EFFECTS OF SUBLEATHAL DOSAGES OF FLUFENOXURON AND CHLORFLUAZURON ON HAEMOLYMPH CHANGES OF *SPODOPTERA LITTORALIS*

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ABSTRACT

The present study aims to investigate the changes of haemocytes in the 6th larval instar of *S. littoralis* haemolymph as a result of treatment of the 3rd larval instar with LC_{25}, LC_{50} and LC_{70} of two insect growth inhibtors, flufenoxuron and chlorfluazuron. Several pathological cases were observed in the haemocytes (plasmatocytes, prohemocytes, granulocytes, spherulocytes and oenocytoids) including variation in the cell volume, vacuolization in the cytoplasm, distortion in the cell membrane and pycnosis of nuclei. At the same time the change in the total and differential haemocytes counts were recorded.

Also, the effects of the two insect growth regulators on the concentrations of different inorganic ions (potassium, phosphorus, sodium, calcium, magnesium) and total protein of haemolymph of the 6th larval instar of *S. littoralis* were evaluated.

INTRODUCTION

Numerous studies have been undertaken for unconventional control agents owing to the hazards of conventional pesticides. Among such agents are the insect growth regulators. Insect cellular defence reactions against invaders include nodule formation and encasulaion (Ratcliffe and Gagen, 1976). The factors controlling these reactions are poorly defined, but many authors reported that the increase of total circulating haemocytes counts could be considered as an immune response against pathogens (Chu *et al.*, 1993; ford *et al.*, 1993 Anderson *et al.*, 1995; Ordas *et al.*, 2000). Also, plasmatocytes, prohemocytes and granulocytes play an important role in phagocytosis, cell clumping and wound healing (Gagen and Ratcliffe, 1976; Barakat, 1997).

The present study aims to investigate the immune response of the cotton leafworm *S. littolarlis* 3rd larval instar towards two insect growth inhibtors. This was attained by determination certain pathological consequence in the defined haemocytes, the total haemocyte count and the percentage of differential haemocyte count in the larvae after treatment with both IGRs. The concentrations of different inorganic ions and total protein were also undertaken. Such studies may be of a salient importance in controlling this insect pest.

MATERIAL AND METHODS

The Experimental Insect

Spodoptera littoralis (Boisd) larvae obtained from the laboratory culture of plant protection Research Institute, Agricultural Research Center (Cairo, Egypt).
Chemical used

Insect growth regulators: Chitin synthesis inhibitors

(1) Flufenoxuron

1-(4- (2- chloro- α, α, α- trifluoro – P – tolyloxy) -2- fluoropheny l- 3- (2 ,6 – difluorobenzyl)Urea.

(2) Chlorfluazuron

N-(4-(3-chloro-5-trifluoromethy-2-Pyridinyloxy] (-3, 5-dich-lorophenyl–amino carbonyl) – 2, 6– diflorobenzamide.

Haematological studies

Collection of hamolymph

Normal and treated six instar larvae resulted from the treated 3rd instar larvae with LC_{25}, LC_{50} and LC_{70} (0.17, 0.335 and 0.60 ppm and 0.082, 0.229 and 0.50 ppm) for flufenoxuron and chlorfluazuron, respectively. The haemolymph was obtained by amputation of one or two prothoracic legs of the larvae with fine scissors. Gentle pressure was done on the thorax until a drop of haemolymph appeared at the point of amputation. The haemolymph from two individuals was never mixed.

Total haemocytes counts (THCs)

The haemolymph was calculated according to the formula of Jones (1962) as follows:

$$\frac{\text{Number of haemocyte counted per chamber X dilution X depth factor}}{\text{Number of 1mm squares counted}}$$

Where the depth factor is usually 10.

Differential haemocyte counts (DHCs)

Examining samples of haemolymph from 10 individuals (6^{th} larval instar) in a given stage made differential haemocyte counts. Whenever possible, a minimum of 100 cells/ 6^{th} larval instar were investigated. Stained preparations, according to Arnold and Hinks (1979). The cell shape, cytoplasmic ratio, cytoplasmic inclusions and shape of nucleus were used for classification of haemocytes using the classification scheme of Brehelin and Zachary (1986). The percentages of haemocyte types were calculated by the formula:

$$\frac{\text{Number of each haemocyte type}}{\text{Total number of haemocytes examined}} \times 100$$

The measurements were replicated 10 times for the 6th larval instar of the control group and the different concentrations of the tested compound.

Chemical analysis of haemolymph

Haemolymph was obtained from the survived 6^{th} instar larvae in LC_{25}, LC_{50} and LC_{70} and control group. Samples of obtained haemolymph were centrifuged at 2500 r.p.m for 10 min, and only the supernatant fractions were used for assay.

Determination of potassium

Potassium concentration was determined using the method of Hillman and Beyer (1967).

Determination of phosphorus

Phosphorus concentration was calculated using the method of Henry (1964).

Determination of sodium

Sodium concentration was determined using the method of Trinder (1951).

Determination of calcium

Calcium concentration was determined using the method of Kessler and Wolfinan (1964).

Determination of magnesium

Magnesium concentration was calculated using the method of Mann and Yoe (1956).

Determination of total protein

Total protein concentration was determined using the method of Weichselbaum (1946).

Data analysis

The data were subject to statistical analysis according to the equation of Dixon and Massay (1957).

RESULTS

Haemocytes were distinguished on the basis of morphological characteristics and staining. Five main haemocytes have been found in 6th larval instar haemolymph. They are plasmatocytes, prohemocytes, granulocytes, spherulocytes and oenocytoids.

Normal plasmatocytes

These cells are polymorphic, ovoid or spindle in shape, and usually have a fusiform configuration. Some of these fusiform cells are truly spindle-shaped with a dense perinuclear and tapering cytoplasmic processes. Others exhibit dense spindle shape in one plane, but as they roll in the haemolymph, they show a broad flat granulated morphology. The majority of the plasmatocytes circulating haemolymph of the 6th larval instar are round dense cells having pale nuclei that contain punctate chromatin granules (PL.1A, B.C). The dense perinuclear region is bounded peripherally by a thin granule, free area that rapidly produced and retracted cytoplasmic blebs (PL.1A). Dense round plasmatocytes are found singly, in pairs, and occasionally in small clusters of four to eight cells (PL.1B, C). Plasmatocytes measure about 18.125±1.737 um in length and 8.25±0.940 um in width. Plasmatocytes represent about 43±3.539% of total haemocytes. Plasmatocytes are capable of phagocytosis, a kind of entocytosis of smaller foreign cells.

Histopathological effects of flufenoxuron and chlorfluazuron on plasmatocytes

The concentration, "0.17 ppm", (LC_{25}) of flufenoxuron caused haemocytic microaggregation of plasmatocytes. Also, plasmatocytes showed vacuolization in their cytoplasm, distortion in cell membrane and lysing cells. Granulosis of nucleus was also observed (PL.1D), while the concentration, "0.335 ppm", (LC_{50}) showed a great destruction and distortion in cell membrane, lysing of the cells and haemocytic microaggregation (PL.1E). The concentration, "0.60 ppm", (LC_{70}) induced great variation in cell volume, vacuolization in the cytoplasm, distortion of cell membrane and granulosis of nucleus. Also, cells of plasmatocytes appeared lysing in addition to small pink and dark staining bodies which represent pieces of denatured nuclear materials (PL.1F).

Histological examination of chlorfluazuron treated larvae with the concentration, "0.082 ppm", (LC_{25}) showed vacuolization in the cytoplasm, granulosis of nucleus and distortion in cell membrane (PL.1G), while the concentration, "0.229 ppm", (LC_{50}) caused great variation in the cell volume, vacuolization in the cytoplasm and distortion of cell membrane (PL.1H).

Under the concentration, "0.50 ppm", of chlorfluazuron (LC_{70}), plasmatocytes showed great variation in cell volume, vacuolization in the cytoplasm, distortion in the cell membrane and granulosis of nucleus. Added to that lysing and degenerated plasmatocytes and vacuolization in the nucleus (PL.1I).

Normal prohemocytes

Prohemocytes are thought to be the stem cells from which all haemocytes arise. These cells are small nearly ovoid or spherical in shape with a very highly nucleus cytoplasm ratio. They have deeply stained cytoplasm that is rich in ribonucleic acid and the nuclei almost fill the cells. These cells are 7.75±0.412 µm in length and 6.25±0.590 µm in width. They are rarely observed in the haemolymph and represent about 9±0.533% of the total haemocytes (PL.2A, B, C).

Histopathological effects of flufenoxuron and chlorfluazuron on prohemocytes

Histological examination of flufenoxuron treated larvae with the concentration, "0.17 ppm", (LC_{25}) showed distortion in cell membrane of prohemocytes and the nuclei were very darkly stained (nuclear hyperchromicity) (PL.2D), while the concentration, "0.335 ppm", (LC_{50}) prohemocyte showed vacuole in the cytoplasm and the nucleus stained very darkly (PL.2E). The concentration, "0.60 ppm", (LC_{70}) of flufenoxuron caused distortion of cell membrane of prohemocytes and very darkly stained nuclei and cytoplasm (PL2F).

The "0.082 ppm", (LC_{25}) concentration of chlorfluazuron, caused distortion in the cell membrane of prohemocytes, very darkly stained nuclei and cytoplasm in some cells. In others the cytoplasm appeared normal but the nucleus stained pink in colour (PL.2G). Under the effect of the concentration, "0.229 ppm", (LC_{50}) the cells appeared binucleated and the cell membrane distorted (PL.2H). At the "0.50 ppm", (LC_{70}) concentration, the prohemocytes showed vacuolation in the cytoplasm, granulosis of nucleus and distortion of the cell membrane (PL.2I).

Normal granulocytes

These cells are abundant and represent about 15±3.590% of the total number of haemocytes. Granulocytes are ovoid, rounded or fusiform-shaped cells, with 9.667±0.898

μm in length and 7.667±0.500 μm in width. The cytoplasm is basophilic and contains large number of acidophilic granules that vary in size within the same cell (PL.3A,B).The nuclei vary in shape from spherical to ovoid and may be centric or eccentric(PL.3A,B). Granulocytes play the major role in encapsulation reactions and haemocytic aggregation.

Histopathological effects of flufenoxuron and chlorfluazuron on granulocytes

The concentration "0.17 ppm" (LC_{25}) of flufenoxuron caused pycnosis in the nuclei and cells were characterized by highly granulated and deeply stained nucleoplasm and vacuolation in the cytoplasm (PL.3C), while the concentration, "0.335 ppm", (LC_{50}) the granulocytes have been affected severely and these effects appeared in the cytoplasm and nucleus. The cells characterized by highly granulated and deeply stained nucleoplasm, distortion of cell membrane and pycnosis appeared in the nuclei (PL.3D). The "0.60 ppm", (LC_{70}) flufenoxuron concentration, caused great effects on granulocytes. Pycnosis appeared in the nuclei and cells were characterized by highly granulated and deeply stained nucleoplasm and cytoplasm. Distortion in the cell membrane, lysed cells and highly vacuolization in the cytoplasm (PL.3E) were also observed.

The concentration, "0.082 ppm", (LC_{25}) of chlorfluazuron caused pycnosis of nuclei and the cells showed deeply stained nucleoplasm and destruction in the cytoplasm (PL.3F). The concentration "0.229 ppm" (LC_{50}) showed highly vacuolization in the cytoplasm that appeared severely affected. Pycnosis appeared in the nuclei and the cells showed highly granulated and deeply stained nucleoplasm and distortion of the cell membrane (PL.3G). Under the effect of the concentration, "0.50 ppm", (LC_{70}) the cytoplasm and nuclei of the granulocytes have been affected severely. Pycnosis appeared in the nuclei, and cells characterized by highly granulated and deeply stained nucleoplasm (PL.3H).

Normal spherulocytes

These cells are the most abundant in circulation following plasmatocytes and represent 32±1.937% of the total haemocytes. Spherulocytes are round in shape and contain several large cytoplasmic inclusions that distinguish them from granular haemocytes. These cells are irregular in shape with large cytoplasmic spherules that obscure the nuclei. Spherulocytes represent 7.833±0.703 μm in length and 6.667±0.715 μm in width (PL.4A, B).

Histopathological effects of flufenoxuron and chlorfluazuron on spherulocytes

Under the effect of the concentration, "0.17 ppm", (LC_{25}) of flufenoxuron, spherulocytes showed distortion in cell membrane, binucleated nucleus and deeply

stained nucleoplasm (PL.4C). Also, the concentration, "0.335 ppm", (LC_{50}) flufenoxuron caused the same effect on spherulocytes (PL.4D). As for the concentration, "0.60 ppm", (LC_{70}) of flufenoxuron it induced great effects on spherulocyte as vacuolization in the cytoplasm, distortion in cell membrane, granulosis and pycnosis of nucleus (PL.4E).

The concentration, "0.082 ppm", (LC_{25}) of chlorfluazuron caused a slight effect on spherulocytes, that was represented in distortion in cell membrane (PL.4F), while under the concentration, "0.229 ppm", (LC_{50}), spherulocytes appeared with bilobed nucleus (PL.4G). The concentration, "0.50 ppm", (LC_{70}) of chlorfluazuron caused great effects on spherulocyte as vacuolization in the cytoplasm and distortion in cell membrane. Also, the nucleus appeared bilobed and granulosis (PL.4H).

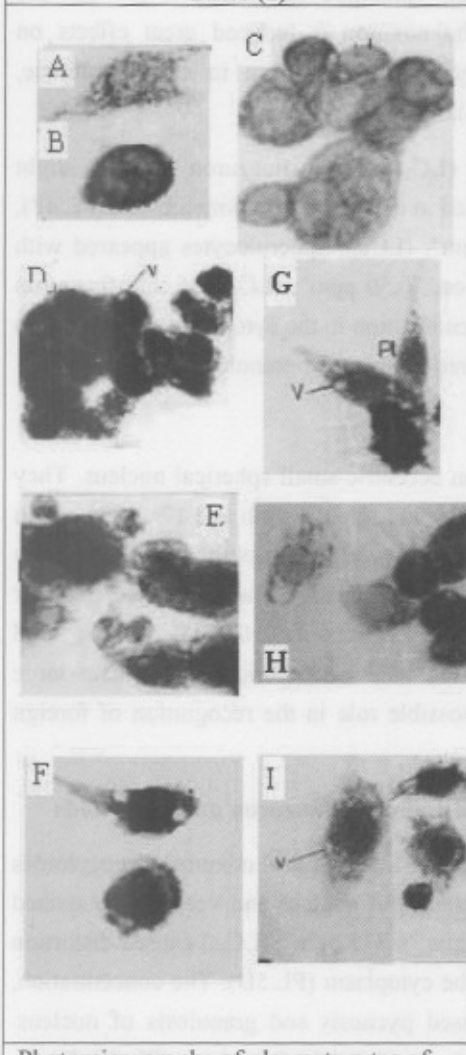
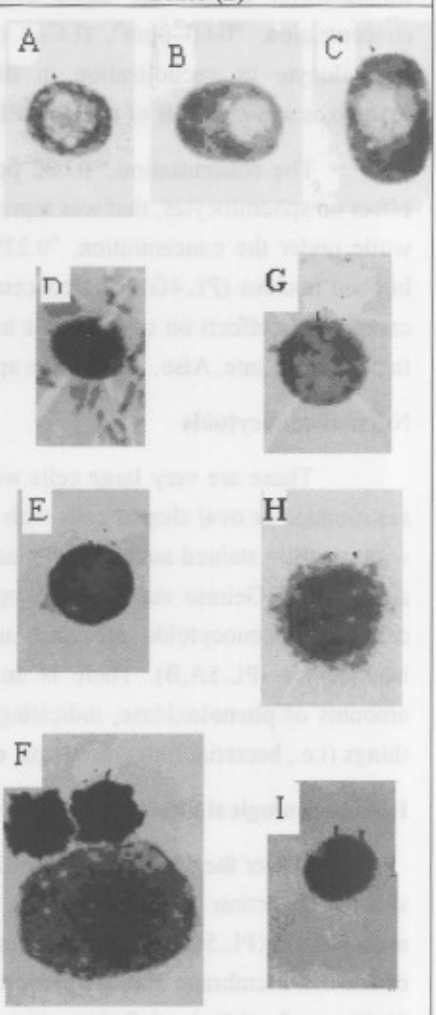
Normal oenocytoids

These are very large cells with an eccentric small spherical nucleus. They are rounded or oval shaped cells with 23.4±0.942 μm in length and 17±1.095 μm in width, darkly stained nucleus and clear, uniform weakly basophilic cytoplasm when stained with Geimsa stain. The cytoplasm has spherical inclusions called granular cells. The oenocytoids are rare and represent about 1±0.271% of the total haemocytes (PL.5A,B). There is an evident that oenocytoid cells contain large amounts of phenoloxidase, indicating a possible role in the recognition of foreign things (i.e., bacteria, fungi, protozoa, etc.).

Histopathological effects of flufenoxuron and chlorfluazuron on oenocytoids

Under the concentration, "0.17 ppm", (LC_{25}) of flufenoxuron, oenocytoides showed distortion in cell membrane, granulosis of nucleus and very darkly stained nucleoplasm (PL.5C), also, the concentration, "0.335 ppm", (LC_{50}) caused distortion of the cell membrane and destruction in the cytoplasm (PL.5D). The concentration, "0.60 ppm", (LC_{70}) of flufenoxuron caused pycnosis and granulosis of nucleus. Also, the nucleus of some cells appeared abnormal (bilobed) in shape (PL.5E, F)

The concentration, "0.082 ppm", (LC_{25}) of chlorfluazuron caused distortion in the cell membrane, destruction in the cytoplasm and granulosis of nucleus (PL.5G). The effects caused by the concentration, "0.229 ppm", (LC_{50}) were similar to that of LC_{25} (PL.5H). As for the effects of the concentration, "0.50 ppm", (LC_{70}) of chlorfluazuron, oenocytoids showed distortion in cell membrane and granulosis of nucleus. Moreover, the nuclei appeared bilobed in shape (PL.5I).

Plate (1)	Plate (2)
	
Photomicrographs of plasmatocytes of normal and treated 6th larval instar of *S. littoralis*.	Photomicrographs of prohemocytes of normal and treated 6th larval instar of *S. littoralis*.

Changes in total haemocytes counting following treatment with flufenoxuron and chlorfluazuron

This part concerns with the changes in total haemocytes counts that occur when third instar larvae were treated with flufenoxuron and chlorfluazuron. The haemolymph was collected from the 6th instar larvae and prepared as described in the materials and methods.

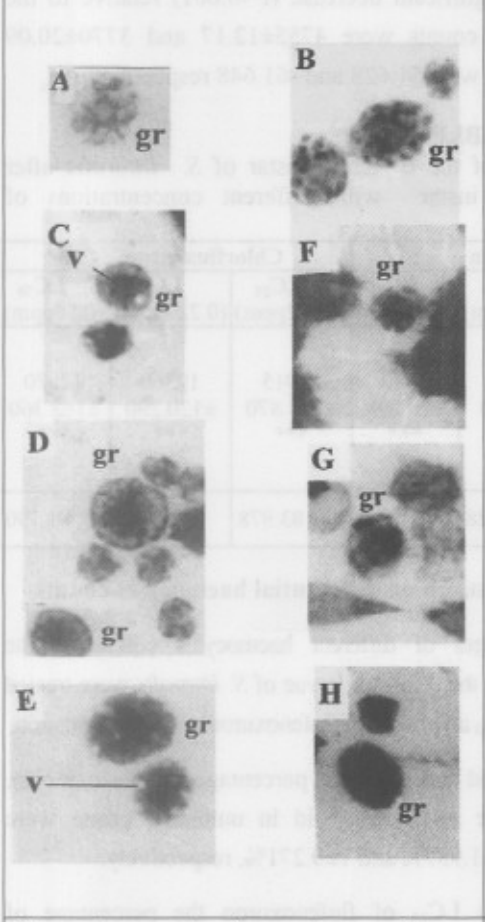
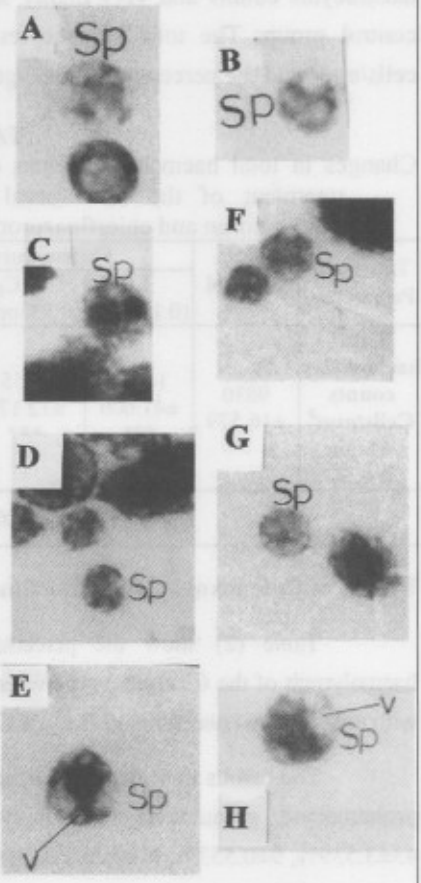
Plate (3)	Plate (4)
	
Photomicrographs of granulocytes of normal and treated 6th larval instar of *S. littoralis*.	Photomicrographs of spherulocytes of normal and treated 6th larval instar of *S. littoralis*.

Table (1) show the total number of haemocytes in haemolymph of the 6th instar larvae at different concentrations of flufenoxuron and chlorfluazuron "LC_{25}, LC_{50} and LC_{70}" when the 3rd instar larvae were treated with the above concentrations. The results indicated that the mean of total haemocytes counts in haemolymph of untreated 6th larval instar was 9830±16.579 cells/mm^3. In treated ones with LC_{25} of flufenoxuron, LC_{25}, LC_{50} and LC_{70} of chlorfluazuron the total number of haemocytes counts were 14165±41.00, 27915±163.87, 19360±120.29 and 17870±163.36 cells/mm^3 respectively These increases were very highly significant ($P<0.001$) as compared to control group with a percentage of change 44.099, 183.978, 183.978 and 81.790 at the previous arrangement of tested compounds.

While the LC_{50} and LC_{70} of flufenoxuron caused high reduction in total haemocytes counts and very highly significant decrease ($P<0.001$) relative to the control group. The total haemocytes counts were 4755±12.17 and 3770±20.09 cells/mm^3 and the percentage of change was -51.628 and -61.648 respectively.

TABLE (I)

Changes in total haemocytes counts of the 6th larval instar of *S. littoralis* after treatment of the 3rd larval instar with different concentrations of flufenoxuron and chlorfluazuron.

Treatment Parameters	Control	Flufenoxuron			Chlorfluazuron		
		LC_{25} (0.17ppm)	LC_{50} (0.335ppm)	LC_{70} (0.60ppm)	LC_{25} (0.082ppm)	LC_{50} (0.229ppm)	LC_{70} (0.50ppm)
Total haemocytes counts Cells/mm^3 Mean ± S.E.	9830 ±16.579	14165 ±41.000 ***	4755 ±12.170 ***	3770 ±20.090 ***	27915 ±163.870 ***	19360 ±120.290 ***	17870 ±163.360 ***
% of change		44.099	- 51.628	- 61.648	183.978	96.948	81.790

Effects of flufenoxuron and chlorfluazuron on differential haemocytes counts

Table (2) show the percentages of different haemocytes counts in the haemolymph of the 6th instar larvae when the 3rd instar larvae of *S. littoralis* were treated with the different concentration "LC_{25}, LC_{50} and LC_{70}" of flufenoxuron and chlorfluazuron.

The results in Table (2) indicated that, the mean percentages of plasmatocyte, prohemocyte, granulocyte, spherulocyte and oenocytoid in untreated group were 43±3.539%, 9±0.533%, 15±3.590%, 32±1.937% and 1±0.271%, respectively.

The larvae were treated with LC_{25} of flufenoxuron the percentage of granulocyte significantly decreased ($P<0.05$) relative to control (5±0.414) with a percentage of change -66.067. Also, the percentage of plasmatocytes count at LC_{50} was significantly decreased ($P<0.05$) relative to the control with a percentage of change -31.953. While the percentage of spherulocyte counts was highly significantly increased ($P<0.01$) with a percentage of change 71.137. The percentage of oenocytoids count increased significantly ($P<0.05$) (2±0.003) with a percentage of change 100.

Under the effect of flufenoxuron LC_{70}, the percentage of prohemocyte count was very highly significant decrease ($P<0.001$) with a percentage of change -44.44. In case of spherulocytes, the percentage of differential count was 39.583±3.928 and increased significantly ($P<0.05$) with a percentage of change 43.75.

In treated ones with all concentrations of chlorfluazuron "LC_{25}, LC_{50} and LC_{70}" the percentage of plasmatocyte increased very highly ($P<0.001$) with a percentage of change 53.488, 44.186 and 55.814 at previous concentrations. On the other hand, the percentage of prohemocytes count had very highly significant decrease ($P<0.001$) at LC_{25} and LC_{70} as compared with the control group and have percentage of changes -99 and –44.44 respectively.

Also the percentage of granulocytes and spherulocytes count decreased significantly ($P<0.05$) with a percentage of change -60, -66.667 and –73.333 at LC_{25}, LC_{50} and LC_{70}. While the percentage of oenocytoid count were increased significantly ($P<0.05$) with a percentage of change 100 and 100 at LC_{25} and LC_{50} of chlorfluazuron.

TABLE (II)

Mean percentage of differential haemocytes counts of the 6th larval instar of *S.littoralis* after treatment of the 3rd larval instar with different concentrations of flufenoxuron and chlorfluazuron.

Treatment		Statistical parameters	Differential haemocytes counts (% + S.E.)				
			Plasmatocyte	Prohemocyte	Granulocyte	Spherulocyte	Oenocytoids
Control			43 ±3.539	9 ±0.533	15 ±3.590	32 ±1.937	1 ±0.271
Flufenoxuron	LC_{25} (0.17 ppm)	Mean ± S.E.	52 ±3.928	11 ±0.131	5 ±0.414	31 ±4.958	1 ±0.003
		% of change	20.930	22.222	- 66.667	- 3.125	0
		Significance	•	•	*	•	0
	LC_{50} (0.335 ppm)	Mean ± S.E.	29.26 ±4.754	6.826 ±1.716	7.15 ±1.64	54.764 ±5.339	2 ±0.003
		% of change	- 31.953	- 24.156	- 52.333	71.137	100
		Significance	*	•	•	**	*
	LC_{70} (0.60 ppm)	Mean ± S.E.	38.417 ±2.5	5 ±0.323	16 ±0.577	39.583 ±3.928	1 ±0.003
		% of change	- 10.658	- 44.44	6.667	43.75	0
		Significance	•	***	•	*	0
Chlorfluazuron	LC_{25} (0.082 ppm)	Mean ± S.E.	66 ±1.8	1 ±0.003	6 ±1.111	25 ±1.530	2 ±0.003
		% of change	53.488	- 99	- 60	- 21.875	100
		Significance	***	***	*	*	*
	LC_{50} (0.229 ppm)	Mean ± S.E.	62 ±2.605	8 ±1.076	5 ±0.783	23 ±2.66	2 ±0.003
		% of change	44.186	- 11.111	- 66.667	- 28.125	100
		Significance	***	•	*	*	*
	LC_{70} (0.50 ppm)	Mean ± S.E.	67 ±4.883	5 ±0.976	4 ±0.796	22 ±3.541	1 ±0.003
		% of change	55.814	- 44.44	- 73.333	- 31.25	0
		Significance	***	***	*	*	0

Significant change in comparison with control group.

• Non significantly different

* Significantly different at $P<0.05$

** Highly significantly different at $P<0.01$

*** Very highly significantly different at $P<0.001$

Effects of insect growth inhibtors on the levels of different haemolymph parameters

Total proteins

Table (3) show the total protein level in serum of the haemolymph of the 6th instar larvae of *S. littoralis* when the 3rd instar larvae were treated with (LC_{25}, LC_{50} and LC_{70}) of flufenoxuron and chlorfluazuron.

The results indicated that the total protein content was significantly reduced ($P<0.05$) as compared with the control group when larvae treated with LC_{25} of flufenoxuron and LC_{70} chlorfluazuron and the percentage of change in the total protein were -30.895 and -30.597 respectively.

As for the concentrations LC_{50} and LC_{70} of flufenoxuron they caused highly significant decrease ($P<0.01$) in the level of the total protein, with a percentage of change was -33.894 and -50.381.

TABLE (III)

Changes in protein levels in haemoymph of the 6th larval instar of *S. littoralis* after treatment of the 3rd larval instar with different concentrations of flufenoxuron and chlorfluazuron.

Treatment Parameters	Control	Flufenoxuron			Chlorfluazuron		
		LC_{25} (0.17ppm)	LC_{50} (0.335ppm)	LC_{70} (0.60ppm)	LC_{25} (0.082ppm)	LC_{50} (0.229ppm)	LC_{70} (0.50ppm)
Total protein gm protein /dL Mean ±S.E.	5.6152 ±1.967	3.8804 ±0.286 *	3.7120 ±0.148 **	2.7862 ±0.235 ***	6.2484 ±0.304 •	6.0126 ±0.452 •	3.8971 ±0.356 *
% of change		- 30.895	- 33.894	- 50.381	11.277	7.077	- 30.597

Inorganic ions

Table (4) show changes in levels of potassium, phosphorus, sodium, calcium and magnesium in haemolymph of the 6th larval instar of *S. littoralis* after treatment of the 3rd larval instar with different concentrations of flufenoxuron and chlorfluazuron (LC_{25}, LC_{50} and LC_{70}).

The results indicated that, the sodium level increased when the larvae were treated with LC_{25}, LC_{50} and LC_{70} of flufenoxuron or chlorfluazuron. This increasing was very highly significant ($P<0.001$) with a percentage of change 386.192, 373.679 and 357.561 in the case of flufenoxuron treatment and 316.1984, 429.000 and 338.944 in the case of chlorfluazuron.

TABLE (IV)

Changes in levels of potassium, phosphorus, sodium, calcium and magnesium in haemolymph of the 6th larval instar of *S. littoralis* after treatment of the 3rd larval instar with different concentrations of flufenoxuron and chlorfluazuron.

Treatment		Statistical parameter	Potassium Mmol potassi./L	Phoshorus Mmol phos./L	Sodium Mmol sod. / L	Calcium Mmol calci./L	Magnesium Mmol mag./L
Control			36.7344 ± 1.967	2.8083 ± 0.093	20.15786 ± 2.375	6.2729 ± 0.272	3.7181 ± 0.034
Flufenoxuron	LC_{25} (0.17 ppm)	Mean ± S.E.	32.7656 ± 1.936	3.1298 ± 0.273	98.00595 ±4.136	3.2035 ± 0.286	3.0846 ± 0.208
		% of change	- 10.804	11.448	386.192	- 48.931	- 17.038
		Significance	•	•	***	***	*
	LC_{50} (0.335 ppm)	Mean ± S.E.	30.2813 ± 1.753	3.5961 ± 0.208	95.4835 ± 2.793	2.2274 ± 0.089	2.9526 ± 0.249
		% of change	- 17.567	28.053	373.679	- 64.492	- 20.588
		Significance	*	*	***	***	*
	LC_{70} (0.60 ppm)	Mean ± S.E.	38.2266 ± 1.951	2.65004 ± 0.262	92.2345 ± 1.962	2.3838 ± 0.129	2.8692 ± 0.246
		% of change	4.062	- 5.635	357.561	- 61.998	- 22.832
		Significance	•	•	***	***	*
Chlorfluazuron	LC_{25} (0.082 ppm)	Mean ± S.E.	43.2499 ± 1.406	3.7451 ± 0.315	83.8967 ±1.162	3.0739 ±0.267	3.1178 ± 0.293
		% of change	17.7367	33.373	316.1984	- 50.997	- 16.145
		Significance	*	*	***	***	•
	LC_{50} (0.229 ppm)	Mean ± S.E.	35.5306 ± 1.755	4.3716 ± 0.158	106.6351±5.807	2.90388±0.303	3.0588 ±0.271
		% of change	-3.315	55.684	429.000	- 53.708	- 17.732
		Significance	•	***	***	***	*
	LC_{70} (0.50 ppm)	Mean ± S.E.	33.7344 ± 1.052	4.6448 ± 0.360	88.4818 ± 3.33	4.0065 ± 0.282	2.9907 ± 0.251
		% of change	-8.1667	65.413	338.944	- 36.130	- 19.564
		Significance	•	**	***	***	*

• Non significantly different P>0.05

* Significantly different at P<0.05

** Highly significantly different at P<0.01

*** Very highly significantly different at P<0.001

In the other hand, the calcium level was reduced at all concentrations of flufenoxuron (LC_{25}, LC_{50} and LC_{70}) and this reduction was very highly significant ($P<0.001$) with a percentage of change -48.931, -64.492 and -61.998, respectively. Also, The data revealed that there was very highly significant decrease ($P<0.001$) in calcium level under the effect of LC_{25}, LC_{50} and LC_{70} of chlorfluazuron as compared with the control group, with a percentage of change -50.997, -53.708 and -36.130, respectively.

The LC_{50} of flufenoxuron caused significant decrease ($P<0.05$) in potassium of the serum of the 6th instar larvae as compared with the control group with a percentage of change -17.567. While, potassium level was significantly increased ($P<0.05$) when the larvae of *S. littoralis* were treated with LC_{25} of chlorfluazuron comparable to the control group and the percentage of change was 17.7367.

At treatment with flufenoxuron, only the LC_{50} has snificantly increased effect on the level of phosphorus relative to the control group with a percentage of change 28.053. Also, the results indicated that LC_{25} of chlorfluazuron caused significant increase ($P<0.05$) in phosphorus level as compared with the control group and the percentage of change was 33.373. While LC_{50} of chlorfluazuron caused very highly significant increase ($P<0.001$) in the phosphorus level with a percentage of change 55.684. At LC_{70} of chlorfluazuron caused highly significant increase ($P<0.01$) in the phosphorus level with a percentage of change 65.413.

The results in the table (4) indicated that the level of magnesium was significantly reduced ($P<0.05$) at all concentrations of flufenoxuron as compared with the control group. The percentage of reduction under the effect of the three concentrations were -17.038%, -20.588% and -22.832%, respectively. While the LC_{25} of chlorfluazuron caused a decrease in the level of magnesium, but this decrease was non-significant ($P>0.05$). The percentage of change was -16.145. As for LC_{50} and LC_{70} they caused significant decrease ($P<0.05$) in the level of magnesium with a percentage of change -17.732 and -19.564, respectively.

DISCUSSION

Haematological studies

There are five distinct haemocyte types in the 6th larval instar of *S. littoralis*: plasmatocytes, prohemocytes, granulocytes, spherulocytes and oenocytoids. Description was based on the basis of cytological parameters such as cell shape, size, nuclear cytoplasmic ratio and cytoplasmic inclusions, as well as

staining affinity. The description of plasmatocytes is similar to that reported by Ralph and William (1965) in diapausing *Hyalophora cecropia* pupae, Richard and jack (1969) in the last instar larvae of the greater wax moth, *Galleria mellonella*. They described five types of haemocytes : prohemocyte, plasmatocytes, adipohemocytes, spherule cells and oenocytoids. David and peter (1982) described the same types of haemocytes in the fifth instar larvae of *Manduca sexta*. However, Osman *et al.* (1984) described four types of haemocytes in the haemolymph of the 4th instar larvae of *S. littoralis* (prohemocytes, plasmatocytes, spherulocytes and oenocytoids). Gurwattan *et al.* (1991) described two types of haemocytes in the male grasshoppers *Melanoplus sanguinipes* ; plasmatocytes and granulocytes. Miller and David (2000) described five types of haemocytes in the 6th instar larvae of tobacco hornworm haemolymph; plasmatocytes, prohemocytes, granulocytes, spherulocytes and oenocytoids. Similar observations were reported by Jian *et al.* (2003) in the larvae of *Ostrinia furnacalis*.

The observed pathological conditions in the infected haemocytes, characterized as (1) changes in the plasma membrane (erosion and extrusion of their cytoplasmic contents), (2) vacuolization and degeneration of the cytoplasm and (3) nuclear changes (pycnosis , karyorrhexis, granulosis and division of the nuclei) that may be induced by the action of flufenoxuron and chlorfluazuron as previously indicated by the work of Miselyunene (1976) on the caterpillar of cabbage butterfly, El-Kattan (1995) on the larvae of Indian meal moth, (*Plodia interpunctella*) and Barakat *et al* .(2002) on the larvae of *Schistocerca gregaria* after injection with *Bacillus thuringiensis* .

The total haemocytes counts (THCs) have been sharply increased after treatments with flufenoxuron and chlorfluazuron. Similar results had been reported by Csman *et al* . (1984) in haemolymph of the larvae of *S. littoralis* after treatment with Dimilin, David and Peter (1982) in larvae of *M. sexta* following injection with bacteria and Barakat *et al.* (2002) in the larvae of *S. gregaria* after injection with *B. thuringiensis*. Contrary to that, Abu El-Magd (1992) found that injection of laminarin to the 5th instar of *S. gregaria* caused a decrease in the total number of haemocytes , in the crab *Carcinus maenas* (Smith *et al* ., 1984) and in the desert locust *S. gregaria* and in the American cockroach *Periplaneta americana* (Gunnarsson and lackie, 1985). This drop in haemocytes number may be due to haemocytes engagement in nodule formation as that recorded by Abu El-Magd (1992). Such a decrease in the total number of haemocytes were reported by Ratcliffe and Gagen (1976) and Chain and Anderson (1982), they found that the

THCs in *Galleria mellonella* dropped rapidly and immediately after injection of bacteria, and they commented that the decrease was almost entirely due to the depletion of plasmatocytes.

The observed remarkable increase in THCs in the 6th larval instar of *S. littoralis* after treatment with flufenoxuron and chlorfluazuron may by due to the release of sessile haemocytes and the activation of mitotic division of the haemocytes. This finding coincided with that reported by Barakat *et al* .(2002) who observed remarkable increase in THCs in the fifth nymphal instar of *S. gregaria* after bacterial injection .

The present results agree with that obtained by Clark and Harvy (1965); Shapiro (1968) on *G. mellonella*; Horohov and Dunn (1982) on *M. sexta* larvae and Guzo and Stoltz (1987) who worked on *Orgyia leucostigma* larvae and found that the nodulation of smaller objects such as yeast cells were accompanied by a rapid and sustained increase in THCs and disagree with the findings of Hoffmann *et al.* (1974) on *L. migratoria*, who found immediate decrease in THCs following injection of *B. thuringiensis* this may be due to species differences.

Studies on changes in the haemocytes population immediately after injection of bacteria in some Lepidoptera have been reported. Decreases were recorded in THCs in *Paseudaletia unipuncta* (Witting, 1965) and in *G. mellonella* (Gagen and Ratcliffe, 1976). The decrease in the former THCs was accompanied by an increase in the proportions of prohemocytes and spherule cells (Witting, 1965). An increase in THCs following the injection of several foreign particles into *P. americana* has been described by (Ryan and Nicholas, 1972).

Many insects posses populations of sessile haemocytes (Wigglesworth, 1972 and Ratcliffe and Gagen, 1976) which might be activated in response to infection. It is reported that *Manduca* posses hemopoietic organs (Monpeyssin and Beaulation, 1978) which produce haemoytes to the circulation and a small measurable population of circulating haemocytes that have been observed undergoing mitosis in other several insects (Jones, 1977).

The present work on the DHCs showed that in the untreated 6th larval instar, the percentage of each type was: plasmatocytes 43 ± 3.539%, prohemocytes 9 ± 0.533%, granulocytes 15 ± 3.590%, spherulocytes 32 ± 1.937% and oenocytoids 1 ± 0.27% of the total cell number. After treatment with flufenoxuron it was recorded that in the 6th larval instar of *S. littoralis*, the proportion of plasmatocytes and prohemocytes increased while the proportion of granulocytes and spherulocytes

decreased at the low concentration (LC_{25}). The increase in number of plasmatocytes and prohemocyes may be attributed to the increase of THCs due to the activation of cell division and the release of sessile haemocytes after treatment. Similar explanation was given by Shapiro (1968) on *G. mellonella* larvae; Gupta (1985) on different insect species and after injection of the 5th nympal instar of *S. gregaria* with bacteria Barakat *et al.* (2002). The decrease in proportion of granulocytes and spherulocytes may be attributed to their involvement in phagocytosis, cell clumping and wound healing. This opinion is supported by Barakat (1997) on *G. mellonella* larvae after injection with *B. cereus* and Barakat *et al.* (2002) on *S. greagaria* nymph after injection with *B. thuringiensis*. Richard and jack (1969) reported that the injection of foreign materials in *G. mellomella* larvae did not change the level of mitotically dividing cells, spherule cells and oenocytoids.

Following treatment of the 6th larval instar of *S. littoralis* with LC_{50} of flufenoxuron, the plasmatocytes, prohemocytes and granulocytes decreased, whereas the spherulocytes and oenocytoids increased. The decrease in plasmatocytes, prohemocytes and granulocytes was mostly due to the initial events of recognition of foreignness, involvement in phagocytosis, cell clumping and wound healing. Same interpreting was said by Barakat (1997) on *G. mellonella* larvae after injection with *B .cereus* and Barakat *et al.* (2002) on the fifth nymphal instar of *S. gregaria* after injection with *B. thuringiensis*.

The increase in spherulocytes and oenocytoids may be attributed to the increase of mitotic index. These results agree with the findings of Abu El-Magd *et al.* (1994) on *S. littoralis* and Barakat *et al.* (2002) on *S. grearia*, and disagree with the results of Abu El-Magd (1992) on fifth nymhal instar of *S. gregaria*, because of different agent (Laminarin) and dose (25ul), besides the differences in rearing conditions, diet and using of unfixed haemolymph.

Granular cells and spherule cells were reported to be the main cell types responsible for the rapid removal of foreign particles from the circulation via nodule formation in *Galleria* sp (Gagen and Ratcliffe, 1976). These cell types have been demonstrated to contact with test particles to spherule cells (Ratcliffe, 1975) and degranulation of granular cells to produce sticky matrix involved in nodule formation (Gagen and Ratcliffe, 1976).

Treatment of the 6th larval instar of *S. littoralis* with LC_{50} and LC_{70} of flufenoxuron caused marked drop in THCs. This reduction may be attributed to the decrease of plasmatocytes, prohemocytes and granulocytes, in addition to the involvement of the haemocytes in phagocytosis and nodule formation, which always

accompanied by the death of defensive haemocytes or may be due to the action of the released toxins. In this respect, our results agree, with the results of some authors who made studies on lepidopterous larvae after injection with live *B. thuringiensis*. (Witting, 1966; Gagen and Ratcliffe, 1976; Faye, 1978; Abu El-Magd *et al.*, 1994). Similar observations were also reported by Hoffman *et al.* (1974) on *L. migratoria*. Barakat (2001) noted the same effect on adult worker of *Apis mellifera* injected with *Pseudomonas aeruginosa*. Contrarily, these results disagree with that of Abu El-Magd (1992) on *L. migratoria* due to species and technique differences.

Treatment of the 6th larval instar of *S. littoralis* with LC_{25}, LC_{50} & LC_{70} of chlorfluazuron caused marked increase in THCs. This may be attributed to the increase in the proportion of plasmatocytes due to the activation of cell division and the release of sessile haemocytes after wounding and the activation of mitotic activity of the haemocytes. Similar explanation was given by Shapiro (1968) on *G. mellonella* larvae, Gupta (1985) on different insect species and Barakat *et al.* (2002) on the 5th nymphal instar of *S. gregaria* after injection with bacteria. The plasmatocytes increased highly in order to compact the toxic effect of Dimilin (Osman *et al.*, 1984). This increase could be attributed to exciting the resting plasmatocytes to enter the circulating haemolymph and the rapid transformation of the prohemocytes (stem cells) into plasmatocytes (Osman *et al.*,1984). The proportional percentage decrease of the prohemocytes in chlorfluazuron fed larvae makes the latter explanation clear.

Following chlorfluazuron treatment of 6th larval instar of *S. littoralis* with the three different concentrations, the granulocytes decreased and this may be attributed to their involvement in phagocytosis, nodule formation and wound healing. This result agrees with the observations of Laigo and Paschke (1966) on caterpillars, *Trichoplus* sp. infected with Nosema. They recorded a decrease in the number of phagocytic cells at least temporary due to phagocytosis. Our results were also in agreement with those of Lia-fook (1968) on *Rhodnius prollixus* after wounding; Horohov and Dunn (1982) on *M. sexta* larvae following the injection with two species of bacteria, Ayaad *et al.* (2001) on *P. surcoufi* larvae infected with a nematode and Barakat *et al.* (2002) on *S. gregaria* nymph injected with bacteria.

The spherulocytes showed a markedly decrease due to chlorfluazuron feeding. Our results agreed with that reported by Osman *et al.* (1984) on *S. littoralis* larvae after treatment with Dimilin. They reported that the proportional of spherulocytes decrease might be returned to their inactivity in the detoxication process; their production in haemopoietic centers would, partially, inhibited in such

toxified larvae. The increase in oenocytoids at LC_{25} and LC_{50} may be attributed to the increase of mitotic index and THCs. These results agree with the findings of Abu El-Magd *et al.* (1994) on *S. littoralis* larvae and opposite to the results of Abu El-Magd (1992) on fifth nymphal instar of *S. gregaria*, because of the different agent (Laminarin) and dose (25 µl), in addition to the differences in rearing conditions, diet and using unfixed haemolymph.

The present study indicated that flufenoxuron and chlorfluazuron caused inhibition in the concentration of total protein. The decrease in the concentration of the total protein in the treated larvae may reflect the inhibition of DNA synthesis and the decrease in the activity of various enzymes that may be related to insect growth inhibitors (IGRs) mechanism as stated by Meola and Mayer (1980) after treatment of stable fly with dimilin. This finding coincided also with that reported by Sokar (1995); Mohamady (2000) in *S. littoralis* after treatment with hexaflumuron and chlorfluazuron. Hamouda (2002) found that the total protein in haemolymph decreased with treatment of the third instar larvae of *S. littoralis* with admiral. Enan (2003) reported the same findings on the fourth nymphal instar of *Oxycarenus hyalinipennis* when treated with LC_{10} and LC_{50} of alsystin (IGR). Also, Bakr *et al.* (2004) reported that the metabolic effects of chlorfluazuron induced decreasing haemolymph total protein of *S. gregaria*.

According to Wilkinson (1976), proteins help insects to synthesis microsomal detoxifying enzymes. Ahmed and Forash (1976) stated that, proteins are the most important compounds present in insects that can bind with foreign compounds; therefore, the decrease in proteins may reflect the decrease in activity of these enzymes.

The quantitative changes in the concentration of haemolymph proteins have been demonstrated by Cölln (1973) who reported that these changes may be partially correlated with the temporal increase in the endogenous titre of ecdyson. He added that exogenous ecdyson and /or juvenile hormone and their analogues have been shown to regulate the concentration of stage specific proteins present in the haemolymph of *Ephestia kühniella*.

Miltin *et al.*, 1977 and Deloach *et al.*, 1981 reported that, the disturbance in total protein content and nucleic acids in treated nymphs and resulted males and females of *Anthonomus grandis* may be due to the inhibition of DNA synthesis and metabolism after treatment with IGR or the interference of ecdyson analogue with protein synthesis which might express the reduction in reproductive potentiality (Padmaje and Rao, 2000).

The inhibition of protein synthesis was recorded as a result of application of the IGRs (Sheble, 1979). Also, these results are confirmed with that obtained by (Scheller and Bodenstein, 1981; Mulla *et al.*, 1985; Bakr, 1986; Mostafa, 1993; Hassanien *et al.*, 1996) they found that the total protein in haemolymph of *Heliothis zea* was decreased after treatment with insecticides and IGR dimilin. Also, Gadallah *et al.* (1990) demonstrated highly pronounced decrease in total protein in the tissues when the 4th instar larvae of *H. armigera* was treated with sublethal concentrations of JHMs-31183 and fenoxycarb.

In the present study, the haemolymph of the 6th larval instar of *S. littoralis* was characterized by low percentage of sodium and high percentage of potassium and this agree with that reported by Salama *et al.*(1994).

Calcium and magnesium are important constituents of the living matter and are present in most living cells. Phosphorus has the smallest percentage in haemolymph (Clark, 1958; Sutcliffe, 1963 and Salama *et al.*, 1994). After treatment of the 3rd instar larvae of *S. littoralis* with flufenoxuron, the concentration of these elements were affected. The concentration of potassium inhibited significantly at LC_{50}, whereas the phosphorus concentration increased significantly at LC_{50}. The Sodium concentration showed very highly significant increase at the three used concentrations, while both calcium and magnesium were inhibited by the three used doses of flufenoxuron.

In case of chlorfluazuron treatment, both LC_{50} and LC_{70} caused an inhibition in potassium concentration but phosphorus concentration was increased with the increase in concentration of chlorfluazuron. Also, sodium concentration increased very highly with application of the three concentrations of chlorfluazuron. Both calcium and magnesium were inhibited by treatment of chlorfluazuron.

In general, calcium and magnesium are essential for maintenance of cellular physiological balance, the stability of intercellular matrices, normal permeability of cells and tissues, and normal activity of the neuromuscular system. Calcium is probably the most influential polyvalention found in the living cell (Heilbrunn, 1943 and Höber, 1945). Without calcium, the present sodium, potassium and magnesium in normal concentrations are very toxic to the cell; calcium is essential in setting off their injurious effects. The calcium ion apparently exerts a greater effect on protoplasmic viscosity than any other ion. In cells small quantities of calcium decrease the viscosity of the interior protoplasm; and conversely, an abundance of calcium causes gelling reactions (Heilbrunn, 1943 and Höber, 1945).

Magnesium in low concentration can also bring about this fluidity of protoplasm. but neither to the degree nor with the ease of calcium.

In the blood of mammals there seems to be a definite concentration of ionic calcium. If this concentration falls, the nervous system becomes hyperirritable and in extreme cases this fall may lead to tetany. Clark (1958) reported that a nerve-muscle preparation loses its indirect excitability after being transferred from Ringer's solution into a normal saline solution and regains its activity after the addition of calcium. He reported also that, the absence of calcium interrupts the pathway of the impulse at the myoneural junction and that there is a calcium containing "cement bridge" allowing the concentration wave to travel across the synapse in some stage of the reversible loosing or tightening of the cement.

In mammals a deficit of magnesium in the diet causes circulatory disturbances, increased irritability and finally convulsions and death. The depressive action of the magnesium ion on nervous tissue, which is opposed to that of the calcium ion, may be observed in both invertebrates and vertebrates Clark (1958). This postulation may explain the mortality rate of larval stage in the present study after treatment of the third larval instar with flufenoxuron and chlorfluazuron.

Calcium is also released during muscle contraction. It may be released in one part of the muscle and immediately bound elsewhere in the same or another muscle (Weise, 1934), or it may be set free as calcium ion in the blood (Wacker, 1929). Calcium can initiate shortening of muscle fibers (Heilbrunn, 1943; Hukuda and Moriya, 1936). It can also initiate a chemical reaction of primary importance in muscle metabolism, namely, the adenosine triphosphate break-down, making a large amount of available energy when calcium activates adenosintriphosphate. It is also mentioning that, although other ions may activates adenosinetriphosphate, the muscle will not contract unless calcium is present in normal state.

Salama *et al.*(1994) reported that the inorganic constituents such as Na^+, K^+, Ca^+ are of vital importance in view of their role in neurophysiology of the insect and their levels inside and outside the nerve membrane and maintained for propagation of impulses. Salama *et al.* (1994) showed that when the larvae of *Phthorimaea operculella* injected with *B. thuringiensis*, the concentrations of different inorganic ions in the haemolymph were changed. The change in the concentrations of those inorganic ions after treatment may be among the factors causing toxicity of *B. thuringiensis*. Salama *et al.* (1994) reported that the absolute concentration of various ions in the haemolymph is not important as the ratio of sodium, potassium, calcium and magnesium.

So, in the present study, general pathological conditions in the haemocytes of the haemolymph may be attributed to the hypermetabolic state of larvae during this period in response to flufenoxuron and chlorfluazuron. especially at higher concentrations and this ensures that these IGRs have a great and strong prove to be used as a controlling agents against *S. littoralis* in this study.

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