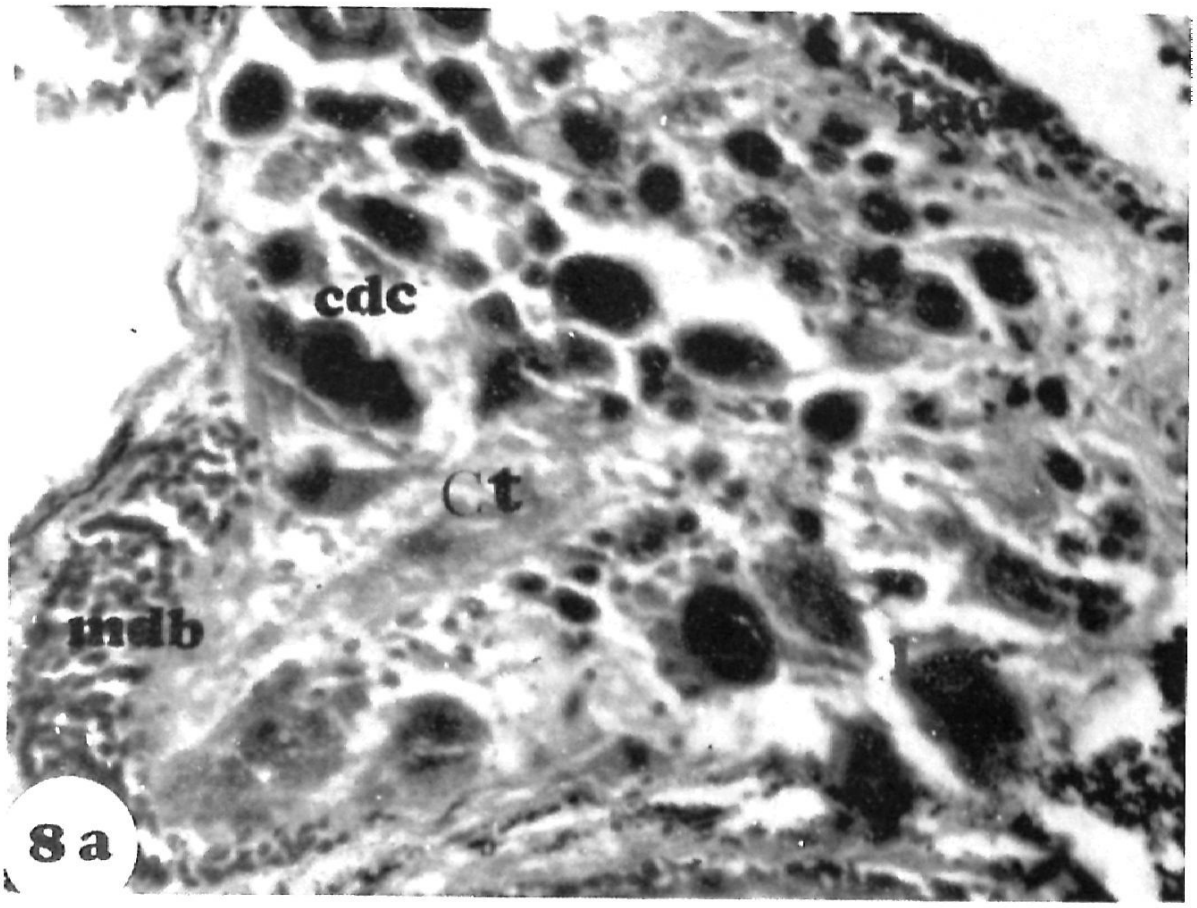
Figure 8 b: Photomicrograph in the neurosecretory gland of treated *B. alexandrina* snails showing vaculated and necrotic cells (nc) of the different cell types mentioned above, X 400.

Figure 9 a: Photomicrograph in the albumen gland of untreated *B. alexandrina* snails showing normal structure of the gland including secretory cells (sc), secretory granules (sg) and inter-connective tissue (ct), X 400.

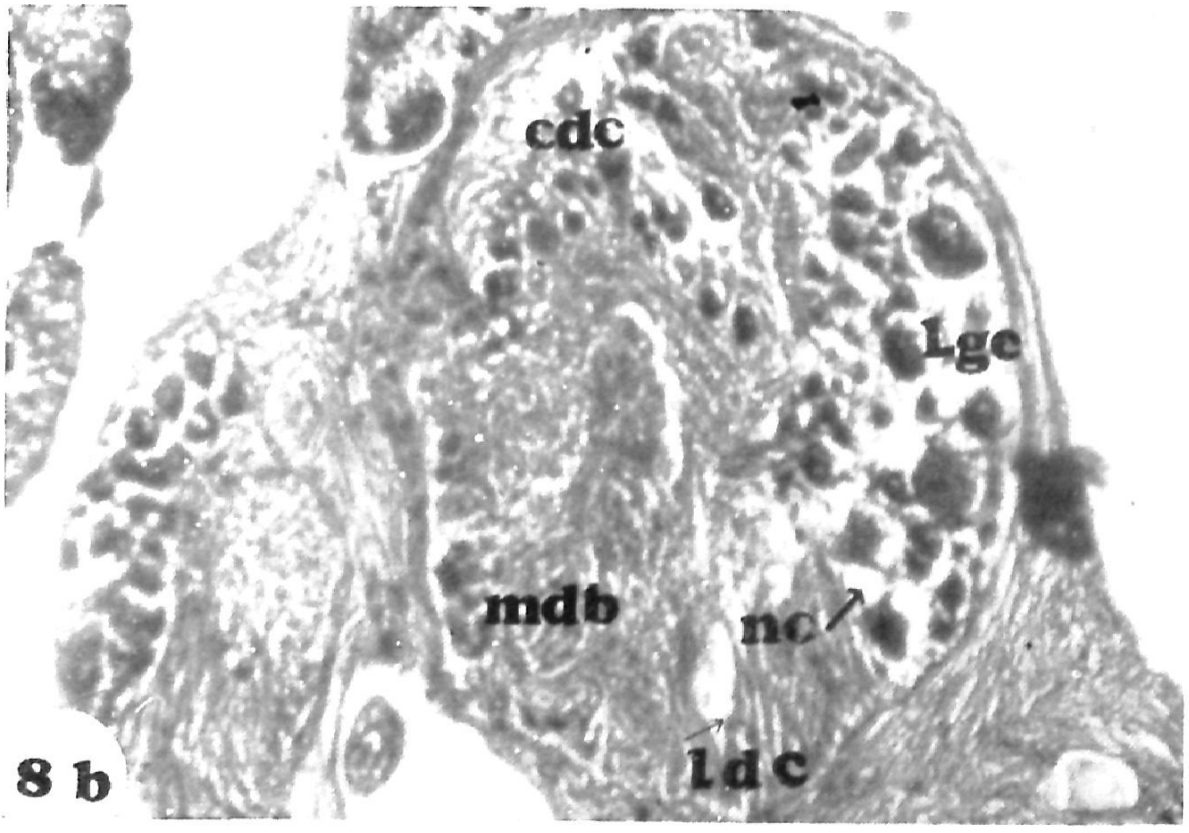
Figure 9 b: Photomicrograph in the albumen gland of treated *B. alexandrina* snails showing vaculated (v) and necrotic epithelial cells (nc) and enlargment of the lumen (L), X 400.

Figure 10 a: Photomicrograph in the hermaphrodite gland of untreated *B. alexandrina* snails showing normal structure of the gland including germinal epithelium (ge), different stages of oocytes and spermatocytes (sp), and mature ova (o) and sperms (s), X 400.

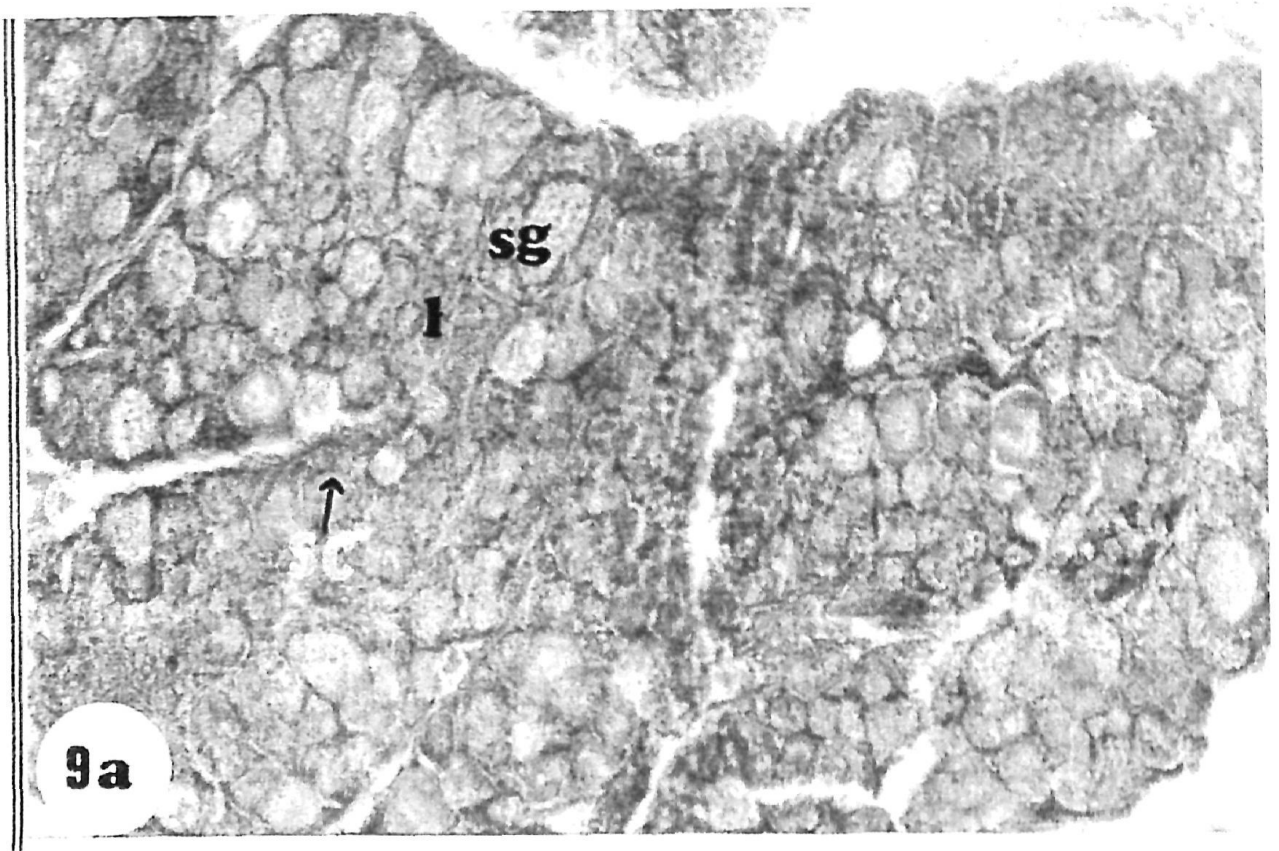
Figure 10 b and c: Photomicrograph in the hermaphrodite gland of treated *B. alexandrina* snails showing destruction of germinal epithelium (ge) oocytes (oc), mature ova (o) appeared to be necrotized and few sperms (s), X 400.

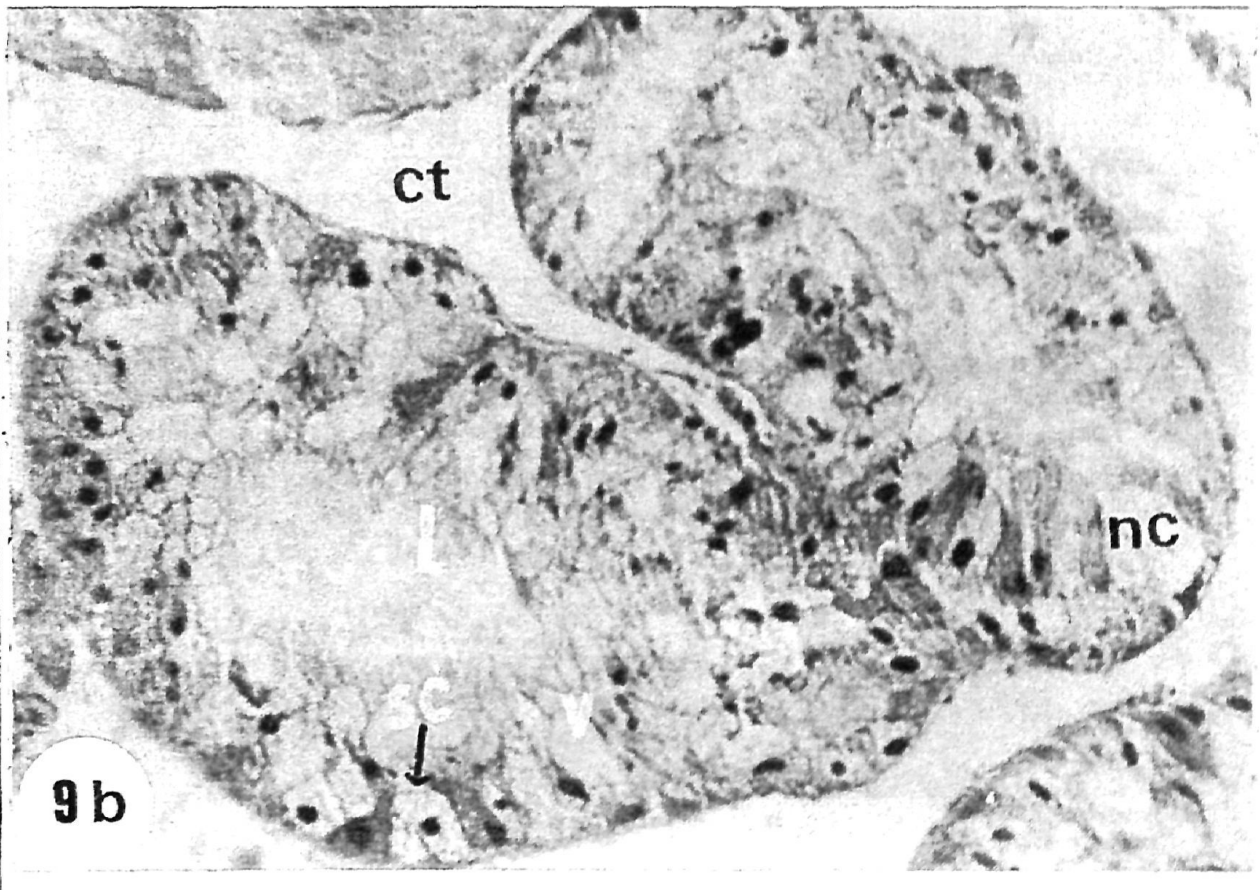


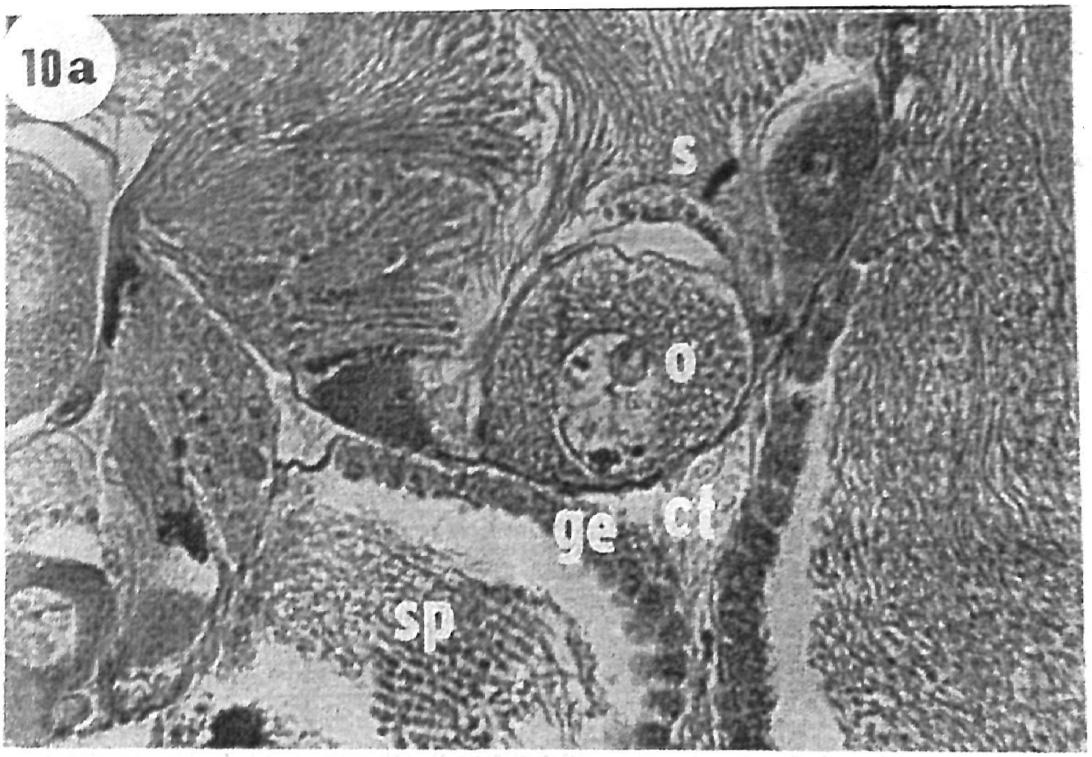
8 a



8 b


sg
l
c
9a


ct
nc
9b


10a
s
o
ge
ct
sp


10 b
o
oc


10c