

**Stimulation of corpora allata by extract from neuroendocrine complex;
comparison of reproducing and diapausing *Pyrrhocoris apterus*
(Heteroptera: Pyrrhocoridae)**

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Abstract. The effect of extracts from neuroendocrine complexes of brain-suboesophageal ganglion-corpora cardiaca-corpora allata (NEC) on synthetic activity of corpora allata (CA) in vitro was investigated by a radiochemical assay in *Pyrrhocoris apterus*. NEC extracts from short-day (diapausing) females and long-day (active) females and males were compared. NEC extracts from both short-day and long-day insects stimulated synthetic activity of the CA from long-day females. The CA from short-day females were refractory to stimulation. The stimulating activity of the NEC extract was retained after removal of proteins using 10,000-molecular weight limit centrifugal ultrafiltration, but was destroyed by treatment with proteinase K. Therefore, the stimulating factor appears to be a peptide. The culture medium containing a mixture of synthetic products of the CA was extracted by hexane and subjected to TLC analysis. It was revealed that the NEC extract stimulated synthesis of a product from radiolabelled methionine which elutes on TLC in the same position as the major product of the CA of four other heteropteran species.

INTRODUCTION

The activity of corpora allata (CA) in *Pyrrhocoris apterus* (L.) is regulated by photoperiod. In long day conditions the CA are active and stimulate reproduction, while in short day conditions reproduction is inhibited. Photoperiodic signals are transmitted via the brain which inhibits the activity of the CA via nervous connections (Hodková, 1976). The inhibition of the CA is stronger under short days than under long days. The internal milieu of feeding insects is stimulating irrespective of photoperiodic conditions and can overcome the weak inhibition of the CA within the long day neuroendocrine complex. By contrast, the strong inhibition of the CA in short day insects cannot be overcome by feeding (Hodková, 1977, 1992). While surgical interventions to the neuroendocrine system in vivo indicate a direct inhibiting effect of the brain on the CA via nervous connections in *P. apterus*, the origin of a stimulating factor in the internal milieu of feeding insects is not known.

The aim of the present paper is to determine whether an allatotropin is present within neuroendocrine complexes of *P. apterus*. Because the chemical nature of juvenile hormone in Heteroptera is not yet known, the synthetic activity of the CA was measured by incorporation of radioactive methionine into products which were shown to have juvenile

hormone-like activity in another heteropteran, *Plautia stali* (Kotaki, 1996). It is demonstrated for the first time that an allatotropin is present in extracts from neuroendocrine complexes of a heteropteran species.

MATERIAL AND METHODS

Insects

Females of *P. apterus* (L.) (Heteroptera) were reared from the egg stage at $26 \pm 1^\circ\text{C}$, either under diapause-preventing long day (18 h light-6 h dark) or under a diapause-inducing short day (12 h light-12 h dark). They were supplied with linden-seed and water ad libitum.

Extract from neuroendocrine complex

The neuroendocrine complex of brain-corpora cardiaca-corpora allatum with attached suboesophageal ganglion and part of aorta (NEC) was dissected in insect saline and extracted by the following methods.

A. Crude extract was prepared by sonication of NECs in 80% methanol. Extracts were centrifuged, and supernatants were concentrated under a stream of nitrogen and diluted with Minimal Eagle's Medium (MEM) (Cosmo Bio) for bioassay.

B. Filtered extract was prepared by homogenization of NECs in methanol/1 N HCl/acetonitrile/water (75:50:50:25) containing 0.05% (by volume) trifluoroacetic acid. Extracts were centrifuged through 0.45 μm filter units, followed by removal of large proteins and proteases using 10,000 NMWL centrifugal ultra-filtration units (Wagner et al., 1993). Filtrates were concentrated under a stream of nitrogen and diluted with MEM for bioassay.

C. To destroy peptides, a part of filtered extract diluted with MEM was treated with proteinase K (Wako) (10 mg/ml) at 26°C overnight. Proteinase K was then removed by centrifugal ultrafiltration as described above.

Synthetic activity of the CA in vitro

The radiochemical assay of juvenile hormone synthesis (Pratt & Tobe, 1974) was used in the modification described by Ferenz & Kaufner (1981). The complex of corpora cardiaca-corpora allatum (CC-CA) was incubated in a siliconized glass tube (6 mm dia., 3 cm length) containing L-[methyl- ^3H] methionine (specific activity of 3.08 TBq/mmol) in 30 μl MEM with Hank's salts and L-glutamate, without sodium bicarbonate, but containing 20 mM HEPES and 5 ppm Tween 80, and adjusted to pH 7.3. After incubation at $29 \pm 1^\circ\text{C}$ for 1 h the medium was replaced and 1, 2 or 3 incubations, each lasting 2 h, followed. After each incubation for 2 h, 100 μl of hexane were added to the medium and the tube was vortexed. The upper hexane phase was collected and divided into two parts. Total radioactivity was measured in an aliquot of 25 or 50 μl . Another aliquot of 25 or 50 μl was applied to a silica TLC sheet (10 cm \times 10 cm) (G 60 F254). Unlabelled standard of JH III (Sigma) was applied simultaneously to the TLC sheet which was then developed with a mixture of hexane and ethyl acetate, 1 : 1. After the development, the JH III standard spot was visualized under UV light. The TLC sheet was cut into sections of 5 mm in length and each section was put into a plastic vial with a scintillation cocktail (ASC II, Amersham). The sample was placed in a dark room for 1 h and then its radioactivity was measured using a liquid scintillation counter (Aloka 7500 or Hewlett Packard).

The complex of CC-CA was used to prevent injury of the CA, but the presence of corpora cardiaca did not influence the radiochemical assay (T. Okuda & M. Hodková, unpubl.). Thus, the measured radioactivity indicates the synthetic activity of the CA only.

The difference between means was evaluated with the Student's t-test.

RESULTS

Synthetic activity of long-day CC-CA

The synthetic activity of 5 CC-CA was measured on days 1–2, 2–3 and 3–4 after adult ecdysis. The radioactivity in the media ranged from about 500 DPM/CC-CA (in all ages)

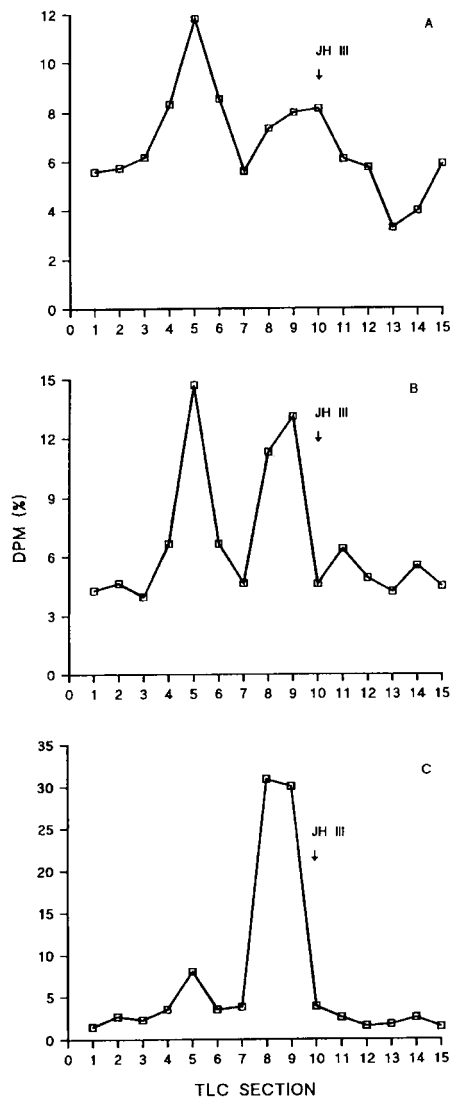


Fig. 1. TLC analysis of the radioactivity in synthetic products of the CC-CA excised from long-day females 1–2 days (A), 2–3 days (B) and 3–4 days (C) after adult ecdysis. Total radioactivities amounted to 630, 1,100, and 3,500 DPM/CC-CA, respectively.

to 1,200, 1,500 and 3,500 DPM/CC-CA on days 1–2, 2–3 and 3–4 after adult ecdysis, respectively.

Synthetic products of 3 CC-CA were analysed by TLC (Fig. 1A, B, C). TLC profiles for the products of the CC-CA with the highest activity showed a major peak of radioactivity in sections 8 and 9 (Fig. 1C). The spot corresponding to JH III standard was located in section 10.

The effect of crude NEC extract

Experiments *in vivo* indicated a direct inhibiting effect of the pars intercerebralis of the brain on the CA (Hodková, 1976, 1977, 1979). Therefore the aim of the first experiment was to search for an allatoinhibiting factor in the NEC extract. NEC extract was prepared from neuroendocrine complexes taken from short-day females 1–2 weeks old. The CC-CA from long-day females 3–4 days old were incubated for the first 2 h without NEC extract. Two NEC equivalents were added before the second incubation for 2 h. The third incubation for 2 h was without NEC extract. In control CC-CA, all incubations were without NEC extract.

The average radioactivity in the media increased 2.3 times during the second incubation with the NEC extract. The radioactivity decreased during the third incubation without NEC extract. In control CC-CA, the radioactivity changed little during the experiment (Fig. 2A).

Synthetic products from two CC-CA that showed the greatest increase (3.6 and 3.5 times) of radioactivity after the addition of NEC extract were subjected to TLC analysis: the radioactivity in section 8 increased 21 (Fig. 3) or 10 times (not shown). A smaller increase of radioactivity was observed in section 9 (Fig. 3). This indicated that synthesis of products migrating to sections 8–9 is specifically stimulated by the NEC extract. Thus, instead of the expected

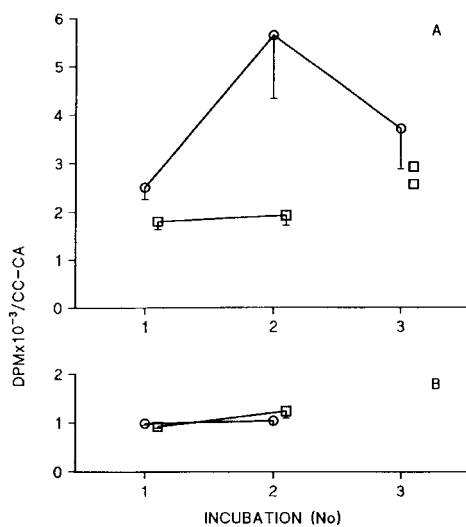


Fig. 2. Effect of crude NEC extract on the incorporation of ^3H methionine by long-day and short-day CC-CA – total radioactivity. A – long-day CC-CA, B – short-day CC-CA. Circle – 2 NEC equivalents from short-day females were added before the 2nd incubation and removed before the 3rd incubation. Values for incubations 1 and 2 in A are different at $p < 0.05$. Square – control. Each point represents mean of 7–9 CC-CA with the exception of the 3rd incubation of control CC-CA where there were only two repeats. Bars represent SEM. For other details, see text.

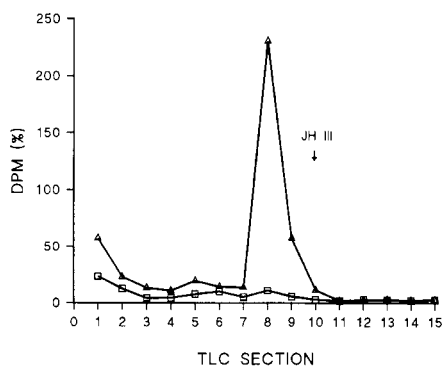


Fig. 3. TLC analysis of the radioactivity in synthetic products of long-day CC-CA before and after incubation with crude NEC extract. Square – 1st incubation without NEC extract. Triangle – 2nd incubation with NEC extract. Total radioactivity after the 1st incubation without NEC extract = 100% DPM. For other details, see Fig. 2.

allatoinhibiting activity, an allatostimulating activity was revealed in the extract from short-day neuroendocrine complexes.

In the next experiment, the effect of the short-day NEC extract on the CC-CA from short-day females 1–2 weeks old was investigated. In this case, however, the NEC extract had no effect on the synthetic activity of the CC-CA (Fig. 2B). This indicates that the short-day CC-CA is insensitive or much less sensitive to the allatostimulating factor(s).

The effect of filtered NEC extract

NEC extracts were filtered to remove proteins and their effect on the CC-CA from long-day females 3–4 days old was studied. NEC extracts were prepared from short-day females, long-day females and long-day males, all 1–2 weeks old. Two or four NEC equivalents were added to the culture medium containing one or three CC-CA, respectively. A part of the extract from short-day insects was treated with proteinase K. Otherwise the experiment was conducted in a similar manner to those previously. The results are summarized in Fig. 4. The average radioactivity increased 2.4 times during the second incubation with extract from short-day females (2 NEC equivalents). Thus the allatostimulating activity of the NEC extract was not lost by removal of proteins. However, the activity of the NEC extract was lost by treatment with proteinase K.

The allatostimulating activity was demonstrated also in NEC extracts from long-day insects. The average radioactivity increased 2.4 or 6.6 times during the second incubation with 2 or 4 brain equivalents respectively. There was no important difference between effects of extracts from males and females.

TLC analysis showed that, as in the previous experiment, the synthesis of products migrating to sections 8–9 was stimulated by

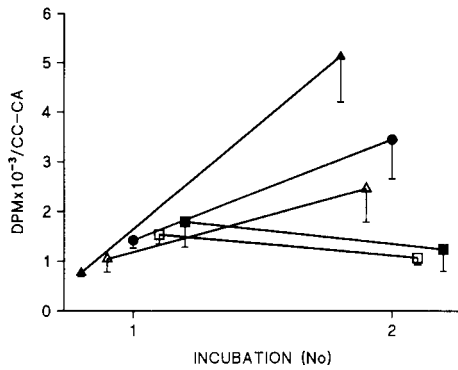


Fig. 4. Effect of filtered NEC extract on incorporation of ^3H methionine by long-day CC-CA – total radioactivity. Solid circle – 2 NEC equivalents from short-day females ($n = 16 \times 1$ CC-CA). Values for incubations 1 and 2 are different at $p < 0.05$. Open triangle – 2 NEC equivalents from long-day females ($n = 6 \times 1$ CC-CA); solid triangle – 4 NEC equivalents from either long-day females or long-day males ($n = 4 \times 3$ CC-CA). Values for incubations 1 and 2 are different at $p < 0.02$. Solid square – 2 short-day NEC equivalents treated with proteinase K ($n = 4 \times 1$ CC-CA), open square – control ($n = 21 \times 1$ CC-CA). Each point represents the mean per 1 CC-CA. Bars represent SEM. For other details, see text.

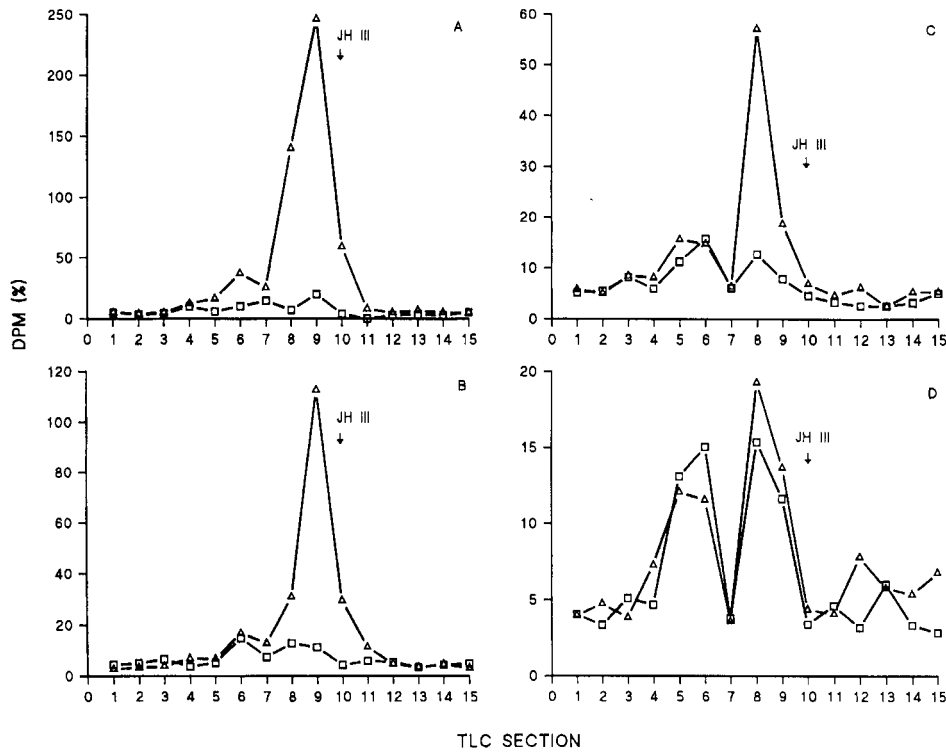


Fig. 5. TLC analysis of the radioactivity in synthetic products of long-day CC-CA before and after incubation with filtered NEC extract. A, B, C, D – four individual CC-CA. A – NEC extract from long-day females; B – NEC extract from long-day males; C – NEC extract from short-day females; D – control (no brain extract). Square – 1st incubation without NEC extract. Triangle – 2nd incubation with NEC extract. Total radioactivity after the 1st incubation without NEC extract = 100% DPM. For other details, see Fig. 4.

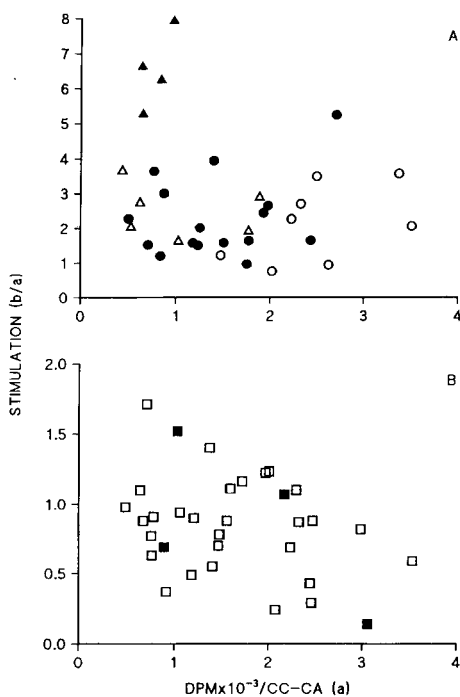


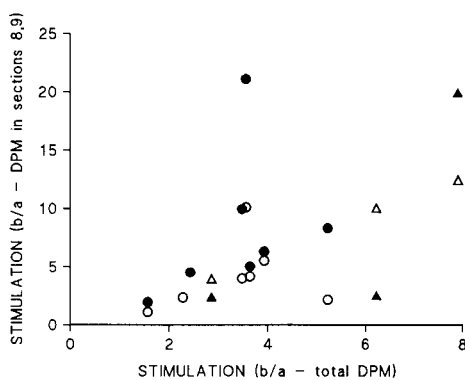
Fig. 6. Relationship between synthetic activity of long-day CC-CA and its stimulation by NEC extract – total radioactivity; x axis – radioactivity after 1st incubation without NEC extract (a), y axis – ratio between radioactivity after 2nd (b) and 1st (a) incubation. A – 2nd incubation with NEC extract. Open circle – crude extract from short-day females (2 NEC equivalents). Solid circle – filtered extract from short-day females (2 NEC equivalents). Open triangle – filtered extract from long-day females (2 NEC equivalents). Solid triangle – filtered extract from either long-day females or long-day males (4 NEC equivalents). B – 2nd incubation without NEC extract (open square) or with NEC extract treated with proteinase K (solid square). Means are given in Figs 2A and 4.

extracts from both short-day and long-day insects. In contrast, the TLC profile of a control sample was unchanged after the second incubation without NEC extract (Fig. 5).

Relationship between the synthetic activity of the CC-CA and its stimulation by the NEC extract

Although there was great variability in the magnitude of stimulation of synthetic activity of the long-day CC-CA during incubation with the NEC extract, the total radioactivity increased in 31 of 34 samples. In four experiments, the maximum increases were 3.6, 3.6, 5.2 and 7.9 times. There was no relationship between the magnitude of stimulation by the NEC extract and the synthetic activity of the long-day CC-CA during incubation without NEC extract (Fig. 6A). However, the short-day CC-CA with relatively low synthetic activity did not respond to the NEC extract.

By contrast to incubations with the NEC extract, the total radioactivity increased during the second incubation only in 9 of 33 control samples. Here the maximum increases in three replicates were only 1.4, 1.5 and 1.7 times. In 24 remaining control samples the radioactivity decreased during the second incubation (Fig. 6B).



In 10 samples analyzed by TLC, synthesis of fractions 8 and 9 was stimulated by the

Fig. 7. Stimulation of synthetic activity of long-day CC-CA by NEC extract; total radioactivity vs. radioactivity in TLC sections 8 and 9. Ratio between radioactivity after incubation with (b) and without (a) NEC extract; x axis – total radioactivity, y axis – radioactivity in sections 8 (solid) and 9 (open). Circle – NEC extract from short-day insects. Triangle – NEC extract from long-day insects.

NEC extract 8.2 and 5.5 times on average, respectively, while the increase of total radioactivity was only 4.1 times on average (Fig. 7).

DISCUSSION

Allatostimulating effect of the NEC extract in vitro versus allatoinhibiting activity within the NEC in vivo

Earlier experiments with *P. apterus* demonstrated that the short-day brain inhibited the activity of the CA in vivo (Hodková, 1976). The inhibiting activity of the brain was maintained in transplanted neuroendocrine complexes (Hodková, 1977, 1992). However, in the present study, instead of the expected inhibitory activity, stimulation resulted from treatment of long-day CA with NEC extracts in vitro. A stimulating factor (or factors) is present in extracts from both short-day and long-day adults. Thus a stimulating factor in the internal milieu of feeding females (Hodková, 1992) may originate in the brain.

It is possible that both stimulating and inhibiting factors are present in NEC extracts, but the response of the long-day CA to stimulating factors may be stronger. The short-day CA are neither stimulated nor inhibited by the NEC extract. It seems that some processes stimulated by long-day conditions, e.g. synthesis of receptors for allatotropins, are prerequisite for stimulation of the CA. These receptors may be missing in the short-day CA. Alternatively, stimulation of the CA may be prevented by a high level of allatoinhibiting factors within the CA or the CC. A possible inhibiting effect of the NEC extract may not be revealed because of the low spontaneous (without NEC extract) activity of the short-day CA. Indeed, extract of NEC from short-day *P. apterus* clearly inhibited the CA from another heteropteran species, *Plautia stali*, which had a high spontaneous activity (T. Kotaki, T. Okuda, M. Hodková, unpubl.).

An allatostimulating factor is released to the internal milieu of both short-day and long-day feeding females of *P. apterus* in vivo; although the CA of starving long-day females is inhibited by the brain, this inhibition within transplanted neuroendocrine complexes is overcome in feeding recipients irrespective of their photoperiodic conditions (Hodková, 1992). However, the inhibition of CA within the short-day neuroendocrine complexes cannot be overcome in feeding recipients (Hodková, 1982). Different responses of short-day and long-day neuroendocrine complexes to the internal milieu in vivo, and different responses of short-day and long-day CC-CA to the NEC extract in vitro, may both be due to strong inhibition of the short-day CA.

Nature of the allatostimulating factor

So far, the primary structure of one allatotrophic peptide in pharate adults of *Manduca sexta* has been published (Kataoka et al., 1989). Allatotrophic peptides with molecular weights of 2kDa (Ferenz & Diehl, 1983) and between 0.7 and 1.5 kDa (Gadot et al., 1987) were reported in *Locusta migratoria migratorioides*. The allatotrophic factor in *Gryllus bimaculatus* also appears to be a peptide (Hoffmann et al., 1994). In larvae of *Galleria mellonella*, the allatotrophic factor was reported to be a polypeptide with molecular weight of 20 kDa (Bogus & Scheller, 1994). Lafon-Cazal & Baehr (1988) described an octopaminergic stimulation of the CA in *L. migratoria migratorioides*.

The allatostimulating activity of the NEC extract in a heteropteran species is reported here for the first time. The activity is not lost after removing of proteins by centrifugal

ultrafiltration but it is destroyed by treatment with proteinase K. Therefore the allatostimulating factor of *P. apterus* appears to be a peptide. Further studies on the isolation and identification of this factor are in progress.

Nature of the juvenile hormone

The chemical nature of heteropteran juvenile hormones is far from clear. JH III was identified in the culture media of the CA of *Oncopeltus fasciatus*, *Dysdercus fasciatus* and *Nezara viridula* (Bowers et al., 1983). However, no significant levels of known JHs were detected in adults of *O. fasciatus* (Baker et al., 1988). Numata et al. (1992) reported that JH I is the principal JH in the hemolymph of *Riptortus clavatus*. However, TLC analysis of the culture media of the CA from four heteropteran species, *Plautia stali*, *Halyomorpha mista*, *N. viridula* and *R. clavatus* revealed no or little products which co-migrated with JH I, JH II or JH III, showing Rf-values of 0.6–0.7. The major peak of radioactivity had an Rf-value of 0.5 (Kotaki, 1993). This material corresponds to the TLC section 8 in the present experiments. A distinct peak of radioactivity in section 8 was observed only exceptionally after incubation of the CC-CA without NEC extract. However, the radioactivity greatly increased in sections 8–9 after incubation of the CA with the NEC extract. Thus it seems that, in *P. apterus*, the NEC extract stimulated synthesis of the substance which was detected as a major synthetic product of the CA in other four heteropteran species. Recent findings indicate that this product has juvenile hormone-like biological activity (Kotaki, 1996).

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