An *in vitro* Culture System for Crayfish Organs

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Organs of crayfishes – mainly integument of *Astacus leptodactylus* – have been kept in an *in vitro* culture for several days. The medium consists of 2 parts of van Harreveld saline and one part of horse serum, supplemented with antibiotics for long term cultures. This medium is very similar to the *Astacus* hemolymph in its ionic content, the pH, the osmolarity, the protein concentration and the cholesterol content. The glucose concentration is somewhat higher and there are differences in the nitrogen excretory end products. Integument of *Astacus* is able to synthesize protein, RNA and DNA *in vitro* for several days. Ecdysone is converted to 20-OH-ecdysone by the integument under *in vitro* conditions.

Introduction

In order to study the physiological effects and the primary action of hormones on different organs, tissue culture is a very useful tool. In the invertebrates such studies have been mainly made in insects [1 – 3] and to a much lesser extent in other invertebrate phyla [4]. This is especially true for studies dealing with the action of molting hormones which have been mostly done on imaginal discs, other epidermal tissue and testes of insects [5 – 7].

In crustaceans tissue culture is restricted to only a few species and organs, mainly testes and ovaries [8 – 10], limb regenerates [11, 12], Y-organs [13, 14] and a few short term cultures [15, 16]. The effect of molting hormones *in vitro* has been described for ovaries [17] and only preliminary for epidermal tissue [18] of cirripeds.

In crayfishes both titer [19] and production [13, 20] and at least some of the initial steps of the molecular action of the molting hormones [21, 22] have been investigated. But in order to elucidate the metabolism and uptake of the molting hormones a tissue culture system is necessary. It was therefore the aim of our investigations to establish an easy and convenient *in vitro* culture for organs of crayfishes.

Materials and Methods

Rearing of the animals and molting stage determination are as described [22]. Before dissection the hemolymph of the animals was withdrawn and the ecdysteroid concentration in a 50 µl aliquot was determined by a radioimmunoassay [23] as a second criterion for the molting stage. The crayfishes were than surface-sterilized with 70% alcohol. For the culture of the integument only parts of the cephalothorax were used. The carapax with the adherent integument was washed several times for short periods in van Harreveld saline [24], containing streptomycin (Serva, Heidelberg), penicillin (Serva, Heidelberg) and Fungizon (Gibco) in a final concentration of 1 mg/l, 1000 E/l and 2.5 µg/l. Between the carapax and the hypodermis a piece of parafilm is carefully inserted and with a sharp round knife pieces of 3 or 4 mm of diameter were cut out. For some experiments bigger pieces were cut out with scissors or a scalpel. In the saline described above the hypodermis separates very well from the hard carapax and from the parafilm. The tissue is then 2 or 3 times washed in the buffer and then transferred to the medium. Each piece of tissue was incubated in 0.5 ml of medium in a small glass vial or for the bigger pieces in 2.5 ml in a sterile Petri-dish (Greiner, 35.0/10 TC). The media were sterilized by filtration (Sartorius filter, pore size 0.2 µm), the sera were first inactivated at 56 °C for 30 min prior to filtration. Hemolymph of intermolt animals was centrifuged at $10\,000 \times g$ for 20 min and the supernatant sterilized by filtration (Sartorius filter, pore size 0.2 µm).

For histological examinations the tissues were fixed in Bouin, sectioned at 7 µm and stained with the Heidenhain-Azan stain [25]. As a rapid test for the viability of the tissues in the different media vital staining with a 0.1% trypan blue solution in the corresponding medium (sterilized by filtration with a Satorius filter, pore size 0.2 µm) were performed.

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Sodium and potassium in the medium and in the hemolymph were analysed by a flame photometer (Corning 450), the osmolarity by a Mikroosmometer (Knauer) and all other analytical data by an automatical clinical analyzer (du Pont Instruments).

Protein synthesis was tested as follows: a piece of integument was incubated in standard medium containing a ^{14}C-labelled amino acid mixture (Amersham Buchler, CFB 104; 52 mCi/milliatom). After incubation the adhering radioactivity was washed away with van Harreveld saline and then with 10% trichloroacetic acid in the cold. The tissue was dried until constant weight, the weight was determined and the tissue homogenized in 0.5 ml 0.5 N NaOH at 60 °C for 90 min, cooled down and centrifuged in order to remove non-solubilized hairs and parts of the cuticle. Proteins were precipitated with TCA in the cold, the precipitate was washed with TCA and then dissolved in 0.5 N NaOH, aliquots of which were taken for the protein determination [26] and for measuring the radioactivity. For measuring thymidine uptake and incorporation into DNA [^3H]thymidine (spec. act. 25 Ci/mmol) was added to the pieces of integument and incubated for different times. The integument was rinsed with van Harreveld saline, dried to constant weight, the weight was determined and the pieces were homogenized and extracted in 0.4 N perchloric acid at 4 °C for 2 h, then centrifuged and the pellet was dissolved in 0.5 N NaOH at 70 °C for 2 h. Both from the supernatant and from the dissolved pellet samples were taken for measurement of the radioactivity and in the pellet fraction DNA was determined [27].

The origin and purification of the molting hormones is already described [22] as well as the procedure for studying the uptake and metabolism of the ecdysteroids [28].

Results

Ten different media have been tested whether they can be applied for an *in vitro* culture of crayfish organs both from intermolt (C 3–C 4) or from premolt (D 1–D 2) animals. Among the different media or combinations of media a mixture of one part of serum – either from the horse or from the calf – and two parts of van Harreveld saline (crayfish saline) (subsequently called standard medium) proved to be best suited for our experiments as tested by microscopical examination and staining with 0.1% trypan blue. For most experiments only the integument of *Astacus leptodactylus* was tested but for comparison also other organs of the same species and organs from Orconectes limosus were tested. The amount of serum necessary was tested in the range from 2.5 to 50%. Below 20% serum concentration the stainability of the tissues with trypan blue is pronounced, decreasing up to about 30% serum concentration. This is true both for horse and for calf serum. The positive effect of serum can not be replaced by adding amino acids since in such a medium – for example M 199 (Gibco, No. 115 E) – when adjusted to the physiological pH and osmolarity 50% or even more of the cells are dead within 6 h, whereas if the medium (M 199) is supplemented with 33% horse serum only few cells are dead in the same time.

The concentrations of several components of the standard medium (2 parts of van Harreveld saline and 1 parts of horse serum) are very similar to the serum of *Astacus leptodactylus* (Table I). There are only a few exceptions, namely a higher glucose concentration in the medium and of course different concentrations of the nitrogen excretory products as compared to the hemolymph as well as an absence of molting hormones in the standard medium.

Integument maintained *in vitro* is able to take up amino acids and to incorporate them into proteins during a relatively long time. In the concentration range tested there is a linear relationship between the amount of radiolabelled amino acids and the incorporation into proteins (Fig. 1). Protein synthesis

Table I. Comparison of some analytical data of the standard medium and *Astacus leptodactylus* hemolymph.

		Standard-medium	Serum of *Astacus leptodactylus* hemolymph
Na^+	mval/l	156	179
K^+	mval/l	4.4	5.8
Ca^{2+}	mval/l	14.6	16.5
Mg^{2+}	mg/100 ml	4.2	3.8
Cl^-	mval/l	160	159
glucose	mg/100 ml	37	9
protein	g/100 ml	2.4	3.6
cholesterol	mg/100 ml	32	29
NH_3	µg/100 ml	0	335
urea	mg/100 ml	24	4
uric acid	mg/100 ml	2.6	1.8
osmolarity	mosmol	349	344
pH		7.6	7.6
ecdysteroids	ng/ml	0	15

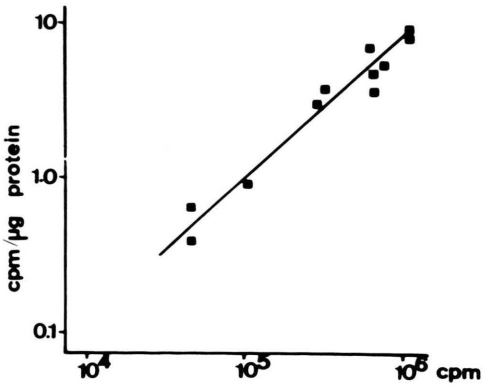

Fig. 1. Relationship between the amount of ^{14}C-labelled amino acids and incorporation into proteins of the integument of *Astacus leptodactylus* after 1 h *in vitro* culture at 25 °C. The line represents a regression line, $r = 0.95$.

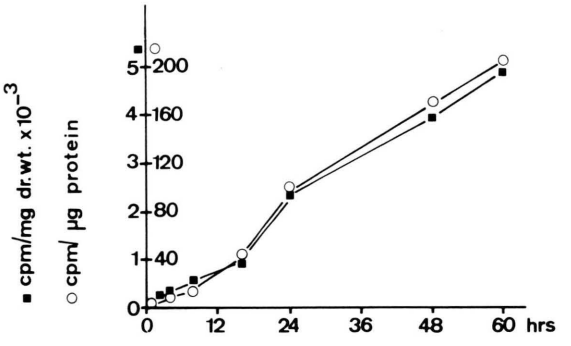

Fig. 2. Incorporation of ^{14}C-labelled amino acids (3.8×10^5 cpm) into protein of *Astacus leptodactylus* integument *in vitro* at 25 °C.

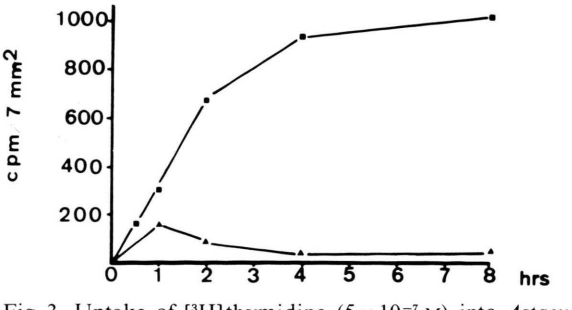

Fig. 3. Uptake of [^{3}H]thymidine (5×10^{-7} M) into *Astacus leptodactylus* integument *in vitro* at 25 °C in the absence (■) or presence (▲) of 2 mM NaCN.

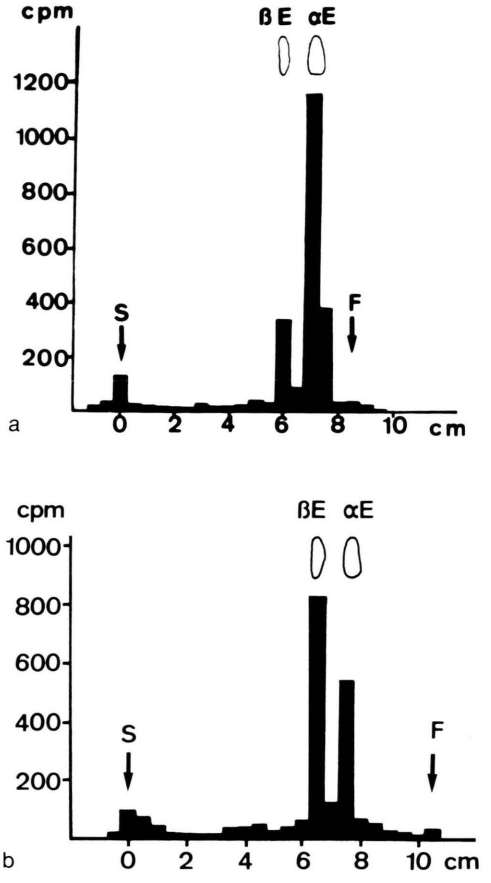

Fig. 4. Conversion of [^{3}H]ecdysone by *Astacus leptodactylus* integument *in vitro* (a) or *in vivo* (b) at 25 °C for 1 h. 1.8×10^{-9} M (a) or 2 µCi (b) of [^{3}H]ecdysone were used. S = start, F = front, α E = ecdysone, β E = 20-OH-ecdysone.

takes place under *in vitro* conditions for at least 3 to 4 days, one of the experiments is shown in Fig. 2. There is often a slower rate of protein synthesis during the first half day of incubation then reaching a higher and linear synthesis rate.

As concerns the DNA-synthesis, [^{3}H]thymidine is taken up and enriched several fold as compared to the medium. The uptake is completely inhibited by 2 mM NaCN (Fig. 3). The rate of DNA-synthesis in the integument is very similar immediately after the beginning of the *in vitro* culture or at the second day in culture (Table II).

Integument *in vitro* converts ecdysone to 20-OH-ecdysone, the amount of 20-OH-ecdysone being higher under *in vivo* conditions than under *in vitro* conditions (Fig. 4 a, b).

Table II. Incorporation of [^{3}H]thymidine into DNA from *Astacus leptodactylus* integument *in vitro*.

Labelling time [h]	cpm/mg dr. wt. first	on the second day
0.5	164	160
1	281	276
2	635	599

Discussion

The sequence of events taking place between secretion of the molting hormones and their final action(s) on target organs has been elucidated at least to some degree in crayfishes. Information exists about the place and rate of synthesis [20, 29], the transport [21], the uptake [28] of the molting hormones and on their binding to cytoplasmic [22] and nuclear receptors [30].

Beside from *in vivo* experiments, changing RNA-, DNA- and protein concentrations and enzymatic activities during a molting cycle (*cf.* [31, 32]) have been described for crayfish integument as well as stimulatory effects of molting hormones on DNA [33] and protein synthesis [31]. In these cases the effect of the molting hormones could be an indirect one, primarily stimulating a different tissue than integument. Thus for studying the biochemical effects of the molting hormones on integument an *in vitro* system is necessary. The same is also true for the determination of the capacities of different organs to convert ecdysteroids. Furthermore an *in vitro* system is also needed for analyzing the uptake of molting hormones (and other compounds) and for an examination of the morphogenetic effects of the molting hormones. Such *in vitro* approaches on the above mentioned topics have been used very successfully in insect endocrinology (*cf.* [1 – 3, 5]).

To determine whether the medium described here is suitable for such studies in crayfishes, several criteria have been used: a) viability tests, b) histological examinations, c) studies on the RNA-, DNA- and protein synthesis, d) studies on the metabolism of the molting hormones and e) a comparison of the medium and the *Astacus* hemolymph. Preliminary light microscopical investigations on the structure of the integument revealed no pronounced differences immediately at the beginning or after 3 days of incubation. These studies have to be repeated in more detail and extended to electron microscopical work. Studying the synthetic capacities of the integument at various times during the *in vitro* culture it could be demonstrated that RNA (data not shown here), DNA and protein synthesis take place for several days. The rate of protein synthesis under *in vitro* conditions is similar to the rate of protein synthesis *in vivo* [31, 34, 35]. This is especially true since different radiolabelled amino acids have been used and also different amounts of amino acids which results in different incorporation rates [28]. Integument (and other organs, too; unpublished results) of crayfishes can convert ecdysone to 20-OH-ecdysone under *in vitro* conditions. If the same temperature and time are used the amount of 20-OH-ecdysone is higher in integument *in vivo* than *in vitro*. This may be due to the fact that the conversion rate is higher *in vivo* or that part of the 20-OH-ecdysone in the integument after an *in vivo* experiment derived from conversion by other organs and has been transported by the hemolymph, which mainly contains 20-OH-ecdysone, and has than been taken up by the integument. This alternative can not be decided at the moment but the experiments clearly show that the target organ integument is able to take up and to convert ecdysone, as it has also been demonstrated in *Carcinus maenas* integument [16]. If we compare the composition of the medium and of *Astacus* hemolymph there are most components in good agreement and also in agreement with already published values [37], with the exception of the nitrogen excretory endproducts. As compared with other media for organ or cell culture of crustaceans [9 – 12, 17, 38, 39] the amount of serum used here is higher but it may be possible to replace some of the serum by adding for example amino acid mixtures, vitamins or cholesterol.

On the basis of these criteria this simple and convenient *in vitro* culture system seems to be useful for further endocrinological work on crayfishes and it is currently used in our laboratory for studies on the uptake and action of molting hormones in crayfish organs.

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