BURSICON, INSECT CUTICLE-SCLEROTIZING NEUROHORMONE – REVIEW ARTICLE

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Abstract: Bursicon is the neuropeptide hormone that is responsible for hardening and darkening of the cuticle in insects. It is one of the five key insect hormones which regulate the proper sequence of the insect molt. Small amount of bursicon could be found in the brain. It could be also detected in the ventral nerve cord 6-10 times more than in the brain. The release time of bursicon is just after ecdysis at each molt. The chemical structure of bursicon is a polypeptide of a single-chain molecule with a molecular weight of 30 kDa. Additionally, nerve cord homogenates of several insect species and the lobster give results in the ligated fly bioassay. These data indicate that bursicon has a conserved structure throughout arthropods.

This article describes the bioassay, chemical identity and gene structure of bursicon. Mode of action and application are discussed.

Key words: bursicon, insect cuticle, sclerotizing neurohormone.

Introduction

Cuticle in insects undergoes many physical and chemical changes. These observable changes were attractants to tens of researchers who extensively studied insect cuticle and the causes for its dramatic behavior.

The most observable process of insect cuticle is the ecdysis process. It includes splitting and shedding of the old cuticle, stretching and expansion of the new cuticle, and stabilization of the stretched cuticle by sclerotization and darkening (Seligman, 1980). The latter was thoroughly investigated by G. Fraenkel as early as 1935 when he concluded that puparium formation of the blowflies *Calliphora erythrocephala* was controlled by a hormone that is produced in the anterior part of the insect (known later as ecdyson). It was assumed that this hormone also controlled the tanning of the cuticle since it accompanies the puparium formation (Fraenkel, 1935a). fraenkel (1935b) also showed that increasing the distance of soil that newly emerged adult blowflies needed to reach the surface resulted in delaying their cuticle sclerotization (tanning) for 24 hr. They remained dull-colored and shrunken until they released off the soil, then quickly expanded and darkened.

This critical finding remained as is and had not been discussed for 27 years until Cottrell (1962a,b) and Fraenkel and Hsaio (1962)

simultaneously but independently demonstrated that tanning in adult blowflies was controlled by a "tanning factor" but not ecdyson. The tanning factor was soon named "Bursicon" from the Greek Bupoa (bursa), meaning hide or skin, and Bupoiko'o (bursikos), pertaining to tanning (Fraenkel and Hsiao, 1965).

Peptide Isolation and Structure

Isolation of bursicon

Fig. (1) show the prepurification flow diagram which was outlined by Kaltenhauser *et al*. (1995). During the development of the prepurification scheme, every step included a bioassay (see below) to find the bursicon activity either in the precipitate or in the supernatant. Accordingly, 8 bioassays were involved and only the buffer was injected in each bioassay as a control. To test for bursicon activity, a 100 µl aliquot was taken and injected into flies. Biotest +: score had to be at least >0.5 points. For controls, only buffers were injected; all control scores were <0.5 points (reproduced from Kaltenhauser *et al*. (1995).

Bioassy

The most recent paper on bursicon isolation was reported by Kaltenhauser *et al*. (1995). They have adapted a procedure originally reported by Fraenkel and Hsiao (1965) and Abboud *et al*. (1983). Hatching blowflies were ligated between head and thorax. These flies do not tan, i.e., no mechanization or sclerotization occurs. If injected with 5 µl of a solution containing bursicon, the flies will tan to a degree dependent on the amount of bursicon in the test solution. At 3 h after the injection, the tanning intensity of the thorax and abdomen were evaluated separately, each of a score of 0-3 points to give a maximum score of 6 points for each fly based on the following scale:

3+++ fully tanned, the cuticle, including the sclerites of the thorax, appears uniformly dark;

2++ the greater part of the cuticle is dark;

1+ tanning is limited to small patches;

0.5± some small indication of tanning;

0- entirely untanned.

In each test, 10-15 flies were injected and the mean score of these flies calculated. In control flies, only the buffer used for the particular purification step was injected. Control groups of flies typically showed a mean score of 0-0.2 points and a maximum score of 0.5 points.

Since, Kostron *et al*. (1995) found that the nerve cords of *Periplaneta Americana* nerve contain about four times as much bursicon as those from other insect species, they were used in our first attempt to sequence bursicon.

An amino acid sequence analysis of the bursicon active 40 kDa protein band excised from one-dimensional

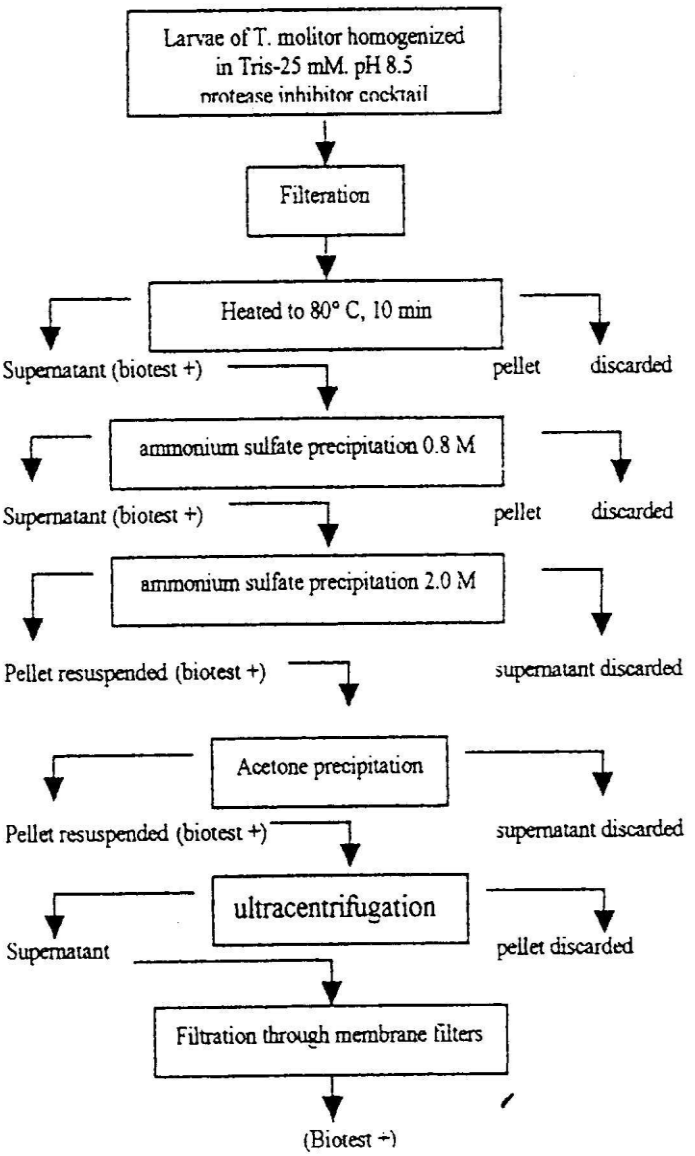

Fig. 1. Flow diagram of crude purification of bursicon from T. molitor larvae.

(ID) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed four partial sequences with a high similarity to Cu, Zn-superoxide dismutase (SOD) of *Drosophila* (Lee *et al*., 1985; Kwiatowski and Ayala, 1989; Kwiatowski *et al*., 1994). SOD catalyzes the removal of metabolically produced superoxides by synthesizing molecular oxygen and hydrogen peroxide, thereby protection cells from oxidative injury.

Chemical identity

Many researchers have attempted to investigate its chemical identity in different insects. Bursicon is released into the hemolymph shortly after eclosion hormone and has a short half-life of 1-2 hr (Abboud *et al*., 1983; Reynolds *et al*., 1979; Truman, 1981). It is destroyed by the proteolytic enzymes trypsin and pronase (Franekel and Hsaio, 1965).

Previous studies have used almost crude purifications to identify the molecular mass of bursicon. Accordingly, different values have resulted for different insects (Kostron *et al*., 1995). Partial purification of bursicon activity indicated that it is a polypeptide of 30-60 kDa (Fraenkel *et al*., 1966; Mills and Lake, 1966; Taghert and Truman, 1982).

The first time, Kaltenthauser *et al*. (1995) were able to purify bursicon to near homogeneity from extract of whole larvae of *Tenebrio molitor*. They showed that *Tenebrio* bursicon is a single-chain polypeptide of 30 kDa molecular mass with anisoelectric point of 5.65. They concluded that, based on whole larvae extractions, this molecular form is the only one exists in the neurons of the larvae and it may be cleaved into active subunits immediately after its release or injection into the hemolymph to exert its effects. As a result, the fate of the 30 kDa bursicon after injection remains unknown. This finding was confirmed by the same lab team, Kostron *et al*. (1995) on four insects species *Calliphora erythrocephala*, *Periplaneta Americana*, *Gryllus bimaculatus* and *Locusta migratoria*. In both papers, bursicon molecular mass was achieved by SDS-PAGE of nervous system homogenates, subsequent division of the gel into slices, protein elution from these slices, and a test for bursicon activity of the eluted proteins in the ligated fly bioassay. The conclusion is that bursiconof 2 holometabolous and 3 hemimetabolous insects was a protein of a molecular mass of 30 kDa. Honegger (1997) reported that a sequence analysis of a 30 kDa region of one dimensional gel yielded 4 partial peptide sequence.

In an effort to characterize the insect molting hormone bursicon from the cockroach, *Periplaneta americana*, amino acid sequences with high identity to Cu, Zn-superoxide dismutase (SOD) of *Drosophila virilis* were identified by

Kostron *et al*. (1999). Antisera against a conserved region of SOD, and a sequence unique to *Periplaneta* SOD were produced and used totest whether bursicon might be a form of SOD. Western blots of one- and two-dimensional gels revealed that the dimeric form of SOD and bursicon have a similar molecular mass (30 kDa). The two proteins can be separated, however, according to their different isoelectric points. Bursicon is identified intwo-dimensional gels by elution from four unique spots not labeled by the anti-SOD antisera. In sections of *Periplaneta* nerve cords the antisera labeled glial material surrounding neuronal somata close to the neural sheath. Bursicon, however, is contained in unique cell pairs in the ganglia of the ventral nerve cord. These neurons were labeled with new antisera produced against novel sequences of one of the four above-mentioned bursicon active spots. The results of Kostron *et al*. (1999) show unequivocally that SOD and bursicon are distinctly different proteins. Furthermore, the anti-SOD antisera provided a tool to isolate and sequence bursicon.

In an attempt to identify bursicon, Honegger *et al*. (2002) were purified it from homogenates of 2,850 nerve cords of the cockroach Periplaneta Americana by using high performance liquid chromatography technology and two-dimensional gel electrophoresis. Bursicon bioactivity was found in four distinct protein spots at approximately 30 kDa between pH 5.3 and 5.9. The protein of one of these spots at pH 5.7 was subsequently microsequenced, and five partial amino acid, sequences were retrieved. Evidence is presented that two of these sequences are derived from bursicon. Antibodies raised against the two sequences labeled bursicon-containing neurons in the central nervous systems of *P. Americana*. One of these antisera labeled bursicon-containing neurons in the crickets Teleogryllus commodus and Gryllus bimaculatus, and the moth Manduca sexta. A cluster of four bilaterally paired neurons in the brain of *Drosophila melanogaster* was also labeled. In addition, this antiserum detected three spots corresponding to bursicon in Western blots of two-dimensional gels.

Using protein elution analysis from SDS gels, Kastron *et al*. (2004) determined the molecular weight of bursicon from different insects to be approximately 30 kDa. Four partial peptide sequences of *Periplaneta Americana* bursicon were obtained from purified nerve cord homogenates separated on two-dimensional gels. Luo *et al*. (2005) show that the first heterodimeric cystine knot hormone found in insects, consists of two proteins encoded by the genes *burs* and *pburs* (*partner of burs*). The pburs/burs heterodimer from *Drosophila melanogaster* binds with high affinity and specificity to activate

the G protein-coupled receptor DLGR2, leading to the stimulation of cAMP signaling *in vitro* and tanning in neck-ligated blowflies. Native bursicon from *Periplaneta Americana* is also a heterodimer. In *D. melanogaster* the levels of *pburs, burs,* and DLGR2 transcripts are increased before ecdysis, consistent with their role in postecdysial cuticle changes. Immunohistochemical analyses in diverse insect species revealed the colocalization of pburs- and burs-immunoreactivity in some of the neurosecretory neurons that also express crustacean cardioactive peptide.

Species variation:

For many insects, bursicon may have conserved structure(Mills, 1965). Ligated blowflies will tan when homogenates of nervous system of different insect orders are injected (Cottrell, 1962; Fraenkel and Hsiao, 1965). As mentioned before, crude purifications were used to determine the molecular mass for bursicon. This has resulted in different values for different insects. Kostron *et al*. (1995) reinvestigate the molecular mass of bursicon using a combination of prepurification step together with electrophoretic separation and elution of bioactive proteins from gel slices. They showed that buriscon of 2 holometabolous and 3 hemimetabolous insects was a protein of a molecular mass of about 30 kDa. Further, the ligated fly bioassay indicated that the abdominal nerve cord of one crustacean (the lobster) contains bursicon-activity. The work is in progress to explore the molecular mass of the protein found in the lobster. However, in a more recent study, Kostron *et al*. (1996) reported that surprisingly, bursicon from the housefly *Musca domestica* is a 56 kDa protein. Since bursicon in various insects including the fly *Calliphora erythrocephala* is a protein of 30 kDa, Song and Ma (1992) raised the possibility of different molecular forms of bursicon that could be found in different insects. Kostron *et al*. (1996) implied the importance of the investigations to uncover whether bursicon has different forms in closely related species.

Gene structure

Gene structure of bursicon would not be known until success is made in determining its molecular structure. An antibody against bursicon is of high demand to answer the question of its molecular structure and cDNA that encodes the peptide, its cellular location and its regulation in different developmental stages of insects and other arthropods (Kostron *et al*., 1995).

Sclerotization has long been known to be controlled by the neuropeptide hormone bursicon, but its large size of 30 kDa has frustrated attempts to determine is sequence and structure. Using partial sequences obtained from purified cockroach bursicon. CG13419 was identified using a modified protein BLAST

search of the *Drosophila* genome using the *P. Americana* partial buriscon peptide sequences obtained by microsequencing (Honegger, 2002). Sequence analysis of the genomic clone and its corresponding cDNA has revealed that the coding sequence is 522 nucleotides long. The gene contains three short exons (130, 125, and 267 uncleotides, respectively) and two introns (64 and 58 nucleotides, respectively). The CG13419 gene product is predicted to be a 173 amino acid preprotein (19 kDa). Removal of the predicted N-terminal signal sequence of 33 amino acids would result in a mature protein of 140 amino acids, approximately 15 kDa. CG13419 is a member of the 10-membered cystine knot protein family (Vitt, 2001), which typically forms dimmers. Members of this family contain six cysteine residues that form the knot and an optional additional cysteine that may be important for dimerization. They include the glycoprotein hormones, TGF-β, platelet-derived growth factor, and the mucins.

Dewey *et al*. (2004) were identified the *Drosophila melanogaster* gene CG13419 as a candidate bursicon gene. CG13419 encodes a peptide with a predicted final molecular weight of 15 kDa, which likely functions as a dimmer. This predicted bursicon protein belongs to the cystine knot family, which includes vertebrate transforming growth factor-beta (TGF-beta) and glycoprotein hormones. Point mutations in the bursicon gene cause defects in cuticle sclerotization and wing expansion behavior. Bioassays show that these mutants have decreased bursicon bioactivity. In situ hybridization and immunocytochemistry revealed that bursicon is co-expressed with crustacean cardioactive peptide (CCAP). Transgenic flies that lack CCAP neurons also lacked bursicon bioactivity. Results of Dewey *et al*. (2004) indicated that CG13419 encodes bursicon, the last of the classic set of insect developmental hormones. It is the first member of the cystine knot family to have a defined function in invertebrates. Mutants show that the spectrum of bursicon actions is broader than formerly demonstrated.

Source

Bursicon is originally produced from neurosecretory cells in the brain. It has neurohaemal organs to be released from. These organs are almost associated with one or more ganglia of the ventral nerve cord. Some variation are present in different species (Table 1).

In many insects, bursicon levels are high only and just after ecodysis, but not before it (Reynolds, 1983) with the exception of *Tenebrio* (Fig. 2) in which bursicon is released before ecdysis (Delachambre, 1971; Grillot *et al*., 1976; Delachambre *et al*., 1979a).

Table(1): Release sites of bursicon from the CNS, from Reynolds (1983).

Species	Stage	Release sites
Periplaneta	Adult	Last abdominal ganglia
Leucophaea	Adult	Thoracic ganglia
Locusta	Adult	Abdominal ganglia
Shistocerca	First instar	Segmental nerves of metathoracic ganglia
Manduca	Adult	Transverse nerves of neurohaemal abdominal ganglia (PVOs)
Tenebrio	Pharate adult	Neurohaemal organs of abdominal ganglia (perisympathetic organs)

however, contrary to the latter findings, Abboud (1983) reported that, using the adult *Calliphora* bioassay, bursicon is found in the haemolymph of pupal and adult *Tenebrio* only at the time of ecdysis when it is released heavily from the thoracic and abdominal central nervous system. No evidence of bursicon before ecdysis were found (Fig. 3). The biological responses have been selected from Seligman (1980) and Reynolds (1983).

Deposition of Endocuticle

In the abdominal sclerites of *Sarcophga bullata*, thickening of the endocuticle was found to be bursicon dependent (Fogal and Fraenkel, 1969a). incorporation of leucine into the epidermis was clearly higher in the presence of the hormone. However, autoradiographic measurement of uridin incorporation into the RNA of hypodermal cells or of fat body cells associated with the hypodermis did not result in definite differences between hormone-treated and control animals. Yet, both actinomycin-D and puromycin (inhibitors of RNA and of protein synthesis, respectively) suppressed endocuticle growth with out affecting in any way the morphological changes that occur in the exocuticle.

Bursicon Control of Cuticle Plasticization

Fogal and Fraenkel (1969) confirmed that the adult blowfly bioassay for bursicon depends on cuticle melanization rather than cuticle tanning. It was believed that the seeable darkening of the cuticle is a sign of its stabilization. In fact, as expected, bursicon does lead the newly emerged fly's cuticle to become stiffer. However, in the same time, it initially causes the cuticle to become more extensible to some degree (Fig. 4) (Reynolds, 1976). This was first mentioned by Cottrell (1962c). He reported that at the time of air swallowing, changes in size and shape are facilitated by the cuticle extensibility. These changes become

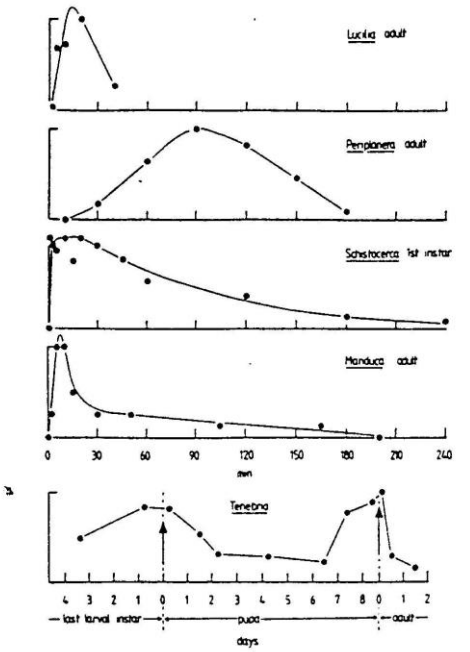

Fig 2. Blood titers of bursicon in five species of insects. Titers are note comparable between the insects, so vertical axes show only relative amounts of hormone. Note that the horizontal axes (time) for Tenebrio is different from that for the other four species. For Lucilia, Periplaneta, and Schistocerca, t = 0 is the time of ecdysis; for Manduca it is the time at which wing-spreading behavior begins (Reproduced after Reynolds, 1983)

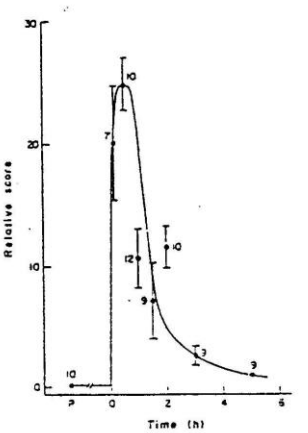

Fig. 3. Bursicon activity in the blood of Tenebrio molitor during pupal ecdysis. Abscissa: time in h after the start of ecdysis. Ordinate: relative score (= actual bioassay score multiplied by dilution of sample) (Abboud et al., 1983)

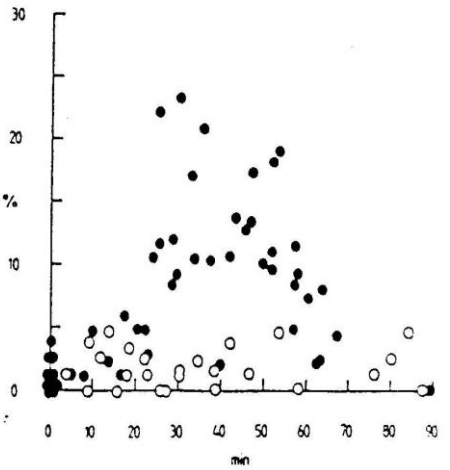

Fig. 4. Cuticle plastcization in newly emerged adult blowflies. Solid circles show the extensibility of the thoracic cuticle in control flies. The open circles show the extensibility of the thoracic cuticle in flies that were neck-ligatured at the moment of emergence in order to prevent bursicon release. Eclosion was timed at t = 0. (Reproduced after Reynolds, 1983)

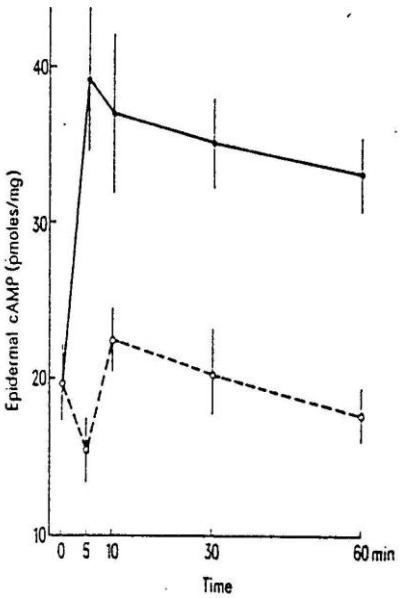

Fig. 5. Epidermal cyclic AMP content of *Tenebrio* host pupae after injection with 3 µl bursicon active haemolymph (•) or bursicon inactive haemolymph (o) (Delachambre *et al.*, 1979b).

permanent as the cuticle stiffness increased. Since plasticization happens only at the onset of cuticle darkening and limited only to subsequently sclerotized parts. Cottrell assumed that plasticization could be an early outcome of tanning that by itself leads to the stiffness of the cuticle. However, plasticization seems to be an independent effect of bursicon because it was not prevented by tanning inhibitors DOPA decarboxilase (Reynolds, 1976).

Bursicon Control of Cell Death

Degeneration process occurred after ecdysis for some of no longer useful structures in the insect's body. In contrast to the mature adults, the wings of the newly emerged blowflies contain epidermal cells between the cuticle they secrete. Muscle insertions for the two layers are resembled by characteristic processes that join the layers together (Seligman *et al*., 1975). After ecdysis, these cells break down and the resulting cellular fragments appear in the blood. If bursicon release is prevented by neck ligature, the wing epidermis does not break down and fragments do not appear in the blood. Injection of blood from expanding flies into the neck-ligatured flies leads to tanning of cuticle and appearance of cellular fragments (Reynolds, 1983).

Cottrell (1962c) showed that the wing epidermis death is programmed by the fly. When the blood of the newly emerged fly was artificially raised before air swallowing began, the wings were extended but did not inflated properly as cuticle did not plasticized yet. When the pressure was applied during air swallowing, the wings inflated normally. But when blood pressure was artificially raised only after air swallowing had finished, the wings became misshaped (the upper and lower lamellae of the wings separated and the wings filled with blood) as a result of the break down of the cellular processes between the two layers by this time.

Mode of Action

Bursicon does not appear to have any effects on the interaction between the tanning substrate, N-acetyldopamine (NADA) and the proteins of the cuticle. Most of the evidence indicates that synthesis rather than utilization of NADA is regulated by the hormone (Seligman, 1980). The rate limiting-step appears to be the first in the sequence, the hydroxylation of tyrosine to form dihydroxyphenylalanine (DOPA) (Seligman *et al*., 1969). Since DOPA is also a precursor for the alternative sequence of reactions that lead to the production of melanin, bursicon also controls melanization (Fogal and Fraenkel, 1969; Padgham, 1976). The mechanism by which bursicon controls the rate of tyrosine hydroxylation is not known. It is still unknown whether tyrosine moves into haemocytes against chemical or electrical gradients, or whether the bursicon-dependent translocation of tyrosine into the cells requires

expenditure of metabolic energy (Seligman, 1980).

Receptors and second messengers

Based on failure to perform ligated flies bioassay in two Lepidopteran species, bursicon might have different receptors than those of successfully responded insects to the same bioassay (Kostron *et al*., 1995). It was confirmed also that cAMP is a second messenger of bursicon. A significant increase of cAMP in the epidermis of *Tenebrio molitor* was recorded 5 min after the injection of bursicon active haemolymph. The cAMP level decreased after that but still at higher level than in the epidermis of noninjected pupae (Delachambre *et al*., 1979a). Also an increase of cAMP level in the epidermis of *T. molitor* pupae is provoked when the pupa was injected by an exogenous bursicon (Delachambre *et al*., 1979b).

Application and future directions

Since bursicon chemical structure is not fully recognized yet and the synthesized bursicon is out of reach, no applications are found for bursicon towards insects control in IPM programs. The full identification of this hormone might yields new ideas that lead to an application of bursicon similar in its importance to that of JH. Contrary, synthesized chemicals (antioxidants) were successfully synthesized, yet not commercially used, to interfere with the process of hardening that is controlled by bursicon.

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" بيورسيكون" هرمون تصلب جليد الحشرات – مقالة مرجعية

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يعرف هرمون " بيورسيكون " بدوره فى تصلب كيوتيكل الحشرات وهو أحد خمسة هرمونات أساسية للحشرات والتى تقوم بتنظيم عمليات الانسلاخ فى الحشرات . وقد قدرت كميات صغيرة من هذا الهرمون فى مخ الحشرة كما قدرت كميات من الهرمون فى الحبل العصبى البطنى تعادل 6-10 أضعاف الموجودة فى المخ . وقد وجد أن وقت افراز الهرمون يعقب مباشرة عملية التخلص من الجلد عقب كل انسلاخ .

فقد وجد أن التركيب الكيماوى لهرمون البيورسيكون عبارة عن جزئ وحيد السلسلة من عديد الببتيد له وزن جزيئى (30 kDa) وقد وجد مهروس الحبل العصبى للعديد من الأنواع الحشرية واللبستر أعطى نتائج مشابهة لما فى الذباب باستخدام التقييم الحيوى وهذه النتائج تدل على أن هرمون البيورسيكون له تركيب ثابت فى مفصليات الأرجل .

ومن ثم خلال ذلك المقال إلقاء الضوء على طريقة التقييم الحيوى للهرمون وتركيبه الكيماوى والجينى كما نوقشت طريقة فعل الهرمون وتطبيقاته .