Isolation of Crustecdysone (20R-Hydroxyecdysone) from a Crayfish (*Jasus lalandei* H. Milne-Edwards)

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1. A small amount (2mg.) of crustecdysone, a moulting hormone of crustaceans, was isolated from 1 ton of crayfish waste. 2. The purification procedure used was developed with the aid of crustacean and insect bioassays. 3. CM-Sephadex was found to be superior to Sephadex and very effective for the chromatographic separation of crustecdysone from other non-ionic compounds. The higher efficiency of CM-Sephadex is attributed to the greater number of carboxyl groups available for hydrogen-bonding. 4. Reversed-phase chromatography, with butan-1-ol-cyclohexane mixtures as the stationary phase and water as the flowing phase, proved superior to countercurrent distribution with these solvents for the fractionation of purified extracts. 5. A second moulting hormone, deoxycrustecdysone, and the red-concentrating hormone were obtained in a partially purified form.

Crustaceans, like insects, shed their external skeletons periodically as they grow. Early experiments by Kopéc (1922) provided the first clue to the hormonal mechanism that controls this process in insects. Gabe (1953) and Echaliér (1955) later proved conclusively that moulting in crustaceans is initiated by a hormone that is produced in the Y-organ. This glandular mass is similar to the prothoracic gland, which is the source of the insect moulting hormone ecdysone (I) (Butenandt & Karlson, 1954). Karlson (1956) showed that an extract of a crustacean (*Crangon vulgaris*) is active in the insect (*Calliphora*) test. It was thus clear that the crustacean moulting hormone, if not identical with the insect hormone, is a very closely related compound. The concentration of hormone at an intermoult stage was found to be very small and an attempt by Karlson & Schmialek (1959) to isolate the active material from 3 tons of shrimps (*Crangon vulgaris*) was unsuccessful.

Crayfish is abundantly available in Australia and the problem of the isolation and determination of the structure of the crustacean moulting hormone was taken up in the Fishermen's Bend Laboratory in 1962. Eventually, by extracting a large quantity of crayfish (1 ton of ventral thorax waste) and using crustacean (Lowe & Horn, 1967) and insect (Fraenkel, 1935; Becker & Plagge, 1939; Karlson, 1956) bioassays, we succeeded in isolating a small amount (2mg.) of the substantially pure hormone. The name crustecdysone (crustacean ecdysis) and

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structure (II) (20R-hydroxyecdysone) was proposed for this hormone (Hampshire & Horn, 1966; Horn, Middleton, Wunderlich & Hampshire, 1966), partly on biogenetic grounds and from a comparison of its properties with those of the insect moulting hormone ecdysone (I), the structure of which was determined by Hoppe & Huber (1965). As an account of the long purification methods used could not be included in the preliminary accounts of structural studies, details of the procedures, which have a number of novel features, are now presented.

METHODS

Chromatographic apparatus

Glass columns fitted with capillary taps with Teflon barrels were used. A plug of cotton wool supported the column filling. The outlets (internal diam. 2mm.) of the

Scheme 1. Flow-sheet of crustecdysone purification steps.

columns were fitted with standard capillary ball joints. The exit of the ball joint was sleeved with Teflon over a short length of stainless-steel tubing (external diam. 1.25 mm.), which enabled it to be connected by means of Teflon tubing to a Uvicord Absorptiometer (LKB Produkter) (wavelength 254 m μ).

Bioassay methods

Calliphora bioassay. The blowfly (*Calliphora erythrocephala*) was used as the test animal and the test was carried out as described by Karlson (1956) except that a smaller volume of test solution (5 μ l. instead of 10 μ l.) was injected into the unpupated posterior portion. Samples for testing were dissolved in saline (0.65% NaCl) together with ethanol (up to 5%) where this was necessary to obtain a solution.

The results were assessed as described by Karlson (1956), the *Calliphora* unit being taken as the amount of hormone that produces 50–70% pupation of the test animals. With the *Calliphora* strain used, 1 *Calliphora* unit was found to correspond to 0.025 μ g. of either pure crustecdysone or synthetic ecdysone, and is higher than the value (0.0075 μ g.) reported for ecdysone by Karlson (1956).

Cherax bioassay. Local freshwater crayfish (*Cherax destructor*) were used as the test animals, and the test was carried out and the results were calculated as described by Lowe & Horn (1967).

Countercurrent distributions

Trial experiments were carried out in centrifuge tubes (15 ml. capacity fitted with ground-glass stoppers, B14).

The two phases (5ml. each) were thoroughly mixed and centrifuged if necessary to permit efficient separation of the phases. After settling, the bottom phases were transferred by means of a Pasteur pipette. At the conclusion of the distribution the fractions were transferred to weighed flasks (50ml.) for evaporation. After the weighing the fractions were dissolved in saline for bioassay.

Chromatography

Gel filtration. (a) Sephadex. The column used (1.6 cm. $\times$ 55 cm.) was packed with Sephadex G-25 (coarse grade; Pharmacia, Uppsala, Sweden) that had been equilibrated with water overnight. The finer particles were removed by stirring the gel with a large volume of distilled water and decanting off the suspension when most of the gel had settled. The column was packed by adding the gel a little at a time with stirring to the column partially filled with distilled water. The column was allowed to settle overnight before being used. The material was eluted with distilled water and 10ml. fractions were collected.

(b) Polyacrylamide gel. A column (1.6 cm. $\times$ 50 cm.) was packed with Bio-Gel P-10 (50–150 mesh; Calbiochem, Los Angeles, Calif., U.S.A.) that had been equilibrated with water overnight. The column was packed and used as described for Sephadex.

(c) CM-Sephadex. Two columns (2.8 cm. $\times$ 42 cm. and 1.6 cm. $\times$ 56 cm.) containing about 200 g. and 50 g. of gel respectively were used. They were prepared from gel that had been thoroughly washed with ethanol and water and then precycled with 0.5 N-HCl, 0.5 N-NaOH, 0.5 N-HCl and finally water until the wash water was neutral. The material to be chromatographed was transferred to the column in water (20ml.), and eluted with distilled water; 10ml. fractions were collected.

Reversed-phase partition chromatography. Three columns were used. The largest column (4.8 cm. $\times$ 82 cm.) was prepared by adding a mixture of hydrophobic Celite (400 g., prepared as described by Howard & Martin, 1950) and butan-1-ol (240 ml., equilibrated with water) to the column partially filled with water saturated with butan-1-ol. To ensure good settling the Celite–butan-1-ol mixture was degassed *in vacuo* (1 mm. pressure) for a short while. The material to be chromatographed was transferred to the column in butan-1-ol (40 ml.) and eluted (15 ml./min.), first with water saturated with butan-1-ol and then with additional, increasing, amounts of methanol (Fig. 3). The eluate was collected in 500 ml. fractions. A small proportion of the eluate was by-passed through the u.v. absorptiometer by means of a stream-splitter. The second column (2.4 cm. $\times$ 84 cm.) was prepared from hydrophobic Celite (150 g.) containing butan-1-ol–cyclohexane (9:1, v/v) (90 ml.). The material to be chromatographed was transferred to the column in stationary phase (20 ml.) and eluted (5 ml./min.) with water equilibrated with stationary phase; 200 ml. fractions were collected. The third analytical column (1.6 cm. $\times$ 20 cm.) was prepared with hydrophobic Celite (10 g.) containing butan-1-ol–cyclohexane (7:1, v/v) (6 ml.) saturated with water. The material chromatographed (0.19 g.) was transferred to the column in stationary phase (0.5 ml.) and eluted (1.0 ml./min.) as shown in Fig. 3; 4 ml. fractions were collected.

Silicic acid chromatography. The column (0.6 cm. $\times$ 18 cm.) was packed with silicic acid (2.5 g.; Mallinckrodt, St Louis,

Mo., U.S.A.), which was prepared as follows. The silicic acid supplied was stirred with N-HCl and washed with water, and the finer suspended particles were decanted off. After being washed with ethanol and water the silicic acid was activated for 24 hr. at 110°. When cool, water was added (15%, by wt.) to the adsorbent and it was stored overnight before use. The column was made up in ethanol–chloroform (1:5, v/v) and the material to be chromatographed was transferred to the column in ethanol–chloroform (1:5, v/v) (1.5 ml.). The column was eluted with the same mixture and collected in 3 g. fractions.

Thin-layer chromatography. The chromatograms were run on unactivated plates [(20 cm. $\times$ 20 cm.) covered with a layer (0.2 mm.) of silica gel (Kieselgel HF254; E. Merck A.-G., Darmstadt, Germany)]. Two solvent systems were used: system A [96% (v/v) ethanol–chloroform (1:4, v/v)] and system B [96% (v/v) ethanol–chloroform (2:3, v/v)]. The spots were located with u.v. light or by spraying the plate with vanillin–H₂SO₄ spray reagent (Stahl, 1962) and heating the plate for 5–10 min. in an oven at 100°. Crustecdysone gave an olive-green spot that faded to yellow in about 10 hr. To preserve the colours the plate was covered with a second plate held tightly in place with rubber bands.

Experimental procedure

A flow-sheet showing the steps used in the purification of crustecdysone is shown in Scheme 1.

Crayfish extraction. Ventral portions of the thorax of the seawater crayfish (*Jasus lalandei* H. Milne-Edwards) were rapidly frozen after the animals were killed and stored frozen till required. For extraction the frozen material (1000 kg.) was milled at –20° in a hammer mill to pass through a screen with holes $\frac{3}{32}$ in. in diameter, the smallest size that could be used without heating up the mill and thawing the material. The powdered crayfish was transferred to an extraction vessel and stirred for 1 hr. with cold 82% (v/v) ethanol (2270 l.) and acetic acid (2 l.). After settling for 30 min. the supernatant liquid (1590 l.) was sucked off through a hessian bag fitted over the end of a flexible rubber hose (2 in. diam.). The residue was then extracted twice in the same way with 65% (v/v) ethanol (1140 l.) and acetic acid (200 ml.). The combined extracts (3860 l.) maintained below 5° were filtered through a filter press (Sperry 0.58 m.²) coated with Supercel (five bags, 93 kg.). The pH of the filtrate was adjusted with acetic acid to neutrality with the aid of a pH-meter and then evaporated in a vacuum still (LTS4; Majonnier Bros., Chicago, Ill., U.S.A.) at below 15° to about 360 l. of aqueous extract (35 kg. of dissolved solids or approx. 70% of the weight extractable with aqueous ethanol). The concentrate was thoroughly stirred with a mixture of propan-2-ol (200 l.) and hexane (70 l.). After settling (30 min.) the mixture was separated into the red upper lipid layer (110 l.) and green aqueous layer (460 l.). The lipid layer was back-washed with propan-2-ol (18 l.) and water (18 l.) and the aqueous layer obtained (55 l.) re-extracted with hexane (23 l.) before being added to the aqueous green layer (460 l.). The combined hexane layers were evaporated to dryness (extract A, 7 kg. of solids). Then (NH₄)₂SO₄ (100 kg.) was added with stirring to the combined aqueous layers. After settling, the propan-2-ol layer (230 l.) that separated was removed and the aqueous residue was again extracted with propan-2-ol (95 l.) and hexane (30 l.). The upper layer formed on

settling (140l.) was combined with the first propan-2-ol layer and concentrated in a vacuum still (LTS3; Majonnier Bros.) at 15° to reduced volume (55l.). Hexane (23l.) and propan-2-ol (27l.) were then added with stirring. After settling, the lipid layer (28l.) was removed and (NH₄)₂SO₄ (11kg.) was added with stirring to the remaining aqueous layer. The upper layer that separated on standing was removed and the remaining aqueous layer was extracted twice more with propan-2-ol (14l.) and hexane (4.5l.). The combined propan-2-ol-hexane extracts (55l.) were concentrated in a vacuum still (LTS1; Majonnier Bros.) to reduced volume (4.5l., 1.3 kg. of solids).

Countercurrent fractionations. (a) Butan-1-ol-water. The above concentrate (4.5l.) was extracted three times with an equal volume of butan-1-ol. The three butan-1-ol extracts after countercurrent extraction with water (4.5l.) were combined and evaporated to an orange syrup (extract B; about 400g.).

(b) Methanol-chloroform-water. The syrup (extract B) was dissolved in methanol (4l.) and added to a mixture of water (4l.) and chloroform (5l.). After thorough mixing, the chloroform layer was removed and extracted twice more with methanol (4l.) and water (4l.). The three aqueous methanol layers obtained were extracted countercurrently with a second portion of chloroform (5l.). When emulsions were formed they were broken up by the addition of small amounts of methanol. The combined chloroform layers were concentrated (extract C; 300g.). The combined aqueous layers and butan-1-ol (10l., added to prevent foaming), were concentrated to an aqueous solution (1.5l.) by 'bleeding in' water during the evaporation. Insoluble material (21g.) was filtered off and the filtrate concentrated to dryness (extract D; 71g., 10 *Cherax* units/mg.).

(c) Ethanol-chloroform-water (1:1:1 by vol.). The above residue (extract D) was transferred with KHCO₃ (20g.) to the first of five separating funnels (2l.) and partitioned between a mixture of ethanol (600ml.), chloroform (600ml.) and water (600ml.). A countercurrent distribution (ten fractions, double withdrawal) was carried out and the five lower layers were combined and evaporated to dryness (extract E; 12g., 30 *Cherax* units/mg.). Then NaCl (50g.) was added to each of the five upper aqueous fractions and the fractions were extracted three times countercurrently with butan-1-ol (600ml.). The butan-1-ol extracts were combined and evaporated almost to dryness. The residue was triturated with ethanol (200ml.) and filtered from salt. The filtrate was evaporated to dryness (extract F; 14g.). This extract was purified as described below for extract E, and was found to contain about 15% of the total amount of crustecdysone present in the first ethanol extract.

Reversed-phase partition chromatography. (a) Butan-1-ol-water. Extract E was chromatographed on a column of hydrophobic Celite (4.8 cm. × 83 cm.) with butan-1-ol as the stationary phase. The fractions (6-9 inclusive) found, from the *Calliphora* bioassay and thin-layer chromatography (*R_F* 0.13, system A), to contain crustecdysone were combined and evaporated to dryness (extract G; 0.9g., 100-200 *Calliphora* units/mg.). The later fractions (14-18 inclusive), also active in the *Calliphora* test, were combined (extract I; 0.7g.) for the isolation of deoxycrustecdysone. Intermediate fractions (10-13; 0.5g.) were combined for the isolation of the red-concentrating hormone.

(b) Butan-1-ol-cyclohexane (9:1, v/v)-water. Extract G was chromatographed on a column (2.4 cm. × 84 cm.) of

hydrophobic Celite with butan-1-ol-cyclohexane (9:1, v/v) as stationary phase. Fractions containing crustecdysone (7-10) were combined (extract J; 355mg.), and fractions containing red-concentrating hormone (11-20; 290mg.) were added to similar fractions (10-13) obtained in the first partition chromatogram (extract H; 0.8g., 100-150 *Cherax* units/mg.).

CM-Sephadex chromatography. Extract J above dissolved in water was introduced on a column of CM-Sephadex (2.8 cm. × 42 cm.) and the eluate was collected in 20ml. fractions. Fractions found by thin-layer chromatography to contain significant amounts of crustecdysone (27-30) were combined and added to similar fractions obtained by fractionating extract F in the same way as described for extract E.

Silicic acid chromatography. The above combined fractions (14.6mg.) were chromatographed on a column (0.6 cm. × 18 cm.) of silicic acid. Fractions 8-15 afforded substantially pure but non-crystalline crustecdysone [2.3mg.; u.v. λ_{max} . 242m μ , ϵ approx. 8000 in ethanol; *R_F* 0.13 in system A, *R_F* 0.50 in system B; the nuclear-magnetic-resonance spectrum measured in deuteropyridine with a Varian A60 spectrometer equipped with a C-1024 time-averaging computer had resonances (3H) at δ 1.56, 1.38, 1.20 and 1.07 p.p.m.]. Crustecdysone (200 μ g.) isolated from pupae (31 kg.) of the oak silkmoth (*Antheraea pernyi*) by the above procedure had the same *R_F* values in these solvent systems and the spots produced with the spray reagent had the same olive-green colour. It also gave the same mass spectrum as crustecdysone from crayfish.

RESULTS

Preliminary experiments

Crayfish waste (ventral thorax; 180kg.) was extracted with ethanol and an attempt made to isolate from the extract compounds active in the *Calliphora* bioassay (Karlson, 1956, and references cited therein). The procedure developed by Butenandt & Karlson (1954) for the isolation of ecdysone was used. However, it was found that the extracts obtained had much less activity (about 1%) in the *Calliphora* bioassays than reported for comparable extracts of silkworm pupae. Also, it was established that considerable loss of activity (75% or more) took place mainly in the step involving chromatography on deactivated alumina. Though losses could be tolerated with insect extracts of higher specific activity, it was clear that a more efficient purification procedure would be necessary if the crustacean hormone was to be isolated in sufficient amount for structural studies. Attempts to develop a better isolation procedure by using the *Calliphora* bioassay were not successful because of the difficulty of measuring the low activity of the crude extracts. As it was considered that a crustacean might be more sensitive to the crustacean moulting hormone than an insect, the possibility of using a crustacean bioassay was explored. Bioassays involving the rate of limb-bud regeneration

(Bliss, 1956), change in blood calcium concentration (Carlisle, 1957) and gastrolith size (McWhinnie, 1959) have been suggested. Attempts to develop a more sensitive test by using the last-mentioned method with freshwater crayfish (*Cherax destructor*) were not successful, but it was found that solutions of both crude and highly purified extracts of moulting hormone injected into this crayfish produced a marked contraction of the red chromatophores in the cuticle and tail fan. It was thus apparent that the purified moulting-hormone extracts contained, besides the moulting hormone, a hormone that regulates the size of the red chromatophores of the cuticle (Lowe & Horn, 1967) and that the two hormones had very similar properties in the countercurrent distributions and column chromatograms used. Further investigation showed that the red chromatophores would respond over a very wide range of hormone concentration and that the effect could be used as a very sensitive and quantitative bioassay for the red-concentrating hormone. As the moulting hormone has similar properties to the red-concentrating hormone it was possible to use the bioassay to locate fractions that also contained the crustacean moulting hormone but that were insufficiently active to produce a response in the *Calliphora* test. Having thus satisfied our need for a more sensitive bioassay it was possible to test the efficiency of a wide variety of purification steps and to develop a better purification procedure.

Purification procedures

Extraction. Extraction of the hormones was readily accomplished with 80% (v/v) ethanol. Removal of the ethanol provided an emulsion that was largely freed from lipids by extraction with light petroleum. Some ethanol or propan-2-ol was added to permit effective mixing of the phases, and thus ensure that all the activity was extracted from the hexane into the aqueous phase. To recover the hormones, the aqueous layer was extracted either with propan-2-ol-hexane (3:1, v/v) after the addition of ammonium sulphate to produce two phases, or with butan-1-ol without addition of ammonium sulphate. The use of propan-2-ol has the great advantage that this solvent can be removed at low temperature (below 40°) more easily than butanol. Propan-2-ol-hexane (3:1, v/v) was found to be more selective than propan-2-ol alone. Two extractions of the crude aqueous extract (4 vol.) with the solvent mixture (1 vol.) recovered more than 90% of the red-concentrating hormone.

Countercurrent distributions. To enrich the propan-2-ol extract further, a number of countercurrent solvent systems were examined, such as methanol-chloroform-water (1:2:1, by vol.),

Fig. 1. Countercurrent distribution of extract B (280 mg.) in methanol-chloroform-water (1:2:1, by vol.) (5 ml. of each phase, six tubes, double withdrawal). ●, Activity (*Cherax* units); the histogram shows the weight (mg.). Aqueous fractions (1-6) and chloroform fractions (7-12) are shown.

Fig. 2. Countercurrent distribution of extract D (180 mg.) and KHCO_3 (300 mg.) in ethanol-chloroform-water (1:1:1, by vol.) (3 ml. of each phase, ten transfers). ●, Activity (*Cherax* units) found; ○, theoretical distribution curve; the histogram shows the weight (mg.). The hormones have a low partition coefficient in this system and travel with the chloroform layers.

Fig. 3. Reversed-phase partition chromatogram of extract E (190 mg.). The column (1.6 cm. $\times$ 30 cm.) contained hydrophobic Celite (10 g.) and butan-1-ol-cyclohexane (7:1, v/v) (6 ml.); 4 ml. fractions were collected and the percentage of methanol added to the eluting phase is indicated by arrows. ●, Weight (mg.); ○, Activity (*Cherax* units); —, E_{254} of the eluate.

Fig. 4. Comparison of chromatography of extract G (53 mg.) on Sephadex G-25 (1.5 cm. $\times$ 56 cm.; 0.01 mequiv./g.) and CM-Sephadex (1.5 cm. $\times$ 56 cm.; 4.5 mequiv./g.). The flow rate was 0.5 ml./min. and 10 ml. fractions were collected. —, E_{254} of the eluate; -----, distribution curve of crustecdysone from thin-layer chromatography.

butan-1-ol-cyclohexane-water (in various proportions) and ethyl acetate-water. Of these the first (see Fig. 1) proved to be by far the most effective.

The hormone extract after extraction with chloroform contained a large proportion of acidic materials. However, as the moulting hormone and especially the red-concentrating hormone are very sensitive to base, it was necessary to use very mild conditions (see Fig. 2) to remove acidic impurities. A countercurrent distribution with methanol-butan-1-ol-water (1:10:10, by vol.) and potassium hydrogen carbonate recovered all the activity of both hormones present. However, this system is less selective and troublesome emulsions were encountered. Some methanol was included in the system to help decrease emulsification. For analytical separations, where the volumes are sufficiently small to permit centrifugation of each tube, a butan-1-ol-water system may be used to advantage. The enrichment of the hormones by chromatography on silicic acid and on neutral or acid-washed alumina with ethanol-ethyl acetate mixtures, on polyamide with ethanol-water in various proportions, on ion-exchange resins and on Sephadex G-25 was also investigated, but none of these procedures gave as good a recovery of hormone as the countercurrent system used.

Reversed-phase chromatography. Attempts to enrich the crude hormone extract by using countercurrent distribution with butan-1-ol-cyclohexane-water (7:3:10, by vol.) as described by Butenandt & Karlson (1954) were unsuccessful because of emulsification, and chromatographic partition systems were investigated. Reversed-phase partition chromatography with hydrophobic Celite (Howard & Martin, 1950) and butan-1-ol-cyclohexane as the stationary phase (Fig. 3) proved more useful than normal partition chromatography with moist silica gel and water-saturated butan-1-ol-cyclohexane mixtures as the flowing phase. The

more polar fractions (15–25, Fig. 3) were used to isolate crustecdysone (Hampshire & Horn, 1966) and the less polar ones (40–60, Fig. 3) to obtain deoxycrustecdysone (Galbraith, Horn, Middleton & Hackney, 1968).

Gel filtration. As the polar fractions obtained by partition chromatography were readily soluble in water, gel chromatography was investigated with dextran (Sephadex G-25) and polyamide (Bio-Gel P-10) gels. The former separated the material into three distinct peaks (Fig. 4). Bio-Gel gave a single broad peak. As Sephadex contains free carboxyl groups (0.01 mequiv./g.), in contrast with Bio-Gel, it was considered that these groups, by providing sites for hydrogen-bonding, were responsible for the superior separating efficiency of the Sephadex gel. Accordingly CM-Sephadex, which has a larger number of carboxyl groups (4.5 ± 0.5 mequiv./g.), was tried and gave a much better separation of the constituents of the mixture than Sephadex G-25 (Fig. 4).

DISCUSSION

Chromatography on CM-Sephadex is very effective in separating crustecdysone from a complex mixture of compounds with very similar chromatographic properties. The superior separation obtained with CM-Sephadex as compared with Sephadex is probably due to the greater number of carboxyl groups available for hydrogen-bonding. Thus it is likely that CM-Sephadex, besides being useful as an ion-exchange medium, will also prove useful as an adsorbent for the chromatographic separation of mixtures of non-ionic polyhydroxylated compounds.

Column chromatography was used for the final purification in preference to thin-layer chromatography, since it was found that with small amounts of material the recovery was higher, there were fewer manipulation losses and the column adsorbent could be more easily washed free from fine particles and soluble impurities.

Crustecdysone was also isolated from pupae of the Japanese oak silkworm (*Antheraea pernyi*) (Horn *et al.* 1966) by the same purification steps. The suggestion that crustecdysone is identical with β -ecdysone (Karlson, 1956) has since been proved correct (Hocks, Schulz, Watzke & Karlson, 1967). Crustecdysone was shown (Galbraith, Horn, Hocks, Schulz & Hoffmeister, 1967) to be identical with the silkworm hormones 20-hydroxyecdysone (Hocks & Wiechert, 1966) and ecdysterone (Hoffmeister & Grützmacher, 1966; Hoffmeister, 1966). The hormone has now been identified in a wide variety of plants and animals (Takemoto, Ogawa & Nishimoto, 1967; Jizba, Herout & Šorm, 1967; Rimpler & Schulz, 1967; Takemoto, Ogawa, Nishimoto & Hoffmeister, 1967; Kaplanis,

Thompson, Robbins & Bryce, 1967; Galbraith & Horn, 1966).

Our structure (II) for crustecdysone was further supported by the oxidation of crustecdysone to a synthetic ketone of known structure (Siddall, Horn & Middleton, 1967) and synthesis of crustecdysone (Hüppi & Siddall, 1967).

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