

Isolation of Nuclei from Crayfish Tissues and Demonstration of Nuclear Ecdysteroid Receptors

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Nuclei from hypodermis and midgut gland of the crayfish, *Orconectes limosus*, have been isolated with a slightly modified Chauveau-method. The isolated nuclei are well preserved and free from cytoplasmic contamination. They are able to incorporate [^3H]UTP into acid-insoluble material. This labelling is α -amanitin sensitive. The isolated nuclei are devoid of the mitochondrial/microsomal ecdysone-C-20-hydroxylase. The DNA:RNA:protein ratio is 1:0.8:3.2 (using Triton X-100 during the isolation procedure the ratio is 1:0.4:4.6) for nuclei from the hypodermis and 1:1:3.8 for nuclei from the midgut gland. The absolute amount of DNA per cell is 1.6, resp. 3.5 pg. The final DNA-recovery is about 20%. The yield increases to about 60% if the final ultracentrifugation step is omitted. The isolated nuclei bind ^3H -ecdysone and ^3H -20-OH-ecdysone specifically. As revealed by Scatchard analysis the affinity of the nuclear 20-OH-ecdysone receptor is $1-2 \times 10^{-9} \text{ M}$.

Introduction

In order to study the molecular action of the arthropod molting hormones, isolated nuclei of high purity from target tissues are necessary. Such target organs have been demonstrated by several physiological and biochemical methods both for insects [1, 2] and crustaceans [3, 4]. In the crayfish, *Orconectes limosus*, a tissue specific pattern of the distribution of the molting hormones [5] and also cytoplasmic receptors for these hormones [6] have been demonstrated, suggesting a mode of action of the molting hormones comparable to the mechanism of the vertebrate steroid hormones, including nuclear binding sites (cf. [7, 8]).

We now report the isolation of nuclei from two different target tissues of the crayfish, *Orconectes limosus*. This method enabled us to study the interactions of molting hormones with nuclear binding sites devoid of contamination with cytoplasmic receptors.

Materials and Methods

For the isolation of the nuclei intermolt *Orconectes limosus* of both sexes were used. Rearing of the animals and molting stage determination are as described [6].

Preparation of nuclei

For isolation of nuclei usually the integument of the carapax and the midgut gland of two animals were used. The integument was stripped from the carapax from caudal and washed several times with an ice cold 0.33 M sucrose solution containing 4 mM CaCl_2 . The integument was cut into small pieces and homogenized in 2 ml of an icecold sucrose solution (0.33 M + 4 mM CaCl_2) with a Dounce homogenizer (pistill A) by about 10 strokes. The homogenate was filtered through a nylon net (48 μm mesh for hypodermis and 100, 75 and finally 48 μm for midgut gland) and centrifuged for 15 min in a Sorvall HB-4 rotor at 2500 rpm (1000 $\times g$) and 4 °C. The pellet was resuspended in the same solution and layered over 1.7 M sucrose (+ 3 mM CaCl_2) and centrifuged under the same conditions. The sediment was suspended in 1.2 ml of the same solution and layered over 2.3 M sucrose (+ 3 mM CaCl_2) and centrifuged

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for 2 h in a Beckman SW 60 Ti rotor at 18000 rpm ($45000 \times g$) at 4 °C. The pellet was washed twice with 0.33 M sucrose (+ 4 mM CaCl_2) and then suspended in the following buffer: 10 mM Tris, 25 mM KCl, 25 mM NaCl, 10 mM MgCl_2 and 1 mM dithioerythrit, pH 7.4 in 0.33 M sucrose (= buffer A).

The first steps in the isolation of the so-called "900 $\times g$ nuclei" are as described, but instead of the ultracentrifugation, the nuclear pellet was resuspended in the 0.33 M sucrose solution (+ 3 mM CaCl_2) and layered over 1.7 M sucrose and again centrifuged in the Sorvall centrifuge at 900 $\times g$ for 15 min at 4 °C. This step was repeated two to three times and the final pellet was suspended in buffer A.

In order to get "Triton-nuclei" the first pellet after the centrifugation with the HB-4 rotor was resuspended in 2 ml 0.33 M sucrose (+ 4 mM CaCl_2) containing 0.1% Triton X-100 at final concentration. The pellet was homogenized by about 5 strokes with the Dounce homogenizer (pistill A), incubated for 15 min at 4 °C and centrifuged for 15 min in a Sorvall HB-4 rotor at 2500 rpm at 4 °C. The final preparation conditions of the "Triton-nuclei" are as described above.

Light and electron microscopy

The nuclei were routinely checked by phase contrast microscopy and counted with a Bürker chamber. For electron microscopy nuclear pellets were resuspended in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, containing 0.18 M sucrose and fixed for 3 h at room temperature. After several washes in the same buffer (a total of 1 h) the nuclei were postfixed in 1% OsO_4 in 0.1 M phosphate buffer, pH 7.4, overnight in the cold. Pellets of washed nuclei (0.1 M phosphate buffer, pH 7.4) were suspended in 2% Agar, dehydrated in graded series of ethanol and finally embedded in Spurr's medium [9]. Corresponding intact tissues (controls) were prepared by using the same fixation and embedding schedule described above, except the Agar preparation. Ultrathin sections were cut with glass knives on a Reichert ultramicrotome OmU 3, stained with 10% uranylacetate in methanol and lead citrate [10] and examined in a Siemens Elmiskop 102 at 80 kV.

[^3H]UTP incorporation in the isolated nuclei

10^7 nuclei were incubated in a final volume of 0.2 ml containing 1 mM of each ATP, GTP, CTP and

0.1 μM [^3H]UTP (= 1 μCi ; Amersham-Buchler, TRK. 412) in buffer B (11): 10 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 5 mM MnCl_2 , 2.5 mM MgCl_2 , 1.3 mM dithiothreitol, 30 mM $(\text{NH}_4)_2\text{SO}_4$. The nuclei were incubated at room temperature. After different times the incorporation was stopped by adding 1.5 ml 10% TCA at 0 °C. After 30 min the precipitate was collected by filtration on glassfiber filters (Whatman GF/A) and washed successively with 10% TCA, ethanol and ether. After drying the radioactivity on the discs was measured with Aqua Luma (Baker) with a counting efficiency of about 40%.

Incubation of purified nuclei with [^3H]ecdysteroids

Isolated nuclei of hypodermis were incubated with either 10^5 cpm of [^3H]ecdysone or [^3H]20-OH-ecdysone (spec. act. ca. 60 Ci/mmol) for 60 min at room temperature in buffer A. After this time maximum uptake of the molting hormones has been reached (unpublished results). The nuclei were carefully rinsed with buffer A in order to remove adhered radioactivity and then extracted with methanol. Each 100 ng of unlabelled ecdysone and 20-OH-ecdysone were added to the sample and then separated by reversed-phase high pressure liquid chromatography using methanol:water (4:6) as eluent (Lichrosorb RP8, 4×500 mm, 130 Bar, 20 drops per fraction, 0.35 ml/min, continuously monitored at 242 nm). The radioactivity of the fractions was determined as mentioned above.

In order to demonstrate nuclear binding, the nuclei were incubated with about 10000 cpm [^3H]ecdysone or [^3H]20-OH-ecdysone for one hour at

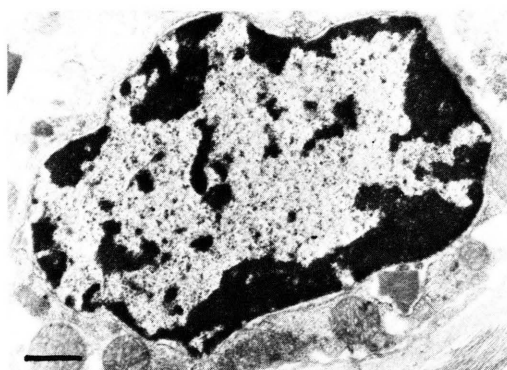


Fig. 1. Electron micrograph of a hypodermal nucleus in intact tissue. The bar indicates 1 μm .

20 °C. The incubation mixture was then filtered with an Amicon vacuum filtration apparatus (VFM2; Sartorius cellulose nitrate filters, pore size 0.2 µm) and three times rinsed with each 500 µl of buffer A.

Quantitation of protein, RNA and DNA

Protein, RNA and DNA were determined according to [12] to [14].

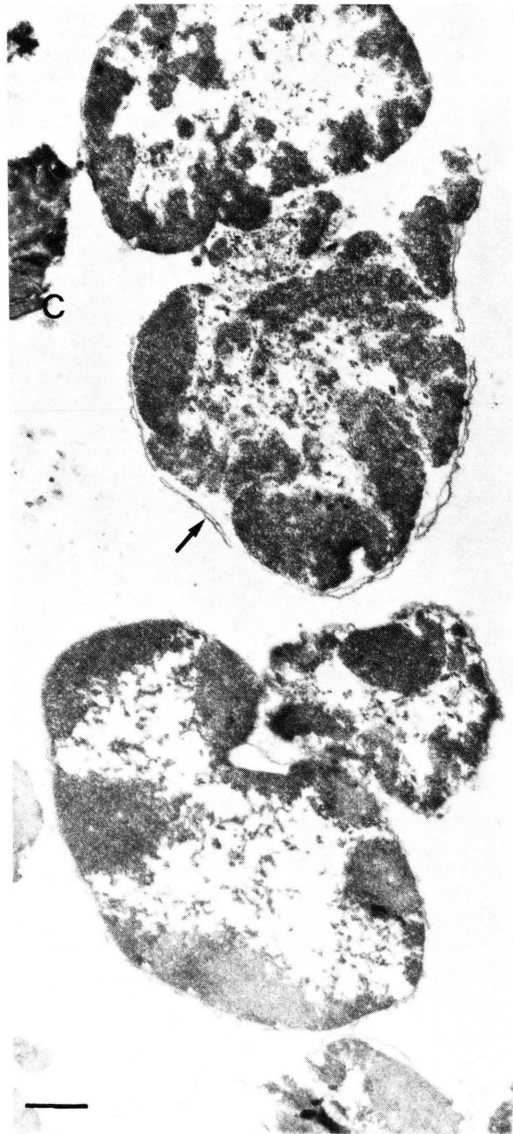


Fig. 2. Electron micrograph of isolated nuclei from hypodermis. C = cuticular remnants, arrow = intact perinuclear cistern. The bar indicates 1 µm.

Results

The native hypodermis (Fig. 1) shows flattened oblong nuclei, those in the midgut gland (Fig. 3) appear flattened too, but more spherical in shape. A typical feature of the hypodermal nuclei is their high amount of peripheral condensed chromatin, being attached to the inner nuclear membrane whereas in the midgut gland the nuclei show less distinct chromatin condensations and their nuclear surface appears less indented than that of the hypodermal nuclei.

With the isolation procedure described nuclei are obtained with a yield of 20% based on DNA re-

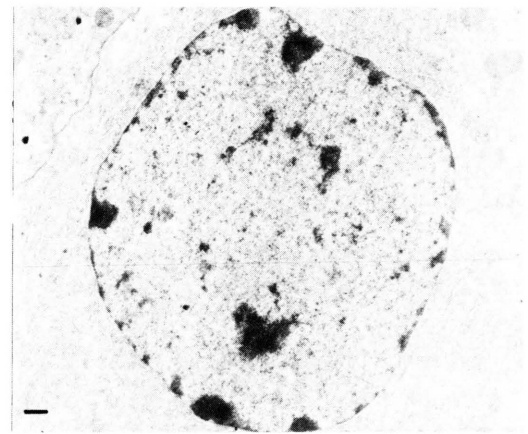


Fig. 3. Electron micrograph of a midgut gland nucleus in intact tissue. The bar indicates 1 µm.



Fig. 4. Electron micrograph of an isolated midgut gland nucleus. Arrow = outer nuclear membrane. The bar indicates 1 µm.

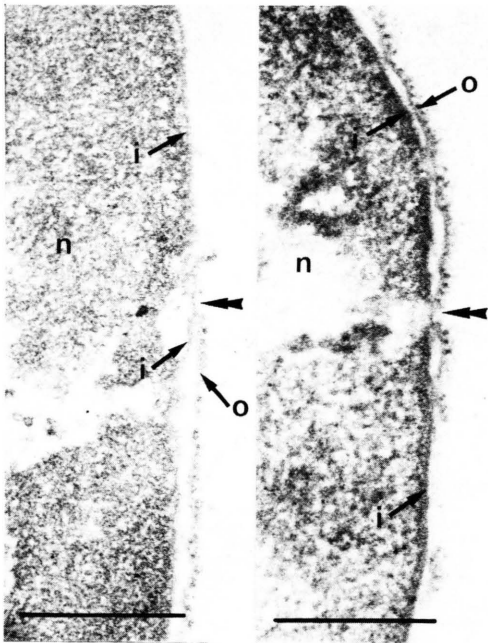
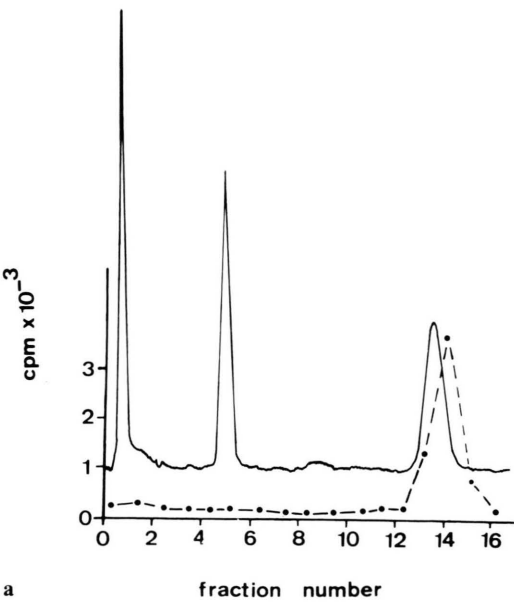


Fig. 5. Electron micrographs of nuclear envelopes from isolated hypodermal (a) and midgut gland (b). The outer nuclear membrane (o) with ribosomal particle coat is partially lost. i = inner nuclear membrane, n = nucleoplasm. Double arrow = nuclear pore. The bar indicates 0.5 μm.

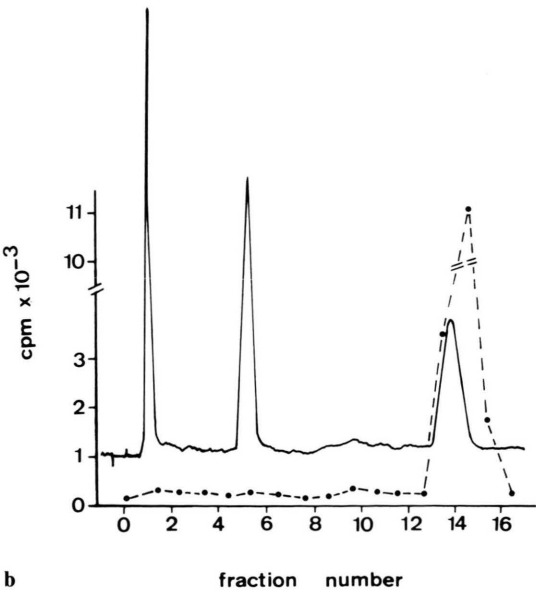
covery. Using Triton X-100 the yield is slightly increased to about 25%. The DNA content per nucleus is between 1.4 and 1.6 pg for hypodermis and 3.5 pg for midgut gland nuclei. If the ultracentrifugation step is ommitted (= “900 × g nuclei”) the yield increases to 58% but the RNA and protein content is twice as high (Table I). Light- and electron microscopical examinations of the “900 × g nuclei” show considerable contaminations of the nuclei with cytoplasm whether they are treated with Triton or not.

Table I. DNA:RNA:protein ratios of isolated nuclei from hypodermis and midgut gland of the crayfish, *Orconectes limosus*. * = Triton X-100 was used during the isolation procedure.

| | Hypodermis | | | Midgut gland nuclei |
|---------|------------|-------|-------------------------|------------------------|
| | Nuclei | | “900 × <i>g</i> nuclei” | |
| DNA | 1 | 1 * | 1 | 1 |
| RNA | 0.8 | 0.4 * | 1.7 | 1.2 |
| protein | 3.2 | 4.4 * | 5.0 | 3.8 |



a



b

Fig. 6. Demonstration of nuclear uptake of molting hormone. Isolated pure nuclei (a) of “900 × g nuclei” (b) were incubated with [³H]ecdysone for one hour at room temperature. The bound hormone was extracted and mixed with unlabelled ecdysone (fractions 12–16) and 20-OH-ecdysone (fractions 4–6) and separated by reversed phase high pressure chromatography. ●—● = cpm, — = O. D. at 242 nm.

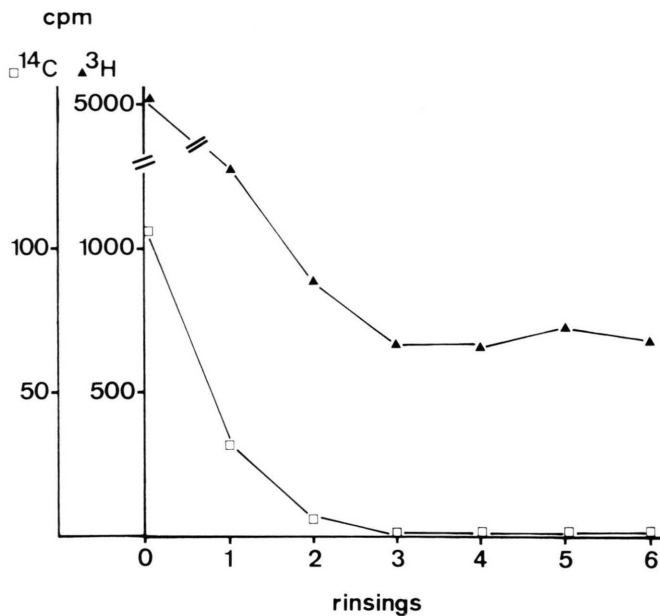


Fig. 7. Specific binding of ecdysone by nuclei. Isolated nuclei ($1 \times 10^7/200 \mu\text{l}$) from hypodermis were incubated with 2.8×10^4 cpm [^3H]ecdysone and 2×10^4 cpm [^{14}C]inulin for one hour at 20°C and then filtrated. The samples were rinsed with each $500 \mu\text{l}$ of buffer A. The radioactivity retained by the filter is shown here. Unspecific adsorption of [^3H]ecdysone (32 cpm per filter) and of [^{14}C]inulin (28 cpm per filter) is already subtracted.

Sectioned nuclear pellets from hypodermal tissue reveal a high purity of the isolated nuclei (Fig. 2); the pellets are contaminated only by cuticular and basal membrane remnants. The isolated nuclei show in some few cases very low, but for the most part no cytoplasmic contaminations. Contrary to the tissue nuclei the nucleoplasm of the isolated nuclei appears less dense and to some degree extracted. The inner nuclear membrane is intact, showing typical chromatin patches attached. The outer nuclear membrane with its ribosomal particle coat is preserved partly (Fig. 5a). The perinuclear cistern appears somewhat swollen, both in the tissue and in isolated nuclei.

Nuclear pellets from the midgut gland show identical morphological criteria for purity (Fig. 4, 5b) when treated the same way as hypodermal nuclear pellets.

After a lag phase of 20 min the isolated nuclei incorporate ^3H -UTP into acid-insoluble material (Table II). The labelling is α -amanitin sensitive since the incorporation can be inhibited by about 90% by $1.5 \mu\text{g}$ α -amanitin/ml.

Nuclei from the hypodermis and the midgut gland are able to take up and bind [^3H]ecdysteroids (Figs. 6–8). No metabolism of both [^3H]ecdysone (Fig. 6) and ^3H -20-OH-ecdysone (data not shown here) can be detected. In Figure 7 resistance to the washing procedure indicated binding of ecdysone with high

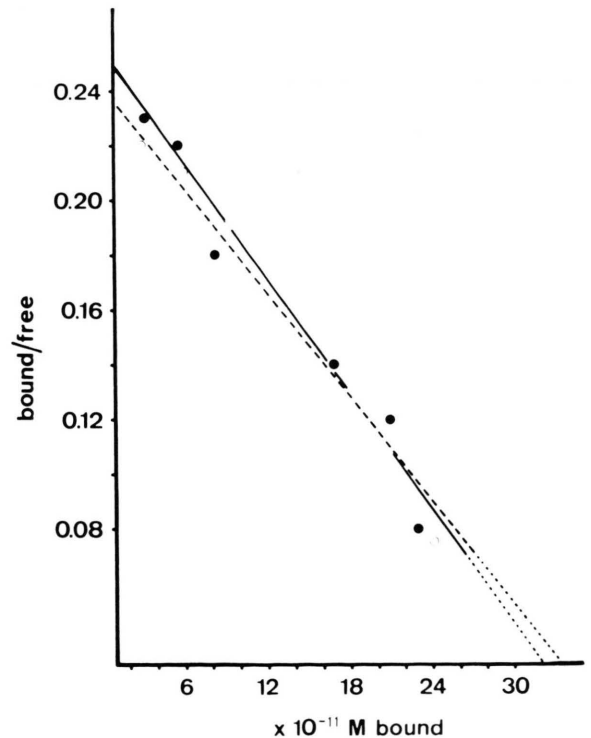


Fig. 8. Scatchard analysis of [^3H]20-OH-ecdysone binding components of isolated nuclei from hypodermis. For each point 0.8×10^8 nuclei were used and incubated with increasing amounts of [^3H]20-OH-ecdysone for one hour at 20°C . ●—● = nuclei isolated without Triton, ○—○ = Triton was used during the isolation procedure. The lines represent regression lines ($r = 0.98$ in both cases).

Table II. Incorporation of [³H]UTP into acid-insoluble material from isolated nuclei of crayfish hypodermis.

| Time [min] | cpm without | cpm with α -amanitin (1.5 μ g/ml) |
|------------|-------------|--|
| 0 | 177 | — |
| 5 | 255 | — |
| 10 | 725 | 94 |
| 15 | 1081 | — |
| 20 | 2008 | — |
| 40 | 2024 | 173 |

affinity. This is confirmed by the Scatchard plot [15] (Fig. 8) from which a dissociation constant K_D of $1.5-1.6 \times 10^{-9}$ M can be calculated. The affinity of [³H]20-OH-ecdysone to isolated nuclei and the number of binding sites per nucleus (5700–6000) is the same whether detergent was used in the isolation procedure or not.

Discussion

For isolation of nuclei a modified procedure of Chauveau *et al.* [16] was chosen. An advantage of this method is the continuous presence of sucrose which stabilizes the ecdysteroid receptors (own unpublished results). The yield of nuclei gained with this method is relatively small compared with isolation procedures using detergents, where 40–60% yields have been reached in *Drosophila* [17] or *Bombyx* [11] or even up to 90% in a very specialized tissue, the salivary glands of *Chironomus* [18]. Addition of Triton X-100 increases the amount of nuclei recovered from crayfish hypodermis not essentially. This agrees with yields gained from a comparable complex tissue, the *Calliphora* hypodermis [19]. Addition of detergent in a concentration of 0.1% at the beginning of the isolation procedure results in only unspecific binding.

The DNA:RNA:protein ratio found with pure nuclei is very similar to data from insects [19] and isolated chromatin from testes and midgut gland from the shrimp, *Palaemon serratus* [20].

Nuclei isolated with the three different methods described here do not metabolize molting hormones,

although both *in vivo* [5] and *in vitro* [21, 22] these hormones are metabolized by crayfish hypodermis. This indicates that the nuclear preparations are free of contaminations with the enzyme ecdysone-C-20-hydroxylase which is located in the mitochondrial [23–26] and/or microsomal [27] fraction.

The morphological data, too, clearly show that the isolation conditions presented here yield nuclear fractions being comprehensively free of cytoplasmic contamination. The isolated nuclei reveal good preservation by the used sucrose concentrations for there exist no drastic ultrastructural changes after the isolation steps described, with the exception of a decreased nucleoplasmic density. Such changes are commonly observed (*cf.* [11, 19]) and are apparently not related to the functional activity of the nuclei. The results obtained support this assumption. The observation that nuclei are ruptured to some degree may be due to the mechanical instability of the prefixed nuclear system during the repeated centrifugation procedures after pre- and postfixation for electron microscopy.

The K_D -value for [³H]20-OH-ecdysone to the nuclear receptors is in the same range as in crayfish hypodermis cytosol [6] but there is an about tenfold or even hundredfold higher affinity in crayfish nuclei than in “900 $\times g$ nuclei” from Kc-cells and imaginal discs from *Drosophila* using the same ligand as well as an about tenfold higher number of binding sites than in the same *Drosophila* tissues [28]. The isolation of nuclei without cytoplasmic contaminations is essential for an unequivocal demonstration of nuclear binding sites for the molting hormones and is a prerequisite for an elucidation of the transport mechanism of ecdysteroids from the cytoplasm into nuclei. These problems as well as a further characterization of the nuclear receptors in crayfishes are areas of current research in this laboratory.

Acknowledgements

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