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Ultrastructural changes induced by precocene II and 3,4-dihydroprecocene II in the corpora allata of *Blattella germanica*

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Summary. Ultrastructural studies on corpora allata (CA) from different stages during the first gonadotropic cycle of the cockroach *Blattella germanica* have shown well defined changes which have a correspondence with oocyte length, CA volume and juvenile hormone (JH) biosynthesis. The most significant variations concern the mitochondria and the endoplasmic reticulum. Topically applied precocene II (P II) at a dose of 200 µg induced a transient arrest of CA function, although cytotoxic effects were occasionally observed. When CA were maintained in vitro with 10^{-3} M of P II, a relationship between the time of treatment (3, 6 or 9 h) and the intensity of the effects was apparent. The 9-h treatment led to an irreversible inhibition of JH production which parallels the severe damages observed in the CA (membrane lysis, nuclear pyknosis, vacuolization). Equivalent studies performed with the chroman derivative 3,4-dihydroprecocene II (DHP II) showed that it is less active than P II. Only treatments as severe as 12 h of incubation with a 10^{-3} M concentration elicited cytotoxic effects which could be due to radical species involved in the in situ oxidative bioactivation of DHP II. Thus, this compound could be regarded as a new type of pro-allatocidin.

Key words: Corpora allata – Ultrastructure – Precocenes – Juvenile hormone – *Blattella germanica* (Insecta)

In 1976 Bowers and coworkers described the symptoms of juvenile hormone (JH) deficiency induced by natural precocenes in the bug *Oncopeltus fasciatus*. Subsequently, Unnithan et al. (1977) demonstrated that these effects were due to a selective destruction of the corpora allata (CA), and further ultrastructural studies on CA from precocene treated specimens of other insects, such as *Locusta migratoria* (Schooneveld 1979) or *Diploptera punctata* (Feyereisen et al. 1981), showed similar effects. Moreover, results from several metabolism studies (Pratt et al. 1980; Soderlund et al. 1980; Hamnett and Pratt 1983) suggested that this action on the CA should be due to the cytotoxic properties of a reactive intermediate formed in situ and postulated to be a 3,4-epoxyderivative.

In *Blattella germanica*, a previous study on virgin females (Bellés et al. 1987b) showed that there is a cycle of JH III release which parallels the development of each batch of oocytes and the dynamics of CA volume changes. After oviposition, the females carry the ootheca until eclosion of nymphs from the egg and during this period the CA release very low levels of JH III, as in freshly ecdysed specimens. In addition, in vivo antigonadotropic activity of precocene in virgin females of this species, and the inhibitory action of such compounds on JH release in CA incubated in vitro have been previously reported (Bellés et al. 1985, 1988).

This paper presents the results of the ultrastructural analysis of CA from in vivo and in vitro precocene II (P II) treated *B. germanica*. In vitro experiments allowed us to study the influence of different periods of incubation with this compound on JH release, thus making possible a detailed correlative analysis between ultrastructural features and functional data. On the other hand, a parallel analysis of the compound 3,4-dihydroprecocene II (DHP II) is also described. Although this derivative lacks the double bond which seems decisive for precocene activity (see above), it induces similar symptoms of JH defficiency in *B. germanica*, either when applied in vivo (Bellés and Messeguer 1981) or in vitro (Bellés et al. 1988). The ultrastructural analysis of DHP II treated CA has been carried out in order to clarify the mode of action of this compound.

Materials and methods

Animals

Cultures of *B. germanica* (L.) were maintained at 27° C. Freshly ecdysed virgin females removed from the colony were used at appropriate adult ages which were additionally assessed by measure of the basal oocyte length.

Test compounds

Synthetic 6,7-dimethoxy-2,2-dimethyl-2*H*-chromene (Precocene II: P II) and 6,7-dimethoxy-2,2-dimethyl-2*H*-chroman (3,4-dihydroprecocene II: DHP II) were prepared in the Department of Biological Organic Chemistry by Dr. A. Messeguer, following previously reported procedures (Camps et al. 1979, 1980).

In vivo experiments

The experimental procedures have been previously described by Bellés et al. (1985). Compounds were applied

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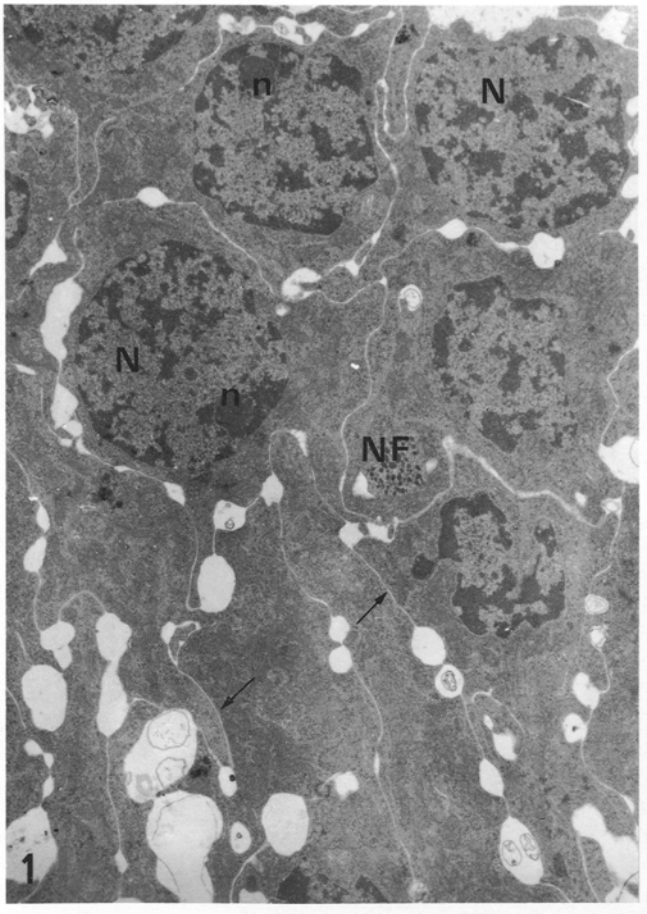
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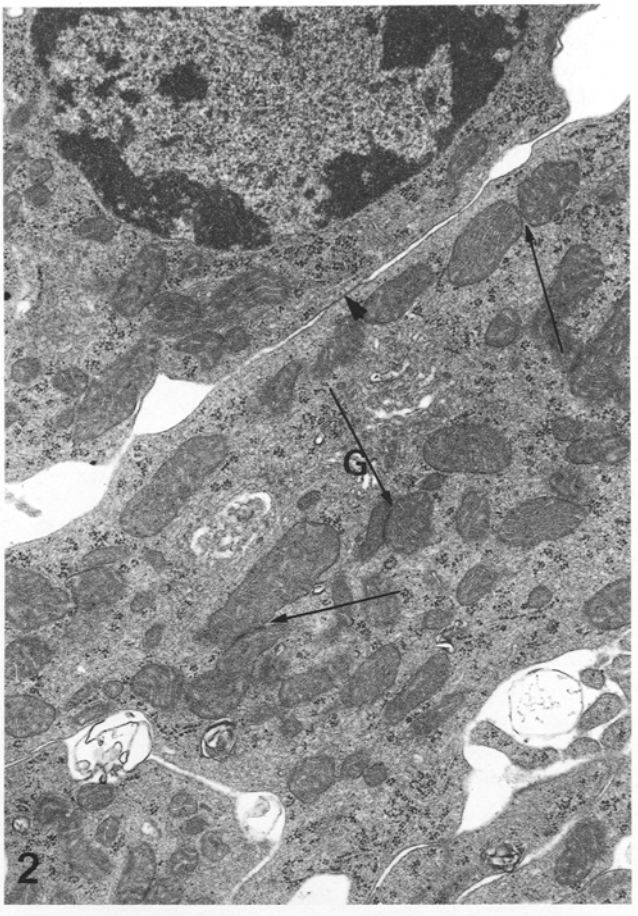
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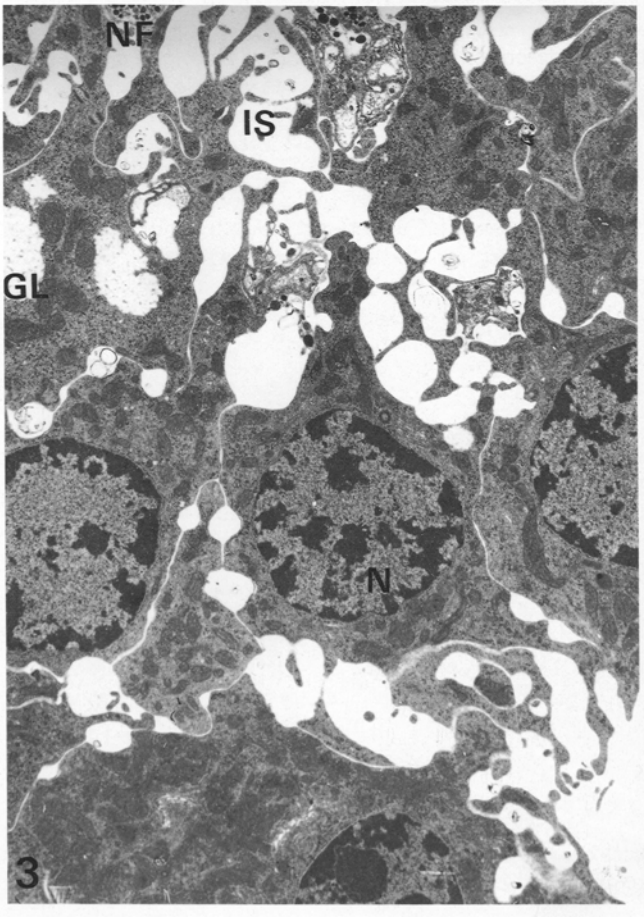
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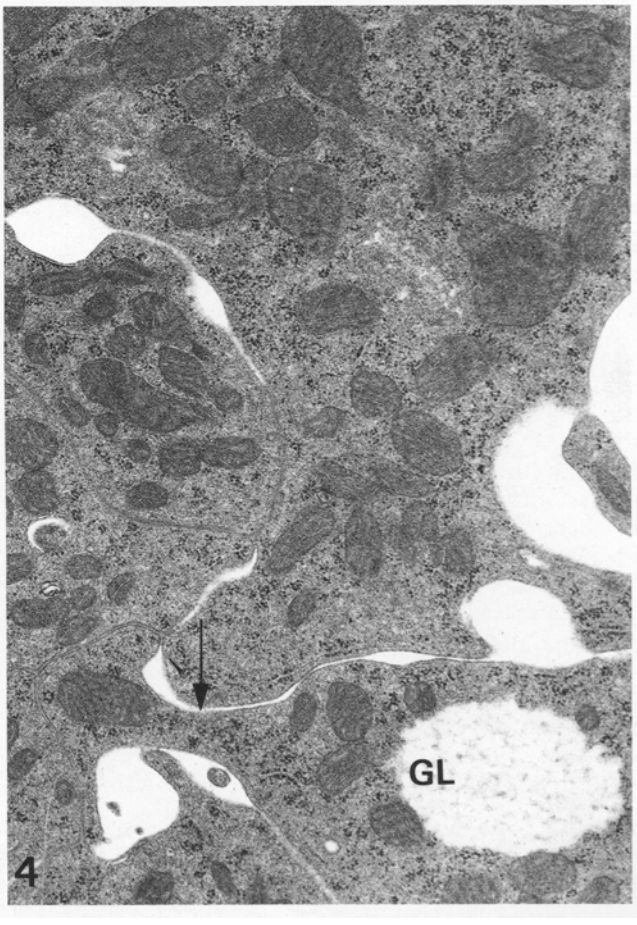
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topically in acetone solution (2 µl) on freshly ecdysed females which were maintained in the absence of males during the experiments. Controls received 2 µl of acetone. In short-term experiments induced effects were checked 6 days after the treatment, by measuring and averaging 3 basal oocytes of each ovary per specimen. In long-term experiments treated and control specimens were maintained under observation until the formation of the first ootheca or until death.

In vitro experiments

Ultrastructural studies were carried out on CA treated in vitro with 10^{-3} M of P II or DHP II. Methods of incubation in the presence of precocenes and techniques for JH III quantification have been reported elsewhere (Bellés et al. 1988). JH III release was determined for individual pairs of CC-CA complexes by the radiochemical method of Pratt and Tobe (1974), which was further adapted to the case of *B. germanica* by Bellés et al. (1987b). Glands from 5- or 6-day-old females were incubated in TC-199 medium (Hank's salts, with glutamine, HEPES buffer, 20 mM; Flow), Ficoll (20 mg/ml; Pharmacia) and [methyl-^{3}H]methionine (final specific activity 100 mCi/mmol; Amersham). After an initial 3-h period of incubation, glands were transferred to fresh medium containing 0.1 µl of ethanolic solution of the test compound and incubation was pursued for 3, 6, 9 or 12 h. Finally, a post-treatment incubation was carried out by transferring the glands to fresh medium. In all cases, control incubations with medium containing 0.1 µl of ethanol were performed.

Electron microscopy

At the end of the in vivo or in vitro assays, the CC-CA complexes were fixed in 0.2 M cacodylate-buffered glutaraldehyde with 2% sucrose for 18 h, then rinsed (30 min) in 0.2 M cacodylate with 5% sucrose. Postfixation was carried out in cacodylate buffered 2% OsO_4 for 75 min. Dehydration was in ethanol and embedding in Epon-Araldite medium. Semithin sections for sample orientation were stained with 1% toluidine blue in 1% sodium borate on a warming plate. Thin sections were contrasted with uranyl acetate and lead citrate (Reynolds 1963). Observations were carried out with a Philips electron microscope (EM 300 or EM 201), at 80 kV voltage.

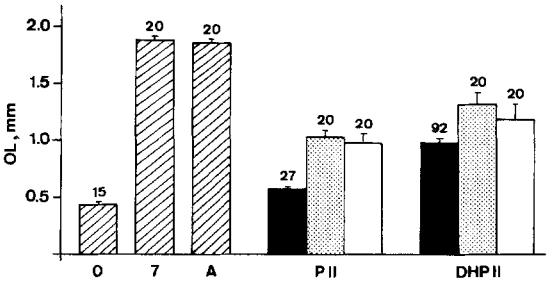


Fig. 5. Basal oocyte length (*OL, mm*) in experimental virgin females of *Blattella germanica*. *O* untreated, freshly ecdysed; *7* untreated, 7-day-old; *A* acetone treated (2 µl), 7-day-old; *P II* and *DHP II*: precocene II and 3,4-dihydroprecocene II treated, 7-day-old (black, dotted and white columns: 200, 100 and 50 µg dose, respectively). Number of individual determinations indicated at top of each column; vertical bars show standard error of the mean (Data of precocene experiments from Bellés et al. 1985)

Results

Ultrastructure of corpora allata during the first gonadotropic cycle

The CA of *B. germanica* are paired, ellipsoidal glands each consisting of parenchymal cells surrounded by a thin basal lamina (Piulachs and Bellés 1985). The ultrastructural studies were carried out on CA from virgin females representing four characteristic developmental stages: (a) freshly ecdysed (previtellogenesis), (b) 4-day-old (beginning of vitellogenesis), (c) 6-day-old (full vitellogenesis), and (d) 10-day-old (post-vitellogenesis, females carrying the ootheca). These stages are well characterised in terms of oocyte length, CA volume and JH biosynthetic activity, according to data described by Bellés et al. (1987b).

The CA from freshly ecdysed females (basal oocyte length: 0.45 ± 0.003 mm, $n=9$) are small (0.94 ± 0.07 µm$^3 \times 10^6$, $n=9$) and release low amounts of JH III (0.09 ± 0.02 pmol/h × pair, $n=9$) when incubated in vitro. The cells are densely packed leaving narrow intercellular spaces (Fig. 1). The cytoplasm contains numerous ribosomes and polysomes. The mitochondrial system is poorly developed and consist of scarce globular units. Spherical nuclei and nucleoli are of small size.

When vitellogenesis takes place (4-day-old females, basal oocyte length: 1.02 ± 0.03 mm, $n=10$), the CA volume has increased (1.49 ± 0.09 µm$^3 \times 10^6$, $n=10$) as well as its synthetic capability (0.58 ± 0.08 pmol JH/h × pair, $n=10$). There are numerous mitochondria, characterized by their large size, their irregular or rod-like shape and by the presence of numerous lamellar cristae; chondriodieresis is frequently observed (Fig. 2). The occurrence of cisternae of rough endoplasmic reticulum associated with the development of glycogen areas, the larger size of nuclei and nucleoli, and the distension of the intercellular spaces are also typical features of this stage.

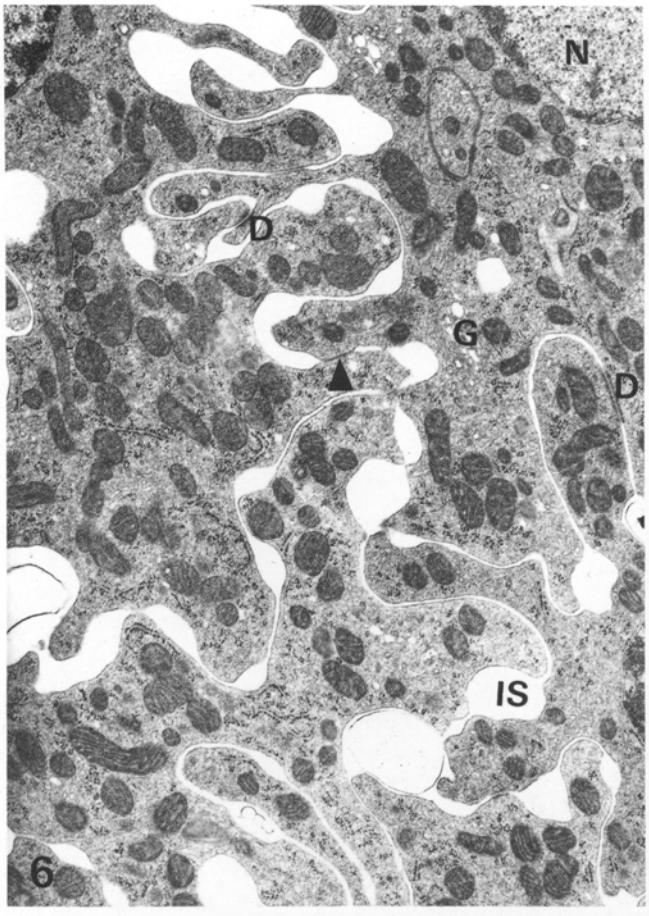
In 6-day-old females (basal oocyte length: 1.81 ± 0.03 mm, $n=10$), the CA are large (1.64 ± 0.08 µm$^3 \times 10^6$, $n=10$) and very active (2.58 ± 0.35 pmol JH/h × pair, $n=10$). The features observed in glands from 4-day-old females are now more conspicuous, including larger intercellular spaces and cytoplasmic infoldings or finger-like projections, enlargement of nuclei and nucleoli, and an increase in mitochondrial number. In addition, the smooth endo-

Fig. 1. CA of freshly ecdysed female. Note densely packed cells and narrow intercellular spaces (*arrows*). *N* nuclei; *n* nucleolus; *NF* neurosecretory fiber. × 9000

Fig. 2. Detail of CA from 4-day-old female showing active chondriodieresis (*arrows*). *G* Golgi apparatus; *black triangle* gap junction. × 16400

Fig. 3. CA from 6-day-old female. Note distended intercellular spaces (*IS*) and numerous areas of glycogen (*Gl*). *NF* neurosecretory fiber; *N* nucleus. × 6600

Fig. 4. Detail of CA from 10-day-old female carrying ootheca. Note narrow intercellular spaces (*arrow*) and aggregated mitochondria. *Gl* glycogen. × 20000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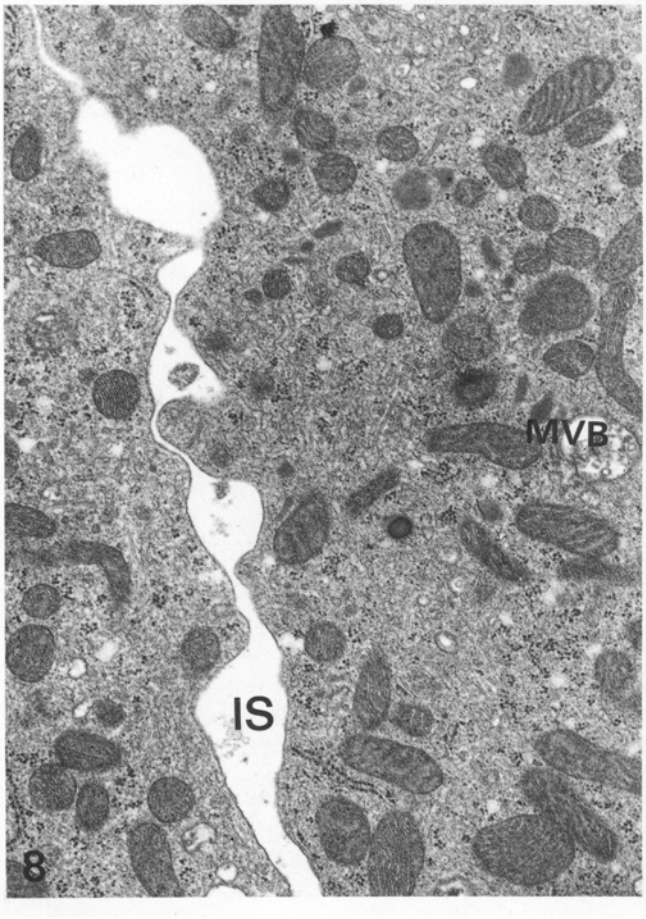
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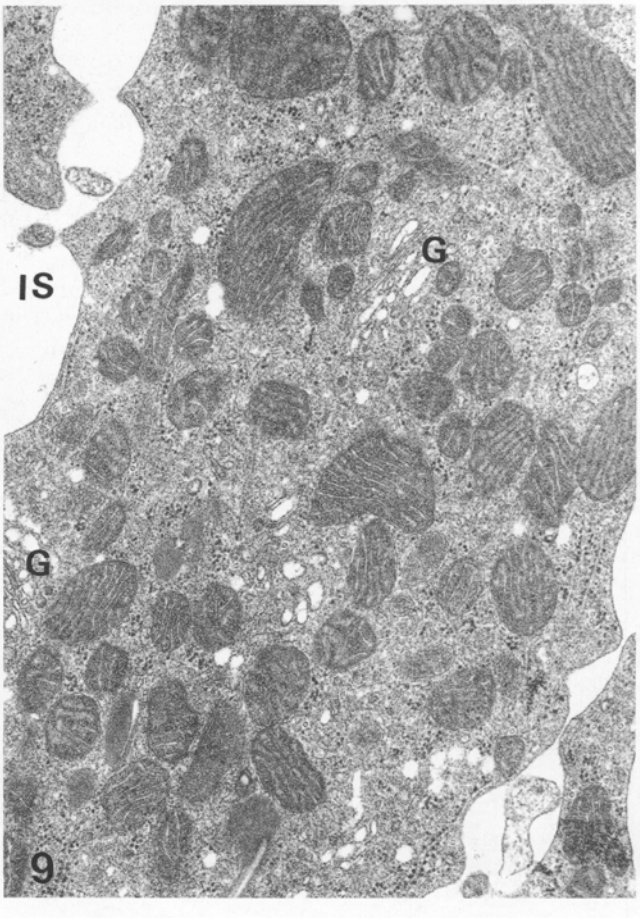
IS
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9

Table 1. Percentage of specimens of *Blattella germanica* treated with precocene II (P II) or with 3,4-dihydroprecocene II (DHP II) in long-term experiments, which formed a first ootheca (%00) and its time of formation (Time, days ± SD). (Results of experiments with P II after Bellés et al. 1985)

Treatment	N	%00	Time
Untreated	40	82.5	9.3 ± 1.7
Acetone 2 µl	30	86.7	12.1 ± 6.3
200 µg P II	30	50.0	13.4 ± 3.5
200 µg DHP II	30	57.0	14.4 ± 8.3

plasmic reticulum and areas of glycogen are now evident (Fig. 3).

At the end of the gonadotropic cycle (10-day-old females carrying an ootheca, basal oocyte length: 0.33 ± 0.005 mm; $n=9$) the CA have a small volume (0.88 ± 0.09 $\mu m^3 \times 10^6$, $n=9$) and a diminished synthetic activity (0.05 ± 0.02 pmol JH/h × pair, $n=9$). This retrogression is related to a decrease of intercellular spaces, of the size of the glandular cells, and of their nucleus and nucleolus. Mitochondria are numerous and frequently aggregated and associated with smooth endoplasmic reticulum. Glycogen areas are, in general, well developed (Fig. 4).

In vivo effects of precocene II and 3,4-dihydroprecocene II

As described by Bellés et al. (1985), a dose of 200 µg of P II induces a nearly complete inhibition of oocyte growth in short-term experiments (Fig. 5). However, long-term experiments revealed that 50% of these specimens were able to produce the first ootheca (Table 1). Concerning the compound DHP II, preliminary data about its antigonadotropic activity in *B. germanica* were reported by Bellés and Messeguer (1981). This activity has now been confirmed, and results of short-term experiments (Fig. 5) indicate that this compound elicits a less pronounced inhibitory activity in comparison with P II (an average of 62% and 92% inhibition of oocyte growth, respectively, at a dose of 200 µg). Conversely, long-term experiments with a dose of 200 µg (Table 1) show more similar results for both compounds in terms of percentage of specimens that formed a first ootheca and its time of formation.

Ultrastructural studies carried out on CA from specimens subjected to short-term experiments (treatment on day 1, dissection on day 7) revealed that the same dose of 200 µg of P II induced distinct degrees of delay in the differentiation of the CA cells, which correlated, in general, with the inhibition observed at the level of oocyte growth. Therefore,

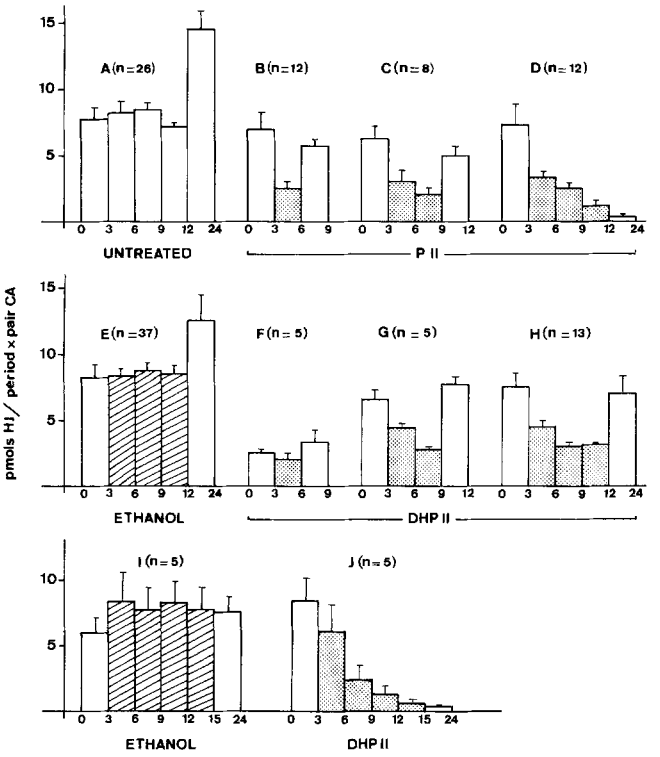


Fig. 10. Effects of different times of incubation with 10^{-3} M P II or DHP II on in vitro JH release (pmols HJ/period × pair CA) by CA from 5-day-old (experiment F) or 6-day-old (remaining experiments) virgin females of *Blattella germanica*. Each column represents absolute value of JH released in corresponding period of incubation, indicated in abscissae. Incubation periods with test compound represented by dotted columns, those with ethanol (controls) by striped columns. Number of individual determinations indicated at top of each experiment; vertical bars represent standard error of mean (data of experiments A–E, G and H from Bellés et al. 1988)

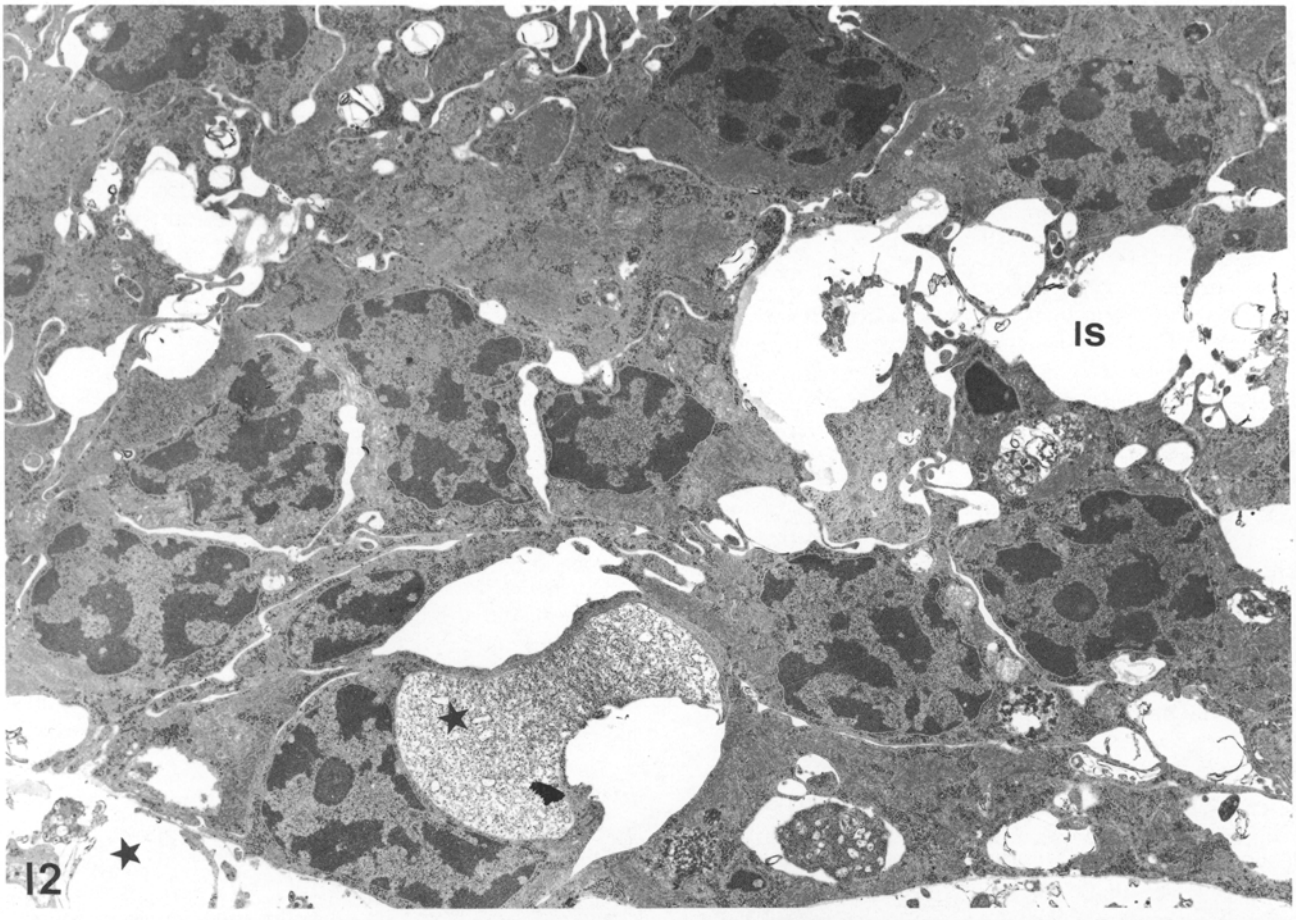
the CA from these PII-treated specimens (7-day-old) (Figs. 6, 7) show intermediate features between those of a freshly ecdysed female and those of a female just beginning vitellogenesis. Moreover, the variability of P II effects also appears at the level of the cellular population of the same corpus allatum, where inactive and incipiently active cells frequently coexist. Cytotoxic effects induced by P II are insignificant or not discernible. Occasionally, some autolytic cells with giant spherical mitochondria appear. Invasion of haemocytes into CA was never observed.

The compound DHP II, applied at the same dose (200 µg) and under the same experimental conditions as P II, induced less pronounced effects, although they were also quite variable. The average of inhibition of oocyte growth was approximately 50% (Fig. 5) and the CA from those specimens corresponding to this level of inhibition (Figs. 8, 9) showed similar characteristics as those of a 4-day-old normal female. Neither cytotoxic effects nor haemocyte invasion were observed.

Glands from control (acetone-treated) specimens show the features of non-treated females just before the end of vitellogenesis (day 7), i.e., hypertrophy of intercellular spaces and cytoplasmic projections, large size of nuclei and nucleoli, and presence of smooth endoplasmic reticulum.

Figs. 6, 7. Detail of CA from 7-day-old female topically treated with 200 µg of P II. Intercellular spaces (*IS*), mitochondrial population and rough endoplasmic reticulum not well developed. *D* desmosome; *G* Golgi apparatus; *N* nucleus; *black triangles* gap junction. **Fig. 6** × 13600; **Fig. 7** × 26000

Figs. 8, 9. Detail of CA from 7-day-old females topically treated with 200 µg of DHP II. Effects of this compound less pronounced in comparison with those of P II. Mitochondria of different sizes more numerous and intercellular spaces (*IS*) more distended. *G* Golgi apparatus; *MVB* multivesicular body. **Fig. 8** × 20000; **Fig. 9** × 26000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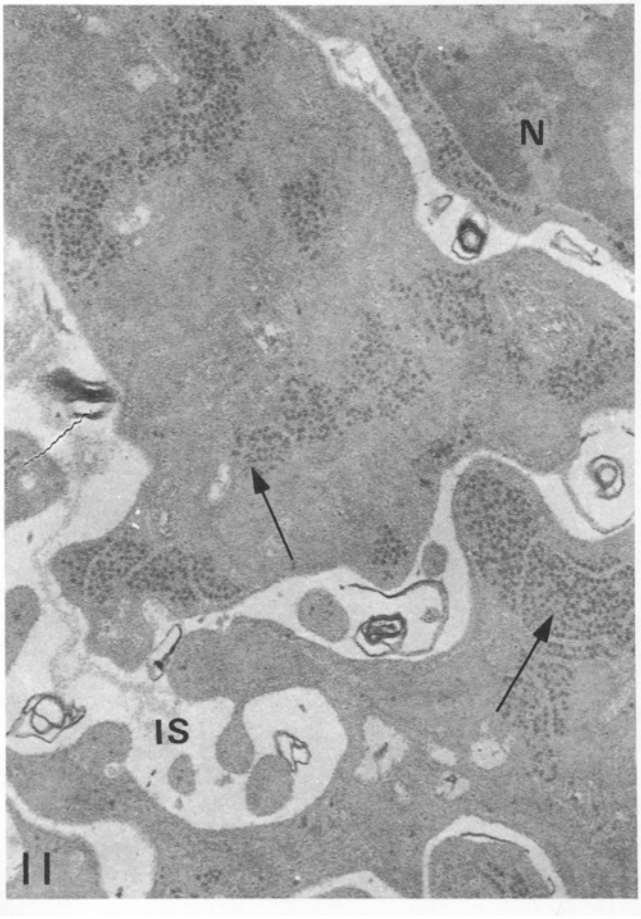
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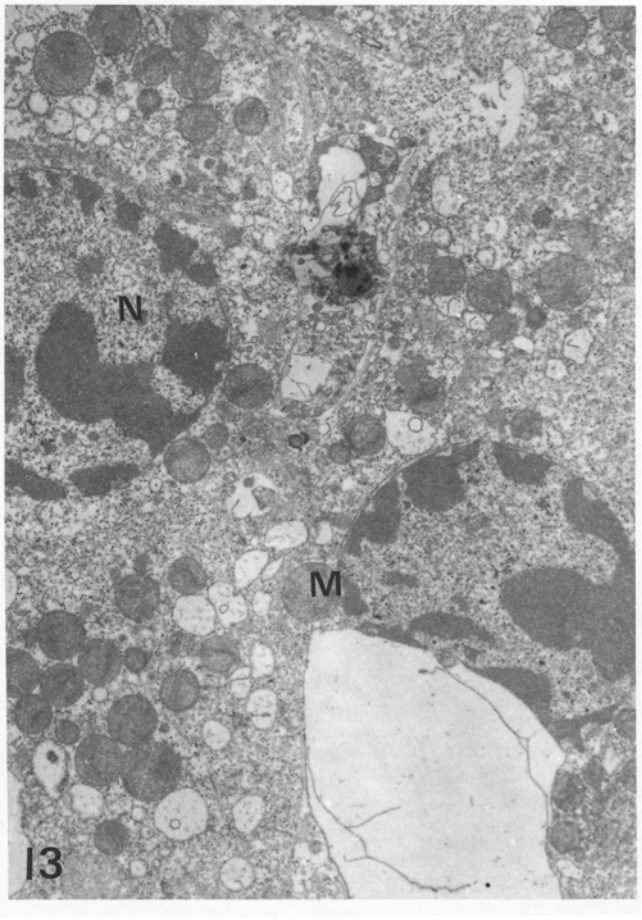
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In vitro effects of precocene II and 3,4-dihydroprecocene II

The effects of different times of incubation with 10^{-3} M of P II or DHP II on JH release have been recently described (Bellés et al. 1988). These data and results of new experiments carried out for purposes of ultrastructural study are summarized in Fig. 10. Ultrastructural studies were performed in glands fixed at the end of each assay. Control glands showed the normal features of CA from freshly dissected specimens of the same age.

Effects of precocene II. Glands subjected to a 3-h treatment (which has a transient inhibitory effect at the level of JH release: Fig. 10), show a very important distension of intercellular spaces. This distension is probably linked to shrinkage of the glandular cells, which show an irregularly shaped nucleus, and a clumping of ribosomes near the nuclei, cisternae of rough endoplasmic reticulum, and plasma membranes. Intercellular spaces frequently contain myeloid structures (Fig. 11). Pyknotic nuclei are infrequent.

From a functional point of view, after 6 h of treatment, the inhibitory effect is more pronounced. However, the CA are still able to increase the levels of JH release in the post-treatment period of incubation (Fig. 10). The ultrastructural changes observed in 3-h treated glands are here more apparent. In addition, numerous areas of cytoplasmic lysis appear, which prove the cytotoxic effect of P II. In the irregularly shaped nuclei, the condensed chromatin clumps suggest a pyknotic process (Fig. 12).

After a 9-h treatment, the biosynthetic activity of CA is irreversibly inhibited (Fig. 10). The glands resulting from these experiments show pyknotic nuclei and spectacular accumulations of cell fragments. Some spherical mitochondria contain numerous tubulo-saccular cristae. The other organelles are vacuolized (Fig. 13).

Effects of 3,4-dihydroprecocene II. The 3-h treatment does not induce any significant inhibitory activity at the level of JH release (Fig. 10). Ultrastructural modifications in these CA are not evident (Fig. 14). The well-developed cytoplasm contains numerous and large mitochondria with lamellar cristae, numerous ribosomes or polysomes and slender rough endoplasmic cisternae. However, nuclei and nucleoli seem shrunken.

The same features were observed in 6-h treated CA (Fig. 15), although the functional capability of these glands was slightly diminished. In 9-h treated CA, the biosynthetic activity is clearly inhibited, although they increase their rates of hormone production after treatment (Fig. 10). This experiment has similar effects on CA to those induced by a 3-h or a 6-h treatment with P II, i.e., distension of intercellular spaces, shrinkage of cells and nuclei, and clumping of ribosomes and mitochondria (Fig. 16). However, no cytotoxic damage was observed in this case.

An additional treatment of 12 h was carried out with DHP II, which caused an irreversible inhibition of JH release (Fig. 10). The CA resulting from this experimental combination show clear cytotoxic damage. The glands display an accumulation of cell fragments, scattered mitochondria and pyknotic nuclei. In addition, numerous myeloid structures and shrunken cells are observed in the enlarged intercellular spaces (Fig. 17). These symptoms of irreversible glandular degeneration are similar to those observed after 9 h in vitro with the same dose of P II.

Fig. 11. CA from 6-day-old female after 3 h of incubation with 10^{-3} M of P II. Note distension of intercellular spaces (*IS*) containing myeloid structures, cell shrinkage and ribosomes clumping (*arrows*). *N* nucleus. ×40000

Fig. 12. CA from 6-day-old female after 6 h of incubation with 10^{-3} M of P II. Note cellular autolysis (*asterisks*), shrinkage of cells and nuclei, hypertrophy of intercellular spaces (*IS*). ×6600

Fig. 13. CA from 6-day-old female after 9 h of incubation with 10^{-3} M of P II. Severe disorganization of glands; note pyknotic and vacuolized nuclei (*N*), cell fragments, some spherical mitochondria (*M*). ×9000

Discussion

Ultrastructural changes of the corpora allata through the first gonadotropic cycle

As expected, during the first gonadotropic cycle of *B. germanica*, CA ultrastructural changes mainly concern two kinds of structures involved in JH synthesis (see Tobe and Stay 1985), the endoplasmic reticulum and the mitochondria. This may be observed in all species studied in this context (see Cassier 1979; Johnson et al. 1985; Sedlak 1985; Tobe and Stay 1985), although interespecific variations concerning the proportionality between both organelles exist.

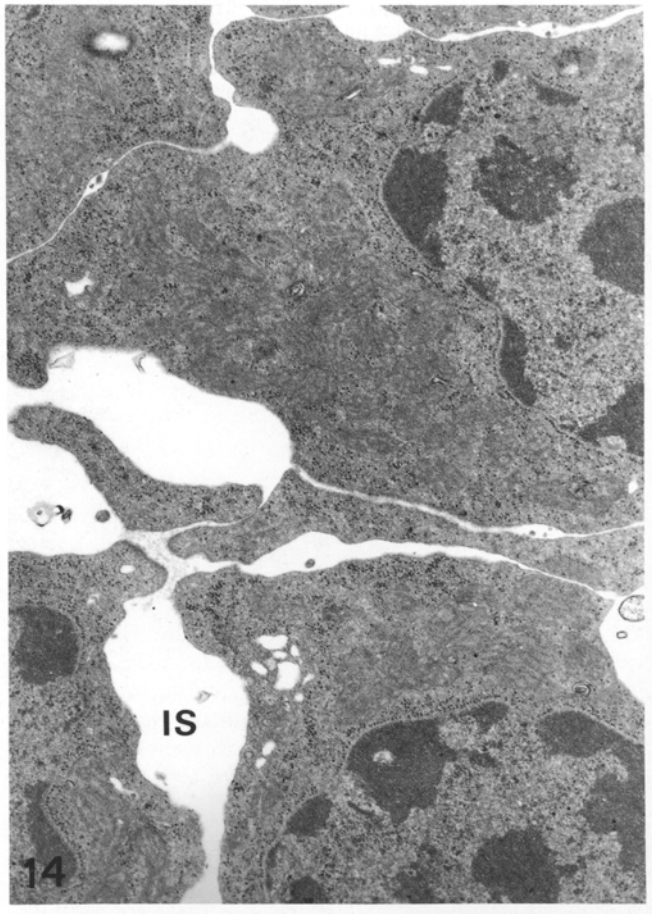
Effects of precocene II

The CA of *B. germanica* show low sensitivity to the characteristic allatocidal effects of P II. Only treatments as severe as 9 h of incubation with a dose of 10^{-3} M, elicited clear symptoms of cytotoxic activity. However, no complete destruction of the glands was reached, either in in vivo or in vitro experiments, as occurs in other insects such as *Oncopeltus fasciatus* (Unnithan et al. 1977) or *Locusta migratoria* (Schooneveld 1979). In *L. migratoria*, Schooneveld (1979) described that in precocene treated specimens the haemocytes invaded the CA in large number, after which the parenchymal cells became increasingly necrotic until the virtual disappearance of the glands. In *B. germanica* no invasion of haemocytes into the CA was observed and this could explain (at least in part) that CA of this cockroach cannot be completely destroyed by these allatocidins.

Effects of 3,4-dihydroprecocene II

Electron micrographs of the CA treated with DHP II, either in vivo or in vitro show similar features as those observed in P II treated glands. However, when equivalent experiments for both compounds are compared, relatively smaller effects are observed in the case of DHP II treated specimens, suggesting that progression of events which leads to the gland's regression caused by this derivative is in some way delayed in comparison with those induced by P II. This fact could be related to a mode of action different from that opperating in the case of P II.

With the data presently available, the one hypothesis that could be postulated to explain the allatocidal action of DHP II is an in situ oxidative bioactivation, at benzylic (C4) position, mediated by the same monooxigenase system of the CA. Therefore, the cytotoxic effects could be induced by radical species involved in this oxidation. In this context, it is worth noting that recent results (Bellés et al. 1987a) about the synergistic action of diethyl maleate – a well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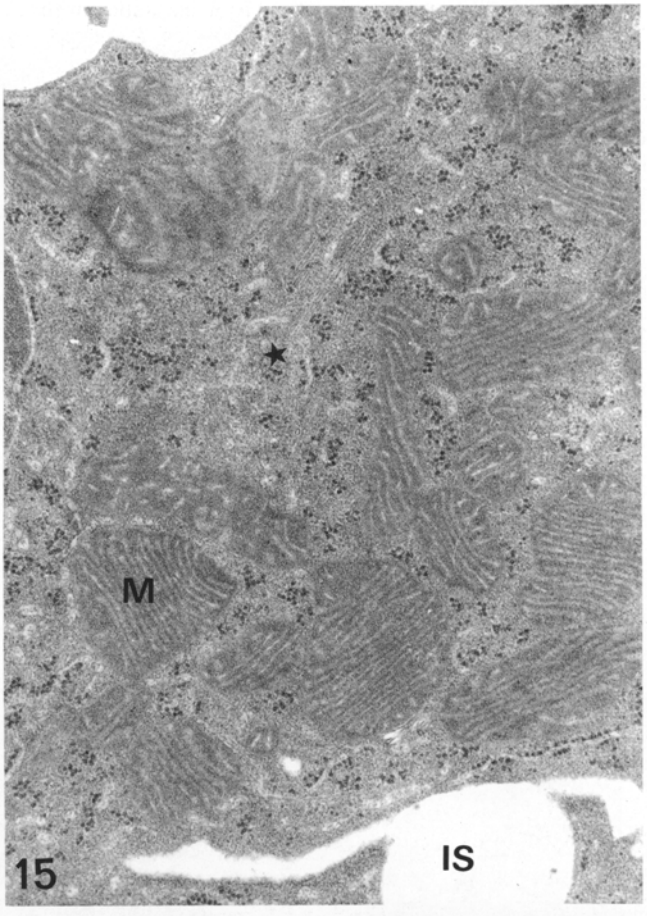
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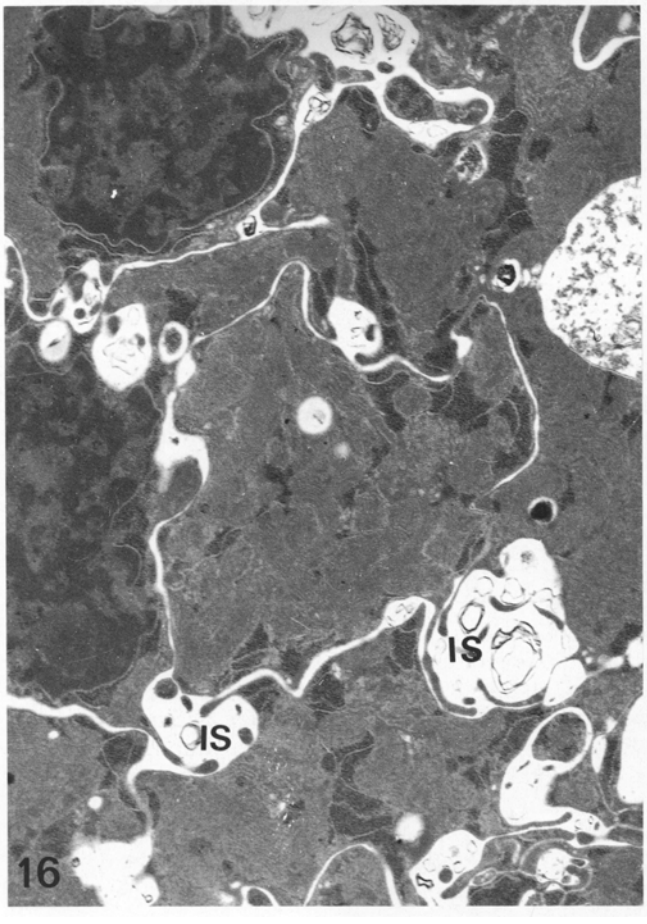
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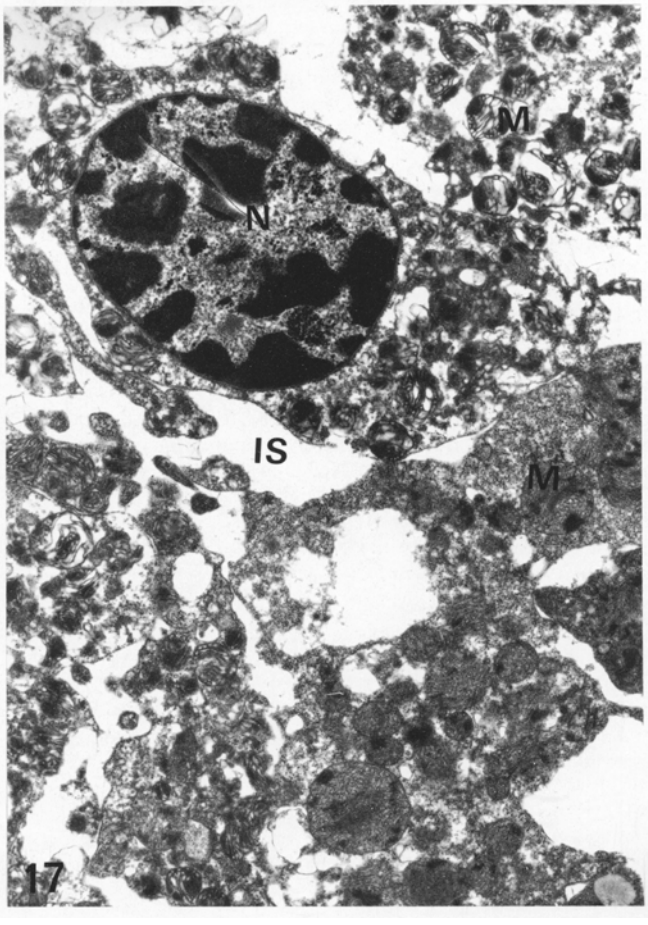
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17

Fig. 14. CA from 5-day-old female after 3 h of incubation with 10^{-3} M of DHP II. Glands show normal organization. *IS* intercellular spaces; *N* nucleus. ×16400

Fig. 15. Detail of cytoplasm of CA from 6-day-old female after 6 h of incubation with 10^{-3} M of DHP II. Glands still exhibit normal organization. *IS* intercellular spaces; *N* nucleus; *asterisk* smooth endoplasmic reticulum. ×32000

Fig. 16. CA from 6-day-old female after 9 h of incubation with 10^{-3} M of DHP II. Effects similar to those observed after 3- or 6-h treatment with P II: cells and nuclei shrinking, ribosomes and mitochondria clumping, intercellular spaces (*IS*) distended. ×13600

Fig. 17. CA from 6-day-old female after 12 h of incubation with 10^{-3} M of DHP II. Most of gland disorganized with numerous cell fragments, pyknotic nuclei (*N*) and some spherical mitochondira (*M*). *IS* intercellular spaces. ×11200

known depletor of glutathione – on the anti-juvenile effects of precocenes, indicated that radical species originated during the biotransformation of these pro-allatocidins could have participated as cytotoxic agents in the degenerative process of CA. Biochemical studies to elucidate these metabolic aspects are now in progress but, in any case, our present data suggest that this chroman derivative can be regarded as a new type of pro-allatocidin which could be of potential interest for insect control and for insect endocrinological studies.

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