Alterations in Ecdysone Content during the Post-Embryonic Development of *Chironomus thummi*: Correlations with Chromosomal Puffing

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The ecdysone titer of larvae and pupae of *Chironomus thummi* was determined by radioimmune assay (RIA) and revealed a concentration of about 150 ng/g fresh weigth in late third instar larvae and a peak of more than 450 ng/g fresh weight just preceding pupation. The ecdysone titer curve shows a high degree of correlation with previously observed puffing activity at the ecdysone sensitive chromosomal site IIId1. Further, β-ecdysone is the only endogenous ecdysone detected by high pressure liquid chromatography during larval-pupal development. It was also observed that developmentally arrested *Chironomus* contain less ecdysone than "normal" larvae.

The advent of micro-analytical procedures and radioimmunoassays for ecdysones [1–3] has resulted in the determination of relatively precise titers of molting hormone in the hemolymph and carcass of a variety of insects, *e. g.* refs. [4, 5]. As these procedures are generally adapted a plethora of publications on ecdysone titers in a multitude of insects can be expected. However, unless these changes in concentration can be correlated with welldefined physiological or biochemical events they add little to our knowledge of either ecdysone action or insect development. Among the best defined phenomena in insects is the puffing of specific gene loci in polytene chromosomes of the salivary glands of *Drosophila* and *Chironomus* [6]. Certain puffs appear only at defined developmental stages; some puffs are elicited by ecdysone; and puffing is probably the visualization of transcriptive activity [6, 7]. For these reasons the chromosomes of *Chironomus* have been studied intensively and we believed that it would be of more than parochial interest to define the endogenous ecdysone titer of *Chironomus thummi*.

Materials and Methods

Larvae of *Chironomus thummi* were raised and staged as described previously; they were predominantly of type R [8]. Pupae were collected at 3 and 6 hour intervals after pupation. The animals were quick frozen, lyophilized and stored at −18 °C. Developmentally arrested animals were collected from cultures 4–6 weeks after eclosion of the main population. The arrested animals were identified morphologically as being in stages eL_4 to mVP [8].

The lyophilized material was extracted with aqueous methanol (50–75%), the extract taken to dryness and the residue suspended in water. This was then partitioned three times against dichloromethane and three times against watersaturated butanol. The pooled dichloromethane and butanol phases were backwashed with water and the butanol fraction dried under vaccum. The resulting material was subjected to thin layer chromatography (TLC) on silica gel plates (F-254: Merck) with a solvent system of dichloromethane: methanol (70:30, v/v) and α- and β-ecdysone as standards. The α- and β-ecdysone (Rohto Pharm. Co.) zones were scraped and eluted with absolute methanol and the eluate analyzed by high pressure liquid chromatography (HPLC). The HPLC equipment is as described by Deininger and Halász [9] and included a high pressure membrane pump (Orlitta: MS4/4) and a UV detector (254 nm). For separation conditions see legend to Fig. 2 while column packing methodology is as described by Strubert [10].

The ecdysone radioimmune assay (RIA) used in these studies has recently been described in detail [1]. The antibody was generated against a C-22 hemisuccinate derivative of α-ecdysone conjugated to thyroglobulin. The characterized antibody has minimal cross reactivity with a variety of other ecdysoids and steroids, except for β-ecdysone [1].

Results

The total ecdysone content of staged larvae and pupae of *Chironomus thummi* was determined by RIA (Fig. 1). The ecdysone titer of late third instar larvae (sL_3) is about 150 ng/g fresh weight but

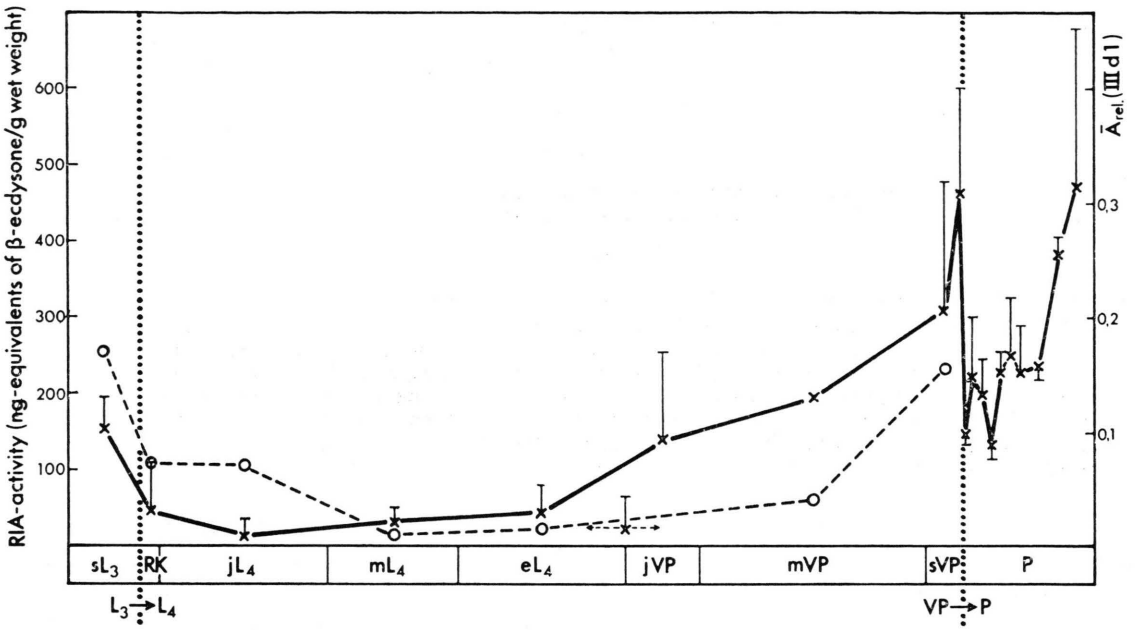

Fig. 1. Ecdysone titers in whole animal extracts and puffing activity at chromosomal site IIId1 during the larval-pupal development of *Chironomus thummi*. Continuous line: ecdysone titer in ng β-ecdysone equivalents/g wet weight with S. E. M. (S. E. M. for mVP=0). Dotted line: puffing activity (A_{rel}) of the puff at IIId1 in the polytene chromosomes of salivary gland cells from the last larval molt to pupation (data from Kroeger [8]: for the sL_3 and L_4 (RK) stages this author gives multiple values; from these we calculated and present the means; from the eL_4 stage onwards we present the values determined for the developmental type R). ←×→: ecdysone content of developmentally arrested animals with approximate spread of their morphological stages. The stage abbreviations follow Kroeger [8]: sL_3=late third larval instar; RK=red head stage; jL_4, mL_4, eL_4=young, middle, late fourth larval instar; jVP, mVP, sVP=young, middle, late prepupa; P=pupa. n for jVP and mVP=2; n for all other stages=3–6. All assays were run in duplicate at two different concentrations.

decreases to one-third that value immediately after ecdysis to the fourth instar and approaches zero in jL_4 larvae. The ecdysone concentration increases only slightly during the remainder of the fourth instar (mL_4, eL_4) but rises further during the prepupal stage. This latter increase is gradual between the early (jVP) and mid-prepupa (mVP) when compared to the sharp rise between the mid- and late prepupa (sVP). A maximum value of 465 ng/g fresh weight was attained just prior to pupation. It is of interest that this is more than double the concentration recently reported for *Drosophila* at a comparable stage [11]. Subsequent to pupation the level decreases to approximately 200 ng/g wet weight in the pupa (P), only to rise again to the previous high during pupal-adult development. Figure 1 also shows the temporal correlation between the *in vivo* ecdysone titer and puffing activity at site IIId1. Developmentally arrested larvae contained very low levels of ecdysone.

The data in Fig. 1 represent *total* ecdysone and are the sum of the amount of α- and β-ecdysone present in the extract as well as possible metabolites that exhibit RIA activity [1]. To identify the ecdysones present during this developmental period seven stages (sL_3, jL_4, mL_4, eL_4, mVP, sVP and P) were analyzed by HPLC. β-ecdysone was observed at stages sL_3, mL_4, mVP and P (Fig. 2) and not at jL_4 and eL_4. In contrast, α-ecdysone was not detected at any of the seven stages analyzed. When [^{3}H] α-ecdysone was subjected to extraction and HPLC analysis, 70% was recovered. Assuming the same recovery from the *Chironomus* samples, the lower detection limit of the techniques used here is below 15 ng/g wet weight. Thus, if present at all, α-ecdysone occurs in only minute quantities, *e. g.* at pupation when about 450 ng β-ecdysone equivalents are noted per g wet weight, there would be at least 30 times more β- than α-ecdysone.

Discussion

The two major insect ecdysteroids present during post-embryonic development are α- and β-ecdysone

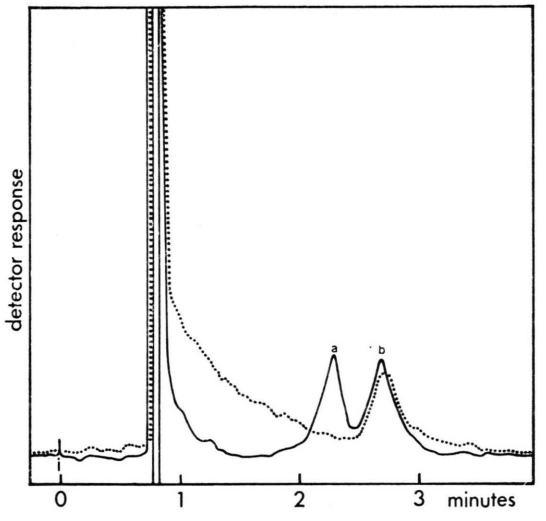

Fig. 2. HPLC separation of α- and β-ecdysone standards (continuous line) and a prepupal extract (dotted line). Column: 10 cm×3 mm, Spherisorb, ⌀ 5 μm (Phase Separation Ltd., Queenferry, Flintshire, UK); mobile phase: dichloromethane/methanol (85:15, v/v); pressure: 50 atm; flow velocity: 1 mm/s; temperature of detector and detector supply: 13 °C; UV detection at 254 nm. Samples were injected in 5 μl of methanol. i: Injection; 1 min.: solvent peak; a: α-ecdysone standard; b: β-ecdysone standard. The peaks of the β-ecdysone standard and prepupal extract were actually coincident but the latter were shifted slightly to the right for easier visualization.

and although it has been suggested that α-ecdysone is a prohormone converted to the actual molting hormone, β-ecdysone, at peripheral sites [1], it is also possible that α-ecdysone has an important physiological role of its own. For example, upon studying the respective effects of α- and β-ecdysone on puffing in *Ch. tentans*, Clever *et al.* [12] concluded that each molecular species has specific effects and that site I-18-C (homologous to site IIId1 in *Ch. thummi*) was sensitive primarily to α-ecdysone. Since it appears that there is virtually no α-ecdysone in *Ch. thummi* during the critical stages L_4 and VP, the above indication of α-ecdysone specificity at site I-18-C is not supported by the present *in vivo* studies. This observed paucity of α-ecdysone in *Ch. thummi* is in accord with studies on another dipteran, *Sarcophaga bullata*, in which it was demonstrated that although α-ecdysone is the secretory product of the ring glands, it is rapidly converted to β-ecdysone by a C-20 hydroxylase [13]. The latter resulted in late last instar *Sarcophaga* larvae containing at least 27 times more β-ecdysone than α-ecdysone.

A tangential but developmentally relevant observation pertains to the arrest of development that can occur late in larval life or during the prepupal stage of chironomids both in nature and in laboratory cultures. They may persist in this state of non-development for months, ultimately to die or to resume development. After observing the so-called ecdysone-specific chromosomal sites of these developmentally arrested *Ch. tentans* larvae, Clever [14] concluded that this chironomid "dormancy" was a result of a lack of ecdysone. The data revealing a lower than normal titer of ecdysone in developmentally arrested *Ch. thummi* support Clever's suggestion, notwithstanding the possibility that this "dormancy" may be brought about by different stimuli in the two species (Ineichen, personal communication). Whether this lower than normal ecdysone titer is a consequence of a decreased rate of synthesis or an increased rate of degradation is conjectural at present.

It is acknowledged that the steroid molting hormone of insects, β-ecdysone, acts at several levels in target cells including the regulation of transcriptive activity [7]. These ecdysone-elicited changes in gene activity can be followed in Diptera by microscopic observation of the puffing activities in polytene chromosomes. Clever, working with *Chironomos tentans* [17] and Ashburner investigating *Drosophila melanogaster* [16] determined certain chromosomal sites to respond rapidly and in a dose dependent manner to experimentally applied ecdysone. The dose dependence suggested that the size of ecdysone specific puffs can be used to monitor the ecdysone titer through the stages of normal development. This possibility could, however, not be adequately tested until methods, such as those employed in the present analysis, became available to quantitatively determine the normal in vivo titers during development. For *Drosophila hydei* the close correlation of the rise and fall of the β-ecdysone titer and the appearance and regression of puff 75 B was demonstrated by Beckers and Emmerich [17]. Our data further substantiate such relationships for *Chironomus thummi* and thus underline the concept that transcriptional activity at chromosomal regions is regulated by the molting hormone.

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