ACTION OF THE HORMONE ECDYSTERONE AND THE RHYTHMICITY OF THE PUPARIUM FORMATION IN DROSOPHILA LEBANONENSIS

J. C. J. EEKEN
Aan

Lotty, Frauke en Wietse
THE ACTION OF THE HORMONE ECODYSTERONE AND THE RHYTHMICITY OF THE PUPARIUM FORMATION IN DROSOPHILA LEBANONENSIS

Proefschrift

Ter verkrijging van de graad van doctor in de Wiskunde en Natuurwetenschappen aan de Katholieke Universiteit te Nijmegen, op gezag van de rector magnificus prof. dr. A.J.H. Vendrik volgens besluit van het college van Decanen in het openbaar te verdedigen op 30 juni 1978 des namiddags te 2 uur precies,

door

Johannes Cornelis Jozef Eeken geboren te Kerkrade

THE ACTION OF THE HORMONE ECDYSSTERONE AND THE RHYTHMICITY OF THE
PUPARIUM FORMATION IN DROSOPHILA LEBANONENSIS

J.C.J. EEKEN
Promotor : prof. dr. W.H.G. Hennig

Co-promotor : prof. dr. J. de Wilde

Subsidie verkregen van de Stichting voor Biologisch Onderzoek (BION) heeft de tot stand koming van dit proefschrift mogelijk gemaakt.
CONTENTS

Chapter I
INTRODUCTION.

Chapter II
THE PROCESS OF PUPARIUM FORMATION OF DROSOPHILA LEBANONENSIS UNDER VARIOUS LIGHT–DARK REGIMEN.

Chapter III
CIRCADIAN CONTROL OF THE CELLULAR RESPONSE TO β-ECYDYSONE IN DROSOPHILA LEBANONENSIS I.
Experimental puff induction and its relation to puparium formation.

Chapter IV
ENDOGENOUS ECDYSTERONE CONCENTRATION OF DROSOPHILA LEBANONENSIS DURING THREE SEQUENTIAL CIRCADIAN CYCLES INCLUDING PUPARIUM FORMATION.

Chapter V
ULTRASTRUCTURE OF SALIVARY GLANDS OF DROSOPHILA LEBANONENSIS DURING NORMAL DEVELOPMENT AND AFTER IN VIVO ECDYSTERONE ADMINISTRATION.

Chapter VI
CIRCADIAN CONTROL OF THE CELLULAR RESPONSE TO ECDYSTERONE IN DROSOPHILA LEBANONENSIS II.
Changes in protein synthesis of salivary glands during puparium formation and after in vivo and in vitro administration of ecdysterone at different phases of the circadian oscillation.

GENERAL SUMMARY.

SAMENVATTING.
INTRODUCTION.

In studying hormone action in insects, it is important to realise that this class of animals was initially chosen for their capacity to survive the rather crude manipulations in early experiments (for review see Wigglesworth, 1954). These involved treatments like ligatures (Kopeč 1917, Fraenkel 1935, Williams 1946, Wigglesworth 1952), decapitation (Wigglesworth 1934), extirpation of endocrine tissues (Hadorn and Neel 1938, Wigglesworth 1940, Thomsen 1952, Highnam 1962, Girardie 1964), tissue transplantation (Stumm-Zolinger 1957) and grafting together more or less whole animals (Wigglesworth 1936, 1940), all of which can be successfully carried out in insects. The biggest problem in pursuing the finer details of the system can be summarised by a second common feature of insects, namely their size. Biochemical work on hormone action in insects has lagged behind, compared to the same field in vertebrates (for review see Jensen and DeSombre 1972, Cuatracasas 1974, Cuatracasas and Hollenberg 1976), simply because insects are too small. Another complication arises from the great physiological diversity of the class of insects, so that results obtained by studying locusts, cockroaches or flies often cannot be added together to generate a common model of endocrinology or the biochemical action of the diverse hormones. However some insects, and in particular the Diptera, do posses another feature, the polyteny of certain tissues (Painter 1933, Heitz and Bauer 1933, Koltzoff 1934, Bauer 1935, Bridges 1935, for review see Beermann 1972), which has become a very useful tool in the study of hormone action at the level of the genome and of gene regulation in general (Beermann 1952, Mechelke 1953, Clever and Karlson 1960, Clever 1961, Becker 1962).
In our study we have used *Drosophila lebanonensis*, a holometabolous insect of the subclass Pterygota. Larvae of holometabolous insects metamorphose completely from the larval form into the adult, whereas the larvae of hemimetabolous insects (the other group of insects within the subclass Pterygota) change gradually through nymphal stages to the adult form. *Drosophila* was chosen because of the possibility to study changes in the activity of the genome in the polytene tissues and the species *Drosophila lebanonensis* was selected in particular because here the onset of metamorphosis is a rhythmic process (Hardeland and Rensing 1967) which might be an additional entrance to the problem of hormone-controlled development.

The following outline of insect development (for reviews see Chen 1971, Anderson 1972, Agerell 1973) is based largely on our knowledge of the genus *Drosophila*, although some of it, especially the endocrinology, is contributed by studies on other insects. After embryogenesis, which itself is being thoroughly investigated (for reviews see Geigy 1931, Sonnenblick 1950, Poulson 1950, Counce 1961, 1973, Wright 1970), *Drosophila* hatches as a larva about 18 hours after the egg is deposited. In common with all other insects, the larval growth of *Drosophila* is limited by its rigid exoskeleton. During growth they change their cuticle twice. The larval stage can thus be divided into three intermoult periods or instars. In the larval-larval moult the processes involved in changing the cuticle (for review see Whitten 1968, Herman 1968, Highnam and Hill 1969, Wyatt 1972, Doane 1973, Gilbert and King 1973, Willis 1974, Novak 1975), start by the synthesis of the brain hormone by the neurosecretory cells (Scharrer 1952, Girardie 1964). The brain hormone is transported through neurons to the corpora cardiaca,
where it is released into the haemolymph. Brain hormone activates the prothoracic glands to synthesize and secrete the moulting hormone (Fukuda 1940, Williams 1949, Wigglesworth 1970). Under the influence of the moulting hormone the epidermal cells are reprogrammed from synthesizing and depositing endocuticular material to synthesizing and secreting the moulting fluid. The first effect of this is the separation of the cuticle from the epidermis, called the apolysis or moulting fluid. The first effect of this is the separation of the cuticle from the epidermis, called the apolysis or moult. Between the moult and the onset of the deposition of new-epi-cuticle mitotic activity and differentiation of the epidermal cells occurs in most insects but not in holometabolous insects. After progressive deposition of subsequently epi-, exo-, and endo-cuticle, the old cuticle, now weakened by the catalytic activity of the moulting fluid, is discarded. The discarding of the old cuticle is called the ecdysis. The fact that no differentiation of the epidermal cells occurs and the fact that after the ecdysis the larval characters are preserved is due to the presence of a hormone produced by the corpora allata, the juvenile hormone. In the absence of juvenile hormone, during a presumed larval-larval moult, precocious pupae and diminutive adult forms are produced (Fukuda 1944, Gilbert and Schneiderman 1959, 1961, Williams 1961). After two larval-larval moult metamorphosis starts in the larvae of Drosophila during the third larval instar. The weight of fullgrown instar larvae is approximately 1.8 - 2.5 mg, depending on the species. Metamorphosis is again induced by the brain. At the end of the third instar the larvae stop feeding and leave the food. They wander around in order to find a suitable place to pupate. The animals begin to contract, evert their anterior spiracles and assume the typical barrallike shape, characteristic for the pupal stage. It is assumed that the pre-
puparium formation behaviour is controlled by the brain through the nervous system. The start of metamorphosis as characterized by contraction and hardening of the cuticle is referred to as puparium formation (Fraenkel and Bhaskaran 1973). The brain again also triggers the synthesis and release of the moulting hormone. The reaction of the third instar larvae to the moulting hormone is different from that of the preceding instars because of the absence of juvenile hormone. The epidermal cells excrete, apart from the moulting fluid, a tanning quinone, which induces the hardening and darkening of the old larval cuticle (for review see Karlson 1967). The old last larval cuticle serves the metamorphosing insect as pupal case. The pupal case is fixed at puparium formation to a substrate with a glue substance originating from the salivary glands (Fraenkel 1952, Fraenkel and Brooks 1953). About 5 to 6 hours after the darkening of the cuticle, the epidermal cells reabsorb the remaining moulting fluid and start to deposit a new cuticle, the prepupal membrane. Since no ecdysis occurs this process is termed the larval-pupal apolysis (Jenkin and Hinton 1966). This new cuticle is built differently from the larval cuticle, although originating from the same larval epidermal cells. Head evagination occurs 6 hours after the larval-pupal apolysis and a recognisable pupa is formed with a pupal cuticle that arises largely from the larval epidermal cells of the abdomen and small areas of the thorax; the remainder being secreted by undifferentiated cells of the different imaginal disks. The adult cuticle is secreted by the adult epidermal cells that originate form the imaginal disks and nests of imaginal cells in the abdomen (Whitten 1968). Most of the larval cell types are, at the beginning of metamorphosis, reprogrammed by the moulting hormone to perform auto histolysis (Boden-
stein 1943, Aizenzon and Zhimulev 1975). Other cell types, the imaginal cells, start to proliferate and differentiate into the adult structure (for review see Ursprung and Nöthinger, 1972). The hormone responsible for this reprogramming of the cells is the moultng hormone.

The first hormone, purified from insects, that showed moultng hormone activity was the steroid ecdysone (Butenandt and Karlson 1954, Karlson 1965, for review see Karlson 1967, Slama 1969). Later a more potent moultng hormone was discovered, the steroid ecdysterone (Othaki et al 1967). Ecdysone, mainly synthetized in the prothoracic glands (Bollenbacher and O'Connor 1973, Bollenbacher et al 1975, 1976, King et al 1974), is converted to ecdysterone in peripheral tissues (King and Siddall 1969, Moriyama et al 1970, Cherbas and Cherbas 1970, King 1972). Ecdysone and ecdysterone are transported through the haemolymph and ecdysterone is accumulated in the target tissues (Yung and Fristrom 1975, Natori and Othaki 1976). Some evidence is available that binding proteins are involved in the transport through the haemolymph (Emmerich 1970a, Thamer and Karlson 1972, Butterworth and Berendes 1974), but this view is not generally accepted (Chino et al 1970). Some specific binding also occurs in the target tissues (Emmerich 1970b, 1972, Thamer and Karlson 1972, Butterworth and Berendes 1974, Gorell et al 1972) but the evidence for specific receptor molecules, such as are known for the steroid hormones in vertebrates (Jensen 1969, Karznia 1969, Teng and Hamilton 1968, Shyamala and Gorski 1969, for review see Jensen and DeSombre 1973, O'Malley and Means 1974), is still rather weak. However, there are indications that an interaction of the cytoplasm with the ecdysterone is necessary before induction of genome activity can occur (Ashburner et al 1973, Brady et al 1974).
The genome activity can be measured in polytene chromosomes. Active genes are expressed by the despiralisation of the compact DNA in the bands. In this way activated genes can be detected by the appearance of a puff at the induced loci (Beermann 1952, 1966, Pelling 1959, 1964, Rudkin and Woods 1959). This change in genome activity can be studied by following the puffing pattern of polytene chromosomes (for review see Ashburner 1970). The changes in the puffing pattern in normal development during puparium formation has been studied in detail in salivary glands (Clever and Karlson 1960, Becker 1962, Berendes 1965a, Ashburner 1967). These changes are the subsequent induction of groups of puffs and repression of others arranged in a temporal sequence (Ashburner 1973, 1974, Ashburner and Richards 1976, Richards 1976). The primary response of the puffing pattern is also studied in other larval tissues (Berendes 1965b, 1966, Berendes and Willart 1971). Induction of puffs involves the accumulation of nonhistone proteins (Swift 1962, Holt 1970, 1971, Helmsing and Berendes 1971, Helmsing 1972). All puffs studied so far are active in RNA synthesis (for review see Pelling 1972). After ecdysterone treatment an increase can be detected in total RNA (Natori and Ohtaki 1975, Scheller and Karlson 1977a), poly-A-RNA (Mascheck et al 1977, Scheller and Karlson 1977b) and specific mRNA's (Fragoulis and Sekeris 1975). Comparison of the primary changes in the puffing patterns of different tissues show that, although they exhibit puffing at a few tissuespecific loci, the change in the puffing pattern just prior to puparium formation is the same. This change can also be induced by administration of ecdysterone (Berendes 1967, Poels 1970, Berendes and Thijssen 1971, Ashburner 1972, Kress 1972, Stocker and Kastritsis 1973). However, the change in function of these tissues during puparium forma-
tion is completely different. This suggests that there exist tissue-specific factors modifying the flow of information from the genome to the cytoplasm at posttranscriptional levels. The result of this modification might be direct or have its impact on the induction of subsequent genes by cytoplasmic inducers. Although nothing is known about processing of puff RNA in Drosophila and little in Chironomus (for review see Daneholt 1975), in some mammalian systems regulation of the information flow can occur at this level (Buckingham et al 1976, Strohman et al 1977). The newly synthesized mRNA in the cytoplasm must be translated into proteins to express the process induced by ecdysone and which leads to a change in function of the cell. At translation a further level of control can exist, where factors can modify hormone induced gene activity (Garren 1964, Tomkins et al 1972, for review see Haselkorn 1973).

This investigation was started to gain more insight in the mode of action of the hormone ecdysone. We studied the role of the circadian oscillation in the puparium formation of Drosophila lebanonensis and tried to use this system as a special tool in the problem of hormone induced change in cell function during metamorphosis. The next chapter will deal with the rhythmic aspect of the puparium formation. Chapter three gives an introduction to the special way in which the circadian oscillation controls the time of puparium formation. The titer of the endogenous ecdysone concentration in relation to the circadian rhythm will be given in chapter four. Chapter five describes, in terms of changes in ultrastructure of the salivary glands, the functional reprogramming of cells initiated by ecdysone. Chapter six gives an analysis of the sequential change in protein synthesis in the salivary
glands during puparium formation and after administration of ecdysterone
in vivo and in vitro. The way in which the circadian oscillator interferes with the action of the hormone is discussed.
References.


Hiñan, K.C. 1962. Neurosecretory control of ovarian development in
Schistocerca meraria. Q. Jl. microsc. Sci. 103, 57-72.

Hiñan, K.C. and Hill, L. 1969. The comparative endocrinology in the

I; Quantitative measurements on dye binding capacity at subsequent
stages of puff formation in Drosophila hydei. Chromosoma 32, 64-78.

II; Interferometric measurements of the amount of solid material
in temperature induced puffs of Drosophila hydei. Chromosoma 32,
428-435.

Jenkin, P.M. and Hinton, H.E. 1966. Apolysis in arthropod moulting

Jensen, E.V., Suzuki, T., Numata, M., Smith, S. and DeSombre, E.R.
1969. Estrogen-binding substances of target tissues. Steroids 13,
417-427.


Science 182, 126-134.

Karlson, P. 1967. The chemistry of insect hormone and insect pheromones.
in Pure Appl. Chem. 14, 75-87.

Karlson, P., Sekeris, C.E. and Maurer, R. 1964. Zum Wirkungsmechanismus
der Hormone. I; Verteilung von tritiummarkiertem Ecdyson in Larven
von Calliphora erythrocephala. Hoppe-Seylers Z. Physiol. Chem. 336,
100-106.


Wigglesworth, V.B. 1952. The thoracic gland in Rodnius prolixus (Hemiptera) and its role in moulting. J. Exp. Biol. 29, 561-570.


THE PROCESS OF PUPARIUM FORMATION OF DROSOPHILA LEBANONENSIS UNDER VARIOUS LIGHT–DARK REGIMEN.

Introduction.

The modern biological study of rhythms was started as early as 1870 by an astronomer, de Marian, who was fascinated by the "nyctinastic movements" of leaves in Kalanchoë. It was shown that this was a persistent rhythm since, independent of the natural light dark cycle, the rhythm of the movements of the leaves continued for several weeks. Another surprising phenomenon was observed and studied in plants by Garner and Allard (1923). They described processes which showed that plants must be able to measure the length of the light period of the day. All such phenomena which involve daylength measurements in plants as well as animals are gathered under the term photoperiodism. Some years later the discovery was made by Beling (1929) and Wahl (1932) that bees were able to return to a special feeding place for several days in succession at exactly the same hour. Not only did the bees remember at what time of the day they had been offered food, they also seemed to know what precisely the time of the day was. Actually Beling and Wahl were the first to recognize that, basically, the phenomenon was a functional chronometer. A fourth class of phenomena, also based on time measurement, was initially described as cases of remarkable orientation and navigation in insects (Frisch, 1950), birds (Kramer, 1952) and crustaceans (Pardi and Papi, 1953).

As can be seen from this very brief historical introduction a number of phenomena as diverse as persistent rhythms, photoperiodism, Zeitgedächtnis, orientation and navigation are all based on the ability to measure time. All phenomena using a time measuring device (TMD) with a period of about the length of the natural day–night cycle are classified
as circadian rhythms. How time is measured and how this information is integrated in the regulation of the diverse rhythmic events is one of the aims of studying circadian rhythmicity.

Once the phenomenon of a TMD was recognised, two theories about its mode of operation were developed. In the first theory, the TMD behaves as an oscillator (Pittendrigh and Bruce, 1957, 1959, Pittendrigh 1960, Klotter 1960 and Wever 1965). According to the second theory the time is measured by an hourglass device. The main difference between an oscillation and an hourglass device is that whereas an oscillation continuously moves from one cycle into the other, an hourglass has to be set once every cycle. The hypothesis that the TMD behaves like an oscillation is most strongly supported by the investigation of persistent rhythms. Most of the evidence in favor of the hourglass model are derived from studying photoperiodism. Evidence is available that in some species photoperiodism can be explained only by an hourglass model (Lees, 1965, 1966 and 1973), whereas in other species this phenomenon can be explained as well by the oscillation model (Pittendrigh and Minis 1964, Tyschenko 1966 as cited by Danilevski et al 1970, Saunders 1970). Truman (1971a, b) showed that photoperiodism, in again another species, must be explained by an hourglass device on top of a TMD that follows the oscillation model. Although photoperiodism is a fascinating phenomenon, it is not the most suitable subject to elucidate the nature of the TMD, since here two kinds of time measurements might be involved: first the basic TMD and second the measurement of the length of a special time interval, the length of the light period each cycle.

From all the evidence available, the most likely model for the TMD is based on an endogenous oscillation. The driving force behind this
endogenous oscillation can be internal or external. That external forces are involved (Brown 1960, 1969, 1976 and Brown et al 1970) is less likely since experiments on the south pole (Hamner et al 1962) showed that circadian rhythms did persist even in conditions where most of the geophysical forces could be eliminated. To investigate the TMD as an endogenous self-sustaining oscillation (ESSO), (Brett 1954, 1955, Pittendrigh 1954, 1960, 1965) started to study the eclosion behaviour in *Drosophila pseudoobscura* pupae. Since eclosion is a developmental event and occurs only once in the life of an insect, the rhythm can only be made visible in a non synchronised population. However it is the expression of the same TMD that is the basis for persistent rhythms like locomotion or feeding. Two aspects of the ESSO, the period and the phase, have been studied most intensively (for review see Saunders 1976, 1977 and Palmer 1976). The period of the ESSO is defined by the length of the interval between two successive peaks of a rhythmic event in constant darkness. This period, the freerunning period, as might be expected of any TMD, is remarkable stable, but never exactly 24 hours (circadian). A few treatments are able to change the period, for example cycloheximide (Feldman 1967) or D$_2$O (Bruce and Pittendrigh 1960, Brenner and Engelmann 1973). The most remarkable and functionally interesting fact is the relative inability of temperature to change the period of the ESSO. The second aspect, the phase, of the ESSO is defined as an instantaneous state of an oscillation within a period. The phase of the ESSO can be studied, if one assumes that any event, controlled by the ESSO, has a fixed relation to a phase of the ESSO. This phase then can be defined as the phase that, each cycle, initiates the start of processes which ultimately, and after a defined time interval, results in the event we are measuring.
In this way, changes in the relation between the rhythmic event and the external light/dark (LD) regimen are reflections of identical changes between the relation of the phase of the ESSO and the external LD regimen. The phase can be set or entrained mainly by two factors or Zeitgebers, light and temperature. Changing the position of a LD regimen relative to an established ESSO will be followed by a process where the ESSO attains again its characteristic phase relation to this particular entraining LD regimen. Likewise a light pulse applied during any phase of a free-running ESSO will induce a permanent shift. The amplitude and sign of this shift depend on the phase of the ESSO when the light pulse is applied. Light pulses applied throughout the complete cycle will give rise to varying responses with respect to the induced phase shifts. The phase response curve obtained in this way is characteristic for all rhythmic events controlled by the ESSO. The phase relation can also be influenced by temperature. A temperature pulse applied at any phase of an established oscillation will give rise to a temporary shift in the phase relation of the ESSO and the entraining LD regimen. However gradually the ESSO will reestablish its former phase relation with the entraining LD regimen.

Although unicellular organisms show many circadian rhythms, implying that single cells can posses an ESSO (Sweeney 1971), in multicellular organisms such as animals, the ESSO is thought to reside in some morphological defined tissue or group of cells. In search for the location of the ESSO, the attention focussed first on the photoreceptors which would provide the ESSO with the essential information of the external LD regimen. Although in insects the compound eyes are the most conspicuous in this respect, only in some insects are these the exclusive photoreceptors
In some insects both compound eyes as well as ocelli are the photoreceptors of the ESSO (Nowosielski and Patton 1963). Extra-optic photoreceptors have been found by a number of investigators (De Wilde & De Boer 1961, Engelmann and Honegger 1966, Truman and Riddiford 1970, Zimmerman and Ives 1971, Dumortier 1972, Godden 1973). It has been shown that the ESSO itself resides within the brain, more specific in the optic lobes (Roberts 1966, 1974, Nishiitsu-Uwo and Pittendrigh 1968b, Truman 1972, for review see Brady 1969, Saunders 1976). However this does not exclude the possibility that all cells within the insect can have their own ESSO. The cellular ESSO's could then be synchronized by a master ESSO, the one experimentally detected in insects. In this way in vitro cultured tissues can maintain a rhythmic behaviour (Rensing 1969, Hardeland 1973) which is gradually lost because of the variance in the periods of the freerunning ESSO of the individual cells.

That the puparium formation of Drosophila lebanonensis is coupled to an LD cycle has been shown (Rensing and Hardeland 1967). The subject of this chapter is to define whether this coupling depends on a regulation by an ESSO.
Methods

The Drosophila used in this study was first described and classified as a subspecies of *Drosophila lebanonensis* (Pipkin 1961). Our laboratory stock, originally labelled *Drosophila victoria* (see also Pipkin 1961), was later identified as *Drosophila lebanonensis casteelli* (Herrebout, unpublished).

Larvae were raised under standard conditions with respect to food (Berendes 1965), temperature (25°C ± 1) and relative humidity (80%). Larvae were kept in a water cooled incubator equipped with lights (Fluorescent white light Code nr 33), automatically controlled by a time switch. During this study "non synchronized" populations were used, which means that eggs were collected over a period of 48 - 72 hours.

In order to follow the time of puparium formation, white prepupae were collected every hour. During dark periods the collecting was performed using a red safety light (Philips PF 712 E). Puparium formation was characterized by the morphology of the animals (the typical "barrellike shape") and the rigidity of the cuticle.
Results and discussion

To investigate if the rhythmic behaviour of the puparium formation of *Drosophila lebanonensis* is controlled by a circadian oscillation, several experiments were set up using different LD regimen. In the first experiment larvae of a non synchronized population were reared under the following LD regimen: LD 4:20, LD 8:16, LD 10:14, LD 12:12, LD 14:10, LD 16:8 and LD 20:4. The time of puparium formation was recorded over at least 5 subsequent days. The mean time of puparium formation in the different populations is expressed in hours circadian time (CT). CT 0 is defined as the time when the light is switched on. Figure 1 shows the mean time of puparium formation under the various light dark regimen. It is clear that the rhythm is entrained by the light dark regimen but there is no direct relation between the time of puparium formation and the time of switching the lights on or off. Rather the relation is based on the ratio between light and dark and is the same as that found for the behaviour of the eclosion rhythm in *Drosophila pseudoobscura* pupae (Pittendrigh 1966). The ultimate proof that the rhythm of puparium formation is independent of environmental clues is to synchronize the circadian oscillation of a "non synchronized" population by entrainment, for several days, to a defined light dark regimen (LD 12:12), and subsequently transferring the population to complete darkness. As can be seen in figure 2 the rhythm in puparium formation is persistent even in complete darkness. By changing the initial entraining LD regimen relative to the external natural light dark cycle, the time of puparium formation in complete darkness is seen to be completely independent of external clues. The period of the free-running oscillation of *Drosophila lebanonensis* is 25.9 hours.
If the rhythm in puparium formation is controlled by a circadian oscillation, then light pulses must induce different phase shifts when given throughout the oscillation period. Such a phase response curve was determined, where we concentrated on those phases of the oscillation where the most dramatic changes in the effect of the entraining light pulses can be detected. The scheme of light interruptions during the subjective night is represented in the upper panel of figure 3. The lower panel of figure 3 shows the phase shifts induced by each light pulse as determined 6 days after the light pulse was applied. Phase delays are induced by light pulses up to CT 16. The jump in phase shifts from delay to advance occurs between CT 16 and CT 17. The obtained phase response curve is the characteristic phase response curve of rhythmic events, controlled by the circadian oscillation.

The results of the experiments described, strongly indicate that the rhythmic event of puparium formation in Drosophila lebanonensis is controlled by the circadian oscillation. The mean value of the freerunning period of the ESSO of Drosophila lebanonensis is 25.9 hours and the phase jump of entraining light pulses occurs between CT 16 and CT 17. In the following chapters an attempt has been made to characterize the process of the induction of the puparium formation in view of our knowledge that this process is controlled by an endogenous self-sustaining oscillation.
Figure 1. The mean value of the time of puparium formation (▼)
of 7 populations of larvae reared under various LD regimen.
CT = Circadian Time; white bars are periods of light,
black bars periods of darkness.
Figure 2. The puparium formation of a population of *Drosophila lebanonensis* reared in complete darkness (DD). The mean time of puparium formation in each "gate" period is indicated (▼).
The phase response curve of the circadian oscillation controlling the puparium formation in *Drosophila lehano-nensis*. The phase response curve is determined by the phase shifts in the puparium formation induced by light pulses applied at 7 different phases of the circadian oscillation.

Upper panel: The light–dark scheme of each of the seven populations.

Lower panel: The phase response curve as determined after the measurement of the phase shifts as induced in the populations by the used light–dark scheme as outlined in the upper panel.
References.


II - 14


II - 17


Circadian Control of the Cellular Response to β-Ecdysone in Drosophila lebanonensis
I. Experimental Puff Induction and Its Relation to Puparium Formation

Jan C. J. Eeken
Department of Genetics, University of Nijmegen

Abstract. Drosophila lebanonensis displays a strict circadian rhythm with regard to the puparium formation and the occurrence of ecdysone-specific puffs in the salivary gland chromosomes. In normal development these puffs occur 3-4 hours before puparium formation. Injection of β-ecdysone at periods before ecdysone puffs are present, induces in all instances their appearance within 30 minutes, irrespective of the phase of the circadian oscillation at the time of injection. In spite of the appearance of the hormone specific puffs following β-ecdysone injection, puparium formation did not occur 3-4 hours after the puffs became active. Depending upon the time of injection within the circadian cycle, puparium formation occurred 5-6 hours after injection (when injection was performed close to a "gate" period), or occurred during the next "gate" of the circadian oscillation.

Introduction
A variety of cell types in Dipteran larvae undergo changes in function and/or cellular organization shortly before and during puparium formation. In a number of cases, it has been shown that the induction of these changes is brought about by the steroid ecdysone (Shaaya and Sekeris, 1965; Oberlander, 1972; Fristrom, 1972; Berendes, 1972; Berendes and Willart, 1971; Poels et al., 1971; Poels, 1972).

In the polytene tissues, the changes in cell function are, in normal development as well as after injection of the hormone, preceded and accompanied by alterations in chromosome activity (Clever and Karlson, 1960; Clever, 1963, 1966; Berendes, 1967; Stocker and Kastritis, 1972; Poels, 1970; Ashburner, 1971; Panitz, 1972).

In several Drosophila species puparium formation occurs independently of the daily light-dark regimen. Moreover, injection of ecdysone into late last instar larvae will at any time elicit almost immediately the hormone specific responses at the level of individual cells as well as puparium formation (Poels, 1970).

However, in Drosophila lebanonensis puparium formation appears to be restricted to a certain period of the circadian oscillation (Rensing and Hardeland, 1967). So far, it is not known whether or not the re-
striction of puparium formation to a certain period in the daily light-dark regimen results from a circadian control of ecdysone release from the ring gland or from other factors which may interfere with the cellular response to ecdysone.

The present study was performed in order to determine whether ecdysone, when injected at different phases of the circadian oscillation, can elicit in all instances the specific cellular responses and puparium formation of the animal.

Material and Methods

Throughout this study a laboratory stock of Drosophila lebanonensis casteel (Pipkin) was used. The larvae were raised under standardized conditions with respect to food, temperature (25°C ± 1°C), humidity and light-dark regimen (LD 12:12).

The larvae used in the experiments were all derived from groups of 100-150 eggs laid by about 40 pairs of 12 to 18 day old flies during the first two hours after lights off (Fig 1a) unless stated otherwise. The development of these larvae is synchronous to the extent that puparium formation of the group is restricted to two subsequent cycles of the circadian oscillation (puparium formation occurring only during the first hours after lights off).

β-Ecdysone (5·10^{-4} M or 5·10^{-5} M) was injected (0·5 μl/larva) as described previously (Berendes, 1967).

Analysis of the chromosomal puffing pattern was performed in squashes of salivary glands fixed in acetic-ethanol (1:3) and stained with aceto-orcein. The estimation of puff sizes is based on the distinction of 4 different size classes (Table 1).

Table 1. Size classes of puffs on the basis of relative diameter values (each range of diameter ratios is based upon at least 5 measurements)

<table>
<thead>
<tr>
<th>Puff locus</th>
<th>Reference bands</th>
<th>Diameter ratios</th>
<th>Ratios of puff/reference band</th>
</tr>
</thead>
<tbody>
<tr>
<td>19C</td>
<td>22C4</td>
<td>1.2-1.7</td>
<td>2.1-2.9</td>
</tr>
<tr>
<td>21A</td>
<td>22C4</td>
<td>1.0-1.6</td>
<td>2.1-2.7</td>
</tr>
<tr>
<td>24A</td>
<td>22C4</td>
<td>1.2-1.7</td>
<td>2.2-2.8</td>
</tr>
<tr>
<td>24B</td>
<td>22C4</td>
<td>1.2-1.8</td>
<td>2.4-2.8</td>
</tr>
<tr>
<td>24C</td>
<td>22C4</td>
<td>1.2-1.7</td>
<td>2.1-2.7</td>
</tr>
<tr>
<td>28B</td>
<td>32A1.2</td>
<td>1.0-1.6</td>
<td>1.9-2.4</td>
</tr>
<tr>
<td>32C</td>
<td>32A1.2</td>
<td>0.7-1.1</td>
<td>1.7-2.2</td>
</tr>
<tr>
<td>54A</td>
<td>55A3.4</td>
<td>0.7-1.1</td>
<td>1.6-2.0</td>
</tr>
</tbody>
</table>

Class values: 0 1 2 3 4

The time of puparium formation was determined by testing the flexibility of the larval cuticle and the position of the mouth parts relative to the wall of the tube. The moment at which the mouth parts are lifted from the wall of the tube and the cuticle is hardened was taken as the moment of puparium formation. In all instances, tanning was completed within two hours after hardening of the cuticle.
Results

I. Puparium Formation and Ecdysone Specific Changes in the Chromosomal Puffing Pattern during Normal Development

a) Rhythmicity in Puparium Formation

Under a LD 12:12, a population of D. labanonensis larvae derived from eggs laid within a 2 hour period, undergoes puparium formation in two distinct groups. One group undergoes puparium formation within the first 6 hours of darkness on the 7th day after oviposition (group I) and the remaining larvae begin puparium formation during the same period of day 8 (group II). The restriction of puparium formation to the first hours after darkness is, for both groups, independent of the time of oviposition (Fig. 1).

Larvae developing from eggs produced at the beginning of a dark period and kept for the first 24 hours under a LD 12:12 and subsequently in the dark, displayed the characteristic biphasic behavior with respect to puparium formation. 62% of the larvae underwent puparium formation essentially during the "gate" period of day 7, the remaining larvae during the "gate" period approximately 24 hours later.

b) Changes in the Puffing Pattern of Larvae of Group II

Puparium formation of larvae of group II peaks at 195 hours after oviposition (top Fig. 1). In order to determine the changes in the chromosomal puffing patterns of these larvae relative to the moment of puparium formation, 8 puffs known to respond to injection of β-ecdysone (19C; 21A; 24B; 24C; 28B; 32C and 54A; Berendes and Thijssen, 1971) were measured over a 28 hour period prior to puparium formation. This period includes the moment at which the larvae of group I undergo puparium formation (peak at 171 hours after oviposition). Since the larvae of group I leave the food medium at around 163 hours, the vast majority of the larvae which are taken from the food after this time are larvae of group II (see Fig. 2). The average sizes of the puffs of group II larvae were investigated over the period from 166-189 hours with intervals of maximally 3 hours, and from 189 to 199 hours with intervals of 1 hour. At each time interval the salivary glands of 5 randomly collected larvae were used to determine the puff size of the hormone-specific loci. Since these larvae may differ 2-3 hours in the time at which they will undergo puparium formation, the most frequently observed puffing pattern at the 8 loci was considered to be representative for that particular time interval.

As shown in Fig. 3 (solid lines) the puffs 19C, 24B, 24C, 24A and 28B display a puffed appearance at 190-192 hours. These puffs have all attained their maximum size at 192-193 hours (Fig. 4). The puffs,
Fig. 1. Puparium formation of *D. lebanonensis* larvae derived from eggs produced during the first two hours after light off (a), and from eggs produced during a two hour period beginning 7 hours before lights off (b). The data of 4 separate experiments are pooled.

Fig. 2. Time table of development of *D. lebanonensis* under the culture conditions used.
32C and 54A also display a puffed appearance around 190-192 hours, but attain their maximum size at 195 hours (the time of puparium formation as defined in Material and Methods). Puff 21A showed a decrease in size during the period from 190-193 hours and, subsequently, an increase in size with a maximum at 195 hours. The observations indicate that puff 24C is the first to become active (190 hours), followed by 19C and 28B (191 hours) and then by 24A, 24B, 32 and 54A (192 hours).

With the exceptions of the puffs 21A and 24C, all other ecdysone-sensitive puffs were absent over the period from 167-188 hours. This finding indicates that in the group II larvae no ecdysone-specific puffs occur at the time during which larvae of group I prepare for, and undergo puparium formation. Larvae of group I display essentially the same changes in puffing pattern prior to puparium formation (see Fig. 3).

2. Hormone Injections into Larvae of Group II at Various Times during the Day-Night Cycle

a) Response at the Genome Level

β-Ecdysone was injected at two different times during the circadian cycle in order to investigate whether or not the specific genome response at the 8 puff loci can occur independently of the circadian rhythm. The salivary gland chromosome of at least 5 larvae were analyzed at 1 and 4 hours after injection and the puff sizes of the 8 loci were estimated. Injection of β-ecdysone was performed at 172 and 180 hours, the middle and end of the dark period preceding the light period at the end of which the larvae prepare for puparium formation (the majority of the larvae have left the food around 187 hours). As shown in Fig. 3 (dotted lines), 7 of the 8 loci (exception: 21A) revealed a positive response (puff formation) to hormone injection. Although the rate of increase in puff size over the 4 hour period varied for the different puffs, four hours after β-ecdysone injection, most loci displayed a similar size as observed in normal development during puparium formation. Puff 21A which is puffed during the dark period from 168-180 hours and during the successive light period, responds to β-ecdysone injection by regressing. This puff displays a similar behaviour during normal development when regression is observed starting at 187 hours (5 hours before the onset of the dark period).

Larvae of group II have also been injected at 163, 171, 173 and 176 hours. Studies on the puffing pattern of the injected larvae at 4 hours after injection revealed that in all larvae studied the sizes of the responsive puffs were in all instances similar to those reported above.

14a Chromosoma (Berl.), Bd. 49

III - 6
Fig. 3
These results indicate that the genome response to β-ecdysone is essentially the same irrespective at what time in the circadian cycle the hormone is administered.

b) Puparium Formation in Response to Hormone Injection

Puparium formation in response to β-ecdysone injection was studied in four groups of animals which were injected with 0.5 μl of a 5–10^{-4} M β-ecdysone solution at 163, 171, 173 and 176 hours, respectively. The number of larvae in which puparium formation was induced by the hormone injection was determined at 180 hours (the end of the dark period), 192 hours (the end of the light period, beginning of the “gate” period), 198 hours (the middle of the dark period, end of the “gate” period) and at 204 hours (end of the dark period). All larvae showing protruded spiraculae and tanning of the cuticle were considered to have undergone puparium formation.

The results of these experiments, shown in Fig. 5, indicate that when β-ecdysone is injected in larvae of group II (puparium formation in non-injected controls of this group occurs between 192 and 193 hours) at 163 hours, 62% of the larvae undergo puparium formation during the following dark period (168–180 hours). Thus, puparium formation of the injected larvae occurs 24 hours earlier than would have occurred during their normal development. Their puparium formation coincides with that of larvae of group I.

Also in larvae of group I puparium formation can be induced 24 hours earlier by injection of the hormone.

When larvae of group II are injected at 171 hours (middle of the “gate” in the dark period), 60% of the larvae responded with puparium formation within the dark period and the remaining 40% underwent puparium formation during the following light and dark period. No peak in puparium formation occurred during this second dark period, although this is the time for puparium formation of uninjected larvae.

When larvae of group II were injected later than at 171 hours (middle of the “gate” period), the majority of the larvae did not respond with puparium formation until about 20 hours later, the moment at which they would have undergone puparium formation in normal development.

Fig. 3. Relative puff sizes of the puffs 19C; 24B; 24C, 24A, 28B, 32C, 54A and 21A in larvae of group II over a period from 167–198 hours after oviposition (---). Relative puff sizes of the same puffs over a 5 hour period from 165–171 hours after oviposition in larvae of group I (--*--). The relative sizes of the same puffs at 1 hour and 4 hours after injection of β-ecdysone at 172 hours and 180 hours after oviposition are also shown (o-o)
Fig. 4a—d. Changes in the sizes of the puffs 2-19C; 2-21A; 2-24A; 2-24B and 2-24C during normal development. The photographs represent: (a) the 2nd chromosome at 168 hours; (b) 192 hours; (c) 193 hours; (d) 194 and 196 hours after oviposition.
Circadian Control of Response to β-Ecdysone in *Drosophila*.

% Puparium formation

- **Fig. 5a—d.** Puparium formation of larvae injected with β-ecdysone at: (a) 163 hours; (b) 171 hours; (c) 173 hours and (d) 176 hours after oviposition. The number of prepupae was counted at the onset of a lights off period following injection and at the onset of a light period following the injection. The distribution of puparium formation observed after the ecdysone injections at different moments of the circadian cycle suggests that injection of the hormone shortly before the middle of the “gate” period results in the majority of the larvae in induction of puparium formation during the dark period including the “gate”. When β-ecdysone is injected during the middle of the “gate” period or shortly afterwards, most of the larvae will become prepupae during the “gate” in the following dark period (17–20 hours later).

It should be pointed out that in the larvae injected with the hormone later than 171 hours, and tested with respect to the genome response, all responsive chromosome loci displayed their characteristic response.

**Discussion**

As has been demonstrated, puparium formation in *D. lebanonensis* occurs exclusively during a definite phase of the circadian cycle (see also: Rensing and Hardeland, 1967). With regard to this developmental process, *D. lebanonensis* behaves like the midge, *Aedes taeniorhynchus* (Provost and Lum, 1967; Nayar, 1967) and the sphinx moth, *Manduca*...
sexta (Truman, 1971). In other Drosophila species, a strict coupling between the onset of puparium formation and a certain phase in the circadian rhythm has not been observed (Rensing and Hardeland, 1967; Pittendrigh and Skopik, 1970), although other developmental processes like emergence of the flies, appear to be strictly controlled by the circadian clock in some species (Pittendrigh, 1960; 1966; Pittendrigh and Minis, 1964).

Since puparium formation is under control of humoral factors, among which the best known is the steroid hormone ecdysone, it has been suggested that the circadian rhythm in puparium formation is a consequence of a circadian rhythm in the release of this hormone from the ring gland (Rensing, 1971).

If the presence of ecdysone-specific puffs in the polytene chromosomes is considered as a suitable parameter for the existence of a certain hormone titer in the hemolymph (which should be at least above the threshold titer required for puff induction; see Ashburner, 1973), it is evident from the present comparison of puffing patterns during the last day of larval development of group II larvae that the threshold titer of ecdysone is strictly coupled to puparium formation. The ecdysone-specific puffs occur, with the exception of puff 21A and 24C, in all instances 3–4 hours before puparium formation. In no case were these puffs observed during periods other than shortly before puparium formation.

The actual hormone titer in the hemolymph, however, is not only a function of the release from the ring gland. It could be modified considerably by a degradation mechanism (Karlson and Bode, 1969; King and Siddall, 1969). If this degradation mechanism is taken into consideration, it could be suggested that not the release of the hormone, but the activity of the degradation mechanism follows a strict circadian cycle. This suggestion, however, finds little support from the results of the injection experiments in which essentially all larvae responded to injected $\beta$-ecdysone with the formation of the hormone-specific puffs.

Although, in normal development, the puffs appear always 3–4 hours before the onset of puparium formation, which occurs within a “gate” of the circadian cycle, the puffs can be induced at other phases of the circadian cycle by injection of ecdysone. Under these conditions, however, puparium formation does not necessarily occur 3–4 hours later. Puparium formation of the majority of the injected larvae still occurs within or close to a “gate” period in the circadian oscillation.

The finding that not all injected larvae display the strict coupling between puparium formation and a “gate” in the cycle (Fig. 5) may be due to the fact that the injections were performed with lights on (during a period of 30 minutes).
The present results, thus, indicate that whereas \( \beta \)-ecdysone injection consequently leads to the appearance of the hormone-specific puffs, it depends on the time of injection in relation to the circadian oscillation as to whether or not puparium formation will occur immediately or with an appreciable delay (20 hours later, during the next "gate" period).

It could be suggested that some other humoral factor interferes with the action of ecdysone (Fraenkel et al., 1972; Price, 1970; Ratnasiri and Fraenkel, 1973). The presence of such a factor could be controlled by the circadian oscillation. If this would occur in \( D. \) lebanonensis, the present data suggest that such a factor should operate at the post-transcriptional level, because the larvae did display, in all instances, the hormone-specific response at the genome level.

Further studies on salivary glands maintained in vitro may reveal in more detail the relationship between the mechanism of hormone action and the circadian oscillation.

Acknowledgement. The author is indebted to Dr. H. D. Berendes for his continuous interest and suggestions and for his help in the preparation of this manuscript.

References
Berendes, H. D., Willart, E.: Ecdysone related changes at the nuclear and cytoplasmic level of Malpighian tubule cells in \( Drosophila \). J. Insect Physiol. 17, 2337–2350 (1971)


Poels, C. L. M.: Mucopolysaccharide secretion from Drosophila salivary glands as a consequence of hormone induced gene activity. Cell Differentiation 1, 63-78 (1972)


Received August 26 — October 15, 1974 / Accepted by W. Beermann
Ready for press October 15, 1974

Drs. Jan C. J. Eeken
Department of Genetics
University of Nijmegen
Toernooiveld
Nijmegen
The Netherlands
Introduction.

Puparium formation in Diptera is controlled by the molting hormone ecdysterone (Karlson and Bode, 1969). The induction of puparium formation by ecdysone is accompanied by changes in the activity of specific gene loci. These changes have been most intensely studied in the polytene chromosomes of the salivary glands of Drosophila melanogaster (Ashburner 1967), Drosophila hydei (Berendes, 1965a), Drosophila pseudoobscura (Stocker and Kastritsis, 1972), Drosophila virilis (Kress, 1972) and Drosophila lebanonensis (Berendes and Thijssen, 1971). Some of the prominent changes in the puffing patterns of the salivary gland chromosomes seen during puparium formation can also be found during this period in the chromosomes of the Malpighian tubule cells (Berendes, 1965b, 1966). However, the subsequent changes at the cytoplasmic level in the salivary glands and Malpighian tubules are completely different (Poels, 1970; Wessing, 1962, 1963), suggesting a control of the expression of the hormone specific genome activity by tissue specific factors.

Puparium formation, as well as the specific changes in the puffing patterns of chromosomes of salivary glands and Malpighian tubules, and the subsequent tissue specific changes at the cytoplasmic level, can be induced experimentally by the injection of ecdysterone (Berendes, 1967; Berendes and Thijssen, 1971; Poels et al, 1971; Berendes and Willart, 1971 and Eeken, 1974, 1977).

In *Drosophila lebanonensis* puparium formation is strictly coupled to the circadian oscillation and occurs only at specific "gate" periods in an artificial 24 hours cycle (Rensing and Hardeland, 1967). Injection
of ecdysterone prior to the "gate" period (G¹) that precedes that in which puparium formation actually occurs (G²) induces the specific changes in the puffing pattern of the salivary gland chromosomes and puparium formation. If ecdysterone is applied after "gate" period (G¹) puparium formation does not occur although the response of the genome can be detected (Esken, 1974). It could be suggested that some other humoral factors interfere with the action of the hormone ecdysterone (Fraenkel et al., 1972; Ratnasiri and Fraenkel, 1973). The presence of such factors could be controlled by the circadian oscillation. If this would occur in Drosophila lebanonensis, the data suggest that such factors should operate at the post transcriptional level, because ecdysterone is able to induce the genome response at all phases of the circadian oscillation (Esken, 1974). However, an alternative explanation is that during the penultimate "gate" period G¹ before puparium formation, the endogenous ecdysterone concentration rises, although not to a sufficient concentration needed for the induction of the puparium formation. In order to investigate this the endogenous concentration was measured during the penultimate "gate" period G¹ before puparium formation and during puparium formation itself.

Although the investigations described so far, favor the universal action of one major moulting hormone, ecdysterone, on all larval tissues during puparium formation, there is some evidence that at least one other ecdysteroid, ecdysone, a precursor of ecdysterone, has hormone activity of its own (Clever et al., 1973). A possible alternative explanation for the observed differences in the reaction at the cytoplasmic level in different target tissues during puparium formation could be the presence of more than one active hormone with different affinities for different
tissues. Further evidence for this view is given by studies on the influence of ecdysterone and ecdysone on fat body in imaginal disc development (Benson et al., 1974; Bergstrom and Oberlander, 1975; Milner and Sang, 1976).

To investigate the presence of different ecdysteroid in Drosophila, extracts of all prepupal and pupal stages, which showed a high radio-immunoassay activity, were analysed using thin layer chromatography.

To ensure that the different reactions to injected ecdysterone before and after the "gate" period are not due to changes in the activity of an inactivating system during the circadian oscillation, the degradation of injected tritium labelled ecdysterone was measured at these two critical phases of the circadian oscillation.
Methods

Larvae of *Drosophila lebanonensis casteelli* (Pipkin) were raised under standard conditions with respect to food, temperature (25°C), humidity (80%) and light–dark regimen (LD 12:12). In a synchronised population, puparium formation occurs in two groups, one group pupating after seven days during a "gate" period in the circadian oscillation 168–174 hours after oviposition, the other group pupating after eight days during the "gate" period 24 hours later. Throughout this study only animals of the second pupating group were used.

To measure the concentration of ecdysterone, extracts of whole animals were used with 20–200 animals in a sample. The weight of the animals changes over the period in which the concentration of ecdysterone was measured from 1.5 mg – 2.0 mg. The increase, due to growth of the third instar larvae, and the decrease, by water loss in the pupal stage, are essentially the same as has been described for *Drosophila melanogaster* (Bakker, 1959). After extraction in 70% methanol, washing with petroleum ether and partitioning in butanol against water, the samples were chromatographed using thin layer chromatography (TLC). TLC was performed on prewashed, precoated TLC plates of silicagel 60 with fluorescent indicator F–254 (Merck) using chloroform/96% ethanol 4:1 as a solvent.

The determination of ecdysteroids in crude 70% methanol extracts as well as in 0.5 mm fractions of the TLC plates were performed with a radioimmunoassay using a highly sensitive antiserum, denoted H21B, developed by Horn et al (1976). Active fractions were rechromatographed on a reversed phase column (Bondapak C–18) using high pressure liquid chromatography (Schooley and Nakanishi, 1973). Activity was again detected by radioimmuno-
assay using an earlier developed antiserum, denoted as M-20, by Borst and O'Connor (1974). The radioimmunoassay is performed as described by Borst and O'Connor (1974). The radioimmunoassay activity of each sample was always detected at two dilutions of the crude extract.

The degradation after injection of $^3$H ecdysterone (ecdysterone ($^3$H(G)), NEN, specific activity of 1.4 Ci/m mole) was followed by extraction and partial purification as described above. TLC fractions were eluted and counted in a Philips Scintillation Analyser. For each time point, two samples of 10 animals were injected with 0.5 µl $^3$H ecdysterone solution (2.10$^{-5}$ M) per animal, supplemented with $^{14}$C inulin (2000 cpm/µl) to enable corrections to be calculated for errors in the injection procedure. Ecdysterone was obtained from Rhoto Laboratories, Japan; α ecdysterone was a gift of prof. Dr. O'Connor; 3-dehydro-ecdysone and 3-dehydro-ecdysterone were gifts from Dr. J. Koolman.
Results.

Ecdysone concentration.

To calculate the concentrations of ecdysterone and ecdysone in extracts of larvae and pupae the efficiency of the method of extraction and purification used was measured in following the amount of radioactivity of $^3$H labelled ecdysterone throughout the procedure. The efficiency of the extraction is about 90% and the efficiency of the purification by TLC is 63%. The efficiency of the total method can also be calculated from the data of the control experiments of the $^3$H ecdysterone injection experiments, where injected larvae were frozen in liquid nitrogen directly after injection. The efficiency of the injection itself is determined by the amount of radioactivity of simultaneously injected $^{14}$C inulin (the efficiency of the $^{14}$C inulin extraction is 90%). The overall efficiency of the method as calculated from the injection experiments, as described above, is 55%.

Extraction of ecdysteroids were made from an average of 400 mg (wet weight) animals when low ecdysone concentrations were expected, and 50-100 mg animals were used when high ecdysone concentrations were expected. In the case of third instar larvae the radioimmunoassay activity was determined in the total crude 70% methanol extract. In all prepupal and pupal stages the radioimmunoassay activity was measured in only a fraction of the crude 70% methanol extracts whereas the remainder of the extract was purified and separated using TLC. Figure 1. illustrates the concentration of radioimmunoassay active material in the crude extracts over the period measured. The data are expressed as picograms ecdysterone equivalents per mg tissue, since other ecdysteroids can be present in the extracts, which can cross react with the antiserum used. During the
measured period the radioimmunoassay activity shows two prominent peaks, the first at puparium formation and the second approximately 48 hours later. No radioimmunoassay activity can be detected during the penultimate "gate" period \( G^1 \) before puparium formation.

To identify the ecdysteroids present in those extracts showing high radioimmunoassay activity, the extracts were purified and the ecdysteroids separated using TLC. Fractions of the TLC plates (5-6 mm) were removed and eluted overnight in ethanol. After TLC three, and sometimes four, fractions can be detected showing radioimmunoassay activity. The \( R_f \) values of these peaks of activity are 0.23, 0.28, 0.43 and 0.50. The active fractions, numbered 1 to 4, cochromatograph with ecdysterone, ecdysone, 3-dehydro-ecdysterone and 3-dehydro-ecdysone. The results of rechromatography of the active fractions in a reversed phase system, using HPLC and determining the activity with a second antiserum M-20), confirmed that fraction 1 consistently cochromatographed with ecdysterone and fraction 2 with ecdysone (Table 1). Fractions 3 and 4 did not cross react with antiserum M-20, and the RIA activity was smeared over a number of fractions when assayed with H21B after elution from a reversed phase column. The fact that antiserum M-20 has a lower affinity to ecdysone and fraction 3 than H21B is easily shown in fig. 2, where the TLC fractions of an extract of zero hours old prepupae were split into two and each fraction was assayed with both H21B and M-20. The amount of ecdysterone and ecdysone expressed as picograms per mg tissue during the prepupal and pupal stages is shown in figure 3. The titer of ecdysterone starts rising approximately 3-4 hours before puparium formation and decreases again 5-6 hours after puparium formation, reaching a maximum of 26 picograms per mg tissue. Starting 24 hours after puparium
formation there is a gradual increase in the amount of ecdysterone which reaches a second prominent peak about 48 hours after puparium formation (68 pg/mg tissue). The concentration of \( \alpha \) ecdysone at the time of puparium formation is \( 1/5 \) of the ecdysterone concentration and also decreases 5-6 hours after puparium formation. The concentration of \( \alpha \) ecdysone remains very low during the following 6-9 hours. During the period 15-27 hours after puparium formation the concentration of \( \alpha \) ecdysone is on the average higher than during the preceding 6-9 hours although it fluctuates somewhat. From 27 hours after puparium formation the \( \alpha \) ecdysone concentration gradually increases to a concentration of 23 pg/mg tissue, virtually as high as the ecdysterone concentration at puparium formation.

Conversion of injected \( ^3\text{H} \) ecdysterone.

The stability of injected \( ^3\text{H} \) ecdysterone was followed after injection before and directly after the penultimate "gate" period \( G^1 \) before puparium formation. In order to work with enough radioactivity so that ecdysterone and its possible conversion products could be detected, and so as not to exceed the ecdysterone concentration injected in previous experiments with non labelled ecdysterone, 10 animals were injected with 0.5 \( \mu \text{l} \) \( ^3\text{H} \) ecdysterone solution (2.10^{-5} \text{ M}) together with \( ^1\text{C} \) inulin for each time point. The distribution of radioactivity on the TLC plate of injected \( ^3\text{H} \) ecdysterone, after extraction of injected animals that had been frozen immediately after injection in liquid nitrogen, is shown in figure 4a. The profiles of the distribution of radioactivity of \( ^3\text{H} \) ecdysterone extracted 1 hour after the injection, performed before the "gate" period (fig. 4b) or directly after the "gate" period (fig. 4c), show that in both cases the initial \( ^3\text{H} \) ecdysterone was reduced to 60-70%
while an additional peak of radioactivity was detected due to a less polar metabolite with the same Rf value as the fraction 3, found by radioimmunoassay in extracts of pharate pupae and pupae.
Discussion.

No endogenous ecdysterone or other radioimmunoassay active material could be detected during the penultimate "gate" period (G^1) before puparium formation. The shape of the curve of radioimmunoassay activity in crude extracts of animals during prepupal and pupal stages is, in general, the same as found in *Drosophila melanogaster* (Hodgetts et al., 1977) and the curve obtained by Shaaya and Karlson (1965) for *Calliphora erythrocephala* using a bioassay. The actual radioimmunoassay activity determined at the time of puparium formation is 400 pg/mg tissue in *Drosophila lebanonensis* and in *Drosophila melanogaster* 180 pg/mg tissue by Hodgetts et al (1977), about 800 pg/mg tissue by DeReggi et al (1975) and 100 pg/mg tissue by Borst et al (1974). Differences in the actual radioimmunoassay activity can be explained by cross reaction of more than one ecdysteroid with the different antisera used. Since TLC analysis of the extracts clearly show that several radioimmunoassay active components are present in a crude extract, (for some of which no data have yet been obtained concerning their cross reactivity with antiserum) calculations of ecdysone concentrations can only be made after separation of the ecdysteroids. In contrast to *Drosophila melanogaster* a substantial amount of \( \alpha \) ecdysone could be detected in *Drosophila lebanonensis*. This may be due to the much higher affinity of antiserum H21B to \( \alpha \) ecdysone in comparison to the antiserum used in the investigation on the ecdysone titers in *Drosophila melanogaster* (Borst et al., 1974).

The concentration of ecdysterone, as calculated from the amount detected after separation on TLC, is lower by about a factor of 5-10 than that calculated from radioimmunoassay activity in crude extracts. That the antiserum H21B detects other ecdysteroids than \( \alpha \) ecdysone and ecdy-
sterone has been shown by figure 2. Additional RIA assay activity might be present in more polar fraction and ecdysteroid conjugates which might have been removed by the extraction procedure. However all the above mentioned titers are in agreement with the dose response curve to ecdysterone of larval puffs in vitro as described by Ashburner (1973). The concentration of ecdysterone as well as αecdysone rises again at 48 hours after puparium formation.

The degradation of injected $^3$H ecdysterone (5000 pg/animal) before or after the penultimate "gate" period is in both cases 30-40% after one hour. This is in agreement with values obtained for Calliphora (Shaaya and Karlson, 1965; Galbraith et al., 1969 and Young, 1976). This result indicates that the degradation mechanism cannot account for the differences in the response, with respect to puparium formation, to ecdysterone injections before or after the "gate" period. However it was not possible to follow the degradation of the initially injected 5000 pg/animal down to the level of the naturally occurring concentration at the puparium formation (40 pg/animal) with the $^3$H ecdysterone at present available. It cannot be excluded that the degradation mechanism working at these high ecdysterone concentrations is not different from that involved in the regulation of the endogenous ecdysterone concentrations. That such differences can exist is indicated by the conversion of low and high concentration of injected $^3$H αecdysone in Calliphora erythrocephala (Young, 1976).

The major in vivo conversion product of $^3$H ecdysterone in Drosophila lebanonensis is a less polar metabolite which, although not found in Calliphora in vivo (Young, 1976) has been detected in vitro (Karlson et al, 1972). Less polar conversion products were also found by Cherbas and
Cherbas (1970) and Moriyama et al (1970). Accumulation of radioactive material with a Rf value similar to 26-OH ecdysterone was not found in *Drosophila lebanonensis*. Conversion to very polar material, at the origin of the TLC plates, can be detected but to a much lesser extent than that described for *Calliphora*. The Rf value of the major conversion product of $^3$H ecdysterone in *Drosophila lebanonensis* is the same as the Rf value of fraction 3 in TLC of extracts of pharate pupae or pupae and as the Rf value of 3-dehydro-ecdysterone. A metabolite with about the same Rf value was found associated with a protein in the hepatopancreas in crustaceans (Gorell et al., 1972). Whether the conversion product of $^3$H ecdysterone or the fraction 3 (and possibly 4) found in extracts of pharate pupae and pupae are really degradation products, in the sense of an inactivation process for a biologically active hormone, depends on the biological activity of these fractions. Indications are available that, for example, 3-dehydro-ecdysone (Rf value equals Rf value of fraction 4) has the same biological activity in *in vitro* puffing assays, using *Drosophila melanogaster* salivary glands, as α ecdysone (Richards, pers. comm.). The cross reactivity of 3-dehydro-ecdysone with H21B antiserum is much lower (5-10 times) than that for ecdysterone (Koolman, pers. comm.) which means for example that at the time of puparium formation the concentration of fraction 4 could be equal or even higher than the concentration of the active hormone ecdysterone. Since the biological activity of 3-dehydro-ecdysterone is the same according to in vitro puffing assays, using *Drosophila hydei* salivary glands (Spindler et al., 1977), the actual amount of fraction 3 in the animal and the localisation of this fraction within the animal is of critical importance, to whether or not ecdysterone is the only actual active hormone in the
process of puparium formation.

No endogenous ecdysteroid is present during the penultimate "gate" period before the puparium formation and the degradation of injected ecdysterone before or after this "gate" period is the same. This eliminates the hypothesis that differences in behaviour, with respect to puparium formation, of animals injected before or after this "gate" period is due to the endogenous ecdysterone concentration or differences in degradation velocities of injected ecdysterone. Since the induction of moulting hormone specific puffs, in contrast to puparium formation, is independent of the phase of the circadian oscillation (Eeken, 1974), the circadian oscillation can interfere with the action of the hormone at the post-transcriptional level.
### Table 1. HPLC pattern of α ecdysone and ecdysterone extracted from third instar larvae of *Drosophila lebanonensis*, preseparated using TLC.

<table>
<thead>
<tr>
<th>sample</th>
<th>elution time (in min.)</th>
<th>fraction</th>
<th>R.I.A. activity (ng β ecdysterone equivalent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β fraction TLC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 - 16</td>
<td>1-4</td>
<td>2,6</td>
</tr>
<tr>
<td></td>
<td>16 - 24</td>
<td>5,6</td>
<td>0,1</td>
</tr>
<tr>
<td></td>
<td>24 - 32</td>
<td>7,8</td>
<td>0,6</td>
</tr>
<tr>
<td></td>
<td>32 - 40</td>
<td>9,10</td>
<td>0,6 β (33')</td>
</tr>
<tr>
<td></td>
<td>40 - 48</td>
<td>11,12</td>
<td>0,4</td>
</tr>
<tr>
<td></td>
<td>48 - 56</td>
<td>13,14</td>
<td>0,0</td>
</tr>
<tr>
<td></td>
<td>56 - 72</td>
<td>15-18</td>
<td>0,1 α (66')</td>
</tr>
<tr>
<td></td>
<td>72 - 80</td>
<td>19,20</td>
<td>0,2</td>
</tr>
<tr>
<td></td>
<td>80 - 88</td>
<td>21,22</td>
<td>0,1</td>
</tr>
<tr>
<td></td>
<td>88 - 96</td>
<td>23,24</td>
<td>0,2</td>
</tr>
<tr>
<td>α fraction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 - 16</td>
<td>1-4</td>
<td>0,3</td>
</tr>
<tr>
<td></td>
<td>16 - 24</td>
<td>5,6</td>
<td>0,2</td>
</tr>
<tr>
<td></td>
<td>24 - 32</td>
<td>7,8</td>
<td>0,2</td>
</tr>
<tr>
<td></td>
<td>32 - 40</td>
<td>9,10</td>
<td>0,4 β (33')</td>
</tr>
<tr>
<td></td>
<td>40 - 48</td>
<td>11,12</td>
<td>0,2</td>
</tr>
<tr>
<td></td>
<td>48 - 56</td>
<td>13,14</td>
<td>0,4</td>
</tr>
<tr>
<td></td>
<td>56 - 72</td>
<td>15-18</td>
<td>1,8 α (66')</td>
</tr>
<tr>
<td></td>
<td>72 - 80</td>
<td>19,20</td>
<td>0,3</td>
</tr>
<tr>
<td></td>
<td>80 - 88</td>
<td>21,22</td>
<td>0,1</td>
</tr>
<tr>
<td></td>
<td>88 - 92</td>
<td>23</td>
<td>0,0</td>
</tr>
</tbody>
</table>

Footnote: Fractions of the HPLC separation were collected (indicated as fractions in column 2 and as elution time in column 1) and assayed for RIA activity using antiserum M-20.
Legends to figures.

Figure 1. The amount of radioimmunoassay activity, expressed as picograms ecdysterone equivalents per mg tissue, in crude extracts of whole *Drosophila lebanonensis* larvae and pupae during three sequential light-dark cycles.

PF, puparium formation. G, "gate" period. Dark bars indicate the periods of darkness during the culture of the larvae.

Figure 2. The distribution of radioimmunoassay activity (in percentage radioimmunoassay activity fraction) of an extract of zero hours prepupae measured by the antiserum H21B (•—•) and M 20 (•—•).

Figure 3. The amount of radioimmunoassay activity of fraction 1 (cochromatographing with ecdysterone •—•) and fraction 2 (cochromatographing with ecdysone ▲—▲) in prepupal and pupal stages, expressed as picograms per mg tissue.

PF, puparium formation. G, "gate" period. Dark bars indicate the periods of darkness during the culture of the larvae.

Figure 4. Distribution of radioactivity after TLC of injected $^3$H ecdysterone.

a. conversion of $^3$H ecdysterone after 0 min.

b. conversion of $^3$H ecdysterone 60 min. after the injection before the "gate" period.

c. conversion of $^3$H ecdysterone 60 min. after injection after the "gate" period.

(cpm. corrected for the efficiency of the injection).

| : indicates the position where fraction 3 of extracts of pharate pupae and pupae would be located.
IA activity/mg tissue

hrs after oviposition

$G^1$, $G^2$, $G^3$, $G^4$

PF

IV - 17
percent RIA act / Fraction

Fractions

IV - 18
CPM/10^{-3}/5\text{mm FRACTION}

- **4a**: 61000 CPM = 100%
  - Total CPM 86000

- **4b**: 44000 CPM = 72%
  - Total CPM 82500

- **4c**: 38000 CPM = 62%
  - Total CPM 91500
References.


Milner, I.J. and Sang, J.H. 1976. The effect of fat body on the differen-
tiation in vitro of wing imaginal discs of Drosophila melano-
caster. Wilhelm Roux' Arch. 180, 73-77.

Moriyama, H., Hakanishi, K., King, D.S., Okauchi, T., Siddall, J.B. and
Hafferl, W. 1970. On the origin and metabolic fate of \( \alpha \) ecdysone

Poels, C.L.M. 1970. Time sequence in the expression of various develop-
mental characters induced by ecdysterone in Drosophila hydei.
Devel. Biol. 23, 210-225.

Poels, C.L.M., Loof, A. de. and Berendes, H.D. 1971. Functional and
structural changes in Drosophila salivary gland cells as a conse-

Ratnasiri, N.B. and Fraenkel, G. 1970. Pupation inhibition factor in
the larvae of the blowfly Calliphora erythrocephala. Nature 228,
876-877.


Shaaya, E. and Karlson, P. 1965. Der Ecdysontiter während der Insekten-
entwicklung. II; Die postembryonale Entwicklung der Schmeissfliege

Catalytical oxidation of ecdysteroids to 3 dehydro products and


Young, N.L. 1976. The metabolism of $^3$H moulting hormone in Calliphora erythrocephala at the mature larval and white puparal stages. Insect Biochem. 6, 1-12.
ULTRASTRUCTURE OF SALIVARY GLANDS OF DROSOPHILA LEBANONENSIS DURING NORMAL DEVELOPMENT AND AFTER IN VIVO ECDYSTERONE ADMINISTRATION

J. C. J. Eeken
Department of Genetics, University of Nijmegen, Toernooiveld, Nijmegen, The Netherlands

(Received 10 February 1977)

Abstract—Changes in the morphology of the salivary glands of Drosophila lebanonensis have been followed at both the light and electronmicroscopic level during a period of 30 hr before puparium formation and during puparium formation itself. Three striking differences were observed in comparison to other Drosophila species studied: (1) the secretion product of Drosophila lebanonensis has a different stainability to PAS reagent and uranyl acetate and no internal structures or “caps” can be observed, (2) the release of this secretion product is not restricted to a time period shortly before puparium formation but is a continuous process starting about 24 hr before puparium formation, and (3) the histolysis of these glands starts immediately after puparium formation, whereas in other Drosophila species this event starts 5 hr later.

Puparium formation of Drosophila lebanonensis is controlled by the circadian oscillation. Injection of ecdysterone before the “gate” period results in changes in the cuticle as observed during normal development, but it is not followed by the histolysis of the glands. Injection of ecdysterone after the “gate” is not followed by changes in the cuticle but histolysis is induced.

INTRODUCTION

Several investigations of the ultrastructure of the salivary glands of Drosophila melanogaster, D. pseudoobscura, and D. hydei have been published during the last years (Lane et al., 1972; von Gaudecker, 1972, Harrod and Kasthuri, 1972a, b; Berendes, 1965; Berendes and Ashburner, 1977). The changes in the ultrastructure of the glands during the third instar, the process of puparium formation and pupation until the histolysis, some 5 to 7 hr after the white pupal stage, are about the same in various species. In these species the cells of the salivary glands show little differentiation of the cytoplasm, which contains free ribosomes, some endoplasmic reticulum, and a few Golgi systems, during the first half of the third instar. Then the appearance of the cytoplasm becomes increasingly complex due to increasing endoplasmic reticulum, often arranged in parallel arrays, and increasing numbers of active Golgi systems. Aside from the fact that some developmental changes move gradually from the posterior to the anterior region of each individual gland, at about the middle of the third instar electron dense, periodic acid Schiff reagent positive granules are formed. These are often in the region of Golgi systems. During the following period of 30 hr the changes in ultrastructure of the glands are mainly due to the increase in number and size of these electron dense granules, which consist of at least two components. Two hours before the white puparial stage most of the granules are found at the apical region of the cells and the product is secreted into the lumen. During the white puparial stage large vacuoles are formed, sometimes associated with Golgi systems, which shortly before the pupal apolysis migrate to the apical region of the cell. The degeneration of the gland cells starts about 5 to 7 hr after puparium formation and is characterized by vesicular endoplasmic reticulum, degenerated mitochondria, autophagic vacuoles and lysosome-like structures.

Some of the ultrastructural changes described have been shown to be inducible in D. hydei by ecdysterone (Poels et al., 1971).

In contrast to the species described, puparium formation of D. lebanonensis is restricted to a certain period of the circadian oscillation (Rensing and Hardehn, 1967). Injection of ecdysterone induces specific changes in genome activity followed by puparium formation only if injected at a specific phase of the circadian oscillation. Injection at other periods during the oscillation does induce puff activity but the puparium formation is delayed and starts at the normal time (Eeken, 1974). This report deals with the ultrastructural changes of the salivary glands of D. lebanonensis during the normal developmental process of puparium formation and after in vivo administration of ecdysterone.

MATERIALS AND METHODS

Larvae of Drosophila lebanonensis casteel (Pipkin) were raised under standardized conditions of food,
humidity, temperature, and light–dark regimen (LD 12:12). As described earlier (EEKEN, 1974), a synchro-
nised population of larvae form puparia in two
distinct groups. The first group pupates during the
“gate” period of the 7th day, the first 6 hr of the 8th
dark period (168–174 hr after oviposition), whereas
the second group does so during the “gate” period
of the 8th day, the first 6 hr of the 9th dark period
(192–198 hr after oviposition). Only animals of the
second group were used. However, the same changes
were observed in salivary glands of animals of the
first group, although 24 hr earlier.

During a period of 30 hr before the white puparial
stage, salivary glands were dissected in Mg-Ringer,
one gland was then prepared for electron-microscopy,
while the other gland was squashed and stained with
acetoorceine to examine the puffing pattern (BER-
ENDES, 1965).

The glands used for electron-microscopy were fixed
in 2.5% glutaraldehyde in 0.1 M sodium-cacodylate
buffer (pH 7.3) for 45 min at 4°C, washed in 0.1 M
sodium-cacodylate buffer (pH 7.3) and postfixed in
2% osmium tetroxide in Veronal-acetate buffer (pH
7.3). After fixation the glands were dehydrated and
embedded in Epon (LUFT, 1961). Sections were
stained with uranyl acetate and lead citrate (VENA-
BLE and COGGESHALL, 1965). In some cases osmium
zinc iode (OZI) technique for staining carbohydrates
according to DAUWALDER and WHALEY (1973) was
used, or staining on block with phosphotungstic acid
(PTA) was used.

Light microscopic examination of the glands was
done on whole mount preparations after staining with
periodic acid Schiff reagent (PAS) according to
McMANUS (1946) or after fixation in picric acid and
staining with aceto-carmine according to KRESS
(1974). Larvae were injected as described by BEREN-
ENDES (1965). Each larva is injected with 0.5 μl ecdysone
solution. The concentrations of ecdysone used are
2 × 10⁻² M or 2 × 10⁻⁴ M.

RESULTS

Light microscope observations

30 hr before puparium formation the salivary
glands of Drosophila lebanonensis of the second pupat-
ing group are still relatively small (Fig. 1a). The lar-
ests of the unequally sized glands, a characteristic of
the subgenus Scaptodrosophila (WHEELER, 1949) is
about 0.6 mm long and is about 1/10 the size of the
glands of larvae of the first pupating group of the
same age, which are, at that time, in their post-feeding
stage. The ecdysone specific loci whose activity is in-
duced by ecdysone are not puffed (9A on the X
chromosome, 19C, 24A, 24B, 24C, 28B, 32C on the
second chromosome and 54A on the fourth chromo-
some), while at locus 21A, which is specifically repressed by ecdysosterone, a puff is present.

During the dark period preceding that in which
puparium formation begins (27–15 hr before the white
puparial stage) the glands gradually increase in size.
This increase is mainly due to an increase in lumen
volume, whereas the cells become thinner (Fig. 1b).
No clear changes in the puffing pattern of the X,
second and fourth chromosome can be detected;
region 24C seems to increase in size during this
period, but no puff activity can be observed at the
other ecdysone specific loci.

By the time the larvae stop feeding and leave the
food (8–10 hr before the white puparial stage) the
glands have reached their maximum size. These large
glands have been examined 5 hr before puparium
formation. The cells are very thin in relation to the size
of the glands (Fig. 1c). A puff at 24C is now clearly
present and still increasing in size. Of the other ecdy-
sone-specific loci, only 19C is becoming active,
whereas 21A starts to decrease in the glands exam-
ined. No activity of the other ecdysone-specific loci
can be detected.

After the extrusion of the secretion product from
the lumen (2–3 hr before the white puparial stage),
the glands have the appearance of flat sacs with the
same length as the glands have just before they empty
(Fig. 1d). At this moment the ecdysone-specific puffs
19C, 24A, 24B, 24C, and 28B have reached their max-
imum size, while 21A is absent. Puffs 32C and 54A
begin to be active.

In glands of white puparia puffs 19C, 24A, 24B,
24C, and 28B are decreasing in size, while 32C and
54A reach their maximum size. Locus 21A begins to
be active again. Throughout the entire period that
the glands were examined, the cells of the glands dis-
play a weak reaction to PAS reagent, whereas the
product in the gland lumen is PAS negative.

Electron microscope observations

On the basis of these light microscope changes in
morphology of the glands and the puffing pattern,
electron microscope preparations were made of
glands of larvae at 30, 21, 5, and 2 hr before the white
puparial stage and of glands of 0 hr old white puparia.

At 30 hr before puparium formation, the distal cells
display a vacuolar cytoplasm. Some of the small
vacuoles appear empty, while others are filled with
flocculent material. The rough endoplasmic reticulum
is evenly distributed over the cytoplasm. At the basa-
lar side of the cell large invaginations of the cell mem-
brane are present, separated from the exterior en-
vironment by a basement membrane. Most Golgi sys-
tems can be found at the basal side of the cell. The
apical region is bordered by long microvilli which
show extensive budding (Fig. 2). Two types of cells
are regularly observed: the most common type is de-
scribed above, in the second type the organelles are
more loosely arranged in the cytoplasm. There is no
obvious difference in the stainability of these two
types of cells as described in other species (LANE
et al., 1972; WIENER et al., 1964) although often a dif-
ference in the stainability was observed for the apical
and the basal region of one and the same cell. The
Fig. 1. Lightmicroscopic survey of developing salivary glands (staining according to KRES, 1974): (a) 27 hr, (b) 15 hr, (c) 6 hr and (d) 2 hr before puparium formation.

Fig. 2. Apical region of salivary gland cells at 30 hr before puparium formation. L, lumen; MV, microvilli. Inlet: Budding occurs at the terminal of the microvilli. Scale = 1 μm on this and all following Figs.
Fig. 3. Apical region of salivary gland cells at 21 hr before puparium formation. Increasing numbers of secretion vacuoles (SG) are present. G, Golgi apparatus; L, lumen.

Fig. 4. Apical region of salivary gland cells at 21 hr before puparium formation. Excretion of secretion vacuoles (SG) into the lumen (L).

Fig. 5. Apical region of two salivary gland cells at 21 hr before puparium formation. Small fields of glycogen-like material (GM) can be observed in both cells. L, lumen.
Fig. 6. Basal region of salivary gland cells at 5 hr before puparium formation. 

a. Large vacuoles (V) appear in the region of the invaginations (I) of the basal cell membrane (BM, basement membrane).

b. Many microtubules (arrow) appear at the cell membranes.
Fig. 7. Central part of the cytoplasm of salivary gland cells 2 hr before puparium formation. Vacuolisation of the cytoplasm. V, vacuoles.

Fig. 8. Central part of the cytoplasm of salivary gland cells of white prepupae. Big vacuoles (V) containing cell remnants.
Fig. 9. Basal part of salivary glands. a. Contractile elements (CE) inside the basal membrane (BM).
b. Contractile elements (CE) in contact with the salivary glands. c. Inlet: actine and myosine elements.
$\times 130,000$. 
Fig. 10. Apical region of salivary gland cells 6 hr after injection with ecdysterone (2 × 10^{-5} M) at 165 hr after oviposition. No cytoplasmic degradation can be observed. A big vacuole (V) is present as can be found frequently 5 hr before puparium formation. L, lumen; MV, microvilli.

Fig. 11. Apical region of salivary gland cells 6 hr after injection with ecdysterone (2 × 10^{-5} M) at 175 hr after oviposition. No cytoplasmic degradation, although protrusions (arrow) of the cytoplasm into the lumen (L) occur, indicating a starting histolysis.
Fig. 12. Apical region of salivary gland cells 6 hr after injection with ecdysterone ($2 \times 10^{-4}$ M) at 165 hr after oviposition. No degradation of the cytoplasm, but increasing numbers of myelinelike structures (arrow) and abnormally shaped and stained mitochondria (M). L, lumen.

Fig. 13. Apical region of salivary gland cells 6 hr after injection with ecdysterone ($2 \times 10^{-4}$ M) at 165 hr after oviposition. In the right cell, already histolysed, no villi border is present. Whereas the other cell only shows minor changes like increased myelinelike structures (arrow). L, lumen.

Fig. 14. Basal region of salivary gland cell 6 hr after injection with ecdysterone ($2 \times 10^{-4}$ M) at 175 hr after oviposition. Complete histolysis. BM, basement membrane; M, mitochondria.
proximal cells have a similar appearance except that there are less vacuoles present.

During the dark period preceding puparium formation, the cytoplasm becomes more differentiated. There is an increase in the amount of endoplasmic reticulum, which partly becomes arranged in parallel arrays. Many active Golgi systems can be observed, most of them in a compact strip at the basal side of the cell. Numerous mitochondria are distributed all over the cell. At the apical region of the cell a new type of vacuole, filled with flocculent material, appears, quickly increases in number and eventually occupies more than 1/3 of the cell volume (Fig. 3). The contents of these vacuoles are emptied into the lumen by exocytosis, between the microvilli (Fig. 4). Even after staining with OZI, alcoholic PTA or aqueous PTA at pH 1, no dense staining can be detected in the vacuoles which empty their contents into the lumen or of the contents of the lumen itself. The process of budding of the microvilli, which was already observed before the secretion vacuoles appeared, continues undiminished during secretion. Apart from these obvious changes in the ultrastructure of the cells, increasing amounts of microtubules are also present, starting around the cell nucleus and later also found centrally in the cytoplasm. In some cells patches of glycogenlike material are present (Fig. 5).

In the cytoplasm of the cells of the very large glands (5 hr before puparium formation) decreasing amounts of rough endoplasmic reticulum and increasing amounts of free ribosomes can be found. The secretion vacuoles have completely disappeared, instead large vacuoles are formed at the basal side of the cells (Fig. 6a). Some of them are optically empty while others are filled with some fibrous material. The microvilli are still present and remain active in the process of budding. The mitochondria, although their morphology is normal, now stain more intensely than the surrounding cytoplasm. Many microtubules can be found centrally in the cytoplasm but also at the cell membrane (Fig. 6b). The basal region surrounding the invaginations stains more densely than the apical region.

In glands examined shortly after they have emptied their lumen, the cytoplasm of the cells is densely packed with free ribosomes and no endoplasmic reticulum can be found. The microvilli, if present, are still active in budding. The basal part of the cell is occupied mainly by large vacuoles separated by small bridges of cytoplasm, multivesicular bodies and increasing numbers of small vacuoles (Fig. 7). Very long microtubules can be observed throughout the cell.

The first signs of histolysis can be detected in the apical region of the proximal cells of glands of white puparia. Large vacuoles dominate the cytoplasmic structures present. These vacuoles contain complete membranes and other cell remnants (Fig. 8). The histolysis of the distal cells starts later than in the proximal cells.

In some cases contractile structures are in close contact with the salivary glands (Fig. 9). The function of these structures is not known; they have not been found in other species of Drosophila.

In vivo administration of ecdysterone

Injection of an ecdysterone solution with a concentration of $2 \times 10^{-5}$ M at 165 hr after oviposition, shortly before a "gate" period, results 6 to 8 hr later in puparium formation (EEKEN, 1974). The ultrastructure of the salivary gland cells, 6 hr after injection, differs from uninjected control cells in various respects: no secretion vacuoles appear, and the stainability of the mitochondria is comparable to that of the salivary gland cells of white puparia. During normal development giant vacuoles are found 3 to 5 hr before puparium formation, only one such vacuole was found in glands from injected larvae (Fig. 10). No degradation of the gland cells can be detected.

Injection of the ecdysterone solution (conc. $2 \times 10^{-5}$ M at 175 hr after oviposition, after the "gate" period, is not followed by the formation of a puparium (EEKEN, 1974). In salivary gland cells 6 hr after injection, as after injection at 165 hr after oviposition, no secretion vacuoles can be detected. The stainability of the mitochondria is also increased. Degeneration features like the absence of microvilli or protrusions of the cytoplasm into the lumen of the salivary glands of some of the injected larvae can be found (Fig. 11).

Injection of an ecdysterone solution with a concentration of $2 \times 10^{-4}$ M at 165 hr after oviposition, shortly before the "gate" period, induces puparium formation after 6 to 8 hr. After 6 hr there is no degeneration of the cells of salivary glands of animals that had not yet formed a puparium. The microvilli appear normal and budding can be found. Although there are many free ribosomes, little rough endoplasmic reticulum is present. Little or no autophagic vacuoles can be observed. No secretion vacuoles are present. Increasing numbers of myelene structures can be observed although they are rather small. The mitochondria often show abnormal shape and the stainability is increased (Fig. 12). Some cells of the salivary glands of animals, that already had formed a puparium after 6 hr, are totally vacuolised and especially the apical region of the cells is degenerated (Fig. 13).

Injection of the ecdysterone solution with a concentration of $2 \times 10^{-4}$ M 175 hr after oviposition, after the "gate" period, is also followed in 6 to 8 hr by the formation of a white puparium. Already 2 to 4 hr after the injection, destruction of the ultrastructure of the salivary gland cells can be detected, and 6 hr after the injection the structure of the cell cytoplasm is completely destroyed (Fig. 14).

DISCUSSION

If one compares the ultrastructure of the salivary gland of Drosophila lebanonensis with D. melanogaster, D. pseudoobscura, and D. hydei, the species studied
in greater detail by von Gaudecker (1972), Lane et al. (1972), Harrod and Kastritis (1972a, b), and Berendes (1965; Berendes and Ashburner, 1977), it becomes evident that, although during the process of puparium formation all species display a similar function with respect to the production and secretion of a glue substance, there are a number of features in this process of secretion production that differ in Drosophila lebanonensis. The secretion product does not react with periodic acid Schiff reagent and also has a different staining behaviour towards uranyl acetate. No internal structures can be observed in the secretion vacuoles. Furthermore, the release of this product into the lumen is not restricted to a short period, namely a few hours before puparium formation, as in the other Drosophila species, but it is a process that starts 30 hr before puparium formation and continues until 10 hr before puparium formation. This is clearly seen after fixation and staining of the glands according to Kress (1974).

These differences in salivary gland behaviour were confirmed by the electronmicroscopical investigation of the glands during this period. The vacuoles with the secretion product emptied out into the lumen by exocytosis, in agreement with observations in the other Drosophila species. Although the process of budding of the microvilli in this period will also contribute to the increase in lumen volume, this process could be seen at all times and could not be related to a defined period in this developmental stage.

The large invaginations at the basal side of the cell are also present, although less pronounced, in the other Drosophila species. In Chironomus these invaginations at the basal side of the cell have been considered as a morphological expression of transport of materials from the haemolymph to the gland (Kloetzel and Lauffer, 1969).

No data are available about the presence of microtubules in the salivary gland cells in other Drosophila species. The function of these organelles, as well as the observed contractile elements in close contact with the salivary gland, need further investigation.

Another obvious difference between the salivary glands of Drosophila lebanonensis and the other Drosophila species is the time when the histolysis of the glands start. In Drosophila lebanonensis the first signs of degeneration can be detected even before puparium formation, whereas in other Drosophila species this occurs some 5 to 7 hr later (Lane et al., 1972). During the period between the secretion of the glue substance and the histolysis large vacuoles occur both in Drosophila lebanonensis and in Drosophila melanogaster (von Gaudecker, 1972) only the period during which they can be observed is for Drosophila lebanonensis shortly before puparium formation whereas in Drosophila melanogaster they occur during a period between puparium formation and the larval-pupal apolysis.

In vivo, ecdysterone is present from 2 hr before the puparium formation until 6 hr after puparium formation (Eeken, unpublished) and ecdysterone can thus induce the changes in the cuticle leading to the puparium as well as the histolysis of the salivary glands. However, if ecdysterone is injected before the "gate" period of puparium formation, no histolysis of the salivary glands occurs although the changes in the cuticle, which accompany puparium formation do occur. If, on the other hand, ecdysterone is injected after the "gate" period of puparium formation, histolysis does occur. Hence the "gate" period in which changes in the cuticle can be induced, occurs earlier in the circadian oscillation than the "gate" period in which histolysis can be induced. The lack of both processes after ecdysterone injection may be due to rapid inactivation of the injected ecdysterone.

Acknowledgements—I am indebted to Dr. N. H. Lubsen for critically reading the manuscript, and I thank Miss E. C. T. Willart for her excellent technical assistance. Financial support was obtained from the Netherlands Organization for pure Scientific Research Z.W.O. (BION).

REFERENCES


Ultrastructure of salivary glands of D lebanonensis


WHEELER M R (1949) The subgenus Pholadoris (Drosophila) with description of two new species Univ Texas Publ 4920. 143-156

CIRCADIAN CONTROL OF THE CELLULAR RESPONSE TO ECODYSTERONE IN DROSOPHILA LEBANONENSIS II. Changes in protein synthesis of salivary glands during puparium formation and after in vivo and in vitro administration of ecdysterone at different phases of the circadian oscillation.

Introduction.

As a result of the increase of moulting hormone in the haemolymph of third instar Drosophila larvae, the larvae transform into pupae. The beginning of this process is characterized by spiracle eversion and the morphological change of the larval shape, by muscle contraction and cuticle hardening, into the typical pupal "barrel" shape and refered to as puparium formation. The change of the function of the salivary glands just prior to the puparium formation from the synthesis and storage of the glue substance to the secretion of this product into the lumen can also prematurely be induced in vivo as well as in vitro by the steroid hormone ecdysterone (Poels 1972, Zhimulev and Kolesnikov 1975, Korge 1975, 1977, Kress 1977, Eeken 1977, Boyd and Ashburner 1977). However the first signs of the start of the reprogramming of the function in the salivary glands can be detected as a changed genome activity by the characteristic changes in the puffing pattern of the polytene chromosomes (Berendes 1965, Ashburner 1967, Eeken 1974). Again these puparium formation specific changes in the puffing pattern can be followed in time during in vivo and in vitro application of ecdysterone (Berendes 1967, Berendes and Thijsen 1971, Ashburner 1972, Kress 1972). It is a plausible assumption that in between the changes in the genome activity and the subsequent changes in the salivary gland function, there will be related changes in protein synthesis (Tissières 1974, Ashburner and Richards 1976).

The time of puparium formation in Drosophila lebanonensis is under the control of the circadian oscillation. The puparium
formation can take place only at a defined "gate" period of 6 hours in an artificial LD regimen. A "synchronized" population of larvae of Drosophila lebanonensis pupates in two distinct groups. The first group pupates during the "gate" period $G^1$ at day 7 after oviposition, the second group during the "gate" period $G^2$ at day 8. The most obvious explanation seemed that the circadian oscillation controls the release of the moulting hormone. However as described in an earlier paper (Eeken 1974), the effect of ecdysterone injections, as detected by puparium formation, differs depending on the phase of the oscillation in which the injection is performed. Animals of the second pupating group injected before $G^1$ induces the puparium formation specific genome response in the salivary glands, followed by the puparium formation within 6-8 hours. Injection after $G^1$, again induces the specific change in genome activity, however is not followed, within 8 hours by puparium formation. The puparium formation in this case follows about 18 hours later, during the next "gate" period ($G^2$).

The assumption that just prior to $G^1$ the endogenous ecdysterone titer in the second group animals rises, although not yet reaching the critical concentration necessary to induce puparium formation, can explain the different reactions to ecdysterone injections at different phases of the oscillation. However a rise in the endogenous ecdysterone titer would result in a change of the relative diameter of the ecdysterone specific loci of the salivary gland chromosomes. No indication of even a small increase in the diameter of the ecdysterone specific loci can be detected during this period (Eeken 1974). An investigation to the ecdysterone concentration and the ecdysterone degradation during the period in which the injections are performed showed that
there was no detectable endogenous ecdysterone nor differences in
the velocity of ecdysterone degradation (Eeken and O'Connor 1977).
Although it is still unknown whether or not the release of ecdoyste-
rone is controlled by the circadian oscillation it is obvious that
the "gating" of the puparium formation is not only determined by
the controlled release of ecdysterone. An alternative explanation
of the observed differences in the effect of ecdysterone injections
at different phases of the circadian oscillation involves a second
control system whereby the circadian oscillation determines the "gate"
period of puparium formation. This second system interferes with the
ecdysterone induced gene activity at the post-transcriptional level.

To study the post-transcriptional control system, the effect
of the hormone, injected at different phases of the circadian oscil-
lation, on the protein synthesis of the salivary glands was determined
and compared with changes in protein synthesis which occur at puparium
formation during normal development. To investigate if the post-trans-
criptional control system originates within the salivary gland itself
or is imposed upon the glands externally, originating elsewhere in
the animal, salivary glands were isolated at different phases of the
circadian oscillation and incubated in vitro with ecdysterone where-
upon its effect on the protein synthesis is determined.
Methods.

Larvae of Drosophila lebanonensis casteeli (Pipkin) were cultured under standardized conditions with respect to food, temperature (25±1 °C), humidity and light-dark regimen (LD 12:12). The population of larvae were synchronized by collecting eggs during a 3 hours period, starting at lights off, which results in a developing population with two pupating groups. The first group pupates during the "gate" period $G^1$ (168-174 hours after oviposition), the second group pupates during the next "gate" period $G^2$ 24 hours later. Both groups can be separated easily on their behaviour, 8-10 hours before the puparium formation of the first group of animals starts. The animals that are going to pupate stop feeding and leave the food. Even before that, both groups can be distinguished by the morphology of their salivary glands as described earlier (Eeken 1977).

Changes in protein synthesis were detected following the technique described by Tissières et. al. (1974). Proteins were labelled by incubating (15') hand isolated salivary glands in incomplete (modified to remove methionine) Poels Medium (Poels 1972) or incomplete modified Grace's Insect Medium (Ashburner 1972) supplemented with 2.4-3.0 μCi/μl $^{35}$S methionine (spec. act. 400-500 Ci/mmol.). The incubation was stopped by replacing the incubation medium by 10% cold TCA (20'). The salivary glands were dehydrated in ethanol 96% / chloroform (1:1) and dried at 37 °C. The dried glands were dissolved by boiling for 2-3 minutes in 25-50 μl phosphate buffer (0.1 M phosphate, pH 6.9, 1% SDS, 4 M urea and 1% B-mercaptoethanol). The labelled proteins were separated electrophoretically on 12.5%
polyacrylamide slab gels in the presence of SDS (Laemmli 1970). After
drying, the gels were exposed to Kodak RP/R 14 X-ray film. Eventually
the autoradiographs were scanned with a Vitatron densitometer.

Ecdysterone ($2 \times 10^{-5}$ M) was injected (0.5 μl/larvae) as described
previously (Berendes 1967). Only animals of the second pupating group
were injected just before or after the "gate" period $\theta$. Salivary glands
were cultured in vitro in Poels' Medium or modified Grace's Insect Medium
supplemented with ecdysterone ($1.5 \times 10^{-6}$ M).

Changes in puffing pattern were determined after staining with
aceto-orceine (Berendes 1967) and expressed as changes in the relative
diameter according to Eeken (1974).
Results.
Changes in protein synthesis pattern of salivary glands during puparium formation in normal development.

To determine the changes in the protein synthesis in the salivary glands during the appearance of the hormone ecdysterone in the haemolymph, glue filled salivary glands of late third instar larvae and empty salivary glands of zero hours old white prepupae were dissected and labelled in vitro with $^{35}$S methionine as described in "Methods". An autoradiogram and its densitometric tracing of the separated labelled proteins of salivary glands of third instar larvae and white prepupae is shown in figure 1. The comparison shows that the most obvious changes are the appearance of two low molecular weight peptides (molecular weight between 12,000 and 18,000 daltons) and the disappearance of a peptide with a molecular weight of approximately 65,000 daltons. This change in the synthetic pattern during puparium formation has a remarkable resemblance in Drosophila lebanonensis, Drosophila melanogaster (see figure 1) and Drosophila hydei (results not shown). Whether these changes are the result of direct or indirect action of the genes induced by ecdysterone cannot be concluded from these results. The assumption however is made that these changes ultimately depend on the presence of the moulting hormone and in that way their presence can be used as an indication of an uninterrupted continuous sequence of events set in motion by this hormone.

Changes in protein synthesis pattern of salivary glands after ecdysterone injection.

Larvae of the second pupating group were injected with ecdysterone
solution just prior to and shortly after "gate" period \( G^1 \). 6 Hours after the injection performed just before \( G^1 \), or just after \( G^1 \), two groups of animals can be observed; pupating larvae (50\% and 10\% respectively of the total of the injected animals) and non pupating larvae (50\% and 90\% respectively of the total of the injected animals). In all four groups the ecdysterone specific genes are activated. Pooling 5 pairs of salivary glands of pupae, the observed protein synthetic pattern was similar to that of white prepupae during normal development. Pooling 5 pairs of glands of injected larvae which were not pupating 6 hours after the injection performed after the "gate" period \( G^1 \) did not show the puparium formation specific change in protein synthesis. However, injection after the "gate" period \( G^1 \) still results in the formation of a puparium in about 10\% of the injected animals. Pooling 5 pairs of salivary glands of these animals showed that in these animals the same change in protein synthesis pattern occurs as in animals during puparium formation in normal development (fig. 2).

Changes in protein synthesis pattern after \textit{in vitro} incubation with ecdysterone.

In this section we tried to establish if \textit{Drosophila lebanonensis} salivary glands posses a circadian oscillator of its own, capable of maintaining \textit{in vitro} the observed "gating" event of the puparium formation specific protein synthesis as has been shown to exist by the \textit{in vivo} administration of ecdysterone during different phases of the oscillation. The changes in protein synthesis pattern was determined after isolation of salivary glands just before and after "gate" period \( G^1 \) and incubating these glands for 2 and 6 hours in the presence of
ecdysterone. The change of the genome activity was checked after 1 and 4 hours after the start of the incubation. An example of the induction of the puparium formation specific puffs in vitro is shown in figure 3. No changes in the protein synthesis pattern as can be detected during puparium formation can be induced in vitro by ecdysterone although in all cases the ecdysterone specific puffs of the polytene chromosomes do appear. No puparium formation specific change can be induced by ecdysone (7.10^{-6} M), a high ecdysterone concentration (10^{-4} M) or even a combination of both (fig. 4a). The same experiment, this time using Drosophila melanogaster instead of Drosophila lebanonensis, results in the appearance of the more prominent of the two low molecular weight peptides (fig. 4b). All experiments were performed in Poels' Medium as well as in Grace's Insect Medium. From these results it was concluded that the interfering system controlled by the circadian oscillation was activating in nature and originated outside the salivary glands. Two experiments were conducted using simultaneous incubation of salivary glands with brains or fat body. Figure 5 shows that no puparium formation specific changes in protein synthesis in salivary glands are induced when 6 pairs of glands are incubated in 50 μl Grace's Insect Medium for 6 hours in the presence of ecdysterone together with 25 white prepupal brains. The incubation was started just before "gate" period G^1. No influence could be detected, by simultaneous incubation of fat body on salivary glands in the presence of ecdysterone, starting the incubation at the same time as the first experiment. Preincubation of the medium with brain or fat body for 6 hours before the incubation of the salivary glands started, all in the presence of ecdysterone, did not have any effect either (fig. 6). However, the protein synthesis pattern
of salivary glands after incubation in medium preincubated with brains is not the same as the protein synthesis pattern of salivary glands after incubation in medium preincubated with fat body.
Discussion.

The protein synthesis pattern of salivary glands of white prepupae of *Drosophila lebanonensis* is different from that of third instar larvae, 6 hours before puparium formation. The most obvious differences as can be detected by electrophoresis in the presence of SDS, are the appearance of two new low molecular weight peptides and the disappearance of a peptide with a molecular weight of 65,000 daltons. Interesting is the observation that at least the two low molecular weight peptides have the same molecular weight as two peptides, whose synthesis is also induced during puparium formation in *Drosophila melanogaster* and *Drosophila hydei* (Tissières et al 1974, Lewis as cited in Ashburner and Richards 1976, Eeken unpublished). Nothing is known about the function of these peptides, although speculations have been made that they are involved in the histolytic process (Ashburner and Richards 1976). The disappearance of the 65,000 molecular weight peptide might be related to the repression of the synthesis of the glue substance. A comparison between the autoradiographs of the pattern of de novo synthesized proteins of third instar larvae, zero hours old white prepupae and the protein pattern of isolated glue substance revealed that none of the glue proteins detected corresponds with the disappearing peptide of 65,000 d. However, it is not excluded that the 65,000 d. peptide is modulated during its processing to glue substance, as has been shown to occur in *Drosophila melanogaster* (Beckendorf and Kafatos 1976).

With the method used it is only possible to establish in time sequential changes in two related processes, transcription and translation, without proving any relationship between them. The detected changes are however an indication to whether or not ecdysterone induced
changes in the activity of the genome is followed by the same changes in protein synthesis, without being interfered with at the post-transcriptional level, as occurs during puparium formation. The earlier postulated hypothesis of a circadian controlled interference system at the post-transcriptional level (Eeken 1974) was based essentially on the detection of ecdysterone induced gene activity in one tissue (puffing patterns in salivary glands) and a change in function of a different tissue (morphological change of the epidermis). In this paper we used the detectable changes in the protein synthesis of the salivary glands as an intermediate step between ecdysterone induced gene activity and the subsequent change in function of this tissue. In this way we restricted the system in which we could study the action of the, by the circadian oscillation controlled, interference system.

In order to investigate whether injection of ecdysterone at different phases of the oscillation induces the puparium formation specific change in protein synthesis, larvae were injected with ecdysterone solution just prior to or after a "gate" period. Even in a "synchronized" population of larvae the width of the "gate" period is not simply the time period in which puparium formation can take place whenever the larvae get ready to pupate, since in that case the distribution of pupating larvae in a "gate" period would not be a normal one, but is rather the expression of the variations in the synchronization of the circadian oscillation of each of the individual larvae. This means that an injection performed at any one stage will be an injection into larvae with a variety of phases of their circadian oscillation. After injection shortly before the "gate" period $G$ not all the animals will be able to respond directly because some of the animals will still be in a phase
of the circadian oscillation which determines that the animal is not
yet in the "right" period to start puparium formation. However since
the relative large amount of the injected hormone, in relation to the
actually necessary amount, at the time the circadian oscillation
reaches the onset of the "right" period there is still enough hormone
left in the animal to induce the puparium formation. The result will
be that all injected animals will pupate in the "gate" period $G^1$.
Injection right after the "gate" period faces the same problem which
might explain the percentage larvae pupating outside the "gate" period
as defined by the behaviour of the population during normal development.
The results indicate that whenever a larva reaches its permissive
"gate" period ecdysterone is able to induce the puparium formation
specific change in protein synthesis in parallel to the activation of
the puparium formation specific genes. In contrast, injection of eco­
ysterone into a larva just leaving its permissive "gate" period is
unable to induce the puparium formation specific change in protein syn­
thesis, although the activation of the puparium formation specific genes
does occur. This means that there exist a control system, coupled to the
circadian oscillation, which interferes with the action of the hormone
ecdysterone at a level somewhere between the induction of puff activity
at the chromosome and the translation at the ribosome.

In order to determine and analyse the interference system, salivary
glands of group II larvae were isolated shortly before $G^1$ and after $G^1$
and incubated in vitro with ecdysterone. The change in protein synthesis,
as observed during puparium formation in normal development was used as
an indication whether or not the interference system, coupled to the
circadian oscillation, allowed the ecdysterone induced reprogramming of
gene activity to be processed and transformed into the intended change in salivary gland function. In the case that all cells possess a circadian oscillation, salivary glands isolated at different phases of the circadian oscillation will behave different with respect to changes in their protein synthesis after incubation in the presence of ecdysterone. In the case that the interference system is controlled by a biological clock that originates outside the salivary glands, the reaction of salivary glands isolated at different phases of the circadian oscillation to ecdysterone will be the same. Since the reaction, with respect to changes in the protein synthesis, of isolated salivary glands to ecdysterone is always the same, it is clear that the interference system originates outside the salivary glands. The puparium formation specific changes in genome activity, however, can be induced in isolated salivary glands, independent of the phase of the circadian oscillation of the donor larvae. The hormone ecdysterone is unable to induce in vitro in Drosophila lebanonensis salivary glands the changes in protein synthesis as they occur during puparium formation in normal development. This result was consistent in different media as well as with different ecdysterone concentrations. This indicates that the interference system is indispensable to process the information provided by puparium formation specific genes in order to establish a change in cell function.

An attempt has been made to identify the tissue responsible for factors interfering with the reaction of salivary gland cells to ecdysterone treatment. The method used was the simultaneous incubation in vitro of salivary glands of larvae of the second pupating group, isolated just prior to "gate" period G with brains or fat body of zero hours old white prepupae, all in the presence of ecdysterone. Brains
were chosen as the first possibility since an extra humoral factor
treated by and directly synthesized at the site of the biological
clock could interfere with the salivary gland cells. According to se-
veral investigations the site of the biological clock is located in the
brain (Williams and Adkisson 1964, Roberts 1965, Truman and Riddiford
1970). Additionally, brain extract is known to enhance puparium forma-
tion (Fraenkel et al 1972). The second tissue tested was fat body,
known to convert ecdysone into the active moulting hormone (Moriyama
et al 1970, King 1972) and to produce "fat body factor" which has a
positive effect on the differentiation of imaginal discs (Benson et al
1974, Milner and Sang 1976). It was not possible in this approach to
induce the puparium formation specific change in protein synthesis of
salivary glands. However no specific conclusion can be drawn from these
results since too many factors might interfere in a simultaneous in
vitro incubation experiment. The fact that the observed change in the
protein synthesis of salivary glands can be modulated by factors added
to the medium by the preincubation of the medium with brains or fat
body is indicative for these interfering factors.
Legend to figures.

Fig. 1. Autoradiograph of labelled proteins after SDS electrophoresis. Lefthand panel, changes in protein synthesis of salivary glands during puparium formation of *Drosophila lebanonensis* and *Drosophila melanogaster*. 6 Hours before puparium formation, *D. lebanonensis* (A), *D. melanogaster* (D). At puparium formation, *D. lebanonensis* (B), *D. melanogaster* (C).

Middle section, a repeat is shown of *D. lebanonensis*, 6 hours before puparium formation (A) and at puparium formation (B).

Righthand panel, densitogram of the autoradiographs shown in the middle section.

Fig. 2. Autoradiograph of labelled proteins after SDS electrophoresis of *Drosophila lebanonensis*.

Patterns of salivary glands, at puparium formation (A), injected larvae 6 hours after injection of ecdysterone, performed just after G₁, that form a puparium (B), control at the time of injection (C), injected larvae 6 hours after injection with ecdysterone, performed just after G₁, that do not form a puparium (D), control 6 hours after the injection (E).

Fig. 3. Induction of the puparium formation specific genes in the salivary gland chromosomes of *Drosophila lebanonensis* after incubation in the presence of ecdysterone. Ecdysterone incubation, ○—○. Control, ■—■.
Fig. 4a. Autoradiograph of labelled proteins after SDS electrophoresis of *Drosophila lebanonensis*. Patterns of salivary glands after 6 hours incubation in the presence of moulting hormone. Concentration ecdysterone $10^{-5}$ M (A), $10^{-4}$ M (B). Concentration ecdysone $7.10^{-6}$ M (D, I), $7.10^{-5}$ M (C, H). Combination of ecdysterone ($10^{-5}$ M) and ecdysone ($7.10^{-6}$ M) (E). Control, pattern at puparium formation (F).

Fig. 4b. Autoradiograph of labelled proteins after SDS electrophoresis of *Drosophila melanogaster*. Patterns of salivary glands after 6 hours incubation in the presence of moulting hormone. Control (A). Concentration ecdysone $7.10^{-6}$ M (B). Concentration ecdysterone $10^{-5}$ M (C). Combination of ecdysone and ecdysterone ($7.10^{-6}$ M and $10^{-5}$ M) (D). Control (E, F).

Fig. 5. Autoradiograph of labelled proteins after SDS electrophoresis of *Drosophila lebanonensis*. Patterns of salivary glands after 6 hours incubation in the presence of ecdysterone, with brains added (A, B), without brains added (C, D). Control incubation without ecdysterone (F, G). Control, pattern at puparium formation (E). Patterns of brains after 6 hours incubation in the presence of ecdysterone (H, I).
Fig. 6. Autoradiograph of labelled proteins after SDS electrophoresis of *Drosophila lebanonensis*.

Patterns of salivary glands after 6 hours incubation in the presence of ecdysterone. Preincubation with brains in the presence of ecdysterone (A, B, C). Preincubation with fat body in the presence of ecdysterone (D, E). Control patterns of salivary glands after incubation for 6 hours after preincubation with brains (C) or fat body (D) without ecdysterone.
SAMENVATTING

Tijdens het popstadium vindt in holometábole insecten de metamorphose plaats. Het begin van het popstadium wordt gekarakteriseerd door een contractie van de wandmusculatuur en een verharding van de cuticula. Dit morphologische proces wordt aangeduid met de term puparium vorming. Gedurende de puparium vorming wordt het insect hergeprogrammeerd van een groei-fase naar een differentiatie-fase. De larvale weefsels moeten worden afgebroken en de volwassen structuren moeten worden opgebouwd uit elementen (o.a. imaginaal schijven) die reeds vanaf de vroege embryogenese als gedefinieerde maar ongedifferentieerde cellen aanwezig zijn. Deze herprogrammering van de functie van alle in de larve aanwezige cel typen wordt geïnduceerd door het vervellingshormoon ecdysteron. De herprogrammering van de functie van cel typen wordt gereguleerd door een verandering in de weefsel specifieke genactiviteit. Deze verandering in genactiviteit kan visueel gevolgd worden door middel van de verandering in het puff patroon van polytene chromosomen. Alhoewel veel larvale weefsels polyteen zijn, wordt de hoogste polyteniegrend bereikt in de speekselklieren. Om deze reden zijn bijna alle studies naar veranderingen in genactiviteit met behulp van het puff patroon in dit weefsel uitgevoerd (Hoofdstuk I). Voorsands werd een onderzoek uitgevoerd naar de morphologie van de polytene speekselklier chromosomen en werd vastgesteld welke de hormoon specifieke veranderingen in het puff patroon van dit specifieke weefsel waren (Hoofdstuk III). Vervolgens werd de functie verandering van de speekselklieren tijdens de puparium vorming gevolgd door bestudering van de verandering van morphologie op licht en electronen microscopisch niveau (Hoofdstuk V). Deze functie verandering in de speekselklieren kan als volgt omschreven worden. Gedurende het einde van het
derde larvale stadium wordt in de speekselklieren een "plak"-substantie geproduceerd die tijdens de puparium vorming de prepop bevestigt aan een ondergrond. Vlak voor de puparium vorming wordt deze synthese stop gezet en enige uren later, vlak na de puparium vorming, kan een begin van histolyse van de speekselkliercellen worden vastgesteld. De veronderstelling is gewettigd dat tussen de verandering van de genoom activiteit en de verandering van de functie, veranderingen zullen optreden in het patroon van de synthese van weefsel specifieke eiwitten. Deze veranderingen in eiwit synthese werden onderzocht met behulp van labelling van de novo gesynthetiseerde eiwitten met $^{35}$S-methionine, waarna de eiwitten electroforetisch gescheiden werden op polyacrylamide gels in de aanwezigheid van SDS. Detectie kan geschieden door middel van autoradiografie van de gedroogde gels. De meest opvallende verandering in het eiwitsynthese patroon is het verschijnen van twee nieuwe peptiden met een laag moleculair gewicht en het verdwijnen van een peptide met een moleculair gewicht van 65.000 d (Hoofdstuk VI).

De puparium vorming van Drosophila lebanonensis is een ritmisch verschijnsel in zoverre dat een niet gesynchroniseerde populatie larven de verpopping begint gedurende een gedefinieerde 6 uur lange "gate" periode in iedere kunstmatige licht/donker cyclus van 24 uur. Dat dit ritme in puparium vorming een circadiaans ritme is, werd bepaald door de puparium vorming te volgen onder verscheidene licht/donker regimes, zoals licht/donker cycli met een constante periode van 24 uur maar een wisselende ratio licht/donker, constant donker en constant donker onderbroken door lichtperiodes van een uur tijdens verschillende fasen van de circadiaanse oscillatie. Het ritme in puparium vorming blijkt te voldoen aan de eisen die inherent zijn aan een circadiaanse oscillatie zoals endogeniteit en
gedefinieerd resetting gedrag van de fase van de oscillatie door licht-interruptions (Hoofdstuk I).

Verdere experimenten werden uitgevoerd met een gesynchroniseerde populatie larven die na 7 dagen uiteenvalt in twee verpoppingsgroepen. Groep I begint verpopping tijdens "gate" periode $G_1$, 7 dagen na ovipositie en Groep II begint de verpopping tijdens "gate" periode $G_2$, 8 dagen na ovipositie. Een manier waarop de circadiaanse oscillatie het tijdstip van de puparium vorming zou kunnen bepalen is de regulatie van de afgifte van het vervellingshormoon door de prothoracale klieren via het hersenhormoon. Om deze hypothese te testen werden Groep II larven geinjiceerd met ecdysteron, vlak voor (tijdstip A) of aan het einde van (tijdstip B) "gate" periode $G_1$, ongeveer 24 uur voordat normaal de puparium vorming in deze larven plaats vindt. Injecties uitgevoerd op tijdstip A resulteren in de puparium vorming binnen 6 uur, gekarakteriseerd door spiercontractie en verharding van de cuticula. Injectie op tijdstip B wordt niet gevolgd, binnen 6 uur, met puparium vorming (Hoofdstuk III). Dit impliceert dat wanneer ecdysteron wordt toegediend op een tijdstip zodanig dat het resultaat een verpopping buiten de "gate" periode zou betekenen, de circadiaanse oscillatie de werking van het hormoon verhindert. Hoe wij dit fysiologisch moeten voorstellen was het doel van dit onderzoek.

Een eerste verklaring voor bovengenoemd verschijnsel kan de endogene regulatie van de ecdysteron concentratie zijn. Zo kan de veronderstelling gemaakt worden dat op tijdstip A de endogene ecdysteron concentratie stijgt maar nog niet die concentratie bereikt die nodig is om de puparium vorming te induceren, terwijl op tijdstip B deze endogene concentratie weer is gedaald. Ook kan er een degradatie mechanisme bestaan.
dat het geïnjecteerde ecdysteron op tijdstip B sneller laat verdwijnen dan op tijdstip A. Gedurende de G periode (inclusief beide tijdstippen A en B) kon geen meetbare hoeveelheid ecdysteron worden aangetoond en ook de snelheid van degradatie op beide tijdstippen was gelijk (Hoofdstuk IV). Een alternatieve verklaring is dat er een interferentie systeem (I.S.) bestaat dat, gekoppeld aan de circadiaanse oscillatie, de werking van het hormoon ecdysteron kan beïnvloeden. Waar dit I.S. aangrijpt binnen de reeks gebeurtenissen gepaard gaand met een functie verandering (transcriptie, translatie dan wel post-translatie niveau), kan aan de hand van de veranderingen in puff patroon en eiwit synthese patroon vastgesteld worden. Met behulp van in vitro incubatie van speekselklieren werd een poging gedaan de oorsprong van het I.S. vast te stellen.

Injecties uitgevoerd op tijdstip A blijken zowel de puparium vorming specifieke verandering in de genoom aktiviteit (Hoofdstuk III) als van het eiwit synthese patroon te veroorzaken (Hoofdstuk VI). Injecties uitgevoerd op tijdstip B blijken wel de puparium vorming specifieke verandering in genoomaktiviteit te induceren (Hoofdstuk III) maar niet de overeenkomstige verandering in het eiwit synthese patroon (Hoofdstuk VI). De volgende conclusie kan hieruit getrokken worden. Ecdysteron is in staat in alle gevallen de puparium specifieke verandering in genoom aktiviteit te induceren, maar het I.S. bepaalt op het post-transcriptie niveau of de door het genoom opgelegde functie verandering inderdaad voltrokken wordt.

De vraag waar het I.S. zijn oorsprong vindt, heeft behalve een fysio-logische tevens een theoretische betekenis in verband met de locatie van de circadiaanse oscillator (Hoofdstuk II). Alhoewel in enkele insecten de hersenen als locatie wordt aangewezen, is het niet uitgesloten dat iedere individuele cel in het bezit is van een eigen oscillator. Om deze vraag
Curriculum vitae

Eeken, Johannes Cornelis Jozef
Houtstraat 5, Nijmegen.
Geboren 10 maart 1949 te Kerkrade.

Afdeling Genetica
Faculteit der Wiskunde en Natuurwetenschappen
Katholieke Universiteit Nijmegen
Toernooiveld
Nijmegen.

Opleiding:
H.B.S. b, september 1961 t/m juli 1966, St. Antonius Doctor, Kerkrade.
Kandidaats examen biologie 12 mei 1970, Katholieke Universiteit Nijmegen.
Doctoraal examen biologie 5 maart 1974, Katholieke Universiteit Nijmegen.
Hoofdvak: Genetica.
Bijvakken: Zoölogie, Microbiologie (Unilever Research Laboratories, Duiven)
NATO Summer School on Molecular and Developmental Biology, 1 - 14 augustus
1974, Erice Sicilië.

Vanaf 2 maart 1974 t/m 31 augustus 1974 in dienst van de Katholieke
Universiteit Nijmegen.
Vanaf 1 september 1974 t/m 31 augustus 1977 in dienst van de Stichting ZWO.
Vanaf 1 september 1977 t/m 31 december 1977 in dienst van de Katholieke
Universiteit Nijmegen.
Per 1 januari 1978 toegekend een EMBO-beurs ter ondersteuning van een
onderzoeksproject uit te voeren aan het C.N.R.S. Marseille, Frankrijk.
Stellingen

1. De aanname dat ecdysteron het enige actieve vervellingshormoon is, moet met de nodige terughoudendheid gehanteerd worden.

Dit proefschrift.

2. Het is onwaarschijnlijk dat het onderzoek naar de invloed van ecdysteron op Drosophila cellinen kan leiden tot een insicht in de fysiologische werking van dit hormoon.


3. Hoewel er een duidelijke correlatie bestaat tussen het verschijnen van de temperatuur-puffs in Drosophila en het optreden van zowel de zogenaamde "heat-shock" eiwitten als de veranderingen in enzym-activiteit van een aantal enzymen uit het energie—metabolisme, moet de relatie tussen deze fenomenen nog experimenteel bewezen worden.


4. Het vinden van verschillen tussen de receptoren voor een bepaald steroïd hormoon in de verschillende doel—organen van dat hormoon, rechtvaardigt nog niet de aanname dat hierdoor, als primaire actie van het hormoon, verschillende genen geactiveerd worden, die de afzonderlijke reacties van deze organen zouden sturen.


5. Het is niet aan te nemen dat een persistent ritme in cellen van een primaire celcultuur van een bepaald orgaan dezelfde ressizing-karakteristieken vertoont als het ritme in het intacte orgaan zelf.


6. De aanname van Hennig en medewerkers, dat in de lussen van het Y-chromosoom van Drosophila een pakkingsratio van drie maal zou bestaan, is onwaarschijnlijk omdat de dichtheid van de RNA-polymerase op de INP-as van deze lussen te laag is om een dergelijk sterke despiralisatie van de as van de lus mogelijk te maken.


7. Om de structurele organisatie van een bepaald gen op te helderen is het niet voldoende alleen de transcriptie produkten van dat gen te analyseren.

Danaholt B.; Cell 4, 1-9 (1975).


8. De localisatie van een gen op een polyteen chromosoom m.b.v. in situ hybridisatie kan leiden tot foutieve interpretaties indien ze niet gecombineerd wordt met een gedetailleerd cytologisch onderzoek naar het locus of de loci waarop de hybridisatie plaats vindt.

Lambert B.; Chromosoma 52, 193-200 (1975).


9. Omdat ouderparticipatie in kindercrèches als een belangrijke verbetering wordt gezien in de opvoeding van de in deze crèches opgevoede kinderen, zou dit ook als criterium moeten worden gebruikt bij het verlenen van subsidies door de overheid.
References.


VI - 24
Holometabolous insects exist during their lives in two completely different forms each adapted to a specific function and as a result living in two complete different ecosystems. These two forms are the larvae and the adult. The change from one form to the other is performed during the pupal stage and is called the metamorphosis. During metamorphosis most of the larval tissues are histolysed and most of the adult structures are formed from undifferentiated cells whose function however is already determined during embryogenesis. The reprogramming of cells from functional units to histolysis and from an undifferentiated, developmentally quiescent, stage to differentiation, each cell with its own program to form the different adult tissues, is initiated by the moultting hormone ecdysterone. The onset of metamorphosis by ecdysterone is morphologically defined by the contraction of the epidermis and the hardening of the cuticle. This step is called puparium formation. The action of the hormone ecdysterone can be summarised as the specific induction and repression of gene activity followed by a changed information flow through mRNA. This is expressed as a changed protein synthesis which results in a change in cell function, followed or accompanied by appropriate changes in ultrastructure of the cells. The change in gene activity can be measured directly in Diptera, because many larval tissues of Diptera are polytene.

To investigate the action of the hormone ecdysterone we studied the puparium formation in *Drosophila lebanonensis*. The puparium formation in *Drosophila lebanonensis* is a rhythmic process which might give an additional tool compared to similar studies in other Diptera. A start was made by investigating the changes in puffing pattern of polytene chromosomes of the
salivary glands during normal development (Chapter III), followed by the changes of the ultrastructure of the salivary gland cells which occur during puparium formation (Chapter V). As a third effect of the hormone on the salivary glands were the changes in protein synthesis recorded during the same period (Chapter VI). The changes in protein synthesis were detected by autoradiography after electrophoretic separation of $^{35}S$ methionine labelled proteins. The most prominent puparium formation specific change in the protein synthesis pattern is the disappearance of a peptide with a molecular weight of 65,000 d., and the appearance of two low molecular weight fractions around 20,000 d.. The characteristic changes in the ultrastructure involve a disappearing of the secretion vacuoles and the appearance of structural changes which are typical for histolysing tissue.

The puparium formation in *Drosophila lebanonensis* is a rhythmic process. This has to be understood in the following way. If a non synchronised population of larvae develops the puparium formation is restricted to a period of 6 hours every artificial light/dark cycle. That this rhythm is a circadian rhythm is proven by testing the rhythm under various light/dark regimen (Chapter II). The rhythm is endogenous and self-sustaining, and the pattern of resetting of the phase shows the typical pattern of all circadian rhythms. Further experiments were conducted with a synchronised population of larvae which is characterised by the presence of two pupating groups. The first group, group I, pupating the seventh day after oviposition during "gate" period $G^1$ and the second group, group II, pupating 24 hours later during "gate" period $G^2$. The control of the timing of puparium formation by the circadian oscillation can be the regulation of the release of the moulting hormone by the
prothoracic glands. To investigate this hypothesis, larvae of the second pupating group were injected just before and after "gate" period G¹ approximately 24 hours before they normally start metamorphosis. Injection just before G¹ results in puparium formation 6 hours later. Injected animals just after G¹ do not start puparium formation within 6 hours but they form a puparium 18 hours later during G², the "gate" period in which they normally would pupate. Examination of the puffing pattern after both injections showed that the puparium formation specific puffs always respond. In the second injection however not followed by puparium formation (Chapter III). To exclude that the endogenous ecdysterone concentration interferes with the injection experiments, this concentration was measured during the penultimate "gate" period G¹ and during puparium formation at G². No detectable amounts of steroids are present during both the injections. The degradation of injected ³H ecdysterone before and after "gate" period G¹ is equally fast. The results so far indicate that the timing of the puparium formation cannot be controlled only by the regulation of the release of the moulting hormone. In the regulation of the timing of puparium formation in Drosophila lebanonensis another system seems to be involved which interferes with the action of the hormone at some step after transcription (Chapter IV). Where the Interfering System (I.S.) is involved in the process triggered by the hormone is studied by comparing the changes in protein synthesis after injection before and after G¹ with the changes that occur in the protein synthesis pattern during puparium formation in normal development. Injection of ecdysterone before the penultimate "gate" period G¹ is followed by the puparium formation specific change in the puffing pattern as well as the characteristic change in the protein synthesis pattern. Injection after
this "gate" period however is only followed by the puparium formation specific genome response (Chapter VI). The I.S. interferes with the ecdysterone induced process of reprogramming after the transcription and before or during translation. To investigate whether the I.S. originates in every single cell or in some central tissue in the insect, salivary glands were isolated just before or after "gate" period G¹ and cultured in the presence of ecdysterone. No changes in the protein synthesis pattern in vitro could be induced comparable to those naturally occurring during puparium formation, although in all cases the genome responds with the puparium formation specific change in puffing pattern (Chapter VI). The I.S. originates from a central tissue in the insect and is indispensable to continue the process of reprogramming initiated by the hormone ecdysterone.