The titers and the clearance of ecdysteroids from the blood of last instar larvae of *Galleria mellonella* L.

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Summary. Mesothoracal ligations of young last instar larvae of *Galleria mellonella* cause the abdominal ecdysteroid blood titer, as determined by radioimmunoassays, to decrease from 48 to 11–17 ng/ml. External application of 13 µg/g, the ecdysterone dose needed to stimulate normal wing growth, raises the titer to 360–1140 ng/ml for 2 days.

The control of growth of imaginal anlagen in insects is not yet fully understood. There is, however, much evidence that ecdysteroids[2] stimulate such growth directly or indirectly[3–9]. Intact imaginal hind wing disks of last instar larvae of *Galleria mellonella* L. stop growing when a ligation is placed between the major endocrine complex of the head and prothorax, and the abdomen[9]. Disk growth, i.e. volume increase by cell proliferation, can to a certain extent be restored by the administration of ecdysone[2] or ecdysterone[2] which must be present continuously to insure continued growth[4,9]. Here we wish to consider the following 2 questions: a) what is the effect of ligation on the ecdysteroid titers in the abdominal blood? Since imaginal disks are presumed to take the hormones up from the blood, hormone titers, and not ecdysteroid contents of whole bodies[10,11], are of interest here. b) to what levels do ecdysteroid titers in ligated abdomens rise upon the administration of exogenous ecdysteroids by injection or topical application?

To answer the first question, we analyzed the blood, drawn with capillaries from ligated, 24–48-h-old last larval abdomens with the help of an ecdysteroid radioimmunoassay (RIA)[12]. The results are given in the table. The values for unligated animals vary between 28 ng and 106 ng ecdysone plus ecdysterone equivalents/ml, depending on the age of the larvae. RIA measurements of the whole body contents of *Galleria* larvae by 2 other research groups yielded values of about 25[10] and 80[11] ng/g fresh weight for 24–48-h-old larvae, and about 25[10] and 400[11] ng/g for 96–120-h-old larvae, respectively.

Ligation causes the concentrations of ecdysteroids detected by RIA in the abdominal blood to become significantly lower compared to unligated controls (see table). Initially the apparent half-time of clearance, established by interpolation of the respective values, is 5–10 h. From 24 to 120 h after ligation, the RIA activity remains between 11 and 17 ng ecdysone plus ecdysterone equivalents/ml. In this context it is useful to recall that ecdysteroid biosynthesis in isolated abdomens of several insect species has been reported[13]. Residual biosynthesis might conceivably be responsible for the low but significant hormone titer present in ligated and isolated abdomens.

The effect of exogenous hormones on the titer of ligated abdomens was investigated by applications of radioactive ecdysteroids and subsequent analyses of the blood. The results are presented in the figure. Part A of the figure shows that ecdysterone is cleared out of the blood with an initial clearance half-time of about 15 min. The type of kinetics in this process has not been elucidated. Clearance might be a complex phenomenon, involving direct transport out of the blood, metabolism in the blood and/or interference with hormone carriers.

Whether the radioactivity found in the thin layer chromatography (TLC) fraction containing ecdysterone represents unmetabolized ecdysterone was tested by converting the compounds of that fraction 1 h after ligation to their trimethylsilyl ethers, and subjecting them to gas chromatography (GC) as described elsewhere[9]. For preparative GC we used a metal splitter 1:5, and reference ecdysteroids were added to the preparation: 90.1% of the recovered radioactivity was associated with the peak of ecdysterone, the rest distributed over the other fractions. Of all known ecdysteroids only the hormonally less active 3-epiecdysterone[14] is thought to behave like ecdysterone in all our chromatographic systems used. In *Manduca sexta,* an enzyme which is responsible for the formation of 3-epiecdys-

Ecdysteroid titers in the abdominal blood of ligated and unligated last instar larvae of *Galleria mellonella,* determined by RIA

		Ecdysone fraction, ecdysone equivalents (ng/ml ± SEM)	Ecdysterone fraction, ecdysterone equivalents (ng/ml ± SEM)	No. of blood samples tested
Larvae	0-h-old	4.4 (−)	84.5 (−)	1
	24 - 48-h-old	8.9 (±2.0)	39.2 (± 7.2)	8
	48.1- 72-h-old	4.5 (±1.3)	23.0 (± 2.7)	5
	72.1- 96-h-old	7.0 (−)	30.0 (−)	2
	96.1-120-h-old	14.0 (±4.8)	91.6 (±22.4)	7
Larval abdomens 24–48-h-old:	ligated for 1 h	7.2 (±1.8)	30.4 (±13.7)	6/4
	24 h	1.3 (−)	9.6 (−)	2
	48 h	2.5 (−)	12.3 (−)	1
	96–120 h	1.1 (±0.3)*	16.3 (± 6.5)*	6

Ligations were made between meso- and metathorax, and the forepart cut off. Each blood sample was prepared from 5–50 larvae (the number of larvae per sample depended on their size and ecdysteroid contents) by drawing off the blood with glass capillaries. The blood was transferred into methanol, bipartitionned with hexane: water 6 times, and the hypophase was fractionated by column chromatography with Sephadex LH-20® and benzene: methanol 85:15. The ecdysteroid fraction was processed by TLC, using the methods of Koolman and Karlson [15]. The zones containing ecdysone or ecdysterone were scraped off separately and dilution series tested for ecdysteroid activity by RIA, using the antiserum H-21[1] and [23,24-^{3}H]-ecdysone[1] with sp. act. 68 Ci/mmole. TLC fractions of blood presumed to contain 3-dehydroecdysteroids or conjugated steroids[15] yielded 4% and 40% respectively of the total RIA positive material, and were disregarded. The titers were estimated by interpolation on standard curves for ecdysone and ecdysterone, and corrected for losses during sample purification (10–20%). The large SEM essentially reflect biological variation and not inaccuracies of the technique, as revealed by parallel processing of divided blood samples. Asterisks indicate titer values that are significantly different from the values of unligated, 24–48-h-old larvae at the 5% or 1% level (Student's t-test).

terone, has been found only in the midgut; in all other organs it appears to be absent[14]. We therefore assume that the majority of the label present in the ecdysterone fraction of TLC (figure, A) is in fact unaltered ecdysterone.

An analysis of the various TLC fractions of blood (only the values for the ecdysterone fraction are depicted in part A of the figure) yielded the following results: the radioactivity in the ecdysone fraction was at all times after ligation in the range of only 1–2% of the activity in the blood, but an apolar fraction corresponding to that of 3-dehydroecdysterone[15] contains up to 70% activity 48 h after ligation. At the same time, only about 7% of the injected label was found in the blood (figure, part A), whereas the feces yielded 38.7% and the carcass (body minus intestine, blood, epidermis and cuticle) 37.5%.

Injecting 11 µg ecdysterone/g yields a rapidly decreasing hormone titer as inferred from the radioactivity measurements (figure, part A): 1 h after injection, it is 6950 ng, after 7 h 537 ng, and after 48 h, 164 ng/ml blood.

The results depicted in part B of the figure have been obtained by injecting ^{3}H-ecdysone. They are similar to those of A, with the following exceptions: a) the initial clearance time appears to be slower (30 min), and b) there is an apparent conversion of ecdysone to ecdysterone as judged from the radioactivity in the respective TLC fraction.

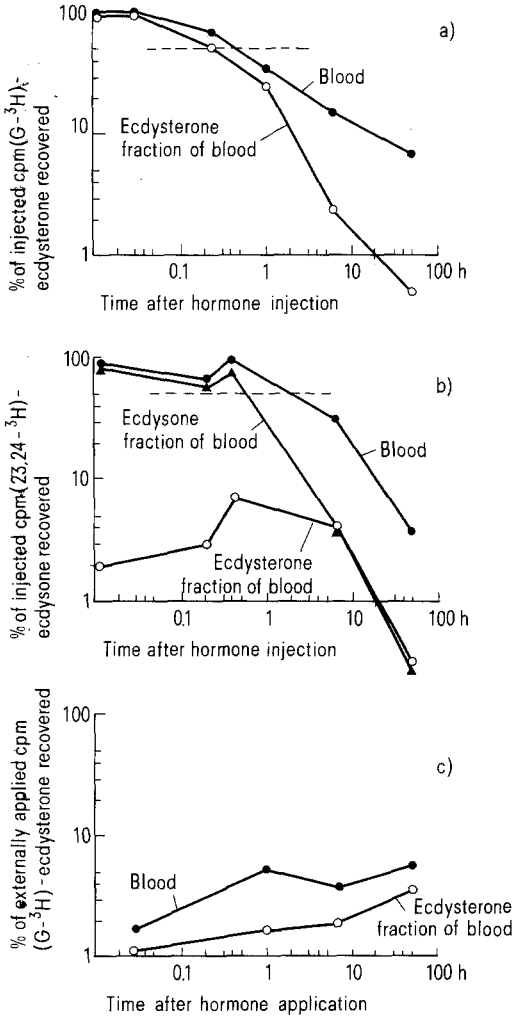
The data presented in part C of the figure have been acquired by a different method: the larvae were dipped in a methanolic solution of labelled ecdysterone[9]. This results in a low, but apparently stable or even increasing radioactivity in the blood as well as in the TLC ecdysterone fraction of blood. Such a pattern of radioactivity is best explained by a hormone deposit effect. Concomitantly the activity washed off the cuticle with methanol decreases with time: 62.8% of the applied activity is washed off 2 min after the hormone treatment; 60 min later it is 58.4%; after 7 h it is 53.6%, after 48 h 25.4%. Methanol-rinsed, macerated cuticle and epidermis did not show more activity than the blood. All TLC fractions of the blood exhibit little activity, and the fraction corresponding to 3-dehydroecdysterone never contains more than 18% of all fractions combined.

The carcass harbors 22.9% of the applied activity, 48 h after hormone application. The distribution of the radiolabel in the body upon topical application of ^{3}H-ecdysterone did not differ significantly in ligated and non-ligated abdomens (data not shown). For example, 48 h after hormone application, we measured 3.6% of the applied activity in the blood of unligated larvae, as compared to 3.7% for the ligated ones (figure, C).

The results given in part C of the figure can be used to calculate the rise of the ecdysterone titer after an external application of 13 µg hormone/g fresh weight. This is the dose needed to bring about a normal growth rate of imaginal disks in ligated hind parts for 2 days[9]. It increases the hormone titer to values between 360 ng and 1140 ng/ml blood.

Galleria wing disks of normal, unligated animals grow continuously throughout the last larval instar, but the smallest growth rate is observed in 24–118-h-old larvae[9]. During this developmental period, we measured hormone titers ranging from 4.4 to 14.0 ng/ml ecdysone and from 23.0 to 91.6 ng/ml ecdysterone equivalents (table). Hence we recognize that the normal, endogenous values are consistently lower than the exogenous titers needed to bring about normal disk growth[9] and cell proliferation in ligated hind parts.

Evidence that ecdysterone is a growth factor has been presented before: in vitro, ecdysone and ecdysterone stimulate DNA synthesis in dipteran[8,16] and lepidopteran[17,18] disks as well as mitoses in wing disks of *Drosophila*[6]. However, these investigations were done with mature rather than with young last larval disks, since the latter do not respond to ecdysteroids in vitro (Kurushima and Ohtaki[17]; D. Bullmore, personal communication).



The clearance of exogenous ecdysteroids from the blood of ligated, 24–48-h-old last larvae, *A* upon injection of ecdysterone, *B* upon injection of ecdysone, *C* upon external application of ecdysterone. [G-^{3}H]-ecdysterone (1.4 Ci/mmole; purchased from New England Nuclear) and [23,24-^{3}H]-ecdysone[1] (68 Ci/mmole) were purified by column chromatography on Sephadex LH-20® in water, and added to unlabelled hormone. The injected dose was 11 µg and 1.2×10^6 cpm/g fresh wt, the dose applied topically, according to methods described elsewhere[9], was 13 µg and $3–8\times10^5$ cpm/g fresh wt. Each measurement was done with batches of 10 animals. The points in diagram A and C represent means from 2–3 batches, in diagram B the values are from 1 batch each. Ecdysteroids were extracted from blood as described in the table. Radioactivity was measured with a spectrometer Packard Mod.2425 in Instagel® (Packard). Blood contents of the larvae were estimated by studying the dilution of ^{3}H-inulin 15 min after injection. Blood was shown to represent 40% of the body weight.

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Influences of dehydroepiandrosterone acetate on ovarian oocytes in mature cycling rats

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Summary. Degeneration of ovarian oocytes occurred to a remarkable extent in rats with polycystic ovaries induced by dehydroepiandrosterone acetate (DHA-Ac) administration. The ratio of degeneration oocytes, compared with the total oocytes examined, finally exceeded 70%.

Experimental induction of polycystic ovaries (P.C.O.) in the rat, in which anovulatory cycles followed by cystic change in the ovarian follicles were observed, was reported by Mahesh et al.[2] and by Ward et al.[3]. The characterization of the cystic follicles was attempted[4]; however, further details of the changes are required, as an animal model for P.C.O. The present study was performed to elucidate the influence of the pathologic environment on the ovarian oocytes.

Wistar rats, 9 weeks of age, were used. The animals were kept in air conditioned rooms at 24 °C with lights switched on at 09.00 h and off at 21.00 h. Solid laboratory chow and water were supplied ad libitum. DHA-Ac was administered s.c. 1 mg/100 g b.wt/day (1-mg group) or 10 mg/100 g b.wt/day (10-mg group) for 4, 7, 11 and 14 consecutive days respectively. A non-treated (control) group received injections of the vehicle only. Vaginal smears were obtained by lavage every day to eliminate non-cycling rats before the treatment and to determine estrus cycles during the treatment. In the 10-mg group 96% of the animals showed constant diestrus smears until day 5 of administration whereas the smears in the 1-mg group were less uniformly constant. Animals showing diestrus smears were preferred for oocyte collection in both the control and DHA-Ac-treated groups.

The ovaries were dissected free of adipose and connective tissue and were transferred to a Falcon dish (3.5 cm in diameter) containing 5.0 ml of physiological saline solution. The oocytes were liberated by random puncturing under a stereoscopic microscope, using 10-fold magnification[5]. The oocyte-containing solution was transferred using a glass micro-pipette to a test tube with several drops of 1% aceto-orcein solution.

The test tube was left for about 30 min. The supernatant was discarded, and the whole sediment was mounted on a glass slide with a cover slip[5]. The oocytes were examined

Degeneration ratio is defined as percent ratio of degenerating oocytes to the total oocytes examined. Note the remarkable increments in the degeneration ratio for the 1-mg group on day 14 and for the 10-mg group on day 11, and that the ratios in both groups exceeded 70% ultimately

Group	Number of animals	Oocyte observed	Physiological stage			Abnormal findings of oocyte				Degeneration ratio (mean ± SD) %
			Dictyate	M-1 to 1-P.B.	Total	Deformation	Fragmentation	Others	Total	
Control	8	357	172	17	189	98	13	57	168	47.1 ± 6.8
1-mg/100 g b.wt/day										
4-d	4	201	80	13	93	77	10	21	108	53.7 ± 5.9
7-d	4	213	75	13	88	58	31	36	125	58.7 ± 3.8
11-d	4	238	82	15	97	69	41	31	141	59.2 ± 3.3
14-d	4	236	52	18	70	81	52	33	166	70.3 ± 4.5
10-mg/100 g b.wt/day										
4-d	4	179	80	10	90	59	4	26	89	49.7 ± 7.0
7-d	4	213	91	14	105	57	17	34	108	50.7 ± 5.3
11-d	4	205	43	13	56	93	28	28	149	72.7 ± 5.6
14-d	4	211	49	15	64	68	30	49	147	70.0 ± 13.0