Chapter 4 Juvenile hormone in heteropteran insects

In this chapter, I will focus on the identity of JH in heteropteran insects. Thus far, seven forms (homologues) of JH have been identified from non-heteropteran insects (Fig. 14). JH I is first identified from a moth, *Hyalophora cecropia* by Röller (1967). In lepidopteran insects, JH II, JH 0, and 4-methyl-JH I are also identified. Among seven forms of JH, JH III is the most widespread and found in not only Lepidoptera but also in other orders. JHB_3 is specific to higher diptera (Richard *et al.*, 1989). Iso-JH II -acid is known as an *in vitro* product by the CA of male adults of *Leucania lorei* (Ho *et al.*, 1995). Methyl farnesoate is an equivalent of JH in crustaceans (Borst *et al.*, 1987; Borst and Tsukimura, 1992).

Although many studies related to the JH have been made with heteropteran insects, the chemical characterization of the JH in this group has not been fully done. JH III was detected in eggs of *O. fasciatus* (Bergot *et al.*, 1981) and in the culture medium of CA obtained from adults of *Dysdercus fasciatus*, *O. fasciatus* and *N. viridula* (Bowers *et al.*, 1983). However, a more recent study by Baker *et al.* (1988) failed to detect any significant levels of JHs or related compounds in adults and eggs of *O. fasciatus*. In *R. clavatus*, JH I was reported to be the main JH homologue in the haemolymph (Numata *et al.*, 1992).

Chapter 3 have shown that diapause is terminated if adults are implanted with CA from reproductively active individuals in *P. c. stali*. Measuring the JH titre is essential to elucidate the hormonal mechanism controlling diapause in this species. Although several techniques have been reported for measuring the amount of JH in

insect samples using a physicochemical method (Gas-chromatograhy/ mass spectrometry) (*e. g.*, Bergot *et al.*, 1981) or radioimmunoassay (*e. g.*, Strambi *et al.*, 1981), these require rigorous and time-consuming purification process or specific antisera against JH that are not commercially available. An alternative method for estimating the JH titre is to determine the rate of JH biosynthesis by the CA *in vitro*, on the assumption that the haemolymph JH titre is positively correlated with the rate of JH biosynthesis (Pratt and Tobe, 1974). This is called radiochemical assay (RCA). No matter which approach to be taken, it is necessary to know which JH homologue occurs in the species in question.

Chapter 3 have also shown that both topical application of synthetic JH III and implantation of CA is effective in terminating diapause. A considerably high dose of JH III is, however, necessary to break adult diapause. In several species of pentatomid bugs, topical application of a powerful JH analogue, methoprene, failed to terminate adult diapause at doses as large as 20μg or more (Tanaka and Kotaki, unpublished data). This may suggest that in these bugs, the sensitivity to those compounds is relatively low. Elucidating chemical identity of the JH in those bugs would provide an explanation for the refractoriness to the synthetic JH and JH analogue.

In this chapter, the CA were cultured in a medium containing radiolabelled precursor and their products were analysed using thin layer chromatography (TLC) according to the method for RCA (Pratt and Tobe, 1974; Ferenz and Kaufner, 1981). For a comparison, not only the CA from *P. c. stali* but also those from three other species of Heteroptera, *H. mista*, *N. viridula* and *Riptortus clavatus,* and from three species belonging to Orthoptera and Diptera, *Locusta migratoria*, *Gryllus bimaculatus*

and *Musca domestica* were incubated and their products were subjected to TLC separation. In the RCA, the radioactivity in the location where a particular JH homologue (usually JH III in non-lepidopteran insects) is supposed to migrate on the TLC plate is measured. In the present study, however, radioactivity in whole range of each TLC lane, from the origin to solvent front, was measured to determine which JH homologue was released by the CA *in vitro*.

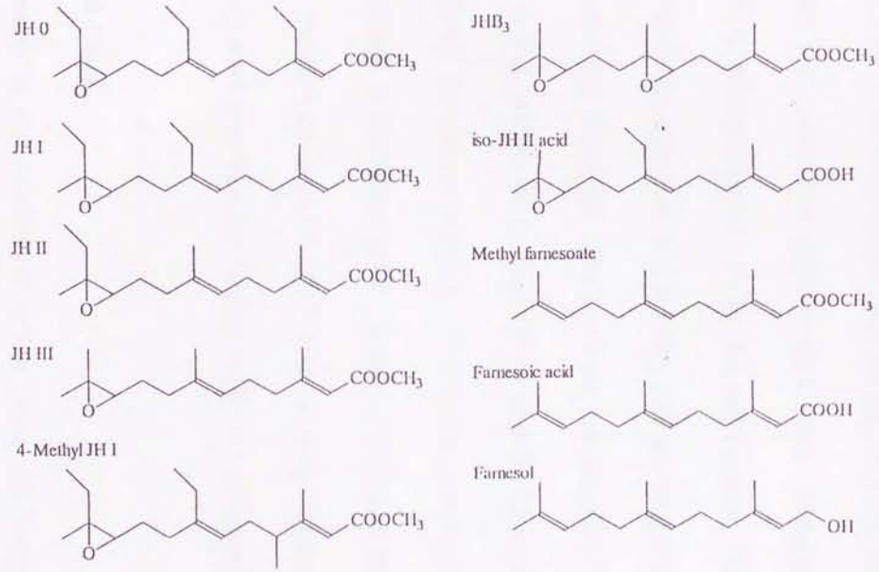

Fig. 14. Homologues of JH, and JH related compounds

Materials and Methods

Insects

Four species of bugs, *P. c. stali*, *H. mista*, *N. viridula* and *R. clavatus* were reared on raw peanuts and dry soybeans in either plastic Petri dishes (9cm diameter, 1.5 cm height) or plastic cups (9 cm diameter, 4.5 cm height) at 25°C and LD 16:8 h. Under such conditions, these bugs reproduce rapidly after adult emergence without entering diapause (Tanaka, 1979; Watanabe *et al.*, 1979; Ali and Ewiess, 1977; Kidokoro, 1978). These species were originally collected in Tsukuba, Ibaraki except for *N. viridula* which was derived from Sadowara, Miyazaki. The methods for handling bugs were similar to those in Chapters 1 and 2. Adults of *L. migratoria* and *G. bimaculatus* were obtained from stock cultures in our laboratory where the former was reared at 30°C and LD 12:12 h according to the method of Hakomori and Tanaka (1992) and the latter at 30 °C and LD 14:10 h by the method of Tanaka (1991) for rearing *Velarifictorus parvus*. *M. domestica* (SRS strain) were provided by National Institute of Health.

CA incubation (RCA)

The method used for CA incubation in this study was basically the same as the one developed for monitoring JH biosynthesis in locusts (Pratt and Tobe, 1974; Ferenz and Kaufner, 1981). A corpus cardiacum-corpus allatum complex (CC-CA) extirpated from adult bugs was incubated in a glass tube (6 mm diameter, 3 cm length) containing 30-50 μl of minimum essential medium (with Hanks' Salts and L-glutamate, without sodium bicarbonate, added 20 mM of HEPES and 5 ppm of Tween 80, adjusted to pH7.2), to which L-[*methyl*-^{3}H]methionine (Amersham) was added to give a final

specific activity of 14.7 MBq/μmol. The principle of RCA is based on the fact that ^{3}H-methyl group of methionine is transferred to methyl ester moiety of JH molecule by the action of *O*-methyl transferase at a fixed ratio (Tobe and Feyereisen, 1983; Schooley and Baker, 1985). Assuming that this holds in heteropteran insects, radiolabelled methionine was used for a tracer in this study. As control incubations, pieces of aorta, fat body, mid gut, flight muscle or the brain were cultured. After incubation at 30°C for 3 h, 60 μl of hexane were added to the medium and the tube was vortexed to extract the biosynthesized products. An aliquot of 30 μl upper hexane phase was collected and applied to a silica TLC plate (10cm x 10cm), Empore® (Analytichem International). Unlabelled standards were applied simultaneously on the TLC plate, which was then developed with a mixture of hexane and ethyl acetate, 1:1, unless otherwise mentioned. As cold standrds, JH I, JH II, JH III (Sigma), the bisepoxide of JH III, methyl 6,7;10,11-bisepoxyfarnesoate (JHB_3) or methyl farnesoate (MF) were used. After TLC development, spots of the cold standards were visualized under a UV light. Cold standard spots were cut out and the remaining part of the plate was cut into sections of 5 mm in length. Each section was put into a plastic vial and its radioactivity was measured using either a liquid scintillation counter (Aloka) after addition of a scintillation cocktail, ACS II® (Amersham).

To examine the effect of precursors of JH III added to the incubation medium on the CA activity, either E,E-farnesol (Aldrich) or farnesoic acid dissolved in ethanol was supplemented to give a final concentration of 100 μM (2 % ethanol). For controls, the CA were incubated in the medium containing 2 % ethanol.

To test the adequacy of the incubation technique and to compare the products by

heteropteran CA with those by the CA of other orders, the CA from reproductively active females of *Locusta migratoria, Gryllus bimaculatus* and *M. demestica* were cultured and hexane extracts of the incubation medium were analysed as mentioned above. In the first two species, JH III has been identified as the major product by CA (Ferenz and Kaufner, 1981; Koch and Hoffmann, 1985) and in *M. domestica*, JHB_3 (Richards *et al.*, 1989). Preliminary experiment showed that the CA from *M. domestica* adults were relatively inactive. To collect more products for TLC analysis, a precursor of JH III, farnesoic acid was added to the medium for the CA of *M. domestica* at a concentration of 100 μM.

To test whether JHs are degraded in the culture medium or not, the CC-CA complexes from *P. c. stali* females was incubated in the medium containing JH III at 30°C for 3 h. ^{3}H-JH III (NEN) and unlabelled JH III were added to the medium to give a final concentration of 16.7 pmol/ml and specific activity of 3.7 MBq/μmol. Hexane extracts of the medium were analysed as described above except that a mixture of CH_2Cl_2: hexane: ethyl acetate, 7:7:1 was used for TLC development.

Synthesis of JHB_3, methyl farnesoate and farnesoic acid

To obtain JHB_3, JH III was oxidized by *m*-perchlorobensoic acid and the oxidized products were separated using TLC and eluted with ethyl acetate. The identity of JHB_3 was checked with a GC/MS system, HP-5972B (Hewlette Packerd) and confirmed by comparing mass-spectra with those for biosynthesized JHB_3 reported by Richard *et al.* (1989). Methyl farnesoate was synthesized from E,E-farnesol by the method of Corey *et al.* (1968). Farnesoic acid was obtained by hydrolysis of methyl

farnesoate.

Results

Products by the CA from adults of four heteropteran species

TLC analysis indicated that CA from reproductively active females of *P. c. stali* released little or virtually no products which co-migrated with the unlabelled standards of JH I, JH II or JH III showing Rf values of 0.7 for JH I and JH II and 0.6 for JH III. However, two major peaks of radioactivity were detected between the origin and the spots of cold standards and their Rf values were 0.3 and 0.5 (Fig. 15A). Similar TLC profiles were obtained for the biosynthesized products by CA from both sexes, although the amounts and ratios of these activities varied with ages (data not shown). None of such substances appeared to be synthesized in control incubations of pieces of the aorta, fat body, mid gut, and flight muscle or brain. When CA from diapausing females were cultured, release of the radiolabel was very low. On the other hand, CA from females which had started oviposition after an extended period of rearing under short-day conditions showed active incorporation (data not shown).

In *N. viridula*, the CA mainly incorporated ^{3}H-methionine into a substance which migrated to the area where one of the major products for *P. c. stali* was found (Fig. 15B). In *H. mista*, on the other hand, the products by CA consisted of at least three peaks (Fig. 15C). One with the smallest Rf value corresponded to that observed for *P. c. stali* and *N. viridula*, and the others behaved like JH III, and JH I or MF, respectively. Using another mixture of CH_2Cl_2: hexane: ethyl acetate, 7:7:1 for development in TLC analysis, it was found that the peak with the largest Rf value was likely to be MF (Rf value: 0.5) rather than JH I (Rf value: 0.4). In *R. clavatus*, a peak was also detected on

TLC plates, but the amount of radioactivity was very low compared with the results from *P. c. stali* and *H. mista* females (Fig. 15D). No significant amount of radioactivity was found in the spots of JH I and JH III. Therefore, these results suggest that main CA product *in vitro* by the CA of four heteropteran species was different from JH I, II, III and MF.

Effects of farnesoic acid and farnesol on biosynthetic activity of CA

Figure 16 indicates that both of JH III precursors, farnesoic acid and farnesol exerted a stimulatory effect on the biosynthetic activity of CA *in vitro*. In the presence of the precursors, a remarkable increase in radiolabelled products was found at an Rf value of about 0.5. The amount of products at an Rf value of 0.3 was smaller in the presence of either farnesoic acid or farnesol than in the control.

Products by the CA from adults of non-heteropteran species

To examine the possibility that the reason why products by stink bug CA did not behave like JHs was artefact, *e.g.*, because of unsuitable sample handling, the CA of non-heteropteran species whose CA products have been already known were incubated in the same way as heteropetran CA. The CA from reproductively active female adults of *L. migratoria* and *G. bimaculatus* produced a radiolabelled substance corresponding to the JH III standard (Fig. 17). In *L. migratoria*, a broad peak of radioactivity between the origin and JH III spot was also detected, and this peak seems to be JH III diol (Gadot et al., 1987) and/or hydroxy JHs (Darrouzet *et al.*, 1997). To test if any of the products by stink bug CA corresponds to JHB_3, the CA of *M. domestica* and *P. c stali* were

incubated at the same time, and their products were applied on the same TLC plate and analysed (Fig. 18). Because of low activity in fly CA, farnesoic acid was added to the incubation medium at a concentration of 100 μM in this experiment. The TLC profile of products by *M. domestica* CA showed several peaks of radioactivity, and two of them were detected at the spot of cold standards of JH III and JHB_3. These results indicated that the method used in this study works well for monitoring CA products. Figure 18 also indicated that products by *P. c stali* corresponded to neither JH III nor JHB_3.

Characteristics of the CA products

To test the possibility that the major peaks of radioactivity revealed by TLC analysis are metabolites of JHs produced during *in vitro* culturing, the CA from *P. c. stali* females were incubated in the medium containing ^{3}H-JH III. TLC resolution of the hexane extract from the incubation medium indicated that little or no conversion of ^{3}H-JH III occurred during the culturing period: almost all radioactivity was recovered from the spot of JH III and only a negligible amount of radioactivity was found at the locations to which the main radiolabelled products of CA migrated (Fig. 19). This showed that JH III added to the incubation medium was not converted to any metabolites during the incubation.

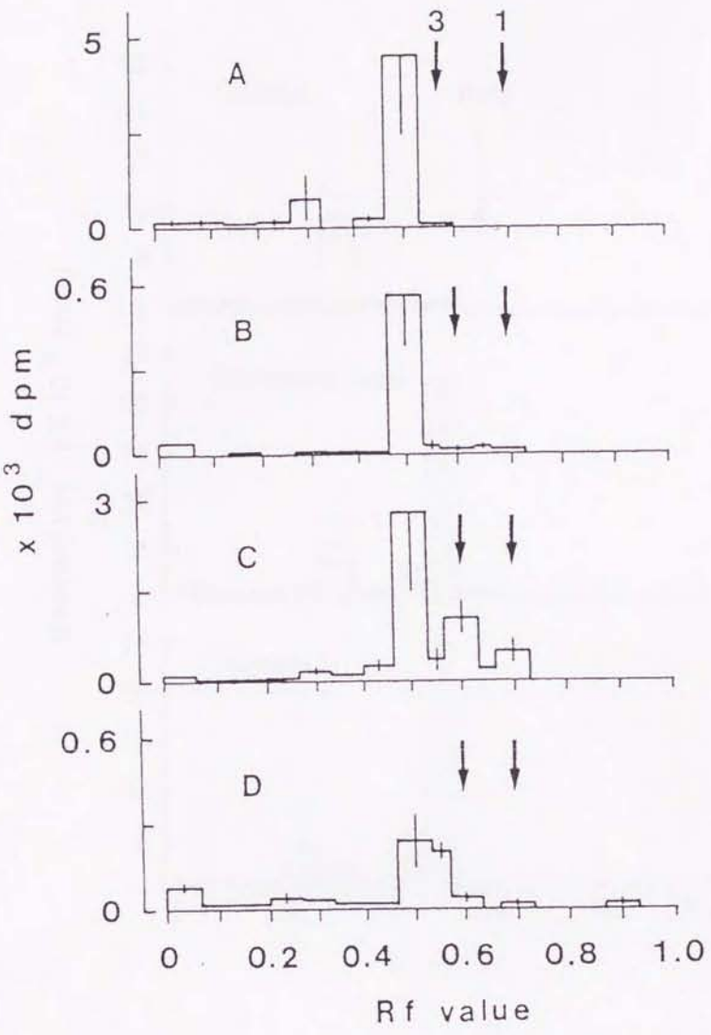

Fig. 15. TLC analysis of hexane-extractable, radiolabelled products biosynthesized *in vitro* by corpora allata from reproductively active day 30 females of *P. c. stali* (A), *N. viridula* (B), *H. mista* (C) and post-diapause females of *R. clavatus* (D). Radioactivities derived from ^{3}H-methionine are indicated as a function of Rf value. Average values of three replicates are shown with SD indicated by vertical bars. Arrows with 1 and 3 show the positions of JH I and JH III spots, respectively.

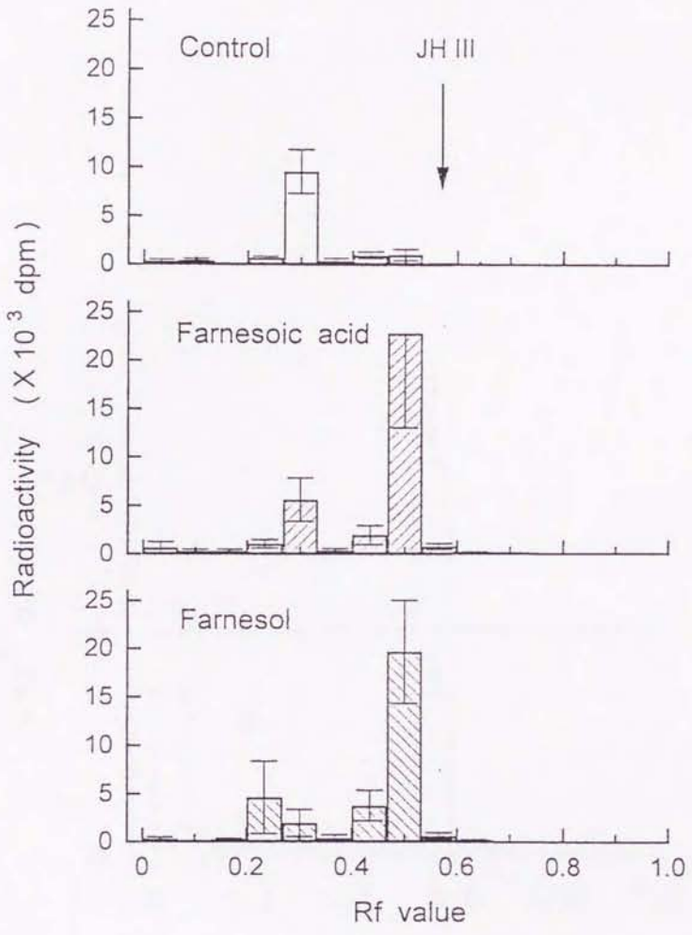

Fig. 16. Effect of farnesoic acid and farnesol on the *in vitro* biosynthetic activity of corpora allata from reproductively active females of *P. c. stali*. Single CC-CA complexes taken from day 10-12 adults reared under 25°C, LD 16:8 h were incubated for 3 h at 30°C in MEM containing ^{3}H-methionine after an addition of 100 μM farnesoic acid (middle), farnesol (lower) or 2 % ethanol (upper) to the medium. Hexane-extracts of the incubation medium were separated by TLC. Radioactivity (average±SD) of each TLC section is indicated as a function of Rf value. Arrow indicates the location where the synthetic standard of JH III migrated. N=5 for each fraction.

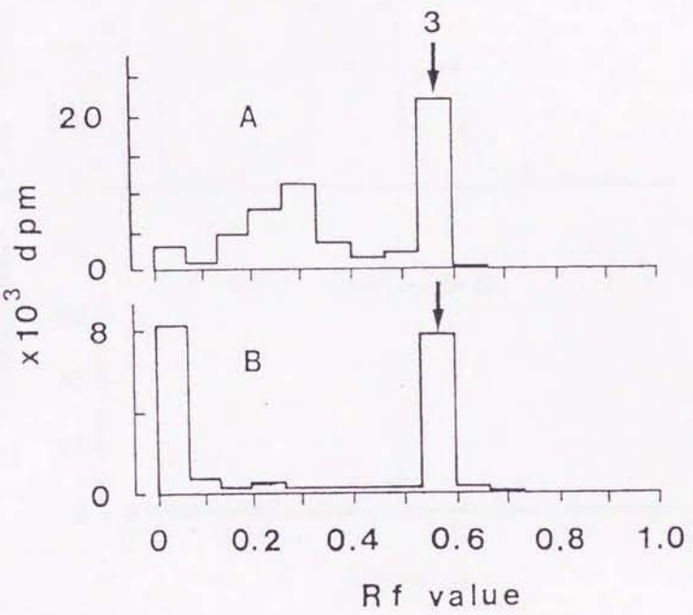

Fig. 17. TLC analysis of hexane-extractable, radiolabelled products biosynthesized *in vitro* by corpora allata from reproductively active females of *L. migratoria* (A) and *G. bimaculatus* (B). Radioactivities derived from ^{3}H-methionine are indicated as a function of Rf value. Arrows with 3 indicate the position of JH III spot.

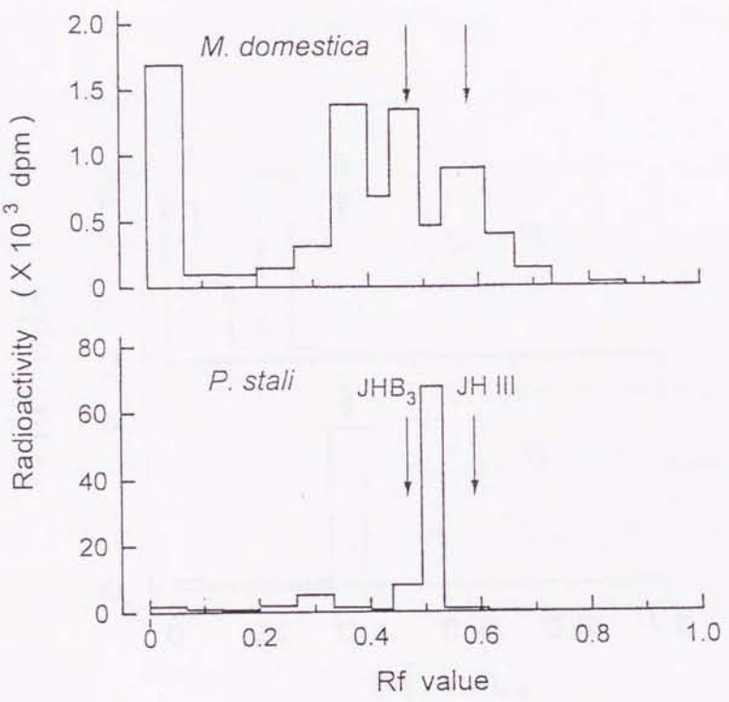

Fig. 18. Comparison of radiolabelled products released *in vitro* by corpora allata of *P. c. stali* and *M. domestica* adults. Groups of 14 and 4 CC-CA complexes from reproductively active adults of *M. domestica* (upper) and *P. c. stali* (lower), respectively, were incubated in MEM containing ^{3}H-methionine and farnesoic acid for 3 h at 30°C. Hexane-extracts of the incubation medium were separated by TLC. Radioactivity of each TLC section is indicated as a function of Rf value. Arrows indicate the locations where synthetic standard of JH III and JHB_3 migrated, respectively.

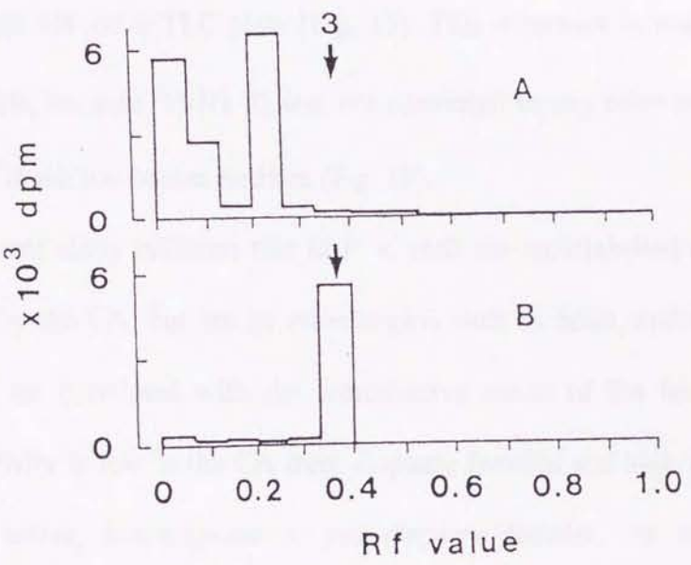

Fig. 19. TLC analysis of hexane extracts of the media containing either ^{3}H-methionine (A) or ^{3}H-JH III (B) where corpora allata from *P. c. stali* females were incubated. Radioactivities derived from ^{3}H-methionine (A) and from ^{3}H-JH III (B) are indicated as a function of Rf value. Arrows with 3 indicate the position of JH III spot. A mixture of CH_2Cl_2: hexane: ethyl acetate, 7:7:1 was used for TLC development. N=3

Discussion

The CA from *L. migratoria* and *G. bimaculatus* females actively biosynthesize JH III and those from *M. domestica* JHB_3 in the present incubation system (Figs. 17 and 18). This is consistent with previous reports by Ferenz and Kaufner (1981), Koch and Hoffmann (1985) and Richards *et al.* (1989), and indicates that the incubation technique used here is adequate for monitoring JH biosynthetic activity. When the same method was applied on four species of bugs, it was found that the CA from reproductively active bugs consistently produced a radiolabelled compound migrating more slowly than JH I, JH II, JH III and MF on a TLC plate (Fig. 15). This substance is not likely to be a metabolite of JHs, because ^{3}H-JH III was not converted to any other substance in the presence of CA in the incubation medium (Fig. 19).

The present study indicates that in *P. c. stali* the radiolabelled compounds are produced only by the CA, but not by other organs such as brain, and the amounts of these products are correlated with the reproductive status of the insects; *i. e.,* the biosynthetic activity is low in the CA from diapause females and high in the CA from reproductively active, non-diapause or post-diapause females. As demonstrated in Chapter 3, the CA plays an important role in control of adult diapause in *P. c. stali*: long-day adults deprived of the CA develop a physiological condition similar to diapause, and diapause is terminated in short-day adults implanted with this gland. Therefore, results obtained in this chapter may suggest the possibility that the CA of *P. c. stali* is responsible for the control of adult diapause and reproduction by producing the substance(s) chemically different from JH I, JH II or JH III.

Precursors of JH often stimulate JH biosynthesis when they are added to the

medium for CA incubation *in vitro* (for reviews, Tobe and Feyereisen, 1983; Feyereisen, 1985). The CA of *P. c. stali* are also stimulated by farnesoic acid and farnesol. The CA of *P. c. stali* have produced at least two radioactive materials *in vitro,* which were found at Rf values of about 0.5 and 0.3, respectively. It seems that the precursors of JH III stimulated the release of only one of the two major products with an Rf value of 0.5. This suggests that the product with an Rf value of 0.5 shares the same carbon skeleton as JH III. The use of radiolabelled methionine as a tracer for monitoring JH biosynthesis is based on the fact that the methyl ester moiety of the JH molecule is derived from methionine by the action of *O*-methyl transferase (for reviews, Tobe and Feyereisen, 1983; Schooley and Baker, 1985). As shown in the present study, the radiolabelled methyl group of methionine was also incorporated into the products by the CA of *P. c. stali*. These results may suggest that CA products have the same sesquiterpenoid skeleton as JH III with a methyl ester moiety and probably unknown polar functional group(s).

In *H. mista*, CA produced additional substances which were found in the areas on a TLC plate corresponding to JH III and MF, respectively. However, it is not possible from the present study alone to conclude that these products are JH III and MF, because different compounds could migrate at similar rates on TLC. The present study failed to confirm the results by Bowers *et al.* (1983) that the CA of *N. viridula* biosynthesize JH III. These authors indicated an additional peak of radioactivity by TLC-autoradiography in *N. viridula* and *O. fasciatus*, and suggested it to be a metabolite of JH. Judging from the migrating behaviour on the TLC plate, this substance might correspond to the unidentified peak found in all bugs tested here. By a physicochemical method Numata *et*

al. (1992) found JH I from the haemolymph of *R. clavatus*, but the present study failed to show that the CA of this and other heteropteran species produce significant amounts of JH I *in vitro*. Thus far, this discrepancy is difficult to explain. Darrouzet *et al.* (1997) have reported that the CA of *L. migratoria* release hydroxylated JH III. In *M. sexta*, Granger *et al.* (1995) have reported the presence of an unidentified product *in vitro* which yields JH III-acid after ester hydrolysis and that this product is not bound to the antiserum against JH III used for radioimmunoassay. Chemical identification of the substances produced by heteropteran CA is necessary for further understanding JH in this insect group.

A test for biological activity as the JH is necessary to determine whether the CA product showing a mobility different from that of known JHs on the TLC plate is heteropteran JH, and this is the subject of the following chapter.

Summary

To identify the JH in heteropteran insects, the CC-CA complexes taken from adults of four heteropteran insects including *P. c. stali*, *Halyomorpha mista, Nezara viridula* and *Riptortus clavatus* were incubated in a medium containing ^{3}H-methionine as a radiolabelled precursor, and extracts of the medium were analysed with thin layer chromatography (TLC). In all species, the CA released radiolabelled products into the incubation medium, but TLC separation indicated that none of them corresponded to JH I, II, III or JHB_3 while the CA taken from adults of *Locusta migratoria, Gryllus bimaculatus* and *Musca domestica* biosynthesized JH III, and JHB_3 for the last species under the same conditions. The biosynthetic activity of the CC-CA complexes taken

from adults of *P. c. stali,* was stimulated by an addition of precursors of JH III, farnesoic acid or farnesol to the incubation medium. This stimulation was mainly attributed to an increase of a product with an Rf value of 0.5 on TLC plates. The substances produced by the CA of *P. c. stali* were unlikely to be metabolites of JH, because ^{3}H-JH III added to the incubation medium was not converted to any metabolite in the presence of CA. These results suggest that the products released by the CA of *P. c. stali* were chemically different from known JHs or metabolites.

Chapter 5 Bioassay for juvenile hormone activity

Chapter 4 has demonstrated that the CA of several species of Heteroptera including *P. c. stali* release products that behave differently from any known JHs on a TLC plate. However, whether these products are JH-active has not yet been determined. This is essential to conclude that the heteropteran CA produce a new JH. Several sensitive bioassay methods such as the *Manduca* black mutant assay (Truman *et al.*, 1973) and *Galleria* wax test (de Wilde *et al.*, 1968) have been developed to monitor JH activity. However, since the aim of this chapter is to examine if CA products is active as a "heteroptera specific" JH, the assay should be done on heteropteran insects themselves. Therefore, in this chapter, an assay system for JH-activity was established, in which samples are topically applied to last instar nymphs of *P. c. stali* and alterations in the morphology of test animals are assessed after the following ecdysis. By this assay, the main radiolabelled product found in the previous chapter was indicated to have biological activity as JH in *P. c. stali*.

Materials and Methods

Nymphs and adults of *P. c. stali* were obtained from the stock culture described in Chapter 2. To develop an assay for JH activity, day 0 or 1 last instar nymphs were implanted with two CC-CA complexes taken from reproductively active female adults. For sham operations, nymphs were implanted with two pieces of the aorta. After the following ecdysis, the lengths of forewing and scutellum were measured with an ocular

micrometer under a stereo microscope. The lengths were divided by the width of the pronotum to compensate for differences in body size between sexes and among individuals on the assumption that the dimensions of the body were the same in the two sexes. Results obtained from nymphs treated on day 0 and those from nymphs treated on day 1 were combined because these results were not significantly different from each other (ANOVA). Other morphological traits such as the number of antennal segments and colour of abdominal terga were also recorded. The effects of synthetic JH III (Sigma), JHB_3 (synthesized as described in Chapter 4), methoprene (provided by Earth Pharmaceutical Co.) and pyriproxyfen (provided by Sumitomo Chemical Co.) on the above morphological traits were examined similarly, but only day 0 last instar nymphs were used in this test. An aliquot of 1 μl acetone or hexane solution of these chemicals was topically applied to the dorsal side of their abdomen with a 10 μl-microsyringe. To examine the JH activity of CA products, a total of 120 CC-CA complexes were extirpated from reproductively active adults. A group of 15-30 CC-CA complexes were cultured in 30-50 μl of MEM supplemented with farnesoic acid (100μM; synthesized as described in Chapter 4) for 3-6 h. After three extractions with hexane (40 μl each), pooled hexane-extracts of the incubation medium were concentrated under a slow nitrogen stream to 20 μl, containing the amount of products from 6 CA per μl solution. An aliquot of 1 μl of this and serially diluted extracts with hexane were applied to day 0 last instar nymphs. To determine the fraction(s) with JH activity, a total of 90 CC-CA complexes were cultured as above and the pooled hexane-extracts of the incubation medium were subjected to TLC separation in the same way as described in Chapter 4. The TLC plate was cut into 5 pieces (1 cm long) and

each was eluted with ethyl acetate. Each eluted fraction was then dissolved in hexane and applied to day 0 last instar nymphs (1 μl per nymph).

Results

Effects of CA implantation, JHs and JH analogues on metamorphosis

To examine morphological traits of bugs of which metamorphosis was inhibited by exogenous JH, two CC-CA complexes taken from reproductively active adults were implanted into last instar nymphs. The operated nymphs moulted to intermediates between the nymph and adult, judging from the size, structure and colouration of the forewing and scutellum (Figs. 20 and 21). The relative lengths of forewing and scutellum of the operated individuals were significantly different from those of fifth instar nymphs and sham-operated adults ($p<0.01$, ANOVA). Normal nymphs had four antennal segments (the basal segment plus three flagella; Fig. 20D) and adults 5 (the basal segment plus four flagella; Fig. 20E). In 10 out of 12 such intermediates, the antennae consisted of 4 segments (Fig. 20F). One of the remaining two intermediates had antennae of the adult type, and the other had one antenna of the adult type and the other of the nymphal type. In most intermediates, body colouration and cuticular texture were similar to those of nymphs; the abdominal terga were not melanized and the pronotum and scutellum did not develop green colour (Fig. 20). Topical applications of synthetic JH III, JHB_3 and pyriproxyfen exerted inhibitory effects on metamorphosis in a dose dependent manner (Figs. 21). The greater the doses applied, the shorter the lengths of forewing and scutellum after the following ecdysis. The percentage of individuals with nymphal type antennae also increased (Fig. 22), and body colouration

and cuticular texture were progressively more juvenilized with increasing doses (data not shown). Small doses produced some normal looking adults, except for unmelanized terga at the site where the chemical was applied. Pyriproxyfen appeared to be the most potent of the four chemicals tested. A dose of 0.1 μg of pyriproxyfen reduced the lengths of forewing and scutellum significantly ($p<0.01$, Tukey HSD test) compared with those of solvent-treated adults, while JH III and JHB_3 induced significant reduction in those lengths at a dose of 10 μg ($p<0.01$, Tukey HSD test). Methoprene did not affect metamorphosis of the bugs even at a high dose of 50 μg; nymphs moulted to apparently normal adults with melanized terga and fully developed forewings, scutellum and antennae. Relative lengths of forewing and scutellum of methoprene-treated bugs were not significantly different from those of solvent-treated controls (ANOVA). These results showed that JH activity could be assayed by measuring the length of forewing and scutellum and the number of antennal segments, as well as looking at cuticular texture.

JH activity of CA products

Last instar nymphs treated with hexane-extracts of the products from farnesoic acid-stimulated CA of *P. c. stali* moulted, at the highest dose (6 individuals equivalents of CA products), into intermediates with significantly reduced forewings and scutellum ($p<0.01$, Tukey HSD test). Bugs treated with CA products at a dose of 0.6 individual equivalent of CA products moulted into apparently normal adults except for their antennae; forewings and scutellum developed normally and terga were melanized as observed in untreated or solvent treated controls. However, 75 % of those individuals

had the nymphal type antennae (Fig. 23). At a dose of 0.06 individual equivalents, juvenilizing effects were no longer evident. Topical applications of farnesoic acid or extracts from the control medium containing farnesoic acid, but no CA, did not affect normal metamorphosis (data not shown).

Using the above method, fractions of the hexane-extracts of the CA products separated by TLC were tested for JH activity. Fig. 24 shows that the JH activity, as determined by morphological traits after the final ecdysis, was detected only in bugs treated with the fraction with Rf values of 0.40-0.53. Those bugs had significantly shorter forewings and scutellum than did bugs applied with other fractions ($p<0.01$, Tukey HSD test). The fraction with Rf values of 0.40-0.53 contained the major CA product in the presence of farnesoic acid. No JH activity was detected from the fraction with Rf values of 0.27-0.40, corresponding to the submajor product, nor with Rf values of 0.53-0.66 where synthetic JH I and JH III standards, if applied, should be found. In another series of bioassays, four fractions with Rf values of 0.39-0.44, 0.44-0.48, 0.48-0.52 and 0.52-0.59, respectively, were tested. The first and last fractions corresponded to the spots of synthetic JHB_3 and JH III standards, respectively, which were applied as lateral markers on the TLC plate. Only the third fraction with Rf values of 0.48-0.52 showed JH activity; four out of five bugs treated with this fraction emerged as adults with nymphal type antennae, while those treated with the other fractions (N=5 for each) moulted to apparently normal adults with adult type antennae. These results indicated that the *in vitro* products by the CA of *P. c. stali* was JH-active and, after TLC separation, the activity was detected only a range of Rf values to which main CA products migrated.

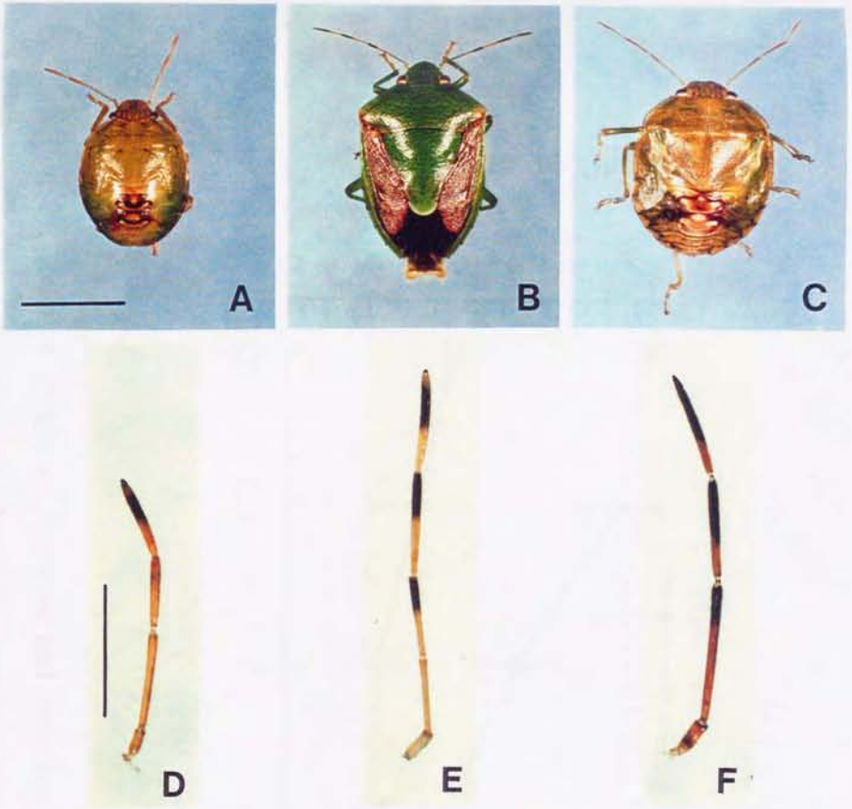

Fig. 20. Effect of implantation of CC-CA complexes on the metamorphosis of last instar nymphs of *P. c. stali*. Implantation of two CC-CA complexes taken from reproductively active females into a last instar nymph (A) induced a nymph-adult intermediate (C) while sham operation, i.e. implantation of pieces of aorta, produced a normal adult (B). Scale bar for A-C: 5 mm. The number of antennal segments was 4 (D) in normal last instar nymphs and 5 (E) in normal adults. In most bugs implanted with CC-CA complexes it was 4 (F). Scale bar for D-F: 2 mm.

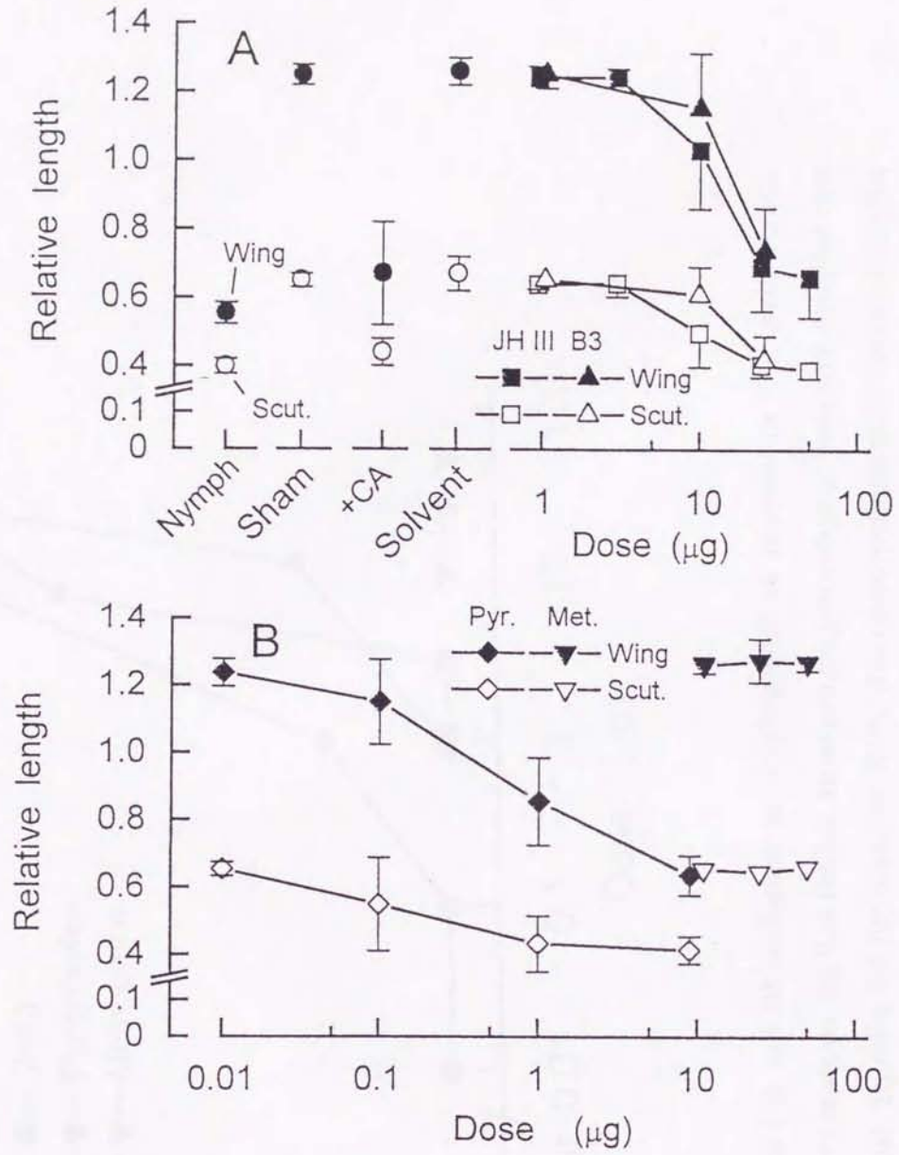

Fig. 21. Relative lengths of forewing and scutellum of bugs moulting after implantation of CC-CA complexes taken from reproductively active females or topical application of synthetic JHs or JH analogues at the beginning of the last instar. Closed and open symbols with vertical bars indicate the average±SD of forewing and scutellum lengths relative to pronotum width, respectively. Nymph, last instar nymphs; Sham, sham-operated control; +CA, CC-CA implanted; Solvent, solvent control. Squares, JH III; triangles, JHB_3; diamonds, pyriproxyfen; inverse triangles, methoprene. Each point is based on at least 10 individuals except for methoprene treatment where N=6.

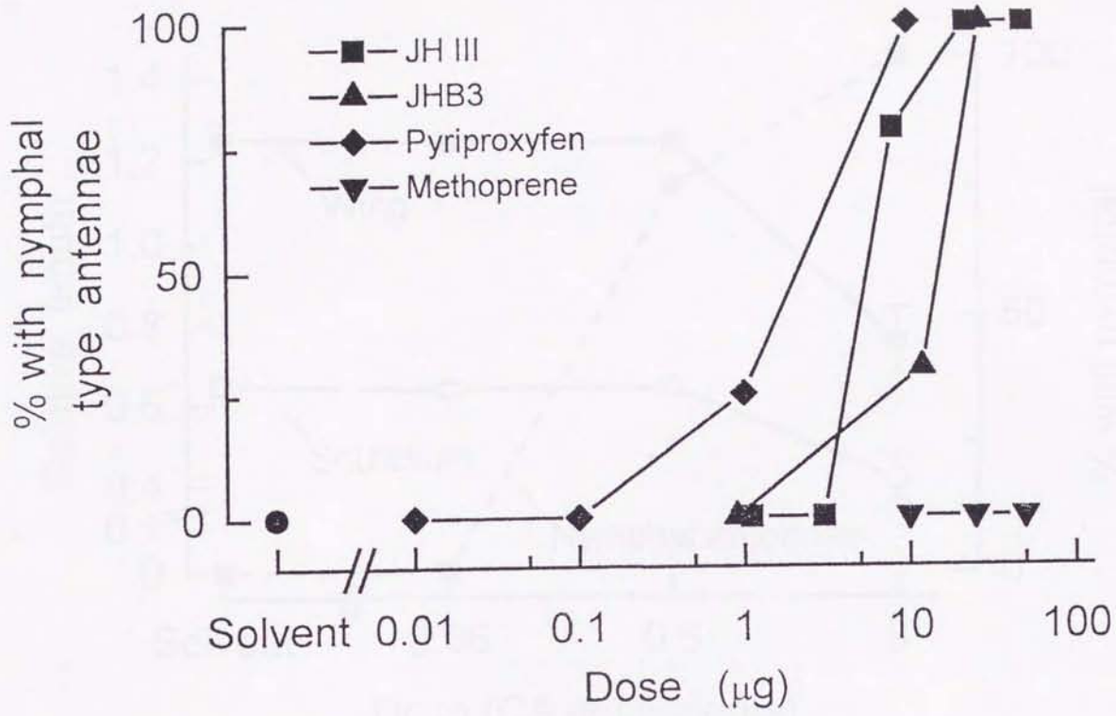

Fig. 22. Effect of synthetic JHs and JH analogues on morphogenesis of antennae in *P. c. stali*. Topical applications of these chemicals were made on day 0 of the last instar and the percentage of bugs with nymphal type (4-segmented) antennae is indicated. Squares, JH III; triangles, JHB_3; diamonds, pyriproxyfen; inverse triangles, methoprene. Each point is based on at least 10 individuals except for methoprene treatment where N=6.

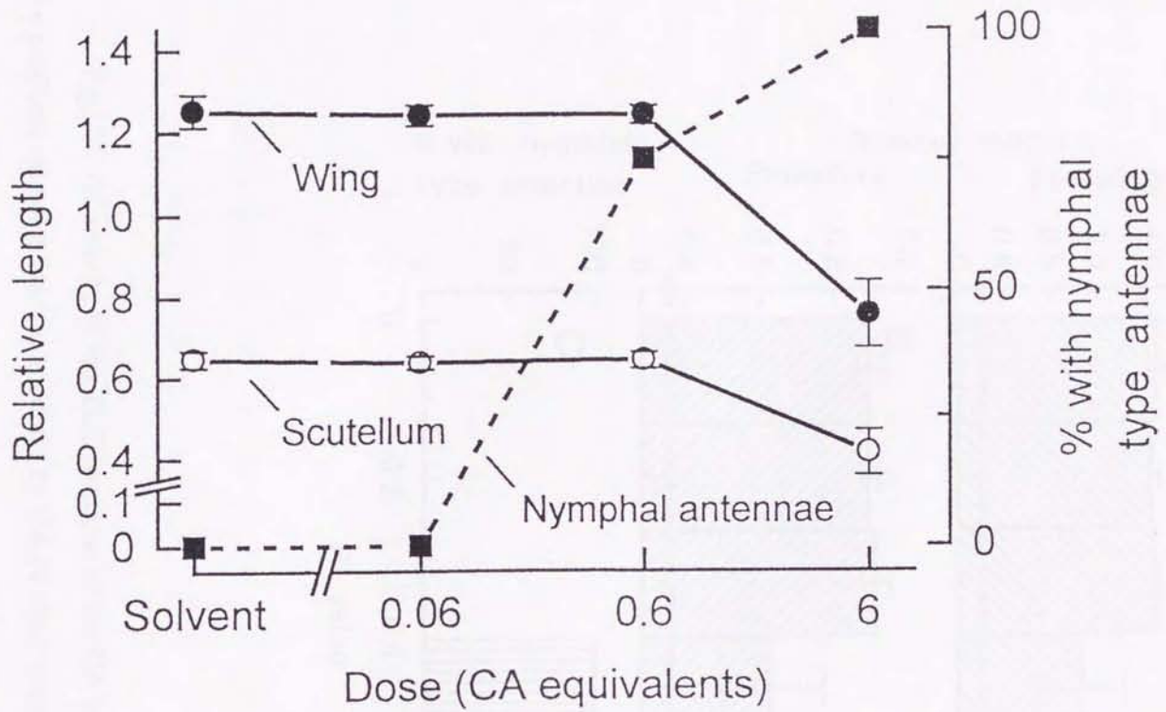

Fig. 23. JH activity of the products released into the incubation medium by CA of *P.* c. *stali* adults. The products released into the medium by CA *in vitro* were extracted with hexane. The serially diluted products were applied to last instar nymphs and the morphology of these bugs was examined after the following ecdysis. Closed and open symbols with vertical bars indicate the average±SD of forewing and scutellum lengths relative to pronotum width, respectively. Squares with broken line indicate the percentage of bugs with nymphal type (4-segmented) antennae. Each point is based on at least 11 individuals.

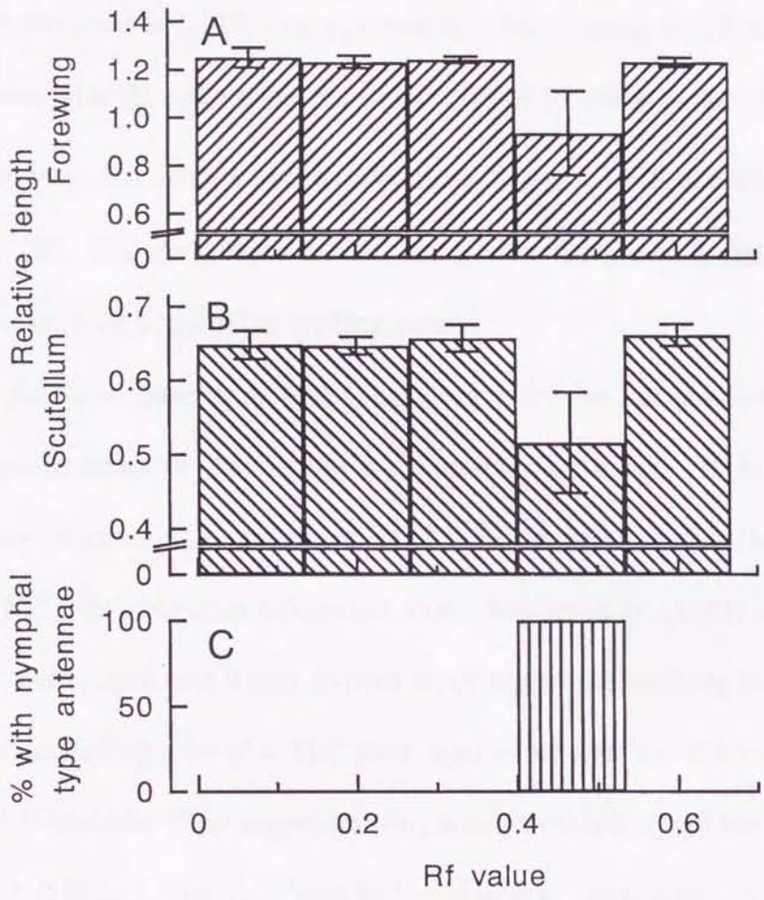

Fig. 24. JH activity of the TLC fractions of the CA products of *P.* c. *stali* adults. The products released into the medium by CA *in vitro* were extracted with hexane and subjected to TLC. Fractions separated were eluted with ethyl acetate and applied as hexane solutions to day 0 last instar nymphs. Relative forewing (A) and scutellum (B) lengths, and the percentage of bugs with nymphal type (4-segmented) antennae (C) were determined after the following ecdysis. At least 9 nymphs were tested for each fraction. Each histogram with vertical bar in A and B shows the average±SD.

Discussion

In Chapter 4, it is demonstrated that the CA of four heteropteran species released a common major product that behaved differently from JH I, II, III, JHB_3 or an unepoxidized precursor of JH III, methyl farnesoate. The bioassay for JH activity shows that the fraction with JH activity has the same range of Rf value as that for the major CA product *in vitro* and that the fractions corresponding to JH I-III or JHB_3 have no JH activity (Fig. 24). Therefore, these results, along with the previous results, strongly suggest the presence of a new JH in the Heteroptera.

In *O. fasciatus*, Baker *et al.* (1988) have suggested that one possible explanation for their failure to detect any known JHs is the presence of a new JH in this insect. Although they reported the production of JH III in heteropterans based on the incorporation of radiolabel from methionine alone, Bowers *et al.* (1983) also observed an additional radioactive spot which showed much higher radioactivity than the JH III spot on their autoradiography of a TLC plate used to separate the CA products of *O. fasciatus* and *N. viridula*. They argued that this was a 'metabolite', but the results given in Chapter 4 indicated that this 'metabolite' seems to correspond to the product commonly found among all these species. It is in this fraction that the bioassay in the present study demonstrated the JH activity in *P. c. stali*.

As discussed in Chapter 4, precursors of JH often stimulate JH biosynthesis when they are added to the medium for CA incubation *in vitro* (for reviews, Tobe and Feyereisen, 1983; Feyereisen, 1985). The CA of *P. c. stali* are also stimulated by farnesoic acid and farnesol. They produced at least two radioactive materials *in vitro,* which were found at Rf values of about 0.5 and 0.3, respectively. It seemed that the

precursors of JH III stimulated the release of only one of the two major products, the JH active products with an Rf value of 0.5. From the present study alone, it is difficult to tell if the CA product at an Rf value of 0.5 is the only product with JH activity; the possibility that the product of the CA with an Rf value of 0.3 is also JH-active can not be ruled out. A further quantitative analysis of JH activity for these products may be necessary.

The JH-active fraction with an Rf value of 0.5 may have the same sesquiterpenoid skeleton as JH III with a methyl ester moiety and polar functional group(s), as discussed in Chapter 4. In other words, the JH in stink bug may be structurally similar to JH III. This is supported by the fact that both synthetic JH III and JHB_3 show biological activity as JH in *P. c. stali*. Chemical identification of the products of heteropteran CA is essential to test the possibility that the product with an Rf value of 0.5 represents the heteropteran JH.

The present bioassay for JH activity uses three morphological traits because they are relatively easy to observe and quantify. When 0.6 individual equivalent of CA products of *P. c. stali* was tested on this bioassay, some of the tested bugs moulted to adults with normal forewings and scutellum, but some of them had 4-segmented antennae, a characteristic of nymphs (Fig. 23). This implies that the antennae are more sensitive to the CA products than the other two organs. After topical application of JH III, JHB_3 and pyriproxyfen, bugs with 4-segmented antennae always occurred among those individuals with reduced forewings and scutellum. In *Bombyx mori*, the commitment time for metamorphosis in last instar larvae varies among different tissues (Ohtaki *et al.*, 1986). Such differences in sensitivity to JH analogue or JHs, and/or

differences in rate of break down between the CA product and JH III, JHB_3 or pyriproxyfen may explain the above phenomenon.

Methoprene was found to have no JH activity in *P. c. stali,* even at a high dose of 50 μg (Figs. 21 and 22). This is consistent with the fact that topically applied methoprene failed to terminate adult diapause in several pentatomid bugs including *P. c. stali* even though JH III was effective (Kotaki and Tanaka, unpublished). Methoprene is known as a potent JH analogue in a variety of insect species including heteropterans such as *R. prolixus* (Nijhout, 1983), *O. fasciatus* (Smith and Nijhout, 1981), *R. clavatus* (Numata and Hidaka, 1984) and *Jadera aeola* (Tanaka *et al.*, 1987). Since all these species do not belong to Pentatomidae, the refractoriness to methoprene may indicate a unique hormonal regulation in this family.

The present study has assessed the JH activity of CA products in terms of inhibition of metamorphosis. Since the CA used here were taken from adults, it will be important to determine if the same active fraction of CA products also functions in the adult stage as a gonadotropic or diapause-terminating hormone in *P. c. stali*.

Summary

A bioassay system was established using last instar nymphs of *P. c. stali* to test the JH activity of the CA products: last instar nymphs either implanted with the CC-CA complexes taken from reproductively active adults or topically applied with synthetic JHs or JH analogues were reared until the following ecdysis, and their forewing and scutellum lengths relative to pronotum width were recorded as well as the number of antennal segments. After CA implantations, bugs developed reduced forewings and

scutellum, and 4-segmented antennae, characteristics of nymphs, instead of the 5-segmented, adult type antennae. JH applications showed similar juvenilizing effects in a dose dependent manner. This assay system revealed that the JH activity of the CA products was limited to the TLC fraction at an Rf value of 0.5 where the major CA product released in the presence of farnesoic acid or farnesol was found. From these results, it was suggested that the JH produced by the CA of *P. c. stali* is different from any known JHs.