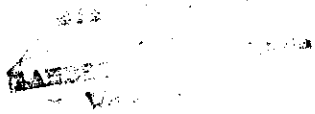
S.J. Kramer

Regulation of the juvenile hormone titre in the Colorado potato beetle

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. H.C. van der Plas,
hoogleraar in de organische scheikunde,
in het openbaar te verdedigen
op vrijdag 14 april 1978
des namiddags te vier uur in de aula
van de Landbouwhogeschool te Wageningen



Stellingen

I

De controle van de activiteit van de corpora allata dient van primair belang te worden geacht in de regulatie van de juveniel hormoon titer in de Coloradokever.

Dit proefschrift.

II

De bewering van Whitmore *et al.* (1972), dat door het juveniel hormoon geïnduceerde carboxylesterasen een belangrijke rol spelen bij de regulatie van de juveniel hormoon titer in *Hyalophora gloveri*, wordt onvoldoende door hun experimenten gesteund.

Whitmore D., Whitmore E., en Gilbert L.I. (1972).
Proc. Nat. Acad. Sci. USA, 69, 1592-1595.

III

De hypothese van Kramer en Childs (1977), dat juveniel hormoon-specifieke dragereiwitten en esterasen een belangrijke fysiologische rol spelen bij de regulatie van de hormoon titer, kan zeker niet betrokken worden op alle insectensoorten.

Kramer K.J. en Childs C.N. (1977).
Insect Biochem., 7, 397-403.

IV

Om de vervroegde rijping van geplukte vruchten en de daarmee gepaard gaande verlaging van de drempelwaarde van ethyleen te verklaren is het niet noodzakelijk om aan te nemen dat een rijping-remmende stof labiel zou zijn.

Sawamura M., Knegt E., en Bruinsma J.
Submitted to Plant and Cell Physiol.
Knegt E., Kramer S.J., en Bruinsma J. (1974).
Colloq. C.N.R.S., 238, 355-358.

V

De opvatting dat de polyedervirussen van insecten de genetische code bevatten voor het eiwit, waaruit de polyeders mede worden opgebouwd, berust op indirecte criteria.

Tinsley T.W. (1977).
In Beltsville Symposia in Agricultural Research: Virology in Agriculture, ed. J.A. Romberger, Abacus Press, Tunbridge Wells, Kent, pp 117-133.

VI

De aanvankelijk grote verwachtingen van de practische toepasbaarheid van juveniel hormoon mimetica als milieuvriendelijke insecticiden in land- en tuinbouw waren meer gebaseerd op fysiologische dan op oecologische overwegingen.

VII

De opvatting dat complexe ecosystemen stabieler zijn dan weinig complexe systemen mag dan wel aantrekkelijk zijn, zij is echter nog nauwelijks geverifieerd.

May R.M. (1976).
In Theoretical Ecology, Principles and Applications, ed. R.M. May,
Blackwell Scientific Publications, Oxford, London, Edinburgh, Melbourne,
pp 142-162.

VIII

Het feit dat een causaal verband tussen de consumptie van bepaalde vetten en het optreden van hartziekten nog niet onomstotelijk is bewezen, impliceert niet dat een overmatig gebruik van vetten de volksgezondheid geen schade zou berokkenen.

IX

In 1977 gaf het Nederlandse volk meer om vuurwerk dan om de natuur.

X

Ook zonder "glaasje op", moeten wij ons meer laten rijden.

Proefschrift van S.J. Kramer
Regulation of the juvenile hormone titre in the Colorado potato beetle
Wageningen, 14 april 1978

Voorwoord

Gaarne wil ik bij het verschijnen van dit proefschrift een ieder bedanken die op enigerlei wijze bij het tot stand komen hebben bijgedragen.
In de eerste plaats wil ik mijn ouders noemen omdat zij mijn studie mogelijk maakten.
Veel dank ben ik verschuldigd aan Prof.Dr. J. de Wilde voor de gelegenheid die hij mij geboden heeft dit onderzoek op het Laboratorium van Entomologie uit te voeren. Zijn grote belangstelling tijdens het onderzoek en zijn opmerkingen bij het doorlezen van de manuscripten worden zeer gewaardeerd.
Stan de Kort, die gedurende het gehele onderzoek de directe supervisie heeft gehad, heeft met zijn grote belangstelling en hulpvaardigheid het onderzoek gestimuleerd. Mijn manuscripten zijn door hem kritisch bekeken en van vele kanttekeningen voorzien.
Veel dank ben ik ook verschuldigd aan Dr. F.J. Oppenoorth voor zijn raadgevingen en discussies.
Margreet Wieten heeft veel bijgedragen aan het tot stand komen van de vierde publikatie. Zij treedt dan ook op als medeauteur.
Helga Privee is mij zeer behulpzaam geweest bij het uitvoeren van alle allatectomie operaties.
Ook mijn collega's Freerk Dortland en Hugo Schooneveld ben ik erkentelijk voor hun kritiek en discussies.
Voor de naar schatting 5000 Colorado kevers, die in de loop van het onderzoek gebruikt zijn, ben ik de heren G. Lubout en Leo Koopman veel dank verschuldigd.
De heren J.W. Brangert, G. Eimers, W.C.T. Middelplaats en F.J.J. Von Planta verzorgden het foto- en tekenwerk.
Ria Cuperus en Rita Schenkhuizen hebben de manuscripten uitgetypt.
De Engelse tekst is, tenzij anders vermeld, gecorrigeerd door de heer J.C. Rigg (Pudoc).
De heer R.J.P. Aalpol (Pudoc) is behulpzaam geweest bij de uitvoering in offset.
De Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek (Z.W.O.) heeft het mij mogelijk gemaakt aan dit projekt te werken en een tweetal buitenlandse reizen te maken.
Als laatsten maar niet onbelangrijksten wil ik mijn vrouw Gea en mijn kinderen Erik en Alexander bedanken voor hun stimulerende invloed en afleiding tijdens het tot stand komen van dit proefschrift.

Contents

General introduction

Hormonal and neuroendocrine control of diapause in the adult Colorado potato beetle, *Leptinotarsa decemlineata*, has been studied for many years. Many workers have studied the juvenile hormone, secreted by the corpora allata (a pair of endocrine glands situated behind the brain).

Diapause in the Colorado potato beetle depends on photoperiod. Beetles reared with a long photoperiod feed and reproduce, whereas short photoperiods induce diapause; the critical photoperiod being about 15 h (de Wilde, 1969). Adult diapause is an adaptive syndrome characterized by immobility, low metabolic rate and failure to reproduce.

There is much evidence that the activity of the corpora allata is controlled indirectly by the photoperiod, via the cerebral neuro-secretory system (de Wilde, 1965; de Wilde and de Boer, 1969; Schooneveld, 1970). In the Colorado potato beetle, titres of juvenile hormone in haemolymph (as assayed by the Galleria wax test) are high under long-day conditions. With short days, an intermediate juvenile hormone peak was found directly after adult emergence, thereafter the titre rapidly decreases. Upon diapause the titre of hormone is below the detection limit of the bioassay (de Wilde *et al.*, 1968; de Wilde *et al.*, 1971). Removal of the corpora allata results in the syndrome of diapause (de Wilde and de Boer, 1961). Probably reproduction, feeding before diapause and diapause are physiologically related to titres of juvenile hormone.

Such studies did not explain how titre of juvenile hormone was regulated. Factors seem to be synthesis and release of juvenile hormone by the corpora allata, and enzymic degradation and protection by juvenile hormone carrier proteins (Akamatsu *et al.*, 1975).

The present study investigated and attempted to measure the factors involved in the titre. The results are presented in six papers. Papers 1 and 4 deal with the enzymic degradation of the hormone *in vitro* and *in vivo*. In paper 2, esterases of juvenile hormone in haemolymph, the main degradative enzymes of the hormone in the Colorado potato beetle, are further characterized. Paper 3 attempts to identify neuro-endocrine factors in esterase activity against juvenile hormone. Paper 5 describes the occurrence and possible role of binding proteins (lipoproteins) in haemolymph. Paper 6 describes corpus allatum activity, as measured *in vitro* by a short-term radio chemical method (Pratt and Tobe, 1974) throughout the adult life span. The general discussion draws together the factors involved in the titre of juvenile hormone in the Colorado potato beetle.

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Tobe S.S. and Pratt G.E. (1975). The synthetic activity and glandular volume of the corpus allatum during ovarian maturation in the desert locust *Schistocerca gregaria*. Life Sci. 17, 417-422.

De Wilde J. (1969). Diapause and seasonal synchronization in the adult Colorado beetle (*Leptinotarsa decemlineata* Say). In: Dormancy and survival. Symp. Soc. exp. Biol. 22, 263-284.

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De Wilde J. and de Boer J.A. (1969). Humoral and nervous pathways in photoperiod induction of diapause in *Leptinotarsa decemlineata*. J. Insect Physiol. 15, 661-675.

De Wilde J., Staal G.B., de Kort C.A.D., de Loof A. and Baard G. (1968). Juvenile hormone titer in the haemolymph as a function of photoperiodic treatment in the adult Colorado beetle (*Leptinotarsa decemlineata* Say). Proc. K. Ned. Akad. Wet. Ser. C. 71, 321-326.

De Wilde J., de Kort C.A.D. and de Loof A. (1971). The significance of juvenile hormone titres. Mitt. schweiz. ent. Ges. 44, 79-85.

Molecular and Cellular Endocrinology 4 (1976) 43–53

AGE-DEPENDENT CHANGES IN JUVENILE HORMONE ESTERASE AND GENERAL CARBOXYESTERASE ACTIVITY IN THE HEMOLYMPH OF THE COLORADO POTATO BEETLE, *LEPTINOTARSA DECEMLINEATA*

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Received 13 June 1975; accepted 15 August 1975

An in vitro method has been developed for the quantitative estimation of juvenile hormone (JH) esterase activity in the hemolymph of the Colorado beetle *Leptinotarsa decemlineata* Say. The apparent K_m value for JH-esterase was determined. JH-esterase and general carboxyesterase activities were estimated throughout the life cycle and under different photoperiodic conditions. High activities were observed in fourth instar larvae and in beetles just before diapause. Lower activities were found in third instar larvae, long-day, diapause and post-diapause beetles. Similar variations were found in general carboxyesterase activity but not in protein concentration. A possible role for the esterase in the regulation of the JH titer is discussed.

Keywords: Colorado beetle; hemolymph; juvenile hormone esterase; carboxyesterase; juvenile hormone titer.

The titer of juvenile hormone (JH) in the hemolymph depends not only on the rates of synthesis and release of hormone (corpus allatum activity) but also on its rate of degradation. The effective titer of JH may not be a reflection of free hormone, but may depend on amounts of JH bound to carrier protein (Kramer et al., 1974). During the life cycle of an insect, dramatic changes occur in the JH titer of the hemolymph. At the end of larval life the JH concentration drops in order to allow metamorphosis (Wigglesworth, 1970; de Wilde et al., 1971). In the adult also JH concentrations are kept at well-defined levels (de Wilde et al., 1968).

There are numerous studies concerned with the state of activity of the corpora allata, but these are mostly based on indirect criteria (see Highnam, 1967). Recently, several investigators have suggested that inactivation mechanisms might be of importance in the control of JH titers (see Gilbert, 1974). Such mechanisms have now been investigated in several insect species. The pathway of JH degradation consists of hydrolysis of the methylester function (carboxyesterase), hydration of the epoxide group (epoxide hydratase), and conjugation of the acid diol as a sulfate for excretion (Ajami and Riddiford, 1971, 1972; Slade and Zibbit, 1971, 1972;

White, 1972; Slade and Wilkinson, 1973, 1974; Erley et al., 1975). In vivo and in vitro studies revealed that esterase attack is one of the main metabolic pathways (Ajami and Riddiford, 1971, 1973).

In the Colorado potato beetle, during adult life, significant changes in JH titer were found, related to the conditions of photoperiod (de Wilde et al., 1968). Under short-day conditions the JH concentration rapidly decreases after adult emergence. This insect is suitable for a study on the regulation of JH titers, because quantitative data of JH concentrations in the hemolymph are available. In order to find out whether inactivation mechanisms play a major role in the control of JH titers, more quantitative data about the rate of JH breakdown are necessary. This paper describes the results of a study concerned with the rate of inactivation of JH by hemolymph esterase at different stages of the life cycle.

MATERIALS AND METHODS

Chemicals

Synthetic juvenile hormone was provided by Hoffmann–La Roche, Basle, Switzerland. The preparation (RO-08-9550) was a mixture of the geometrical isomers of methyl-10-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate. Labelled Cecropia JH([7-ethyl-1,2-^{3}H]JH, 20 Ci/mmole) was purchased from New England Nuclear Corp. The reaction product JH-acid was a gift of Dr. H.W.A. Biessels, Laboratory of Organic Chemistry, Utrecht, The Netherlands. The solutions used for the biuret reaction were obtained from C.F. Boehringer & Soehne, GmbH, Mannheim, G.F.R. α-Naphthylacetate and α-naphthol were purchased from Sigma Chemical Co., St. Louis, Mo., USA. Diazoblue salt was from Edward Gurr, Ltd., London. All the other chemicals and the TLC plates were from E. Merck, Darmstadt, G.F.R.

Insects

Larvae and adults of the Colorado beetle, *Leptinotarsa decemlineata* Say, were obtained from the internal laboratory breeding stock. The beetles were reared at 25°C on fresh potato foliage as described by de Wilde (1957) and de Kort (1969). The adults were exposed to two different photoregimes: long-day (18 h) and short-day (10 h). Larvae were reared under long-day conditions. Post-diapause beetles were obtained by removing the beetles from the soil after being in diapause for 3 months.

Collection of hemolymph

Hemolymph was collected in a capillary pipette from larvae by a dorsal abdominal incision and from adult beetles by clipping the hind legs. The hemolymph was diluted in 0.1 M phosphate buffer pH 7.5 containing 1 mM reduced glutathion, and centrifuged for 10 min at 4000*g*. The diluted hemolymph could either be used

immediately or stored at −20°C, since esterase activity is not affected under these conditions of storage.

Determination of JH-esterase activity

All incubations were carried out in a shaking water bath at 30°C in stoppered incubation tubes. To each tube was added 1.7 nmoles (0.5 μg) [^{3}H] JH (25 Ci/mole) dissolved in 500 μl ethanol. After evaporation of the ethanol, 1 ml 0.1 M phosphate buffer, pH 7.5, was added and thoroughly shaken. The incubations were started by the addition of the hemolymph sample and terminated by addition of 2 ml ethyl acetate. After mixing, the ethyl acetate was removed and the incubation mixture re-extracted with a further 2 ml ethyl acetate. The combined ethyl acetate extracts were reduced to a small volume under a stream of air and spotted on a silica gel thin-layer plate (type 60 F_{254}, layer thickness 0.25 mm). The thin-layer plates were developed in toluol/ethyl acetate/acetic acid (70 : 30 : 1, v/v). Zones 0.5 cm wide were scraped off the plate and transferred to scintillation vials with an automatic zonal scraper (modification of an apparatus designed by Snyder and Kimble, 1965). Standards of C_{18}-JH and C_{18}-JH-acid were used for identification.

After addition of 10 ml scintillation mixture, 1.0% PPO, 0.05% POPOP and 5.0% naphthalene in dioxane/methylcellosolve, 5 : 1 (Bruno and Christian, 1961), the radioactivity was counted with a Nuclear-Chicago Mark I liquid scintillation counter. The counting efficiency was 27–30%. The JH preparation was checked for radiochemical purity. JH-acid formed (μmol/ml hemolymph/h) was calculated from the radioactivity recovered from the area corresponding to the JH-acid region (dpm) and the specific activity of the JH solution added to the incubation medium.

Determination of general carboxyesterase activity

General carboxyesterase activity was measured spectrophotometrically using the method of van Asperen (1962). The substrate solution was 3×10^{-4} M α-naphthyl-acetate in 0.04 M sodium phosphate buffer, pH 7.0. Hemolymph samples (0.1–0.5 μl) were incubated in 1 ml substrate solution for 30 min at 30°C.

Incubations were terminated by addition of 200 μl diazoblue–lauryl sulphate solution. The intensity of the blue color was measured at 600 nm after exactly 10 min.

Protein determination

Protein was determined by the biuret method (Gornall et al., 1948) using bovine serum albumin as a standard. A blank reaction was carried out to correct for the absorbance of hemolymph pigments.

RESULTS

Thin-layer chromatography

As shown in fig. 1 JH-acid and JH are well separated by the method described in

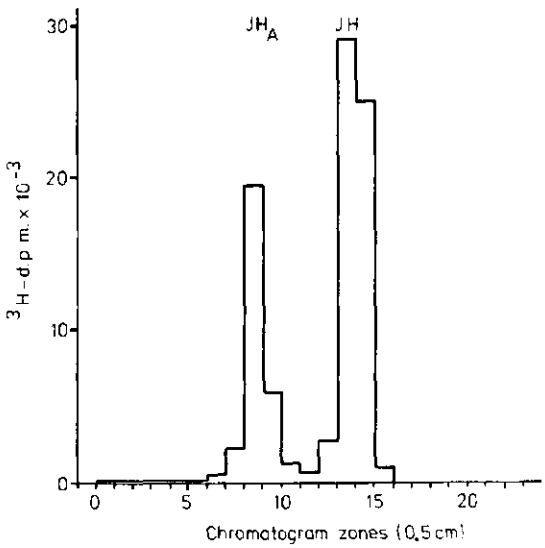

Fig. 1. The distribution of Cecropia JH and the metabolite JH-acid (JH_A) on a silica gel thin-layer chromatography plate. The incubation and the separation were carried out as described in Materials and Methods. A 1 µl hemolymph sample derived from 4-day-old long-day females was incubated for 40 min.

Materials and Methods. About 97% of the radioactivity was recovered from the zone corresponding to JH after zero time incubation. The remaining 3% was distributed over the plate. In all incubations the total JH-recoveries averaged 95%, and were corrected to 100% recovery. Other possible degradative products, diol (R_f = 0.29) and diol-acid (R_f = 0.11) do not account for significant amounts of radioactivity (Ajami and Riddiford, 1973).

Biochemical properties of the hemolymph JH esterase(s)

The results of Weirich et al., (1973) suggested that normal standard techniques could not be applied to study the enzyme kinetics of JH-esterase, because of the hydrophobic nature of the substrate. However, recent results of Kramer et al. (1974) revealed that C_{18}-JH is quite soluble in water. We therefore re-examined this question using hemolymph derived from 4-day-old females, reared under a long-day photoregime, and measured different biochemical properties of JH-esterase. Fig. 2 illustrates that only minute amounts of hemolymph are needed for the estimation of JH-esterase activity and that the enzyme(s) are very efficient in converting JH into JH-acid even when offered sub-optimal substrate concentrations.

Fig. 3 shows the effect of increasing JH concentration on JH hydrolysis. The relationship is a rectangular hyperbole, showing maximum hydrolysis at JH concentrations of 1.7×10^{-6} M. This JH concentration is used in all following experiments.

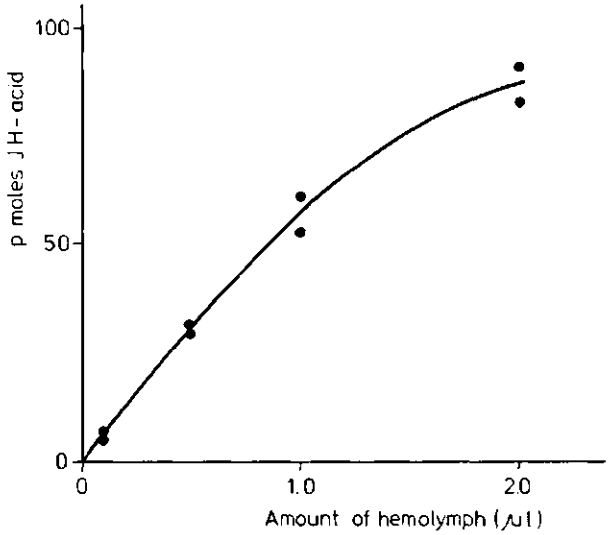

Fig. 2. Effect of hemolymph concentration on JH hydrolysis. 0.1 µg [^{3}H]JH (85,000 dpm) was incubated for 15 min at 30°C with indicated amounts of hemolymph derived from 4-day-old long-day females in 1.0 ml 0.1 M phosphate buffer, pH 7.5.

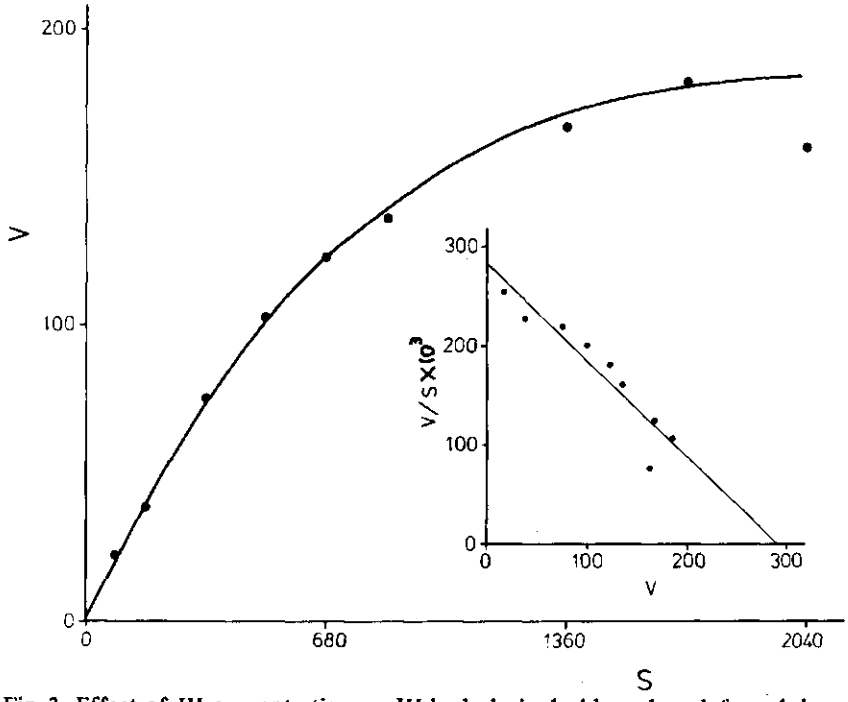

Fig. 3. Effect of JH concentration on JH hydrolysis. 1 µl hemolymph from 4-day-old long-day females was incubated with indicated amounts of [^{3}H]JH in 1.0 ml 0.1 M phosphate buffer, pH 7.5. Substrate concentration (s) expressed in pmoles JH/ml incubation mixture, the rate of hydrolysis (v) in pmoles JH-acid/15 min/1 µl hemolymph. Insert: Eadie and Hofstee plot from v versus s curve.

Fig. 3 also shows that a Eadie and Hofstee transformation of these data resulted in a straight line. From this plot an apparent K_m value of 1.0×10^{-6} M can be calculated. In a similar experiment with hemolymph derived from 8-day-old females reared under short-day conditions an apparent K_m of 0.6×10^{-6} M was found.

Under optimal substrate concentration the rate of hydrolysis is linear with time during the first 15 min and declines thereafter (fig. 4). Under these incubation conditions (substrate concentration 1.7×10^{-6} M and 10-min incubation time) the rate of hydrolysis is directly proportional to the amount of hemolymph, provided that the total hydrolysis does not exceed 15% of the amount of JH present.

JH-esterase activity during the life-cycle

After we had developed the conditions necessary for the quantitative assay of JH-esterase we estimated the JH-esterase activity in hemolymph derived from beetles at different developmental stages. For these experiments both larvae and adults were used. The estimations on hemolymph from adult beetles were carried out separately in males and females. In addition, we compared the activities in beetles reared under long-day and short-day photoregimes, since under these conditions significant differences in hemolymph JH-titers have been shown (de Wilde et al., 1968).

The whole life-cycle experiment was carried out as two independent series. Each point in the life cycle was measured in duplicate, with two different hemolymph concentrations. The results are depicted in fig. 5.

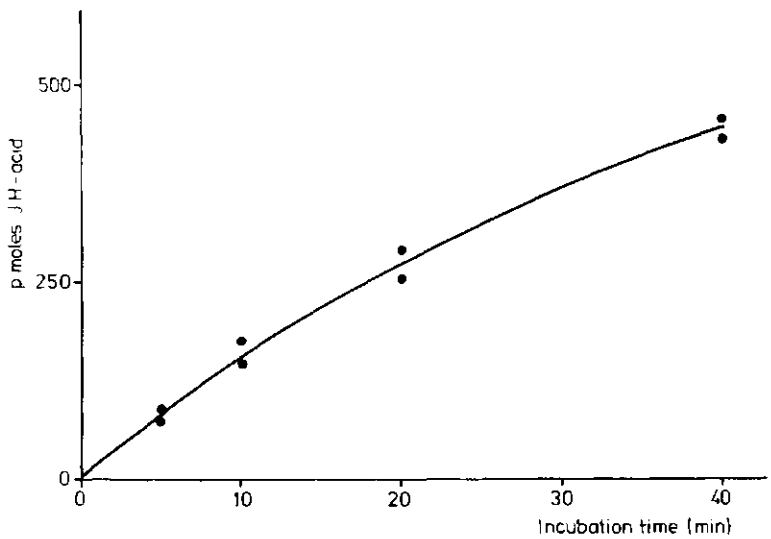

Fig. 4. JH hydrolysis as a function of time. JH-esterase determinations were carried out as described in Materials and Methods. Incubations were terminated at indicated times. Hemolymph concentration: 1 µl (4 long-day females).

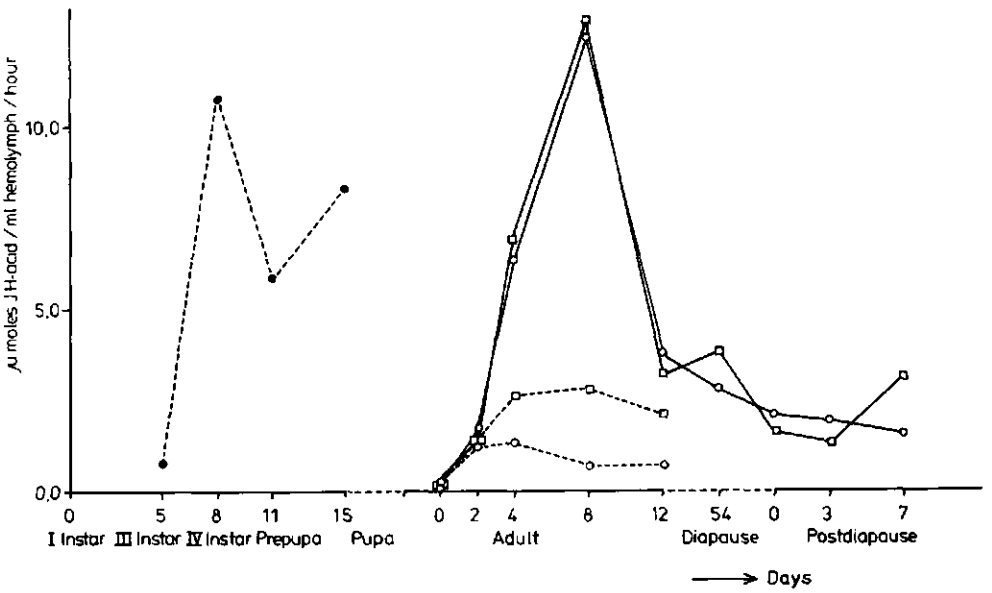

Fig. 5. JH-esterase activity in hemolymph during life cycle of the Colorado beetle. JH determinations were carried out as described in Materials and Methods. Incubation time: 10 min. Each point was estimated in duplicate, using two different hemolymph concentrations. Hemolymph concentration range: 0.05–2.0 µl. Symbols; ------, long-day animals; ———, short-day animals; circles, data obtained from females; squares, data obtained from males.

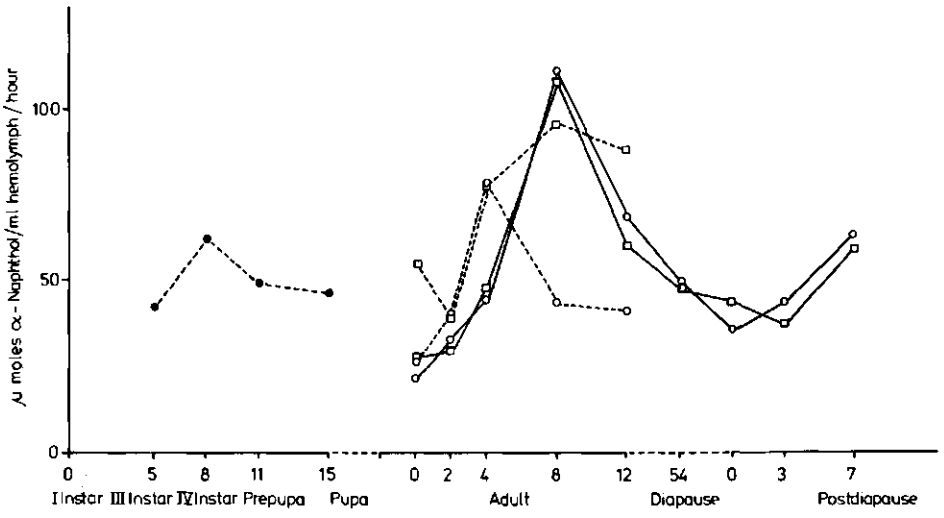

Fig. 6. α-Naphthylacetate esterase activity in hemolymph during life cycle of the Colorado beetle. α-Naphthylacetate esterase activity was measured as described in Materials and Methods. Symbols as indicated in fig. 5.

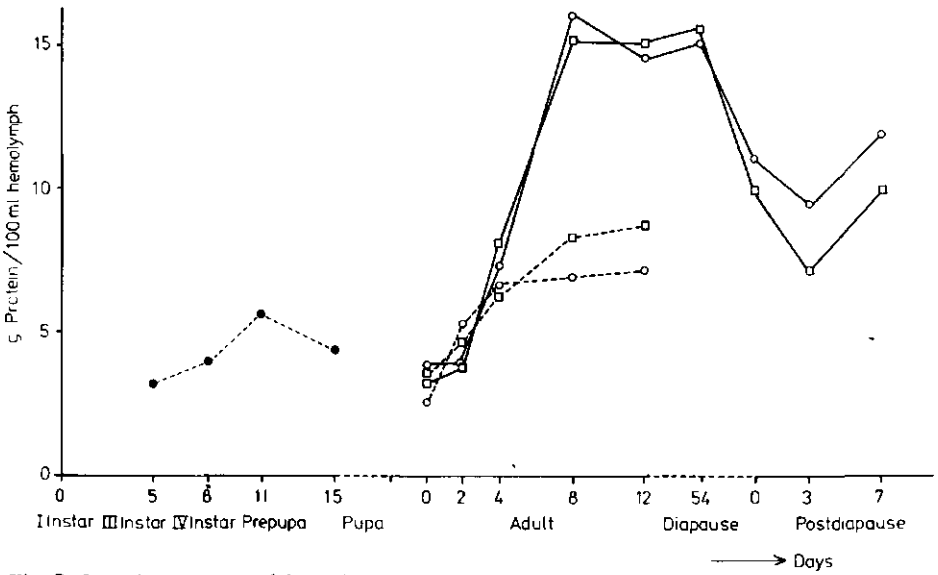

Fig. 7. Protein content of hemolymph during life cycle of the Colorado beetle. Symbols as indicated in fig. 5.

General carboxyesterase activity and protein content during the life cycle

In order to determine whether or not general carboxyesterase activity undergoes changes similar to JH-esterase activity during the life cycle, general carboxyesterase activity was measured in the same hemolymph samples as were used for the JH-esterase determinations.

The total protein content in the hemolymph samples was measured, since significant changes in protein concentration could be expected (de Loof and de Wilde, 1969, 1970). The results are given in figs. 6 and 7, respectively.

DISCUSSION

The results presented describe kinetic properties of the enzymic hydrolysis of JH I and in addition quantitative data on the rate of JH I degradation in the hemolymph of the Colorado beetle at different stages of its life cycle. JH I is the main corpus allatum hormone in the adult Cecropia silkmoth (Röller and Dahm, 1968), but not in other insects. Recent studies by Trautman et al., (1974) showed that JH III occurs in the Colorado potato beetle. The measurement of JH breakdown is limited by the availability of labelled JH. At the present time JH I is the only labelled hormone readily available. Once JH III becomes available, the enzymic degradation of this hormone should also be tested.

Nevertheless, we believe our data are of interest for an understanding of the regulation of JH titers. The great similarity in chemical structure between JH I

(C_{18}) and JH III (C_{16}), together with the similarity in the type of degradation pathway studied, make it very likely that similar kinetics of degradation exist for JH III and JH I, although perhaps absolute values of hydrolysis may differ. JH I and JH III are both biologically active in the Colorado beetle (Bartelink and de Kort, 1975). Moreover, most other studies on JH breakdown also used JH I (Ajami and Riddiford, 1973; Weirich et al., 1973; Erley et al., 1975), which permits direct comparison of results. Only in the very recent study of Sanburg et al. (1975) on JH-specific esterases in the hemolymph of the tobacco hornworm, *Manduca sexta*, has the intrinsic JH (i.e., JH III) been used as a substrate.

Our assay system for determining esterase activity is not basically different from that described by Weirich et al. (1973). The results illustrated in figs. 2–4 show that our method is suitable for accurate and reproducible estimations of JH-esterase activity and that the system behaves according to normal enzyme kinetics. Weirich et al. (1973) did not find normal kinetic properties and suggested that their experimental limitations were due to the hydrophobic nature of the substrate (see also Sanburg et al., 1975). However, recent studies of Kramer et al. (1974) revealed that JH I is soluble in aqueous solutions up to a limit of 5×10^{-5} M. This solubility is high enough to permit determinations of apparent K_m values (fig. 3). The apparent K_m values found were 0.6×10^{-6} M and 1.0×10^{-6} M for 4-day-old long-day and 8-day-old short-day females, respectively. These results are in clear contrast to that of Sanburg et al. (1975) who, using JH III, were not able to determine K_m values. It can be suggested that the affinity of the Colorado beetle esterase enzyme for JH I is much higher than that of *Manduca* for JH III. The failure of Weirich et al. (1973) to demonstrate normal enzyme kinetics for their preparations might be due to the high amounts of hemolymph used in their incubations. This can be inferred from the fact that 40–60% of the JH was hydrolysed within the first 5 min of incubation (Weirich et al., 1973). It is also possible that the use of relatively large (20 μl) volumes of hemolymph may have significantly affected their results because of the presence of large amounts of binding proteins in insect hemolymph.

Our results in fig. 4 exhibit a linear rate of hydrolysis during the first 15 min, provided that not more than 15% of the substrate was hydrolysed. The small differences between the apparent K_m values measured in 4-day-old long-day and 8-day-old short-day females does not exclude the possibility that more than one type of JH-esterase is present in the hemolymph of the Colorado beetle. Investigations into the identity of the JH-esterases are now in progress.

The JH-esterase activity in the hemolymph undergoes striking changes during the life cycle. In the third instar (24 h) the JH-esterase activity is low while it is high in the fourth instar (24 h) and is newly moulted pupae. The high JH-esterase activities in IV instar larvae can be correlated with the steep decline in the JH titer thought to occur during the last larval instar of many insects (see Wigglesworth, 1970). We have made several efforts to estimate the JH-titer in third and fourth instar larvae of the Colorado beetle, but only trace amounts of JH could be detected (unpublished observations).

In the adult, a significant decline in JH titer has been shown in beetles reared under short-day conditions (de Wilde et al., 1968). Also here the sharp decline can be correlated with the increase in JH-esterase activity. At stages where the JH titer is high, i.e., in the third larval instar, the adult under long-day conditions and after termination of diapause, the JH-esterase activity is relatively low. Such correlations might suggest that JH-esterases play a crucial role in the regulation of hormone titers. However, there is no direct evidence to show that high esterase activity is primarily responsible for a low JH titer. Recently Whitmore et al. (1972, 1974) showed that in pupae of *H. gloveri* JH administration causes the appearance of fast-migrating esterases in the hemolymph. They suggest that these carboxyesterases function in degrading JH. Whether or not an induction phenomenon is involved in the regulation of JH titers in the Colorado beetle is at present under investigation.

In the Colorado beetle, de Wilde et al. (1971) showed that under short-day conditions except for a slight activity during the first 4 days of adult life, the corpora allata remain inactive after adult emergence. Also at the end of larval life the corpora allata of several insect species have been shown to become inactive (see Highnam, 1967).

Although most studies on the activity of corpora allata used indirect criteria as a parameter, control of the activity of the corpora allata is still a most likely candidate for the regulation of JH titers. It is likely that by some overall control mechanism the JH-esterase activities fluctuate in conjunction with the changes in activity of the corpora allata. In our opinion the esterases protect the animal against circulating JH at a stage when a low titer of the hormone is required.

In contrast with *Manduca sexta* (Weirich et al., 1973) there is quite a close relationship between the changes in JH-esterase and general carboxyesterase activities in the Colorado beetle. The differences in general carboxyesterase activities between short- and long-day beetles are not so spectacular as differences in JH-esterase activities. However, it is possible that JH-esterase activity comprises a substantial portion of general carboxyesterase activity. There is no correlation between the protein content and the JH-esterase activity of the hemolymph during the life cycle of the Colorado beetle. Still, significant differences in activity remain between short- and long-day beetles if the JH-esterase activity is expressed in μmoles/g protein.

ACKNOWLEDGEMENTS

The authors wish to thank Professor Dr. J. de Wilde for suggesting the problem and for his critical remarks, Professor Dr. F.J. Oppenoorth for valuable advice, Mr. N.W.H. Houx, who developed the thin-layer chromatography technique, and Dr. W. Mordue and Dr. V.J. Brookes for critical reading of the manuscript and correcting the English text. This work was supported by the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

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Life Sciences Vol. 19, pp. 211-218, 1976.
Printed in the U.S.A.

Pergamon Press

SOME PROPERTIES OF HEMOLYMPH ESTERASES FROM LEPTINOTARSA DECEMLINEATA SAY.

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(Received in final form June 3, 1976)

Summary

Hemolymph JH esterases, capable of hydrolyzing C-18 and C-16 juvenile hormones (JH I and JH III respectively), are present in the hemolymph of the Colorado potato beetle Leptinotarsa decemlineata Say. Similar kinetics of degradation were found for JH I and JH III. The apparent Km value for JH III esterase was determined. Two JH analogues, an ethylester without the 10, 11-epoxide group (ZR512) and an isopropylester (ZR515) were not hydrolyzed by hemolymph esterases. JH-esterases were found to be insensitive to 10^{-3}M diisopropylfluorophosphate (DFP), while general carboxy-esterases were totaly inhibited. Triton X-100 (0.1%) appeared to have the opposite effect. An electrophoretic study of the esterases showed that a number of JH-specific esterases exist in the hemolymph. JH-specific hemolymph esterases occur during all the stages tested, but show characteristic changes in activity during development.

In the hemolymph of various insects esterases are present, which are able to hydrolize juvenile hormone (1-4). In the Colorado potato beetle incubation of C_{18} juvenile hormone (JH I) with hemolymph in vitro also produces a high rate of JH breakdown. Significant changes in the activity of these esterases have been reported during the life cycle of the Colorado potato beetle and the tobacco hornworm (4,5).

The exact function of this JH degradation mechanism in the control of JH titers in the hemolymph is not yet clear, although a crucial role of these esterases has been suggested (4). In order to assess the role of esterases in the regulation of JH concentrations in the hemolymph more knowledge concerning two important aspects is necessary. Firstly, it should be established that these esterases play a role in vivo in the regulation of the JH titer. The recent finding that specific carrier proteins occur in the hemolymph suggests that these may protect JH from being inactivated by hemolymph esterases (4,6,7,8,9). Secondly, it is important to know whether the esterases are specific towards JH. In hemolymph of fifth instar larvae of Manduca sexta two families of esterases were distinguished by their reactivity with diisopropylfluorophosphate (DFP) (4). One group consisted of general esterases which were capable of hydrolyzing free JH but not JH complexed to the carrier protein and were completely inhibited by DFP.The other group (JH-specific esterases) were relatively DFP resistant and could also hydrolyze JH bound to the carrier protein.

This paper deals with the further characterization of JH hydrolytic activity in Colorado potato beetle hemolymph using different substrates; the sensitivity of hemolymph esterases towards DFP and Triton X-100 and an electrophoretic study of hemolymph esterases.

Material and Methods

Insects: Larvae and adults of the Colorado potato beetle, Leptinotarsa decemlineata Say were reared as described previously(5). The adults were exposed to two different photoregimes: long day (18 h) treatment results in reproductive animals, short day (10 h) induces diapause 10 days after adult emergence. Manduca sexta larvae were obtained from the Laboratory of Animal Physiology, Wageningen, The Netherlands. The method for collecting hemolymph has been described elsewere (5).

Determination of JH-hydrolytic activity: The method for measuring the hydrolysis of Cecropia C_{18} JH (JH I) has been described previously (5). The breakdown rates of the JH-analogues ZR-512 and ZR-515 were measured identically. C_{16} JH hydrolytic activity was measured by monitoring the release of radioactive methanol (4). The procedure was partially modified to meet optimal conditions. The reaction mixture normally composed 2.0 nmoles ^{3}H-C_{16} JH (20 mCi/nmole) in 950 µl 0.1 M phosphate buffer, pH 7.5 and 50 µl of diluted (10^{2}-10^{3} times) hemolymph. At the end of the incubation period (10 min) the reaction was stopped by the addition of 100 µl of glacial acetic acid. Subsequently, free ^{3}H-JH and JH- acid was adsorbed onto charcoal by the addition of 0.45 mg charcoal suspended in 1.0 ml distilled water. After vigorous shaking and 10 min standing the sample was centrifuged at 5,000 g for 10 min. One ml aliquots of the supernatant were taken for determination of the amount of radioactive methanol by liquid scintillation counting (5). JH hydrolytic activity was expressed in µmoles JH-acid formed/ml hemolymph/hour.

Electrophoresis: Polyacrylamide gel electrophoresis was carried out using a slab gel apparatus (modification of an apparatus designed by Reid and Bieleski (10)). The slabs were made of 7% gels. Routinely, 1-10 µl of hemolymph were placed in each well on the gels. The buffers used were those of Davis (11). Proteins were stained in 0.2% Coomassie Brilliant Blue solution in methanol, water and acetic acid (45:45:10, v/v) for 2 hr at 25°C. The gels were destained by several changes in a solution of methanol, water and acetic acid (5:87.5:7.5, v/v). Esterases were visualized by first incubating the gel in 0.04 M phosphate buffer, pH 7.0 during a few minutes at 25°C. Subsequently the gel was incubated in a reaction mixture containing 78 ml 0.04 M phosphate buffer, pH 7.0 and 12 ml 0.03 M α-naphthylacetate for 5 min at 25°C. The gel was stained with fast blue RR salt (180 mg/90 ml distilled water) during 5-10 min and destained in a solution of methanol, water and acetic acid (45:45:10 v/v).
Esterases were extracted from the gel by grinding slices of the gel in 2 ml cold 0.1 M phosphate buffer, pH 7.5 with a Virtus S 45 mixer. After centrifugation of the slurry at 20,000 for 30 min 0.5 ml of the supernatant was used for the determination of JH-hydrolytic activity.

Chemicals: C-16 JH (methyl trans, trans, cis, 3,7,11-trimethyl-10,11-epoxydodeca-2,6,dienoate) with tritium label in the ester methyl group (20 mCi/mmole) was a generous gift from Dr. J.H.Law, The University of Chicago. The JH-analogues (labelled and unlabelled), 5-^{14}C hydroprene (ZR-512, ethyl, trans, trans, 3,7,11-trimethyl-2,4-dodecadienoate) and 5-^{14}C methoprene (ZR-15, isopropyl, trans, trans-11-methoxy-3,7,11,trimethyl-2,4-dodecadienoate) were kindly provided by Zoecon Corporation, Palo Alto, California. The inhibitor diisopropylfluorophosphate (DFP) was purchased from Sigma Chemical Co., St. Louis, Mo, USA. Triton X-100 and carbowax-20M were obtained from Packard Instrument Company, Inc., USA. The sources of other chemicals have been described previously (5).

Results

In a previous paper only JH I was used as a substrate to determine JH-hydrolysis. In this paper we repeated the enzymic degradation with JH III as substrate, as described in Materials and Methods. Fig. 1. shows the effect of increasing concentration of JH III upon JH hydrolysis. The relationship is a rectangular hyperbole, showing maximum hydrolysis of JH III at concentrations of 2.0×10^{-6}M. Fig. 1 also shows that a Eadie and Hofstee transformation of these data resulted in a straight line. From this plot an apparent Km value of 0.5×10^{-6}M can be calculated.

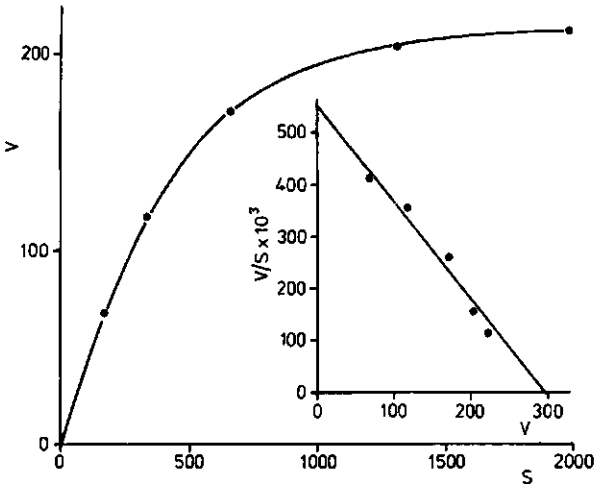

Fig. 1

Effect of JH III concentration on JH hydrolysis.
0.05 µl hemolymph from 8 day old (short day) adults was incubated with indicated amounts of ^{3}H-JH III in 1.0 ml 0.1 M phosphate buffer, pH 7.5. Substrate concentration (s) expressed in pmoles JH/ml incubation mixture, the rate of hydrolysis (v) in pmoles JH-acid/10 min/0.05 µl hemolymph.
Insert: Eadie and Hofstee plot derived from v verses s curve.

Estimations of the JH-hydrolytic activity in different hemolymph samples with JH I and JH III as substrates show that the maximum rates of hydrolysis of JH III are approximately twice as high as those for JH I (see also table 1). The observed differences between the rate of hydrolysis of JH I and JH III could be due to the different solubilities of the two compounds or to differences in their surface activity. The solubility of JH I has been reported to be 5×10^{-5}M (7). JH III has a higher solubility than JH I (12), although Pratt (12) reported only $3.0\text{-}3.5 \times 10^{-5}$M. The concentrations of JH employed in our experiments are however at least one order of magnitude below this limit of solubility. We therefore checked the tendancy of the two hormones to stick to glass over a range of concentrations. The results are given in Fig. 2. Both JH I and JH III adsorb to glass in the concentration range used for the determination of JH hydrolysis. JH I and JH III show 50% and 30% adsorption respectively. Coating of the glasstubes with carbowax (0.1% in chloroform) reduced the adsorption of juvenile hormone to less than 10%. JH-

hydrolytic activity determined with carbowax coated glasstubes was not different from JH-hydrolytic activity determined with uncoated ones, even if sub-optimal substrate concentrations were used.

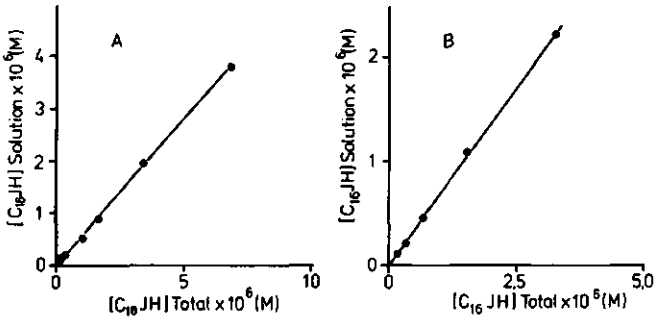

Fig. 2

A and B: Amounts of ^{3}H-JH I and ^{3}H-JH III present in solution in 1ml 0.1M phosphate buffer, pH7.5.

In order to check the specificity of the hydrolytic activity two JH-analogues, an ethylester without the 10,11-epoxide group (ZR512) and an isopropylester (ZR515), were tested for their suitability as a substrate for hemolymph esterases. In contrast to JH I and JH III neither of these analogues were affected by hemolymph esterases. Even large amounts of hemolymph (5 µl) did not cause hydrolysis. Moreover, addition of equimolar amounts of the analogues to an incubation medium containing ^{3}H-JH I did not alter the rate of JH I hydrolysis. Since it has been found that the ethylester (ZR 512) was susceptible to hemolymph esterases from Manduca sexta (13) we tested ZR-512 degradation with hemolymph from 2-3 day old Vth instar larvae under our conditions. No hydrolytic activity against the ethylester analogue was detected, while JH I was rapidly hydrolized under these conditions.

Recently Sanburg et al.(4) reported that DFP inhibited the general esterases in the hemolymph of Manduca sexta, but did not affect the specific JH-esterases. We therefore tested whether DFP could be used to distinguish JH-specific from general esterases in the hemolymph of Leptinotarsa. Hemolymph samples were preïcubated for 15 min with DFP. Fig. 3 illustrates that the hemolymph JH-hydrolytic activity is not affected by high DFP concentrations (10^{-3}M), while the α-naphthylacetate hydrolysis was almost completely inhibited.

In hemolymph derived from insects at different stages of development a high percentage of the JH-hydrolytic activity appeared to be resistent to 10^{-3}M DFP (Table 1). Maximally 30% of the activity was inhibited using either JH I or JH III. On the other hand Triton X-100 (0.1%) appeared to have an opposite effect: the JH-hydrolytic activity was completely inhibited while general carboxyesterase activity was not affected.

In order to characterize the number of esterases present in the hemolymph of the Colorado potato beetle, the hemolymph proteins were subjected to slab-gel electrophoresis. Usually 6 bands were detected after staining for esterases with α-napthylacetate as substrate. In long-day and in young short-day beetles the bands were difficult to distinguish because of their low esterase activity. However in hemolymph with high JH-hydrolytic activity 3-4 additional very faint bands became visible after prolonged staining. These bands correspond with 3 protein bands visible after Coomassie-blue staining (Fig.4).

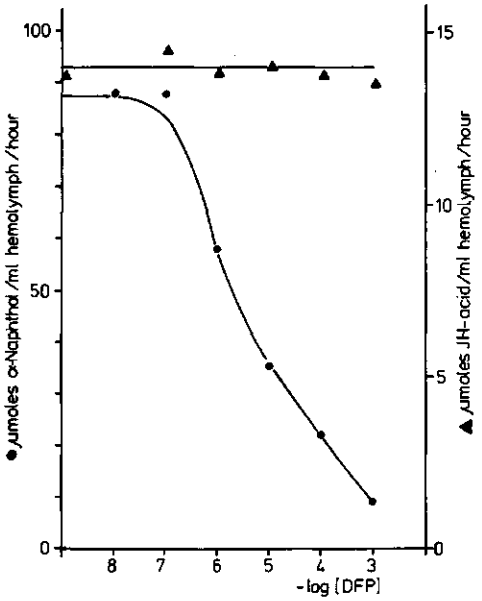

Fig. 3

Inhibition of hemolymph JH I esterases and α-Naphthyl acetate esterases from 8 day old (short day) adults by varying concentrations of DFP.
Symbols: ▲——▲ JH esterase activity, ●——● α-NA esterase activity.

In order to see which esterases exhibited JH-hydrolytic activity the unstained gel was sliced up and the esterases extracted with buffer. These extracts were assayed for JH-degradation. The results are illustrated in Fig.4. Only the group of 3-4 faint bands showed JH-hydrolytic activity. Similar results were obtained with hemolymph derived from 4 day old long day Colorado potato beetles.

Discussion

Recent studies by Trautmann et al. (14) showed that JH III occurs in several species of different insect orders e.g. Leptinotarsa decemlineata and Nauphoeta cinerea. In addition to these investigations the simultaneous occurence of the three JH's was demonstrated in the adult stage of Nauphoeta cinerea (15). Calculations of JH concentration in adult females of the Colorado potato beetle from the results of the Galleria waxtest (16) based on the assumption that JH III is the only hormone results in 10^{-6}M JH, which is about a hundred times higher than the concentration determined in Manduca sexta larvae (17). This calculation and the findings in Nauphoeta cinerea (15) may indicate that the other two JH's are also synthesized in the Colorado potato beetle.

Very similar kinetics were found for the degradation of JH III as compared to JH I. In hemolymph of 8 day old short day females an apparant K_m value of 0.5×10^{-6}M was found which is of the same magnitude as the apparant K_m values determined in hemolymph of 4 day old long day and 8 day old short day female Colorado potato beetles with JH I as substrate (5). This means that the affinity of the Colorado potato beetle hemolymph esterases for JH I and JH III

Table 1.

Effect of 10^{-3}M DFP on JH I- and JH III- hydrolytic Activity (in µmoles/ml/hr)

Stage	- DFP		+ DFP	
	JH I	JH III	JH I	JH III
IVthinstar larvae	12.1	24.8	9.3	16.9
4 long day adults	1.6	3.4	1.4	3.1
0 short day adults	1.1	-	0.9	-
8 short day adults	14.2	28.8	13.4	20.8
7 postdiapause adults	2.8	-	1.9	-

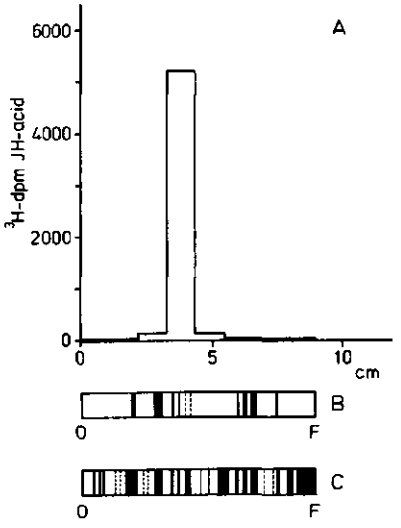

Fig. 4

A) Degradation of ^{3}H-JH I by hemolymph carboxy-esterases of 8 day old (short day) Colorado beetles extracted from polyacrylamide gel slices.
B) Diagrammatic representation of hemolymph α-Naphthyl acetate esterases.
C) Diagrammatic representation of hemolymph proteins after staining with Coomassie Blue.

is about equal. These apparent K_m values are about 10 times lower than the apparent K_m value derived from the quasi Michaelis-Menten curve determined in Schistocerca gregaria (12).

The aim of studying the rate of hydrolysis of different JH analogues was

to characterize the substrate specificity of the hemolymph esterases. The two JH mimics, contained different ester groups, and neither was hydrolyzed under our conditions. In contrast to the results of Weirich and Wren (13) hemolymph derived from V^{th} instar larvae of Manduca did not affect ZR 512 either. This discrepancy is difficult to explain. The only difference in these two studies was that Weirich and Wren used a ZR 512 preparation labelled in the ethyl-ester group. It should be stressed that in their system no radioactive metabolite could be detected and metabolism of ZR 512 was indicated solely by a difference in counts recovered versus counts applied. It is a possibility that the difference in hydrolysis could be due to the fact that we used a different strain of Manduca sexta. Even though the range of JH analogues tested was limited it may be concluded that the hemolymph esterases of the Colorado potato beetle involved in JH-hydrolysis, have a high substrate specificity.

The results from the DFP inhibition studies are very interesting. In accordance with the results of Sanburg et al. (4) we confirmed a high resistancy against DFP of the hemolymph JH esterases. In contrast other carboxy-esterases are very sensitive towards DFP. Both types of esterases are inhibited by paraoxon (S.J.Kramer, unpublished), which was also shown for hemolymph esterases of Schistocerca gregaria (12). On the other hand JH-hydrolysis is completely inhibited by 0.1% Triton X-100, while α-naphthyl-acetate hydrolysis is not affected by this detergent. In the Colorado potato beetle it seems therefore justified to distinguish JH-specific from general carboxyesterases. This conclusion is further substantiated by the results of slab-gel electrophoresis (Fig 4). A group of JH-specific esterases are clearly separated from the other (carboxy) esterases. The JH-specific esterases stain very weakly with the α-naphthylacetate/Fast blue RR procedure and thus we can infer that α-naphthylacetate is a poor substrate for these JH esterases.

We have shown previously that significant changes occur in the activity of JH-esterases throughout the life cycle of the Colorado potato beetle (5). It is clear from Table 1 that these changes are primarily due to marked changes in the activity of JH-specific esterases. Moreover, in vitro (carboxy-) esterases do not contribute significantly in the JH-breakdown (Fig. 4). In the tobacco hornworm JH-specific esterases are barely detectable throughout the larval stages, until the fourth day of the fifth instar when they suddenly appear at a high concentration (4). However in the Colorado potato beetle JH-specific esterases occur during all the stages tested, even when JH concentrations in the hemolymph are high.

Acknowledgement

We thank Professor Dr.J.de Wilde and Professor Dr. F.J. Oppenoorth for valuable advice and critism, and Dr. W.Mordue for a critical reading of the manuscript and correcting the English text. This work was supported by The Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

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METABOLISM OF JUVENILE HORMONE IN THE COLORADO POTATO BEETLE, *LEPTINOTARSA DECEMLINEATA*

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(*Received* 27 *October* 1976)

Abstract—The *in vitro* and *in vivo* degradation of ^{3}H- Cecropia C_{18} juvenile hormone has been studied in the Colorado potato beetle, *Leptinotarsa decemlineata*, reared under different physiological conditions. Degradation of the hormone involved hydrolysis of the ester function mainly by haemolymph esterases and hydratation of the epoxide group by tissue epoxide hydratases. Injected exogenous JH was degraded very rapidly *in vivo* in beetles reared under both long and short day conditions. The half-life of JH was estimated to be between 25 to 30 min. Injection with Triton X-100 resulted in a three fold increase of the half-life. The results suggest a major rôle of haemolymph JH-specific esterases in the degradation of JH.

INTRODUCTION

JUVENILE hormone plays a key rôle in the regulation of development and reproduction in insects (GILBERT, 1974). The hormone titre shows dramatic changes during the life cycle (FAIN and RIDDIFORD, 1975; DE WILDE *et al.*, 1971). Although the precise mechanisms regulating hormone titres are still not established, it is believed that a complex of factors is involved. These factors are considered to be on one hand the synthesis and release of JH by the corpora allata, and on the other the enzymic degradation and protection by JH-carrier proteins (AKAMATSU *et al.*, 1975).

The metabolic fates of juvenile hormone have been investigated the last few years in several insect species. In general it has been established that JH-degradation occurs via two major pathways: ester hydrolysis by esterases and hydratation of the epoxide group by epoxide hydratases (SLADE and ZIBITT, 1972; AJAMI and RIDDIFORD, 1973; ERLEY *et al.*, 1975; SLADE and WILKINSON, 1974, SLADE *et al.*, 1976). In addition, in higher Diptera e.g. *Musca domestica*, JH is metabolized mainly by microsomal oxidases (YU and TERRIERE, 1974, 1975).

In vitro studies with *Manduca sexta* (SANBURG *et al.*, 1975) and *Leptinotarsa decemlineata* (KRAMER and DE KORT, 1976a) showed that dramatic changes in activity of JH-specific esterases occur during the life cycle. The exact function of these JH-specific esterases *in vivo* was not clear, although a crucial rôle for these esterases has been suggested (AKAMATSU *et al.*, 1975). In the present paper the degradation pathways of JH in the Colorado potato beetle are investigated *in vivo* and *in vitro*. The relative importance of the degradative enzymes is estimated.

MATERIALS AND METHODS

Chemicals

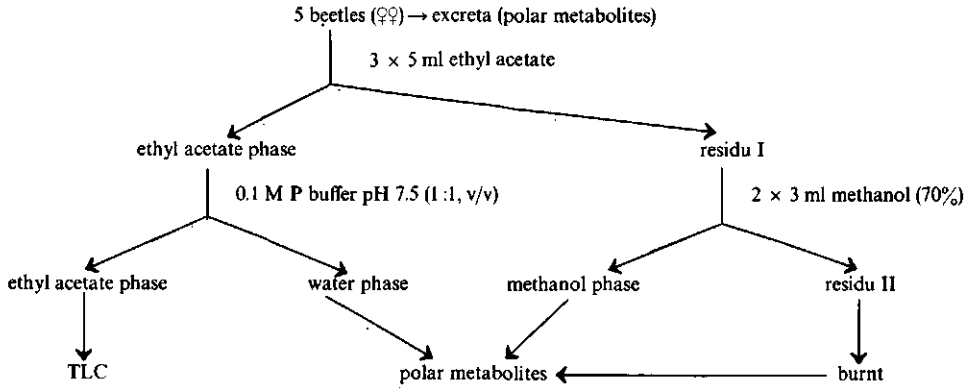
Labelled Cecropia C-18 JH (7-ethyl-1.2-^{3}H JH, 13.5 Ci/mmole) was purchased from New England Nuclear Corp. Dimethylsulfoxide (DMSO) was obtained from E. Merck, Darmstadt, G.F.R. The sources of other chemicals have been listed previously (KRAMER and DE KORT, 1976b).

Insects

Adults of the Colorado potato beetle, *Leptinotarsa decemlineata* were reared at 25°C on fresh potato leaves under two different photoregimes as described previously (DE KORT, 1969). Houseflies, *Musca domestica*, strain Fc, were obtained from the Laboratory for Research on Insecticides, Wageningen, the Netherlands. Haemolymph was collected as described by KRAMER and DE KORT (1976a).

In vivo *experiments*

Long day (LD), and short day (SD) beetles were injected with ^{3}H labelled JH I (respectively 0.03 μCi and 0.05 μCi per beetle) dissolved in a volume of 1 μl DMSO as described by BARTELINK and DE KORT (1973). After injection the beetles were kept at 25°C for the prescribed time. The beetles were then subjected to an extraction and fractionation procedure according to the following scheme:



Juvenile hormone and its metabolites (ethyl acetate phase) were separated by thin layer chromatography (TLC) and the radioactivity measured by liquid scintillation counting as described previously (KRAMER and DE KORT, 1976a). The water phase and the methanol phase were checked on TLC. All the radioactivity remained on the origin (polar metabolites). The residue II was burnt in a LS sample oxidizer (Intertechnique 4104) and the radioactivity counted in 10 ml scintillation mixture, containing 0.7% Butyl-PBD and 2.0% naphthalene in dioxane/toluene, 7:3 (v/v). In every experiment the TLC yield was estimated by counting a fraction of the ethyl acetate phase. The average yield (± S.E.) from TLC was 99.7% ± 10.0 ($\eta = 41$). The amounts of JH and its metabolites in the ethyl acetate fraction were corrected for 100% yield using an average yield (± S.E.) after zero time incubation of 89.2% ± 3.5 ($\eta = 8$).

In vitro *experiments*

Microsomes were isolated from abdomens of long day Colorado potato beetles and houseflies. The abdomens were homogenized in 0.15 M phosphate buffer pH 7.5, containing 0.25 M sucrose and 1.5% bovine serum albumine fraction V, in a Dounce homogenizer. The suspension was centrifuged for 30 min at 17,000 *g* (4 C). The supernatant was centrifuged for 60 min at 150,000 *g* in an ultracentrifuge (Beckman-model L5-65). The pellet, containing the microsomes, was resuspended in 0.1 M phosphate buffer pH 7.5.

To distinguish between microsomal oxidase and epoxide hydratase activity incubations were carried out in the presence and absence of NADPH (1 mg/ml).

Thoraces from long day beetles were prepared as described by BARTELINK and DE KORT (1973). The isolated thoraces were first washed in 0.1 M phosphate buffer pH 7.5, containing 0.25 M sucrose and then homogenized in 0.1 M phosphate buffer pH 7.5 in a Dounce homogenizer (10 thoraces/4 ml).

Fat body from long day beetles was isolated as described by DORTLAND and DE KORT (in preparation). The isolated fat body was homogenized in an identical manner to thoraces.

The preparations (microsomal suspensions of 2.5 abdomens of the housefly and 0.25 abdomens of the beetle and homogenates of 0.05 thorax and 0.10 fat body of the beetle) were incubated with 0.5 μg ^{3}H JH I in 1.0 ml 0.1 M phosphate buffer pH 7.5 for 10 min at 30°C. The incubation mixtures were extracted with ethyl acetate and analyzed on TLC using the same procedure as for the *in vivo* experiments.

Determination of JH III-esterase activity

The method for measuring the hydrolysis of C_{16} JH (JH III) has been described elsewhere (KRAMER and DE KORT, 1976b).

RESULTS

Four-day old long day and 4- and 8-day old short day adult beetles were injected with ^{3}H JH I. The degradation of the hormone was stopped at 0, 10, 30, and 60 min after injection by homogenizing the whole beetles in ethyl acetate. Figure 1 shows the distribution of JH I and its metabolites (ethyl acetate fraction) in TLC diagrams at different times after injection. The formation of polar metabolites (P.M.), calculated from the radioactivity in the excreta, water phase, methanol phase and residue II as described in Materials and Methods, are included in the figures. The acid, acid-diol and diol metabolites reach a low steady state level within 10 min (about 10% of total radioactivity), while the polar metabolites accumulate (about 30% of total radioactivity) in this time. Identical degradation patterns were obtained with beetles reared under either long day or short day conditions.

From the analysis of the metabolism *in vivo* it seems that the degradation of JH I occurs via two major pathways, ester hydrolysis by JH-esterases and epoxide cleavage by epoxide hydratases. Another possible degradation mechanism is the oxidation of JH by microsomal oxidases (NADPH requiring enzymes) as shown in the housefly (YU and TERRIERE, 1974). To investigate whether microsomal oxidation occurs in the beetle, microsomes were isolated from the abdomens and incubated with JH I in the presence and absence of NADPH. As can be seen from Fig. 2a there is no effect of NADPH upon JH-degradation by beetle microsomes. However, addition of microsomes isolated from abdomens of houseflies to these incubation mixtures resulted in a stimulation

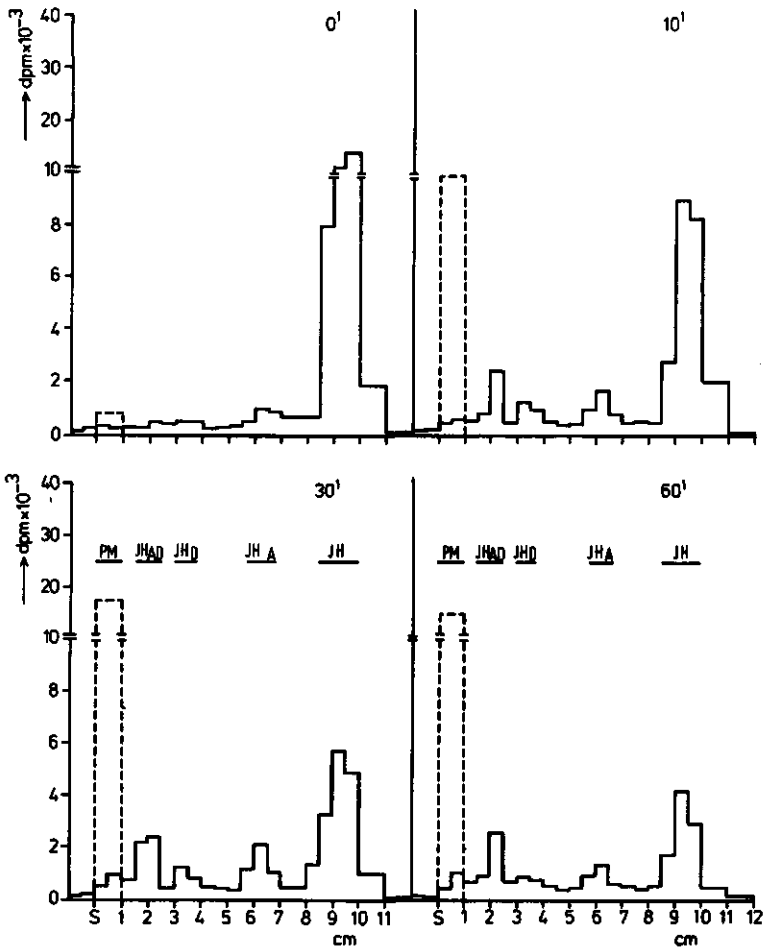


Fig. 1. TLC analysis of the degradation of injected ^{3}H JH I different times after injection. The beetles used were 4-day-old females reared under short day regime. Abbreviations for different metabolites are as follows: JH_A, JH-acid; JH_D, JH-diol; JH_{AD}, JH-acid-diol; PM, polar metabolites.

in the formation of the diol and acid-diol in the presence of NADPH (Fig. 2b).

The *in vivo* experiments with exogenous JH I show clearly that JH-degradation is extremely rapid in the beetle. The fractionation scheme, as described in Materials and Methods, allows us to measure quantitatively the extent of JH-degradation *in vivo*. In Fig. 3 the changes in the amount of JH I have been plotted semi-logarithmically against time. From the slopes of the lines the half-life can be calculated. Table 1 represents the half-life of JH I in beetles reared under long day and short day conditions. For each group three independent experiments were performed.

The experiments described above establish the route of JH-degradation in the adult Colorado potato beetle, but, because none of the JH metabolites accumulated to any extent, except the polar metabolites, these experiments do not permit any conclusion about the relative importance of the two main degradation pathways. In order to obtain this information we used two approaches. Firstly, we tried to inhibit "selectively" one of the two pathways. Secondly, we established degradation by homogenates of tissues qualitatively and quantitatively.

In a previous study (KRAMER and DE KORT, 1976b) it was found that JH-esterase activity in the haemolymph was almost completely inhibited by 0.1% Triton X-100, while general carboxyesterase activity was not affected. Figure 4 shows that Triton X-100 is indeed a potent inhibitor of haemolymph JH-esterase

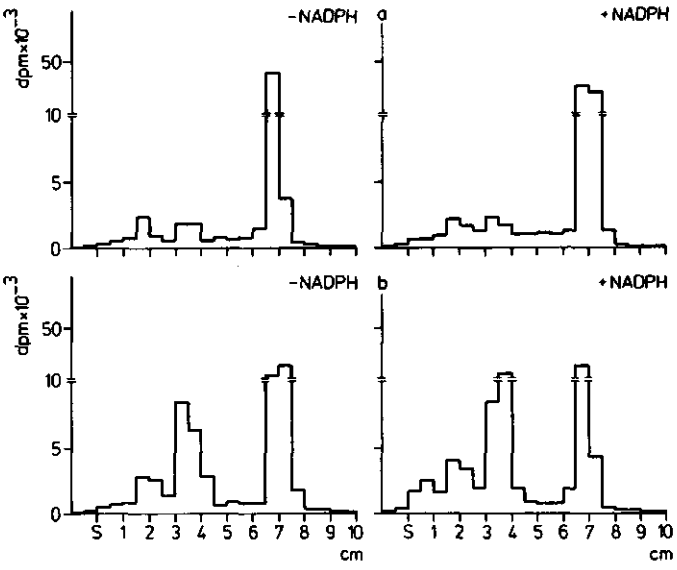


Fig. 2a and b. TLC analysis of the degradation of ^{3}H JH I after incubation with a microsomal suspension isolated from Colorado potato beetles (a) and co-incubation with a microsomal suspension isolated from houseflies (b) as described in the Materials and Methods.

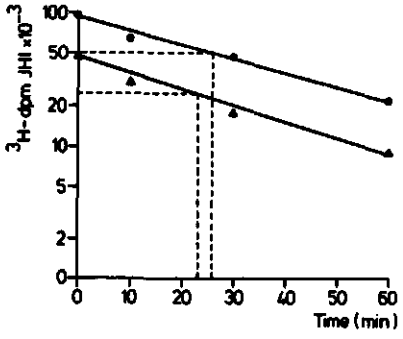


Fig. 3. Changes in concentration of JHI as a function of time. Symbols: ●——●, 4-day-old short day females; ▲——▲, 4-day-old long day females.

activity *in vitro*. Subsequently we determined whether Triton X-100 is also effective *in vivo*. Triton X-100 was shown to be non-toxic. Two µl 7.5% Triton X-100 was injected and after 15 min haemolymph samples were collected. The JH III-esterase activity was measured in the haemolymph samples and compared with control haemolymph and haemolymph inhibited with 0.1% Triton X-100 *in vitro*. JH III-esterase activity was inhibited both *in vivo* and *in vitro* for 90 and 96% respectively. After it was established that Triton X-100 also inhibits haemolymph esterases *in vivo*, we looked at the effect of Triton X-100 injection

Table 1. Half-life of injected JH I in the Colorado potato beetle

Age in days	Half-life in minutes
4 LD	24
4 SD	29
8 SD	27
4 SD + Triton X-100	91

on the half-life of JH. The results are shown in Table 1. Three independent experiments show that the half-life of JH after injection of Triton X-100 increased 3-fold. However, Triton X-100 did not appear to inhibit JH-esterases selectively, because addition of

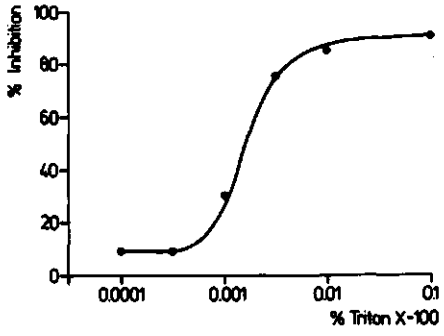


Fig. 4. Inhibition of haemolymph JH III-esterase activity by varying concentrations of Triton X-100.

0.1% Triton X-100 to an incubation mixture containing microsomes and ^{3}H JH I resulted in a total inhibition of the epoxide hydratase activity as well.

The next step was to establish whether JH-specific esterases in the haemolymph or JH-specific and general carboxyesterases in the tissues contribute significantly to the total breakdown of JH. Therefore, we measured JH-esterase activity in total homogenates of thoraces and fat body using labelled JH I and JH III as a substrate. In both tissues JH III-esterase activity was extremely low. The highest activity was 36 nmole/thorax/hr and 11 nmole/fat body/hr. Epoxide hydratase activities were of a similar low level in both tissues.

DISCUSSION

The results presented in this paper show that two main pathways of JH-degradation, established for other insects (SLADE and ZIBITT, 1972; AJAMI and RIDDIFORD, 1973; ERLEY *et al.*, 1975; SLADE and WILKINSON, 1974; SLADE *et al.*, 1976), also occur in the Colorado potato beetle. The primary products of the esterase and the epoxide hydratase, the acid and the diol, can be detected, although they do not accumulate to a great extent. Moreover, microsomal oxidases known to play a major rôle in JH-metabolism in the housefly *Musca domestica* (YU and TERRIERE, 1974) could not be shown to affect JH-breakdown in the beetle (Fig. 2a). The possibility exists that the microsomal preparations are damaged during isolation. However, the results presented in Fig. 2b make it unlikely that microsomal oxidases play an important rôle in JH-degradation in the beetle.

Although JH-breakdown is rapid, only the polar metabolites accumulate to a large extent, which suggests that these are the end products of the degradation of JH. This can be also inferred from the fact that the radioactivity found in the excreta remains at the origin in our TLC system and can only be detected a long time after injection of hormone. The lack of accumulation of intermediates also suggests that the conversion to polar metabolites is rapid. This, together with the rapid accumulation of the polar metabolites might indicate that conjugation occurs not only for the acid-diol, but that also the acid and the diol can be converted directly to polar conjugates. This was suggested also by SLADE and ZIBITT (1972), but direct proof in the case of the Colorado potato beetle needs further experimentation.

The results presented demonstrate that JH is rapidly metabolized *in vivo* by the Colorado potato beetle. In both long day and short day beetles a half life of exogenous JH I of about 25 min was found. This value is in agreement with that determined by NYHOUT (1975) for the decay of endogenous JH in Vth instar larvae of *Manduca sexta* and those of SLADE and ZIBITT (1972) and AJAMI and RIDDIFORD (1973) for the loss of exogenous JH activity in pharate pupae and pupae of *Manduca sexta*. In contrast a half-life for endogenous JH of about 1.5 hr was found in IVth instar larvae of *Manduca sexta* and in adult male locusts. (FAIN and RIDDIFORD, 1975; JOHNSON and HILL, 1973). A similar half-life was determined for exogenous JH in early last instar larvae of *Galleria* (REDDY and KRISHNAKUMARAN, 1972). Thus in IVth instar larvae of *Manduca sexta* the half-life is longer than in Vth instar which may correlate with the rapid increase in JH-specific esterases during the Vth instar. These JH-specific esterases are capable of hydrolyzing both free JH and JH complexed to a high affinity carrier protein (SANBURG *et al.*, 1975). In contrast to *Manduca sexta* JH-specific esterases occur in the Colorado potato beetle during the whole life cycle and under both long day and short day conditions (KRAMER and DE KORT, 1976a, b). Thus, the short half-life of JH may be caused by the continuous presence of these JH-specific esterases. The rôle of such specific esterases in regulating the JH-titre is supported by the effects of Triton X-100, which causes a significant increase in the half-life. Furthermore Triton X-100 inhibits the JH-specific esterases *in vivo*. However, it is an unwarranted conclusion that the esterases are solely responsible for the short half-life of JH, because *in vitro* experiments show that Triton X-100 also affects epoxide hydratase activity.

Only few data are available concerning the degradation capacity of insect tissues. Larval fat body of *Manduca sexta* has been found to degrade JH rapidly to the acid, diol and diol-acid (HAMMOCK *et al.*, 1975), whereas mid-gut homogenates of the last larval instar of the southern armyworm, *Prodenia eridania*, possessed a high JH-hydratase activity (SLADE *et al.*, 1976). In contrast, homogenates of fat body and thorax of the beetle have a very low capacity for degrading exogenous JH. The contribution of the tissue esterases to the total esterase activity is very low. The haemolymph volume of fullgrown beetles is about 60 µl (DE KORT, unpublished). Thus the total capacity of the haemolymph of one long day beetle to degrade JH III is approximately 250 nmole/hr. This is high compared with the capacity of thorax and fat body homogenates described in this paper. These findings, together with the inhibition studies with Triton X-100 suggest a major rôle for the haemolymph JH-specific esterases in controlling the JH-titre in the Colorado potato beetle. The similarity in the half-life of JH in beetles reared under different photo-regimes, would seem contradictory to the differences in haemolymph JH-specific esterase activities. Binding and protection of JH by a carrier protein may also play a rôle in the regulation of the JH-titre. The occurrence and the rôle of such carrier proteins is at present under investigation.

Acknowledgements—The authors wish to thank Dr. J. DE WILDE and Dr. F. J. OPPENOORTH for valuable advice and criticism, and Dr. W. MORDUE (London) for critically reading the manuscript and correcting the English text. Thanks are also due to Dr. H. SCHOONEVELD for critically

reading the manuscript, and to the K.A.B.O. (Wageningen) for using the LS sample oxidizer. This work was supported by the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

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Key Word Index: Colorado potato beetle, JH-degradation, JH-specific esterase, JH-epoxide hydratase

J. Insect Physiol., in press

REGULATION OF THE ACTIVITY OF JH-SPECIFIC ESTERASES IN THE COLORADO POTATO BEETLE, *LEPTINOTARSA DECEMLINEATA*

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Abstract

The regulation of haemolymph JH-esterase activity, the main degradation pathway of JH in the Colorado potato beetle, *Leptinotarsa decemlineata*, has been studied by means of JH application and microsurgical techniques. Treatment of diapause beetles with JH I and different JH analogues resulted in an increase in JH-esterase activity within 24 hr. Puromycin or actinomycin D treatment prevented the appearance of these enzymes. Ligation and allatectomy experiments performed on diapause beetles suggest, but do not prove conclusively, that this induction phenomenon is an indirect effect of JH. Transfer of short day beetles to a long day photoregime and treatment of short day beetles with JH prevented the occurrence of high levels of JH-esterase activity. On the other hand also allatectomy of short day beetles resulted in an identical effect. It is very likely that in short day beetles the occurrence of a low JH titre during the first days after adult emergence is necessary for the induction of the high JH-esterase peak, prior to entering diapause. Whether the effect of JH is direct or indirect still remains unknown. The results suggest that the regulation of haemolymph JH-esterase activity is conducted by a complex of neuro-endocrine factors.

INTRODUCTION

The juvenile hormone titre at any point in the insect's life cycle is believed to be the result of synthesis and release of JH by the corpora allata on one hand and its enzymic degradation and protection by JH carrier proteins on the other (Akamatsu *et al.*, 1975).

In the Colorado potato beetle it has been established previously that JH is metabolized mainly by JH-esterases (Kramer *et al.*, 1977). *In vitro* studies showed that dramatic changes in the activity of JH-esterases occur during the life-cycle of the Colorado potato beetle (Kramer and De Kort, 1976a). For instance in beetles reared under a short day photoregime high JH-esterase activities occur just before entering diapause. At this stage a low titre of the hormone is required. It is likely that a control mechanism exists which regulates the JH-esterase level in the haemolymph in conjunction with the changes in activity of the corpora allata.

In *Hyalophora gloveri* pupae it has been shown that juvenile hormone can induce several carboxylesterases (Whitmore *et al.*, 1972; Whitmore *et al.*, 1974). These esterases degraded JH, but were sensitive to diisopropylfluorophosphate (DFP), an inhibitor used to

distinguish between general- and JH-specific esterases (Sanburg *et al.*, 1975). It was suggested that this induction of esterases is one mechanism insects use to regulate the titre of circulating JH.

This present paper is an attempt to elucidate which neuro-endocrine factors are involved in the control of JH-esterase activity in the haemolymph of the Colorado potato beetle.

MATERIAL AND METHODS

Chemicals

Synthetic juvenile hormone (JH I) was provided by Hoffmann-La Roche, Basle, Switzerland. The preparation (RO-08-9550) was a mixture of the geometrical isomers of methyl-10-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate. The JH analogues hydroprene (ZR 512, ethyl, trans, trans, 3,7,11-trimethyl-2,4-dodecadienoate) and methoprene (ZR 515, isopropyl, trans, trans, 11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate) were provided by Zoecon Corporation, Palo Alto, California. The JH analogue (NH) JH II (methyl-3,7,11-trimethyl-10,11-imino-2,6-tridecadienoate) was a gift from Dr. W.A. Biessels, Laboratory of Organic Chemistry, Utrecht, The Netherlands. JH III (methyl-3,7,11-trimethyl-10,11-epoxy-2,6-dodecadienoate) with tritium label in the ester methyl group (20 mCi/mmole) was a generous gift from Dr. J.H. Law, The University of Chicago. Puromycin was purchased from Sigma Chemicals Co., St. Louis, Mo., USA. Actinomycin D was obtained from Merck Sharp and Dohme Research Lab. Rathway N.J. USA. All other chemicals were of reagent grade.

Insects

Adults of the Colorado potato beetle, *Leptinotarsa decemlineata* Say were reared at 25°C on fresh potato leaves under two different photoregimes as described previously (De Kort, 1969). Beetles reared under short day photoregime (10 hr) enter into diapause 10 days after adult emergence.

Administration of compounds

JH I was either injected in paraffin oil (50 µg/µl/beetle) as described by Bartelink and De Kort (1973) or applied topically in acetone (50 µg/µl) on the dorsal part of the abdomen. JH analogues were applied in acetone in a similar manner. Puromycin was injected in water (200 µg/g fresh weight → 35 µg/µl water/beetle). Actinomycin D was dissolved in 95% ethanol (2 µg/µl) and diluted appropriately in water before injection (3 µg/g fresh weight → 0.5 µg/µl/beetle). Beetles were anaesthetized with CO_2 before administration. Unless otherwise stated all treatments were performed on groups of 10 beetles.

Surgical techniques

Allatectomy and allatocardiacectomy were performed by removing the glands through a slit in the neck membrane (De Wilde and Stegwee, 1958). The beetles were operated 0-12 hrs after adult emergence. A survival rate of approximately 90 percent in LD beetles and 60 percent in SD beetles was obtained.
Ligation was performed with silk thread by tying a knot round the neck. In order to achieve this the head of the beetle was pulled slightly by means of a tube attached to it by applying vacuum.

Determination of esterase activities

JH III-esterase activity was measured as described elsewhere (Kramer and De Kort, 1976b).

General esterase activity was measured with α-Naphthylacetate as a substrate as described by Van Asperen (1962).

RESULTS

In order to investigate which factors are involved in the regulation of JH-esterase activity we interfered with the endogenous hormone content by changes in the photoperiod, allatectomy, ligation and JH administration.

Induction of JH esterases in diapausing beetles

Diapausing Colorado potato beetles, a stage devoid of juvenile hormone and with relatively low JH-esterase activity (De Wilde *et al.*, 1968; Kramer and De Kort, 1976a), were treated with JH I as described in the Materials and Methods. Preliminary experiments showed that injection or topical application of the hormone results in identical

Table 1. JH III-esterase and general carboxylesterase activities in pooled haemolymph samples of ten 2-4 week old diapausing Colorado potato beetles at different times after topical application of JH I or acetone (control).

Time (hr) after treatment	General esterase activity (μmoles α-Naphthol/ml haemolymph/hr)		JH III-esterase activity (μmoles JH acid/ml haemolymph/hr)	
	Acetone	JH	Acetone	JH
6	55	78	5.2	5.2
24	68	72	5.2	8.8
48	64	66	5.2	14.0
72			4.6	12.8
96			3.4	12.6

effects. Table 1 shows that JH-esterase activity increases significantly 24 hr after topical application of JH I, while after 48 hr the maximum response is obtained (an increase of 270% compared with control animals). The higher level of JH-esterase activity is not inhibited by DFP. In contrast, α-naphthylacetate-esterase activity was not significantly affected. In an attempt to investigate the specificity of this "induction" phenomenon three JH analogues, an ethylester without the 10,11-epoxide group (ZR 512), an isopropylester (ZR 515) and an imino analogue of JH II, were applied to diapause beetles. The effect was measured 24 hr after treatment. Table 2 shows that induction is also achieved with different JH analogues, which means that the reaction is not specific for juvenile hormone.

Table 2. JH III-esterase activities in pooled haemolymph samples of ten 2-4 week old diapausing beetles 24 hours after application of 3 different JH analogues or acetone (control).

JH analogue	JH III-esterase activity (μmoles JH acid/ml haemolymph/hr)
Control	6.2
ZR 512	13.2
ZR 515	12.0
(NH) JH II	14.0

Although we have used the term induction, it is possible that injection or topical application of juvenile hormone increases the activity of existing enzymes, or causes the selective release of esterases from its tissue of origin.

To determine whether protein synthesis and RNA synthesis are essential for the induction, we repeated the experiment with JH I but in the presence of puromycin and actinomycin D. Puromycin (0.2 mg/g) was administrated with JH I simultaneously, while actinomycin D (3 μg/g) was injected 6 hr before application of JH I. Haemolymph was collected 24 hr after application of JH I and JH III-esterase activity measured. Table 3 shows that both puromycin and actinomycin D suppress the induction of JH-esterases.

Table 3. JH III-esterase activity in pooled haemolymph samples of ten 2-4 week old diapausing beetles 24 hours after injection of puromycin, actinomycin D and topical application of JH I or acetone (control).

JH III-esterase activity (μmoles JH acid/ml haemolymph/hr)					
Acetone	JH I	Puromycin	Puromycin + JH I	Actinomycin	Actinomycin + JH I
2.3 (100)*	3.4 (148)	1.6 (70)	1.7 (74)	2.2 (96)	2.1 (91)

* Values in parentheses represent the percentage of control activity.

These data suggest that juvenile hormone may be acting at the transcriptional level to induce these enzymes.

Effect of ligation on induction of JH esterases in diapausing beetles

SD beetles which had just entered diapause were head-ligated as described in Material and Methods. Subsequently the beetles were treated with JH I and haemolymph was collected and tested 48 hr later. Table 4 illustrates that in three independent experiments JH-esterase activity does not increase significantly after application of JH I as compared with the control experiments (without ligation). However, the activity is a little higher than the control. This can be explained by the assumption that possibly not all the beetles were completely ligated.

Table 4. JH III-esterase activity in pooled haemolymph samples of ten head-ligated diapausing beetles 48 hours after topical application of JH I or acetone (control).

	JH III-esterase activity (µmoles JH acid/ml haemolymph/hr)		
	Untreated	Acetone	JH I
- Ligation	3.7	3.4	9.3
+ Ligation			
I	2.7	1.8	3.1
II	3.7	2.8	4.1
III	2.2	2.9	3.5
Average	2.9	2.5	3.6

Effect of JH I treatment on level of JH-esterase activity in short day beetles

In beetles reared under short day photo-regime a sharp increase of JH-esterase activity occurs with its maximum at day 8 (Kramer and De Kort, 1976a). To investigate whether this JH-esterase peak can be influenced by exogenous JH application, SD beetles were treated with JH I (50 µg/beetle) at 0, 1 or 2 days after emergence. At day 8 haemolymph was collected and tested for JH III-esterase activity. Table 5 shows that application of JH I at day 0 and 1 results in low JH-esterase activities at day 8, comparable with the values found in beetles reared under long day photo-regime. These two groups of beetles were ovipositing on day 8. Beetles treated at day 2 still had not begun oviposition, while JH-esterase activity was intermediate (Table 5).

Table 5. JH III-esterase activity in pooled haemolymph samples of ten 8 day old short day beetles after application of JH or acetone (control) at day 0, 1 and 2 after emergence.

	JH III-esterase activity (µmoles JH acid/ml haemolymph/hr)		
	Time of application (days after emergence)		
	0	1	2
Untreated	23.9	17.9	17.9
Acetone	24.2	16.7	19.6
JH	4.9	6.6	9.8

Effect of change of photo-period on level of JH-esterase activity in short day beetles

In order to assess the effect of photo-period on JH-esterase activity SD beetles were transferred to long day photoregime different times after adult emergence (0, 2, 3

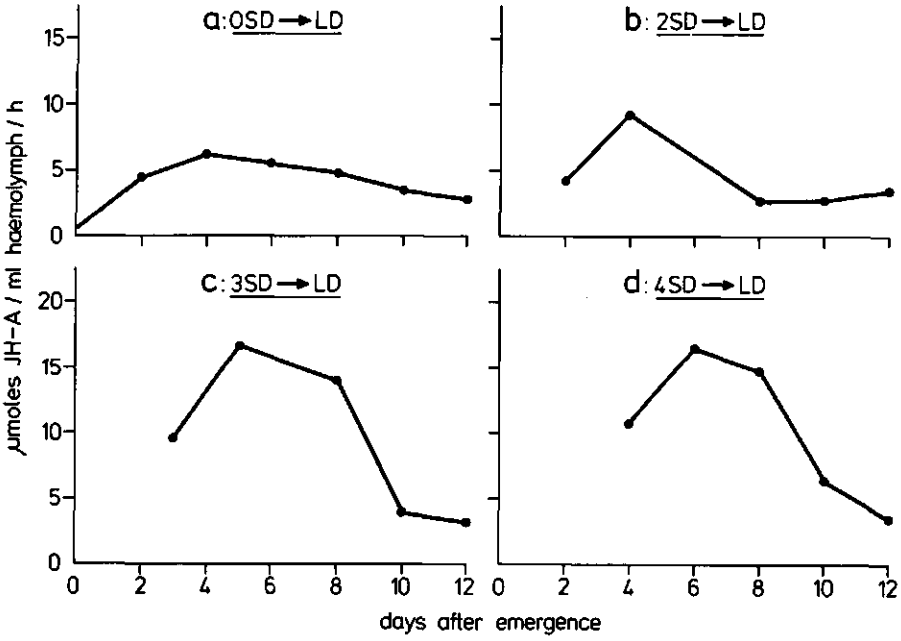

Fig. 1 a-d. Transfer of short day beetles at different times after adult emergence to long day photo-regime and its influence on JH III-esterase activity. Esterase activities were measured in pooled haemolymph samples derived from ten beetles.

and 4 days). Haemolymph was collected at intervals of 2-3 days and JH III-esterase activity measured. Figure 1 a-d shows the course of JH-esterase activity until 12 days after adult emergence. Transfer to long day conditions directly after emergence resulted in a JH-esterase pattern the same as that found in normal LD beetles (Fig. 1a). Transfer 2, 3 and 4 days after SD adult emergence showed an increase of JH-esterase activity during the first two days (Fig. 1 b-d), afterwards JH-esterase activity declined. In all the experiments the females started oviposition about 5-6 days after transfer to LD conditions, which is about 1 day longer than in normal LD females.

Effect of allatectomy on the level of JH-esterase activity

Beetles were operated on directly after adult emergence and at intervals of 2-4 days JH-esterase activity was measured in the haemolymph. Allatectomy of SD beetles prevents the occurrence of the high JH-esterase peak at day 8 (Fig. 2a). Sham operation of SD beetles results in JH-esterase activities similar to those found in normal SD beetles (Fig. 2c).

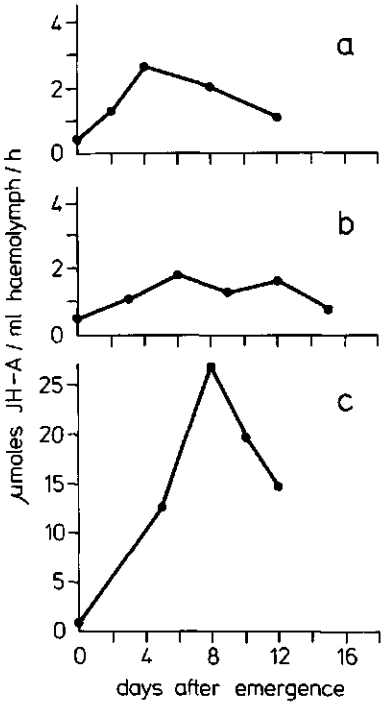

Fig. 2 a and b. Allatectomy of short day and long day beetles respectively at adult amergence and its influence on JH III-esterase activity. Esterase activities were measured in pooled haemolymph samples derived from five beetles.

Fig. 2 c. JH III-esterase activity of short day beetles sham operated at adult emergence. Esterase activities were measured in pooled haemolymph samples derived from five beetles.

If allatectomy was postponed till day 1 or day 2 after emergence, the esterase levels at day 8 show low and intermediate activity respectively (Table 6). Allatectomy of LD beetles results in relatively low esterase levels (Fig. 2b).
If allatectomized SD beetles were allowed to enter diapause and subsequently treated

Table 6. JH III-esterase activity in pooled haemolymph samples of ten 8 day old short day beetles after allatectomy or sham operation at day 1 and 2 after adult emergence.

	JH III-esterase activity (µmoles JH acid/ml haemolymph/hr)	
	Time of allatectomy (days after emergence)	
	1	2
sham	27.8	24.2
allatectomy	5.8	12.8

with JH I, the JH-esterase activity increases identically as with unoperated control beetles (Table 7). The experiments mentioned above show that allatectomy of young adults prevents the occurrence of high JH-esterase levels under SD conditions. Nevertheless corpora allata are not necessary to enhance JH-esterase levels after the beetles enter diapause (table 7). Subsequently, we tried to induce high esterase levels in young allatectomized beetles by hormone therapy. However, we failed to induce high JH-esterase levels when allatectomized SD beetles were treated with 50, 10, 1 or 0.1 µg JH I per beetle, within 48 h of the operation.

Table 7. JH III-esterase activity in pooled haemolymph samples of diapausing beetles (with or without corpora allata) 48 hours after topical application of JH I or acetone (control). Each group consisted of 5 beetles.

	JH III-esterase activity (µmoles JH acid/ml haemolymph/hr)		
	Untreated	Acetone	JH I
+ CA	3.3	3.1	8.5
- CA	2.7	2.1	8.9

DISCUSSION

The present paper attempts to investigate which factors are involved in the regulation of JH-specific esterases, in the Colorado potato beetle.

In the literature little data are available concerning the mechanisms involved in the control of JH degrading enzymes. Whitmore *et al.*, (1972) have demonstrated that a class of carboxylesterases (sensitive to DFP) appear in the haemolymph of *H. gloveri* pupae (a stage normally devoid of JH) when JH is injected, and that inhibition of protein and m-RNA synthesis prevents this induction. In the housefly Terriere and Yu (1973) have found that β-ecdysone, JH I and JH analogues stimulate microsomal oxidase

activity. Microsomal oxidase is an important pathway of JH-degradation in this insect and probably also in other Dipterans. However, this microsomal oxidase system is not specific for JH and is involved in the metabolism of many endogenous and foreign compounds. In contrast to these findings application of JH I and three JH analogues to diapausing Colorado potato beetles (a stage devoid of JH) results in a significant increase of JH-specific esterase activity (intensitive to DFP). In a previous paper (Kramer and De Kort, 1976b) it has shown that two JH analogues (ZR 512 and ZR 515) are not degraded by JH-esterases. Therefore it is most likely that this induction phenomenon is not specific for JH. The induction effect was blocked when puromycin and actinomycin D were administered together with the hormone. This suggests that *de novo* protein synthesis is required for the induction process. *De novo* synthesis of JH-specific esterase has not been shown (see also Whitmore *et al.*, 1974).

In order to investigate whether this induction phenomenon is a direct or an indirect effect of JH, diapausing Colorado potato beetles were headligated (exclusion of the cerebral neuro-endocrine system) prior to application of JH. No significant change in JH-esterase activity was obtained and therefore it is likely that the effect on the fat body, the site of origin of JH-esterases (unpublished observation), is an indirect one. In contrast to these results, injection of JH I into brainless *H. gloveri* pupae yielded the same results as injection into animals with intact brains (Whitmore *et al.*, 1972). This induction phenomenon was also achieved when fatbody was cultured *in vitro* and stimulated by JH (Whitmore *et al.*, 1974). These authors therefore suggests that JH is acting directly and not secondarily through another hormone.

Little is known about the role of neurosecretion in the control of JH breakdown enzymes. Retnakaran and Joly (1976) have shown that in *Locusta migratoria* the JH-specific esterase level drops after electrocoagulation of the A and B neurosecretory cells, although a drop of only 40% was obtained. Histology studies of Schooneveld *et al.* (1977) have shown that JH treatment of diapausing Colorado beetles results in a release of secretion from A-type neurosecretory cells, followed later by an increase in nuclear size reflecting increased synthetic activity of the perikarya. Our ligation experiments also suggest, but do not conclusively prove, that JH may act via the cerebral neurosecretory system. This is also substantiated by the finding that application of JH to diapause beetles, which were allatectomized directly after adult emergence, resulted in an induction of JH-esterase activity.

In order to obtain a better understanding of the role of JH in the regulation of JH-esterases, hormone applications, change of photo-period and allatectomy experiments were performed on newly emerged adult long day (LD) and short day (SD) beetles. The experiments concerning the transfer of SD beetles to a long day photo-regime and the treatment of SD beetles with JH clearly show that the occurrence of high JH titres results in low-esterase activities, as is found in normal LD beetles. On the other hand, allatectomy of newly emerged adult SD beetles prevented the appearance of high JH-esterase activity at day 8. This experiment leads to the conclusion that in SD beetles the occurrence of a low JH titre (below a certain level) during the first days after adult emergence is necessary for the appearance of a high JH-esterase peak, prior to entering diapause. Indeed this conclusion is substantiated by the recent finding that the corpus allatum remains active

during the first 6 days after adult emergence under SD conditions (Kramer, in preparation).

Allatectomized SD beetles treated with JH 2 days after the operation did not show any induction of JH-esterases. An explanation for this finding could be that the JH titre raises above a certain level. This is substantiated by the fact that most of the females showed reproductive development. However, administration of lower JH concentrations did not lead to a different result. The discrepancy between these findings and the induction effects in diapausing beetles still remains difficult to explain.

The different effects of JH on haemolymph esterases might be related with the competence of the fat body to react (direct or indirect) on the titre of JH. The results presented show that regulation of haemolymph esterases is conducted by a complex of factors.

ACKNOWLEDGEMENTS

The author is indebted to Drs. C.A.D. de Kort and F.J. Oppenoorth for valuable advice and criticism, to Dr. J. de Wilde for his interest and to Dr. W. Mordue (London) and Dr. H. Schooneveld for critically reading of the manuscript. Many thanks are due to Mrs. H. Privee who performed all the allatectomy operations. Dr. W. Mordue corrected the English text. The work was supported by the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

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Insect Biochemistry, in press

JUVENILE HORMONE CARRIER LIPOPROTEIN(S) IN THE HAEMOLYMPH OF THE COLORADO POTATO BEETLE, *LEPTINOTARSA DECEMLINEATA*

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Abstract

The haemolymph of larvae and adults of the Colorado potato beetle, reared under two different photoregimes, contains large molecular weight lipoprotein(s) (M.W. > 100,000) capable of binding C18 juvenile hormone. The lipoprotein(s) were separated by gelpermeation chromatography and polyacrylamide gelelectrophoresis. The binding characteristics of the lipoprotein(s) indicate low affinity ($K_d \approx 10^{-5}$M), low specificity, (JH analogues ZR 512 and ZR 515 and fatty acids such as palmitic acid were also bound) and high capacity. The carrier protein(s) provide to some extent protection for JH against haemolymph JH-specific esterases. A possible role of these lipoprotein(s) in the regulation of JH titre is discussed.

INTRODUCTION

Juvenile hormone, synthesized and secreted by the corpora allata, reaches the target tissues via the haemolymph. In insect haemolymph lipoproteins are known to transport neutral lipids, sterols, and a number of other lipophilic compounds (Gilbert and Chino, 1974). Therefore it was suggested that the lipophilic JH molecule is also transported by lipoproteins. Several investigators have reported the binding of JH and JH analogues to high molecular weight lipoproteins in different insect species e.g. pupae and adults of saturniid moths, *Hyalophora gloveri*, *H. cecropia*, and *Antheraea polyphemus* (Whitmore and Gilbert, 1972), larvae of the yellow mealworm, *Tenebrio molitor* (Trautmann, 1972), adult females of the locust, *Locusta migratoria* (Emmerich and Hartmann, 1973) and adults of *Tenebrio obscurus* and *Oncopeltus fasciatus* (Kramer *et al.*, 1976).

In contrast, larvae of the tobacco hornworm, *Manduca sexta*, the Indian mealmoth, *Plodia interpunctella*, and thirteen other insect species from three different orders contain a relatively low molecular weight protein (2.8×10^4 mol wt) which binds JH with high affinity, high specificity and low capacity (Kramer *et al.*, 1974; Gilbert *et al.*, 1976; Ferkovich *et al.*, 1975; Kramer *et al.*, 1976). Haemolymph lipoproteins were also present in these insects, but a complex with JH was only formed when the high affinity binding protein was saturated with JH. In *Manduca sexta* it was demonstrated that the JH binding protein allowed protection against general esterases while JH-specific esterases could degrade

both the bound and free hormone (Sanburg *et al.*, 1975). This paper describes the occurrence and possible role of binding proteins in the haemolymph of the Colorado potato beetle. The relationship between these JH binding proteins and the degradation of JH by haemolymph JH-specific esterases (Kramer *et al.*, 1977) will be discussed.

MATERIALS AND METHODS

Chemicals

Labelled Cecropia C-18 JH (7-ethyl-1,2-^{3}H -JH,13.5 Ci/mmole) was purchased from New England Nuclear Corp. The JH analogues (labelled and unlabelled), 5-^{14}C hydroprene (ZR-512, ethyl, trans, trans, 3,7,11-trimethyl-2,4-dodecadienoate), and 5-^{14}C methoprene (ZR-515, isopropyl, trans, trans-11-methoxy-3,7,11 trimethyl-2,4-dodecadienoate) were both provided by Zoecon Corporation, Palo Alto, California. Palmitic Acid-1-C14 (S.A. 44.3 mCi/mmole) was derived from the Radiochemical Centre, Amersham, England. Sephadex G-100 was obtained from Pharmacia Fine Chemicals AB, Uppsala Sweden. Cellulose polyacetate electrophoresis strips (sepraphore III) were obtained from Gelman Instrument Company, Ann Arbor, Michigan, USA. The sources of the other chemicals have been described previously (Kramer and De Kort, 1976a).

Insects

Larvae and adults of the Colorado potato beetle, *Leptinotarsa decemlineata* Say were reared at 25°C on fresh potato leaves under two different photoregimes as described previously (De Kort, 1969). Adult beetles were collected within 16 hr after emergence from the soil and kept until the desired age (young adults emerge from the soil at the second day of the imaginal stage). The method for collecting haemolymph has been described elsewhere (Kramer and De Kort, 1976a). *Manduca sexta* larvae (Vth instar) were obtained from the Laboratory of Animal Physiology, Wageningen, The Netherlands. To inhibit JH-specific esterases, centrifuged haemolymph was incubated at 4°C for 20 min with 10^{-4}M paraoxon.

Gelfiltration

Haemolymph was treated with paraoxon and diluted, if necessary, with 0.1 M phosphate buffer pH 7.5. After incubation with ^{3}H-JH I (10^{-6}-10^{-8}M, $\pm$ 200,000 dpm) for 30 min at 4°C, the haemolymph was applied to a 1.5 x 30 cm column of Sephadex G-100 at 4°C and eluted with 0.1 M phosphate buffer pH 7.5. The flow rate was 25 ml/hr and 1 ml fractions were collected. Blue Dextran 2000 was used to determine the void volume. The elution patterns of the proteins were determined by monitoring the eluant at 254 nm using a UV absorption meter. The radio-labeling pattern was determined by mixing 0.5 ml of each fraction with 10 ml scintillation solution and counting the radioactivity as described previously (Kramer and De Kort 1976a). To separate the macromolecular fraction, haemolymph was applied to a 1.5 x 90 cm column of Sepharose 4-B at 4°C and eluted with 0.1 M phosphate buffer pH 7.5. The flow rate was 20 ml/hr and 2-5 ml fractions were collected.

Electrophoresis

Polyacrylamide gel electrophoresis was carried out using slabs as described previously (Kramer and De Kort, 1976a) and preparative discs (1.5 x 10 cm). The slabs and discs were made of 7% gels. Proteins were stained as described elsewhere (Kramer and De Kort, 1976a). Lipoproteins were stained with 0.5% Sudan Black B in acetone-acetic acid-water (20:15:65,v/v) overnight and destained with acetone-acetic acid-water (20:15:65, v/v) (Maurer, 1971).

Gel slices were counted for radioactivity in 10 ml scintillation mixture, containing 0.3% PPO in Triton X-100/Xylol (25:75,v/v) after overnight incubation at 60°C in 1 ml 30% H_2O_2.

Proteins were extracted from the gel discs by means of diffusion. Gel slices were incubated in 1.5 ml 0.1 M phosphate buffer pH 7.5 overnight in a shaking bath at 4°C. The gel slices were removed and the solution used for the determination of the binding capacity. Cellulose-acetate electrophoresis was performed according to Kohn (1964).

Charcoal adsorption assay

In order to minimize the adsorption of the JH-lipoprotein complex to the charcoal, activated charcoal was washed with 6N HCl, followed by water until the washings were neutral and then suspended in water (ca. 50 mg/ml) (Rapaport *et al.*, 1975). For routine analysis, the binding assay mixture consisted of 3-66 x 10^{-7}M-JH I and 5-75 ul haemolymph (10^{-4}M paraoxon treated) in a total volume of 500 ul 0.1 M phosphate buffer pH 7.5. After a 30 min binding period at 4°C, unbound JH I was removed by adding 100 ul charcoal suspension. The mixture was thoroughly agitated (Vortex mixer), allowed to stand for 10 min and centrifuged at 8,000 g for 1 min in an Eppendorf 3200 centrifuge. To determine the amount of bound JH I, an aliquot of the supernatant was assayed for radioactivity by scintillation counting.

Diethylaminoethyl (DEAE)-cellulose filter binding assay

The filter assay described by Kramer *et al.* (1976) was used.

Thin-layer chromatography

JH I and its metabolites were identified by TLC as described previously (Kramer and De Kort, 1976a).

Determination of JH III-esterase activity

JH III-esterase activity was measured as described elsewhere (Kramer and De Kort, 1976b).

Degradation of ^{3}H-JH I by haemolymph JH-specific esterases in vivo and in vitro

In vivo. Long day and short day beetles (5 ♀♀ per group) were injected with ^{3}H-JH I (about 150,000 dpm) dissolved in a volume of 1 ul acetone. After injection the beetles were kept at 25°C for the prescribed time. Four ul haemolymph were collected from each beetle. Juvenile hormone and its metabolites were extracted from the pooled haemolymph samples and identified by TLC (Kramer and De Kort, 1976a). The radioactivity was expressed per bloodvolume of each beetle (75 ul in LD beetles and 63 ul in SD beetles, Dortland and De Kort, in preparation).

In vitro. 250 ul haemolymph from long day and short day beetles was incubated with ^{3}H-JH I (about 150,000 dpm) at 25°C. At the prescribed times 50 ul haemolymph samples were taken and extracted as described above.

RESULTS

Sephadex chromatography and electrophoresis of haemolymph proteins

Figure 1a shows the radioactivity and elution pattern of paraoxon inhibited haemolymph from 8 day old short day beetles, containing 10^{-7}M ^{3}H-JH I. Two major UV absorbing peaks were resolved, a macromolecular fraction and a low molecular weight absorption peak. The macromolecular fraction eluted in the exclusion volume, which indicates that the proteins possess an apparent molecular weight > 10^{5}. All the radioactivity was associated with the macromolecular fraction, containing the yellow colour of the haemolymph due to carotenes associated with the lipoproteins. TLC analysis revealed that the radioactivity in the macromolecular fraction was due to unchanged Cecropia JH I. Approximately 80% of the radioactivity applied to the column was recovered in the first 60 fractions. A control experiment with an aqueous solution of ^{3}H-JH resulted in a recovery of approximately 30% in the same eluent volume (Fig. 1b). Identical radioactivity and elution patterns were obtained with haemolymph from beetles reared under long day conditions. Haemolymph inhibited with 10^{-3}M di-isopropylfluorophosphate (DFP) overnight showed two peaks of radioactivity, the first was associated with the macromolecular fraction, the second eluted in fractions 40 to 50. TLC analysis revealed that the radioactivity in the macromolecular fraction was due to unchanged Cecropia JH I. The radio-activity in the second fraction consisted of JH acid. Figure 1c shows that JH I is bound unspecifically to bovine serum albumin (BSA) (10 mg per incubation mixture).

In order to check the specificity of the binding to the macromolecular fraction, haemolymph of the beetle was incubated with two JH analogues, an ethylester without the 10,11-epoxide group (ZR 512) and an isopropylester (ZR 515), and palmitic acid. The elution patterns on Sephadex G-100 were identical to the pattern presented in Fig. 1a. In agreement with Kramer *et al.* (1974). Fig. 1d shows that in the haemolymph of Vth instar larvae of *Manduca sexta* JH is not bound to the macromolecular fraction, containing lipoproteins, but to a low molecular weight protein. Figure 2 illustrates that haemolymph applied to a Sepharose-4B column results in 3 major UV absorbing peaks. The radioactivity

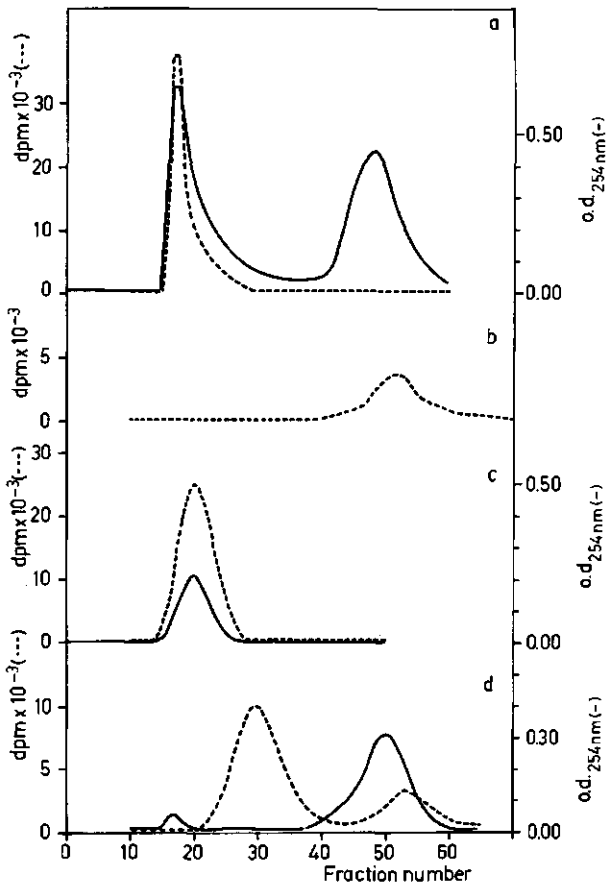

Fig. 1. Gel filtration patterns obtained by chromatography on Sephadex G-100 in 0.10 M phosphate buffer, pH 7.5 of a) 0.10 ml 10^{-4} M paraoxon-treated haemolymph from 8 day old short day beetles containing 10^{-7} M ^{3}H-JH I (about 200,000 dpm), b) 10^{-7} M ^{3}H-JH I (about 200,000 dpm), c) 0.5 ml BSA (20 mg/ml) containing 10^{-7} M ^{3}H-JH I (about 200,000 dpm) and d) 0.10 ml 10^{-4} M paraoxon-treated haemolymph from Vth instar larvae of *Manduca sexta* containing 10^{-7} M ^{3}H-JH I (about 200,000 dpm). See methods for experimental details.

was associated with the first which contained the yellow colour of the haemolymph. Electrophoresis of this binding fraction revealed that only a few proteins were present (Fig. 3b, bands 1, 2, 3, 4 and 7). The macromolecular fraction from the Sephadex G-100 column was further analysed by slabgel polyacrylamide electrophoresis (Fig. 3b). The gels were stained with Coomassie Blue and Sudan Black. Comparison with total haemolymph (Kramer and De Kort, 1976b) showed that most of the proteins were still present in the macromolecular fraction from the Sephadex G-100 column. Five of the bands appeared to be lipoprotein (Fig. 3b, bands 1, 2, 3, 7, and 16). The lipoprotein bands 1, 2, and 16 (Fig. 3b) were associated with the yellow pigments of the haemolymph. However, most of the yellow pigments remained on top of the spacergel. When the macromolecular fraction labelled with ^{3}H-JH I was subjected to polyacrylamide gel electrophoresis, the radioactivity was found almost exclusively on top of the spacergel (Fig. 3a). To ensure that JH I did not disso-

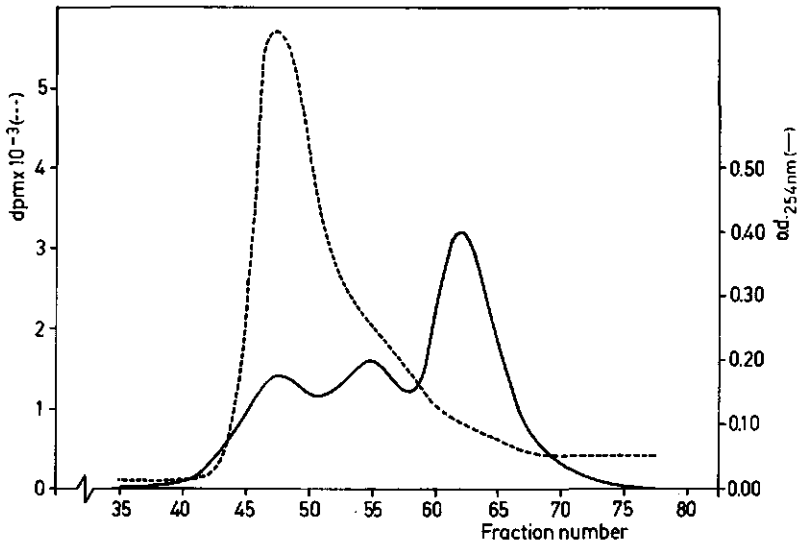

Fig. 2. Gel filtration pattern obtained by chromatography of 0.2 ml 10^{-4} M paraoxon-treated haemolymph from 8 day old short day beetles containing 10^{-7} M ^{3}H-JH I (about 200,000 dpm) on Sepharose 4-B in 0.1 M phosphate buffer, pH 7.5.

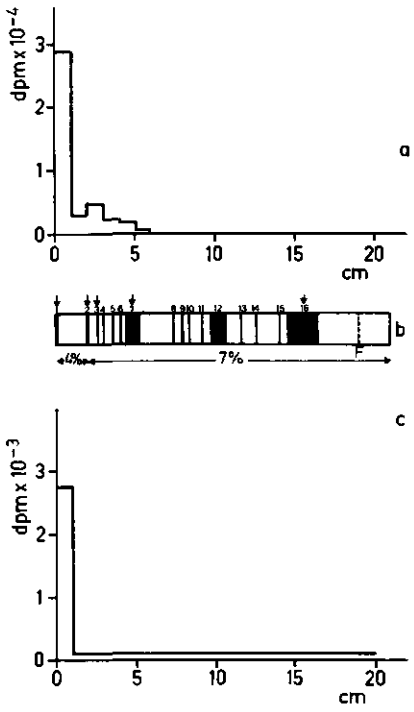

Fig. 3. a) Radioactivity pattern of a 0.1 ml macromolecular fraction of haemolymph from 8 day old short day beetles (Sephadex G-100) containing 10^{-7} M ^{3}H-JH I (about 200,000 dpm) and subjected to polyacrylamide slab gel electrophoresis. b) Diagrammatic representation of proteins and lipoproteins (indicated with arrows) in the macromolecular fraction used in Fig. a. c) Binding of ^{3}H-JH I by haemolymph proteins of 8 day old short day beetles extracted from preparative polyacrylamide gel slices.

ciate from the macromolecular fraction during electrophoresis, the run was performed on cellulose-acetate. With this technique two lipoprotein bands were found, one on the anionic and one on the cationic side of the application line (De Loof and De Wilde, 1970). After electrophoresis of JH I labelled haemolymph on cellulose-acetate strips, the radioactivity was recovered from the application line and not associated with the two lipoprotein bands. Apparently JH dissociates from its macromolecular carrier protein during electrophoresis. Therefore, another approach had to be followed to demonstrate which lipoprotein bands are involved in JH binding. Haemolymph (pretreated with paraoxon) was subjected to preparative polyacrylamide gels and subsequently the unstained gels sliced and extracted with buffer as detailed in Materials and Methods. The gel extracts were tested for JH binding capacity using the charcoal adsorption assay. Figure 3c shows that the lipoprotein band on top of the spacer gel is the only band capable of binding JH. Identical results were obtained with the macromolecular fractions eluted from the Sephadex G-100 and the Sepharose 4-B columns.

Binding assay procedures

Detection of the lipoprotein-JH complex using Sephadex column chromatography is a tedious and time consuming method. Therefore, two other binding assays were tested: (1) DEAE-cellulose filter paper disc adsorption assay, and (2) charcoal adsorption assay. The filter paper assay was originally developed for steroid hormone receptors (Santi *et al.*, 1973) and modified for the JH carrier protein complex in the haemolymph of *Manduca sexta* (Kramer *et al.*, 1976). This method could not be used for the lipoprotein-JH complex in the Colorado potato beetle. After washing with Triton X-100 no radioactivity was retained on the filter discs. In contrast, haemolymph of Vth instar larvae of *Manduca sexta* did not show high binding under the same experimental conditions. Method (1), a common procedure used in vertebrates, allowed satisfactory detection of the hormone-lipoprotein complex. The charcoal was pretreated with 6 N HCL to avoid adsorption of the complex to the charcoal (Sherline *et al.*, 1974). The binding values determined with this method were in agreement with those determined by gel filtration on Sephadex G-100. The binding capacity of haemolymph of adult beetles reared under different photoregimes did not show any significant differences. Figure 4 represents a saturation curve and a Scatchard plot (Scatchard, 1949) of the interactions between JH I and haemolymph lipoproteins of short day beetles. From the slope of the line an apparent dissociation constant of $\pm$ 3.6 x 10^{-5}M can be calculated.

Biological role of lipoproteins

To investigate the biological role of these low specific lipoproteins, *in vitro* experiments were designed to test whether the lipoproteins provide protection against JH-specific esterases present in the haemolymph. Two approaches were followed. Firstly, varying amounts of the Sephadex G-100 macromolecular fractions were added to a JH III-esterase incubation mixture, containing 0.4 nmoles ^{3}H-JH III and 0.1 ul haemolymph from 8 day old short day beetles in 1.0 ml 0.1 M phosphate buffer pH 7.5. The macromolecular

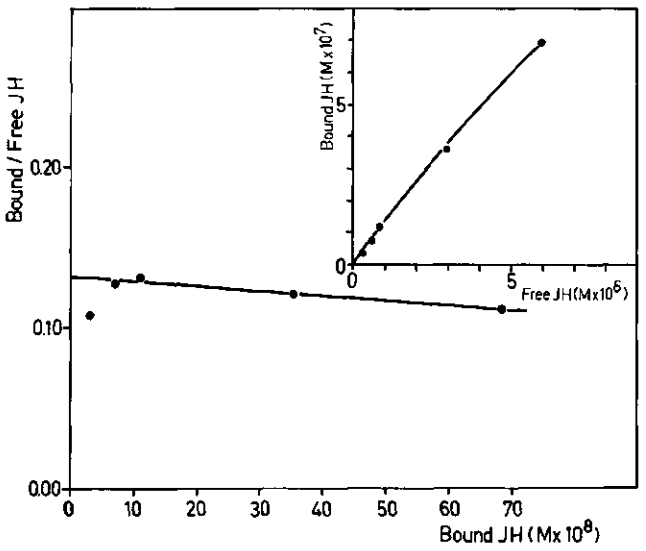

Fig. 4. Scatchard plot analysis of ^{3}H-JH I binding to haemolymph from 8 day old short day beetles. The saturation curve is shown in the insert. The incubation mixture consisted of 25 µl 10^{-4} M paraoxon-treated haemolymph and different concentration of ^{3}H-JH I in 0.5 ml 0.1 M phosphate buffer, pH 7.5. See methods for further experimental details.

fraction was either derived from haemolymph of 8 day old short day or from 4 day old long day beetles (Table 1). Secondly, varying amounts of haemolymph from long or short day beetles were added to an incubation mixture containing JH I complexed to the macromolecular fraction of the same haemolymph. All the free hormone was removed with activated charcoal (Fig. 5).

Table 1 illustrates that addition of increasing amounts of the macromolecular fraction results in an increasing protection of the hormone against JH-specific esterases. It should be noted that the amount of protein added with the carrier lipoprotein fraction is extremely high as compared with the amount of protein in the haemolymph, containing the

Table 1. The effect of addition of carrier lipoproteins derived from haemolymph of long and short day beetles on the hydrolysis of JH III by JH-specific esterases present in 0.1 µl haemolymph from 8 day old short day beetles.

	% Protection of JH hydrolysis	
amount of carrier lipoprotein fraction (µl)	8 SD (1.5 mg protein/100 µl)	4 LD (2.5 mg protein/100 µl)
0	0	0
5	6	-
10	8	8
25	58	40
50	82	81
75	91	93

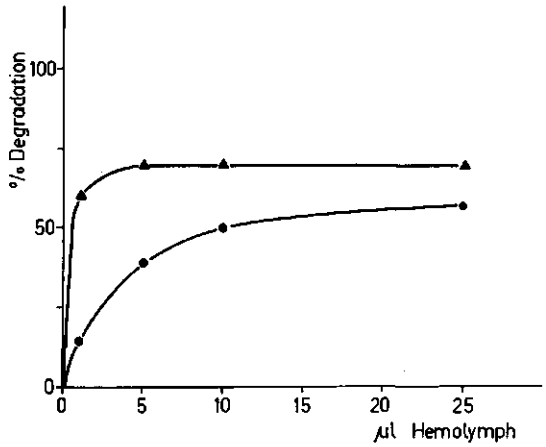

Fig. 5. Degradation of 0.3 x 10^{-6}M free ^{3}H-JH I (▲——▲) and 0.3 x 10^{-6}M ^{3}H-JH I bound to carrier lipoproteins present in the macromolecular fraction of 32 µl haemolymph (4 LD) obtained by chromatography on Sephadex G-100 (●——●) by increasing amounts of haemolymph JH-specific esterases (4 LD). Incubation time: 10 min.

JH-specific esterases. In contrast, 1 mg BSA does not protect JH from being degraded by JH-specific esterases.

Figure 5 demonstrates that JH bound to carrier lipoproteins is less degraded by haemolymph JH-specific esterases than the free hormone, especially at the lower concentrations of the esterases. In the control experiment maximal degradation of JH is achieved after addition of more than 5 ul haemolymph to the incubation mixture. This may indicate that some of the free hormone is complexed to carrier lipoproteins present in the added haemolymph. An identical experiment performed with haemolymph possessing a high JH-specific esterase activity (8 SD) resulted in higher maximal degradation levels. The results concerning the binding of JH to carrier lipoproteins do not satisfactorily explain the recent findings of Kramer *et al.* (1977) that although the half-life of JH in beetles reared under different photoregimes is similar, there exist very large differences in the level of JH-specific esterase in the haemolymph. An endeavour to resolve this problem two additional experiments were performed. The half-life of exogenous JH I was measured *in vivo* and *in vitro* in the haemolymph of both long and short day beetles. Figure 6 shows that JH I is degraded extremely rapid *in vitro*. The half-life of JH in the haemolymph is calculated to be 4½ and 9 min in short and long day beetles respectively. The results presented in Table 2 illustrates that the injected radioactivity can only be recovered partially from the haemolymph. At zero time (which is actually about 90 sec after injection) approximately 50% of the total injected radioactivity can be recovered, while after 30 min only 6% of the radioactivity can be detected in the haemolymph of both long and short day beetles.

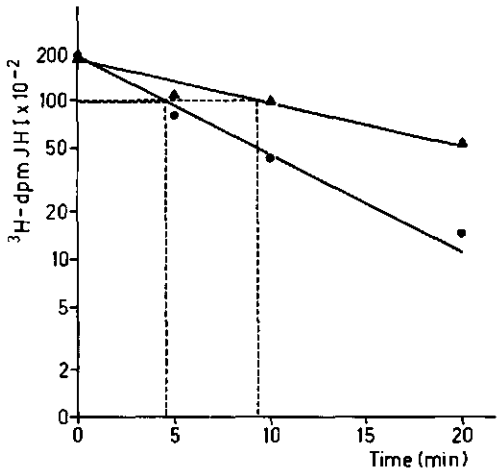

Fig. 6. Changes in concentration of ^{3}H-JH I in the haemolymph *in vitro* as a function of time. Symbols: ●——● haemolymph from 8 day old short day beetles, ▲——▲ haemolymph from 4 day old long day beetles. See methods for experimental details.

Table 2. Distribution of injected JH I in the haemolymph of the Colorado potato beetle.

Time after injection (min)	4 LD		8 SD	
	% of injected radioactivity recovered per total blood-volume	% of injected radioactivity recovered as ^{3}H-JH I per total blood-volume	% of injected radioactivity recovered per total blood-volume	% of injected radioactivity recovered as ^{3}H-JH I per total blood-volume
0	50.9	36.7	53.8	26.9
5	31.7	24.4	14.9	7.6
10	18.1	12.3	17.0	8.0
30	6.1	3.4	6.3	2.5

DISCUSSION

In the literature concerning JH carrier-proteins two types of proteins are indicated as candidates for JH binding: (1) lipoproteins (Whitmore and Gilbert, 1972; Trautmann, 1972; Emmerich and Hartmann, 1973; Kramer *et al.*, 1976), and (2) low molecular, high-affinity binding proteins (Kramer *et al.*, 1974; Hammock *et al.*, 1975; Nowock *et al.*, 1975). The results described in this paper clearly show that high-affinity carrier-proteins do not occur in the Colorado potato beetle. In agreement with findings in *Hyalophora* and *Locusta migratoria* (Whitmore and Gilbert, 1972; Emmerich and Hartmann, 1973), high molecular weight lipoproteins are found in the haemolymph of *Leptinotarsa* which are able to

bind JH. The binding characteristics of these lipoprotein(s) for JH differ significantly from those of the low molecular weight carrier-proteins found in *Manduca sexta* and in some other insect species. Figure 4 clearly shows that the affinity of the lipoproteins for JH is rather weak. An apparent dissociation constant of $\pm$ 3.6 x 10^{-5}M could be calculated. Moreover, the binding is of low specificity for JH and JH analogues and palmitic acid are also bound. The binding capacity is, however, high.

These results, together with the fact that bovine serum albumine also binds JH (Fig. 1c) lead to the question whether these lipoproteins play any role in JH regulation. The results given in Table 1 and confirmed in Fig. 5 show that in contrast to bovine serum albumine the haemolymph lipoprotein fraction protects JH to some extent from being degraded in a system containing potent JH-esterases. The very short half-life of JH in haemolymph *in vitro* (Fig. 6) is in agreement with the low rate of protection. This together with the binding characteristics makes it very likely that these carrier lipoproteins are of minor importance in the total regulation of the JH titre in the Colorado potato beetle. The binding results described in this paper do not provide an explanation for the finding that the half-life of injected JH I in long day and short day beetles is the same (Kramer *et al.*, 1977), despite the fact that a much higher JH-esterase activity occurred in haemolymph from 8 day old short day animals (Kramer and De Kort, 1976a). However, the experiments concerning the *in vitro* and *in vivo* degradation of JH by haemolymph JH-specific esterases do provide an explanation for this discrepancy. After injection of ^{3}H-JH I most of the radioactivity could not be recovered from the hemolymph within a short time (Table 2). Therefore it is possible that the free hormone which is not bound to the haemolymph carrier lipoproteins sticks to lipophilic parts of general body tissues, membranes etc., where it is perhaps less susceptable to attack by haemolymph JH-specific esterases. It is only during stages when a low titre of JH hormone is required, for instance in beetles reared under a short day photoregime, that high esterase activities are found. These elevated levels of JH-specific esterase occur in order to eliminate all the free and bound hormone before the insect enters diapause. The rapid degradation of JH by haemolymph JH-specific esterases *in vitro* and *in vivo* suggests that the high titres of JH present in the blood of long day beetles must be maintained by high rates of JH synthesis and release by the corpora allata.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. F.J. Oppenoorth for valuable advice and criticism, to Dr. J. de Wilde for his interest and to Dr. W. Mordue (London) and Dr. H. Schooneveld for critically reading of the manuscript. Dr. W. Mordue corrected the English text. The co-operation of Mrs. M. Wieten in part of the experiments was highly appreciated. This work was supported by the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

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AGE-DEPENDENT CHANGES IN CORPUS ALLATUM ACTIVITY IN VITRO IN THE ADULT COLORADO POTATO BEETLE, *LEPTINOTARSA DECEMLINEATA*

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Summary

The synthesis of JH by isolated corpora allata (CA) of the Colorado potato beetle was determined *in vitro* by means of a short-term, radiochemical assay, throughout the adult life cycle and under different photoperiodic conditions. High activities were observed during reproduction in long day and post-diapause beetles. In beetles reared under short day conditions intermediate activities were found during prediapause until day 6 after emergence; thereafter and during diapause CA activity was barely detectable. It is postulated that CA activity is one of the main factors controlling the JH titre.

During the life cycle of an insect, dramatic changes occur in the JH titre of the haemolymph (1-3). In the Colorado potato beetle JH is rapidly degraded by haemolymph JH-specific esterases (4,5) while binding by carrierproteins (lipoproteins) was considered to give only minor protection against breakdown (6). The rapid degradation of the hormone together with the occurrence of high titres in the haemolymph during reproduction (7) suggests high rates of JH synthesis and release by the corpora allata. Until now the state of activity of the corpora allata in the adult Colorado potato beetle was based only on indirect criteria, namely on the measurements of corpus allatum volumes (8). In this study a close positive correlation was found between changes in corpus allatum volume throughout adult life and changes in the JH titre as determined by de Wilde *et al.* (7).

With the development of a short-term *in vitro* method for assaying the synthesis and release of JH by the CA (9, 10) it has become possible to measure the activity of the glands. In *Schistocerca gregaria*, *Periplaneta americana* and *Diploptera punctata* extensive studies have been performed to relate the synthetic activity of the corpora allata to the events in oocyte development (11-13). These studies revealed that large changes in JH synthesis occur during the course of sexual maturation in the species.
In the present investigation, the *in vitro* rate of synthesis of JH by CA of the Colorado potato beetle has been determined during the adult life cycle.

MATERIAL AND METHODS

Insects

Adults of the Colorado potato beetle, *Leptinotarsa decemlineata* were reared at 25°C on fresh potato leaves under two different photoregimes as described previously (14). Adult beetles were collected within 16 hr after emergence from the soil and kept until the desired age (young adults emerge from the soil at the second day of the imaginal stage). Newly ecdysed adults (0-6 hr old) were obtained by digging pupae from the soil one day before pupal-adult ecdysis and transferring them to moist filter paper.

Measurement of JH biosynthesis by CA

Corpora allata with corpora cardiaca attached were dissected from unanaesthetized beetles under Ringer solution (15). Three to five pairs of CA were incubated in 100 ul Millipore-filtered MEM medium (Gibco Bio-Cult, Scotland; with Hank's Salts, with L-glutamine and without Sodiumbicarbonate), containing 20 mM Hepes buffered at pH 6.5 and fortified with 20 mg/ml Ficol (Sigma). Methyl-^{14}C methionine (Radiochemical Centre, Amersham, Bucks) was incorporated into the incubation medium at final specific radioactivities which ranged from 30-37 mCi/mmol. Incubations were carried out in the dark at 30°C with vigorous shaking for 3-5 hr. Preliminary experiments showed that the hormone is not stored to any appreciable extent within the glands (see also 10). Therefore, the medium was extracted together with the glands. In all samples, the amounts of radiolabeled JH and methylfarnesoate were determined; the procedure for the extraction, separation and quantitation of the incubation products using standard thin-layer chromatography (tlc) and liquid scintillation spectrometry were as described by Tobe and Pratt (10) and Pratt and Tobe (9). Radioactivity was determined in a Nuclear Chicago Mark III liquid scintillation spectrometer.

RESULTS

Corpus allatum activity in different tissue culture media

As a preliminary investigation corpus allatum activity was measured in three different commercial tissue culture media, TC 199, Grace's and MEM (Gibco Bio-Cult). The pH of the media was adjusted to 7.0. Table 1 shows that MEM culture medium results in the highest JH synthetic activity. Therefore in the standard procedure the glands were incubated in MEM medium.

Effect of pH on JH synthesis

The influence of extra-glandular pH on the rate of synthesis of JH was investigated by adjusting the pH of the medium over the range 6.0 to 8.5 (Fig. 1).

Table 1. JH synthetic capacity of corpora allata from 2 day old long day Colorado potato beetles in 3 different tissue culture media. Three pairs of corpora allata were used per incubation.

	JH synthesis (pmol hr^{-1} pair CA^{-1})		
	TC 199	Grace's	MEM
1.	0.54	0.94	2.20
2.	0.65	1.17	2.16

It can be seen that the synthetic activity of the glands is more or less constant between pH 6 and 7.5. Higher pH values results in a decrease of the synthetic activity.

The results indicate that the synthetic activity of the glands may be determined over a broad pH range. However, in the standard procedure the glands were incubated at pH 6.5.

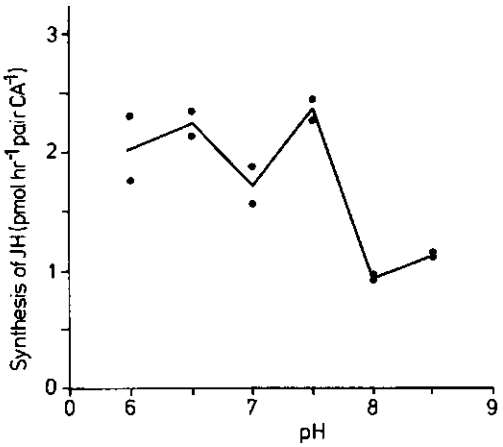

Fig. 1. Influence of H^+ concentration on the rate of synthesis of juvenile hormone. In each experiment three pairs of corpora allata from 2 day old long day Colorado potato beetles were incubated for 3 hr in medium containing [methyl-^{14}C] -methionine. The relationship shown in fig. 1 is the arithmetic mean of two separate experiments.

Time Course of JH synthesis

To investigate the linearity of JH biosynthesis during the course of time separate incubations were terminated at various intervals up to 4 hr. Fig. 2 shows that the rate of JH synthesis is linear over a period from 1 to 4 hr and that a lower rate of release occurs during a period of approximately 1 hr.

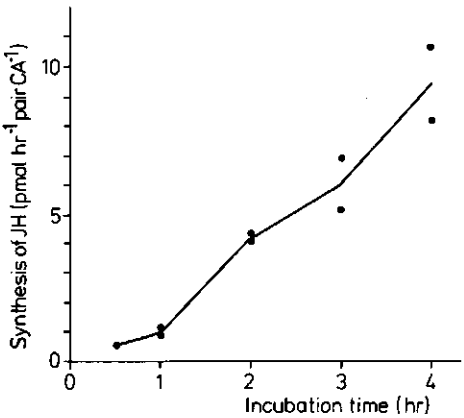

Fig. 2. Progress of synthesis of radiolabeled JH from three pairs of corpora allata from 2 day old long day Colorado potato beetles. Each point represents an individual determination.

Corpus allatum activity during the adult life cycle

After development of the conditions necessary for the quantitative assay of corpus allatum activity the rate of synthesis of JH was estimated in beetles, reared under different physiological conditions. Unless otherwise stated all estimations were carried out with corpora allata from females. The activities were compared in beetles reared under long day and short day photoregimes, since under these conditions significant differences in haemolymph-JH titres have been shown (7).
At each point in the life cycle CA activities were measured at least in triple, using 3-5 pairs of corpora allata per incubation. The results are shown in fig. 3. The curves are drawn through the arithmetic means of the different determinations.

DISCUSSION

The results presented in this paper show that the short term method for assaying the synthesis and release of JH by the corpora allata is suitable to measure the biosynthetic activity of CA from adult Colorado potato beetles. In spite of the fact that the biosynthetic rates in the beetle are one magnitude or more lower than those measured in three other insect species (i.e. in *S. Gregaria* (11), in *P. americana* (12), and in *D. Punctata* (13), this method permits quantitative measurements under our conditions.

The CA from the Colorado potato beetle synthesize JH at a linear rate after a lag period of approximately 1 hr for at least 4 hr after extirpation. This supports the idea that the rate of synthesis measured *in vitro* is a reflection of that *in vivo*.

Isolated CA are capable of synthesizing JH at the same rate over a wide range of pH's. This in contrast to *Schistocerca gregaria* (10), in which insect a peak was found at pH 8.

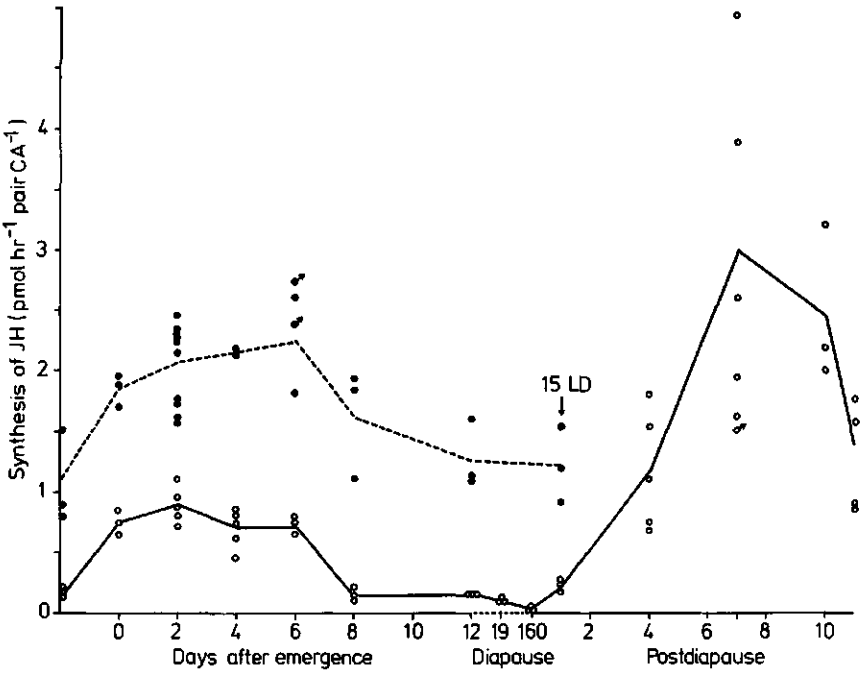

Fig. 3. Rates of *in vitro* synthesis of JH by corpora allata from adult female beetles reared under different physiological conditions. Incubation conditions were as described in Materials and Methods. Each point represents an individual determination, using 3-5 pairs of corpora allata per incubation. Symbols: ——— short day females; - - - - long day females; ♂ short day females; ♂ long day males.

The nature of the JH produced was not analyzed. Studies by Trautmann *et al.* (16) showed that JH III is the only hormone which occurs in total body extracts of reproductive adults of the Colorado potato beetle. Recent determinations in haemolymph extracts of adult beetles partially confirmed this finding (Schooley, personal communication). Besides the occurrence of JH III small amounts of JH I and JH II were detected in a few adult stages. In all the incubations with CA from LD and SD pre-diapause beetles the intermediate methylfarnesoate could not be detected. However, in post-diapause beetles with active CA some methylfarnesoate (as determined by co-chromatography with synthetic methylfarnesoate in a tlc system) has been observed (maximally 0.2 pmol hr^{-1} pair^{-1}).

This paper shows remarkable differences in JH synthetic activity of CA from beetles reared under different photoregimes (fig. 3). Under SD conditions CA activity is low at adult ecdysis, but rises within two days to an intermediate level at adult emergence which remains constant until day 6. Thereafter the CA activity declines to a low level and the beetle enters diapause. With regards to the haemolymph JH titre as measured by de Wilde *et al.* (7) the increase in JH during the first two days after adult ecdysis under SD conditions correlates well with the CA activity measured *in vitro*. After day 3, only traces of JH are shown in the haemolymph, while CA activity is still intermediate This difference can possibly be explained by the rapid increase in JH-specific esterases

in the haemolymph (4).

Under LD conditions CA activity shows an intermediate level at adult ecdysis. The level increases during the first few days and then remains constant until day 6; afterwards a slight decrease in CA activity occurs (fig. 3). The JH titre curve shows a more or less identical course, except that at adult ecdysis only traces of JH are shown. In agreement with these findings JH esterase activity is relatively low in adults reared under LD conditions (4). During diapause CA activity remains low and after termination of diapause the activity increases rapidly to a high level at day 7. The mean of this level is higher than the maximal activity in reproducing beetles under LD conditions, although large variations were found between different incubations. An explanation for these large variations might be large individual differences in the onset and rate of reactivation of the CA. In agreement with this high CA activity, the JH titre and the JH concentration as measured by gas liquid chromatography show corresponding higher values than those measured in normal reproducing LD beetles (Schooley, personal communication). The biological significance of this phenomenon is not clear. CA activity of LD and post-diapause males did not differ significantly from females.

Changes is size of CA have often been observed to be associated with differences in the synthetic activity of the glands. In *S. gregaria* however no correlation was found between CA volume and JH synthetic capacity (17,18) while in *D. punctata* a good correlation was observed (13). In the Colorado potato beetle changes in CA activity (fig. 3) correlate fairly well with changes in CA volume (8). However, the first days after adult emergence are exceptional: no differences in CA volumes exist between beetles reared under different photoregime, whereas the CA activity differs by more than twofold.

We tried to make a JH balance sheet for reproducing LD beetles, using the CA activity data presented in this paper, the half life of JH measured *in vivo* (5) and the amount of endogenous hormone present in the haemolymph. A theoretical CA activity of 9 pmol/hr/per pair CA can be calculated, if we consider a half life of JH of 30 min and a endogenous JH content of 3.6 ng (Schooley, personal communication). This means that with the short-term radiochemical *in vitro* incubation assay we measure approximately 20% of the theoretical value. However, it should be stressed that the half life of JH measured by injection of exogenous JH (5) is not necessarily a true reflection of the half life *in vivo*.

Apparently, the conditions used in the short-term *in vitro* incubation assay are not ideal. Nevertheless the differences in CA activities measured *in vitro* are most likely a reflection of the changes in synthetic activity *in vivo*. It can therefore be concluded that this technique has provided a tool in obtaining a better insight in the regulation of the JH titre. The results presented in this paper support the idea that the control of the activity of the CA is probably of prime importance in the regulation of the JH titre in the Colorado potato beetle.

ACKNOWLEDGEMENTS

The author wishes to thank Dr. G.E. Pratt of the Agricultural Research Council, University of Sussex, Brighton, for the opportunity to learn the *in vitro* CA assay at his laboratory, for critically reading the manuscript and correcting the English text.

Thanks are also due to Drs. C.A.D. de Kort, J. de Wilde, F.J. Oppenoorth and H. Schooneveld for critically reading the manuscript.

The investigations were supported (in part) by the Foundation for Fundamental Biological Research (BION), which is subsidized by the Netherlands Organization for the Advancement of Pure Research (ZWO).

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General discussion

Juvenile hormones play a key role in the development of insects from embryo to adult. Sharp changes occur in the titre of juvenile hormone of the haemolymph during the life cycle of an insect (Fain and Riddiford, 1975; Hsiao and Hsiao, 1977; de Wilde *et al.*, 1971). As indicated in the general introduction, the titre can be subject to several regulatory mechanisms.

The results presented in Paper 3 show that two main pathways of juvenile hormone degradation, established for other insects (Slade and Zibitt, 1972; Ajami and Riddiford, 1973, Erley *et al.*, 1975; Slade and Wilkinson, 1974; Slade *et al.*, 1976) also occur in the Colorado potato beetle. The primary degradation products from esterase and epoxyhydratase activity, the acid and the diol of the juvenile hormone, were detected *in vivo*. In the haemolymph of many insects, including the Colorado potato beetle, juvenile hormone is solely degraded by esterases (Ajami Riddiford 1973; Paper 1). Esterase activity against the hormone in the haemolymph undergoes striking changes during the life-cycle of the beetle (Paper 1, Fig. 5). Activity of juvenile hormone esterase correlates fairly well with the titre of juvenile hormone found by de Wilde *et al.* (1968).

The short half-life of exogenous juvenile hormone measured *in vivo* (Paper 3) and *in vitro* (Paper 5), and inhibition studies with Triton X-100 suggest the major role of esterases in breakdown of the hormone.

Juvenile hormone I (C_{18}JH) and juvenile hormone III (C_{16}JH) were inactivated according to similar kinetics, whereas two juvenile hormone mimics, containing two different esters were not hydrolysed under the same conditions. Though the range of analogues was limited, one may conclude that the esterases in haemolymph involved in hydrolysis of juvenile hormone are highly specific. Inhibition studies with diisopropylfluorophosphate (DFP), an inhibitor used to distinguish between general carboxylesterases and esterases specific to juvenile hormone (Sanburg *et al.*, 1975), showed that during all developmental stages juvenile hormone esterases are specific; this contrasts with larvae of *Manduca sexta*, in which juvenile hormone esterases are specific only in the 5th instar (Sanburg *et al.*, 1975).

The rapid changes in activity of juvenile hormone suggests the existence of a control mechanism. There are few published data on this topic. Whitmore *et al.* (1972, 1974) induced carboxylesterases in pupae of Hyalophora gloveri after treatment with juvenile hormone. These induced esterases were sensitive to DFP. I introduced hormone, altered the photoperiod, removed the corpora allata and head ligated beetles to investigate whether juvenile hormone esterases were regulated directly or indirectly by the hormone (Paper 4). Removal of corpora allata from beetles kept under short day, transferrence from short day to long day, or treatment of young beetles before diapause with juvenile hormone all prevented high activity of juvenile hormone esterase. Treatment of diapau-

sing beetles with juvenile hormone or juvenile hormone analogues significantly increased activity of juvenile hormone esterase. These effects might be associated with the ability of the fat body (the source of juvenile hormone esterases) to react to the hormone titre (indirectly) at different physiological stages. It is very likely that with short days a low titre of juvenile hormone during the first days after adult emergence is necessary for the induction. Hormone supplied to diapausing beetles with corpora allata removed or head ligated suggested that juvenile hormone may act through the cerebral neurosecretory system. All the experiments together suggest that the activity of juvenile hormone esterases in haemolymph is most likely controlled indirectly by the hormone, through a centre in the brains (hormostate). The activity of esterase is probably dependent on the sensitivity of this hormostate and on the titre of juvenile hormone.

A protein of low molecular weight has been found in several insects with high affinity for juvenile hormone, high specificity, low capacity and protective against general carboxyesterases (Kramer *et al.*, 1976). It was not detected in the Colorado potato beetle. In agreement with findings in *Hyalophora gloveri* and *Locusta migratoria* (Whitmore and Gilbert, 1972; Emmerich and Hartmann, 1973) lipoproteins of high molecular weight were detected in the haemolymph of the beetle, capable of binding juvenile hormone and analogues. The binding characteristics of these lipoproteins showed low affinity ($K_d \approx 10^{-5}$M), low specificity and high capacity. The low protection for complexed juvenile hormone (Paper 5, Fig. 5) and the binding characteristics suggest that these lipoproteins are of minor importance in the regulation of the hormone titre in the beetle.

The rapid degradation of juvenile hormone by juvenile hormone esterases from haemolymph *in vitro* and *in vivo* suggests that the high titres of hormone in haemolymph with long day must be maintained by high rates of synthesis and release by the corpora allata. Paper 6 examines these processes in the corpora allata. There were significant differences between beetles at different stages and reared under different photoregimes. The curves of synthesis parallel reasonably the titre of juvenile hormone as measured by de Wilde *et al.* (1968). Except with short day, for which there were only traces of hormone after Day 3, while the corpus allatum activity is still intermediate. This difference can possibly be explained by the rapid increase in juvenile hormone esterases in haemolymph (Paper 1, Fig. 5). The calculated balance (Paper 6) suggested that synthesis by corpus allatum *in vitro* was not identical with that *in vivo*. Perhaps conditions *in vitro* did not reflect natural conditions. Nevertheless, the differences in synthesis by corpus allatum *in vitro* probably reflect changes *in vivo*.

Thus, the titre of juvenile hormone in the Colorado beetle is the result of synthesis and release of hormone by the corpora allata and enzymic degradation of hormone by esterases mainly in haemolymph. Binding and subsequent protection against haemolymph esterases by carrier lipoproteins are of minor influence. Control of synthesis and release from the corpora allata probably primarily governs the titre of juvenile hormone. Only during stages when a low titre of juvenile hormone was required, for instance in beetles reared with a short day were activities of juvenile esterase high, so eliminating free and bound hormone, before the insects entered diapause.

This pattern is not valid for all insects. As indicated in the Papers 3 and 5, large differences in degradation and binding of juvenile hormone can exist between species. The

degradation pathways can differ from insect to insect; in higher Diptera, the cleavage of the epoxide derivative is the major metabolic pathway (Slade and Zibbitt, 1972). Juvenile hormone may be inactivated in different parts of an insect: in haemolymph of larvae of honey bee and adults of honey bee, housefly and cockroach, juvenile hormone is hardly degraded (de Kort, personal communication). Apparently in these insects, juvenile hormone is mainly inactivated in the tissues. Slade *et al.* (1976) showed that in the last larval instar of the southern armyworm, *Prodenia eridania*, homogenates of mid-gut had a high catalytic activity of hydratase against juvenile hormone (Slade *et al.*, 1976). Several species contain carrier proteins of high affinity that play an important role at those stages where juvenile hormone esterases are absent. These proteins possibly play a role in distribution of juvenile hormone in the target tissues. There are no published data about activity of the corpora allata in relation to the titre of juvenile hormone.

However, the results presented and the extensive literature about this problem suggest that the titre of juvenile hormone is regulated by a complex of factors. Regulation of the corpora allata remains an unsolved problem in insect endocrinology. Several mechanisms have been suggested, including neurosecretion, neural control from the brain, suboesophageal ganglion or both, changes in concentration of metabolites or any combination of these (Doane, 1973).

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Summary

Three main topics were investigated in regulation of the titre of juvenile hormone in haemolymph of the Colorado potato beetle (*Leptinotarsa decemlineata* Say): enzymic breakdown of the hormone; binding and protection of the hormone by carrier proteins; the synthetic capacity of the corpora allata.

Juvenile hormone was broken down by two major pathways: ester hydrolysis by esterases and hydration of the epoxide group by epoxide hydratases in tissue. In haemolymph of the beetle, juvenile hormone is solely broken down by juvenile hormone esterases. An *in vitro* method was developed to measure the catalytic activity of juvenile hormone esterase from haemolymph. High activities were observed in fourth-instar larvae and in beetles just before diapause. Lower activities were found in third-instar larvae and in beetles reared with long days, at diapause and after diapause. The juvenile hormone esterase was insensitive to diisopropylfluorophosphate (DFP), an inhibitor used to distinguish between carboxylesterases and esterases specific to juvenile hormone. Electrophoresis of the esterase from haemolymph showed one or more esterases specific to juvenile hormone.

The short half-life of juvenile hormone measured *in vivo* and *in vitro* in the haemolymph and inhibition studies with Triton X-100 suggests that juvenile hormone esterases in haemolymph govern breakdown. Activities of juvenile hormone esterase correlate well with the juvenile hormone titre.

The sharp changes in juvenile hormone esterase suggest that esterase activity is regulated. The mechanism was studied by supplying juvenile hormone and by microsurgery. Treatment of diapausing beetles with juvenile hormone itself or analogues caused an increase in activity of juvenile hormone esterase within 24 h. Ligation or removal of corpora allata suggested that this induction was an indirect effect of juvenile hormone. Transfer from short day to long day and treatment with hormone of beetles reared with short days prevented high activity of juvenile hormone esterase. Removal of corpora allata at emergence from beetles reared with short days resulted in the same. In beetles reared with short days the titre of hormone during the first days after adult emergence probably induces the rise in esterase. Esterase activity is thus most likely controlled indirectly by the hormone, via a centre in the brains (hormostate). The level of esterase activity is probably dependent on the sensitivity of this hormostate and on the titre of the juvenile hormone.

In several insects juvenile hormone is transported bound to carrier proteins. In haemolymph of larval and adult Colorado potato beetles lipoproteins of high molecular weight (> 100,00 daltons) were found, capable of binding juvenile hormone, its analogues, and palmitic acid. The lipoproteins were partially separated by gel permeation chromatography and electrophoresis on polyacrylamide gel. The binding characteristics of the lipoproteins indicate low affinity ($K_d \approx 10^{-5}$M), low specificity and high binding capacity.

The juvenile hormone complexed to lipoproteins was protected against esterases from haemolymph to some extent. Thus these carrier lipoproteins probably play little role in the regulation of the titre of juvenile hormone.

In the last part of our investigations the activity of the corpora allata was measured *in vitro*. High activities were observed in beetles reared with long days and in beetles after emergence. In beetles reared with short days, amounts of hormone produced were intermediate until Day 6 after emergence, thereafter declining to a low value. During diapause, production remained low. The production by corpus allatum and the activity of juvenile hormone esterase were in good agreement with the titre of juvenile hormone. The corpora allata are probably the primary regulator of the hormone titre in the Colorado potato beetle.

Samenvatting

Dit proefschrift betreft een onderzoek naar de regulatie van de juveniel hormoon titer in de Coloradokever (*Leptinotarsa decemlineata* Say).

Drie deelonderwerpen zijn onderzocht, namelijk a) de enzymatische afbraak van het hormoon, b) de binding van het hormoon aan drager eiwitten en het beschermende effect hiervan en c) de synthese capaciteit van de corpora allata.

In de Coloradokever is aangetoond dat juveniel hormoon op een tweetal manieren afgebroken kan worden: hydrolyse van de ester groep d.m.v. esterasen en hydratatie van de epoxygroep d.m.v. weefsel epoxyhydratasen. In het bloed van de kever wordt juveniel hormoon alleen afgebroken door juveniel hormoon esterasen. Een *in vitro* methode werd ontwikkeld ten einde de juveniel hormoon esterase aktiviteit kwantitatief te kunnen meten. Hoge esterase aktiviteiten werden waargenomen in larven van het vierde stadium en in kevers vlak voordat ze in diapause gaan. Lagere aktiviteiten werden gevonden in larven van het derde stadium, lange dag, diapauze en postdiapauze kevers. Deze bloed esterasen waren ongevoelig voor diisopropylfluorofosfaat (DFP), een remstof die gebruikt wordt om onderscheid te kunnen maken tussen algemene carboxyesterasen en juveniel hormoon specifieke esterasen. Een electroforese studie van de bloedesterasen toonde het bestaan van een of meer juveniel hormoon specifieke esterasen aan. Zowel de korte halfwaarde tijden van juveniel hormoon gemeten *in vivo* en in het bloed *in vitro*, als de remmingsproeven met Triton X-100 suggereren het grote belang van de bloed juveniel hormoon esterasen in de totale afbraak van het hormoon. Bovendien vertonen de juveniel hormoon esterase aktiviteiten een goede correlatie met de hormoon titer.

De spectaculaire veranderingen in juveniel hormoon esterase aktiviteit en het instellen van bepaalde niveaus vormen een aanwijzing voor het bestaan van een regulatie mechanisme. Dit mechanisme is bestudeerd met behulp van juveniel hormoon toediening en microchirurgische technieken. Behandeling van diapauze kevers met juveniel hormoon en verschillende juveniel hormoon analoga resulteerde in een toename van de juveniel hormoon esterase aktiviteit binnen 24 uur. De resultaten van ligatuur en allatectomie experimenten leidden tot de veronderstelling dat dit inductie fenomeen misschien een secundair effect van het juveniel hormoon is. Het overzetten van korte dag kevers naar lange dag omstandigheden en de behandeling van korte dag kevers met hormoon resulteerde in het uitblijven van de hoge juveniel hormoon esterase piek. Daarentegen verhinderde allatectomie van korte dag kevers ook de hoge esterase aktiviteit op dag 8. Het is erg waarschijnlijk dat het voorkomen van een beperkte juveniel hormoon titer gedurende de eerste dagen na het uitkomen van de kevers noodzakelijk is voor de inductie van de hoge esterase aktiviteit. Deze resultaten ondersteunen de hypothese dat de esterase aktiviteit waarschijnlijk indirect gereguleerd wordt door het hormoon, via een centrum gelegen in de hersenen (hormostaat). Het niveau van de esterase aktiviteit is waarschijnlijk afhankelijk van de gevoeligheid

van de hormostaat en de juveniel hormoon titer.

Naast de afbraak van het hormoon is ook aandacht besteed aan het transport van het hormoon door dragereiwitten. In het bloed van larven en volwassen kevers zijn hoogmoleculaire lipoproteinen (MW > 100.000) gevonden, die juveniel hormoon, juveniel hormoon analoga en het vetzuur palmitine zuur kunnen bieden. De lipoproteinen zijn gedeeltelijk gescheiden m.b.v. gelpermeatie chromatografie en polyacrylamide elektroforese. De bindings eigenschappen van de lipoproteinen vertoonden lage affiniteit ($K_d \approx 10^{-5}$M), lage specificiteit en hoge capaciteit. Beschermings experimenten uitgevoerd met juveniel hormoon gebonden aan lipoproteinen toonden aan dat het hormoon enigszins beschermd wordt tegen bloed juveniel hormoon esterasen. Geconcludeerd is dat deze drager lipoproteinen van geringe betekenis zijn in de totale regulatie van de juveniel hormoon titer.

In het laatste gedeelte van dit proefschrift is de corpus allatum aktiviteit *in vitro* gemeten. Hoge aktiviteiten zijn gevonden in lange dag en post-diapauze kevers. In kevers gekweekt onder korte dag lengte zijn intermediaire aktiviteiten gevonden tot 6 dagen na het uitkomen uit het zand, daarna nam de aktiviteit van de corpora allata af tot een lage waarde. Gedurende diapauze bleef de aktiviteit laag.

De corpus allatum - en juveniel hormoon esterase aktiviteiten gemeten in dit proefschrift vertonen een goede correlatie met de juveniel hormoon titer. Gesuggereerd wordt dat de controle van de aktiviteiten van de corpora allata waarschijnlijk van primair belang is voor de regulatie van de juveniel hormoon titer in de Coloradokever.

Curriculum vitae

Steven Jan Kramer werd op 1 september 1948 te Durban (Zuid Afrika) geboren. Hij bezocht het Lorentz Lyceum in Eindhoven, waar hij in 1967 het einddiploma HBS-B behaalde. In aansluiting hierop ging hij studeren aan de Landbouwhogeschool te Wageningen. In juni 1968 is het propadeutische examen, in januari 1971 het kandidaatsexamen richting Planteziektenkunde en in juni 1974 het doctoraalexamen richting Planteziektenkunde met als hoofdvak Entomologie en als keuzevakken de Fysiologie der Planten en de Biochemie afgelegd.

Van 1 oktober 1974 tot 1 oktober 1977 is hij werkzaam geweest als Wetenschappelijk ambtenaar aan het Laboratorium voor Entomologie van de Landbouwhogeschool in dienst van de Nederlandse Organisatie voor Zuiver-Wetenschappelijk Onderzoek (Z.W.O.).