Control of corpus allatum activity in the adult Colorado potato beetle

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STELLINGEN

I

De corpus allatum activiteit in de volwassen Coloradokever wordt gereguleerd door neurale en humorale factoren.

Dit proefschrift

II

Humorale factoren die leiden tot activering van de corpus allatum zijn, in de Coloradokever, ondergeschikt aan neurale inhiberende factoren vanuit de laterale neurosecretorische cellen van de hersenen.

Dit proefschrift

III

De bestaande hypothesen betreffende de allatocide werking van precocenen zijn onbevredigend.

Pratt et al., Nature, 284, 320-323 (1980).
Sodurlund et al., Juvenile Hormone Biochemistry, 353-362 (1981).

IV

De regulatie van een hormonaal effect op receptor niveau kan als een endocrinologisch veiligheidsmechanisme beschouwd worden.

V

De suggestie, dat ecdysteroiden betrokken zouden zijn bij de inductie van diapauze in de Coloradokever, wordt onvoldoende door experimentele gegevens ondersteund.

Briers et al., Physiological Entomology 7, 379-386 (1982).

VI

De ontwikkeling van insektenplagen in de landbouw is net zo voorspelbaar als het weer.

VII

De bewering, dat verhoogde resistentie van Anopheline muggen tegen bepaalde insecticiden en de daarmee gepaard gaande heropleving van malaria in Zuid-India en andere tropische gebieden mede veroorzaakt is door intensief gebruik van deze bestrijdingsmiddelen in de landbouw, dient serieus onderzocht te worden.

Chapin, G. and Wasserstrom, R., Nature 293, 181-185 (1981).

VIII

De opvatting dat economisch herstel in westerse industrielanden gunstig zal zijn voor derde wereldlanden is net zo bedenkelijk, als de opvatting dat het bezit van kernwapens de wereldvrede kan waarborgen.

IX

Het bedankje "voor technische hulp" in wetenschappelijke publicaties doet meestal tekort aan de bijdrage van wetenschappelijke analisten.

X

Het huidige kabinet zal aanzienlijk meer vertrouwen kweken, als het de "sense" in zijn beleid beter zou belichten dan de "no-nonsense".

M.A. Khan
Control of corpus allatum activity in the adult Colorado potato beetle.
Wageningen, 6 October, 1983.

M.A. KHAN

CONTROL OF CORPUS ALLATUM ACTIVITY IN THE ADULT COLORADO POTATO BEETLE

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
Dr. C.C. Oosterlee,
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To Aba and Ami

CONTENTS

GENERAL INTRODUCTION

More than 20 years ago, it was established that photoperiod played a crucial role in the induction of diapause in the Colorado potato beetle (*Leptinotarsa decemlineata* Say; Coleoptera: Chrysomelidae). Since then this insect has been reared under two different photoregimes in our laboratory (De Wilde *et al.*, 1959). Under an 18 h photophase (long-day) the physiology of the adults is directed towards reproduction whereas under an 8 h photophase (short-day) it is converged upon preparations for diapause. These two populations of our laboratory strain have been subjected to numerous investigations in order to clarify the processes involved in the translation of an environmental signal such as day-length into physiological and behavioral responses. The neuroendocrine complex, comprising the central nervous system and the paired corpora cardiaca/allata, was an obvious candidate for such studies. Indeed, as early as 1961, the involvement of the corpora allata became apparent, when it was shown that extirpation of these glands from long-day beetles inhibited reproduction and induced diapause (De Wilde and De Boer, 1961). Subsequent studies revealed that these glands, which synthesize juvenile hormone, were fairly active in long-day females but under short-day they became inactivated, leading to, respectively, high and very low juvenile hormone titres in the haemolymph (De Wilde *et al.*, 1968; Kramer, 1978a; De Kort *et al.*, 1982). Although the rate of hormone synthesis was the main factor influencing the titre in the haemolymph, high rates of hormone breakdown by specific juvenile hormone esterases may also contribute significantly towards this end at certain stages during adult development (Kramer, 1978b). The extreme differences in juvenile hormone titre under the two photoregimes are partly responsible for the different physiological and morphological responses of the ovaries, fat body and flight muscle tissue in these beetles (de Wilde *et al.*, 1959; De Kort, 1969; Dortland, 1979).

The control of corpus allatum activity by the higher centres remained an intriguing problem. The innervation between the gland and the brain did not appear to play a role in its regulation (De Wilde and De Boer, 1969; Schooneveld *et al.*, 1979). It was speculated that, under long-day, the median

neurosecretory cells of the brain continuously released an allatotropin which activated the corpora allata via the haemolymph (De Wilde and De Boer, 1969). However, cauterization studies (De Wilde and De Boer, 1969) and histological attempts to correlate the activities of neurosecretory centres in the brain with those of the corpus allatum (Schooneveld, 1970) did not yield any conclusive results. Of necessity, these studies relied on indirect criteria, such as oviposition or gland volume, for expressing corpus allatum activity and this approach had obvious limitations.

The introduction of a short-term, radiochemical *in vitro* assay for direct measurement of corpus allatum activity (Tobe and Pratt, 1974; Pratt and Tobe, 1974) opened new possibilities for investigating regulation of juvenile hormone synthesis. Kramer (1978a) demonstrated the applicability of this method in the Colorado potato beetle. The aim of the present studies was to utilize this technique, together with measurements of juvenile hormone titre in the haemolymph, for clarifying whether neural or humoral factors regulated corpus allatum activity in *L. decemlineata*. The results are presented in five chapters. The first chapter outlines the conditions and characteristics of the *in vitro* corpus allatum assay so as used by us. The second, third and fourth chapters investigate the significance of corpus allatum innervation for its control during starvation, during experimentally elevated juvenile hormone titres and during short-day photoperiod respectively. Chapter five describes the innervation of the corpus allatum utilizing the neuronal marker, horse radish peroxidase. The main findings from these experiments are integrated and discoursed upon in the General Discussion.

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1. *IN VITRO* ASSAY CONDITIONS FOR CORPUS ALLATUM ACTIVITY

ABSTRACT

Assay conditions for the short-term, radiochemical, *in vitro* determination of the spontaneous rate of juvenile hormone biosynthesis by isolated corpora allata from *Leptinotarsa decemlineata* have been further improved permitting the measurement of juvenile hormone biosynthesis by individual pairs of corpora allata. The final incubation product has been identified as juvenile hormone III with the aid of High-performance liquid chromatography (HPLC) and juvenile hormone esterase degradation. Using the new assay conditions, the activities of adult corpora allata during maturation were found to be significantly higher in reproductive, long-day animals than in pre-diapause, short-day beetles. During diapause no activity was detectable, whereas corpora allata from post-diapause beetles were reactivated totally after 5 days. Simultaneous determination of the *in vitro* rates of juvenile hormone biosynthesis and corpus allatum volumes revealed no clear correlation although the results suggest that the volume may be indicative of the maximal capacity for juvenile hormone production. Corpora allata from a population of beetles did not display any synchronous diurnal rhythmicity.

INTRODUCTION

Since its inception about 7 yr ago, the short-term radiochemical *in vitro* assay for measuring the spontaneous rate of juvenile hormone biosynthesis (PRATT and TOBE, 1974; TOBE and PRATT, 1974a) has found various applications. It has been used to study the relationships between juvenile hormone biosynthesis and: (a) juvenile hormone release (PRATT and TOBE, 1974; TOBE and PRATT, 1974b; PRATT *et al.*, 1975; TOBE and STAY, 1977), (b) oöcyte development (TOBE and PRATT, 1975a; TOBE and STAY, 1977; WEAVER *et al.*, 1978; ROSELER *et al.*, 1980), (c) juvenile hormone titre in the haemolymph (LANZREIN *et al.*, 1978; DE KORT and GRANGER, 1981) and (d) hierarchy in social insects (ROSELER *et al.*, 1980). In addition, the technique has been employed to study the regulation of corpus allatum activity (STAY and TOBE, 1977; TOBE *et al.*, 1977; STAY and TOBE, 1978; SCHOONEVELD *et al.*, 1979; TOBE and STAY, 1980; STAY *et al.*, 1980; FRIEDEL *et al.*, 1980), and has contributed towards a better understanding of the juvenile hormone biosynthetic pathway in the glands (PRATT and TOBE, 1974; TOBE and PRATT, 1976; KRAMER and LAW, 1980). The attributes of this method as compared with other available techniques have been outlined in a recent review (DE KORT and GRANGER, 1981).

In our laboratory, this assay was first adopted by KRAMER (1978) to follow the corpus allatum activity during adult maturation, under different photo-regimes, of the Colorado potato beetle. However, under the conditions employed, the incorporation of radioactive methionine into labelled product was rather low and necessitated the pooling of 3-5 pairs of glands per incubation. The purpose of the present investigation was to identify the final radioactive product of the *in vitro* incubation and to establish assay conditions that would allow the activity of individual pairs of corpora allata to be measured accurately. This is an essential prerequisite for our intended studies on the regulation of the corpus allatum activity in *Leptinotarsa decemlineata*.

MATERIALS AND METHODS

Insects

Adults of the Colorado potato beetle, *Leptinotarsa decemlineata* Say, were exposed to either an 18-hr photophase (long-day) or a 10-hr photophase (short-

day) (temperature = 25°C; r.h. = 65-75%). Routinely, within 16 hr after emergence from the soil, the beetles were collected and reared on fresh potato foliage until the desired age. Diapause of 90-day-old, short-day beetles was broken by transfer from short-day to long-day conditions.

Unanaesthetized beetles were decapitated and the corpora allata/corpora cardiaca complexes dissected under a Ringer solution containing 1.57 mM $CaCl_2$, 6.71 mM KCl, 0.55 mM KH_2PO_4, 1.01 mM $MgSO_4.7H_2O$, 172 mM NaCl, 5.21 mM $NaHCO_3$ and 0.42 mM $Na_2HPO_4.2H_2O$ (pH = 7.2). After a brief rinse in sterile Minimum essential medium with Hank's salts (without L-Glutamine and sodium bicarbonate), containing 20 mM Hepes buffer (Flow laboratories) and fortified with 10 mg/ml Ficoll 400 (Pharmacia), pH 6.9, the glands were transferred to incubation tubes containing 100 µl of the same medium plus 1 µCi L-[Me-^{14}C]-methionine (Radiochemical Centre, Amersham, Bucks). The final specific radioactivity of methionine ranged from 34 to 37 mCi mmol^{-1}. The tubes were incubated routinely for 2-4 hr (unless otherwise mentioned) in the dark at 30°C with gentle shaking. In experiments where the time course of juvenile hormone biosynthesis was measured, the incubation medium was removed every 2 hr by aspiration and replaced with fresh medium. The procedure for extraction and thin-layer chromatographic (TLC) separation of the biosynthesized product was essentially similar to that described by WEAVER *et al.* (1980). The juvenile hormone spot on the TLC plates was visualized under an ultra-violet-(UV) lamp, scraped off and added to 10 ml of scintillation medium (Hydroluma, Baker Ltd.). The radioactivity was measured in a Searle Mark III liquid scintillation counter. The procedure is illustrated in Fig. 1.

Osmolarity measurements were carried out with a Knauer vapour pressure osmometer at 37°C. HPLC was performed with a Chromatronix 3500, equipped with a variable UV detector, Schoeffel SF 770 adjusted to 220 nm and 0.04 AUFS. A reverse phase column, Nucleosil 10-C-18 (dimensions: 250 x 4.6 mm) was used for separation. Methanol/water (80:20) served as eluent and the flow rate was 1.2 ml/min. Purified juvenile hormone I, juvenile hormone II and juvenile hormone III (Calbiochem.) were used as references.

To find out whether the *in vitro* biosynthesized product was degraded by *Leptinotarsa* haemolymph containing juvenile hormone esterases (KRAMER and DE KORT, 1976) the following experiment was performed. After HPLC purification, 5000 dpm of the *in vitro* biosynthesized product was mixed with 1 µg unlabelled juvenile hormone III and incubated in 500 µl phosphate buffer (pH 7.5) together with 100 µl haemolymph from 5-day-old, short-day beetles

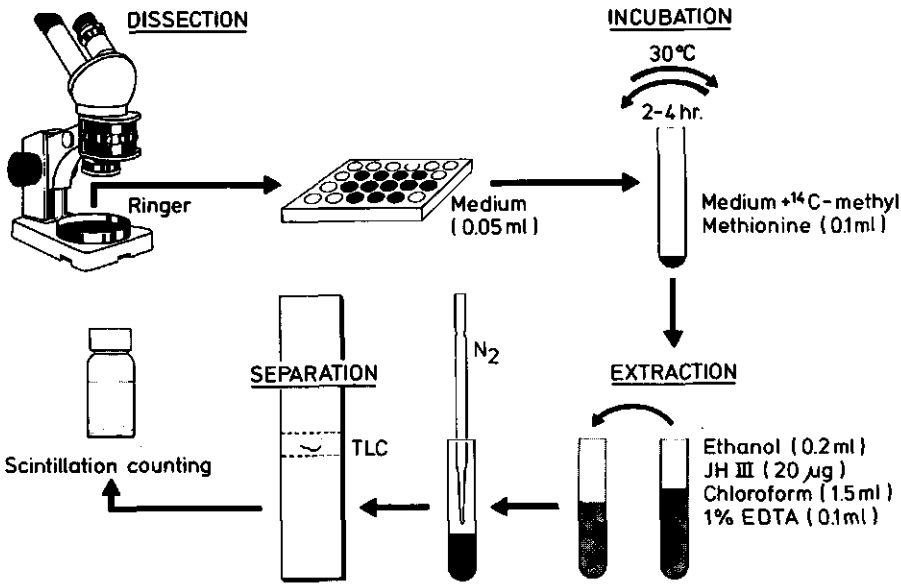

Fig. 1. The steps involved in the *in vitro* assay.

for 45 min at 4°C. For comparison, 10,000 dpm of [^{3}H]-juvenile hormone III (specific activity = 11.0 Ci/mmol, New England Nuclear) was mixed with 1 µg unlabelled juvenile hormone III and treated similarly. The degraded juvenile hormone was separated and estimated according to the procedure described by KRAMER and DE KORT (1976).

RESULTS

Recovery of the radiolabelled incubation product and its identification

An average recovery of 95% was obtained when [^{3}H]-juvenile hormone III was included in corpus allatum incubations and afterwards extracted from the medium as described in the Materials and Methods section. HPLC analysis of the biosynthesized radioactive product is shown in Fig. 2. This shows clearly that almost all radioactivity eluted with juvenile hormone III. In addition, Table 1

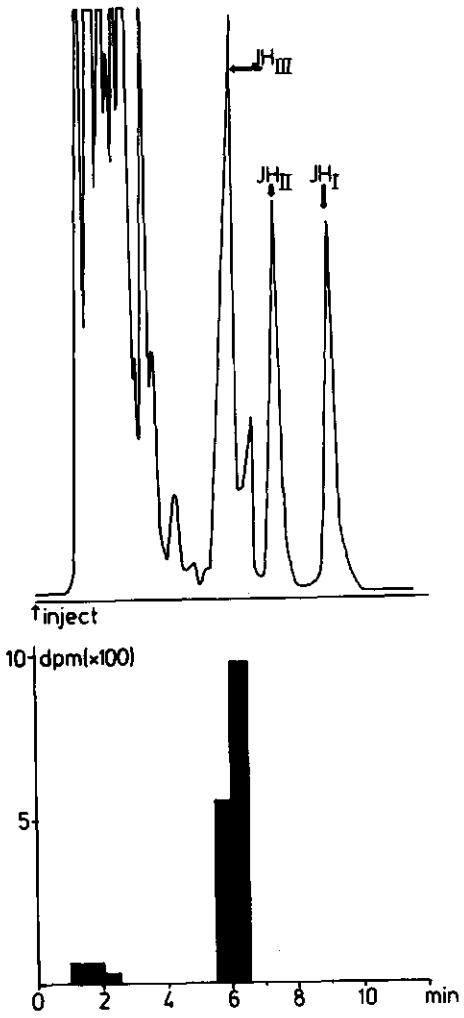

Fig. 2. HPLC profile of reference juvenile hormones (top) and radiochromatogram of product extracted from corpus allatum culture medium (bottom). After separation by thin-layer chromatography as described in Materials and Methods, the radioactive incubation product was extracted with ethyl acetate (4 x 1.0 ml) which was subsequently evaporated to dryness under nitrogen. After the addition of 0.2 mg juvenile hormone II and juvenile hormone I, this extract was taken up in 0.01 ml methanol and applied to the HPLC column. Fractions were collected every 30 sec and subjected to liquid scintillation counting.

shows that the degradation of the radioactive HPLC fraction and [^{3}H]-juvenile hormone III by beetle haemolymph is similar.

Selection of incubation conditions

The optimal methionine concentration in our assay was about 0.25 mM (Fig. 3.).

Osmolarity measurements indicated that the Ringer solution routinely used

Table 1. Esterase degradation of [^{3}H]-juvenile hormone III and HPLC purified radioactive product extracted from corpus allatum culture medium

Incubation	(%) degradation (in duplo)	
(1) Buffer + [^{3}H]-juvenile hormone III	1.3	2.7
(2) Buffer + [^{3}H]-juvenile hormone III + 0.1 ml Haemolymph	47.4	48.0
(3) Buffer + [^{14}C]-HPLC fraction	5.5	5.4
(4) Buffer + [^{14}C]-HPLC fraction + 0.1 ml Haemolymph	53.6	52.2
Average degradation of [^{3}H]-juvenile hormone III	45.7	
Average degradation of [^{14}C]-HPLC fraction	47.6	

in our laboratory for corpora allata dissection (EPHRUSSI and BEADLE, 1936) was considerably hypo-osmotic (250 mOsmol) compared with the haemolymph of the beetle (400 mOsmol) and the incubation medium (360 mOsmol). A new Ringer solution was therefore formulated in which the salt composition of the incubation medium was maintained but the concentrations of these salts was proportionally increased so that the Ringer was isotonic to the incubation medium (see Materials and Methods).

Of the three media tested by KRAMER (1978), Minimum essential medium with Hank's salts gave the highest rate of juvenile hormone biosynthesis. We first investigated the possibility of enhancing this medium. For example, Hank's salts were replaced by a salt solution based on the salt composition of *Leptinotarsa* haemolymph, and the amino acid fraction was supplemented with

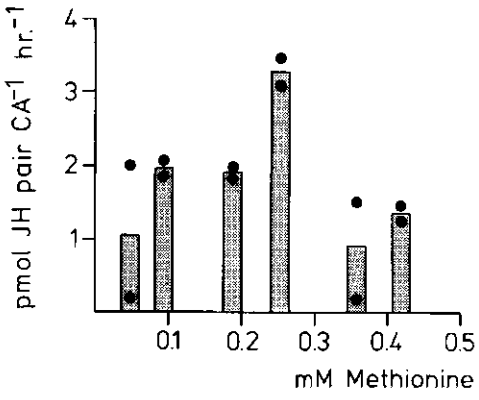

Fig. 3. Influence of L-[Me^{14}C]-methionine concentration on the rate of juvenile hormone biosynthesis by corpora allata from five-day-old, long-day females. Hank's basal salt solution (Flow laboratories) was used as incubation medium with varying concentrations of L-[Me^{14}C]-methionine spec. act. 60 mCi/mmol (2.23 GBI/nmol). Each point represents the mean activity of three pairs of corpora allata pooled together and incubated for 4 hr.

proline and serine, because these are also present in high concentrations in the beetle haemolymph (DE KORT and KRAMER, 1976). These alterations did not result in any increase in juvenile hormone biosynthesis. Basal medium with Hank's salts (Flow laboratories) was just as effective as Minimum essential medium.

The viability of the corpora allata and the rate of juvenile hormone biosynthesis was improved slightly by a reduction in the amount of Ficoll in our assay. This effect was revealed during our studies on the time course of juvenile hormone biosynthesis by individual pairs of glands. When these glands were incubated in medium containing 2% Ficoll for 2, 4 and 6 hr without interruption, the mean rate of juvenile hormone biosynthesis remained constant for up to 6 hr (Fig. 4a). This is in agreement with KRAMER's (1978) finding of a linear rate of incorporation for 4 hr using this method. However, when the rate of juvenile hormone biosynthesis of individual pairs of glands was monitored by replacement of the medium every 2 hr, only glands with low activities maintained their initial rates of synthesis, whereas those with high activities did not (Fig. 4b). In medium containing 1% Ficoll, even highly active corpora allata displayed a constant rate of juvenile hormone biosynthesis for up to 6 hr (Fig. 4c). Moreover, as shown in Table 2, the mean corpus allatum activity was slightly but significantly higher (Wilcoxan-Mann Whitney two-sample statistic $P < 0.1$) after 5 hr incubation in medium with 1% Ficoll than in medium with 2% Ficoll.

Table 2. The effect of Ficoll concentration of the incubation medium on the rate of juvenile hormone biosynthesis*

Ficoll concentration	1%	2%
pmol JH pair CA^{-1} hr^{-1}	4.3 ± 1.5 (9)	3.4 ± 1.2 (10)

*Corpora allata from 5-day-old, long-day females were incubated for 5 hr. The mean corpus allatum activities ± standard deviations are shown. The number of determinations is indicated in parentheses.

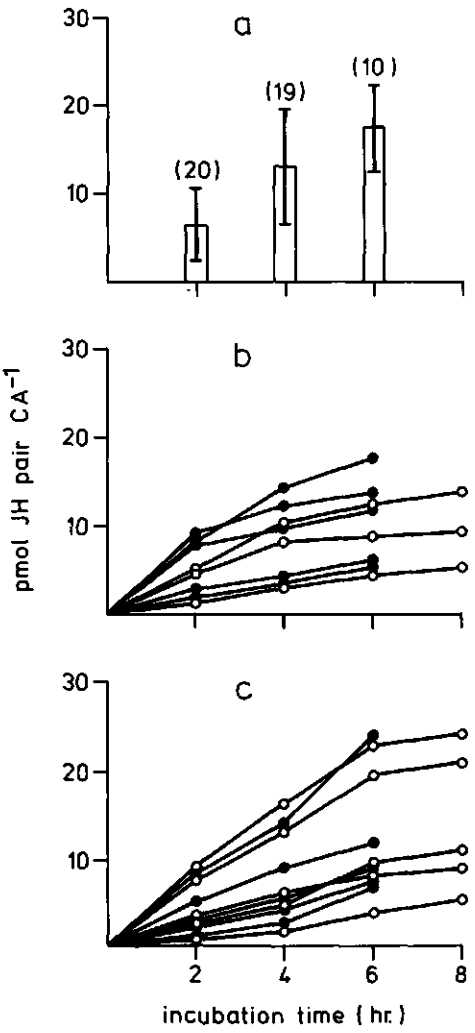

Fig. 4. (a) Time course of juvenile hormone biosynthesis by corpora allata from five-day-old, long-day females in medium containing 2% Ficoll 400. Incubations of individual gland pairs were stopped after 2, 4 and 6 hr. The number of determinations is indicated in parentheses. The columns and bars represent the means and standard deviations respectively. (b) Incubations of individual gland pairs in which the medium is changed after every 2 hr. During each replacement the glands were also washed briefly with 100 µl unlabelled medium. The replaced medium was pooled with the washing and extracted. Different symbols indicate separate experiments. (c) Same as (b) but with a concentration of 1% Ficoll 400 in the incubation medium.

Corpus allatum activity during maturation of the adult beetle under different photoregimes

The results of these studies are shown in Fig. 5. Individual pairs of corpora allata from long-day females displayed mean rates of juvenile hormone biosynthesis of around 5 pmol/pair/hr from day 0-9. The activities of long-day male corpora allata did not differ significantly from long-day female corpora allata during this period. The variation in corpus allatum activities

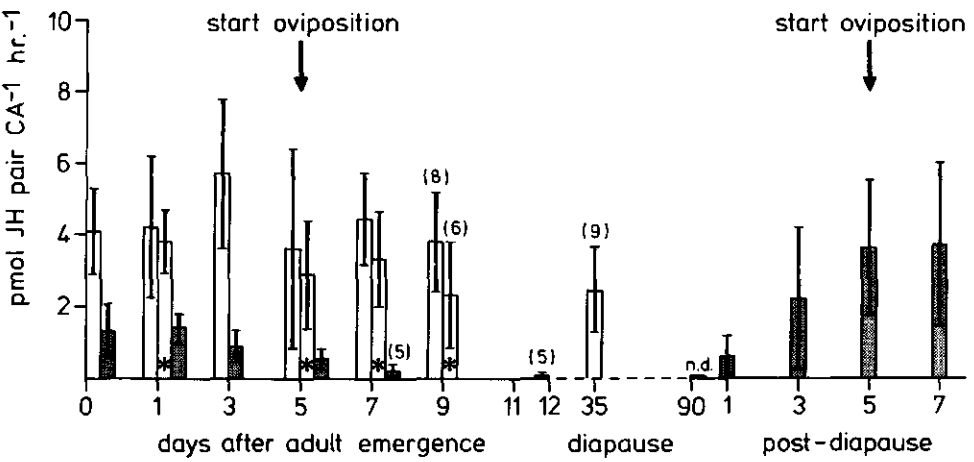

Fig. 5. The mean rates of juvenile hormone biosynthesis by corpora allata from long-day (open columns) and short-day (shaded columns) beetles during the adult life cycle. Male corpus allatum activities are shown with an asterisk. Each column represents the mean of ten individual determinations unless otherwise mentioned in parentheses. Bars represent the S.D.; n.d. = not detectable.

between individuals in long-day beetles was rather large and ranged from 2 to 9 pmol/pair/hr. Corpora allata from short-day beetles displayed a mean activity of about 1.5 pmol/pair/hr at emergence, gradually declining to 0.2 pmol/pair/hr 7 days later. On day 11, these beetles entered diapause. Corpora allata from post-diapause females reached the same mean values as long-day females on the fifth day after the break of diapause.

Relationship between corpus allatum activity and volume in long-day females

As illustrated in Fig. 6(a) there was no clear correlation between the corpus allatum volumes and their spontaneous rate of juvenile hormone biosynthesis. However, it is interesting to note that corpora allata smaller than 800 x 10^{-6} mm^3 have a mean activity of around 2 pmol/pair/hr (ranges 1-5 pmol/pair/hr) whereas larger glands have a mean activity of about 5 pmol/pair/hr (range: 1-9 pmol/pair/hr).

Figure 6(b) shows that on day 5, the glands attained their maximal size, which is about twice that at emergence. The corpus allatum activity, expressed per gland volume decreased slightly after the third day.

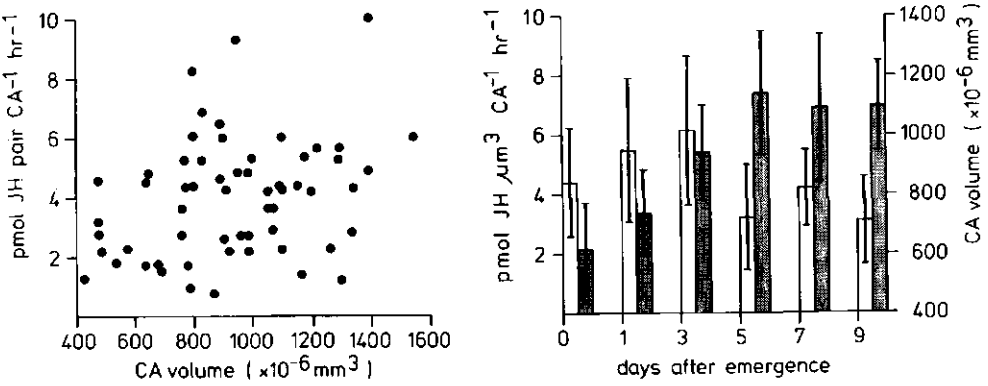

Fig. 6.(a) The rate of juvenile hormone biosynthesis by corpora allata from long-day females of various ages in relation to the volume of the paired glands. (b) The volume of paired corpora allata from long-day females (shaded columns) and the rate of juvenile hormone biosynthesis per paired gland volume (open columns) in relation to age. Bars represent standard deviations.

Corpus allatum activity during a 24-hr *cycle*

The mean rates of juvenile hormone biosynthesis by corpora allata from eight-day-old females, dissected every 4 hr, remained constant during one daily cycle (Fig. 7).

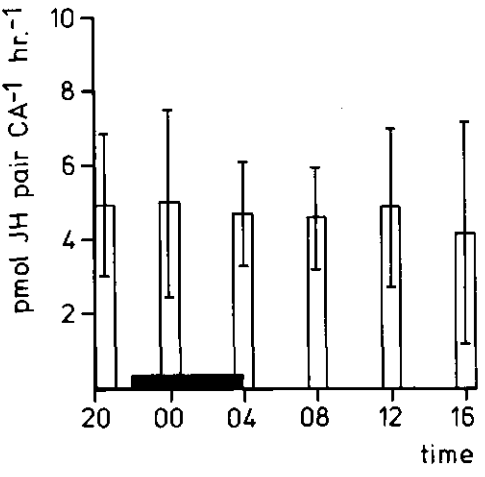

Fig. 7. The rate of juvenile hormone biosynthesis by corpora allata from 8-day-old, long-day females dissected every 4 hr during 24 hr. Each column represents the mean of five individual determinations. Bars represent the standard deviations. The 'lights out' period is indicated from 22.00 to 04.00 hr.

DISCUSSION

The values for *Leptinotarsa* corpus allatum activities obtained by the radiochemical *in vitro* procedure described in this paper are at least three-fold higher than those reported by KRAMER (1978). This enables the measurement of activities of individual pairs of corpora allata to be made. The rates of juvenile hormone biosynthesis measured *in vitro* now approach much closer to the calculated rate of juvenile hormone synthesis *in vivo* in the Colorado potato beetle (KRAMER, 1978) lending additional validity to this assay.

It appears that the Ficoll concentration of the incubation medium may affect the viability of *Leptinotarsa* corpora allata. Especially glands with high rates of biosynthesis seemed to be influenced. A reduction in the Ficoll concentration from 2 to 1% appeared to improve the linearity of juvenile hormone biosynthesis by these glands and subsequently resulted in slightly higher mean corpus allatum activities. How this effect is exerted is not clear. Since the introduction of this assay the amount of Ficoll in the incubation media used by the majority of investigators is 2% and until now there has been no report of any influence that this polysaccharide may have on the incubations. However, it should be pointed out that a reduction in the amount of Ficoll cannot be the sole factor responsible for the improved rates of juvenile hormone biosynthesis in the present assay. Apparently, an isotonic dissection Ringer and better extraction of the final radioactive product have also contributed towards this end.

Identification of the incubation product by HPLC and esterase degradation leaves little doubt that *Leptinotarsa* corpora allata produce only juvenile hormone III *in vitro*. The hormone synthesized *in vivo* is also juvenile hormone III as analysed by physico-chemical methods (DE KORT *et al.*, 1981).

The pattern of corpus allatum activities of individual insects during maturation confirms that reported by KRAMER (1978). The rates of juvenile hormone biosynthesis by corpora allata of long-day males did not differ significantly from those of long-day females. This is in contrast to the recent findings in *Diploptera punctata* where the female corpora allata are much more active and it has been suggested that the low rates of juvenile hormone biosynthesis by male corpora allata are an intrinsic property of the glands themselves (TOBE *et al.*, 1979; SZIBBO and TOBE, 1981).

The volume of the corpora allata has often been used as a criterion for their activity in various insects, including *Leptinotarsa decemlineata*

(SCHOONEVELD *et al.*, 1979). Simultaneous measurement of the corpus allatum activity and their volume has, however, been rare. One recent study with females of the wasp, *Polistes gallicus*, reports a good correlation between volume and activity during the period of egg maturation but in overwintering or ovariectomized females, very large glands appeared to produce little juvenile hormone (ROSELER *et al.*, 1981). In *Schistocerca gregaria*, no direct correlation was found between the spontaneous rate of juvenile hormone biosynthesis and the corpus allatum volume but a relationship between glandular volume and maximum activity could be demonstrated (TOBE and PRATT, 1975b). This is supported by our present observations with *Leptinotarsa decemlineata* where there seems to be no clear correlation between the spontaneous rate of juvenile hormone biosynthesis and corpus allatum volume, although larger glands appear to be capable of higher activities than smaller ones. The activities of larger glands are, however, highly variable. One possible explanation may be that these glands synthesize juvenile hormone in pulses, as has been suggested earlier (LANZREIN *et al.*, 1978). If this is true, then it would appear that in the Colorado potato beetle, there is no synchronisation of these pulses of activity in a population during a diurnal cycle.

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2. REGULATION DURING STARVATION

ABSTRACT

When two-day-old female *Leptinotarsa decemlineata* were starved, their corpus allatum activity, as measured by the radiochemical *in vitro* assay, was significantly reduced after 24 hr. Such a reduction was not observed when the nerve connections between the central nervous system and the retrocerebral complex were severed and the beetles starved up to 5 days. In some experiments, the rate of juvenile hormone biosynthesis *in vitro*, was substantiated by measurement of the juvenile hormone titre in the haemolymph by physico-chemical methods. It is concluded that intact nervous connections between the central nervous system and the corpora allata are essential for restraining the juvenile hormone biosynthesis during the initial stages of starvation.

Corpora allata from 1-day starved insects were considerably stimulated *in vitro* by farnesenic acid indicating that juvenile hormone synthesis is controlled enzymatically at a stage prior to the final two steps in the pathway. However, on day 5 of starvation, rate-limitation may occur after formation of this intermediate, since farnesenic acid stimulation was much less at this time.

Corpora allata of adult females newly emerged from the soil were activated within 4 hr regardless of feeding.

INTRODUCTION

The Colorado potato beetle is a voracious feeder. When reared under long-day conditions, most of the metabolic resources are invested in reproductive activity, and it is therefore hardly surprising that the quality as well as quantity of food strongly influences the rate of oviposition in this insect. Beetles fed on physiologically aged potato leaves display reduced reproductive activity and, in some cases, a complete standstill of egg production, followed by diapause (GRISON, 1957; DE WILDE and FERKET, 1967). Corpus allatum volume measurements, and implantation experiments led DE WILDE and FERKET (1967) to conclude that the lack of reproduction in these beetles is due to an endocrine deficiency.

In most insect species, starvation of adult females suppresses their fecundity and often the primary cause is not a nutritive deficiency but a hormonal one involving the corpora allata (JOHANSSON, 1955; MORDUE, 1967; ENGELMANN, 1970; BELL, 1971). Recently, in adult *Schistocerca gregaria* and *Periplaneta americana* direct evidence was presented showing that starvation suppresses the corpus allatum activity (TOBE and CHAPMAN, 1979; WEAVER and PRATT, 1981).

The mechanisms by which the corpora allata are inactivated during starvation are still uncertain. In insects such as *Rhodnius prolixus* (WIGGLESWORTH, 1936), *Culex pipiens* (LARSEN and BODENSTEIN, 1959) and *Oncopeltus fasciatus* (JOHANSSON, 1958), the brain seemed to restrain these glands. In *Oncopeltus* it appeared that intact nervi corporis allati were essential for this process. That the central nervous system may somehow be involved was also derived from histological data revealing an accumulation of granules in the neurosecretory cells and axonal tracts during starvation in various species including *Schistocerca* (HIGHNAM *et al.*, 1966), *Tenebrio* (MORDUE, 1967), *Periplaneta* (QUAYYUM and VIDYASAGAR, 1977) and *Leptinotarsa* (DE WILDE *et al.*, 1969). However, a clear relationship between this phenomenon and inactivation of the corpora allata is presumptive. Another mechanism may be that the haemolymph-protein concentration influences the corpora allata directly, e.g. as suggested for *Leucophaea maderae* (ENGELMANN, 1965, 1970).

The present study was undertaken to investigate whether the corpus allatum activity of *Leptinotarsa* was affected by starvation and whether this effect was mediated neurally or humorally. For this purpose, denervation and implantation techniques were combined with measurement of corpus allatum

activity *in vitro* using the short-term radiochemical assay (PRATT and TOBE, 1974; TOBE and PRATT, 1974) as adapted for *Leptinotarsa* (KRAMER, 1978; KHAN *et al.* 1981). In addition, juvenile hormone titres in the haemolymph were determined according to the method of BERGOT *et al.* (1981). The effect of exogenous farnesenic acid on the rate of juvenile hormone biosynthesis by isolated corpora allata was also studied to see whether the final two steps in the biosynthetic pathway of this hormone, which are not rate-limiting, were affected by starvation (TOBE and PRATT, 1976).

MATERIALS AND METHODS

Insects

Adults of the Colorado beetle, *Leptinotarsa decemlineata* (Say) were obtained from the internal laboratory breeding stock which was reared as described previously (DE KORT, 1969). The present investigations involved only long-day beetles (18-hr photophase, 25°C).

The experimental animals, males and females, were reared together in rectangular glass cages (volume *ca.* 8l) on fresh potato foliage. Starved animals were kept in similar cages containing only moist sand and were checked regularly for incidences of cannibalism.

The incorporation of L-methyl [^{14}C]-methionine (Radiochemical Centre, Amersham, England, final specific activity, 34-36 mCi/mmol) into juvenile hormone by individual pairs of female corpora allata *in vitro* was utilized to estimate their activities (PRATT and TOBE, 1974; TOBE and PRATT, 1974). The biochemical steps involved are summarised in Fig. 1. *Leptinotarsa* glands biosynthesize only juvenile hormone III (KHAN *et al.*, 1981; DE KORT *et al.*, 1981). The assay conditions and sources of chemicals have been described previously (KHAN *et al.*, 1981).

In certain experiments, a mixture containing 57% *trans*, *trans* Δ2 Δ6 and 31% Δ2 *cis* Δ6 *trans* farnesenic acid (donated by Zoecon, Palo Alto, California) dissolved in acetone (which was subsequently evaporated under nitrogen) was included in the incubation mixture.

Prior to surgical operations, the beetles were anaesthetized with carbon dioxide and surface sterilized with ethanol. Denervation of the corpora allata occurred as follows: The corpus allatum/cardiacum complex of the experimental animal was removed via the neck membrane (DE WILDE and STEGWEE, 1958) and

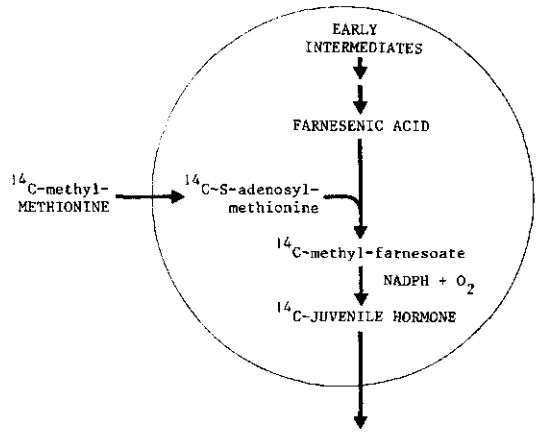

Fig. 1. The final steps in the juvenile hormone biosynthetic pathway, concerning the incorporation of ^{14}C-methyl-methionine.

immediately, a retrocerebral complex from another animal of the same age was implanted dorsally in the thorax (Fig. 2a). Sham-operated animals underwent

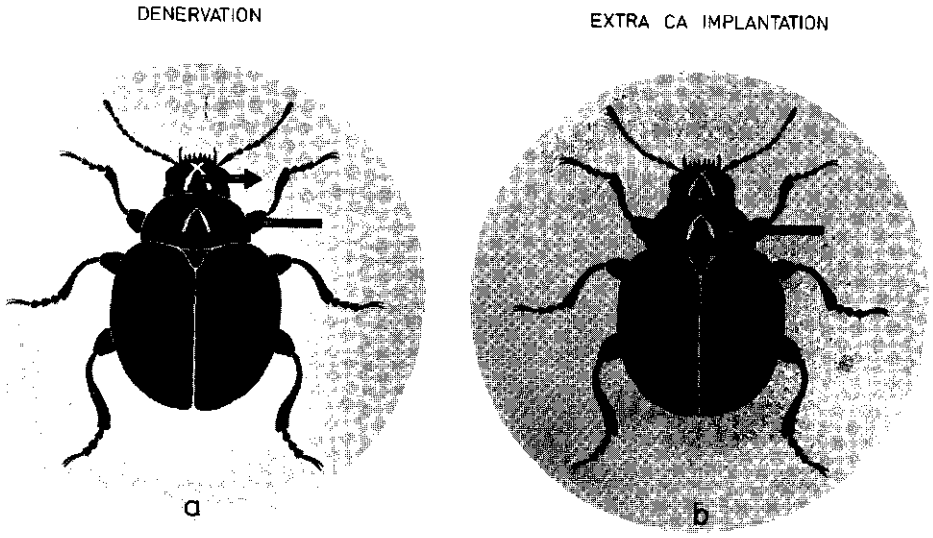

Fig. 2. Surgical operations for investigating significance of intact corpus allatum innervation.

similar treatment except that the connections between the central nervous system and glands were left intact and pieces of flight muscle substituted as implants.

Haemolymph was collected with glass capillary pipettes, after clipping off the wings, and each animal yielded about 0.005-0.010 ml of blood. Fractions of 0.050 ml haemolymph were pooled together, in ice, and transferred to 6.0 ml acetonitrile containing appropriate concentrations of [^{3}H]-internal standard (BERGOT *et al.*, 1981). The total amount of haemolymph required per analysis varied from 0.3 to 1.0 ml. The acetonitrile extracts were stored at -20°C until further analysis according to the method of BERGOT *et al.* (1981).

Blood volumes were determined using the isotope dilution method (WHARTON *et al.*, 1965). Anaesthetized beetles were injected just under one wing base with 0.005 ml *Leptinotarsa* Ringer (DORTLAND and DE KORT, 1978) containing 60,000 dpm [^{3}H]-Inulin (specific activity = 300 mCi/mmol, Radiochemical Centre, Amersham, England). Thirty minutes later, 0.005 ml of haemolymph was collected after clipping off the other wing and subjected to liquid scintillation counting.

For statistical evaluation, the Wilcoxon-Mann-Whitney two sample test was employed, unless otherwise mentioned.

RESULTS

Optimal concentration of farnesenic acid

The spontaneous rate of juvenile hormone biosynthesis by fully developed glands from 5-day-old normally fed females was stimulated about 5-fold when 20-60 µM of the farnesenic acid mixture was included in the incubation medium (Fig. 3). Higher concentrations of the farnesenic acid mixture (120 µM) seemed to offset any stimulation. We therefore selected 40 µM as the optimal concentration in our assay system.

Effect of starvation

Beetles in this experiment were supplied with fresh potato foliage until 2 days after emergence from the soil, at which age the corpora allata are fully activated (KRAMER, 1978; KHAN *et al.*, 1981). Thereupon, these animals were starved for various periods. After 1 day of starvation the rate of

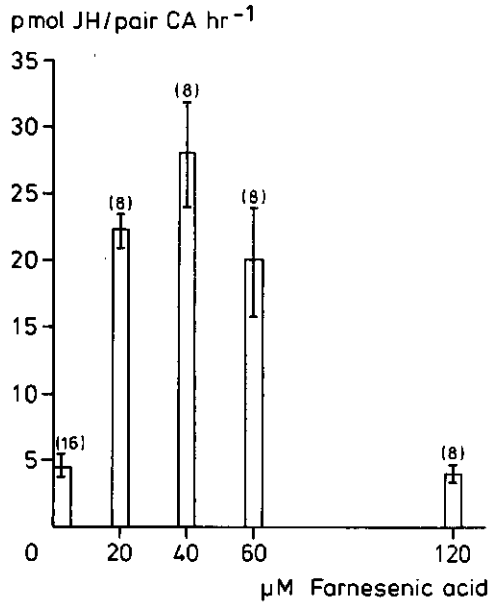

Fig. 3. The influence of various concentrations of farnesenic acid *in vitro* on the rate of juvenile hormone (JH) biosynthesis by corpora allata from 5-day-old ovipositing females. In all figures, the columns and bars represent the means ± S.E.M., respectively. Number of individual determinations are indicated in parentheses.

juvenile hormone biosynthesis decreased to about half that of the fed controls ($P < 0.005$) and this reduced rate was maintained on the 3rd ($P < 0.05$) and 5th ($P < 0.005$) day of starvation (Fig. 4). The hormone titre in the haemolymph of 5-day starved animals was also lowered when compared with fed controls of the same age (Table 1). Fed beetles oviposited normally at day 5, whereas starvation completely inhibited egg-laying. When 5-day starved females were refed, oviposition was resumed 5 days later. After 3-4 weeks of starvation most animals died and none entered diapause.

Farnesenic acid stimulation of isolated corpora allata from 1-day starved beetles resulted in a 3-fold increase and from 5-day starved beetles, a 2-fold increase in activity (Fig. 4). The stimulated activity of glands from 5-day starved beetles did not exceed the spontaneous corpus allatum activity of fed controls at this time (Fig. 4).

In order to check whether starvation also caused dehydration, the haemolymph volumes were compared. Beetles starved for 5 days had a haemolymph volume of 54.8 ± 4.9 µl (means ± S.D. $n = 8$) and this value did not differ significantly from that of normally fed controls of the same age (50.9 ± 4.1 µl, $n = 8$).

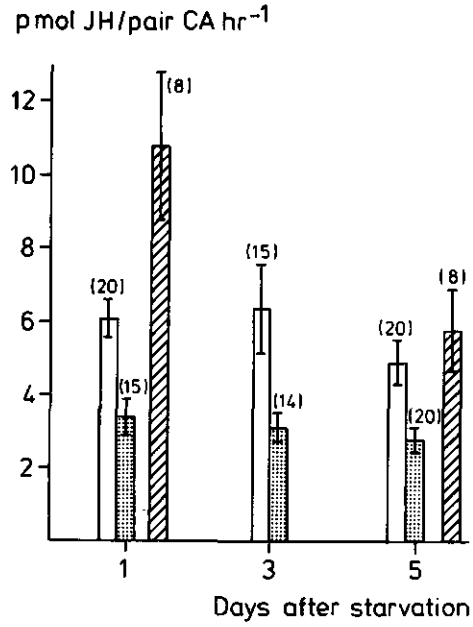

Fig. 4. The effect of varying periods of starvation on corpus allatum activity of 2-day-old females (open columns-fed controls; shaded columns-starved animals). Striped columns show the stimulated rate of juvenile hormone biosynthesis by glands from starved females in the presence of 40 μM farnesenic acid.

Table 1. The juvenile hormone III (JH) titre in the haemolymph of female beetles, 5 days after various treatments

Experimental group	JH titre (ng/ml)
(1) Fed females	(i) 80.4 (ii) 45.3
(2) Starved females	(i) 12.4 (ii) 25.1
(3) Fed females + implanted CA pair	(i) 62.1 (ii) 51.2
(4) Starved females + implanted CA pair	(i) 45.5 (ii) 27.5

Animals were 2 days old when starvation was started. Corpus allatum (CA) implantation experiments were similar to those described in the legend of Fig. 6. Duplicate determinations are indicated by (i) and (ii).

Effect of starvation after denervation of the corpus allatum/cardiacum complex

As in the previous experiment, the beetles were fed until 2 days old. Next, the retrocerebral complexes of the experimental animals were denervated, controls sham-operated (see Materials and Methods), and immediately thereupon, both groups starved for various periods. As shown in Fig. 5, after 1 day of starvation, there was apparently no difference in the rate of juvenile hormone biosynthesis between the denervated and innervated (sham-operated) glands. However, on day 3 ($P < 0.005$) and day 5 ($P < 0.025$) of starvation, the activity of loose corpora allata was normally maintained whereas the innervated glands were significantly restrained. Oviposition was not observed in either of the two groups within the experimental period.

Effect of starvation on host (innervated) and implanted (loose) corpora allata in the same individual

Single pairs of active corpus allatum/cardiacum complexes from 2-day-old female donors were implanted in the thoraces of female recipients of similar

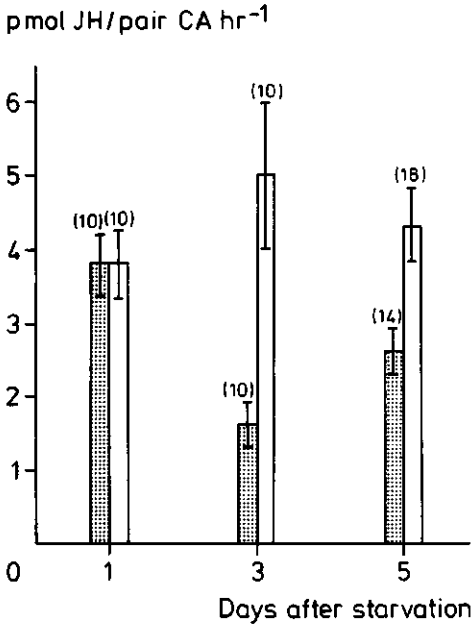

Fig. 5. The effect of varying periods of starvation on the rate of juvenile hormone biosynthesis of 2-day-old females after denervation of the corpora allata (open columns, denervated; shaded columns, sham-operated controls).

age which retained their own glands (Fig. 2b). After various periods of starvation or feeding the activities of the host as well as implanted glands were measured (Fig. 6).

In the fed group, the activities of the host and implanted glands did not differ significantly from each other throughout the experimental period ($P < 0.1$, analysis of variance, whereby the activity of an implanted pair of corpora allata is compared with the corresponding host pair in individual beetles). In the starved beetles, the implanted glands were significantly more active than the host glands on day 1 ($P < 0.05$), day 3 ($P < 0.05$) and on day 5 ($P < 0.01$). In addition, there was a striking increase in the activity of the implanted glands from day 1 to day 5 ($P < 0.05$) when they attained normal rates of synthesis whereas the host glands were restrained throughout this period (Fig. 6). The hormone titres in the haemolymph after 5 days feeding or starvation are shown in Table 1. The titres from starved females in this experiment were distinctly higher than those from starved females lacking an active pair of implanted corpora allata (see Table 1). On

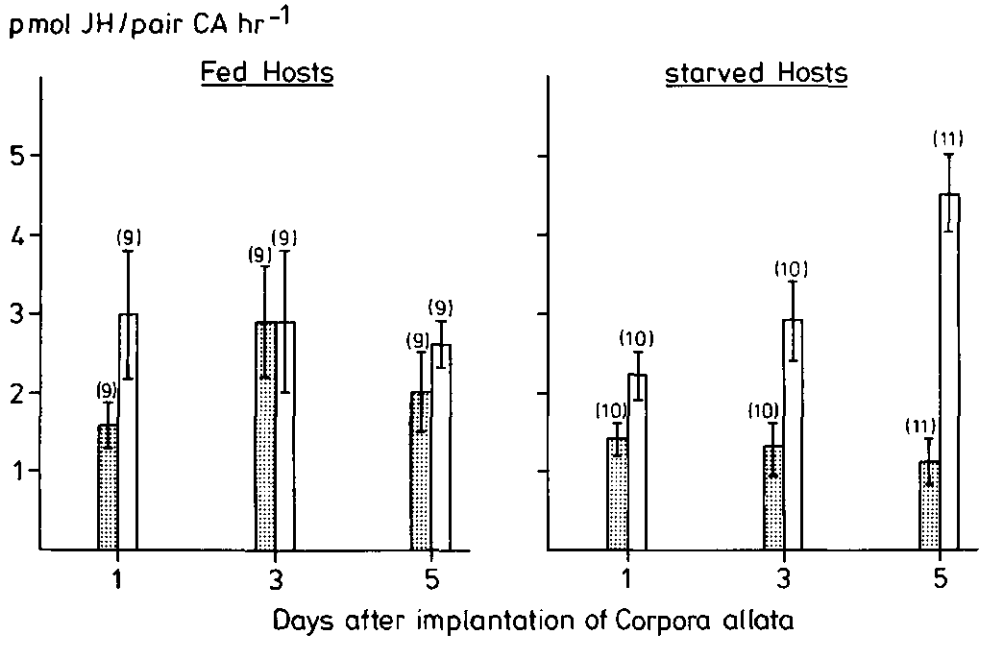

Fig. 6. The rate of juvenile hormone biosynthesis by host corpora allata (shaded columns) and implanted corpora allata (open columns) in fed and starved beetles after varying periods. The gland donors as well as hosts were 2-day-old females and starvation started immediately after the operations.

the other hand, the titres of fed females having an extra pair of glands were not higher than normal values. Again, starved females did not lay any eggs whereas fed females oviposited normally.

Effect of feeding on newly emerged females

Most adult females on the verge of emergence, when removed from the soil and provided with fresh potato foliage started feeding within two hours. That ingestion of food had actually occurred was verified by examination of the gut contents. Four hours after removal from the soil, both the corpus allatum activities of the fed as well as unfed beetles were similar but had increased significantly ($P < 0.005$) when compared with beetles at 0 hr (Fig. 7). The activities of these starved and fed groups remained similar up to 16 hr and only 24 hr after removal from the soil were glands from starved animals significantly restrained ($P < 0.05$).

DISCUSSION

The evidence presented in this paper strongly suggests that, in starved *Leptinotarsa* females, the corpus allatum activity is restrained and that this effect is mediated via nervous pathways from the brain and/or suboesophageal ganglion. Intact corpora allata displayed reduced activities whereas loose glands maintained normal rates of hormone biosynthesis indicating that there

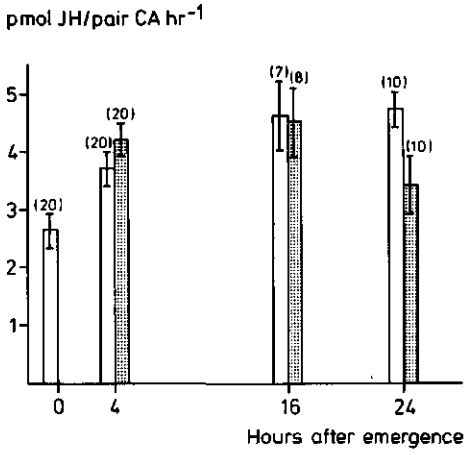

Fig. 7. The effect of feeding and starvation on the corpus allatum activity of newly emerged females in relation to time in hours (open columns, fed animals; shaded columns, starved animals). The activity of beetles which were dissected immediately after removal from the soil is shown at 0 hr.

was no direct influence of the humoral milieu during the initial stages of starvation (Fig. 5). These conclusions are presented schematically in Fig. 8. The corpus allatum activities measured *in vitro* were, in several experiments, substantiated by haemolymph hormone titres.

The effect of starvation on 2-day-old *Leptinotarsa* was quite rapid. Apparently, the lifestyle of this insect, which lives on its host plant feeding regularly, makes it highly susceptible to the withdrawal of food. Starved beetles never oviposited during the experimental period. Even females with active denervated corpora allata and sufficient juvenile hormone in the haemolymph did not lay any eggs when starved, indicating that these animals apparently lacked other factors required for successful oviposition. Blood volume measurements showed that no significant dehydration occurred during the first 5 days of starvation, so that any effects due to this condition may be ruled out.

In the fourth experiment (see Fig. 6), where each beetle received an additional (implanted) pair of glands, a negative feedback effect of the raised juvenile hormone titre on corpus allatum activities must also be considered (SCHOONEVELD *et al.*, 1979; TOBE and STAY; 1980). In the fed beetles, both the innervated host glands and the denervated implanted glands displayed

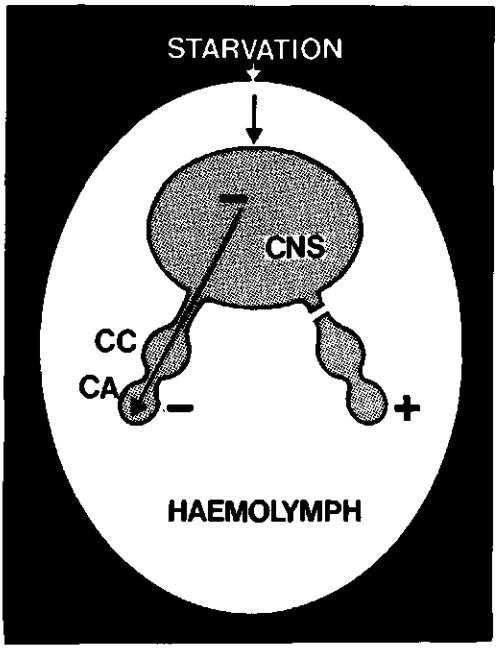

Fig. 8. Schematic representation of corpus allatum (CA) regulation under the influence of starvation. Intact CA are restrained (-) by neurally mediated signals originating in the central nervous system (CNS). Loose glands remain active (+).
CC (corpus cardiacum).

subnormal activities (Fig. 4). This situation is different from that in *Diploptera punctata* where only the innervated host glands were restrained in the presence of a pair of supernumerary corpora allata of similar age, although when the host glands were also denervated, both pairs displayed reduced rates of juvenile hormone biosynthesis (TOBE and STAY, 1980). The existing evidence clearly indicates that the negative feedback loop may operate via neural and/or humoral pathways (TOBE, 1980). It is interesting to note that the juvenile hormone titre in the fed beetles with a supernumerary pair of corpora allata, 5 days after implantation was not elevated above normal values (Table 1). This suggests that the titre has been regulated at this time to normal values through a reduction in synthesis by both pairs of glands. In the starved beetles, the juvenile hormone titre was presumably elevated only shortly after implantation of the supernumerary corpora allata, because the host (innervated) glands become restrained in their activity due to starvation (Figs. 4 and 6). Indeed, our results in Fig. 6 revealed that the implanted glands displayed subnormal activities on day 1 after implantation, but gradually attained higher rates of juvenile hormone synthesis 5 days later, possibly through a positive feedback effect of the declining rate of juvenile hormone synthesis by the host glands.

The concentration of farnesenic acid required for maximal stimulation of the corpus allatum activity in our assay was in the same range as that found by others, taking into consideration that apparently only the *trans, trans* isomer is metabolised (TOBE and PRATT, 1974; WEAVER *et al.*, 1980). Glands from 1-day starved beetles were considerably stimulated by exogenous farnesenic acid. Similar findings were reported for *Schistocerca* and *Periplaneta*, implying that, at this stage, the rate-limiting step(s) in the pathway of juvenile hormone biosynthesis occur prior to the formation of farnesenic acid (TOBE and PRATT, 1976; TOBE and CHAPMAN, 1979; WEAVER and PRATT, 1981). However, in the present investigations the corpora allata from 5-day starved animals were stimulated much less by farnesenic acid than those from normally fed 5-day-old females (Fig. 3) or those from 1-day starved beetles (Fig. 4). The possibility that enzymatic steps occurring after formation of farnesenic acid may also contribute to rate limitation on day 5 cannot be excluded.

In anautogenous insects such as *Rhodnius prolixus* and *Culex pipiens*, mechanical distension of the midgut, caused by a blood meal under natural circumstances, appeared to trigger the activation of the corpora allata

(WIGGLESWORTH, 1936; LARSEN and BODENSTEIN, 1959). In *Leptinotarsa*, our data suggest that the rates of juvenile hormone biosynthesis increased significantly 4 hr after emergence from the soil. This rapid increase may be due to either the removal of an inhibitory signal or the release of a stimulatory signal and it is clear that this activation is not dependant on the availability of food for at least the first 16 hr. After 24 hr, the glands from starved beetles displayed reduced activity.

It is perhaps worthwhile pointing out that in *Pyrrhocoris apterus* it has been suggested that the inhibition of the corpora allata by short-day photoperiod occurs via intact nerve connections with the brain (HODKOVÁ, 1977). In the Colorado potato beetle, the restrained corpus allatum activity as well as the juvenile hormone titre was still considerably higher after 5 days of starvation than that of normally fed short-day (pre-diapause) beetles of the same age in which these glands are almost totally inhibited (KRAMER, 1978; KHAN *et al.*, 1981; DE KORT *et al.*, 1981). This implies that, in *Leptinotarsa*, the mode of regulation under the influence of photoperiod may involve additional or different pathways to those described above for starvation. Indeed, denervation alone never activated the glands in pre-diapause insects of this species (DE WILDE and DE BOER, 1969). In *Oncopeltus*, it has been suggested that starvation leads to levels of juvenile hormone which are below those required for ovarian development but sufficient to induce migratory flight (RANKIN and RIDDIFORD, 1977). These 'intermediary' levels of juvenile hormone also appear to occur in *Leptinotarsa* during starvation and probably prevent diapause under these conditions.

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3. NEGATIVE-FEEDBACK REGULATION

ABSTRACT

When the titre of juvenile hormone III in female *Leptinotarsa decemlineata* was elevated by the implantation of supernumerary corpora allata or by the injection of the hormone, the rate of endogenous hormone production by the host glands was significantly restrained, as determined by the short-term *in vitro* radiochemical assay. From denervation studies, it is suggested that during phases of elevated juvenile hormone titre, the corpus allatum activity is regulated via humoral as well as neural factors requiring intact nerve connections. Restrainment of gland activity appears to be mainly via the neural pathway. Isolated corpora allata were not influenced by 10^{-5}M juvenile hormone III added to the incubation medium *in vitro*.

Studies with farnesenic acid revealed that the final two enzymatic steps in the biosynthetic pathway of juvenile hormone are also diminished during prolonged neural inhibition of the corpora allata.

20-Hydroxyecdysone and precocene II had no apparent effect on the corpus allatum activity of *Leptinotarsa decemlineata*.

INTRODUCTION

In the adult Colorado potato beetle, the titre of juvenile hormone is controlled predominantly by the rate of hormone synthesis by the corpora allata (KRAMER, 1978; DE KORT and GRANGER, 1981). The mechanisms by which these glands are regulated in *Leptinotarsa decemlineata* are still largely unknown. The intimate association of the corpora allata with the central nervous system leaves open the possibility of both neural and humoral control (TOBE, 1980). DE WILDE and DE BOER (1969) observed the effects of innervated and denervated corpora allata on reproduction and diapause under the influence of photoperiod, and suggested that these glands are regulated mainly by the humoral pathway in *L. decemlineata*.

The short-term *in vitro* radiochemical assay is a convenient direct method for estimating the spontaneous activity of the corpora allata (PRATT and TOBE, 1974; TOBE and PRATT, 1974). This assay, adapted for *L. decemlineata*, has been suitably employed recently to show that, during starvation of adult females, these glands are restrained via neural pathways and not humorally (KHAN *et al.*, 1982a, b).

It has been reported that the corpus allatum activity *in vitro* and their volume decreased following treatment of long-day *L. decemlineata* with juvenile hormone I (SCHOONEVELD *et al.*, 1979). In the present paper, these observations are reinvestigated, using an improved *in vitro* assay whereby the activities of individual pairs of glands can be determined, and employing juvenile hormone III, the natural homologue in *Leptinotarsa* (DE KORT *et al.*, 1981; KHAN *et al.*, 1982a). In order to determine whether the last two enzymatic steps in the biosynthetic pathway of juvenile hormone, which are not rate-limiting normally, were also affected, corpora allata from treated animals were incubated with farnesenic acid (TOBE and PRATT, 1976). Denervation and implantation experiments were performed to study the possible mechanisms of negative-feedback regulation. The results of hormone treatment on the corpus allatum activity of short-day beetles are also described. Finally, the effects of 20-hydroxyecdysone and precocene II, which have been reported to influence the corpora allata of other insect species (BOWERS, 1976; STAY *et al.*, 1980), were included in the present study.

MATERIALS AND METHODS

Adults of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) were reared either under long-day (18 L:6 D, 25°C) or short-day photoregimen (10 L:14 D, 23°C) and fed *ad libitum* on fresh potato foliage as described previously (DE KORT, 1969). Routinely, the beetles were collected within 16 hr after emergence from the soil and timed from then onwards. Under these conditions, long-day beetles begin oviposition on day 5 whereas short-day beetles do not lay any eggs but enter diapause on day 11 or 12.

The experimental animals were narcotized with carbon dioxide prior to all surgical operations. Denervation of the corpora allata was performed as follows: the insects were allatectomized through a slit in the neck membrane as described by DE WILDE and STEGWEE (1958) and thereupon a corpus allatum/corpus cardiacum complex from another female of similar age implanted dorsally in the thorax, using only intact and undamaged glands.

The corpus allatum activity was determined by using the *in vitro* assay introduced by PRATT and TOBE (1974) and TOBE and PRATT (1974) and adapted for *L. decemlineata* (KRAMER, 1978, KHAN *et al.*, 1982a). This method relies on the incorporation of the methyl moiety of L-(Me^{14}C)-methionine (Radiochemical Centre, Amersham, U.K., final specific activity, 34-37 mCi/mmol) into juvenile hormone. The corpora allata of *L. decemlineata* synthesize only juvenile hormone III (DE KORT *et al.*, 1981; KHAN *et al.*, 1982a). The assay conditions and sources of chemicals were similar to those described by KHAN *et al.* (1982a).

Farnesenic acid (consisting of 57% *trans, trans* and 31% *cis, trans* farnesenic acid), ZR 512 and ZR 515 were donated by Zoecon, Palo Alto, California. Juvenile hormone III was purchased from Calbiochem, 20-hydroxyecdysone from Organon (Oss, Holland) and precocene II from Sigma Chemicals. Pure olive oil was a gift from Prof. M.P. Pener.

For statistical evaluation, the Wilcoxon-parameter-free test was employed, unless otherwise mentioned.

RESULTS

Effect of elevated juvenile hormone III titre on innervated corpora allata

Two days after emergence from the soil, the corpora allata of long-day *Leptinotarsa decemlineata* are fully activated (KRAMER, 1978; KHAN *et al.*,

1982a). Females of this age were implanted with a pair of supernumerary corpora allata. After 72 hr, the activities of the host (innervated) glands were slightly but not significantly lower than those of glands from sham-operated controls (Fig. 1). However, when 3 pairs of supernumerary corpora allata were implanted in each individual, the activities of the host glands were significantly reduced ($P < 0.005$) (Fig. 1). This later effect was confirmed when, instead of supernumerary corpora allata, the animals received a single injection of 50 µg juvenile hormone III in olive oil. As shown in Fig. 2, a significant decrease in the gland activity was already apparent 12 hr after injection ($P < 0.05$), and the activity declined even further after 24 hr ($P < 0.005$) and 72 hr ($P < 0.005$).

The activity of corpora allata from juvenile hormone-injected animals, 12 hr after treatment, was considerably stimulated *in vitro* in the presence of 40 µM farnesenic acid mixture (this is the optimal concentration for corpus allatum stimulation in the *L. decemlineata* assay, Khan *et al.*, 1982b). However, 72 hr after treatment, the stimulated activity was only slightly higher than the spontaneous rate of juvenile hormone synthesis by control corpora allata at this time (Fig. 2).

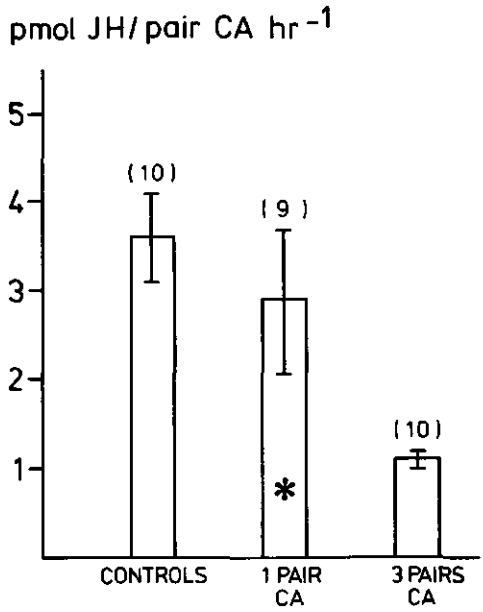

Fig. 1. The effect of implanting various numbers of supernumerary corpora allata (CA) on the rates of juvenile hormone biosynthesis by host glands after 72 hr. Gland donors as well as recipients were 2-day-old females. Controls were implanted with flight muscle tissue. *Data from Khan *et al.* (1982b). Number of determinations are indicated in parentheses. Columns and bars represent means ± S.E.M., respectively.

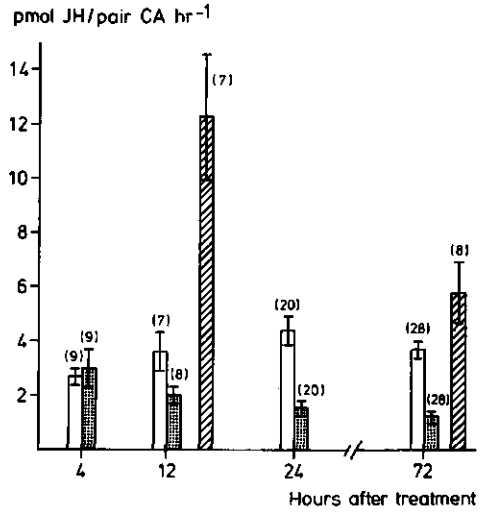

Fig. 2. The effect of juvenile hormone III injections on the rates of juvenile hormone biosynthesis of 2-day-old females after various periods. Experimental animals were injected once, just under the abdominal integument with 50 µg juvenile hormone III in 1 µl olive oil. Controls received only olive oil (open columns, controls; shaded columns, hormone treated). In addition the striped columns represent the stimulated rates of synthesis by isolated glands from juvenile hormone-treated animals in the presence of 40 µM farnesenic acid. Number of determinations are indicated in parentheses. Columns and bars represent means ± S.E.M., respectively.

Effect of elevated juvenile hormone III titres on denervated corpora allata

Two different approaches were used to study this. In the first experiment, the corpora allata of 2-day-old females were denervated. Immediately thereupon, the experimental group was injected with 50 µg juvenile hormone III in 1 µl olive oil and the control group only with olive oil. After 72 hours, the mean corpus allatum activity of treated animals was about half that of the controls ($P < 0.1$, Fig. 3a).

In the second experiment 2-day-old females were implanted with a supernumerary pair of corpora allata from female donors of similar age. The gland recipients were then injected with juvenile hormone III, and after 72 hr the activities of the host and implanted glands were measured, together with those of intact glands from sham-operated controls injected with olive oil. The results are shown in Fig. 3(b). Both the host and implanted glands were significantly restrained in their activities with respect to controls ($P < 0.001$, analysis of variance). In addition, the host (innervated) glands were significantly more restrained than the implanted (loose) corpora allata ($P < 0.05$, analysis of variance).

Direct effect of juvenile hormone III on corpus allatum activity in vitro

In order to test whether the hormone influenced denervated glands

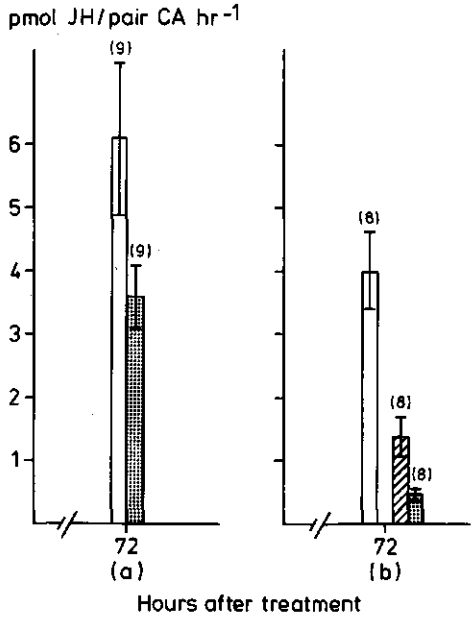

Fig. 3 (a) The effect of elevated hormone titre, after denervation of corpora allata. The retrocerebral complexes of both experimental and control animals (2-day-old females) were denervated and, immediately thereupon, they were injected with either juvenile hormone III or only olive oil, as described in the legend of Fig. 2 (open column, controls; shaded column, hormone treated). (b) The effect of elevated juvenile hormone titres on host (shaded column) and implanted (striped column) corpora allata in the same animal. Donors as well as recipients were 2-day-old females. The open column represents the rates of juvenile hormone biosynthesis by intact corpora allata from control animals implanted with flight muscle tissue and injected with only olive oil. Number of determinations are indicated in parentheses. Columns and bars represent means ± S.E.M., respectively.

directly, we included 10^{-5}M juvenile hormone III in the incubation medium. As shown in Table 1 this apparently had no effect on the rates of juvenile hormone biosynthesis during 5 hr incubation.

Table 1. The effect of various concentrations of juvenile hormone III in the incubation medium on the rate of juvenile hormone biosynthesis by isolated corpora allata from 3-day-old females

JH III conc. of incubation medium	0	10^{-5}M	$2x10^{-5}$M
pmol JH/pair glands hr^{-1}	5.8 ± 0.9 (8)	5.3 ± 0.6 (8)	6.7 ± 1.1 (8)

Corpus allatum activities are displayed as means ± S.E.M. Number of determinations are indicated in parentheses.

Effect of juvenile hormone III in short-day females

The corpus allatum activity as well as the juvenile hormone titre in the haemolymph of short-day beetles is lower than that of long-day beetles at emergence from the soil and declines rapidly thereafter (DE WILDE *et al.*, 1968; KRAMER, 1978; KHAN *et al.*, 1982a; DE KORT *et al.*, 1981). These beetles do not oviposit and normally enter diapause on day 11 or 12. Table 2 shows the effect of treatment of 2-day-old males and females with 50 µg of various juvenile hormones and their analogues. The substances were applied topically in acetone. About 7 days after application, oviposition was induced in 55% of the females treated with juvenile hormone III wherease juvenile hormone I and ZR 512 were less effective and ZR 515 had no effect at all. However, oviposition was not maintained and females which had laid eggs entered diapause 5 to 10 days later than normal. An identical effect was obtained when 50 µg juvenile hormone III was administered by injection in olive oil instead of topical application (results not shown). None of the controls laid any eggs and 100% of them entered diapause by day 12.

In order to ascertain whether the oviposition induction of the juvenile hormone-treated females was entirely due to the exogenously administered hormone or whether the corpora allata of these individuals were activated by hormone treatment, we measured their gland activities. As shown in Table 3,

Table 2. The effect of various juvenile hormone homologues and analogues on oviposition induction of 2-day-old prediapause beetles

Analogue applied		Acetone (controls)	ZR-512	ZR-515	JH I	JH III
N	♀	21	15	15	10	9
	♂	22	15	15	6	6
total eggs/female		0	3	0	8	25
% ovipositing females		0	15	0	30	55

Each animal was applied topically with a single dose of 50 µg compound dissolved in 2 µl acetone. Oviposition, where induced, usually commenced 9 days after treatment.

Table 3. The effect of exogenous juvenile hormone III on the corpus allatum activity of 2-day-old short-day females

Days after JH III treatment		5	8	11
pmol JH/pair glands hr^{-1}	Controls	0.9 ± 0.3 (10)	0.7 ± 0.2 (7)	0.3 ± 0.2 (5)
	JH treated	0.5 ± 0.1 (10)	0.3 ± 0.1 (13)	0.2 ± 0.2 (5)

The hormone was administered as described in the legend of Fig. 2. The hormone treated animals assayed on day 11 were still active whereas controls had already entered diapause.

there was apparently no activation of these glands.

Effect of 20-hydroxyecdysone

20-Hydroxyecdysone injection (5 µg per animal) in 1-day-old immature females as well as in 5-day-old ovipositing females had apparently no effect on their corpus allatum activity up to at least 48 hours after treatment (Table 4).

Effect of precocene II

50 µg precocene II, dissolved in olive oil, was injected into 2-day-old females. Their corpus allatum activity, measured 2 and 4 days later, remained similar to that of controls (Table 5). In addition, glands from precocene II-treated animals could be fully stimulated with exogenous farnesenic acid. Topical application of 100 µg precocene II also had no effect on the rate of juvenile hormone biosynthesis (Table 5).

Finally, isolated corpora allata were incubated for 5 hours in 10^{-4}M precocene II. Again, no effect was apparent on the spontaneous, as well as the farnesenic acid-stimulated, rate of juvenile hormone biosynthesis (Table 5).

Table 4. Effect of 20-hydroxyecdysone administration on corpus allatum activity of females of various ages

	Age of animals	1 day old		5 days old	
	Hours after treatment	16	48	12	48
pmol JH/ pair glands hr^{-1}	Controls	2.5 ± 0.5 (7)	3.0 ± 0.2 (9)	4.0 ± 0.6 (10)	3.5 ± 0.7 (8)
	20-hydroxy-ecdysone treated	3.5 ± 0.5 (7)	3.9 ± 0.6 (9)	4.7 ± 0.8 (10)	3.5 ± 0.5 (10)

The hormone was dissolved in 5% ethanolic HEPES-buffered minimum essential medium (Flow laboratories) and injected just under the abdominal integument. Each animal received 5 µg 20-hydroxyecdysone in 2 µl medium. Controls were injected with only medium.

DISCUSSION

Previous observations on the effect of experimentally elevated juvenile hormone titres on corpus allatum activity in the Colorado potato beetle (SCHOONEVELD *et al.*, 1979) are confirmed, clarified and extended by the evidence presented here.

The results appear to indicate that during experimental elevations of the juvenile hormone titre, negative-feedback regulation may occur via an humoral, as well as a more pronounced, neural pathway (Figs 1, 2 and 3). These conclusions are presented schematically in Fig. 4. It has been proposed that humoral regulation may be mediated by an inhibitory factor or by the absence of a stimulatory factor (TOBE and STAY, 1980). Since isolated corpora allata were unaffected by the presence of 10^{-5}M juvenile hormone III in the incubation medium (Table 1), end-product inhibition by the hormone itself seems unlikely. This supports earlier suggestions (PRATT and FINNEY, 1977; TOBE, 1980).

During relatively high elevations in the hormone titre (e.g. by implantation of 3 pairs of supernumerary glands or by injection of 50 µg

Table 5. The effect of precocene II treatment on corpus allatum activity of 2-day-old females

	Type of treatment	*in vivo*			*in vitro*
		Injection		Topical applic.	Incubation medium
	Time after treatment	2 days	4 days	1 day	5 hr
pmol JH/ pair glands hr^{-1}	Controls	4.6 ± 0.7 (9)	3.9 ± 0.7 (6)	5.5 ± 1.0 (10)	3.8 ± 0.6 (8)
	Precocene II treated	3.9 ± 2.0 (7)	3.3 ± 0.8 (7)	3.9 ± 0.5 (10)	3.6 ± 0.6 (7)
	Precocene II treated + farnesenic acid.		23.3 ± 4.6 (8)		19.2 ± 3.7 (8)

Whole animals were either injected once with 50 µg precocene II in 1 µl oil or topically applied twice with 50 µg precocene II in 2 µl acetone. Controls were injected with only olive oil or topically applied with only acetone, respectively. Also shown is the effect of 10^{-4}M precocene II on isolated corpora allata from 3-day-old females during 5 hr incubation *in vitro* and the stimulated rate of juvenile hormone biosynthesis by isolated glands in the presence of 40 µM farnesenic acid after *in vivo* and *in vitro* precocene treatment.

juvenile hormone III, Figs. 1, 2 and 3), in addition to humoral modulation, a pronounced neural pathway of negative-feedback regulation becomes apparent. This later effect was manifested within 12 hours (Fig. 1). Whether such high titres occur under physiological conditions in *Leptinotarsa decemlineata* is debatable. Nevertheless, these studies give us an insight into possible mechanisms of corpus allatum regulation. Clearly in *L. decemlineata*, innervation of the corpora allata seems to be essential for effectively restraining their activity under various circumstances, such as during starvation (KHAN *et al.*, 1982b) and in the present studies, during experimentally elevated juvenile hormone titres.

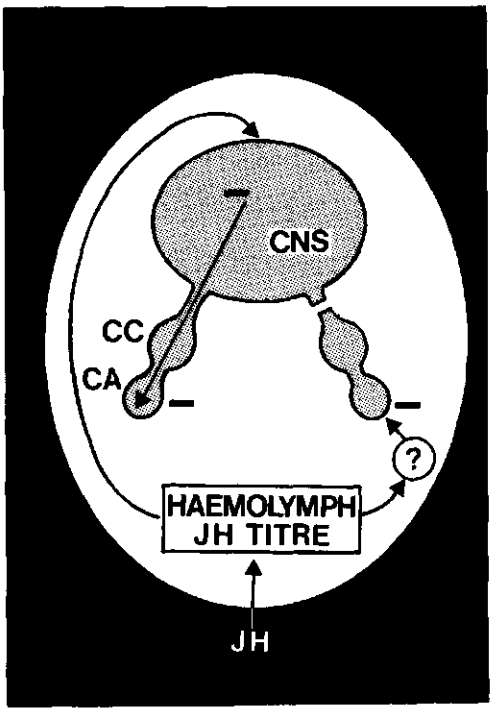

Fig. 4. Negative-feedback regulation during experimentally elevated juvenile-hormone (JH) titres. Inhibitory signals (-) from the central nervous system (CNS) restrain the activity of intact corpora allata (CA). Loose glands are also restrained (-). The source of this humoral factor, indicated by a question mark, remains unknown. CC (corpus cardiacum).

These observations are in good agreement with the well-documented work on negative-feedback regulation by juvenile hormone in *Diploptera punctata*. The corpus allatum activity of this cockroach was distinctly restrained when whole animals were treated topically with the juvenile hormone analogue. ZR 512 (TOBE and STAY, 1979) or implanted with supernumerary corpora allata (STAY and TOBE, 1978; TOBE and STAY, 1980). Denervation studies revealed that the negative-feedback loop in this cockroach operates predominantly via neural pathways, although a slight humoral effect was also apparent (TOBE and STAY, 1980).

It is noteworthy that, in *L. decemlineata*, 5 days after implantation of a supernumerary pair of corpora allata, direct determination of the haemolymph juvenile hormone titre by physico-chemical methods revealed that the titre was not elevated above that of beetles with only one pair of glands (KHAN *et al.*, 1982b). This indicated that the titre may be temporarily elevated shortly after treatment and partly through reduced hormone synthesis by the host and implanted glands; it was probably regulated to normal values by day 5. A few juvenile hormone-treated individuals did oviposit a day earlier than

controls in our experiments. In *D. punctata* it has been implied that acceleration of spermatophore release and oviposition is indicative of elevated titres (STAY and TOBE, 1978; TOBE and STAY, 1980). It should be pointed out that a temporary elevation in juvenile hormone titre at the time of treatment may be sufficient to cause the above effects, and whether the titre remains elevated throughout the experimental period can only be verified conclusively by its direct measurement.

The spontaneous rate of juvenile hormone biosynthesis by isolated corpora allata can be stimulated many-fold by including *trans, trans* farnesenic acid in the incubation medium, suggesting that the rate-limiting step(s) in the biosynthetic pathway of juvenile hormone occur prior to the formation of this intermediate (TOBE and PRATT, 1976). Fully developed corpora allata from 5-day-old ovipositing *L. decemlineata* displayed a 5-fold increase in activity when incubated *in vitro* with 20-60 μM of a farnesenic acid mixture also used here (KHAN *et al.*, 1982b). In the present studies, 12 hr after juvenile hormone injection (Fig. 2), the glands of hormone treated animals, although restrained in their spontaneous activity could be stimulated considerably by exogenous farnesenic acid. However, 3 days after treatment, the activity of restrained glands was stimulated only slightly above the spontaneous activity of corpora allata from controls (Fig. 2). Similar observations were made during restrainment of the corpora allata under the influence of starvation (KHAN *et al.*, 1982b). These results suggest that under prolonged neural inhibition the final two enzymatic steps in the biosynthetic pathway of juvenile hormone are also diminished.

Previous workers observed that treatment of prediapause *L. decemlineata* with an isomer mixture of juvenile hormone I did not induce oviposition but only delayed the onset of diapause (SCHOONEVELD *et al.*, 1977). We found that juvenile hormone III, the natural homologue in *L. decemlineata*, induced oviposition in about 50% of the short-day females and was much more affective than juvenile hormone I, ZR 512 or ZR 515. It is interesting that a single application of exogenous juvenile hormone III was sufficient to induce partial oviposition 9 days later. The corpora allata of hormone-treated beetles were never activated throughout this period and this is probably the reason why oviposition induction was only temporary and diapause was not prevented.

In cockroaches, the corpus allatum activity follows a cyclical pattern of increase and decrease closely correlated with ovarian cycles (STAY and TOBE, 1977; LANZREIN *et al.*, 1978), and apparently stimulatory and inhibitory

signals from the ovary are partly responsible for this synchronization (STAY and TOBE, 1978; TOBE, 1980; LANZREIN *et al.*, 1981). It has been suggested that the ovarian factors causing a decline in corpus allatum activity towards the end of a gonotrophic cycle may be ecdysteroids (STAY *et al.*, 1980). In *L. decemlineata* , 20-hydroxyecdysone had no effect on the gland activity. Interestingly enough, females of this species display no obvious cyclicity in oöcyte development (DORTLAND and DE KORT, 1978) or corpus allatum activity (KHAN *et al.*, 1982a) and besides, the ovary does not appear to be the main site of ecdysteroid synthesis (BRIERS and DE LOOF, 1981).

The precocenes are chromenes extracted from plants of the genus *Ageratum* and reportedly interfere with juvenile hormone-mediated processes in several, primarily hemimetabolous, insect species (BOWERS, 1976; PENER and ORSHAN, 1977; MACKAUER *et al.*, 1979; UNNITHAN *et al.*, 1980). We have tested precocene II using identical methods as those described above for demonstrating the effects of exogenous juvenile hormone III on corpus allatum activity. Such an approach is useful since comparisons between various studies on precocenes are difficult due to the range of indirect criteria employed to indicate corpus allatum integrity and the enormous variation in susceptibility of the insects (SODERLUND *et al.*, 1981). In *L. decemlineata*, precocene II has been reported to induce diapause in long-day beetles and the implication was that the corpora allata of the treated animals were inhibited (BOWERS, 1976). This effect could not be reproduced with our laboratory strain of *L. decemlineata* in spite of different modes of precocene II and III treatment, including topical application and fumigation (DE KORT, unpublished). The present results clearly show that the corpora allata of treated animals were unaffected (Table 5).

It has been reported that corpora allata of insect species which are not susceptible to precocene II treatment *in vivo* may be affected when exposed to the compound *in vitro* (PRATT and BOWERS, 1977; BOWERS and FELDLAUFER, 1981; SODERLUND *et al.*, 1981). For example, the corpora allata of *Periplaneta americana* exposed *in vitro* to more than 10^{-4}M precocene II for 30 minutes displayed reduced activities (PRATT and BOWERS, 1977). In our experiments, exposure of *Leptinotarsa* glands *in vitro* to 10^{-4}M precocene II during 5 hours incubation had no apparent effect on their activities. In addition, the integrity of these glands was confirmed by the fact that they could be maximally stimulated by exogenous farnesenic acid (Table 5). We must therefore conclude that *Leptinotarsa* corpora allata are relatively insensitive to the fairly high precocene II concentrations used in our studies.

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4. RELATIVE IMPORTANCE OF NEURAL AND HUMORAL PATHWAYS

ABSTRACT

To elucidate corpus allatum (CA) regulatory mechanisms under the influence of photoperiod and starvation in *Leptinotarsa decemlineata*, gland activities were measured *in vitro* by the short-term radiochemical assay. This data was substantiated with juvenile hormone (JH) titre determinations by using a physico-chemical method or a radio-immuno-assay. Under short-day conditions both neural and humoral factors may be involved in CA inhibition. This was indicated by the temporary activation of short-day glands after denervation in early pre-diapause and the gradual restrainment of active CA from long-day females implanted into short-day hosts. Studies with farnesenic acid as a precursor indicated that the last two steps in the biosynthetic pathway of JH are also diminished during humoral restrainment of the CA. Transfer of short-day beetles to long-day photoperiod completely activated the CA and this process was independent of CA innervation. Starvation leads to neural restrainment of intact glands but to possible stimulation of loose CA since implanted glands in starved hosts were fully activated irrespective of the photoperiod.

INTRODUCTION

Photoperiod has a profound effect on the life cycle of the adult Colorado potato beetle. Long-days induce reproductive activity whereas short-days lead to diapause (DE WILDE *et al.*, 1959). When long-day beetles were allatectomized, their reproductive activity ceased and they subsequently entered diapause, suggesting that the lack of juvenile hormone (JH) may be responsible for the diapause syndrome in this insect (DE WILDE and DE BOER, 1961). Measurement of the JH titre of the haemolymph by the Galleria bioassay as well as by physico-chemical methods revealed high levels of this hormone circulating in long-day beetles and extremely low amounts in pre-diapause beetles, the only hormone being JH III (DE WILDE *et al.*, 1968; DE KORT *et al.*, 1981). The suggestion that the corpora allata (CA) of prediapause animals were relatively inactive (DE WILDE and DE BOER, 1961) was verified conclusively by direct measurement of the activity of these glands *in vitro* (KRAMER, 1978b; KHAN *et al.*, 1982a).

In order to determine the factors responsible for inactivity of the CA under short-day, the effects of denervation or gland implantation on reproduction and diapause were observed (DE WILDE and DE BOER, 1969). These authors concluded that the CA of *L. decemlineata* are regulated mainly by the humoral pathway. They presumed that a neuroendocrine factor from the medial protocerebral neurosecretory cells was required for CA stimulation and that, under short-day this factor was absent.

At present, we are studying the regulation of CA activity in *L. decemlineata* by employing the short-term radiochemical *in vitro* assay for determining the activity of these glands (PRATT and TOBE, 1974; TOBE and PRATT, 1974). This assay has been adapted for *L. decemlineata* and been successfully used to study the regulation of the CA during starvation and during experimentally elevated JH titres in long-day females (KRAMER, 1978b; KHAN *et al.*, 1982a,b,c). This paper describes experiments concerning the influence of photoperiod and starvation on CA activity and the relative importance of the nervous and humoral pathways in regulating this activity. In addition to CA activity measurements *in vitro*, JH titres in the haemolymph were also determined in some experiments by using either a physico-chemical method (BERGOT *et al.*, 1981) or a radioimmunoassay (STRAMBI *et al.*, 1981).

MATERIALS AND METHODS

Adults of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) were reared either under long-day (18 h photophase, 25°C) or short-day (10 h photophase, 23°C) on fresh potato foliage as described previously (DE KORT, 1969). The beetles were collected routinely within 16 hours after emergence from the soil and timed from then onwards. Under these conditions long-day beetles begin oviposition on day 5 whereas short-day beetles do not lay eggs but enter diapause on day 11 or 12.

The experimental animals, males and females, were reared together in rectangular cages (volume ca. 8 l). Starved animals were provided with water and checked regularly for incidences of cannibalism.

Prior to all surgical operations the beetles were narcotized with CO_2 and surface sterilized with ethanol. The corpus allatum/cardiacum complexes were implanted via an incision dorsally in the thorax.

The CA activity was determined *in vitro* by using the short-term assay whereby the rate of incorporation of the methyl moiety of L-(Me-^{14}C)-methionine (Radiochemical Centre, Amersham, U.K., final specific activity 34-37 mCi/mmol) into juvenile hormone is used to estimate the rate of hormone synthesis (PRATT and TOBE, 1974; TOBE and PRATT, 1974). The assay conditions for *L. decemlineata* CA were similar to those described previously (KHAN *et al.*, 1982a).

One series of haemolymph samples was collected and extracted as described earlier (KHAN *et al.*, 1982b) and analysed qualitatively and quantitatively for JH at Zoecon Corporation, Palo Alto, California, using the gas chromatographic/mass spectroscopic method according to BERGOT *et al.* (1981).

Another series of haemolymph samples was extracted twice with methanol: water (70:30 v/v). The pooled extracts were mixed with 4 volumes of hexane and after partioning the hexane phase was transferred to appropriate glass ampoules. This was done thrice and the pooled hexane phase was evaporated to dryness under nitrogen. The residue was dissolved in 400 µl acetonitril and the ampoules sealed. These were transported in dry ice to Centre National de la Recherche Scientifique in Marseille, France for JH-radioimmunoassay (STRAMBI *et al.*, 1981).

Farnesenic acid mixture (consisting of 57% *trans, trans* and 31% *cis, trans* farnesenic acid) was a gift from Zoecon, Palo Alto, California.

The Wilcoxon-parameter-free test was employed for statistical evaluation.

RESULTS

The restrainment of active CA from long-day females implanted into short-day female hosts

Fully activated CA from 3 day old long-day females (KRAMER, 1978b; KHAN *et al.*, 1982a) displayed a slight, but not significant, decrease in activity one day after implantation into 1 day old short-day female hosts (Fig. 1). From 4 days onwards the implanted glands were significantly restrained ($p < 0.05$) when compared with intact CA of similar age from females kept under long-day. The implanted glands were never completely inactivated but maintained a basal rate of synthesis of about 1.5 pmol JH/pair/hr even in hosts which had been in diapause for 10 days (Fig. 1).

The addition of farnesenic acid to CA *in vitro* stimulates the rate of JH synthesis many-fold, implying that the rate limiting steps in the biosynthetic pathway of JH occur prior to the formation of this intermediate (TOBE and PRATT, 1976). Active CA from *L. decemlineata* were stimulated about five-fold

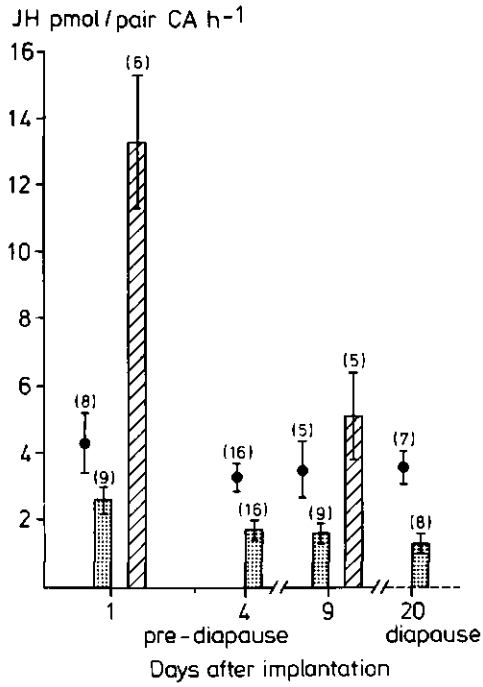

Fig. 1. The rate of JH biosynthesis of loose CA from 3-day-old, long-day females various periods after implantation into 1-day-old, short-day female hosts reared under short-day conditions (shaded columns). As controls, activities of intact CA of similar age from females maintained under long-day photoperiod are shown (●). Striped columns show the stimulated rate of JH biosynthesis by the restrained, implanted CA in the presence of 40 µM farnesenic acid in the incubation medium. Number of determinations are indicated in parentheses. Columns and bars represent means ± S.E.M.

in the presence of 20 to 60 µM of a farnesenic acid mixture which is also used in the present studies (KHAN *et al.*, 1982b). With this approach it was demonstrated that the last two enzymatic steps in the JH biosynthetic pathway are diminished during prolonged neural restrainment of the CA in *L. decemlineata* (KHAN *et al.*, 1982b; 1982c). This also appears to be the case during humoral restrainment of the glands since the stimulated rate of JH biosynthesis by the implanted glands in the presence of 40 µM farnesenic acid mixture *in vitro* also decreased significantly 9 days after the implantation (Fig. 1).

The role of CA innervation under short-day photoperiod

CA from short-day females display relatively low rates of JH biosynthesis at emergence, and are gradually inactivated just before diapause (KRAMER, 1978b; KHAN *et al.*, 1982a). The gland activities correlate well with the JH titres in the haemolymph of these insects (DE KORT *et al.*, 1981). In order to study the importance of CA innervation in the inactivation process, we implanted 1-day-old short-day female hosts with CA from similar donors and followed the activities of the implanted and host glands under short-day rearing conditions. One day after the operation, the implanted glands were significantly ($p < 0.05$) more active than the host glands and this difference was maintained until at least 5 days later (Fig. 2). These results indicate that innervation plays an important role for the maintenance of low (host) gland activity. However, the implanted CA did not achieve high rates of hormone biosynthesis (e.g. as displayed by similar implanted glands in hosts which were transferred to long-day photoperiod, see experiments below) and their activity declined from day 3 onwards, suggesting that humoral factors were also restraining the glands. The experimental animals never oviposited and entered diapause one day later than normally. In 50-days diapausing female hosts, the implanted as well as host CA were completely inactive (Fig. 2).

Effect of transference of short-day beetles to long-day photoperiod

When 2 day-old short-day beetles were transferred to long-day conditions they started oviposition 5 days later. Three days of exposure to long-day fully activated the CA of these females ($p < 0.005$) (Fig. 3a). The JH titre

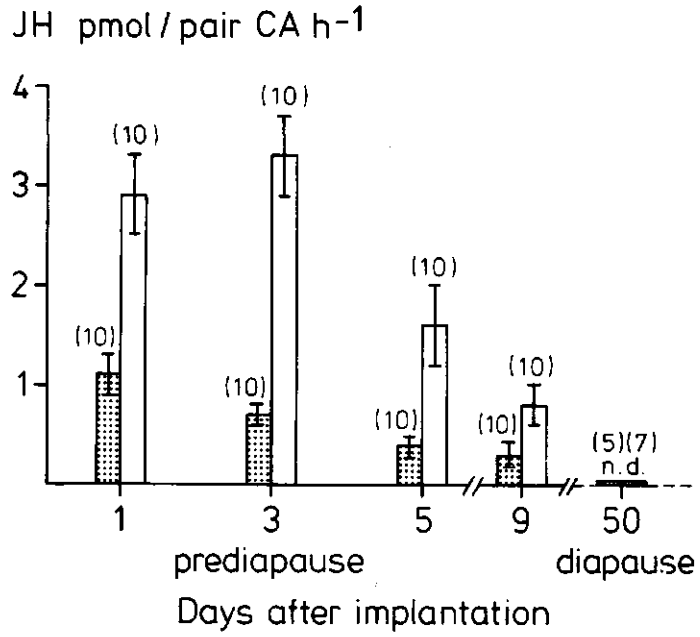

Fig. 2. The activities of intact host (shaded columns) and implanted CA (open columns) in relation to time under short-day conditions. Both gland donors as well as recipients were 1-day-old, short-day females at the time of implantation. n.d. = not detectable. Number of determinations are indicated in parentheses. Columns and bars represent the means ± S.E.M.

in the haemolymph of these insects, as determined by physico-chemical means, also showed an increase, reaching high levels 5 and 7 days after exposure to long-day (Table 1). The low JH titre on day 3, in spite of the high CA activity, may have resulted from the relatively high JH-esterase activity present in the haemolymph at this time (KRAMER, 1978a).

The CA of short-day females which were simultaneously starved after transference to long-day were not activated. In addition, the haemolymph JH titre, as determined by RIA, of starved females was also much lower than that of normally fed animals after 5 days in this experiment (Fig. 3a). In order to investigate the role of innervation under these circumstances, 1-day-old short-day female hosts were implanted with CA from similar donors. The operated animals were fed and kept another day under short-day before being transferred

Table 1. The JH III titre in the haemolymph of 2-day-old, short-day females after various periods of exposure to long-day photoperiod.

Days of Exposure to long-day	0	1	3	5	7
JH titre ng/ml	2.40	1.23	3.20	57.0	111.0

The titres were determined with the aid of physico-chemical methods and only JH III was detected. Each titre value represents pooled haemolymph collected from 15 to 20 females.

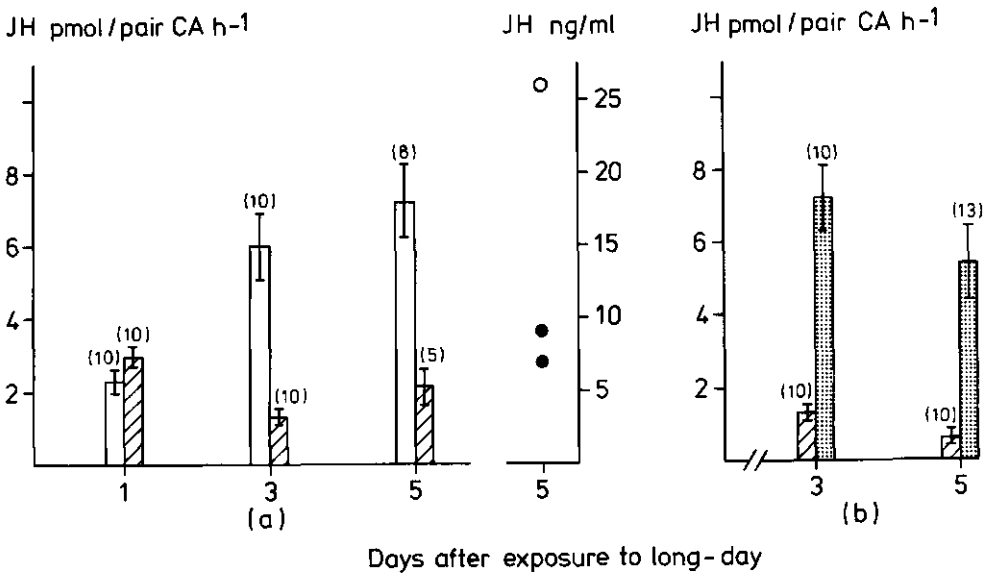

Fig. 3. (a) The effect of transferring 2-day-old, short-day females to long-day photoperiod on their CA activity. After transference the animals were either fed (open columns) or starved (striped columns). The JH haemolymph titres of these beetles, determined by RIA, 5-days after transference are displayed on the right (open circle, fed; closed circles, starved). Each circle represents an individual titre determination with pooled haemolymph from about 12 females.

(b) The activities of intact host (striped columns) and implanted CA (shaded columns) after transference of 2-day-old, short-day female hosts to long-day photoperiod and simultaneous starvation. Both gland donors and recipients were 1-day-old short-day females at the time of implantation. Number of CA activity determinations are indicated in parentheses. Columns and bars represent means ± S.E.M.

to long-day and starved. Three days after exposure to long-day, the loose CA were fully activated and remained so until at least 5 days whereas the intact (host) CA were restrained during this period ($p < 0.005$) (Fig. 3b). The starved beetles did not oviposit, nor was any egg development apparent after dissection. The fat body was almost depleted when examined visually.

Starvation-induced activation of loose CA

In the previous experiment, one cannot distinguish whether the activation of the implanted CA was due to the change in photoperiod or to starvation. To clarify this, another experiment was performed. After implantation of CA from one day old short-day female donors into females of similar age, the hosts were provided with fresh potato foliage and kept under short-day for another day. Thereupon, the experimental animals were randomly divided into 2 groups, one was transferred to long-day while the other remained under short-day. At the same time food was withheld from both groups and after 3 and 5 days the activities of the implanted CA were compared. As shown in Fig. 4a these glands were fully activated under both photoregimes, indicating that starvation itself leads to a humoral milieu in which loose CA can reach optimal activities.

For confirmation, the above experiment was repeated, but this time both experimental groups were reared under short-day conditions and food was withheld from only one group. The loose CA in starved females were significantly more active ($p < 0.025$) than the loose glands in fed females 3 and 5 days after treatment (Fig. 4b). In addition, the JH titre, as determined by RIA, was much higher in starved females than in fed females of this group on day 5 (Fig. 4b).

Intact CA from sham-operated fed and starved females were not activated after 5 days under short-day conditions. In addition, the JH haemolymph titres of such beetles remained low (Fig. 4c).

DISCUSSION

The experiments presented in this paper reveal some hitherto undescribed aspects of CA regulation in the adult Colorado potato beetle. The data obtained from direct measurement of CA activities *in vitro* correlated very well with the JH titre determinations under various experimental conditions.

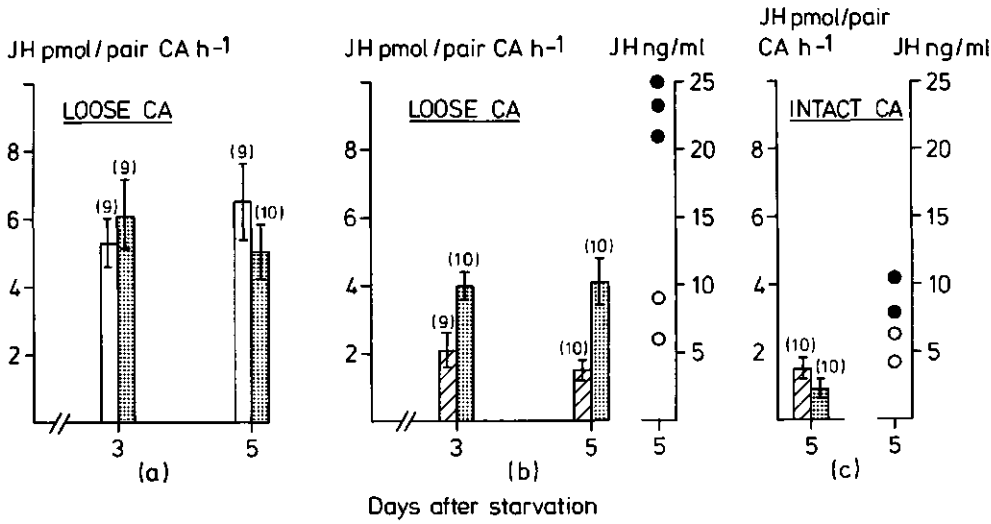

Fig. 4. (a) The effect of various periods of starvation on the activities of loose implanted CA in 2-day-old, short-day female hosts, maintained continuously under short-day (shaded columns) or transferred to long-day photoperiod (open columns). The CA donors and recipients were 1-day-old, short-day females at the time of implantation.

(b) The effect of various periods of starvation (shaded columns) or feeding (striped columns) on the activities of loose implanted CA in 2-day-old, short-day female hosts reared under short-day photoperiod. The gland donors as well as recipients were 1-day-old, short-day females at the time of implantation. Also shown on the right are the JH haemolymph titres of these females, determined by RIA, on the fifth day of starvation (closed circles) or feeding (open circles).

(c) The effect of 5-days starvation (shaded columns) or feeding (striped columns) on the activities of intact CA from 2-day-old, short-day sham operated females reared under short-day conditions. The haemolymph JH titres in these females, as determined by RIA are also shown on the right (closed circles, starved; open circles, fed). Each circle represents an individual titre determination with pooled haemolymph from about 12 females. Number of CA activity determinations are indicated in parentheses. Columns and bars represent means ± S.E.M.

The titre values obtained by the RIA were somewhat different from those estimated by physico-chemical analysis. A discussion on the possible causes of such differences between the two methods is beyond the scope of this paper.

The inhibition of the CA under short-day photoregimen

In *Pyrrhocoris apterus* it was suggested that neurally mediated signals were responsible for CA inhibition under short-day (HODKOVA, 1977) whereas, in the Colorado potato beetle, the humoral pathway appeared to play a predominant role in this process (DE WILDE and DE BOER, 1969). Our results imply that, under short-day conditions, the CA of *L. decemlineata* are regulated by both neural and humoral factors. During the early stages of pre-diapause, intact innervation played an important role in restraining the CA since loose glands were significantly activated at this time.
However, these loose glands did not maintain their activated states and their rate of JH biosynthesis started to decline 3 days after implantation, indicating a humoral influence (Fig. 2). The temporarily activated CA did not induce oviposition or prevent diapause in their short-day hosts. Indeed severing of the nervi corpori allati never prevented diapause in short-day beetles (DE WILDE and DE BOER 1969). In fact the induction of temporary oviposition in short-day females under short-day photoperiod was only achieved in a fraction of treated beetles by either implantation of large numbers of active CA (DE WILDE and DE BOER, 1969) or administration of high doses of JH III (KHAN *et al.*, 1982c). This relative insensitivity to JH in these insects may be due to several possibilities including the following: (1) High JH esterase activity in the haemolymph of short-day females may prevent high JH titres (KRAMER, 1978a; KRAMER and DE KORT 1976). (2) The reproductive target organs may not be sufficiently sensitized e.g. due to lack of JH receptors. (3) In addition to JH another neuroendocrine factor may be necessary to induce reproduction or prevent diapause. Further experimental work should clarify this issue.

The humorally mediated effect on the CA is also apparent from the observation that active glands from long-day animals were restrained after implantation into short-day females (Fig. 1). Factors in the haemolymph of the pre-diapause hosts must be responsible for the reduction in gland activity since active CA have been shown to maintain their rates of JH biosynthesis when implanted into long-day hosts using similar techniques (KHAN *et al.*, 1982b; 1982c). It is noteworthy that long-day CA implanted into short-day hosts did not become totally inactivated as did short-day glands.

The above evidence strongly suggests that in pre-diapause *L. decemlineata* complete inactivation of the CA occurs by the dual action of neural and

humoral factors (Fig. 5). This mode of action closely resembles that proposed for CA inactivation in larval insects. Direct measurements of CA activity in larval insects are scarce, but the available evidence indicates that both neural and humoral factors facilitate CA inactivation in the last larval instar although the contribution of each pathway may vary between species (GRANGER and SEHNAL, 1974; PIPA, 1980; BHASKARAN *et al.*, 1980). An additional similarity between adult *L. decemlineata* and larval insects is that in both cases CA inactivation is accompanied by high JH esterase activity in the haemolymph. (for review see DE KORT and GRANGER, 1981). Well-documented studies on the adult cockroach, *Diploptera punctata*, also demonstrate neural and humoral regulation of CA activity (for review see TOBE, 1980).

Activation of CA from short-day females

Exposure of short-day beetles to long-day photoperiod resulted in complete activation of their CA (Fig. 3). In contrast to gland restrainment, intact innervation does not appear to play a role during activation of short-day CA (Fig. 3b and 4). This evidence is supported by the finding that inactive CA from 7 day old short-day females when implanted into 1-day-old

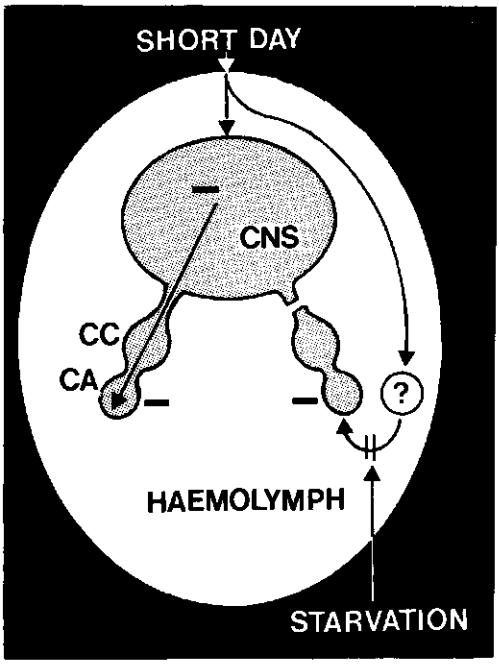

Fig. 5. Under short-day photoperiod, in pre-diapause females, both intact corpora allata (CA) as well as loose glands are restrained (-) in their activities. The neurally mediated inhibitory signals probably originate in the central nervous sytem (CNS) but the source of the humoral factors remains a question mark. When such beetles are simultaneously starved, the humoral restrainment is lifted, activating the loose glands.

allatectomized long-day females prevented diapause and restored oviposition (unpublished observations). Indeed, it has been reported that denervated glands could be repeatedly activated or inactivated by manipulating the photoperiod (DE WILDE and DE BOER, 1969).

When short-day beetles were also starved after transference to long-day, their intact CA remained restrained whereas implanted CA became fully activated (Fig. 3). This is in agreement with earlier conclusions that intact CA innervation is essential for restraining JH biosynthesis during starvation (KHAN *et al.*, 1982). However during the present starvation experiments, denervated CA became activated, regardless of the photoregimen to which the hosts were exposed (Fig. 4). Thus starvation overcomes the humorally mediated restrainment of loose CA implanted into short-day females. It is perhaps interesting to speculate on the physiological significance of this phenomenon. If food supplies are restricted, the neural restrainment of the CA together with humoral stimulation in short-day females may ensure intermediary JH-titres so that diapause can be avoided under circumstances whereby the insect has not built up sufficient nutrient reserves. In larval *Manduca sexta* there are well documented studies indicating that starvation leads to CA activation presumably via an allatotrophic factor (BHASKARAN and JONES, 1980) but this phenomenon has not yet been described in adult insects.

WIGGLESWORTH's (1952) conclusion on the importance of intact innervation for CA regulation is clearly supported by the present studies and previous observations in *L. decemlineata* (KHAN *et al.*, 1982b,c). The present findings also provide additional evidence for the suggestion that neural inhibitory signals take precedence over any stimulatory signals (TOBE *et al.*, 1981). In *L. decemlineata*, the central nervous system influences JH synthesis by either releasing or blocking inhibitory signals by way of the nervous tracts. In the absence of neural inhibition humorally mediated factors determine the extent of CA activation. The nature and source of the humoral factors still remains to be conclusively established. The bulk of circumstantial evidence points towards a CA stimulatory substance, an allatotropin, produced in the neurosecretory cells of the protocerebrum (GRANGER and SEHNAL, 1974; BHASKARAN *et al.*, 1980; REMBOLD *et al.*, 1981; TOBE *et al.*, 1981). Nevertheless, the existance of an humorally mediated allatohibin cannot be completely ruled out at this stage.

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5. INNERVATION OF THE CORPUS ALLATUM

ABSTRACT

The application of horse radish peroxidase for tracing the cells innervating the corpus allatum in adult *Leptinotarsa decemlineata*, using simple instruments, is described. The corpus allatum in this insect appears to be innervated by a single group of about 8 cells. The size and position of these cells indicate that they belong to the L-type lateral neurosecretory cells. The axons from each of these cells split into two pathways: one pathway arborizes extensively in the dorsal part of the brain adjacent to the pars intercerebralis whereas the other passes unbranched through the neuropile of the protocerebral lobe and corpus cardiacum into the corpus allatum. Additional cell groups in the pars intercerebralis which were stained when the corpus cardiacum was also contaminated with horse radish peroxidase are illustrated. The possible involvement of the lateral neurosecretory cells in the regulation of juvenile hormone biosynthesis is discussed.

INTRODUCTION

The juvenile hormone titre in the haemolymph as well as the corpus allatum activity is influenced by a variety of external and internal factors. Recent studies revealed that neural as well as humoral signals are involved in the regulation of the corpus allatum activity in the adult Colorado potato beetle, *Leptinotarsa decemlineata*. Intact innervation of the corpus allatum seemed to be essential for restraining their activity in starved females (KHAN *et al.*, 1982b). Such neural restrainment was also apparent when the juvenile hormone titres were experimentally elevated, although under these circumstances humoral factors too contributed towards gland restrainment (KHAN *et al.*, 1982a). Under short-day photoperiod, both neural and humoral factors appeared to act together to inactivate the corpus allatum in pre-diapause females (KHAN *et al.*, 1983). In order to locate the source of the neurally mediated inhibitory signals it is important to know the precise innervation of the glands. Previous studies indicated that the retrocerebral complex received two main tracts of axons, the nervi corporis cardiaci (NCC) I and the NCC II, emanating in the brain from the median and the lateral neurosecretory cells (NSC) respectively (Schooneveld, 1970; 1974). However, with traditional histological methods, it was not possible to deduce conclusively which group(s) of NSC had axons terminating in the corpus cardiacum and which ones in the corpus allatum. The advent of highly specific neuronal markers enables us to overcome this difficulty. The cobalt chloride diffusion and precipitation method has been used to trace the axonal pathways between the brain and the retrocerebral complex in several insect species (MASON, 1973; NIJHOUT, 1975; FRASER and PIPA, 1977; PIPA, 1978). In the present paper we describe a simple technique for visualising cells and axons in the brain which specifically innervate the corpus allatum in the adult Colorado potato beetle, utilizing retrogade diffusion with horse radish peroxidase (HRP). The position and nature of these cells is analysed histologically at the light microscope level.

MATERIALS AND METHODS

Insects

Adult males and females of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), were reared on fresh potato foliage as described previously (DE KORT, 1969). Routinely, 1 to 2 weeks old post-diapause females, which had been in diapause for 3 months, were used because of their rather well-developed corpora allata. Diapause was terminated by digging the insects out of the soil and rearing them further under long-day (18 hr) photophase.

Application of horse radish peroxidase

The heads of unanaesthesized beetles were separated from the body and mounted under a Ringer solution described previously (KHAN *et al.*, 1982a). This saline was modified by the addition of 2% glucose and adjustment of its pH to 6.9. The brain, suboesophageal ganglion (SOG) and retrocerebral glands were carefully dissected out, keeping the nerve connections between these structures intact. This complex, hereafter referred to as brain complex, was maintained further under Ringer and the HRP introduced into the corpus allatum by one of the following methods:

a) The proximal one-quarter part of a corpus allatum was cut off with a micro-dissection scissors. A bottleneck was created at the tip of a microcapillary (Clark GC-200F-6) by holding it for a few seconds in a gas flame. A number of such micro-capillaries were kept at hand and a suitable one selected according to the size of the corpus allatum. The micro-capillary, supported on a piece of moulding plasticine, was positioned next to the damaged corpus allatum which was then sucked up into the bottleneck (diameter about 50 to 100 µ) by mouth via a rubber tube. A good fit was considered to be one whereby the corpus allatum formed a water-tight plug in the bottleneck, anchoring the rest of the brain complex firmly to the capillary tip (Fig. 1). The Ringer in the capillary was removed with a fine plastic canula attached to a syringe and replaced by a few microlitres of a 5% aqueous solution of HRP (Grade II, Boehringer, Mannheim). After allowing to stand undisturbed for about 4 hr at room temperature, the brain complex was carefully loosened from the capillary tip and subsequently submerged in Ringer containing 5 µg/10 ml

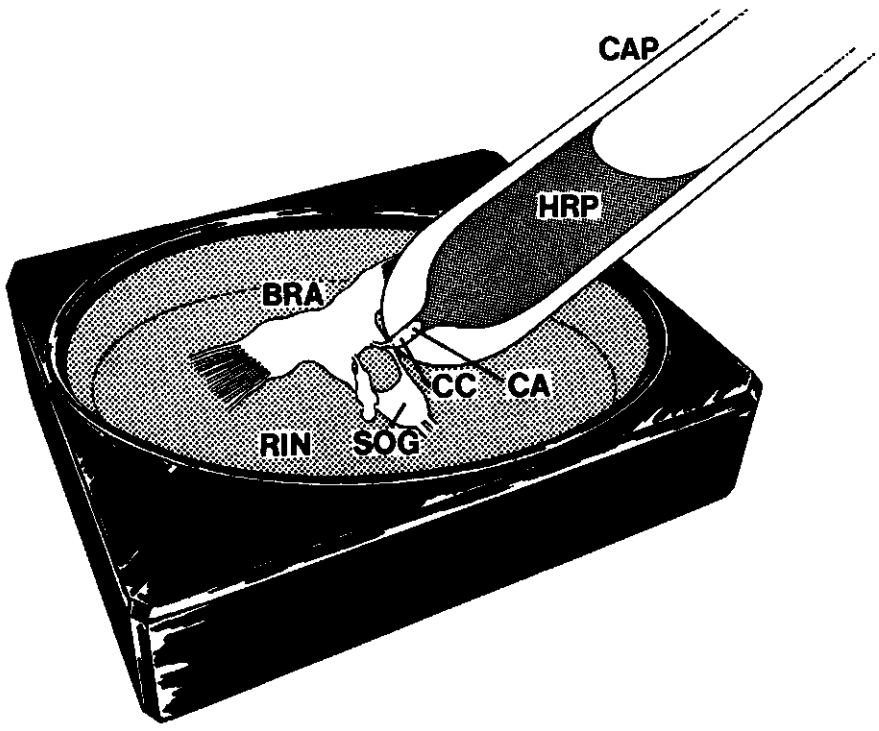

Fig. 1. Method for infusing horse radish peroxidase (HRP) into corpus allatum (CA). After damaging the allatal membrane, the gland was sucked up into the tip of a specially prepared micro-capillary (CAP), forming a water-tight plug. Subsequently, the capillary tip was filled with the enzyme. Throughout, the brain (BRA) complex was kept under Ringer (RIN). The length of the CA/CC complex is about 200 µ in reality. CC (corpus cardiacum); SOG (suboesophageal ganglion).

3,3'-Diaminobenzidine tetrahydrochloride (Grade II, Sigma) and 20 µl/10 ml hydrogen peroxide (BDH Chemicals Ltd.) for about 20 minutes. During this period, cell bodies and axons, which had been filled by retrograde diffusion of the HRP from out the corpus allatum, were stained dark brown and became visible under the dissection microscope. Although this method gave excellent results, seepage of the HRP along the outer allatal membrane and subsequent contamination of the adjacent corpus cardiacum could not be completely ruled out. In order to be absolutely sure that only axon terminals within the corpus allatum came into contact with the enzyme another method was used.

b) Glass micro-capillary (Clark GC-200F-15) tips of about 30 μ were prepared with the aid of a pipette puller (Model 700c, David Kopf Instruments). The tips were filled with a 7% HRP solution in Ringer and mounted on a micromanipulator (Prior). Using minute pins and avoiding damage, the brain was carefully positioned under Ringer, in such a manner that the corpora allata were readily accessible and pressed against the bottom of the container. This position restricted movement of the gland and facilitated penetration of the allatal membrane by the capillary tip. The horse radish peroxidase solution was slowly injected into the corpus allatum by slight pressure applied with a syringe via a rubber tube. The brownish solution could be seen entering the semi-transparent corpus allatum. After 4 hr incubation under Ringer at room temperature, the HRP in the brain complex was stained as described above.

About 4 brain complexes could be incubated daily by using either of the 2 methods and the rate of successful fillings was ± 30%. About 40 successfully filled complexes were analysed for this study.

Histological procedures

The brain complexes were subsequently fixed in either 5% formaldehyde or in Heidenhain's susa for 12 to 24 hr. After dehydration and clearing, they were preserved in depex (Fluka) as whole mounts, or embedded in paraffin (Paraplast plus) and serially sectioned (7 μ thick). These sections were counterstained with haematoxylin-eosin (Mayer).

RESULTS

Cells in the brain innervating the corpus allatum

The two methods of HRP application described above gave similar results. Findings whereby the enzyme was specifically injected into the gland only confirmed what was already revealed by passive infusion of HRP into a damaged corpus allatum. It was noticed that when the allatal membrane was left undamaged, infusion of HRP into the gland did not occur so readily.

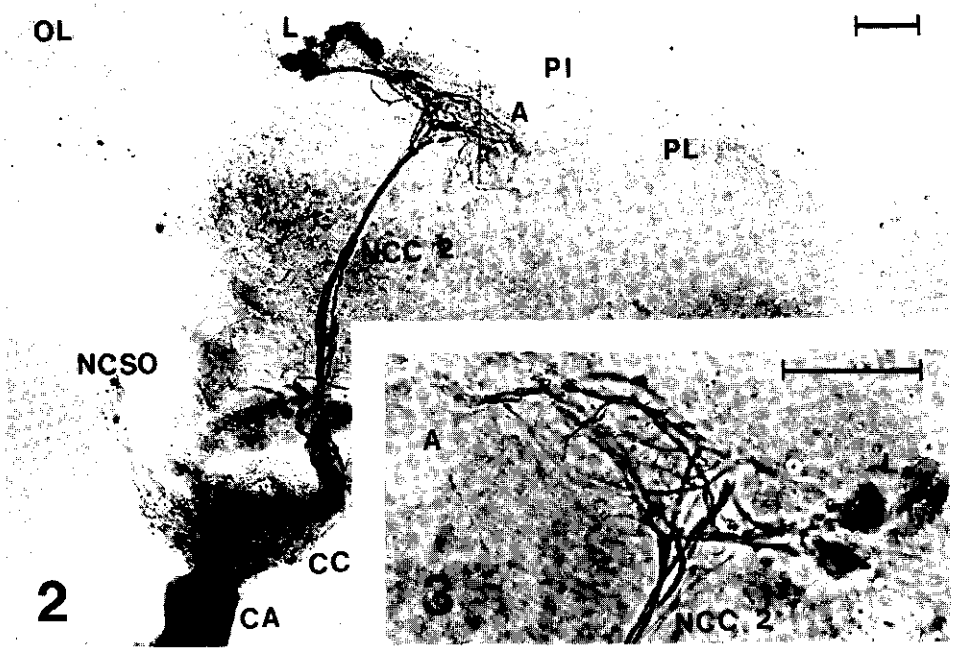

Fig. 2. A montage foto of the structures visualised in a whole mount preparation after infusion of HRP into a left corpus allatum according to the first method.

Fig. 3. Detail of the axons from the lateral NSC (right corpus allatum filled) at the junction where they split into two pathways: one leads to the NCC 2 and the other arborizes extensively in the dorsal region of the protocerebral lobe. Each horizontal bar represents 50 µ.
Abbreviations: A (Arborizations); CA (corpus allatum); CC (corpus cardiacum); L (lateral neurosecretory cells); NCC (nervus corporis cardiaci); NCSO (nervus cardiaco suboesophagealis); OL (optic lobe); PI (pars intercerebralis); PL (protocerebral lobe).

A typical result obtained is shown in Fig. 2. Filled axons and the cell bodies appeared intense dark brown after treatment and were distinctly visible even in fresh uncleared preparations. Most prominent was a compact group of 8±2 cell bodies lying just under the dorso-caudal surface of the ipsilateral protocerebral lobe, adjacent to the optic lobe. The shape of the perikarya was ellipsoid and maximum dimensions ca. 25 µ by 15 µ. The axons from these cells ran laterally towards the pars intercerebralis for a short distance and then divided into 2 pathways. One pathway continued laterally before branching extensively. These fine arborizations penetrated a large area of the dorsal surface of the protocerebral lobe and extended as far as

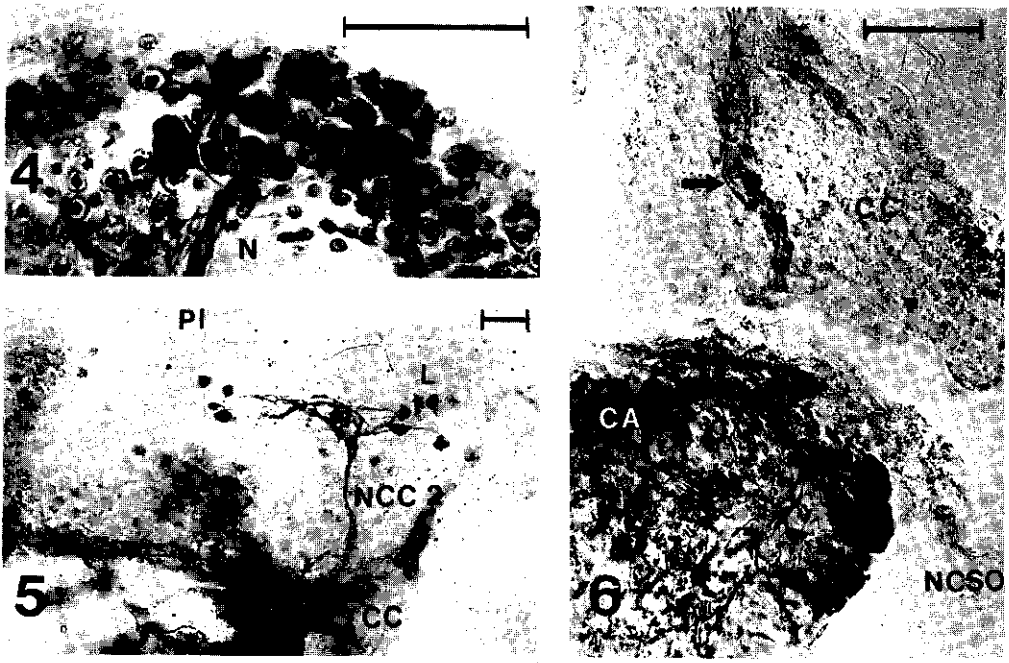

Fig. 4. The dark, HRP filled, cells are distinctly visible in histological sections counter-stained with haemotoxylin-eosin.

Fig. 5. A whole mount preparation in which the corpus cardiacum is also contaminated with horse radish peroxidase. An additional group of 4 cells in the ipsilateral pars intercerebralis is seen. No other axonal pathway except the NCC 2 was apparent.

Fig. 6. A whole mount preparation of the corpus allatum/cardiacum complex whereby the corpus allatum was injected with HRP. The axons passing through the corpus cardiacum on their way to the brain are indicated (arrow). The NCSO, leading to the SOG, remained unstained. Each horizontal bar represents 50 μ.
Abbreviations: CA (corpus allatum); CC (corpus cardiacum); L (lateral neurosecretory cells); N (Neuropile); NCC (nervus corporis cardiaci); NCSO (nervus cardiaco suboesophagealis); PI (pars intercerebralis).

the pars intercerebralis, and in some cases, below the pars intercerebralis into the contralateral protocerebral lobe (Fig. 2 and 3). The other pathway of the axon turned ventrally and traversed the neuropile of the protocerebral lobe to the NCC without branching. This is probably the axonal pathway known as NCC 2 (Fig. 2 and 7; SCHOONEVELD, 1970; 1974). From the NCC these axons passed unbranched through the corpus cardiacum and entered the corpus allatum via the Nervus corporis allati (NCA) (Fig. 6). The nature of the filled

perikarya was determined by examining histological sections counter-stained with haemotoxylin-eosin. Neurosecretory cells, which are stained blue by this procedure, can be distinguished amongst ordinary neurons. As shown in Fig. 4 the dark brown HRP filled cells were distinctly visible in such sections. They appeared to belong to L-type NSC cells, forming part of the group of lateral NSC. These histological sections further revealed that some of the fine axonal arborizations described above (Fig. 2 and 3) extended up to the median NSC lying in the ipsilateral pars intercerebralis although it could not be ascertained whether there was intimate contact between them. No collaterals from the NSC 2 into the neuropile could be seen.

Additional cell groups filled via the corpus cardiacum

In a few cases (5%), about 3 to 4 cells in the ipsilateral region of the pars intercerebralis were also distinctly filled. These cells were presumably filled via the NCC 2 since no other axonal pathways were apparent (Fig. 5). Such a lateral pathway of median NSC has been described before (SCHOONEVELD, 1974). However, in the present preparations, it was not possible to determine in what part of the retrocerebral complex these axons terminated because the corpus cardiacum was also slightly stained. When the corpus cardiacum was heavily stained with HRP, the axonal pathway known as NCC 1, together with a group of cells in the contralateral pars intercerebralis were visualised. These cell groups were not studied in detail. Fig. 7 is a schematic representation of the various cell groups and their axons visualised as a result of filling the corpus allatum specifically and as a result of corpus cardiacum contamination with HRP.

Connections with the sub-oesophageal ganglion

A delicate nerve, the Nervus cardiaco-suboesophageal is (NCSO), runs between the corpus cardiacum and the SOG. About 15 carefully dissected complexes, whereby this nerve was kept intact, were observed after filling the corpus allatum by HRP injection. No distinctly filled perikarya could be distinguished in the SOG, even after 6 hr incubation in some cases. Detailed examination of the retrocerebral complex revealed that the NCSO remained clear even though axons from the corpus allatum passing through the corpus cardiacum towards the brain could be distinguished (Fig. 6).

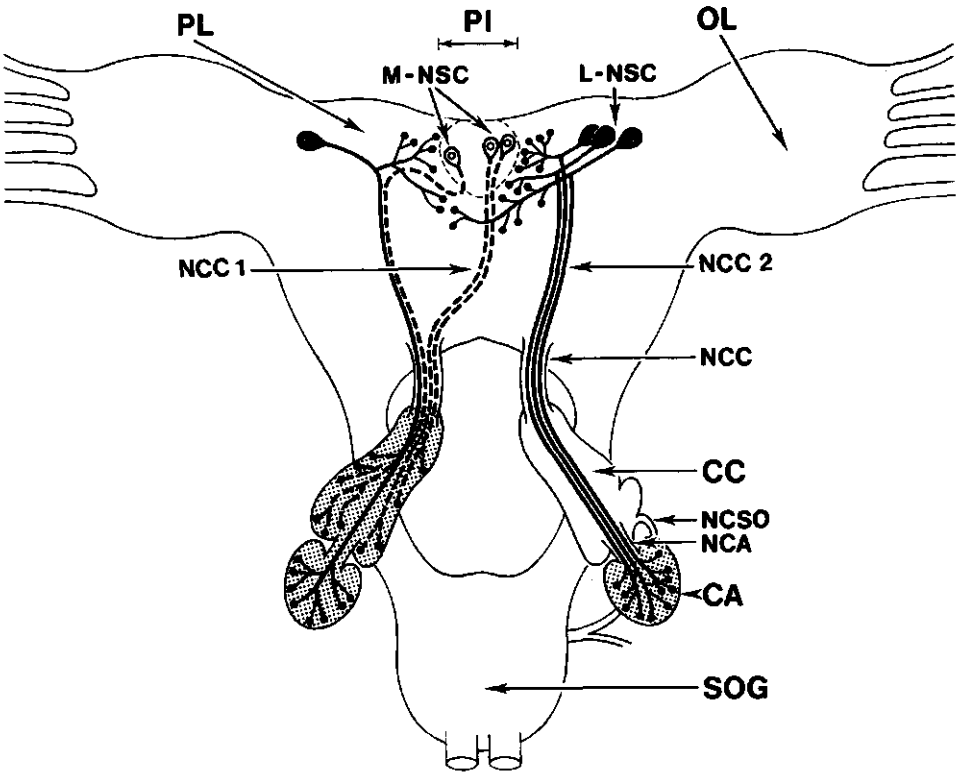

Fig. 7. Schematic representation of the various cell groups and axonal pathways visualised when only the corpus allatum was filled with HRP (right) and when the corpus cardiacum was also contaminated with the enzyme (left). Only a few cells in each group are represented and the size of the retrocerebral complex has been exaggerated.
Abbreviations: CA (corpus allatum); CC (corpus cardiacum); L-NSC (lateral neurosecretory cells); M-NSC (median neurosecretory cells); NCA (nervus corporis allati); NCC (nervus corporis cardiaci); NCSO (nervus cardiaco suboesophagealis); OL (optic lobe); PI (pars intercerebralis); PL (protocerebral lobe); SOG (suboesophageal ganglion).

DISCUSSION

With the methods described in this paper, it has been possible to follow the innervation of the corpus allatum specifically, in spite of the close proximity of this gland to the corpus cardiacum in *L. decemlineata*.

Innervation of each corpus allatum seems to be limited to a compact group of about 8 L-type lateral NSC in the ipsilateral protocerebral lobe of the brain. A rather unexpected finding because, until now, it was suspected that the corpora allata in this insect were innervated by the A-type median NSC from the pars intercerebralis and that the axons of the L-type lateral NSC terminated in the corpus cardiacum (SCHOONEVELD, 1970; 1974). This contradiction illustrates the need for caution when interpreting histological data based on one method. Although our results clearly show a direct connection between lateral NSC and the corpus allatum, the remote possibility that other cells innervating the gland may have escaped detection with the present approach cannot be completely excluded.

The number of lateral NSC which were filled by HRP was variable. This may be related to the extent of corpus allatum filling with the enzyme, an event which could not be controlled precisely. On the other hand, large variability in lateral NSC number (range 5 to 25) has been reported in previous studies (SCHOONEVELD, 1970).

A prominent feature of the present study is the presence of intricate arborizations displayed by the axons emanating from the lateral NSC. These arborizations are restricted to the dorsal part of the protocerebral lobe. Similar arborizations in *P. americana* extended into the neuropile of the cerebral lobe and even the NCC 2 displayed collaterals into the neuropile (PIPA, 1978). It has been proposed that such axonal branching from NSC may provide sites for synaptic communication (ADIYODI and BERN, 1968).

In very few cases we have observed median NSC being filled by way of the NCC 2 (Fig. 2). This confirms the previous finding that some ipsilateral A-type median NSC have axons running ipsilaterally via the NCC 2 (SCHOONEVELD, 1974). Some of these axons appear to terminate in the corpus cardiacum, very close to the corpus allatum and it is probable that the rare filling of these cells in the present experiments was due to contamination of these. In addition, the NCC 1 and cell bodies in the contralateral pars intercerebralis (SCHOONEVELD, 1970), also became apparent when the corpus ~~allatum~~ cardiacum was heavily stained. Although these cells were not studied in detail, we mention them in order to illustrate the possibility of employing this technique for studying NSC with axon terminals in the corpus cardiacum.

The present findings are in good agreement with those in adult *Schistocerca paranensis* (STRONG, 1965b) and adult *S. vaga* (MASON, 1973). In these locusts the NCC 2, which innervates the corpus allatum with about 10-12

lateral NSC in the brain, is morphologically separate from the NCC 1 and can therefore be approached more specifically. In adult *Periplaneta americana*, in addition to lateral NSC, 2 clusters of median NSC were suggested to innervate the corpus allatum, but in this study, contamination of axon terminals in the corpus cardiacum could not be completely ruled out (FRASER and PIPA, 1977; PIPA, 1978). Similarly, in larval and pupal *Manduca sexta* several clusters of NSC in the brain, including lateral NSC, were proposed to innervate the corpus allatum (NIJHOUT, 1975). However, as pointed out by the author, the corpus cardiacum of Lepidopteran species is relatively poorly supplied with axon terminals, so that the corpus allatum in *Manduca sexta* may supplement the role of the corpus cardiacum as neurohaemal organ. Indeed, it has been suggested that the corpus allatum is the neurohaemal organ for prothoracicotrophic hormone in *Manduca* (AGUI *et al.*, 1980).

In *S. paranensis*, *S. vaga* and *P. americana* the NCA 2 connects the corpus allatum with the SOG, but this pathway does not seem to be involved in the control of corpus allatum activity (STRONG, 1965b; MASON, 1973; FRASER and PIPA, 1977; PIPA, 1982). The present findings in *L. decemlineata* do not reveal any direct connection between the corpus allatum and the SOG via the NCSO, in agreement with earlier suggestions (SCHOONEVELD, 1970). If this is true then the proposal that the NCSO may mediate inhibitory signals from the SOG to the CA in this insect seems unlikely (DE WILDE and DE BOER, 1969).

The involvement of lateral NSC in corpus allatum regulation in adult insects was first proposed by STRONG (1965b) in female *S. paranensis*. He found that sectioning the NCC 2 had a negative effect on gland volume as well as the length of the terminal oocyte and deduced that stimulatory signals from the lateral NSC to the corpus allatum were mediated via this pathway. The significance of intact corpus allatum innervation for its control by the brain has been implied in many adult insect species including *Rhodnius prolixus* (WIGGLESWORTH, 1952), *Diploptera punctata* (ENGELMAN, 1959), *Pyrrhocoris apterus* (HODKOVÁ, 1977) and *Periplaneta americana* (PIPA, 1982). In *D. punctata* (STAY and TOBE, 1977; TOBE, 1981) and *L. decemlineata* (KHAN *et al.*, 1982b; 1983), direct measurement of the corpus allatum activity *in vitro* revealed that this gland is regulated by the dual action of neurally and humorally mediated factors. In *L. decemlineata* and most other species, contrary to the situation in *Schistocerca* (TOBE *et al.*, 1977), denervation seems to lift inhibitory signals. The source of these neurally mediated signals has yet to be located. In the light of the present findings, it is justified to assume

that some, if not all, the lateral NSC innervating the corpus allatum in *L. decemlineata* may constitute such an inhibitory centre, analogous to the stimulatory centre suggested in *S paranensis* (STRONG, 1965b). This argument is further supported by ultrastructural examination which shows that axon terminals in the corpus allatum are widely distributed inbetween the gland cells and contain electron dense granules. The presence of synaptic vesicles seems to indicate synaptic connections between these axon terminals and the gland cells (SCHOONEVELD, unpublished).

In conclusion, although the histological relationship between the lateral NSC and the corpus allatum needs to be confirmed by physiological studies, it is perhaps appropriate to recall STRONG's (1965a) warning, that conclusions concerning corpus allatum regulation, drawn from cauterization studies of the pars intercerebralis, whereby the state of the lateral NSC has been ignored, need to be viewed with caution.

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GENERAL DISCUSSION

Insect endocrinologists have pondered over the subject of corpus allatum regulation ever since Wigglesworth's (1936; 1948; 1952) classic experiments with *Rhodnius prolixus*. His original statement that, "normal regulation (of the corpus allatum) is dependant on its connexion with the central nervous system and that the signal to the corpus allatum to change its secretory activity comes from the brain" (Wigglesworth, 1952) has stood the test of time, although during the past decade the involvement of several additional factors has come to light (For review see Tobe, 1980).

The introduction of an *in vitro* assay for direct measurement of corpus allatum activity has, no doubt, enriched our knowledge on this subject (chapter 1). Using this technique some previous opinions have been confirmed and extended whereas others have been invalidated. For example, juvenile hormone is not stored in the corpus allatum to any appreciable extent (Tobe and Pratt, 1974; Pratt *et al.*, 1975; Kramer, 1978). Therefore the major regulatory mechanisms of this hormone must occur at the level of synthesis, which in turn is dependant on the activity and/or concentration of critical enzymes in the biosynthetic pathway. These rate limiting enzymes in the biosynthetic pathway occur prior to the final two steps in juvenile hormone biosynthesis, namely the methylation and epoxidation of the farnesyl precursor (chapter 2). For all its attributes, it remains an *in vitro* method, and in order to have some notion of the *in vivo* situation we also measured the juvenile hormone titres in various experiments. This approach has clearly shed new light on corpus allatum regulation in *L. decemlineata*. The most significant is perhaps the finding that intact innervation between the gland and the central nervous system is essential for its normal regulation (chapters 2, 3 and 4). It is noteworthy that this neural effect e.g. during starvation (chapter 2) and short-day treatment (chapter 4) would not be apparent if oviposition was used as criterium for active corpora allata as was done previously (de Wilde and de Boer, 1969).

This thesis clearly shows that both neural and humoral factors are involved in the regulation of corpus allatum activity (chapter 3 and 4). It is interesting how these two factors interact to achieve different levels of

gland activity. For fully active corpora allata (e.g. under long-day) there is neither neural nor humoral restrainment present. For "intermediary" levels of activity (e.g. during starvation) humoral factors enhance gland activity but are predominated by neurally restraining factors (chapters 2 and 4). This mechanism ensures that, under these circumstances, the corpora allata do not become completely inactive, and this is obviously vital for the survival of the insect. For complete inhibition of corpus allatum activity (e.g. in pre-diapause beetles) both neural and humoral factors must exert a restraining influence (chapter 4). However, it should be kept in mind that there may be inherent differences in the basal rates of juvenile hormone biosynthesis between long-day and short-day beetles because, under experimental conditions, corpora allata from long-day females did not become completely inactivated (chapter 4). Our experiments also reveal that innervation is only essential for restraining corpus allatum activity and not for activation of the glands (chapter 4).

The existance of a negative feedback loop regulating the corpus allatum in *L. decemlineata* has been confirmed (chapter 3). Restrainment of gland activity occurred mainly via nervous pathways. Apparently, a regulatory centre in the brain is capable of integrating such divergent stimuli as starvation and elevations in juvenile hormone titres and translating them via nerve connections into reduced corpus allatum activity. However, considering the diversity of response of the denervated corpora allata under these circumstances, it is clear that the system is quite complex and cannot be explained solely by the "final common pathway" concept of neuroendocrine regulation (Scharrer and Scharrer, 1963).

According to Tobe (1980), regulators of juvenile hormone synthesis which originate outside the corpora allata may be divided into two groups: the allatotropic factors (allatotropins) which stimulate or enhance juvenile hormone biosynthesis, and the allatostatic factors (allatostatins) which inhibit or reduce juvenile hormone biosynthesis. As described above, these factors may be neurally or humorally mediated. The source(s) and nature(s) of the humorally mediated factor(s) in our studies with *L. decemlineata* remain unknown. This humoral factor may be allatotropic or allatostatic. Possible sources of such factor(s) include the central nervous system and target organs such as ovary and fat body. As discussed in chapter 4, in several insect species, there is circumstantial evidence implicating the

median neurosecretory cells of the brain as the source of an allatotropic factor. In some cockroaches the ovary may be an important source of humorally mediated allatotropins as well as allatostatins (chapter 3). The influence of the ovary on corpus allatum activity in *L. decemlineata* has not been investigated. It is perhaps worthwhile to discuss this relationship on the basis of available evidence. The fact that the corpora allata are fully active in ovipositing females and inhibited in pre-diapause females, together with the finding that gland activities of males and females are not significantly different (chapter 1), render it unlikely that the fully developed ovary is the source of allatostatic factors. Could the ovary be the source of allatotropic factors? There are several arguments against this being so: The corpora allata of pre-diapause beetles in which temporary oviposition had been induced by treatment with juvenile hormone never became activated (chapter 3). On the other hand, in starved, pre-diapause females, loose corpora allata became activated (chapter 4). It is possible that the influence of the ovary on corpus allatum activity under all these conditions is obscured by other factors. However, it would appear that, compared with the brain, the ovary seems to play a relatively minor role in corpus allatum regulation in *L. decemlineata*.

As for the fat body, there is nothing known about its role in corpus allatum regulation, although this tissue synthesizes juvenile hormone esterases and therefore influences the final hormone titre at certain stages during adult development (Kramer and de Kort, 1976). It is interesting that in all insects studied so far an increase in juvenile hormone esterase activity in the haemolymph is accompanied by a decrease in corpus allatum activity (for review see de Kort and Granger, 1981). Could juvenile hormone esterase be the elusive humoral factor directly inhibiting corpus allatum activity? This speculation is worthy of consideration, although, it is experimentally difficult to measure the direct effect of juvenile hormone esterase on corpus allatum activity.

The evidence presented in chapter 5 indicates that the source of the neurally mediated factors may be located amongst the lateral neurosecretory cells of the brain. The corpus allatum may act as a neurohaemal organ for some of these neurosecretions while others may regulate the activity of gland cells via neurosecretomotoric innervation. The denervation experiments seem to indicate that allatostatic factors are mediated neurally. These findings remain to be confirmed by cauterization studies.

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SUMMARY

Control of corpus allatum activity was investigated in adult *Leptinotarsa decemlineata* (Say) under various external conditions. Gland activity was measured *in vitro* utilizing the incorporation of (^{14}C)-methyl-Methionine into juvenile hormone III by individual pairs of corpora allata.

The activities of corpora allata during adult maturation were found to be significantly higher in reproductive, long-day animals than in pre-diapause, short-day beetles. The activities of male and female glands were similar. During diapause no activity was detectable, whereas corpora allata from post-diapause beetles were totally reactivated within 5 days. There was no clear correlation between the spontaneous rate of juvenile hormone biosynthesis and gland volume although larger glands appeared to be capable of higher activities. Corpora allata from a population of beetles of similar age did not display any synchronous diurnal rhythmicity.

During starvation, young long-day females displayed reduced corpus allatum activities as well as low juvenile hormone titres in the haemolymph. Denervation and implantation studies revealed that intact nervous connections between the central nervous system and corpora allata are essential for restraining juvenile hormone biosynthesis during a 5 days starvation period. The initial activation of corpora allata from newly-emerged long-day females was not dependent on the first feeding.

When the titre of juvenile hormone III in long-day females was elevated by implantation of 3 pairs of supernumerary corpora allata or by injection of 50 µg of the hormone dissolved in 1 µl olive oil, the rate of endogenous hormone production was significantly restrained. Denervation experiments indicated that this restrainment occurred predominantly via the neural pathway but humoral factors were also implicated. There was no direct effect of 10^{-5}M juvenile hormone III on gland activity *in vitro*. 20-Hydroxyecdysone tested *in vivo* and precocene II tested *in vivo* and *in vitro* had no apparent effect on corpus allatum activity.

Corpus allatum activities *in vitro*, substantiated by juvenile hormone titres (determined by gas chromatography/mass spectroscopy and radioimmuno-

assay) showed that under short-day conditions both neural and humoral factors are involved in corpus allatum inhibition. This was indicated by the temporary activation of short-day glands after denervation in early pre-diapause and the gradual restrainment of active corpora allata from long-day females implanted into short-day hosts. Transfer of short-day beetles to long-day photoperiod completely activated the corpora allata and this process was independent of gland innervation. Starvation leads to neural restrainment of intact glands but to possible stimulation of loose corpora allata since implanted glands in starved hosts were fully activated irrespective of the photoperiod.

Studies with farnesenic acid as a precursor in the *in vitro* assay indicated that the rate limiting enzymes in the biosynthetic pathway probably occur prior to the last two steps in juvenile hormone biosynthesis, namely the methylation and epoxidation of the farnesyl precursor. However, the final 2 enzymatic steps were also diminished during prolonged neural or humoral restrainment of the corpora allata.

The innervation of the corpus allatum as revealed by retrograde diffusion with horse radish peroxidase seems to be restricted to a group of about eight cells in the ipsilateral protocerebral lobe of the brain. It is suggested that the neurally mediated inhibitory signals controlling corpus allatum activity may originate from these lateral neurosecretory cells.

SAMENVATTING

Dit proefschrift beschrijft de regulatie van de corpus allatum activiteit bij imagines van de Colorado kever, *Leptinotarsa decemlineata* Say, onder invloed van verschillende uitwendige omstandigheden. De klier activiteit werd *in vitro* bepaald door gebruik te maken van de incorporatie van (^{14}C)-methyl-Methionine in juveniel hormoon III bij individuele paren corpora allata. Gedurende het volwassen leven waren de activiteiten van de corpora allata van reproductieve, lange-dag dieren significant hoger dan die van pre-diapauze, korte-dag kevers. Er was geen verschil in klier activiteit tussen mannelijke en vrouwelijke dieren. Tijdens de diapauze was geen JH synthese waarneembaar, maar na het verbreken van de diapauze waren de corpora allata binnen vijf dagen volledig geactiveerd. Er bestond geen duidelijke correlatie tussen de spontane snelheid van juveniel hormoon biosynthese en het corpus allatum volume, hoewel grotere klieren in staat waren tot hogere activiteiten dan kleinere. Corpora allata van een groep kevers van dezelfde leeftijd vertoonden geen synchrone dag/nacht ritmiek.

Voedselonthouding bij 2 dagen oude lange-dag wijfjes verlaagde de corpus allatum activiteiten en tevens de juveniel hormoon titer in de haemolymph. Microchirurgische experimenten wezen uit, dat intacte zenuwverbindingen tussen de corpora allata en het centrale zenuwstelsel essentieel waren voor het tot stand komen van deze remming van de corpus allatum activiteit. De activering van corpora allata van pas uitgekomen wijfjes werd niet beïnvloed door de aan- of afwezigheid van voedsel.

De snelheid van endogeen hormoon synthese werd significant geremd wanneer de juveniel hormoon III titer van lange-dag wijfjes was verhoogd door implantatie van 3 paar extra corpora allata of door injectie van 50 µg hormoon opgelost in 1 µl olijfolie. Experimenten, waarbij de zenuwen tussen de corpora allata en het centrale zenuwstelsel werden doorgesneden, toonden aan, dat deze remming voornamelijk via neurale weg geschiedde, maar dat ook humorale factoren een rol speelden. De corpora allata werden blijkbaar niet direct beïnvloed door de aanwezigheid van 10^{-5}M juveniel hormoon III in het *in vitro* medium. Toevoeging van 20-hydroxyecdysone, *in vivo*, of precocene II *in vivo* of *in vitro* bleek eveneens geen effect op de corpus allatum

activiteit te hebben.

Onder korte-dag omstandigheden zijn zowel neurale als humorale factoren betrokken bij de remming van de corpora allata. Deze conclusie berust op metingen van corpus allatum activiteit *in vitro*, ondersteund door juveniel hormoon titer bepalingen met behulp van gas chromatografie/mass spectroscopie of radioimmunologische bepalingen. Denervatie van de corpora allata van korte-dag kevers leidde tot tijdelijke activering van de klieren onmiddellijk na de operatie. Actieve corpora allata van lange-dag wijfjes geïmplanteerd in korte-dag kevers werden geleidelijk geremd. Deze klieren werden echter nooit volledig inactief. Overplaatsing van korte-dag kevers naar lange-dag fotoperiode leidde tot volledige activering van de corpora allata en dit proces werd niet beïnvloed door intacte zenuwverbindingen. Voedselonthouding veroorzaakte neurale remming van klieren, maar niet geënerveerde corpora allata werden gestimuleerd, aangezien geïmplanteerde klieren in hongerige dieren volledig geactiveerd werden, zowel onder korte- als onder lange-dag omstandigheden.

Experimenten, waarbij farnezeen zuur toegevoegd werd aan het *in vitro* medium, toonden aan dat de sleutelenzymen in de biochemische keten van juveniel hormoon synthese zich waarschijnlijk vóór de laatste 2 enzymatische stappen namelijk de methylering en epoxidering van het farnezeen zuur bevinden. Deze laatste 2 enzymatische stappen werden echter ook in activiteit verlaagd onder aanhoudende neurale en/of humorale remming van de corpora allata.

De innervatie van de corpora allata is bestudeerd met behulp van retrograde diffusie met het enzym horse radish peroxidase. Hieruit bleek dat het corpus allatum direct is verbonden met een beperkte groep van ca. 8 cellen in de hersenen, die gelegen zijn in de ipsilaterale protocerebrale lob aan het caudo-dorsale oppervlak. Door hun positie, grootte en vorm is het aannemelijk dat deze cellen de bekende laterale neurosecretorische cellen zijn. Deze cellen zouden de oorsprong kunnen zijn van de inhiberende signalen die de corpora allata via de zenuwverbindingen bereiken.

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Mohamed Aslam Khan werd geboren op 4 maart 1949 te Nairobi, Kenia. In deze vroegere kolonie van Groot-Brittannië genoot hij onderwijs volgens het Britse systeem. Hij behaalde zijn Cambridge School Certificate in november 1966 en zijn Cambridge High School Certificate in november 1968. Vervolgens ging hij naar de Makerere Universiteit te Kampala in Oeganda, waar hij in oktober 1972 in de richtingen Zoölogie/Biochemie zijn Bachelor of Science graad ontving. In 1973 was hij als student-assistent werkzaam aan de Universiteit van Nairobi. In oktober 1974 zette hij zijn studie biologie voort aan de Vrije Universiteit te Amsterdam. Na aanvullende cursussen legde hij in juni 1975 het kandidaatsexamen (B4) af en in oktober 1978 het doctoraalexamen (cum laude) met als hoofdvak Vergelijkende Endocrinologie en bijvakken Clinische Chemie en Medische Parasitologie. In het jaar 1979 was hij tijdelijk wetenschappelijk medewerker aan de medische faculteit van de Universiteit van Nairobi. Van februari 1980 tot juni 1983 werkte hij als wetenschappelijk assistent op het laboratorium voor Entomologie aan de Landbouwhogeschool te Wageningen, alwaar dit proefschrift is gerealiseerd. Het onderzoek is gefinancierd door de Stichting voor Biologisch Onderzoek (BION), die wordt gesubsidieerd door de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek (ZWO).