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CHARACTERIZATION OF A SEX PHEROMONE IN THE BLUE CRAB, *Callinectes sapidus:* Crustecdysone Studies

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Abstract—The molting hormone/sex pheromone hypothesis of Kittredge and Takahashi (1972) and Kittredge et al. (1971) was explored in *C. sapidus*. Two concentrations of crustecdysone (5×10^{-5} M and 5×10^{-6} M) were presented to male crabs in a bioassay system in which courtship behavior was monitored. The results demonstrate that crustecdysone does not stimulate courtship in this species. The physical properties of crustecdysone were also compared to those of the partially purified sex pheromone derived from pubescent females. Using HPLC and mass spectral analysis, no correspondence of crustecdysone with the bioactive material could be shown. These results, in conjunction with the findings of others, do not support an evolutionary relationship between the molting hormone and sex pheromone communication in the Crustacea.

Key Words—Crab, *Callinectes sapidus,* sex pheromone, crustecdysone, molting hormone, HPLC, mass spectrometry, bioassay, DCI, desorption chemical ionization.

INTRODUCTION

Chemical communication functions importantly in coordinating the mating behavior of a number of crustacean species (Dunham, 1978). Although several studies have examined the chemical nature of the signal compounds

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involved, to date none have been isolated and structurally characterized (e.g., Christofferson, 1970; McLeese et al., 1977).

Kittredge et al. (1971) reported that the crustacean molting hormone, crustecdysone (Figure 1; synonyms: β-ecdysone, 20-hydroxyecdysone, ecdysterone, isoinokosterone), induces precopulatory behavior in males of *Pachygrapsus crassipes, Cancer antennarius,* and *Cancer anthonyi.* In addition, although details of the experiments are not presented, they note an overlap in pheromone activity among different crab species; namely, the pheromone released by *P. crassipes* stimulates courtship behavior in *C. antennarius,* and *Cancer magister* males are excited by the pheromone of *Cancer productus.* Based on this apparent lack of pheromone specificity and the induction of precopulatory behavior by crustecdysone, they conclude that either crustecdysone is the sex pheromone for these species or has a structure which is sufficiently similar to the natural compounds to mimic their actions. From these findings, the investigators postulate that the molting hormone served as a substrate for sex pheromone evolution (Kittredge and Takahashi, 1972; Kittredge et al., 1971). They speculate this was effected via release of molting hormone by females to the external environment with a concomitant externalization of crustecdysone receptors by males. These two events permitted pheromone communication which, because of its reproductive advantage, was subsequently fixed in the genome.

Although the concept of this evolutionary link between pheromone communication and an internal (molting hormone) communication system is frequently cited in the literature (e.g., Shorey, 1976), the supporting evidence is nevertheless controversial. In addition to the Kittredge et al. (1971) report, two other studies have described apparent mating-related behaviors in other crustacean species following crustecdysone exposure. In the first of these, Hammoud et al. (1975) examined the effects of crustecdysone on male precopulatory behavior (coupling) in the amphipods *Gammarus pulex* and *Gammarus fossarum.* They noted that males exhibited precopulation towards previously "nonattractive" females which had been treated by the external application of a crustecdysone solution. The behavior was main-

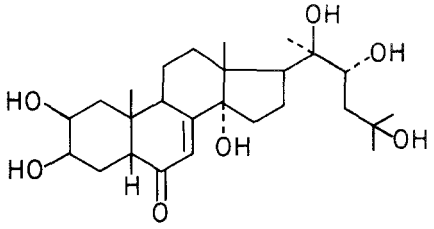

FIG. 1. Structure of crustecdysone.

tained for only a few minutes, however, and was species specific (i.e., males did not couple with treated females of the other species). In another study, Rudd and Warren (1976) tested male rock lobsters, *Jasus lalandii,* with crustecdysone and found that a "searching" activity was induced. This "searching" they consider to be similar to that exhibited by males exposed to water from tanks containing females in proecdysis. In contrast to these reports, the studies of Atema and Gagosian (1973) and Gagosian and Atema (1973) with *Homarus americanus* do not support the crustecdysone hypothesis. When known ecdysones and ecdysone metabolites were presented to males of this species, although alert responses were observed in some test animals, none of the compounds elicited definitively sexual behaviors. Similar findings were reported for the shore crab, *Carcinus maenas;* Seifert (1982) was unable to demonstrate any behavioral responses to crustecdysone (10^{-7} M) in this species. Moreover, contrary to the hypothesis that crustecdysone should only be released from females just prior to a nuptial molt (Kittredge and Takahashi, 1972), Seifert (1982) found that ecdysone excretion (as measured by radioimmunoassay) occurs in both sexes of *C. maenas* juveniles throughout the molting cycle.

Dunham (1978) has argued that important controls were not included in the Kittredge et al. (1971) study, and therefore their conclusion of a molting hormone/sex pheromone relationship in Crustacea was premature. In particular, it was not unambiguously shown that the behaviors exhibited with exposure to crustecdysone were, in fact, premating behaviors as opposed to novel stimulus responses. [A similar criticism is applicable to the Rudd and Warren (1976) study as well.] Indeed, Takahashi (1974) has subsequently noted that *P. crassipes* exhibits an "alert" posture which closely resembles the mating stance used in the Kittredge et al. (1971) bioassay. Since this "alert" posture is apparently stimulated by "glutamate and asparate as well as other substances," it would seem particularly important to control for such responses and to use blind observation procedures. Another subsequent finding which confounds the crustecdysone hypothesis is that, whereas *C. antennarius* and *C. anthonyi* were reported to exhibit concentration vs. "mating stance" relationships for crustecdysone which were similar to those determined for *P. crassipes* (Kittredge et al., 1971), Takahashi (1974) was unable to clearly demonstrate any sexual responses with crustecdysone exposure in *C. magister.*

As has been previously shown (Gleeson, 1980), males of *C. sapidus* display a specific courtship behavior when stimulated by a pheromone released by pubescent females. In light of the controversy surrounding the molting hormone/sex pheromone hypothesis, the objective of the present study was to examine the behavioral effects of crustecdysone on *C. sapidus* males. In addition, as a part of a project to characterize the structure of the

pheromone(s) released by the female of this species, we have compared a number of the physical properties of crustecdysone with those of the partially purified pheromone fraction derived from the urine of pubertal females.

METHODS AND MATERIALS

Urine Collection. Urine from pubertal females was used as a raw material source of the pheromone. Collections were made by directly aspirating urine from the antennal gland pores using Pasteur pipets which had been modified by tapering and fire polishing the tips. Samples collected were immediately frozen for storage in glass vials. Several vials of urine were subsequently pooled to provide sufficiently large batches of homogeneous material for chemical fractionations and bioassay tests.

Bioassay Procedures. All behavioral studies were performed in a semi-enclosed wet lab located adjacent to the Little Annemessex River at the University of Maryland's Marine Products Laboratory in Crisfield, Maryland. Water was continuously pumped from the river for circulation through the holding and bioassay tanks. During these tests salinities ranged from 15‰ to 19.5‰ and water temperatures ranged from 22 to 31°C.

Animals were obtained from local commercial sources and sustained on a diet of fish. Males used for bioassay were initially screened for responsiveness in a holding tank into which water from a tank containing pubertal females was introduced. Only those males exhibiting courtship behavior, as defined previously (Gleeson, 1980), were held for the subsequent bioassay testing. The criteria used in defining a courtship response were: (1) a courtship display: chelae extended in the lateral position, swimming appendages (fifth pereopods) rotated anterodorsally and waved from side to side above the carapace, and walking legs (second to fourth pereopods) extended such that the body is elevated to a near maximum height above the substrate; or (2) an approach towards another crab with chelae extended in the lateral position followed by an attempt to cradle-carry the approached individual.

Bioassays were performed using batteries of 6 to 12, 30-liter, glass aquaria each of which was fitted with a flow-through water system in which filtered (to 10 μm), aerated water was introduced at the surface and siphoned out at the bottom. The flow rate was approximately 1.4 liters/min. Each aquarium contained a single "test" male, previously screened for responsiveness to the pheromone, and an additional unresponsive male which had been rendered anosmic by antennule ablation (Gleeson, 1980, 1982). The latter animal served as an object for the orientation of courtship display and/or coupling behavior exhibited by the test crab. The aquaria were screened from

the observer by a partition with small viewing ports for observing behavior in each tank. All tests were performed between 0700 and 1800 hr under ambient daylight conditions.

Samples to be screened by bioassay were examined using 50-μl aliquots, each of which was brought to a volume of 1 ml in filtered seawater and presented to a test male via ejection through a standardized length of polyethylene tubing. The end of the tube was affixed directly to the carapace of the test animal with wax (Tackiwax, Central Scientific Co.) and oriented such that the ejected sample was expelled immediately beneath and between the antennules. Using this approach the experimenter had relatively precise control over the time and placement of the stimulus. Test animal activity was noted over a 2-min period following ejection of a water blank, then observed for another 2-min period following stimulus ejection, and the presence or absence of courtship behavior recorded.

The protocol used in guiding the chromatographic isolation of the pheromone from urine has been to present a series of six different samples to each test animal over an 8-hr period (one sample every 1.5 hr). The order in which these presentations are made to individual animals is randomized and not known to the observer. A series is presented to at least 24 different crabs over a 2 to 4-day period, and the number of pheromone responses associated with each of the six samples cataloged. In each test series, unfractionated urine is run as an "internal standard" against which four fractionated samples plus a recombined fraction (combined aliquots from the fractionated urine) can be compared. Since all fractionated samples are lyophilized and reconstituted to volumes equivalent to unfractionated urine, this approach permits direct comparison of response frequencies for monitoring activity in the fractions. The recombined material serves as a check on potential synergistic effects and/or activity losses resulting during the fractionation process.

For the crustecdysone assays, two solutions (10^{-3} M and 10^{-4} M) were made up in glass-distilled water using crystalline β-ecdysone (Sigma Chemical Co.). These solutions were then divided into 50-μl fractions which were immediately frozen and held at $-70°$C until bioassayed. Since each of the 50-μl aliquots was diluted with seawater just prior to presentation to a test crab, the maximum concentration of crustecdysone expelled in the region of the antennules was 5×10^{-5} M and 5×10^{-6} M, respectively. In addition to the two crustecdysone solutions, three urine standards and a distilled-water control were presented to each animal. As in the protocol described above, the order in which samples were presented to individual animals was randomized and unknown to the observer.

Pheromone Purification. Partial purification of the pheromone was accomplished by high-pressure liquid chromatography (HPLC) of raw,

bioactive urine on a Whatman reverse-phase silica preparative column (M-9, ODS 3, 25 cm) using various mixtures of water and methanol as eluant. Organic compounds were detected by ultraviolet (UV) absorbance at 220 nm, and all fractions were monitored for pheromone activity using the bioassay described above. Analytical HPLC was performed using a Whatman PXS 10/25 ODS 3 column (25 cm), eluting with 70:30 methanol–water at a flow rate of 1 ml/min. Thin-layer chromatography was carried out on Merck cellulose plates (0.25 mm thick × 173 mm long) which were developed with 70:30 methanol–water. Visualization was accomplished with ultraviolet light, sulfuric acid charring, or vanillin–sulfuric acid–ethanol spray followed by heating.

Mass Spectrometry. Mass spectra were determined on a Finnigan model 4500 mass spectrometer equipped for obtaining desorption ionization (DCI) spectra. DCI spectra were obtained using methane as the ionizing reagent gas; methane pressure was maintained constant between 0.1 and 0.3 torr. The ionizer temperature was 80° C and spectra were measured from 50 to 800 amu with an acquisition rate of one spectrum per second. Samples to be analyzed were dissolved in water and applied to a rhenium filament which was inserted, after the water had evaporated, into the ionization chamber of the mass spectrometer. Data acquisition was begun just prior to insertion of the filament into the ionization chamber. The filament was heated by passing a current through it at a rate of increase of 50 mA/sec to a final value of 1000 mA. This corresponds to a heating rate of approximately 50° C/sec with a final temperature of 1000° C. The rhenium filament was cleaned between runs by passing a high current (1350 mA) through it for approximately 10 sec.

RESULTS AND DISCUSSION

Repeated chromatography of pheromone-containing *C. sapidus* urine by preparative HPLC produced a fraction with all the biological potency of raw urine and which contained three substances as detected by UV absorbance. Thin-layer chromatography confirmed this finding; the components had R_f values of 0.23, 0.65 and 0.69. Comparison of the HPLC retention time of crustecdysone with the retention times of components of the purified pheromone fraction showed no correspondence between crustecdysone and the bioactive fraction (Figure 2). Under the analytical conditions used, crustecdysone had a retention time of 4.4 min, whereas the bioactive fraction elutes as an unresolved cluster with a retention time of 3.6 min.

Mass spectrometry was used to search for the presence of crustecdysone in the partially purified bioactive material obtained from preparative HPLC. The mass spectrum of crustecdysone was determined by the DCI technique, a

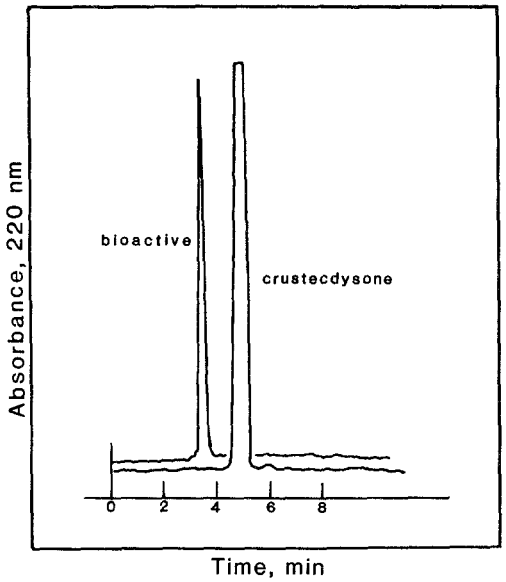

FIG. 2. Comparison of the HPLC retention time of crustecdysone with that of the partially purified pheromone from pubertal *C. sapidus* females.

method known to facilitate ionization of unstable and/or polar, nonvolatile substances of relatively high molecular weight (Cotter, 1980). Under DCI conditions, crustecdysone displayed a mass spectrum characterized by a strong pseudomolecular ion [$(P^+ + 1)$, 481 amu] and by successive losses of water molecules from the parent ion [$(P^+ + 1) - 18$, $(P^+ + 1) - 36$, $(P^+ + 1) - 54$, $(P^+ + 1) - 72$]. Components of the purified bioactive HPLC fraction were partially separated by heating on the DCI filament during the course of the mass spectral run. Comparison of the fragmentation patterns of crustecdysone with fragmentation patterns of components of the bioactive fraction revealed no correspondence between the two sets of spectra. A limited mass search was performed by computer to examine each spectrum determined for the bioactive mixture for the simultaneous occurrence of the five major fragment ions of crustecdysone (481, 463, 445, 427, and 409 amu). If a spectrum were found to contain these fragments, it would have been strong evidence that crustecdysone might be present in the bioactive fraction. In fact, with a detection threshold of at least 10^{-6} M, no spectrum was found to contain that particular combination of fragment ions or any subset of the ion group. This lack of spectral correspondence indicated that crustecdysone could not be detected in the bioactive HPLC fraction by this very sensitive mass spectrometric technique.

TABLE 1. COURTSHIP RESPONSES OF *C. sapidus* MALES FOLLOWING CRUSTECDYSONE PRESENTATION

Stimulus[a]	Courtship responses (No. of animals)
Urine stock A	12
Urine stock A (replicate)	10
Urine stock B	12
Distilled H_2O blank	0
Crustecdysone (5×10^{-5} M)	0
Crustecdysone (5×10^{-6} M)	0

[a] Each stimulus was presented to each test male. N=27.

The results of experiments in which the behavioral effects of crustecdysone were examined are shown in Table 1. Samples from two different stocks of urine (derived from pubertal females) were incorporated as internal standards to monitor the courtship response frequencies of test males. Samples from urine stock A were presented twice to each animal. A total of 27 males were tested, 17 of which exhibited definitive courtship behavior at least once following a urine sample presentation. No courtship responses to the distilled water blank nor to either of the crustecdysone samples were observed. An analysis of these response distributions using the Cochran Q test (Siegel, 1956) yielded a highly significant difference ($P < 0.001$) among the stimuli tested. "Alert" responses were occasionally seen following crustecdysone presentation. These responses were defined as an obvious change in behavior (excluding a courtship response) which occurred immediately following sample ejection; e.g., an increase in the rate of antennule flicking. Four animals exhibited low-intensity "alert" responses at each of the crustecdysone concentrations tested; however, a comparable "alert" frequency (five animals) was observed for the distilled-water blank. The "alert" responses are not surprising in that distilled water represents a potential chemical stimulus in seawater (i.e., via dilution of salts and/or other constituents of seawater).

These behavioral results, in conjunction with our data on the physical/chemical characteristics of the partially purified pheromone of *C. sapidus*, do not support the hypothesized molting hormone/sex pheromone role of crustecdysone (Kittredge and Takahashi, 1972; Kittredge et al., 1971), and therefore ally with other studies (Atema and Gagosian, 1973; Gagosian and Atema, 1973; Seifert, 1982) which do not substantiate such an evolutionary relationship in the Crustacea.

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