

Secretion and Metabolism of Ecdysteroids by Oenocyte-Fat Body Complexes (OEFC) in Adult Males of *Gryllus bimaculatus* DEG (Insecta)

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Extracts of whole adult males of *Gryllus bimaculatus* show distinct maxima of free ecdysteroids on the 8th, 12th and 14th day after imaginal moult at a breeding temperature of 25 °C. Isolated OEFC of 11 days old males secrete *in vitro* free as well as conjugated ecdysteroids, although the latter predominate. The bulk of free ecdysteroids seems to consist of E and 20E according to their retention times in two HPLC systems. Apart from the polar ones, apolar metabolites appear during incubation. By esterase the apolar metabolites are mostly split into polar ones, partly into 20E and E. Further treatment of the polar metabolites with helicase leads again to E and 20E. OEFC transform [³H]E preferably into apolar and polar conjugates, with dominance of the apolar ones at the beginning and of polar ones in the further course of incubation. Only a small quantity of free 20E is found during this experiment, but it can be produced in significant quantities by splitting the polar conjugates. No conversion of [³H]cholesterol into [³H]ecdysteroids could be detected.

Introduction

In most insects, PTG degenerate after metamorphosis, so that the original source for the formation of moulting hormones is exhausted. Nevertheless, female insects generally produce a high hormone titer, most probably in connection with reproduction. The follicle cells in their ovaries represent safe sources for the ecdysone-biosynthesis (Lagueux *et al.*, 1976; Weidner *et al.*, 1992). Except for *Gryllus bimaculatus*, no production of comparable ecdysteroid titers in male adults has been found. Nevertheless the presence of ecdysteroids in males seems necessary, because they trigger spermatogenesis (Dumser, 1979; Friedländer and Reynolds, 1988; Loeb *et al.*, 1988). Thus, in most insects with an extensive imaginal period the production of spermatozoa occurs over longer periods of adult life.

The extremely high titers of ecdysteroids in adult male *Gryllus* appear at constant times during the imaginal period (Shahab and Romer, 1982; Behrens and Hoffmann, 1982). The intermediate periods between these maxima are characterized by rather low titers of free ecdysteroids. Concerning male crickets the tergites appear to be possible sources of the moulting hormones (Bressel *et al.*, 1990; Weidner *et al.*, 1992). Keeping in mind the larvae of *Tenebrio molitor*, for which the synthesis of ecdysteroids in the oenocytes had been asserted earlier (Romer *et al.*, 1974), it should be tested, whether this type of tissue could be likewise the source of hormone synthesis in *Gryllus*. Unfortunately, the anatomic conditions of *Gryllus* are less convenient: the oenocytes are not connected to the epidermic cells or the tracheae, but are placed in the fat body above the sternites in the neural sinus. Within the frame of this study the secretion activities of the OEFC of adult males of *Gryllus bimaculatus* shall be described. The secretion capacity at a typical minimum (day 10) is compared with that of a growing maximum (day 11). The hormones secreted shall be quantified by radioimmunoassay and qualified by HPLC analysis. Another experiment shall help to analyze the metabolization of ecdysone *in vitro* with isolated OEFC.

Abbreviations: E, ecdysone; 20E, 20-OH-ecdysone; 3d20E, 3-dehydro-20-OH-ecdysone; HPLC, high performance liquid chromatography; TLC, thin layer chromatography; PTG, prothoracic glands; 50% MeOH, 50% methanol in water (representative for other concentrations).

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Materials and Methods

Gryllus bimaculatus were reared under mass-breeding conditions at room temperature with a light/darkness rhythm of 14:10 h. For the individual experiments the insects were isolated after imaginal moult in separate containers at 25 °C. They were fed with porridge oats, dried milk and fresh salad leaves.

In vitro incubation of tissues

For most of the experiments 11 days old insects were used. The insects were narcotized with CO₂. Their surface was then sterilized with ethanol and the OEFC dissected. After washing in Ringer solution and approximate 15 min in Grace's medium the OEFC were incubated in 900 µl Grace's medium with the addition of 0.1 mg/ml penicilline G and 0.5 mg/ml streptomycine-sulphate. After 1, 2, 4 and 8 h, respectively, 300 µl of the incubation medium were redrawn and fixed with 500 µl 100% MeOH. The incubate was each time replenished with the same quantity of fresh medium. The incubation was stopped after 23 h. Organs and incubation media had been separated.

Processing the samples

The incubation media removed and the organs were centrifuged at 10,000×g. The sediments were washed again with 60% MeOH, the surmounts collected, evaporated and taken up with 500 µl 60% MeOH. After filling up with *aqua dest.* to 5 ml they were applied onto a Sep-Pak C₁₈ cartridge (Lafont *et al.*, 1982). The ecdysteroids, including polar products, held back by the cartridge were first eluted with 60% MeOH. The eluate with 100% MeOH was collected as well and stored until further use.

The extraction of ecdysteroids from whole insects was performed by the method of Karlson and Shaaya (1964). Lipids were removed by separation between dichloromethane, methanol and water (pers. communication by Prof. Spindler). E and 20E were separated by TLC. Standards were established with both ecdysteroids (E and 20E) and the extracts of whole insects were tested with the respective standard curve.

Radioimmunoassay (RIA)

After separation with the C₁₈ cartridges the ecdysteroid contents from the incubations were defined by RIA following the protocol of Spindler *et al.* (1978). The antiserum was induced in a rabbit against 20E-2-hemisuccinate coupled with thyroglobulin (Soumoff *et al.*, 1981). The cross reaction between E and 20E rated 1:2.2. The [³H]ecdysone used was provided by Dupont/U.S.A. (specific activity: 45.0 Ci/mmol). The quantity of ecdysone bound by the antibody was measured with a liquid scintillation spectrometer. As a rule ecdysteroid equivalents are based on 20E as standard (exceptions see Figs. 1 and 3).

Secretion profiles

According to Hirn *et al.* (1979) the ecdysteroids secreted into the medium were added up to total curves based on the values taken from the samples after 1, 2, 4, 8 and 23 h, respectively. As it does not take into account metabolization and/or decomposition, this method does not allow an absolutely exact statement about the quantity of ecdysteroids secreted into the medium, but it helps defining the minimum amount.

HPLC

To cover the spectrum of metabolites a part of the extracts purified beforehand was fractionated by HPLC. An isocratic system was applied for detection. Chromatographic conditions: wavelength 242 nm, elution rate 1 ml/min, maximum pressure 1000 psi, elution medium 50% MeOH, room temperature, separation column either Lichrosorb RP 18 (10 µm) or alternatively Lichrosorb RP 2 (10 µm) by Merck. Apolar components were washed from the column by a purge with 100% MeOH. The fractions gathered in microvials with the help of a fully automatic fraction collector (LKB Multirac 2111) were stored at -28 °C until further processing.

Digestion by enzymes

Polar conjugates were split by a mixture of β-glucuronidase and arylsulfatase from *Helix pomatia* (Merck) and apolar conjugates by esterase EC 3.1.1.1 (Sigma). Both enzymes did not contain

considerable amounts of ecdysteroids binding to the antibody.

Results

Ecdysteroid titers during the imaginal period

The titer course established from whole insects (Fig. 1) presents a remainder of approx. 200 ng ecdysteroid equivalents (E + 20E/g wet weight) at the beginning of the imaginal period. A first maximum on the 4th day is followed by other ones after 8, 12 and 14 days. The maximum of 1600 ecd. equiv. after 8 days is followed by 1000 ng/g on the 12th and 14th day, respectively. Separations with thin layer chromatography assert the quantitative dominance of 20E over E. Due to the fact that on the 11th day the maximum of the 12th day starts building up, this day is most suitable for establishing of secretion profiles. At that point of time the tissues offer the best prospects with regard to the graphs.

Secretion profiles

The secretory capacity is registered in form of total profiles (Fig. 2) assembled from the values taken from $\frac{1}{3}$ of the incubates after 1, 2, 4 and 8 h and those from the remnants of the incubation fluid after 23 h. Consequently all ecdysteroids secreted into the medium were measured. The

total values range between 8.3 ng and 16.6 ng per incubate, averaging 12.5 ng. After incubation, the organs themselves contain between 2.6 and 10.0 ng, averaging 4.5 ng. The so-called average diagram contains the mean secretion values of ecdysteroids. Accepting the quantity of ecdysteroid equivalents secreted into the medium within only 1 h (3×2.17 ng) as a basis, the total amount of 12.5 ng after 23 h admits the consideration that the ecdysteroids found cannot originate from those accumulated before dissection.

Estimating the capacity with regard to time units, the first hour rates with 2170 pg and the second with 1790 pg/h. The ensuing two hours average 1025 pg, the next four hours still 610 pg and the remaining hours including the 23rd 275 pg per hour. Even though a continuous secretion activity could be observed, it declined during the course of incubation. Attention must be paid to the fact that retention in the tissues of the individual samples varies and may reach rather high values (see animal III).

The analysis of the apolar fraction (100% MeOH) in the RIA (lower half of Fig. 2) leads to even higher values than those of the free ecdysteroids (average 17.5 ng). As a rule those insects with a high concentration of free ecdysteroids show also elevated amounts in the apolar fraction. Details of the analysis of the apolar fraction will be discussed later.

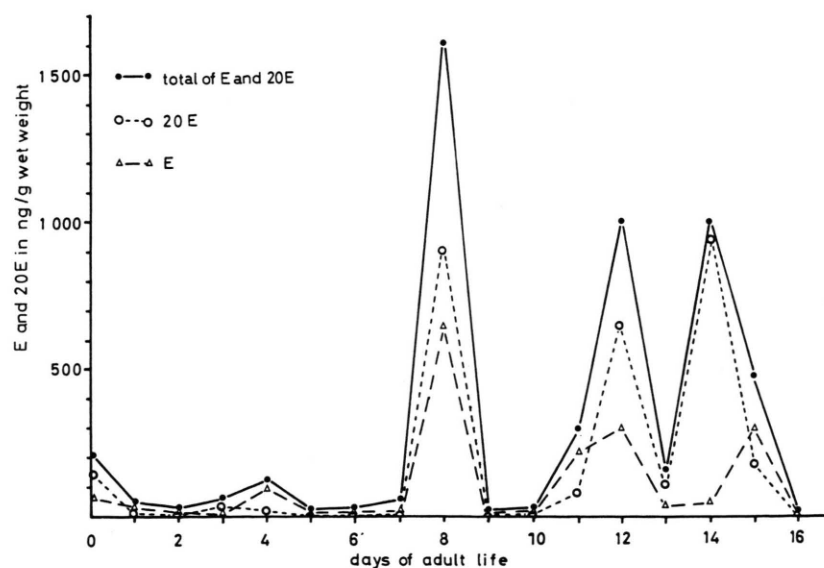


Fig. 1. Ecdysteroid titers of adult males of *Gryllus bimaculatus* (whole animals). The separation was performed by thin layer chromatography; 20E dominates over E.

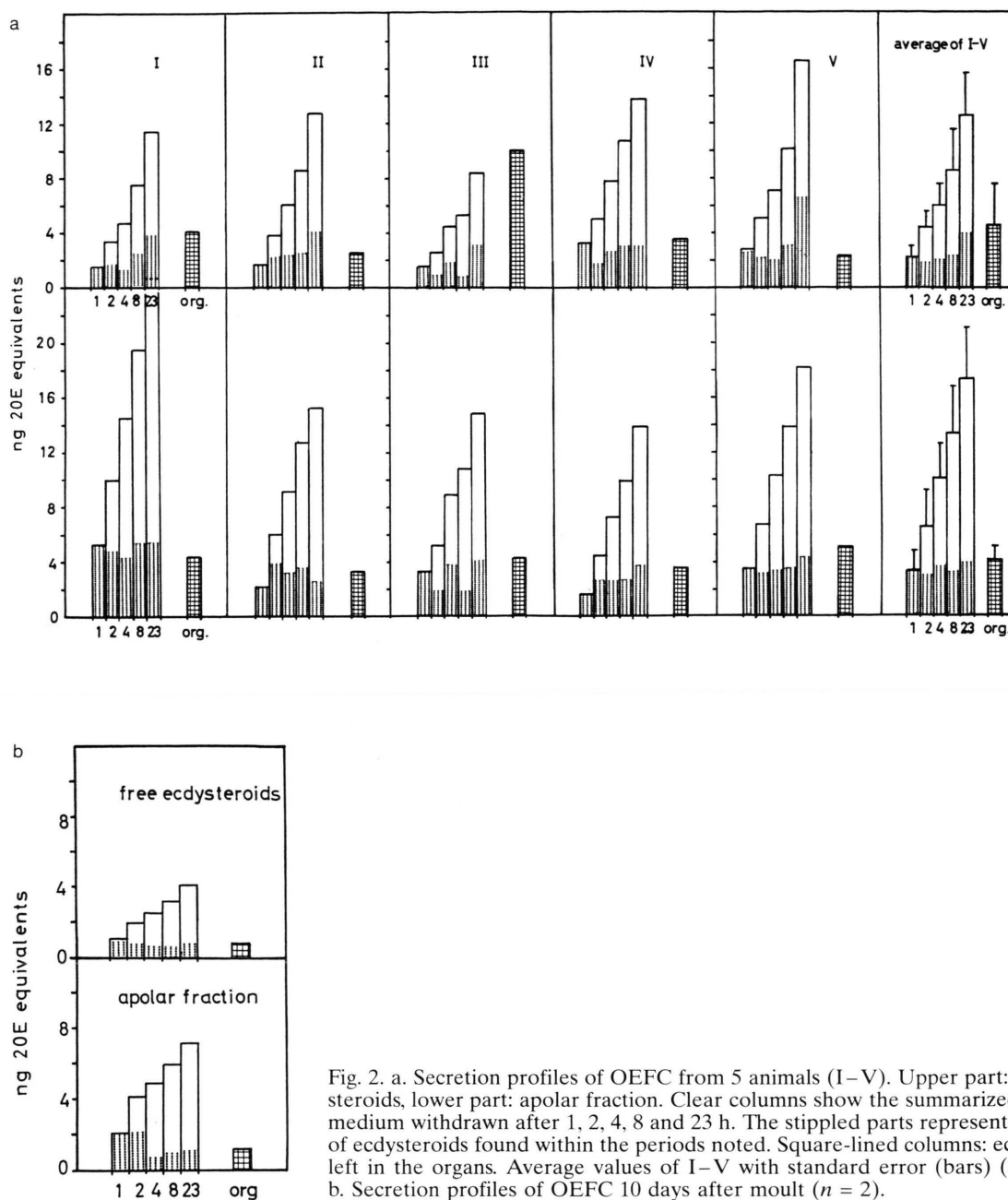


Fig. 2. a. Secretion profiles of OEFC from 5 animals (I-V). Upper part: free ecdysteroids, lower part: apolar fraction. Clear columns show the summarized values of medium withdrawn after 1, 2, 4, 8 and 23 h. The stippled parts represent the values of ecdysteroids found within the periods noted. Square-lined columns: ecdysteroids left in the organs. Average values of I-V with standard error (bars) (right box). b. Secretion profiles of OEFC 10 days after moult ($n = 2$).

Zero values: Before incubation the ecdysteroid contents reached after repeated washing a mean value of 1.14 ng/organ ($n = 7$). Most of the values rated below 1 ng, only one out of seven came far above (3.85 ng). Fig. 2b shows the average values of incubations of 10 days old insects. They come to

4.1 ng for free ecdysteroids and 7.2 ng for apolar ecdysteroids. The residual quantities in the organs come to 0.9 and 1.2 ng/animal, respectively. They are generally much lower than those in the 11 days old males.

HPLC analysis of secretion products after different periods of incubation

After elaboration of the secretion profiles the remnants of the incubates I–V were pooled and fractionated by HPLC in order to assess the different metabolites (Fig. 3). Due to the fact, that the above mentioned remnants were not of similar quantity, it is not possible to simply compare the absolute values. E as well as 20E were found in all samples, with E lightly dominating. 2 h after the beginning of the incubation components with varying elutive behaviour could still be observed besides E and 20E. From the 4th hour onward, they had disappeared. After 20 min of fractionation a rather large quantity of apolar components could be washed off the column with a purge of 100% MeOH. But after 23 h of incubation only 7 ng could be measured. Polar products coupling to the antibody appeared also during the course of the incubation. Their quantity reaches a maximum in the 8 h old incubates. In that case the fraction 5 (2–2.5 min) almost equals the amount of 20E. With regard to elution profiles, the same results were obtained in parallel analyses with a Lichro-

sorb RP 2 column, which is less lipophilous due to shorter C-chains.

Metabolism

The OEFC of 11 days old insects were incubated in 1.2 ml Grace's medium together with 1.8 μCi [^3H]E. In order to determine the respective degree of metabolization, an aliquot (100 μl) was drawn and fixed in accordance with the same time scheme as was used in establishing the secretion profiles (Fig. 4). Comparable sample portions were given to HPLC. 1 h after the beginning of the incubation 75% of the ecdysone had not changed at all. Significant quantities (11.9%) could only be asserted for apolar metabolites. Lightly increased values could be found between the fractions 5–8. In the course of the incubation this overall pattern showed little variations, except for the fact that the peaks in the apolar fraction continuously decreased. Total activity came to 5.1% during 23 h. 4 h after start of incubation fraction 5 represented 3.5% of the activity. After 23 h this value had increased up to approx. 25.4%. Correspondingly, the portion of unchanged ecdysone had been reduced to 50.8%. Only 3.2% could be identified as 20E. This led to the assumption, that after the synthesis of ecdysone was finished, hydroxylation at C-20 is only possible to a small extent (in contrast to the larvae).

Splitting the apolar fraction of incubates from the OEFC with the help of esterase (Fig. 5a)

Concerning the total of 44.5 ng ecdysteroid equivalents the resulting products represent mostly 3 components: a very polar fraction of approx. 20 ng, 7.4 ng of 20E and 5.4 ng of E. About 2 ng of apolar components remained. With regard to the small portion of 20E found after the incubation of [^3H]E, the value of 7.4 ng seems to be rather high. The initial apolar fraction probably consisted of conjugates of ecdysteroids with fatty acids (Whiting *et al.*, 1993; Hoffmann *et al.*, 1985).

Splitting the polar fraction with glucuronidase-sulphatase

The treatment of the polar conjugates (Fig. 5b) resulted in a surprisingly high share of 20E

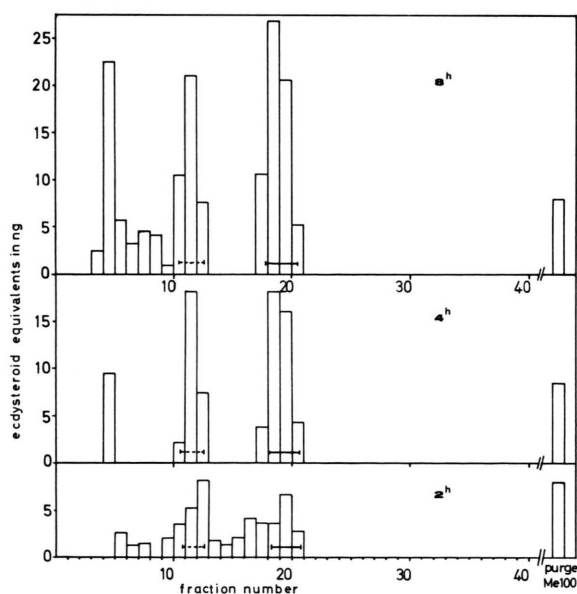


Fig. 3. HPLC analysis of media redrawn after 2, 4, and 8 h. Main components are E (—) and 20E (---); fractions 19–21 are referred to E as standard, the rest to 20E. A part of apolar components appears after purge with 100% MeOH. In the 8 h fraction the polar components are already remarkable.

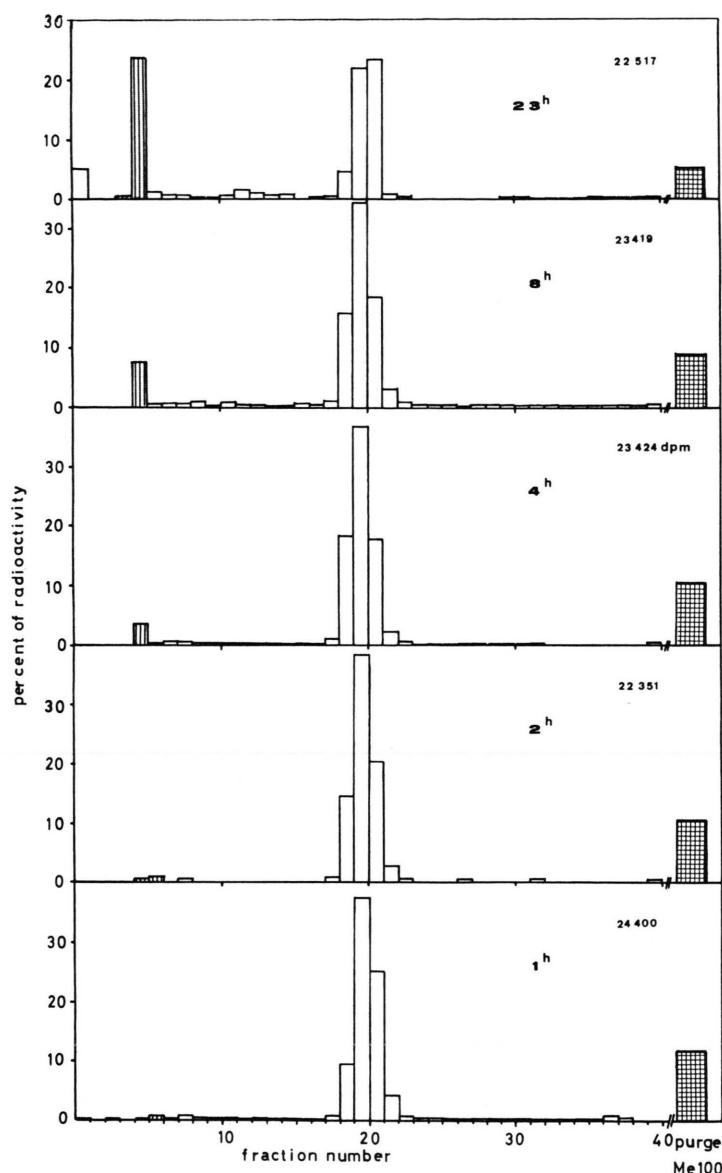


Fig. 4. Metabolism of $[^3\text{H}]\text{E}$ in the course of 23 h. Aliquots were redrawn at the times given and separated by HPLC. 1 h after start of the incubation the apolar fraction is rather high, it is diminished at the cost of the polar fraction (5). The part of 20E remains rather low.

(37.8%). Furthermore a peak at E (22.7%) was found as well as a rest of 23.3% in the fractions 5, 6 and 7, which had not been attacked by the mixture of enzymes.

An application with 100% MeOH was necessary to wash the apolar compound(s) from the column. It seems that it represents a double conjugate, which was at first reduced by esterase to a polar one and furthermore by helicase to the initial steroids 20E and E.

Experiments on retention

Three crickets each 11 days old were injected with $0.5 \mu\text{Ci } [^3\text{H}]\text{E}$. 2 h later the OEFC were dissected, washed and incubated in 600 μl medium. After 1, 2, 4, 8 and 23 h respectively the medium was completely exchanged and its radioactivity measured. At the end of the incubation the residual activity of each of the organs was also evaluated. The total activity of each organ was taken as 100% and the activity passed into the medium as

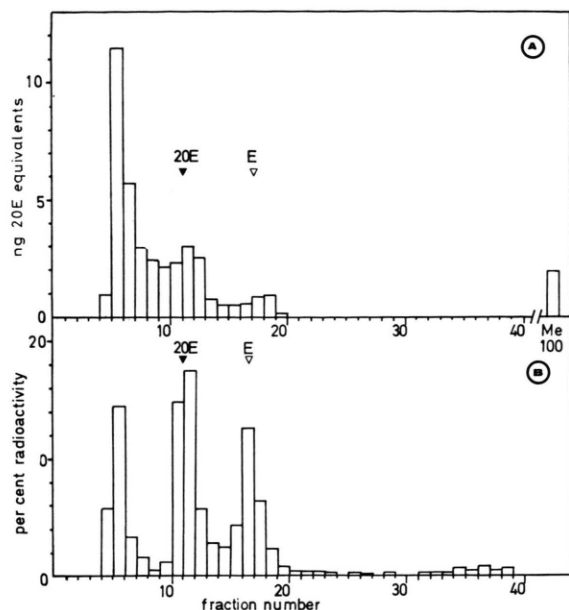


Fig. 5. a. Digestion of apolar components eluted with 100% MeOH 1 h after start of incubation. The results are polar components (fraction 5–7), 20E (dark arrow) and to a lower extent E (clear arrow). b. Digestion of the polar peak with helicase after incubation of OEFC with [^3H]E for 23 h. There is mainly 20E beside unchanged material and E. Free 20E occurs only in small amounts therefore it is obviously metabolized toward polar components.

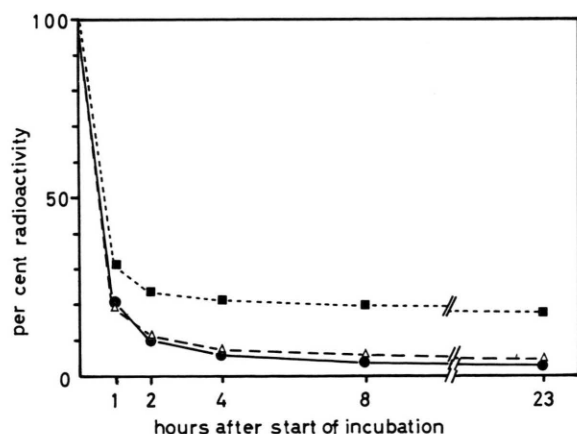


Fig. 6. Elution profiles of OEFC preincubated with [^3H]E. Medium was totally changed in the pattern given at the abscissa and replaced by fresh one. Only in the tissue of one animal (dark squares) remained about 18% of the radioactivity, in the two others there were less than 5%.

well as the residual activity were set against the time (Fig. 6). Most of the activity had been secreted during the first hour (mean 76.5%). After 23 h the tissue samples of two out of the three insects contained less than 5% of the original radioactivity, those of the third one 17.5%. In the tissue complex isolated, the tracer has a half-time period of 40 min. The observation, that within a short period of time large quantities of the hormone are found in the medium supports the idea, that ecdysteroids are not stored in the OEFC. Only small quantities of the hormones remain in the tissues.

Discussion

The prothoracic glands play an important role in the realization of the hormone titer. In most insects examined the glands degenerate after the imaginal moult. Only in *Periplaneta americana* the persistence of the gland until the 15th day and its capacity to secrete ecdysteroids at least until the 10th day had been asserted (Richter, 1984). Up to now such results could not be established for *Gryllus bimaculatus*. Our own preliminary research seems to indicate the degeneration of the gland at the end of the last larval instar.

It is to point out that the results of whole animals and those of single organ incubations were performed by different methods (extraction with butanol/water *versus* separation by Sep-Pak cartridges). Thus, it is not to exclude that the older method yielded higher titers than the Sep-Pak separation. Therefore, both results should be compared with care.

The question whether the secretion products observed are really E and 20E can be answered in so far as they are identical to them with high probability, because both show the same behaviour in two different HPLC systems like the original compounds. In addition, extracts of whole animals that had been precleaned by TLC showed exactly the same retention times in the following gas liquid chromatography as authentic E and 20E did.

The hormone titers determined in male adult insects rarely surpass 10 ng/g wet weight (*Blaptica* (Romer and Shahab, 1984), *Tenebrio* (Briers and DeLoof, 1981), *Bombyx* (Shaaya and Karlson, 1965), *Drosophila* (Handler, 1982), *Calliphora* (Koolman *et al.*, 1979), *Sarcophaga* (Briers and

DeLoof, 1980). In contrast, values of 1000–1600 ng/g wet weight were found on the 8th, 12th and 14th day in *Gryllus bimaculatus*. The fact that the minima between these maxima do not come above 50 ng/g and then increase within 24–36 h to reach these maxima is of greatest interest, because it implies the secretion of 30–42 ng/g of free ecdysteroids per hour. Experiments on elimination asserted the continuous excretion through the faeces up to the hormone maximum (Eibes and Romer, 1990). A newly published study (Weidner *et al.*, 1992) fails to explain this dramatic increase of ecdysteroids. In the best of all cases presented within this study values of 3 ng/animal×h for the epidermis and the adjacent fat body were achieved. Possibly the epidermal cells work much more efficiently at the beginning of the incubation and slow down in the course of it (like OEFC). A long-term incubation over 16 h would in that case result in a distorted image of the secretion capacity of epidermal cells *in vivo*.

The secretion profiles of the OEFC show a continuous release of ecdysteroids into the medium with declining values in the course of the incubation. Several reasons could be discussed:

1) The Grace's medium used as incubation medium is possibly less suitable for *Gryllus* and leads to a continuous reduction of the secretion capacity.

2) The effect of the ecdysiotropic factors decrease, *i.e.* the amount of PTTH-like compounds available is not sufficient to stimulate a possible synthesis or secretion.

3) The precursors for the formation of ecdysteroids, whatever their nature may be, are either too few or become extracted from the system *via* the exchange of the medium.

Before the start of the incubation the ecdysteroid content of the OEFC reaches 1.1 ng/animal after twofold washing. Taking into account the quantities secreted into the medium (12.5 ng) and those residual in the organ (4.5 ng), a new formation of 15.9 ng per insect can be asserted.

The other recent observation concerns the massive secretion of apolar products by the OEFC of *Gryllus*. It surmounts the secretion of free ecdysteroids by almost 50%. This accounts for some part of each hormone peak, though not for the total amount. Apart from free ecdysteroids the epidermal cells of *Gryllus* contained polar and apolar products, which together sometimes sur-

mounted the quantities of free ecdysteroids (Weidner *et al.*, 1992).

Metabolism of [^3H]ecdysone

When adding tritiated ecdysone to freshly prepared OEFC, a metabolite appears after 1 h, which has to be purged off the column with 100% MeOH. This apolar component covers about 12% of the activity. During the further course of incubation the balance shifts insofar, as the quantity of this metabolite decreases and a more polar one appears after approx. 4 h. In 23 h old incubates this polar metabolite accounts for approx. 25% of the input radioactivity. This increase is realized not only at the expense of the apolar peak, but also at that of E. At retention times, where 20E normally eluates, only a few percent of radioactivity can be detected after 8 and 23 h, whereas fractioning of products from the secretion profiles results in a 20E to E ratio of 40:60. There are two possible explanations:

1) Either hydroxylation does not follow the order established so far: C₂₅, C₂₂, C₂ (Meister *et al.*, 1985). Some of the ecdysteroids ought to be hydroxylated at C₂₀ before the secretion of E. Precondition would be the presence of 3d20E as a precursor (Lafont and Connat, 1989), as is the case with *Drosophila*. Or more probably:

2) Conjugation occurs in the incubate immediately after hydroxylation at C₂₀. Polar and then apolar conjugates emerge. This hypothesis would explain the large amount of 20E appearing after the enzymatic splitting of the polar conjugates (Fig. 5b). The subsequent application of esterase and helicase on apolar conjugates (not represented here) leads to products similar to those obtained during the analysis of polar conjugates with the help of helicase. A similar formation of double conjugates with fatty acids and *via* glycosylation or sulphatation can be observed during the embryonic development of *Acheta domesticus* (Whiting *et al.*, 1993).

The OEFC are certainly not capable of synthesizing ecdysteroids from cholesterol. Rather massive input of [^3H]cholesterol did not lead to any transformation into E or 20E, the products of which would have presented a typical acetylation pattern of the two ecdysteroids. The OEFC must be classified as a secondary source of ecdysteroids

(Delbecque *et al.*, 1990). Up to now the nature of the precursors for the ecdysteroids *in vivo* could not be described. The idea of a partial recycling of the free ecdysteroids found during the periods of maximum hormone titers cannot be lightly rejected, because after enzymatic digestion E and 20E always appear.

Except for 20E the secretion products of the OEFC and the spectrum of metabolites correspond well with the products of exogenously added ecdysone. The enzymatic digestion of both polar and apolar secretion products also shows principally the same pattern of metabolites as can

be observed after 4 or 8 h of incubation. E and 20E can be found as well as a polar compound (retention time 2.5–3 min), which may be interpreted as a consecutive product of the decomposition of ecdysone, that is apparently finally esterified with long-chained fatty acids.

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