Comprehensive survey of endogenous regulators of crustacean moulting

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Abstract. An investigation at SUNBOR into moulting regulation of crustaceans provided the background for the current study using crayfish *Procambarus clarkii*. Our present finding is that 3-dehydroecdysone (3-dhE) is a major biosynthetic product in the Y-organ *in vitro*, and that 3-dhE physiologically functions as the moulting hormone. The alteration in ecdysteroidogenesis were investigated by immunological, chemical and spectroscopic methods. After bilateral eyestalk ablation, ecdysteroidogenesis in the Y-organ generally increased and was accompanied by an increase of the major free ecdysteroid, 20-hydroxyecdysone (20-E), in the hemolymph. However, the increase of the later was not always due to biosynthesis of ecdysone in the Y-organs. The hormonal response of 3-dhE, upon injection into *P. clarkii*, was comparable to those of ecdysone and 20-E. Metabolic pathway of 3-dhE *in vivo* was focused through a time-course experiments using its radioactive tracer; counts were apparent in 3-dhE, ecdysone and 20-E within 3 h in the target tissues. The physiological significance of 3-dhE produced by Y-organ of *P. clarkii* will be discussed.

The trend in monitoring the isolation of natural products by specific assays related to the biological activities or the physiological importance is becoming increasingly popular. However, isolation and characterization of biological active compounds based on *in vitro* assay is merely the first step; further clarification of its mode of action in the complex cascade of biological reactions rely on a series of *in vivo* experiments. We have performed such studies aimed towards the understanding of hormonal regulation in crustacean moulting. In this presentation, I would like to review our efforts on this subject.

MOULTING CONTROL

The periodic shedding of the exoskeleton is essential for the development of many crustaceans and insects. The physiological events, including those before and after ecdysis, are called moulting, which is currently believed to be controlled by at least two major types of hormones. In crustaceans (ref. 1, 2), this process is controlled by ecdysteroids, the moulting hormones, and the moult-inhibiting hormone (MIH). Ecdysone is synthesized in the pair of Y-organs located in the cephalothorax, released into the hemolymph and converted into the major circulating hormone, 20-hydroxyecdysone (20-E). The physiological role of the

Y-organ is similar to that of the prothoracic gland in insects (ref. 3). MIH is released from the X-organ/sinus gland complex located in the eyestalks of crustaceans. It has been known since early in this century that removal of eyestalks shortens the intermolt period (C) and causes precocious ecdysis, whereas re-implantation of eyestalks reverses this effect. Injection of eyestalk-extract into the eyestalk-ablated animals delayed the onset of moulting and lengthened the moult-cycle. This effect had been defined as the MIH effect.

Recent reports (ref. 4) indicated that peptides with molecular weight ranging from 4000 to 8000 Da exhibited MIH activity. The MIH activities were not entirely species-specific, and the mode of action is obscure. We propose that the MIH effect is generated only in part by these peptides as Y-organotropic hormones, through negative control, in contrast to prothoracicotropic hormones (PTTHs) with positive control (ref. 5). The most interesting feature of the regulatory peptides is the homology (ref. 6, 7) among MIHs, crustacean hyperglycemic hormones (CHHs) and vitellogenesis inhibiting hormones, and also that (ref. 8) between PTTHs and insulin.

In 1977, Soyez and Kleinhorz reported (ref. 9) the presence of a small molecule exhibiting MIH activity *in vivo*. We isolated an active compound and characterized as 3-hydroxy-L-kynurenine (3-OHK) based on chemical and spectroscopic data (ref. 10). This compound's electrophorogram was identical to that of the one reported by Soyez. Thus, the compound reported as indole alkylamine (ref. 9) was revealed to be 3-OHK. In our study, the isolation of an active compound was monitored by HPLC analysis of ecdysone produced in the medium of Y-organ culture, in the presence or absence of eyestalk extracts. The isolated compound inhibited ecdysteroidogenesis *in vitro* in a wide range of crustacean Y-organs, and exhibited MIH effect *in vivo* upon injection into the de-eyestalked crayfish *Procambarus clarkii*.

This regulation mechanism of crustacean moulting (ref. 11) was proposed as follows. L-3-OHK was released from the eyestalks and accumulated in the Y-organs through a chemo-reception system, whose presence was proven by autoradiography (Fig. 1). In the Y-organs, L-3-OHK was enzymatically [transaminase with pyridoxal phosphate (V B_6)] transformed into xanthurenic acid (XA). XA interfered with cytochrome P450s, the mediators in ecdysteroidogenesis (ref. 12), and suppressed ecdysteroidogenesis. It was shown that the 8-OH of XA was essential to ligand exchange at the iron porphyrin, by using cytochrome P450$_{PB}$ (ref. 11) as the model. ESR also exhibited interaction between XA and another model compound, cytochrome c (ref. 10).

In addition, the inhibition of ecdysteroidogenesis by XA was also reproduced by use of the prothoracic glands taken from silk-worms *Bombyx mori* (ref. 12). Thus, it was considered that the same kind of biochemical reactions were involved in both the crustacean and insect moulting glands.
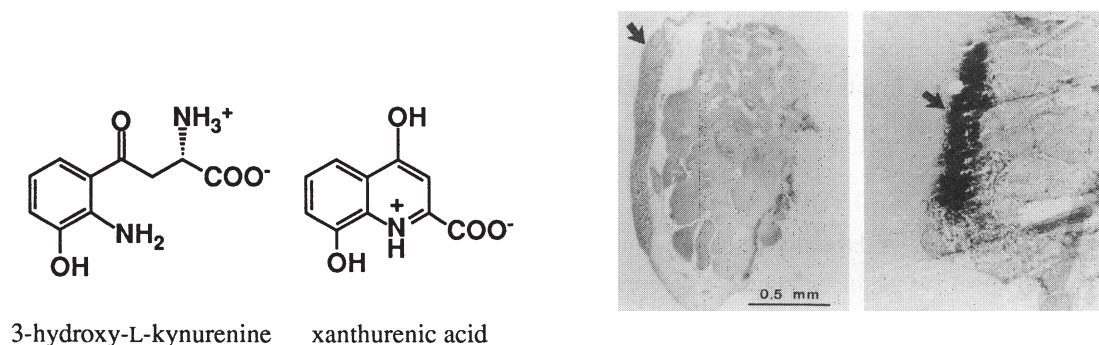
3-hydroxy-L-kynurenine   xanthurenic acid

Fig. 1. Vertical section of Y-organs, *P. clarkii*.
left: intact; right: [^{3}H]-3-hydroxy-L-kynurenine incorporated.

One of the most difficult problems encountered in this study was securing reliable assay animals. Only when ecdysteroidogenesis was potent, suppression by the ecdysone biosynthesis inhibitor EBI was evident by HPLC analysis. The change of ecdysteroidogenesis in Y-organ was attributable to the physiological stage of the animal. The moulting is, thus, controlled by multiple factors, even though the negative-regulation of ecdysteroidogenesis seems to have a great impact on the moulting hormone titer in crustaceans. Complexity of the hormonal events and improvement in laboratory techniques prompted us to make a more comprehensive survey of the hormone control system.

ANALYTICAL RESULTS

In order to investigate changes of ecdysteroidogenesis in Y-organs, juvenile male crayfish, *P. clarkii* (7.5-8.5 cm in length) were used. As a function of days after eyestalk removal, Y-organs were removed and incubated in the culture medium at 25°C for 12 h (ref. 14). The major ecdysteroid [ca. 85 % of the total radioimmunoassay (RIA)-reactive ecdysteroids] produced in the Y-organs was characterized as 3-dehydroecdysone (3-dhE) by reverse-phase HPLC in combination with RIA (ref. 14) using two different types of antisera (ref. 15), S-3 and H-22 (Fig. 2), and also by mass spectrometry. The structure was further confirmed on the basis of $NaBH_4$ reduction which gave ecdysone and 3-epi-ecdysone in the same ratio.
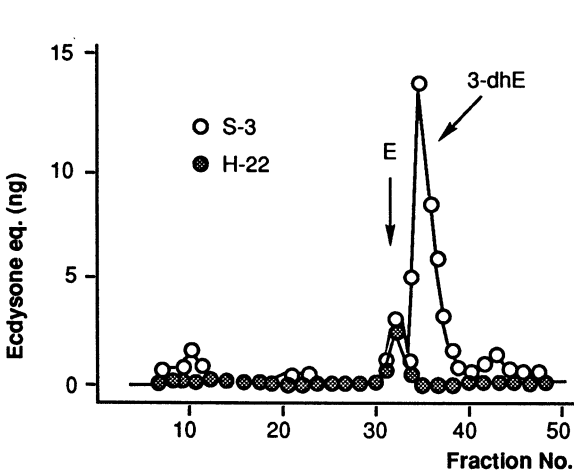

Fig. 2. S-3 & H-22 antisera reactive ecdysteroids. Each fraction was quantified by RIA as ecdysone equivalents. Arrows indicate the retention times of authentic standards.
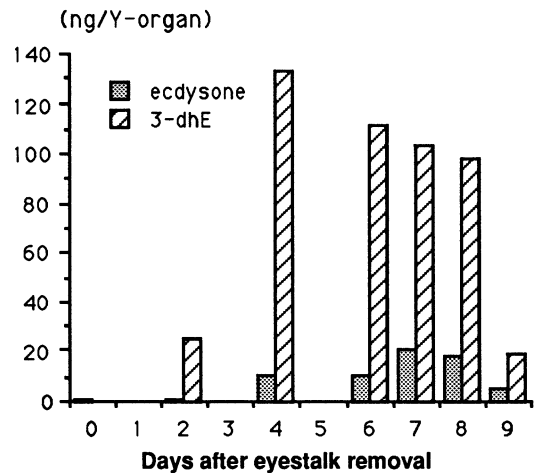E: ecdysone, 3-dhE : 3-dehyroecdysone.

Fig. 3. Ecdysone and 3-dhE in Y-organ culture. Y-organs were excised from the donors, as a function of days after eyestalk removal, and cultured at 25°C for 6 h.
Each ecdysteroid was determined by HPLC.

The antiserum S-3 (20-E-3-succinylthyrogloblin amide) has affinity mainly for ecdysteroids modified at the A-ring, and the antiserum H-22 (20-E-22-succinylthyrogloblin) retains affinity to ecdysteroids having a modified side chain (ref. 16, 17); the cross-reactivity of 3-dhE to S-3 and H-22 was 1:116, whereas that of ecdysone was 1:1 (ref. 15). Thus, S-3 led us to find 3-dhE, a dominant product in the Y-organs of *P. clarkii*. It should be noted that 3-dhE was also found in the crab Y-organs of *Cancer antennarius* (ref. 18) and in the insect prothoracic glands of *Manduca sexta* (ref. 16).

The question has been raised whether 3-dhE functions as a molting hormone in the crayfish. The injection of 3-dhE into *P. clarkii* shortened the moult cycle to the levels of the duration induced by injections of ecdysone and 20-E. Time-course analysis of the Y-organ products exhibited that 3-dhE was produced prior to ecdysone as illustrated (Fig. 3). Ecdysteroidogenesis in Y-organ was generally enhanced by eyestalk removal and resulted in an increase of 20-E in the hemolymph. It was also found that ecdysteroidogenesis in Y-organ *in vitro* plays an alterable physiological function which is represented by specimen-1 and -2 as indicated in Fig. 4. Even when ecdysteroidogenesis in the Y-organ (specimen-2) was not enhanced (Fig 4a), an increase of 20-E in the hemolymph and the development of gastrolith were obviously induced by eyestalk removal (Fig. 4b) and not necessarily the results of ecdysone biosynthesis.
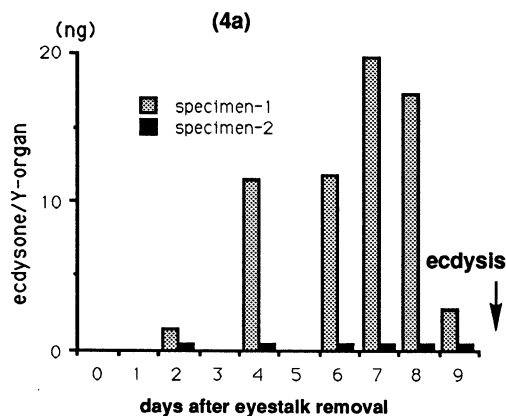

Fig. 4. (4a): Change of ecdysone biosynthesis in Y-organ. Specimen-1 and -2 represent physiological variation of animals.
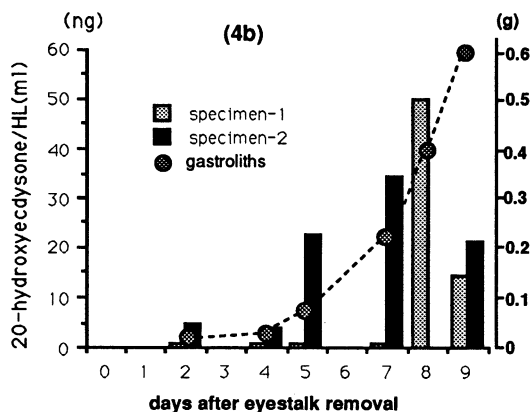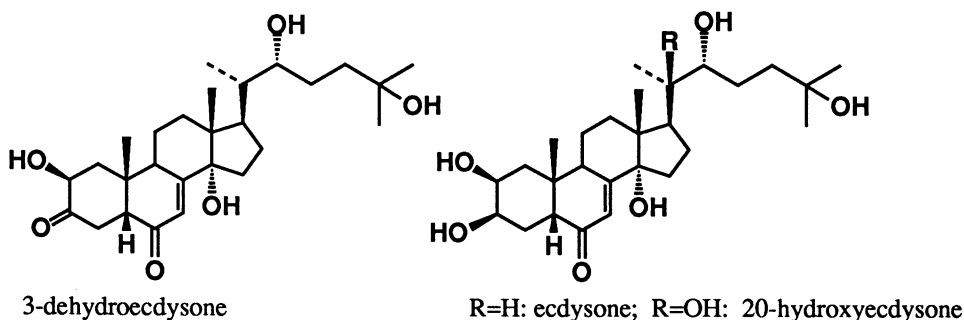
Fig. 4. (4b): increase of 20-hydroxyecdysone and gastroliths induced by eyestalk removal. Development of gastroliths was almost same in between two specimen.

3-dehydroecdysone

R=H: ecdysone; R=OH: 20-hydroxyecdysone

From the above results, it is difficult to rule out the following possibilities: 1) 3-dhE is a precursor of ecdysone and therefore 20-E; 2) 3-dhE is involved in the conjugated-ecdysteroid's storage and related to release of 20-E into the hemolymph; 3) Removal of eyestalk stimulates unknown target tissues, other than Y-organs, and causes an increase of 20-E in the hemolymph, that is independent from 3-dhE; 4) 3-dhE is a new molting hormone and interacts with the ecdysteroid receptor(s).

PHYSIOLOGICAL SIGNIFICANCE

In order to determine the hypothetical role of 3-dhE, the metabolic pathway of radio-labeled 3-dhE, prepared from [3H]ecdysone (ref. 19), was investigated through injection into the crayfish and incubation

with excised tissues. Significant interconversion *in vitro* between ecdysone and 3-dhE was not observed in either the Y-organ or its homogenate, in the presence or absence of peripheral tissues with hemolymph, in contrast to the result obtained from an insect *Manduca sexta* (ref. 16). When the crayfish were injected with [3H]3-dhE, the epidermis with carapace concentrated more of the radiolabel (3-dhE, ecdysone and 20-E) than any other tissue. Time-course profile of [3H]3-dhE pathway indicates that it is a precursor in the ultimate production of 20-E *via* ecdysone at the peripheral tissues of Y-organs. Around 66% of radiolabel of the total injection was excreted after 1h and 89% after 72h, when 100μg (5 μCi) per animal of the 3-dhE was injected at the base of the first periopod. In the hemolymph at 1h after injection, [3H]3-dhE was only detected (about 10 % of the body radiolabel), followed by labeled 20-E (dominant) and ecdysone appearing within 3h (1-2% of the body radiolabel). About 80% of the body radiolabel was found, after 3h of injection, in the epidermis with carapace, whereas about 50% of the radiolabel was shifted after 72h into the hepatopancreas, gut and stomach; noxious compounds, such as a large excess of hormone, were detoxified through conjugated forms. Ecdysteroid metabolism and excretion changes over the moult cycle as shown by Snyder and Chang (ref. 20).
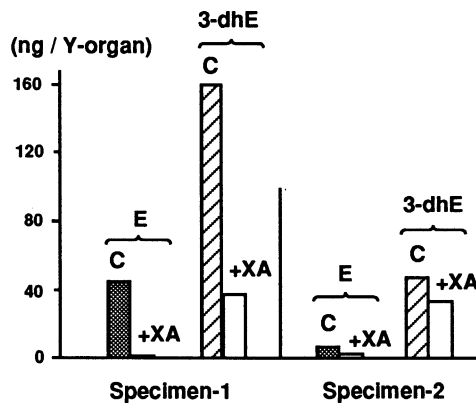

Fig. 5. Inhibition effects of xanthurenic acid on ecdysteroidogenesis. Specimen-1 and -2 are representative for the physiological variation of animals. E: ecdysone; 3-dhE: 3-dehydroecdysone; C: control; +XA: addition of xanthurenic acid.

It is interesting to find that Y-organ culture produced dominantly 3-dhE with a samll proportion of ecdysone, while Y-organ homogenate produced only ecdysone with little 3-dhE. The dehydration to afford 3-dhE may occur by interaction with a membrane protein, although the subcellular site of ecdysteroidogenesis in Y-organ is far from understood. In addition, ecdysone biosynthesis *in vitro* was significantly inhibited in comparison to 3-dhE, when xanthurenic acid (10^{-6} M) was added to the culture medium. The threshold value of 3-dhE for the inhibition by XA was much higher relative to ecdysone, and the inhibition was obvious whenever ecdysteroidogenesis was potent (Fig. 5). The above results may suggest a specific distribution of cytochrome P-450s, mediators of ecdysteroidogenesis, either in the cytoplasmic fraction or within the cell membrane of Y-organ. More studies are under way to understand the hormone control system involved in the moulting of *P. clarkii*.

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