

Distribution and Transport of Molting Hormones in the Crayfish, *Orconectes limosus*

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Z. Naturforsch. **33 c**, 437–441 (1978) ; received April 24, 1978

Molting Hormones, Crayfish, Ecdysteroid Tissue Distribution, Ecdysteroid Hemolymph Transport

When [^3H]ecdysone is injected in intermolt crayfishes, about 60–70% of the total radioactivity are excreted within one hour as the unchanged hormone, but nearly no [^3H]ecdysterone is excreted. The remaining [^3H]ecdysone in the body is effectively converted to [^3H]ecdysterone. In addition polar and apolar metabolites of both molting hormones can be extracted in an organ specific pattern. On the basis of radioactivity per g fresh weight hypodermis and male gonads and to a lesser extent hindgut and midgut gland contain most of the injected radioactivity. It can be demonstrated from *in vitro* experiments using the charcoal adsorption test and equilibrium dialysis that the cytosol of target tissues is able to bind both hormones with the highest capacity for hypodermis and male gonads. Both molting hormones do not appear to be bound to carrier proteins in the hemolymph.

Introduction

Steroid hormones are transported by the blood or hemolymph either in a free state or bound to a carrier protein, taken up by the target tissue and eventually metabolized and then bound to a steroid specific receptor protein. This hormone receptor complex migrates to the nucleus and there binds to the chromatin evoking a specific response of the target cell [1, 2]. Whereas this basic scheme has been generally accepted for vertebrate steroid hormones, most of these points are controversial or unknown in invertebrates [3–5]. The aim of this paper is therefore to deal with some aspects of the action of a steroid hormone in an invertebrate. The crayfishes are well suited for such investigations since their molting physiology is relatively well known [6–8] and they have long intermolt stages with a low titer of molting hormones [7] thus enabling distribution-, transport- and especially binding studies with only a small dilution of exogenously administered radiolabeled molting hormones.

Material and Methods

Rearing of the animals, molting stage determination, origin and purification of the chemicals and the *in vitro* binding assays are as described in [9].

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** Supported by the Deutsche Forschungsgemeinschaft (Sp 140/2 and Sp 140/3).

For the *in vivo* experiments 2 μCi of either [$^{23,24}\text{H}$]ecdysone (spec. act. = 68 Ci/mmol) or [^3H]ecdysterone (prepared by *in vitro* transformation of [^3H]ecdysone according to [10]), dissolved in 100 μl van Harreveld's solution [11] were injected into the ventral abdominal sinus. The crayfishes were kept individually in 100 ml water at room temperature (20–22 $^{\circ}\text{C}$) for one hour. After this time hemolymph samples (50 or 100 μl) were taken with calibrated siliconized glass capillaries and counted in Unisolve 1 (Zinsser, Frankfurt). Then as much hemolymph as possible was taken through the coxal membrane of a walking leg and mixed with methanol. The animals were sacrificed and the different organs dissected out. All tissues and the remaining carcass were thoroughly rinsed with ice-cold van Harreveld solution and aliquots of the rinsing solution were counted in the liquid scintillation counter. The wet weights of the different organs and of the carcass were determined and each tissue homogenized in 25 ml methanol with an Ultra-Turrax-Homogenizer. The homogenates were extracted over night at 4 $^{\circ}\text{C}$, centrifuged at 5000 rpm for 15 min, the supernatant was decanted and the extraction was repeated three times. The combined supernatants were evaporated to dryness and dissolved in 100–500 μl methanol. An aliquot of 10 to 50 μl of the solution was used for determining the radioactivity in the different organs. Prior to counting the samples were incubated over night in 0.5 ml 30% H_2O_2 then saccharose was added and the solution was counted in 5 ml of cocktail. The counting efficiency was determined by the external

standard channel ratio. The remaining solution was used for studying the metabolism by thinlayer-chromatography on silica gel plates with fluorescence indicator (Merck, Darmstadt) using dichloromethane-methanol = 8:2 and internal standards of ecdysone and ecdysterone. The chromatograms were scanned with a Berthold thinlayer-scanner (Berthold and Frieske, Karlsruhe). 98% of the injected radiolabeled hormones were recovered (average of 15 experiments).

Sera of *Orconectes* hemolymph or from rabbit blood were incubated with radiolabeled molting hormones for 30 min at room temperature. Samples of 200 μ l were sedimented on linear 10–35% glycerol gradients (in van Harreveld solution, SW 60Ti rotor, 60 000 rpm, 12 hours at 4 °C). The tubes were fractionated by a density gradient fractionator (ISCO 640) and fractions of 200 μ l were collected and counted in 5 ml of scintillation cocktail. 600 μ l serum of *Orconectes limosus* were chromatographed on a Sephadex G-25 column (7.3 $\times$ 2.0 cm in van Harreveld saline). The void and retained volumes were determined using bovine hemoglobin and DNP-alanine. The sera were eluted from the column using van Harreveld saline. Fractions of 50 drops were collected, the optical density at 280 nm was determined and all fractions were extracted three times with each 1 ml of water-saturated n-butanol. The butanol was evaporated to dryness, two times extracted with methanol and aliquots were assayed with a radioimmunoassay [12].

Another 1 ml of serum was incubated with 36 000 dpm [3 H]ecdysterone for 60 min and then fractionated on a Sephadex G-25 column as before.

Results

Ecdysone and ecdysterone show different distribution patterns when injected in intermolt crayfishes. About two third of the injected ecdysone is excreted within one hour (Fig. 1) whereas only a small amount of ecdysterone is excreted (or leaked out during the injection). Most of the injected ecdysterone is found in the hemolymph and in the carcass which account for 81% of the total body weight. All other organs investigated so far contain between 1 and 6% of the total radioactivity. On the basis of radioactivity per g fresh weight (Fig. 2) ecdysterone seems to accumulate slightly more than ecdysone. On the contrary more [3 H]ecdysone is concentrated

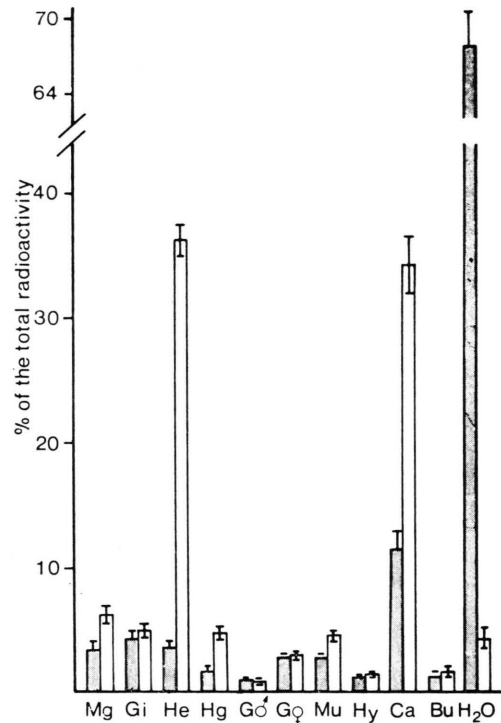


Fig. 1. Distribution of radioactivity in intermolt crayfishes, *Orconectes limosus* of both sexes one hour after injection of either 2 μ Ci [3 H]ecdysone or [3 H]ecdysterone. Each bar represents the mean of 9 ([3 H]ecdysone) or 6 ([3 H]ecdysterone) animals. I = standard deviation. ▨ = [3 H]ecdysone injected, ▤ = [3 H]ecdysterone injected. Mg = midgut gland, Gi = gills, He = hemolymph, Hg = hindgut, G♂ = male gonads, G♀ = female gonads, Mu = muscle, Hy = hypodermis, Ca = carcass, Bu = buffer rinse, H₂O = water in which the animals were kept after injection.

in the male gonads, whereas in the female gonads both radiolabeled hormones are found in the same amounts.

The two hormones are effectively metabolized (Fig. 3 a) yielding both polar (in gills, midgut gland, muscle and hemolymph) and apolar products. When ecdysone is injected, only in the midgut gland an apolar metabolite can be seen by thinlayer-chromatography (Fig. 3 b). After injection of ecdysterone an apolar metabolite was detected in gills, hemolymph, muscle and hindgut.

The *in vivo* experiments described above suggest that there may be different tissue specific binding capacities for the molting hormones. This could be demonstrated by *in vitro* experiments using binding assays. When the binding capacities of the different organs of the crayfish are compared on the basis of the same protein content the highest values

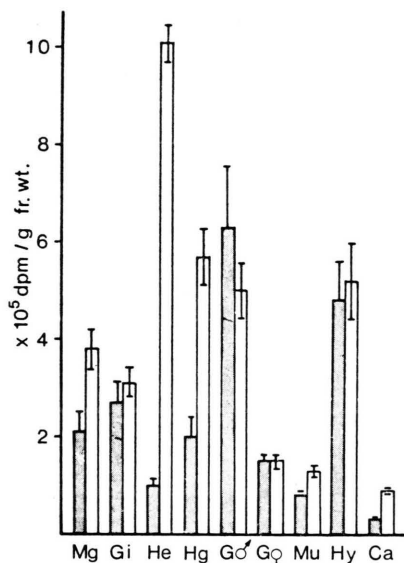


Fig. 2. The distribution of radioactivity per g fresh weight. The same animals and symbols as in Fig. 1.

are reached by the hypodermis and the male gonads (Table I). The most effective binding of [³H]ecdysone was found in the hypodermis whereas in the male gonads [³H]ecdysone was bound to the highest extent. Very similar relations in the binding capacities of the different organs could also be demonstrated by equilibrium dialysis (unpublished results).

The hemolymph of *Orconectes* does not seem to bind the molting hormones. This has been found using either the charcoal adsorption test, equilibrium dialysis (Fig. 4), density gradient centrifugation (Fig. 5), chromatography on Sephadex-G 25 (Fig. 6) or a filter assay (unpublished results), whereas with these methods binding of radiolabeled molting hormones in target tissues could be demonstrated [9]. Chromatography of serum and deter-

Table I. Binding of [³H]ecdysone and [³H]ecdysterone to homogenates (20000×g supernatant) of various tissues of the crayfish *Orconectes limosus*. Total binding in percent per 100 µg protein. Mean ± Standard Deviation.

Tissue	n =	[³ H]ecdysone		[³ H]ecdysterone	
		M	SD	M	SD
Midgut gland	9	8	1	8	2
Hindgut	8	14	5	15	4
Male gonads	5	53	23	44	18
Hypodermis	10	46	7	58	8

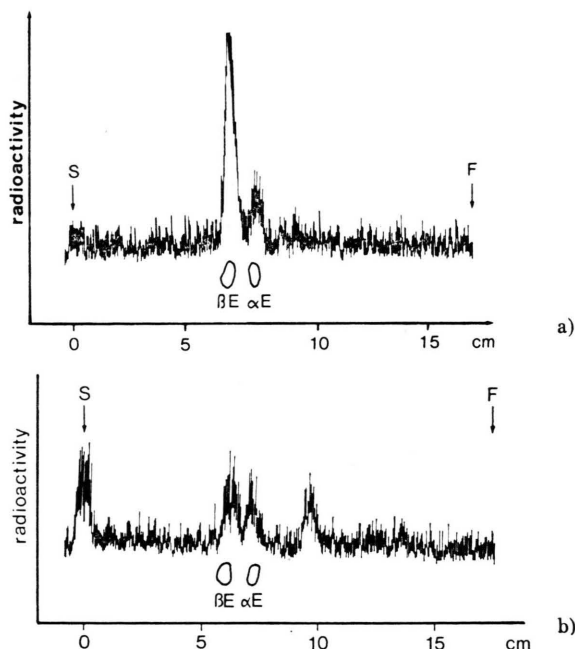


Fig. 3. Radiochromatogram of methanol extracts of homogenates from *Orconectes limosus* (intermolt stage), a) hindgut and b) midgut gland one hour after injection of [³H]ecdysone. Unlabeled ecdysone (αE) and ecdysterone (βE) were added as internal standards. S = start, F = front.

mining the RIA-activity in the single fractions shows less than 3% of the total RIA-activity in the macromolecular fraction (Fig. 7).

Discussion

From our experiments it seems obvious that both ecdysone and ecdysterone circulate in a free state and are not specifically bound to hemolymph proteins in intermolt *Orconectes*. Using the charcoal adsorption test the extremely low values of "binding" for hemolymph samples are in the same range as the values for the binding of the molting hormones to rabbit serum or bovine serum albumin as a control or even to van Harreveld saline and there is no correlation between "binding" and protein content. This was demonstrated using equilibrium dialysis, density gradient centrifugation or a filter assay. Transport of the molting hormones in a free state was shown in the crab, *Pachygrapsus*, using density gradient centrifugation or column chromatography [13], whereas in insects both bound [14–18] and freely circulating molting hormones [19, 20] have been demonstrated. The mechanism of the uptake of the

molting hormones in the tissues of the crayfish *Orconectes* has not been studied. But preliminary experiments showed that maximum uptake is reached in

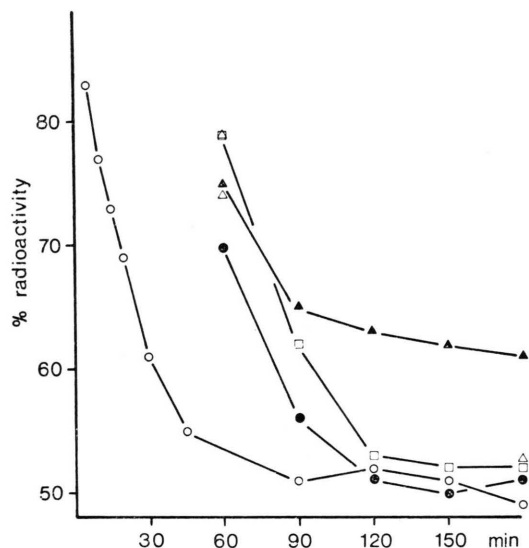


Fig. 4. Equilibrium dialysis of [^3H]ecdysone (ca. 3000 dpm) and various solutions in van Harreveld saline against van Harreveld saline at 20 °C. Both the samples and the radio-labeled hormone were in one dialysis chamber. At the given times the two chambers and the membrane were counted. The percentage of the total radioactivity in the one chamber is shown. ○ = van Harreveld saline, □ = ovalbumin, △ = homogenate (20000×g supernatant) of rabbit liver, ● = serum of *Orconectes limosus* hemolymph, ▲ = homogenate (20000×g supernatant) of hypodermis from *Orconectes limosus*.

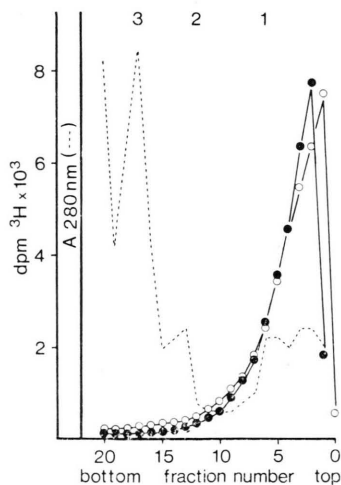


Fig. 5. Glycerol density gradient centrifugation of *Orconectes limosus* hemolymph sera samples incubated with [^3H]ecdysone without (●) or with (○) a 100 fold excess of ecdysterone for 30 min at room temperature. — — — represents the optical density at 280 nm. Marker proteins: 1 = cytochrome C, 2 = bovine serum albumin, 3 = aldolase.

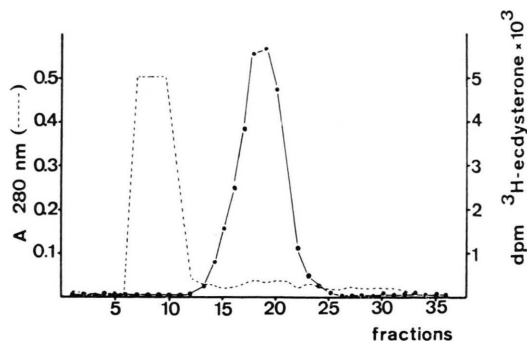


Fig. 6. Separation of serum from intermolt *Orconectes limosus* on Sephadex-G25. Serum preincubated with 30000 dpm [^3H]ecdysterone. Profiles show optical density at 280 nm (— — —) and dpm per fraction (●).

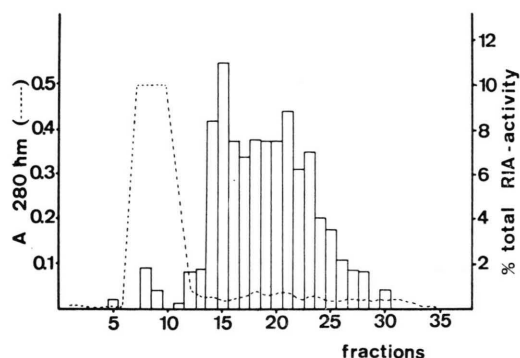


Fig. 7. Radioimmunoassay of intermolt *Orconectes limosus* serum eluted from Sephadex-G25. — — — = optical density at 280 nm, □ = RIA activity per fraction in per cent of the total RIA-activity.

about half an hour, at the temperature we used in the experiments described above (unpublished results).

From the distribution pattern of the two radio-labeled hormones it can be clearly seen that all organs contain both steroids, but not in the same ratio. The most obvious difference is the high excretion rate of ecdysone as compared to the low level of excreted ecdysterone. The effective excretion of ecdysone by intermolt *Orconectes limosus* may be the reason why the level of ecdysone in the hemolymph of this animal is much lower than in *Orconectes virilis* [21], or in the insects *Hyalophora cecropia* [22] and *Antheraea polyphemus* [20]. But also in insects an excretion of the injected [^3H]ecdysone up to 80% has been demonstrated [23].

On the basis of radioactivity per g fresh weight there is an accumulation of the molting hormones in hypodermis, male gonads and the hindgut of *Orconectes limosus*. From our data it is not yet

possible to determine the accumulation of the molting hormones by different organs on a molar basis since the endogenous levels of these hormones are only known for the whole animal [7] and not for single organs, since the specific activity of the [^3H]ecdysterone is unknown and since there may be a contamination of the different organs with hemolymph even after a thorough rinsing.

Concerning the metabolism of the injected radio-labeled hormones in *Orconectes limosus* there are several features in common when compared with the metabolism of the molting hormones in other crustaceans [24–29, 13]: ecdysone is rapidly converted to ecdysterone and there are both apolar and polar derivatives of the molting hormones in an organ specific pattern. For example there is only one apolar metabolite in *Orconectes limosus* in only one organ (midgut gland) when ecdysone is injected, similar to the findings of Gorell *et al.* [25] using *in vitro* experiments. After an injection of [^3H]ecdysterone apolar derivatives have been found in several organs. But since the metabolites have been separated solely by thinlayer chromatography and have not been further characterized and since no *in vitro* experiments have been done in *Orconectes limosus* so far, the metabolizing capacities of the different organs remain unknown.

The investigation about the distribution of injected [^3H]ecdysone and [^3H]ecdysterone led to the

conclusion that different organs may have quite different capacities for a binding of the molting hormones. This could be verified by *in vitro* experiments directly studying the binding. The highest binding capacity for [^3H]ecdysterone has the hypodermis and a very high binding is also exerted by homogenates of male gonads. [^3H]ecdysone is effectively bound to the male gonads and also to the hypodermis. The same relations were found using the charcoal adsorption test and equilibrium dialysis, but also with a filter assay according to Beckers (personal communication) or with density gradient centrifugation (unpublished results).

All four methods quite clearly showed that molting hormones are bound to the cytosol of different tissues to different degrees. This can be interpreted in terms of target or non-target organs but the problem seems to be that in arthropods this is not a clear cut alternative [20, 21] since nearly all organs must respond to one and the same hormonal signal during a molting cycle and there may be only gradually or time-shifts in responding to the molting hormones or there may even be different kinetics of the uptake of the hormones in different organs.

The crayfishes were kindly provided by Prof. Dr. R. Keller (Bonn). We gratefully acknowledge the *in vitro* transformation of [^3H]ecdysone by Drs. Feyer-eisen and J. Hoffmann (Strasbourg).

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