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Ultrastructural changes in integument of *Tribolium castaneum* (Coleoptera: tenebrionidae) induced by chitin synthesis inhibitor (IGR) chlorfluazuron.

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ABSTRACT

The present study was planned to investigate the histopathological effects of the chitin synthesis inhibitor chlorfluazuron against the 4th larval instar of *Tribolium castaneum* (Coleoptera: Tenebrionidae) under electron microscope level. Toxicity of chlorfluazuron was evaluated under three different temperatures. The sublethal concentrations LC_{40} were 2.118, 39.76 and 1.207 ppm, at 20, 29 and 38˚C respectively. Toxicity of this compound increased at relatively high temperature. Ultrastructural studies were carried out on the integument of the prepupae treated as larvae with LC_{40} of chlorfluazuron. The treated samples revealed that exuviae of the old cuticle were existed above the new formed integument, some densely–stained material was deposited in endocuticle, procuticle lamellae were disorganized and partially degenerated. Disappearance of moulting fluid. The hypodermis was destructed and detached from endocuticle. Mitochondria were swollen and fusion. Lysis of lysosomelike bodies.

Key words: Chitin synthesis inhibitor, *Tribolium castaneum*, ultrastructural studies, integument.

INTRODUCTION

Tribolium castaneum is common pest found in indoor food storage facilities. Many research studies in which IGRs have been tested against this species and other indoor stored-product pests have involved mixing the chemical with insect diet (Oberlander *et al.*, 1997). With this technique, the IGR is often effective because the earlier an insect is exposed to IGRs in the larval life cycle; the less likely it is to reach the adult stage.

Insect cuticles form an exoskeleton that exhibits only a limited capacity to keep space with body growth because it is a more or less rigid structure due to the presence of chitin and sclerotized proteins. To allow growth and development, insects are therefore periodically forced to replace their old cuticle with a new and looser one during moulting (ecdysis). Ecdysis is initiated by apolysis, the process that separates epidermal cells from the old cuticle by moulting fluid secretion and ecdysial membrane formation. The moulting fluid contains proteases and chitinases enzymes that digest the main constituents of the old endocuticle (Reynolds and Samueles, 1996). Shortly before ecdysis, the moulting fluid, which has accumulated in the apolysial space, is reabsorbed, allowing the recycling of old cuticle components.

Insect growth regulators (IGRs) are third- generation insecticides less toxic and compatible with insect pest management that were developed to reduce the pollution of food and environment. These compounds have a specific mode of action on insects and a lower toxicity against vertebrates than conventional insecticides (Grenier and Grenier,1993). IGR's include compounds that affect moulting and metamorphosis by mimicking juvenile hormone (JH agonists) or usually antagonizing

JH activity, ecdysteroid agonists, or by interfering with cuticle formation ,chitin synthesis inhibitors, (Smet *et al*., 1990; Oberlander *et al*., 1991 and Oberlander *et al.*, 1997).

The chitin synthesis inhibitor benzoylphenylurea (BPU) insecticides prevent the moulting process by inhibiting chitin synthesis, thereby causing abnormal endocuticular deposition and abortive moulting, (Ishaaya, 1990 and Dhadialla *et al*., 2005). Chlorfluazuron is an insect growth regulator inhibiting chitin synthesis and acts as an anti-moulting agent, inhibits biosynthesis of chitin of an important constituent in insect cuticle, loses cuticle elasticity and results in abortive moulting.

The objective of this study were to determine the efficacy, through laboratory bioassays of chitin synthesis inhibitor chlorfluazuron against the fourth larval instar of *T. castaneum* under different temperatures, and determine some ultrastructural changes induced in the prepupal integument treated as larvae with sublethal dose at 29°C

MATERIALS AND METHODS

Insect colony:

A laboratory colony of the red flour beetle, *T. castaneum* was maintained for many generations under constant conditions 29°C ±1 and 65-75% R.H. in the Department of Entomology, Ain Shams University. The rearing medium was wheat flour mixed by weight with Brewer's yeast (19:1, w: w).

The chitin synthesis inhibitor:

The chitin synthesis inhibitor (5% E.C.), chlorfluazuron (Atabron) was tested in the present study. Its chemical name is 1- [3, 5 –dichloro – 4 - (3 - chloro - 5-trifluoromethy l-2- pyridyloxy) phenyl] -3- (2,6- difluorobenzoyl) urea.

Bioassay test:

A preliminary experiment was carried out to determine the sublethal concentrations of chlorfluazuron as a chitin synthesis inhibitor against *T. castaneum* at different temperatures of 20, 29 and 38°C. The feeding technique used was according to Oberlander *et al.,* (1997). Fourth instar larvae were fed on treated flour till pupation. A control experiment was also performed using flour alone. Four replicates each containing 25 larva / jar was used for each concentration and for the control. The effect of temperatures on the inhibition of adult emergence by chlorfluazuron at the LC_{40} was determined.

Histopathological studies and ultrastructural techniques:

The effect of a sublethal dose LC_{40} at temperature 29°C on the integument of normal and treated prepupae was examined by the use of electron microscope. Samples were prepared one day after pupation. The control and treated prepupae were fixed in 2.5% glutaraldehyde (0.1 M Sorensen phosphate buffer, pH7.2) for 2 hrs at 4°C. They were rinsed overnight in 0.1 M phosphate buffer: 12.5% sucrose (1:1) and post fixed in 1% osmium tetroxide (prepared in 0.1 M phosphate buffer, pH7.2). Samples were dehydrated through ethanol series, and twice in propylene oxide and then embedded in ERL (Spurr, 1969), as generally described by Smagghe *et al*. (1996). Ultrathin sections were prepared with an ultramicrotome and stained with uranyl acetate and lead citrate in LKB automatic stainer prior to examination by JEOL electron microscope. Electron microscope studies were conducted at the electron Microscopy Unit, Ain Shams University, Cairo, Egypt.

Statistical analysis

The mortality in susceptibility bioassay was corrected by Abbott's formula, Abbott (1925). F Probit analysis was used to determine the sublethal concentrations as described by Finney (1971).

RESULTS AND DISCUSSION

Susceptibility test:

Larvae of *T. castaneum* were susceptible to chlorfluazuron at all the tested temperatures (Table 1). However, the Lc40 was 39.76 ppm at 29˚C, lower and higher temperature increase the sensitivity of insects to the (IGR). While the sensitivity of insect much higher at high temperature, $LC_{40's}$ were 2.118 and 1.207 at 20˚C and 38˚C, respectively. A significant difference ($P< 0.05$) were found among the sublethal concentrations at each tested temperatures. According to the obtained results, it is clear that chlorfluazuron can be used to control *Tribolium castaneum* at relatively high temperature. The same conclusion was achieved by El Shazly and Refaie, (2002) in case of (IGR) pyriproxyfen on *Culex pipiens*. Also, this finding is in agreement with Arthur (2003) who reported that the (IGR) hydroprene may effectively control *T. castaneum* and *T. confusum* in confined spaces inside food storage facilities. However, environmental conditions may affect control of these insects. More larvae of both species were arrested in the larval stage and more adults died after they emerged in exposure times conducted at 32˚C and 75% R.H. He also reported that as temperature increase, larvae would be expected to complete development in a shorter time, thereby decreasing the time in which they are exposed to the chemical.

Table (1) Toxicity of chlorfluazuron against last instar larvae of *T. castaneum* at three constant temperatures

Temperatures	Lc10ppm	Lc20ppm	Lc30ppm	Lc40ppm
20	0.503	0.928	1.450	2.118
29	8.57	20.16	26.57	39.76
38	0.161	0.380	0.710	1.207

Data on emergence inhibition of adult *T. castaneum* by chlorfluazuron are presented in Table (2). It is noticed that the emergence inhibition from the treatment of survivors with Lc40 generally increased at low temperature. Similarly, decreased adult emergence rate in *T. castaneum* due to larval treatment with chlorfluazuron was also reported by Degheele, (1990); Mostafa, (2002) and Abdel Fattah and Khaled, (2008) as a result of blocking of the imaginal discs or due to deformation of adult chitin.

Table (2) Emergence inhibition of *T. castaneum* by sublethal concentration Lc40 of chlorfluazuron at three constant temperatures.

Temp	control		Treated	
	% adult emergence	% adult inhibition	% adult emergence	% adult inhibition
20	80	20	5	95
29	97.5	2.5	22	78
38	90	10	7	93

Histology and Ultrastructure of the integument of untreated prepupae.

The integument of untreated prepupae consists of, cuticle, hypodermis(or epidermis), and basement membrane from outside to inside. The cuticle is differentiated into an outer epicuticle and an inner procuticle (exocuticle and

endocuticle) with clear lamellae. The hypodermis consists of a single layer of epidermal cells with large nuclei and mitochondria that are scattered through the cytoplasm. In the epidermal cells, there is also, some scattered lysosomelike bodies. The epidermal cells are held together near their apices. At greater distances from the cuticle, were no tightly to each other, and the spaces between them called lateral lymph spaces, Locke, (1991). Gap junctions between epidermal cells probably provide a pathway for the movement of low molecular weight substances, such as hormones. This enhances coordination between cells, reducing any minor differences that might occur due to slight differences in the timing or amounts of hormonal signals carried by the hemolymph (Chapman, 2002). Because the samples examined were in prepupal stage during moulting time. The moulting fluid appeared in the apolysial space between the cuticle and the epidermal cells. Figs.(1, 3, 4 and 5).

Histology and Ultrastructure of the integument of treated prepupae.

Histopathological examination of prepupal instar of *T. castaneum*, resulting from 4th larval instar fed on Lc40 of chlorfluazuron was illustrated in Figs. (2, 6, 7 and 8). Treated specimen, revealed detachment of the epidermal cells from the endocuticle (Fig. 2). Also, the epidermal cells are not arranged in the form of a single layer epithelium, but rather undistinguishable and almost completely destroyed (Figs. 2 & 8). Ultrastructural examination of the treated specimen also showed absence of moulting fluid in the apolysial space and adherence of the old larval cuticle (exuviae) to the newly formed cuticle, Fig. (6). In the endocuticle, some densely – stained material were deposited, and adjacent lamellae were partially digested. Fig. (7). On the other hand, chlorfluazuron caused drastic effect on the epidermal cells where the nuclei shrank; vacuoles appeared in the cytoplasm; the mitochondria completely lose their normal form and being swollen and fused and lysis of lysosomelike bodies, Fig. (8). Liu, (1989) suggested that juvenile hormone III activated the lysosome system on the hypopharyngeal glands of *Apis mellifera*, which caused cytolysis. Cytolysis could eventually lead to degeneration of the epidermal layer.

In general, the IGR chlorfluazuron interferes with the normal development and disrupts the structure of the different cuticular components in treated prepupae of *T. castaneum*. The undistinguishable and destroyed epidermal cells may be prevent the pathway of hormonal secretion and thus prevent the coordination between cells. The effect of chlorfluazuron on the treated samples of *T. castaneum* , may be also explicated by causing an imbalance in the hormone titers at critical times of moulting because the proper balance in the hormone titers is necessary for normal growth and transformation into the pupal and adult stage, and this may be led to failure of epidermal cells to secrete the moulting fluid. In addition, this may induce a great disturbance in the moulting process to produce the new cuticle and shed out of the old one. Similar results have been reported by Abdel Fattal and Khaled (2008). In addition the observed damage of the mitochondria in epidermal cells may lead to a great lack of energy production in the treated samples. All these factors may give some interpretations for the failure of the IGR treated insects to complete their life cycles. These histopathological changes by chlorfluazuron to great extent, agree with the results recorded for the same IGR on *Musca domestica* Bakr and Hussein (1988). In agreement with our obtained results, for some other IGRs on some insect species as *Schistocerca gregaria* Bakr *et al.*, (2008), *Lucilia cuprina* Binnington *et al.*, (1987).

Finally, two conclusions can be reached from this investigation. First, comparison of my results with those obtained with chitin synthesis inhibitors indicated that treatment with chlorfluazuron results at least partly, in similar

symptoms. Second, the chlorfluazuron can be used as a larvicidal agent against *Tribolium castaneum* at relatively high temperature.

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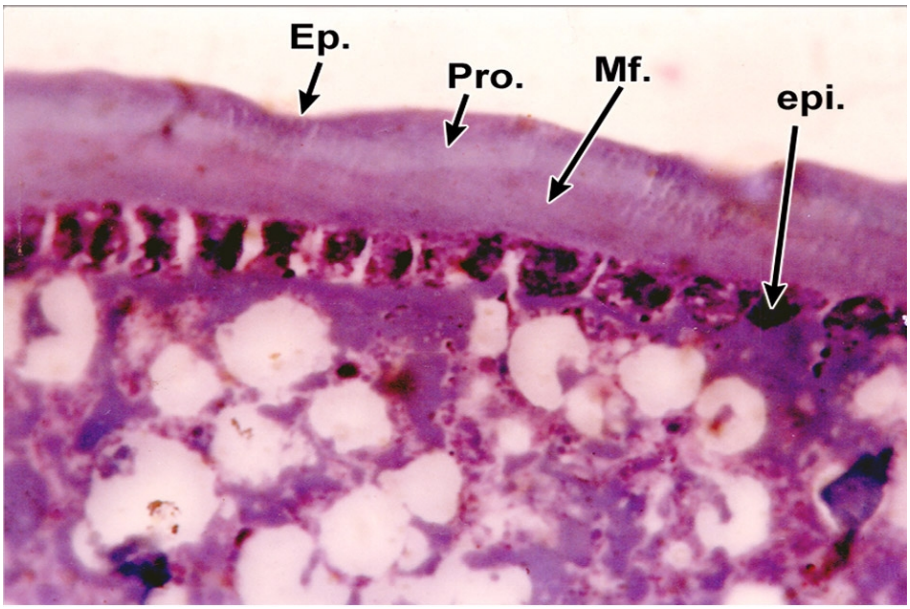

Fig. (1): Transverse section of integument in untreated prepupa of *Tribolium castaneum* showing:
- epicuticle (Ep.), procuticle (Pro.), moulting fluid (Mf) and epidermis (epi.) X= 1000

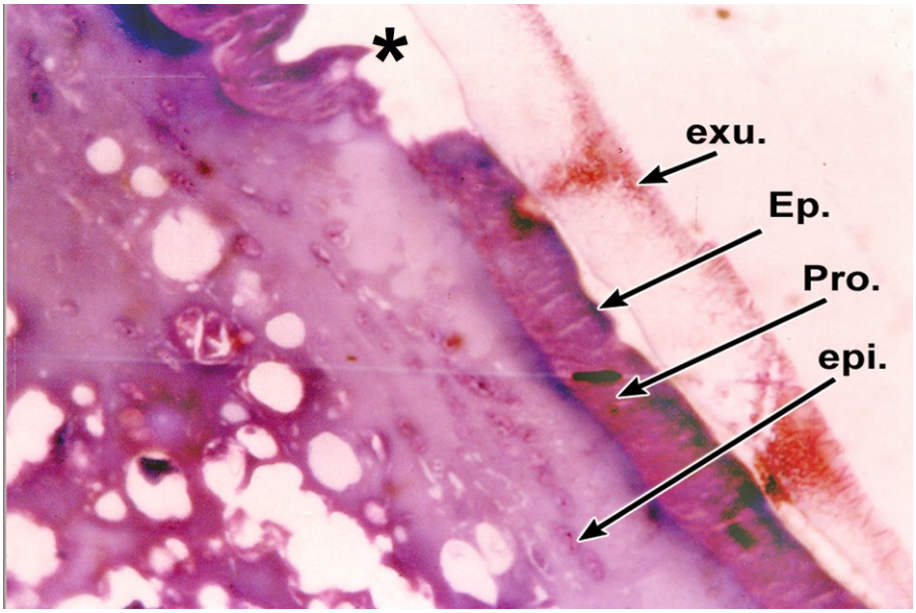

Fig. (2): Transverse section of prepupal integument of *Tribolium castaneum* treated as larva with LC_{40} of chlorfluazuron showing:
- exuvia (exv.), epicuticle (Ep.).
- Incomplete formation of cuticle (*).
- detachment between epidermal cells (epi.) and procuticle (Pro.). X= 1000

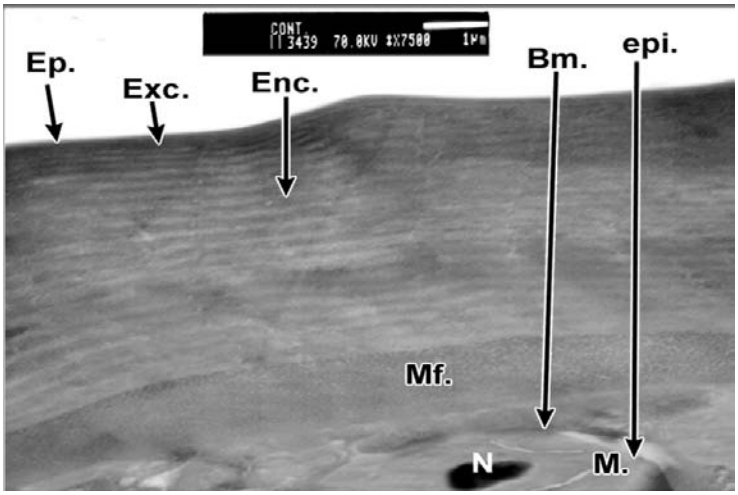

Fig. (3): Electron micrograph of integument in untreated prepupa of *Tribolium castaneum* showing:
- epicuticle (Ep.), exocuticle (Exc.),endocuticle (Enc.), moulting fluid (Mf.).
- epidermis (epi.), basement membrane (Bm.), Nucleus (N.) Mitochondria (M.) X=7500

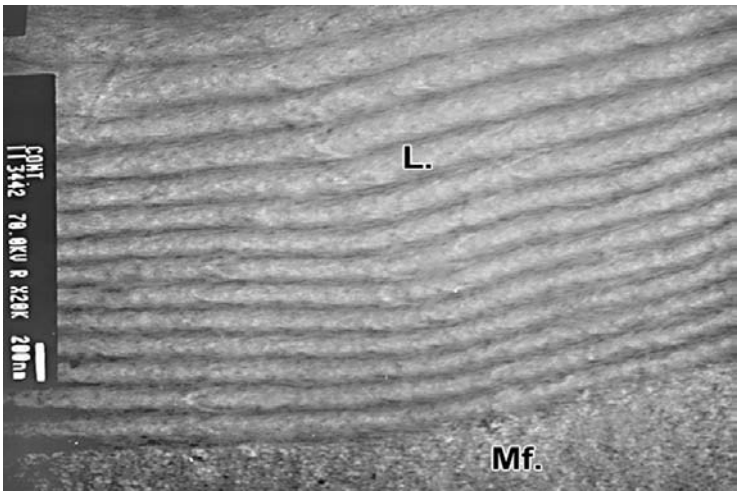

Fig. (4): Electron micrograph of integument in untreated prepupa of *Tribolium castaneum* showing: Lamellae of endocuticle (L.) and moulting fluid (Mf.). X= 20000

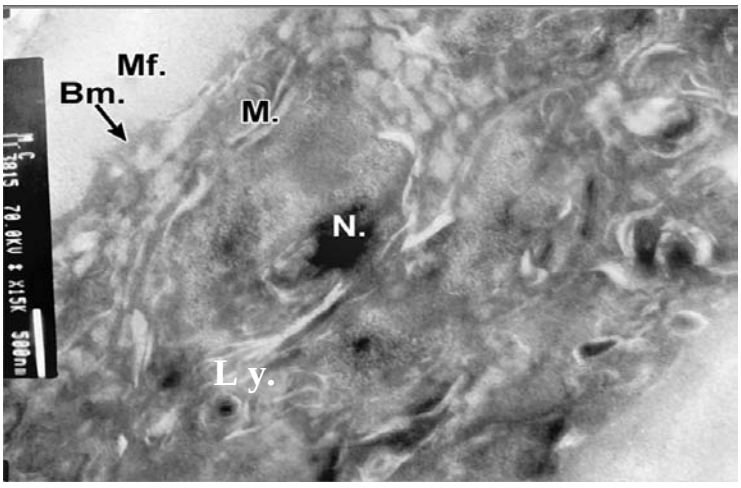

Fig. (5): Electron micrograph in the epidermal layer in untreated prepupa of *Tribolium castaneum* showing:
- moulting fluid (Mf.), basement membrane (Bm.).
- Nucleus (N.) Mitochondria (M.) Lysosomelike bodies (LY.) x= 15000

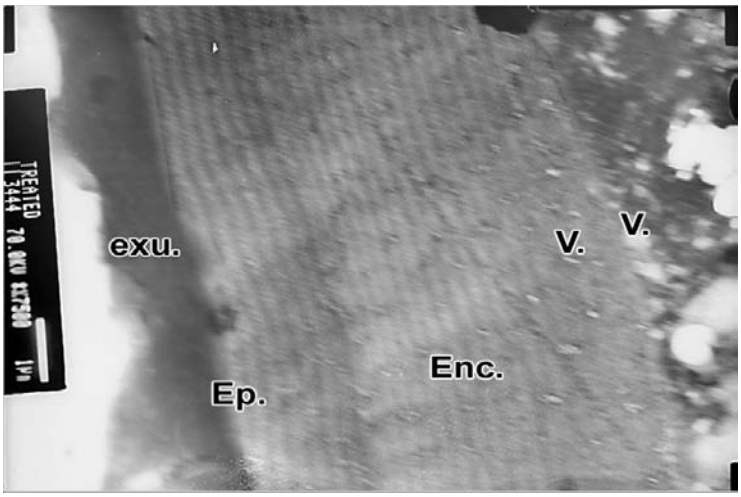

Fig. (6): Electron micrograph of integument in prepupa of *Tribolium castaneum* treated as larva with LC_{40} of chlorfluazuron showing:
- Exuvia of old cuticle (exu.) epicuticle (Ep.), endocuticle (Enc.) and vacuoles. X=7500

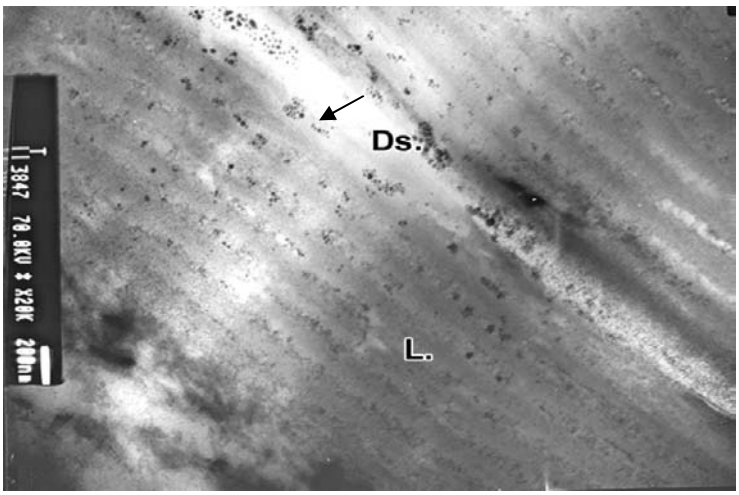

Fig. (7): Electron micrograph of lamellae in prepupa of *Tribolium castaneum* treated as larva with LC_{40} of chlorfluazuron showing:
- disorganized and degeneration of lamellae (L.) →.
- dense-stained material (Ds.) X= 20000

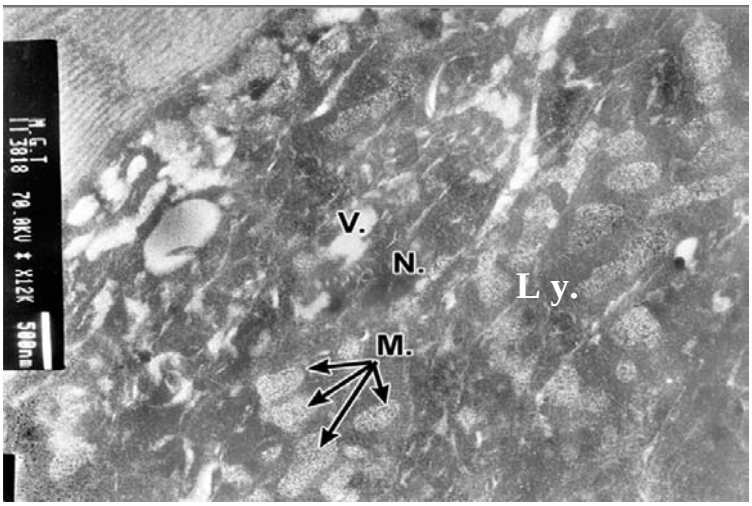

Fig. (8): Electron micrograph of epidermis in prepupa of *Tribolium castaneum* treated as larva with LC_{40} of chlorfluazuron showing:
- disappearance of moulting fluid in apolysis space (→). (X=12000)
- appearance of vacuoles (V.).
- degeneration of nucleus (N.).

RABIC SUMMARY

تغيرات في التركيب الدقيق لجليد خنفساء ترايبوليم كستانيم بواسطة مثبط تكوين الكيتين كلورفلوزيورون

أماني سليمان خالد

قسم علم الحشرات- كلية العلوم- جامعة عين شمس- القاهرة- **مصر**

استهدفت هذه الدراسة معرفة التأثيرات الهستولوجية المرضية لمثبط تكوين الكيتين كلورفلوزيورون على جليد الطور اليرقي الرابع لخنفساء ترايبوليم كستانيم (غمدية الأجنحة : **السوس**) في المعمل تحت تأثير ثلاث درجات حرارة مختلفة تزداد سمية هذا المركب في درجة الحرارة العالية . التركيز تحت المميت Lc40 **كان**2.118 ، 39.76 ، **و** 1.207**في درجات حرارة** 20، 29 **و** 38 درجة مئوية على التوالي . **دراسة** التركيب الدقيق أجريت على جليد العذراء الناتجة من معاملة الطور اليرقي الرابع بالتركيز تحت المميت Lc40 من الكلورفلوزيورون . وقد أوضحت الصور في العينات المعاملة وجود الجليد القديم أعلى الجليد الجديد ، **وجود** بعض البقع القاتمة في الكيتين الداخلي ، عدم انتظام وتحلل جزئي لطبقة الألياف في الكيتين الداخلي ، غياب سائل **الانسلاخ** ، طبقة خلايا البشرة الداخلية يوجد بها تحلل ومنفصلة عن طبقة الكيتين الداخلية . خلايا الميتوكوندريا **منتفخة وملتحمة** .